Prevalence of *Dirofilaria immitis* in Dogs from Shelters in Vojvodina, Serbia

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ABSTRACT

Background: *Dirofilaria immitis* is vector borne parasite of carnivores, with zoonotic potential, endemic in many parts of the world, including Europe. The aim of this study was to determine the prevalence of *Dirofilaria immitis* infection in dogs from shelters, especially compared to their lifestyle. Dogs living in shelters in Serbia may be at high risk of acquiring vector borne pathogens, mainly because most of them live outside in pens and backyards, in contact with vectors. Also, dogs in shelters are not always regularly treated against ectoparasites, thus, representing an easy feeding source for the vectors. The objective of this study was to determine the prevalence of *Dirofilaria immitis* infection in dogs from 5 shelters in South Bačka and Central Banat districts, in Autonomous Province of Vojvodina, Northern part of Serbia. Also, the objective was to compare the relation of infection with *Dirofilaria immitis* with age, sex, type of keeping the animals and preventive treatment in dogs. **Materials, Methods & Results:** Between May 2017 and October 2019, blood samples were collected from 336 randomly selected dogs from 5 shelters in 2 districts, South Bačka and Central Banat districts, in Autonomous Province of Vojvodina, Northern part of Serbia. The epidemiological survey has been conducted with all of the dogs involved in this research. The survey was designed to collect data about sex, age, lifestyles, food type, treatment against mosquitoes with insecticides and filarioid worms with macrocyclic lactones, regular testing for *Dirofilaria* infections. The presence of circulating microfilariae was examined using a modified Knott's test. For the presence of circulating adult female *Dirofilaria immitis* antigen, serum samples were tested by commercially available enzyme-linked immunosorbent assay, which reacts to antigen of female *Dirofilaria*. In total, 336 dogs were examined for the presence of *Dirofilaria immitis* antigen. For that dog population which came from 5 shelters, total prevalence was 25.30%. Most of the positive findings were observed in a shelter where dogs lived exclusively outdoors in fenced yards in big groups and they were partly tested for heartworm infections from time to time. These dogs were not treated with macrocyclic lactones, against mosquitoes with insecticides or filarioid worms. The prevalence in this shelter was 56.36%. On the contrary to that, the lowest positive findings were detected in the shelter, where dogs were allowed to move freely between outside and indoors and they were also provided with accommodation indoors. These dogs have been regularly tested for *Dirofilaria* infections and treated against mosquitoes with insecticides and filarioid worms with macrocyclic lactones. In this shelter the seroprevalence was 7.69%. Microfilariae of *Dirofilaria immitis* were detected, by modified Knott's test, in all of the antigen positive dog samples; except in 2 dogs from one shelter.

Discussion: This study shows persistence of cardiopulmonary dirofilariosis in shelter dogs under different maintaining conditions. By comparing the data during the last 17 years, it can be stated that there is a constant increase of prevalence for *Dirofilaria immitis* in dogs in northern part of Serbia over the years. The results gained in this study are important from the veterinary point of view, but also from the Public Health point of view.

Keywords: *Dirofilaria immitis*, prevalence, Heartworm disease, dogs, shelter, diagnosis.

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INTRODUCTION

Cardiopulmonary dirofilariosis caused by *Dirofilaria immitis* is a vector borne disease transmitted by mosquitos, with zoonotic potential [1]. *D. immitis* resides in dogs and wild canids as definitive hosts [25]. Mosquito species that have been identified as vectors of dirofilariosis in Europe are *Aedes vexans*, *Aedes albopictus*, and *Culex pipens* complex [3,23]. *D. immitis* infections were first found in Mediterranean countries, but later the disease has spread over the Balkans and Central Europe [7-9,24,34], due to climate change, density of the vectors and travel of infected dogs [17].

Diagnostic methods available for infections with *D. immitis* are based on detection of microfilaria (modified Knott's test or filtration test), antigen (ELISA), antibodies (ELISA) or specific genes (COI by PCR) of *D. immitis* [18,29,32].

The first report of cardiopulmonary dirofilariosis in dogs, in Serbia (Yugoslavia) was in 1989, during necropsy of three dogs and then in 1999 which influenced the constant diagnosis in dogs in Serbia [5,18,22,35,36]. During the last two decades dirofilariosis has spread over Serbia and multiple studies on determination and differentiation *Dirofilaria* spp. were conducted in hunting dogs, pet owned dogs, military dogs of Serbian army, stray dogs, dogs from shelters [26,28,40-42].

The objective of this study was to determine the prevalence of *Dirofilaria immitis* infection in dogs from five shelters in South Bačka and Central Banat districts, in Autonomous Province of Vojvodina, Northern part of Serbia. Also the objective was to compare the relation of infection with *Dirofilaria immitis* with age, sex, type of keeping the animals and preventive treatment in dogs.

MATERIALS AND METHODS

Study areas, animals and sampling

Between May 2017 and October 2019, blood samples were collected from 336 randomly selected dogs from 5 shelters in 2 districts, South Bačka and Central Banat districts, in Autonomous Province of Vojvodina, Northern part of Serbia. Samples distribution according to shelters was 94 for A, 52 for B, 65 for C, 55 for D and 70 for E. Sample size was calculated using OpenEpi software (<https://www.openepi.com/SampleSize/SSPropor.htm>) [4]. Most of the dogs were medium-sized mixed breed, aged between 6 months and 15 years. In shelters B and partly D when dogs enter the shelter they are checked for *D. immitis* infection and if positive, they are in most cases treated. Epidemiological data about dogs from shelter are shown in Table 1.

Table 1. Epidemiological data on origin, lifestyle, food type, treatment and age of tested dogs from 5 shelters located in South Bačka and Central Banat districts in Autonomous Province of Vojvodina, Serbia

Shelter	N°	Dog's lifestyle	Food type	Treatment ¹	Regular testing ²	Age			
						≤ 2y	2-5y	5-8y	>8y
A	94	In pens	Commercially produced dog food	No	No	59	32	3	0
B	52	Indoor and outdoor	Commercially produced dog food	Yes	Yes	21	12	8	11
C	65	Outdoor	Commercially produced dog food	No	No	40	24	1	0
D	55	Outdoor	Commercially produced dog food	No	Partly	34	16	2	3
E	70	Outdoor	Commercially produced dog food, bread and slaughterhouse leftovers	No	No	51	19	0	0

¹Treatment against mosquitoes with insecticides and filarioid worms with macrocyclic lactone. ²Regular testing for *Dirofilaria immitis* infections.

With the consent of persons responsible for managing the shelters, a 4 mL blood sample was withdrawn from the cephalic vein of each dog using 2 labelled tubes, one without anticoagulants and the other with Heparin. After centrifugation, the serum samples were stored at -20°C until further processing.

Detection of microfilariae

The presence of circulating microfilariae was examined using a modified Knott's test. The test was

performed according to Bazzochi *et al.* [2]. The differentiation of microfilariae was done based on morphological characteristics of two *Dirofilaria* species microfilariae - the shape of cephalic and caudal ends of *D. immitis* and *D. repens* [10].

Detection of adult female *D. immitis* antigen

Serum samples were tested for the presence of circulating adult female *D. immitis* antigen by commercially available enzyme-linked immunosorbent assay

VetLine *Dirofilaria* Antigen ELISA¹ in accordance with the manufacturer's instructions.

The point prevalence was calculated within the cross-sectional design of epidemiological study.

Statistical analysis

All data were analyzed using statistical program Past 4². The chi-square test and Fisher's test were used to estimate statistical differences between groups. The level of statistical significance was set at $P < 0.05$.

RESULTS

For population of dogs from 5 shelters from the territory of South Bačka and Central Banat districts total *D. immitis* antigen prevalence was 25.30% (85/336) found by ELISA method. The highest number of positive findings was observed in shelter D, where 31 were positive out of 55 tested blood sera of dogs (56.36%). Shelter C identified 27 antigen reactive blood serum samples from dogs from 65 (41.54%) examined. In shelter E, a total of 70 blood sera of dogs were examined, of which 12 gave a positive result, respectively a prevalence for *D. immitis* antigen of 17.14% was recorded. A prevalence for *D. immitis* antigen of 11.70% was obtained in shelter A, and a total of 94 samples were examined, of which 11 gave a positive finding. The lowest prevalence for *D. immitis* antigen was recorded in shelter B 7.69% where a total of 52 blood sera of dogs were tested and a positive result was obtained in 4 samples.

Microfilariae of *D. immitis* were detected, by modified Knott's test, in all of the antigen positive dog samples; except in 2 dogs from shelter B. The results of prevalence of *D. immitis* in 5 shelters are shown with Figure 1.

The results were also analysed by the age and sex (male/female) of the dogs and the data are shown in Table 2. There was no significant difference between males and females in any of the shelters. In all of the shelters there was a significant difference between the prevalence for *D. immitis* in dogs under 2 years of age, compared to all the other age groups.

Figure 1. Total prevalence of *Dirofilaria immitis* in 336 randomly selected dogs from 5 shelters located in South Bačka and Central Banat districts, Autonomous Province of Vojvodina in Northern part of Serbia, from May 2017 to October 2019.

Table 2. Distribution of *Dirofilaria immitis* antigen positive dogs among different shelters (A-E) and according to the age and sex of dogs.

Statistical significance	age				Statistical significance	Sex		Statistical significance
	≤ 2 god.	2-5y	5-8y	>8y		M	F	
$\chi^2=57.346$ $P < 0.0001$	7 (7.45%)	3 (3.19%)	1 (1.06%)	0	$\chi^2 = 1.5277$ $P = 0.46586$	5 (5.32%)	6 (6.38%)	$P = 1$
	2 (3.85%)	0	2 (3.85%)	0	$\chi^2 = 5.3909$ $P = 0.14531$	2 (3.85%)	2 (3.85%)	$P = 1$
	17 (26.15%)	9 (13.85%)	1 (1.54%)	0	$\chi^2 = 1.5838$ $P = 0.45298$	14 (21.54%)	13 (20.00%)	$P = 0.8036$
	14 (25.45%)	13 (23.64%)	1 (1.82%)	3 (5.45%)	$\chi^2 = 9.573$ $P = 0.022568$	18 (32.73%)	13 (23.64%)	$P = 0.42237$
	7 (10.00%)	5 (7.14%)	0	0	$\chi^2 = 1.5448$ $P = 0.2139$	6 (8.57%)	6 (8.57%)	$P = 0.75436$
	47 (13.99%)	30 (8.93%)	5 (1.49%)	3 (0.89%)	$\chi^2 = 2.3234$ $P = 0.50806$	45 (13.39%)	40 (11.90%)	$P = 0.209$

DISCUSSION

In this study the prevalence for *D. immitis* of 25.30% was confirmed for Northern part of Serbia. The results gained in this study are important from the veterinary point of view, but also from the Public Health point of view. This study shows persistence of dirofilariosis in shelters under different maintaining conditions. By comparing the data during the last seventeen years, it can be stated that there is a constant increase of prevalence for *D. immitis* antigen in dogs in northern part of Serbia over the years. Retrospectively, in 2003-2004, prevalence for *D. immitis* in dogs was 5.9-7% [39], in 2006-2007, prevalence for *D. immitis* in dogs with clinical symptoms was 80%, but in dogs with no clinical symptoms it was 10-11% [33]. During the period from 2009 to 2013, prevalence for *D. immitis* in dogs with or without clinical symptoms was 27.6% [37]. Pajković *et al.* [26] reported that the prevalence for *D. immitis* in military dogs was 14%, in time period from 2004 to 2010. Savić *et al.* [36] in a study from 2013-2014, showed that the prevalence for *D. immitis* in hunting and military dogs was 22.78% and for pet dogs prevalence for *D. immitis* was found to be 22%. In the same study, the authors have found a lower prevalence for *D. immitis* in dogs from asylum, namely only 3.12%, but in this study only one asylum was analysed. The total prevalence in pet, military and asylum dogs reported by the authors [36] for *D. immitis*, was 15.29%, and in 92.3% of positive samples, *D. immitis* were confirmed by PCR. During the period 2015-2017, Savić *et al.* [38] analysed 482 dog blood samples from dogs entering the shelters and asylums, and they concluded that the prevalence for *D. immitis* among stray dogs in Vojvodina region is between 5% and 8%, that more prevalent is *D. immitis* than *D. repens*. In the latest published research in Vojvodina, Potkonjak *et al.* [29] detected infection with *Dirofilaria* spp. in 27.1% of the stray dogs. *Dirofilaria* spp. infection is widespread in the countries surrounding Serbia also. The prevalence for *D. immitis* in dogs from Hungary was reported to be 8.1% [6], 3.3%-7.2% in Romania [13,21], 7.4%-9.2% in Bulgaria [11,15], but Panayotova-Pencheva *et al.* [27] found adult *D. immitis* during the necropsies of dogs and reported the prevalence for *D. immitis* in dogs of 33.3%. The prevalence for *D. immitis* in dogs from North Macedonia was reported to be 12.5% [16]. Rapti & Rehbein [30] reported the prevalence for heartworm in dogs from

Albania as 13.5%. The prevalence for *D. immitis* in dogs from Bosnia and Herzegovina was reported to be 3.1% [43], and 0.6%-7.5% in Croatia [12,14].

The prevalence found in this study for the Northern part of Serbia is rather high compared to the neighboring countries. It is also higher than in the Southern part of the country. One example is the wider area of Leskovac city where the prevalence of *D. immitis* is 8.51% [19]. This could also be due to the fact that much more research has been done in dogs and wild canids in the Northern part of Serbia, but it also is because of the different landscape. The South of the country is more hilly and not as humid as the Northern part. It is also interesting that South Bačka region has a total prevalence for *D. immitis* of 27.44% and the Middle Banat region has a lower prevalence of 17.14%. This could be due to the fact that less samples were analysed from the Middle Banat region, but it can be also a consequence of the geographic factors. In 2 dogs from ayzlum B microfilariae of *D. immitis* were not detected, even though they were positive by ELISA. After analyzing the results it was found out that those dogs were actually treated with a larvicidae therapy for one time before the sampling. So, those two dogs were positive, since they were not yet cured from the adult forms of *Dirofilaria*. These dogs were treated with a "slow kill" therapy which is based on elimination of larval stages and adults remain live for some time [20]. The possibility of occult infection is about 20 % in dogs and it means infection with adult *Dirofilaria immitis* in the absence of circulating microfilariae. It can be detected in dogs with prepatent infection, unisexual heartworm infection, drug-induced sterility of adult heartworms, and an immune-mediated infection [31]. In our case it was a consequence of drug treatment.

In all the shelters there is a significant difference between the prevalence for *D. immitis* in dogs under 2 years of age, compared to all the other age groups. This was expected even though it is more possible that older dogs have adult forms of *D. immitis*. There are two reasons for this situation that especially refers to shelters. The first one is that there are a lot more young dogs in the shelters than the old ones (over 60% were dogs under 2 years of age in all the shelters except in shelter B). For that reason there were more young dogs available for sampling then the older ones. The other reason is that in some shelters (B and partly D) when dogs enter the shelter they are checked for *D. immitis*

infection and if positive, they are in most cases treated with larvicide therapy for some time [20].

In relation to the sex of dogs which were analysed, approximately half were males and half were females in every shelter. There was no significant difference between males and females in any of the shelters, meaning that the prevalence for *D. immitis* was similar for both sexes in all of the shelters. There is no evidence in the literature that sex of dog influences in any way the infection with *Dirofilaria immitis*.

CONCLUSIONS

The results of this study indicate the persistence of *Dirofilaria immitis* infection in dogs from 5 shelters. The shelters are placed in South Bačka and Central Banat districts, in Autonomous Province of Vojvodina, Northern part of Serbia. The study was done during the period from May 2017 to October 2019. These results indicate that dirofilariosis should always be considered in this region, which is endemic

for the disease during the past years. This also means that shelters can serve as sentinels of *D. immitis* infection. They can be reservoirs for the disease in dogs but also in humans. Dogs from shelters rarely have preventive methods applied against *D. immitis* infection and in most cases they are without any protection against vector-borne diseases. Due to public health threats, it is necessary to enable routine diagnostic testing for dogs in shelters and treatment of infected animals, as well as prophylactic treatment of healthy dogs to reduce the spread of infection to other dogs and humans.

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