Kafkas Univ Vet Fak Derg
22 (5): 751-755, 2016
DOI: 10.9775/kvfd.2016.15330

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

The Serologic and Molecular Prevalence of Heartworm Disease in Shelter Dogs in the Thrace Region of Turkey [1]

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[1] *This study was supported by the Research Fund of Istanbul University (Project Number: 625/28062007)*

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Article Code: KVFD-2016-15330 Received: 12.02.2016 Accepted: 17.04.2016 Published Online: 18.04.2016

Abstract

Dirofilaria immitis is an important nematode of dogs and cats which causes fatal heartworm disease in their hosts. The aim of this study was to investigate the prevalence of *D. immitis* by serologic and molecular methods in shelter dogs, in the Thrace Region of Turkey. Blood samples were collected from 402 dogs in shelters which were located in four cities (Istanbul, Edirne, Tekirdag and Kirklareli) in this region. The blood smears were examined for the presence of *D. immitis* microfilariae under the light microscope. The commercial Snap3Dx test kit and PCR assay for amplifying the ITS-2 gene region were used for the serological and molecular analyses, respectively. The serologic and molecular prevalence of *D. immitis* was determined as 6.7% and 2.7%, respectively. *D. immitis* microfilariae were also determined in the blood smears of three dogs (0.8%). The distribution of the infection according to the provinces was 14.7% in Edirne, 11% in Kirklareli, 1% in Tekirdag and 0% in Istanbul. The occult infection rate was determined as 59.3%. This study revealed the presence of *D. immitis* for the first time in the Thrace Region of Turkey. This region is the transition point to Europe and obtained data in this study could contribute to the control of heartworm disease in the area.

Keywords: *Heartworm Disease, Dog, Serology, PCR, Thrace Region, Turkey*

Türkiye'nin Trakya Bölgesindeki Barınak Köpeklerinde Kalp Kurdu Hastalığının Serolojik ve Moleküler Prevalansı

Özet

Dirofilaria immitis, konaklarında ölümcül Kalp Kurdu Hastalığı'na neden olan köpek ve kedilerin önemli bir nematodudur. Bu çalışmanın amacı, Türkiye'nin Trakya Bölgesi'ndeki barınak köpeklerinde *D. immitis*'in prevalansının serolojik ve moleküler yöntemlerle araştırılmasıdır. Kan örnekleri, bu bölgedeki dört şehirde (İstanbul, Edirne, Tekirdağ ve Kırklareli) lokalize olmuş barınaklardaki 402 köpekten alındı. Kan yaymaları *D. immitis*' in mikrofilerlerinin varlığı için ışık mikroskobu altında incelendi. Snap 3Dx test kiti ve ITS-2 gen bölgesini çoğaltmak için PCR yöntemi sırasıyla serolojik ve moleküler analizlerde kullanıldı. *D. immitis*'in serolojik ve moleküler prevalansı sırasıyla %6.7 ve %2.7 olarak belirlendi. Ayrıca 3 köpeğin kan yaymalarında *D. immitis'* in mikrofilerleri saptandı (%0.8). İllere göre enfeksiyonun dağılımı Edirne'de %14.7, Kırklareli'nde %11, Tekirdağ'da %1 ve İstanbul'da %0 olarak bulundu. Gizli enfeksiyon oranı %59.3 olarak saptandı. Bu çalışma *D. immitis*'in varlığını, Trakya Bölgesinde ilk kez ortaya koydu. Bu bölge Avrupa'ya geçiş noktası olup, bu çalışmada elde edilen veriler Kalp Kurdu Hastalığı'nın bölgedeki kontrolüne katkı sağlayabilir.

Anahtar sözcükler: *Kalp Kurdu Hastalığı, Köpek, Seroloji, PCR, Trakya Bölgesi, Türkiye*

INTRODUCTION

Dirofilaria immitis is the causative agent of canine heartworm disease which leads to serious health problems even death in dogs. It threatens not only the animals' health but also the humans as it is a zoonotic disease. While adult worms are found in pulmonary arteries, heart and vena cava, microfilariae are found in the blood of dogs. Different species of mosquitoes belonging to the family of Culicidae (e.g. *Anopheles* spp., *Aedes* spp., *Culex* spp.,) transmit the infection between the dogs [1]. *Aedes vexans* and *Culex pipiens* were reported as the potential vectors of *D. immitis* in Central Turkey [2]. Temperature has influence not only on the survival of mosquitos but also on

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the development of infective microfilariae (L3) in them. The amount of microfilariae in the peripheral blood of the dogs varies daily and seasonally [3]. Occult (amicrofilaremic) infections can occur in consequence of the presence of only the same gender, prepatent infection or sterile adult worms depending on drug-induced [4].

There are kind of methods for detection of adult *D. immitis* and their microfilariae in peripheral blood of dogs. Many commercial serologic kits detecting the antigen of adult female *D. immitis* can be used for diagnosis. Microfilariae of the nematode can be determined in the blood samples by the concentration methods, filter tests, histochemical staining and PCR assay [5]. Among these techniques, PCR assay is a sensitive and rapid assay for differentiation of *D. immitis* microfilariae from the microfilariae belong to other filarial worms living in dogs [6,7].

Heartworm disease has worldwide distribution and the rate of this infection has been increasing through the central and northern Europe [1]. Many researchers [8-10] reported the presence of the infection in different countries around the world. In Europe, the prevalence range of the infection reported between 0.2% [11] and 28.7% [12]. *Dirofilaria immitis* also reported from different cities of Turkey by serologic and molecular assays and the prevalences were between 1.5% [13] and 46.2% [14].

Best of the authors' knowledge, *D. immitis* has not been reported in the Thrace Region of Turkey. This region is border to Europe and has an intensive movement of pet animals. The aim of this study was to determine the prevalence of *D. immitis* in shelter dogs in four provinces of the Thrace Region of Turkey.

MATERIAL and METHODS

This study has been approved by the ethical committee of Istanbul University (ref: 2006/168).

A total of 402 blood samples were collected from the shelter dogs in Istanbul (Avcilar, Altinsehir, Bakirkoy, Beyoglu, Gurpinar, Kemerburgaz, Silivri, Zeytinburnu districts), Edirne (center, Kesan district), Tekirdag (center, Corlu, Cerkezkoy, Saray districts), and Kirklareli (center, Luleburgaz, Igneada districts) provinces located in the Thrace Region of Turkey. The blood samples were taken from the cephalic vein of the dogs into the tubes containing EDTA. Blood smears were prepared and stained with May-Grünwald Giemsa. The blood smears were examined for the presence of the microfilariae of *D. immitis* under the light microscope. Identification of the microfilaria species was based on morphological and morphometric characteristics according to Genchi et al.[5].

Serologic Assay

A commercial kit SNAP3Dx (IDEXX Laboratories, Westbrook, ME, USA) was used to detect *D. immitis* antigens. This kit includes two antibodies (one for capture and the other for detection) which are specific to antigens of adult female *D. immitis*. Whole blood was used according to the kit manufacturer's instructions.

PCR Assay

DNA extraction was carried out with a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. PCR assay was performed using the primers D.imm-F1 CAT CAG GTG ATG ATG TGA TGA T and D.imm-R1 TTG ATT GGA TTT TAA CGT ATC ATT T which targeted to the ITS2 region of *D. immitis* [6]. The PCR reaction mix consisted of 2.5 µl 10X Taq polymerase buffer, 1.5 µl $MgCl_2$ (25 mM), 0.5 µl of each primer (200 mM), 0.12 µl (5 U) Taq polymerase (MBI, Fermentas, Lithuania), 0.5 µl dNTPs (200 mM each) and 5 µl template DNA with a final volume of 25 µl. All reagents except DNA were added to negative control. PCR was carried out in a thermal cycler (MaxyGene Gradient Thermal Cycler, Axygen Scientific, USA) with the following conditions: initial denaturation 3 min at 94°C, followed by 35 cycles of denaturation 45 sec at 94°C, annealing 45 sec at 60°C, extension 45 sec at 72°C and a final extension 7 min at 72°C. PCR products were electrophoresed through 1.5% agarose gels containing ethidium bromide (10 mg/ml) and the expected DNA fragments of 302 bp were visualised under UV.

Sequencing

All positive PCR products were sent to a commercial company (BGI, Shenzhen, China) for sequencing in order to confirm the validity of PCR amplifications. Sequences were compared with sequences of *D. immitis* available in the GenBank using Blast analysis.

Statistical Analysis

Haematological parameters of the infected dogs were compared with non-infected dogs using independent samples t-test. The differences of prevalence with serologic and molecular assays according to the cities were analysed with Chi- square test. $P<0.05$ was considered as significance level in both statistical analyses.

RESULTS

The results of serologic, molecular and microscopic analyses according to the provinces are given in *Table 1*. Out of 402 blood samples, 27 (6.7%) were positive serologically and 11 (2.7%) were positive by molecular analysis. While there was no significant difference in prevalence between Edirne and Kirklareli, these two cities had significant differences between Istanbul and Tekirdag with both serologic and molecular assays (P=0.001 and P=0.009 respectively) *(Table 1)*. The seropositivity was higher in Edirne (14.7%) and Kirklareli (11%) than Tekirdag (1%). None of the dogs from Istanbul was found to be

Table 1. *Results of serologic, PCR and microscopic examination assays for the detection of D. immitis in examined dogs according to the provinces in the Thrace Region*

Tablo 1. *İncelenen köpeklerde D. immitis'in belirlenmesinde kullanılan serolojik, PZR, mikroskobik inceleme yöntemlerinin Trakya bölgesindeki illere göre sonuçları*

Sampling Provinces	Number of Seropositive Dogs by Serologic Test	Number of Positive Dogs by PCR	Number of Positive Dogs by Microscopic Examination
Edirne (n = 102)	15 (14.7%) [a]	5 (4.9%) [a]	0
Kırklareli (n = 100)	11 (11%) [a]	6 (6%) [a]	3 (3%)
Tekirdag (n = 100)	1 (1%) [b]	0 [b]	0
İstanbul (n = 100)	0 [b]	0 [b]	0
Total (n = 402)	27 (6.7%)	11 (2.7%)	3 (0.8%)
X^2	26.174	11.498	
P	0.001	0.009	

[a, b] *Different letters within same column indicate significant differences between cities*

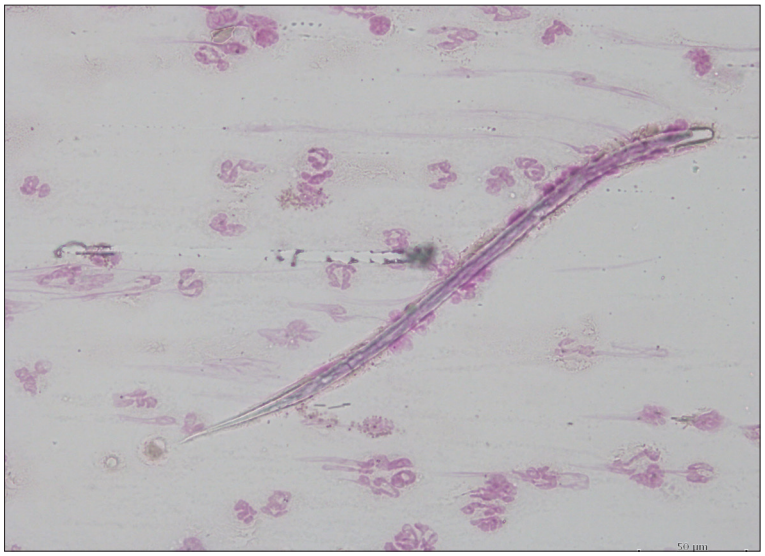

Fig 1. Microfilaria of *Dirofilaria immitis*

Şekil 1. *Dirofilaria immitis'*in mikrofileri

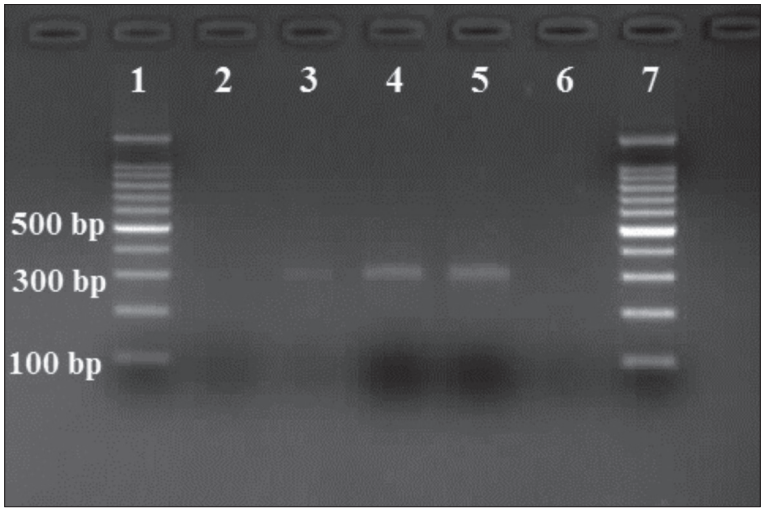

Fig 2. Agarose gel images of positive PCR products belong to *D. immitis*. Lane 1: 100 bp DNA marker, Lane 2: negative control, Lane 3, 4, 5: *D. immitis* positive samples (302 bp), Lane 6: negative control, Lane 7: 100 bp DNA marker

Şekil 2. *D. immitis'*e ait pozitif PZR ürünlerinin agaroz jel görüntüleri. şerit 1: 100 bp DNA marker, şerit 2: negatif kontrol, şerit 3, 4, 5: *D. immitis* pozitif örnekler (302 bp), şerit 6: negatif kontrol, şerit 7: 100 bp DNA marker

positive either by serologic or molecular assays. 16 of 27 seropositive blood samples were negative by both PCR and microscopic analyses result in 59.3% occult infection. Microfilaria *(Fig. 1)* was only found in blood smears from three dogs (0.8%) which were also positive with serologic and PCR assays by microscopic examination. The microfilariae were 290-295 µm in length and 6-7 µm in width with straight tail. The positive samples gave the expected amplification products of 302 bp for *D. immitis* by PCR and the gel images of DNA are shown in *Fig. 2*.

Sequences of positive samples were analysed by BLAST and were found closely related to DNA sequences of *D. immitis* available in GenBank (accession numbers: JX481279.1, EU182329.1, AF217800.2). There were no significant differences in haematological parameters between infected and non-infected dogs (data not shown).

DISCUSSION

Dirofilariasis is a fatal disease in dogs with worldwide distribution. In the current study, this infection was investigated for the first time in shelter dogs located in the Thrace Region of Turkey by serologic, molecular and microscopical assays. The seroprevalence of the infection was reported as 1% in Iran [15], 18% in India [16], 13.5% in China [10] and 55.3% in Russia [8]. In Europe, the seroprevalence was recorded as 9.6% in Italy [17], 2.4% in Spain [18] and 2.1% in Portugal [19]. The first case report of *D. immitis* was notified in an imported dog in Turkey, in 1951 [20]. Tasan [21] reported the prevalence of the infection as 5% for the first time in native dog breed. Until the year 2000, the studies on the prevalence of *D. immitis* had been performed using necropsy and/or Knott technique [21-27]. After development of commercial serologic tests for the diagnosis of *D. immitis,* many researchers used them in Turkey. The seroprevalence was 2% in Şanlıurfa [28] and 2.4% in Diyarbakır [29] which were lower than this study (6.7%). Considering the other provinces, the seroprevalence was found to be 9.1 % in Elazıg [30], 9.3% in Ankara [31], 9.6% in Kayseri [32], 17.8% in Van [33], 26% in Hatay [34], 34.5% in Kırıkkale [35], 40% in Igdır [36], 46.2% in Van [14] which were higher than the seroprevalence determined in the current study. There are limited studies on the diagnosis of this nematode in dogs by molecular techniques in Turkey. The frequency of the infection was reported as 5.4% in Kars [37], 19.6% in Igdır [37] and 8.1% in Erzurum [38] by PCR which were higher than the current study. PCR assay was also used for determination of *D. immitis* microfilariae in the mosquitos as the vectors in Turkey and the ratio of infected pools in *Aedes vexans* and *Culex pipiens* were reported as 0.32% and 1.24%, respectively [2].

First case report of *D. immitis* in three dogs in Istanbul was reported in 2005 [39]. In another study [13], in Istanbul the seroprevalence of the infection was reported as 1.5%. However, no infection was found in the examined dogs from Istanbul in the current study. To our knowledge, there was no information about the heartworm disease in the provinces of Edirne, Kirklareli and Tekirdag until this study. The seroprevalence of the dirofilariasis was 16.2% in Bulgaria [40], 17.9% in Greece [41] and 28.7% in Romania [12]. These countries have borders to the provinces of Edirne and Kirklareli and the seroprevalence in these countries were higher than the rate obtained with this study.

Occult infection rates were reported as 61.4% [34], 36.1% [37], 35.5% [42], 29.6% [32] and 27.5% [35] in other studies conducted in Turkey. The high rate of occult infections (59.3%) was also observed in this study. The reason of this high rate could be attributed to the low parasite burden, the presence of only the same genders of nematodes in the host, the prepatent infection or the treatment with a microfilaricide in the area which were also mentioned by several researchers [4,32,34]. The time of the blood collection in this study might also affect the results of the microfilariae detection assays. The amount of the microfilariae in the circulating blood varies daily [3] and the blood of dogs were not taken at a certain time interval in this study.

It was reported that the prevalence of *D. immitis* was higher in outdoor, stray and suburban dog population than the indoor, owned and urban dogs [9,33,34]. Parallel to these reports, the dog population was consisting of stray dogs which were living in shelters in the present study.

In conclusion, the prevalence of *D. immitis* was determined in dog populations for the first time in the Thrace Region of Turkey with this study. This region has border to Europe and is the transition point. The results of this study might contribute to prevention and control measures against the spreading of the disease.

Acknowledgments

We would like to thank all veterinarians, personnel in dog shelters for helping us during the collection of blood and ticks. We are also grateful to Prof. Dr. Sami Simsek from Veterinary Faculty of Fırat University for the positive samples.

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