Evaluation of *Leishmania infantum* in cat by PCR-RFLP in an endemic region of visceral leishmaniasis in meshkin-shahr, Iran

Soraya Mohamadzadeh Dorbadam¹, Lame Akhlaghi¹, Behnaz Akhondi², Homa Hajjaran², Zabihollah Zarei², Ramtin Hadighi*¹

(1) *Iran University of Medical Science, Medicine Faculty, Parasitology Department*
(2) *Tehran University of Medical Science, Health Faculty, Parasitology Department*

Abstract

*Leishmania infantum*, is an endemic zoonosis cause of visceral leishmaniasis in Iran. Although dogs is a main reservoir host for visceral leishmaniasis, Several case of feline leishmaniasis have been reported in previous studies. serological, parasitological and molecular study was performed on 50 cats living in this area. in one cat anti-*Leishmania* antibody’s titer higher than 1:20480 revealed by DAT and *L. infantum* infection confirmed using parasitological and molecular techniques. This is the first survey of feline leishmaniasis (FL) in Meshkin-shahr endemic area. Further investigation is needed because of cats may have playing a role as a secondary host in the transmission of Visceral leishmaniasis in Iran.

Keywords: Feline leishmaniasis; PCR-RFLP; DAT; Culture; ITS1; meshkin-shahr ; Iran.

1 Introduction

Visceral leishmaniasis (VL) caused by protozoan parasite *Leishmania Infantum* is an important zoonotic disease transmitted to the human and animals by sand flies in some area in Iran. The annual report of human Visceral leishmaniasis (HVL) in worldwide is estimated to be more than 500,000 and accounts for 75,000 painful deaths [1, 2]. meshkin-shahr district which is located in the central northern part of the Ardebil province is considered as a one of the main endemic regions for VL in Iran with a more than 2000 reported human cases of visceral leishmaniasis since 1998. dogs are main reservoir of parasite in this region [3, 4, 5]. Several vertebrates are considered as a reservoir host around the word. Few reports of cats, may indicate that a probable role of this animals in the parasite life cycle. first record of feline leishmaniasis (FL) was revealed in 1912 [6] also sporadic case of leishmania infantum have been reported in cats from different contries such as italy [6, 7], france [8], spain [9], portugal [10, 11, 12] and in Middle
East (Israel, Iran) [13,14]. This study was performed Because of the importance of identifying of reservoir hosts in endemic areas of visceral leishmaniasis.

2 Material and Method

This study was performed during 2012 in the Meshkin-Shahr district, which is located in the central northern part of the Ardebil province with a cold predominantly dry climate, annual rainfall between 300 and 385 mm and 61% to 70% relative humidity.

Sampling was performed in endemic villages. Based on previous studies some villages such as Alni, Ballujeh, Kojenagh, Parikh and Aghbolagh with higher than 10% of dog’s positive for anti-Leishmania antibody considered as endemic areas [15, 16, 17].

3 Animals and Samples

50 stray cats without any clinical sign captured using live traps. Blood samples were collected by saphenous venipuncture. These animals were among various sexes and ages which were selected by simple random sampling. Serum specimens were separated and stored at -20 °C until examination.

4 Direct agglutination test (DAT):

Direct agglutination test for titration of Leishmania-specific antibodies performed based on general procedures described by Harith (1989) [18, 19, 20, 21]. The L. infantum antigens for this study were prepared in the protozoology unit of the School of Public Health in the Tehran University of Medical Sciences. The principal phases of the procedure for making DAT antigen were mass production of promastigotes of L. infantum Lon49 (Iranian strain) in RPMI 1640 plus 10% fetal bovine serum, trypsinization of the parasites, staining with coomassie brilliant blue and fixing with formaldehyde 2% [21, 22, 23]. Antigen concentration for the DAT was 5×10⁷ promastigotes/ml. The serum samples were diluted in physiological saline (0.9% NaCl) containing 1.56% β-mercaptoethanol. Two-fold dilution series were made from 1:80 to 1:20480 in V-shaped micro titer plates (Greiner, Germany) and incubated for 1 hour at 37 °C. Fifty microlitres of reconstituted DAT antigen was subsequently added to each well containing 50 μl of diluted serum. Quantitative results obtained with DAT are expressed as an antibody titer, i.e. the reciprocal of the highest dilution at which agglutination (large diffuse blue mats) was still visible after 18 h incubation at room temperature, compared with negative control wells, which had clear blue dots. The positive standard control serum prepared from dogs with L. infantum infection from the endemic areas confirmed by microscopy, culture and animal inoculation with 1:20480 titers. Cat negative control serums prepared from Veterinary Faculty of Tehran University. We considered specific anti-Leishmania antibodies titers for ≥1:320 as Leishmania infection in this investigation to maximize sensitivity and specificity of the test [21, 22, 23].

4.1. Parasitological study

Seropositive cases (1:320 and higher) were considered as leishmanial infection and then animals were euthanized, using ketamin plus xylocaine and specimen of liver and spleen were taken from each animal. Infection with Leishmania in cats was evaluated through touch smear preparation of the liver and spleen samples for microscopy examination, also these samples were cultured in RPMI 1640 medium, and incubated at 26°C. The cultures were checked regularly for 2 months before being considered negative. Mass cultivation of positive cultures was made in RPMI 1640 medium supplemented with 10% fetal Bovine serum.
4.2. Molecular characterization
PCR-RFLP used for molecular characterization of infected animals. ITS-1 fragment was amplified using primers LITSR (F) & 5.8S (R) as described by Schoenian et al [24]. According to the manufacture’s instruction PCR product was digested using BsuR1 (Hae III) (fermentaz) and the restriction fragment were analyzed using 2.5% agarose gel electrophoresis. Also, the PCR-product of the samples was sequenced.

5 Results
In this study, 50 cats were evaluated to detect Leishmania infection. The seropositive rate in titers higher than 1: 3200 was 2%. Only one cat showed anti-Leishmania antibodies in titers of ≥1: 20480 in DAT, and parasite was detected in Gimsa-stained spleen and liver tissues. Moreover, Leishmania parasites was isolated and mass cultured from spleen and liver of infected cat.

PCR-RFLP
ITS1 PCR was carried out and 300-350 bp which is specifying of genus leishmania was detected for identification of leishmania species.

The species of this sample identified as L. infantum by RFLP (figure 1). Also, the PCR-product of the sample was sequenced with more than 95% homology with L. infantum, and then registered in NCBI Gen bank with KC355188 accession number.

![Figure 1: PCR-RFLP obtained with DNA samples from infected cat](image)

As can be seen in the figure, Column (1) is of the negative control and no DNA. Column (2) is the standard strain of L. tropica with three bands about 50,70 and 180 bp are clear. Column 3 and 4 are strains L. major with creating two bands 180 and 220 bp and Column 5 is the standard of L. infantum Strain and have 3 specific bands 50,90 and 200 bp and is separable from other species and Column (6) is L. infantum isolated from the cat. (M) Marker
6 Discussion

This survey is the first study was carried out on feline Leishmania infection in the Meshkin-Shahr district. 50 stray cats captured from five (Alni, Ballujeh, Kojenagh, Parikhan and Aghbolagh) villages around Meshkin-shahr, by live trap that showed a low sero-prevalence of infection with Leishmania by serological, parasitological and molecular techniques.

In recent studies the seropositive prevalence of leishmania infection has been assessed in cats from southern Europe, which disclosed antibody titers generally lower than in Canine Leishmania infection. Seropositive prevalence have stretched between 0.9 and 68.0% in different endemic areas [25, 26]. Many feline leishmanial infection was reported 20 years ago, clinical characterize Related with Leishmania infection were explained and parasite factors were correctly confirmed using parasitological or molecular techniques [27]. Five class of Leishmania have been recognized in this animal group [28].

Many coetaneous form of leishmaniasi in cats are usual, such as nodular form, ulcerative, harsh or papular lesions, But involvement of spleen and liver or kidney has been less reported [5].

Infection of sandflies from cats naturally infected with Leishmania was recently approved in Italy and Brazil [29, 30], and it is suggest that cats maybe play a role as secondary reservoir host for L. infantum. Also 24 feline leishmaniosis in Europe reported since 1980s that all of them were infected with L.infantum and most of them belonging to zymogene MON-1 [31].

According to this study and comparison with previous studies, it can be concluded that, in the endemic area of visceral leishmaniasis in human and dogs, this disease is observed in cat. Regardless of high or low rate of infection, cats maybe play a role in the transmission of disease. Needless to say, for control and eradication of any infectious disease, recognition of vector and reservoir host is very important. Because of that considering the cats as a probable reservoir host in endemic areas such as meshkin-shahr for controlling program is very important. We recommend further studies in other endemic areas for visceral leishmaniasis in Iran to determine a role of cats as a reservoir host.

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