

## Original Article

# Canine Visceral Leishmaniasis in Wild Canines (Fox, Jackal, and Wolf) in Northeastern Iran Using Parasitological, Serological, and Molecular Methods

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(Received 30 Nov 2014; accepted 8 Nov 2015)

## Abstract

**Background:** Although many studies had been conducted on various aspects of canine visceral leishmaniasis (CVL) in domestic dogs in the endemic areas of Iran, investigations on CVL in wild canines are rare.

**Methods:** This is a cross-sectional study was conducted from December 2012 to 2013 in northeast of Iran where human VL is endemic. Wild canines were trapped around the areas where human VL cases had been previously identified. Wild canines were collected and examined both clinically and serologically using direct agglutination test (DAT). Microscopically examinations were performed in all the seropositive wild canines for the presence of the amastigote form of *Leishmania* spp. Some *Leishmania* sp. which had been isolated from the spleens of wild canines, were examined analyzed by conventional PCR and sequencing techniques using  $\alpha$ -tubulin and GAPDH genes.

**Results:** Altogether, 84 wild canines including foxes (*Vulpes vulpes*, n=21), Jackals (*Canis aureus*, n=60) and wolves (*Canis lupus*, n=3) were collected. Four foxes and seven jackals showed anti-*Leishmania infantum* antibodies with titers of 1:320–1:20480 in DAT. Furthermore, one fox and one jackal were parasitologically (microscopy and culture) positive and *L. infantum* was confirmed by sequence analysis.

**Conclusion:** The present study showed that sylvatic cycle of *L. infantum* had been established in the studied endemic areas of VL in northeastern Iran.

**Keywords:** Canine visceral leishmaniasis, Wild canines, Iran

## Introduction

Visceral leishmaniasis (VL) is one of the most important infectious diseases in human and canines. Mediterranean type of VL which caused by *Leishmania infantum* is commonly seen in children less than 10 years old. Domestic and wild canines are known animal reservoir hosts and some genus and species of sandflies are the main vectors of the disease (WHO 2010). Wild canines including fox, jackal and wolf were infected by *L. infantum* and it seems that these carnivores have the

potential role in sylvatic transmission cycle of *L. infantum* in endemic areas of VL particularly in villages located in mountainous regions, where the transmission cycle was established (WHO 2010). Determination of prevalence of canine visceral leishmaniasis particularly in endemic areas is necessary to define control measures for zoonotic visceral leishmaniasis (Tesh 1995). Based on annual reports of Bojnurd Health Centre from northeastern Iran, 164 cases of human VL were

microscopically diagnosed during two last decades which are higher than the average reported VL cases of Iran (Arzamani 2012). Based on a sero-epidemiological study that was done on 1385 children up to 12 year ages, VL was known as an endemic disease in some areas of North Khorasan Province from northern Northeastern Iran (Mohebali et al. 2011). In cause Because some of limitations in diagnosis and reporting of VL in the studied areas, it seems that real numbers of VL are being higher than registered cases. Considering that epidemiological aspects on sylvatic cycle of VL are unknown in endemic areas of the disease thus, this study was conducted on wild canines. The results of this study can be help to health authorities to make special managements for prevention and control of the disease.

## Materials and Methods

### Study areas

This cross-sectional study was conducted for a period of 1 year from 2012 to 2013. Seven villages in North Khorasan Province were selected, where human VL had been reported in the last 10 years. Altogether, 21 foxes (*Vulpes vulpes*), 60 Jackals (*Canis aureus*) and 3 wolves (*Canis lupus*) were trapped around the villages after obtaining necessary permits from the Directorate General for the Environment (Fig. 1).

### Serological test

All suspected canines were physically examined by a veterinary doctors and then blood samples (2 ml) were taken from them and processed 4–10 h after collection. The collected blood samples were centrifuged at 800 g for 5–10 min, and the sera were separated and stored at -20 °C until tested by DAT.

The *Leishmania infantum* antigens were prepared in the leishmaniasis Lab. of proto-

zoology unit at the School of Public Health of Tehran University of Medical Sciences. The procedure for making DAT antigen were mass production of promastigotes of Iranian strain of *L. infantum* [MCAN/IR/07/Mohebgh. (GenBank accession no FJ555210)] in RPMI1640 medium (Biosera, South America) plus 10% fetal calf serum (Biosera, South America), following tripsinization of the parasites, staining with coomassie brilliant blue R-250 (Sigma, USA) and fixing with formaldehyde 1.2% (Harith et al. 1989, Edrissian 1996a, Mohebali et al. 2005, 2006).

All collected serum samples were tested by DAT. Samples were diluted from 1:40 to give end-point titers of 1: 20480. One Negative and one positive serum controls were included in each plate daily for comparing of the agglutination phenomena among all examined sera in each 96 wells of each V shaped plate. The titer was defined as the highest dilution at which agglutination was still visible, as blue dot, compared to negative control wells, which showed clear blue dots. Two individuals read the tests independently. Specific antibodies against *Leishmania infantum* at a titer of 1:320 were considered as positive based on previous studies (Edrissian 1996a, Boelaert et al. 1999, Mohebali et al. 2005).

### Parasitological study

Parasitological examinations were performed in symptomatic canines (ie hair shedding, skin lesions and cachexia) with DAT positive results ( 1:320) after their euthanization with Ketamin and acepromizine. Microscopical smears were prepared from any skin lesion, liver, spleen and large lymph nodes of all autopsied canines. All of the prepared smears were fixed with absolute methanol, stained with Giemsa 10% and examined microscopically for the demonstration of amastigote forms of *Leishmania* spp. Biopsy specimens were collected aseptically from the spleen and liver of the infected ca-

nines, then cultured into Novy Mac-Neal and Nicolle (NNN) culture media (prepared from nutrient agar containing 10% whole rabbit blood overlaid with normal saline containing 100–200 UI/ml penicillin G and 1 mg/ml streptomycin). The cultures were incubated at 23 °C for up to six weeks and examined weekly for the demonstration of promastigotes.

### Molecular Characterization

Some *Leishmania* spp. isolated from infected wild canines, were checked by conventional PCR using  $\alpha$ -tubulin and GAPDH genes. DNA was isolated from cultured promastigotes and Giemsa-positive slides (amastigotes) using a commercial DNA extraction kit (Roche Diagnostics GmbH, Mannheim, Germany, Lot No: 13779500 high pure PCR template preparation) according to the manufacturer's instructions.

PCR was done with  $\alpha$ -tubulin and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) genes (Sheppard and Dwyer 1986, Kazemi-Rad et al. 2013) Gene and primers used in this study summarized in Table 1.

Amplification was conducted using the PCR PreMix (Roche) in a 25  $\mu$ l total reaction volume. 12  $\mu$ l Master mix, 1  $\mu$ l of each primer (10 pmol), 1  $\mu$ l DNA and for the rest Distilled Water were used. The Both amplicons, 154 bp for  $\alpha$ -tubulin and 119 bp for GAPDH, were analysed on 2% agarose gels and visualized by UV light after staining with GelRed stain. Parasite species were determined by comparing the profiles of the samples with those of the reference species (Fig. 2).

The results were compared with standard species of *L. infantum* (MCAN/IR/97/LON49), *L. tropica* (MHOM/SU/74/K27) and *L. major* (MRHO/IR/75/ER) at the School of Public Health, Tehran University of Medical Sciences. The PCR products of one fox and one jackal samples were purified using an Accuprep Gel Purification kit (Bioneer, Deajeon, Korea), then sequenced (MWG-Biotech, Ebersberg,

Germany) by the primers employed in the PCR. Sequence alignments were constructed using the program ClustalW version 1.83. (<http://www.ddbj.nig.ac.jp/search/clustalwe.html>). ClustalW alignment and phylogenetic analysis with the construction of a gene tree were performed using the Tamura 3-parameter model.

### Ethical approval

The trial was reviewed and approved by ethical committee from vice-chancellor for research, Tehran University of Medical Sciences as well as Vector-borne Diseases Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran.

## Results

### DAT results

Among the 84 wild canines examined, the sera of six *Vulpes vulpes* (foxes), and seven *Canis aureus* (jackals) showed antibodies against *Leishmania infantum* with titers ranging from 1:80–1:20480 indicating that these animals were infected with *Leishmania* spp.

### Parasitological (microscopic and culture) results

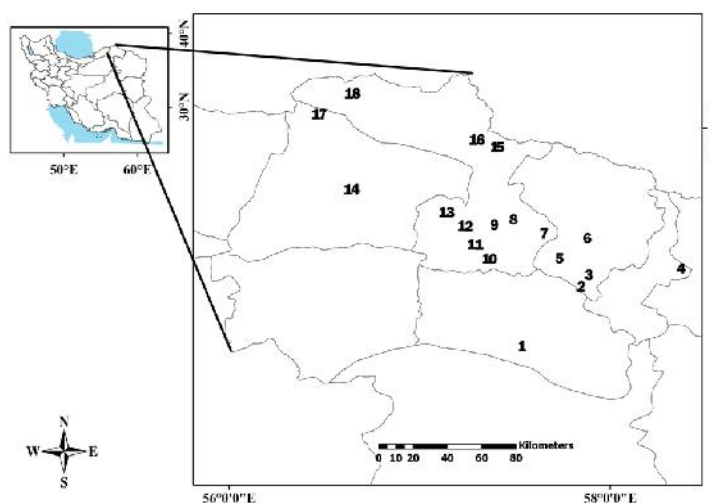
Necropsy was performed on all seropositive *Vulpes vulpes* (n=6) and *Canis aureus* (n=7) accompanied by symptomatic *Vulpes vulpes* (no.2) and symptomatic *Canis aureus* (no.4). *Leishmania* spp. was found in one *Vulpes vulpes* (12.5%) and one *Canis aureus* (9.09%) using parasitological methods. All of the three wolves were dead and no clinically signs and symptoms were found.

All of the three wolves were dead and had not appropriate samples for finding of *Leishmania* infection.

**Molecular results (conventional PCR and sequencing)**

Two positive parasitological samples including one *Canis aureus* (12.5%) and one *Vulpes vulpes* (9.09%) subjected to conventional PCR using  $\alpha$ -tubulin and GAPDH genes (Fig. 2A and B) and *L. infantum* was identified by sequencing results.

All the DNA sequences were aligned using Multalin and analyzed with Mega 6 software (Fig. 3). The GAPDH sequence was submitted in GenBank (Accession No. KM350534).



**Fig. 1.** Geographical locations of collected wild canines (fox, jackal, and wolf) of North Khorasan Province, Northeastern Iran, 2012-2013. 1. Gerati, Esfraien, 2. Estarkhi Shirvan, 3. Gelian, Shirvan, 4. Titkanloo, Faruj 5. Zoeram Shirvan, 6. Shirvan, 7. Sisab, Bojnurd, 8. Naveh, Bojnurd, 9. Babamoosa, Bojnurd, 10. Asadli, Bojnurd, 11. Mehnan, Metranloo, Bojnurd, 12. Bidak, Bojnurd, 13. Tatar, Bojnurd, 14. Ashkhaneh, Mane and Samalghan, 15. Gifan, Bojnurd, 16. Jodar, Bojnurd, 17. Kohne Jolge, Mane and Samalghan, 18. Yekeh Suod, Raz and Jargalan

**Table 1.** Gene and primers used for conventional PCR assay

Gene	Primer designations and sequences (5–3) <sup>1</sup>	Cycling Conditions <sup>2</sup>	Amplicon size (bp)
<b>-tubulin</b>	F:CAGGTGGTGTCTCTCTGAC	d:96 <sup>o</sup> c, 4min c:30	119
	R:TAGCTCGTCAGCACGAAGTG	d:94 <sup>o</sup> c, 30sec a:60 <sup>o</sup> c, 30sec e:72 <sup>o</sup> c, 45sec	
<b>GAPDH</b>	F: GCATGTGCTGACAAAGGAGA	d:96 <sup>o</sup> c, 4min c:30	154
	R:GGTCGTACTCGGGATGATGT	d:94 <sup>o</sup> c, 30sec a:60 <sup>o</sup> c, 30sec e:72 <sup>o</sup> c, 45sec	

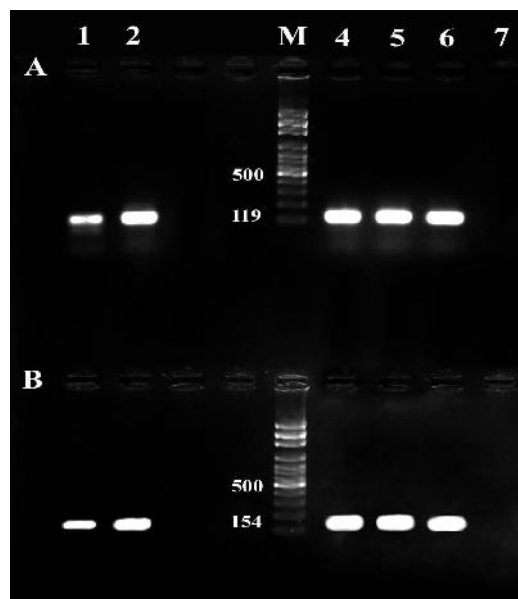
<sup>1</sup>F: forward, R: reverse.

<sup>2</sup>d: denaturation, c: cycles, a: annealing, e: extension. Final elongation for all assays was at 72 C for 10 min.

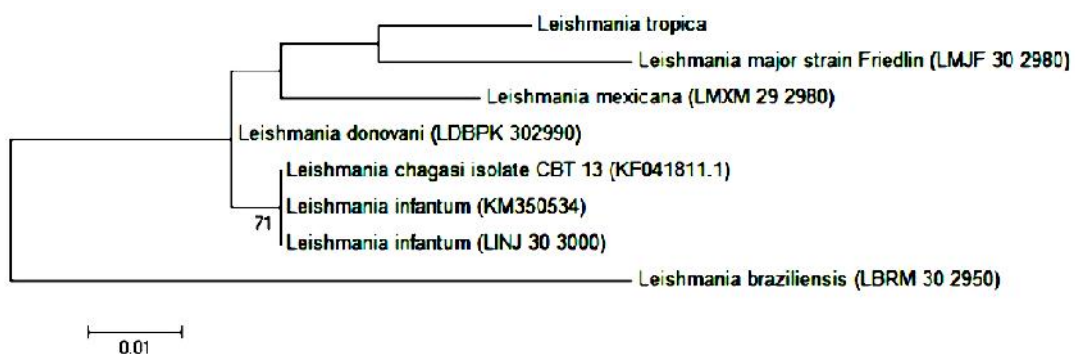
**Table 2.** Parasitology, serology and molecular results in wild canines (fox, jackal) trapped in northeastern Iran during 2012–2013

Animal	Microscopic examination		Culture		Serologic examination		Molecular Examination		Total
	Examined	Positive	Examined	Positive	Examined	Positive	Examined	Positive	
Fox	8	1	8	1	21	6	0	0	21
Jackal	11	1	11	1	60	7	1	1	60

All of the three wolves were dead and had not appropriate samples for finding of *Leishmania* infection.



**Fig. 2.** Conventional PCR Patterns of  $\alpha$ -tubulin and GAPDH genes obtained from test samples and standard *Leishmania* stocks A:  $\alpha$ -tubulin. B: GAPDH. Lane 1 and 2 are Samples of one jackal and one fox. M: 100bp size marker. Lane 4: *Leishmania major* (MRHO/IR/75/ER), Lane 5: *Leishmania tropica* (MHOM/SU/74/K27) and Lane 6: *Leishmania infantum* (MCAN/IR/97/LON49) as positive controls. Lane 7: Negative control



**Fig. 3.** Dendrogram based on the sequence of the GAPDH gene from species of the Genus *Leishmania* (sequences from this study and retrieved from GenBank), with standard *Leishmania tropica* sequence for comparing. The access numbers for sequences retrieved from Gen Bank are given in brackets. The numbers under the branch indicate bootstrap

## Discussion

VL Visceral leishmaniasis is a potentially fatal protozoan infection that is endemic in some parts of Iran (Mohebbali 2013). Domestic dogs (*Canis familiaris*) are the principal reservoir hosts that can carry either *L. infantum* or *L. chagasi* (WHO 2010). It has been reported that our review indicates DAT is an easy-to-perform, highly sensitive, specific, reliable, and cost-effective technique for the diagnosis and sero-epidemiological study of VL in humans and canines across different geographical regions. Even a small amount of serum or plasma specimen, or a drop of dried blood taken from the tip of the finger on a filter paper could be used for DAT (Harith et al. 1986, Edrissian et al. 1996b, Mohebbali et al. 2006, 2011, Mohebbali 2013).

VL Visceral leishmaniasis as an important vector borne disease is endemic in some parts of north east, north west and south areas of Iran and the infections caused by *L. infantum* were reported in infected humans, domestic canines and phlebotomine vectors (Mohebbali et al. 2005, Rassi et al. 2009, Oshaghi et al. 2009, Yaghoobi-Ershadi 2012, Hajjaran et al. 2013). In recent years many studies had been performed on various aspects of VL in domestic dogs in the endemic areas of Iran but investigations on VL in wild canines were rare.

In the present study, DAT was applied to determine the circulating *Leishmania* spp isolated from animal reservoirs. Samples from one fox and one jackal that showed positive results through parasitological methods were subjected to molecular methods. Conventional PCR was performed with GAPDH as the housekeeping gene (Kazemi-Rad et al. 2013). Although the results confirmed microscopic detections, the electrophoretic models were identical for all the tests and controls because of high similarity in various species (difference in a few nucleotides), (Fig. 2). Hence, for species identification, sequencing technique

was employed. The results were analyzed using Mega 6 software and the sequence derived was compared with other reference species in GenBank.

Molecular phylogenetic analysis using Mega 6 software was conducted by applying the Maximum Likelihood method based on the Kimura 2-parameter model. The findings showed that most of the *Leishmania* spp. belonged to the monophyletic group, and the sequence determined in the present study (Accession No. KM350534.1) had 100% homology with *L. infantum* /*L. chagasi* (XM\_001467109/KF041811.1) and 99% with *L. donovani* (XM\_003862963) and presented phylogenetic relationships (Fig. 3). Similarly, the results of another study on *Leishmania* spp. based on *trypanosomatid* barcode (SSU rDNA) and gGAPDH genes also revealed phylogenetic relationships (Marcili et al. 2014). However, although a previous study on *Trypanosoma* spp. using gGAPDH and SSU rDNA demonstrated phylogenetic relationships, a similar study on *Leishmania* spp. showed unrelated results (Hamilton et al. 2004, 2007). Similarity of our study strain sequence with documented GAPDH sequence of *Leishmania infantum* in GenBank (XM\_001467109) that derived JPCM5 strain isolated from a naturally infected dog and its ability for infecting human macrophage reveals and also this study strain can infect human (Peacock et al. 2007).

## Conclusion

Our findings indicate that wild canines have potential role in sylvatic transmission cycle of VL similar to other Mediterranean regions and the disease is turning among domestic dogs and wild canines in endemic areas of VL in Iran.

## Acknowledgements

This study received financial support from Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences (Project No: 91-04-160-20312) and also Vector-borne Diseases Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran. We wish to thank Dr E Kazemi-Rad, Mr MT Satvat and Mrs. Sorour Charehdar for laboratory helps.

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