Feline visceral leishmaniasis in Kerman, southeast of Iran: Serological and molecular study

Baharak Akhtardanesh1–2, Iraj Sharifi2, Ali Mohammadi2–3, Mahshid Mostafavi2, Mojdeh Hakimmipour1 & Neda Ghasemi Pourafshar1

1Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman; 2Leishmaniasis Research Center, Kerman; 3Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran

ABSTRACT

Background & objectives: Visceral leishmaniasis (VL) is a fatal zoonotic disease in tropical and sub-tropical countries including Iran. Dogs constitute the main domestic reservoir for VL (kala-azar) in Iran but incidence of the disease in cats from Fars and East Azerbaijan provinces has led to propose them as secondary reservoirs, and possible expansion of the feline role in the transmission of disease. The aim of this study was to evaluate the prevalence of Leishmania infantum infection in stray cats in Kerman City by ELISA and PCR methods.

Methods: In this cross-sectional descriptive study, 60 stray cats were randomly live trapped from different parts of Kerman City during a six month period between March and September 2014. About 3 ml blood samples were drawn from jugular vein of captured cats and a detailed questionnaire about demographic characteristics and clinical status of each cat was recorded by attending veterinarian. The complete blood counts and biochemistry analysis were performed for all cats. Finally collected sera samples were tested by an indirect enzyme-linked immunosorbert assay (ELISA) kit and PCR amplification method.

Results: Prevalence of Leishmania infantum infection was 6.7 and 16.7% by ELISA and PCR assays, respectively. Infection rate was significantly higher in leukopenic cats, which were older than 3 yr.

Interpretation & conclusion: The results of the study indicate that stray cats are at risk of L. infantum infection in Kerman City. Further, studies are required to elucidate the role of cats as potential reservoir host in the epidemiology of VL in endemic regions.

Key words Cat; Iran; PCR; serology; visceral leishmaniasis

INTRODUCTION

Leishmaniasis represents a spectrum of disease condition with significant health impacts, caused by different species of Leishmania genus. This disease is currently endemic in 98 countries of the world. Overall, annual prevalence is 12 million and the population at risk is approximately 350 million1. Cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) are present in 14 of the 22 countries of the Eastern Mediterranean Region2. Iran is an endemic country for both CL and VL3.

Leishmania infantum is transmitted by various species of female phlebotomine sandflies4. Domestic dogs (Canis familiaris) are major reservoir for L. infantum which play a key role in transmission of infection to humans, whereas cats have been suggested as a secondary reservoir in endemic areas5–6. Unfortunately, there are still several obscure points about host factors, clinical outcome and transmission of VL to other vertebrates7–8; hence it is necessary to evaluate their infection status and role in the epidemiology of zoonotic VL. The main foci of kala-azar in southern Iran are Kazeroun, Nourabad, Firouzabad and Darab districts in Fars province, where dogs are the principle domestic reservoir9–12. Based on a hospital record, 260 cases of VL have been recorded during 2001–2009 from these regions12. Reports of feline VL from the aforementioned areas indicate that cats might also be involved as potential hosts in the epidemiology of the disease13–14.

Detection of antibodies to Leishmania by different diagnostic tests consisting of enzyme-linked immunosorbert assay (ELISA), indirect fluorescent antibody (IFA), and the direct agglutination test (DAT), proved suitable for screening canine and human population in different studies15–18. In the last decade, the use of polymerase chain reaction (PCR) for detection of Leishmania DNA was shown to be highly sensitive and specific. A variety of canine tis-
sues, including bone marrow, spleen, lymph nodes, skin, and conjunctival biopsy specimens and peripheral blood have been used for the identification purpose 19.

Cats might be implicated as a secondary reservoir in Fars and Azerbaijan provinces in Iran where kala-azar is endemic, and hence evaluation of their infection status and role in the epidemiology of zoonotic leishmaniasis is very important 8. The aim of this study was to assess the prevalence of *L. infantum* infection within the population of stray cats in Kerman City using ELISA and PCR methods. The results of the present study identified another potential animal reservoir host of *L. infantum* (other than dog) and this data could be used for planning an effective future control strategy for VL in Iran.

**MATERIAL & METHODS**

**Study area**

This epidemiological study was conducted in the City of Kerman, the center of Kerman province. Kerman is located in the southeast of Iran, the second largest province of the country with an area of 180,726 km² and population of over 800,000 people (2015 census).

**Sampling**

In total 60 stray cats were captured by a double door live trap cages containing baits by a volunteer cat rescue group in a Trap-Neuter-Return program. The cages were placed at five different locations of Kerman City near garbage dumpsters. These cats were randomly selected with no limitation for age, sex, and clinical status between September and March 2014. Before neutering surgery, all animals were clinically monitored for three consecutive days and a detailed questionnaire consisting of age, sex and clinical status of each animal was recorded by attending veterinarian. From each cat 3 ml blood samples were collected from jugular vein and transferred into falcon tubes containing anticoagulant, ethylenediaminetetraacetic acid (EDTA).

All clinical procedures were performed by appropriately qualified scientific colleagues and the project underwent ethical review, and was given approval by an Institutional Animal Care (Approval number: C/93.6.14).

**Haematology, biochemistry and serology**

Complete blood counts were performed manually for all cats and the presence of haematological disorders such as anaemia (haematocrit <20), leucopenia or leucocytosis (<5500 to >19500 leukocyte/µl of blood) and changes in differential leukocyte count were recorded 20. Serum samples were separated by centrifugation at 3000 rpm for 3–5 min and stored at −20 °C for serological and biochemical examination. Total protein, globulin, blood urea nitrogen (BUN), creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level were measured by an autoanalyser (Autolab, AMS-18A, China) 21. All the serum the samples were tested by an indirect ELISA kit (ID Screen Canine Leishmaniasis, ID-Vet Company, France) following the manual’s instruction of the manufacturing company, and the samples were read at 450 nm by an ELISA reader (ELX800 BioTek, USA).

The ELISA test was validated if the mean value of the positive control optical density (ODPC) was > 0.350 (ODPC > 0.350), and the ratio of the mean values of the positive and negative controls (ODPC and ODNC, respectively) was > 3 (ODPC/ODNC > 3). The proportion rate of each sample over positive (S/P) control was calculated by the following formula:

\[
S/P = \frac{OD \text{ (Sample)} - OD \text{ (NC)}}{OD \text{ (PC)} - OD \text{ (NC)}} \times 100
\]

The sample was interpreted as positive if the rate was ≥50%. The ratio >40 and <50% was considered doubtful and ≤40% was recorded as negative.

**Molecular identification**

**DNA extraction**: DNA extraction from blood samples of cat was carried out by Viral Gene-spin™ Viral DNA/RNA Extraction kit (VeTeK™, South Korea) according to manufacturer’s instructions. Template DNA was measured using a NanoDrop—2000 spectrophotometer (Thermo Fisher Scientific) and extracted DNA was stored at −20°C.

**Nested PCR assay**: The two step of nested PCR were used on extracted DNA to amplify variable fragments of kinetoplast DNA (kDNA) of the *Leishmania* species. The chosen primers were designed specifically for this PCR analysis as previously described by Noyes *et al* 22. In the first-step of the PCR, external primers CSB2XF (5’-CGAGTAGCAGAAACTCCCGTTCA-3’) and CSB1XR (5’-ATTTTTTCGCGATTTTTCGAGAACG-3’) were used. In the second-step internal primers 13Z (ACTGGGGGTTGTTAAAATAG) and LiR (TCGCCAGACCGCCTT) were used. In the first round of each 25 µl reaction mixture of PCR 5 µl template DNA, 12.5 µl Taq DNA Polymerase Master Mix Red (Ampliqon, Denmark) and 30 picoM of each CSB2XF/CSB1XR primers were used. The ther-
mocycler program consisted of an initial step at 95°C for 5 min, followed by 35 cycles, 30 sec each at 94°C, 1 min at 55°C and 1 min at 72°C, and then a final extension for 5 min at 72°C. For the second round of PCR, 1 μl of diluted (1 : 9) PCR product of the first round was used as template and this step was performed with the same conditions and reaction mixture as in the first one, but with different specific primers LiR and 13Z. The amplified DNA was subjected to electrophoresis in a 2% agarose gel, pre-stained with ethidium-bromide and viewed under ultraviolet light. The presence of specific bands was recorded in positive samples.

**PCR assay for sequencing and phylogenetic analysis:** Specific amplification was used on extracted DNA to amplify partial 7SL RNA gene of the *Leishmania* species as described previously in a study by Zelazny *et al.* The PCR amplification with primers TRY7SL (5'-TGCTCT-GTAACCTTGCCGGCT-3') and TRY7SL (5'-GGCT-GCTCGTYNCCGGCCTGACCC-3') generated a product of 137–139 bp (excluding the primers). The PCR assay was performed in 25 μl reaction mixture containing 5 μl template DNA, 12.5 μl Taq DNA Polymerase Master Mix Red (Ampliqon, Denmark) and 10 picoM of each primer. The PCR program was set for initial step at 95°C for 5 min, followed by 34 cycles at 95°C for 30 sec, 65°C for 30 sec, and 72°C for 45 sec and a final incubation at 72°C for 5 min. All amplicons were sequenced in both directions by an ABI-3730XL capillary machine (Macrogen Inc., South Korea). Nucleotide sequence analysis was performed by the basic local alignment search tool (BLAST) from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov) and Bioedit software (ver.7.2). All individual sequences from partial 7SL RNA region of *Leishmania* species (KMC3, KMC4, and KMC7 and KMC8) were identified and submitted to GenBank under accession numbers KU870696 to KU870699.

Evolutionary analyses for partial 7SL RNA region were conducted in MEGA 6. The analyses of multiple sequence alignments for evolutionary history were inferred employing the maximum-likelihood (ML) method based on the Kimura 2-parameter model. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-join (NJ) and bio-NJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with a superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

**Data analysis**

The PCR method was considered as a gold standard for determining the sensitivity, specificity, positive and negative predictive value of ELISA. The degree of agreement between ELISA, and kDNA PCR was determined by calculating Kappa. Finally positive PCR test was set as an outcome variable while sex, age, health status, biochemical and haematological alterations were considered independent variables. Analysis of prevalence values relative to independent variables was conducted using the statistical package for the social sciences (SPSS) software version 15 (SPSS Inc., Chicago, IL) considering a probability (p) value of <0.05 as statistically significant.

**RESULTS**

By using ELISA, anti-*Leishmania* antibody was detected in 4 (6.7%) of the studied cats, whereas by using PCR method, 10 cats (16.7%) were found infected by *L. infantum* and one (1.7%) by *L. tropica*. The specific kDNA amplicon lengths of 650 bp for *L. infantum* were amplified from nine samples but the suspected mixed 750 bp band for *L. tropica* was amplified from one of the blood samples. Amplification was not detected in 2 (2%) cases and in the negative controls (Fig. 1). All infected cats were apparently healthy at clinical examination. The factors associated with positive PCR test were age (p = 0.01), elevated creatinine (p = 0.01) and globulin

![Fig. 1: Agarose gel electrophoresis.](image-url)
level ($p = 0.03$) and leukopenia ($p = 0.05$). There were no other significant alterations in haematological and biochemical findings in the infected cats. The sensitivity and specificity of ELISA method was 44.4 and 100%, respectively while positive and negative predictive values for ELISA method were 100 and 90.9%, respectively (considering PCR as gold standard). Mild agreement ($Kappa = 0.368$) was seen between these diagnostic methods.

Multiple sequence alignments compression of the amplificons of partial 7SL RNA region showed much more polymorphism in KMC3 sequence than in other Leishmania species (Fig. 2). A consensus tree with the highest log likelihood ($-556.2154$) is shown in Fig. 3. The 7SL RNA phylogeny analysis of three individual sequences showed high similarity to the other $L. infantum$ whereas the KMC3 was clustered in a separate branch next to the other $L. infantum$ and as sister clade of $L. donovani$.2\textsuperscript{23-24}

The numbers on the branches represent the percentage of 1000 bootstrap samples supporting the branch. Initial tree(s) for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. All positions containing gaps and missing data were eliminated. There were total 134 phylogenetic positions in the final dataset.

**DISCUSSION**

In Iran, visceral leishmaniasis is caused by $L. infantum$ and the main reservoirs of the disease are dogs; however, infection in other animals such as cats has also been reported.\textsuperscript{13} The cats ($Felis domesticus$) are still regarded as unusual hosts for VL, although the first record of feline leishmaniasis dates back to 1912 in Algeria, in a bone marrow sample from a 4-month-old house hold kitten living in the same house with a dog and a child which together were affected by CL and VL.\textsuperscript{26}

In recent years, asymptomatic or symptomatic infection caused by $L. infantum$ in cats has been reported in several countries where zoonotic VL is prevalent; however, the actual susceptibility of cats to infection by Leishmania spp. is still poorly understood.\textsuperscript{14}

The common techniques for detecting VL are indirect fluorescent antibody test (IFAT), ELISA, direct agglutination test (DAT) and PCR. ELISA has been widely used as serodiagnostic method, with high sensitivity in dogs, but its specificity depends upon the antigen. Earlier studies on dogs have reported moderate agreement between results obtained by serological and molecular methods.\textsuperscript{27} Serological investigations in endemic VL areas revealed different seroprevalence in cats ranging from 0.6% in Portugal to as high as 69% in southern Italy.\textsuperscript{26-28}

In Middle East, in a nonendemic place of Jerusalem, seroprevalence of 6.7% was reported among cats.\textsuperscript{29} In a previous study carried out on felines for detection of VL, 28 and 25% of sampled cats revealed antibodies reacting with $L. infantum$ by DAT and IFAT method, respectively in the endemic provinces of Fars and East Azerbaijan, Iran, whereas in another study in the same regions, prevalence of feline VL was estimated to be 10% when determined by parasitological and molecular methods using liver and spleen samples.\textsuperscript{13-14}

![Alignment of partial 7SL RNA sequences from Leishmania spp. Dots indicate identity with the Leishmania infantum sequence. Since all isolates belonging to the same species had an identical 7SL RNA sequence, only KMC3 sequence showed highly nucleotide polymorphism in some positions.](image)
Although, nucleotide polymorphism was observed among samples, phylogenetic analysis did not show a significant difference between positive samples in the present study and other *L. infantum* records. However, due to the presence of more nucleotide differences in KMC3 sample, the sample was placed in a separate branch from the *L. infantum* cluster.

Eslami et al. have reported similar results from the conserved region inside kDNA when compared with other genomic DNA regions. They had suggested further studies to find more significant molecular markers to differentiate the *Leishmania* species. Thus, based on the results of the two amplification region and phylogenetic analysis, although KM3 was considered as positive sample, in the absence of a clear species border within suspected samples, it was named as *Leishmania* spp. according to Auwera et al. studies. The species characterization for this suspected sample is essential for further study on other gene regions.

The specificity and sensitivity of the commercial ELISA test used in other studies have been reported as high as 99.1 and 98.5% in the detection of canine VL in endemic areas. In this study, the sensitivity and specificity of ELISA method was 44.4 and 100% respectively in cats. Based on the considerable specificity and predictive value of ELISA, this method could be effectively used to rule out disease in suspected feline cases.

*Leishmania infantum* grows poorly in culture media, and culture method has low sensitivity; therefore culture and isolation were not preformed in the present study. On the other hand, for ethical consideration, noninvasive methods were used for primary investigations.

Till date, 28 clinical confirmed cases of feline visceral leishmaniasis (FVL) have been reported across the world, 11 (39.3%) of them occurred in the New World (10 cases in South America) and 17 (60.7%) in the Old World. The most common reported clinical finding in infected cats was skin lesions which was not detected in the current study. In addition, it is important to emphasize that previous studies have shown that cats are more attractive and are more preferred as blood meal sources by phlebotomine sandflies.

As there are limited studies in the field of feline leishmaniasis, hematological and biochemical alteration have not been obviously defined, though elevated creatinine and globulin levels and leucopenia have been reported in infected VL cases. Elevated creatinine level might not be directly related to leishmaniasis as most of elderly cats are predisposed to chronic renal failure.

In the present study, there was a significant asso-
ociation between age of cats and disease prevalence. This finding is consistent with other reports which noted that seropositivity is higher in geriatric animals\textsuperscript{14-42}. However, there was no significant association between prevalence and sex which is in agreement with the findings of other studies\textsuperscript{43-45}.

**CONCLUSION**

In conclusion, the results of the study indicate that domestic cats might serve as potential secondary reservoirs for *Leishmania*. The lack of accurate diagnostic method for feline leishmaniasis in endemic areas might imply that the animal will continue to be a potential reservoir for *L. infantum*. Further, epidemiological studies are necessary to determine the exact role of cats in the transmission of leishmaniasis in endemic areas of Iran.

**Conflict of interest**

None of the authors of this paper have any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

**ACKNOWLEDGEMENTS**

The support and facilities provided by the Faculty of Veterinary Medicine, Shahid Bahonar University Kerman, Iran are gratefully acknowledged.

**REFERENCES**


*Correspondence to:* Dr Akhtardanesh Baharak, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman; and/or Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran.
E-mail: bakhtardanesh@yahoo.com; akhtardanesh@uk.ac.ir

*Received:* 27 September 2016  *Accepted in revised form:* 25 November 2016