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## **Dirofilariosis and Leishmaniasis in the Northern Region of Serbia**

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Additional information is available at the end of the chapter

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### **Abstract**

Research in the field of vector borne diseases and zoonoses became a topic of interest in Serbia, during the last decade. Climate changes in the country (and the region) are evident. Also, significantly is higher frequency of human and animal movement and travel, especially of dogs, within the European countries, but with overseas countries as well. The presence of vectors has already been confirmed in the country and all the surrounding countries. Current research in the domain of infectious diseases in dogs mostly includes diseases which drastically endanger health and population of dogs. Some of those infectious diseases, like dirofilariosis and leishmaniasis, which are found more or less often in dogs, cause clinical symptoms which are not obvious and therefore they represent a danger for public health with dogs acting as reservoirs of the infection.

Vectors necessary for the transmission of dirofilariosis are mosquitoes and for leishmaniasis are sand flies. Vectors of dirofilariosis are mosquitoes. Female mosquitoes which feed on mammals can transfer microfilaria from one infected organism to another non infected one. *Dirofilaria immitis* is a nematode, intravascular parasite that lives in bloodstream of host, usually pulmonary vessels. Prepatent period is at least 6-7 months in definitive hosts. Maturation of organisms in mosquitoes is temperature dependant and over 14oC is needed. Diagnostic methods for dirofilariosis are many, but several serological methods can be used: ELISA modified Knott test, immunoenzyme fast test and then molecular method (PCR), etc.

Leishmaniasis is a vector borne zoonotic disease caused by a pathogen of *Leishmania* species. For the transmission of the disease, sand flies are needed as vectors from

*Lutzomyia* spp. Female sand flies are bloodsucking organisms which can transfer the pathogen from one host to another during their feeding time. The presence of *Phlebotominae* (commonly known as “sand flies”) has been identified in Serbia. The most certain method for diagnostic is demonstration of the parasite from bone marrow, splenic or lymph node aspirates, but there are other less invasive methods, like IFAT (immunofluorescent test) and ELISA (enzyme-linked immunosorbent assay).

Material for the research were samples from dogs and samples of vectors. In total, 292 samples of mosquitoes were collected and identified and 170 of blood samples from dogs were examined for dirofilariosis and leishmaniasis. Methods used in the study were modified Knott test and PCR for dirofilariosis and ELISA test for leishmaniasis. For dirofilariosis a total prevalence of the disease in dogs was found to be 15,29%, (with different values from 3-22%) for different groups of dogs (hunting and military dogs, dogs from asylum and pet dogs). Total seroprevalence for all 170 blood samples was 10,59% for leishmaniasis. Overall, there is actually no difference in seroprevalence for leishmaniasis, between different groups of dogs (hunting and military dogs, dogs from asylum and pet dogs). There is a reasonable doubt that leishmaniasis appears as a disease in the Northern part of Serbia, in region of Vojvodina. The presence of vectors has been identified (*Phlebotomus papatasi*, *Laroussius tobbi*) as well as existing seroprevalence in dogs with and without clinical symptoms. All of this suggests that there is an existence of the reservoirs of infection.

**Keywords:** dirofilariosis, leishmaniasis, diagnostics, dogs

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## 1. Introduction

Research in the field of vector-borne diseases and zoonoses became a topic of interest in Serbia during the last decade. Climate changes in the country (and the region) are evident, compared to the weather conditions from 10 or more years ago in terms of higher temperatures during the summer, higher humidity in summer, shorter spring and autumn periods, and shorter period of low temperature during winter. The influence of climate change has already been highlighted [1]. Also, the frequency of human and animal movement and travel, especially of dogs, is significantly higher not only in European countries but also in overseas countries. The importation of dogs is done on a pretty flexible basis with health status analysis only for rabies. The presence of vectors has already been confirmed in the country and all the surrounding countries. Current research in the domain of infectious diseases in dogs mostly includes diseases that drastically endanger health and population of dogs. Some of those infectious diseases, like dirofilariosis and leishmaniasis, which are found more or less often in dogs, cause clinical symptoms that are not so characteristic and expressed. These diseases are zoonoses, and therefore they represent a danger for public health with dogs acting as reservoirs of the

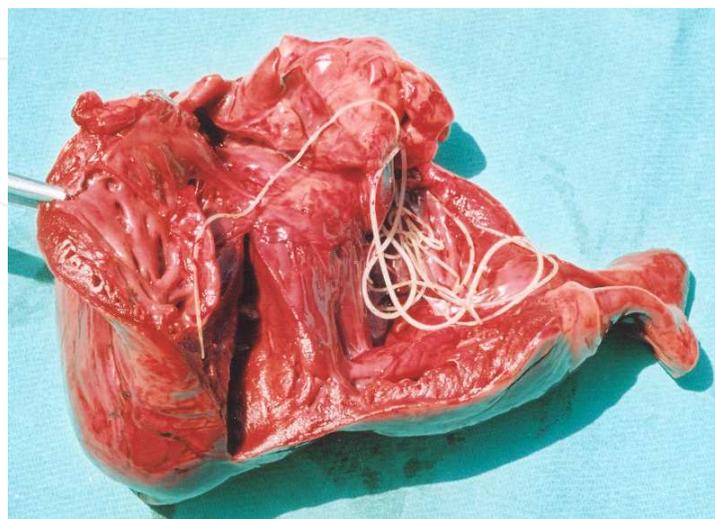
infection. For a transmission of vector-borne diseases among dogs and from dogs to humans, vectors are essential because a part of the pathogen's life cycle takes place in vectors.

Dirofilariosis and leishmaniasis were earlier recognized as Mediterranean vector-borne diseases. They both have a zoonotic potential. Vectors necessary for the transmission of dirofilariosis are mosquitoes and for leishmaniasis are sand flies. Today there is evidence of dirofilariosis in different countries around the world and also evidence of presence of vectors for dirofilariosis and leishmaniasis in countries other than Mediterranean [2–5].

Dirofilariosis is a vector-borne zoonosis mostly caused by *Dirofilaria immitis* and *Dirofilaria repens*. Even though dirofilariosis was primary known as a disease found in Mediterranean countries only, it has spread out to the North and West of Europe through the years, so now clinical cases of dirofilariosis can be found in middle Europe, including Serbia [6–12].

The first published research on dirofilariosis in Serbia (ex, like previously known as Yugoslavia) was done during the 1990s, when the first cases were discovered in humans and dogs [13–16]. Since that time, there is a follow-up on dirofilariosis in several regions of Serbia. Diagnostics of dirofilariosis in Serbia has started approximately 10 years ago. Since 2004 until nowadays, veterinary services have started a regular, routine check up in dogs for dirofilariosis. Cases of dirofilariosis (*Dirofilaria immitis* and *Dirofilaria repens*) in Serbia have been found so far both in humans and dogs. Several cases of dirofilariosis in humans have been represented, and few studies have been done [17–22].

The first cases of dirofilariosis in Serbia, in dogs, were discovered as a side finding during dissections [43]. The actual first case of canine dirofilariosis in Serbia was considered to be in a dog imported from USA. A number of studies were done on the outbreaks of dirofilariosis in dogs and seroprevalence in different regions [23–27]. In the northern part of Serbia, Vojvodina province, several studies have been done during the previous period on seroprevalence and diagnostic methods [28–32]. Some research was also done on seroprevalence to dirofilariosis in working and military dogs and in pet dogs [33] (Figure 1).



**Figure 1.** *Dirofilaria immitis* found in heart at dissection of a dog.

Vectors of dirofilariosis are mosquitoes. Female mosquitoes that feed on mammals can transfer microfilaria from one infected organism to another noninfected one. Female mosquitoes are vectors that can be found in high numbers in Serbia during the warm period of the year, from May to October. Over 70 mosquito species can be vectors of dirofilariosis out of 3000 mosquito species worldwide. Three of those species can be found in Serbia – *Aedes*, *Anopheles*, and *Culex* [34].

Dirofilariosis can appear with different severity, from asymptomatic to mild, or it can also progress to fatal. Definitive hosts of the parasite can be domestic dogs and wild canines, such as wolves, coyotes, and foxes. Reservoirs of dirofilariosis in wildlife are raccoons, wolverines, coyotes, deers, and bears. Dirofilariosis has a zoonotic potential. Humans are not definitive hosts for *Dirofilaria*, but occasionally the disease can occur, most usually under the skin or in the eye.

*Dirofilaria immitis* is a nematode, intravascular parasite that lives in bloodstream of host, usually pulmonary vessels. Prepatent period is at least 6–7 months in definitive hosts. The maturation of organisms in mosquitoes is temperature dependant, and over 14°C is needed. When mosquitoes feed on the blood of an infected dog, they ingest first-stage (L1) larvae (microfilariae), which are produced over many years by the mature heartworm in the dog. Within the mosquito, larvae mature from stage 1 to stage 3. Most of their development takes place in the malphigian tubes of the mosquito. Once developed to the infective (L3) larval stage, they migrate through the body to the head cavities of the mosquito, where they wait to infect another host by leaving the mosquito during the blood meal. The prepatent period between initial infection of the dog and the maturation of the worms into adults living in the heart takes 6 to 7 months in dogs. The (L3) larvae of heartworms deposited by the mosquito into dog's skin grow for a week or two and then molt to the next larval stage (L4) under the skin at the site of the mosquito bite. Then they migrate to the muscles of the chest and abdomen, and 45 to 60 days after infection, they molt to the next larval stage (L5). Between 75 and 120 days after infection, these immature heartworms then enter the bloodstream and are carried through the heart to reside in the pulmonary artery. Over the next 3 to 4 months, they increase in size. Seven months after infection, the adult worms have mated, which has a consequence of the appearance of microfilariae in the blood stream of the host. Microfilariae may circulate in the bloodstream for up to 2 years, waiting for a bloodsucking mosquito. The extrinsic incubation period required to reach the stage when microfilariae become transmittable to another host can vary from 2 to 6 weeks, depending on the temperature. It is possible that there are no evident clinical symptoms in a host for even a year after infection. In humans, *Dirofilaria immitis* never reaches the adult stage, and they can never be found in the heart of humans because humans are accidental hosts [34] (Figure 2).

Dirofilariosis in dogs is most frequently located in the right side of the heart, pulmonal arteries, and rarely in the lungs. Clinical symptoms in dogs are unspecific: lethargy, weakness, fatigue, exercise intolerance, dyspnea, cough, anorexia, weight loss, vomiting, diarrhea, collapse, seizures, and sudden death.



**Figure 2.** *Dirofilaria immitis* taken out from the heart of a dog.

Diagnostic methods for dirofilariosis are many, but several serological methods can be used: ELISA modified Knott test, immunoenzyme fast test, and then molecular method (PCR). Antibodies formed against the antigens of *Dirofilaria* sp. can be detected by ELISA method.

ELISA is a very sensitive and specific test, easy to perform, but it has to be done in a laboratory. There can be a false-positive reaction if there is a cross reaction with another antigen. Also, there can be a false-negative finding, if the analysis is performed too early after the infection and the dog still does not have a level of antibodies high enough (Figure 3).



**Figure 3.** ELISA method for diagnostics—positive and negative control and positive and negative samples.

Antibodies formed against *Dirofiliria* sp. can also be detected by an immunoenzyme test, usually called “fast” or “snap” tests. It is a user-friendly one- or two-step test that can be performed anywhere. No laboratory conditions are needed for the performance of the test, so it can be done at veterinary practice or even in the field. The results of the tests are ready to be read within 10–15 minutes, and the sensitivity and specificity of fast tests is good compared to the other available tests (Figure 4).



**Figure 4.** Immunoenzyme fast test—positive (two dots) and negative (one dot) findings.

The most “popular” and most used diagnostic test for dirofilariosis among veterinarians is the modified Knott test. With this test, circulating microfilaria from the blood stream can be found, colored, and seen with a microscope. The procedure is not complex but requires some laboratory equipment; time and skills are also needed, with a good knowledge of microfilarial morphology. This method is highly specific and sensitive in dogs, and microfilariae belonging to different species can be determined [35].

PCR is a molecular method with which DNA of *Dirofilaria immitis* is detected. This is a sensitive and accurate method to discriminate microfilariae from other different filarial worms in dogs. It is a good confirmation test and a research tool. If dirofilariosis is detected by snap tests, ELISA, or modified Knott test, the presence of the DNA of pathogen can be confirmed by PCR method [36].

Leishmaniasis is a vector-borne zoonotic disease caused by a pathogen of *Leishmania* species. For the transmission of the disease, sand flies are needed as vectors from *Lutzomyia* spp. Female sand flies are bloodsucking organisms that can transfer the pathogen from one host to another during their feeding time. In the overview of human leishmaniasis from 2009, in Europe there are cases described in Greece, Cyprus, France, Italy, Malta, Portugal, Spain, FYROM, and Albania [5]. Later on, there are data published on cases of leishmaniasis in humans in Bulgaria [37], in dogs in Romania [38], and in dogs in Hungary [39].

In Serbia, leishmaniasis is considered, so far, an imported disease, and there is no official data that the disease exists as an autochthonous infection in humans or in animals. There are cases of humans with leishmaniasis in Serbia, but all of them were imported from Montenegro, FYOM (Former Yugoslavia Republic of Macedonia), or Greece during holiday season [40].

There were some notifications about leishmaniasis in dogs during the last several years. Three separate cases of dogs were found with clinical symptoms that could indicate leishmaniasis, and they were found seropositive to leishmaniasis. After therapy, their condition has improved [41, 42].

The presence of *Phlebotominae* (commonly known as “sand flies”) has been identified in the southern part of Serbia a long time ago, and these vectors are known in Mediterranean countries as vectors of leishmaniasis. During the late 1950s and early 1960s, studies were done on the presence of *Phlebotominae* in the southern part of Serbia, but after that period, nothing else was published [43]. During a previous period, several dogs were found in the region, as clinical cases suspicious to leishmaniasis (epistaxis, cachexia, pale mucosa, skin problems, blindness, and lethargy), with seropositive findings for this disease [42].



**Figure 5.** Bitch with skin lesions, positive serological finding for leishmaniasis.



**Figure 6.** Dog with skin lesions, positive serological finding for leishmaniasis.

The first clinical cases of leishmaniasis in humans and dogs in Serbia were infections coming from abroad (Montenegro, Greece, Former Yugoslavia Republic of Macedonia, and Croatia), mostly after summer holidays. Within the last 3 years, positive findings were identified in dogs that have never left their homes in Serbia (Figures 5 and 6) [41, 42].

Domestic and wild canines are the main host species for leishmaniasis, but the domestic dog is the only epidemiologically important reservoir. Causative organisms are protozoa *Leishmania donovani* (in Asia, Middle East, and Africa) and *Leishmania infantum* (in Asia, Middle East, Europe, and South America). The transmission of the disease occurs via sand fly bites, and dogs are the reservoir hosts. Humans are accidental hosts. Transmission of the disease between dogs and humans directly is not possible.

Clinical symptoms of leishmaniasis in dogs are nonspecific. They can be as fever, weakness, lethargy, weight loss, muscle wasting, lymphadenopathy, pallor, anemia, thrombocytopenia, conjunctivitis and eye problems, skin lesions and alopecia, etc.

Diagnostic procedures for leishmaniasis are several. The most certain method is the demonstration of the parasite from bone marrow, splenic, or lymph node aspirates, but there are other less invasive methods too. Serologic tests are most commonly immunofluorescent test (IFAT) and enzyme-linked immunosorbent assay (ELISA) [44].

## 2. Materials and methods

Materials for the research were samples from dogs and samples of vectors. The research was planned as serological examination of dog blood samples for dirofilariosis and leishmaniasis. The vectors (mosquitoes) were collected, identified, and analyzed for the presence of causative agents of dirofilariosis in the northern part of Serbia.

During spring and summer of 2014 (May–September), 292 samples of mosquitoes were collected and identified. Collecting was done with lamps with dry ice. The identification of mosquitoes (for gender and species) was done at Faculty of Agriculture, University of Novi Sad. Vector identification was done with microscopic observation. The analysis of vectors for the presence of causative agent for dirofilariosis was done by a molecular method (PCR). PCR analysis were performed at the Scientific Veterinary Institute of “Novi Sad.” The samples were pooled as 20 mosquitoes into one pool. In collected mosquitoes, a molecular method of PCR was performed. DNA extraction was done with commercial kits from Quiagen (QIAmp), during a 2-day protocol. PCR was done according to the prescription of Rishniw et al. [36]. Primers used for PCR analysis were primers 5′–3′, forward: DIDR-F1\_for AGTGCGAATTG-CAGACGCATTGAG and reverse: DIDR-R1\_rev AGCGGGTAATCACGACTGAGTTGA. Determination was done based on 542 bp for *D. immitis*.

In total, 170 of blood samples from dogs were examined for dirofilariosis and leishmaniasis. Serological analysis for dirofilariosis and leishmaniasis were done from blood samples of dogs obtained by venous punctation. The blood samples were divided into three groups, according to the way of life of the dogs:

- Group of hunting and military dogs (79 samples)—in this group, samples were analyzed from dogs that are actively used for hunting. They had their owners, and they mostly did

not receive any preventive treatment against parasites. Not one of these dogs has ever left Serbia.

- Group of dogs from asylum for homeless dogs (64 samples)—in this group were dogs kept in the asylum, but for a long time, and they have all received preventive treatment against parasites annually. Not one of these dogs has ever left Serbia since they were in asylum, but for many of them, the history of their previous life and origin is unknown.
- Group of pet dogs (27 samples)—in this group were dogs that came to veterinary practice for numerous reasons, with nonspecific clinical symptoms, or no clinical symptoms at all. Not one of the owners thought that their dog has dirofilariosis or leishmaniasis. Some of the dogs have received antiparasitic prevention and some did not. Even the ones which did receive preventive treatment did not receive it annually, only during spring and summer. Not one of these dogs has ever left Serbia.

Methods used in the study were the following: modified Knott test and PCR for dirofilariosis and ELISA test for leishmaniasis.

Analysis for dirofilariosis was done with the modified Knott test for the detection of microfilaria in circulation. Analysis for all the samples was done on the same day of sampling or the next day. Samples were taken with anticoagulant. The procedure of the modified Knott test was performed according to the instructions of Genchi et al. [35]. The modified Knott test was done in all the samples—from dogs with clinical symptoms as well as from dogs without any clinical symptoms for dirofilariosis. Positive samples found by the modified Knott test were then selected for molecular analysis to be done by PCR. DNA extraction was done with QIAmp commercial kits for DNA extraction (Quiagen, by the instructions of the producer). After that, a PCR was performed according to the protocol from Rishniw et al. [36]. The same primers were used in the protocol of *Dirofilaria* DNA detection in blood samples as in mosquito samples (primers 5'–3'):

Forward: DIDR-F1\_for AGTGCGAATTGCAGACGCATTGAG and

Reverse: DIDR-R1\_rev AGCGGGTAATCACGACTGAGTTGA.

Determination was done based on 542 bp for *Dirofilaria immitis*.

For diagnostics of leishmaniasis, same blood samples were used taken from the same dogs. Analysis for leishmaniasis was done by ELISA method (commercial kit by Ingenaza, done by the prescription of the producer). From blood samples, sera samples were obtained by centrifugation. After that, blood sera samples were kept on  $-20^{\circ}\text{C}$  until ELISA was performed.

### 3. Results and discussion

#### 3.1. Dirofilariosis

Analysis of vectors for dirofilariosis: In total, 292 samples of mosquitoes were collected. After determination, it was found that they belong to *Culex* (*Culex pipiens* and *Culex culex*) and *Aedes* species. The samples were collected as random samples from different locations of the northern

part of Serbia. In 292 mosquitoes randomly collected, the presence of DNA of causative agent for dirofilariasis (*Dirofilaria immitis*) was not found. Mosquitoes were collected randomly, and weather conditions during the collection of samples were not favorable. Weather conditions were bad for the lamps and the collection process because there was a lot of wind and often rain during the time of collection. Also, the outside temperature was lower than usual for that the time of the year. The results found after the analysis of mosquito samples indicate that sampling was perhaps not done in the best way. The samples should have been collected at the residence of positive dogs and not from several randomly chosen locations. Also, the weather conditions may have influenced the development of L3 larval stage of microfilariae in the mosquito because the temperature for many days was not too much above 14°C. All of these may have influenced the absence of *Dirofilaria* sp. DNA in mosquito samples.

The results of analysis of blood samples from three groups of dogs (170 samples in total) to dirofilariasis by the modified Knott test are shown in Table 1.

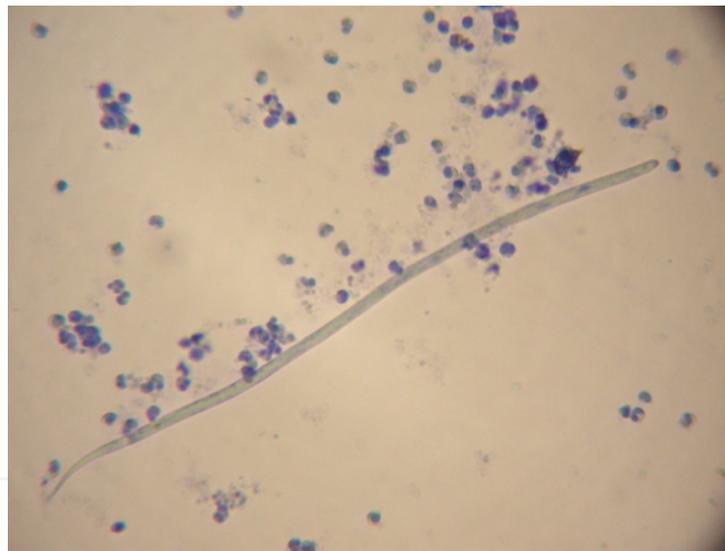
| Group of dogs                      | Total number of examined dogs | Number of positive dogs | Percentage of positive dogs |
|------------------------------------|-------------------------------|-------------------------|-----------------------------|
| Hunting and military dogs          | 79                            | 18                      | 22.78                       |
| Dogs from asylum for homeless dogs | 64                            | 2                       | 3.12                        |
| Pet dogs                           | 27                            | 6                       | 22                          |
| Total                              | 170                           | 26                      | 15.29                       |

**Table 1.** Results of the analysis of blood samples from three groups of dogs to dirofilariasis

In the group of hunting and military dogs, a seroprevalence for dirofilariasis was found to be 22.78%. In the group of dogs from asylum, a lower seroprevalence for dirofilariasis was found —3.12% and in the group of pet dogs, and seroprevalence for dirofilariasis was found to be 22%. In the case of dirofilariasis, the seroprevalence of the disease in different groups was different. It depended on the received prevention treatment against parasites and the lifestyle of dogs. Seroprevalence was the lowest (3.12%) in dogs living in asylum with regular prevention care. These dogs received preventive treatment monthly during the whole period when mosquitoes can be found (March/April–October). It is important to highlight that even two positive dogs found in asylum were new dogs that came from another place, less than 1 month previously to the sampling. The highest seroprevalence (22.78%) was found in hunting dogs with no prevention treatment in most of the cases. Seroprevalence found in pet dogs was not much different than the one found in hunting dogs. This would refer to the fact that not many pet dogs are under preventive treatment, or even if they are, it is not being repeated enough times. Most of the pet owners are not aware enough of the existence of dirofilariasis as a disease in dogs, and so they believe that it is enough if they give the preventive treatment to their pet dogs once or rarely twice during the whole period of the year when mosquitoes are present (March/April–September). Also, the fact that there are no clinical symptoms in dogs usually for a long time after infection makes the owners believe that their dog is healthy.

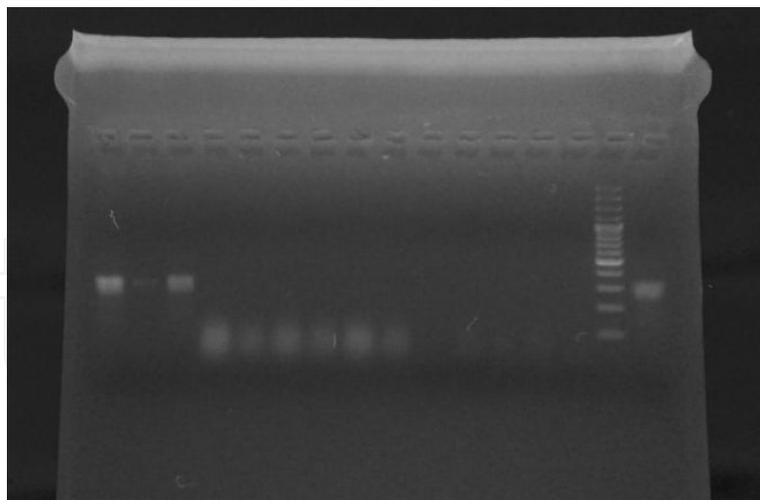
During a 2-year period, 170 dog blood samples were analyzed. Most of the dogs did not have any clinical symptoms. Only several dogs had clinical symptoms such as cough, lethargy, tiredness, and heart failure symptoms. The modified Knott test gives us a direct overview into the existence of larvae of *Dirofilaria* (microfilaria) in dogs' circulation. A total average seroprevalence for the whole three groups of dogs was found to be 15.29%, but the highest seroprevalence was found to be in hunting and military dogs, followed very closely by pet dogs. Hunting and military dogs live in most cases outside in backyards and are in constant contact with vectors. They have a long time of outside activities in the regions where vectors can be found. Also, quite a lot of dogs from this group is not protected constantly with preventive ectoantiparasitic treatment.

The modified Knott test is a fast and reliable diagnostic tool recognized in the world as a method for the detection of microfilaria in circulation of dogs. In veterinary practices, fast tests can be used for routine checkup of patients. However, in the case of positive finding or if there are recognizable clinical symptoms in a dog, a confirmation of diagnosis has to be done with the modified Knott test. With this test, an identification of *Dirofilaria* can be done with distinction between *Dirofilaria immitis* and *Dirofilaria repens* [35] (Figure 7).



**Figure 7.** Diagnostics of dirofilariosis by the modified Knott test.

After positive samples were found by the modified Knott test, a PCR analysis was done for the conformation of *Dirofilaria immitis*. PCR analysis was done from blood samples of dogs in which microfilariae were found (26 samples). The isolation of *Dirofilaria*'s DNA from 200  $\mu$ l of blood samples was done with QIAmp set kit (Quiagen). PCR procedure was done as described by Rishniw et al. [36]. PCR is a very sensitive, specific, and accurate method with which determination of *Dirofilaria* species is possible. It is more a research tool than a diagnostic tool because it is a demanding procedure in equipment and skills. From 26 blood samples from dogs in which *Dirofilaria* was found by the modified Knott test, a positive result was found by the PCR method in 24 samples (92.3%) (Figure 8).



**Figure 8.** PCR reaction for *Dirofilaria immitis* in blood samples of dogs positive to dirofilariosis by the modified Knott test, determination based on 542 bp.

These data can be compared to the data collected during the last several years in the same region by the same authors, shown in Table 2. The first official acknowledgment of dirofilariosis in Serbia was published by Dimitrijevic in 1999 [43]. After that time, several authors have been following the development of this disease in dogs in different regions of Serbia. For the northern part of Serbia, data have been collected for more than 10 years now.

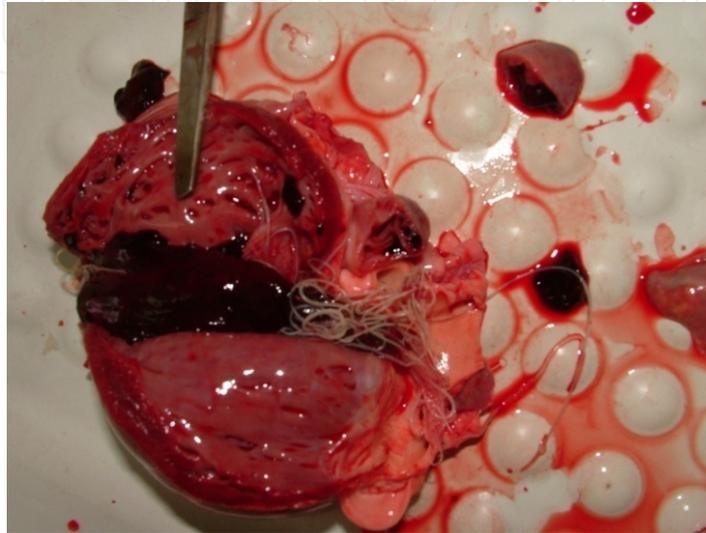
| Year   | Percentage of positive dog samples |
|--|------------------------------------|
| 2003–2004  | 5.9–7                              |
| 2006–2007, dogs with no clinical symptoms                          | 10–11                              |
| 2006–2007, dogs with clinical symptoms                             | 80                                 |
| 2010   | 14                                 |
| 2010, only pet dogs  | 11                                 |
| 2011–2013  | 5 human cases                      |
| 2013–2014 hunting and military dogs, dogs from asylum and pet dogs | 15.29                              |

**Table 2.** An overview of data collection during a 10-year period on the seroprevalence of dirofilariosis in dogs in northern Serbia

By comparing the data during the last decade, it can be stated that there is a constant increase of seroprevalence for dirofilariosis in dogs in Serbia over the years. After these findings were published, diagnostic methods for dirofilariosis were introduced into the routine checkup of dogs in veterinary practices—modified Knott test, fast test, and ELISA test. Also, fast tests became available to the practitioners and became the mostly used diagnostic tool in veterinary practices. Preventive treatment is present and offered to the owners, but the awareness of the owners is not quite high enough. Further research on the presence of causative pathogen (*Dirofilaria immitis*) must be done in vectors so that a risk estimation can be made. Definitely,

dirofilariosis is present in the northern part of Serbia in the percentage that justifies the fact that this disease should always be considered when looking at a patient in veterinary practice. Also, there are already human cases in the region, so attention should be paid to this disease in the meaning of “One Health” point of view.

Clinical cases of canine dirofilariosis in Serbia are still often found after dissections, and still mostly as a side finding (Figure 9).



**Figure 9.** Dirofilariosis in one of the military dogs from the survey.

It appears that dirofilariosis is a disease more and more frequent in dogs, so there is more demand for control of health status for dirofilariosis within a routine checkup in dogs. The owners are not enough aware that disease can occur without any clinical symptoms for a certain period of time. During the period of our study, seropositive findings for dirofilariosis were present all the time in dogs, which makes therapy and prevention necessary in the region.

The awareness of the fact that dirofilariosis is a zoonotic disease is higher over the time, and this makes the disease a danger for public health. Cases of human dirofilariosis are also present in the northern part of Serbia but are still neglected within diagnostic procedure. Medical doctors are still not completely aware of the diagnostics of dirofilariosis in humans, and there are still no reliable, noninvasive diagnostic methods on the market [21].

Apart from the modified Knott test done from the blood samples of dogs, an identification of the pathogen has been confirmed by PCR method too. Positive findings were gained by PCR method, at the matching rate of 92.3% with the modified Knott test.

### **3.2. Leishmaniasis**

During the same period of study, 170 blood samples were examined for leishmaniasis from dogs that did or did not have clinical symptoms of the disease. After serological testing of the samples, positive findings for leishmaniasis were gained. Blood samples were analyzed for

the presence of specific antibodies against *Leishmania* sp. with the ELISA method (Ingezim Leishmania, Ingenasa, 1.5.LSH.K.1). From total number of samples, in 10.59% of samples, the presence of specific antibodies against *Leishmania infantum* was found. It is important to highlight that not one of the examined dogs has ever left their dwelling place. In 18 dogs, positive serological findings for leishmaniasis were obtained. Three of the examined dogs had skin lesions that would not heal and bad skin condition in general.

The findings after the analysis of blood samples from three groups of dogs (170 samples in total) for leishmaniasis with ELISA test are shown in Table 3.

| Group of dogs                      | Total number of examined dogs | Number of positive dogs | Percentage of positive dogs |
|------------------------------------|-------------------------------|-------------------------|-----------------------------|
| Hunting and military dogs          | 79                            | 8                       | 10.12                       |
| Dogs from asylum for homeless dogs | 64                            | 7                       | 10.33                       |
| Pet dogs                           | 27                            | 3                       | 11.11                       |
| Total                              | 170                           | 18                      | 10.59                       |

**Table 3.** Results of the analysis of blood samples from three groups of dogs for leishmaniasis

In the group of hunting and military dogs, a seroprevalence for leishmaniasis was found to be 10.12%. In the group of dogs from asylum, seroprevalence was found to be 10.33%, and in the group of pet dogs, seroprevalence for leishmaniasis was 11.11%. The total seroprevalence for all 170 blood samples was 10.59% for leishmaniasis. Overall, there is actually no difference in seroprevalence for leishmaniasis between different groups of dogs. There is a similar seroprevalence in all three groups of dogs, unlike the seroprevalence to dirofilariosis. There is a constant presence of causative pathogen among the dog population in the northern part of Serbia. There is a reasonable doubt that leishmaniasis appears as a disease in the northern part of Serbia, in Vojvodina. The presence of vectors has been identified (*Phlebotomus papatasi* and *Laroussius tobbi*) (Vaselek, unpublished data), as well as the existing seroprevalence in dogs with and without clinical symptoms. All of this suggests that there is an existence of the reservoirs of infection. Leishmaniasis in humans has been identified so far only in people who have traveled to Mediterranean countries and not as an autochthonous infection. Due to climate changes, summer temperatures and conditions in the northern part of Serbia are more and more in favor of the life cycle of vectors—sand flies.

In our history, there is evidence of leishmaniasis in humans and in dogs in Serbia, but over 60 years ago. The first autochthonous cases of visceral leishmaniasis were found in the southern part of Serbia (region around city of Nis) back in 1945. During the period of 1946–1949, there were 350 registered cases of human visceral leishmaniasis in Serbia, and some cases were even registered around city of Belgrade [45]. At that same time, about 2% of dogs in the region around city of Nis were found to have asymptomatic leishmaniasis, and dogs were identified as main reservoir of infection [45]. During the period from 1968 to 1969, rare cases of autochthonous visceral leishmaniasis were reported in the southern part of Serbia. At that time, the

vectors of leishmaniasis were detected: *P. major*, *P. simici*, and *P. perfiliewi* [46]. In the northern part of Serbia, the disease or vectors have never been identified before. After this period of studies and interest of public into leishmaniasis, no more data were found, and no research has been done until now. No vectors have been identified any more, or they were just not looked for until 50 years later. There is a question on the existence of leishmaniasis in Serbia, but at the moment, there is evidence of the existence of vectors and clinical disease in dogs, with serological confirmation of the disease and successful therapy. Today, Serbia is surrounded with several countries that have leishmaniasis for sure (Croatia, Montenegro, and FYROM), countries where vectors are identified so far (Hungary), and countries in which there is also a reasonable doubt that leishmaniasis exists in dogs (Romania). More research has to be done, especially on vectors and reservoirs of the infection, with a precise identification of the pathogen.

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