

Investigation of Anti-*Toxoplasma gondii* and Anti-*Leishmania infantum* Antibodies among Sivas Kangal Dogs*

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Abstract: The present study was carried out to determine the seroprevalence of toxoplasmosis and leishmaniosis among Kangal dogs on breeding farms in Sivas, Turkey. Serum samples from 50 Kangal dogs without clinical symptoms of disease were tested for anti-*Toxoplasma gondii* antibodies with the Sabin Feldman Dye Test (SFDT) and for anti-*Leishmania* antibodies with the Indirect Fluorescent Antibody Test (IFAT). Forty-six out of the 50 dogs were seropositive for *Toxoplasma gondii*, with titers ranging from 1:16 to 1:1024. Only 1 of the 50 dogs was seropositive for leishmaniosis (titer: 1:128) and 5 had anti-*Leishmania* antibodies with a titer of 1:64, which was considered uncertain. There were no statistically significant differences in the seroprevalence of toxoplasmosis and leishmaniosis based the gender and age of the dogs ($P > 0.05$). In conclusion, the study revealed that *Toxoplasma gondii* is widely distributed in Kangal dogs. Although the seroprevalence of canine leishmaniosis among Kangal dogs was low and no clinical signs were observed, asymptomatic animals can be significant reservoirs for the spread of the disease.

Key Words: *Toxoplasma gondii*, *Leishmania infantum*, seroprevalence, Kangal dogs, SFDT, IFAT

Sivas Kangal Köpeklerinde Anti-*Toxoplasma gondii* ve Anti-*Leishmania infantum* Antikorlarının Araştırılması

Özet: Bu çalışma, Sivas ilinde Kangal köpeği yetiştirme çiftliklerindeki köpeklerde *Toxoplasma gondii* ve *Leishmania infantum* seroprevalansının saptanması amacıyla yapılmıştır. Klinik belirtileri bulunmayan 50 Kangal köpeğinden alınan kan örnekleri anti-*Toxoplasma gondii* antikorlarının varlığı yönünden Sabin-Feldman Dye Test, anti-*Leishmania infantum* antikorlarının varlığı yönünden ise İndirekt Floresan Antikor Testi ile araştırılmıştır. İncelenen 50 köpeğin 46'sında 1:16 ile 1:1024 arasında değişen titlerde anti-*Toxoplasma gondii* antikorları saptanmıştır. Sadece bir örnekte 1:128 titrede anti-*Leishmania infantum* antikorları bulunurken, beş örnekte şüpheli pozitif olarak kabul edilen 1:64 titrede antikorlar tespit edilmiştir. Her iki testte elde edilen seropozitiflik oranları, yaş ve cinsiyet açısından değerlendirildiğinde aradaki farklılık istatistiksel olarak anlamlı bulunmamıştır ($P > 0,05$). Sonuç olarak, bu çalışma Kangal köpeklerinde *Toxoplasma gondii*'nin çok yaygın olduğunu göstermektedir. Leishmaniosis seroprevalansının düşük olması ve seropozitif köpeklerde klinik belirtilerin olmamasına rağmen, asemptomatik hayvanların hastalığın yayılımında rezervuar olmaları nedeniyle önemlidir.

Anahtar Sözcükler: *Toxoplasma gondii*, *Leishmania infantum*, seroprevalans, Kangal köpeği, SFDT, IFAT

Toxoplasmosis and leishmaniosis are important zoonotic infections that affect both humans and dogs worldwide. Toxoplasmosis is caused by the obligate intracellular parasite *Toxoplasma gondii*, which parasitizes members of the *Felidae*, which serve as definitive hosts, and has a wide range of intermediate hosts, including humans, domestic and wild animals, as well as birds (1). The disease is mainly spread to canines through the

consumption of undercooked or raw meat containing parasite cysts, or by ingestion of food, water, or sand contaminated with sporulated oocysts (1,2).

Toxoplasma gondii produces a wide range of clinical syndromes in humans, other mammals, and a variety of bird species, but seldom causes significant clinical symptoms (2). The majority of infections in dogs and other animals are asymptomatic. Toxoplasmosis is seen mainly

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in young dogs between 7 and 12 months of age, but the pathogenesis of the infection is not well understood. Clinical toxoplasmosis is occasionally encountered in dogs and produces symptoms resembling those of viral or canine distemper diseases characterized by fever and signs of gastrointestinal, respiratory, and central nervous system disturbance (1,3). The seroprevalence rate of such parasitic infection is of epidemiological interest when dogs act as indicators of *Toxoplasma* circulation among the hosts in a given geographical area.

Visceral leishmaniosis (VL), which is caused by *Leishmania infantum*, is a zoonotic infection and domestic dogs are the main reservoir of the parasite. Dogs are the primary cause of the persistence of VL in most urban areas in the Mediterranean region (4). Infection with *Leishmania* often leads to clinical symptoms in dogs, especially in tropical, sub-tropical, and temperate regions (5). Male and female dogs from all age groups and of all breeds are reported to be infected with *Leishmania* (6). The clinical pattern of the disease in dogs may vary considerably, from asymptomatic infection to severe systemic disease (4-6).

In some endemic areas 20%-40% of seropositive dogs are asymptomatic carriers of *Leishmania* and can frequently act as an unrecognized reservoir of infection for other dogs and humans (4,6). Because the clinical signs of toxoplasmosis and leishmaniosis in dogs are variable and asymptomatic infection is common, serology is the most widely used tool for their diagnosis (1,3-6).

In Turkey, VL is mainly observed in the western and southern regions, along the Aegean and Mediterranean coasts. Recently, human VL cases in Central Anatolia have been reported (7), indicating that the disease is more widespread in the country than was previously assumed.

The Kangal dog is the national dog breed of Turkey. The breed takes its name from the Kangal district of Sivas province in the Eastern Anatolian Plateau, where it probably originated. It is estimated that 1500 Kangal dogs are present in Sivas province located in the central part of Anatolia. This province has a harsh continental climate (mean temp.: 8.6 °C) and high altitude (> 1200 m). Kangal dogs are accustomed to withstand the extremes of temperature of the area, conditions that are usually not suitable for parasites. The relative isolation of the Sivas-Kangal region has kept the Kangal dog free of cross-breeding and has resulted in a natural pure breed of remarkable uniformity of appearance, disposition, and behavior. Recently, the Turkish government has identified

the breed as a national treasure in order to protect and conserve the gene pool; therefore, the Turkish government and academic institutions have built kennels and fund the breeding of Kangal dogs in government breeding farms; villagers have also established small breeding units.

The survey was performed in the Kangal region of Sivas, a place where no human VL cases have ever been reported and the data indicate the absence of these parasitic infections in Kangal dogs. The aim of the present study was to determine the seroprevalence of toxoplasmosis and leishmaniosis among the Kangal dogs kept at breeding farms. Fifty asymptomatic dogs (30 females and 20 males) were subjected to physical examination. Sera were collected from dogs at 2 government sponsored breeding farms (Sivas-Ulaş Agricultural Administration Directorate and Kangal District Breeding Farm) and from 7 villages (Havuz, Humarlı, Çayırova, Tahtalı, Çat, Arpalı, and Kızılınış) in Kangal district. These villages have special agreements with the Kangal district for breeding Kangal dogs. Peripheral blood was drawn for serological surveys and serum samples were stored at -20 °C until examination.

Serum samples were tested for toxoplasmosis with the Sabin Feldman Dye Test (SFDT), using live tachyzoites and methylene-blue dye. This test was performed as previously described (8). In short, positive and negative controls, and test sera were diluted with saline in a series of 4-fold serial dilutions (1:4-1:1024). Each dilution, 25 µl, was transferred to a tube and an equal volume of activator sera rich in C2, C3, C4, and properdin was added. After 48 h, tachyzoites (a number sufficient to produce a final concentration of approximately 25 tachyzoites per 400 microscope field) were added per tube. The tubes were then incubated at 37 °C for 50 min. Then, additional 10-min incubation at 37 °C was performed in the presence of 25 µl of alkaline methylene blue (pH 11). After incubation, 20 µl of each sample was examined under a 40 objective. Dilutions for which ≥ 50% of the observed *T. gondii* tachyzoites remained unstained were considered positive. An antibody titer of 1:16 or higher was considered positive (8).

The Indirect Fluorescent Antibody Test (IFAT) was carried out using the standard procedure described by De Korte et al. (9). Antigen was prepared from promastigotes of *L. infantum* (MON-1) maintained in NNN medium and grown in RPMI 1640 containing 10% FCS. Parasites were harvested from 4-6-day-old cultures, washed by

centrifugation, and then resuspended at a concentration of 2×10^6 per ml of saline. Then, 10 ml of this suspension was transferred per spot onto multi-spot IFAT slides. After air-drying, slides were stored at -20°C until used. Two-fold serial dilutions of dog sera (ranging from 1:16 to 1:256) were prepared. The positive control was diluted 1:64 in PBS. Aliquots of 10-12 ml were applied onto the antigen-coated wells. Following incubation in a humid chamber for 30 min at 37°C , the slides were washed. Fluorescent staining was performed using FITC-labeled anti-dog IgG (Sigma F-4012, Germany) diluted 1:100. The highest serum dilution showing an evident yellow-green fluorescent signal upon microscopic examination was regarded as a positive IFAT titer. A titer of 1:128 was considered indicative of *Leishmania* infection in the canine and 1:64 was considered uncertain (9).

A chi-square test (χ^2) was used to detect significant differences between proportions, and a probability of < 0.05 was considered to be statistically significant (Minitab Statistical Software, 2000).

In all, 50 dogs were examined, representing approximately 3.3% of the estimated total dog population of the area. The epidemiological data and seroprevalence in the dogs tested for toxoplasmosis are shown in Tables 1

and 2. Forty-six of the 50 dog serum samples (92%) were seropositive.

Regarding the seroprevalence of toxoplasmosis, no statistically significant differences were found between male and female dogs (90% and 93.3%, respectively, were seropositive). Two-year-old dogs had the lowest seroprevalence (80%), whereas dogs from all other age groups were 100% positive. *T. gondii*-seropositive animals were observed to be normal at the time of physical examination.

With a cut-off titer of 1:128, only 1 female (9 years old) (2%) was found to be seropositive. Five dogs (10%) were found to have anti-*Leishmania* antibodies at a titer of 1:64, which was considered uncertain. In addition, 9 dogs (18%) had anti-*Leishmania* antibodies at a titer of 1:16. Seropositive animals appeared healthy when examined physically and there were no statistically significant differences in the prevalence based on sex or age.

In 5 dogs that were positive for both anti-*Leishmania* and anti-*Toxoplasma* antibodies, WBC counts were within normal limits, except for monocytes. Compared to the non-infected dogs, a significant increase ($P < 0.05$) in the mean monocyte count ($0.1-1.4 \times 10^3/\text{ml}$) was observed in the seropositive dogs.

Table 1. Distribution of SFDT titers according to gender.

Gender	Dogs Tested n	Seropositivity n (%)	SFDT Titers			
			1:16	1:64	1:256	1:1024
Female	30	28 (93.3)	4 (14.3%)*	10 (35.7%)	10 (35.7%)	4 (14.3%)
Male	20	18 (90)	2 (11.1%)	9 (50%)	6 (33.3%)	1 (5.6%)
Total	50	46 (92)	6 (13%)	19 (41.3%)	16 (34.8%)	5 (10.9%)

*Percent positive.

Table 2. Distribution of SFDT titers according to age.

SFDT Titers	Age							Total
	2	3	4	5	8	9	10	
1:16	4	1	-	-	1	-	-	6
1:64	5	6	4	4	-	-	-	19
1:256	6	4	4	-	1	-	1	16
1:1024	1	2	-	1	-	1	-	5
Positive/No. of dogs	16/20	13/13	8/8	5/5	2/2	1/1	1/1	46/50

In the present study the seroprevalence of *T. gondii* was 92% in Kangal dogs and there were no statistically significant differences in prevalence according to age or gender ($P > 0.05$). In previous studies carried out in different parts of Turkey the seroprevalence of toxoplasmosis among dogs varied between 11.7% and 85.6%, based on SFDT (10-16). These differences could be explained by the diagnostic methods used, the number of animals examined, and the geographical areas surveyed (1,17). Previous studies in Sivas province found that the seroprevalence in cats and healthy women was 78% (18) and 47.7% (19), based on the Indirect Hemagglutination Test. Antibody titers increased to 77.1 in a group of patients that suffered from abortus, abnormal birth, and death (19).

Adult Kangal dogs on breeding farms are fed, on average, a 2-kg daily diet consisting of a mixture of meat, grain cereals, vegetables, and water. In the villages, dogs were fed dough prepared from barley flour and the addition of meat and scalded bones. The higher seroprevalence rate observed in this study might also have been related to the fact that Kangal dogs on both the government and village breeding farms might have been fed raw or undercooked meat that harbored *T. gondii* cysts (1-3), and Kangal dogs on village breeding farms may have consumed the after-birth at lambing time, or taken off surreptitiously with an already dead lamb to consume at leisure. The dogs on village breeding farms might also have hunted, killed, and eaten small game. Another possible explanation for the high toxoplasmosis seroprevalence rate is that the dogs may have come in contact with cat feces contaminated with *T. gondii* oocysts. It is worth mentioning that high prevalence rates in Kangal dogs, cats, and humans (18,19) may be an indication that the domestic environment is contaminated by *T. gondii*, and as both humans and Kangal dogs are exposed to common sources of infection, the infection rate among Kangal dogs could serve as a risk indicator for human infection.

There were no statistically significant differences in prevalence between male and female dogs, which is in accordance with the results obtained by other researchers in Turkey (12-14). Regarding the different age groups, we found a higher rate among older dogs (> 2 years of age), which is in agreement with other studies (11-14); however, the small number of older dogs in this study did not permit statistical analysis of these results.

Dogs are the main reservoir host for *L. infantum* in the Mediterranean region and in some endemic areas the

seroprevalence of canine VL varied between 1.1% and 37% (4,5,20). In Turkey, infantile VL is mainly observed in the western and southern regions along the Aegean and Mediterranean coasts. Characteristic of this disease, infection rates may reach high levels among dog populations, with very few human cases (21-26). The seroprevalence of canine VL ranges from 0%-7% (21-25); however, in areas where cases of human VL are common the seroprevalence of canine VL increases up to 25% (26). The VL prevalence rate observed in the present study (2%) was not as high as that found in endemic areas, which might have been due to the harsh climatic conditions of the study area shortening the sand fly's active season.

Since approximately 50% of all *Leishmania*-infected dogs lack clinical signs of the disease, serodiagnosis is frequently used to evaluate the prevalence of leishmanial infections (7,21-26). IFAT is widely accepted as a highly sensitive serological test for ZVL (7,25). None of the seropositive Kangal dogs had clinical signs of infection. It should be considered, however, that symptomatic as well as asymptomatic animals could harbor the parasite and may act as carriers of *L. infantum*, which may be transmitted to humans by the Phlebotominae sand fly vector (4-6).

The age of infected dogs varied between 9 months and 15 years (median: 5 years). Due to a long incubation period, the disease is rarely seen in very young dogs. No age or gender preferences were observed in the seroprevalence of canine VL, which is in accordance with other studies conducted in Turkey (7,21-25).

One of the striking observations made in this study was that the animals seropositive both for toxoplasmosis and leishmaniosis had monocytosis, while seronegative dogs had normal monocyte counts. Although normocytic normochromic anemia, moderate leukopenia, leukocytosis with neutrophilic left shift, lymphopenia, and lymphocytosis have been reported in canine VL, monocytosis in canine VL has rarely been reported (27,28). Monocytosis in normal WBC counts may be related to either concomitant infection of 2 intracellular parasites, *L. infantum* and *T. gondii*, or other infectious agents such as *Ehrlichia canis* and *Hepatozoon canis*. We carried out further investigation to determine which organism caused monocytosis in the seropositive dogs. Dogs that were seropositive for toxoplasmosis and leishmaniosis were negative for *H. canis* and did not have detectable levels of antibodies against *E. canis*, based on IFAT. We conclude

that monocytosis was likely to have been related to intracellular protozoon infection.

In conclusion, the findings of this survey indicated that toxoplasmosis is very common in Kangal dogs. Accordingly, the potential risk to humans for acquiring toxoplasmosis is very high in this area. From a public health point of view, asymptomatic dogs with positive serology to *L. infantum* could be a reservoir for human VL. Domestic dogs can attract sand flies into houses, which could transmit the disease to humans.

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