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# Molecular and serological evaluation of visceral leishmaniasis in domestic dogs and cats in Maragheh County, north-west of Iran, 2018–2021

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## Abstract

**Objective:** Zoonotic visceral leishmaniasis (VL) is caused by *Leishmania infantum*, of which dogs are the main reservoir. VL is endemic in the Middle East, also in some parts of Iran. Following reports of new cases of VL in children in Maragheh County, the non-endemic area of the disease, we encouraged to conduct a preliminary study on domestic dogs and cats to identify their potential role as reservoirs for the disease.

**Materials and Methods:** This study was conducted during a period of 3 years from 2018 to 2021. Two hundred ownership dogs and 25 cats from Maragheh County, north-west of Iran, were randomly screened. Blood samples were collected. A direct agglutination test (DAT) was used for the detection of anti-*L. infantum* antibodies. Furthermore, buffy coat samples from the *L. infantum* seropositive animals were examined to detect parasite presence using polymerase chain reaction.

**Results:** Out of the total of 200 ownership dogs evaluated, 170 (85%) were male and 30 (15%) were female with a mean age of 4.3 years. Anti-*L. infantum* antibodies (IgG cut-off $\geq$ 1:320) were observed in 3.5% of dogs (7/200) by the DAT test. All seropositive dogs were identified in the first year of examination. Regarding molecular approaches in seropositive dogs, two samples were positive for a 565 bp kDNA minicircle gene specific for *L. infantum*. During the study period, no seropositive case was detected in the cats examined.

**Conclusions:** This study shows that the domestic cycle of *L. infantum* has been established in the studied region. It is necessary to increase the awareness and monitoring of the disease with the study of wild reservoirs periodically.

KEYWORDS
cat, dog, Iran, molecular identification, seroprevalence, visceral leishmaniasis

## 1 | INTRODUCTION

In Iran and throughout the Middle East, *Leishmania infantum* is the cause of visceral leishmaniasis (VL), a systemic infection which is fatal if left untreated. The vectors are female sand flies (Diptera: Psychodidae) of the genus *Phlebotomus* and infected dogs (*Canis familiaris*) are the main reservoirs. More infected dogs do not present clinical signs, but they are seropositive and maybe the reservoirs in endemic areas of canine visceral leishmaniasis (CVL) in the world (Rostamian et al., 2021; Mohammadiha et al., 2013; Moshfe et al., 2009). Some studies have shown that asymptomatic individuals are involved in the transmission chain, even in areas where they are a breeding ground for dogs. Therefore, determining the prevalence and distribution of asymptomatic cases helps to better identify the status of commodity harassment and control methods (Moshfe et al., 2012, Rostami et al., 2020).

Serological tests for identifying anti-*Leishmania* antibodies, like the direct agglutination test (DAT) and ELISA, are useful because of their reliability and validity. In addition to these methods, identifying *Leishmania* species in VL is essential for controlling the disease (Ribeiro et al., 2019, Brianti et al., 2016, Paltrinieri et al., 2016). Polymerase chain reaction (PCR)-based methods are applied to isolate and differentiate *Leishmania* species in infected dogs, especially in areas where both cutaneous leishmaniasis and VL existed (Rostami et al., 2020, Cavalera et al., 2021).

VL is endemic in Iran especially in two known old foci including Ardabil and Fars provinces. The pooled prevalence of human visceral leishmaniasis (HVL) infection based on seroepidemiology has been estimated to be 2% (95% confidence interval: 1%–2%) in the general population of Iran. Although the incidence of HVL has decreased in Iran over the last decades, new human cases of the disease have been reported in new areas where it was non-endemic previously, such as Razavi Khorasan, Alborz, Kohgiluyeh, and Boyer-Ahmad. CVL infection is prevalent in rural areas of the Kaleybar and Khoda-Afar districts located in East Azerbaijan Province, north-west of Iran (Behniafar et al., 2019). Also, *L. infantum* was isolated from 10% of the cats studied in this area (Hatam et al., 2010). These studies show that the disease is endemic in East Azerbaijan province.

In our study area, positive clinical cases of CVL under 5 years of age have been reported and introduced in specialized hospitals in the last 3 years. In the first year, due to the existence of seven positive clinical samples, clinical and epidemiological studies were performed on children in the study area to diagnose the disease and positive cases were introduced for treatment. The studied areas are geographically mountains and are prone to the growth of carriers (Zakharov & Goryacheva, 2020, Fatollahzadeh et al., 2016). Infected dogs may develop clinical symptoms of the disease, or remain asymptomatic throughout the infection which can play a role in transmitting this disease. This study aimed to investigate the possible reservoir role of VL in the Maragheh due to new cases of VL in humans.

## 2 | MATERIALS AND METHODS

### 2.1 | Study area

The present study was carried out in Maragheh County at a 37°23′N longitude and 46°24′E latitude, in East Azerbaijan province, northwestern Iran (Figure 1). The population of the area is approximately 262,604 inhabitants, most of them living in urban areas. The study area has cold temperate and relative humidity. The average annual temperature in the city is 12.5°C. Maragheh has many green areas and gardens, and the Sufi Chai River runs through the Maragheh city centre. In this area, there are a lot of dogs which either live with herds of sheep or look after the house or farm.

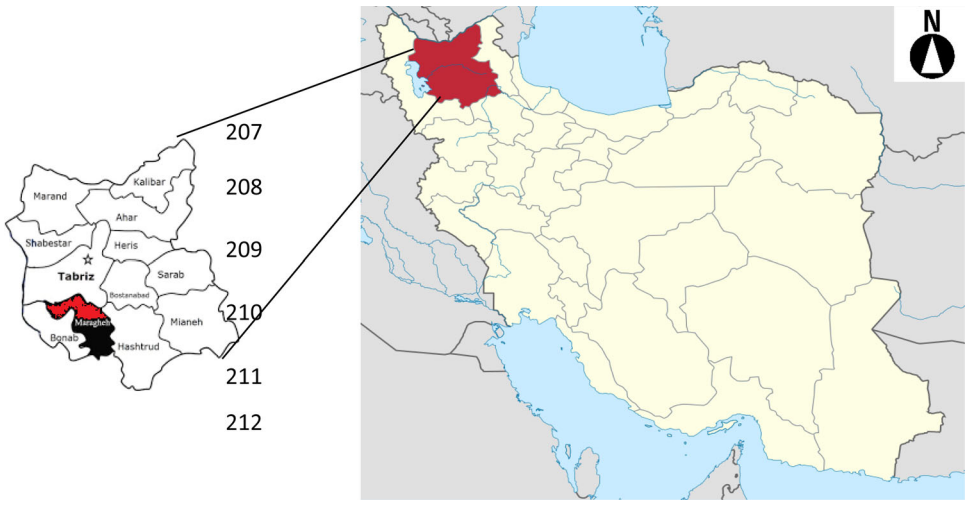


**FIGURE 1** Sampled regions from owner dogs and cats in this study. Maragheh County at a 37°23′N longitude and 46°24′E latitude, in East Azerbaijan province. Maragheh lies to the south of Sahand Mountain in northwestern Iran

## 2.2 | Sampling

Our study was based on a randomized sample of ownership 200 dogs and 25 cats originating from four rural districts, in Maragheh County during a 3-year period from spring 2018 to spring 2021. Animals are kept outdoors and roam freely in the village. In the first year, we selected 150 ownership dogs and 10 cats, and in the second and third years, 25 dogs were selected. Also, 10 cats in the second year and five cats in the third year were examined. Demographic characteristics including age and sex were recorded. At first, the animals were surveyed for the presence of any VL clinical signs including lymphadenopathy, skin lesions, weight loss, hair loss, hepatosplenomegaly, and so forth.

The blood samples were collected from the jugular vein of domestic dogs and cats. The samples were transported to the laboratory under cool conditions and were allowed to clot at room temperature. The blood clots were centrifuged for 5–10 min at 800 × *g*; the sera were put into a microtube and stored at −20°C until the serological examination. For molecular tests, the buffy coat was separated from peripheral blood by centrifugation at 2000 × *g* for 20 min and stored at deep-freeze temperature until use. Clinical examination for VL and blood sampling were performed after obtaining informed consent from each dog or cat owner.

## 2.3 | Serological diagnosis

The DAT antigen used in this study was prepared in the leishmaniasis Laboratory, Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Iran. The principal phases of the procedure for making DAT antigen were mass production of promastigotes of *L. infantum* (Iranian strain) in RPMI1640 medium plus 10% foetal bovine serum, trypsinisation of the parasites, staining with Coomassie brilliant blue, and fixing with formaldehyde 2% (el Harith et al., 1989, Mohebali et al., 2006). Anti-*Leishmania* antibodies detection was performed by DAT. DAT was performed according to the modified method of Mohebali et al. (2006). *Leishmania* antibody titres at ≥1:320 titre were taken as the cut-off. Both negative control (from diluent and antigen) and positive control (from confirmed CVL) were applied (Mohebali et al., 2006).

## 2.4 | DNA extraction

Total DNA was extracted from buffy coats with a High Pure PCR Template Preparation Kit (Dynabio, Takapouzist, Iran), according to the manufacturer's instructions, and stored at −20°C until use. Briefly, the samples were transferred to microtubes. Then, 20 μl proteinase K and 200 μl lysis buffer were added to the samples, mixed, and incubated at 60°C for 15 min to be completely lysed. Afterwards, 200 μl of absolute ethanol was added to the samples and mixed by pulse-vortexing for 30 s. The mixtures containing some precipitates were carefully transferred to column microtubes, centrifuged at 8000 rpm for 1 min, and washed several times using buffers to remove impurities from the column microtubes. Finally, 100–200 μl of elution buffer or dd$H_2O$ was added to the membrane centre of the column tubes and kept for 3 min. The tubes were centrifuged at 14,000 rpm for 2 min to elute the DNA and were stored at −20°C for PCR amplification.

## 2.5 | DNA amplification by PCR and phylogenetic analyses predicated

The Kinetoplastic minicircle DNA (kDNA minicircle) of *L. infantum* was amplified with specific primers—forward 5′-TCGCAGAACGCCCCTACC-3′ and reveres 5′-GGGGTTGGTGTAAAATAGGC-3′—as described by Mahboudi et al. (2002). The 25 μl amplification DNA fragments were analysed by 1.5% agarose gel electrophoresis. Samples were considered positive when a PCR product of 565 bp was detected. Both strands of the amplified fragments were sequenced by Bioneer Company using the same primers. PCR products were sequenced for both directions with the same primers used in a PCR (Bioneer Company, Korea).

## 2.6 | Phylogenetic analysis

Forward and reverse sequences were assembled using ChromasPro version 1.7.5 (Technelysium, Tewantin, Australia). Sequences were aligned using ClustalW as implemented in BioEdit v.7.2 software and then checked by eye. Ambiguous (heterozygous) sites were coded using the standard IUPAC codes for combinations of two or more bases. Sequences obtained in this study were manually trimmed and edited with previously published kDNA minicircle sequences from *L. infantum* isolates in BioEdit v.7.2 software. The maximum-likelihood (ML) phylogram was constructed in MEGA (version X; Biodesign Institute, Tempe, USA) using HKY+G (Hasegawa-Kishino-Yano) for kDNA minicircle, which was chosen as the most appropriate substitution model. Node support was assessed with 1000 bootstrap replicates.

## 2.7 | Statistical analysis

Statistical significance was analysed using the chi-square test or Fisher's exact test ($p < 0.05$).

# 3 | RESULTS

Out of the total of 200 ownership dogs evaluated, 170 (85%) were male and 30 (15%) were female with a mean age of 4.3 years. Anti-*L. infantum* antibodies (IgG cut-off ≥ 1:320) were observed in 3.5% of dogs (7/200) by DAT. All infected dogs were identified in the first year of examination. For the next two consecutive years, seroepidemiological examination to evaluate the parasite transmission cycle in the dogs of this area was negative (Table 1). The highest seroprevalence (six cases) was found in dogs aged 3–4 years and one of them was 5 years old. All of the seropositive dogs were asymptomatic and not showed clinical signs

**TABLE 1** Number of seroprevalence (titre ≥ 1:320) of *Leishmania* infection in dogs and cats

| Year | Number of dogs (positive) | Number of cats (positive) |
|---|---|---|
| 2018–2019 | 150 (7) | 10 (0) |
| 2019–2020 | 25 (0) | 10 (0) |
| 2020–2021 | 25 (0) | 5 (0) |

of disease including lethargy, cachexia, skin lesions, alopecia, epistaxis, and myopathy. With regard to exposure to *Leishmania* infection, no statistically significant difference was found between males and females ($p = 0.911$). In this study, 10 female cats and 15 male cats were also examined. However, during the 3-year study period, no seropositive case was detected in the cats examined.

Regarding molecular approaches in seropositive dogs, two samples were positive for a 565-bp kDNA minicircle gene specific for *L. infantum*. The species of the parasite were confirmed by sequencing. The obtained sequences from isolated *Leishmania* were aligned and compared with the sequences in GenBank and had 100% nucleotide similarity with *L. infantum* isolates (Figure 2). The nucleotide sequence data were submitted to the GenBank database under the accession number: OL589130. All blood samples taken from cats were examined using molecular methods and no positive samples were obtained.

## 4 | DISCUSSION

Following reports of new cases of VL in children in Maragheh County, we encouraged to conduct a preliminary study on the circulation of *L. infantum* in domestic dogs and cats using a valuable tool for the serodiagnosis with high sensitivity and specificity methods which can be useful in control measures and managing the zoonotic VL (Mohebali et al., 2020). At the same time, in the following years, clinical and epidemiological studies were performed on children in the study area, and in the following years, there were no positive human clinical cases (the results are not included in this study). As far as we are aware, the present study was the first CVL serosurvey conducted in Maragheh County: a non-endemic area for VL but bordering with endemic foci of disease in northwestern Iran.

PCR revealed the amplification of the 565 bp specific *L. infantum* minicircle kDNA fragment in two of seven seropositive samples in dogs. Although PCR on aspirates of popliteal lymph node and bone marrow is more sensitive than blood samples (buffy coat) for detection of *L. infantum* amastigotes, it is an invasive method and sometimes difficult in healthy asymptomatic dogs. Also, there may be PCR inhibitors in blood samples. These restrictions may affect the sensitivity of the PCR method in our study (Moshfe et al., 2009, Barati et al., 2015). Overall, the detection of *L. infantum* DNA in dogs with high antibody titres (1:320) could suggest the occurrence of subclinical infection or a recent infection.

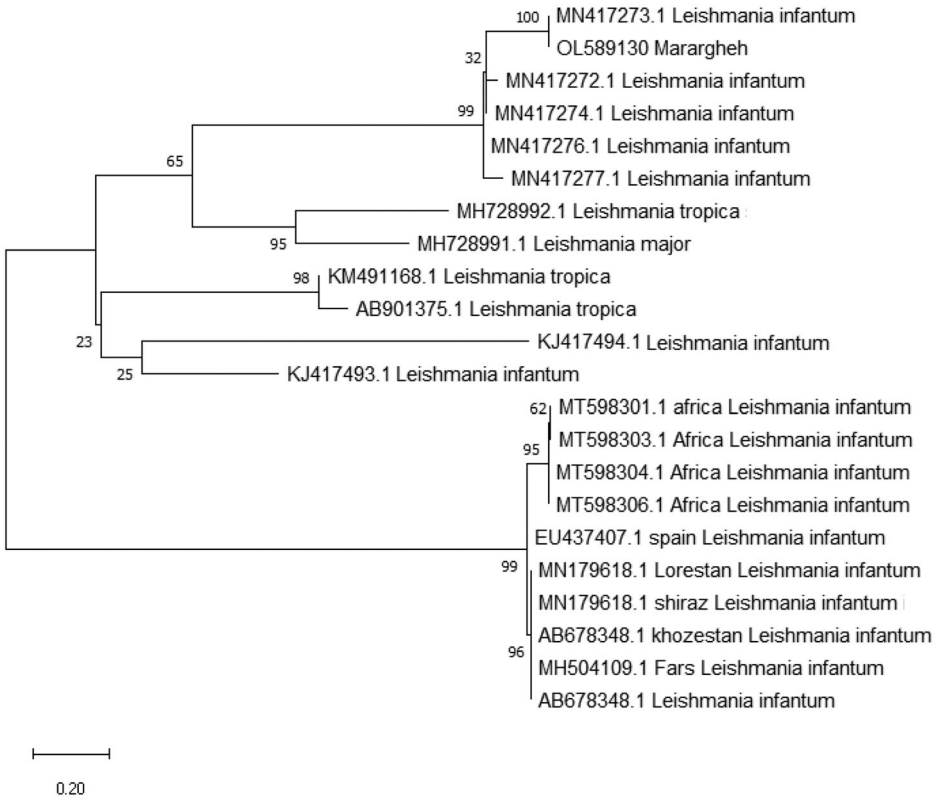


**FIGURE 2** Phylogenetic relationship based on Kinetoplastic minicircle DNA (kDNA minicircle) of *Leishmania* species from the north-west of Iran. Phylogenetic tree of *Leishmania* species according to the maximum likelihood (ML) method conducted based on the multiple sequence alignment by MEGA X without outgroup. The high similarity of the sequence of studied samples with other sequences from Iran can be observed.

Among the 200 dogs evaluated, 3.5% were positive using the DAT test. In a rural area of Kazeroun County, a focus of VL in Fars province, the reported seroprevalence for CVL is 46.7% (Najafi et al., 2021). In Hamedan province, a survey of 170 stray and 210 owned shepherd dogs showed a seroprevalence of 6.47% and 1.9%, respectively (Gharekhani et al., 2016). In Meshkin-Shahr district, a focus of VL in Ardabil province, the reported seroprevalence for CVL is 23.4% (Barati et al., 2015). However, the high seroprevalence rate is to be related to weather conditions and humidity in endemic areas of Iran.

In the present study, no *Leishmania* infection was found in the serological and molecular survey on 25 cats during a period of 3 years. The lack of prevalence of *Leishmania* infection in cats in our study is in line with other investigations. By contrast, Mohebali et al. (2017) showed that 24.27% of asymptomatic free roaming cats in the Meshkin-Shahr district are located in Ardabil province, which is a well-known endemic region for VL revealed positive by DAT test. Furthermore, a systematic review and meta-analysis study suggests that cats could play a role in the maintenance of the *Leishmania* spp. life cycle at least in endemic foci (Asfaram et al., 2019). Further studies are needed to assess the role of cats in maintaining the life cycle of VL. On the other hand, it is worth noting that Oshaghi et al. (2009) previously reported for the first time the occurrence of *Leishmania donovani* in *Phlebotomus perfiliewi transcaucasicus*, in the Germi region, northwestern Iran (Oshaghi et al., 2009). Due to the importance of VL in this region for the first time, one of the study's limitations was the importance of the subject and the disease, and the involvement of dog owners. A sampling of dogs and the availability of animals were other limitations. Due to the time-consuming nature of this model of the study, environmental factors can affect the results. The potential risks during the plan's implementation, such as rabies, are not unexpected.

## 5 | CONCLUSION

This finding is important because the VL transmission cycle by *L. donovani* is mainly anthroponotic, whereas *L. infantum* is anthropozoonotic with a dog reservoir. The detection of *L. infantum* in ownership dogs in Maragheh County suggests the possible existence of *L. infantum* transmission cycle in this area. Other investigations like as entomological check are required to fully understand the epidemiology.

### ACKNOWLEDGEMENTS

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### CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

### ETHICAL APPROVAL

The Ethical Committee of the Maragheh University of Medical Sciences approved this project (Ethics no. IR.MARAGHEHPHD.REC.1397.006).

### AUTHOR CONTRIBUTIONS

Ali Soleimani performed formal analysis. Mehdi Mohebali designed methodology. Saber Gholizadeh wrote the original draft. Arezoo Bozorgomid reviewed and edited the manuscript. Reza Shafiei designed methodology. Saber Raeghi performed supervision, designed methodology and wrote the original draft.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.846

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