Original Article

Neospora caninum and *Leishmania infantum* Co-Infection in Domestic Dogs (*Canis familiaris*) in Meshkin-Shahr District, Northwestern Iran

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Abstract

Background: Mediterranean visceral leishmaniasis (MVL) is an infectious disease that affects both human and animals. Domestic dogs (*Canis familiaris*) are principal reservoir hosts of MVL caused by *Leishmania infantum*. Dogs are definitive hosts for *Neospora caninum* and a risk factor for infecting intermediate hosts. The immunosuppression caused by visceral leishmaniasis (VL) can promote the occurrence of co-infections with other agents such as neosporosis. This study aimed to determine the frequency of co-infection of the both protozoan parasites in the endemic areas of VL from Meshkin-Shahr District, north-west of Iran.

Methods: Altogether, 171 serum samples were collected from domestic dogs of Meshkin- Shahr District by multistage cluster sampling from October 2008 to August 2009. The collected serum samples were tested for the detection of simultaneous infection of *L. infantum* and *N. caninum* using direct agglutination test (DAT) and indirect ELISA, respectively.

Results: Of the 171 domestic dogs, 27 (15.8%) and 52 (30.4%) were showed antibodies against *L. infantum* and *N. caninum*, respectively. Simultaneous infections of *N. caninum* and *L. infantum* was found in 16 (9.4%) of the dogs. In VL-positive and VL-negative dogs, *N. caninum* infection was found in 59.3% and 25.0%, respectively. A statistically significant difference was found between VL-positive and VL-negative dogs with *N. caninum* infection (P= 0.001).

Conclusion: These findings indicate that Meshkin-Shahr District in northwestern Iran is an active focus of canine visceral leishmaniasis (CVL). *Neospora caninum* and *L. infantum* co-infection is prevalent in the area and infection by *L. infantum* seems to enhance susceptibility to *N. caninum* infection in domestic dogs.

Keywords: *Neospora caninum, Leishmania infantum,* Co-infection, Seroepidemiology, dog, Iran

Introduction

Canine visceral leishmaniasis (CVL) is an infectious disease that affects both human and canines, and is transmitted by phlebotomine sand flies (Alvar et al. 2004). *Leishmania infantum* is the agent of Mediterranean visceral leishmaniasis and domestic dogs are efficient reservoir hosts for this infection (WHO 1990). Asymptomatic infected dogs can remain infected without displaying apparent clinical manifestations of visceral leishmaniasis for years and even for their whole life (Moshfe et al. 2009). Many countries have re-emergence of visceral leishmaniasis (VL) during recent years, in both aspects of geographically

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and the number of cases (Arias et al. 1996). For a long time, kala-azar seems to be endemic in Meshkin-Shahr areas from the northwest of Iran (Moshfe et al. 2008). Infected dogs are important risk factors for human infection in the endemic areas of Iran (Gavgani et al. 2002). The prevalence of *L. infantum* infection in the domestic dogs in Meshkin-Shahr areas is high and its prevalence has been reported from 14.2% to 17.4% (Bokaei et al. 1998, Mohebali et al. 2005, Moshfe et al. 2008).

Neospora caninum is a coccidian intracellular parasite from the Sarcocystidae family with a wide host range (Dubey and Lindsay 1996). Dogs, coyotes, and dingoes are both the definitive and intermediate hosts for *N. caninum* that excreted oocysts in their feces and shedding oocysts in the environment is a risk factor for the occurrence of miscarriages and stillbirths associated with *N. caninum* in cattle and probably other intermediate hosts (Dubey 2003, King et al. 2010). The parasite can be transmitted transplacentally in dogs for several generations (Dubey and Lindsay 1989). Neosporosis can cause severe neuromuscular disorders as ascending paralysis with hyperextension of the hind limbs, especially in congenitally infected dogs (Dubey 2003). A few studies on *N. caninum* infection in dogs have been carried out in Iran (Haddadzadeh et al. 2007, Malmasi et al. 2007, Hosseininejad et al. 2010a, 2010b, Yakhchali et al. 2010). Studies on *N. caninum* and *L. infantum* co-infection in the world are very rare (Andreotti et al. 2006, Cringoli et al. 2002).

This is the first study in Iran, aimed to determine simultaneous infection of *N. caninum* and *L. infantum* in domestic dogs from Meshkin-Shahr District as CVL endemic area in north-west of Iran.

Materials and Methods

Animals

A total of 171 domestic dogs of mix breeds from some villages of Meshkin-Shahr areas including Ahmad abad, Paikhan, Kojanagh, Mazraeh khalaf, Mirak and Ourkandi were selected by multi-stage cluster sampling from October 2008 to August 2009. Information on the dogs such as sex and age were taken from the owners and physical examination was performed by a doctor of Veterinary Medicine (DVM).

Preparation of the serum samples

All blood samples were prepared from the cephalic vein by venapuncture, poured into 10 ml polypropylene tubes, and processed 4–10 h after collection. The collected samples were centrifuged at 800 g for 5-10 min, and the separated sera were kept at -20° C until tested.

Direct agglutination test

Serum samples were tested using direct agglutination test (DAT) for the detection of anti-*L. infantum* antibodies.. DAT antigen was prepared at the School of Public Health, Tehran University of Medical Science (Mohebali et al. 2006).

The main phases of the process of making DAT antigen were mass cultivation of promastigotes of Iranian strain of *L. infantum* (MCAN/IR/96/Lon49) in RPMI1640 plus 10% fetal bovine serum, tripsinization of the parasites, staining with Coomassie brilliant blue and fixing with 1.2% formaldehyde (Mohebali et al. 2006).

To screen the serum samples, initially dilutions were made 1: 80 and 1: 320 and samples with titers 1: 80 were diluted further to give the end-point dilution of 1: 20480. Negative control (antigen only) and known positive control serum samples were included on each plate. 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 (Mohebali et al. 2005). The positive control serum was produced from dogs with *L. infantum* infection (at 1: 20480) and confirmed by microscopy, culture and animal inoculation (Harith et al.

1989). Based on previously studies in Iran, Anti-*Leishmania* antibodies at a titer of 1: 320 and higher were considered as positive in this investigation (Mohebali et al. 2006).

Indirect ELISA

For the detection of *N. caninum* infection, sera were tested with an indirect ELISA using an affinity purified surface antigen (P38). The performance of indirect ELISA was based on the method of Hosseininejad et al. (Hosseininejad et al. 2010a). Optical density (OD) values were measured at 450nm on an ELISA reader (Labsystems Multiskan ® plus).

An optimal serum dilution of 1:100 was established by checkerboard titration for the purpose of the test. Index values were calculated by the formula; SIn= (Sn−N)/(P−N) where SIn is the individual ELISA index value, Sn is the OD value obtained for the mean of tested samples, N is the OD value obtained for the negative serum, and P represents the OD values obtained for the positive serum. Anti- *Neospora* antibodies at OD≥ 0.23 by ELISA were considered as *Neospora* sp. positive results (Hosseininejad et al. 2010 a).

Parasitological study

To confirm visceral leishmaniasis in dogs, necropsy was performed in some of high seropositive dogs with specific clinical signs and symptoms. Liver and spleen samples of the infected dogs cultured in specific media such as NNN and RPMI1640 and then a few touch smears were prepared from the liver and spleen of them. All the prepared smears were fixed with methanol, stained with Giemsa stain 10% and examined microscopically for the presence of amastigotes.

Ethical approval

This research project was reviewed and approved by the Ethics Committee of Tehran University of Medical Sciences, Iran.

Statistical analysis

Statistical test including Chi-squared (X^2) and MacNemar were used to compare seroprevalence values relative to gender, age and clinical signs of *N. caninum* and *L. infantum* co-infection in the domestic dogs. Statistical analyses were performed using SPSS software version 13.5 (SPSS Inc, Chicago, IL, USA), with a probability (*P*) value of <0.05 as statistically significant.

Results

All of the dogs were of mixed breed and all of them were kept as guard dogs and sheep-dogs. From 171 serum samples that were tested by DAT, 27 (15.8%) of them were positive in with DAT (≥1:320). The seroprevalence values among male and female animals were 16.4% and 12.0%, respectively (Table 1). No statistically significant difference was seen between canine *L. infantum* infection and the gender (*P*= 0.574). The highest sero-prevalence rate 9/36 (25.0%) was observed among the domestic dogs of more than five years age and the lowest values 3/52 (5.8%) were seen in dogs under 2 years old (Table 2). Clinical signs including dermatitis, alopecia, keratitis, cachexia and weakness were seen in 27/171(15.8%) dogs. Only 12 (44.4%) from 27 DAT- seropositive dogs (≥1:320) showed clinical signs (Table 3). In this survey, 8 out of 12 necropsied dogs were positive for *L. infantum* infection with DAT (≥1:320) and impression smear that prepared from their liver and spleen. Only 3 out of 12 necropsied dogs showed positive results in *Leishmania* culture media about 2 weeks post inoculation.

Altogether, 52 (30.4%) of the dogs showed anti *N. caninum* antibodies by indirect ELISA test using an affinity purified surface antigen (P38). No significant statistically difference was seen between *N. caninum* infection and both sex and age groups (*P*> 0.05) (Table 1 and 2). None of the infected dogs had clinical signs including neurologic and cutaneous neosporosis disorders.

The presence of antibodies against *N. caninum* and to *L. infantum* was detected in 16 from 171 dogs (9.4%) among six villages of Meshkin-Shahr, which were selected by multi-stage cluster sampling (Fig.1).

L. infantum infection seems to enhance susceptibility to *N. caninum* infection in domestic dogs.

[κ^2= 10.26, *P*< 0.01, odds ratio= .43 (95% CI, 0.25–0.75).

Table 4 and 5 show the distribution of the *N. caninum* infection in VL-negative dogs and VL-positive dogs, respectively. In DAT-positive dogs (≥1:320), the prevalence of *N. caninum* infection was calculated 59.3% and in DAT-negative dogs (<1: 320) the prevalence of the infection was 25.0%. A statistically significant difference was found between DAT-positive and DAT-negative dogs compared with *N. caninum* infection (*P*= 0.001). In 58.3% of males and 66.7% of female in the DAT-positive dogs, showed anti *N. caninum* antibodies (OD≥ 0.23). No statistically significant difference was found in the both groups (*P*= 0.781). Also, 25.4% of males and 22.7% of female in the DAT- negative dogs, showed anti *N. caninum* antibodies but no statistically significant difference was found between in the both groups (*P*= 0.789).

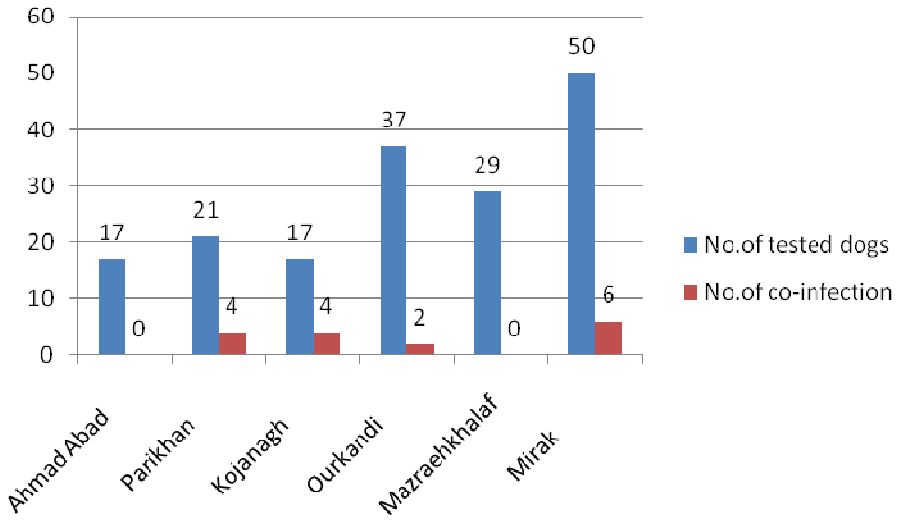

Fig. 1. Frequency of *Neospora caninum* and *Leishmania infantum* co-infection among the domestic dogs were selected by multi-stage cluster sampling in Meshkin-Shahr District, northwestern Iran

Table 1. Sero-prevalence of canine *L. infantum* and *N. caninum* co-infection by gender in Meshkin-Shahr District, northwestern Iran

Gender	n of dogs (%)	*Leishmania* Positive*		*Neospora* positive**	
		n	(%)	n	(%)
Male	146 (85.4)	24	16.4	45	30.8
Female	25 (14.6)	3	12.0	7	28.0
Total	171 (100)	27	15.8	52	30.4

* *anti-Leishmania* antibodies *detection* by DAT at titers ≥1:320

** anti-*Neospora* antibodies detection by ELISA at OD≥0.23

No statistically significant difference between DAT positive dogs (≥1:320) and gender was observed (*P*= 0.574).

No statistically significant differenc between *N. caninum* infection and gender was observed (*P*= 0.777).

L. infantum infection could increase *N. caninum* infection in the domestic dogs [κ^2=10.26, *P*<0.01, odds ratio=0.43 (95%CI, 0.25–0.75)]

Table 2. Sero-prevalence of canine *L. infantum* and *N. caninum* co-infections by age group in Meshkin-Shahr District, northwestern Iran

Age group (years)	n of dogs (%)	*Leishmania* Positive*		*Neospora* positive**	
		n	(%)	n	(%)
< 2	52 (30.4)	3	5.8	12	23.1
2-5	83 (48.5)	15	18.1	29	34.9
> 5	36 (21.1)	9	25.0	11	30.6
Total	171(100)	27	15.8	52	30.4

* anti-*Leishmania* antibodies detection by DAT at titers ≥1:320
** anti-*Neospora* antibodies detection by ELISA at OD≥0.23
Statistical difference between DAT positive dogs (≥1:320) and different age groups is significant (*P*= 0.038). No statistically significant difference between *N. caninum* infection and different age groups was observed (*P*= 0.345).

Table 3. Distribution of symptomatic and asymptomatic dogs by sero-prevalence rate of *L. infantum* infection in Meshkin-Shahr District, northwestern Iran

DAT test (≥1:320)	n of dogs (%)	Symptomatic		Asymptomatic	
		n	%	n	%
Positive	27 (15.8)	12	44.4	15	55.6
Negative	144 (84.2)	15	10.4	129	89.6
Total	171 (100)	27	15.8	144	84.2

Statistical difference between DAT positive (≥1:320) symptomatic and asymptomatic dogs was significant (*P*=0.001).

Table 4. Distribution of *N. caninum* infection by gender and age in DAT- dogs from Meshkin-Shahr District, northwestern Iran

Variables	*N. caninum* Positive		*N. caninum* Negative		Total
	n	%	n	%	
Sex					
Males	31	25.4	91	74.6	122
Females	5	22.7	17	77.3	22
Total	36	25	108	75	144
Age					
< 2	10	20.4	39	79.6	49
2-5	21	30.9	47	69.1	68
> 5	5	18.5	22	81.5	27
Total	36	25	108	75	144

In DAT negative dogs**,** no statistically significant difference was found between *N. caninum* infection with gender (*P*= 0.789) and age groups (*P*= 0.299).

Table 5. Distribution of *N. caninum* infection by gender and age in DAT^+ dogs from Meshkin-Shahr District, northwestern Iran

Variables	*N. caninum* Positive		*N. caninum* Negative		Total
	n	%	n	%	
Sex					
Males	14	58.3	10	41.7	24
Females	2	66.7	1	33.3	3
Total	16	60	11	40	27
Age					
< 2	2	66.7	1	33.3	3
2-5	8	53.3	7	46.7	15
> 5	6	66.7	3	33.3	9
Total	16	60	11	40	27

In DAT positive dogs**,** no statistically significant difference was found between *N. caninum* infection with gender (*P*= 0.781) and age groups (*P*= 0.782).

Discussion

For diagnosing canine leishmaniasis, DAT is considered a sensitive and applicable technique and is well correlated with clinical signs (Moshfe et al. 2008). According to previous studies (Harith et al. 1989, Edrissian et al. 1996, Mohebali et al. 2004) the performance of the DAT for recognizing of VL in dogs was desirable. Hence, we utilized DAT for the determination of sero-prevalence of canine *L. infantum* infection in this investigation.

Based on the present study, sero-prevalence of canine *Leishmania* infection in the domestic dogs of Meshkin-Shahr District was determined 15.8% using DAT with cut-off value of 1:320. Based on three studies that designed for sero-prevalence of canine leishmaniasis in northwest of Iran, sero-positivity rates of the infection were found 21.6%, 18.2% and 17.4%, respectively (Gavgani et al. 2002, Mohebali et al. 2005, Moshfe et al. 2008)**.** In this study, 55.6% of the DAT- positive dogs did not show any clinical signs. In Iran, it found asymptomatic *Leishmania*-infected dogs as well as symptomatic ones, have a potential role in the maintenance of *L. infantum* infection and probably the establishment of domestic transmission cycle of the parasite in the VL endemic areas (Moshfe et al. 2009).

The seroprevalence values among male and female animals were 16.4% and 12.0%, respectively No statistically significant difference between canine *Leishmania* infection and gender was observed (P=0.083).

Similar findings were reported by other Iranian authors (Bokaei et al. 1998, Mohebali et al. 2005, Moshfe et al. 2008). Our findings indicated that the chance of having *L. infantum* antibodies increases with age of the dogs is in agreement with previous studies (Mohebali et al. 2005, Moshfe et al. 2008). Statistical analysis revealed greater sero-prevalence in older dogs (5 years and above), indicating that the chance of exposure to the bite of sand flies infected with *L. infantum* increases when the dogs become older (Cardoso et al. 2004).

In the current study, the prevalence of anti -*N. caninum* antibodies was determined 30.4% in dogs of Meshkin-Shahr District. This sero-prevalence was reported to be 33%, 28% and 27% in Iran, respectively (Haddadzadeh et al. 2007, Malmasi et al. 2007, Yakhchali et al. 2010). These higher seroprevalences in dogs can be due to the close

contact with placenta, materials of aborted fetuses, and the uterine discharge of the intermediate hosts of the parasite as cattle (Dijkstra et al. 2002, Fernandes et al. 2004). No significant difference was found between *N. caninum* sero-positivity male dogs and females ($P > 0.05$). This finding has agreement with the results of other studies (Haddadzadeh et al. 2007, Malmasi et al. 2007, Yakhchali et al. 2010). In the present study, significant difference was not detected among the age groups ($P > 0.05$) but in other surveys in Iran the sero-positivity rates increased with age, suggesting postnatal exposure to *N. caninum* by means of horizontal transmission (Haddadzadeh et al. 2007, Yakhchali et al. 2010).

For the first time in Iran, we found the co-presence of antibodies to *N. caninum* and to *L. infantum* in 9.4% of domestic dogs. Cringoli et al. (2002) reported that 46 (4.3%) of 1058 dogs had co-presence of antibodies to *N. caninum* and to *L. infantum.* Based on these findings *N. caninum* and *L. infantum* co-infection is prevalent in this area and significant difference was found when sero-positive and sero-negative dogs for *L. infantum* were compared with *N. caninum* sero-prevalence. Sero-epidemiological surveys that were done in dogs from the Campania region of southern Italy indicated that the co-presence of antibodies of *N. caninum* and *L. infantum* is prevalent in dogs and infection by one protozoan seems to enhance the susceptibility to the other one (Cringoli et al. 1996, 2002). Andreotti et al. (2006) reported that *N. caninum* and *L. infantum* co-infection is common in dogs in Campo Grande area, Brazil but there was no significant difference when VL-positive and VL-negative dogs were compared with *N. caninum* sero-prevalence and VL does not appear to enhance susceptibility to *N. caninum* infection. In addition, Tarantino et al. (2001) have reported *N. caninum* and *L. infantum* simultaneous skin infection in a young dog in Italy.

In our study *L. infantum* infection seems to enhance susceptibility to *N. caninum* infection in domestic dogs (odds ratio=0.43) because canine visceral leishmaniasis may lead to immune system suppression and T-cell mediated immune response to parasite antigens can be damaged. CD21+ and CD4+ lymphocytes decline in the peripheral blood of infected dogs with CVL. Immune system suppression caused by CVL can be increased dog's susceptibility to *N. caninum* infection (Tarantino et al. 2001).

In conclusion, *N. caninum* and *L. infantum* co-infection in domestic dogs is prevalent in the CVL endemic areas of northwestern Iran and the infection by *L. infantum* seems to enhance susceptibility to *N. caninum* infection.

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