

Seroepidemiological investigation of visceral leishmaniasis in dogs of Ahvaz district, Iran

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ABSTRACT

Visceral leishmaniasis is a parasitic infectious disease from dogs and canids to human that is caused by protozoans of the genus *Leishmania*. Information on the prevalence of canine leishmaniasis is necessary to define control measures for zoonotic leishmaniasis. This seroepidemiological survey was performed in dogs from Ahvaz district using by DAT and ELISA. Blood was randomly collected in 38 pure or mixed breed dogs presented to veterinary hospital of Shahid Chamran university (urban dogs) and 172 mongrel dogs of 10 villages around Ahvaz city (rural dogs). A high level of concordance (98%) was found between the titers measured by DAT and ELISA then DAT selected as valid and simple test. The detected seroprevalences based on DAT were 2.6% and 16.3% in urban and rural dogs respectively. No statistically significant differences were observed between male and female seroprevalences in each groups and among various villages ($P>0.05$). Regarding age-groups of rural dogs, the lowest of seroprevalence (5.3%) was found in dogs younger than one year of age and the highest (33.3%) in dogs older than seven years. Only between of these two groups was statistically significant difference ($P<0.05$). This study revealed the importance of the dog as a reservoir for visceral leishmaniasis in Ahvaz district. It seems that seroprevalence of disease in rural dogs from Ahvaz district is similar to endemic area as of Mediterranean countries.

Keywords: Visceral leishmaniasis, ELISA, DAT, Dog, Ahvaz

INTRODUCTION*

Canine visceral leishmaniasis (CVL) is a severe systemic disease of dogs caused by the protozoan parasite *Leishmania infantum*. Clinical signs usually include lymphadenopathy, dermatitis, alopecia, cutaneous ulcerations, onychogriposis, lameness, an-

orexia, weight loss, cachexia, ocular lesions, epistaxis, anemia, diarrhea and renal failure. Dogs are the chief reservoir of this parasite, which is transmitted among canines and to humans by phlebotomine sandflies (Slappendel & Ferrer 1998). The percentage of infected dogs living in an area where CVL is endemic has major public health implications. It was demonstrated that infected, but asymptomatic, dogs were sources of the parasite for

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phlebotomine vector sandflies and as a consequence play an active role in the transmission of *Leishmania* (Molina *et al* 1994). Information on the prevalence of CVL is necessary to define control measures for zoonotic leishmaniasis. Due to the high proportion of asymptomatic infected dogs and parasitological diagnosis, including direct examination and polymerase chain reaction (PCR), is neither not sensitive nor practical enough, detection of specific antibodies remains the method of choice for mass-screening of dogs in epidemiological surveys and evaluation of prevalence (Quinnell *et al* 2001, Reithinger *et al* 2002). Several diagnostic tests are available to detect anti-*Leishmania* antibodies in canine sera. The present study was designed to investigate the prevalence of the infection in a canine population living in Ahvaz (a tropical area in south west of Iran). We have chosen to use the direct agglutination test (DAT) and enzyme-linked immunosorbent assay (ELISA) for our survey as these tests have proven to be very suitable for the sero-diagnosis of canine *Leishmania* infections (Schallig *et al* 2002). Visceral leishmaniasis is dispersly endemic in at least four provinces of Iran including East Azerbaijan, Ardabil, Fars and Bushehr (Fallah *et al* 2006). This is the first report in seroepidemiology of CVL in Ahvaz.

MATERIALS AND METHODS

Animals and Samples. The survey carried out in 38 urban dogs (pure or mixed breeds) that referred to veterinary hospital of Shahid Chamran University and 172 rural dogs (mongrel) in 10 villages around Ahvaz city using by DAT and ELISA. Blood samples were collected by cephalic or saphenous venipuncture. These animals were among various sexes and ages which were selected by simple random sampling (Tables 1-4). Serum specimens were separated by centrifugation at 800g for 5-10 minutes. The sera were heat-inactivated at 56 °C for 30 minutes and stored at -20 °C until examined

through School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences. Heat inactivation does not affect the results of the employed serological test (Schallig *et al* 2002). Information on animal gender, age and home village was recorded, and prior to sampling, all dogs clinically examined.

Direct agglutination test (DAT). Direct agglutination test for titration of *Leishmania*-specific antibodies followed the general procedures described by Harith (1989). 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% (Harith *et al* 1989, Edrissian *et al* 1996, Mohebbali *et al* 2006). Antigen concentration for the DAT was 5×10^7 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 μ l 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. 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, Mohebbali *et*

al 2006). Therefore, we considered anti-*Leishmania* antibodies titers at $\geq 1:320$ as *Leishmania* infection in this investigation to maximize sensitivity and specificity of the test.

Enzyme-linked immunosorbent assay (ELISA). *Leishmania*-specific IgG antibodies in dogs were detected by ELISA as performed according to Hommel *et al* (1978). The whole promastigotes of *L. infantum* Lon49 were used as antigen in this study. Briefly, antigen of *L. infantum* promastigote stages were diluted (5 $\mu\text{g/ml}$) in 0.1 ml of coating buffer (0.1 M carbonate/bicarbonate, pH 9.6) containing 0.02% NaN_3 . One hundred microliters per well were used to coat 96-well microtiter plates (Nunc, Roskilde, Denmark) overnight at 4 °C. Plates were washed three times with NaCl-Tween 0.3% before saturation for 20 min at 37 °C with phosphate-buffered saline (pH 7.2) containing 0.02% NaN_3 , 0.05% bovine hemoglobin (Fluka, Italy) and 0.2% v/v Tween-20 (PBS-Tween-20). Each dog serum was diluted 1:400 in PBS-Tween-20 and 100 μl diluted serum per well were used. After an incubation period of 55 min at 37 °C plates were washed three times and 100 μl conjugate (rabbit anti-dog IgG, conjugated to alkaline phosphatase, Sigma Co., USA) diluted 1:1000 in PBS-Tween-20 was added per well. After an incubation of 55 min at 37 °C the plates were washed and 100 μl of a 1 mg/ml solution of *p*-nitrophenyl phosphate (Sigma Co., USA) in 0.05 M carbonate/bicarbonate plus 1 mM MgCl_2 buffer (pH 9.8) was added to each well. Plates were incubated for 20 min at 37 °C. The reaction was stopped by addition of 100 μl of 3 M NaOH to each well and absorbance values were read at the wavelength 405 nm in an automatic ELISA reader (Labsystem, Netherlands). Positive control sera of dogs with parasitologically proven *Leishmania* infections and negative control sera of *Leishmania*-free dogs were included in all test plates.

Sera from 50 dogs not infected with *L. infantum* that were not living in an endemic region were tested to set up a cut-off to IgG-specific ELISA

determinations. The cutoff absorbance was established as the mean plus 2 standard deviation in normal dog population, resulting in 0.042 for IgG (Mohebbali *et al* 2006).

Data analysis. The agreement between DAT and ELISA results was assessed by dividing of number of positive results for both tests plus number of negative results for both tests to total samples. Fisher's exact tests were used to compare seroprevalence values relative to gender, age, and village. Analyses were done with SPSS version 10 software for Windows, with a probability (*P*) value < 0.05 as statistically significant.

RESULTS

Level of concordance between the titers measured by DAT and ELISA. With a cut-off titer of 320, both types of samples of 28 dogs were found positive and 21 negative. None of the DAT negative animals had an ELISA positive result; and one DAT positive dogs were negative on ELISA. The observed agreement of DAT and ELISA tests was 98% based on following formula:

$$\text{The agreement between DAT and ELISA results} = \frac{28 + 21}{50}$$

Seroepidemiological survey. The prevalence for visceral leishmaniasis was found to be 13.8% (29 out of 210) in Ahvaz district, being 16.3% (28 out of 172) in the rural area and 2.6% (1 out of 38) in the urban area. The difference between rural and urban areas was statistically significant ($P < 0.05$). The seroprevalence values among male and female animals (both rural and urban) are presented in Tables 1 and 2, respectively. No statistically significant difference in the levels of canine *Leishmania* infection was found for gender ($P > 0.05$). The seroprevalence values of rural and urban dogs in various age groups are presented in Tables 3 and 4 respectively. Regarding age-groups, the highest value of seroprevalence (33.3%) was found in rural dogs older than 7 years of age. There

seems to be an increased prevalence for *L. infantum* associated with the animals' growth in rural areas.

Table 1. Seroprevalence of canine *Leishmania* infection by gender in rural dogs from Ahvaz, Iran by DAT.

Gender	Dogs tested, (n)	Relative distribution (%)	DAT-positive (n)	Prevalence (%)
Male	114	66.3	17	14.9
Female	58	33.7	11	18.9
Total	172	100	28	16.3

Table 2. Seroprevalence of canine *Leishmania* infection by gender in urban dogs from Ahvaz, Iran by DAT.

Gender	Dogs tested, (n)	Relative distribution (%)	DAT-positive (n)	Prevalence (%)
Male	26	68.4	1	3.8
Female	12	31.6	0	0
Total	38	100	1	2.6

Statistically significant difference in antibody titer against *L. infantum* was found between rural dogs over 7 years old and under 1 year ($P < 0.05$). But no statistically significant difference in the prevalence of antibody was found in urban dogs for age ($P > 0.05$).

Table 3. Seroprevalence of canine *Leishmania* infection by age-group in rural dogs from Ahvaz, Iran by DAT.

Age-group (years)	Dogs tested (n)	Relative distribution (%)	DAT-positive (n)	Prevalence (%)
>1	18	47.4	0	0
1-3	12	31.5	1	8.3
3-5	4	10.5	0	0
5-7	2	5.3	0	0
>7	2	5.3	0	0
Total	38	100	1	100

Table 4. Seroprevalence of canine *Leishmania* infection by age- group in urban dogs from Ahvaz, Iran by DAT.

Age-group (years)	Dogs tested (n)	Relative distribution (%)	DAT-positive (n)	Prevalence (%)
>1	19	11.1	1	5.3
1-3	78	45.3	12	15.4
3-5	53	30.8	10	18.9
5-7	13	7.5	2	15.4
>7	9	5.3	0	33.3
Total	172	100	28	100

The canine seroprevalence in the 10 villages of the Ahvaz district ranged from 12.5 to 18.7%. In this survey, we found seropositive dogs in all of the 10 villages of the Ahvaz district. No statistically significant differences were found between seroprevalence values for area ($P > 0.05$).

DISCUSSION

Canine visceral leishmaniasis constitutes a considerable veterinary medical problem since 2.5 million, or more, out of 15 million dogs may be infected in the south-western European countries (Moreno & Alvar 2002). Information on the prevalence of canine *Leishmania* infection is necessary to define control measures for zoonotic leishmaniasis and simple but accurate diagnostic tests are essential for large-scale screening of dog populations. In the present study, DAT and ELISA performed on canine serum samples gave highly concordant results. This study further demonstrates that DAT can be used as a simple, rapid and sensitive screening test for canine *Leishmania* infection and, together with ELISA, is a valuable tool in the assessment of CVL. The overall prevalence for visceral leishmaniasis was found to be 13.8%. Observed seroprevalence specially in rural dogs (16.3%) were similar or higher than that found in other Mediterranean areas: e.g. 5.3% in southern Spain (Acedo Sanchez *et al* 1996), 13–26%

in Mallorca (Solano-Gallego *et al* 2001), and 15.6-17.1% in central Italy (Moretti *et al* 1996). Therefore it seems that Ahvaz district (Iran) is among endemic areas of the world, or the disease may become endemic in this region.

According to our results, the prevalence of CVL in rural and urban dogs was 16.3 and 2.6%, respectively. These results agree with those from other investigators reporting that canine leishmaniasis is more common in rural than urban sites (Zaffaroni *et al* 1999, Courtenay *et al* 1994). This difference may be due to general physical and climatological characterization of rural areas, so that ecological conditions were suitable and ready for abundance of phlebotomine sandflies. In this study, presence of farm animals, chickens and stagnant water in rural areas attract sandflies into houses. On the other hand, this difference can be accounted by the fact that dogs in suburbs live in closer association with phlebotomine sandflies, in places such as quarries, as well as sheep and goat corrals. In addition, our data show that the principal risk factor for infection with *L. infantum* is a dog's lifestyle. Rural dogs that spend the night outdoors show higher prevalence and seroconversion rates than urban dogs that spend the night indoors. Sideris *et al* (1996) in Athens, Greece, showed that the short-furred animals (such as Collie dogs) were more easily bitten by sandflies. Because all of the rural dogs in this study were among short-furred animals, it seems clear that the characteristics of the fur may play an important role in the higher prevalence rate of infection in rural dogs. This phenotype is certainly very conspicuous but literature almost always fails to consider the possible relationship between fur length and risk of infection by viscerotropic *Leishmania*.

In this study, all of the seropositive dogs were asymptomatic. The unspecificity of clinical signs (Ciamarella *et al* 1997) and a high proportion (>90%) of asymptomatic among the seropositive animals underlines the usefulness of serology as a diagnostic tool. Furthermore, the ability to infect

sandflies does not depend on the clinical status of the *Leishmania* infected dog (Guarga *et al* 2000). However, the meaning of asymptomatic but seropositive dogs is difficult to explain without a follow-up study. 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. The eradication of *Leishmania* infection in dogs will not be reached without taking effective control measures on seropositive asymptomatic animals.

Based on results of this study, no statistically significant difference in the levels of canine *Leishmania* infection was found for gender. No specific patterns were found for CVL as for gender distribution of those dogs in Portugal (Abranches *et al* 1992), Greece (Sideris *et al* 1996) and Iran (Bokai *et al* 1998). However, Fisa *et al* (1999) found higher significant prevalence of CVL in male dogs in Spain. One possible explanation could be an increase in female mortality, in which pregnancy and nursing may play an important role. In this sense, the owners of seropositive females repeatedly reported a close relationship between the death of the bitch and pregnancy, delivery and nursing.

Seropositivity for CVL in rural dogs was found at all ages. Also there seems to be an increased prevalence for *L. infantum* associated with the animals' growth in rural areas. In the Old World different investigations have reported an increase of prevalence following aging of the animals (Rab *et al* 1995 and Abranches *et al* 1992). Rab *et al* (1995) suggested that the increasing prevalence of the infection with the age can be related to the time exposed to the phlebotomine activity. Another possible explanation for the conflicting results might be due to the different stratification of the ages into intervals. The differences could also be related to the distinctive epidemiological environments, but still unexplained. Statistical significant differences were not found between seroprevalence values of 10

villages. This resemblance could be related to similar climatic conditions particularly mean monthly temperature and mean monthly relative humidity as well as similar population of dogs.

Although the direct role of infected dogs in the epidemiology of human VL is controversial (Federico *et al* 1991, Evans *et al* 1990), canine leishmaniasis (CVL) is an important veterinary and public health problem. Until effective vaccines become available, epidemiological surveillance and reservoir management will be among the practical measures for prevention and control of this zoonotic disease. Elimination of dogs as a control measure is resisted by dog owners. Finally we recommend dissemination of information to dog owners suggesting periodical serological control of their pets and adequate measures to reduce the risk of exposure (keeping dogs indoors at night and utilizing chemical repellents against the sandfly vectors).

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