Seroprevalence of *Neospora caninum* and *Toxoplasma gondii* infection in dogs from west and central parts of Iran using two indirect ELISA tests and assessment of associate risk factors

Hosseininejad, M.* **and Hosseini, F.**

Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran and Research Institute of Zoonotic Diseases, Shahrekord University, Shahrekord, Iran

***Correspondence:** M. Hosseininejad, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran and Research Institute of Zoonotic Diseases, Shahrekord University, Shahrekord, Iran. E-mail: hosseini-m@vet.sku.ac.ir

(Received 8 Jun 2010; revised version 25 Oct 2010; accepted 30 Oct 2010)

Summary

To determine the seroprevalence of anti-*Neospora caninum* and anti-*Toxoplasma gondii* antibodies in dogs in Iran and to investigate related risk factors to the infection, a study was conducted in Chaharmahal va Bakhtiari, Isfahan and Khoozestan provinces, locate in west central parts of Iran. For this, 548 serum samples were collected from dogs during an 18 month period from July 2007 to February 2009. Serodiagnosis of *N. caninum* was carried out using a homemade indirect ELISA test and of *T. gondii* using an optimized indirect ELISA designed using an affinity purified 30 kDa *T. gondii* surface antigen (SAG1). From a total of 548 dogs, 159 (29%) were positive for *N. caninum* and 147 (26.8) for *T. gondii*. 49 (8.94%) dogs had detectable antibodies against both *N. caninum* and *T. gondii*. No sex predisposition was detected in the examined animals, but age and living places were of high importance for both *N. caninum* and *T. gondii* infections.

Key words: *Neospora caninum*, *Toxoplasma gondii*, Dogs, Iran, ELISA

Introduction

Toxoplasma gondii and *Neospora caninum* are two closely related protozoan parasites that are distributed worldwide and that cause neurologic disease in dogs. Both organisms can infect a wide range of animal species and have an indirect life-cycle with carnivores as the definitive hosts; domestic cats and other felidae for *T. gondii* and dogs and coyotes for *N. caninum* (Frenkel, 1970; McAllister *et al*., 1998; Gondim *et al*., 2004).

In general, clinical findings of neosporosis in dogs are similar to those of toxoplasmosis, but neurologic deficits and muscular abnormalities predominate. Likely, many dogs diagnosed with toxoplasmosis before 1988 actually had neosporosis (Dubey and Lappin, 2006).

While *T. gondii* has an important role in public health and causes congenital defects or abortion and fatal diseases in immune compromised people, *N. caninum* is known as a major cause of bovine abortion and canine neuropathies. Ingestion of food or water contaminated with oocysts from faeces of definitive hosts is of major modes of transmission (Jackson and Hutchison, 1989; Dubey and Lindsay, 1996).

Although felines are the only definitive host of *T. gondii* and only they have been linked to faecal transmission of the infection, dogs have been suspected as possible mechanical carriers of *T. gondii*. A role for dogs in the transmission of *T. gondii* to humans has been postulated based on serological surveys and observations that dogs ingest cat faeces and often roll in cat faeces and other foul-smelling substances (Frenkel *et al*., 2003). In one study, viable sporulated oocysts were detected in dog faeces for up to 2 days after feeding on sporulated *T. gondii* oocysts (Lindsay *et al*., 1997). Schares *et al*. (2005) have detected *T. gondii* oocysts in faeces of two of 24.089 dogs in Germany.

Serological surveys suggest that *N.*

caninum and *T. gondii* infections are widespread throughout the world in carnivores as well as in intermediate hosts. Several serological tests were used for sero-diagnosis of *N. caninum* and *T. gondii* infections among which ELISA tests are superior in aspects of possibility to analyse several samples more quickly and at lesser expenses (Schares *et al*., 1999a; Schares *et al*., 2001; Azevedo *et al*., 2005; Malmasi *et al*., 2007; Wapenaar *et al*., 2007).

The aim of this study was to evaluate the serological prevalence of *N. caninum* and *T. gondii* infections in dogs in three Iranian provinces (Chaharmahal va Bakhtiari, Isfahan and Khoozestan) and to investigate the possible risk factors.

Materials and Methods

Parasites

The NC-1 strain of *N. caninum* (Dubey *et al*., 1988) was maintained as previously described (Schares *et al*., 1999b). Tachyzoites were freeze-dried until used for ELISA.

Toxoplasma gondii RH strain tachyzoites were grown *in vitro* using Vero cell monolayers in RPMI-1640 supplemented with 2% fetal bovine serum and a mixture of 50 U/ml penicillin and 50 mg streptomycin. The cultures were incubated at 37°C in a 5% CO_2 environment. Tachyzoites were frozen at -80°C until used.

Animals

Blood samples were collected randomly from the saphenous vein of 548 healthy dogs during an 18 month period from July 2007 till February 2009 in three Iranian provinces, Chaharmahal va Bakhtiari (248 samples), Isfahan (200 samples) and Khoozestan (100 samples) locate in the west central parts of Iran. Information about age, sex and living condition (stray or shepherd and household) were obtained for further processing. Serum samples were aliquoted and stored at -80°C until used.

From 548 sampled dogs, 300 (54.74%) were males and 248 (45.26) were females; 113 were less than 12 months of age and 435 were 12 months and more; 317 were stray or shepherd dogs and 231 were household dogs.

Serologic tests

N. caninum ELISA test

Serodiagnosis of *N. caninum* infection was performed using a homemade indirect ELISA test as described previously (Ortega-Mora *et al*., 2007) with some modifications. A suspension of approximately 1×10^8 tachyzoites in 1 ml of PBS was frozen and thawed (-80°C and +37°C) three times repeatedly. This was then ultrasonicated and centrifuged at 10,000 g at 4°C for 30 min. The supernatant was diluted (1:1000) in 0.1 M sodium bicarbonate, pH = 8.3 and used to sensitize polysorp 96-well micro-titre plates (Nunc-Immuno (polysorb), Denmark) by 1 h incubation at 37°C, followed by overnight incubation at 4°C overnight.

Wells were then emptied, washed and incubated with blocking solution (PBS-T, 20% horse serum) at 37°C for 0.5 h. All washing procedures were done three times, using PBS-T (PBS, pH = 7.2, 0.05% Tween-20). Wells were then emptied and the serum samples (diluted 1:100 in PBST, 20% horse serum) added. 30 negative serum samples (confirmed using an indirect fluorescent antibody test) were used to optimize a cut-off point.

After 30 min of incubation and three times washing, an anti-dog IgG; whole molecule peroxidase conjugate (A9042, Sigma-Aldrich, USA) diluted 1:10,000 in PBS-T 2% horse serum was added to the wells (37°C, 30 min). Sera and conjugate dilutions were optimized using a checkerboard titration. Washing was performed three times with PBS-T and two times with distilled water. Bound antibodies were detected by incubation with 100 µl 3,3′,5,5′-tetramethylbenzidine substrate (T2885, Sigma-Aldrich) prepared in DMSO (100 µg/ml), phosphate citrate buffer and 0.02 of 30% hydrogen peroxide (30%). After 15 min, 50 micro-liters of 2N sulfuric acid was used to stop the reaction and optical density (OD) values were measured at 450 nm on a microplate reader. A sample was regarded as positive when its OD was more than the mean OD of the negative samples plus 5 standard deviations (Ortega-Mora *et al*., 2007).

T. gondii ELISA test

Toxoplasma gondii ELISA test was

performed using an affinity purified surface antigen SAG1 as described previously by Hosseininejad *et al*. (2009).

Statistical analysis

The prevalence was estimated from the ratio of positive results to the total number of dogs examined. Assessment of association between the seroprevalence of anti-*T. gondii* and anti-*N. caninum* antibodies in dogs and selected risk factors was made by logistic regression test with a confidence interval of 0.95 using SPSS 16 (SPSS Inc. Headquarters USA) software. To assess any possible correlation between the seroprevalence of the two investigated parasites, the results were subjected to the McNemar test in SPSS 16 software.

Results

From a total of 548 dogs, 159 (29%) were positive for *N. caninum* and 147 (26.8) for *T. gondii*. 49 (8.94%) had detectable antibodies against both *N. caninum* and *T. gondii*. The seroprevalence of the infections are summarised in Table 1 in detail.

No sex predisposition was seen, neither for *N. caninum* (P=0.857) nor for *T. gondii* (P=0.768) infections. *Neospora caninum* and *T. gondii* were of higher seroprevalence in dogs of 12 months and more in comparison with younger dogs. Household dogs had a lower rate of infection than stray or shepherd dogs (P<0.001).

From the total of the serum samples analyzed, 49 (8.94%) were reactive to both parasites, but without significant correlation (McNemar test showed no agreement between these two tests).

Discussion

Neospora caninum crude antigen has previously been used in ELISA tests with acceptable results. In previous studies, the optimal correlation between ELISA using crude antigen, IFAT, and Western blot confirmed the suitability of this ELISA for large-scale seroepidemiologic studies (Gottstein *et al*., 1998; Naguleswaran *et al*., 2004). Using Mean±5SD for cut off ensures high specifity of the test to minimize non specific reactions due to cross reactivity or other possible factors. Other ELISA tests using affinity purified or recombinant antigens have also been used for serological diagnosis of this disease, although antigen purification and production are time consuming procedures and necessitate expensive procedures (Hosseininejad *et al*., 2010).

Previously done SAG1 ELISA for serodiagnosis of *T. gondii* in dogs showed a relative sensitivity and specificity of 94.52 and 93.60%, respectively using an index of 0.790 as ELISA index cut-off point (Hosseininejad *et al*., 2009).

Some serological assays have been done in Iran to clarify the extent of *N. caninum* infection. These studies were performed in a limited population of dogs in Tehran and Urmia (Malmasi *et al*., 2007; Yakhchali *et al*., 2010) as well as in cattle (Razmi *et al*., 2006) and camel populations (Sadrebazzaz *et al*., 2006).

Detection of specific antibodies in canine serum samples by means of the indirect ELISA tests provided evidence that dogs in these parts of Iran have had contact

Table 1: Comparison of *N. caninum* and *T. gondii* seroprevalence in different sex, age and living places

	Sex		Age		Living place	
	Male	Female	Under 12 months	12 months and more	Stray or shepherd	Household
Total	300	248	113	435	317	231
N. caninum positive (%)	88 (29.33)	71 (28.62)	6 (5.3)	152 (34.94)	138 (43.35)	20 (8.65)
Statistical difference	P=0.857		P<0.001		P<0.001	
T. gondii positive (%)	84 (28)	67 (27.01)	5 (4.42)	146 (33.56)	135 (42.58)	16 (6.92)
Statistical difference	(P=0.768)		P<0.001		P<0.001	

S: Significant difference, and NS: No significant difference (P≤0.05)

with *N. caninum* and *T. gondii*, in relatively high rates.

Neospora caninum infection has been estimated in different countries. The prevalence of 29% for *N. caninum* found in our study was higher than that reported for dogs from Turkey (10% of 150 dogs) (Coskun *et al*., 2000), Brazil (6.7% of 163 dogs) (Mineo *et al*., 2001), Brazil (10% of 500 pet dogs and 25% of 611 stray dogs) (Gennari *et al*., 2002) and Italy (6.4% of 1,058 dogs) (Cringoli *et al*., 2002). The high prevalence of infection in Iran indicates the necessity of control strategies to be performed in dogs and cattle populations. A previously done serologic test performed in Tehran, Iran has estimated the seroprevalence of this infection as 33% of 100 dogs (Malmasi *et al*., 2007).

The prevalence of 26.8% for *T. gondii* found in our study was less than what has been estimated in Sweden (30%) (Bjoerkman *et al*., 1994) and Brazil (76.4%) (Canon-Franco *et al*., 2004) and approximately identical to what has been detected in Austria (26%) (Wanha *et al*., 2005).

Our finding that the chance of having *T. gondii* and *N. caninum* antibodies increases with age of dogs is in agreement with previous findings (Lin, 1998; Azevedo *et al*., 2005; Malmasi *et al*., 2007; Yakhchali *et al*., 2010) and has been attributed to a greater probability for exposure to these protozoan parasites over time, increasing the susceptibility in older dogs.

Higher seroprevalence of *N. caninum* and *T. gondii* in stray or shepherd dogs may be linked to their accessibility to risk factors of infection including raw meat, abortion materials from intermediate hosts (cattle and sheep, respectively) and oocysts contaminated water and food (Dubey, 1987; Dijkstra *et al*., 2002). No significant association of the infections with sex has been reported by others (Lin, 1998; Malmasi *et al*., 2007).

Concordant occurrence of *N. caninum* and *T. gondii* infections was seen with different percentages in different studies and reported as 14 of 286 (4.9%) from Brazil (Azevedo *et al*., 2005) and 1.7% from Austria (Wanha *et al*., 2005).

This study shows a relatively high infection of the dogs with *T. gondii* and *N. caninum* in the three mentioned Iranian provinces and emphasizes the necessity of regarding these two diseases in clinical cases and to perform serological tests in dogs suspected to have these two infections.

Acknowledgements

This study was financially supported by the research deputy of Shahrekord University, Iran. The authors thank Dr. G. Schares and his group for their help in providing antigens used for serodiagnosis and the scientific information.

References

Azevedo, SS; Batista, CS; Vasconcellos, SA; Aguiar, DM; Ragozo, AM; Rodrigues, AA; Alves, CJ and Gennari, SM (2005). Seroepidemiology of *Toxoplasma gondii* and *Neospora caninum* in dogs from the state of Paraiba, northeast region of Brazil. Res. Vet. Sci., 79: 51-56.

Bjoerkman, C; Lunden, A and Uggla, A (1994). Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in Swedish dogs. Acta Vet. Scand., 35: 445-447.

Canon-Franco, WA; Bergamaschi, DP; Labruna, MB; Camargo, LM; Silva, JC; Pinter, A and Gennari, SM (2004). Occurrence of anti-*Toxoplasma gondii* antibodies in dogs in the urban area of Monte Negro, Rondonia, Brazil. Vet. Res. Commun., 28: 113-118.

Coskun, SZ; Aydyn, L and Bauer, C (2000). Seroprevalence of *Neospora caninum* infection in domestic dogs in Turkey. Vet. Rec., 146: 649.

Cringoli, G; Rinaldi, L; Capuano, F; Baldi, L; Veneziano, V and Capelli, G (2002). Serological survey of *Neospora caninum* and *Leishmania infantum* co-infection in dogs. Vet. Parasitol., 106: 307-313.

Dijkstra, T; Barkema, HW; Eysker, M; Hesselink, JW and Wouda, W (2002). Natural transmission routes of *Neospora caninum* between farm dogs and cattle. Vet. Parasitol., 105: 99-104.

Dubey, JP (1987). *Toxoplasma gondii* cysts in placentas of experimentally infected sheep. Am. J. Vet. Res., 48: 352-353.

Dubey, JP; Hattel, AL; Lindsay, DS and Topper, MJ (1988). Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc., 193: 1259-1263.

Dubey, JP and Lappin, M (2006). Toxoplasmosis and neosporosis. In: Greene, C (Ed.), *Infectious diseases of the dog and cat*. (3rd Edn.), Toronto, W. B. Saunders Co., PP: 754-775.

Dubey, JP and Lindsay, DS (1996). A review of *Neospora caninum* and neosporosis. Vet. Parasitol., 67: 1-59.

Frenkel, JK (1970). Pursuing toxoplasma. J. Infect. Dis., 122: 553-559.

Frenkel, JK; Lindsay, DS; Parker, BB and Dobesh, M (2003). Dogs as possible mechanical carriers of toxoplasma, and their fur as a source of infection of young children. Int. J. Infect. Dis., 7: 292-293.

Gennari, SM; Yai, LE; D'Auria, SN; Cardoso, SM; Kwok, OC; Jenkins, MC and Dubey, JP (2002). Occurrence of *Neospora caninum* antibodies in sera from dogs of the city of Sao Paulo, Brazil. Vet. Parasitol., 106: 177-179.

Gondim, LF; McAllister, MM; Pitt, WC and Zemlicka, DE (2004). Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. Int. J. Parasitol., 34: 159-161.

Gottstein, B; Hentrich, B; Wyss, R; Thur, B; Busato, A; Stark, KD and Muller, N (1998). Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. Int. J. Parasitol., 28: 679-691.

Hosseininejad, M; Azizi, HR; Hosseini, F and Schares, G (2009). Development of an indirect ELISA test using a purified tachyzoite surface antigen SAG1 for sero-diagnosis of canine *Toxoplasma gondii* infection. Vet. Parasitol., 164: 315-319.

Hosseininejad, M; Hosseini, F; Mosharraf, M; Shahbaz, S; Mahzounieh, M and Schares, G (2010). Development of an indirect ELISA test using an affinity purified surface antigen (P38) for sero-diagnosis of canine *Neospora caninum* infection. Vet. Parasitol., 171: 337-342.

Jackson, MH and Hutchison, WM (1989). The prevalence and source of *Toxoplasma* infection in the environment. Adv. Parasitol., 28: 55-105.

Lin, DS (1998). Seroprevalences to *Toxoplasma gondii* in privately-owned dogs in Taiwan. Prev. Vet. Med., 35: 21-27.

Lindsay, DS; Dubey, JP; Butler, JM and Blagburn, BL (1997). Mechanical transmission of *Toxoplasma gondii* oocysts by dogs. Vet. Parasitol., 73: 27-33.

Malmasi, A; Hosseininejad, M; Haddadzadeh, H; Badii, A and Bahonar, A (2007). Serologic study of anti-*Neospora caninum* antibodies in household dogs and dogs living in dairy and beef cattle farms in Tehran, Iran. Parasitol. Res., 100: 1143-1145.

McAllister, MM; Dubey, JP; Lindsay, DS; Jolley, WR; Wills, RA and McGuire, AM (1998). Rapid communications: dogs are definitive hosts of *Neospora caninum*. Int. J. Parasitol., 28: 1473-1478.

Mineo, TW; Silva, DA; Costa, GH; von Ancken, AC; Kasper, LH; Souza, MA; Cabral, DD; Costa, AJ and Mineo, JR (2001). Detection of IgG antibodies to *Neospora caninum* and *Toxoplasma gondii* in dogs examined in a veterinary hospital from Brazil. Vet. Parasitol., 98: 239-245.

Naguleswaran, A; Hemphill, A; Rajapakse, RP and Sager, H (2004). Elaboration of a crude antigen ELISA for serodiagnosis of caprine neosporosis: validation of the test by detection of *Neospora caninum*-specific antibodies in goats from Sri Lanka. Vet. Parasitol., 126: 257-262.

Ortega-Mora, L; Gottstein, B; Conraths, FJ and Buxton, D (2007). *Protozoal abortion in farm ruminants: guidelines for diagnosis and control*. 1st Edn., Cambridge, CABI Publishing. PP: 276-289.

Razmi, GR; Mohammadi, GR; Garrosi, T; Farzaneh, N; Fallah, AH and Maleki, M (2006). Seroepidemiology of *Neospora caninum* infection in dairy cattle herds in Mashhad area, Iran. Vet. Parasitol., 135: 187-189.

Sadrebazzaz, A; Haddadzadeh, H and Shayan, P (2006). Seroprevalence of *Neospora caninum* and *Toxoplasma gondii* in camels (*Camelus dromedarius*) in Mashhad, Iran. Parasitol. Res., 98: 600-601.

Schares, G; Conraths, FJ and Reichel, MP (1999a). Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. Int. J. Parasitol., 29: 1659-1667.

Schares, G; Pantchev, N; Barutzki, D; Heydorn, AO; Bauer, C and Conraths, FJ (2005). Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int. J. Parasitol., 35: 1525-1537.

Schares, G; Rauser, M; Zimmer, K; Peters, M; Wurm, R; Dubey, JP; de Graaf, DC; Edelhofer, R; Mertens, C; Hess, G and Conraths, FJ (1999b). Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. J. Parasitol., 85: 688-694.

Schares, G; Wenzel, U; Muller, T and Conraths, FJ (2001). Serological evidence for naturally occurring transmission of *Neospora caninum* among foxes (*Vulpes vulpes*). Int. J.

Parasitol., 31: 418-423.

Wanha, K; Edelhofer, R; Gabler-Eduardo, C and Prosl, H (2005). Prevalence of antibodies against *Neospora caninum* and *Toxoplasma gondii* in dogs and foxes in Austria. Vet. Parasitol., 128: 189-193.

Wapenaar, W; Barkema, HW; Schares, G; Rouvinen-Watt, K; Zeijlemaker, L; Poorter, B; O'Handley, RM; Kwok, OC and Dubey, JP (2007). Evaluation of four serological techniques to determine the seroprevalence of *Neospora caninum* in foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*) on Prince Edward Island, Canada. Vet. Parasitol., 145: 51-58.

Yakhchali, M; Javadi, S and Morshedi, A (2010). Prevalence of antibodies to *Neospora caninum* in stray dogs of Urmia, Iran. Parasitol. Res., 106: 1455-1458.