# Geographic distribution of canine heartworm (*Dirofilaria immitis*) infection in stray dogs of eastern Romania

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## Abstract

A survey was conducted in the eastern part of Romania to assess the prevalence and geographical distribution of *Dirofilaria immitis* in dogs. Plasma samples were collected from 458 stray dogs hosted in shelters in 8 counties and tested serologically for the presence of heartworm. In addition, 45 blood samples from dogs of a shelter in Galati city were examined by the modified Knott and multiplex polymerase chain reaction (PCR) techniques. The immmunological assay showed a heartworm infection prevalence of 8.9% in the dogs. Optical density results for positive samples ranged between 0.217 and 2.683. Geographical information systems (GIS) were used to produce overlays of distribution maps of *D. immitis* prevalence and predictive maps based on temperature suitability. High prevalence of *D. immitis* was found in the central East up to the northern border of the country, *i.e.* Galati county (60%), followed by the counties of Vaslui (12.0%) and Iasi (7.7%). Out of 45 samples examined using the Knott test, 23 were positive for circulating microfilariae (51.1%), while 19 dogs were positive for *D. immitis* and 4 for both *D. immitis* and *D. repens* with the multiplex PCR test. The high prevalence for *D. immitis* shown in dogs in the Southeast (Galati, 42.2%) also by multiplex PCR gave strong support to the results achieved by the serological tests. The present study confirms the ability of GIS to predict the distribution and epidemiology of dirofilariosis in different geographical territories as has been already demonstrated by the empirical epidemiological data obtained at the continental, national and intraregional levels.

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## Introduction

In Europe, the most common canine filarial species producing blood-circulating microfilariae are: *Dirofilaria immitis, D. repens, Acanthocheilonema (syn. Dipetalonema) dracunculoides* and *A. (syn. Dip.) reconditum* (Magnis *et al*., 2013). Cardiopulmonary dirofilariosis or heartworm disease (HWD) is caused by *D. immitis*, which is the one of the most pathogenic parasites of dogs with an increasing distribution, possibly due to climatic changes as well as animal and human movements (Genchi *et al*., 2011). Suitable hosts, *i.e*. domestic dogs and some wild canids serve as the main infection reservoirs. The intermediate hosts and vectors are different species of culicid mosquitoes of the genera *Culex, Aedes* and *Anopheles* (Cancrini and Kramer, 2001). Immature *D. immitis* first reach the lung and as they grow they affect larger and larger pulmonary arteries, eventually spilling over into the right chamber of the heart, where the worm burden can be high (Furlanello *et al*., 1998). *D. immitis* can be transmitted to humans inducing pulmonary dirofilariosis with benign pulmonary nodules which may be misidentified as malignant tumours (Simon *et al*., 2005). In spite of the availability of reliable diagnostic tools and effective prophylaxis, HWD is still prevalent in dogs in large areas (McCall *et al*., 2008). Dirofilariosis in animals and humans is being detected more and more frequently in the Mediterranean countries (Genchi *et al*., 2005). Spain, Portugal, Italy and France were endemic before 2001 and still remain so. In these regions, however, the distribution of cardiopulmonary dirofilariosis is generally only reported as sporadic cases, or not reported at all (Morchon *et al*., 2012). *Dirofilaria* spp. have spread towards the eastern and north-eastern European countries, but only limited epidemiological information is available from there (Genchi *et al*., 2009, 2011). Spread of cardiopulmonary dirofilariosis in Europe could be due to several factors, such as global warming (Genchi *et al*., 2001; Sassnau *et al*., 2014), the presence of vectors as well as adequate climate conditions needed for its development, new species of competent mosquitoes (Madon *et al*., 2002; Cancrini *et al*., 2003; Roiz *et al*., 2007), increasing number of dogs travelling with their owners, *e.g*., for holiday trips, and a growing role of reservoirs such as jackals and foxes (Tolnai *et al*., 2014).

The presence of canine and human *Dirofilaria* infections in the Balkan Peninsula suggests that climate, ecology factors and mosquito abundance are suitable for the full development and transmission of the infection (Tasić-Otaševi *et al*., 2015). However, the prevalence and distribution of *Dirofilaria* spp. infections in dogs in Romania are still unclear. The highest reported prevalence figures range from 3.6 to 14% in Tulcea county to 3.3% in the South, Southwest and Southeast of the country (Mircean *et al*., 2012). Another study conducted in different areas of Romania has shown 23.7-35% seroprevalence for *D. immitis* (Coman *et al*., 2007), while information on *D. repens* has only been recorded in the western (Ciocan *et al*., 2010, 2013) and southern (Tudor *et al*., 2013) regions. In a recent study conducted by Ionică *et al*. (2015), the seroprevalence of *D. immitis* was 7.1% in the East and the South. By continuing monitoring of HWD in Romania, the current study was aimed at determining the prevalence of *D. immitis* in the eastern part of the country using geographical information systems (GIS) to compare current data with prevailing temperatures as described in the predictive model by Genchi *et al*. (2005).

## Materials and Methods

### Study area and sample collection

Between 2013 and 2014, a cross-sectional survey was carried out based on plasma collected from 458 stray dogs (245 females and 213 males) of ages varying from 9 months to 12 years. The dogs were hosted in shelters from 8 different counties (Suceava, Botosani, Neamt, Iasi, Bacau, Vaslui, Galati, Vrancea) in the eastern part of Romania. The animals' coat length was recorded as short in 289 dogs and long in 169 at the time of sample collection. In addition, 45 blood samples were collected from dogs from another shelter, located in Galati city. These dogs (28 females and 17 males) were of ages varying from 2 to 10 years and the samples were kept in ethylenediaminetetraacetic acid (EDTA) until use to counteract coagulation. For each dog, data about the potential risk factors (sex, age, hair coat) were recorded. All dogs were kept outdoors and did not generally receive any kind of regular antiparasitic treatment. All samples used in this study were collected under standard protocols for management of dogs at participating in animal shelters and by the Institutional Animal Care and Use committees at the University of Iasi (Romania).

### Laboratory techniques

At least 2 mL of blood from each of the 458 analysed dogs was collected in a plasma separator tube and stored at -20°C until tested for the presence of *D. immitis* antigen using a commercial ELISA test kit based on heartworm antigen (HA) available from PetCheck® (Westbrook, ME, USA) according to the manufacturers' instructions. Measurements of the optical density (OD) were done spectrophotometrically at 650 nm. For the assay to be valid the OD positive control value minus the OD negative control value (P-N) was set to be greater than 0.150 with the negative control OD value ≤0.150.

The additional 45 blood samples in EDTA were tested by the modified Knott test performed as described previously (Knott, 1939; Magnis *et al*., 2013). Briefly, 9 mL of 2% formalin was added to 1 mL of whole blood and agitated to lyse the red blood cells. The sample was then centrifuged at 1200 rpm, and the pellet stained with 1% methylene blue. Circulating microfilariae were identified based on morphology (Magnis *et al*., 2013) using a standard microscope equipped with calibrated measuring eyepieces giving a final 200-400× magnification. Samples reacting positively (23) were subjected to molecular diagnosis, *i.e*. genomic DNA was extracted from 100 μL of each positive sample using the DNeasy blood and tissue kit (Qiagen, Leipzig, Germany) following the manufacturer's instructions. An equimolar combination of general and specific primers (12SF/12SRdeg/12SF2B/12SR2) to amplify a portion of the small subunit ribosomal RNA gene of the mitochondrion (12S rDNA) were used to perform a multiplex polymerase chain reaction (PCR) according to Gioia *et al*. (2010). The PCR products were detected on a 2.5% ethidium bromide-stained low-melting agarose gel (BIO-RAD, Madrid, Spain). The amplification of the conserved region was 500 bp (12SF/12SRdeg) for canine filarial samples, while the simultaneous amplification of the specific fragments was 204 bp (12SF2B/12SRdeg) and 327 bp (12SF/12SR2) for *D. immitis* and/or *D. repens,* respectively. The final PCR volume used was 20 μL (19 μL mix + 1 μL DNA) for each sample. The thermal profile used was 95°C for 10 min; 40 cycles at 92°C for 30 sec, at 49°C for 45 sec, at 72°C for 1 min with the final elongation step at 72°C for 10 min.

### Geographical information system and statistical analysis

All data were georeferenced (at the county level) and the spatial distribution of *D. immitis* was displayed on a map constructed within the GIS using ArcGIS, 10.3 software (ESRI, Redlands, CA, USA). The geographical distribution of *D. immitis* in the study area was compared with the map obtained by the HWD predictive model based on temperature suitability by Genchi *et al*. (2005, 2009). Univariate statistical analysis (chi-squared test) was performed using SPSS software (IBM, Armonk, NY, USA) to study the association between the data found, particularly dog seropositivity and *D. immitis* presence.

## Results

Table 1 reports the ELISA results for *D. immitis* with regard to the 458 analysed dogs hosted in the eastern Romania shelters. The mean prevalence of *D. immitis* was 8.9% and the OD results for the positive samples ranged from 0.217 to 2.683. The *D. immitis* prevalence was higher in dogs over 2 years of age ($P<0.05$) but there were no significant differences between dogs with short or long coat ($P>0.05$). Both sexes had similar prevalence ($P>0.05$).

The highest prevalence of HWD was seen in the central East of Romania with 60% recorded near the northern border of Galati county followed by Vaslui county (12%) and Iasi county (7.7%). Out of the 45 samples examined with the Knott test, 23 were positive for circulating microfilariae (51.1%). Nineteen dogs were positive for *D. immitis* and 4 were positive for both *D. immitis* and *D. repens* according to the PCR test (Table 2). The high prevalence for *D. immitis* in dogs in the Southeast (Galati county, 42.2%) was confirmed by multiplex PCR providing strong support to the results achieved by the serological tests.

## Discussion

There were no significant differences between dogs with short or long coat (P>0.05), as also found by Vieira *et al*. (2014). Both sexes had similar prevalence (P>0.05) in agreement with several authors (Song *et al*., 2003; Montoya *et al*., 2006; Rapti and Rehbein, 2010), which is in contrast to other studies (Yldrim *et al*., 2007; Traversa *et al*., 2010), where significantly higher prevalence rates in males were shown. It has been suggested that the higher infection rate in male dogs could be due to them attracting vectors more strongly (Montoya *et al*., 1998). Further studies are needed to resolve this question.

For some authors, age represents an important risk factor (Köse *et al*., 2012) and the present study did indeed find higher *D. immitis*

**Table 1. Seroprevalence of *Dirofilaria immitis* in dogs from the eastern part of Romania.**

| County | *D. immitis* | | Gender | | | | Coat length | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Female | | Male | | Short | | Long | |
| | Positive° (%) | 95% CI | Positive° (%) | 95% CI | Positive° (%) | 95% CI | Positive° (%) | 95% CI | Positive° (%) | 95% CI |
| Suceava | 0/100 | - | 0/38 | - | 0/62 | - | 0/57 | - | 0/43 | - |
| Botosani | 0/40 | - | 0/17 | - | 0/23 | - | 0/11 | - | 0/29 | - |
| Neamt | 0/33 | - | 0/19 | - | 0/14 | - | 0/22 | - | 0/11 | - |
| Iasi | 5/65 (7.7) | 2.9-17.8 | 2/32 (6.3) | 1.1-22.22 | 3/33 (9.1) | 24-25.5 | 4/43 (9.3) | 3.0-23.1 | 1/22 (4.5) | 0.2-24.9 |
| Bacau | 0/70 | - | 0/46 | - | 0/24 | - | 0/44 | - | 0/26 | - |
| Vaslui | 6/50 (12) | 5-25 | 3/24 (12.5) | 3.3-33.5 | 3/26 (11.5) | 3.0-31.3 | 5/36 (13.8) | 5.2-30.3 | 1/14 (7.1) | 0.4-35.8 |
| Vrancea | 0/50 | - | 0/31 | - | 0/19 | - | 0/37 | - | 0/13 | - |
| Galati | 30/50 (60) | 45.2-73.30 | 26/38 (68.4) | 51.2-82 | 4/12 (33.3) | 11.3-64.6 | 25/39 (64.1) | 47.2-78.3 | 5/11 (45.4) | 18.1-75.4 |
| Total | 41/458 (8.9) | 6.6-12.0 | 31/245 (12.7) | 8.9-17.6 | 10/213 (4.7) | 2.4-8.7 | 34/289 (11.8) | 1.8-16.2 | 7/169 (4.1) | 1.8-8.7 |

*D. immitis, Dirofilaria immitis;* CI, confidence interval. °Positive out of total investigated.



**Table 2. Results of Knott test and multiplex polymerase chain reaction for 23 out of 45 dogs sampled from Galati city in Romania positive for circulating microfilariae.**

| Galati sample | Gender | Knott *D. immitis* | Knott *D. repens* | PCR° *D. immitis* | PCR° *D. repens* |
|---|---|---|---|---|---|
| 1 | F | + | - | + | - |
| 2 | F | + | - | + | - |
| 3 | F | + | - | + | - |
| 4 | M | + | - | + | - |
| 5 | F | + | + | + | + |
| 6 | F | + | - | + | - |
| 7 | M | + | - | + | - |
| 8 | M | + | + | + | + |
| 9 | M | + | - | + | + |
| 10 | F | + | - | + | - |
| 11 | M | + | + | + | - |
| 12 | F | + | - | + | - |
| 13 | F | + | - | + | - |
| 14 | F | + | - | + | - |
| 15 | F | + | - | + | - |
| 16 | F | + | - | + | - |
| 17 | F | + | - | + | - |
| 18 | F | + | - | + | - |
| 19 | F | + | - | + | - |
| 20 | M | + | + | + | + |
| 21 | M | + | - | + | - |
| 22 | M | + | - | + | - |
| 23 | F | + | - | + | - |

PCR, polymerase chain reaction; *D. immitis, Dirofilaria immitis; D. repens, Dirofilaria repens.* °Multiplex PCR.

prevalence in dogs over 2 years of age. Several studies found older dogs having an increased risk of *D. immitis* infection that was thought to be due to the prepatency period of the parasite (Song *et al*., 2003; Tudor *et al*., 2009; Lim *et al*., 2010; Vieira *et al*., 2014). According to the literature (Montoya *et al*., 1998; Yildirim *et al*., 2007) another important risk factor is the time of exposure to the vector; in fact these dogs were kept in outdoor shelters, where they were more likely to acquire this infection.

Romania is the country with the largest area of the Danube Basin. Galati county is located in the Centre-east of the country and belongs to the series of counties bordering the Carpathian Mountains and the Danube River. The counties that include the floodplains are endemic areas for both *Dirofilaria* species due to the presence of confirmed vector species, such as *Anopheles maculipennis* and *Culex pipiens* (Nicolescu *et al*., 2002, 2003a, 2003b). Therefore, this could be an explanation for the high prevalence in the southeastern Romania as detected in this study.

A previous study from Romania showed no positive cases for HWD in Iași county (Mircean *et al*., 2012). By contrast, our study found a 7.7% prevalence of heartworm infection there. A possible explanation for the previous failure to detect positive cases here is that the antigen test may have given false negatives in dogs with low parasitic burdens or in blood samples from those infected only by male worms (American Heartworm Society, 2014). However, also the low-level prevalence in Iași county revealed in our study is important because, as reported by Miro *et al*. (2013), *D. immitis* may serve as a reservoir of infection for other animals in low-incidence areas.

The present study showed negative results in some counties, such as Botosani, Suceava, Neamt, Bacau and Vrancea. Given that transmission of dirofilarial infections by mosquitoes is strictly dependant on the climate and the larvae will only develop in the vectors if the temperature is suitable (Kalluri *et al*., 2007; Medlock *et al*., 2007), these areas are characterized by a temperate-continental climate (cool and moist) with eastern aridity and Baltic-Scandinavian influences in the north. The presence of *D. immitis* in the eastern part of the country extends the areas positive for the parasite from the southern, southwestern and southeastern parts of the country. Moreover, the maps agree well with the distribution of HWD presented by Genchi *et al*. (2005).

GIS has been used for epidemiologic mapping of human and animal



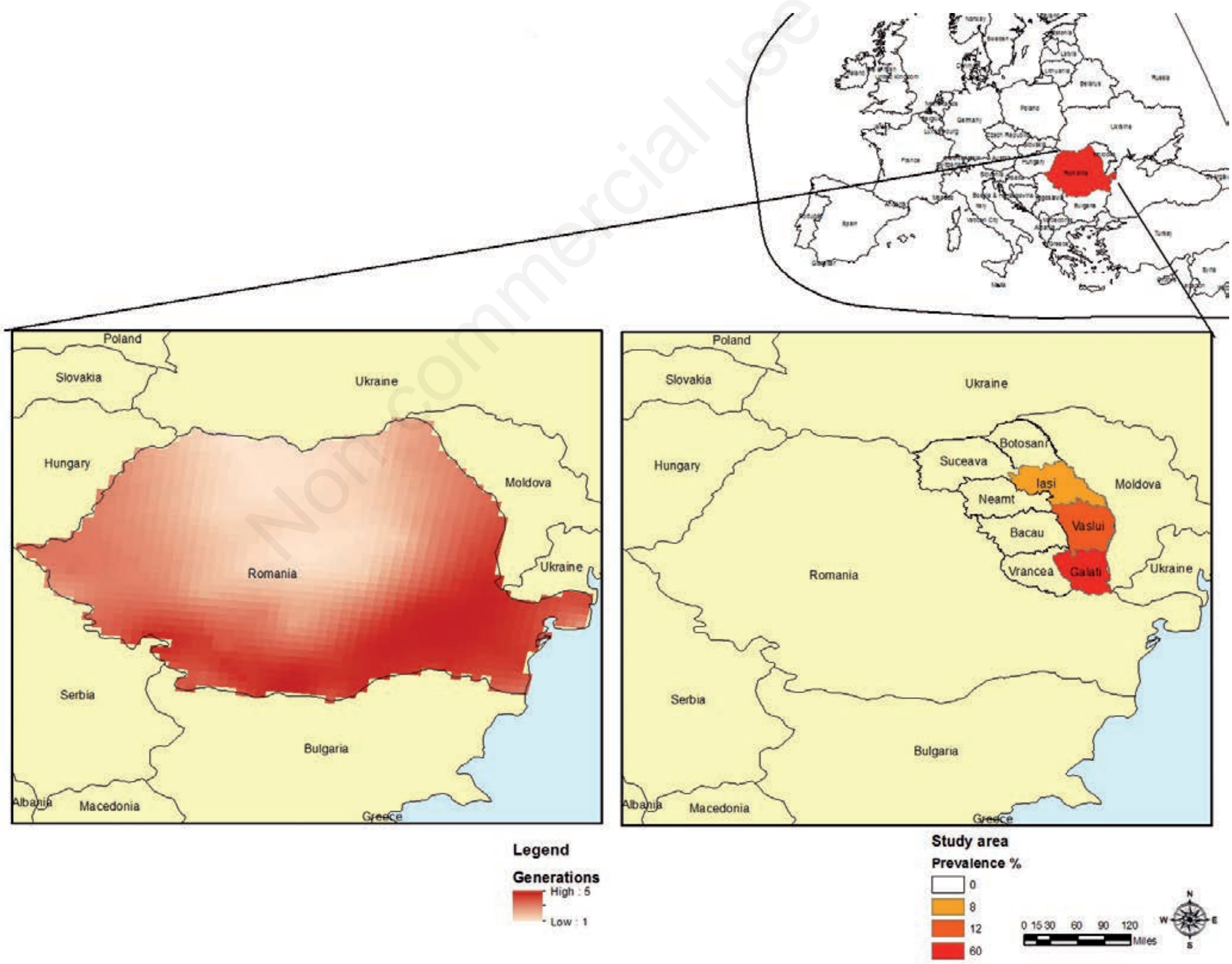


**Figure 1. Distribution of heartworm infection in dogs in eastern Romania and comparison with the predictive model by Genchi *et al.* (2005).**

filariosis (Noma *et al*., 2002; Genchi *et al*., 2005, 2009, 2011; Simon *et al*., 2012) enabling the impact risks involved to be managing and controlling its spread. In fact, apart from the studies carried out in Romania (Mircean *et al*., 2012; Ionică *et al*., 2014), few studies have focused on mapping the risk areas. Here, GIS showed points of transmission period and temporal dynamics of heartworm development units (HDU), a temperature-dependent measure of the time it takes to reach maturity of the parasite (Genchi *et al*., 2005), which indicated HWD accumulation in the eastern part of Romania. This model confirms that the period when the development of *D. immitis* occurs is the longest in Bucharest (21 May - 20 September). Furthermore, published prevalence data presented by Trotz-William and Trees (2003) based on a systematic review of the literature suggest that the HWD should be more prevalent in dogs from East-European countries involving Romania (Genchi *et al*., 2005). The climate of Romania is temperate–continental of the transitional type with four clearly defined seasons. Therefore, the periods for the development of *Dirofilaria* spp. infections are the longest in May-October in the South and Southeast, followed by the West and Southwest (May-September) and shorter (June-August/September) in the rest of the country (Genchi *et al*., 2005, 2011). Finally, the results achieved by the serological tests are in line with the predictive map based on HDUs in the eastern part of Romania (Figure 1).

In the present study, HWD has been found in dogs living in areas with favourable environmental temperatures and humidity factors required by the life cycle of this parasite. Thus, the infection seems to expand in the northeastern areas of the country where the climate conditions support the transmission of this infection.

## Conclusions

The ability of GIS to predict the distribution and epidemiology of dirofilariosis in different geographical areas has been already demonstrated by the empirical epidemiological data obtained at the continental (Genchi *et al*., 2009; Kartashev *et al*., 2014), national (Medlock *et al*., 2007; Simón *et al*., 2014), and regional (Mortarino *et al*., 2008; Montoya-Alonso *et al*., 2015) levels. As demonstrated in our present study, GIS could become an important tool for the management of HWD in endemic and non-endemic countries.

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