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Dogs may carry *Leishmania tropica* and *Leishmania major* in their blood circulation: a molecular and hematological study

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Abstract

Background Dogs may be infected with species of *Leishmania* parasites that are disseminated through blood circulation and invade the internal organs. In this study, we aim to detect the parasite in the blood of dogs using the PCR technique. The present work was performed from February 2022 to May 2023 in Fars Province, southern Iran, where the disease is endemic.

Results In total, 7(5.1%) out of 135 blood samples, six were identified as *Leishmania tropica* and one as *Leishmania major*. We found no trace of *Leishmania infantum*, which is always known for visceral infection. In addition, no sign of cutaneous lesions or a significant disease was seen in the animals infected with both species. Of 48 dogs with anemia, two were *Leishmania* positive. The mean value of hematological parameters in the infected dogs was within the normal range except for a significant reduction in the platelet measures ($p < 0.05$).

Conclusions Our data revealed that both *Leishmania* species, *tropica* and *major*, may manifest as viscerotropic leishmaniasis. More investigations are needed to understand the conditions under which these species choose the type of infection. Moreover, our data emphasize the role of asymptomatic dogs in carrying these parasites, a crucial factor in spreading the disease.

Keywords *Leishmania*, Dog, Iran, PCR, Hematologic parameters, Thrombocytopenia

Background

Leishmaniasis is a contagious disease that infects both people and domestic and wild dogs worldwide. The disease has been epidemiologically expanded, and new methods of diagnosis have corroborated that the *Leishmania* species are progressively spreading to different hosts [1, 2]. Compared to conventional diagnostic methods, the molecular techniques are very accurate, sensitive, and reliable ways to find infections at the early stage, especially in people and reservoirs that are hiding infections or without any clinical signs [3, 4]. The usefulness of the PCR-based methods has been assessed for a range of samples, such as skin, bone marrow and blood. Among different genes, the internal transcribed spacer (ITS) region has different sizes and nucleotide sequences for each species. Therefore, it is a suitable molecular marker for tracking and identifying *Leishmania* species in dog hosts [5].

Based on the clinical manifestations, domestic dogs may show cutaneous symptoms, mainly associated with *Leishmania tropica* and *Leishmania major*, while *Leishmania infantum* is the main cause of visceral leishmaniasis (VL). Dogs are usually thought to be the principal

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domestic vectors of *L. infantum*, which causes zoonotic VL in humans [6].

Leishmaniasis, due to the three species mentioned, has been frequently reported and is endemic in the Middle East region of Asia [7]. A recent systematic review in Iran showed that canine visceral leishmaniasis (CVL) is endemic in at least half of the country's provinces, with an estimated overall prevalence of 16% [8]. According to the available molecular studies, *L. infantum* is the main species involved in visceral infection in canid hosts [8, 9]. In other research, molecular data implicated that dogs with visceral or blood infection may carry the other species, particularly *L. tropica* [10–12]. In addition, the detection of *Leishmania* in the blood could be explained by the fact that they should be suspected as a reservoir for cutaneous disease. Such findings help to understand the transmission of this disease (as the reservoir host) and, therefore, to prevent and control the spread of leishmaniasis [13].

The present cross-sectional study aimed to investigate and identify the *Leishmania* parasite in dogs' blood circulation using the molecular approach. The sequence data of the two highly variable ITS sections (ITS-1 and ITS-2) were used to characterize the *Leishmania* species. In addition, we attempted to determine any possible associations between the parasite contamination and the hematological alterations.

Materials and methods

Sample collection and DNA extraction

This study was conducted on domestic dogs referred to the Clinic of Veterinary Medicine, Shiraz University, Fars Province, Iran. This area is located in an arid and semi-arid region of southern Iran and covers a 52,069-square-mile area (8.09% of the entire country) at 27°03′ and 31°42′N latitudes and 50°30′ and 55°36′E longitudes (Fig. 1).

A total of 135 blood samples were taken through the jugular vein and stored in EDTA tubes. Sampling was done without anesthesia and with the owner's consent. A complete blood count was performed by a Veterinary Hematology Analyzer (Nihon Kohden, MEK-6450 Celltac Alpha, Tokyo, Japan). Standard blood smears were also prepared, and the remaining blood samples were used for DNA extraction. Genomic DNA was extracted from 200 µl of whole blood using the commercial Blood Genomic DNA Purification Kit (Parstous®, Iran). The quantity and purity of the extracted DNA were checked using a NanoDrop spectrophotometer (Thermo Scientific, NanoDrop, USA). The collected DNA was stored at − 20 ∘C until use.

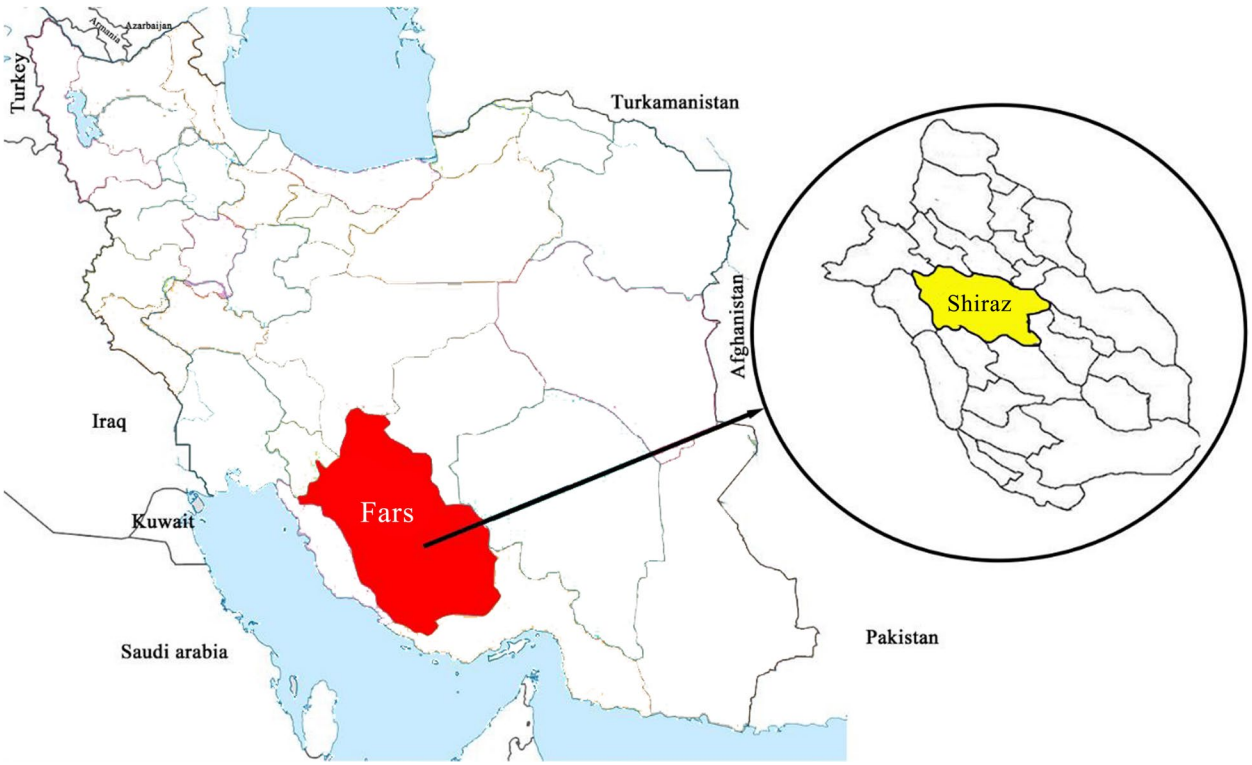

Fig. 1 Map of sampling area in Fars province, southern Iran

Polymerase Chain Reaction (PCR)

In this study, the infection with *Leishmania* was determined by PCR reactions targeting the ribosomal DNA regions. Primers LSGITS1-F1: 5'-CATTTTCCGATG ATTACAC-3', LSGITS1R1: 5'-CGTTATGTGAGCCGT TATC-3', and LGITSF2: 5'-GCATGCCATATTCTC AGTGT-3', LGITSR2: 5'-GGCCAACGCGAAGTTGAA TTC-3' were used for the identification of ITS1 and ITS2 regions [14]. The amplification reaction was carried out in a total volume of 20 µl containing 10 µl PCR premix (Ampliqon, Denmark), 1 µl (10 pmol) of each primer, 5 µl H2O, and 3 µl (~30 ng) of DNA as a template. In this study, a touchdown PCR program was used to delete irrelevant products or smears. The amplification condition was as follows: An initial denaturation at 95°C for 5 min, then one cycle of denaturation (1 min at 94°C), annealing (1 min at 58°C), and extension (45 s at 72°C), continued with three PCR cycles again with annealing temperatures at 57°C, 56°C, and 55°C (for 1 min), followed by 35 cycles as above except a final annealing temperature at 54°C (for 1 min). After the last cycle, an extension step was applied for a further 5 min at 72°C. The PCR products were subjected to electrophoresis on a 3% agarose gel stained with Safe DNA Gel Stain (Sinaclone®, Iran) and visualized under ultraviolet light.

Products were directly sequenced (Pishgam Biotech Co., Tehran, Iran), and the results were compared with other available sequences in the NCBI using the BLAST search. Our sequences were aligned with homologous sequences existing in the GenBank database using the Clustal W program performed by MEGA software (version 11). The phylogenetic tree was constructed based on the maximum likelihood method, and analyses were carried out using the Kimura 2-parameter distance estimate [15].

Statistical analysis

Animals included in this study were assigned to *Leishmania* positive and negative groups, according to the PCR results. The mean values of the haematological parameters were compared between the groups. In order to test whether the *leishmania* infection may relate to anemia in dogs, the data were also divided into two groups according to the hematocrit (Hct) being under or above the normal percentage of 37. Based on the normal or abnormal spread of each data set, a parametric or non-parametric statistical approach was applied using an independent sample t-test and Mann–Whitney analysis, respectively.

Results

Among the 135 dogs sampled, seven (5.1%) were positive for *Leishmania* DNA. Of 48 anemic dogs, only two cases showed the parasite in their blood circulation. The Hct Value (%) in those two dogs were 31.2% and 26.7%. The infected animals did not show any signs of cutaneous invasion.

The results of measured hematological parameters in *Leishmania* positive and negative dogs are represented in Tables 1 and 2. Analyses on RBC-related parameters did not accompany with significant differences. In the *Leishmania* positive group, means of RBC, PCV and hemoglobin were all in the range of normal values (Table 1). In this study, the platelet count was the only hematological factor significantly lower in the infected dogs ($p < 0.05$). However, compared to the non-infected

Table 1 Mean ± SEM of the hematological parameters related to the RBC (Red blood cell) value

	Hb (g/dl)	RBC ($\times 10^9$ L^{-1})	Hct (%)	MCHC (%)	MCH (pg)	MCV (fl)	RDW (%)
Leishmania positive	13.71 ± 1.87^a	5.88 ± 0.61^a	40.57 ± 4.56^a	33.27 ± 0.95^a	22.94 ± 1.16^a	68.94 ± 2.51^a	14.56 ± 0.65^a
Leishmania negative	13.88 ± 0.38^a	5.5 ± 0.15^a	39.83 ± 1.07^a	34.90 ± 0.26^a	25.40 ± 0.31^a	72.75 ± 0.76^a	15.88 ± 0.37^a

The same letters in each column indicate no statistical difference ($p > 0.05$)

Table 2 Mean ± SEM of Platelet count, Mean Platelet Volume (MPV), and the total WBCs with its differential measurements

	Platelet ($\times 10^3$ µL^{-1})	MPV (fl)	WBC ($\times 10^9$ L^{-1})	Neutrophil ($\times 10^9$ L^{-1})	Lymphocyte ($\times 10^9$ L^{-1})	Monocyte ($\times 10^9$ L^{-1})	Eosinophil ($\times 10^9$ L^{-1})	Band Neutrophil ($\times 10^9$ L^{-1})
Leishmania positive	**208.57± 36.93^a**	10.66 ± 2.06^a	15.48 ± 3.09^a	12.64 ± 2.10^a	2.70 ± 0.60^a	0.21 ± 0.21^a	0.62 ± 0.56^a	0.29 ± 0.17^a
Leishmania negative	**344.75 ± 15.47^b**	8.15 ± 0.13^a	12.33 ± 0.73^a	9.32 ± 0.65^a	1.87 ± 0.10^a	0.43 ± 0.04^a	0.25 ± 0.33^a	0.64 ± 0.12^a

Different letters indicate the statistical difference ($p < 0.05$)

group, we did not conclude any remarkable difference in white blood cell counts of the infected dogs (Table 2).

Molecular analysis

The PCR amplification based on the ITS region revealed seven *Leishmania*-positive samples. Fragments about 260 and 450 bp were successfully amplified and sequenced using primers specific to ITS-1 and ITS-2 regions, respectively. The sequence analysis of the ITS-1 region showed that six samples were identified as *L. tropica* and one was *L. major*. The ITS-1 sequences obtained for *L. tropica* (252 bp) and *L. major* (266 bp) were recorded in the GenBank under the accession numbers OR237833 and OR237832, respectively.. No intraspecific variation was observed among Iranian obtained sequences. The infection with both species was also corroborated by the sequencing of ITS-2 amplicons, which were recorded in the GenBank as OR229790 (*L. tropica*) and PP094785 (*L. major*). The expected size for the ITS-2 bands of *L. tropica* (415 bp) and *L. major* (434 bp) was observed on the agarose gel. However, *L. infantum* yields 418 bp-sized fragments, close to *L. tropica*. Here, sequencing helped us to distinguish the species.

The phylogenetic trees inferred from data on ITS-1 and ITS-2 are demonstrated in Fig. 2 (A and B, respectively). Both the cladograms show that the ITS region has separated different species well and placed them in a different clade. According to the pairwise distance analysis of the ITS-1 region, the present *L. tropica* strains had a distance value of 0.00 to 0.016 from the other reported Iranian isolates. This value reached the 0.025 with other GenBank records (Table 3). Similar results were obtained considering the ITS-2 region, except for much higher distance values of 0.078 and 0.041 with records from human specimens in Ethiopia and *Phlebotomus sergenti* from Israel, respectively. The analysis of the ITS region showed that the only *L. major* specimen identified in this study resembled other reports from geographical and host resources (Table 4).

Discussion

In the present study, the infection rate of the blood samples was 5.1%. In terms of species involved, *L. tropica* and *L. major* were confirmed using the molecular test. In a previous study in southern Iran, 23% of blood samples of domestic dogs were PCR-positive and the only identified species was *L. infantum* [16]. A few years later, the infection with *L. infantum* and *L. tropica* was detected in three (5%) buffy coat samples of dogs living in a similar region [17]; however, the sera infection rate of those animals was 46.7%. In the North of Iran, three species of *L. infantum* (18%), *L. tropica* (4%), and *Crithidia* spp. (3%)

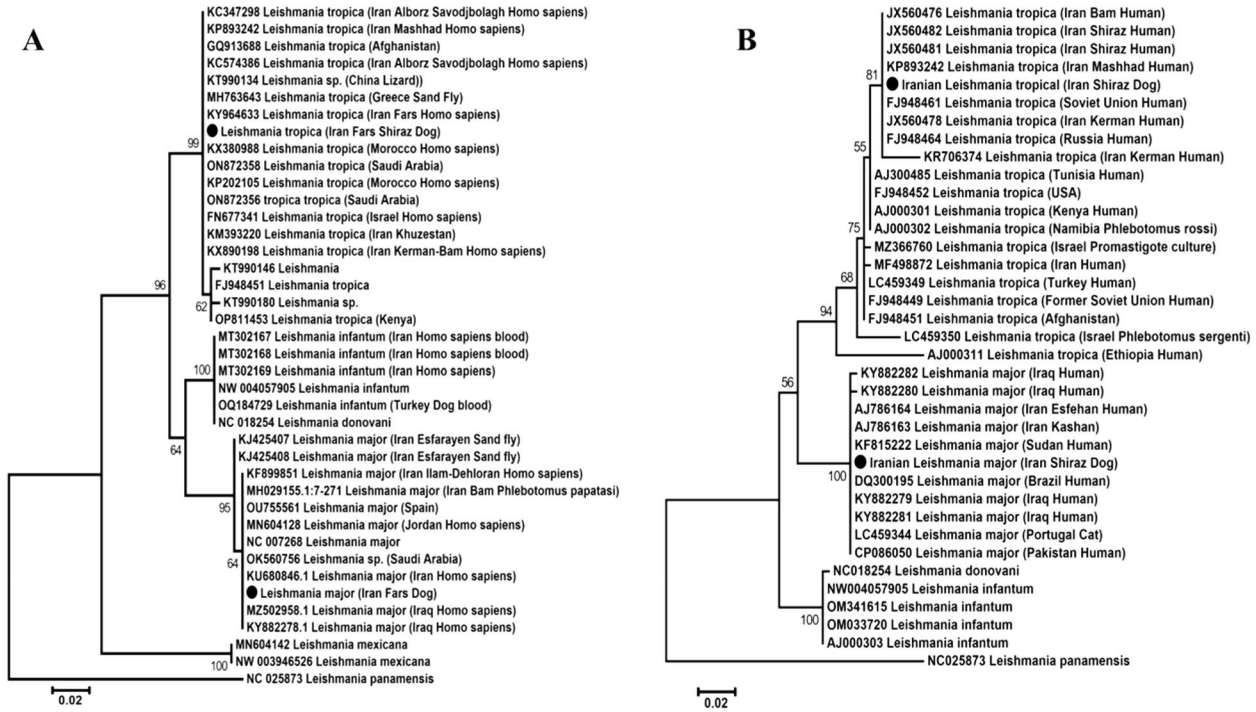

Fig. 2 Phylogenetic tree of the *Leishmania* species found in blood of dogs based on analyzes on the ITS1 (**A**) and ITS2 (**B**) regions using the Maximum likelihood method. The numbers associated with nodes represent the percentage of 2000 bootstrap reps and the horizontal distance is proportional to hypothesized evolutionary change (scale bar)

Table 3 Pairwise distance between sequences of the *L. tropica* obtained in the present study and some of the similar species recorded before in the GenBank. Other *Leishmania* species are included for more comparisons

GenBank records (ITS-1 region)		**GenBank records (ITS-2 region)**	
***Leishmania* tropica (this study)**	Distance	***Leishmania* tropica (this study)**	Distance
KX890198 L. tropica (Iran Bam Homo sapiens)	**0.000**	**JX560481 L. tropica (Iran Shiraz Human)**	**0.000**
KM393220 L. tropica (Iran Khuzestan Homo sapiens)	**0.016**	**JX560476 L. tropica (Iran Bam Human)**	**0.000**
KY964633 L. tropica (Iran Fars Homo sapiens)	**0.000**	**JX560482 L. tropica (Iran Shiraz Human)**	**0.000**
KC574386 L. tropica (Iran Alborz Homo sapiens)	**0.000**	**JX560478 L. tropica (Iran Kerman Human)**	**0.000**
KC347298 L. tropica (Iran Alborz Homo sapiens)	**0.000**	**KP893242 L. tropica (Iran Mashhad Human)**	**0.000**
KP893242 L. tropica (Iran Mashhad Homo sapiens)	**0.004**	**MF498872 L. tropica (Iran Human)**	**0.015**
ON872356 L. tropica (Saudi Arabia)	0.016	**KR706374 L. tropica (Iran Kerman Human)**	**0.015**
OP811453 L. tropica (Kenya Homo sapiens)	0.021	FJ948449 L. tropica (Former Soviet Union Human)	0.013
KP202105 L. tropica (Morocco Homo sapiens)	0.004	FJ948461 L. tropica (Soviet Union Human)	0.000
ON872358 L. tropica (Saudi Arabia)	0.017	FJ948464 L. tropica (Russia Human)	0.000
KX380988 L. tropica (Morocco Homo sapiens)	0.004	AJ000311 L. tropica (Ethiopia Human)	0.078
KT990146 *Leishmania* sp. (China Lizard**)**	0.025	LC459350 L. tropica (Israel *Phlebotomus sergenti*)	0.041
MH763643 L. tropica (Greece Sand Fly)	0.000	AJ300485 L. tropica (Tunisia Human)	0.008
KT990134 Leishmania sp. (China Lizard)	0.000	LC459349 L. tropica (Turkey Human)	**0.013**
KT990180 *Leishmania* sp. (China Lizard)	0.025	FJ948452 L. tropica (USA,human)	0.010
GQ913688 L. tropica (Afghanistan BALB/c mice)	0.000	MZ366760 L. tropica (Israel Promastigote culture)	0.015
FJ948451 L. tropica (USA CDC approved sp.)	0.025	FJ948451 L. tropica (Afghanistan)	0.013
FN677341 L. tropica (Israel Homo sapiens)	0.017	AJ000301 L. tropica (Kenya Human)	0.010
OQ184729 L. infantum (Turkey Dog blood)	0.056	AJ000302 L. tropica (Namibia *Phlebotomus rossi*)	0.008
MN604142 L. mexicana	0.210	NW004057905 L. infantum	0.063
NC 018254 L. donovani	0.055	NC018254 L. donovani	0.063
NC 025873 L. panamensis	0.302	NC025873 L. panamensis	0.205

were also detected in the blood of dogs [12]. However, Bamorovat et al. [10] detected only two *L. tropica*-positive specimens from visceral tissue samples taken from stray dogs [10]. These variations in the frequency of the diseased animals and the species detected may originate from differences in sample size, geographical location and climate, method of detection and gene regions used, season of sampling and activity of vectors [18].

A comparative investigation of the mentioned studies reveals that *L. tropica* and, according to our study, *L. major* are specified to establish visceral infection. Dogs have been regarded as the significant reservoirs of VL for human infection [9, 19]. In older literature, the etiology of CVL in domestic and wild canines was mainly attributed to *L. infantum* in the old and new worlds [20]. This species was not traced in the current study. Therefore, the presence of *L. tropica* and *L. major* in blood circulation

Table 4 Pairwise distance between sequences of the *L. major* obtained in the present study and some of the similar species recorded before in the GenBank. Other Leishmania species are included for more comparisons

GenBank records (ITS-1 region)		**GenBank records (ITS-2 region)**	
***L. major* (this study)**	Distance	***L. major* (this study)**	Distance
KF899851 L. major (Iran Dehloran Homo sapiens)	**0.000**	**AJ786164 L. major (Iran Esfehan Human)**	**0.000**
MH029155 L. major (Iran Bam *Phlebotomus papatasi*)	**0.000**	**AJ786163 L. major (Iran Kashan)**	**0.000**
KJ425407 L. major (Iran Esfarayen Sand fly)	**0.004**	KF815222 L. major (Sudan Human)	0.000
KU680846.1 L. major (Iran Homo sapiens)	**0.000**	DQ300195 L. major (Brazil Human)	0.000
KJ425408 L. major (Iran Esfarayen Sand fly)	**0.004**	KY882279 L. major (Iraq Human)	0.000
OU755561 L. major (Spain)	0.000	KY882281 L. major (Iraq Human)	0.000
MN604128 L. major (Jordan Homo sapiens)	0.000	LC459344 L. major (Portugal Cat)	0.000
NC_007268 L. major (USA Referenced specimens)	0.000	CP086050 L. major (Pakistan Human)	0.000
MZ502958 L. major (Iraq Homo sapiens)	0.000	KY882282 L. major (Iraq Human)	0.005
KY882278 L. major (Iraq Homo sapiens)	0.000	KY882280 L. major (Iraq Human)	0.005
OQ184729 L. infantum (Turkey Dog blood)	0.062	NW004057905 L. infantum	0.067
NC 018254 L. donovani	0.061	AJ000303 L. infantum	0.067
MN604142 L. mexicana	0.328	NC018254 L. donovani	0.067
NC 025873 L. panamensis	1.484	NC025873 L. panamensis	0.223

should be highlighted because both species are often detected in skin scrapes. This is a crucial result, as those infected dogs could be a suspected reservoir for cutaneous leishmaniasis and form a basis for transmission of the parasite to canine and human hosts. In our study, an animal with blood infection with *L. major* was detected. However, the animal had no sign of skin lesions. According to the literature at least in Iran, this was a new finding that *L. major* is detected in the blood of infected dogs. Previously, *L. tropica* was reported to have the potential to be an etiologic agent of visceral Leishmaniasis in Morocco [21, 22]. This species has been shown before for its capability of organ invasion known as the Kala-azar in humans [23].

The *Leishmania*-positive animals in the present study represented alterations in thrombocyte count. Also, mild anemia was seen in two animals, but no evident cutaneous lesion or significant visceral disease was seen in them. In line with our observations, meta-analysis of studies in Iran indicates that 81% of the infected dogs were asymptomatic [8]. Other surveys from other parts of the world also show that exposure of dogs to *Leishmania* is mostly not coincident with clinical disease [24]. Thus, in asymptomatic dogs, the focus should be on their possible role in parasite transmission. In addition, the low infection rate in the present study is maybe because the animals were households and probably had much less exposure to the vector [25].

In the present study, the values of most hematological parameters were within the normal range. This finding is consistent with the lack of significant clinical signs in the infected dogs. Of all parameters measured, only the platelet value was significantly lower in PCR-positive dogs. In several clinical and experimental studies of *Leishmania*-infected dogs with or without epistaxis [26, 27], hemostatic abnormalities and thrombocytopenia was not severe enough to cause spontaneous bleeding. Still, one of the observed hemostatic abnormalities was described as thrombocytopathy [27, 28]. A number of previous studies reported a low frequency of thrombocytopenia in the infected dogs, especially in asymptomatic cases [29–31]. In contrast, thrombocytopenia and anemia were more common in dogs with acute leishmaniasis and the clinical symptoms were due to bone marrow and renal and other organ dysfunctions [32]. Since anti-platelet antibodies have been found in dogs with leishmaniasis, an immune-mediated peripheral elimination of circulating platelets is the most plausible cause

of thrombocytopenia [33–35]. In this study, a significant decrease was seen in platelet numbers in three out of seven infected dogs. They were clinically healthy, but no information was available regarding the co-infection disease. Therefore, additional studies are suggested to investigate the extracted DNA material for the presence of other protozoans using the appropriate molecular approach.

Conclusion

To estimate the prevalence of *Leishmania* parasite infection, our study used the PCR method based on the ITS region, investigating visceral infection in blood samples of dogs in an endemic area in Fars Province, southern Iran. The results of this study did not show any trace of *L. infantum*. However, the other two detected species, *L. tropica* and *L. major* were found in the blood specimens with no history of notable disease. Therefore, clinically healthy dogs can transfer the parasite to other hosts and owners in areas with densities of sandflies.

Abbreviations

VL Visceral Leishmaniasis
CVL Canine Visceral Leishmaniasis
ITS Internal Transcribed Spacer

Authors' contributions

AR helped to perform hematological and molecular analysis; ER and HSY contributed to conceptualization, methodology, writing the manuscript, reviewing and editing; TY contributed to hematological and molecular analysis, writing the initial manuscript and editing. ND: supervised fieldwork and contributed to sample collection and technical assistance.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request. In addition, the sequence data of the ITS1 and ITS2 regions were deposited in the GenBank under the primary accession numbers, OR237833 and OR229790 (*Leishmania tropica*) and OR237832 and PP094785 (*Leishmania major*).

Declarations

Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All methods are reported in accordance with ARRIVE guidelines. This study has been approved by the Ethics Committee of the Faculty of Veterinary Medicine, Shiraz University, Iran. Informed permission/consent was obtained from dog owners before blood sample collection.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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