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Full Length Research Paper

Seroepidemiological survey of canine *Visceral leishemanisis* in Sarab District, East Azerbaijan Province, Northwest of Iran in 2009

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***Visceral leishmaniasis* (VL) is one of the most important endemic parasitic diseases in different parts of Iran. It is also a health problem in some tropical and subtropical countries. The purpose of the present study was to determine the seroprevalence of canine VL (CVL) in owned dogs of Sarab and to identify the isolated *Leishmania* species. Sera samples were taken from 384 dogs from 30 random villages around Sarab and tested by enzyme-linked immunosorbent assay (ELISA) . A total of thirty five dogs were seropositive. The seroprevalence rate (SPR) of CVL was 9.1% (95 % CI; 6.6 - 12.4). Out of 361 (94%) asymptomatic dogs, 31 (8.6%) were seropositive, and out of 23 (6%) symptomatic dogs, 4 (17.4%) were seropositive. 306 (79.7%) dogs were male and 78 (20.3%) were female. 28 (9.2%) male dogs and 7 (9%) female dogs were seropositive. There was no statistically significant relation between sex and seroprevalence (p = 0.962). The largest age group was 2 - 4 years, with 170 (44.3%) dogs out of which only 18 dogs (10.5%) were seropositive. Agreement between the ELISA test and clinical signs was 86.7%. A significant difference in the presence of antibodies against *Leishmania* was seen between symptomatic and asymptomatic dogs (p = 0.015) . ELISA was efficiently sensitive in the identification of infected dogs and seems to be an appropriate tool in rapid diagnostic screenings. ELISA is highly recommended to be applied as a standard test for routine CVL diagnosis.**

Key words: Dogs, enzyme-linked immunosorbent assay (ELISA), epidemiology, Iran, *Visceral leishmaniasis,* prevalence, serology.

INTRODUCTION

Infection with the protozoan parasite *Leishmania* spp. (Kinetoplastida: Trypanosomatidae) appears in three different forms: cutaneous, mucocutaneous and *Visceral leishmaniasis* (VL). VL, also called Kala-azar, is the most severe form of *leishmaniasis* caused by the *L. donovani* complex species of *Leishmania* (*L. donovani, L. d. infantum, and L. d. chagasi*) (Farajnia et al., 2008). VL is a systemic zoonotic infection endemic in more than 80 tropical and subtropical countries. Up to half a million new cases of VL occur worldwide every year (Desjeux et al., 2004). A prevalence of 1 - 37% has been reported in

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Mediterranean areas, and the World Health Organization reported VL disease, as one of the six most important human infectious diseases in the world (Fakhar et al., 2004; Gradoni et al., 1995). Canine VL (CVL) is a zoonotic disease in endemic areas, and the disease is more common in Mediterranean regions and Middle East (Mohebali et al., 2005; kanmohammadi et al., 2010). The incidence of *leishmaniasis* depends on a variety of ecological and biological factors. Since the presence of various *Leishmaniasis* species depend as much on the specific reservoirs as on the specific vector species, a focal *Leishmania* can only exist if suitable ecological conditions are present for both the host animal species and the vector sandfly species.

The topography and the climate are essential for the maintenance of the parasite life cycle. The transmission of the parasite and main-tenance of the infectious cycle are only possible if the reservoir and the vector live close enough together. VL in the countries of Mediterranean basin and Middle East including Iran is caused by *Leishmania infantum* (Mohebali et al., 2005). Dogs (*Canis familaris*) act as domesticated hosts, and jackals, foxes and wolves are the major wild reservoir hosts for CVL (Mohebali et al., 2005; Fakhar et al., 2004; Mohebali et al., 2002 and Moshfe et al., 2008). Sandfly vectors belonging to *Phlebotomus* spp. and *Lutzoumyia* spp. are responsible for the transmission of *Leishmania* spp. between humans and animal reservoirs (Abranches, 1991; Belazzoug, et al., 1992 and Mohebali, et al., 2005). Asymptomatic dogs are the most important source for parasite transmission to humans (Mohebali et al., 2005). Up to now, at least four endemic disease foci have been studied and approved in Ardebil province (Meshkin shahr and Moghan, Ghermi, Pars Abad and bille-savar), east Azerbaijan province (Kaleybar, Ahar and Azarshahr), Fars province (Jahrom, Ghir and kazeron), Semnan province, Bushehr province, Qom province, Kerman province and Karaj (Fakhar et al., 2004) . Sporadic cases of VL are reported from other parts of Iran every year (Mohebali et al., 2005). *L. infantum* LON49, was determined as the species responsible for the occurrence of *Leishmania* in animal reservoirs of Ardebil and east Azerbaijan (Mohebali et al., 2005).

This *Leishmania* strain was mostly the same strain isolated from many cases of people with human *Visceral leishmaniasis* (HVL), it may then be concluded that canines are important animal reservoirs in HVL (Abranches et al., 1991; Mohebali et al., 2005). Because of the similarity of a wide spectrum of clinical signs and symptoms between HVL and other etiologic factors, the diagnosis of VL may be challenging (Harris et al., 1998). In fact, microscopic detection of the parasite, routine Giemsa staining, or cultivation tests are the easiest diagno-stic approaches. The microscopic examination is easy, fast and cheap; however, its sensitivity is decreased when there are few parasites in tissues. On the other hand, it was not possible for us to differentially diagnose *Leishmania* species, since bone marrow, lymph node, and spleen biopsies are very aggressive approaches and require animal sacrifice (Reale et al., 1999; Singh et al., 1999; Aransay et al., 2000; Da silva et al., 2001 and Nasereddin et al., 2006). ELISA test is widely used to detect CVL and HVL, but its sensitivity and specificity depends on the type of antigen used. Different types of antigens, including crude and recombinant proteins (dp72, gp70, rK39, and HSP70), have been reported (Nasereddin et al., 2006). Despite the observation of cross-reactions with other diseases, crude antigen solutions are still widely used (Ashford et al., 1995). Anti-*Leishmania* antibody titers are typically high during the acute stages of the disease, and this feature has been exploited for the serodiagnosis of VL using different methods (Farajnia et al., 2004).

MATERIALS AND METHODS

Study area, dog population and sampling

Sarab District is located in east Azerbaijan province in northwestern Iran with moderate mountainous climate. It covers an area of approximately 18/3452 km², including 168 villages, and its population is estimated to be 148,831 of which 43% is settled in urban areas and 57% is settled in rural areas. Most of the inhabitants of Sarab District are involved in agriculture and animal husbandry, live in mud or stone houses, and maintain domestic animals, such as sheep, goats, chickens, and dogs. The city of Sarab is situated at an altitude of 1650 m above the sea level and is the closest city to Sabalan Mountain (Figure 1).

The present cross -sectional study was performed using multi-stage cluster random sampling. Out of 168 villages in Sarab District, 30 villages (cluster) were selected randomly. Based on a previous study, the least percentage of serologicaly symptomatic dogs was reported to be 4% in endemic area (Meshkin shahr) (Moshfe et al., 2008; Mohebali et al., 2005). Since this percentage seems to be very low in value, the maximum possible number of samples was attempted to be included in the present study (one possible dog per ten possible human) in order to obtain the most reliable data. With a 95% confidence interval and a marginal error of less than 2%, 384 dogs were selected. The following information was obtained for each dog using a questionnaire: Owner's name; dog's age; gender; hair colour; size; habitancy location; presence or absence of VL symptoms (skin abnormalities including dry exfoliative dermatitis, periorbital alopecia, hair loss, cachexia, lethargy and onychogryphosis, local or general lymphadenomegaly, keratoconjunctivitis, big belly, diarrhoea, and splenomegaly); and other distinctive characteristics of each animal owner. Each dog was assigned a number for identification purposes. A 10 cc sample of peripheral blood was taken from each dog. Three to four smears were quickly prepared prior to coagulation. Also 5 ml of each blood sample was dispensed into polypropylene tubes. To prevent lysis of blood samples after 6 to 10 h, blood samples were immediately taken to the Sarab Hospital laboratory, and sera were isolated by centrifugation at 2000 rpm for 20 min.

Enzyme-Linked Immunsorbent Assay (ELISA)

ELISA Indirect kits (ID Screen® Leishmania Indirect, Code: LEIS, Paris, France) were used for detection of canine antibodies direct against *L. infantum*. First, the *L. infantum* diagnostic kits were retrieved from refrigerator and antigen-coated micro plates prepared in raised laboratory temperature (5 ± 21˚C). A Biolinx

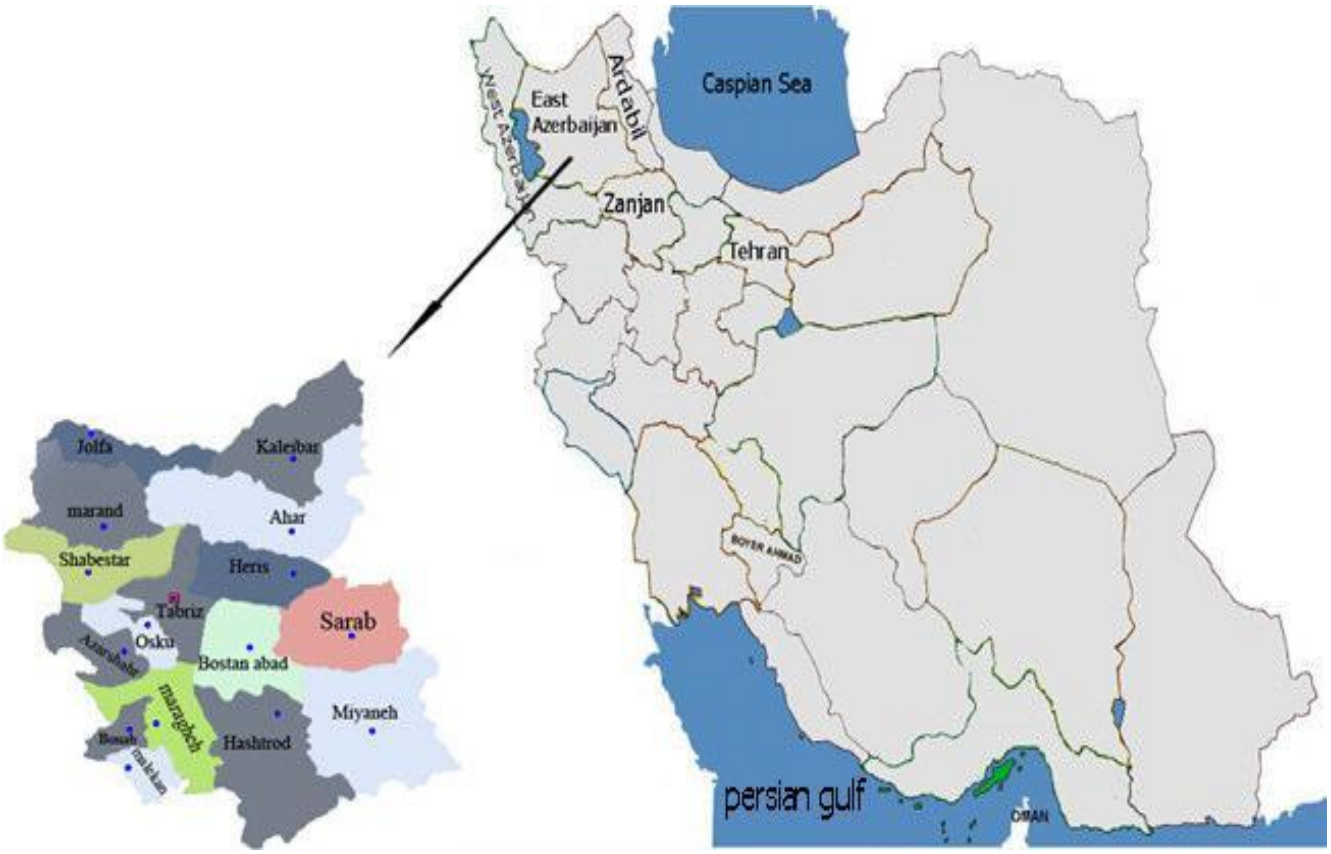

Figure 1. Geographical map of Sarab District in East Azerbaijan Province, northwestern Iran, showing the study area.

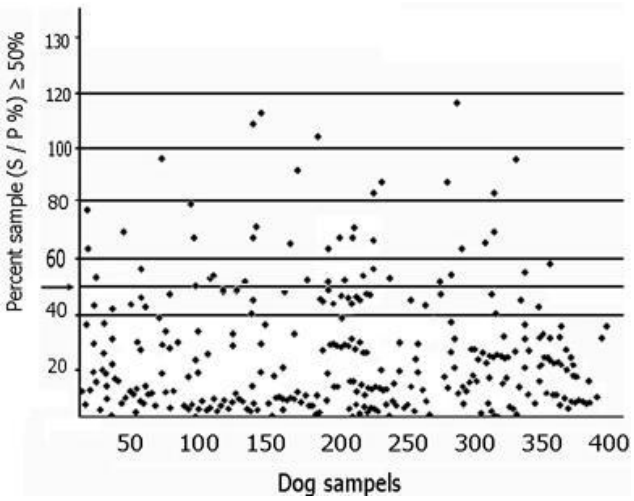

Figure 2. ELISA for antileishmanial antibodies against *L. infantum* antigen in sera of 384 dogs from 30 villages in the Sarab district, Arrow indicates the cut-off value.

Dynatech ELISA reader (Figure 2) (Dynatech Laboratories, Roseville, Canada) was then used to read the plates at 450 nm. The final optical density of each sample was calculated according to the manufacturer's instructions using the following formula:

OD positive control / OD negative control > 3 and percent sample (S / P %) = OD sample / OD positive control × 100.

Percent sample (S / P %) 50% was positive (Table 1).

Parasite isolation

Seropositive dogs were autopsied to determine parasite species and strains. Spleen biopsies and bone marrow aspirates were cultured in specific media for *Leishmania*, including NNN medium supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich, Dorset, UK), RPMI 1640 or Schneider's Drosophila Medium (Gibco, Invitrogen, USA). Cultures were examined after 48 h to assess the presence and growth of promastigotes. This procedure was repeated once a week for five weeks if results were negative. When no growth was observed after this period, then cultures were presumed to be negative. Positive NNN cultures were transferred to -MEM (minimum essential medium) fluid medium for large-scale cultures. These cultures were transferred to the Applied Drug Research Centre at Tabriz University of Medical Sciences for PCR testing (kDNA, ITS-18sRNA). The primers used to amplify kDNA were 5'-TCGCAGAACGCCCCTACC-3' (forward) and 3 -AGGGG-TTGGTGTAAAATAGG (reverse), and the primers used to amplify ITS-18sRNA were 5'-CTGGATCATTTTCCGATG-3' (forward) and 3 -ACACTCAGGTCTGTAAAC (reverse). DNA from *L. infantum* was used as a positive control and sample containing all PCR materials without DNA used as a negative control. ITS-18sRNA PCR products were separated on 1.5% polyacrylamide gels in 1X Tris-acetate-EDTA (TAE) buffer at 12 V/cm, (Figure 4). and kDNA PCR

Table 1. Result of ELISA Indirect kits.

Position	Result percent sample (S/P%)
Negative	40
Doubtful	> 40
Positive	50

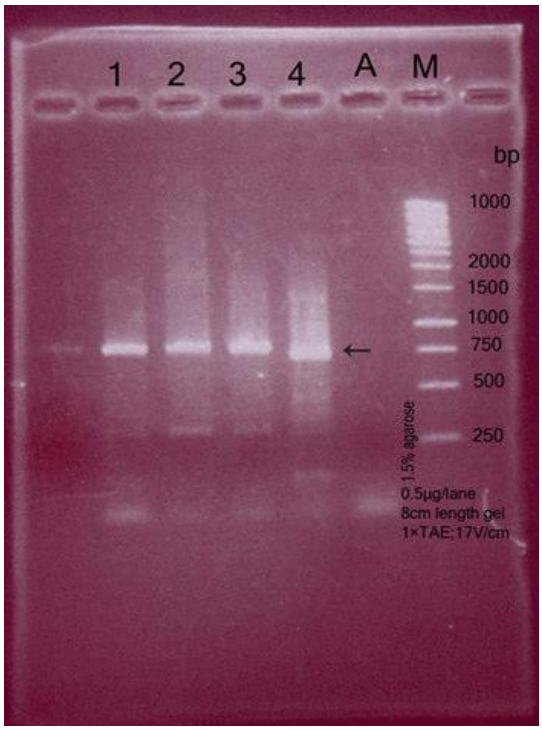

Figure 3. Amplification of parasite DNA of PCR products (KDNA), DNA (1 ng) extracted from cultures of parasite isolates was used for PCR. Lane M: Marker, 100 bp ladder. Lane A: Negative Control. Lane 4 *L. infantum* (Standard strain *L. infantum,* (MCAN/IR/96/LON49) School of Public Health, Tehran University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

products were separated 17 V/cm (Gen Ruler, Fermentas). All gels were stained with ethidium bromide (10 mg/ml in ddH2O), visualized, and images were captured using a UV transilluminator imaging system (UV- GENTM; Bio-Rad Laboratories) (Figure 3 and 4) . A suitable marker was used to determine the molecular weights of the PCR products. Parasite species were identified by comparing the electrophoresis pattern (fingerprint) with reference strains of *L. infantum*, (MCAN/IR-/96/LON49), *L. tropica*, (MHOM/IR102/Mash4) and *L. major* (MRHO/IR/75/ER) . Seropositive samples were then confirmed using primers specific for *L. infantum*, and PCR products were analyzed by electrophoresis.

DNA isolation and PCR amplification

Genomic DNA was isolated as described. Briefly, logarithmic phase promastigotes were disrupted in lysis buffer (50 mM NaCl, 50 mM

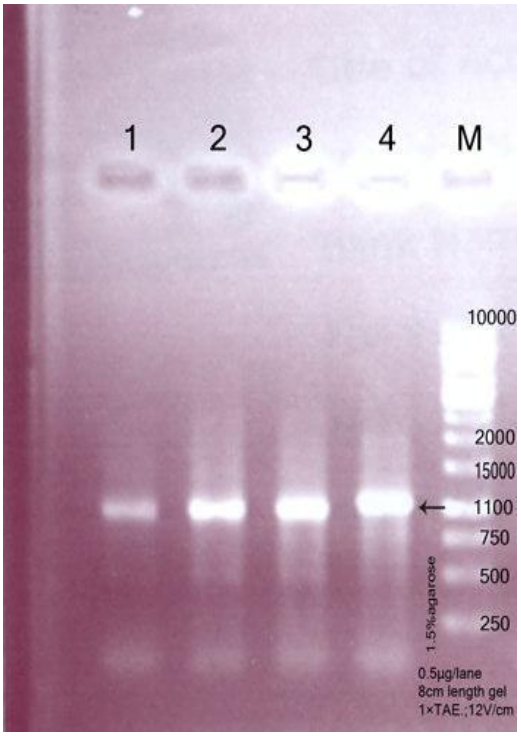

Figure 4. Amplification of parasite DNA of PCR products (ITS-18sRNA), DNA (1 ng) extracted from cultures of parasite isolates was used for PCR. Lane M: Marker, 100 bp ladder. Lane 4 *L. infantum* (Standard strain *L. infantum,* (MCAN/IR/96/LON49) School of Public Health, Tehran University of Medical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

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 further purified by phenol-chloroform extraction and ethanol precipitation. An Eppendorf DNA thermal cycler and Taq DNA polymerase (Roche, Mannheim, Germany) was used to amplify the desired gene. The reaction mixture included 10 pmol of each primer, 200 mM dNTPs and 1.5 mM MgCl2. PCR conditions were as follows: initial 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).

Statistic analysis

SPSS software version 13.5 (SPSS Inc., Chicago, Ill) was used for statistical analysis. A probability (p) value of < 0.05 was considered as statistically significant. Kappa (k) was measured to determine the consistency between the results of ELISA and the clinical symptoms. Sensitivity and specificity of tests were examined using chi-square (2) and Fisher's exact tests.

RESULTS

Seroepidemiological survey

In the present study, sera samples were taken from 384

Table 2. Seroprevalence of CVL infection by gender in Sarab district (2008-2009)

Gender	No. of dogs (%)	*ELISA test positive	
		No	Seroprevalence (%)
Male	306 (79.7)	28	9.2
Female	78 (20.3)	7	9
Total	384	35	9.1

*Enzyme-linked immunosorbent assay.

Table 3. Sero-prevalence (Elisa) of CVL infection by sings and symptoms in Sarab district (2008-2009).

Single symptoms	No. of dogs (%)	ELISA Test Positive	
		No. of dogs	Seroprevalence (%)
Symptomatic	23(6)	4	17.4
Asymptomatic	361(94.)	31	8.6
Total	384(100)	35	9.1

dogs from 30 random villages around Sarab and tested by enzyme-linked immunosorbent assay (ELISA). A total of thirty five dogs were seropositive. The seroprevalence rate (SPR) of CVL was 9.1% (95% CI; 6.6 - 12.4). 306 (79.7%) dogs were male and 78 (20.3%) were female. 28 (9.2%) male dogs and 7 (9%) female dogs were seropositive (Table 2). Out of 361 (94%) asymptomatic dogs, 31 (8.6%) were seropositive, and out of 23 (6%) symptomatic dogs, 4 (17 .4%) were seropositive (Table 3). There was no statistically significant relation between sex and seroprevalence (p = 0.962). The largest age group was 2 - 4 years, with 170 (44.3%) dogs out of which only 18 dogs (10.5%) were seropositive (Table 4). Agreement between the ELISA test and clinical signs was 86.7%. A significant difference in the presence of antibodies against *Leishmania* was seen between symptomatic and asymptomatic dogs (p = 0.015). Based on Kappa meas-urement, ELISA test and clinical signs were 86.7% con-sistent. The highest rate of infection was seen in Jalde Bakhan village, with 9 (33.3%) infected dogs. The lowest rate of infection was detected in Asb Froshan village, with only 1 infected dog (1.4%) (Table 5). Because some dog owners were disinclined to perform test or sampling, we tested 28 animals from between 35 volunteered seropositive dogs for parasitological examination. For each dog, 20 impression smear slides from spleen, liver and bone marrow tissues were prepared, Giemsa-stained and examined for Leishman-Donovan bodies. These were observed in the autopsy slides of liver and spleen tissues from 21 (75%) dogs. Of the impression smear slides, 10-0% of spleen and 85.7% of liver samples were positive.

DISCUSSION

At the present, VL is known as an endemic disease in some areas of Iran and also as a sporadic disease in all parts of the country. Many factors such as environmental conditions, density of dogs as the reservoir host, and *Phlebotamus* as the vector are effective on the occurence of VL (Fakhar et al., 2004). Epidemiological studies on VL hosts have been discouraged by the lack of sufficiently sensitive and practical methods to detect infections of various species. Negative parasitological results do not rule out *Leishmania* infection in dogs. In many cases, a combination of clinical, parasitological, serological, and therapeutical tests has to be done to confirm *Leishmania* infection in the reservoir host (Harith et al., 1989). Several diagnostic tests, such as the indirect immunofluorescence antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), microscopic examination of smears, and cultural isolation, are routinely used for diagnosis. The gold standard for VL diagnosis is the detection of parasite in specimens of infected organs. For this purpose, invasively obtained samples such as bone marrow (BM), lymph nodes, or spleen aspirates are typically needed (Mohebali et al., 2004) . Blood Sampling is less invasive, easily repeatable, and more readily accepted by dog owners than bone marrow, spleen, or lymph node aspiration. The overall seroprevalence rate (SPR) of CVL was 9.1% in Sarab District. Similar findings were reported by Abranches et al. (1991) in Portugal, Poaio et al. (1981) in Italy, Sideris et al. (1996) in Greece, Molina et al. (1994) in Portugal, Hamidi et al.(1982), Soleimanzadeh et al. (1993), Bokai et al.(1998), Gavgani et al. (2002) Mohebali et al., and Moshfe et al. (2008) in Iran. In a study on the dog infections in Baft (Kerman province), IFA and ELISA showed estimated values of 18 and 14.5%, respectively (Sharifi et al., 1994). In another study in Qort Tappeh village of Meshkin Shahr, there were 12.2 and 16.4% positive serums, respectively from 164 examined dogs according to DAT and ELISA methods (Mohebali et al., 2005). In other studies by the same

Table 4. Seroprevalence of CVL infection by age in dogs in Sarab district (2008-2009)

Age group (years)	No, of dog (%)	ELISA Test positive	
		No. of dogs.	Seroprevalence (%)
0 – 2	126 (32.8)	9	7.1
2 – 4	170 (44.3)	18	10.5
4 – 6	87(22.6)	8	9.1
≥ 7	1(0.3)	-	-
Total	384 (100)	35	9.1

Table 5. Distribution of CVL infection different villages of Sarab district.

Village	No. of dog (%)	Elisa test positive	
		No. of dogs	Seroprevalence (%)
Ardeha	40 (10.4)	5	12.5
Jahizdan	15 (3.9)	3	20
Bahraman	16 (4.1)	4	25
Arzang	31 (8.1)	2	6.4
Baraghosh	22 (5.7)	5	22.7
Asbfroshan	70 (18.2)	1	1.4
Jaldbakhan	27 (7.03)	9	33.3
Khaki	30 (7.8)	1	3.3
Asfestan	28 (7.2)	5	17.8
Total	279 (72.6)	35	12.5

group in 2000 in Parikhan village of Meshkin Shahr, 4.9 and 9.8% positive serum results were detected in 344 dogs, respectively with DAT and ELISA tests (Mohebali et al., 2005). In a study on the dogs of Meshkin Shahr city, only 13.6% positive serums findings were associated with positive clinical signs. From 22 dogs whose antibody titer anti *Leishmania* with DAT method reached 1:2048, only 12 dogs (54.5%) had clinical symptoms (Bokai et al., 1998). The most important serological result was a high proportion of seropositivity for *leishmaniasis* (8.6%) 31/-361 among asymptomatic dogs. These findings are con-sistent to those of other similar studies (Sideris et al., 1996, Bokai et al., 1998, Gavgani et al., 2002, Mohebali et al., 2005, Moshfe et al., 2008).The concept presented hereby is therefore very important because asymptomatic house-dogs can play an important role in the epidemiology of this zoonosis. In a previous study in Spain, asymptomatic dogs as well as symptomatic cases could be a cause when ability of sandflies to pick up infection is not dependent on clinical manifestations (Molina et al., 1994). Undoubtedly, this condition indicates previous contact with the parasite, but we do not know whether these dogs are immune resistant animals or whether they will subsequently develop the disease (Moshfe et al., 2008). Thus, the present study and others confirm that the prevalence of *Leishmania* infection in dogs has been underestimated. So, the decrease in domestic dog population may have a key role in controlling the epidemiology and the transmission of VL to human beings. It seems that both symptom-free dogs and dogs with clinical symptoms potentially transmit VL to human beings. In the present study, *L. infantum* infection was more prevalent in 2 to 4 years old dogs (mean age) than other age groups. Accordingly, Bokai showed the highest infection in average-age dog groups (Bokai et al., 1998). In the studies of Mohebali, dogs aged 8 and more showed the highest seroprevalence (Mohebali et al., 2005). Consistent to other studies, no statistically significant relations were found between *Leish-mania* infection and gender in our study (Abranches et al., 1992 in Portugal, Poaio et al., 1981 in Italy, Sideris et al., 1996 in Greece, Bokai et al., 1998, Mohebali et al., 2005 and Moshfe et al., 2008 in Iran). Additional surveys are needed to establish and confirm the trend of CVL incidence in this region since the annual prevalence of CVL in end-emic regions fluctuates freq-uently and can vary among adjacent villages. Results imply that the prev-alence of CVL may be an important risk factor for the incidence of HCL in this region (Gavgani et al., 2002). Therefore, in order to control VL in epidemic areas, it is suggested that all the stray dogs be Collected and the owned ones be monitored with serolo-gical tests and be killed in the cases of positive results in above-mentioned test. It should be noted that, screening programs on infected dogs will be almost impossible without reliable serological assessments measures to detect the

asymptomatic reservoirs. Essentially, elimini-nation of infected animals has been recommended, but alternative control measures should be recommended for ethical and social reasons (Moshfe et al., 2008).

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