

Short Communication

Filarial infections in dogs in Cyprus, an apparently heartworm free island

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ABSTRACT

The current study investigated for the first time the occurrence of filarial infections in dogs in Cyprus. Blood samples of dogs from five districts of Cyprus (Lefkosia, Lemessos, Larnaka, Pafos and Ammochostos) were examined by the modified Knott's method and by serology, and the morphological classification of microfilariae was confirmed by molecular methods. A total of 200 dogs, 153 living in shelters and 47 owned dogs, were included in the study. *Acanthocheilonema reconditum* microfilariae were found in 9 (4.5%) samples and one (0.5%) sample was seropositive for *D. immitis*. No statistical significance was observed between microfilaraemic samples and the evaluated variables apart from the district ($p = .005$). The present study showed that dogs in Cyprus can be infected with blood circulating microfilariae and for the first time *A. reconditum* was reported as autochthonous infection in dogs in the country. No microfilariae of *Dirofilaria* spp. were detected. However, veterinarians should remain vigilant regarding *Dirofilaria* infections and should consider preventive protection to the animals, at least in case of travel in enzootic areas.

1. Introduction

Filarial nematodes (Superfamily Filarioidea) are vector-transmitted parasites affecting vertebrates. Among them, the mosquito transmitted genus *Dirofilaria* includes two species with great veterinary and medical importance: *Dirofilaria immitis*, the agent of heartworm disease and *Dirofilaria repens* that causes subcutaneous nodules in dogs, cats and other carnivores (Bowman and Atkins, 2009), both able to affect also humans (Genchi et al., 2011). Apart from these nematodes, two more filarial species i.e., *Acanthocheilonema reconditum* and *Dipetalonema* (Syn. *Acanthocheilonema*) *dracunculooides* release microfilariae into the bloodstream but involve other blood sucking arthropods (fleas and lice for *A. reconditum* and ticks for *D. dracunculooides*) as vectors. These latter species live in the subcutaneous connective tissue (*A. reconditum*) or body cavity (both species) of dogs and other wild canids and are considered less pathogenic than *Dirofilaria* spp. (Otranto et al., 2013).

South Europe is considered an enzootic area for *Dirofilaria* spp. and particularly Spain (Montoya-Alonso et al., 2017), Portugal (Alho et al., 2018), France (Pantchev et al., 2009), Italy (Genchi et al., 2011) and Greece (Diakou et al., 2016), where the parasites' distribution covers a

considerable area of the countries. There are reports of *D. immitis* infection also in other Mediterranean countries (Rjeibi et al., 2016; Khataat et al., 2017; Tahir et al., 2017) and Balkans (Rapti and Rehbein, 2010; Ionică et al., 2015; Pantchev et al., 2015; Mrljak et al., 2017), while the parasite progressively spreads over Europe (Morchón et al., 2012), establishing new enzootic areas in formerly heartworm-free regions (Traversa et al., 2010). Simultaneously, *D. repens* shows a more aggressive spreading pattern with the northernmost areas reporting autochthonous cases being Northern Russia, Finland and Estonia (Jokelainen et al., 2016; Pietikäinen et al., 2017).

Despite the accumulating information from many parts of Europe, there are no available published data about the epizootiology and prevalence of dirofilariosis, and in particular heartworm disease, in Cyprus, the islandic state in the eastern Mediterranean Sea. Among local veterinarians, Cyprus is considered a heartworm free state; however, recently there were a few sporadic anecdotal, not confirmed reports of suspected cases. In this context and considering the veterinary and medical importance of *Dirofilaria* infections, the aim of the present study was to investigate for the first time the prevalence of *D. immitis* and other filarial nematodes with blood circulating microfilariae in

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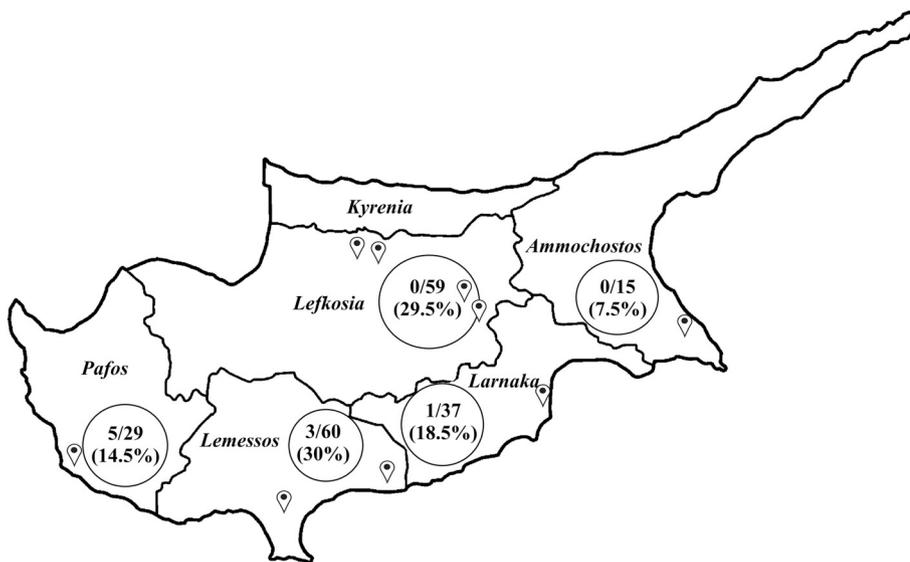


Fig. 1. Map of Cyprus showing the distribution of dog shelters (📍) involved in the study. In the circle, the microfilaraemic samples out of the total population per District is recorded.

dogs in Cyprus, and to confirm or reject the anecdotal information that this area is heartworm free.

2. Materials and methods

2.1. Study areas

In order to create a basic and representative profile of the potential prevalence of filarial infections in different areas of Cyprus, blood samples from 5 different districts across the country were collected, i.e. Lefkosia, Lemesos, Larnaka, Pafos and Ammochostos (Fig. 1). Samples were collected from dogs living in urban, but mainly in semi-urban and rural areas. The study areas covered the regions where anecdotal and unverified filarial cases had previously been reported.

2.2. Sampling population

Two hundred blood samples were collected from respective number of dogs. These dogs were sheltered (153/200), residing in outdoor shelters, as well as owned (47/200). The animals included in the study were at least 12 months old, had overcome the expected prepatent period since the last transmission season for *Dirofilaria* spp., and were not receiving any/regularly preventative chemoprophylaxis for heartworm disease (inclusion criteria). More precisely, in most animals ectoparasiticide and endoparasiticide chemoprophylaxis was applied on a monthly and three-monthly basis, respectively. Regarding macrocyclic lactones as substances that may prevent *Dirofilaria* spp. infections, 43 dogs were receiving milbemycin oxime and 14 ivermectin once every 3 months, i.e. not regular preventative chemoprophylaxis for heartworm disease, according to the guidelines of the American Heartworm Society (American Heartworm Society, 2018) and the European Society of Dirofilariosis and Angiostrongylosis (ESDA, 2017). Some previous data about the regional distribution of dogs' population in Cyprus reported approximately 31, 23, 16, 10 and 4 thousand dogs in Lefkosia, Lemesos, Larnaka, Pafos and Ammochostos respectively, as retrieved from the information provided by the Veterinary Services, of the Republic of Cyprus (Ministry of Agriculture, Rural Development and Environment). This information is in agreement with the regional odds of human's population according to the 14th Census of Population of Cyprus conducted by the Statistical Service of Cyprus in October 2011. Thus, the general rounded ratio of 4:4:2:2:1 for the above regions was used in the present study as sampling regional odds.

A questionnaire was designed including information about sex, age,

weight, breed, hair length, travelling abroad history, recent anti-parasitic treatment, other known infectious diseases, and lifestyle (outdoor living, usage) of the dog and the environment (type of vegetation and existence of water collection of any type). The sample size of the animals ensures the rule of the existence of at least 10 observations per variable ($n = 200$ for the above 12 factors) and at least 5 observations per level of each factor (Zar, 2010).

2.3. Sample collection and examination

All animals were examined with the consent of either their owner or the administrator of the shelter, while for two public shelters written consent was given from the Director of Cyprus Veterinary Services. From each dog, 4 ml of blood were collected from a peripheral vein (jugular or cephalic), 2 ml in EDTA tube and 2 ml in plain tube. The samples were processed within 3 h from collection as follows: 1 ml from EDTA tube was transfused to Eppendorf tube and then preserved in freezer (-20°C) for molecular analysis. The other 1 ml from EDTA tube was prepared for the Knott's method, i.e. mixed with 9 ml of 2% formalin and after centrifugation, the sediment was transfused in Eppendorf tube and kept refrigerated (4°C) until further processed. The 2 ml blood in plain tube were centrifuged and the extracted serum was collected in Eppendorf tube and kept frozen (-20°C) for serological examination.

All tubes were sent within a period of maximum 2 months to the Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki. All the samples were examined by two methods: the modified Knott's test for the detection and identification of microfilariae and the serological test DiroCheck® (SYNBIOTICS, San Diego). The microfilariae retrieved by the Knott's test were identified under light microscopy at $100\times$ and $400\times$ magnifications on the basis of their morphometric (i.e. length and width) and morphological (i.e. anterior and posterior extremities) features, according to the international literature (McCall et al., 2008; Magnis et al., 2013).

2.4. Molecular analyses

The whole blood aliquot of the microfilariae positive samples was sent to IDEXX Laboratories, Germany, for molecular confirmation of the morphological identification of microfilariae. The negative to microfilariae blood samples were not subjected to molecular analysis, as it has been shown that PCR requires sufficient DNA to be present in order to detect it, that is only available when microfilariae are present in the

blood (Simsek et al., 2008; Pantchev et al., 2011; Simón et al. 2012).

For the molecular identification of microfilariae total nucleic acid was extracted from whole blood using the QIAamp DNA Blood BioRobot MDx kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Screening reaction was performed as multiplex PCR and 4 species specific real-time PCR assays were performed using the LightCycler 480 (Roche, Mannheim, Germany) with proprietary forward and reverse primers and hydrolysis probes. The target gene for *D. repens* used was the cytochrome oxidase subunit I (COI; accession number AJ271614.1), for *D. immitis* the ITS-1 (AB973231), and for *A. reconditum* and *A. dracunculoides* the ITS-2 (AF217801 and DQ018785, respectively). All four real-time PCR assays have been shown to have a reproducible average analytical sensitivity of 10 DNA molecules per reaction, and a comparable performance to the assays applied by Pantchev et al. (2011). The analytical specificity of the real-time PCR assays was assessed with DNA samples of 40 isolates (10 of each species *D. immitis*, *D. repens*, *A. reconditum* and *A. dracunculoides*) derived from routine diagnostics and characterized through sequencing of the 5.8S-ITS2-28S region (DIDR-F1 and DIDR-R1 primers from Rishniw et al., 2006) as described by Pantchev et al. (2011) and was found to be 100%.

2.5. Statistical analysis

The relation of the filarial infection with various animal's characteristics, vegetation, life style and treatment (effect factors) was statistically analysed using the Chi-square independence test or Fisher's exact test (according to the number of animals in each cell of contingency table); a value of $p < .05$ was considered significant. Furthermore, the filarial infection was also tested for possible combined relations with the above factors using a general linear model GLM test (Zar, 2010). The software used for the statistical analysis was IBM SPSS Statistics V.23.

3. Results

According to the questionnaire data analysis, forty-seven of the examined dogs were owned, living exclusively indoors (8/47) or outdoors, either constantly (26/47) or intermittently (13/47), while 153 were dogs living in shelters. The results of the recorded data about the animals are shown in Table 1.

Microfilariae were detected by the Knott's method in 9 (C.I. $4.5\% \pm 3.8\%$) of the animals and were all morphologically identified as *A. reconditum*. All positive samples were from dogs kept in shelters. The prevalence of infection in the different districts was 17.2% in Pafos ($n = 5$), 5% in Lemesos ($n = 3$), 2.7% in Larnaka ($n = 1$), and 0% both in Lefkosia ($n = 0$) and Ammochostos ($n = 0$) (Fig. 1). In all cases, the morphological identification was molecularly confirmed.

Furthermore, in the serological examination, 1 (0.5%) sample was positive for *D. immitis* antigen. The same sample was also positive for *A. reconditum* microfilariae. Unfortunately, it was not possible to confirm this evidence of heartworm infection; however, the animal was not showing any signs of heartworm disease at the time of sampling.

Regarding the 9 microfilaraemic dogs, 6 (66.7%) were males and 3 (33.3%) females, 4 (44.4%) were ≤ 3 years old and 5 (55.6%) > 3 years, 2 (22.2%) weighed ≤ 15 kg and 7 (77.8%) > 15 kg. Also, 8 (88.9%) dogs received ectoparasitocidal and 3-month endoparasitocidal chemoprophylaxis, though 3 (33.3%) of the latter did not include macrocyclic lactones, whereas 1 (11.1%) dog did not receive any parasitocidal treatment (Table 1). The blood samples from 5 (55.6%) dogs were collected between 15:00–17:59 h, from 3 (33.3%) dogs between 12:00–14:59 h and 1 (11.1%) dog between 9:00–11:59 h.

According the results of the statistical analysis the only factor associated with a positive Knott's test (*A. reconditum* infection) was the district where the animal was living ($p < .05$), with the district of Pafos having the highest prevalence of positive animals (17.2% of the animals

examined in this particular district were positive) (Table 1). Furthermore, the GLM analysis showed no effect of the filarial infection with the factors ($p > .05$). However, the small number of affected animals (only 9) cannot provide reliable diagnostics on the factors affecting the filarial infection (lack of models fit) (Warton et al., 2016). Indeed, in this study the number of affected animals (replicates) is rather small to estimate possible differences between the levels of a factor.

4. Discussion

This study aimed to investigate the presence and prevalence of filarial parasitic nematodes with blood circulating microfilariae in dogs in Cyprus, as to date no studies have been conducted to confirm or rule out the estimation that this country is heartworm free, despite the temperate climate and the presence of suitable vectors (Violaris et al., 2009).

Interestingly, two of Cyprus neighbouring countries, i.e. Greece and Turkey, are enzootic for both *Dirofilaria* species (Diakou et al., 2016; Guven et al., 2017). However, a recent prevalence study in Turkey revealed that a province on the south coast had the lowest prevalence among the regions in which *Dirofilaria* spp. were detected, despite the warmer climate (Simsek et al., 2008). Similarly, in Crete, the biggest and southernmost island of Greece, also neighbouring to the enzootic countries of South-Eastern Europe, no *Dirofilaria*-positive dog was recorded (Diakou et al., 2016).

Cyprus has common geographical characteristics with Crete as both islands are situated in eastern Mediterranean Sea, surrounded by enzootic regions. Temperatures in Cyprus would facilitate short extrinsic development, and thus high number (> 10) of transmission cycles of the mosquito transmitted filariae, according to the Linear Kriging interpolation predicting the distribution of these parasites in relation to climate (Genchi et al., 2009). However, as it is evidenced, the sole presence of the intermediate hosts in geographical regions with temperate climates is not enough to promote the enzootic character and outspread of mosquito transmitted filariae (Morchón et al., 2012). The particularities of various factors involved in the transmission and settlement of *Dirofilaria* spp. in each area, as e.g. the availability of reservoir hosts and the abundance and stability of vectors, may finally determine its epizootiology (Diakou et al., 2014).

One of the examined samples was positive for *D. immitis* antigen, but the infection could not be confirmed, as the dog was no longer resident of the shelter soon after sampling. In some cases, false-positive results in the detection of *D. immitis* antigen have been reported due to cross-reactions in the presence of other nematodes, i.e. *Angiostrogylus vasorum* and *Spirocerca lupi* (Schnyder and Deplazes, 2012; Aroch et al., 2015). However, dogs infected with *A. reconditum* did not cross react to a heartworm antigen test (Weil et al., 1985). A possible explanation of the latter result, that was also confirmed in a more recent study both for *A. reconditum* and *D. repens* (Pantchev et al., 2011), could be that these filariae are mainly located in subcutaneous tissue and thus no, or minimum antigen passes into the blood circulation. A cross reactivity in antigen detection between *D. immitis* and *Acanthocheilonema ordendhali* was reported by Krucik et al. (2016) in sea lions, but these results are not directly comparable to the findings of the present study, because they are associated with a different parasite and host species. Altogether, a false positive result of the test applied in the present study cannot be fully excluded, especially as there is no available information about the presence and prevalence of *Angiostrogylus vasorum* or *Spirocerca lupi* in dogs in Cyprus.

The results of the present study seem to confirm the existent impression that Cyprus is practically a heartworm free country. Although negative results in each of the three different examinations applied to the samples (Knott's test, serology and PCR) could give a false negative result, the combination of all these methods is expected to significantly diminish the chance of missing a positive sample and thus provides high accuracy of the combined result. For example, it is well known that

Table 1Recorded data of Cyprus dogs examined for filarial infections. The end-right column shows the *p* value of each factor in relation to the positive Knott's test.

Factor		Results (%)	Microfilaraemic dogs (%)	<i>p</i> value		
Sex	Male	107 (53.5)	6 (66.7%)	.42		
	Female	93 (46.5)	3 (33.3%)			
Age (years)	1 to 3	120 (60)	4 (44.4%)	.33		
	> 3	80 (40)	5 (55.6%)			
Body weight (kg)	≤ 15	58 (29)	2 (22.2%)	.647		
	> 15	142 (71)	7 (77.8%)			
Ownership status	Stray (sheltered)	153 (76.5)	9 (100%)	.089		
	Owned	47 (23.5)	0			
Hair type	Short	137 (68.5)	8 (88.9%)	.386		
	Semi-long	58 (25.5)	1 (11.1%)			
	Long	12 (6)	0			
Lifestyle	Indoors	8 (4)	0	.394		
	Outdoors	25 (12.5)	0			
District	Shelter	167 (83.5)	9 (100%)	.005		
	Lefkosa	59 (29.5)	0			
	Lemessos	60 (30)	3 (33.3%)			
	Larnaka	37 (18.5)	1 (11.1%)			
	Pafos	29 (14.5)	5 (55.6%)			
Parasitocidal chemoprophylaxis	Ammonochoistos	Endo-	15 (7.5)	0	.27	
		N	29 (14.5)	1 (11.1%)		
		Y (ML)	56 (28)	5 (55.6%)		
		Y (no ML)	97 (48.5)	3 (33.3%)		
	n/a	18 (9)	0			
	Ecto-	N	33 (16.5)	1 (11.1%)		.53
		Y	149 (74.5)	8 (88.9%)		
n/a		18 (9)	0			
Trip abroad	Y	4 (2)	0	.386		
	N	46 (23)	0			
	n/a	150 (75)	9 (100%)			

Y = yes; N = no; n/a = not available, ML = macrocyclic lactones.

occult (amicrofilaraemic) *D. immitis* infections are common (McCall et al., 2008) and in these cases the Knott's test cannot detect the infection, and also that in some samples false negative antigen tests is recorded due to blocked antigens (Little et al., 2018). However, the combination of both tests is the best approach to diagnosis, as recommended both by the American Heartworm Society (American Heartworm Society, 2018) and by the European Society of Dirofilariosis and Angiostrongylosis (European Society of Dirofilariosis and Angiostrongylosis (ESDA), 2017). In addition, the identification of microfilariae found in the Knott's test was confirmed by molecular tools, excluding any chance of morphological misdiagnosis.

In our study, microfilariae in nine samples were confirmed to be *A. reconditum*. Although the morphometric analyses of filariae enables diagnosis between *D. immitis*, *D. repens* and *Acanthocheilonema* spp., due to overlapping characteristics of *A. reconditum* and *A. dracunculoides*, biochemical or molecular techniques are required to distinguish these two species (Magnis et al., 2013). Furthermore, it has been shown that acid phosphatase staining has limitations for species diagnosis and molecular methods are more reliable (Pantchev et al., 2011). The distribution of *A. reconditum* is herein investigated for the first time in Cyprus and was found in 4.5% of the dogs.

Acanthocheilonema reconditum has a global distribution and, in many geographical areas of the world is the sole or the most prevalent filarioid species in dogs (Brianti et al., 2012). In our study, all the microfilaraemic samples were collected from the south coastal regions of the island (3 out of 5 districts, Table 1). Differently from other filarioids, *A. reconditum* is transmitted with fleas (*Ctenocephalides canis*, *Ctenocephalides felis*, *Pulex irritans*, *Pulex simulans*, *Echidnophaga gallinae*) (Newton and Wright, 1956; Nelson, 1962; Bain and Beaucournu, 1974; Brianti et al., 2012) or lice (*Heterodoxus spiniger*, *Linognathus setosus*) (Nelson, 1962; Pennigton and Phelps 1969). The prevalence of infection in fleas was 5% in a survey conducted in a shelter in Sicily, Italy (Brianti et al., 2012). There is evidence that the transmission of this nematode requires proximity between the infected and non-infected dogs (Brianti et al., 2012). This is probably due to the fact that adult fleas and lice do

not move in long distances away from their host, and thus vector transmission between and among individuals is more likely when animals are co-housed (Rust, 1994). This ecological aspect makes the epidemiology of *A. reconditum* very different from that of other filarioids affecting dogs such as *D. immitis* and *D. repens*, since mosquitoes can spread the infestation to long distances (McCall et al., 2008)

The parasites were detected in canine blood samples collected between 11:00 and 17:00 h in all cases. Despite contrasting results about periodicity of microfilariae of *A. reconditum* in blood in the past (Newton and Wright, 1956; Pennigton and Phelps, 1969; Korkejian and Edeson, 1978; Bobade et al., 1981), a more recent study indicated that there is no defined periodicity of these microfilariae in dogs (Brianti et al., 2012), which is also in line with the absence of defined circadian rhythm of blood feedings for its intermediate hosts (Koehler et al., 1989).

No microfilaraemic dog displayed clinical signs suggestive of infestation by *A. reconditum* in our study, which is indeed considered one of the less pathogenic filarioids of dogs (Bobade et al., 1981). Accordingly, a study in dogs experimentally infected with *A. reconditum* showed that this nematode is not a parasite of clinical significance even though some of the experimentally infected dogs presented significantly greater counts of leukocytes and eosinophils than control dogs (Lindemann and McCall, 1983). However, further studies should be conducted to better understand the real pathogenic role of this filarioid in dogs, and its possible indirect immunological effects, as proposed for *A. dracunculoides* (Albrechtová et al., 2011).

Regardless the clinical insignificance of *A. reconditum* infesting dogs, microfilaricide treatment should be always advocated to limit the reservoir role of infected hosts. Though reports on microfilaricide treatment for minor species are scant, evidence suggests macrocyclic lactones are effective against first stage larvae (Lindemann and McCall, 1983). Pantchev et al. (2011) found that selamectin was not able to clear microfilariae in two dogs with *A. reconditum* and one dog with *A. dracunculoides*, but moxidectin was successful in treating *A. reconditum* on one dog. Furthermore, the vector control with appropriate

ectoparasitocides must not be overlooked. Interestingly, in our study, 8 out of 9 dogs had received ecto- and *endo*-parasiticidal chemoprophylaxis. More specifically, regarding deworming, 5 of these dogs received milbemycin oxime once every three months; however, the facts that all microfilaraemic dogs were in shelters, i.e. previously untreated for parasites, and afterwards only intermittently treated with macrocyclic lactones (3-monthly), potentially justifies our findings.

5. Conclusion

In conclusion, to the author's best knowledge this is the first epizootiological study for the investigation of filarial infections in Cyprus. Heartworm infection was not confirmed in any case and no infections with *D. repens* were identified. Nevertheless, microfilariae of *A. reconditum* were found in autochthonous dogs and therefore Cyprus can now be considered enzootic for the flea-transmitted filaria. More prevalence studies in the future are needed in order to investigate the presence of the *Dirofilaria* species, as are those with greater veterinary and zoonotic importance. Practitioners should remain vigilant regarding these infections, and consider preventive protection to the animals, at least in case of travel in enzootic areas.

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Declaration of competing interests

The authors declare no conflict of interest.

Ethical statement

No experiments on animals have been performed in the study. Dog blood samples used for the examinations in this survey have been collected with the consensus of the owners or of the authorised person for sheltered animals.

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