Epidemiological Survey of Dirofilaria Spp. and Acanthocheilonema Spp. in Dogs from the Republic of Moldova

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Abstract

Background: During the last decades, the spread of the infection with filarial nematodes *Dirofilaria* spp. in dog's population in various European countries has been dynamic and rapid. The increased interest of the scientific community as well as the availability of new diagnosis tools, allowed a better knowledge on the biology, morphology and epidemiology of different species of filarial worms. However, in some countries, data are still scarce on this topic. To address some knowledge gaps, we assessed the epidemiological status of different canine filariasis in the Republic of Moldova.

Methods: A total of 120 blood samples were collected between June 2018 and July 2019 from dogs originating from Cahul and Chișinău cities. The samples were microscopically examined and multiplex PCR was performed to evaluate filarioid species diversity.

Results: Microscopic examination revealed that 12 dogs (10%) were positive for circulating microfilariae. Molecular test showed that 1 dog was positive for *Acanthocheilonema reconditum* (0.83%), 1 for *D. immitis* (0.83%), 6 for *D. repens* (5%), and 4 (3.33%) harboured a coinfection with *D. immitis* and *D. repens*.

Conclusions: This study is the first epidemiological survey of filaroid worms in dogs from the Republic of Moldova and the first to show the presence of *D. repens* and *A. reconditum* in this species.

1. Introduction

Filaroids (superfamily Filaroidea) are vector-borne nematodes that pose a risk for domestic and wild animals, and for the human health (1). During the last decades, numerous filarial species have been recognised and underwent morphological and molecular characterization (2–4). Moreover, evolution and availability of new diagnosis tools allowed an increasing knowledge on the epidemiology and eco-biology of many filarial species (1). While some species are intensively studied and the awareness of filarioid infections in various mammals is high (e.g. *Dirofilaria immitis, D. repens*), other filarial species are rather neglected (e.g. *Cercopithifilaria bainae*, *C. grassii*) or less known (e.g. *Acanthocheilonema reconditum*, *A. dracunculoides*, *Onchocerca lupi*) (4). Despite the difference in available data on various filarial species, it is undeniable that this group of parasitic nematodes has gained the interest of the scientific community.

Dirofilariases are one of the most studied and the best-known parasitic diseases. They are caused by mosquito-borne nematodes, of which *D. immitis* and *D. repens* are the most relevant (4, 5) due to their high pathogenicity, negative impact that they may have on the public health, and their wide distribution and endemicity (5). Both *D. immitis* and *D. repens* can be transmitted by several genera of culicids (*Anopheles, Aedes, Ochlerotatus, Culex, Culiseta* and *Coquillettidia*), which are recognised as vectors (6). *D. immitis* causes a severe cardiopulmonary condition in dogs and other domestic and wild carnivores (5). Although humans are considered as accidental hosts, infections occur occasionally and can cause pulmonary life-threatening conditions and, in some instances, even ocular or subcutaneous diseases (7–9).

D. repens is the agent of subcutaneous dirofilariasis in animals and usually has a mild or unrecognised clinical form. In humans, this species is the main agent of dirofilariasis and most frequently causes ocular and subcutaneous disease. Other localisations (e.g. pulmonary, oral cavity, eyelid) have also been reported in the literature (9). In Europe, it is recognised as an emergent pathogen. Despite its zoonotic and veterinary importance, and its continuously expanding distribution area, *D. repens* seems to be a less interesting topic for the scientific community as compared to *D. immitis.* For both *Dirofilaria* species, Microfilariaemic dogs represent the main reservoir for animal and human infections (10). Another species of filaroid parasitizing in dogs, which may have a zoonotic impact, is *Acanthocheilonema reconditum* (11). Even if this filarial worm species is the most widely spread and has a global distribution, it is one of the less pathogenic filaroid in dogs (4, 12). The life cycle of *A. reconditum*, unlike any other filaroids, depends on several species of fleas (*Ctenocephalides canis, C. felis, Pulex irritans, P. simulans*, and *Echidnophaga gallinacea*) or lice (*Linognathus setosus* and *Heterodoxus spiniger*), which serve as vectors and intermediate hosts (4).

D. immitis, D. repens and *A. reconditum* have the highest veterinary importance (13), posing various difficulties for their diagnosis and threatening the human health.

Even though information on filarial pathogens exponentially increased, there are still some gaps in knowledge of their epidemiology and biology.

Two recent epidemiological studies suggested the presence and the circulation of dirofilariasis in humans (14) and in arthropod vectors (15) in the Republic of Moldova, but no correlation between the prevalence and geographical distribution of these nematodes in the canine and human population are possible due to the lack of epidemiological studies in dogs. To address this need, our study assessed the prevalence and the risk factors of the infections with canine filariasis in dog populations from the Republic of Moldova.

2. Materials And Methods

2.1. Sampling

A total of 120 dogs were sampled between June 2018 and July 2019. The dogs originated from public dog shelters located in the South and Central part of the country: Cahul (n = 42) and Chișinău (n = 48), respectively, and from some local clinics from Chișinău (n = 30). Informed consent was obtained from the owner or manager of the shelter before dogs' inclusion in the study.

Blood samples were collected from the cephalic vein of each dog (2 ml), in a labelled tube with anticoagulant (EDTA). Location, sex, age, breed, origin, and travel history were recorded for each dog, to assess the risk factors for *Dirofilaria* spp. and *Acanthocheilonema reconditum* infections.

2.2. Microscopical examination

From each dog, 1 ml of blood was processed by modified Knott's test following the standard procedure (16). The sediments were examined using an Olympus BX61 microscope. If present, the microfilariae were identified based on morphology (16). Photographs and measurements for morphological identification were taken using a DP72 camera and Cell^F software (Olympus Corporation, Tokyo, Japan).

2.3. Molecular analysis

Genomic DNA was extracted from 200 µL of whole blood using a commercial kit (Isolate II Genomic DNA Kit, Bioline, London, UK) according to the manufacturer's instructions. Multiplex PCRs amplifying partial regions of the *cytochrome c oxidase* subunit 1 (*cox*1) gene of different sizes of 3 filarioid species (*D. immitis*, 169 bp; *D. repens*, 479 bp; and *A. reconditum*, 589 bp) were performed using species-specific forward primers and the reverse primer NTR, as described in literature (17). The PCR products were visualized by electrophoresis in a 2% agarose gel stained with RedSafe™ 20000x Nucleic Acid Staining Solution (Chembio, Hertfordshire, UK), and their molecular weight was assessed by comparison to a molecular marker (HyperLadder™ 100 bp, Bioline, London, UK). All corresponding bands were excised from the gel and purified using a commercially available kit (Isolate II PCR and Gel Kit, Bioline, London, UK). The purified products were sequenced using an external service (performed by Macrogen Europe B.V., Amsterdam, The Netherlands). The attained sequences were compared to those available in GenBank™ data base by means of Basic Local Alignment Search Tool (BLAST) analysis.

2.4. Statistical analysis

Data analysis was performed using EpiInfo 7 software (CDC, USA). The frequency of infection, prevalence with 95 % confidence intervals (CIs) were tabulated, and the risk factors (locations, sex, age, breed and origin of the animals) were assessed using chi square testing. The differences were considered significant if *P* value was lower than 0.05.

3. Results

Microscopic examination revealed that 12 dogs (10%; 95% CI: 5.27-16.82) were positive for circulating microfilariae. There were no significant differences among locations, sex, age, breed and origin of the animals (Table 1).

Among the microfilariaemic dogs, 1 was positive for *Acanthochilonema* spp. (0.83%; 95% CI: 0.02-4.56), 1 for *D. immitis* (0.83%; 95% CI: 0.02-4.56), 6 for *D. repens* (5%; 95% CI: 1.86-10.57), and 4 (3.33%; 95% CI: 0.92-8.31) harboured a coinfection with *D. immitis* and *D. repens*. *Acanthocheilonema* spp. infection was identified only in Cahul, while *Dirofilaria* spp. were detected at both locations. Although the relative prevalence tended to be higher for both *Dirofilaria* spp. in Cahul, no difference was statistically significant. No other significant risk factors were identified (Tables 2 and 3).

The molecular analysis confirmed the microscopy outcomes. In the case of *Acanthocheilonema* spp. infection, the sample was PCR positive for *A. reconditum*, while the BLAST analysis revealed a 100% nucleotide similarity to an *A. reconditum* sequence from a dog from Italy (JF461456). For *D. immitis*, all 5 sequences were identical, having a 100% nucleotide similarity to other isolates from Europe and Asia (e.g. LC107816, KF692101, MK250715, KR870344). From the *D. repens* isolates, 2 different sequences were obtained. The first one, identified from 9 dogs, was 100% identical to 3 other European isolates from human cases (KR998257, KX265049) and mosquitoes (MF695085). The second sequence, identified from a dog originating in Cahul, was 100% similar to a different human case isolate (AB973225), from a Japanese woman after having travelled to Europe.

The 4 sequences were deposited in Genbank^{TM} database under the Accession Numbers: MW656248-MW656251.

4. Discussion

The Republic of Moldova is one of the countries were few data is available on the epidemiology of *Dirofilaria* spp. and *A. reconditum*. The lack of diagnostic tools, the misdiagnosis, and the low awareness of medical doctors and veterinarians are considered as the main factors leading to this gap (15). In a molecular study from 2016, of 347 pool of female mosquitos analysed, 109 and 30 tested positive for *D. repens* and *D. repens*, respectively. The geographic distribution of the positive sample sites and temperature analyses allowed the authors to conclude that the entire country has favourable climatic conditions for the transmission of *Dirofilaria* spp. (15). The results of our study confirm this hypothesis. Although both *D. immitis* and *D. repens* have been diagnosed in the human population from the Republic of Moldova, most of the available information is represented by several case reports, where the nematodes were identified based on microscopic examination only. Only 5 cases of human dirofilariasis have been reported until 2016 (15). However, from the provided data, it is not clear whether the cases were autochthonous or imported (15). The only extensive study on the human population from the Republic of Moldova was performed in 2018, when 263 serum samples were screened for exposure to *Dirofilaria* spp. One sample was positive for *D. repens* antigens, 36 were positive for anti-*D. immitis* IgG, while 3 samples reacted for both antigens of *D. immitis* and *D. repens* (14). Only one study reported the presence of *D. immitis* in the local canine populations. A total number of 13 shepherd dogs originating from 2 counties, Ialoveni and Criuleni, located in the Central part of Moldova, were evaluated for the presence of various parasite species by necropsy. Three of the examined dogs were infected with *D. immitis* (18). The identification method of the parasites is not mentioned. It is not clear whether the positive dogs originated from the same county or not. Infection(s) with *D. repens* and/or *A. reconditum* has not been reported so far in the Republic of Moldova. However, in Ukraine that is bordering the Republic of Moldova to the East, North and South, and Romania, the western neighbour state, many infections with these pathogens have been reportedin human and animal populations. In Ukraine, dirofilariasis caused by *D.* repens in dogs was first reported in 1904, while human infection in 1927 (19). Between 1997 and 2013, 1465 cases of human infections with *D. repens* were confirmed. The incidence rate of dirofilaria infection ranged between 0.07–3.71 per 100000 persons in the geographical areas neighbouring the Republic of Moldova. The presence of the pathogen in all the oblasts of Ukraine as well as the high incidence registered in many regions, allowed the authors to conclude that in Ukraine, dirofilariasis due to *D. repens*, is an emergent zoonosis (19). In 2015, Rossi et al. demonstrated the implication of both *D. immitis* and *D. repens* in ocular and subcutaneous pathology in humans from Ukraine (20). In Romania, although sporadic infections with *D. immitis* in dogs were reported since the beginning of the 1900s', more recent studies revealed prevalence values ranging from 23.07–38% by use of various diagnostic methods (21, 22). In 2014, both *D. immitis* and *D. repens* were categorized as endemic species in areas from Southern and South-eastern Romania. In the same study, a first extensive epidemiological overview of the prevalence and distribution of *A. reconditum* was provided the first time in this country (23). Analysing the epidemiological situation in Europe, with emphasis on the two neighbouring countries, Romania and Ukraine, as well as the presence of favourable climatic conditions for the transmission of *Dirofilaria* spp., it is highly probable that the limited reports of these pathogens in the Republic of Moldova is the result of the lack in targeted epidemiological studies.

During the last decades, the infection with *Dirofilaria* spp. in dogs has spread form the traditionally endemic regions from Italy, Spain, France, (24) towards central, eastern and north-eastern European countries such as Germany (25), Austria (26), Czechia (27), Poland (28), Romania (22), etc. Interestingly, more recently, a new trend of the distribution of these pathogens has been observed: in areas of high endemicity from the Western European countries, the prevalence of dirofilariasis has decreased in the last few years. This aspect was attributed to the increased awareness on the diseases and therefore the acceptance and widespread use of the prevention measures (29). Contrary, in non-endemic regions or where the parasites have not been reported (Eastern European countries and not only), recent data has shown the first cases and/or an increase in the prevalence of these diseases. The spreading of the pathogens was likely facilitated by the climate changes, the lack of experience of veterinary practitioners on the diagnosis and treatment of these diseases, the decreased awareness on the diseases of both medical personnel and dog owners, and the high number of stray dogs (29). Austria, a country that reported the presence of autochthonous *D. repens* for the first time in 2012 (30), was announcing in 2018 that the infections with *D. immitis* and *D. repens* tripled their number, and today it is wondering if they are facing a pre-endemic status (31). A similar pattern for associated infections with 2 pathogens was registered in Romania and Ukraine.

Dogs are the main reservoir for the human and other mammals' infection (5). Monitorization of the canine population would be one important step for the prevention programmes and could c decrease the zoonotic risk. The need for more solid information and development of monitoring programmes and epidemiological studies on dirofilariasis and other zoonotic vector-borne pathogens in dogs from the Republic of Moldova has been highlighted before (14, 15). Our study is the first one to provide data that could be a starting point for future more extensive epidemiologic research. More data on the prevalence and geographical distribution in dogs, humans and vectors would allow a better understanding of the circulation of these pathogens in the Republic of Moldova. Our results should raise the awareness of veterinarians and physicians. The circulation of dogs between different countries, together with or heading to their owners is more and more common. In the light of the current study, dogs originating from the Republic of Moldova should be screened for *Dirofilaria* spp. and *Acanthocheilonema* spp..

Nevertheless, for the dogs entering this country, preventive measures should be taken by veterinarians and dog owners.

The role of the stray dogs in the circulation of these filarial worms and the high risk posed for the human health by this category of animals, has been previously demonstrated (32). Although origin was not identified as a risk factor in our study, possibly due to the low sample size, these dogs could act as an important source for other carnivores and human contamination. The responsible authorities and institutions should gather their efforts on decreasing the stray dog population, controlling and applying preventing measures to limit dirofilarial infections spread. To the best of our knowledge, our findings on *A. reconditum* and *D. repens* represent the first report of these pathogens in the canine population from the Republic of Moldova.

5. Conclusions

The current study is the first epidemiological survey of filaroids in dogs from the Republic of Moldova, confirming the presence *D. immitis, D. repens and A. reconditum* in different canine populations. These data extend the knowledge on the geographical distribution of these nematode, emphasizing the need of development of prevention programmes to avoid the negative implications that these diseases could have on animal and public health.

Declarations

Ethics approval and consent to participate: The study was approved by the Ethical committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Cluj, Romania. Informed consent was obtained from the owner or manager of the shelter before dogs' inclusion in the study.

Consent for publication: Not applicable

Availability of data and materials: Some datasets generated, used and analysed during the current study are included in this published article and some data are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no financial and non-financial competing interests.

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Authors' contributions: MOD: Conceptualization, Funding acquisition, Investigation, Writing - original draft, Formal analysis, Methodology, Project administration. **GD:** Investigation, Writing - review & editing. **EV:** Investigation. **SM:** Investigation. **VM:** Methodology, Writing - review & editing. **AMI:** Writing - original draft, Writing review & editing. Formal analysis, Data curation.

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Tables

Table 1: The prevalence of filarioid infection in dogs sampled in the Republic of Moldova

		Frequency	**Prevalence**	**95% CI**	X^2 (*d.f.*=)	*P*
Location	Chișinău	7/78	8.97%	3.68-17.62	0.036 (1)	0.751
	Cahul	5/42	11.90%	3.98-25.63		
Sex	M	2/50	4%	0.49-13.17	2.381 (1)	0.072
	F	10/70	14.29%	7.07-24.71		
Age	<2 years	0/29	0%	0-11.94	5.837 (2)	0.054
	2-4 years	4/44	9.05%	2.53-21.67		
	≥4 years	8/47	17.02%	7.65-30.81		
Breed	Pure breed	1/11	9.09%	0.23-41.28	0 (1)	1
	Mixed breed	11/109	10.09%	5.15-17.34		
Origin	Shelter	11/90	12.22%	6.26-20.82	1.111 (1)	0.290
	Owned	1/30	3.33%	0.08-17.22		
TOTAL	-	12/120	10%	5.27-16.82	-	-

Table 2: The prevalence of *D. repens* infection in dogs sampled in the Republic of Moldova

		Frequency	Prevalence	95% CI	X^2 (*d.f.*=)	*P*
Location	Chișinău	6/78	7.69%	2.88-15.99	0 (1)	1
	Cahul	4/42	9.52%	2.66-22.62		
Sex	M	2/50	4%	0.49-13.17	1.246 (1)	0.19
	F	8/70	11.43%	7.07-24.71		
Age	<2 years	0/29	0%	0-11.94	5.416 (2)	0.066
	2-4 years	3/44	6.82%	1.43-18.66		
	≥4 years	7/47	14.89%	6.20-28.31		
Breed	Pure breed	1/11	9.09	0.23-41.28	0 (1)	1
	Mixed breed	9/109	8.26	3.85-15.10		
Origin	Shelter	9/90	10%	4.68-18.14	0.581 (1)	0.448
	Owned	1/30	3.33%	0.08-17.22		
TOTAL	-	10/120	8.33%	4.07-14.79	-	-

Table 3: The prevalence of *D. immitis* infection in dogs sampled in the Republic of Moldova

		Frequency	Prevalence	95% CI	X^2 (*d.f.*=)	*P*
Location	Chișinău	2/78	2.56%	0.31-8.96	0.516 (1)	0.341
	Cahul	3/42	7.14%	1.50-19.48		
Sex	M	2/50	4%	0.49-13.71	0 (1)	1
	F	3/70	4.29%	0.89-12.02		
Age	<2 years	0/29	0%	0-11.94	3.877 (2)	0.143
	2-4 years	1/44	2.27%	0.06-12.02		
	≥4 years	4/47	8.51%	2.37-20.38		
Breed	Pure breed	0/11	0%	0-29.49	0 (1)	1
	Mixed breed	5/109	4.59%	1.51-10.38		
Origin	Shelter	5/90	5.56%	1.83-12.49	0.626 (1)	0.329
	Owned	0/30	0%	0-11.57		
TOTAL	-	5/120	4.17%	1.37-9.46	-	-

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstract.png