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Original Article

Leishmania tropica in Stray Dogs in Southeast Iran

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Abstract

Background: Cutaneous leishmaniasis (CL) caused by *Leishmania tropica* is endemic in Kerman, southeastern Iran. While dogs have long been implicated as the main domestic reservoirs of *L. infantum*, etiological agent of zoonotic visceral leishmaniasis (ZVL), they can also carry *L. tropica* infection. The objective of the present study was to determine molecular identity and to evaluate histopathological changes due to CL in dogs in a well-known focus of anthroponotic CL (ACL) in Kerman, southeastern Iran.

Methods: This study was carried out in three prospective series from 1994 to 2013 on dogs. Tissue samples were taken from 471 stray dogs. Pathological specimens including skin, spleen, liver and lymph nodes were prepared for paraffin blocks, sectioning and staining for further histopathological examination. PCR amplification of kDNA was performed to identify the causative agent and sequencing. Overall, two out of 471 stray dogs were infected with *L. tropica*. Hyperplasia of red pulp by the proliferation of histiocytes, lymphocytes, plasma cells and cytoplasm of histiocytes collection of amastigotes was noted.

Results: Based on the results of PCR products and sequencing analysis, the parasites isolated from the lesions of two dogs were characterized as *L. tropica*, corresponding to a band of 830 bp

Conclusion: This finding revealed infection with *L. tropica* in stray dogs in the city and suburbs of Kerman. This information is essential for public health concerns and planning effective future control programs. The role of dogs as potentional reservoir in the epidemiology of ACL needs further investigation.

Keywords: *Leishmania tropica*, Dog, Histopathology, Molecular, Epidemiology, Iran

Introduction

Leishmaniasis is one of the world's most neglected vector-borne diseases with different clinical features and diverse epidemiology (1, 2). Ninety-eight countries and territories on 4 continents have reported endemic leishmaniasis transmission. Overall, official case estimates totaled more than 220,000 cutaneous leishmaniasis (CL) and 58,000 visceral leishmaniasis (VL) cases per year (1). This disease is among the nine infectious diseases with significant health impacts in terms of medical burden, while it is ignored in tropical diseases priorities (3, 4).

Dogs have long been implicated as the main domestic reservoirs of *L. infantum*, the etiological

agent of zoonotic VL (ZVL). Not only domestic dogs, but also canids in general, fulfill the required attributes to be efficient reservoirs of *L. infantum* (5, 6), although; dog has been demonstrated to carry *L. tropica* (7, 8). Due to close relationship with humans, domestic dog has long been involved as the principal reservoir of *L. infantum* in China, the Mediterranean basin and the Americas (6, 9-11).

The present study was performed in a focus of anthroponotic CL (ACL) in Kerman, southeast of Iran (12, 13). Global reports on the infection of dogs with *L. tropica* are scarce. Hajjaran et al. (14) reported a case of *L. tropica* from viscera of a domestic dog with typical signs of canine VL in Iran. Mohebali et al. (15) described detection of *L. tropica* from organs of a puppy by PCR-RFLP technique. In addition, few reports from Morocco showed two dogs infected with *L. tropica* presenting clinical manifestations compatible to those found in canine with *L. infantum* infection (7, 8). The general CL lesion is a non-itchy dermatitis located on different areas of the body such as ears, feet, face and particularly around the eyes (16).

In dogs, CL is usually diagnosed by direct observation of the parasites, using Giemsa or proprietary quick stains, in smears from tissue biopsies and skin scrapings. In addition, histopathology and polymerase chain reaction (PCR) techniques could be used for diagnosis (17). Biopsy specimens of various canine tissues, including reticuloendothelial system (RES) and skin have been used for diagnostic purposes(18).

In the last decade, the use of PCR for identification of *Leishmania* DNA has shown to be highly sensitive and specific. Conventional and nested PCR have proven to be sensitive methods for diagnosing CL and often-used in epidemiologic surveys (19). Fresh, formalin-fixed tissues and blood have been used to assess the presence of the parasite (18, 20).

The objective of this study was to determine the molecular identity and histopathological evaluation of CL in Kerman, southeastern Iran. This information is essential for public health concerns, planning an effective future control program and identifying the potential animal hosts of the disease.

Material and Methods

Study area

This study was performed in the city and suburbs of Kerman, center of Kerman Province, southeastern Iran. It is located at 30°17 13 N and 57° 04 09 E. The mean elevation of the city is 1,755 m above sea level. Kerman has a hot and arid climate and the average annual rainfall is 135 mm. The city and suburbs of Kerman with a population of about 800,000 people (21). It is the largest and most developed city in the Province and the most populous city in the southeast of Iran. The population of stray dogs in Kerman Province is estimated at 145 000–480 000 (22).

Ethical consideration

This project (no. 91/56) was reviewed and approved by the Ethics Committee (K/91/47) of the Kerman University of Medical Sciences and Leishmaniasis Research Center. This study was undertaken in coordination with the health authorities and municipality office for rabies control. Dogs are the main reservoir of the disease. Control of human rabies is dependent on the elimination of dogs. In Iran, rabies is highly prevalent in the province of Kerman (23). Recently, efforts have been made to control the disease in this area. In accordance with the WHO guidelines, free-roaming stray dogs were randomly selected among the animals and euthanized in dog population control program (24). The action plan was organized by the municipality office. In this regard, dogs were randomly killed each year in prospective manner. Heads of the dogs were sent to the Pasteur Institute in Tehran for rabies examination, during which we obtained our sample population for this study.

Sampling

The survey was carried out from January 1994 to August 2013. The authorization for shooting dogs and necropsy was obtained from the Kerman mu-

nicipality office. In fact, the phenomenon of stray dogs and its related public health concern are responsibility of the local municipalities in the country. Dogs were clinically examined for suspected CL lesions (Fig. 1) and enlargement of RES.

Fig. 1: Cutaneous lesions by *Leishmania tropica* in a stray dog

Necropsy specimens were taken from stray dogs in the city and suburbs of Kerman. A questionnaire was completed for each dog recording sex, age and any clinical manifestation of CL including skin lesions, alopecia, hyperkeratosis, cachexia and enlargement of RES organs. Samples were transferred to the Pathology and Molecular Laboratory at School of Medicine in Kerman University of Medical Sciences for further histopathology and molecular examinations. This work has been performed in three series from 1994 to 2013 in dogs.

Histopathological study

At necropsy, suspected dogs were inspected for skin lesion and RES organs such as spleen, liver and lymph nodes. Tissue slices of 1×1cm were preserved in 10% formalin and embedded in paraffin. Four mm thick tissue sections were stained with Haematoxylin and Eosin (H&E) for further histopathological examination.

DNA extraction: DNA from tissues of suspected samples and positive amastigotes in histopathology examination were extracted using high pure PCR template purification kit (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'-TCGCAGAACGCCCCTACC-3' and downstream 5'-AGGGGTTGGTGTAAAATAGGC-3' according to the method described by Mahboudi et al. (25, 26). 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 primer. The mixture was incubated in a thermo cycler (FelxCycler, Analyticgena) at 94°C for 5 min followed by 16 cycles, each was consisting of 30s at 94°C, 30s at 72°C and 30s at 72°C. The annealing temperature was decreased by 0.5 degree in each cycle. An additional 15 cycles consisting of 30s at 94°C, 30s at 64°C, 30s at 72°C and with final extension at 72 °C for 10 min. Then 6 µl of amplicons was visualized in 2% agarose gel which stained with ethidium bromide. The parasites isolated from the lesion were identified as *L. tropica* with a band corresponding to 830 bp and *L.infantum* with a band corresponding to 650 bp (25, 26). Afterwards, PCR products were purified and sequenced by Macrogen Company, Korea. The DNA sequences of each individual species edited with BioEdit software (27), then clustalW alignment and Phylogenetic analyses were performed with Mega 6 software based on UPGMA method (28).

Results

Epidemiological survey

Two dogs of the total 471 stray dogs were infected with *L. tropica*. Out of 471 stray dogs examined, 229 (48.61%) males and 242(51.38%) fe-

males were categorized into four age groups (< 1 years, 1-3 years, 3-5 years and >5 years) (Table 1).

Clinical findings

CL is usually chronic and sometimes subclinical, later evolving toward overt clinical disease. Clinical examination of the two dogs showed alopecia, footpad hyperkeratosis and pustular dermatitis of snout, co-infected with myiasis in one dog. Other clinical signs included weight loss, cachexia, splenomegaly, lymphadenomegaly especially of the prescapular and popliteal lymph nodes manifestations.

Table 1: Age and sex distribution of dogs examined for anthroponotic cutaneous leishmaniasis in the city and suburbs of Kerman, southeastern Iran, 1994-2013

Age (yr)	Female n (%)	Male n (%)	Total n (%)
<1	49 (20.2)	41 (17.9)	90 (19.1)
1- 3	68 (28.1)	84 (36.7)	152 (32.2)
3-5	66 (27.3)	59 (25.8)	125 (26.5)
>5	59 (24.4)*	45 (19.6) *	104 (22.1)
Total	242 (51.4)	229 (48.6)	471 (100)

*One female and one male dog in age group>5 years were infected with *Leishmania tropica*

Histopathological findings

The results obtained from the slides prepared from spleen, lymph nodes and skin lesions of these infected stray dogs were as following: spleen showed hyperplasia of red pulp by proliferation of histiocytes, lymphocytes and plasma cells. Within cytoplasm of histocytes collection of amastigotes (Leishman Bodies) was noted. Lymph nodes showed reactive lymphoid follicles with active germinal center along with sinus histiocytosis in medullary and cortical sinuses. Presence of intracytoplasmic amastigotes in parasitophorous vacuoles was noted in medullary sinuses. Skin tissue revealed multifocal and diffuse lymphohistiocytic infiltrates in dermis even deep close to the hypodermis. High power field revealed energic histiocytic reaction with many intracytoplasmic Leishman Bodies of the infected macrophages (Fig. 2).

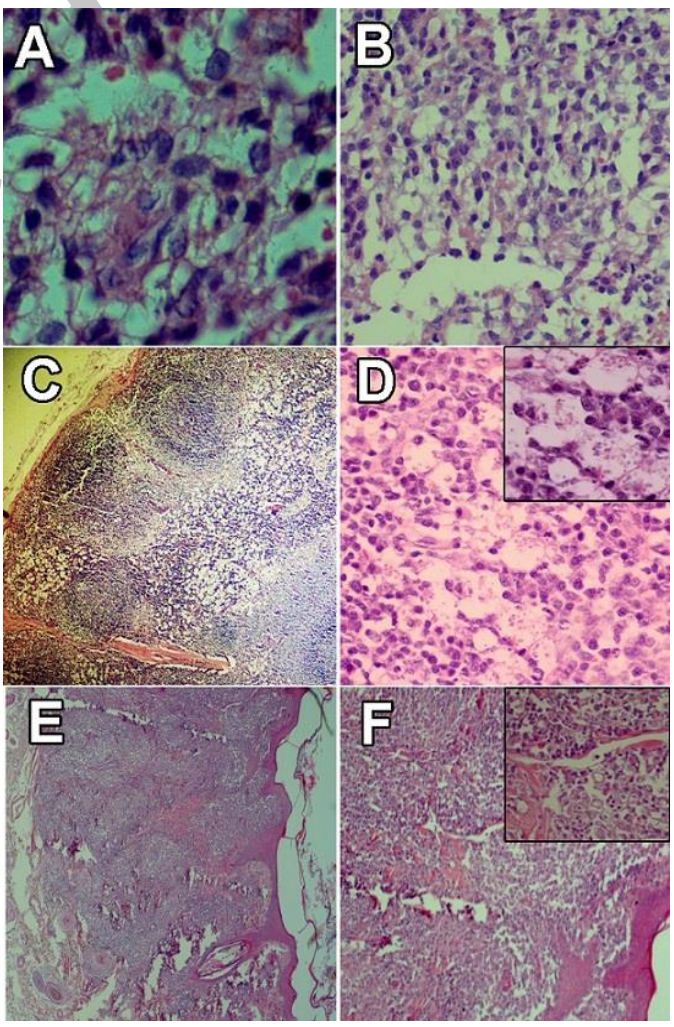

Fig. 2: A. Hyperplasia of red pulp by proliferation of histiocytes, lymphocytes and plasma cells **B**. Cytoplasm of histiocytes collection of Leishman Bodies **C.** Reactive lymphoid follicles with active germinal center along with sinus histiocytosis in medullary and cortical sinuses **D.** Intracytoplasmic Leishman Bodies in parasitophorous vacuoles **E.** Multifocal and diffuse lymphohistiocytic infiltrates in dermis even deep close to the hypodermis **F.** Energic histiocytic reaction with many intracytoplasmic Leishman Bodies of the infected macrophages

Molecular study

Based on PCR findings, among total specimens two positive samples were characterized as *L. tropica* (Fig. 3).

Results of sequence analysis were directly submitted to GenBank with accession number KM491168. For phylogenetic analyses a set of kDNA sequences of *Leishmania* were retrieved from GenBank, included 11 sequences of *Leishmania* records. The phylogenetic analysis revealed that the positive sample in this research was closely related to the Iranian *L. tropica* kDNA with accession number AB678350 (Fig. 4).

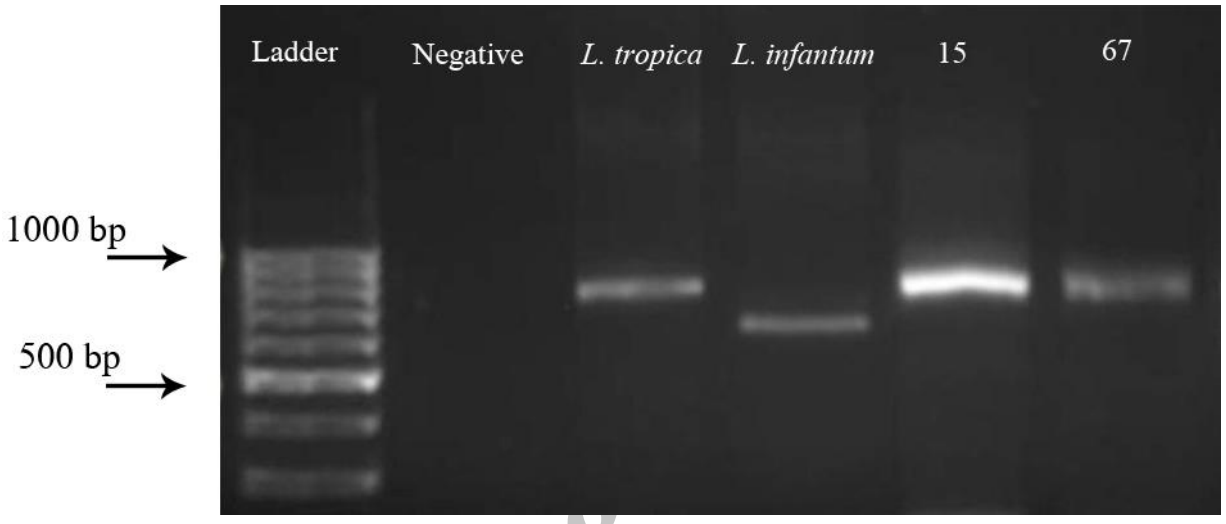

Fig. 3: Agarose gel electrophoresis of PCR amplification of extracted kDNA of suspected CL lesions. Lanes: 1, DNA size marker 100 bp (Thermo Scientific); 2, Negative control; 3, Positive control, *L. tropica* (MHOM /Sudan/ 58 OD strain); 4, Positive control *L. infantum* (MHOM/ TN/ 82/ IPT1strain); 5, 6, Isolates 15and 67 obtained from lesions of the stray dogs *L. tropica*

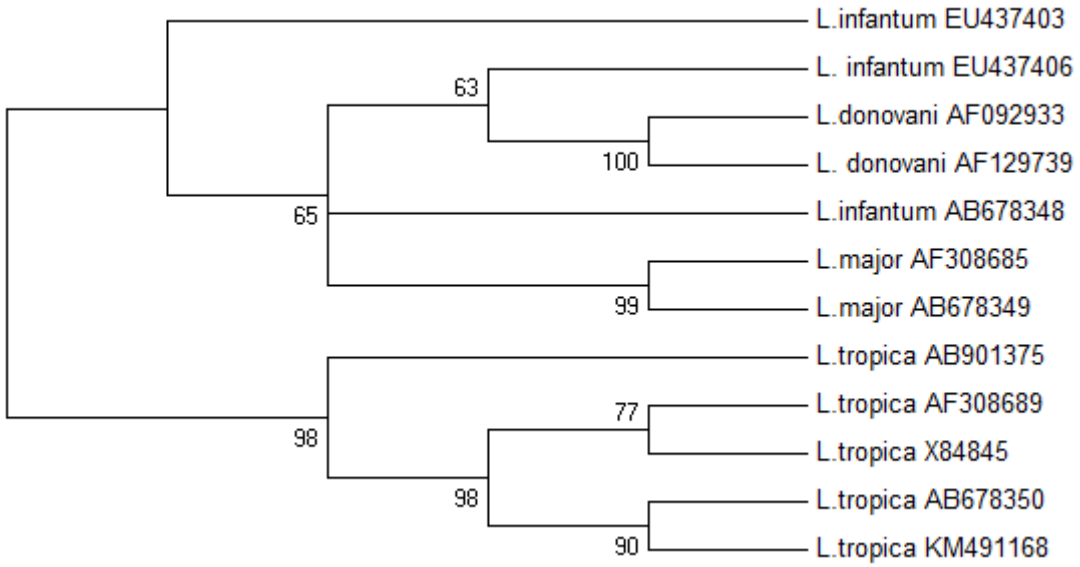

Fig. 4: Phylogenetic analysis indicating genetic diversity between isolates based on kDNA sequence analysis

Discussion

Leishmaniasis is an important vector-borne disease with significant morbidity and mortality in numerous countries across the world including Iran (2, 17). *L. tropica* is the principal causative agent of ACL in different parts of the Old World including the Middle East such as Iran, North Africa, Central Asia and some parts of southern Europe (29, 30). The species has recently been isolated from a few cases of canine VL and human VL (HVL) in Africa and some parts of the Middle East (8, 22, 31, 32). The epidemiology of leishmaniasis involves a dynamic network of highly complex interactions among *Leishmania* parasites, phlebotomine sand fly vectors and susceptible hosts (33).

Canine and HVL are endemic in Northwest and southern parts of Iran, with *L. infantum* as the main causative agent (34). The primary reservoir hosts of *Leishmania* are domestic dogs and sylvatic mammals, such as wild canids (10, 35). Dereure et al. (36) reported seven canine CL cases caused by *L tropica*. These dogs showed no lymphadenopathy or splenomegaly and they were identified in a known focus of human *L. tropica* in central Morocco.

Based on the finding, the overall prevalence of CL in dogs in Kerman was determined to be 2/471 dogs. The role of dog as the probable secondary reservoir host, in which an infectious agent including *L. tropica* normally lives and reproduces itself in such a manner that it can be transmitted to a susceptible host is not well established. However it is generally accepted that dogs are implicated as an incidental host (17), may play some role in transmission of the causative agent. The role of dog as positional reservoir host in the epidemology needs further investigation.

Histopathology of leishmaniasis is not specific and consists of granulomatous to necrotizing granulomatous with predominance macrophages and variable numbers of lymphocytes, plasma cells and neutrophils in the skin, spleen, lymph nodes, liver, kidneys, bone marrow, intestine and conjunctiva (20, 37, 38). We also observed that proliferation of infected macrophages by intracytoplasmic Leishman Bodies was the predominant histopathological confirmation of leishmaniasis in the lesions of skin, splenomegally and lymphadenopathy of the infected stray dogs.

Molecular findings of our study revealed two positive samples infected with *L. tropica* based on their relative specific electrophoretic pattern. In endemic areas, the prevalence of infection of dogs is much high than the prevalence of disease, many dogs can harbor the parasite without ever showing clinical disease. These findings should help in greater understanding of epidemiological aspects of leishmaniasis in a global scale.

Conclusion

This finding revealed infection with *L. tropica* in stray dogs in the city and suburbs of Kerman, a well-known focus of ACL. This information is essential for public health concerns and planning future control programs. The role of dogs as the secondary reservoir host needs further investigation. To our knowledge, this is the first extensive and documented report of *L. tropica* infection in stray dogs in Iran.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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