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Original Article

Canine Visceral Leishmaniasis in Kerman, Southeast of Iran: A Seroepidemiological, Histopathological and Molecular Study

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Abstract

Background: Canine visceral leishmaniasis (CVL) is a systemic disease with a high mortality rate, caused by a diphasic protozoan parasite, *Leishmania infantum/chagasi* in the world. The objective of the present study was to determine the presence of CVL in the city and suburbs of Kerman, using a range of serological, histopathological and molecular methods.

Methods: Blood samples were taken from 80 clinically symptomatic stray dogs All the collected blood samples were tested by direct agglutination test (DAT) to detect the anti-*Leishmania* antibodies in dogs, using a cut-off value of ≥1:320. Pathological specimens including spleen, liver and lymph nodes were prepared for paraffin blocks, sectioning, staining and final microscopic examination in the pathology laboratory. PCR amplification of kDNA from 9 samples of DAT positive stray dogs was studied.

Results: The anti-*Leishmania* antibody was detected in 9 dogs (11.25 %) of the total 80 studied dogs. No significant difference was found between VL infection and gender. In contrast, there was a significant difference between seropositivity and age ($P<0.05$). Pathological samples showed changes including hyperplasia of infected macrophages and inflammatory cells that occupied sinusoids and splenic cords. Among the samples which was characterized by PCR, only one specimen revealed to be mixed infection between *L. infantum* and *L. tropica*.

Conclusion: The results revealed a high prevalence of *L. infantum* infection in stray dogs in Kerman. This kind of information is needed for implementation of future control programs.

Introduction

Leishmania infantum is the main etiological agent of canine visceral leishmaniasis (CVL) around the Mediterranean Basin including Iran (1). CVL due to *L. infantum*, is transmitted by different species of *Phlebotomus* sand flies which is considered one of the most important canine protozoal diseases of zoonotic concern (2). Dog has also been shown to carry *L. infantum*, main causative agent of human visceral leishmaniasis (HVL) in Iran (3). Eventually, VL results in death if left untreated. The majority of leishmaniasis deaths go unrecognized, and even with treatment access, VL may result in case-fatality rates of 10–20% (4-7).

National experts provided leishmaniasis case data for the last 5 years and information regarding treatment and control in their respective countries and a comprehensive literature review was conducted covering publications on leishmaniasis in 98 countries and territories (8). The prevalence and incidence of canine infection are important parameters and the estimation of which depend on the reliable identification of infected dogs (9). In humans, symptoms of the disease include fever, spleen and liver enlargement, immunosuppression, anemia and weight loss (10). VL is the most severe form of leishmaniasis in the world, which is responsible for approximately 500,000 cases each year, worldwide (11). The parasite migrates to the reticuloendothelial system (RES) including liver, spleen and bone marrow (12).

VL is endemic in various parts of Iran including Ardabil, East Azerbaijan, Fars and to some extent sporadic in Boushehr, Kerman and recently in Qom and northern Khorasan province. Sporadic cases of VL are also reported from other parts of the country (7). CVL is not only a veterinary problem but is also a serious public health concern. Serologic testing can identify exposure to the parasite. Rapid detection of CVL is highly important for controlling HVL. Serological methods are highly sensitive and fairly non-invasive and they are appropriate to be used in field conditions (13). In this study, direct agglutination test (DAT) was used as a sero-diagnostic tool because it is a simple and valid test and does not require sophisticated equipments (3, 14,15). 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 tissues, including bone marrow, spleen, lymph nodes, skin, and conjunctival biopsy specimens, have been used for identification (16).

The objective of this study was to determine the seroepidemiological, histopathological and molecular identity of CVL in the city and suburbs of Kerman in southeastern Iran. The reason for selection of this place was due to the previous reported cases of VL from this area (17). This kind of information is essential for planning an effective future control strategy and to identify the animal reservoir hosts of the disease for implementation of control programs.

Material and Methods

Study area

This study was carried out in the city and suburbs of Kerman. This province is located in southeastern Iran with arid and semiarid climate which is the largest province of Iran and constitutes 11% of the total area of the country. The province has a population of about 2.5 million.

Sampling

The survey was carried out from January 2012 to April 2013. Blood samples were taken from 80 clinically symptomatic stray dogs in the city and suburbs of Kerman. The authorization for shooting dogs and necropsy was obtained from the Kerman municipality office. In fact, the phenomenon of stray dogs and its related public health concern are respon-

sibilities of the local municipalities in the country. A considerable number of dogs more frequently in the winter will be sacrificed each year by shooting and their corpse will be buried in a defined area.

A questionnaire was completed for each dog, recording sex, age and any clinical manifestations of VL including skin lesions, alopecia, nose hyperkeratosis and cachexia. Five ml peripheral blood samples were taken from the cephalic vein of each dog and transported to the Leishmaniasis Research Center at School of Medicine in Kerman University of Medical Sciences. Blood samples were centrifuged at 3000 rpm for 3-5 min and the separated sera were stored at -20°C for serological examination.

Serological test

The sera were tested by DAT. An initial screening DAT was performed at dilutions of 1:80 and 1:320. Samples with titers of 1:320 in dogs were further diluted to endpoint titer of 1:20480 in dog's samples. Control wells (antigen only on each plate) with confirmed negative and positive control sera were tested in each plate daily. The cut- off value was defined as the highest dilution at which agglutination was visible, as a blue mat, compared with negative control wells, which had clear blue dots. The positive standard control serum was prepared from dogs with *L. infantum* infection in an endemic area and confirmed by parasitological methods. Quantitative results obtained with DAT are expressed as an antibody titer, the reciprocal of the highest dilution at which agglutination (large diffuse blue mats) was visible after 12-18 h incubation at room temperature (18, 19). The cut-off was based on previous studies (3, 20-22). The anti-*Leishmania* antibody titers at ≥1:320 were considered positive for *L. infantum* infection in dogs.

Histopathological study

At necropsy, suspected dogs were inspected and evaluated for enlargement of reticuloendotelial system (RES) organs such as spleen, liver and lymph nodes. The RES organs were transported to the pathology laboratory at School of Medicine in Kerman University of Medical Sciences. Tissue slices of $1cm^3$ were preserved in 10% formalin and embedded in paraffin. Four µm thick tissue sections were stained with routine Haematoxylin and Eosin (H&E) for histopathological study.

Molecular identification

DNA extraction

DNA from tissues of samples which were positive for DAT test extracted by using DNA extraction kit for cells and tissues (Roche, Germany, Product No. 11814770001) and quantified with a spectrophotometer (Nano Drop-2000c; Thermo Scientific).

Amplification

Kinetoplastic minicircle DNA was amplified with specific primers, upstream 5'-TCG-CAGAACGCCCCTACC-3' and downstream 5'-AGGGGTTGGTGTAAAATAGGC-3' according to the method described by Mahboudi et al. (23). The 25 µl amplification reaction was carried out with 12.5 µl master mix (Ampliqon, Product Number, 160301) and 50 ng/reaction of DNA extract and 1 µl of 10 picoM of each primers. The mixture was incubated in a thermo cycler (Felx Cycler, Analyticgena) for 5 min at 94°C followed by 16 cycles, each was consisting of 30s at 94°C, 30s at 72°C, and 30 s at 72°C. Annealing temperature was decreased to 0.5 degree in each cycle. Then, new 15 cycles were consisted of 30s at 94°C, 30s at 72°C, and 30s at 72°C. Extension was continued for a further 10 min and 6 µl of the amplification reaction product was visualized in 2% agarose gel and stained with ethidium bromide.

Data analysis

Analyses were performed using SPSS software ver. 15 and positive DAT test was set as an outcome variable. Sex and age were used as independent variables. A primary screening was performed using two K contingency table

(cross-tab) of exposure variables by chi-square and fisher exact tests and $P<0.05$ was defined significant.

Results

Serological examination

In this study, anti-*Leishmania* antibodies at the titers of ≥1:320 were detected in 9 (11.25 %) out of 80 studied dogs (Table 1). No significant difference was found between VL infection and gender. In contrast, there was a significant difference between seropositivity and age ($P<0.05$).

Histopathological finding

Samples prepared from spleen, liver, lymph nodes and skin of 9 sero-positive dogs showed the following pathological changes: proliferation of amastigote laden macrophages (leishman bodies) (Fig.1). Focal periadnexal and perivascular lymphocytic infiltration of the skin (dermatitis) (Fig. 2), relative lymphoid hyperplasia of the spleen and lymph nodes, inflammatory cell infiltration and vasculitis in various organs.

Molecular study

PCR amplification of kDNA from samples of DAT positive stray dogs was studied. Among the samples only one dog was identified as positive for *L. infantum,* although molecular study revealed mixed infection between *L. infantum* and *L. tropica* with corresponding electrophoretic bands. (Fig.3). Based on expectation pattern on bands of *Leishmania* species, *L. infantum* with a band at 650 bp and *L. tropica* with a band at 830 bp could be differentiated at species level (23). In this study, the ampliqon reaction from positive sample shows both bands (Fig. 3).

Table 1: Seroepidemiology of canine visceral leishmaniasis caused by *Leishmania infantum* in stray dogs in the city and suburbs of Kerman, Kerman province, southeast of Iran, 2012-2013

Characteristic		Seropositive no. (%)	Seronegative no. (%)	Total no. (%)
Age (yr)	<1	1(1.25)	24 (30)	25(31.3)
	1-3	1 (1.25)	24 (30)	25(31.3)
	3-6	4 (5)	17(21.25)	21(26.3)
	>6	3 (3.75)	6 (7.5)	9 (11.3)
Sex	Male	4 (5)	27 (33.75)	31 (38.75)
	Female	5 (6.25)	44 (55)	49 (61.25)
	Total	9(11.25)	71 (88.75)	80 (100)

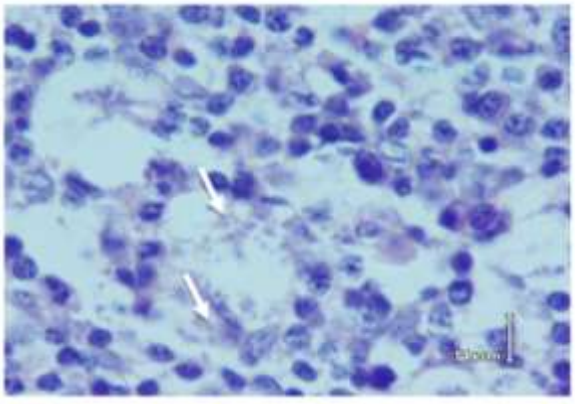

Fig. 1: Multiple amastigote laden macrophages in the spleen (H&E, ×1000)

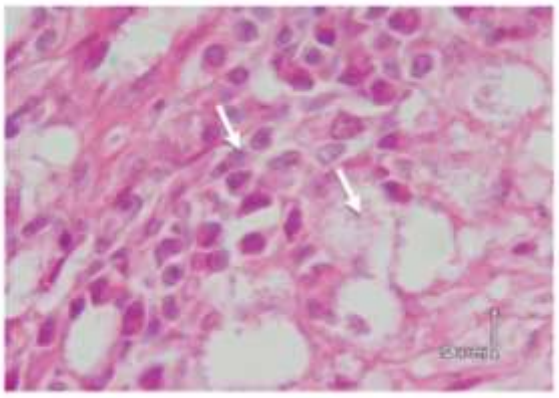

Fig. 2: Periadnexal and perivascular lymphocytic infiltration of the skin with amastigote (leishman bodies) (H&E, ×1000)

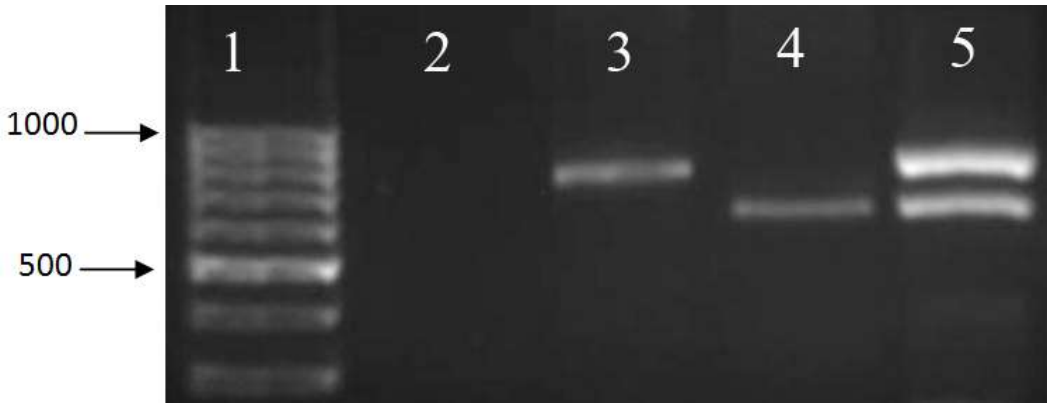

Fig. 3: Agarose gel electrophoresis of PCR amplification of extracted kDNA of DAT positive samples. Lanes: 1, DNA size marker 100 bp (Thermo Scientific Cat. No SM0243); 2, Negative control (distilled water); 3, Positive control, *L. tropica* (MHOM/Sudan/58/OD strain); 4, Positive control *L. infantum* (MHOM: TN:82:IPT1strain); 5, Sample 66 shows *L. infantum* (650 bp band) mixed with *L. tropica* (830 bp band). Dogs with negative samples are not shown

Discussion

Visceral leishmaniasis remains a significant disease with high mortality rate (11). This disease is endemic in some parts of Iran including Ardabil, Fars, East Azerbaijan and to some extent sporadic in Bushehr, North Khorasan, Kerman and Qum provinces (13, 24), and sporadic foci of the disease are also reported from other parts of the country (7). Based on these results, seroprevalence of CVL in Kerman was determined to be 11.25% using the cut-off value of ≥1:320. The seropositivity rate was lower than those found by Gavgani et al (25), and Mohebali et al (3), who reported 21.6% and 18.2%, respectively, seroprevalence in dogs from northwest of Iran.

Dogs and wild canines are the animal reservoir hosts for *L. infantum* in both Old and New Worlds (26). Determination of the prevalence of canine *Leishmania* infection is necessary to define and plan control measures for zoonotic visceral leishmaniasis (27). According to the previous studies (20, 28, 29) the performance of DAT for detection of *L. infantum* infection in humans and dogs was desirable. Therefore, we used DAT to determine seroprevalence of canine *Leishmania* infection. In our study, no statistical difference was found between gender and seropositivity. This finding is consistent with those reported in Portugal (30), Greece (31) and Iran (32, 33).

In our study 5% of the sero-positive cases (≥1:320) were male and 6.25% female dogs. No significant difference was observed among male and female animals. In the current study, we found canine *Leishmania* infection mostly in older dogs (≥3 years of age). In contrast, there was a significant difference regarding the age and seropositivity. Statistical analysis revealed a greater seroprevalence rate in the groups of older dogs, suggesting that the probability of exposure to the bite of sand flies infected with *L. infantum* enhanced with increasing age of dogs (30, 34).

The pathology of CVL shows that resistant animals exhibit low parasite burden and reduced inflammatory responses, making it difficult to find parasites in the mononuclear phagocytic cells of RES organs (35, 36). According to the study of Sanchez et al. (37), organic response in CVL varies from individual to individual and within the same individual, with a strong evidence of being organ-specific. The pathological finding of the present study showed hyperplasia of macrophages, inflammations, vasculitis in various organs and dermatitis by focal lesions.

Molecular study revealed mixed infection of *L. infantum* and *L. tropica* in one dog with their relative specific electrophoresis bands. Madeira et al (38) reported the first case of co-infection with *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) chagasi* in a naturally infected dog from Rio de Janeiro, Brazil. Bakhshi et al. (39) reported that in their molecular detection on sand fly specimens against *Leishmania* parasites, two of their positive samples revealed mixed infection of both *L. turanica* and *L. gerbilli*. Although some studies report mixed infection with other species, there is no report on mixed infection between *L. tropica* and *L. infantum* in literature. To our knowledge this is the first documentation report indicating the concurrent infection between *L.tropica* and *L. infantum* in dog. The results of the present study indicated a high prevalence of *L. infantum* infection in stray dogs in Kerman, especially in dogs older than 3 years of age, indicating a high public health concern due to their contribution to the transmission of infection to humans by sand fly vectors. Therefore, it is necessary to prevent CVL by controlling animal reservoirs and sand fly vectors and taking steps to avoid exposure to sandflies bites.

Conclusion

This finding revealed a high prevalence of *L. infantum* infection in stray dogs in the city and suburbs of Kerman. Mixed infection with *L. infantum* and *L. tropica* could be occurred. This kind of information is required for implementation of future control programs.

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