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Serological and molecular studies on *Dirofilaria immitis* in dogs from Turkey

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Abstract

We estimated the prevalence of *Dirofilaria immitis* infection in domestic dogs in five Turkish provinces – Sakarya, Kocaeli, Ankara, Elazig and Mersin – using a commercial ELISA kit for detecting circulating antigen and a PCR test for detecting circulating microfilarial DNA. A total of 211 whole-blood and serum samples were collected from dogs of various breeds, ages and life status (owned or stray). Sample population characteristics were recorded and examined for differences in prevalence. Additionally, we collected 15 blood samples from cats (14 owned and 1 stray) from Ankara province and used PCR to detect *D. immitis* infection. Twenty-seven (12.8%) of 211 dog samples were positive for *D. immitis* antigen by ELISA. No differences in prevalence were observed by sex (female: 14.4%; male: 10.7%; $P > 0.05$). The prevalence of *D. immitis* infection varied with age: 11.8% in younger dogs (0.5–2 years) and 17.5% in older dogs (3–5 years). Prevalence between stray dogs (15.2%) and owned dogs (9.3%) did not differ ($P > 0.05$). Prevalence rates were highest in Kocaeli province (18.3%), followed by Ankara (14.8%), Sakarya (12.3%) and Mersin (10.5%) provinces. Prevalence in Elazig province was 0%. No dogs or cats had microfilarial DNA detectable by PCR.

Introduction

Dirofilariosis, caused by *Dirofilaria immitis,* is found worldwide, but the most endemic areas are those with high temperatures and appropriate mosquito vector populations. *Dirofilaria immitis* is one of the most pathogenic nematode parasites of dogs. Adult nematodes are normally found in the pulmonary arteries of dogs. Adult heartworms may cause clinical signs ranging from mild cough to congestive heart failure, intravascular haemolysis and pulmonary thromboembolism, which are often fatal if untreated (Soulsby, 1986; Kassai, 1999). The infective larval stage (L_3) can develop in approximately 70 species of mosquitoes. Since these vectors are widespread throughout the world and are commonly found near human habitats, human infection, although rare, is nevertheless also possible, creating concern about potential zoonosis (Fan *et al.*, 2001; Simon *et al.*, 2005). *Dirofilaria immitis* in dogs can be diagnosed through careful morphological examination of circulating microfilariae, detection of circulating antigens, histochemical or immuno-histochemical staining of circulating microfilariae or, more recently, through molecular approaches. Morphological identification of circulating microfilariae, however, is not always easy and is potentially misleading (Casiraghi *et al.*, 2006; Rishniw *et al.*, 2006).

Few studies have been published regarding the distribution and prevalence of *D. immitis* in dogs in Turkey. *Dirofilaria immitis* was first reported in a dog in Turkey in 1951 (Guralp, 1981). Tasan (1983) detected microfilaraemia in 53/283 (18.7%) stray dogs in Elazig. Coskun *et al.* (1992) documented infection in 5/168 dogs (2.98%) in Bursa. The prevalence was recorded in Sivas (Atas *et al.*, 1997), Van (Agaoglu *et al.*, 2000), Ankara (Oge *et al.*, 2003), Istanbul (Oncel & Vural, 2005) and Kayseri

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(Yildirim *et al.*, 2007) as 6%, 46.2%, 9.3%, 1.52% and 9.6%, respectively. These different prevalence rates may reflect different testing methodologies or true regional differences. The prevalence of *D. immitis* in dogs has been determined traditionally by postmortem inspection, detection of microfilariae and serological testing. However, dogs with occult heartworm infections are amicrofilaraemic. In addition, some antiparasitic treatments such as macrolides may render an infected dog amicrofilaraemic for 6–9 months (Hoover *et al.*, 1996). Thus, serological and microfilarial examinations should be applied together for screening *D. immitis* in dogs. DNA-based diagnostic tests for *D. immitis* infections have been shown to overcome some deficiencies of parasitological and serological diagnosis, and specific and sensitive polymerase chain reaction (PCR)-based assays have been reported (Cancrini *et al.*, 2000; Mar *et al.*, 2002; Rishniw *et al.*, 2006).

The present study was performed to investigate the prevalence of canine *D. immitis* infection with antigen-detecting enzyme-linked immunosorbent assay (ELISA) and PCR in Turkey and to compare the prevalence in five enviromentally distinct regions of Turkey. Furthermore, this study is the first to use PCR-based detection of microfilaria in peripheral blood of dogs and cats in Turkey.

Materials and methods

Sample collection and study area

The study was carried out in five environmentally distinct provinces in Turkey (fig. 1). Environmental conditions of these areas, such as climate, altitude, temperature, relative humidity in the summer and throughout 2005 are provided in table 1. Of the 211 dogs used in the current study, 93 (44.1%) were males and 118 (55.9%) were females; 143 (67.7%) were 0.5–2 years old, 57 (27%) were 3–5 years old and 11 (5.2%) more than 6 years old; 125 (59.2%) were strays and 86 (40.7%) were owned dogs. Blood samples were collected between March 2005 and March 2006 from randomly selected dogs in five different provinces: Kocaeli ($n = 71$), Sakarya ($n = 65$), Elazig ($n = 29$), Ankara ($n = 27$) and Mersin ($n = 19$). Demographic information for each dog, including age, sex, breed, life status (stray or owned) was recorded. Serum was separated by centrifugation and stored at −20°C in a freezer for subsequent serological analysis. Whole-blood samples were collected for PCR assay from each dog and stored at −80°C until analysis. Fifteen cats (14 owned and 1 stray) from Ankara province were examined by PCR for *D. immitis* infection.

Control samples

A total of three different *D. immitis* genomic DNA samples were used as controls in this study. One DNA control sample was extracted from an adult *D. immitis* parasite (this worm was collected from a dog that had been necropsied at Firat University, Faculty of Veterinary Medicine, Department of Pathology). The second and third DNA control samples were extracted from microfilariae present in the blood of infected dogs (one sample was obtained from another research group (Yildirim *et al.*, 2007) and microfilariae were visualized with Giemsa staining; while the other sample was provided by one of the authors, M.R.).

Serological analysis

Circulating *D. immitis* antigen was detected using a commercial antigen ELISA kit (DiroCHEK, Synbiotics Corp., USA), according to the manufacturer's instructions. DiroCHEK Canine Heartworm Antigen Test kit is an enzyme immunoassay designed to detect the presence of circulating antigen from adult females of *D. immitis* in serum. All sera were tested with this kit, and sera were re-tested when inconclusive results were obtained.

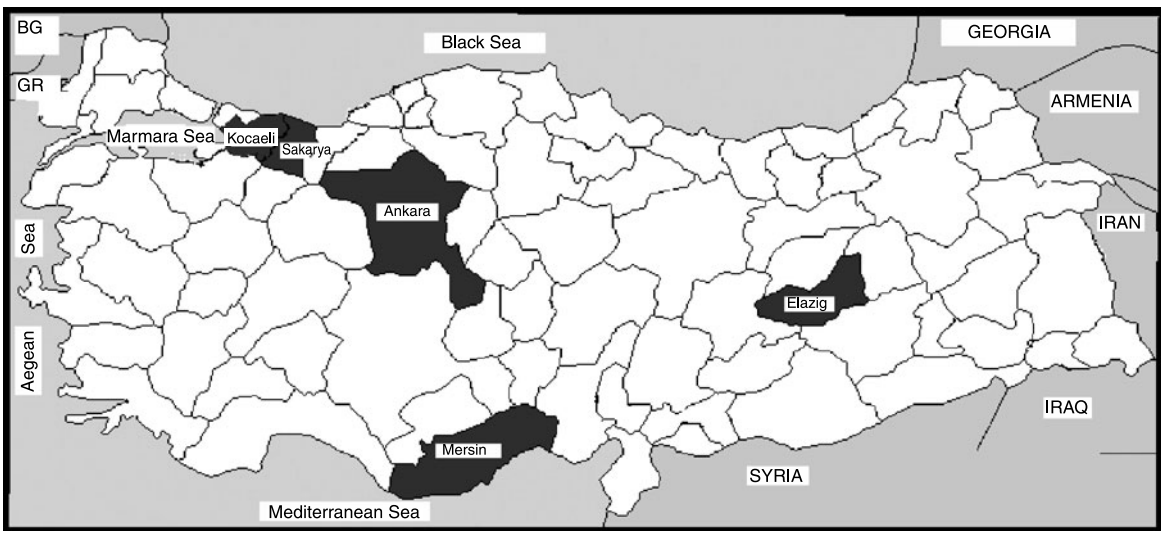


Fig. 1. Location of study areas (shaded in black) in Turkey.

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Table 1. Environmental and climatic conditions of five Turkish provinces in which dirofilarial testing was performed.

Provinces	Area (km^2)	Geographic condition	Altitude (m)	Mean temperature from May to September (°C) (range)	Mean temperature in 2005 (°C) (range)	Mean relative humidity between May to September (%) (range)	Mean humidity in 2005 (%) (range)
Sakarya	4.880	Shoreline	31	21.72 (17.6–25.1)	15.05 (7.4–25.1)	71.88 (69.7–73.3)	72.41 (66.1–78.4)
Kocaeli	3.625	Shoreline	25	21.82 (17.8–25.2)	15.05 (7.3–25.2)	68.22 (67.2–69.3)	69.65 (62.6–78.5)
Ankara	25.401	Urban	891	21.04 (16.6–25.4)	12.4 (2.5–25.4)	54.9 (51.1–58.7)	61.7 (51.1–69.8)
Elazig	9.281	Highland	1067	23.40 (17.8–27.7)	13.30 (0.1–28.5)	36.02 (28.8–46.4)	51.47 (28.8–77.3)
Mersin	15.512	Shoreline	0 (sea level)	26.36 (21.7–29.4)	19.87 (11.6–29.4)	70.54 (62.1–75.4)	64.96 (54.4–75.4)

DNA isolation and PCR

DNA extraction from canine and feline blood was performed according to the method described by Clausen *et al.* (1999) with slight modifications. Briefly, 125 μl of blood were added to 250 μl of lysis mixture (0.32 M sucrose, 0.01 M Tris, 0.05 M $MgCl_2$, 1% TritonX-100, pH 7.5) and the mixture was centrifuged at 11,600 *g* for 30 s. The pellet was washed five times with lysis buffer. Following this, 250 μl PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8, 0.1% TritonX-100 pH 8.3) containing 50 $\mu g\,ml^{-1}$ proteinase-K (Sigma, St Louis, Missouri, USA) was added to the pellet and incubated at 56°C for 3 h. At the end of this period, samples were boiled at 95°C for 10 min. Five microlitres of the supernatant were used as templates in the PCR. This method was used for both whole blood and adult worm extraction procedures. For DNA extraction from the adult worm, a small section of worm was macerated and this tissue was then used for genomic DNA extraction as described above. The PCR was performed using two sets of primers. Initially, we used one primer pair: 5′-CATCAGGTGATGATGTGATGAT-3′ (forward), and 5′-TTGATTGGATTTTAACGTATCATTT-3′ (reverse), which have been described by Mar *et al.* (2002) for the amplification of a 302 bp ITS-2 gene segment. Unexpectedly, this primer pair non-specifically amplified a 350 bp product from all samples, including all positive controls. Therefore, a second set of primers was used: 5′-AGTGTAGAGGGTCAGCCTGAGTTA-3′ (forward) and 5′-ACAGGCACTGACAATAC CAAT-3′ (reverse), identical to those described in a previous study (Rishniw *et al.*, 2006) for the amplification of a 203 bp cytochrome oxidase subunit 1 (CO1) gene fragment. The PCR was performed in a touchdown thermocycler in a total reaction volume of 50 μl containing 4 μl $MgCl_2$ (25 mM, Promega, Madison, Wisconsin, USA), 4 μl dNTP (10 mM dNTP mixture, Promega), 5 μl 10 × PCR buffer (Promega), 1.2 μl Primer F (68.2 nmol, lyophilized) and 1.2 μl Primer R (64.8 nmol, lyophilized), 0.2 μl *Taq* DNA polymerase (5 U μl^{-1}, Promega), 29.4 μl distilled water and 5 μl template DNA. Positive control DNA was used at each PCR and distilled water was used as a negative control. The PCR procedure consisted of a predenaturing step at 94°C for 1 min and 32 cycles of denaturing (30 s at 94°C), annealing (30 s at 63°C), extension (30 s at 72°C) and a final extension (7 min at 72°C). The amplified products were separated by gel electrophoresis in 1.5% agarose gel with a Tris–boric acid–EDTA (TBE, pH 8.3) buffer at 90 V for 45 min. Following electrophoresis, the amplified products were visualized with ethidium bromide (0.5 $\mu g\,ml^{-1}$) staining for 45 min at room temperature. A visible band of 203 bp in the PCR was considered to be a positive result.

Statistical analysis

Pearson's chi-square (χ^2) test was used to evaluate the differences between proportions of infected dogs or cats detected by each method. Statistical comparisons were carried out using SPSS 11.0 statistical software (SPSS Inc., Chicago, Illinois, USA). A $P < 0.05$ was considered significant.

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Results

All dogs and cats were negative by PCR analysis. Figure 2 summarizes our findings with a variety of negative and control DNA templates amplified by the *D. immitis* specific primer pairs.

The results of the ELISA examination on the canine dirofilariosis and the status (owned or stray) are shown in table 2. Twenty-seven (12.8%) of 211 samples tested positive with the antigen-detecting ELISA kit. There were no differences in ELISA-positive results between sexes (females, 14.4%; males, 10.7%; $P > 0.05$). The mean overall positivity rates of *D. immitis* infection were 11.8% in the 0.5–2-year-old group, 17.5% in 3–5-year-old group. The prevalence rates between stray dogs (15.2%) and owned dogs (9.3%) did not differ ($P > 0.05$). Highest seropositivity rate was detected in Kocaeli province (18.3%), followed by Ankara (14.8%), Sakarya (12.3%) and Mersin (10.5%) provinces. No dogs from Elazig province tested positive.

Discussion

Our study further documents the prevalence of *D. immitis* in dogs in Turkey, complementing and expanding the data of previous studies (Oge *et al.*, 2003; Oncel & Vural, 2005; Yildirim *et al.*, 2007). This study demonstrates that the overall prevalence is relatively low (12.7%), varies regionally (18.3%, 14.8%, 12.3%, 10.5% and 0% in Kocaeli, Ankara, Sakarya and Mersin and Elazig provinces, respectively), and may be increasing in some areas – in the one province we examined where previous serological data were available (Ankara), antigen positivity increased from 9.3 to 14.8% over the past 4 years (Oge *et al.*, 2003). Whether this apparent increase is due to random sampling differences, differences in methodology, or an actual increase in prevalence, is unknown.

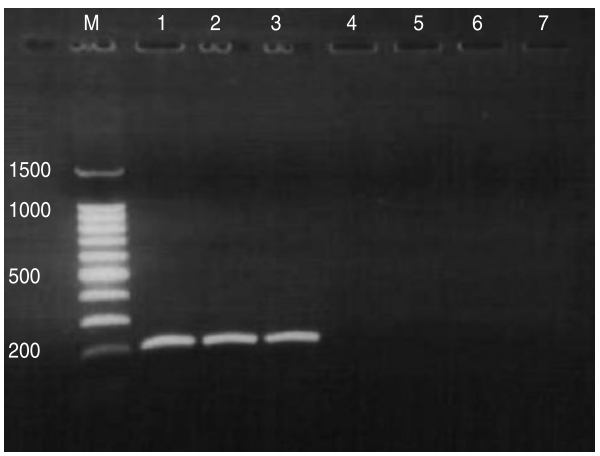


Fig. 2. Species-specific PCR amplification of *Dirofilaria immitis CO1* (203 bp). Lanes: M, molecular weight marker; 1, positive control (template DNA isolated from adult *Dirofilaria immitis*); 2, positive control (template DNA isolated from infected dog blood); 3, positive control, (template DNA isolated from infected dog blood); 4, negative control (distilled water); 5–7, randomly selected test samples.

Table 2. Seroprevalence (%) of ELISA by sex, age, life status and provinces.

	Shoreline area										Urban area				Highland area				Total			
	Sakarya				Kocaeli				Mersin						Elazig							
	No.	P	%	χ^2	No.	P	%	χ^2	No.	P	%	χ^2	No.	P	%	χ^2	No.	P	%	χ^2	No.	P	%	χ^2
Sex																						
Male	37	5	13.5	0.116	22	3	13.6	0.131	8	–	–	1.626	16	2	12.5	0.167	10	–	–	–	93	10	10.7	0.622
Female	28	3	10.7	$P > 0.05$	49	10	20.4	$P > 0.05$	11	2	18.1	$P > 0.05$	11	2	18.1	$P > 0.05$	19	–	–	–	118	17	14.4	$P > 0.05$
Age																						
0.1–2	51	5	9.8		46[a]	11	23.9		5	–	–		12	1	8.3		29	–	–	–	143	17	11.8	
3–5	14	3	21.4	1.375	18[b]	2	11.1	22.03	14	2	14.2	0.789	11	3	27.2	2.440	–	–	–	–	57	10	17.5	2.87
≥6	–	–	–	$P > 0.05$	7[c]	–	–	$P < 0.001$	–	–	–	$P > 0.05$	4	–	–	$P > 0.05$	–	–	–	–	11	–	–	$P > 0.05$
Life status																						
Stray	26	3	11.5	0.024	48	11	22.9	2.102	18	2	11.1	0.117	6[a]	3	50	7.56	27	–	–	–	125	19	15.2	0.717
Owned	39	5	12.8	$P > 0.05$	23	2	8.6	$P > 0.05$	1	–	–	$P > 0.05$	21[b]	1	4.7	$P < 0.01$	2	–	–	–	86	8	9.3	$P > 0.05$
Total	65	8	12.3		71	13	18.3		19	2	10.5		27	4	14.8		29	–	–	–	211	27	12.7	

[a,b,c] Values with different letters within the same column are significantly different ($P < 0.01$).
No., Number of dogs examined; P, number of dogs positive by ELISA; %, positivity rate.

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It is not surprising that regional differences in prevalence exist. A previous study of dirofilariasis in Korea demonstrated that prevalence was significantly higher in coastal areas (69.5%) than in urban and mountain areas (Song *et al.*, 2003). Sakarya, Kocaeli and Mersin are coastal areas and have warmer climates than Ankara and Elazig. We expected the highest prevalence to be in Mersin, due to the warmest climate; however, Mersin had the lowest prevalence of any region in which dirofilaria were detected. Thus, high ambient temperatures are necessary but not sufficient to predict prevalence rates for canine dirofilariasis. Other factors influence prevalence, most important of which is a suitable mosquito population, which may depend on non-thermal factors, such as humidity (Ahid *et al.*, 1999). We could find no difference in seropositivity between stray and pet dogs. Previous studies have examined seropositivity in indoor and outdoor dogs with and without chemoprophylaxis, and suggested that outdoor dogs have a tenfold increased prevalence due to increased exposure to mosquitoes (Byeon *et al.*, 2007). In our study population, all pet dogs received monthly prophylaxis and stray dogs in Kocaeli, Sakarya and Ankara provinces received monthly ivermectin injections. This would eventually render dogs amicrofilaraemic and may explain why we could not detect any microfilarial DNA in peripheral blood samples by PCR.

Prevalence showed an initial increase with age, followed by a decline: prevalence was 11.8% in the 0.5–2-year age group, 17.5% in 3–5-year age group and 0% in dogs >6 years old. This is similar to previous findings of an increase in prevalence until middle age (about 4–8 years), with a subsequent decline occurring in later years (Glickman *et al.*, 1984; Capelli *et al.*, 1996; Byeon *et al.*, 2007), and is suspected to be the consequence of prophylaxis in older dogs (Grieve *et al.*, 1991; Byeon *et al.*, 2007). Additionally, we included some dogs that may have been too young to seroconvert, although all dogs were more than 6 months old. We did not find any differences in prevalence between genders. This is similar to the results of some previous studies (Oge *et al.*, 2003; Song *et al.*, 2003), but different from those of others (Montoya *et al.*, 1998; Byeon *et al.*, 2007; Yildirim *et al.*, 2007) where prevalence rates in males were significantly higher than in females.

The lack of any PCR-based positivity in our study is a little perplexing. This method requires sufficient DNA to be present in the sample to act as a template and is usually provided by circulating microfilariae. Several possibilities for our results exist. First, it is possible that the seropositive dogs in this study had occult infections. As stated earlier, pet dogs were all receiving chemoprophylaxis, and stray dogs were being given monthly ivermectin injections. Alternatively, the blood collection method may have affected our study – blood for PCR was collected into EDTA tubes and DNA was subsequently extracted using the procedure described above. A critical component of PCR reactions, Mg^{2+}, which acts as a cofactor for *Taq* polymerase, can be chelated by EDTA. It is possible that the Mg^{2+} was chelated by residual EDTA from the blood sample. However, the DNA extraction procedure should have diluted and removed the majority of the EDTA, and the amount of DNA added to a PCR reaction is on the order of 5% of the total reaction volume, so this effect of EDTA is extremely unlikely. Additionally, dirofilarial PCR from blood samples collected into EDTA has been performed routinely by one of the authors without complications.

Feline dirofilariosis is now recognized as a potential cause of serious disease and can present both a clinical and diagnostic challenge to practising veterinarians. Feline heartworm infection can be symptomatic in some cats. However, it is possible that the presence of only one or two adult worms in the lungs and/or heart may cause severe illness (Borgarelli *et al.*, 1997). We could not find any studies of prevalence of feline dirofilariosis in Turkey. We examined 15 cats by PCR in this study but had no positive results. This is not surprising, since the PCR reactions require infected animals to be microfilaraemic – a state that cats rarely achieve for any length of time. Further studies of the prevalence of feline dirofilariosis may require serological testing; however, given the prevalence of canine dirofilariosis in Turkey, the predicted prevalence in cats would be extremely low.

In conclusion, *D. immitis* is prevalent at relatively modest rates throughout several Turkish provinces, but appears to be absent in others. Therefore, heartworm prophylaxis is advisable for dogs in certain areas (which should be determined by further regional prevalence studies) or for dogs travelling to endemic areas.

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