Survey of *Dirofilaria immitis* in dogs from Sivas Province in the Central Anatolia Region of Turkey

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Abstract: In this research, we investigate the prevalence of *Dirofilaria immitis* infection in dogs using microscopical, serological, and molecular methods from the Central Anatolia Region (Sivas) of Turkey and reveal the associated risk factors. Whole blood and serum samples were obtained from 306 dogs older than 7 months. Polymerase chain reaction (PCR) and the modified Knott's method (Knott) were used for detecting the microfilaria and enzyme-linked immunosorbent assay (ELISA) was applied for the presence of *D. immitis* antigens. The prevalence of *D. immitis* in dogs was detected as 2.9% (9/306) by PCR and ELISA and as 1.3% (4/306) by Knott. All Knott-positive samples were also found positive by both PCR and ELISA. According to ELISA and PCR results, no statistically significant difference was found between the prevalence in males (4.2%) and females (1.5%); dogs 0.7–2 years old (2.5%), 3–5 years old (2.2%); and more than 6 years old (7.1%); or stray (3.7%) and owned (1.7%) dogs (P > 0.05). One of the PCR products was selected for sequence analyses of the 16S small subunit rRNA gene and the obtained sequence was submitted to GenBank (KJ183078). The 16S rRNA sequence exhibited 100% identity to other *D. immitis* sequences available in GenBank (FJ799911, FJ799917, FJ799916). This research provides the first data on *D. immitis* infections in dogs from Sivas Province by using the combined identification techniques.

Key words: *Dirofilaria immitis*, dogs, enzyme-linked immunosorbent assay, microscopy, polymerase chain reaction, Turkey

1. Introduction

The heartworm *Dirofilaria immitis* is a vector-borne filarioid parasite of dogs. This parasite particularly settles in the right ventricle and pulmonary arteries. The parasite can infect dogs, cats, and other carnivores. Humans could also be aberrant hosts, as well (1–3).

Various techniques are used for performing the diagnosis of canine dirofilariasis, such as morphological parameters of microfilariae by blood smear, the modified Knott's method (Knott), histochemical reactions of microfilariae, and immunoassays like enzyme-linked immunosorbent assay (ELISA) tests (4). Some particular hardships can occur when interpreting the results by these techniques (4,5).

Correct detection of filarioid species is clinically important due to zoonotic worries and therapeutic effects (5,6). Molecular methods like polymerase chain reaction (PCR) have been recommended for detecting and differentiating filarioid nematodes in dogs based on their supremacy in specificity and sensitivity (6).

Dirofilaria immitis infections are widespread in tropical and subtropical regions. Turkey has intermediate hosts and

suitable climatic conditions for the development of this parasite (1,7). In this regard, many studies from Turkey have been reported. In studies conducted with different diagnostic methods in various areas, the prevalence of *D. immitis* was between 1.5% and 46.2% (5,8–10).

Territorial factors and diagnostic tests can cause distinctions among research results. In recent years, it is proposed to use an integration of serologic and parasitologic methods for the diagnosis of *D. immitis* (5,6).

A postmortem study of street dogs has been the only report until today about the prevalence of *D. immitis* in dogs from Sivas (11). Our research was conducted to investigate the situation of *D. immitis* infection between stray and owned dogs of different ages and sexes by the methods of ELISA, PCR, and Knott.

2. Materials and methods

2.1. Sample collection

The research was conducted in Sivas Province, an area of about 28,000 km^2 in the central part of Turkey (38°32′N to 40°16/N and 35°50′E to 38°14′E). Sivas is located at an

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altitude of 1278 m in the broad valley of the Kızılırmak River.

Permission for the study was received from the Cumhuriyet University Animal Experiments Local Ethics Committee (27.02.2012/304) before the experimental process.

Blood samples with EDTA and sera samples were collected between April and October 2012 from dogs in six districts of Sivas Province (Sivas Center, Ulaş, Kangal, Yıldızeli, Hafik, and Suşehri) (Table 1).

All the dogs in the experiment were older than 7 months of age, indigenous breeds, and did not have prophylactic treatment for dirofilariasis based on owners' testimonies. The demographic data of age, sex, and origin (stray or owned) of each dog were recorded. About 8 mL of whole blood was drawn from the cephalic vein of each dog between 1000 and 2000 hours. Half of the sample was put into a tube containing EDTA to avoid coagulation and the other half was permitted to clot. Serum was separated by centrifugation and stored at –20 °C until use (Table 1)

2.2. Microscopic examination (modified Knott's method)
Knott's technique was used for the analysis of *Dirofilaria* microfilariae. The Knott test was applied following Panchev et al. (12). One milliliter of whole blood and 9 mL of 2% buffered formalin were mixed and centrifuged at 2000 rpm for 5 min. The supernatant was removed and sediment was stained using 1% methylene blue. The sediment was transmitted to object slides, coated with coverslips, and analyzed by light microscopy. Filarial species characterization was performed based on well-known morphological criteria (2).

2.3. Serological analysis
All of the sera samples were subjected to ELISA for determining the existence of circulating antigens of *D. immitis*. A commercial ELISA test kit (DiroCHEK, Synbiotics Corp., San Diego, CA, USA) was used. The procedure was according to the instructions provided in the kit. Kit samples were used as positive and negative controls.

2.4. DNA extraction and polymerase chain reaction
Total DNA was separated from blood samples using the DNeasy Blood & Tissue Kit (GeneJET Genomic DNA Purification Kit, K0722, Fermentas, St. Leon-Rot, Germany) based on the manufacturer's instructions with slight modifications. First, 200 μL of blood was incubated overnight at 56 °C within 200 μL of the lysis buffer including 20 μL of proteinase K (20 mg/mL) (Sigma, St. Louis, MO, USA). At the end of the digestion phase, the kit's directions were followed and then the pellet was diluted by 50 μL of sterile distilled water, and DNA samples were stored at –20 °C until use in the final stage.

The 16S SSU rRNA gene region of *D. immitis* was amplified with the Di-16S-rRNA-F (5'-AGCTCGTAGTTGGATCTGCAT-3') and Di-16S-rRNA-R (5'-CGTCAAGGCGTATTTACCG-3') primers (13). Amplification was carried out in a total reaction volume of 50 μL including 10X PCR buffer [100 mM Tris-HCl (pH 9), 500 mM KCl, 1% Triton X-100], 1.5 mM MgCl$_2$, 250 μM of each dNTP, 1.25 U of Taq DNA polymerase (Fermentas), and 2 μL (20 pmol/μL) of each of the primers. Five microliters of the eluted DNA was used in the PCR.

The amplification was performed in a thermal cycler (Multigene Thermal Cycler TC9600-G, Labnet International, Woodbridge, NJ, USA) for 35 cycles using the following reaction conditions: predenaturation of DNA at 95 °C for 10 min, denaturation of DNA at 95 °C for 1 min, primer annealing at 55 °C for 1 min, primer extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Five microliters of the amplified PCR products was loaded onto ethidium bromide-stained 1.5% agarose gel.

Table 1. Distribution of the examined dogs according to the location, age, sex, and origin.

Location	Age			Sex		Origin		Total
	0.7–2 years	3–5 years	≥6 years	♂	♀	Owned	Stray	
Sivas Center	24	112	21	73	84	9	148	157
Ulaş	10	22	1	20	13	28	5	33
Kangal	10	14	6	15	15	25	5	30
Yıldızeli	20	14	7	28	13	27	14	41
Hafik	2	9	-	5	6	-	11	11
Suşehri	14	13	7	27	7	26	8	34
Total	80	184	42	168	138	115	191	306

A 100-bp DNA ladder (Promega, Madison, WI, USA) was used to confirm the expected molecular weight of the amplification product. The samples that demonstrated visible bands of roughly 453 bp were accepted as positive for *D. immitis* (13).

2.5. DNA sequencing
One positive PCR product was purified by Wizard SV gel and a PCR clean-up system (Promega), and the amplicon was sequenced by the Sanger method. The DNA sequence was compared with present databases in GenBank using the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov.

2.6. Statistical analysis
SPSS 15.0 for Windows was used for statistical analysis. As a reference diagnostic test, the results of the antigen ELISA and PCR were utilized for exploring statistical assessments. The chi-square test was used to evaluate the differences among different parameters. The value of $P < 0.05$ was accepted as statistically significant.

3. Results
Overall prevalence of *D. immitis* in the Sivas region was found as 2.9% using a combined screening with Knott, ELISA, and PCR examinations. The prevalence of *D. immitis* according to diagnostic test was 1.3% by Knott, 2.9% by ELISA, and 2.9% by PCR. Five samples were found as negative by Knott but positive by both ELISA and PCR.

According to results of ELISA and PCR, there were no statistically significant differences in the distribution of *D. immitis* between males and females, owned and stray dogs, or among the age groups of dogs based on the chi-square test (Table 2).

A 453-bp fragment of the 16S SSU rRNA gene of *D. immitis* was successfully amplified with PCR using the Di-16S-rRNA-F and Di-16S-rRNA-R primers. The obtained 16S rRNA DNA sequence was submitted to the GenBank with accession number KJ183078. The sequence was found to share 100% identity with the other *D. immitis* sequences (FJ799911, FJ799917, FJ799916) registered in GenBank.

4. Discussion
The prevalence of *D. immitis* in dogs was specified in the range of 1.1%–46.2% by using various diagnostic techniques from different geographical areas of the world (4,8,12,14–22). There have been different prevalences for a single country depending on many factors such as the number of samples, selection method of samples, preselection of samples, and applied methods (14,17). Different prevalences have been reported in several studies with microscopy, necropsy, and serology-based research from Turkey (3,5,7–11,23–27). The highest prevalence in Turkey was reported to be 46.2% in Van Province (8). Previously, the prevalence of *D. immitis* in a postmortem survey of 50 street dogs in the Sivas city center was

Table 2. The prevalence of *D. immitis* in dogs by diagnostic tests included in the study. The statistical analyses of the distribution of infection according to sex, age, and origin of dogs based on ELISA are also presented.

		n	Knott		ELISA		PCR	
			+	%	+	%	+	%
Sex	Male	168	4	2.4	7	4.2	7	4.2
	Female	138	-	0.0	2	1.5	2	1.5
					$\chi^2 = 1.953$, P = 0.16			
Age	0.7–2 years	80	-	0.0	2	2.5	2	2.5
	3–5 years	184	1	0.5	4	2.2	4	2.2
	≥6 years	42	3	7.1	3	7.1	3	7.1
					$\chi^2 = 1.335$, P = 0.25			
Origin	Owned	115	-	0.0	2	1.7	2	1.7
	Stray	191	4	2.1	7	3.7	7	3.7
					$\chi^2 = 0.930$, P = 0.33			
Total		306	4	1.3	9	2.9	9	2.9

n: Number of dogs examined; +: Number of dogs positive by Knott, ELISA, and PCR; %: Positivity rate; Knott: Modified Knott's method; ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction.

determined as 6.0% by Ataş et al. (11). The prevalence in the center of Sivas and its districts was found here as 2.9%, which was lower than that reported by Ataş et al. (11). Whether this obvious difference is due to the random exemplification differences, dissimilarities in techniques, or a current reduction in prevalence is unclear.

Climatic and environmental circumstances, vector populations, used diagnostic techniques, and status of diseases should be related to the diversity of the prevalence of the parasite (1). Compared to other parasite prevalences in Turkey, a low prevalence of 2.9% was found in our study. The climatic conditions of Sivas Province, which has snowy and long winters and dry, warm summers, could be associated with the previous situation. Mosquito activity lasts less than approximately 3 months. The environmental temperature is the critical factor for *D. immitis* maturation in terms of infecting third-stage larvae (L3) in the mosquito (1,23).

This research is the first exhaustive study generated by utilizing a combination of serological, conventional, and molecular methods for determining the distribution and occurrence of *D. immitis* in Sivas Province. ELISA (2.9%) and PCR (2.9%) methods detected the prevalence of the disease in a higher range than Knott (1.3%). The PCR assay is accepted as more reliable and reproducible when compared with microscopic inspection (4,6,13,17,21). The ELISA test is commonly used to detect *D. immitis*, especially for occult infections (10,23). Moreover, a false negative result could occur by microscopic inspection for characterization of microfilariae, as well (17). This study shows that ELISA and PCR are more sensitive and useful than microscopy for *D. immitis*. We speculate that the sensitivity of the method is important in epidemiological studies because the potential risks are shown by the existence of asymptomatic and microfilaremic animals.

It could be mentioned that when interpreting the prevalence of *D. immitis* in terms of sex that male dogs (4.2%) have a higher rate than females (1.5%), although statistically no significant differences were observed (P > 0.05). This condition was almost identical to results of some previous studies reporting that the frequency of the disease

is connected with lifestyle and male dogs become more frequently infected than female dogs (4,17,18,20,24,25).

The prevalence of *D. immitis* was found as 7.1% in dogs 6 years and older, 2.2% in the age group of 3–5 years, and 2.5% in the age group of 0.7–2 years by both ELISA and PCR methods. The differences between all ages were accepted as statistically insignificant by both ELISA and PCR methods ($\chi^2 = 1.335$, P > 0.05). Most previous papers reported similar findings in Turkey and other countries (7,18,20,23,25,26). On the other hand, there are different studies in which the age of the dog was accepted as a risk factor for the prevalence of *D. immitis* (5,14).

In this study, it was detected that the prevalence of *D. immitis* was 1.7% in owned dogs and 3.7% in street dogs, but there was no statistically significant difference between origins (P > 0.05). Interacting with the external environment of both stray dogs and owned dogs could be the explanation for this. It was usually presumed that mosquitoes more often bit outdoor dogs than indoor ones (14). All stray dogs within the scope of the research lived in a shelter or on the streets, but the owned dogs also lived in outdoor circumstances. Yildirim et al. (10) and Simsek et al. (9) stated similar conclusions. Other investigators (3,17,20,24) confirmed that being stray or owned were the major risk factors, specified by the exposure time to the endemic area.

These results indicate that a more precise indication of the level of disease could be seen by using together the methods of Knott, PCR, and ELISA. This research is the first detailed study about *D. immitis* in dogs from the Sivas region of Turkey and more studies must be carried out in other nonstudied areas to complete the epidemiological map of *D. immitis* in Turkey.

Acknowledgments

References

1. Genchi C, Mortarino M, Rinaldi L, Cringoli G, Traldi G, Genchi M. Changing climate and changing vector-borne disease distribution: the example of *Dirofilaria* in Europe. Vet Parasitol 2011; 176: 295-299.

2. Furtado AP, Melo FTV, Giese EG, dos Santos JN. Morphological redescription of *Dirofilaria immitis*. J Parasitol 2010; 96: 499-504.

3. Yaman M, Guzel M, Koltas IS, Demirkazik M, Aktas H. Prevalence of *Dirofilaria immitis* in dogs from Hatay province, Turkey. J Helminthol 2009; 83: 255-260.

4. Furtado AP, Do Carmo ES, Giese EG, Vallinoto ACR, Lanfredi RM, dos Santos JN. Detection of dog filariasis in Marajo Island, Brazil by classical and molecular methods. Parasitol Res 2009; 105: 1509-1515.

5. Simsek S, Ozkanlar YE, Balkaya I, Aktas MS. Microscopic, serologic and molecular surveys on *Dirofilaria immitis* in stray dogs, Turkey. Vet Parasitol 2011; 183: 109-113.

6. Rishniw M, Barr SC, Simpson KW, Frongillo MF, Franz M, Dominguez-Alpizar JL. Discrimination between six species of canine microfilariae by a single polymerase chain reaction. Vet Parasitol 2006; 135: 303-314.

7. Öncel T, Vural G. Seroprevalence of *Dirofilaria immitis* in stray dogs in İstanbul and İzmir. Turk J Vet Anim Sci 2005; 29: 785-789.

8. Ağaoğlu Z, Akgül Y, Ceylan E, Akkan H. The incidence of *Dirofilaria immitis* in dogs in Van Province. Yüzüncü Yıl Üniversitesi Veteriner Fakültesi Dergisi 2000; 11: 41-43 (in Turkish with abstract in English).

9. Simsek S, Utuk AE, Koroglu E, Rishniw M. Serological and molecular studies on *Dirofilaria immitis* in dogs from Turkey. J Helminthol 2008; 82: 181-186.

10. Yildirim A, Ica A, Atalay O, Duzlu O, Inci A. Prevalence and epidemiological aspects of *Dirofilaria immitis* in dogs from Kayseri Province, Turkey. Res Vet Sci 2007; 82: 358-363.

11. Ataş AD, Özçelik S, Saygı G. The occurence of helminth species in stray dogs, their prevalence and significance of public health in Sivas. Türkiye Parazitoloji Dergisi 1997; 21: 305-309 (in Turkish with abstract in English).

12. Pantchev N, Etzold M, Daugschies A, Dyachenko V. Diagnosis of imported canine filarial infections in Germany 2008–2010. Parasitol Res 2011; 109: 61-76.

13. Vezzani D, Mesplet M, Eiras DF, María F, Fontanarrosa MF, Schnittger L. PCR detection of *Dirofilaria immitis* in *Aedes aegypti* and *Culex pipiens* from urban temperate Argentina. Parasitol Res 2011; 108: 985-989.

14. Song KH, Park JE, Lee DH, Lee SH, Shin HJ. Serological update and molecular characterization of *Dirofilaria immitis* in dogs, South Korea. Res Vet Sci 2010; 88: 467-469.

15. Bolio-Gonzalez ME, Rodriguez-Vivas RI, Sauri-Arceo CH, Gutierrez-Blanco E, Ortega-Pacheco A, Colin-Flores RF. Prevalence of the *Dirofilaria immitis* infection in dogs from Merida, Yucatan, Mexico. Vet Parasitol 2007; 148: 166-169.

16. Cardoso L, Mendao C, Carvalho LM. Prevalence of *Dirofilaria immitis*, *Ehrlichia canis*, *Borrelia burgdorferi* sensu lato, *Anaplasma* spp. and *Leishmania infantum* in apparently healthy and CVBD-suspect dogs in Portugal - a national serological study. Parasite Vector 2012; 5: 62.

17. Hou H, Shen G, Wu W, Gong P, Liu Q, You J, Cai Y, Li J, Zhang X. Prevalence of *Dirofilaria immitis* infection in dogs from Dandong, China. Vet Parasitol 2011; 183: 189-193.

18. Magi M, Guardone L, Prati MC, Tozzini G, Torracca B, Monni G, Macchioni F. Canine filarial infections in Tuscany, central Italy. J Helminthol 2012; 86: 113-116.

19. Morchon R, Moya I, Gonzalez-Miguel J, Montoya MN, Simon F. Zoonotic *Dirofilaria immitis* infections in a province of Northern Spain. Epidemiol Infect 2010; 138: 380-383.

20. Nematollahi A, Barazandeh MAJ. A survey on *Dirofilaria immitis* occurence in stray dogs of Tabriz (Iran). Acta Vet Brno 2010; 79: 449-451.

21. Pantchev N, Schaper R, Limousin S, Norden N, Weise M, Lorentzen L. Occurrence of *Dirofilaria immitis* and tick-borne infections caused by *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato and *Ehrlichia canis* in domestic dogs in France: results of a countrywide serologic survey. Parasitol Res 2009; 105: 101-113.

22. Sun M, Zhuo W, Guo S, Liao S, Shi D, Liu J, Cheng Z, Liu Y, Niu X, Wang S et al. Serological survey of canine dirofilariosis in Chongqing, Kunming, Nanchang, Fuzhou, Guangzhou, Shenzhen, and Nanning in Southern China. Vet Parasitol 2012; 185: 225-228.

23. Adanır R, Sezer K, Köse O. The prevalence of *Dirofilaria immitis* in dogs with different breed, ages and sex. Ankara Univ Vet Fak 2013; 60: 241-244.

24. Göz Y, Koltaş İS, Altuğ N, Demirkazık M, Yüksek N, Ağaoğlu Z. Seroprevalance of *Dirofilaria immitis* in dogs in Van District. Yüzüncü Yıl Üniversitesi Veteriner Fakültesi Dergisi 2007; 18: 5-8 (in Turkish with abstract in English).

25. Kozan E, Sevimli FK, Birdane FM. Incidence of *Dirofilaria* sp. in stray dogs in the Afyonkarahisar and Eskisehir provinces. Ankara Univ Vet Fak 2007; 54: 117-119 (in Turkish with abstract in English).

26. Taşçı GT, Kılıç Y. The prevalance of *Dirofilaria immitis* (Leidy, 1856) in dogs and investigations on potential vector mosquito species in Kars and Iğdır. Kafkas Univ Vet Fak 2012; 18 (Suppl. A): A29-A34 (in Turkish with abstract in English).

27. Yıldırım A. The prevalence of filarial agents in dogs in Ankara and vicinity. Ankara Univ Vet Fak 2004; 51: 35-40 (in Turkish with abstract in English).