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Review

A case report of visceral leishmaniasis in red fox (*Vulpes vulpes*)

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A survey of 52 red foxes, a single two year old male weighing about 6 kg showed clinical signs including hair loss, impotence, local or general lymphadenopathy, keratitis, hepatosplenomegaly, lymphadenopathy, hair shedding, dermal lesions, onychogriposis and cachexia. The studied fox IFA titer was larger or equal to 1:1280 and light absorption in enzyme liked immunosorbent assay (ELISA) was equal to 2.127. In the slides prepared from impression smear of the liver and spleen, Leishman body was observed. The culture results were positive. Parasites isolated from seropositive fox with specific primers K-DNA and ITS-18sRNA was tested and polymerase chain reaction (PCR) identification of parasites isolated from seropositive fox were 99% homologous with Donovani complex (*Leishmania infantum, Leishmania donovani, Leishmania chagasi*).

Key words: Visceral leishemanisis, *Leishmania infantum,* ELISA, IFAT, fox, sarab.

INTRODUCTION

There are many fox species such as red fox (*Vulpes vulpes*). The golden jackal (*Canis aureus*) are reported to be a reservoir hosts of *Leishmania infantum* (Nicolle, 1908) in rural areas of Central Asia where they are considered a potential source of human infection (Lainson and Shaw, 1987). However, the literature contains no reports of the lesions associated with Leishmania species infestation in the jackal. Visceral leishmaniasis has been described in dogs and other members of the Canidae family in the Mediterranean basin. Canine visceral leishmaniasis (CVL) is caused by *L. infantum* and is endemic throughout the northwestern and southern parts of the Islamic Republic of Iran (Edrissian, 1990). Dogs appear to be the chief source of infection for human visceral leishmaniasis. Unfortunately, too many seropositive dogs exist in the endemic areas and anti vector measures have been largely unsuccessful (Mohebali et al., 2005). Visceral leishmaniasis is one of the most important parasitic diseases worldwide and remains a challenge to public health in at least 80 countries (Fallah et al., 2011; Khanmohammadi et al., 2010; Moreno and Alvar, 2002; Desjeux, 2004). The agent of disease in Iran is the Mediterranean type of *L. infantum*. Dogs (*Canis familaris*), as the main domestic host, golden jackals (*Canis aureus*), foxes (*V. vulpes*) and wolves (*Canis lupus*) are the main wild reservoir hosts for CVL (Mohebali et al., 2006; khanmohammadi et al., 2011). Rodents are also another reservoir hosts for CVL in Iran (Fallah et al., 2006). These animals are assumed to be reservoirs for parasites, particularly in the regions where sporadic cases of disease have been reported. So far, at least four endemic focal areas of disease in the provinces of Ardebil (Meshkinshahr, Moghan and Bilesuvar), East Azerbaijan (Kaleibar, Ahar and Azarshahr), Fars (Jahrom, Ghir, Kazeroun), Semnan, Bushehr, Qom (Fakhar et al., 2004) Karaj, Kerman, have been identified, and there have been sporadic reports about the disease in some other provinces of Iran (Mohebali et al., 2001, 2005, 1998; Moshfe et al., 2008; Edrissian et al., 1988; Fallah et al., 2006). This short communication describes lesions associated with infestation by Leishmania species in organs and identifies red foxes as a wildlife reservoir of the disease in Sarab District of Iran.

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Having got permission from the health deputy, veterinary organization and environment agency organization of east Azerbaijan province, 52 foxes were hunted by gun. The foxes were mainly hunted around Sarab and a nearby village. Immediately after the hunt, foxes underwent necropsy, and 10 ml of each one's heart blood was extracted into polypropylene tubes. 6-10 hours later, they were centrifuged in 800 g for 10 to 15 min, and serums were isolated. The sera was divided into micro tubes 1 ml (Ependroff) and kept in -20°C. F. Then it was ready for ELISA and Indirect Immuno Fluorescence test. For indirect immuno fluorescence and ELISA test, *L. infantum* diagnostic kits (IDvet France -Id screen ®, Paris, France) were used. In this study, Titer of anti lgG conjugated dogs with Isothiocyanate fluorescein (Sigma ® F4012) was 1:50. Based on the previous studies of researchers and antibody geometric mean image and considering other studies in the area, positive titer was determined equal or larger than 1:80 (Mohebali et al., 2006; khanmohammadi et al., 2011; Hamidi 1982). On the basis of the criteria presented by the kit manufacturing company, if the percentage of OD sample/OD positive control is equal to or larger than 50%, the sample is accepted as positive. Finally the samples were examined by ELISA reader (Dynatech Laboratories, Roseville, Canada) as well as the microscope fluorescent (Olympus B × 50, Japan). To demonstrate *L. infantum* in the autopsied fox, the impression smear was taken from the spleen and liver, immediately fixed with methanol 95%, and finally stained with Giemsa 10%. The samples were studied by the microscope for Leishman bodies. For the pathological studies, small parts of spleen, liver, kidney, lung, heart were removed and mesenteric samples were taken. More small parts of liver and spleen were cultured in specific culture medium *L. infantum* in Nicole-Novy_McNeal (NNN), RPMI 1640 medium and Schneider with 20% fetal gulf serum. Isolated Promastigotes were mass production in culture.

Case presentation

Of the 52 red foxes screened, a single two year old red fox weighing about 6 kg showed clinical signs of leishmaniasis. These symptoms included hair loss, impotence, local or general lymphadenopathy, Keratitis, Hepatosplenomegaly, lymphadenopathy, hair shedding, dermal lesions, Onychogriposis and Cachexia (Figure 3). The fox IFA titer was larger or equal to 1:1280 and light absorption in ELISA was equal to 2.127 (138% = S/P% percent of samples).

Parasite isolation

The Seropositive fox was autopsied and spleen biopsy, bone marrow aspirate were cultured in specific media for Leishmania such as NNN and RPMI 1640 or Schneider medium. Cultures were studied after 48 h to assess the existence and growth of Promastigotes. This practice was repeated once a week for 5 weeks. NNN positive cultures are transferred to α- MEN fluid medium for mass growth. This stage of the parasites was used for PCR (k-DNA, ITS-18sRNA). Pair of primers specific oligonucleotide was used for K-DNA F1, TCGCAGAACGCCCCTACC R1, AGGGGTTGGTGTAAAATAGG and F1, CTGGATCAT-TTTCCGATG R1, ACACTCAGGTCTGTAAAC (Gen Ruler-Fermentas) for ITS. Standard DNA *L. infantum* and *L. major* Institute Pasteur (MRHO/75/ER) were used as a positive control and samples containing all PCR materials without DNA as negative control. PCR products on Agarose gel 1.5% for ITS-18sRNA, PCR 1× TAE buffer 12V/cm and K-DNA, PCR 1× TAE buffer 17 V/cm was electrophoresis. All the gels were stained with ethidium bromide (0.25 mg/ml- Bio-Rad Laboratories), visualized, and images captured using UV Transilluminator imaging system (UV-GENTM; Bio-Rad Laboratories).

To do electrophoresis, a suitable marker with definite and specified molecular weights of sediment was used. For electrophoresis, a suitable marker with sediment bands was used and molecular weight was determined. Bands created by samples were compared with marker bands and approximate molecular weight was determined.

After analyzing the electrophoresis pattern (finger print), the results were compared with standard species of *L. infantum*, (MCAN/IR/96/LON49), *L. tropica* (MHOM/IR102/Mash4) and *L. major* (MRHO/IR/75/ER) in the School of Public Health, Tehran University of Medical Sciences.(Fallah et al., 2011; Khanmohammadi et al., 2011; Mohebali et al., 2005; Mohebali et al., 2006). Parasite species were identified. Seropositive samples after PCR with *L. infantum* specific primers and results of electrophoresis analysis PCR products were evaluated.

DNA isolation and PCR amplification

Genomic DNA was isolated as described previously. Briefly, logarithmic phase promastigotes were disrupted in lysis buffer (50 mM NaCl, 50 mM EDTA,1% SDS, 50 mM Tris–HCl, pH 8.0), and then incubated overnight with proteinase K (100 mg/ml, Sigma-Aldrich) at 37°C. DNA was purified further by phenol-chloroform extraction and ethanol precipitation. An Eppendorf DNA thermal cycler and Taq DNA polymerase (Roche Mannheim, Germany) were used for amplification of desired gene. The reaction mixture included 10 pMol of each primer, 200 mM dNTPs and 1.5 mM MgCl2. PCR conditions were: primary denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min (Farajnia et al., 2004; Noyes et al., 1996).

RESULTS

Some of the positive cultures promastigote were used for

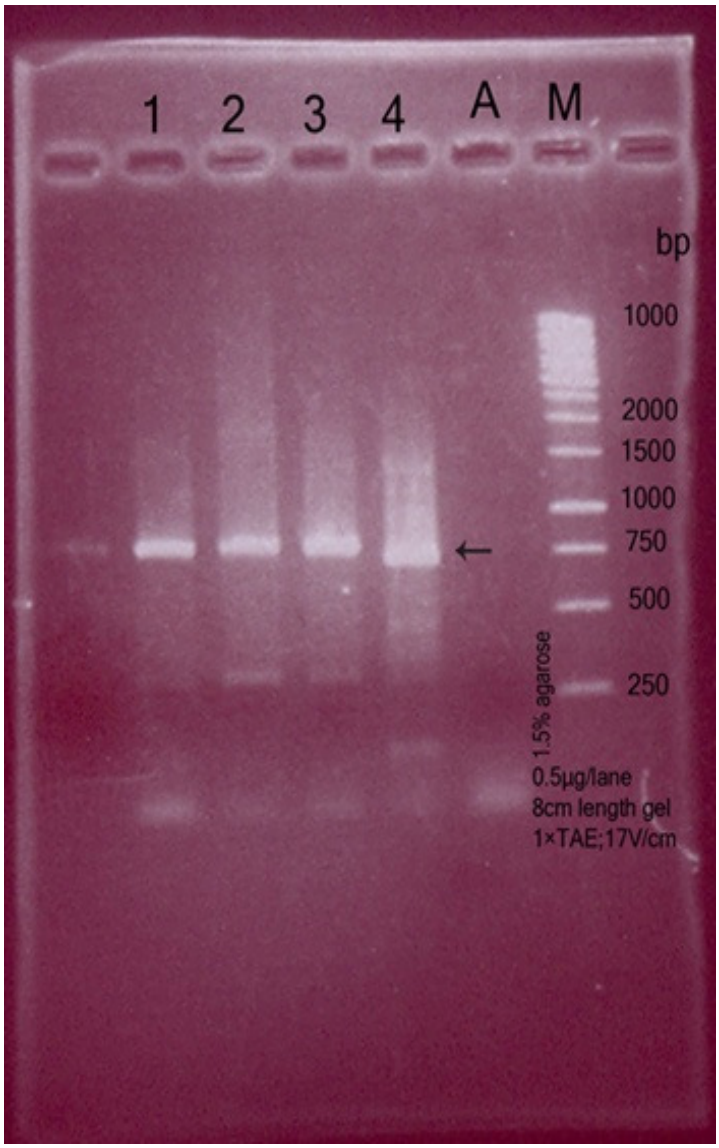

Figure 1. Amplification of parasite DNA of PCR products (KDNA), DNA (1 ng) extracted from cultures of parasite isolates was used for PCR. Lane M: Marker, 100 bp ladder. Lane A: Negative control. Lane 4, *L. infantum*, (Standard strain *L. infantum*, MCAN/IR/96/LON49) School of Public Health, Tehran University of Medical Sciences), and lanes 1, 2 DNA (1 ng) *L. infantum* isolated from positive dogs; lane 3, *L. infantum* isolated from infected fox (750bp).

sequencing. Solution containing the DNA amplified after coding was sent to the MWG Company for sequencing. The sequences of results were compared using BLAST software with Leishmania sequences recorded in gene banks and was compared homology rate with standard strains. The impression smear slides prepared from liver and spleen, the Leishman body observed and the result was positive in both cultures. Fox parasites were isolated for the PCR and transferred to the Applied Research Center of Tabriz University of Medical Sciences and tested with K-DNA and ITS18SRNA specific primers and reported specifically belong to genus Leishmania (Figures 1 and 2). To determine sequence, positive samples for PCR were sent to the MWG research institute in Germany. Sequences results was evaluated with BLAST software and PCR identification of parasites isolated from seropositive fox were 99% homologous with Donovani complex (*Leishmania infantum, Leishmania donovani, Leishmania chagasi*). To have definite sequencing, other gene segments or other specific methods like isoenzyme must be used. In this study, it was determined that wild carnivores are the main

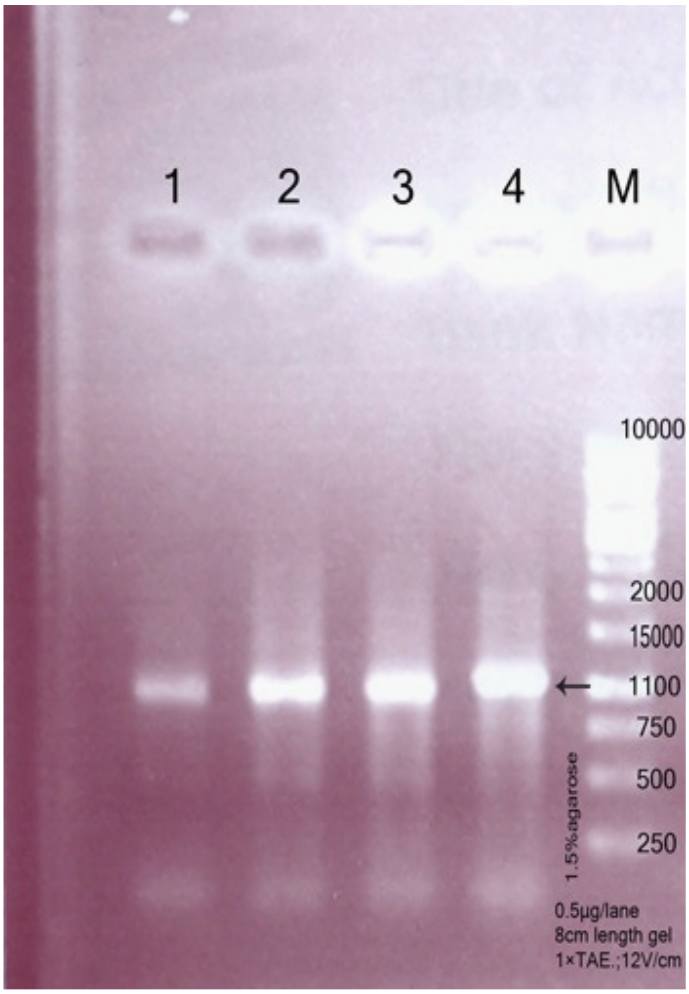

Figure 2. Amplification of parasite DNA of PCR products (ITS-18sRNA), DNA (1 ng) extracted from cultures of parasite isolates was used for PCR. Lane M: Marker, 100 bp ladder. Lane 4, *L. infantum*, Standard strain *L. infantum,* (MCAN/IR/96/LON49) School of Public Health, Tehran University of Medical Sciences, and lanes 1, 2 DNA (1 ng) *L. infantum* isolated from positive dogs and lane 3, *L. infantum* isolated from infected fox (1100 bp).

hosts of visceral leishmaniasis in Sarab, located in the East Azerbaijan Province. The most significant finding an examination was a stomach full of food; the liver was enlarged, and some areas were discoloured; the spleen was considerably enlarged and black. The referred material was routinely processed for light microscopy and embedded in paraffin. Sections 4 pm thick were cut from these blocks, and stained with Haematoxylin & Eosin and Giemsa. Sinusoidal space of spleen was larger and macrophage containing the organisms into the cytoplasm was seen. C form shape sometimes was available. Edema and interstitial infiltrates were seen. The spleen exhibited lymphocyte depletion in per arteriolar lymphoid sheaths, proliferation of infested macrophages in the same region, hyperplasia of lymphoid structures and enlargement of red pulp, with clusters of macrophages containing numerous amastigote forms of Leishmania species. Necrosis and fibrosis in T cell areas of splenic white pulp were extended and in lymphocytes, severe accumulation of PAS positive internal parts was reported. Phagocytosis within mononuclear cells, containing forms of Leishmania amastigot were seen, and these organisms include core vesicular with a kinetoplast inside cells (Figure 4). The number of macrophages hemo-siderin pigmentation was evident. White pulp was swollen and filled with lymphoid follicle, red pulp with high cells density and some blood was seen. Increased macro-phage was remarkable. In the lymph nodes, lymphocyte decreased and the liver showed evidence of multifocal granulomatous hepatitis; the periportal areas contained clusters of macrophages with abundant amastigote forms. There was also intense infestation of circulating

Figure 3. The Infected fox Hunted in Sarab district.

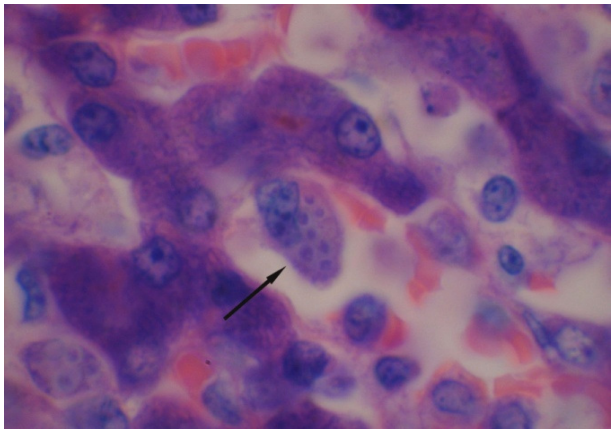

Figure 4. Red pulp with clusters of macrophages containing numerous amastigote forms of Leishmania species in spleen. spleen. Giemsa x 100.

monocytes and kupffers cells.

DISCUSSION

Mediterranean type of visceral leishmaniasis is one of the most dangerous diseases which is transmitted from animals to humans. Dogs and wild canines (fox and jackals) are considered the most important animal reservoirs of visceral leishmaniasis in endemic regions of Iran (Mohebali et al., 2005). Dogs are the main source of infection in endemic regions of visceral leishmaniasis in Iran (Mohebali et al., 2006; Moshfe et al., 2008; Edrissian et al., 1988; Khanmohammadi et al., 2011; Fallah et al., 2001). Routine diagnosis of the parasite in smears is observed in amastigot parasites obtained from spleen and bone marrow; however, although the microscopic examination is rapid, easy and cheap, it lacks the necessary sensitivity when the number of parasites in tissue is low and cannot detect the species of parasites. IFA test is completely sensitive and specific as a qualitative test used in the diagnosis of leishmaniasis. Studies show that the *L. infantum* Lon49 is the main cause of the disease in humans and animals in different parts of the reservoir in Iran (Mohebali et al., 2005). The results of our study indicate that the endemic focus of VL in East Azerbaijan province is similar to the endemic foci of Kalaybar and Ahar in this province, Jahrum, Ghir and Firooz Abad in Fars province, Meshkin-shahr (Moshfe et al; 2008; Mohebali et al; 2005) in Ardabil province and other endemic areas of the Islamic Republic of Iran. In a study conducted by Nadim et al. in 1982 in Northern Iran

to review reservoir animal visceral leishmaniasis, 20 jackals and 10 foxes were taken and autopsied.

A golden jackal (*Canis aureus*) had clinical symptoms (weakness, impotence, hair loss, muzzle wounds), respectively. In another apparently healthy jackal with no clinical symptoms (hunted in the eastern part of the Alborz mountain range, 15 km east of Shahroud), a low number of Leishmania parasites were seen). In the study by Mohebali et al. (2001) on 30 wild canines (10 foxes, 10 jackals, 10 wolves) only a fox, a jackal and a wolf were reported to have developed *L. infantum*. In a study by Mohebali et al. (2001) in Dashti and Dashtestan located in Bushehr Province, having studied four foxes, 10 jackals were designated one of which was serological and parasitological positive. 37 foxes 29 dogs were reported to be serum positive by Courtenay et al. (2002). In Brazil, Dipineto et al. (2007) diagnosed 50 foxes in southern Italy fox as positive using PCR. In the study done by Mancianti et al. (1994) in Italy, the test of IFA and ELISA showed 18% of the foxes had antibodies against Leishmania. In Bettini et al. (1980) study in Italy, out of 35 hunted foxes, only 4 were reported positive using PCR-RFLP method to assess Sobrino et al. (2008) who studied on 39 wolves and 162 foxes in Spain, 8 and 23 cases were reported positive. By separating parasites from the spleen and liver of the fox using parasitology methods, and by identifying the species of parasites with molecular techniques, the wild canine, as one of the reservoirs of the disease must be taken into account in controlling programs.

New theories that can express the different strains of *L. infantum* parasites are this area. So, the strains of the disease or antigenic characteristics are different with each other. Finally, the infected people should be cured along with controlling the vector mosquitoes provided that hygienic standards are observed and no damage is caused to biological environment. Planning controlling programs is a proper step in the prevention of Visceral Leishmaniasis.

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