ISSN 1735-1383

Iran. J. Immunol. December 2011, 8 (4), 244-250

Behnam Mohammadi-Ghalehbin, Gholam Reza Hatam, Bahador Sarkari, Mehdi Mohebali, Zabih Zarei, Mansoureh Jaberipour, Shahab Bohlouli

A *Leishmania infantum* FML-ELISA for the Detection of Symptomatic and Asymptomatic Canine Visceral Leishmaniasis in an Endemic Area of Iran

Article Type: RECEARCH ARTICLE

The *Iranian Journal of Immunology* is a quarterly Peer-Reviewed Journal Published by Shiraz Institute for Cancer Research and the Iranian Society of Immunology & Allergy, Indexed by Several World Indexing Systems Including:
ISI, Index Medicus, Medline and Pubmed

For information on author guidelines and submission visit:

www.iji.ir

For assistance or queries, email:

iji@sums.ac.ir

A *Leishmania infantum* FML-ELISA for the Detection of Symptomatic and Asymptomatic Canine Visceral Leishmaniasis in an Endemic Area of Iran

Behnam Mohammadi-Ghalehbin[1], Gholam Reza Hatam[2*], Bahador Sarkari[2], Mehdi Mohebali[3], Zabih Zarei[3], Mansoureh Jaberipour[4], Shahab Bohlouli[5]

[1]Department of Parasitology and Mycology, School of Medicine, [2]Center for Basic Researches in Infectious Diseases, Shiraz University of Medical Sciences, Shiraz, [3]Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, [4]Institute for Cancer Research, Shiraz University of Medical Sciences, Shiraz, [5]Department of Physiology and Pharmacology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran

ABSTRACT

Background: Visceral leishmaniasis (VL) is caused by *Leishmania infantum* in Mediterranean basin and is an endemic disease in some parts of Iran. Canines are the main reservoirs of VL in most of the endemic areas. Different serological methods have been introduced for diagnosis of canine visceral leishmaniasis (CVL). **Objective:** In this survey a Fucose-Mannose Ligand (FML) ELISA, using native *L. infantum* antigen, was developed and its validity for detection of infected dogs in comparison with direct agglutination test (DAT) and PCR was evaluated. **Methods:** Blood samples of sixty ownership dogs (≤ 3 years old) were collected from Meshkin-shahr district in Ardabil province, North-west of Iran. Sera were separated for serological assays (DAT and FML-ELISA) and the buffy coats were collected for molecular evaluation. **Results:** Two out of the 60 (3.33%) samples were found to be positive (antibody titer of ≥ 1/320) in DAT while seven of the 60 (11.66%) samples were positive by FML-ELISA. Nine out of 60 (15%) buffy coat samples showed a band about 680 bp indicative of *L. infantum* in PCR. Three out of 60 dogs had Kala-azar symptoms and were positive by PCR and FML-ELISA, while two of these three dogs had antibody titers >1/320 in their serum samples. The sensitivity and specificity of FML-ELISA for the detection of CVL in both symptomatic and asymptomatic dogs were found to be 77.8% and 100%, respectively. **Conclusion:** Considering the acceptable sensitivity and high specificity of FML-ELISA, use of this serological method can be recommended for epidemiological surveys of CVL.

Keywords: Canine, DAT, ELISA, Iran, PCR, Visceral Leishmaniasis

---

*Corresponding author: Dr. Gholam Reza Hatam, Center for Basic Researches in Infectious Diseases, Shiraz University of Medical Sciences, Shiraz, Iran,e-mail: hatamghr@sums.ac.ir

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is an infectious disease caused by *L. donovani* complex and is fatal if left untreated. VL is caused by *L. infantum* in Mediterranean basin and is an endemic disease in a few provinces of Iran including Fars province in the South, Ardabil and East Azerbaijan provinces in the North-west and in Kohkilooyeh and Boyerahmat province in the Southwest of the country (1-5).

Domestic dogs (*Canis familiaris*) play the main role as reservoir hosts for human visceral leishmaniasis (6). Control of reservoirs for visceral leishmaniasis is somehow based on the culling of seropositive dogs (7). Common serological methods for epidemiological studies of canine visceral leishmaniasis (CVL) are indirect immunofluorescent antibody test (IFA), enzyme-linked immunosorbent assay (ELISA) and direct agglutination test (DAT). The definite diagnosis of CVL is based on the finding of leishmanial amastigotes in biopsy materials from spleen, bone marrow, lymph nodes or liver. However such approaches are invasive and have relatively low sensitivity. High validity of DAT can be considered for detection of VL associated with clinical symptoms (8). Different studies have confirmed the appropriate performance of DAT for detection of VL and CVL (8-12).

Although the sensitivity of DAT might be sufficient for detection of asymptomatic infected dogs (13), due to batch to batch variability of DAT antigens, development of a sensitive and specific serological method is still required for screening and diagnosis of symptomatic and asymptomatic infected reservoirs.

High sensitivity and specificity of Fucose-Mannose Ligand-ELISA (FML-ELISA) for diagnosis of CVL and human visceral leishmaniasis (HVL) has been previously documented in Brazil (14,15). Here we purified the Fucose-Mannose Ligand (FML) antigen from native *L. infantum* and examined the validity of FML-ELISA in comparison with DAT and PCR for detection of CVL.

MATERIALS AND METHODS

Study Area and Sample Collection. Blood samples of sixty ownership dogs (≤ 3 years old) were collected from Agh-bulagh village in Meshkin-shahr district in Ardabil province, North-west of Iran where VL is a major health problem (1,5). Meshkin-shahr is the center of Meshkin-shahr County, and is 839 kilometers far from Tehran, the capital of Iran. Being near the high Sabalan mountains, it enjoys a moderate mountainous climate. Agh-bulagh village is located in south of Meshkin-Shahr city and north of Sabalan high mountain.

All of the dogs were examined for clinical signs such as hair loss, cachexia and dermal lesions. The serum of collected blood samples were used for serological assays (DAT and ELISA) and the buffy coats were tested by molecular methods. All of the buffy coats and serum samples were stored at -20°C until use.

Direct Agglutination Test (DAT). Antigen for Direct Agglutination Test (DAT) was kindly provided by Protozoology Unit of the School of Public Health and Institute of Public Health research, Tehran University of Medical Sciences.

The serum samples from sixty dogs were tested by DAT for detection of anti-*Leishmania* antibody. Three dilutions of 1/80, 1/160 and 1/320 were examined for each

sample. Positive and negative controls were tested simultaneously in each plate. The titer of 1/320 and higher was considered as positive for CVL (5,12).

FML Antigen Preparation. FML antigen of native *L. infantum* was prepared as originally described by Palatnik et al. (16). Briefly, native *L. infantum* (MCAN/IR/07/Moheb-gh) was mass cultivated in BHI broth (37g/l) (Himedia, India) supplemented with 10% FCS (Biocera), hemin (0.01g/l) and folic acid (0.02 g/l) (Sigma, USA) at 25°C. The promastigotes were harvested after 5-7 days in stationary phase and washed three times with PBS. The pellet was mixed with cold distilled water and centrifuged and the supernatant was collected. This step was repeated once and the two supernatants were mixed and boiled for 15 minutes at 100˚C. The sample was then centrifuged and the supernatant was lyophilized for chromatography. The size exclusion chromatography was used with 1.6×100 cm column (Pharmacia, USA) and P10 Bio Gel (Biorad, UK). FML antigen was collected in void volume, lyophilized and stored at -20˚C until use.

Fucose-Mannose Ligand-ELISA (FML-ELISA). ELISA was carried out in flat-bottom 96-well microplates (Corning, USA). The microplates were coated with 5µg/ml of purified FML, diluted in phosphate-buffered saline (PBS), and incubated at 4°C overnight. Excess antigen was removed by washing with PBS. Blocking was performed with blocking buffer [3% skimmed milk in phosphate-buffered saline containing 0.05% Tween 20 (PBST)] and the plates were incubated at room temperature for 1.5 hours. The plates were washed twice with PBST. 100 µl of diluted sera (1/100 dilution in PBST) were added to the plates followed by their incubation at room temperature for two hours. After washing with PBST, 100 µl of conjugated anti-dog (HRPO-conjugated) diluted 1/3000 in PBST was added to each well and the plates were incubated at room temperature for another 1 hour. Plates were then washed with PBST, incubated with 100 µl of substrate solution (Ortho phenylenediamine, 100mg in 250ml citrate phosphate buffer and 125 µl hydrogen peroxide) at room temperature for 30 minutes. The optical density was determined at 490 nm (OD_{490}) using an ELISA plate reader. The cut off point was set as 3SD above the mean of control samples.

DNA Extraction. DNA was extracted from buffy coat samples of 60 dogs as described previously (17). Briefly, 50 µl of buffy coat and 150 µl of lysis buffer (Tris base, EDTA, Tween 20; pH=8) was mixed with 5 µl of proteinase K and incubated at 37°C for 24 hours. Then 75 µl of phenol: chloroform: isoamyl alcohol was added and mixed well. The sample was centrifuged at 15000 ×g for 10 minutes. Aqueous top phase was removed and absolute alcohol was used to precipitate the DNA.

kDNA Amplification by Nested PCR. Amplification of the kDNA of *Leishmania* was performed using a two-step nested PCR as originally described by Noyes et al. (18). The external primer pair, CSB2XF (C/ GA/GTA/ GCAGAAAC/ TCCCGTTCA) and CSB1XR (ATTTTTCG/ CGA/TTTT/ CGCAGA ACG) were used for the first-round of PCR amplification and the internal primer pair, 13Z (ACTGGGGGTTGGTGTAAAATAG) and LiR (TCGCAGAACGCCCCT) were used for the second-round of PCR amplification.

Electrophoresis of PCR Products. PCR products were electrophoresed on 1.5% agarose gel and positive samples were compared with DNA ladder for identification of *Leishmania spp*. In this nested PCR method, the size of PCR products varies in different species of *Leishmania*. It is 560 bp for *L. major*, 680 bp for *L. infantum* and 750 bp for *L. tropica* (18).

RESULTS

Antibodies to *L. infantum* (titer ≥ 1/320) were detected in serum samples of two out of 60 (3.33%) dogs by DAT. Both animals had cachexia and hair loss and one had dermal wounds as well. Moreover, one of the samples had antibody titer of 1/160 in DAT and this was the sample from a symptomatic dog with cachexia, hair loss and dermal wounds. Furthermore, six of animals had antibody titer of 1/80 but they were all asymptomatic. No antibody was detected in the rest of 51 animals and none of these animals had any symptoms of CVL.

When the samples were tested by FML-ELISA, seven out of 60 (11.6%) serum samples were positive by this assay. A sensitivity of 77.8% (95% CI=40.2%-96.05%) and a specificity of 100% (95% CI=91.3%-100%) were calculated for the FML-ELISA in comparison to PCR, as the golden standard. Positive predictive value (PPV) and negative predictive value (NPV) of the test were 100% (95% CI=56.1%-100%) and 96.2% (95% CI=85.9%-99.3%), respectively. Three of these seropositive dogs were symptomatic while the rest (four dogs) had no symptoms related to CVL upon clinical examination. Two of these seven seropositive dogs had antibody titers of ≥ 1/320 by DAT and one had serum antibody level of 1/160.

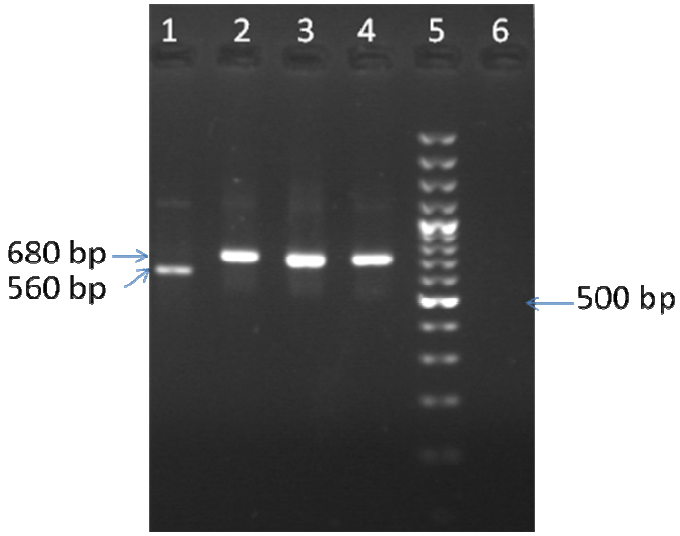

Figure 1. *Leishmania* minicircle kDNA amplification by nested PCR in dogs buffy coat samples. The size of PCR products varies in different species of *Leishmania* by this method. It is 560 bp for *L. major*, 680 bp for *L. infantum* and 750 bp for *L. tropica*. lane 1; *L. major* (control), lane 2; *L. infantum* (control), lane 3 and 4; *L. infantum* from infected dogs, lane 5; 100 bp ladder and lane 6; negative control.

All of the seven FML-ELISA positive samples were positive by PCR. When the buffy coat samples of the animals were tested by PCR, a 680 bp fragment corresponding to *L. infantum* was detected in nine out of 60 (15%) samples (Figure 1). Seven out of nine (77.7%) PCR-positive samples were positive by FML-ELISA while only two of them (22.2%) were found to be positive by DAT. Besides, six of PCR-positive dogs had no

CVL-related symptoms. Table 1 summarizes the performance of FML-ELISA, DAT and PCR in the diagnosis of symptomatic and asymptomatic CVL.

Table 1. Comparison of FML-ELISA, DAT and PCR in diagnosis of CVL in symptomatic and asymptomatic dogs.

Samples \ Methods	Symptomatic dogs 3(5%)		Asymptomatic dogs 57(95%)		Total 60 (100%)
	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)	
FML-ELISA	3 (100)	0 (0)	4 (7.02)	53 (92.98)	60 (100)
DAT	2 (66.67)	1 (33.33)	0 (0)	57 (100)	60 (100)
PCR	3 (100)	0 (0)	6 (10.53)	51 (89.47)	60 (100)

DISCUSSION

FML-ELISA has been used for diagnosis of human visceral leishmaniasis in Brazil and has showed 100% sensitivity and 96% specificity for identifying patients with overt Kala-azar (15). It has also been reported that FML-ELISA could identify subclinical VL infections (15). The same group used FML-ELISA for detection of canine visceral leishmaniasis in Brazil with a sensitivity and specificity of 100%. The assay could also identify the asymptomatic dogs, with 100% predictive value (15).

In this study we prepared FML antigen from native *L. infantum* and evaluated the performance of FML-ELISA for detection of VL-infected dogs in Meshkin-shahr, an area in the Northwest of Iran which is known as one of the main foci of VL. Seven out of 60 ownership dogs were FML-ELISA positive in this study whereas only three of the dogs had clinical signs. On the other hand, four out of seven FML-ELISA positive dogs were asymptomatic. These findings are in agreement with the results of Borja Cabrera et al. (15) study in Brazil. We compared the results of our FML-ELISA with DAT and PCR and found that FML-ELISA is a suitable assay for detection of CVL in both symptomatic and asymptomatic dogs. Performance of serological methods, such as DAT and IFA, for identification of VL and CVL has been evaluated in several studies. Zijlstra et al. reported a sensitivity of 94% and specificity of 72% for DAT in comparison with tissue direct microscopy for diagnosis of Kala-azar (19). In a study by Mikaeili et al. (20), sensitivity and specificity of three serological methods for detection of anti-*Leishmania* antibodies in VL patient were found to be 70.5% and 100% for DAT, 83.6% and 90.5% for ELISA, and 80.3% and 90.5% for IFA, respectively. In a similar study, Sarkari et al. (21) have reported a sensitivity and a specificity of 83.6% and

90.5% for ELISA and 70.5% and 100% for DAT, respectively. Similar results for ELISA (sensitivity = 86.2%, specificity = 90.5%) and DAT (sensitivity = 93.1%, specificity = 100%) were reported by Ghatei et al. (22). Sera samples from parasitologically confirmed VL patients have been used in all of the aforementioned studies.

Performance of DAT for detection of *L. infantum* infection in dogs has been assessed in few studies. Mohebali et al. found a sensitivity of 70.9% and a specificity of 84.9% at a 1/320 cut off point for detection of anti-*Leishmania* antibody in dogs (11). In another study in Sudan, sensitivity of 100% and specificity of 98.9% have been reported for DAT in the diagnosis of CVL (9).

Recent molecular studies on the diagnosis of CVL showed considerable discrepancies between the results of serological and molecular methods (13,23,24). In the study of Moshfe et al. which has been performed in the same area as our study, sixteen out of 66 symptomatic and asymptomatic dogs were reported to be positive by DAT whereas 37 of them were found to be positive by PCR (13). Our findings are in agreement with previous studies (13,23,24,25) which show that molecular methods have higher sensitivity for diagnosis of symptomatic and asymptomatic infection in dogs. In the study of Moshfea et al. DAT has not been able to detect all of the asymptomatic infected dogs (13). Asymptomatic dogs, as reservoirs, can play an important role in the epidemiology of human and canine VL (26).

Considering the results of this study, the performance of the FML-ELISA for the detection of CVL in both symptomatic and asymptomatic dogs is significant. Seven out of nine PCR-positive dogs were positive by FML-ELISA and all of the FML-ELISA positive samples were positive by PCR. Considering PCR as a gold standard method, sensitivity and specificity of present FML-ELISA for the detection of CVL in both symptomatic and asymptomatic dogs would be 77.8% and 100%, respectively. It should be noted that PCR can detect both infection and the disease in the animals. Therefore PCR might not be a suitable gold standard method for diagnosis of CVL. Other serological methods are unable to detect the infected dogs in the early stages and may show low sensitivity and specificity if PCR is considered as a gold standard and that is what we are seeing in our study.

In view of the high diagnostic performance of FML-ELISA, this serological method can be recommended for epidemiological surveys of CVL in any VL endemic areas, especially in Iran. Further studies are needed for proper evaluation of FML-ELISA in comparison with other serological and parasitological assays for the diagnosis of human VL and also CVL in our country.

ACKNOWLEDGMENTS

This article represents part of the Ph.D. thesis written by Behnam Mohammadi-Ghalehbin at the School of Medicine. The study was financially supported by the office of Vice-chancellor for research of Shiraz University of Medical Sciences (Grant No: 5182) and also technically supported by Meshkin-Shahr station for Training and Research, National Institute of Health Research (NIHR), as well as School of Public Health, Tehran University of Medical Sciences. The authors thank Mrs. P. Habibi, Mr. M. Kalantari, Mrs. B. Akhoundi, Mrs. S. Charehdar, Mrs. S. Molaei, and Mr. Khanmohammadi for their technical assistance.

REFERENCES

1 Edrissian GhH, Nadim A, Alborzi AV, Ardehali S. Visceral leishmaniasis: the Iranian experiences. Arch Iranian Med. 1999; 1:22-6.
2 Mohebali M, Hamzavi Y, Edissian GH, Frouzani A. Seroepidemiological studies of visceral leishmaniasis in Bushehr province, Islamic Republic of Iran. East Mediterr Health J. 2001; 7:912-7.
3 Mohebali M, Hamzavi Y, Edissian GH, Frouzani A. Seroepidemiological studies of visceral leishmaniasis in Bushehr province, Islamic Republic of Iran. East Mediterr Health J. 2001; 7:912-7.
4 Sarkari B, Pedram N, Mohebali M, Moshfe AA, Zargar MA, Akhoundi B, et al. Seroepidemiological study of visceral leishmaniasis in Booyerahmad district, south-west Islamic Republic of Iran. East Mediterr Health J. 2010; 16:1133-6.
5 Mohebali M, Edrissian Gh H, Shirzadi M R, Akhoundi B, Hajjaran H, Zarei Z, et al. An observational study on the current distribution of visceral leishmaniasis in different geographical zones of Iran and implication to health policy. Travel Med Infect Dis. 2011; 9: 67-74.
6 Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis. 2004; 27:305-18.
7 WHO. Manual on visceral leishmaniasis control; Geneva, WHO/LEISH/96.40. 1996.
8 Boelaert M, El Safi S, Jacquet D, de Muynck A, van der Stuyft P, Le Ray D. Operational validation of the direct agglutination test for diagnosis of visceral leishmaniasis. Am J Trop Med Hyg. 1999; 60:129-34.
9 el Harith A, Salappendel RJ, Reiter I, van Knapen F, de Korte P, Huigen E, et al. Application of a direct agglutination test for detection of specific anti-Leishmania antibodies in the canine reservoir. J Clin Microbiol. 1989; 27:2252-7.
10 Edrissian GhH, Hajjaran H, Mohebali M, Soleimanzadeh G, Bokaei S. Application and evaluation of direct agglutination test in ser-diagnosis of visceral leishmaniasis in man and canine reservoirs in Iran. Iran J Med Sci. 1996; 21:119-24.
11 Mohebali M, Taran M, Zarei Z. Rapid detection of Leishmania infantum infection in dogs: comparative study using an immunochromatographic dipstick rk39 test and direct agglutination. Vet Parasitol. 2004; 121:239-45.
12 Mohebali M, Hajaran H, Hamzavi Y, Mobedi I, Arshi Sh, Zarei Z, et al. Epidemiological aspects of canine visceral leishmaniosis in the Islamic Republic of Iran. Vet Parasitol. 2005; 129:243-51.
13 Moshfe A, Mohebali M, Edrissian G, Zarei Z, Akhoundi B, et al. Canine visceral leishmaniasis: Asymptomatic infected dogs as a source of L. infantum infection. Acta Trop. 2009; 112:101-5.
14 Palatnik de Sousa CB, Gomes EM, Paraguai de Souza E, Palatnik M, Luz K, Borojevic R. Leishmania donovani: titration of antibodies to the fucose-mannose ligand as an aid in diagnosis and prognosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 1995; 89:390-3.
15 Cabrera GP, Da Silva VO, Da Costa RT, Reis AB, Mayrink W, Genaro O, et al. The Fucose-Mannose Ligand-ELISA in the diagnosis and prognosis of canine visceral leishmaniasis in Brazil. Am J Trop Med Hyg. 1999; 61:296-301.
16 Palatnik CB, Borojevic R, Previato JO, Mendonça-Previato L. Inhibition of Leishmania donovani promastigote internalization into murine macrophages by chemically defined parasite glycoconjugate. Infect Immun. 1989; 57:754-63.
17 Mehrabani D, Motazedian MH, Oryan A, Asgari Q, Hatam GR., Karamian M. A search for the rodent hosts of Leishmania major in the Larestan region of southern Iran: demonstration of the parasite in Tatera indica and Gerbillus sp, by microscopy, culture and PCR. Ann Trop Med Parasitol. 2007; 101:315-22
18 Noyes HA, Reyburn H, Bailey JW, Smith D. A nested-PCR based schizodeme method for identifying Leishmania kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of Leishmania tropica in Pakistan. J Clin Microbiol. 1998; 36:2877-81.
19 Zijlstra EE, Ali MS, el-Hassan AM, el-Toum IA, Satti M, Ghalib HW, et al. Direct agglutination test for diagnosis and sero-epidemiological survey of kala-azar in the Sudan. Trans R Soc Trop Med Hyg. 1991; 85:474-6.
20 Mikaeili F, Fakhar M, Sarkari B, Motazedian MH, Hatam G. Comparison of serological methods (ELISA, DAT and IFA) for diagnosis of visceral leishmaniasis utilizing an endemic strain. Iran J Immunol. 2007; 4:116-21
21 Sarkari B, Hatam GR, Mikaeili F, Sadeghi H, Ebrahimi S. A comparative study of antigen and antibody detection in visceral leishmaniasis using serum and urine-based ELISA. Trop Biomed. 2008; 25:96-9.
22 Ghatei MA, Hatam GR, Hossini MH, Sarkari B. Performance of latex agglutination test (KAtex) in diagnosis of visceral leishmaniasis in Iran. Iran J Immunol. 2009; 6:202-7.
23 Nasereddin A, Ereqat S, Azmi K, Baneth G, Jaffe CL, Abdeen Z. Serological survey with PCR validation for canine visceral leishmaniasis in northern Palestine. J Parasitol. 2006; 92:178-83.
24 Ashford DA, Bozza M, Freire M, Miranda JC, Sherlock I, Eulalio C, et al. Comparison of the polymerase chain reaction and serology for the detection of canine visceral leishmaniasis. Am J Trop Med Hyg. 1995; 53:251-5.
25 Fallah E, Khanmohammadi M, Rahbari S, Farshchian M, Farajnia S, Hamzavi F, et al. Serological survey and comparison of two polymerase chain reaction (PCR) assays with enzyme-linked immunosorbent assay (ELISA) for the diagnosis of canine visceral leishmaniasis in dogs. Afr J Biotechnol. 2011; 10:648-56.
26 Alvar J, Molina R, San Andres M, Tesouro M, Nieto J, Vitutia M, et al. Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy. Ann Trop Med Parasitol. 1994; 88:371-8.