Domestic dogs carriers of Leishmania infantum, Leishmania tropica and Crithidia fasciculata as potential reservoirs for human visceral leishmaniasis in northeastern Iran

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Abstract
Background: In recent years, cases of human visceral leishmaniasis (HVL) have been reported in some districts of Golestan Province, northeastern Iran, particularly in rural areas. Recent epidemiological evidence in Leishmania infantum endemic regions of Iran indicates approximately 50%–80% of seropositive dogs are asymptomatic for Leishmania infection.

Objective: The goal in this study was to determine Leishmania species infecting domestic dogs in Golestan Province, Iran.

Methods: Between 2015 and 2016, blood samples were obtained from 100 domestic dogs in rural regions of Golestan Province, northeastern Iran. All samples were tested for anti-Leishmania antibodies using a direct agglutination test (DAT), and for Leishmania spp. kinetoplast DNA (kDNA) using PCR.

Results: Seven (7%) dogs were antibody positive and 25 dogs (25%) were Leishmania spp. DNA positives by PCR positive for leishmaniasis. Four of the seven (71%) antibody-positive dogs and 19 of the 25 (76%) PCR-positive dogs were asymptomatic. The rate of infection detected by PCR was significantly higher in male dogs (21/75, 28%) than that in female dogs (4/25, 16%). The ITS1 PCR-RFLP assay identified the presence of L. infantum, L. tropica or Crithidia spp. in the 25 PCR-positive samples.

Conclusions: The high proportion of asymptomatic dogs in the study areas represent they act as potential reservoirs in the transmission cycle of Leishmania spp. and also

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Crithidia fasciculata as an emerging agent for the first time. Moreover, our data showed that PCR is a more reliable assay than DAT for detecting Leishmania spp. infection among asymptomatic dogs.

KEYWORDS
canine visceral leishmaniasis, Crithidia fasciculata, DAT, dog, Iran, PCR

1 | INTRODUCTION

Visceral leishmaniasis (VL), also known as kala-azar, is a neglected and poorly reported tropical disease that is caused by the Leishmania donovani complex. The annual global incidence of human visceral leishmaniasis (HVL) is approximately 50,000–90,000 cases (WHO, 2021). In the countries of the Mediterranean basin, South America, and the Middle East, including Iran, HVL is caused by Leishmania infantum/chagasi (Alvar et al., 2012; Fakhar et al., 2014b). In Iran, there are at least eight main endemic foci for HVL: Fars, Bushehr and Khuzestan provinces in the south-west, Ardabil and East Azerbaijan provinces in the north-west, Golestan and North Khorasan provinces in the northeast and Qom Province in the centre of Iran (Fakhar et al., 2014a; Fakhar et al., 2014b; Mohebali, 2013; Mohebali et al., 2011; Mohebali et al., 2005; Shokri et al., 2017). Cases of HVL are most frequent in rural areas and in nomadic tribes (Asgari et al., 2006; Fakhar et al., 2014a; Fakhar et al., 2014b).

The Canidae family are susceptible reservoirs for VL as the Leishmania parasite proliferates in their skin macrophages and is transmitted by sand flies. Domestic dogs (Canis familiaris) are the most significant reservoir hosts for VL in Iran (Shokri et al., 2017). The results of a recent systematic review in Iran showed that canine visceral leishmaniasis (CVL) is endemic in at least half of the provinces of the country, with an estimated, overall prevalence of 16% (Shokri et al., 2017).

In endemic areas, most seropositive dogs are asymptomatic. The symptomatic canine cases frequently have cachexia, weight loss, lymphadenopathy, hepatosplenomegaly, skin and mucosal inflammation, alopecia and epistaxis. (Fakhar et al., 2014b). Recent epidemiological evidence in L. infantum endemic regions of CVL in Iran indicates that approximately 50% to 80% of seropositive dogs are asymptomatic (Fakhar et al., 2014b; Fakhar et al., 2012b; Mohebali, 2013; Moshefe et al., 2008).

Current lab diagnostic methods for CVL include direct smear, culture, serology and polymerase chain reaction (PCR) assay (Fakhar et al., 2014b; Fakhar et al., 2012a). As dogs with CVL may be asymptomatic and seronegative (Molina et al., 1999), PCR can assist in early diagnosis of infection.

Recent reports of cases of HVL from parts of Golestan Province, northeastern Iran, especially among rural poor communities, suggest that this may be an emerging focus for HVL in Iran (Asfaram et al., 2016; Fakhar et al., 2014a; Fakhar et al., 2014b; Ghaee et al., 2020).

Dogs are so important in the life style of these people, and there is a close association between human and dog. In our previous study, during 2011/2012, the seroprevalence of HVL and CVL in the Maraveh Tappeh district of the Golestan Province was 1.3% and 32%, respectively (Fakhar et al., 2014a; Fakhar et al., 2014b). However, no recent data regarding the prevalence of Leishmania infection among domestic dogs in the province are available. The aim of this study was to determine Leishmania species infecting domestic dogs in Golestan Province, Iran using the direct agglutination test (DAT) and PCR-based assays.

2 | MATERIALS AND METHODS

2.1 | Study area

This study was undertaken during 2015 and 2016 in the eastern regions of Golestan Province, within nine villages of the Maraveh Tappeh district, where human VL cases had previously been diagnosed. Golestan Province is located in the northeastern Iran (54°26′E, 36°50′N). It has a humid climate with a mean annual rainfall of 556 mm (Saiedian, 2009).

2.2 | Blood sample collection

Blood samples were randomly obtained from 100 domestic dogs (25 females and 75 males) from rural parts of Golestan Province, an endemic area of HVL. In EDTA-containing tubes, two millilitres of blood were collected from saphenous or cephalic veins. The blood samples were centrifuged at 1000×g for 5 min. Then, the plasma and buffy coat were separated and kept at –20°C. No transmission of Trypanosoma cruzi was described in dogs from the studied areas.

2.3 | DAT (direct agglutination test)

DAT was performed on canine plasma samples as described by Mohebali et al. (2015). The plasma was diluted to 1:160 for initial screening. The positive samples were serially diluted up to 1:1240 to find the exact titre for IgG antibody titration. The cut off was considered 1:320 for CanL. The highest titres that agglutination was still visible were reported.
2.4 DNA extraction

Total DNA was extracted from the blood buffy coat using a phenol-chloroform: isooamy lalcohol (PCI) (Pasteur, Amol, Iran) method. A 200 μl sample of buffy coat mixed with 200 μl lysis buffer [1 mM EDTA, 50 mM Tris-HCl (pH 7.6), and 1% Tween 20], was incubated by overnight with 12 μl proteinase K solution (CinnaGen, Tehran, Iran) (20 mg/ml) at 45°C. Then, 200 μl PCI mixture was added. After a vigorous shaking, the tube was centrifuged (12,000 rpm for 10 min). Then the DNA in the supernatant was precipitated with 500 μl cold 100% ethanol and washed twice in cold absolute and once in cold 70% ethanol. Samples were dried on a hot plate, resuspended in 50 μl sterile distilled water and stored at 4°C.

2.5 Kinetoplast DNA (kDNA) PCR assay

The PCR protocol was used to amplify a fragment of 145 bp present on the highly multicopy kDNA minicircles employing RV1(5-CTTTTCTGGTCCCGGGTAGG-3) and RV2(5-CCACCTGCGCTATT TTACACCA-3) primers as previously described by Lachaud et al. (2001). PCR reactions were carried out in a 25 μl reaction mixture, including 1 μl of template DNA, 25 pmol of each primer and 12.5 μl of PCR premix (2× Master Mix RED; Amplicon, Denmark). The kDNA amplification was performed with 4 min of initial denaturation at 94°C, and 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C and 30 s elongation at 72°C followed by a final extension at 72°C for 5 min. All PCR in the present study was conducted using a Mastercycler ep Gradient S thermocycler (Eppendorf, Hamburg, Germany) and negative (no-DNA) and positive controls were included for each PCR set.

2.6 Internal transcribed spacer 1 genes PCR - restriction fragment length polymorphism (ITS1 PCR-RFLP) assay

The samples testing positive in the kDNA PCR assay were subsequently tested by the ITS1 PCR-RFLP assay in order to identify the species. The LITSR forward primer (CTG GAT CAT TTT CCG ATG) and the L5.8S reverse primer (TGA TAC CAC TTA TCG CAC TT) were used as described by Schönian et al. (2003) to amplify partial 18S, complete ITS1 and partial 5.8S regions of the Leishmania ribosomal DNA. The contents of the ITS1-PCR mixture were as mentioned for kDNA PCR. The temperature conditions in ITS1-PCR were as follows: an initial denaturation of 95°C for 2 min, then 35 cycles of 95°C, 20 s (denaturation), 53°C, 30 s (annealing), 72°C, 1 min (extension), followed by a final extension at 72°C for 6 min. The ITS1 PCR products were subjected to RFLP analysis employing BshFI restriction enzyme (Jena Biosciences, Germany), a HaelII prototype, based on the manufacturer’s protocol. The restriction fragments were run through a 2% agarose gel by electrophoresis and detected using a UV transilluminator after staining with SYBR green. The PCR products of three isolates were submitted for sequencing using the same PCR primers in both directions, employing an Applied Biosystem automated sequencer (3730 XL) via Bioneer Company (South Korea). The sequences were inserted into the GenBank database under the accession numbers KX808125 and KX808126.

2.7 Phylogenetic analysis

Species identification of the samples was performed by comparison with published ITS1 sequences of trypanosomatids in a public sequence database employing the BLAST system (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was carried out employing the Clustal W program. All of the consensus sequences were aligned to each other and nucleotide variability was obtained by using BioEdit software (version 7.2.5; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic tree was inferred using our representative ITS1 sequence data as well as relevant Leishmania tropica (L. tropica) and Leishmania major (L. major), as well as all available good quality Crithidia spp. in the GenBank database. The phylogenetic trees were performed using the maximum likelihood (ML) method implemented in MEGA11 software (Tamura et al., 2021). The robustness of the extracted tree was investigated by applying 1000 bootstrap replicates of the data.

2.8 Statistical analysis

The data were analysed using a chi-square statistical test in SPSS 19.0 to determine the significance of the variables’ prevalence. When p ≤ 0.05, differences were considered statistically significant.

3 RESULTS

3.1 DAT

Of the 100 dogs (25 female; 75 male) tested, 7 (7%) were antibody-positive on DAT screening. All positive samples were from male dogs. The highest prevalence (43%, 3/7) in dogs less than 2 years of age, was not statistically significant. Only 3/7 (29%) DAT-positive dogs had clinical signs, which included cachexia, alopecia and other skin changes, including onychogryphosis. There was no statistically significant difference between DAT-positive and clinical signs (p = 0.54) (Table 1).

3.2 kDNA PCR assay

Twenty-five (25%) of the 100 dogs tested were PCR positive with the RV1 and RV2 primers for leishmanial DNA (Figure 1). Only two (8%) of the 25 PCR-positive dogs were also DAT-positive. The proportion of the PCR-positive dogs in male (28%, 21/75) was significantly
TABLE 1  The relationship of clinical signs, direct agglutination test (DAT) and PCR results in studied domestic dogs from Golestan Province, northeastern Iran

| Clinical signs   | No. of examined dogs | DAT positive (titre ≥ 1:320) | PCR positive |
|------------------|----------------------|------------------------------|--------------|
| With clinical signs | 9                    | 3                            | 6            |
| Alopecia         | 3                    | 1                            | 2            |
| Cachexia         | 2                    | 1                            | 1            |
| Skin Lesion      | 2                    | 0                            | 2            |
| Onychogryphosis  | 2                    | 1                            | 1            |
| Without clinical signs | 91               | 4                            | 19           |
| Total            | 100                  | 7                            | 25           |

FIGURE 1  1.2% agarose gel electrophoresis of kDNA-PCR products from buffy coat DNA of dogs in Golestan province, Iran to detect leishmanial DNA. Lane M: 100 bp DNA marker, Lanes 1, 2, 3, 4: positive dogs’ samples, Lane 5: Negative control, Lane 6: standard Leishmania infantum (145 bp)

higher than female (16%, 4/25) (p < 0.05). The highest prevalence (44%, 11/25) in dogs less than 2 years of age was not statistically significant; infection in dogs 2 to 4 years of age and over 4 of age was 36% (9/25) and 20% (5/25) respectively.

Furthermore, only 24% (6/25) of the PCR-positive dogs had clinical signs. No statistically significant differences were obtained between PCR-positive and clinical signs (p = 0.84) (Table 1). Most infected dogs were asymptomatic according to the DAT (71%) and PCR (76%) tests.

3.3  | ITS1 PCR-RFLP assay

By the ITS1 PCR-RFLP assays of the samples from the 25 PCR-positive dogs, identified the species of Leishmania parasites as L. infantum (18/25), L. tropica (4/25) and Crithidia spp. (3/25) (Figure 2).

3.4  | Phylogenetic analysis

To analyse the phylogenetic position of L. tropica (AN: KX808125) and C. fasciculata (AN: KX808126) from our study in comparison to relative

FIGURE 2  2% agarose gel electrophoresis of ITS1-RFLP products (HaellI) from buffy coat of dogs in Golestan province, Iran to detect Leishmania species. Lane M: 100 bp DNA marker, Lane 1: standard L. tropica (300, 190 bp), Lane 2: standard L. infantum (200, 100 bp), Lane 3: negative control (H2o), Lane 4: L. tropica-positive dog (300, 190 bp), Lane 5: L. infantum-positive dog (200,100 bp), Lanes 6, 7: Crithidia-positive dogs (330,430 bp)
kinetoplastids, a phylogenetic tree was inferred based on maximum likelihood method (Figure 3).

The highest similarity of the nucleotide sequence of L. tropica from our study was with L. tropica from Afghanistan (AN: KJ420583) with only a single nucleotide difference. Analysis of the ITS1 sequences revealed that Crithidia spp. identified in our study belongs to C. fasciculata. This sequence has a maximum similarity with C. fasciculata (AN: HM004585) previously isolated from bone marrow of known human VL case in Yasuj, Western Iran (Mahmoudzadeh-Niknam et al., 2011) (Figures 4 and 3). The ITS1 DNA sequencing of L. infantum isolates was not completed due to technical problems.

4 | DISCUSSIONS

In the present study, we used the direct agglutination test (DAT) since as it is a simple, cost-effective, and reliable antibody-detection test suitable for screening dog populations for leishmaniasis (Fakhar et al., 2014a; Fakhar et al., 2008; Mohebali et al., 2015; Shokri et al., 2017) and found 7% antibody-positive rate. As PCR has a high sensitivity and specificity (90-100%) for epidemiological field studies (Fakhar et al., 2008; Fakhar et al., 2012b; Moreira et al., 2007; Shokri et al., 2017) and complements serological results (Maia et al., 2009), it was used in our present study to detect asymptomatic and symptomatic canine leishmaniasis and indicated 25% positive rate. There was a poor correlation between antibody and PCR positivity (2 antibody-positive among 25 PCR-positive samples). This finding is in concordance with other studies, including a canine study from Spain that reported only 22 seropositive among 67 PCR-positive samples (Solano-Gallego et al., 2001) and a canine study in Greece that reported 12.3% (9/73) IFAT-positive and 63% (46/73) PCR-positive (Leontides et al., 2002).

Dogs are considered to be the main reservoir host for CVL in the Mediterranean countries, including Iran (Mohebali et al., 2005; Shokri et al., 2017). The results of a previous study in Iran (Gavgani et al., 2002)
showed that seropositivity of VL in children increased significantly with density of dogs in villages. However, the rate of the anti-leishmanial antibody detected in dogs in different parts of Iran was reported to be between 3.5% and 51% (Shokri et al., 2017). In our previous study (Fakhar et al., 2014a), the rate in some villages in Maraveh Tappeh district, was 32% (16/50), which is significantly higher than our study (7%). The rate of antibody-positive samples in this study is also lower than in other countries such as Spain (Fisa et al., 1999), Greece (Sideris et al., 1999) and Italy (Rombolà et al., 2021), which reported a Leishmania infection rate in dogs of 10.2%, 22.4% and 74.3%, respectively.

In our study, the rate of infection in male dogs was higher than in female dogs using both antibody detection and PCR assays. Similar findings were reported by Mohebali et al. (2005) in Iran and Tamponi (2021) in Italy. Although, the highest rate of infection observed in dogs less than 2 years of age in this study, was not statistically significant, a high frequency of PCR positives in the young dogs has been attributed to their greater susceptibility to infection or vertical transmission (Fakhar et al., 2012b).

In our study, 71% (4/7) of DAT positive and 76% (9/25) of PCR-positive dogs did not have clinical signs. The PCR positive asymptomatic dogs are likely to have *L. infantum* and *L. tropica* amastigotes in the peripheral blood and therefore may play a role as reservoir hosts in the transmission of *L. infantum* and *L. tropica* to people and other dogs via sand flies (Fakhar et al., 2012b). These results are in concordance with CVL studies in other endemic countries in the Mediterranean region, where most infected dogs were asymptomatic. In a study in Spain, 57.7% (15/26) of seropositive dogs were asymptomatic, and can act as active reservoirs in the life cycle (Solano-Gallego et al., 2001). In another study in Italy, 44.1% of Leishmania infected dogs showed clinical signs (Tamponi et al., 2021). In a study of the dog population in Romania using ELISA and real-time PCR, none of seropositive and PCR-dogs had clinical signs of CVL (Dumitrache et al., 2016). Our previous study in Iran showed that 67% of DAT-positive and 88% of PCR-positive dogs were asymptomatic (Fakhar et al., 2012b). DAT can be used for seroprevalence studies of symptomatic CVL in endemic areas and can help to confirm the diagnosis of symptomatic infected dogs, but is not suitable to detect *L. infantum* infection in all asymptomatic dogs (Fakhar et al., 2012b).

The report of *L. tropica* in the blood samples of 16% of PCR-positive dogs in this study is remarkable. In the phylogenetic tree, employing ITS1-rDNA sequences, the *L. tropica* sequence from a dog in this study was very similar to *L. tropica* sequences originating from Western Afghanistan and Eastern Iran (Figure 3). *C. fasciculata* from the present study was placed in a cluster with other *C. fasciculata* reported from Iran and in a larger group with *Crithidia* spp. from other countries (Figure 3). *Crithidia* spp. are considered insect parasites, but in recent years, cases of infection with this agent have been reported in human patients with suspected cutaneous leishmaniasis (Barazesh et al., 2019; Doudi et al., 2010; Ghobakhloo et al., 2019) as well as phlebotomine sand flies (Kalantari et al., 2018b) and *Tatera indica* rodents (Kalantari et al., 2018a) in southern Iran. The isolation of *Crithidia* spp. for the first time from the blood samples of dogs (12% of PCR-positive dogs) in this study highlights the need to determine the pathogenesis and clinical importance of *Crithidia* spp. infection in dogs and other vertebrates. This also could be the base stone for further investigations to detect and identify *Crithidia* infection among dogs population in other endemic regions of ZVL in the world.

There were some limitations in the current study. Two main limitations included the small sample size due to the remote rural areas in northeastern Iran which impacts on results interpretation. Accordingly, the number of examined asymptomatic dogs in our study compared to the small number that are symptomatic for each of the testing methods could account for why there are no statistically significant differences observed on diagnostic tests comparisons. Also, the DNA sequencing of *L. infantum* isolates and their comparison with other similar species to determine phylogenetic position was failed due to technical problems. The main power of the present study is the application of two DNA targets (kDNA and ITS1) for characterising the
causative agents of CVL and also analysing the phylogenetic position of them.

5 | CONCLUSION

Our study, for the first time, showed that domestic dogs can be asymptomatic carriers of *L. infantum*, *L. tropica* and *C. fasciculata* and consequently have a role as reservoirs for human infection in Golestan Province, which is an emerging focus for HVL in Iran. Moreover, our data revealed that PCR is a more reliable assay than DAT for detecting *Leishmania* spp. infection among asymptomatic dogs. As a whole, the present work attempted to provide a molecular clue of *Crithidia* infection as an emerging agent among dogs population in Iran. Control strategies for HVL, including control of the dog reservoirs and sandfly vectors, as well as proper methods for diagnosis, are warranted.

AUTHOR CONTRIBUTIONS

MF, MK and HZH designed the study. MF and MS contributed to all parts of the study. MD, SG, MM and BA collaborated in study implementation. MS and MK contributed in the analysis and interpretation of data. MF, MK, HZH and MS collaborated in the manuscript writing and revision. All the authors commented on the drafts and accepted the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

DATA AVAILABILITY STATEMENT

All data of this article are fully available without restriction on request from the corresponding author.

ETHICAL STATEMENT

This study was reviewed and approved by the ethical committee of Mazandaran University of Medical Sciences (IR. MAZUMS.REC.1390.149).

PEER REVIEW

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