Annals of Parasitology 2020, 66(2), 143–156
doi: 10.17420/ap6602.249

Original papers

Alarming: high prevalence of *Leishmania infantum* infection in cats from southern Iran based on molecular and serological methods

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ABSTRACT. Recently, *Leishmania infantum* has increasingly been detected in stray cats in endemic regions of the world. Cats have been considered playing a role in the epidemiology of visceral leishmaniosis, an endemic zoonosis in Iran. The studies concerning feline leishmaniosis (FeL) allow the hypothesis that cats can be considered as potential reservoirs. The investigations on *Leishmania* infection in cats are very few in Iran and therefore we aimed to assess the *L. infantum* infection in stray cats and its possible role in transmission of the disease to human by direct agglutination test (DAT), ELISA, nested-PCR and confirmation via sequencing and phylogenetic analysis in Fars province, Iran. Whole blood samples were obtained from 174 stray cats. Anti-*Leishmania* antibodies were detected in the sera using DAT and ELISA. DNA was extracted from the buffy coat of each subject and PCR amplified, targeting *Leishmania* kDNA gene. PCR results were confirmed by sequence analysis. Prevalence of clinical signs in positive cats was 19.0%. Anti-*Leishmania* antibodies with different titers were detected in 48 (27.59%) and leishmanial DNA in 36 (20.69%) of the cats. The sequencing of PCR-positive cats revealed the parasite as *L. infantum*. A high seroprevalence of *L. infantum* was revealed, with higher levels in males, adult cats, and those living in rural districts and southern zones. Despite the reservoir task of cats in nature is still ambiguous, the high serological and molecular detection of *L. infantum* in stray cats indicates that cats are regularly bitten by infected sand flies in Fars province, southern Iran, and may have a potential reservoir role in the maintenance of *L. infantum* in the endemic areas of zoonotic visceral leishmaniosis in Iran. Anyway, *Leishmania* infection must be appraised in the differential diagnosis of cutaneous or systemic clinical signs in cats.

Keywords: *Leishmania infantum*, stray cats, ELISA, DAT, nested-PCR, Iran

Introduction

Leishmaniosis is a vector-borne zoonotic infectious disease caused by the obligate intracellular protozoa of the genus *Leishmania* (order Kinetoplastida) [1]. In humans, visceral leishmaniosis (VL) is the most severe clinical manifestation of *Leishmania infantum* infection, being observed mainly in children and immunocompromised adults [2]. It is presumed that VL is transmitted by the bite of infected phlebotomine sand flies [3,4]. The disease is endemic in 98 countries across the four continents and > 90% of the 500,000 cases annually worldwide occur in Iran, India, Bangladesh, Nepal, Sudan and Brazil [5]. It is estimated that VL leads to approximately 59,000 mortalities per year [1]. In Iran, the disease is an endemic zoonosis caused by

L. infantum [6].

Dogs are considered the main reservoir of *L. infantum*, playing an active role in the transmission of the disease [7,8]. Moreover, canine leishmaniosis (CanL) is a systemic, chronic, and even fatal clinical condition that represents an important veterinary medical and public health problem in southern Iran [9]. A large majority of the infected dogs does not develop clinical signs but they may still be capable of transmitting the parasite to the vectors. The prevalence of infection in rural dogs reaches a remarkable 36%, much higher than urban dogs that reaches 19% [9].

Feline leishmaniosis (FeL) caused by *L. infantum* is an emergent feline disease more frequently reported in endemic areas [10]. In Iran, large numbers of cats are found roaming residential streets, so they can be an important potential source of transmission of infection to humans and other animals [11]. The unusual clinical cases of FeL are characterized predominantly by cutaneous lesions, although visceral involvement has also been described [12]. Cats have been suggested as a secondary reservoir in areas where *L. infantum* is endemic, and as they live close together with humans and dogs, it is necessary to evaluate their infection status and role in the epidemiology of zoonotic leishmaniosis [13].

The low prevalence of FeL could be explained by under-reporting or by a natural resistance of the cat to this infection. Some authors consider that the cat is an incidental host for this infection, while others suggest that felids can act as a potential peri-domestic reservoir hosts. However, the epidemiologic role of the cat remains to be clarified [14].

Clinical cases of FeL have been reported mostly in areas where the organism is endemic. Nonetheless, the real susceptibility of cats to infection by *Leishmania* spp. and the outcome of leishmaniosis in them are poorly understood [15,16]. Clinical signs caused by this infection in cats are similar to those observed in dogs. Leishmaniosis in cats can cause both cutaneous and visceral forms of the disease, although the cutaneous pattern seems to be the most common [15,16]. Ocular alterations are also frequent, especially uveitis [17]. In some reports, FeL has been related to feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) infections [18].

Molecular investigations of *Leishmania* DNA and anti-*Leishmania* antibody detection have proved to be suitable for the evaluation of large

Figure 1A. Map of Iran showing the location of Fars province

Figure 1B. Counties and central cities in Fars province, southern Iran, from where the cat's serum samples were collected

numbers of serum samples in dogs and cats [19,20]. In Iran, the northern Ardabil and the southern Fars province have the highest endemicity levels for CanL [9]. Considering the fact that there is a large number of stray cats in or around the cities and villages, it seems necessary to determine the epidemiology of FeL and its role in transmission of VL disease to human population in each endemic areas of the country.

The aim of the present study was to assess the

prevalence of feline *Leishmania* infection in a large and representative sample of stray cats from Fars province, Iran, where human VL and CanL is endemic, since very limited data is available about epidemiology of FeL caused by *L. infantum* among the feline population.

Materials and Methods

Study area. Fars province is one of the thirty-one provinces of Iran and known as the cultural capital of the country. It is in the south of the country, and its administrative center is Shiraz. It has an area of 122,608 km². In 2011, this province had a population of 4.6 million people, of which 67.6% were registered as urban dwellers (urban/suburbs), 32.1% villagers (small town/rural), and 0.3% nomad tribes. Based on the 2010 census Shiraz, the administrative center of Fars province, had a population of 1,749,926 which constitutes 39% of the population in the province. It is the most populous city in the province and the sixth most populous one in the country. According to the latest divisions, the province contains 29 counties. This province also contains 102 cities and 2000 villages (Fig. 1A, B).

Ethics statement. All animal experiments and care procedures were arranged under the instructions and endorsement of the Institutional Animal Care Committee (IACC) of Shiraz University of Medical Sciences (approval no. 90-01-01-2817) for animal ethical and welfare standards.

Sample collection. A total of 174 urban and rural stray cats from different regions of Fars province, Iran, were investigated on the basis of serum and plasma samples obtained year-round between May 2016 and July 2018. Cats were enticed alive using Sherman baited cage-traps with tinned fish. Supplemental general data on these cats including age, gender, and health status were estimated based on body condition and examination of dentition (data not shown). A dermatologic examination for changes compatible with FeL (e.g., alopecia, nodulo-ulcerative plaques, and crusty or scaly dermatitis) was also conducted. Cats were categorized as young (<1 year), adult (between 1–7 year) or old (8–17 year).

Zoletil® (Virbac, Carros, France, 10 mg/kg, comprise of Tiletamine and Zolazepam) were injected for anesthetic and sedative effects. Nearly, 3 ml whole blood were collected from the saphenous veins of the cats into both EDTA-anticoagulant and sterile plain collection tubes. The blood left to clot at room temperature for 3 hours, and then centrifuged at 1500 × g for 10 min. The separated sera were then aliquoted and stored at −20°C until processed. In addition, buffy coat samples of cats were applied for the analysis of *L. infantum* DNA by nested-PCR. All clinical procedures were performed by appropriately qualified scientific colleagues.

Direct agglutination test (DAT). Direct agglutination antigen was prepared in the Culture Laboratory of the School of Medicine at Shiraz University of Medical Sciences. The main phases of the procedure for making DAT antigen were mass cultivation of *L. infantum* isolated from a domestic dog affected CanL from Meshkin-Shahr district (MCAN/IR/14/M14) in RPMI-1640 medium (Gibco, Frankfurt, Germany) supplemented with 15% heat-inactivated FBS (Gibco, Frankfurt, Germany), 2 mM L-glutamine, 100 U/ml Penicillin, and 100 µg/ml Streptomycin (Gibco, Frankfurt, Germany), trypsin treatment of the parasites (at a concentration of 1×10^8 promastigotes/ml), fixing with 2% formaldehyde in Locke solution for 20 h, staining with 0.1% Coomassie® Brilliant blue (Merck, Darmstadt, Germany), and finally washing in cold citrate-saline solution.

DAT was carried out as previously described with some modifications [21,22]. Briefly, the serum samples were diluted in 0.9% normal saline containing 1.56% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). Twofold dilution series were made ranging from 1:100 to an end-point titer of 1:102,400 in Corning® polystyrene V-bottom 96-well microplates (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 1 h at 37°C. Fifty-µl of reconstituted DAT antigen was then added to each well containing 50 µl of diluted serum. Results obtained with DAT are expressed as an antibody titer, which is the reciprocal of the highest dilution at which agglutination is still clearly visible after 18 h incubation at room temperature. Compact blue dots were scored as negative and large diffuse blue mats as positive.

To maximize sensitivity of the test, cats were considered seropositive at a dilution of 1:100. Positive controls for the DAT consisted of serum samples from four cats with confirmed FeL.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed by using *L. infantum* (MCAN/IR/14/M14) crude soluble

antigen (CSA). The cultured promastigotes were collected by centrifugation, washed three times with cold PBS, and pellets were re-suspended in 1×PBS and an equal volume of complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Lysate was prepared by six alternate cycles of freezing (at −196°C) in liquid nitrogen and thawing (at 25°C), followed by three rounds of sonication for 30 s at 10 Hz. Supernatants were collected as CSA by further centrifugation at 800 × g for 45 min. The protein content was estimated by Bradford method and the extracted antigen was stored at −20°C until used.

ELISA was executed as previously described for cat sera with some modifications [23−25]. Briefly, the Corning® 96-well polystyrene flat-bottom microplates (Sigma-Aldrich, St. Louis, MO, USA) were coated with 100 µl/well of 20 µg/ml of purified CSA in 0.1 M sodium carbonate-sodium bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Unbound antigens were removed by washing the plates 5 times in PBS-0.05% Tween 20 (PBST, pH 7.4). Blocking was carried out with 200 µl of 5% non-fat dried skimmed milk in PBST (PBST-M) for 2 hours at 25°C. Afterwards, the wells were washed 5 times with washing buffer. One hundred µl per well of diluted cat sera (1:100 in PBST) were applied to the plates and incubated for 1 h at 37°C in moist chamber. After three washes with PBST and one wash with PBS, 100 µl per well of goat anti-cat IgG (Bio-Rad, Hercules, CA, USA) diluted 1:4000 in PBST conjugated to horseradish peroxidase (HRP) was added and incubated for 1.5 h at 37°C in moist chamber. Then, the plates were rewashed as above and incubated with 100 µl per well of substrate solution (0.4 mg/ml OPD; Sigma, St. Louis, MO, USA), plus H_2O_2 (0.4 µl/ml) in 0.1 M citrate buffer (pH 5.0) for 30 min at 25°C in the dark. The reaction was stopped with 100 µl of 2 M H_2SO_4. The optical density (OD) was read at 492 nm in an automatic ELISA microplate reader (Bio-Tek® ELx800, Sursee, Switzerland). The reaction was quantified as ELISA units (EU) related to a positive cat sera used as a calibrator and arbitrarily set at 100 EU. The calibrator cat had confirmed FeL by serology and PCR and serum from this cat has been used in previous studies [26,27]. All determinations included the calibrator serum as a positive control and serum of a cat from an area where leishmaniosis is not endemic as a negative control. The cutoff was established at 68 EU (Mean + 4 SD of sera of 75 cats from an area where leishmaniosis is not endemic).

DNA extraction. Total genomic DNA was extracted from the buffy coat of the cat's blood samples, using the QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to manufacturer's instructions. Following the centrifugation and washing steps, total DNA was eluted from the spin columns with 50-µl elution buffer and stored at −20°C until be used. The quantification and quality control of the DNA extraction procedures were determined by measuring optical absorbance at 260 nm using a Nano® spectrophotometer (NanoDrop® 2000, Thermo Fisher Scientific, Wilmington, DE, USA). All samples for PCR assays were prepared with aerosol-guard pipette tips to avoid contamination. All reactions were performed in appropriated places, following the good practice of laboratories to avoid sample contamination.

Nested-PCR assay. The conserved sequence blocks of the minicircle kDNA from the *Leishmania* species were amplified by nested-PCR [28].

The external primers CSB2XF (5'-CGA GTA GCA GAA ACT CCC GTT CA-3') and CSB1XR (5'-ATT TTT CGC GAT TTT CGC AGA ACG-3') were used in the first round, and the internal primers 13Z (5'-ACT GGG GGT TGG TGT AAA ATA G-3') and LiR (5'-TCG CAG AAC GCC CCT-3') were used in the second round of nested-PCR.

Each 25-µl, first-round reaction mixture contained 0.2 mM of each dNTPs (Roche, Alameda, CA, USA), 1.0 U Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 2-µl template DNA, 1.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.6), and 10 pmol of each forward and reverse primers.

The Eppendorf Mastercycler Gradient (Hamburg, Germany) was set to 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. The products of the first round of PCR were diluted 1:9 with HPLC pure water and then 1 µl of these dilutions were used as templates for the second round of PCR under the same conditions as those for the first round, except with primers LiR and 13Z. PCR products were visualized by UV light (Uvitec, Cambridge, UK) after electrophoresis on 1.5% agarose gels (Cleaver Scientific, Rugby, UK) using TAE buffer and staining with GelRed® (Biotium, Hayward, CA, USA). The size of each detected amplicon was estimated by comparison with a 100- to 1500-bp molecular marker (Fermentas, Vilnius, Lithuania) run on the same gel.

Table 1. Seroprevalence of *Leishmania infantum* infection in stray cats of different regions of Fars province, determined by DAT and ELISA

Districts	Central		Northern		Southern		Western		Eastern		Total	
Results	F	P	F	P	F	P	F	P	F	P	F	P
Positive	6	20	12	28.6	12	100	6	25	12	9.1	48	27.59
Negative	24	80	30	71.4	0	0	18	75	54	90.9	126	72.41
Total	30	100	42	100	12	100	24	100	66	100	174	100

Explanations: F=frequency; P=prevalence (%)

All primers were synthesized by Roche Molecular Diagnostics (Roche, Penzberg, Germany).

Reference strains of *L. infantum* (MCAN/IR/14/M14), *L. tropica* (MHOM/IR/89/ARD-L2), and *L. major* (MHOM/IR/54/LV39) were used as positive controls and run on each gel. Also, double D.W. was included in each run as negative control.

Sequencing and phylogenetic analysis. The 680-bp amplicons of kDNA from positive samples were purified using the Roche High PCR Purification Kit (Roche, Mannheim, Germany), cloned in the pGEM-T vector (Promega, Madison, WI, USA) and sequenced with the LiR and 13Z primers. Sequencing was carried out on an ABI PRISM® 3730*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) through the sequencing service of Roche Molecular Diagnostics (Roche, Mannheim, Germany). The resulting sequences were aligned and compared with those of existing homologous sequences related to *Leishmania* in the GenBank database using the BLAST program. The sequences were assembled and edited with the BioEdit (7.2.6) [29]. Multiple alignments were performed with data related to *Leishmania* species from Iran and other countries deposited in GenBank. Phylogenetic tree was constructed by the Maximum-Likelihood (ML) method and genetic distances were calculated with Kimura 2-parameter model using MEGA-X [30]. The reliability of the ML tree was assessed by the bootstrap method with 1,000 replications. *Endotrypanum monterogeii* (MCHO/CR/62/A9) was treated as out-group.

Nucleotide sequence accession numbers. The partial sequences of the kDNA gene of *L. infantum* obtained in this study were deposited in the GenBank database under accession number MH504109.

Statistical analysis. The agreement beyond chance between PCR and serology results was measured using the Cohen's kappa (κ) index (GraphPad Prism®, Melbourne, Australia) and results were interpreted as follows: no agreement ($\kappa < 0$), slight agreement ($0 < \kappa < 0.2$), fair agreement ($0.2 < \kappa < 0.4$), moderate agreement ($0.4 < \kappa < 0.6$), substantial agreement ($0.6 < \kappa < 0.8$), and almost perfect agreement ($\kappa > 0.8$) [31].

Chi-square (χ^2) analysis and Fisher's Exact Test were used to test for associations between all of the parameters (gender, age, origin, habitat, and clinical status). Subsequently, any statistically meaningful associations were evaluated by Logistic Regression analysis. Analyses were done with SPSS (SPSS 24.0, Chicago, IL, USA) with a P-value < 0.05 as statistically significant.

Results

The cats were aged between 2 months and 204 months (17 years). Seroreactivity to DAT antigen was found in 30 cats, at the dilutions of 1:100 (n = 20) and 1:200 (n = 10). Forty-eight cats were found positive in the ELISA (68-109 EU), including those 30 DAT-positive animals. The serology and PCR results were positively associated by χ^2 analysis ($P < 0.001$). A κ-value of 0.706 was found between two serological tests and PCR, which demonstrated a substantial agreement. Considering that 48 cats were seropositive, either by DAT or ELISA, the overall seroprevalence of *L. infantum* infection was set at 27.59% (Table 1). A total of 36 out of 174 cats (20.69%) were positive by nested-PCR (Table 2).

The prevalence of clinical signs in positive cats either by serology or PCR was 19.0%. The most shared clinical outcomes were ulcerative or nodular dermatitis, and alopecia and scaling.

A meaningful difference was found between the seroprevalence values in females and males ($P < 0.05$). Adult cats presented the highest level of seropositivity (40.90%). A significant difference (P

Table 2. kDNA detection of *Leishmania infantum* in stray cats of different regions in Fars province by nested-PCR

Districts	Central		Northern		Southern		Western		Eastern		Total	
Results	F	P	F	P	F	P	F	P	F	P	F	P
Positive	0	0	6	14.3	12	100	12	50	6	9.1	36	20.69
Negative	30	100	36	85.7	0	0	12	50	60	90.9	138	79.31
Total	30	100	42	100	12	100	24	100	66	100	174	100

Explanations: F=frequency; P=prevalence (%)

< 0.05) was found comparing the seroprevalences in cats aged less than 12 months (2.5%) and in those more than 12 months. A consequential difference was found (P < 0.05) between the seroprevalences in cats living in rural districts (villages or towns) and those living in urban areas (cities). No important differences (P > 0.05) were detected between serological status to *L. infantum* in cats that lived entirely indoors and those that had approach to outdoors, and allegedly healthy or clinically ill cats by Fisher's Exact Test (Table 3).

The specific kDNA amplicon lengths of 680-bp fragment was amplified for *L. infantum*, whereas a 560-bp and 750-bp fragments were amplified for *L. major* and *L. tropica*, respectively. No amplification was detected in the negative control (Fig. 2).

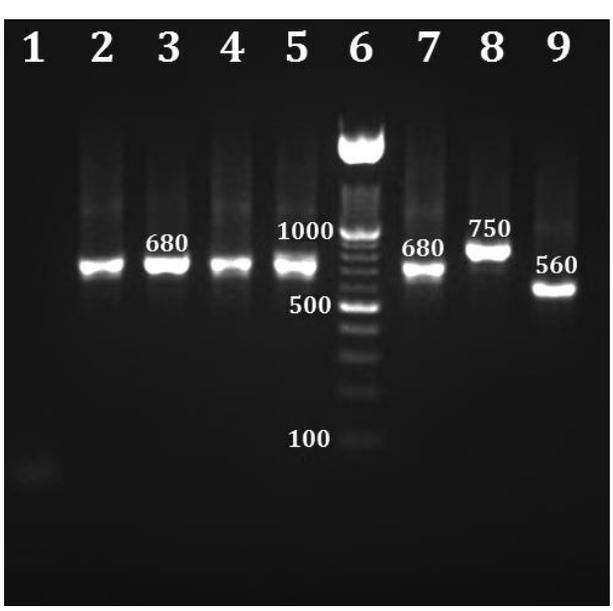

Figure 2. Electrophoresis of PCR products of DNA extracted from the buffy coat of the cat's blood samples. The nine lanes contained the products from positive controls of *L. major* (lane 9), *L. tropica* (lane 8), and *L. infantum* (lane 7); positive samples of *L. infantum*, which shows a specific diagnostic band of 680-bp fragment (lanes 2–5), negative control (lane 1), and a molecular size marker (lane 6).

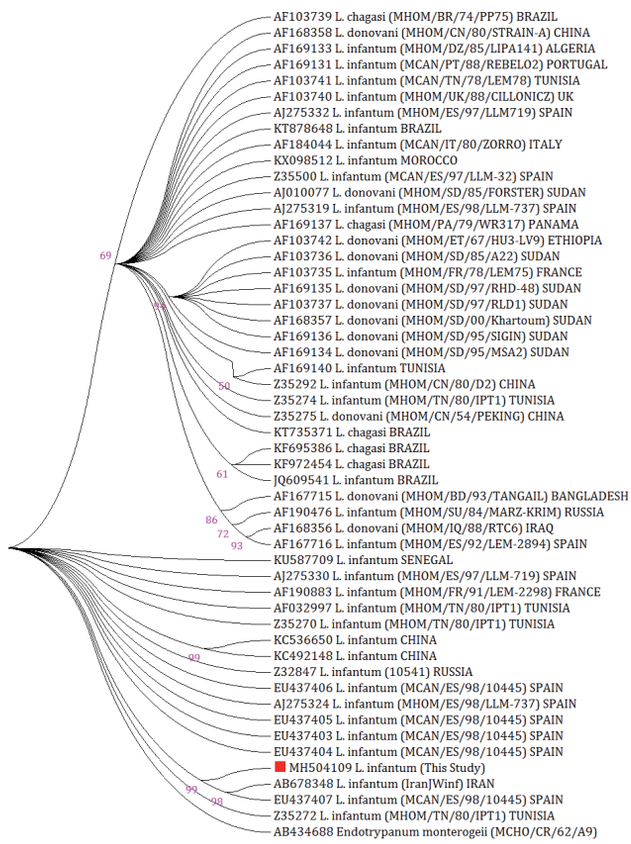

Figure 3. Molecular phylogenetic relationship among various *Leishmania* isolates to each other as inferred by Maximum-Likelihood tree based on minicircle kDNA gene. Numbers on branches are percentage bootstrap values of 1,000 replicates. The evolutionary distances between sequences were computed using the Kimura 2-parameter model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The reference sequences accession numbers are inserted. *Endotrypanum monterogeii* (MCHO/CR/62/A9) was treated as out-group. Evolutionary analyses were conducted in MEGA-X.

Table 3. Seroprevalence of *Leishmania infantum* infection in cats from southern Iran by gender, age group, origin, habitat, and clinical status

	Cats assessed No.	Relative distribution (%)	DAT and/or ELISA positive No.	Seroprevalence (%)
Gender				
female	73	41.9	9	12.33
male	101	58.1	39	38.61
Age Group				
young	40	22.99	1	2.5
adult	110	63.22	45	40.90
old	24	13.79	2	8.33
Origin				
urban	107	61.49	18	16.82
rural	67	38.51	30	44.78
Habitat				
entirely indoors	44	25.29	14	31.81
approach to outdoors	130	74.71	34	26.15
Clinical Status				
healthy	129	74.14	38	29.46
ill	45	25.86	10	22.22
Total	174	100	48	27.59

The analysis of the phylogenetic tree revealed two distinct clades. The kDNA sequence analysis of positive cats showed 99.7% identity to the Iranian *L. infantum* (MCAN/IR/97/LON49), isolate IranJWinf (AB678348). Plus, this isolate showed 98% similarity to the *L. infantum* (MCAN/ES/98/10445) from Spain (EU437407) (Fig. 3).

Discussion

Human VL is caused by *L. infantum* in Iran. The main reservoir of the disease in this region is dog, with a seroprevalence ranging from 19% to 36% [9]. However, infection in other animals such as cats has also been reported [26,27]. Feline leishmaniosis was first described in 1912 in Algeria in a sample of bone marrow from a 4-month old kitten living as a pet in the same house where a dog suffered from CanL and VL affected a child [23]. The cats are still contemplated as uncommon hosts for VL [12,32].

The first aim of the present study was to detect the presence of anti-*Leishmania* antibodies using serological methods in stray cats in Fars province, Iran. The current study was the largest one accomplished on FeL in southern Iran. A high seroprevalence of *L. infantum* in stray cats in Fars province, Iran was disclosed (27.59%), with higher levels in males, adult cats, and those living in rural districts and southern zones. The expanding prevalence of the infection with the age can be connected to the time exposed to the sand flies' activities. Furthermore, a substantial agreement was found between serology and PCR results in combination to diagnose FeL.

In recent years, asymptomatic or symptomatic infection caused by *L. infantum* in cats has been reported in several countries where zoonotic VL is prevalent. Anti-*Leishmania* antibody detection has been extensively used in cats for research and clinical aims by means of IFA, ELISA, DAT and western blot (WB) techniques [21,25,33,34]. FeL cannot be excluded only based on a negative serological test because, as in dogs, discrepancies are known between IFA, ELISA, DAT and WB [25]. It has been displayed that DAT is a very applicable and credible procedure with high sensitivity and specificity in the diagnosis of VL in humans and animals, especially in seroepidemiological studies [21]. Recently, diagnostic performance of ELISA, IFA and WB were compared in cats and WB proposed best sensitivity and specificity [25].

The combination of ELISA and DAT have been utilized for the detection of antibodies to *Leishmania* in cats [21]. In this study, the titers or concentrations of antibodies to *L. infantum* detected in cats, by either DAT or ELISA, were lower than those commonly perceived in dogs. DAT and ELISA results from the present study support the

assumption that the immune response to *Leishmania* infection in cats is different from the one observed in dogs. A higher grade of natural resistance to infections with the protozoan in cats is probably determined by the feline genetic background and related immune responses [21].

Moreover, a wide range of specific seroprevalences has been reported in cats globally using serological methods. In countries of southern Europe including Spain [14,23,24,35,36], Portugal [21,37–39], Italy [20,40–44], Greece [45–48], Turkey [49], Cyprus [50], Israel [51], and Brazil [33,52–55], serological investigations performed among feline populations by different techniques have revealed seroprevalences ranging from less than 0.6% to more than 68% in different regions and groups of cats. In Iran, in a previous study in Fars province, prevalence of FeL was appraised to be 10% when determined by parasitological and molecular methods using liver and spleen samples [27]. A recent study was carried out in Kerman city, southeast of Iran, wherein the overall seroprevalence of FeL by ELISA and PCR was 6.7% and 16.7%, respectively [11]. In two prior studies executed in cats from Ahar and Azarshahr districts, East Azerbaijan province, Northwest of Iran, using DAT, 23% to 28% of cats were seropositive, respectively, but their infections were not confirmed through parasitological and molecular methods [56,57].

The different results from the same or distinct geographical regions may proclaim the real endemicity levels of FeL among the local feline populations, but might also express the differences in the sampled populations, as well as differences in the serological techniques and their cutoff values or positivity thresholds [21,43,44]. In addition, disparate results obtained in the same biogeographical area could be related to the ELISA conditions used. The higher dilutions of sera and conjugate possibly hindered the detection of the low rate of antibodies present in feline infections [23]. Besides, based on previous studies, the seroprevalence of *L. infantum* infection in cats has been found lower than in dogs from the same areas [21,41,45,46]. This seems to correspond well with our study. Cats coming from a rural zone had a higher seroprevalence of infection compared to those animals from urban regions. This may be due to a higher rate of exposure to vectors of *Leishmania* in the rural fields. Researchers have suggested the involvement of synanthropic mammals (such as rats), and domestic cats as alternative reservoirs of *L. infantum* in urban areas, and optional sources of blood meals for sand flies, respectively [41,58,59].

Globally, serosurveys have proposed that *L. infantum* circulates among cats. Although epidemiological position of cats in leishmaniosis is disputed, cats are considered as secondary, alternative or incidental reservoir host for *Leishmania* species [14,37,60]. In addition, two different experimental studies have showed that cats may operate as a potential reservoir, rather than just an incidental one, for *L. infantum* by virtue of the demonstration of infectivity of sand fly vectors from a chronically infected cat in areas where VL is endemic [58,61]. A “Good” reservoir should be in close contact with human via the sand fly, it should be susceptible to the pathogenic agent, and it should make it available to the vector in sufficient quantities to cause infection. Disease should present a chronic evolution allowing the animal to survive at least until the next transmission season [37]. Taken together, all these data allow us to imply that cats can act as a potential reservoir host of *L. infantum*, rather than just an incidental one, since they (i) can be infected by *Leishmania* and develop disease; (ii) present parasites in peripheral blood; (iii) are a blood source and can transmit parasites to competent vectors; and (iv) cohabited with humans. Moreover, naturally infected cats do not recuperate without special anti-leishmanial therapy [34,62–64].

Other potential vectors that frequently spread infections in cats (e.g., ticks) should also be investigated for their role in leishmaniosis transmission. New tick-borne pathogens have been found that affect cats, such as *Ehrlichia* spp., *Rickettsia felis*, *Anaplasma phagocytophilum*, and *Babesia* spp. that might be able to cross-react with *Leishmania*. In a recent study conducted in southern Italy, 11 of 132 (8.3%) ticks of various species removed from stray and owned cats tested positive for *L. infantum* DNA by PCR analysis [65]. This has been demonstrated in dogs in that IFA cross-reactivity has been reported for *L. infantum* and *T. cruzi*, *L. braziliensis*, and *Ehrlichia canis* infection [53,66].

Asymptomatic infection or clinical disease in cats caused by *L. infantum* have been proclaimed in ecoregions around the Mediterranean basin. The most common clinical signs reported in FeL include cutaneous and mucocutaneous lesions (ulcerative or nodular, focal alopecia, and scaling) on the head or distal limbs, lymphadenomegaly, ocular lesions

(uveitis), and chronic gingivostomatitis. Visceral forms with liver, spleen, lymph nodes, and kidney involvement have been less commonly described [34,41,67]. An epidemiologic study in cats have reported significant association between infection with *L. infantum* diagnosed by serology or PCR and cutaneous conditions especially of the face and ears [24]. In the present study, no significant association was observed between seropositivity and any clinical variable. Moreover, feline *L. infantum* coinfections with FeLV, FIV, feline coronavirus (FCoV) and/or *Toxoplasma gondii* have been reported lately [18,24,36], but a significant association was found only between *L. infantum* positivity (molecular or serological) and FIV [68].

The other aim of the present study was to screen the presence of *Leishmania* DNA by nested-PCR targeting kDNA gene in blood samples of cats. Molecular investigations of *Leishmania* DNA are largely used for both CanL and FeL diagnosis [23,65]. In feline epidemiological surveys, most studies were performed on EDTA-blood and usually a lower positivity rate was found in cats compared to dogs from the same area [65,69]. Blood sampling is less invasive and is easy-to-perform, especially for epidemiological studies concerning a great number of subjects, as in our investigation. Studies among feline [70] and human blood donors [71] have illustrated a high rate of infection by PCR in blood samples. Limited data are available about other tissues (skin, lymph node, bone marrow) or non-invasive sampling (conjunctival or oral swabs) [65,69,72,73].

The frequency of *Leishmania* DNA in cats was between 0% and 26% in Spain and 5.76%, 10%, and 30.4% in Brazil, Iran and Portugal, respectively [14,27,35–38,70,74,75]. In a previous study in Turkey, 8.84% of 147 domestic cats were found to be positive for *Leishmania* DNA using real-time PCR [76]. In our study, the *L. infantum* DNA was detected in 36 out of 174 stray cats (20.69%). The high prevalence of *Leishmania* in peripheral blood implied that cats living in endemic regions are regularly exposed to infection through vectors. In other studies, *L. braziliensis*, *L. amazonensis*, and *L. chagasi* have been characterized in cats that live in different countries [18,77–79]. Recently, *L. tropica*, *L. infantum*, and *L. major* were confirmed in cats in Turkey [49,76].

The PCR has been used alone or in combination with serology to assess the prevalence of FeL. In our study, seropositivity was higher than PCR-positivity to *Leishmania* in cats. This is in accordance with previous studies [14,24,35,37,70]. We recommended that serologic assessment could overvalue the real or active degree of infection.

Our results demonstrate the presence of sera- and PCR-positive cats to *L. infantum* in southern Iran, an endemic area for this disease in dogs. Seroprevalence of *L. infantum* in cats is lower than in dogs from the same area. In Brazil, from 13.3% to 23% of the cats were positive in ELISA while the seroprevalence in dogs was 40.3% [80]. In Greece, 3.87% of the cats were positive in ELISA while the seroprevalence in dogs was 21.3% [45]. Differences in the immune response (mainly cellular immunity), host preference of vectors, the natural resistance of the cat to this infection, and diminished body surface area exposure to the vector (considering the fur density present on cats) could elucidate these deviations with CanL [70].

Divergent results when analyzing samples from the same animals using serology and PCR can be due to the sensitivity and specificity of the tests. The lack of correlation between PCR and serologic results could reflect an ineffective immune response or could be explained by the absence of antibody production during an early stage of the infection [45,70].

The diagnosis of FeL could be difficult because the physical signs and symptoms can be similar to other pathologies and inasmuch, there are no commercially available serologic diagnostic tests [32]. Information on the prevalence of *L. infantum* infection is necessary to define control measures for zoonotic leishmaniosis. Although dogs are the main reservoir in Iran, the role of cats in the epidemiology of *L. infantum* needs further attention.

In conclusion, our results demonstrate high levels of seroreactivity to *L. infantum* in cats living in Fars province, Iran, where zoonotic leishmaniosis is endemic. Nevertheless, *Leishmania* infection must not be underestimated and leishmaniosis may be included in the differential diagnosis of cutaneous or systemic clinical signs in cats. Despite the fact that no official program exists in Iran to research other reservoirs of *L. infantum* besides dogs, we believe that our findings could be helpful to guide veterinary surgeons in Fars province, southern Iran with high rates of prevalence and incidence of VL and CanL to consider the existence of the possibility of cats infected with the parasite in these areas. Taking into account the high rate of FeL in our study, it can be suggested that the cat acts as

a good reservoir host of *L. infantum* infection. Further parasitological, serological, molecular, and xenodiagnostic studies are required in order to comprehend the epidemiologic role of cats in the transmission of VL disease, to characterize clinically this infection in cats, and to investigate the frequency of FeL among cats. This study will have important consequences for public health.

Acknowledgments

We thank and appreciate all the veterinary staffs in every place of the Fars province, Iran that contribute in collection of cat's blood samples. This study was funded supported by the office of Vice-Chancellor for Research of Shiraz University of Medical Sciences, Shiraz, Iran. The university had no role in the study design, collection/analysis/interpretation of data, writing of the manuscript, or the decision to submit the manuscript for publication.

Authors' contributions. QA, IM, MHM, and FBG contributed in conception, study design, and methods used. IM, FBG, MK, and SH contributed in acquisition and collation of data, and analysis of data. IM wrote the manuscript. All the authors read and approved the final version of the manuscript.

Disclosure statement. The authors declare that they have no conflict of interests.

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Received 13 May 2019
Accepted 04 April 2020