Epidemiological aspects of canine visceral leishmaniosis in the Islamic Republic of Iran

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Abstract

An epidemiological study to examine the sero-prevalence of zoonotic visceral leishmaniosis (ZVL) among domestic and wild canines in endemic foci of Iran was carried out during 1999–2003 to assess the distribution of the disease and the possible association between infection in dogs, wild canines and people. Anti-leishmanial antibodies were detected by the direct agglutination test (DAT). Parasitological study was performed for all captured wild canines and were detected in some of the seropositive dogs with specific clinical signs ($n = 107$). Serum samples ($n = 1568$) were collected from domestic dogs in villages that are known endemic foci of human visceral leishmaniosis (HVL). Wild canine sera were collected from jackals (*Canis aureus, n = 10*), foxes (*Vulpes vulpes, n = 10*) and wolves (*Canis lupus, n = 10*). Of the 1568 serum sampled collected from domestic dogs, 222 (14.2%) were positive by DAT (1:320 and above). No statistically significant difference was found between male (15.2%) and female (11.8%) sero-prevalence ($P = 0.083$). Dogs of 8 years and above showed the highest sero-prevalence (40.6%). Only 23.9% of the seropositive domestic dogs had clinical signs. Parasitology and serology tests that were performed in 30 wild canines showed 10% these animals were infected by *Leishmania infantum*. Ten out of 11 *Leishmania* spp. isolated from the dogs and wild canines were identified as *L. infantum* and one other as *L. tropica* by molecular and biochemical techniques. For the first time in Iran, *L. infantum* and *L. tropica* were isolated from viscera of both a wolf and a domestic dog.
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Keywords: Canine visceral leishmaniosis; Epidemiology; Domestic dogs; Wild canines; Iran

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1. Introduction

Visceral leishmaniosis is an infectious disease transmitted by sand flies and caused by various species of *Leishmania* parasites. Distinct species occur in parts of the old world and new world, and can infect people, domestic and wild animals. Domestic dogs (*Canis familiaris*) are principal reservoir hosts of Mediterranean type of visceral leishmaniosis caused by *Leishmania infantum* (WHO, 1990). These parasites cause a wide spectrum of clinical manifestations in humans and it is estimated that the annual occurrence of human visceral leishmaniosis (HVL) cases worldwide is 500,000 (CSR, WHO, 2000).

Canine leishmaniosis was discovered by Nicole and Comte (1908) and since then, reports of infected dogs have come from almost all human visceral leishmaniosis foci in the Mediterranean area (Kirmse et al., 1987; Manciati et al., 1986). The canidae family especially domestic dogs are efficient reservoir hosts because parasites of fixed macrophages are so abundant in the dermal layer that the parasites are readily taken up by feeding sand flies (Ashford and Bettini, 1987; Nash, 1993).

The first case of canine leishmaniosis was reported from Tehran in 1913 (Neligan, 1913) and subsequently, was reported by Pouya (1949) in a dog from the Caspian area (Pouya, 1950), where he also reported the first human case of visceral leishmaniosis in Iran. Three cases were also reported in domestic dogs in the vicinity of Tehran (Mobedi et al., 1968). Subsequently, a few other infected dogs were found in other parts of Iran (Hamidi et al., 1982; Mohebali et al., 2001a,b; Bokai et al., 1998). The wild carnivores such as jackals and foxes that have been found infected with *Leishmania* spp. are considered the reservoirs of visceral leishmaniosis in Iran, particularly in the areas where sporadic cases of disease have been found (Nadim et al., 1978; Hamidi et al., 1982; Mohebali et al., 2001b).

The objectives of this study were to determine the sero-prevalence of canine visceral leishmaniosis in various parts of Iran especially in endemic foci of HVL to identify the natural reservoir of human kala-azar in these areas. Focus was particularly on dogs, with a view to determining infection rates among them and their role in transmission of the disease to humans.

2. Materials and methods

2.1. Study area

The investigation was conducted over a period of 4 years (from 1999 to 2003) in northwest, south and central parts of Iran where HVL is endemic (Edrissian et al., 1999; Mohebali et al., 2001b). Dog populations in villages were selected by simple random sampling and wild canines were shot around the areas where case of human visceral leishmaniosis had been identified previously. A field laboratory was set up at each location for the purpose of providing a base where the animals could be examined. Three distinct geographical zones in Iran, where HVL is endemic and samples were collected are shown in Fig. 1.

2.2. Blood sampling

Blood samples (2.5 ml) were taken from 1568 domestic dogs from Ardebil and east Azerbaijan (northwestern), Bushehr and Khozestan (southern), Chaharmahal and Ghom (intermediate) provinces where HVL is endemic. Additionally, samples were taken from 30 wild canines in these areas: 10 jackals, 10 foxes and 10 wolves. Blood samples were taken by venapuncture and put into 10 ml polypropylene tubes and processed 4–10 h after collection. The blood was centrifuged at $800 \times g$ for 5–10 min and sera were separated and stored at -20°C. Dog age was
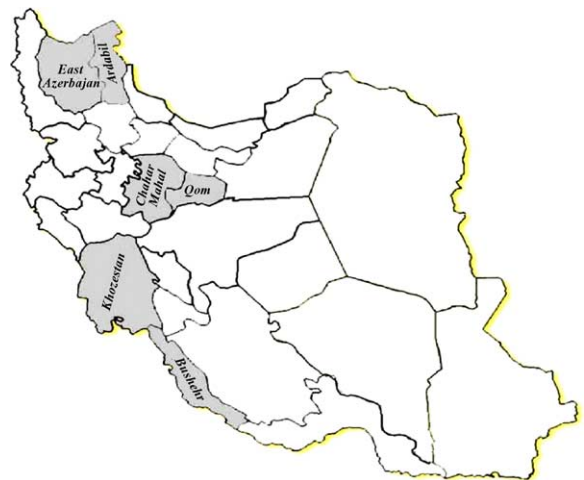

Fig. 1. Geographical locations where this study was carried out.

determined by interviewing dog owners. Male dogs constituted 69.7% of the sampled population and females 30.3%. No transmission of *Trypanosama cruzi* was reported in dogs in the studied areas. All the serum samples were tested by direct agglutination test (DAT).

2.3. Serological tests

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 RPMI1640 plus 10% fetal bovine serum, tripsinization of the parasites, staining with coomassie brilliant blue and fixing with formaldehyde 2% (Harith et al., 1989; Edrissian et al., 1996).

The dog and wild canine serum samples were tested by DAT according to the methods described by Harith et al. (1989). Initially, for screening purposes, dilutions were made from 1:80 and 1:320. Samples with titers 1:80 were diluted further to give end-point titers 1:20480. Negative control wells (antigen only; on each plate) and known negative and positive controls were tested in each plate daily. The 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 from the endemic areas confirmed by microscopy, culture and animal inoculation with 1:20480 titers. Two individuals read the tests independently. The cut off was determined in previous study by experimental infection (Mohebali et al., 2001c). In based on that study, 16 seronegative dogs were randomly divided into two groups. All of the eight dogs of group 1 received an intraperitoneal challenge of 2.5×10^6 infective promastigotes of *L. infantum* in stationary phase (MCAN/IR/94/LON49). All dogs of groups 1 and 2 were tested from 1 to 7 months after challenge by DAT, ELISA and IFA techniques for detecting anti-*Leishmania* antibodies. Necropsy was performed on all dogs to investigate for parasites.

Of the eight dogs in interventional group, seven had DAT titers of ≥1:320 and other one had 1:160.

Serum samples from control group had titers of <1:40. Thus, a complete correlation was observed between DAT (1:320 and above) and ELISA (OD ≥0.04) with parasitological procedures after 3 months post-infection (submit for publication). Sensitivity and specificity of the DAT were estimated with exact binomial 95% confidence limits. To study the optimal DAT cut off level, a receiver-operator characteristics (ROC) curve was constructed according to the method Fletcher et al. (1982). Moreover, specific *Leishmania* antibodies at a titer of 1:320 and above were considered as positive in previous studies too (Harith et al., 1989; Edrissian et al., 1996). Therefore, we considered anti-*Leishmania* antibodies titers at ≥1:320 as *Leishmania* infection in this investigation.

For ELISA and IFA techniques the whole promastigotes of *L. infantum* Lon49 were used as antigen in our laboratory and all wild canine and suspected clinically serum samples were tested by these techniques. The ELISA was performed according to Hommel et al. (1978). The conjugate was rabbit anti-dog IgG alkaline phosphates obtained from Sigma Co., USA. In the preliminary check board titration, the batch of conjugate used in this study was found to be optimal at a dilution of 1:1000. The results were read by ELISA reader (Labsystem, Netherlands) at the wavelength of 405 nm. The cut off point was calculated by $\bar{X} + 2$ S.D. in normal dog population. The IFAT was performed according to the method of Edrissian et al. (1981). The conjugate was rabbit anti-dog IgG fluorescein obtained from Sigma Co., USA. The cut off point was calculated with comparing the results of suspected serum with standard positive and negative control samples after calculating Geometric Mean Reciprocal of Titer (GMRT). The titers of 1:160 and above were considered as positive.

2.4. Parasitological study

The dogs and wild canines were examined externally for signs of *Leishmania* infection; afterwards, all the captured wild canines and some of suspected dogs were dissected for the following investigation:

- Smears were prepared from any skin lesion and from the liver and spleen of each dog. All the prepared

smears were fixed with methanol, stained with Giemsa stain 10% and examined microscopically for the presence of amastigotes.

- Biopsy specimens were collected aseptically from spleen and liver, then cultured into biphasic culture media (prepared from nutrient agar containing 10% whole rabbit blood overlaid with liver infusion tryptose broth (LIT) containing 100–200 UI/ml penicillin G and 1 μg/ml streptomycin). The inoculation cultures were incubated at 21°C for up to 6 weeks and examined weekly for the presence of promastigotes. Meanwhile, for mass production of promastigotes, Schneider Insect (HIMEDIA) and RPMI1640 (GIBCO) media were used.

2.5. Molecular characterization

Some *Leishmania* promastigotes which had been isolated from spleens of domestic dogs and wild canines following mass production in RPMI1640 media, were analyzed by RAPD-PCR techniques and compared the results with standard species of *L. infantum*, (MCAN/IR/96/LON49), *L. tropica* (MHOM/SU/74/K27) and *L. major* (MRHO/IR/75/ER) in School of Public Health, Tehran University of Medical Sciences and izoenzyme analysis at the London School of Hygiene and Tropical Medicine, United Kingdom and Shiraz University of Medical Sciences. (Evans, 1989; Motazedian et al., 1996; Noyes et al., 1996; Mohebali et al., 2002). Dr. K.P.

Chang from Chicago University, USA, confirmed some of the *Leishmania* identification with PCR-RFLP.

2.6. Data analysis

Chi-squared (χ^2) was used to compare sero-prevalence values relative to gender, age, geographical zone and tribe. Analyses were with Epi-Info software, with a probability (P) value of <0.05 as statistically significant.

3. Results

3.1. Sero-epidemiological survey

Anti-*Leishmania* specific antibodies, using the cut-off value of 1:320 and above were detected in male and female domestic dogs. The sero-prevalence values among male and female animals were 15.2 and 11.8%, respectively (Table 1). No statistically significant differences between canine *Leishmania* infection and gender were observed ($P = 0.083$).

Referring to animal age groups, the highest sero-prevalence (40.6%) was found in dogs greater than 8-year-old and the lowest values (9.9%) in dogs less than 3-year-old (Table 2). Strong statistical significance was observed between age groups ($P < 0.001$). Three distinct geographical zones of Iran could be defined

Table 1
Sero-prevalence of canine *Leishmania* infection by gender in endemic areas of Iran

Gender	No. of dogs tested	No. of DAT[a] positive (≥1:320)	Prevalence (%) ($P = 0.083$)
Male	1093 (69.7)	166	15.2
Female	475 (30.3)	56	11.8
Total	1568 (100.0)	222	14.2

[a] Direct agglutination test.

Table 2
Sero-prevalence of canine *Leishmania* infection by age groups in endemic foci of Iran

Age group (years)	No. of dogs tested	No. of DAT[a] positive	Prevalence (%)
0–3	931 (59.4)	92	9.9
4–7	509 (32.5)	78	15.3
≥8	128 (8.1)	52	40.6
Total	1568 (100.0)	222	14.2

[a] Direct agglutination test.

Table 3
Sero-prevalence of canine *Leishmania* infection by geographical zones of the country

Zones	No. of dogs tested	No. of DAT[a] positive	Prevalence (%)
Northwest	916 (58.4)	167	18.2
Intermediate	333 (21.2)	41	12.3
Southwest	319 (20.4)	14	4.4
Total	1568 (100.0)	222	14.2

[a] Direct agglutination test.

including: northwestern (mean sero-prevalence 18.2%), intermediate (12.3%) and southwestern (4.4%) (Fig. 1; Table 3). A highest significant difference was observed between the northwest and the other two locations ($P < 0.013$).

If in shown in Table 4 that 68 (10%) of domestic dogs belonging to nomads, had *Leishmania* infection, while 154 (17.3%) from settled populations were infected and CVL cases were much more frequent in settled population dogs. Statistically significant differences between these two groups of dogs were seen ($P < 0.001$).

Fifty-three of the 222 sero-positive domestic dogs showed at least one clinical sign including lymphadenopathy, hair shedding, dermal lesions, onychogriposis and cachexia which may be regarded as symptomatic form of the disease, while the other 169 dogs (76.1%) were asymptomatic. The proportion of symptomatic infections in sero-positive dogs was 23.9% (53/222) and overall prevalence of CVL was 3.4% (53/1568).

3.2. Parasitological study

During this study, 77 of the suspected domestic dogs were dissected after obtaining owner consent. The results are summarized in Table 5. Results of parasitological and serological tests in the wild canines that were captured around the villages from endemic foci of HVL in Iran are shown in Table 6. Visceral leishmaniosis in a wolf from northwestern Iran was reported for the first time.

3.3. Characterization

Ten out of 11 isolates from infected dogs, were identified as *L. infantum* by RAPD-PCR and isoenzyme analysis techniques. The only other isolate was determined as *L. tropica* by RAPD-PCR and confirmed by RFLP analysis of PCR amplified from the sample using specific restriction enzyme discrimination under supervision of Dr. K.P. Chang from the University of Chicago. This was the first

Table 4
Sero-prevalence of canine *Leishmania* infection by nomadic tribal regions and settled areas

Nomad/non-nomad	No. of dogs tested	No. of DAT[a] positive	Prevalence (%)
Nomadic	678 (43.2)	68	10.0
Settled	890 (56.8)	154	17.3
Total	1568 (100.0)	222	14.2

[a] Direct agglutination test.

Table 5
Clinical, serological and parasitological results[a] of dogs by geographical zones of Iran

Zones	No. of dogs	DAT positive	Parasitological positive		*Leishmania* species
			Smear	Culture	
Northwest	60	42	43	21	*L. infantum, L. tropica*
Intermediat	8	8	8	7	*L. infantum*
Southwest	9	2	1	1	*L. infantum*

[a] Postmortem examination.

Table 6
Results of parasitology and serology tests in wild canines collected in northwestern and southern parts of Iran

Type of animal	No. of animal	Parasitological positive	Serological positive			*Leishmania* species
			DAT[a]	ELISA	IFA[a]	
Foxes	10	1	1	1	0	*L. infantum*
Jackals	10	1	1	1	1	*L. infantum*
Wolves	10	1	1	1	1	*L. infantum*
Total	30	3	3	3	2	*L. infantum*

[a] Direct agglutination test and indirect fluorescence assay.

report for isolation of *L. tropica* from viscera of dogs in Iran.

4. Discussion

Dogs and wild canines are the domestic reservoir for *L. infantum* in both the old and new worlds. Determination of the prevalence of canine *Leishmania* infection is necessary to define control measures for zoonotic visceral leishmaniosis (Tesh, 1995). A simple and suitable diagnostic test is essential for large-scale screening of dog populations. According to previous studies (Saul and Semiao, 1996; Harith et al., 1989; Edrissian et al., 1996; Boelaert et al., 1999; Mohebali et al., 2004a,b) the performance of the DAT for detection of *L. infantum* infection in humans and dogs was excellent. Therefore, we use of the DAT for the determination of sero-prevalence of canine *Leishmania* infection. 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; Pozio et al. (1981) in Italy; Sideris et al. (1996) in Greece; Bokai et al. (1998) in Iran.

In the current study, we found canine *Leishmania* infection mostly in older dogs (8 years and above). There seems to be an increased sero-prevalence of the infection associated with animal growth and aging. (Abranches et al., 1992; Cardoso et al., 2004). The high prevalence of *Leishmania* infection appears to be due to high exposure with *Leishmania* parasites both in villages in the wild.

The three distinct established zones of mean sero-prevalence (northwestern 18.2%), (intermediate 12.3%) and (southwestern 4.4%) show the geographical regions with various weathers.

Increased levels of canine *Leishmania* infection in the cold northwestern part of Iran were shown to be the most important focus of visceral leishmaniosis in the country. Dogs from this zone seem to have the most important role with the disease because of large dog populations (7 dogs/100 humans) and their heavy infections that sometimes reached to 20% in some of the villages (Mohebali et al., 1999, 2001a; Bokai et al., 1998). The dog populations and *Leishmania* spp. infection rate of dogs in the hot south zone was low (Mohebali et al., 2001b). The reason for low seroprevalence of canine *Leishmania* infection in nomadic locations, may be related to short activities of sandflies and high mountain slope, for example, sand fly season in nomadic regions in northwestern Iran runs from July to September (Nadim et al., 1978; Rassi and Javadian, 1997) and nomadic populations usually lives in high mountain slope (Sabalan in north-west and Zagros in south of Iran), where the weather is cold and density and activity of sandflies are low.

The proportions of symptomatic cases in infected domestic dogs and the overall prevalence of canine *Leishmania* infection, respectively, 23.9 and 3.4% are higher than values found by other investigators (Franca-Silva et al., 2003; Bokai et al., 1998; Sharifi and Daneshvar, 1996; Edrissian et al., 1993). Such a high numbers of infected dogs lacking clinical signs may be related to development of protective immunity especially in older dogs and their high exposure to *Leishmania* parasites (Pinelli et al., 1994). In 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.

In this study, we isolated *L. infantum* from 10 domestic dogs, 1 jackal, 1 fox and 1 wolf. Previous studies in the same areas have found that parasites

infecting humans and dogs are the same zymodeme of the parasite (Edrissian et al., 1999; Mohebali et al., 2001a). Therefore, the Canidae family especially domestic dogs are the most important source of *L. infantum* infection for humans. One of the *Leishmania* sp. that were isolated from a domestic dog in northwestern Iran near the Azerbaijan country was identified as *L. tropica*. This species of *Leishmania* is reported in Iran for the first time. This *Leishmania* species as a cause of visceral leishmaniosis in humans and dog was described recently (Guessous-Idrissi et al., 1997).

Results of the present study demonstrate that wild canines including jackal, fox and wolf were infected by *L. infantum* and it seems that these carnivores have the possible role of secondary reservoirs in endemic areas particularly in villages located in mountainous regions, where the transmission cycle takes place. In previous studies, *Leishmania* spp. were isolated from the jackal and fox (Nadim et al., 1978; Hamidi et al., 1982; Mobedi et al., 1968) but species of the isolated parasites had not been determined. In this survey, we have identified all the *Leishmania* spp. that were isolated from wild canines and this is the first record of *L. infantum* infection in a wolf out of the10 which were shot and examined around Ahar city, where HVL and CVL are highly endemic (Mohebali et al., unpublished data).

Regarding to highly *L. infantum* infection of domestic dogs, similarity between *L. infantum* isolated from infected humans (Edrissian et al., 1999) and domestic dogs in the studied areas, no veterinary preventive measure for infected dogs, domestic dogs seem to be the main reservoirs of infection in endemic foci of VL focusing in northwest and central parts of Iran.

In conclusion, the eradication of *L. infantum* in 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 (Palatnik-de-Sousa et al., 2001), but alternative control measures should be recommended for ethical and social reasons. The only hope at present is to find an effective vaccine for prevention and control of CVL. Vaccination would not only prevent clinical manifestations of CVL but would also break the transmission cycle of the parasites to dogs and humans

(Gradoni, 2000). Some studies have been conducted in various parts of the world including Iran to find an effective vaccine against CVL that would provide some protection (Duan et al., 1989; Genaro et al., 1996; Mohebali et al., 1998, 2004b).

Acknowledgements

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