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The first molecular identification of *Dirofilaria immitis, Dirofilaria repens* and *Leishmania infantum* in dogs from Central Balkan

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Simple Summary: The current high mobility of pets in Europe demands ~~a safe management and~~ more detailed knowledge of the geographical distribution and prevalence of major health threatening pathogens. Amongst them are *Dirofilaria immitis* and *Dirofilaria repens*, respectively the causative agents of cardio-pulmonary and subcutaneous dirofilariosis in dogs and cats. Both *Dirofilaria* species can cause human infections. In addition, leishmaniosis is the only tropical vector-borne disease that has been considered endemic in southern Europe, primarily in the Mediterranean. Results of this survey showed that Central Balkan is an endemic region for canine dirofilariosis. Coupled with our detection of leishmaniosis in dogs from North Macedonia and its' ability to spread to neighboring countries, results of this study warn us that adequate preventive measures are needed to combat the spread of dirofilariosis and leishmaniosis and preserve the health of both dog and human population of this region.

Abstract: Dirofilariosis and leishmaniosis are severe parasitic diseases in dogs, and their causative agents can also be pathogenic to humans. In this study, we conducted a multicentric survey in the regions of Serbia and North Macedonia, with the goal to establish an epidemiological scenario of dirofilariosis and leishmaniosis on the territory of Central Balkan. Using molecular analyses, a total of 535 dogs from Northern Serbia (NS), Southern Serbia (SS) and North Macedonia (NM) were screened for the presence of *Dirofilaria* spp. and *Leishmania* spp. We confirmed that Central Balkan is an endemic region for dirofilariosis in dogs. *Dirofilaria (D.) immitis* was found to be the dominant species in this area with the highest prevalence of 8.75% in NM, followed by NS (6.68%) and a significantly lower prevalence in SS (1.51%). Two dogs (2.5%) from NM were positive for leishmaniosis. None of the dogs from Serbia tested positive for *Leishmania* spp. High prevalence and dominance of *D. immitis* species, and the rising threat of leishmaniosis spread to the territory of Serbia suggest that preventive measures are of a great necessity to combat the spread of these vector-borne zoonoses.

Keywords: Dirofilaria spp., Leishmania spp., Central Balkan., Dogs

1. Introduction

Determining geographical distribution of infective agents, their reservoir, and their source in particular countries is a prerequisite for creating a proper strategy and effective preventive measures that will preclude the spread of vector-borne zoonoses (VBZ) in Europe. ~~Furthermore, successful disease prevention and management primarily depend on the data obtained by continuous research.~~

~~Dirofilariosis~~ and leishmaniosis are severe parasitic diseases in dogs, with a high zoonotic potential. *Dirofilaria (D.) immitis* is commonly known as heartworm because when found in the heart and large blood vessels, this parasite can cause life-threatening disease in dogs, inducing severe damage to arteries and right cardiac chambers [1]. On the contrary, *D. repens* causes mild infection of cutaneous/subcutaneous tissue of the animals [2]. However, this species is the dominant etiological agent of human dirofilariosis, usually manifested as superficial infection (subcutaneous or ocular) [3, 4, 5]. On the other hand, *D. immitis*, with rising prevalence in Europe, may cause visceral disease in humans, most frequently presented as lung nodules [6].

In addition, leishmaniosis is the only tropical vector-borne disease that has been considered endemic in southern Europe, primarily in the Mediterranean [7]. *Leishmania (L.) infantum* is a causative agent of visceral and cutaneous form of the infection. High prevalence of asymptomatic ~~patients~~ and wide distribution of natural hosts represent crucial predispositions for the occurrence of leishmaniosis, but also provide a possibility of spreading the disease to non-endemic regions in the north of the continent [8]. Consequently, based on literature data, there has been an increase of imported cases of leishmaniosis in non-endemic regions of Europe. Canine leishmaniosis is a systemic disease with lymphadenomegaly, skin and eye damage, epistaxis, onychogryphosis, loss of body weight and weakness as the most common clinical manifestations, followed by less frequent symptoms. In humans, visceral leishmaniosis is a severe systemic disease presented with a wide spectrum of clinical manifestations depending on the host's immune response including fever, headache, weakness, weight loss, splenomegaly, hepatomegaly, lymphadenopathy, anemia, thrombocytopenia, petechial hemorrhage and agranulocytosis in advanced cases [9].

From the epidemiological point of view, the prevalence of VBZ correlates with climatic and geographic characteristics of the particular region and, more importantly, the presence of competent vectors. Recently, entomological collection carried out on the territory of the Central Balkan region detected biological vectors for *Dirofilaria* species, such as *Culex pipiens*, biotype *molestus*, and *Aedes vexans*, as well as for *Leishmania* sp., such as *Phlebotomus (P.) papatasi*, *P. perfiliewi*, *P. neglectus* and *P. mascittii*, in Serbia [10]. Competent vectors for *Dirofilaria* spp.-microfilariae were also collected on the territory of North Macedonia (unpublished data from the National project of Macedonian Ecological Society).

These results inspired us to conduct a systemic survey in the regions of Serbia and North Macedonia, try and get results from non-investigated areas, in order to establish an epidemiological scenario of dirofilariosis and leishmaniosis on the territory of Central Balkan region.

2. Materials and Methods

2.1. Study design

The survey was designed as a systemic, prospective, multicentric, international, laboratory-based study conducted from 2018**.** to the end of 2019. In 2018, the Public Health Institute of Niš sent the invitation to veterinarians from Northern Serbia (NS), the southern part of the country (Southern Serbia - SS), and from North Macedonia (NM) to obtain samples from dogs for molecular analyses. The research was conducted at Public Health

Institute, Faculty of Medicine, University of Niš; Department of Public Health and Infectious Diseases, "Sapienza" University of Rome; Veterinary institute Novi Sad, Serbia; Veterinary Faculty, University of Skopje, North Macedonia.

2.2. Dog population

Until the end of 2019, we successfully collected blood samples of 535 dogs, mainly from the territory of Northern Serbia (389 samples), Southern Serbia (66 samples), and North Macedonia (80 samples). A total of 535 dogs were screened for *Dirofilaria* spp. and *Leishmania* spp. (54.9% male, 45.1% female, aged 6 months to 14 years). We examined dogs from dog shelters, as well as privately-owned dogs. Both were randomly enrolled in the study using the following criteria: (1) never moved outside the study area; (2) healthy, asymptomatic; (3) kept outdoors. Blood (300–500 μL) was extracted from the earlobe of each animal after cutting the hair, shaving, and disinfecting the area, and placed on filter papers for molecular analyses. Samples from these dogs were taken during a regular annual checkup of the dog's health status, and no additional sampling was done. All dogs were handled according to the demands of animal welfare.

2.3. Molecular analyses

Genomic DNA was isolated from dried blood spots using a commercial kit (Dried Blood Spot DNA Isolation Kit, Norgen Biotek Corporation, Canada) and submitted to a PCR targeting a 650-bp fragment from the filariod-cox1 gene using the primers COIintF (5′-TGATTGGTGGTTTTGGTAA-3′) and COIintR (5′-ATAAGTACGAGTATCAATATC-3′) as previously described [5]. All specimens were successively amplified by conventional PCR using primers L5.8S/LITSR targeting partial region of the ITS1 (~300bp) gene of *Leishmania* spp. [11].

PCR amplifications were performed in a final volume of 25 μL under the following final conditions: 10X buffer including 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1 mM each of forward and reverse primers, and 1 unit of polymerase (BIOTAQ, Bioline, UK). To test the specificity of the reaction, DNA extracted from *Dirofilaria repens* or from *in vitro* culture of *Leishmania infantum* promastigotes, and an equivalent volume of double distilled water were included in each PCR run as positive and negative controls, respectively. The amplification was performed in a thermocycler (Bio-Rad, Des Plaines, IL, USA) using cyclic profiles previously published [5, 12]. The PCR amplification products were separated by stained (SafeView Biologicals, UK) 1.2% agarose gel electrophoresis at 100 V for 45 min and visualized on a UV transilluminator (BIO-RAD, USA). Amplicons of the expected size were directly sequenced (BioFab Research, Roma, Italy), and the resulting chromatograms were analyzed and edited using the software Chromas version 2.33 (Technelysium Pty Ltd., South Brisbane, Australia). The sequences obtained were compared to those at the same gene previously deposited in GenBank and available at the website (http://www.ncbi.nlm.nih.gov/genbank , accessed on December 20, 2020) by using the BLAST application.

2.4. Statistical analyses

The data are presented as the arithmetic mean and standard deviation, i.e., count and percentage. The comparison of the frequency of variables was performed by the Chi-square test, i.e., the Fisher test. The Mann-Whitney test performed to compare continuous data between the two groups was performed by the Mann-Whitney test. Univariate and multivariate logistic regression analysis was used to test risk factors for infections in dogs. The null hypothesis was tested with a significance level of 0.05. Epi Info version 7.2.2.6 (CDC, Atlanta, US) was used for statistical analyses.

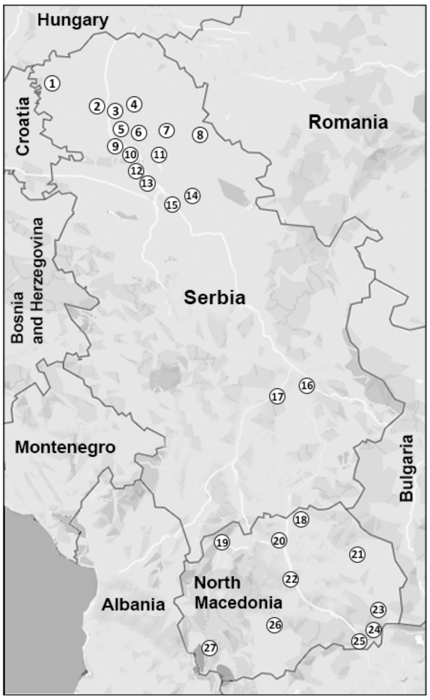
3. Results

This first molecular investigation confirmed that Central Balkan is an endemic region for ~~dirofilariosis~~ in dogs (Figure 1).

Indeed, a total of 38/535 (7.10%) dogs were found positive for Dirofilaria DNA, and sequence analysis evidenced D. immitis to be the dominant species in this area, identified in 6.35% of the positive dogs. The highest prevalence of dirofilariosis, all caused by D. immitis, was established in NM (8.75%). Similarly, a high prevalence of dirofilariosis in dogs due to D. immitis infection was found in NS (6.68%). D. repens was detected on the territory of NS as well, but only in 2 dogs. The lowest prevalence of dirofilariosis, although with detection of both D. immitis and D. repens, was found in SS (3.02%), a region where dirofilariosis in dogs had not been recorded previously.

As for leishmaniosis, only two dogs from North Macedonia were positive. Sequence analysis of the amplicons confirmed the identification of Leishmania infantum. Regardless of the considerable number of examined dogs from Serbia, none tested positive for Leishmania spp.

Statistical analysis shows that infected dogs were significantly older than uninfected ones (p = 0.037), with female dogs being infected more frequently (p = 0.022). Surprising fact is that urban or rural residence (p = 0.162), controlled conditions (p = 0.617) and the presence of ectoparasites (p = 0.561) ~~are not~~ associated with the occurrence of the infection.



Sample locality	N° of samples	N° of positive results (isolated species [N° of isolates])
1. Stapar	1	/
2. Vrbas	12	/
3. Srbobran [1]	4	1 (D. immitis [1])
4. Becej	6	/
5. Temerin	4	/
6. Zabalj [2]	125	12 (D. immitis [12])
7. Zrenjanin	60	5 (D. immitis [5])
8. Jasa Tomic	1	/
9. Novi Sad [3]	72	1 (D. immitis [1])
10. Sremski Karlovci	10	1 (D. repens [1])
11. Titel [4]	6	/
12. Indjija [5]	6	/
13. Pazova	1	/
14. Pancevo	61	9 (D. immitis [7]; D. repens [2])
15. Beograd	20	/
16. Nis	22	1 (D. repens [1])
17. Prokuplje	44	1 (D. immitis [1])
18. Kumanovo	1	/
19. Tetovo	3	/
20. Skopje	58	5 (D. immitis [4]; L. infantum [1])
21. Vinica	1	/
22. Veles	2	/
23. Strumica	1	/
24. Bogdanci	1	/
25. Gevgelija	2	2 (D. immitis [2])
26. Prilep	9	2 (D. immitis [1]; L. infantum [1])
27. Ohrid	2	/

1 - includes data from Srbobran and Nadalj; 2 - includes data from Zabalj, Djurdjevo and Curug; 3 - includes data from Novi Sad, Kovilj, Kac, Futog and Budisava; 4 - includes data from Titel and Sajkas; 5 - includes data from Cortanovci and Beska locality

Figure 1. Prevalence of infected dogs collected from different areas of Central Balkan.

4. Discussion

As forecast models predict, epidemiological and epizootiological scenarios of vector-borne diseases constantly change [1]. Climate change in regions, economic problems, migration of people, and animals and changes in vector ecology significantly influence the need for constant monitoring of zoonotic pathogens. The territory of Europe is endemic for ~~dirofilariosis~~ in dogs, with main distribution in Mediterranean region, where in several areas both *D. repens* and *D. immitis* coexist [13]. Additionally, reports from every Balkan country classify this area as an endemic zone [3]. Based on literature data, more than 200 cases of human dirofilariosis were diagnosed on this territory, mostly in the form of superficial infection caused by *D. repens*, except for the three cases, two pulmonary and one subcutaneous form of human dirofilariosis due to *D. immitis* infection [4].

In Serbia, after a long time, the first systemic research of dirofilariosis in dogs started at the beginning of the 21st century. This investigation demonstrated that Northern part of Serbia (Vojvodina Province) was hyperendemic and endemic for *D. repens* and *D. immitis* infection in dogs, respectively [14, 15]. In the same period, studies from Southeastern Serbia, previously hyperendemic region for *D. repens* infection, found that some areas are still endemic (with significantly lower prevalence of *D. repens* infection detected only in dogs), but declared certain parts of the territory *Dirofilaria*-free [16]. In the following years, *Dirofilaria* infection was detected in different carnivore hosts and present competent biological vectors [17, 18]. Although North Macedonia shows epidemiological features for dirofilariosis onset, data on *Dirofilaria* infection from this country are scant, not easily found in the international literature, and mainly performed using traditional techniques [19]. There are some recorded cases of dirofilariosis caused by *D. repens* (21%) and *D. immitis* (12.5%) in small number of examined dogs [19], followed by new data of recently reported sporadic *D. repens* infection in wolves [20].

Recently, in addition to dirofilariosis, leishmaniosis caused by *L. infantum* has been diagnosed repeatedly among dog and the human population of the Balkan Peninsula countries, after decades with sporadically reported cases [21].

Since 1999, the number of reported cases of canine and human visceral leishmaniosis in Romania started to rise, with confirmation of autochthonous disease at the same time [22, 23]. Leishmaniosis was diagnosed in dogs and humans in Bulgaria as well. First clinical cases of canine leishmaniosis in this country were reported in 2006, and at least ten new ones were found in the following years [24]. Most of the human cases were autochthonous, with some rare imported from an endemic area of Southern Europe [25, 26]. Several studies report positive results in a serological survey of dogs, mostly in rural areas of Albania [27, 28]. In addition, cases of human visceral leishmaniosis have occurred in 35 of 36 districts in the country, with rare diagnosed cutaneous form. According to the WHO, human leishmaniosis in North Macedonia has also been reported, along with high seroprevalence of leishmaniosis in dogs (unpublished data). As for Montenegro and Croatia, it is well known that the area of the Adriatic coast is endemic for leishmaniosis, which was proven in the last decade by diagnosed human cases, and, in Croatia, by serological testing of local human (11.4%) and dog (42.9%) population [20, 30, 31]. In addition, species *P. neglectus*, a confirmed vector for *L. infantum*, was identified, mainly in the coastal regions of Croatia and Montenegro [21].

Greece is the only country on the Balkan Peninsula considered endemic for both forms of the disease, with the visceral form being more frequent. Seropositive dogs were found in nearly all surveyed prefectures, with seropositivity as high as 50.2% in the island of Corfu [32]. Additionally, *Leishmania* spp. has been detected in stray cats of insular and continental parts of Greece [33, 34]. Autochthonous human cases have now been reported in 41 of the 54 prefectures, with an average of 47 leishmaniosis cases reported annually since 1998 [35].

Considering the findings in the surrounding countries, Serbia, as a former endemic area of visceral leishmaniosis, is again at risk of infection spread. The history of leishmaniosis in Serbia is significant, with the first confirmed cases of human infection reported in 1945, over 930 cases of visceral leishmaniosis in the following period, and confirmed infection in dogs as the reservoir of infection [36, 37]. However, in 1969, leishmaniosis was declared eradicated in Serbia, which led to the cessation of epidemiological research in this area.

After many years of negligence, this vector-borne disease was determined on the territory of Northern Serbia. *L. infantum* was determined in vectors [38] and among the golden jackals (*Canis aureus*) [20]. Moreover, there are reports of dogs seropositive for leishmaniosis, and among them were infected dogs that never left the country [39]. From

2001 to 2007, 22 human cases were diagnosed, whereby most of the patients were found to have previously resided on the Montenegrin coast or other endemic areas [40].

Using molecular analyses for the first time, we investigated the prevalence of ~~dirofilariosis and leishmaniosis~~ in asymptomatic dogs from the territory of the Central Balkan region. Besides dogs from Northern Serbia, the study included dogs from Southern Serbia, the territory which previously conducted investigation point as *Dirofilaria*-free zone, and from North Macedonia, from where we have insufficient data.

Results from this study show the highest prevalence of ~~asymptomatic dirofilariosis~~ (8.75%) in North Macedonia, ~~where all dirofilariosis cases were due to *D. immitis* infection~~. Similarly, 7.45% of infected dogs were found in Northern Serbia, with heartworm being the primary culprit as well. The region of Southern Serbia had the lowest prevalence of ~~dirofilariosis,~~ although with both *D. immitis* and *D. repens* species being detected. All the territory of the Central Balkan region is endemic for dirofilariosis, but more interesting is that the epizootiological scenario has changed. Now *D. immitis* dominates in the area. An overall prevalence of about 8.8% for *D. immitis* was shown, suggesting a noticeable risk of heartworm infections in the study region. Recently, human infections by *D. immitis* were observed and confirmed by molecular methods in the European region, demonstrating that this species, although with low prevalence, should not be of zoonotic concern only in America anymore [13].

In our survey, none of the 455 dog blood samples collected on the territory of Serbia tested positive for *L. infantum*, and this parasite was identified in only 2 samples from North Macedonia, with a prevalence of 2.5%. Considering that *L. infantum* was previously detected in competent vectors [10], among the jackals [20] and in human population of the region [39], as well as the reports of seropositive dogs from Northern Serbia [38], our findings are unexpected and certain considerations about the limits of this study should be discussed. Despite the blood sample being a reliable clinical material for detecting *Leishmania* DNA from asymptomatic hosts, it showed extremely low levels of parasitemia compared to bone marrow aspirate. To overcome this critical issue, an RT-PCR-based approach is suggested instead of a conventional PCR protocol to detect the parasite in peripheral blood, due to the ability of qPCR to quantify extremely low levels of parasitemia [40]. Thus, the low prevalence of *L. infantum* detected in this study can be attributed to the limited sensitivity of the detection method performed, i.e., conventional PCR from dried blood samples which cannot detect low parasite load.

5. Conclusions

To conclude, we can emphasize that Central Balkan is an endemic region for dirofilariosis ~~and leishmaniosis~~. Our findings of the high prevalence and dominance of *D. immitis* species show that we can expect a significant impact on dog health going forward. ~~Moreover, domination of *D. immitis* in the study area allows the assumption that more frequent visceral forms of human dirofilariosis may be anticipated. Even though we did not determine leishmaniosis in investigated dogs, spreading of infective agents could be a threat to the territory of Serbia in the foreseeable future.~~ All these facts suggest that preventive measures and One health approach are of great necessity to combat the spread of this infection in Serbia.

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