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Original Article

Seroepidemiological Study on Canine Visceral Leishmaniasis in Meshkin-Shahr District, Ardabil Province, Northwest of Iran during 2006-2007

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Abstract

Background: This study aimed to determine the seroprevalence of canine visceral leishmaniasis in Meshkin-Shahr district as endemic areas of human visceral leishmaniasis (HVL) for presenting control program of HVL to health authorities.
Methods: A seroepidemiological study to determine seroprevalence of canine visceral leishmaniasis (CVL) among ownership dogs using direct agglutination tests (DAT) in 21 villages of Meshkin-Shahr district, Ardabil Province was carried out from June 2006 to August 2007. Three hundred and eighty four ownership dogs were selected by multi-stage cluster sampling. Chi-square and Fisher exact tests were used to compare seroprevalence values relative to gender, age and clinical signs.
Results: Of the 384 serum samples tested by DAT, 17.4 %(95%C.I, 13.2%-20.8%) were positive (1:320 and higher). No statistical significant difference was found between male (16.5%) and female (20.2%) seroprevalence (P=0.416). The highest seroprevalence rate (64.2%) was observed among the ownership dogs of three years age and above. Only 25.4% of the seropositive dogs had clinical signs and symptoms. The most clinical signs among symptomatic dogs were cachexia (75%) and alopecia (36.5%).
Conclusion: The majority of seropositive dogs (74.6%) lived in endemic areas of Meshkin-Shahr district were asymptomatic. It seems that all symptomatic and asymptomatic infected dogs are the most important risk factors for human infection in VL endemic areas.

Keywords: *Canine visceral leishmaniasis, Seroepidemiology, Direct agglutination test, Iran*

Introduction

Every year, approximately 500,000 new cases of VL (1, 2) which cause 59,000 human deaths annually, are reported from various parts of the world (3) and incidence rate of the disease is increasing in some countries (1).
Since 1980, annually more than 200 human cases have been diagnosed from North West of

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Iran, mostly, from Meshkin-Shahr areas (Fig. 1). It seems, that kala-azar has being endemic in this area for a long time (4). Canine leishmaniasis is not only a veterinary problem but is also a serious public health problem. Rapid detection of canine visceral leishmaniasis (CVL) is highly important for control of human visceral leishmaniasis.

Domestic dogs (*Canis familiaris*) are the most important animal reservoir hosts of CVL, which is transmitted among canines and to humans by phlebotomine sand flies (5-8). Dog ownership is considered as an important risk factor for human infections in the endemic areas of the disease in Iran (9). In a seroepidemiological study, the seroprevalence of infection was obtained 14.8% in the studied dogs in Meshkin-Shahr district (10).

The percentage of infected dogs living in an area where canine visceral leishmaniasis is endemic has major public health implications. It was demonstrated that infected, but asymptomatic dogs were sources of the parasite for phlebotomine vector sandflies and consequently play an active role in the transmission of *Leishmania infantum* (11).

CVL caused by *L. infantum* is an endemic zoonotic disease in the Mediterranean basin and Middle East, including Iran where seroprevalence rate of disease has been reported from 10 to 37% (12-14).

As the high proportion of infected dogs is asymptomatic, therefore, detection of specific antibodies remains the method of choice for mass screening of dogs in epidemiological surveys and evaluation of prevalence (15-19).

Serological methods are highly sensitive and non-invasive, so they are best suited for use in field conditions (20, 21). Several diagnostic tests are available to detect anti-*Leishmania* antibodies in canine sera. In the present study, the direct agglutination test (DAT) was used as sero-diagnostic tool, because it is a simple as well as valid test and does not require specialized equipments (12, 22-25).

This study aimed to determine the present status of seroprevalence of CVL in various parts of Meshkin-shahr district especially in endemic foci of HVL to more identifying the role of dog as natural reservoir of human kala-azar in these areas to presenting effective control programme of HVL to health authorities.

Materials and Methods

Study area

Meshkin Shahr district is located in Ardabil Province, north-west of Iran. It was calling "Khiav", "Orami", "Varavi" in the past. The city of Meshkin-Shahr is situated at an altitude of 1490m above sea level and is the nearest city to the Sabalan high mountain (Fig.1). The weather of this district is moderate mountainous. It covers an area of approximately 1530 km^2 including 323 villages and its population is estimated to be 169967 among which 42% was settled in urban areas and 58% in rural areas. Out of which a small part belongs to nomad tribes.

Sampling

The method of this study was descriptive cross-section and the sampling method was multi stage cluster random sampling. Out of 323 villages in Meshkin- Shahr district, 21 villages (cluster) were selected randomly and in each cluster, serum samples were taken from 10 dogs randomly. The investigation was carried out over a period of 15 months from June 2006 to August 2007 on 384 ownership dogs. All the selected dogs were physically examined by a doctor of veterinary medicine. Dog age was determined by interviewing dog owners. Blood samples were taken from the selected dogs by venapuncture in villages of Meshkin- Shahr where HVL is endemic, poured into 10 ml polypropylene tubes and processed 4-10 h after collection. The collected blood samples were centrifuged at 800 ×g for 5-10 min, and the separated sera were stored at -20°C. All the serum samples were tested by DAT in the Leish-

maniasis Laboratory in the School of Public Health, Tehran University of Medical Sciences.

Direct Agglutination Test

The *L. infantum* antigens for this study were prepared in the Leishmaniasis Laboratory, Protozoology Unit of the School of Public Health, 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 RPMI1640 plus 10% fetal bovine serum, tripsinization of the parasites, staining with coomassie brilliant blue and fixing with formaldehyde 2% (24-26).

The dog serum samples were tested by DAT, initially, for screening purposes; dilutions were made 1:80 and 1:320. Samples with titers 1:320 were diluted further to end-point titer 1:20480. Negative control, wells (antigen only; on each plate) and known negative and positive control serum samples were tested in each plate daily. The cut off titer was defined as the highest dilution at which agglutination was still visible, as blue dot, compared with negative control wells, which had clear blue dots. The positive standard control serum prepared from dogs with *L. infantum* infection (at 1:20480) in an endemic area and confirmed by microscopy, culture and animal inoculation. 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) is still visible after 18 h incubation at room temperature (23). Two individuals read the tests independently. The cut off was determined in previous studies by experimental infection (12, 24).

We considered anti-*Leishmania* antibodies titers at ≥ 1: 320 as canine *Leishmania* infection in this investigation.

Parasitological study

To confirm *Leishmania* infection in dogs, necropsy was performed on two DAT highly positive dogs, liver and spleen samples of these dogs cultured in specific media for *Leishmania* such as NNN and RPMI 1640.

Some *Leishmania* promastigotes, which had been isolated from spleens of domestic dogs following mass production in RPMI1640 media, were analyzed by PCR technique. The primers, LeiRNAF (5'-CAC CAC GCC GCC TCC TCT CT-3') and LeiRNAR (5'-CCT CTC TTT TTT CNC TGT GC-3') were used to amplify the genes coding for internal transcribed spacer 2 (ITS2) and compared the results 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 (27,28).

Data analysis

Chi-square and Fisher exact tests were used to compare seroprevalence values relative to gender, age and clinical signs. Analyses were conducted using SPSS software version 13.5, with a probability (P) value of <0.05 as statistically significant.

Results

The sero-prevalence rate (SPR) in titers 1: 320 and above was 17.4 % (95% C.I, 13.2-20.8). Seventy (25.4%) of the seropositive dogs showed at least one clinical sign including skin lesions, such as exfoliative dermatitis and ulcerations, local or generalized lymphadenopathy, cachexia, low appetite, alopecia, ocular lesions, epistaxis and lameness. No clinical signs and symptoms were seen in 50 (74.6%) of seropositive dogs.

Anti-*Leishmania* specific antibodies, using the cut-off value of 1:320 and above were detected in male and female domestic dogs. The seroprevalence values among male and female animals were 16.5 % and 20.2%, respectively (Table 1). No statistically significant differences between canine *Leishmania* infection and gender were observed.

Table 2 shows that 32.7% of symptomatic dogs were seropositive whereas 15.1% of asymptomatic dogs were Ab negative. The most symptomatic dog (No. 33) had 3 years old age and

higher. Referring to animal age groups, the highest seroprevalence (39.4%) was found in dogs greater than 8 years old and the lowest values (10%) in dogs under 3 years old (Table 3). Strong statistical significance was observed between ≥8 and <8 dog age groups.

Of the 384 dogs, 52(13.5%) dogs had at least one clinical sign and 50% of them did not have titer of antibody while 50% had antibody titer detected by DAT (Table4).

The titers of antibody in symptomatic dogs were 1:320 to 1:20480. The most clinical sign among symptomatic dogs was cachexia (75%) and alopecia (36.5%).

Both two DAT positive dogs were parasitologically positive and amastigotes were observed in the viscera of them. Promastigotes were seen in NNN and RPMI1640 culture media after 2 weeks. *L. infantum* was identified by PCR technique. Samples were scored as positive when a PCR product of 565 bp was detected (Fig.2).

The PCR products were sequenced at MWG Company, Germany. Nucleotide sequence data submitted to the GenBank database with Accession Number EU680963 and EU680962**.**

Table 1: Sero-prevalence of canine *Leishmania* infection by gender in Meshkin-Shahr district (2006-2007)

Gender	No. of dogs (%)	DAT* test Positive (≥1:320)	
		No.	Seroprevalence (%)
Male	290 (75.5)	48	16.5
Female	94 (24.5)	19	20.2
total	384 (100)	67	17.4

* Direct agglutination test

Table 2: Sero-prevalence of canine *Leishmania* infection by signs and symptoms in Meshkin-Shahr district (2006-2007)

Signs & Symptoms	No. of dogs (%)	DAT test Positive (≥1:320)	
		No.	Seroprevalence (%)
Symptomatic	52 (13.5)	17	32.7
Asymptomatic	332 (86.5)	50	15.1
total	384 (100)	67	17.4

Table 3: Sero-prevalence of canine *Leishmania* infection by age in ownership dogs in Meshkin-Shahr district (2006-2007)

Age group(years)	No. of dogs (%)	DAT test Positive (≥1:320)	
		No.	Seroprevalence (%)
0-3	239 (62.2)	24	10.0
4-7	112 (29.2)	30	26.8
≥8	33 (8.6)	13	39.4
total	384 (100)	67	17.4

Table 4: Distribution of titers of anti *Leishmania* antibodies in symptomatic and asymptomatic ownership dogs by DAT in Meshkin-Shahr district (2006-2007)

Titer of Ab	No. of dogs (%)	Symptomatic		Asymptomatic	
		No.	%	No.	%
< 1:80	240 (62.5)	26	10.8	214	89.2
1:80	37 (9.6)	5	13.5	32	86.5
1:160	40 (10.4)	4	10.0	36	90.0
1:320	22 (5.7)	6	27.3	16	72.7
1:640	9 (2.3)	1	11.1	8	88.9
1:1280	13 (3.4)	4	30.8	9	69.2
1:2560	9 (2.3)	1	11.1	8	88.9
1:5120	6 (1.6)	0	0.0	6	100
1:20480	8 (2.1)	5	62.5	3	37.5
total	384 (100)	52	13.5	332	86.5

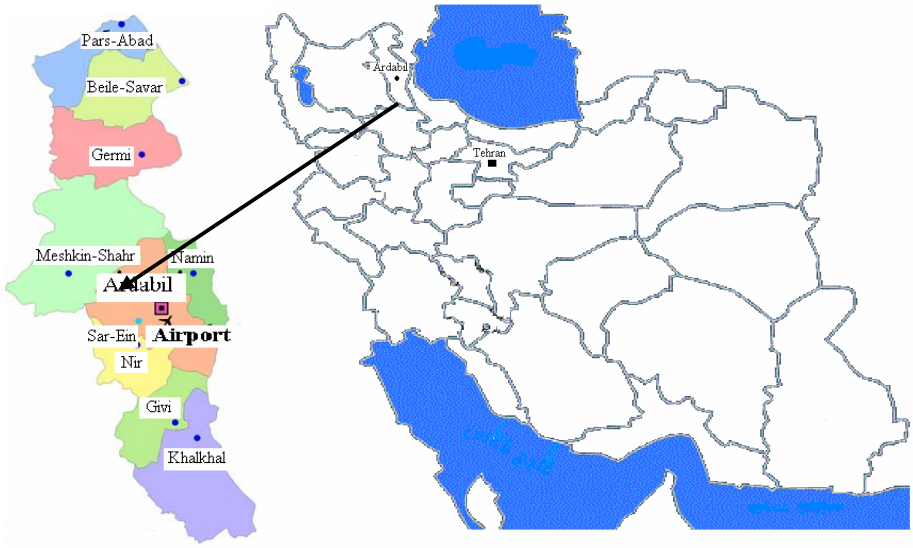

Fig 1: Geographical situation of Meshkin-Shahr district in Ardabil Province, northwest of Iran

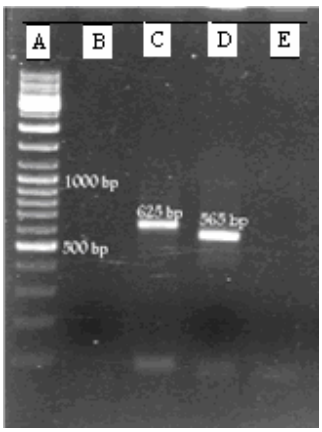

Fig. 2: Gel Electrophoresis of PCR products: A: Marker, 100 bp. B و E: Negative Control. C: *Leishmania major*, 625 bp. D: *Leishmania infantum*, 565 bp

Discussion

Dogs and wild canines are animal reservoir hosts for *L. infantum* in both old and new worlds (3).Determination of the prevalence of canine *Leishmania* infection is necessary to define control measures for zoonotic visceral leishmaniasis (29).

Our studies in the last decade showed that *L. infantum* Lon49 was the principal agent of the disease in human and animal reservoirs in different parts of Iran (28, 30, 31). Dogs and wild carnivores such as jackals, foxes and wolves, which have been found, infected with *L. infantum* (32, 33) and domestic dogs are considered the most important source and reservoirs of *L. infantum* infection particularly in the endemic areas of Iran(9, 12).

Visceral leishmaniasis, caused by *L. infantum,* is endemic in Meshkin-Shahr district where almost 40% of human VL cases in Iran were reported in recent years (25, 31, 34).

Some scientists believe that nomadic from Moghan district may have introduced the disease into the area from the northeast of Ardabil Province (12). A number of studies have carried out on diagnosis and epidemiologic surveys to identify potential risk factors of the diseases in the area (9, 10). For canine leishmaniasis, serology is considered a sensitive and useful technique and is well correlated with clinical signs. According to previous studies (24, 26, 35, 36) the performance of the DAT for detection of *L. infantum* infection in humans and dogs was desirable. Therefore, we use DAT for the determination of sero-prevalence of canine *Leishmania* infection.

With consideration to ecological and epidemiological changes in the studied areas during last decade, this study was designed.

Based on our results, seroprevalence of CVL in Meshkin-Shahr was determined 17.4% using the cut-off value of 1:320 and above. A seroepidemiological study was carried out by Bokai *et al*. on 303 serum samples of ownership dogs in Meshkin-Shahr district in 1998, the seroprevalence of infection was obtained 14.8% in the studied dogs, and only 13.6% of the seropositive dogs were symptomatic (10). Based on two studies that designed for CVL seroprevalence determination in northwest of Iran, seropositivity rate was achieved 21.6% and 18.2% in the northwest of Iran, respectively (9, 12).

No statistical differences were found among *Leishmania* infection with regard to gender in our study. Similar results were found by Abranches *et al*. (1992) in Portugal; Poaio *et al*.,(1981) in Italy; Sideris *et al*.,(1996) in Greece ,Bokai *et al*.,(1998) and Mohebali *et al*.(2005) in Iran.(10, 12, 37-39).

In the current study, we found canine *Leishmania* infection mostly in older dogs (8 years and above). Statistical analysis revealed greater seroprevalence in groups of older dogs, indicating that the probability of exposure to the bite of sand flies infected with *L. infantum* increased with age of infected dogs (37, 40). The high prevalence of *Leishmania* infection in dogs appears to be due to high exposure with *Leishmania* parasites both in villages and in the wild areas.

Dogs from Meshkin-Shahr district seem to have the most important role for the disease because of large dog populations (7 dogs/100 humans) and their heavy infections that sometimes reached to 20% seropositive in some of the villages (10, 41, 42).

However, the role of asymptomatic but seropositive dogs (50 out of 67) is difficult to explain without a follow-up study. In a previous study, Molina *et al*. (1994) found in Spain that asymptomatic dogs as well as symptomatic cases could be a cause when ability of sandflies to pick up infection is not dependent in clinical manifestations (11). 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 (43). Thus, our study and others confirm that the prevalence of *Leishmania* infection in dogs has been underestimated (43, 44).

The high proportions of asymptomatic to symptomatic in infected domestic dogs lacking clinical signs may be related to development of protective immunity especially in older dogs and their frequent exposure to *Leishmania* parasites (45). In the compartmental mathematical model of canine leishmaniasis, it was assumed that asymptomatic dogs were not infectious for sandflies (46, 47). However, other authors showed that infectivity of dogs presenting *Leishmania* infection is not exclusively linked to the symptomatic stage of the disease. They found that three out of five asymptomatic but seropositive dogs transmitted the parasite to the sandfly vectors (11).

In conclusion, the most important result was a high proportion of seropositivity for leishmaniasis (15.1%) among dogs without clinical signs of canine leishmaniasis. These data are very important because ownership dogs can play an important role in the epidemiology of this zoonotic disease. Furthermore, the domestic dog population could be helpful sentinels to follow the progress of the disease in endemic areas.

Control programs on infected dogs will be almost impossible without taking effective measures to determine the status of sero–positive in asymptomatic dogs. Essentially, elimination of infected animals has been recommended (48), but alternative control measures should be recommended for ethical and social reasons.

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