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Methods: The epidemiological research was conducted between February 2008 and October 2010, on a total of 457 dogs.

The modified Knott's method was used to identify the *Dirofilaria* spp. microfilariae. Blood samples positive for microfilariae were examined using the polymerase chain reaction (PCR), at the "Szent István" University, Faculty of Veterinary Science in Budapest, Hungary in two periods 2009 and 2010.

Results: Using the modified Knott's method, 33 dogs were positive for microfilariae (7.2%, 33/457) and using molecular technique, the following species were identified: *D. repens* (18/28) and *D. immitis* (1/28). Five samples (5/33) positive for microfilariae, not examined by PCR were examined by the *Diro Speed®* Test / Heartworm (Bio Veto Test, France).

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Conclusions: This is the first report of canine dirofilariosis in the Timiș County, Romania. This survey shows that there is potential environmental condition for the development of intermediate host mosquito species and the spread of *D. repens* and *D. immitis* at the Western part of Romania, too.

Keywords: *dirofilaria immitis*, *dirofilaria repens*, dog, timiș county, romania.

I. BACKGROUND

Dirofilaria immitis and *Dirofilaria repens* represent the most important filarioid species in Europe because of their pathogenicity on dog's health and because of their zoonotic potential [11]. *D. immitis* is commonly found in the pulmonary arteries and the right ventricle of dogs and causes canine heartworm disease, but it also occurs in cats and humans [18].

D. repens is the less pathogenic form, infesting subcutaneous tissues [14], occasionally, dermal

swelling and subcutaneous nodules containing adult worms may be observed [22].

In the past decade the infections caused by filarioid nematodes in dogs and cats are apparently spreading in different geographic areas [10].

The distribution of *Dirofilaria* spp. in different European countries has been attributed to several factors including the introduction of new species of mosquitoes like *Aedes albopictus* [5], and consequence of climate changes together with increased pet travel [23].

Cutan dirofilariosis is considered an emerging zoonosis in Europe, though most cases of *D. repens* infections are benign in humans because the adult nematode is localized mainly invisible way in subcutaneous tissue. Occasionally this parasite can cause subcutaneous nodules or ocular lesions sporadically it can reach deeper tissues. In lesions the immature or adult nematode can be formed nodules, which can mimic a tumor [15, 17]. Human *D. repens* infection cases have been described from northeastern countries such as Hungary [19], Slovak Republic [16], Poland [24] and Russia [12]. Recent reports are from Croatia [4], Romania [13], Russia [9] and France [15].

II. MATERIALS AND METHODS

a) Study Area

Timiș County is located at the Western Romanian border with Hungary and Serbia, and has an area of 8.696 km². The Timiș landscape is predominantly flat and is crossed from East to South-West by the Bega and Timiș rivers. The climate is classified as moderate continental, with Mediterranean influences. The average annual temperature is 10.7 °C.

b) Blood Sampling

The epidemiological study was carried out from February 2008 to October 2010, with 457 asymptomatic dogs, of different breeds, aged between 6 months and 15 years. In this group of dogs 183 were females and 274 males. Dogs examined were from 42 localities in Timiș County. Some of them belonged to different owners and some dogs were from shelters. Blood samples were collected into vacutainers with EDTA anticoagulant.

c) Modified Knott's test

For this test, 1 ml of blood was used on the EDTA, adding approximately 9 ml of 2% formaldehyde.

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The liquid was centrifuged, and the sediment was mixed with equal parts of 1:1000 methylene blue dyes. The colored sediment was spread on a slide, and covered with coverslip, and was examined under the microscope, using the 10x and 40x light microscope objective.

d) Molecular Techniques

Positive blood samples with microfilariae were examined using molecular techniques, at the Department of Parasitology and Zoology of Faculty of Veterinary Science "Szent István" University in Budapest, Hungary, in 2009 and 2010. The DNA extraction was performed in 100 μ l of blood collected on EDTA, for each positive sample. We used the Blood and Tissue Dneasy kit (QIAGEN). The general primers and the thermal profile described by Casiraghi et al., 2006 [6] were used for the PCR, with species specificity for detecting *D. repens* and *D. immitis* presence. The migration of amplicons was performed by electrophoresis in 1.5% agarose gel. The ethidium bromide dye was used for preparing the agarose gel. After the migration of PCR products through the agarose gel, the gel image with the migrated DNA fragments was photographed using a Kodak EDAS 290 Polaroid system. The PCR-products were sequenced by Biomi Ltd. (Gödöllő, Hungary) directly using the ABI technology, in order to verify the specificity of PCR reactions.

e) *Diro Speed® Test / Heartworm (Bio Veto Test, France)*

Five samples (5/33) positive for microfilariae, not examined by PCR, were investigated to detect *D. immitis* antigen. The examination of the samples was processed according to the manufacturers description.

III. RESULTS

As a result of the examination of blood samples, using the modified Knott's method, the microfilariae were identified in 33 of the 457 dogs examined (Table 1). The prevalence of *Dirofilaria* spp. in dogs, according to the modified Knott's method was 7.2%. The microfilariae examined using the Olympus microscope with video extension, and 400 x objectives, showed the morphology of *D. repens* microfilariae. This means the absence of the cephalic hook, the front end having a slightly tapered shape, and the filiform caudal end presented an "umbrella handle" shape (Figure 1,2,3). The length of the microfilariae observed was between 330-380 μ m.

The dogs infected with *Dirofilaria* spp. belonged to 16 breeds and 17 of them being males and 16 females aged between 2 and 13 years (Table 2, 3).

The size of the fragments amplified for the 12SrDNA in 18 samples (18/28) were ~ 390 base pairs and suggested that the samples isolated from dogs with

microfilaremia belonged to the *D. repens* species, and ~ 450 base pairs at a sample of 28, that suggested an infection with *D. immitis*. An example is shown in Figure 4.

Only one dog tested positive for infection with *D. immitis*, a Rottweiler breed female, aged 11 years, from Timișoara. The result of the infection with *D. immitis*, obtained by PCR, is questionable. At this patient further investigation were not possible. The number of microfilariae (mf) / ml of blood in dogs with dirofilariosis ranged from <30 to 5000 mf / ml of blood. This molecular biology study, performed for the first time in Romania, shows that the dogs diagnosed with microfilaremia were infected with *D. repens* and *D. immitis*. Tested with the Speed®Diro / Heartworm (Bio Veto Test, France), all 5 dogs (5/33) examined were negative for antigens of *D. immitis*.

IV. DISCUSSIONS

Most dog of diagnosed with microfilariae of *Dirofilaria* spp. came from localities crossed by a river, such as Timiș, Albina, Bega Veche, Behela rivers and the Bega Channel (Figure 5). The rivers, the lakes and the ponds are considered as preferred habitats for mosquito's larval development. So far, there are no statistics available concerning the species of mosquitoes identified in Timiș County. The increasingly high temperatures in summer, the mild winters, the large number of vectors, and numerous stray dog and the dog imports, has led to dirofilariosis spread in the Western part of Romania.

The dogs with microfilaremia were considered local cases because they have not left our country and have not been imported from Europe. Taking into consideration the results of this research we concluded that the infection with *D. repens* is endemic in the Western part of Romania.

The number of dogs carried subcutaneous dirofilariosis is growing, and this is why this study should be continued. The *D. immitis* parasitism was not identified so far in the Western part of Romania. The case tested positive by PCR in this survey, is the first one found.

Between 2008 and 2010, several necropsies in dogs, were achieved at the Necropsy Pathology Laboratory in Timișoara, for "finding" *D. immitis*. Special attention was given to the thoracic cavity and heart. Using this technique, only adult *D. repens* were found, located in the subcutaneous tissue in certain body regions. An interesting case was represented by a dog, which presented a *D. repens* nematode in the scrotum after orchidectomy. This surgical procedure was performed in a veterinary clinic EUROPET-FV in the city. In Hunedoara County (central-western part of the country) 92 dogs were tested at the shelter. Two dogs were diagnosed with *D. repens* (2.2%, 2/92) [7]. In 2009

– 2010, in Arad, 30 dogs were tested for microfilariae. A single dog was diagnosed with subcutaneous dirofilariosis (3.3%, 1/30) (Ciocan, unpublished observations).

In Bucharest (Southeast part of the country) the infection with *D. immitis* and *D. repens* of dogs had been confirmed long time ago. Coman et al. (2007) from the "Spiru Haret" University of Veterinary in Bucharest confirms the presence of *D. immitis* in 12 dogs examined (23.1%, 12/52) [8]. Tudor et al. (2008) reported a prevalence of 29.3% (34/116) for the *D. immitis* infection in dogs in the Bucharest area [21]. A year later, in Bucharest the diagnosis of *D. immitis* was confirmed again, after the parasitological examination of 35 dogs [20].

In Iași (Northeastern part of Romania) in March 2009 was reported the first case of infestation with *D. immitis* in dogs, and afterwards 27 new cases were diagnosed in dogs raised by owners and 41 cases in dogs from shelters. A total number of 303 dogs were examined and 68 of them tested positive for microfilariae (Knott's method), with a prevalence of 22.4% (*D. immitis*). Using the Snap Heartworm rapid test, 16 dogs tested positive for *D. immitis* (5.3%). The first case of *D. repens* infestation was diagnosed in February 2009 [1, 2]. Acatrinei et al. (2008) confirms the presence of *D. immitis* in four dogs from the southeast part of the country (Tulcea) [3].

V. CONCLUSIONS

Following the research, we concluded that the prevalence of *D. repens* infection is high in Timiș region. The Western Region meets all the conditions for developing of Culicidae larvae and therefore canine dirofilariosis may increase in the future.

The presence of stray dogs in many places represents a reservoir in the spread of both dirofilarioses in other animals and humans. It is pointed out that Timiș County bordered with Hungary and Serbia where the subcutaneous dirofilariosis cases in dogs are very frequent. A special attention should be given on imported dogs. Veterinarians and dog owners should be informed of the presence of *D. immitis* and *D. repens* in this part of Romania and therefore, it is very important to inform the dog owners about the available prevention possibilities.

VI. COMPETING INTERESTS

The author(s) declare that they have no competing interests.

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Figure 1 : *D. repens* microfilaria in a dog, modified Knott's method (400x objectives)



Figure 2 : *D. repens* microfilaria in a dog, modified Knott's method (400x objectives)



Figure 3 : *D. repens* microfilaria in a dog, modified Knott's method (400x objectives)

Table 1 : The *D. repens* infection of dogs (n=457) correlated with breed, age and gender No 33/457

Breed	No. Pos	Age (years) 1-5	Age (years) 6-10	Age (years) ≥ 11	Sex	
					Male	Female
Half breed	8/33	6	1	1	4	4
Caucasian Shepherd	3/33	1	2	0	2	1
Bernese Shepherd	1/33	0	1	0	1	0
Amstaff	2/33	0	2	0	2	0
Bordeaux Dog	1/33	1	0	0	0	1
American Cocker	1/33	1	0	0	0	1
Cocker Spaniel	1/33	0	1	0	1	0
Doberman	2/33	0	2	0	1	1
German Wirehaired Pointer	1/33	1	0	0	1	0
Poodle	1/33	0	0	1	0	1
Rottweiler	2/33	0	1	1	0	2
German Shepherd	6/33	3	3	0	1	5
Mioritic Sheepdog	1/33	1	0	0	1	0
Labrador	1/33	1	0	0	1	0
Cane Corso	1/33	1	0	0	1	0
Dalmatian	1/33	0	1	0	1	0
Total	33	16	14	3	17	16

Legend: n: number of examined dogs; No. Pos: Number of positives dogs

Table 2 : The prevalence of *D. repens* in dogs correlated with age

Age	n	Knott's test positive	Prevalence (%)
1-5	279	16/279	5.7%
6-10	133	14/133	10.5%
≥11	45	3/45	6.6%
Total	457	33/457	7.2%

Legend: n: number of examined dogs

Table 3 : The prevalence of *D. repens* in dogs correlated with gender

Sex	n	Knott's test positive	Prevalence (%)
Males	274	17/274	6.2%
Females	183	16/183	8.7%
Total	457	33/457	7.2%

Legend: n: number of examined dogs

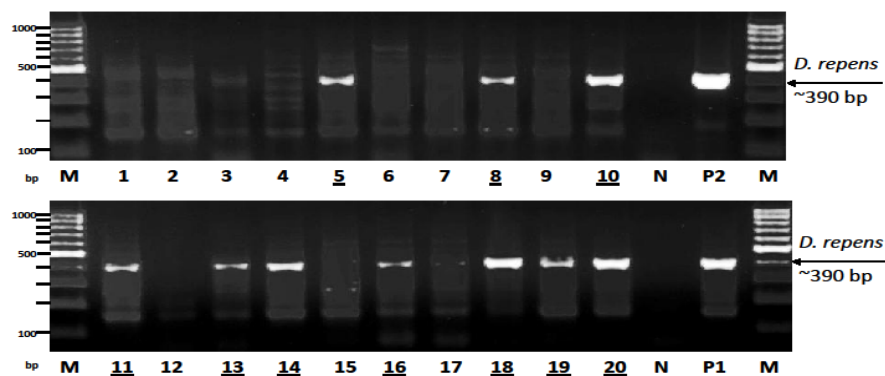


Figure 4 : The DNA products amplified with the specific *D. repens* primers show ten positive samples (underlined numbers)

Legend: M: molecular marker 100-1000 bp; bp: base pairs; *D. repens*: *Dirofilaria repens*; N: negative controls; P1, P2: positive controls.

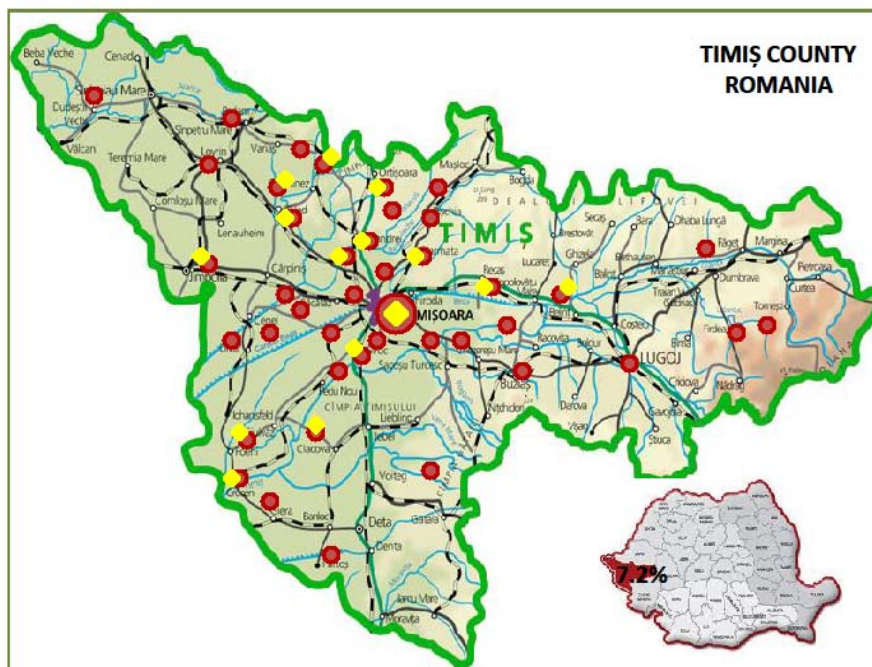


Figure 5 : The regional distribution of dogs examined is demonstrated by red circles, and origin of the positive dogs is indicated by yellow rhombus