

Enzyme-linked immunosorbent assays using recombinant TgSAG2 and NcSAG1 to detect *Toxoplasma gondii* and *Neospora caninum*-specific antibodies in domestic animals in Turkey

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ABSTRACT. Considering the scarce information on occurrences of *Toxoplasma gondii* and *Neospora caninum* in domestic animals from Turkey, the aim of this study was to investigate the seroprevalence of these parasite infections in cattle, horses, sheep, goats and dogs in Turkey. The specific antibodies against *T. gondii* and *N. caninum* were detected by iELISAs based on the recombinant TgSAG2 or NcSAG1 in a total of 2,039 serum samples from eleven provinces. The seroprevalence of *T. gondii* infections was 46.3%, 4.0%, 20.0%, 12.9% and 19.8%, that of *N. caninum* infections was 0.3%, 7.4%, 2.1%, 3.2% and 16.6% in the horses, cattle, sheep, goats and dogs, respectively. These results indicated that *T. gondii* and *N. caninum* infections are prevalent in Turkish domestic animals.

KEY WORDS: ELISA, *Neospora caninum*, *Toxoplasma gondii*, Turkey

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Toxoplasma gondii (*T. gondii*) and *Neospora caninum* (*N. caninum*) are two closely related apicomplexan protozoa that infect many warm blood animals. *T. gondii* shares structural, genetic and immunological similarities to *N. caninum*, but they are antigenically different [10, 23]. *T. gondii* infections cause abortion or neonatal mortalities in human and warm-blooded animals [7, 28]. *N. caninum* is the causative agent of neosporosis, an infection that always causes reproductive failure in cattle, sheep, goats and horses, and neurological alterations in dogs [1, 10, 26]. Numerous epidemiologic studies of toxoplasmosis and neosporosis have been reported in many areas worldwide [2, 6, 20, 27]. However, the epidemiological information about the seroprevalence of *T. gondii* and *N. caninum* is limited in Turkey [19]. Therefore, the objective of this study was to determine the seroprevalence of *T. gondii* and *N. caninum* in a wide range of domestic animals in Turkey. Recombinant antigens are usually available in pure form, which provide better options in serological diagnosis [5, 16]. Surface antigen 2 of *T. gondii* (TgSAG2) and surface antigen 1 of *N. caninum*

(NcSAG1) have been identified as important candidate of serological diagnosis for toxoplasmosis and neosporosis, respectively [5, 13, 15–17, 21]. In this study, we determined the seroprevalence of *T. gondii* and *N. caninum* in a wide range of domestic animals in Turkey using ELISA based on the recombinant TgSAG2 and NcSAG1, respectively.

The field samples analyzed in this study were collected from 11 provinces that located in the 6 regions of Turkey (Fig. 1). The serum samples were obtained from a total of 2,039 animals (616 horses, 377 cattle, 610 sheep, 249 goats and 187 dogs) within the study area. The horses were adult draft type belonging to the farmers in Adana, Bursa, Gaziantep, Istanbul, Izmir and Konya provinces. The cattle that raised primarily for milk production (dairy breed/cross breed) were selected from Adana, Afyon, Diyarbakir, Karaman, Kirklareli, Konya and Zonguldak provinces. Sheep that raised principally for the meat, wool and breeding were selected from Karaman, Konya and Zonguldak provinces. Most of the selected sheep were females and over one year old. The goats selected from the herds in Karaman and Konya provinces were reared for family milk and meat consumption. The dogs in the present study were house and stray dogs across the urban-rural areas of Konya province. All animal experiments were approved by the Scientific and Technological Research Council of Turkey. Care of animals and animal experimentation were performed in accordance with Animal Welfare Approved Standards for Turkeys (<http://animalwelfareapproved.org/>).

The recombinant TgSAG2-GST and NcSAG1-GST proteins used in this study were generated according to

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Fig. 1. Regions for collecting samples in Turkey. The serum samples from six historical regions were studied: Karaman and Konya (Central Anatolia region); Zonguldak (Black sea region); Bursa, İstanbul and Kırklareli (Marmara region); Afyon and İzmir (Aegean region); Adana and Gaziantep (Mediterranean region); Diyarbakir (Southeastern Anatolia region). The provinces, from which the serum samples were collected, are presented with grey.

the method described previously [5, 16]. In brief, the PCR products of truncated TgSAG2 and NcSAG1 were inserted into the pGEX-4T vector (Amersham Pharmacia Biotech, San Francisco, CA, U.S.A.) and expressed in an *E. coli* BL-21 strain. A fresh 10 ml overnight culture of transformed *E. coli* was grown in 1 L of LB base broth containing 50 µg/ml of ampicillin at 37°C with shaking at 250 rpm until the optical density (OD) at 600 nm reached to 0.5. The expression of these proteins was induced by 5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 27°C overnight. The *E. coli* culture was centrifuged at 8,000 g for 15 min, and the cell pellet was then suspended in TNE buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA and 1% Triton X-100) containing 50 mg/ml lysozyme, 1% (w/v) N-Lauroylsarcosine sodium and protease inhibitors. These recombinant proteins were purified from the soluble fractions using Glutathione-Sepharose 4B beads, according to the manufacturer's instructions (Amersham Pharmacia Biotech). Protein expressions were verified by SDS-PAGE stained with Coomassie blue. The concentration of the expressed protein was measured using the BCA assay.

Indirect ELISAs were carried out to detect specific antibodies to *T. gondii* (rTgSAG2-ELISA) and *N. caninum* (rNcSAG1-ELISA) according to the previous reports, with modifications [5, 16]. Briefly, rTgSAG2-GST, rNcSAG1-GST and rGST were diluted to a final concentration of 4 µg/ml, 4 µg/ml and 4 µg/ml, in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6), respectively. The wells of the ELISA plate were coated with 100 µl of these antigens and incubated overnight at 4°C. Typically, after removing the coating solution, the plates were then blocked with PBS containing 3% (w/v) skim milk for 1 hr at 37°C. After washing, the plates were incubated with serum samples (diluted 1:100). The bound antibody was detected by treating with horseradish peroxidase (HRP)-conjugated (Bethyl, Montgomery, AL, U.S.A.) to anti-horse IgG, anti-bovine IgG, anti-sheep/goat IgG or anti-dog IgG (1:4,000) and ABTS

[2,2'-azinobis (3-ethylbenzthiazolinesulfonic acid)] (Sigma, St. Louis, MO, U.S.A.). The color was allowed to develop at room temperature. And, 50 µl stop solution was added (2 M sulfuric acid) to each well to stop the action of horseradish peroxidase in the substrate. Optical density (OD) was measured by an MTP-500 microplate reader (Corona Electric, Hitachinaka, Japan) at 415 nm. The ELISA results were determined for each sample by subtracting OD₄₁₅ value of GST protein from OD₄₁₅ value of recombinant TgSAG2 or NcSAG1 proteins. The cut-off values were determined using the *T. gondii* or *N. caninum*-negative sera from different animals and calculated as the mean of OD₄₁₅ value of negative sera plus three standard deviations. The cut off values were determined as 0.098, 0.103, 0.107, 0.102 and 0.118 for TgSAG2-ELISA; and 0.118, 0.093, 0.102, 0.141 and 0.124 for NcSAG1-ELISA in horses (n=30), cattle (n=30), sheep (n=50), goats (n=50) and dogs (n=20), respectively (Fig. 2). A sample was considered positive when the absorbance value of sample was higher than the cut-off value. The prevalence of anti-*T. gondii* and anti-*N. caninum* antibodies was estimated from the ratio of positive results to the total number of different animal examined with the exact binomial confidence interval of 95% (95% CI). Odds ratio (OR) with 95% confidence intervals based on likelihood ratio statistics is reported. Difference in the prevalence of these pathogens among different provinces was calculated using the binary logistic regression in SPSS (Release 18.0 standard version, SPSS Inc., Chicago, IL, U.S.A.).

The prevalence of *T. gondii* and *N. caninum* in horses, cattle, sheep, goats and dogs from 11 provinces of Turkey is summarized in Table 1. The overall seroprevalence of *T. gondii* (24.1%, 95% CI: 22.3–26.0%) is significantly higher than *N. caninum* (4.0%, 95% CI: 3.3–5.0%) in the surveyed regions ($P < 0.0001$; OR: 7.49; 95% CI: 5.88–9.56). Furthermore, the anti-*Toxoplasma* antibodies were detected in all of the 11 provinces (100%). The overall seroprevalence of *T. gondii* in horses (46.3%, 95% CI: 42.4–50.2%) was

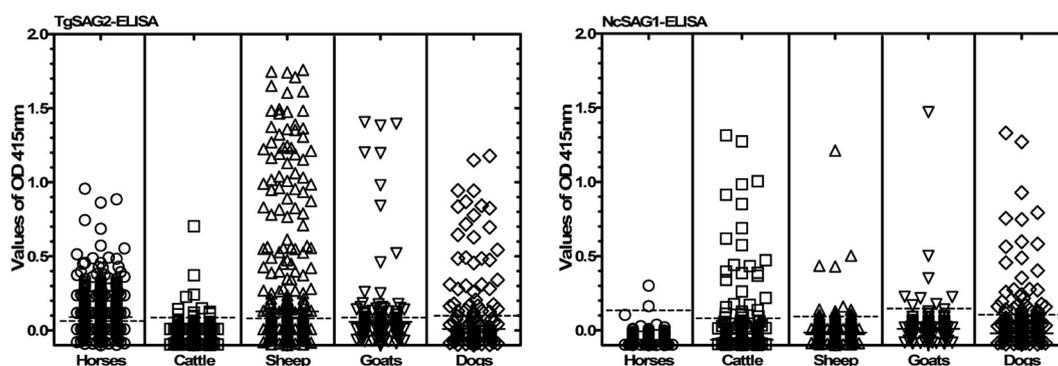


Fig. 2. Enzyme-linked immunosorbent assay (ELISA) results of the recombinant TgSAG2 and NcSAG1.

higher than that of sheep (20.0%, 95% CI: 17.0–23.4%), dogs (19.8%, 95% CI: 14.7–26.1%), goats (12.9%, 95% CI: 9.3–17.6%) and cattle (4.0%, 95% CI: 2.4–6.5%). This investigation revealed a high *T. gondii* seroprevalence of horses in Turkey, indicating a potential threat to equine health in this country. Under natural conditions, seroprevalence of *Toxoplasma* in horses may vary from 0 to 90% [25]. In this study, the seroprevalence of *T. gondii* was detected in all the surveyed regions ranged from 3.7 to 56.5%, which was consistent with a previous study. Cattle have a high natural resistance to *T. gondii*, and infection in them does not usually cause clinical symptoms [8]. Therefore, the seroprevalence of *T. gondii* in cattle was low. Seroprevalence of *T. gondii* infection in cattle in Brazil and Iran was 1.03 and 4.0%, respectively, in a previous study [20, 22]. The present study also showed a low seroprevalence (4.0%) of *T. gondii* in cattle. The role of canine toxoplasmosis in the epidemiology of this parasitic disease has not been clarified, however, these animals can act as sentinels of environmental contamination with oocysts. Seroprevalence of *T. gondii* infections in free-living animals, such as stray dogs, has been investigated worldwide [4, 18, 29]. However, the information about the distribution and density of *T. gondii* in dogs from Turkey is limited. Therefore, we estimated the distribution and transmission dynamics of *T. gondii* in the environment by examining the seroprevalence of stray dogs as indicators in Konya province of Turkey. The seroprevalence of *T. gondii* antibodies in dogs was 19.8% in this study, which was lower than the previous reports in other countries [4, 18, 29].

The specific antibodies of *N. caninum* were detected in the sera of 82 out of 2,039 (4.0%) animals in this study. Neosporosis is a serious disease of cattle and dogs worldwide, and it also affects sheep, goats, water buffaloes and horses [3]. The overall seroprevalence of *N. caninum* in dogs (16.6%; 95% CI: 11.9–22.6%) was higher than that of cattle (7.4%; 95% CI: 5.2–10.5%), goats (3.2%; 95% CI: 1.6–6.2%), sheep (2.1%; 95% CI: 1.3–3.6%) and horses (0.3%; 95% CI: 0.1–1.2%), which demonstrated that the infection of *N. caninum* is not common in horses, sheep and goats in Turkey. Many studies about the prevalence of *N. caninum* in sheep and goats have been reported. The *Neospora* was reported at a prevalence of 3.3% and 6% in goats from Brazil and Czech Republic,

respectively. Furthermore, 8.1% of sheep from Uberlandia, and 16.8% of sheep and 6.9% of goats from Greece showed seropositive for anti-*N. caninum* antibodies in a previous study [2, 12, 24]. The seroprevalence of *N. caninum* in sheep and goats obtained by us is similar with other reports from different countries. Antibodies to *Neospora* species in equine populations were reported in many parts of the world, such as Brazil [7, 9], U.S.A. [11] and Iran [14]. However, little is known on the prevalence of antibodies to *Neospora* in subclinically infected horses in Turkey. Several studies have detected the antibodies against *N. caninum* even more than 30% of horses [11]. The current study found that the proportion of horses with antibodies against *N. caninum* was quite low (0.3%), which was lower than previously reports for other regions. The finding of this study indicates that the horses in Turkey are at risk of neosporosis.

The highest prevalence of toxoplasma in cattle was found in Konya province (9.2%; 95% CI: 5.4–15.4%), and followed by Kırklareli (2%; 95% CI: 0.7–15.4%) and Afyon (1.3%; 95% CI: 0.2–6.8%). The specific antibody against *T. gondii*s could not be detected in other four provinces. For neosporosis, Zonguldak showed the highest prevalence (39.1%; 95% CI: 22.2–59.2%) in cattle. The seroprevalence of *N. caninum* in cattle was 7.7% (95% CI: 2.7–20.3%), 3.8% (95% CI: 1.3–10.5%), 5.9% (95% CI: 1.1–27.0%), 7.7% (95% CI: 3.6–15.8%) and 4.6 (95% CI: 2.1–9.7%) in Adana, Afyon, Diyarbakir, Kırklareli and Konya provinces, respectively. Mixed infection was found in one cow. The sheep from Konya showed the highest prevalence (24.0%; 95% CI: 20.3–28.2%) of toxoplasma, and followed by Karaman (9.2%; 95% CI: 5.2–15.7%) and Zonguldak (7.5%; 95% CI: 2.6–19.9%) provinces. The seropositivity of *neospora* in sheep from Karaman and Konya was 0.8% (95% CI: 0.2–4.6%) and 2.7% (95% CI: 1.5–4.6%). Mixed infection was found in 3 sheep. In goat samples, the antibodies against *T. gondii* were found to be 8.3% (95% CI: 3.6–18.1%) in Karaman and 14.3% (95% CI: 10.0–20.0%) in Konya. The specific antibodies against *N. caninum* were detected in the goats only from Konya province showing low level of infection rate with 4.2% (95% CI: 2.2–8.1%). Mixed infection was found in 2 goats. The seroprevalence of *T. gondii* (19.8%; 95% CI: 14.7–26.1) and *N. caninum* (16.6%; 95%

Table 1. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* among horses, cattle, sheep, goats and dogs in Turkey

Animal species	Regions	No. of animals tested	<i>T. gondii</i> -seropositive		<i>N. caninum</i> -seropositive		Both seropositive	
			Frequency	Prevalence (95% CI)	Frequency	Prevalence (95% CI)	Frequency	Prevalence (95% CI)
Horses	Adana	54	17	31.5 (20.7–44.7)	0	0 (0–6.6)	0	0 (0–6.6)
	Bursa	370	209	56.5 (51.4–61.5)	1	0.3 (0.1–1.5)	1	0.3 (0.1–1.5)
	Gaziantep	19	5	26.3 (11.8–49.0)	0	0 (0–16.8)	0	0 (0–16.8)
	Istanbul	39	19	48.7 (33.9–63.8)	0	0 (0–9.0)	0	0 (0–9.0)
	Izmir	54	2	3.7 (1.0–12.5)	0	0 (0–6.6)	0	0 (0–6.6)
	Konya	80	33	41.3 (31.1–52.2)	1	1.3 (0.2–6.8)	0	0 (0–4.6)
	Total	616	285	46.3 (42.4–50.2)	2	0.3 (0.1–1.2)	1	0.2 (0–0.9)
Cattle	Adana	39	0	0 (0–9.0)	3	7.7 (2.7–20.3)	0	0 (0–9.0)
	Afyon	80	1	1.3 (0.2–6.8)	3	3.8 (1.3–10.5)	0	0 (0–4.6)
	Diyarbakir	17	0	0 (0–18.4)	1	5.9 (1.1–27.0)	0	0 (0–18.4)
	Karaman	10	0	0 (0–27.8)	0	0 (0–27.8)	0	0 (0–27.8)
	Kirklareli	78	2	2.6 (0.7–8.9)	6	7.7 (3.6–15.8)	0	0 (0–4.7)
	Konya	130	12	9.2 (5.4–15.4)	6	4.6 (2.1–9.7)	1	0.8 (0.1–4.2)
	Zonguldak	23	0	0 (0–14.3)	9	39.1 (22.2–59.2)	0	0 (0–14.3)
	Total	377	15	4.0 (2.4–6.5)	28	7.4 (5.2–10.5)	1	0.3 (0.1–1.5)
Sheep	Karaman	120	11	9.2 (5.2–15.7)	1	0.8 (0.2–4.6)	0	0 (0–3.1)
	Konya	450	108	24.0 (20.3–28.2)	12	2.7 (1.5–4.6)	3	0.7 (0.2–2.0)
	Zonguldak	40	3	7.5 (2.6–19.9)	0	0 (0–8.8)	0	0 (0–8.8)
	Total	610	122	20.0 (17.0–23.4)	13	2.1 (1.3–3.6)	3	0.5 (0.2–1.4)
Goats	Karaman	60	5	8.3 (3.6–18.1)	0	0 (0–6.0)	0	0 (0–6.0)
	Konya	189	27	14.3 (10.0–20.0)	8	4.2 (2.2–8.1)	2	1.1 (0.3–3.8)
	Total	249	32	12.9 (9.3–17.6)	8	3.2 (1.6–6.2)	2	0.8 (0.2–2.9)
Dogs	Konya	187	37	19.8 (14.7–26.1)	31	16.6 (11.9–22.6)	8	4.3 (2.2–8.2)
	Total	2,039	491	24.1 (22.3–26.0)	82	4.0 (3.3–5.0)	15	0.7 (0.5–1.2)

CI: 11.9–22.6) was similar in dogs. In this study, the dog samples were collected from 50 stray and 137 registered dogs in Konya province. The seroprevalence of these two parasites in stray dogs was higher than that of registered dogs (data not shown). Mixed infection was detected in 8 dogs. These results indicated that the prevalence of these parasites was different among the different animal species and regions. Furthermore, the co-infection may affect transmission and recovery from infection, and therefore, the co-infection should be considered in a disease control and treatment decisions.

In summary, the results of this study confirm that *T. gondii* and *N. caninum* affect some species of domestic animals in Turkey. In the case of toxoplasmosis, the relative high prevalence was evidenced, emphasizing the necessity of more detailed studies for the control of this infection in these regions. In respect to neosporosis, the results obtained in this study showed a higher prevalence of dogs and cattle than those of horses, sheep and goats. For preventing *N. caninum* infection, the livestock need to be quarantined with dogs, which play an important role in the transmission and dissemination of the disease.

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