Original Article

Serological and Molecular Detection of *Dirofilaria* Species in Stray Dogs and Investigation of *Wolbachia* DNA by PCR in Turkey

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Abstract

Background: *Dirofilaria immitis* and *Dirofilaria repens* are the most common species of filarial nematodes described in the dogs. A single-step multiplex PCR was applied to detect and differentiate simultaneously and unequivocally *D. immitis* and *D. repens* on DNA extracted from canine peripheral blood and besides to detect the seroprevalance of *D. immitis* by ELISA in Elazig Province, Turkey. A PCR detection of the *Wolbachia*, which plays an important role in *D. immitis* biology and contributes to the inflammatory pathology of the heartworm, was also applied for the first time in Turkey.

Methods: A total of 161 whole blood and sera samples were collected from stray dogs and stored at -20 °C until used. After DNA extraction, all samples were processed with *Dirofilaria* primers by multiplex-PCR and *Wolbachia* primers by conventional PCR besides ELISA for serology. The amplification was performed using a set of primers designed on a portion of the small subunit ribosomal RNA gene of the mitochondrion (12S rDNA).

Results: Three of the examined dogs (1.8%) were found to be infected with only *D. immitis*, one (0.6%) with *D. repens* and three (1.8%) with both parasites. Besides, 10 out of 161 dogs (6.2%) were found infected with *Wolbachia* sp. Finaly, the seroprevalence of dirofilariosis in the examined dogs was found to be 3.7% (6/161).

Conclusion: Although dirofilariosis is not a serious problem in the region, the stray dogs still continue to be a source of infection.

Keywords: *Dirofilaria immitis*, *Dirofilaria repens*, *Wolbachia*, Multiplex-PCR, ELISA

Introduction

Dirofilariosis, caused by *Dirofilaria immitis*, is found world-wide, but the most endemic areas are those with high temperatures and appropriate mosquito vector populations. *Dirofilaria immitis* typically inhabits the right ventricle and pulmonary arteries of dogs. "This vector-borne parasite can cause patent infections in dogs, cats and wild canidae" (Dillon 2000). It is one of the most pathogenic nematode parasite of dogs. Adult heartworms may cause clinical signs ranging from mild cough to congestive heart failure, intravascular hemolysis and pulmonary thromboembolism which are often fatal if untreated (Soulsby 1986). *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 recent-ly, through molecular approaches. Morphological identification of circulating microfilariae, however, is not always easy and is potentially misleading (Rishniw et al. 2006). *Dirofilaria repens*, a filarial parasite of canids, is transmitted by mosquitoes. The adult worms are observed mainly in the subcutaneous tissue of dogs, and produce microfilariae that circulate in the blood stream of infected dogs. Dignosis of it can be done by blood smear evaluation for the presence of microfilariae, serologic detection

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antigen or antibodies and detection of microfilarial DNA by PCR (Lee et al. 2004).

Dirofilaria immitis is transmitted by several culicid mosquito species belonging to a wide range of genera, including *Culex*, *Aedes*, *Ochlerotatus*, *Anopheles*, *Armigeres* and *Mansonia* (Cancrini et al. 1995). *Aedes vexans* and *Culex pipiens* were detected as the potential vectors of *D. immitis* in Turkey (Yildirim et al. 2011). For the first time, cytochrome c oxidase I (COI) sequences were obtained from Iranian specimens of *An. hyrcanus*, *An. pseudopictus*, *Cx. theileri* and *Oc. caspius* s.l. Only *Culex theileri* were found naturally infected with third-stage (infective) larvae of *D. immitis* (Azari-Hamidian et al. 2009).

DNA-based diagnostic tests for *D. immitis* and *D. repens* 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 (Mar et al. 2002, Rishniw et al. 2006). The usefulness of different PCR methods for the identification of *Dirofilaria* spp microfilaria in dog blood (the definitive host) has been reported in recent publications (Gioia et al. 2010, Simsek et al. 2011, Giangaspero et al. 2012, Latrofa et al. 2012).

Dirofilaria immitis is one of the several species of parasitic nematodes that hold the obligate symbiont bacteria *Wolbachia* spp. large colonies of *Wolbachia* live in the subdermal lateral cords of both female and male nematodes, as well as in the reproductive structures of females (McHaffie 2012).

The aim of the current study was to performe a single-step multiplex PCR to detect and differentiate *D. immitis* and *D. repens* on genomic DNA isolated from dog blood and also detect the seroprevalance of *D. immitis* by ELISA. The amplification was performed using a set of primers designed on a portion of the small subunit ribosomal RNA gene of the mitochondrion (12S rDNA). The other aim of this work was to PCR detection of the *Wolbachia* which is play an important role in *D. immitis* biology and contributes to the inflammatory pa-thology of the heartworm.

Materials and Methods

Samples collection

A total of 161 whole blood and sera samples were obtained from stray dogs in Elazig Province of eastern Turkey within 2010. These dogs had been captured from suburbs by the local authorized for the aim of spaying and during this procedure the blood samples were acquired under anesthesia. The blood and sera samples were stored in -20 °C untill use and age, breed and genders were recorded.

DNA (gDNA) Isolation, PCR amplification and sequencing

The blood samples were removed from freezer and waited at room temperature untill thawed. Then 1 ml blood sample was putted into an eppendorf tube and centrifuged during 5 min by 5000 rpm for sink to the bottom of possible microfilaria. Supernatant was removed and prior to gDNA isolation pellet was digested overnight at 56 °C with 600 µl lysis buffer of the kit to which 20 µl Proteinase-K (20 mg/ml) (Sigma, USA) were added. The tubes were incubated at 56 °C for overnight and the kit procedure was followed and at the last step the pellet was resuspended in 80 µl sterile distilled water, and the gDNA samples were stored at -20 °C until use.

The multiplex-PCR reactions for *D. immitis* and *D. repens* were performed using two sets of primer in the same reaction. General primer pairs 12SF (5'-GTTCCAGAATAATCGGCTA-3') and 12SRdeg (5'-ATTGACGGATG(AG)TTTGTACC-3') were used previously designed on the 12S rDNA region (Casiraghi et al. 2004). Besides we used a

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specific forward primer for *D. immitis* (12SF2B 5'-TTTTTACTTTTTTGGTAATG-3') and a specific reverse primer for *D. repens* (12SR2 5'-AAAAGCAACACAAA-TAA (CA)A-3') previously designed by Gioia et al. (2010). The PCR reactions were carried out in a total volume of 50 µl containing 5 µl of genomic DNA for each sample amplification, 5 µl of $MgCl_2$, 1.25 mM of each dNTP, 5 µl 10 X PCR buffer, 0.5 IU Taq DNA polymerase and 20 pmol of each primers. The thermal profile used was 92 °C for 1 min; 40 cycles of 92 °C for 30 s, 52 °C for 45 s, 72 °C for 1 min and final elongation step at 72 °C for 10 min. The amplified products were separated by electrophoresis in 2% 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 µg/ml) staining for 45 min at room temperature. *Dirofilaria immitis* genomic DNA positive control sample was extracted from microfilariae present in the blood of infected dogs (gifted from another research group) (Yildirim et al. 2007). Another gDNA control sample was extracted from an adult *D. repens* parasite (this worm was gifted by Luigi Venco (Veterinary Hospital "Citt'a di Pavia", Viale Cremona Pavia, Italy).

Extracted DNA was also tested for the presence of *Wolbachia* using a PCR-based assay and the gene primer wsp. A specific primer sets (Forward 5'-TGGTCCAATAA GTGATGAAG AAACTAGCTA-3', reverse 5'-AAAATTAAACGCTACTCCAGCTTCT GCAC-3') previously described by Zhou et al. (1998) were used for the amplification of gDNA. The PCR mixtures were composed of 5 µl of 10X PCR buffer, 5 µl of $MgCl_2$, 125 µM of each dNTPs, 20 pmol of each primers, 0.2 µl (5 IU) Taq-DNA Polymerase and 5 µl of genomic DNA was used for each PCR reaction. The reactions were performed on a PCR thermal cycler (Thermo Electron Corporation, Waltham, MA, USA) under the following conditions: 94 °C for 3 min و 40 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 5 min. PCR products were analyzed on 1.4% agarose gels stained by ethidium bromide and visualized under ultraviolet light.

Randomly selected six *Dirofilaria* and two *Wolbachia* samples were sequenced for confirmation of the PCR results.

Serological Analysis

Filarcheck (Agrolab, Italy) kit was used for working the dog sera for serological analysis. The test is based on a sandwich ELISA technique. Microplate wells were coated with a monoclonal antibody against the circulating antigen of *D. immitis*. Canine serum was added into the wells. If the serum contained the antigen, wells gave blue colour otherwise colorless.

Statistical Analysis

The data were evaluated by SPSS 15.0 programme using of 2X2 Fischer's Exact test and Pearson's Chi square test.

Results

Multiplex-PCR reaction showed the expected amplification products of app-roximately 500 bp for the genus *Dirofilaria*, 327 bp for *D. repens* and of 204 bp for *D. immitis.* (Fig. 1). *Wolbachia* surface protein PCR amplified 630 bp band as shown in Fig. 2.

The results of the PCR assay according to the ages and gender of filarial agents and *Wolbachia* are shown in Table 1. Thirty five male dogs were examined by multiplex-PCR and the prevalance values were 5.7% for *D. immitis*, 2.8% for *D. repens* and 2.8% for mixed infection (both *D. immitis* and *D. repens*). On the other hand, 126 female dogs were examined by PCR and only one case was *D. immitis* (0.8%) and two cases were

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D. repens (1.5%). There was no any mix infection in female dogs.

Among the 161 samples screened by the ELISA, 6 samples (3.7%) tested positive for the *D. immitis*. There was no significant difference in the number of positive *D. immitis* infection among female dogs (4 out of 126, 3.2%) and male dogs (2 out of 35, 5.7%). Only 2 out of the 69 dogs belonging to the 0–1 yrs old group were positive (2.9%), while 4 out of 71 dogs belonging to the 2–4 yrs old group were positive (5.6%). A total of 21 dogs belonging to the >4 yrs old group showed no seropositivity of *D. immitis* infection.

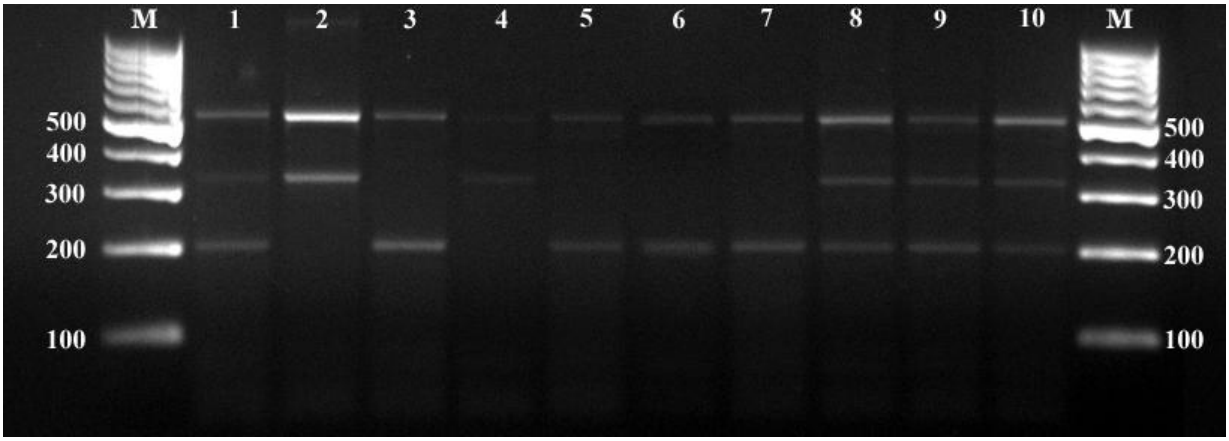

Fig. 1. Multiplex-PCR bands of samples. M: Molecular weight marker (100 bp), 1: Positive control of mix infection (500 bp, 327 bp and 204 bp), 2: Positive control of *Dirofilaria repens* (500 bp and 327 bp), 3: Positive control of *Dirofilaria immitis* (500 bp and 204 bp), 4: Only *D. repens* detected sample, 5, 6, 7: Only *D. immitis* detected samples, 8, 9, 10: Mix infected samples.

Table 1. Positivity of filarial agents and *Wolbachia* according to ages and gender

		Inspected	Only *D. immitis*				Only *D. repens*				Mix				*Wolbachia*			
		Dog (n)	n	%	*P*		n	%	*P*		n	%	*P*		n	%	*P*	
Gender	Male	35	2	5.7	0.119		1	2.8	0.217		1	2.8	0.523		4	11.4	0.226	
	Female	126	1	0.8			-	-			2	1.5			6	4.7		
					χ^2	*P*			χ^2	*P*			χ^2	*P*			χ^2	*P*
Ages	0-1	69	-	-	3.875	0.144	-	-	1.276	0.528	-	-	2.628	0.269	3	4.3	1.098	0.577
	2-4	71	3	4.2			1	1.4			2	2.8			6	8.4		
	4>	21	-	-			-	-			1	4.7			1	4.7		
Total		161	3	1.8			1	0.6			3	1.8			10	6.2		

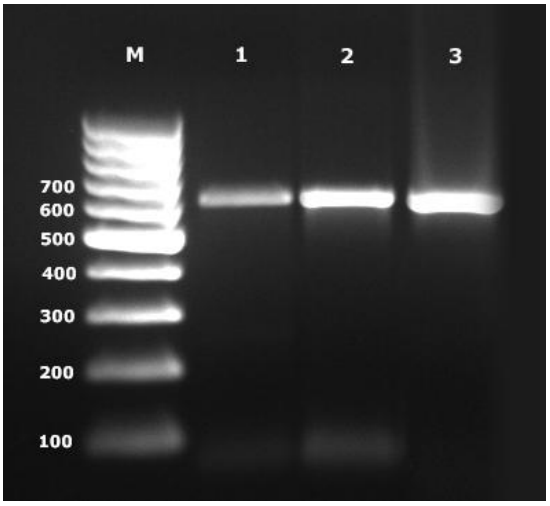

Fig. 2. PCR bands of *Wolbachia* surface protein. M: Molecular weight marker (100 bp), 1, 2: Positive samples (630 bp), 3: Positive control

Discussion

Adult *D. immitis*, inhabit the right ventricle of the heart the pulmonary arteries where they cause canine heartworm disease while the adults *D. repens* usually inhabit the subcutaneous tissue. In addition, it is well-known that *D. immitis* and *D. repens* produce microfilariae that circulate in the blood of dogs (Soulsby 1986). *Dirofilaria immitis* occurs worldwide in tropical, sub-tropical and temperate climates however *D. repens* occurs in the oldworld, in particular, throughout the Mediterranian sub-region, South Asia and sub-Saharan Africa (Cringoli et al. 2001).

Vector borne pathogens are sensitive to climatic condition, and there are some evidence that climate change may increase the incidence and dentensity of the diseases transmission (Purse et al. 2005). By altering the global environment, climate change has significant potential to intensify the vector borne diseases (Khasnis and Nettleman 2005). *Dirofilaria immitis* vectors are mosquitoes of Culicidae family with nearly 70 species susceptible for developing of parasite and thus considered potential vectors (Vezzani and Carbajo 2006). *Aedes albopictus* is reported as the primary potential vector of *D. immitis* in Italy (Cancrini et al. 2003). Whereas, *Cx. theileri* was detected as a vector of *D. immitis* in Portugal (Santa-Ana et al. 2006). There are limited study about vectors of *Dirofilaria* species in Turkey. Yildirim et al. (2011) determined that *Ae. vexans* and *Cx. pipiens* are the main potential vectors for *D. immitis* in Central Turkey. In the current study we could not investigate the potential vectors of *Dirofilaria* species.

Several studies have been published regarding the distribution and prevalence of *D. immitis* in dogs in Turkey. It was first reported in a dog the year of 1951 in Turkey (Guralp 1981). Tasan (1983) detected microfilaraemia in 53/283 (18.7%) stray dogs in Elazig. The prevalence was recorded as 1.52% in Istanbul (Oncel and Vural 2005), 9.6% in Kayseri, (Yildirim et al. 2007), 8.1% in Erzurum (Simsek et al. 2011) besides 12.3%, 18.3%, 10.5% and 14.8% in Sakarya, Kocaeli, Mersin and Ankara, respectively (Simsek et al. 2008). 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 (Rishniw et al. 2006). The current study describes a quick and accurate molecular method for the

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simultaneous detection of the *D. immitis* and *D. repens* for the first time in Turkey.

Although there have been some records about variability between age and dirofilariosis prevalence (Montoya et al. 1998, Song et al. 2003, Fan et al. 2003), some authors (Rowley 1981, Martin and Collins 1985) have reported that age has no effect on dirofilariosis and it can be occur in all ages dog. While the others (Song et al. 2003, Fan et al. 2001) have stressed that ages and positivity, have positive relation and especially 3–7 ages group have higher risk than the others. Fan et al. (2001) found the lowest prevalence in 1–3 ages (6.3%) and following 3–6 ages (14.1%) while the highest rates has been found in up to 6 ages (23.7%). Simsek et al. (2008) determined the highest percent 3-5 ages dog (17.5%) while there was no positivity in up to 6 ages. In the current study, all dogs that were defined only *D. immitis* by PCR were in 2–4 ages group (4.2%). Similarly, the ELISA seropositivity was 2.9% in 0–1 ages and 5.6% in 2–4 ages dog. A possible explanation for higher seroprevalence of *D. immitis* infection in older dogs might be due to their longer exposure to the risk factor like mosquito (Fan et al. 2001). Selby et al. (1980) also indicated that the age of dogs was an important risk factor and determined by time of exposure in the endemic area.

In the present study, ELISA and PCR positivity were higher in male than female dogs. Similarly, Montoya et al. (1998) indicated that heartworm infection was more common in male dogs than female dogs, and the generally higher infection rate in male dogs had been postulated to be due to their stronger attraction to mosquitoes. However, Simsek et al. (2008), reported 10.7% for males and 14.4% for females. More male dogs live in the outdoor, due to their use in defence of property. They are, therefore, more likely to be bitten by mosquitoes (Montoya et al. 1998). However, all studied stray dogs in this work were living in outdoor. Thus, living conditions are not unique on the prevalence. We believe that, some individual parameters like hormonal changes and immune deficiencies in female dogs may more tend to dirofilariosis.

Canine heartworm disease is generally diagnosed by antigen testing for *D. immitis*, and/or identification of microfilariae in the blood of infected dogs. However, some other filariae, including *Dipetalonema reconditum*, *D. repens* and approximately 1% of *D. immitis* infestations, can produce persistent microfilaremias with negative heartworm antigen tests (Rishniw et al. 2006). Thus, serological and molecular techniques should be use as combined. In this study, the seroprevalence with ELISA was 3.7% while the positivity was 1.8% by PCR. This differences might be related with some possible cross reactions with the other nematodes, single-sex adults and/or possible treatment of microfilaria by macrolids.

Dirofilaria repens is transmitted by mosquitoes. The adult parasites are observed in the subcutaneous tissues of dogs and produce microfilariae that circulate in the perifer blood of infected dogs. It is less remarkable than *D. immitis* due to the lower pathogenicity (Soulsby 1982). *Dirofilaria repens* was detected first time in Turkey in 1962 (Merdivenci 1970). Tasan (1984), necropsied 120 dogs and found the occurence of *D. repens* as 2.5% in Elazig province of Turkey. Whereas, Yildirim (2004) examined a total of 300 dog blood by modified knott and membrane filtration tests and no detected any *D. repens* micofilaria in Ankara. In the current study, one of the examined dogs (0.6%) was infected with only *D. repens* by PCR and three of them (1.8%) were infected with both *D. immitis* and *D. repens*. These rates are close to reported by Tasan (1984). Microfilariae of *D. repens* are difficult to discriminate from *D. immitis*, since they have similar morphology. Staining of microfilaria was widely used for dis-

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criminate the both species. In the last years, PCR analysis was reported to be quite sensitive and specific for the differentiate the species (Lee et al. 2004). Gioia et al. (2010) designed a single-step multiplex PCR was based on the amplification of a partial 12S rRNA gene of the mitochondrion with a mix of general and species-specific filarial primers in a single reaction. We also used the same primers for the amplification of *D. immitis* and *D. repens* 12S rRNA genes by PCR in a single tube. Thus, the simultaneous detection of both *D. immitis* and *D. repens* in naturally infected dogs has been achived for the first time in Turkey.

Wolbachia is an intracellular alphaproteobacteria endosymbionts sheltered in a broad range of insects and nematodes (Pfarr and Hoerauf 2007). According to reports based on DNA amplification, one in five of the arthropods are infected with *Wolbachia*, rendering this bacterium the most ubiquitous intracellular symbiont yet described (Bourtzis 2008). *Dirofilaria immitis* and *D. repens* harboured the *Wolbachia* endosymbiont (Kozek 2005). We amplified the wsp (*Wolbachia* surface protein) gene by PCR and detected 6.2% (10/161) positivity in the current study. There are very limited study about this subject in Turkey. Sarali et al. (2009) collected 150 dogs blood from Izmir and Aydın provinces and detected the prevalences as 12.3% for both *D. immitis* and *Wolbachia* sp. In the current work, we determined the *Wolbachia* in 6 samples together with *D. immitis* and *D. repens* and in 4 samples without *Dirofilaria* spp as well. In this instance, either those 4 samples were infected with any *Dirofilaria* species and the PCR could not detect or those dogs had another residence for *Wolbachia* in the dogs. It is widely accepted that *Wolbachia* is released into the tissues of the infected host following worm death and that bacteria derived molecules provoke innate inflammatory responses (Saint Andre et al. 2002). Thus, doxycycline treatment may reduce *Wolbachia* levels in adultworms and less severe pathology as well.

Conclusion

This is the first study on the detection of *Dirofilaria* species using of multiplex-PCR in Turkey. Besides, it was attentioned to neglected filarial nematod which is *D. repens* in Turkey and obtained actual prevalence data about *D. repens*, *D. immitis* and *Wolbachia*, as well. Besides, the seroprevalence of *D. immitis* was determined by ELISA. Those results have been shown that canine dirofilariosis still prevalent and there is no effective reduction yet.

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