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Article

The occurrence of *Dirofilaria repens* in dogs in the southern Transdanubia

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ABSTRACT – *Dirofilaria repens* causes skin worm infection in canid sepcies and humans. This parasite needs mosquitoes as intermediate hosts in their lifecycle. By warming of the global climate, more and more areas become suitable mosquito habitats. Therefore, mosquito transmitted diseases can emerge in new areas. In this study, the authors investigated the dog population of the south Transdanubia, the southernmost part of Hungary to determine the average *D. repens* prevalence. By Knott's test and molecular confirmation of infected status, 11.7% prevalence could be detected in 95 domestic dogs. In risk analysis, urban residence and female sex proved to carry high risk of infection. On the other hand, age, breed, hair length showed no impact on infection status. Though all these features seemed important in mosquito biting success, this study could not support the relevance of them.

Keywords: *Dirofilaria repens*, dog, southern Transdanubia

INTRODUCTION

Of the 27 species of *Dirofilaria* currently known, *Dirofilaria immitis* and *Dirofilaria repens* are the most important. The species of worms that cause heartworms (*D. immitis*) and skinworms (*D. repens*) in dogs are also of significant public health importance on the European continent. These *Dirofilaria* species develop indirectly, developing into adults through 5 larval stages in their biological cycle, which requires both final and intermediate hosts. Infectious larvae of helminths develop in mosquitoes. About 60-70 species of the *Culicidae* family belonging to several genera are considered as potential intermediate hosts and vectors worldwide (*McCall et al.,* 2008). The role of the final host for both parasites is predominantly played by canine predators (dog, wolf, fox, jackal), but also occurs in cats, weasels, and bears (*Otranto and Deplazes*, 2019).

Humans can also become infected, but the worm, with rare exceptions, remains infertile (*Pampiglione et al.,* 2001; *Petrocheilou et al.,* 1998; *Negahban et*

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al., 2007). Infections in humans usually manifest as a single subcutaneous nodule caused by filaria trapped by the immune system (*Pampiglione et al.,* 2000, 2001). Subcutaneous migration of the worm can cause local swellings with varying localization. In addition, rare organ manifestations involving the lungs, male genitals, female breast, or eyes have been reported (*Pampiglione et al.,* 2001).

Until the last decade of the twentieth century, *Dirofilaria* repens occurred mainly in southern European countries, such as Italy, Portugal, Spain, France, and Greece, where dirofilariosis was historically present (*Trotz-Williams and Trees,* 2003; *Genchi et al.,* 2005). In addition to Europe, *D. repens* also occurs in Asia Minor, Central Asia, and Sri Lanka (*Simón et al.,* 2012). Recent epidemiological studies have confirmed that *D. repens* has appeared and become endemic in many countries in Central and Eastern Europe, where more and more infections have been found in local dogs (*Tasić-Otašević et al.,* 2015; *Capelli et al.,* 2018).

The rapid geographical spread of these parasites to areas of Europe where they were not previously present is explained by several factors. It should be emphasized that increasing warm periods due to climate change can be considered significant for mosquito development activity and seasonal survival, this also contributes to the development of *Dirofilaria* larvae in vectors (*Medlock et al.,* 2007; *Genchi et al.,* 2009, 2011).

The first domestic *D. repens* infections in dogs were reported in Hungary in the late 1990s (*Fok et al.,* 1998). In a decade, the first nationwide *Dirofilaria* survey revealed that the prevalence of *D. repens* infection was 14% (*Fok et al.,* 2007). A bit higher infection level (19%) was observed after a few years (*Jacsó,* 2014). In the end of the second decade of 2000s, the reported prevalence of *D. repens* infection was 14.2% in Hungarian dogs (*Farkas et al.,* 2020).

In addition, the presence of the parasite has been registered in neighbouring countries such as Germany, the Czech Republic, Slovakia (Figure 1) (*Bocková et al.,* 2013, *Sassnau et al.,* 2009, *Kronefeld et al.,* 2014, *Czajka et al.,* 2014, *Svobodvá et al.,* 2006).

The country most affected south of Hungary is Serbia, where *D. repens* was found in dogs with a frequency of 17-49% (*Tasić et al.,* 2012). The infection has also been found to be widespread in wildlife (*Ćirović et al.,* 2014). Several *D. repens* infections have also been reported in humans (*Ćirović et al.,* 2014, *Dzamić et al.,* 2004, *Tasić et al.,* 2011), and a recent survey of canine and human cases has shown the endemic status of dirofilariosis in parts of Serbia (*Krstić et al.,* 2017). Croatia (*Janjetović et al.,* 2010, *Marusić et al.,* 2008, *Bezić et al.,*

2009) and, less frequently, Bosnia and Herzegovina (*Ritter et al.,* 2012), Montenegro (*Dzamić et al.,* 2004, *Radovanovic et al.,* 2015), and Slovenia (*Pampiglione et al.,* 1995) have also been reported in human infection. In addition, infection with *D. repens* in dogs in the Balkans is spreading, and several human cases have been reported accordingly (*Janjetović et al.,* 2010).

Depending on this, we consider it is essential to examine the southern counties of the ransdanubia in Hungary, such as Baranya and Somogy, for *D. repens*.

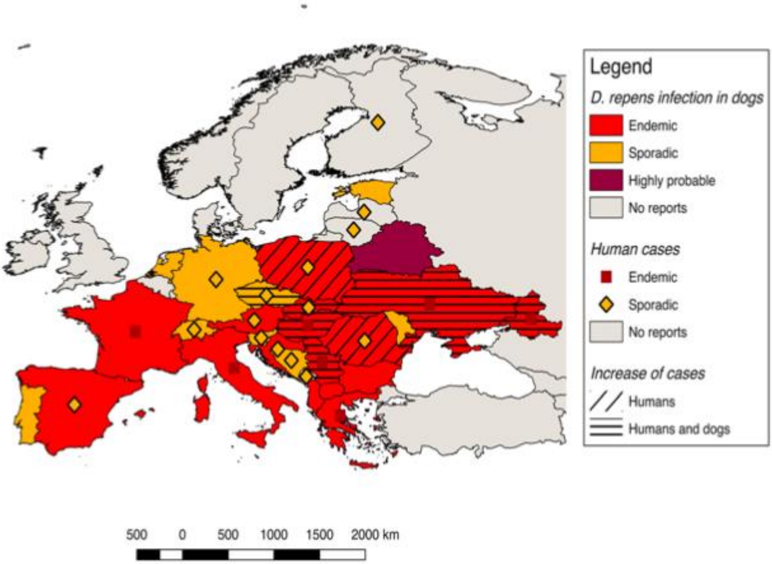

Figure 1. Map showing the prevalence of *Dirofilaria repens* across Europe in dogs and humans (*Capelli et al.*, 2018)

MATERIAL AND METHODS

Parasitological analysis

Dog blood samples (n=95) were collected for the owners' request in veterinary clinics in the southern Transdanubian region of Hungary (Supplementary Table 1). The samples were taken by veterinarians in the daily routine. The aim of the animal health targeted sampling was to confirm the presence of *Dirofilaria immitis* as the causative agent of canine heartworm disease in the animals. All the samples were cooled at 4 °C for few days (maximum 5 days) till the analysis. Modified Knott's test was used to determine the *Dirofilaria* infection

(*Zajac and Conboy,* 2012). For morphological identification of observed microfilariae, we used *Traversa's et al.* (2010), *Liotta's et al.* (2013) and *Magnis's et al.* (2013) works.

Molecular analysis

Because the morphological overlapping of microfilariae between the two *Dirofilaria* species did not make possible the exact differentiation, we applied a molecular identification, as well. In this approach, we adapted a highly sensitive multiplex PCR. The method allowed then simultaneous detection and discrimination of *Dirofilaria immitis* and *Dirofilaria repens* from the peripheral blood samples (*Gioia et al.,* 2010). Briefly, 1mL homogenised blood was diluted and centrifuged with 9 mL distilled water. After removing the supernatant, the dilution-centrifugation process repeated two times. After the final centrifugation the microfilariae were collected from the sediment and lysed using 5% Chelex 100 (Bio-Rad, Hercules, USA) suspension to purify the DNA. For the identification, we applied two sets of primer in the same mixture reaction. On the one hand, we used a general primer pair 12SF (5′-GTTCCAGAATAATCGGCTA-3′) and 12SRdeg (5′-ATTGACGGATG(AG)TTTGTACC-3′), which were designed on the 12S rDNA highly conserved region. On the other hand, we used a specific forward primer for *D. immitis* (12SF2B 5′-TTTTTACTTTTTTGGTAATG-3′) and a specific reverse primer for *D. repens* (12SR2 5′-AAAGCAACACAAATAA(CA)A-3′) to differentiate the two related species. The adapted thermal cycling conditions is shown in Table 1.

Table 1
Steps and conditions of thermal cycling for differentiation of *Dirofilaria* spp.

Steps	Temperature	Time	Cycles
Initial denaturation	92 °C	10 min	1
Final denaturation	92 °C	30 s	
Annealing	49 °C	45 s	40
Extension	72 °C	1 min	
Final extension	72 °C	10 min	1

In this reaction the amplification resulted a 500 bp length product in the conserved region, 204 bp sized fragment in the presence of *D. immitis* and 327 bp fragment if *D. repens* was present in the sample (*Gioia et al.,* 2010).

Statistical analysis

We used the online 1.0.15 version of QPweb parasitological software to determine the prevalence with 95% confidence interval (CI95%) of *Dirofilaria* spp, *D. repens*, and *D. immitis* infection (URL1; *Reiczigel et al.,* 2019). Because some of the samples were confirmed as mixed infection, we did not calculate the mean intensity.

Binary logistic regression model was built to determine the role of some possible driving factors. The chosen variables are shown in Table 2. For analysis, SPSS 27.0 statistical software was used. The approach was applied only in the case of *D. repens* infection.

Table 2
Variables were used in the binary logistic regression

Name	Abbreviation	Coding
age	AGE	age in month
sex	SEX	1=male; 2=female
breed	BREED	FCI standard weight of the breed (kg) 1= 1-10; 2=10.1-25; 3=25<
coat	COAT	Hair length (cm) 1=0.1-3; 2=3-6; 3=6<
location	LOCALITY	Type of the settlement, where the animal lives. 1=city; 2=village

The best fitted model was chosen by the smallest Akaike's Information Criterion (AIC) (*Field,* 2017) and its performance was assessed by using the area under the curve (AUC). We considered the AUC score as a fair one, if it was above 0.7 (*Swets,* 1988).

RESULTS

By the investigation of 95 dog samples, the overall *Dirofilaria* prevalence proved to be 15.8% (CI95%=9.4-24.6%). From the dogs 11 had *D. repens* infection (11.7%; CI95%=6.3-20.1%), and 6 were *D. immitis* infected (6.3%; (CI95%=2.8-13.03%). We found three animals with mixed infection (Figure 1 and Figure 2).

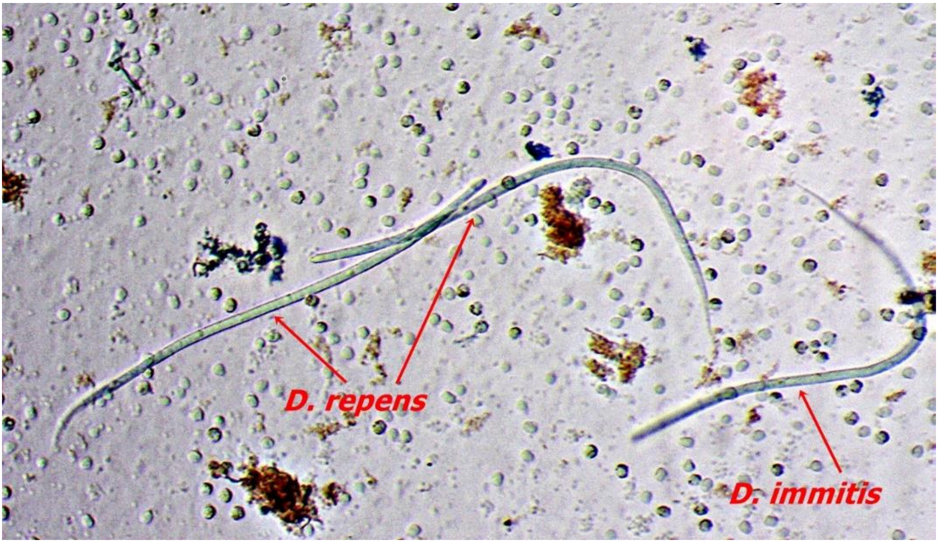

Figure 1. Mixed *Dirofilaria* infection in a sampled dog.

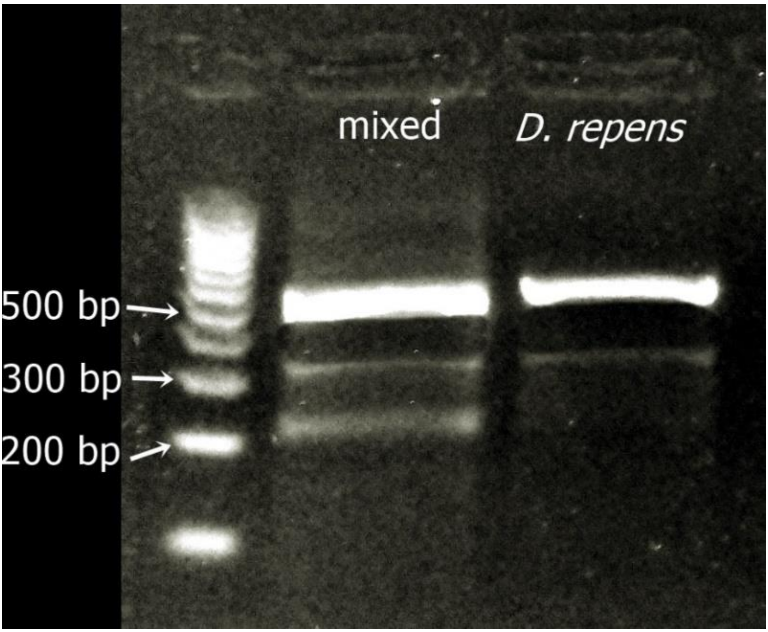

Figure 2. Result of molecular analysis (**mixed**: mixed infection; ***D. repens*:** only *D. repens* DNA detected in the sample).

The binary logistic model revealed that only LOCALITY and the SEX had affected the *D. repens* infection. Our best model had a 0.69 AUC score (p=0.043), while the smallest AIC value was 49.26. In the final model, the settlement type (LOCALITY) had negative regression coefficient, which suggested that in the rural settlements the animals could become infected with a smaller probability than in a city. The analysis of our data predicted that infection in female dogs is more likely than in males (Table 3). The age, breed, and hair-length showed no correlation with infection.

Table 3

Binary logistic regression for *D. repens* infection of badger

Predictor	β[1]	p-value	OR[2]	OR CI95%	
				Lower	Upper
$LOCALITY_{(city)}$[3]	-2.319	0.008	0.067	0.098	0.539
$SEX_{(male)}$[4]	2.261	0.044	9.59	1.07	86.23
CONSTANT	2.837	0.006			

[1] regression coefficient; [2] odds ratio; [3]the reference category=city; [4] the reference category=male

DISCUSSION

Our study surveyed the occurrence of *D. repens* in the southern Transdanubia by the investigation of dogs' whole-blood samples. This area is the southernmost part of Hungary with sub-Mediterranean climate. Our hypothesis was that the effect of the global climate change would be detected here first in the country. We aimed to determine the prevalence and to compare it with previous surveillance data, and to identify the risk factors of infection for a dog.

This study detected 11.7% average prevalence for *D. repens* in the region, which was lower than the previously reported Hungarian dog data. Previous surveys showed that most of the infected dogs were kept by the two largest rivers, the Danube and Tisza where environmental conditions for mosquito breeding are ideal (*Farkas et al.,* 2020). Our study site, the southern Transdanubia also provides appropriate conditions for the intermediate hosts. Large forest-covered areas, wet grasslands, plenty of fishponds characterise the studied sites. Despite the humid environment and the warm climate, our data fell behind the prevalence measured during the first two decades of the century.

Moreover, our data suggest that urban areas carry higher risk of *D. repens* infection for dogs than rural areas, though the latter ones lie closer to wetlands, which are ideal mosquito habitats. The epidemiological role of cities needs further research, but two possible explanations emerged. Urbanised areas can form heat islands, which have higher average temperature than the surrounding natural or semi-natural areas (*Chick et al.,* 2019; *de Jesús Crespo et al.,* 2021). In these circumstances, yearly breeding periods of intermediate hosts can extend, and more generations of mosquitos can develop and act as a disease transmitter.

The very dense dog population of the cities can provide another explanation for the increased health risk of the urban areas. Stray dogs, shelter dogs, and backyard guard dogs might form a reservoir that more important than the natural populations of red foxes and golden jackals.

Our findings confirmed that the age of an adult animal could not influence the infection status. We hypothesised that the risk of infection increased by age. In adult animals, this hypothesis could not be supported, whereas both young and very old animals were found to be positive. Further research should be extended to subadult animals to determine the beginning of the risk period of a dog's life exactly.

The apparent susceptibility of female animals is an interesting phenomenon, which is hardly explicable. The distinction between certain breeds seems more relevant than the uncommonness of females and males. Difference between body size, hair length, skin thickness of different breeds, which characteristics might influence biting success of mosquitoes, proves to be bigger than difference between an 'average' female and male dog. Therefore, susceptibility of the two sexes will need further research with larger sample size.

In our study, neither body size nor hair length proved to have an impact on the infection status. Though both of these factors might affect the biting frequency of mosquitoes. Among backyard guard dogs, which are kept outdoor all-year round, more large breed individuals can be found than among house pets. Though, most outdoor dogs have longer hair than their pet counterparts. The exact role of hair length, body size should be investigated considering also husbandry attributes, which are also very important in infection risk assessment.

We did not get information on preventive actions applied by the owners to protect their dogs against insect bites and/or dirofilariosis. Antiparasitic treatments could also modify the impact of other risk factors such as body size, hair length, breed, age etc. In future research, it would be worth investigating the efficacy of different protective attempts.

CONCLUSIONS

As a result of the recent study, we can conclude that by parasitological investigation of 95 dog blood samples, a 11.7% *D. repens* prevalence could be detected. This value was lower than the prevalence that was confirmed previous studies. Our additional finding was that urban environments posed higher risk of *D. repens* infection for dogs than rural areas. Based on this experience, we hypothesised that urban dog populations could be more important reservoirs of *D. repens* than natural populations of wild canids. This hypothesis will need further research to be confirmed. For analysing the other important risk factors, more information on husbandry and antiparasitic actions should be collected because these conditions can modify the epidemiological impacts of the dogs' natural features.

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