Canine dirofilarioses in two noninvestigated areas of Serbia: epidemiological and genetical aspects

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| Keyword: | Filaria, Epidemiology, Genetics, Diagnosis, Zoonosis |

Abstract:

In 2009 canine filarioses were investigated in two northern areas of Serbia (Pančevo and Veliko Gradište) applying morphometry, biochemical staining, kit to detect Dirofilaria immitis antigens, and 3 experimental ELISAs to detect antibodies to D. repens and D. immitis somatic/metabolic polyproteins, and to the recombinant Wolbachia Surface Protein (rWSP). Moreover, molecular tools were applied to analyze the phylogenetic relationships of the isolated strains with that already studied. Microfilariae, detected in 21/122 dogs (17.2%), were identified as D. repens (n=21) and D. immitis (n=2). Neighbor-joining analysis matched D. immitis isolates to other European strains deposited in GenBank, whereas for D. repens suggests the need of further researches on the intraspecific variability. D. immitis antigens were found in further 13 animals with occult infection; all the above 15 heartworm positive dogs had, in addition, antibodies to this parasite, which were detected in further 13 subjects, indicating an overall heartworm disease seroprevalence of 22.9%. Serology for D.repens evidenced antibodies in 42,6% of the dogs, but failed to recognize as positive 4 microfilaremic dogs. Serology against WSP proved positive in only 5.7% of the dogs. As for the two different areas, the prevalence of microfilariae and/or D. immitis antigens, mainly due to D. repens microfilaremic animals, was not-significantly higher in Veliko Gradište.
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(33.3%) than in Pančevo (22%). However, serology evidenced a different epidemiological picture: heartworm infection occurred more often than it appeared in both areas, and antibodies to dirofilarial nematodes were detected in 72.9% of dogs living in Pančevo, therefore more than living in Veliko Gradište (57.1%). No risk factors for infection were evidenced, confirming data on the uselessness of prophylactic drugs at least against D. repens, and suggesting the presence, in these areas, of by day, sunrise or sunset biting mosquitoes as important vectors. The need of appropriate entomological studies is stressed.
Canine dirofilarioses in two noninvestigated areas of Serbia: epidemiological and genetical aspects

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Running title: Canine dirofilarioses in Serbia

Key words: Dirofilaria, Serbia, epidemiology, genetics
Abstract

In 2009 canine filarioses were investigated in two northern areas of Serbia (Pančevo and Veliko Gradište) applying morphometry, biochemical staining, kit to detect *Dirofilaria immitis* antigens, and 3 experimental ELISAs to detect antibodies to *D. repens* and *D. immitis* somatic/metabolic polyproteins, and to the recombinant *Wolbachia* Surface Protein (rWSP). Moreover, molecular tools were applied to analyze the phylogenetic relationships of the isolated strains with that already studied. Microfilariae, detected in 21/122 dogs (17.2%), were identified as *D. repens* (n=21) and *D. immitis* (n=2). Neighbour-joining analysis matched *D. immitis* isolates to other European strains deposited in GenBank, whereas for *D. repens* suggests the need of further researches on the intraspecific variability. *D. immitis* antigens were found in further 13 animals with occult infection; all the above 15 heartworm positive dogs had, in addition, antibodies to this parasite, which were detected in further 13 subjects, indicating an overall heartworm disease seroprevalence of 22.9%.

Serology for *D. repens* evidenced antibodies in 42.6% of the dogs, but failed to recognize as positive 4 microfilaremic dogs. Serology against WSP proved positive in only 5.7% of the dogs. As for the two different areas, the prevalence of microfilariae and/or *D. immitis* antigens, mainly due to *D. repens* microfilaremic animals, was not significantly higher in Veliko Gradište (33.3%) than in Pančevo (22%). However, serology evidenced a different epidemiological picture: heartworm infection occurred more often than it appeared in both areas, and antibodies to dirofilarial nematodes were detected in 72.9% of dogs living in Pančevo, therefore more than living in Veliko Gradište (57.1%). No risk factors for infection were evidenced, confirming data on the uselessness of prophylactic drugs at least against *D. repens*, and suggesting the presence, in these areas, of by day, sunrise or sunset biting mosquitoes as important vectors. The need of appropriate entomological studies is stressed.
Introduction

Dirofilarioses are mosquito-borne diseases, endemic in areas with temperate, tropical and subtropical climatic conditions. The species of major interest in veterinary and public health are *Dirofilaria immitis* (cosmopolitan) and *Dirofilaria repens* (found only in the Old World). *D. immitis*, well known as heartworm due to its location in the vertebrate host (it inhabits the right ventricle and pulmonary arteries), can cause patent infection in dogs, cats and foxes, but also in lions, ferrets, sea lions, and even in horses, bears, orangutans and humans (Duran-Struuck et al. 2001). Most infected dogs do not show any symptoms for months or years, but the infection can became fatal when the parasitic burden induces severe damages to arteries and right cardiac chambers (Bolio-Gonzales et al. 2007). On the contrary, *D. repens* resides in the cutaneous/subcutaneous tissue of the animals (but often also humans) where it induces mild infection, whose severity is related only to the parasitic load and to the work of the animal (Cringoli et al. 2001). This species is the most frequent etiological agent of human dirofilariosis (Pampligione et al. 2000; Džamić et al. 2009; Genchi et al. 2011).

In usual hosts dirofilariae produce circulating in the peripheral blood microfilariae, source of infection for the female vector mosquitoes, and useful to diagnostic purposes. Searching for microfilariae may be fruitless in case of low parasitaemia or of occult infection (adults are present, but microfilariae can not be found in the blood because the dog harbors only female or only male specimens, or in case of female old age or sterility). Luckily, infections induced by *D. immitis* can be immunologically evidenced, using commercial kits available for the detection of both parasite antigens and host antibodies against the filarial worm. Such diagnostic tools do not exist for the other species that can be evidenced only in experimental assays (Cancrini et al. 2001; Marcos-Atxutegi et al. 2004).

In Serbia, dogs are affected by both *Dirofilaria* species and by *Acanthocheilonema reconditum* (Tasić et al. 2008). The severity of heartworm disease, the need of information about the
geographical areas involved in canine filarioses and, in addition, the recent report of further human infections (Tasić et al. 2011) urged us to carry on with investigations in Serbia, applying microscopy and both commercial and non-commercial immunological diagnostics, and estimating the risk factors for dirofilariosis in asymptomatic dogs. A last aim was the characterization of the isolated strains by the genetical point of view and their phylogenetic relationships with other studied strains.

**Materials and methods**

**Study area**

The study was conducted in northern Serbia, which proved endemic for dirofilarioses. The chosen areas were Pančevo (44,86°N; 20,64°E, in Vojvodina region) and Veliko Gradište (44,76°N; 21,51°E, at the boundary between Vojvodina and Central Serbia) (Fig.1). The cities are included in areas considered at risk for dirofilariosis on the basis of the climate, the geographical characteristics and the presence of many mosquito species possibly implicated in the parasite transmission.

**Dog population**

From Jun until September 2009, a total of 122 animals (59 resident in Pančevo and 63 in Veliko Gradište areas) were randomly selected. For each animal, dog owners and shelter attendants filled an anamnestic form collecting data on the dog age, sex, breed, type of housing, dog profile, and prophylaxis anti-heartworms. Dogs included in the study population never moved away from the study area.

**Blood samples**

Blood samples (10 mL) were drawn from the cephalic vein of each animal between 9 am and 15 pm. Knott technique and a commercial filtration test (Difil-test®, Evsco Pharmaceuticals, Buena, NJ, USA) were applied to concentrate, respectively, a volume of 1 mL and 4 mL of blood before the microscopic analysis. Remaining 5 mL were used for the antigen detection and, after blood coagulation, for serological tests. Clots of positive samples were submitted to genetic analyses.
Microfilariae identification

Microfilariae were identified on the basis of morphological and morphometric characteristics calculated by Laboratory Universal Computer Image Analysis system (Lucia M, 1996, Czechoslovakia). Then, they were further analysed to evidence the somatic distribution of acid phosphatase activity (Chalifoux and Hunt, 1971) and biochemically discriminate between *D. repens* and *D. immitis*.

Immunological investigations

Blood from each dog was examined by means of commercial kits available to detect adult female *D. immitis* circulating antigens (Witness Dirofilaria®, Synbiotics, Lyon, F), and according to the manufacturer’s instructions. In addition, 3 experimental ELISAs that use as antigen *D. repens* (Dr) and *D. immitis* (Di) somatic/metabolic polyproteins (Cancrini et al. 2001), and the recombinant *Wolbachia* Surface Protein (rWSP) (Bazzocchi et al. 2000) were applied to each serum to detect the infection. In fact, bacteria belonging to the genus *Wolbachia* are filarial endosymbionts present in lateral cordae of all developmental stages and in reproductive organs of both sexes of almost all filarial species, *D. repens* and *D. immitis* included (but absent in *Acanthocheilonema* species). Therefore, the presence of antibodies to WSP in dogs is indicative of dirofilariosis.

Molecular analyses

Genomic DNA was extracted from positive blood clots (NucleoSpin tissue, Macherey-Nagel, Germany), submitted to PCR amplification with specific for *D. repens* (R1-R2) and *D. immitis* (I1 - I2) primers (Favia et al. 1996), and then sequenced (Eurofins MWG Operon, Italy). Sequences were assembled, corrected by visual analysis of the electropherogram (Bioedit v.7.0.2, Hall, 1999) and subjected to Blast Identity Search (NCBI) to study their identity with sequences deposited in GenBank. Nucleotide sequences were aligned with corresponding sequences of *D.immitis* (5Sribosomal RNA) and *D.repens* (DNA repeat region) to construct phylogenetic trees (neighbor-
joining method, MEGA 5.0 software package, Tamura et al. 2011) that were rooted by including as outgroup *Toxocara leonina* (GenBank accession no. HM800922.1).

**Statistical analyses**

Data are presented as mean ± SD or median and range, with P ≤ 0.05 indicating statistical significance. Comparisons between groups were made using the Mann-Whitney test and the Fisher exact test (the chi-square test for more than two groups) for nominal variables and for non-parametric data, respectively. The individual dog data were analysed by the logistic regression model, using both *D. repens* and *D. immitis* status as a dependent variable. The independent variables were tested in a multivariate model by stepwise forward method. At each step, the least significant variable was removed from the model until all remaining variables were significant at p < 0.05. All analyses were performed using the SPSS 14.0 software.

**Results**

The animals checked (56 males and 66 females), 1-12 years old, were grouped in hunting, watch, stray, service dogs and pets. Considering their shelter, nutrition, care, training, working, health protection, sanitary controls, 38 of them were dogs bred in partially controlled life conditions (PCLC) and the remaining 84 subjects lived in uncontrolled life conditions (ULC),

Parasitological results are summarized in Table 1. Microfilariae (mf) were detected in 21/122 dogs (17.2%), which harbored *D. repens* (n=21) and *D. immitis* (n=2), also in co-infection (2 cases). Mean body length and width of 2,719 measured microfilariae were: 360.9 ± 8.5 µm x 7.48 ± 0.35 µm for *D. repens* (n=2,400), and 305.38 ± 12.51 x 6.43 ± 0.42 for *D. immitis* (n=319).

Histochemical staining showed the expected acid phosphatase distribution: one red-stained spot at the excretory pore and one at the anal pore in *D. immitis*, only one red spot at the anal pore in *D. repens* (Figure 2).

*D. immitis* antigens were detected in the blood of 15 (12.3%) dogs (two of them with a confirmed presence of circulating microfilariae, and the remaining 13 as occult infection). All the above 15
positive animals had also antibodies to this parasite, which were detected in further 13 subjects, indicating an overall heartworm disease seroprevalence of 22.9%.

Antibodies against *D. repens* poliproteins were found in 52/122 (42.6%) dogs (17 microfilaremic and 35 amicrofilaremic subjects), and that against WSP in only 7 (5.7%) dogs (2 amicrofilaremic and without dirofilarial antibodies, 4 having *D. repens* microfilariae and antibodies to both *D. repens* and *D. immitis*, and 1 showing antibodies to *D. repens*). However, serology proved negative to *D. repens* in 4 microfilaremic dogs.

Therefore, the overall dirofilariosis seroprevalence was 63.1% (77/122), and the overall prevalence evidenced by all the analyses amounted to 68%. Serology indicated that contacts with *D. repens* occur markedly more frequently (p=0.001) than with *D. immitis*.

As for the two different areas, the prevalence of proven dirofilariosis (microfilariae and/or *D. immitis* antigens), mainly due to *D. repens* microfilaremic animals, was higher in Veliko Gradište (33.3%) than in Pančevo (22%), but not-significantly (p=0.234); heartworm (microfilariae/antigens) showed a not significantly lower than *D. repens* prevalence (13.9% vs 17.2%; p=0.101), without difference in the two studied areas. However, serology evidenced a different epidemiological picture: heartworm infection occurred more often than it appeared, and most dogs living in Pančevo (72.9%) were reactive (≈56% to *D. repens*, and ≈19% to *D. immitis*), therefore more than living in Veliko Gradište (57.1%), where the two dirofilariae showed a more balanced seroprevalence (≈30% and 27%, respectively). The overall infection rate evidenced by all diagnostic tools was 79.7% in Pančevo and 57.1% in Veliko Gradište, suggesting no significant epidemiological differences (p=0.103).

No significant differences were found between infected and uninfected dogs by any considered factor. The presence of ectoparasites proved significant at the 10% level in the univariate analysis but, when included in the multivariate regression model, turned out a no significant risk factor (Tables 2-3).
Finally, *D. immitis* proved closely related to the strains reported in Italy (EU360965-64) and Japan (D87041) (100%, 99.5% and 99.7% identity, respectively); *D. repens* totally matched with an Indian strain and is less related to other strains described in the same country (identity: 86.6% and 87.9%).

**Discussion**

The present study aimed to extend researches on dirofilarioses in one more area of Vojvodina (a region proven hyperendemic for *D. repens* and endemic for *D. immitis* in 2008), and in the territory between Vojvodina and the Central Serbia. *D. immitis* and *D. repens* were identified in both areas, whereas *A. reconditum*, formerly reported in Serbia, was not discovered. *D. immitis* proved closely related to the strains present in Genebank, whereas *D. repens* (DNA repeat region) clusters only with one out of the Indian strains, therefore it requires further researches on the intraspecific variability.

*D. repens* was more prevalent (17.2%) than *D. immitis* (1.6%), even if the detection of its antigens in further 13 occult infections sent the *D. immitis* prevalence up 12.3%. Serology, positive in 63.1% of the examined dogs, confirmed the above trend: the contacts with *D. repens* occur markedly more frequently than with *D. immitis* (42.6% versus 22.9%). Differences in the spreading of the two dirofilarial species could be ascribed to the mosquito population present in the studied areas, which could include vectors more efficient for *D. repens* than *D. immitis* (Cancrini et al. 1992), or to ivermectin that prevents heartworm infection. The analysis of the last factor applied to dogs without considering serological results, which are of difficult interpretation (development stopped by the drug or recent infection in progress?), proved it is non-influential, and the poor efficacy of ivermectin to prevent *D. repens* infections, suggested by laboratory data (Cancrini et al. 1989), seems confirmed. By the technical point of view, the assay that recognizes antibodies to *Wolbachia* proved less sensitive and not-matching in two cases, probably because antibody production depends on the *Wolbachia* quantity released in the blood (therefore on the parasitic burden and the
larval/adult stage of the present dirofilaria) and because the cytokine synthesis patterns activated by this bacterial antigen is different from that activated by the worms (Simon et al. 2009).

Concentration techniques and searching for antigens evidenced infection rates high (22-33.3%) but lower (p=0.001) than observed in other regions of Vojvodina, like the cities of Kikinda and Zrenjanin (>60%, Tasić et al. 2008). However, the very high seroprevalence of antibodies against dirofilarial nematodes here evidenced suggests that in the study area many dogs become infected, even if the infection remains undetected by circulating microfilariae and/or by antigens. Since serological tests we applied should be scarcely affected by cross-reactions (specificity 90%, positive predictive value 75%) and are sensitive (can detect 2-months old infections), we suppose that the age of the checked animals (average≈4 years) may have affected the microscopy results. In fact, young animals have been exposed to the infection for a short time and, in addition, the worms could not have completed their slow development to mature and mated adults producing circulating microfilariae. Furthermore, occult/abortive infections could have been favoured by the use of antibiotic drugs as tetracyclines that, reducing or killing the obligate endosymbionts wolbachiae, indirectly inhibit the development and reproduction of filarial nematodes (Casiraghi et al. 2001).

The unexpected uselessness of life controlled conditions (at least a protection from the mosquito bites during the night) lead to suspect that by day, sunrise or sunset biting mosquitoes can actively participate to the infection, and opens the questions about the vectors. In detail, also the differences observed between the studied areas, which seem unrelated to dog samplings, climatic and geographic characteristics, mosquito abundance, could be rather ascribed to the mosquito species that act as vectors, an epidemiological aspect about undigested. In fact, to date only Anopheles maculipennis, among the many Culicidae present and recognized elsewhere as experimental or natural vectors, has been proposed as a possible vector for D.immitis in Serbia (Simić, 1928). Therefore, entomological researches based on dog-baited traps and molecular diagnostics, which can recognize -for each dirofilaria species- mosquito vector species and their relative importance, are advisable, as proven by the results obtained in other countries (Cancrini and Gabrielli, 2007).
Conclusions

Canine dirofilariosis is endemic also in this area of Serbia, even if with decreasing prevalence when Central Serbia is reached; luckily for the dogs, infections are mainly due to *D. repens*, a less pathogen filarial species. However, the application of non-commercial serological tests detected a very active transmission level of both species, an alarming result that matches with the increasing number of detected human infections. Therefore, entomological investigations are needed to identify the mosquito species actually involved in the parasites transmission and the vector efficiency of each of them, which are data essential to start appropriate control programs. Finally, the intraspecific genetic heterogeneity evidenced in *D. repens* DNA repeat region merits further investigations.

Authors Disclosure Statement

The authors disclose any commercial associations and declare that no competing financial interests exist.

References


Cancrini G, Montoya MN, Prieto G & Bornay-Linares FJ. Current advances in experimental
diagnosis of human and animal dirofilariosis. In: Heartworm infection in humans and animals:
Simon F. and Genchi C. Eds; 2001: 103-120.

Cancrini G, Della Torre A, Coluzzi M. Different probabilities of transmission of *Dirofilaria immitis*
and *D. repens* by *Culex pipiens*. Proc VIth EMOP 1992; 90.

Parassitologia 1989; 31: 177-182.

Casiraghi M, Mazzocchi G, Sacchi L, Bandi C. The *Wolbachia* endosymbionts of *Dirofilaria*

Chalifoux L, Hunt RD. Histochemical differentiation of *D. immitis* and *D. reconditum*. JAVMA,

Cringoli G, Rinaldi L, Veneziano V, Capelli G. A prevalence survey and risk analysis of filariosis

Duran-Struuck JC, Hernandez HA. *Dirofilaria immitis* prevalence in a canine population in Samana

Džamić AM, Arsić-Arsenijević V, Radonjić I, Mitrović S, Marty P, Kranjčić-Zec, IF. Subcutaneous

Favia G, Lanfrancotti A, Della Torre A, Cancrini G, Coluzzi M. Polymerase chain reaction-
identification of *Dirofilaria repens* and *Dirofilaria immitis*. Parasitology 1996;113:567-71.

2011;11:1307-17.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for


Table 1. Results of microscopic and immunological analyses carried out on the checked dogs, by area.

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<thead>
<tr>
<th>Dogs positive to</th>
<th>Pančevo</th>
<th>V. G. n=</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>mf</td>
<td>6 (10.2)</td>
<td>15 (23.8)</td>
<td>21 (17.2)</td>
</tr>
<tr>
<td>Di Ag</td>
<td>8 (13.6)</td>
<td>7 (11.1)</td>
<td>15 (12.3)</td>
</tr>
<tr>
<td>Microscopy/antigens</td>
<td>13 (22.0)</td>
<td>21 (33.3)</td>
<td>34 (27.9)</td>
</tr>
<tr>
<td>Dr Ab</td>
<td>33 (55.9)</td>
<td>19 (30.2)</td>
<td>52 (42.6)</td>
</tr>
<tr>
<td>Di Ab</td>
<td>11 (18.6)</td>
<td>17 (27.0)</td>
<td>28 (22.9)</td>
</tr>
<tr>
<td>WSP Ab</td>
<td>1 (1.7)</td>
<td>6 (9.5)</td>
<td>7 (5.7)</td>
</tr>
<tr>
<td>Serology</td>
<td>43 (72.9)</td>
<td>34 (57.1)</td>
<td>77 (63.1)</td>
</tr>
<tr>
<td>Overall prevalence</td>
<td>47 (79.7)</td>
<td>36 (57.1)</td>
<td>83 (68.0)</td>
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</table>
Table 2. Univariate logistic regression analysis of risk factors for infection. B: B coefficient; SE: standard error; Wald: Wald chi-square test; OR: odds ratio, 95%CI: confidence interval.

<table>
<thead>
<tr>
<th>Factor</th>
<th>B</th>
<th>SE</th>
<th>Wald</th>
<th>p</th>
<th>OR</th>
<th>(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.034</td>
<td>0.071</td>
<td>0.229</td>
<td>0.632</td>
<td>1.035</td>
<td>(0.900-1.189)</td>
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<tr>
<td>Female</td>
<td>0.137</td>
<td>0.391</td>
<td>0.123</td>
<td>0.725</td>
<td>1.147</td>
<td>(0.553-2.467)</td>
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<tr>
<td>Race</td>
<td>-</td>
<td>-</td>
<td>15.065</td>
<td>0.447</td>
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<tr>
<td>Purpose</td>
<td>-</td>
<td>-</td>
<td>9.669</td>
<td>0.041</td>
<td></td>
<td>-</td>
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<tr>
<td>ULC</td>
<td>-0.390</td>
<td>0.434</td>
<td>0.806</td>
<td>0.369</td>
<td>0.677</td>
<td>(0.289-1.586)</td>
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<tr>
<td>Presence of ectoparasites</td>
<td>0.839</td>
<td>0.441</td>
<td>3.619</td>
<td>0.057</td>
<td>2.313</td>
<td>(0.975-5.487)</td>
</tr>
</tbody>
</table>

Table 3. Multivariate logistic regression analysis of risk factors for infection (stepwise forward method). B: B coefficient; SE: standard error; Wald: Wald chi-square test; OR: odds ratio, 95%CI: confidence interval.

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<th>p</th>
<th>OR</th>
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<tr>
<td>Purpose</td>
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<td>-</td>
<td>6.870</td>
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<td>Presence of ectoparasites</td>
<td>0.317</td>
<td>0.640</td>
<td>0.245</td>
<td>0.621</td>
<td>1.372</td>
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<tr>
<td>Constant</td>
<td>0.486</td>
<td>1.295</td>
<td>0.141</td>
<td>0.708</td>
<td>1.625</td>
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Figure 1. Study area
Figure 2. *D. repens* (left) and *D. immitis* (right) microfilariae, showing different phosphatase activity.
Figure 3. Phylogenetic relationships of *D. immitis* (5S ribosomal RNA) and *D. repens* (DNA repeat region) strains found in Serbia and that deposited in GenBank.
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<tr>
<td>N (%) (95%CI)</td>
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<td>N (%) (95%CI)</td>
<td></td>
</tr>
<tr>
<td>mf</td>
<td>6 (10.2) (2.5-17.9)</td>
<td>15 (23.8) (13.3-34.3)</td>
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</tr>
<tr>
<td>Di Ag</td>
<td>8 (13.6) (4.8-22.4)</td>
<td>7 (11.1) (3.3-18.9)</td>
<td>15 (12.3) (6.5-18.1)</td>
</tr>
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<td>Microscopy/antigens</td>
<td>13 (22.0) (11.4-32.6)</td>
<td>21 (33.3) (21.7-44.9)</td>
<td>34 (27.9) (19.9-35.9)</td>
</tr>
<tr>
<td>Dr Ab</td>
<td>33 (55.9) (43.2-68.6)</td>
<td>19 (30.2) (18.9-41.5)</td>
<td>52 (42.6) (33.8-51.4)</td>
</tr>
<tr>
<td>Di Ab</td>
<td>11 (18.6) (8.7-28.5)</td>
<td>17 (27.0) (16-38)</td>
<td>28 (22.9) (15.4-30.4)</td>
</tr>
<tr>
<td>WSP Ab</td>
<td>1 (1.7) (0.3-8.9)</td>
<td>6 (9.5) (2.3-16.7)</td>
<td>7 (5.7) (1.6-9.8)</td>
</tr>
<tr>
<td>Serology</td>
<td>43 (72.9) (61.6-84.2)</td>
<td>34 (57.1) (44.9-69.3)</td>
<td>77 (63.1) (54.5-71.7)</td>
</tr>
<tr>
<td>Overall prevalence</td>
<td>47 (79.7) (69.4-89.9)</td>
<td>36 (57.1) (44.9-69.3)</td>
<td>83 (68.0) (59.7-76.3)</td>
</tr>
</tbody>
</table>
Table 2. Univariate logistic regression analysis of risk factors for infection. B: B coefficient; SE: standard error; Wald: Wald chi-square test; OR: odds ratio, 95%CI: confidence interval.

<table>
<thead>
<tr>
<th>Factor</th>
<th>B</th>
<th>SE</th>
<th>Wald</th>
<th>p</th>
<th>OR</th>
<th>(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.034</td>
<td>0.071</td>
<td>0.229</td>
<td>0.632</td>
<td>1.035</td>
<td>(0.900-1.189)</td>
</tr>
<tr>
<td>Female</td>
<td>0.137</td>
<td>0.391</td>
<td>0.123</td>
<td>0.725</td>
<td>1.147</td>
<td>(0.553-2.467)</td>
</tr>
<tr>
<td>Race</td>
<td>-</td>
<td>-</td>
<td>15.065</td>
<td>0.447</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Purpose</td>
<td>-</td>
<td>-</td>
<td>9.669</td>
<td>0.041</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ULC</td>
<td>-0.390</td>
<td>0.434</td>
<td>0.806</td>
<td>0.369</td>
<td>0.677</td>
<td>(0.289-1.586)</td>
</tr>
<tr>
<td>Presence of ectoparasites</td>
<td>0.839</td>
<td>0.441</td>
<td>3.619</td>
<td>0.057</td>
<td>2.313</td>
<td>(0.975-5.487)</td>
</tr>
</tbody>
</table>
Table 3. Multivariate logistic regression analysis of risk factors for infection (stepwise forward method). B: B coefficient; SE: standard error; Wald: Wald chi-square test; OR: odds ratio, 95%CI: confidence interval.

<table>
<thead>
<tr>
<th>Factor</th>
<th>B</th>
<th>SE</th>
<th>Wald</th>
<th>p</th>
<th>OR</th>
<th>(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purpose</td>
<td>-</td>
<td>-</td>
<td>6.870</td>
<td>0.143</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Presence of ectoparasites</td>
<td>0.317</td>
<td>0.640</td>
<td>0.245</td>
<td>0.621</td>
<td>1.372</td>
<td>(0.392-4.808)</td>
</tr>
<tr>
<td>Constant</td>
<td>0.486</td>
<td>1.295</td>
<td>0.141</td>
<td>0.708</td>
<td>1.625</td>
<td></td>
</tr>
</tbody>
</table>
Study area
50x57mm (300 x 300 DPI)
D. repens (left) and D. immitis (right) microfilariae, showing different phosphatase activity

50x16mm (300 x 300 DPI)
Phylogenetic relationships of D. immitis (5S ribosomal RNA) and D.repens (DNA repeat region) strains found in Serbia and that deposited in GenBank.
Figure 1. Study area

Figure 2. *D. repens* (left) and *D. immitis* (right) microfilariae, showing different phosphatase activity

Figure 3. Phylogenetic relationships of *D. immitis* (5S ribosomal RNA) and *D. repens* (DNA repeat region) strains found in Serbia and that deposited in GenBank.