Original Article

The Seminested PCR Based Detection of *Leishmania infantum* Infection in Asymptomatic Dogs in a New Endemic Focus of Visceral Leishmaniasis in Iran

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ABSTRACT

Visceral Leishmaniasis (Kala-azar) is a serious health problem in some northern and south western parts of Iran. The incidence of kala-azar caused by *Leishmania infantum* has recently increased in Nourabad-Mamassani district of Fars Province, in the south of the country. This study was designed to determine the role of asymptomatic dogs as host reservoir of *L. infantum* in this new formed focus and detection of prevalence of infection near them. A total of 20 asymptomatic stray and sheep dogs were randomly sampled. The Buffy coat layer of their peripheral blood was used for DNA extraction and PCR. A species specific seminested PCR was used for DNA amplification using LINR4, LIN17 and LIN19 primers. These primers amplified variable area of the minicircle kDNA of *Leishmania* parasites. Of the 20 sampled dogs checked for leishmanial kDNA, six (30%) were found naturally infected. It is concluded that, dogs (*Canis familiaris*) even if asymptomatic, is considered as the domestic host reservoir of kala-azar in this endemic focus.

Key words: *Leishmania infantum*, Dogs, Iran

INTRODUCTION

The leishmaniasis is parasitic diseases widespread in the old and new world with great epidemiological diversity. They are caused by about 20 species of *Leishmania*, protozoa transmitted by the bite of female sandflies (Diptera: Psychodidae). Visceral leishmaniasis which its causative agent is *Leishmania infantum* (Syn. *L. chagasi*) is a severe, often fatal disease common in the Mediterranean region and in Latin America (Peters 1987). In the Mediterranean region, domestic dogs (*Canis familiaris*) are the main domestic reservoir hosts and sandflies be-longing to the subgenus *Larroussius* of the genus *Phlebotomus* are the principal vectors (WHO 1990). In Iran, there are currently two main endemic foci of kala-azar caused by *L. infantum:* one in the north-west and the other, within Fars Province, in the south (Edrissian 1999). Dogs, red foxes (*Vulpes vulpes*), golden jackals (*Canis aureus*) and wolves (*Canis lupus*) are thought to be the reservoir hosts (Hamidi 1982, Edrissian 1993, Mohebali 2005). Besides, *L. infantum* has been isolated from some rodents such as *Meriones persicus* and *Mesocricetus auratus* and these animals are considered as secondary reservoirs of kala-azar in Iran (Mohebali 1998). Two sandfly species (*Phlebotomus alexandri* and *Ph. Kandelakii*) have been recently introduced as the proven vector of disease in south and northwest of Iran, respectively (Azizi 2006, Rassi 2005). In the last few years the incidence of VL in Mahoor-Milaty district, Nourabad-Mamassani county

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which lies, at about 50° 35'E, 29° 51' N and 1500 m above sea level, in Fars Province (southern Iran), has suddenly increased. In 2003 there were eight cases of the disease (all of them aged< 10 yr) in villages that had a combined population of only about 4000 person (Unpublished data).

The main aim of the present study was to determine the prevalence of leishmanial infection in dogs and investigation on the role of asymptomatic ones in being host reservoir of VL in this new focus.

MATERIALS AND METHODS

Sampling

The study was carried out in the Mahoor-Milaty District, Nourabad-Mamassani County in Fars Province, southwest Iran. Peripheral blood samples were collected, randomly from 20 asymptomatic stray and owner dogs (about 20% of dogs population in the study area). The Buffy coat layer was separated from whole blood after being centrifuged at 2500 rpm for 10 min.

DNA extraction

A 5µL of each sample was used for DNA extraction. DNA was extracted as described elsewhere (Motazedian 2002). Briefly, 100µL of lysis buffer [50mM Tris-HCl (pH 7.6), 1mM EDTA and 1% Tween20] and 12µL of a Proteinase K solution (containing 19 µg of the enzyme/ml) were added, in a 1.5ml microcentrifuge tube. The homogenate was then incubated at 37 °C overnight before 300 µl of a Phenol: Chloroform: Isoamyl alcohol mixture (25:24:1, by Vol.) were added. After being shaken vigorously, the tube holding the mixture was centrifuged (10000 g for 10min) and then the DNA in the supernatant solution was precipitated with 400 µl cold, pure ethanol, resuspended in 50µl double-distilled water, and then stored at -20 °C until it could be tested for leishmanial kDNA.

PCR

An assay based on the seminested PCR was used for amplification of variable area of the minicircle kDNA (with a slight modification) as described elsewhere (Aransay 2000). The combination of primers LINR4 (forward), LIN17 (reverse) and LIN19 (reverse) was used in a seminested PCR technique (Table 1). These primers were designed within the conserved area of the minicircle and contained conserved sequence blocks (CSB), CSB3, CSB2 and CSB1, respectively (Brewster 1998).

The first amplification reaction was carried out in a total of 25µl contained 250µM of each deoxynucleoside triphosphate (dNTPs), 1.5mM $MgCl_2$, 1U Taq polymerase (Cinagene, Tehran), 1µM LINR4, 1µM LIN17 and 5µl of DNA extract, in 1X PCR buffer (Boehringer Mannheim, Mannheim, Germany), overlaid with mineral oil. The mixture was incubated in a CG1-96 thermocycler (Corbett Research, Sydney, Australia) at 94 ºC for 5 min followed by 30 cycles, each consisting of 30s at 94 ºC, 30s at 52 ºC and 1 min at 72 ºC. After the last cycle, the extension was continued for a further 5 min. The second round was carried out, with the addition of a 40 µl solution containing buffer, $MgCl_2$, dNTPs and Taq polymerase as described above for the first round and 1µM LIN19 primer for 33 cycles (94 ºC for 30s, 58 ºC for 30s and 72 ºC for 1min) and final extension at 72 ºC for 10 min. A 5µl sample of each PCR product was resolved in a 1.5% agarose gel. The bands were then stained with ethidium bromide and visualized under ultraviolet trans-illuminator.

RESULTS

A total of 20 collars of asymptomatic stray and owner dogs were sampled. Infection to *Leishmania* kDNA was observed in 6 (30%) cases. Species specific primers identified the parasites as *L. infantum*, the causative agent of kala-azar. Parasites were identified by comparing the size of the band produced from a test sample with those produced from the reference strains. Reference strains of *L. infantum* (MCAN/IR/96/Lon49), *L. tropica* (MHOM/IR/89/ARD2) and *L. major* (MHOM/IR/54/LV39) were used as standards. Bands of 720, 760 and 560bp, in-

dicated the above standards, respectively. The visualized obtained bands of positive samples were similar to standard *L. infantum*, which was equal to 720bp (Fig. 1).

Table 1. Sequences of the primers used in this study

Primer	Sequence
LINR4 (Forward)	5'- GGGGTTGGTGTAAAATAGGG-3'
LIN17 (Reverse for 1st round)	5'- TTTGAACGGGATTTCTG -3'
LIN19 (Reverse for 2nd round)	5'- CAGAACGCCCCTACCCG -3'

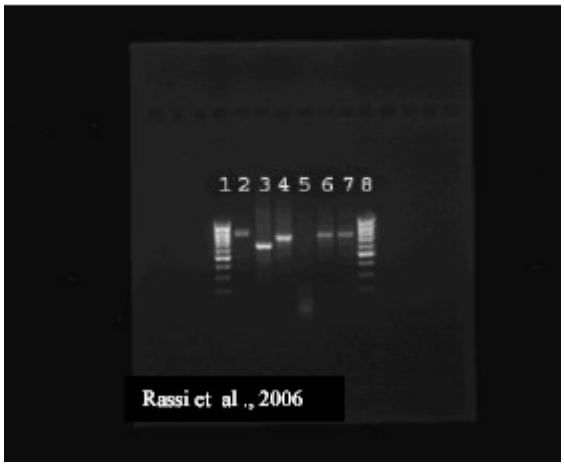

Fig. 1. The results of Seminested PCR based of DNA extracted from Buffy coat of whole blood of dogs. The bands shown on 1.5% agarose gel stained with ethidium bromide, correspond to molecular weight markers (Lanes 1and 8), Reference strains of *Leishmania tropica* (Lane 2), *L. major* (Lane3), *L. infantum* (Lane 4), Blank (Lane 5), Two infected dog samples (Lanes 6 and 7).

DISCUSSION

A thorough knowledge of *Leishmania* ecology and epidemiology is required for control programs of leishmaniasis especially in endemic areas. Identification of reservoir hosts as well as detection of probable or proven vectors are the main problems which epidemiologists faced to. In many areas, however, despite considerable research on VL, the main reservoir hosts and the species of sandfly responsible for most transmission have still to be identified. *L. infantum* infections are responsible for VL in at least 70 countries. In most endemic areas it is widely believed that domestic dogs are the principal hosts.

The main evidences are as follows: isolated parasites from dogs are indistinguishable from those in humans; relatively high infection rates in dogs; positive correlation between dogs and human prevalence, and occurrence of asymptomatic infected dogs (Mazloumi Gavgani 2002).

Characterization of parasites in the past was mainly based on the clinical manifestations, geographical foci of distribution of the disease in humans and biological characteristics of the parasites in laboratory animals; however more recently new methods such as isoenzymes and PCR based techniques have been used for characterization (Ardehali 2000).

The high sensitive technique of PCR has been used formerly for detection of *Leishmania* in sandflies (Aransay 2000, Kato 2005, Rassi 2005, Azizi 2006) and reservoirs (Le Fichoux 1999, Lachaud 2002). Aransay et al. in 2000, used the LINR4, LIN17 and LIN19 primers (which have been used in this study) for detection of *leishmania* infection in sandflies of Greece (Aransay 2000). Our results showed that specific PCR on Buffy coat was a sensitive and suitable method for detection of *Leishmania* infections. Working with peripheral blood is non-invasive, straightforward and easy to repeat which easily can be used for owner dogs (Lachaud 2002).

In Iran, there are many evidence, for natural *Leishmania* infections in domestic dogs, golden jackals and red foxes (Hamidi 1982, Edrissian 1993, Mohebali 2005) and some rodents (Mohebali 1998). The infection rate of dogs in other well-known endemic foci of disease of country, mainly based on serological DAT method, is lower than our finding (Nadim 1978, Edrissian 1993, Mohebali 2001).According to our results, consist of high infection rate of dogs to *L. infantum* (30%), this animal is introduced as the

main reservoir host of kala-azar in Mahoor-Milaty district, Noorabad-Mamassani county of Fars Province in south west of Iran and asymptomatic infected dogs should be considered as an important reservoir of infection for vectors. Undoubtedly, this region can be considered as a new formed endemic focus of kala-azar in Iran. Recently, we found *Ph. alexandri* (Azizi 2006) and fox [unpublished data] infected with *L. infantum* in this area.

The importance of the current method for detection of infectivity in asymptomatic dogs is apparent. Our finding could provide a clue for planning of disease control in the endemic areas by the authorities.

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