SHOULD GLOBAL WARMING INFLUENCE THE OCCURRENCE OF CANINE DIROFILARIOSIS INFECTION IN ROMANIA?

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Abstract

In order to evaluate the prevalence of heartworm disease in the South-East of Romania, we collected blood from 40 dogs from a shelter in Galati, which were examined by several methods for the *Dirofilaria immitis* and *Dirofilaria repenes* microfilariae identification, and for the detection of *Dirofilaria immitis* antigen respectively. Eleven dogs were positive for microfilariae (27.5%), of which 3/11 were co-infections and only 1/40 had *D. repens* microfilariae detected. Following serological test, *D. immitis* antigen was detected in 30% of the investigated serum samples (12/40). The high prevalence of heartworm disease requires continuous monitoring of symptomatic dogs, but also of the asymptomatic ones from shelters or of those from veterinary clinics in endemic areas. Furthermore, the mosquito populations control in endemic areas is mandatory.

Key words: canine dirofilariosis; shelter; Galati; global warming;

Canine dirofilariosis is a potentially zoonotic disease transmitted through mosquito bites, which is produced by the nematodes *Dirofilaria immitis* and *Dirofilaria repens* (Capelli et al., 2018). The intermediate hosts (the vectors) involved in the transmission of these nematodes are *Culex*, *Anopheles* and *Aedes* genus (Mirahmadi et al., 2017; Adebayo et al., 2020).

Concerns in recent years regarding the expansion of filarial nematodes in the northern areas, from the endemic areas of Europe (southern, central and eastern) are reinforced by the multitude of studies conducted on the prevalence of heartworm disease cases in domestic and wild animals (Sassnau et al., 2014).

Heartworm disease produced by the species *Dirofilaria immitis* is considered a fatal pathology, due to its pulmonary and cardiac location (Genchi et al., 2007; Ciucă et al., 2020a). Clinical manifestations occur due to the large number of adult nematodes, which causes antigenic reactions and mechanical irritation, clinically expressed by a chronic pulmonary hypertension (Beugnet et al., 2018; Burton et al., 2020). In the literature, the clinical stage of cardiopulmonary dirofilariosis is represented as follows: 1) asymptomatic or with very mild manifestations, such as apathy, dyspnea following physical effort and cough; 2) anemia, dyspnea, progressive weakness, chronic cough, vomiting; 3) syncope, increased heart and respiratory rate (Sonneberger et al., 2020).

The second species, *Dirofilaria repenes* causes an asymptomatic and non-pathogenic infection in the subcutaneous tissue in pets, but in humans this is considered the main species involved in the production of the disease (Otranto et al., 2013; Capelli et al., 2018). The life cycle of these two species consists of five larval stages that develop in both hosts, and of the adult forms which have different locations depending on the species (*D. immitis* – right heart and pulmonary arteries; *D. repenes* – subcutaneous tissue) (Genchi et al., 2009).

The female mosquitoes become infected with microfilariae when feeding on the blood of infected definitive hosts, after which they transform into stage 3 larvae. These are considered the infective forms for the subsequent hosts of the mosquitoes (Hess et al., 2023).

The definitive hosts are represented by more than 30 species of domestic (dogs, cats) and wild animals (foxes, ferrets, coyotes, wolves, badgers, etc.) (Adebayo et al., 2020; Ionică et al., 2022).

Recent studies carried out in Romania have highlighted the presence of both species of heartworm in dogs, cats or wild animals (Hamel et al., 2012; Girdan et al., 2015; Ionică et al., 2015; Ciucă et al., 2016a; Ciucă et al. al., 2016b; Ionică et

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al., 2016; Ionică et al., 2017a; Ionică et al., 2017b; Mircean et al., 2017; Ciucă et al., 2020b; Cîmpan et al., 2022; Ionică et al., 2022).

The *Wolbachia* endosymbiont is found in almost all filaria, having the role of helping the nematodes with their long survival, development and fertility (Ciucă et al., 2018).

The aim of this study was to obtain a current prevalence of canine heartworm disease in Galați county, Romania, comparing current data with studies carried out in previous years, from the same area.

MATERIAL AND METHOD

This study included 40 dogs from a shelter in Galați, Romania. Data on age, gender, hair coat length and medical history were recorded for further processing. Their age range was between 1 and 16 years, with varied hair coat length, and according to medical records, none of the dogs received antiparasitic treatment. Based on the age, the dogs were grouped as follows: young (1-3 years); adults (4-7 years); seniors (8-12 years); and geriatrics (13-18 years). Therefore, 40 blood samples were collected in tubes with anticoagulant (ethylenediaminetetraacetic acid- EDTA) and 40 samples in tubes without anticoagulant. The samples were transported at temperatures of 4ºC, and analyzed in the Clinical Laboratory of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine in Iasi.

All samples used in this study were collected under standard protocols for management of dogs participating in animal shelters and by the Institutional Animal Care and Use committees of the Faculty of Veterinary Medicine Iasi (Romania).

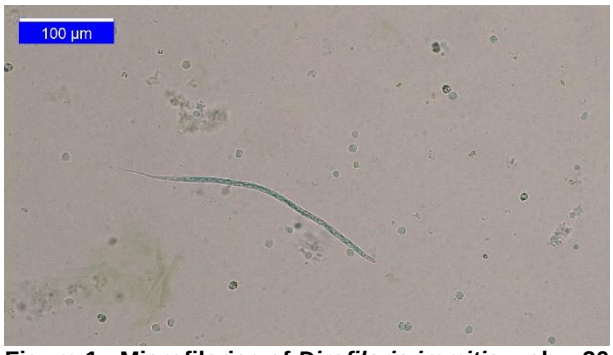

Figure 1 - Microfilariae of *Dirofilaria immitis* – ob. x20

Figure 2 - Microfilariae of *Dirofilaria repens* – ob. x20

Laboratory techniques

Blood samples were tested for the identification of circulating microfilariae using the modified Knott method, as well as the detection of antigens, using the PetCheck and SNAP 4DX Plus serological tests. For the serological tests, canine blood samples were collected in anticoagulant-free tubes, which were after centrifuged, and the serum obtained was transferred to Eppendorf tubes and labeled until use.

Modified Knott test

The technique consists in adding to a 15 ml tube, 1 ml of canine blood sample, which was mixed with 9 ml of 2% formalin. The tube was centrifuged for 5 minutes at 1500 rpm and the supernatant was removed, adding 1% methylene blue to the sediment. After gentle mixing, from the resulting sediment, 1 drop was transferred to a coverslip and examined under an optical microscope (x20-x40) (Gioia et al., 2010; Magnis et al., 2013; Ciucă et al., 2016b). Circulating microfilariae were morphologically identified using specialized

literature (Magnis et al., 2013). the whole sediment was analyzed.

SNAP 4DX Plus immunochromatographic rapid test (Idexx Laboratories)

The first serological test used was the commercial SNAP 4DX Plus kit (Idexx). This qualitative method is used for the detection of circulating *Dirofilaria immitis* antigens, but also for the *Anaplasma platys*, *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Ehrlichia ewingii* and *Borrelia burgdorferi* antibodies detection. Testing was performed according to the manufacturer's instructions. The purpose of using this kit was to perform a more complex screening in order to identify possible co-infections.

PetCheck Heartworm Testing (Idexx Laboratories)

The second serological test used was the PetCheck HW kit (Idexx). This test detects *Dirofilaria immitis* antigens in canine serum or plasma, using the ELISA technique. Testing was performed according to the manufacturer's instructions.

The results of the both serological tests were scored as positive or negative.

RESULTS

Of the 40 dogs, all were homeless (n=40), and regarding the gender ratio, 62.5% were female (25/40) and 37.5% were male (15/40).

The 40 blood and serum samples were tested using the methods described previously. Thus, the modified Knott test showed a total of 11/40 samples positive for microfilariae (27.5%), of which 10/11 samples were positive for *D. immitis* microfilariae (*figure 1*) and 1/11 for *D. repens* microfilariae (*figure 2*). Also, *D. immitis* antigens were detected in 12/40 samples using the PetCheck ELISA (30%), and no samples were positive for SNAP 4DX Plus (*table 1*).

The highest prevalence was detected in the age group 4-7 years (adults), followed equally by the groups 8-12 (senior) years, respectively 13-18 years (geriatrics). In addition, in the gender ratio, 33.4% (25/40) were female and 37.5% (15/40) were male (*table 2*).

The results of the positive samples, tested by the PetCheck HW and modified Knott methods are reported in *Table 3*. Thus, of the 12 samples positive for *D. immitis* Ag. (in Petcheck HW), in 3 of them the microfilariae of both species were identified by the Knott test (*Dirofilaria immitis* and *Dirofilaria repens*), in 5 samples only the microfilariae of the species *D. immitis* were identified, and in 4 samples no microfilariae were identified. Furthemore, from the samples found negative for *D. immitis* Ag., the results from modified Knott method proved that 2 more blood samples were positive for *D. immitis* microfilariae and one for *D. repens* microfilariae.

Table 1 – The results of analyzed samples

Test	Positive samples	
	Total positive/total sampled	**%**
PetCheck HW	12/40	30%
Knott Test	11/40	27.5%
SNAP 4DX Plus	0/40	0%

Table 2 - Comparison of age groups and gender in dogs tested positive for *Dirofilaria* spp. by at least one method

AGE	**No. of dogs tested**	**No. of dogs positive**
1-3 years	8	0 (0%)
4-7 years	14	7 (50%)
8-12 years	10	4 (40%)
13-18 years	8	4 (50%)
Total	**40**	**15 (37.5%)**
GENDER		
Males	15	5 (33.4%)
Females	25	10 (40%)
Total	**40**	**15 (37.5%)**

Table 3 - Comparative results of positive samples

	PetCheck HW	Knott test		Total no. of canine samples
		Dirofilaria immitis	*Dirofilaria repens*	
Sample 8	positive	negative		40
Sample 11	positive	mf	mf	
Sample 12	positive	mf	mf	
Sample 14	positive	mf	mf	
Sample 21	positive	mf	-	
Sample 22	positive	negative		
Sample 24	positive	negative		
Sample 27	positive	mf	-	
Sample 28	negative	mf	-	
Sample 29	negative	mf	-	
Sample 30	positive	mf	-	
Sample 31	positive	mf	-	
Sample 33	positive	negative		
Sample 35	negative	-	mf	
Sample 40	positive	mf	-	

Note: mf - microfilariae

DISCUSSIONS

In this study, are presented the results obtained from the examination of blood samples of 40 dogs randomly chosen from Galați using parasitological and serological methods, in order to establish the prevalence of canine heartworm in the county.

Following studies carried out in previous years, it was found that the highest prevalence of canine heartworm disease is found on the South-East of Romania (Poliana et al., 2013; Ionică et al., 2015; Ciuca et al., 2016; Cimpan et al., 2022). Galați County is located in the South-East of Romania, near the Danube river and other important rivers, thus generating an optimal habitat for the development of vectors. In a research study conducted at the Danube Delta Reservation, certain mosquitoes and domestic dogs were found to exhibit a higher prevalence of *Dirofilaria immitis* and *Dirofilaria repens* infections. (Tomazatos et al., 2018). The study carried by Ciucă et al. in 2016 showed a 60% prevalence of heartworm disease in Galati, the highest rate from all the investigated areas from Eastern Romania.

In the literature, there are described several risk factors involved in the persistance of the disease, including dog gender (males are more susceptible than females in contracting the disease), size (larger dogs are more prone than small ones), habitat, definitive host species, as well as age (older dogs are more susceptible than young ones) (Fan et al., 2001). Comparing these factors with the data presented in this study, it is founded that the prevalence of heartworm disease was increased in females (10 positive samples/25 analyzed samples), and adult dogs, aged between 4-7 years.

The analysis of the 40 blood samples by the Knott method revealed that 11/40 samples were positive for microfilariae, of which co-infections with both species (*D.immitis* and *D.repens*) were detected in 3 blood samples. This fact was also reported by Ionică et al. in 2017 (2017a). They considered that, in endemic areas, the risk of co-infections with both species is very high, especially because these parasites are having common vectors for transmission. In addition, 2 samples positive for *D. immitis* microfilariae were negative on serological testing, most likely due to a low number of females (Rojas et al., 2015). *Dirofilaria immitis* microfilariae can persist for over 2 years in the bloodstream, even after the death of the adults (Ionică et al., 2015).

From the total of 40 serum samples analyzed, 4 samples were positive for *Dirofilaria immitis* Ag., but they were negative in the modified Knott test. According to literature in the field, this situation can be encountered in cases of subclinical infection or during the incubation period, as well as if adult sterility has been previously induced by some drugs used (Ionică et al., 2015). Serological testing by antigen identification is frequently used for the detection of asymptomatic (occult) infections, but the disadvantage of this method consists in false positive results, due to cross-reactions with other filariae (Laidoudi et al., 2019). False negative serological results could occur in cases where the number of female nematodes is low (Schnyder and Deplazes, 2012; Ionica et al., 2015).

According to the manufacturer's specifications, the sensitivity and specificity of the SNAP 4DX Plus and PetCheck HW tests, which detect the antigen of the species *Dirofilaria immitis*, are identical, more precisely 98% sensitivity, respectively 100% specificity (IDEXX Laboratories). In the study conducted by Courtney and Zeng (2001), the PetCheck test was considered the most sensitive compared to the other serological tests used in the study. However, in our study 12/40 serum samples were positive in the PetCheck test and negative in the SNAP 4DX Plus test. The authors of other studies in the branch consider that if the rapid test SNAP 4DX Plus for the detection of *D. immitis* Ag. is elected, it is necessary to use a second serological test, to increase the predictive value (Pantchev et al., 2009).

In Europe, the presence of several filariae in the blood of dogs has been reported, and filariae of the genus *Acanthocheilonema* are also frequently reported. Differentiation of microfilariae is carried out by the modified Knott method, analyzing the morphological characteristics as well as their sizes. However, sometimes there can be uncertainties regarding the species of microfilariae, and the use of other diagnostic methods (serological or molecular) is also recommended (Little et al., 2018; Laidoudi et al., 2020).

Due to the subclinical form of heartworm disease produced by the species *D. repens*, dogs infected with this species remain undiagnosed, indirectly participating in the maintenance of the pathogen (Capelli et al., 2018).

The accuracy of diagnostic methods is extremely important for veterinarians, thus being able to determine the most effective plan for each patient (Panarese et al., 2020). The main reservoir of *Dirofilaria immitis* and *Dirofilaria repens* is considered to be the dog, although heartworm disease can still be prevented by using macrocyclic lactones (Sassnau et al., 2014).

Global warming is thought to be the main cause of the spread of heartworm disease, closely followed by the transport of untested dogs from endemic to non-endemic areas (Genchi et Kramer, 2020).

Dogs in shelters should undergo periodic prophylactic treatments due to prolonged exposure to tick and mosquito infestations, due to the fact that most animal shelters are built on the outskirts of towns. Animal welfare organizations should also be regularly trained on the treatment and testing of dogs for vector-borne diseases, especially those to be imported from endemic areas.

CONCLUSIONS

The high prevalence of heartworm disease in the South-East of Romania should raise an alarm, making the monitoring and prophylactic treatment of dogs mandatory. Also, heartworm disease should be included in the differential diagnosis of veterinarian clinicians, which need to properly inform the owners about the risks associated with canine dirofilariosis. Taking into consideration the evolution of dirofilariosis cases in dog shelters from Romania in the last years, it should be possible that Global Warming influences the increase in the case numbers of this disease, in accordance with abundance of etiological agents.

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