Parasitological screening on canine dirofilariosis, Southern Romania: preliminary data

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Abstract. *Dirofilaria immitis* and *Dirofilaria repens*, mosquito-borne helminthes (Nematoda: Filarioidea), are the causative agents of cardiopulmonary (known also as heartworm disease) and subcutaneous dirofilariosis, respectively. Canids are the predominant definitive hosts; however, other mammalian species, including humans are aberrant hosts. Regarding the aberrant hosts, particular symptomatology can be found. Currently, canine and human dirofilariosis are considered emerging diseases in Europe. However, little is known on the current prevalence and geographical distribution of canine dirofilariosis in Romania. Therefore, the present study aimed to perform a parasitological screening on *Dirofilaria* infection of dogs in Southern Romania. For this, a total of 227 dogs, including shelter (n=100) and owned (n=127) dogs, of 1 year to 17 years old (average 6.36 yr; SD=4.11) originating from two areas in Southern Romania were included in the study. Canine EDTA-anticoagulated whole blood samples were collected and tested for the presence of circulating microfilariae using a modified Knott test and for the presence of *D. immitis* antigen using a rapid immunochromatographic (SNAP 4Dx) kit; of them, a subset of 101 samples was tested by both methods. The overall prevalence of *D. immitis* was 14.53% (33/227), the infection rate in shelter and owned dogs being close, of 16.0% and 13.4%, respectively (p=0.576). Moreover, 11.6% occult infections were detected. Additionally, 1.8% and 2.0% of dogs were positive for *D. repens* and mixed infection (*D. repens* plus *D. immitis*), respectively. Additionally, *Anaplasma* spp. (1.0%) and *Ehrlichia* spp. (12.9%) infections have been also registered. The findings show significant exposure to *Dirofilaria* infection in dogs from Southern Romania. However, further more extended epidemiological studies are planned to identify the risk factors for animal and public health.

Keywords: Dirofilariosis; Knott test; Snap 4Dx; Microfilariae; Dogs; Romania.

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Introduction

Dirofilariosis is a vector-borne parasitic disease caused by *Dirofilaria immitis* and *Dirofilaria (Nochtiella) repens* (Nematoda: Filarioidea). *D. immitis* resides in the pulmonary arteries and the right heart chambers and is the causative agent of cardiopulmonary or heartworm disease (HWD), while *D. repens* is located in
subcutaneous tissues inducing nodular dermatitis (subcutaneous dirofilariosis). The parasites are transmitted by different culicid mosquitoes, such as species of the genera *Aedes*, *Anopheles*, *Culex*, some of which feed on both animals and humans. Canids are the predominant definitive hosts, but these parasites can also infect cats and other mammalian species, including humans, in which infection occasionally may cause severe clinical findings (Genchi et al., 2011; Mitrea, 2011).

Dirofilariosis affects canine, feline and human populations with an increased incidence in temperate and tropical areas of the world (Simón et al., 2009). Both *D. immitis* and *D. repens* are considered species with zoonotic potential. In human pathology granulomas have been noticed, which can be confused with tumors (mimicking tumors) (Pampiglione et al., 2009; Genchi et al., 2011). Despite the fact that there are several methods for diagnosis and prevention which can be successfully applied, dirofilariosis is still spread over large areas (McCall și col., 2008).

The increasing temperatures caused by global warming can affect the distribution and abundance of mosquito populations, and subsequently the transmission and spread of mosquito-borne diseases (Khasnis and Nettleman, 2005; Otranto et al., 2009a). Additional, other social factors are involved, such as increased mobility of both humans and dogs (Genchi et al., 2011).

Recently an alarming increase in the prevalence of canine cardio-pulmonary dirofilariosis is being reported in Romania. The epidemiological status of dirofilariosis shows a rapid evolution; the dynamics of this disease has changed from sporadic to emerging (Ionita et al., 2012). However there are limited information regarding the prevalence and distribution of canine dirofilariosis in Romania. A recent study on canine vector-borne diseases suggested clustered foci for *D. immitis* in southern regions of the country (Mircean et al., 2012). Therefore, the present study aimed to perform a parasitological screening on *Dirofilaria* infection of dogs in some areas in Southern Romania.

### Materials and methods

#### Study area

During January 2017 to July 2018, a total of 227 blood samples were collected from dogs originating from two cities in Southern Romania, namely Bucharest (44.26 N, 26.6 E) and Râmnicu Vâlcea (45.10 N, 24.36 E).

#### Animals

The animals selected for the study were shelter and owned dogs, all having access outdoor. All dogs taken in the present study had no recorded history of travelling outside Romania.

#### Blood sampling technique and investigation methods

EDTA-anticoagulated whole peripheral blood samples were collected from dogs and subjected for detection of *Dirofilaria* infection. For this, two investigation methods were used:

(i) a rapid immunochromatographic test (SNAP 4 DX; IDEXX Laboratories, Inc., Westbrook, Maine, USA), to detect circulating antigens of *D. immitis* (n=101) and (ii) a modified Knott technique, to identify and differentiate between *D. immitis* and *D. repens* microfilariae (n=221). A subset of 101 samples was tested by both methods.

(i) The immunocromatographic SNAP-4Dx test is a qualitative ELISA based technique and is able to detect the circulating antigen of *D. immitis*. The testing was performed according to the manufacturer’s instructions. The test is able to detect also antibodies of *Anaplasma phagocytophilum* and *Anaplasma platys*, *Borrelia burgdorferi*, *Ehrlichia canis* and *Ehrlichia ewingii*.

(ii) The modified Knott test is a concentration method in which the red blood cells are lysing and the microfilariae (mf) are fixed and stained as such they can be further differentiated based on morphological criteria. The method is used successfully in dogs, being very specific (Magnis et al., 2013). Briefly we describe here the technique: 1.0 mL of whole EDTA-venous blood was mixed with 9 mL of 2.0% formaline and then centrifuged for 3 minutes at 1500 g.
The supernatant was discarded and 1-2 drops of methylene blue was added to the sediment and mixed gentle (Ionita and Mitrea, 2013); 1-2 drops of the sediment were placed on a glass slide, covered with a cover slip then the slides were further subjected for microscopic examination for detection (at 10x to assess the presence of microfilariae) and identification of microfilariae (at 40x to observe the morphological characters) (Genchi et al., 2005). The whole sediment was analyzed for each sample.

**Results**

Of the total dogs (n=227) investigated in this study for *Dirofilaria* infection, 44.1% were from an animal shelter and 55.9% were owned dogs, with the age varying from 1 year to 17 years old (average 6.36 years; SD=4.11).

Subsequently to the parasitological testing, an overall prevalence of *D. immitis* of 14.53% (33/227) was found, with similar infection rate in shelter and owned dogs, of 16.0% and 13.4%, respectively (p=0.576).

However, different prevalence values for *D. immitis* infection were obtained, according to the investigation method used. Therefore, the Ag test showed a prevalence of 28.7% (29/101), while the Knott test a prevalence varying from 3.4% (4/126) to 17.8% (18/101). Within the dogs tested by both methods, 11.6% (11/101) of dogs were only Ag positive; for these no microfilariae were detected (occult infection) (table 1).

Of the total dogs tested, 1.8% of dogs were positive for *D. repens* mf, while 2.1% showed mixed infections (*D. repens* plus *D. immitis*) (table 1).

Microfilariae of *D. immitis* showed a characteristic straight tail and a spindle-shaped cephalic extremity, while microfilariae of *D. repens* have a curved tail and rounded cephalic extremity (Genchi et al, 2005) (figure 1).

Additionally to *D. immitis* Ag, the SNAP test also showed infection of dogs with *Ehrlichia* spp. (12.9%; 13/101) and *Anaplasma* spp. (1.0%; 1/101 (table 2) but no one positive for *B. burgdorferi* sensu lato infection.

**Discussions**

In the present study we used two diagnostic approaches for a parasitological screening on canine dirofilariosis. The detection of circulating Ag of *D. immitis*, by using a commercial immunochromatographic kit is commonly recommended for the screening of asymptomatic dogs. Therefore, in endemic areas, an annual serological screening would be recommended to promote early detection and treatment (Otranto et al, 2009b).

<table>
<thead>
<tr>
<th>Table 1. Prevalence of <em>Dirofilaria immitis</em> and <em>Dirofilaria repens</em> infection in dogs, Southern Romania (two investigation methods have been used: Knott¹ and Ag² tests)</th>
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<tbody>
<tr>
<td><strong>Number of tested dogs</strong></td>
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<tr>
<td><strong>Total</strong></td>
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<tr>
<td><strong>Life style</strong></td>
</tr>
<tr>
<td>Shelter</td>
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<td>Owned</td>
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<td><strong>Gender</strong></td>
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<tr>
<td>Male</td>
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<tr>
<td>Female</td>
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¹: test used for detection and differentiation of *D. immitis* and *D. repens* microfilariae - mf;
²: test used for detection of *D. immitis* Ag;
Figure 1. Microfilariae of: A: *Dirofilaria repens* [the anterior end is obtuse; the posterior extremity like an umbrella handle]; B: *Dirofilaria immitis* [the anterior end is sharp; the posterior extremity straight]
Table 2. Sero-prevalence of selected arthropod-borne pathogens (by an immunochromatographic test) of dogs in Southern Romania

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Number of Positive dogs (percentage)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>D.im. (Ag)*</td>
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<tr>
<td><strong>Total</strong></td>
<td>101</td>
<td>29 (28.7%)</td>
</tr>
<tr>
<td><strong>Life style</strong></td>
<td></td>
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<tr>
<td>Shelter dogs</td>
<td>55</td>
<td>13 (23.6%)</td>
</tr>
<tr>
<td>Owned dogs</td>
<td>46</td>
<td>16 (34.8%)</td>
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<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>47</td>
<td>18 (38.2%)</td>
</tr>
<tr>
<td>Female</td>
<td>54</td>
<td>11 (20.3%)</td>
</tr>
</tbody>
</table>

*Ag: antigen of Dirofilaria immitis; Ab: antibody to Ehrlichia spp.; A.ph: antibody to Anaplasma spp.; B.b. sl: antibody to Borrelia burgdorferi sensu lato.

When comparing the two tests, an important advantage of the Ag test is highlighted by the identification of occult infection, when adult worms are present but no circulating microfilariae (Genchi et al., 2009), as showed here, too. In this study, for 11.6% of the tested dogs, occult infections were detected. Similar findings were reported recently by a similar study on dogs originating from the urban area of Bucharest which showed a prevalence rate of 21.14% for D. immitis antigens but only 16.57% for microfilariae of which 10.28% were D. immitis mf 4.57%, D. repens mf and 1.71% Acanthocheilonema spp. mf (Girdan et al., 2017). So it is confirmed the importance of using both diagnostic methods as microfilaricide drugs may induce a false negative result and also as in the case of mixed infections the morphological recognition of microfilariae proved to be difficult (Genchi et al., 2011).

The overall prevalence of D. immitis found in this study in shelter and owned dogs of 16.0% and 13.4%, respectively, is supported by other studies on canine dirofilariosis in Southern Romania reporting prevalence rates of 18.68% (in stray dogs), 21.14% and 23.03% (Ionita et al., 2012; Girdan et al., 2015; Anghel et al., 2016). However, lower prevalence values of 2.1% and 3.3% have been reported in dog populations in Eastern (Moldova) and western Romania, respectively (Mircean et al., 2012; Ciucă et al., 2014). These could be explained based on the temperate-continental climate which provides favourable conditions for allowing development of the mosquito populations with important clustered foci in Southern areas of the country (Nicolescu et al., 2003; Tomazatos et al., 2018).

Moreover, in the present study, males showed a higher prevalence (38.2%) than females (20.3%), which confirms similar studies (Yildirim et al., 2007; Traversa et al., 2010), where male's prevalence was demonstrated. A higher rate of infection in males has been suggested due to their stronger attraction to vectors (Montoya et al., 1998) but also this can be attributed to the fact that more male dogs are kept outdoors, because they are considered to be more suitable for defending property (Song et al., 2003).

When searching for literature reports, D. immitis shows a global distribution, being endemic in several countries. The prevalence reported for D. immitis in some European countries are extremely variable, from 0.6 to 80% in Italy, 0.6 to 6.8% in France, 1.6% in Switzerland, 6.2% in Serbia, or from 10.0 to 34.0% in Greece (Traversa et al., 2010).

D. repens is considered endemic in Southern Europe (McCall et al., 2008), with infection rates varying form 20.0 to 30.0% (Otranto et al., 2013) and higher prevalence rates reported in Italy (Pampiglione et al., 2001). In our study, 1.8% of dogs were positive for D. repens mf and 2.1% of dogs have mixed infection, D. repens plus D. immitis.

Altogether, these findings provide new evidences for the co-circulation of the two Dirofilaria species in Romania, as previously reported (Mircean et al., 2012; Ionica et al., 2015; Ciucă et al., 2016) and highlight that...
dogs from Southern Romania are at risk of acquiring *Dirofilaria* infection. Furthermore, clinical cases on *D. repens* infection have been also reported for both humans and dogs (Popescu et al., 2012; Macarie et al., 2017; Mircean et al., 2017).

Apart from *Dirofilaria* spp., two other canine tick-borne pathogens have been detected in this study: *Ehrlichia* spp. (12.9%) and *Anaplasma* spp. (1.0%). Both pathogens are of veterinary interest and have been previously reported in dogs, with infection rate varying from 2.25% to 4.0% and from 1.14% to 16.0%, respectively (Ionita et al., 2012; Gîrдан et al., 2015; Morar et al., 2015; Anghel et al., 2016) and the tick-vector (infection rates of 2.0% an 7.5% and of 6.7%, respectively) (Ionita et al., 2013; 2016). Therefore, these results confirm the risk also for tick-borne diseases in the investigated area, some of them of public health concern.

In conclusions, the findings of the present study show significant exposure to *Dirofilaria* infection of dogs in Southern areas of Romania and highlight the importance for constant screening of dogs for early detection of infection but as well as the need for an increased awareness of veterinarians and dog owners concerning dirofilariosis and other vector-borne disease.

**References**


