

Review

by Prof. Rayko Dimitrov Peshev, Ph.D., head. Of Department "Epizootology and Infectious Animal Diseases" in NDNIVMI, Sofia, on a dissertation on the topic: „Studies on cardiac dirofilariasis in dogs in Bulgaria“ for the award of a scientific and educational degree "Doctor" in the field of higher education: 6. Agricultural sciences and veterinary medicine, Professional direction: 6.4. Veterinary medicine, scientific specialty: "Epizootology, infectious diseases and prevention of infectious diseases in animals", presented by Radoslav Mitkov Rafailov, from the department: "Infectious pathology, hygiene, technology and control of food of animal origin" from the Faculty of Veterinary Medicine at Forestry University in Sofia.

Autobiographical data of the candidate: Radoslav Mitkov Rafailov was born on February 7, 1990 in the town of Lovech. He started his secondary education in 2005 and graduated in 2009 in the science and mathematics high school with English language studies in the town of Lovech. From 2009 to 2015, he graduated as a Master of Veterinary Medicine at the Faculty of Veterinary Medicine at the Forestry University of Sofia. For two months he worked as a veterinarian in the pig complex of the Doverie cooperative, in Lesidren vilage. After that, he worked at the St. George veterinary clinic Estrella Vet OOD as a veterinarian. From 2016 to December 2020, he worked as an assistant at the Faculty of Forestry. There he prepares and conducts exercises, seminar classes, practical and semester exams in the disciplines: Parasitology, Diseases of bees, fish, game, a mobile clinic and conducting scientific research activities.

The dissertation work submitted to me for review on the topic: Studies on cardiac dirofilariasis in dogs in Bulgaria is written on 187 pages, and is designed in accordance with the requirements for a dissertation work and includes: Introduction - 2 pages, Literature review - 32 pages. , Own research, Aim and tasks -1 p., Materials and methods - 18 p., Results - 66 p., Discussion -19 p., Conclusions - 2 p., Contributions - 1 p., Recommendations for practice - 1 p., Participation in scientific conferences and published articles related to the dissertation - 2 pages, Acknowledgments - 2 pages, References 32 pages. The dissertation is illustrated with 13 tables and 105 figures.

Cardiac heartworm disease is a disease caused by the nematode *D.Immitis*, which parasitizes the right atrium, right ventricle, and pulmonary artery, and in massive invasion specimens are found in the posterior vena cava. Intermediate hosts are many species of mosquitoes in the genera *Aedes*, *Culex*, *Anopheles*, *Culiseta* and *Coquilletidia*. The disease is widespread throughout the world, and in our country, in previous studies, it was found that the prevalence ranges between 6.6 and 34.3%. Climate change and the ban on the use of insecticides, which have an adverse effect on the environment, have caused an increase in the mosquito population worldwide, which is associated with an increase in mosquito-borne pathogens. *Dirofilariasis* is no exception to this. In the dissertation work, through the conducted epizootological and parasitological studies, the prevalence and characteristics of the disease are studied, the types of mosquitoes involved in its transmission are studied, and molecular biological studies are applied with a view to improving diagnostics and quickly proving the causative agent.

In the literature review section, the doctoral student provides historical facts about the species of parasites of the genus *Dirofilaria* and the prevalence of cardiac heartworm disease worldwide, in Europe and Bulgaria. The general characteristics of *D.Immitis*, the morphology and the biological cycle of the causative agent are given. The following is a description of the

disease in the dog, pathogenesis, clinical picture and methods for diagnosing the disease. Methods and schemes for the treatment of cardiac dirofilariasis in dogs are given. There are several ways to treat heartworm disease with different combinations of antiparasitics and drugs. Treatment according to the American Heart Parasite Association and methods of surgical extraction of adult forms of *D. Immitis* in dogs, autopsy findings, pathoanatomical and pathohistological changes are also described. Mosquitoes are vectors of the disease, their species composition in Europe and in Bulgaria and the types of mosquitoes that carry microfilariae are described, the factors on which their spread depends, as well as important climate-geographic indicators and data on their development are indicated. From the literature review thus made, it can be seen that the biological cycle and morphology of heartworms have been partially studied. The timing of initiation and completion of microfilariae transmission from the intermediate to the definitive host is not yet clear. It is also not well understood which mosquito species are potential carriers of invasive larvae to invade the definitive hosts. There is insufficient data on mosquito invasions and their summer dynamics, such as numbers and distribution, and how climato-geographical features affect recent disease distribution. There is little data on the relationship of infected dogs with the causative agent, their habitat, their breed affiliation, sex, age, weight, which would clarify the predisposition of individuals within a breed, gender and age predisposition to the disease. There is little worldwide data on the pathoanatomical and pathohistological changes in various organs and systems of dogs suffering from dirofilariasis. Clarifying these features will improve prevention and reduce the spread of the disease in our country.

The literature review shows that Dr. Rafailov deals very well with the modern and older literature concerning the causative agent itself, the intermediate host, the diagnostic methods of proof, the ways of treatment and prevention of the disease. From the literature review made in this way and the unclear points regarding the disease, the goals and tasks set before the dissertation work are derived, namely: To conduct a study of the prevalence of cardiac dirofilariasis and to specify the possibilities of diagnosis of the disease, to deepen the knowledge of the pathological changes in the final hosts, the seasonal dynamics of mosquitoes in different areas of the country and to make a correlation between their number and the carrier of microfilariae of the type of *D. Immitis*. For the realization of this goal, 6 tasks have been set for implementation. I believe that the goal and tasks are correctly set, which will result from the further correct study of the disease.

In the results section, Dr. Rafailov, in chronological order, gives data from studies of the prevalence of the disease in dogs from different regions of the country after the implementation of the rapid antigen tests for diagnosis. A comparative analysis was made of the data obtained with the rapid antigen tests in comparison with the Knott method and the conventional PCR test. The correct interpretation of the results of these studies is impressive. The section follows the determination of the species composition of mosquitoes in our country depending on the climatic and geographical conditions of the studied regions, and the beginning and end of the period of effectively infection of mosquitoes (PESK) is determined. By polymerase chain reaction in real time, a comparison was made between the summer seasonal monthly dynamics of mosquitoes in different cities of the country, and the degree days and the beginning and end of the PESK were established. Finally, the patho-anatomical and histological assessment of the changes in the internal organs of heartworm-infected dogs is presented. I want to note that the section is very well illustrated, both with the intermediate hosts and with the pathological and histological changes in the final hosts.

In the discussion section, the research results are chronologically discussed, comparing them with the data of other researchers and properly discussing the data obtained by the author. The results of studies of the prevalence of the disease in different regions of the country, after the use of rapid antigen tests, are discussed, and the differences between his studies and those of other authors working on the problem are critically discussed. Male dogs of large breeds between the ages of 2 and 7 are most often affected by heartworm disease, that is, they have had enough years to become infected. The author concludes that a possible reservoir and source of the invasion are stray dogs that have not been treated with specific antiparasitic agents. The increase in prevalence compared to previous years is discussed and an explanation given. The results of the laboratory tests obtained by the rapid antigen tests in comparison with the Knott method and the conventional PCR test are critically discussed and the reasons for the differences resulting from the application of the various tests are discussed, the sensitivity and specificity of the tests are discussed. During the analysis of the results obtained from determining the species composition of mosquitoes in our country, it was found that they are four species, one of the species *Culex pipiens*, three of the species *Aedes-vexans*, *cinereus* and *caspius*, one *An.maculipennis* complex and one of the genus *Culiseta*, as they are distributed in different percentages in different regions and cities of the country. The author also discusses the results for the beginning and the end of the period of effective infection of mosquitoes with the causative agent, and so far no such study has been done in our country, which, in turn, is important for starting preventive measures at the most appropriate moment. Data using real-time polymerase chain reaction and their correlation with monthly peak catches of *Culex* and *Aedes* mosquitoes are discussed. In the last part of the discussion, the data from the pathological and histological changes in the internal organs of dogs with dirofilariasis are discussed, comparing them with the results of other authors and expressing an opinion, which organs and systems are most strongly and severely affected, apart from those belonging to circulatory system. From the discussion, it is clear that the doctoral student knows the literature well, can correctly interpret the results obtained by him and express his own opinion on the developed problem.

I believe that the dissertation reflects a complex study of the spread of cardiac heartworm disease in regions and cities in our country, with the different types of mosquitoes, vectors of the disease and the dependence between their spread and temperature and seasonal characteristics being established. *D. Immitis microfilariae* were found in 6 species of mosquitoes inhabiting our country. The period of effective infection of mosquitoes during the summer months in 9 cities located in different parts of the country has been proven. The method for proving microfilariae by polymerase chain reaction in real time has been developed and adapted to the conditions of the laboratory, which will speed up the establishment of an accurate diagnosis and hence the appointment of adequate treatment and prevention.

Fourteen conclusions are given, which fully correspond to the results of the research. I think that the conclusions could have been combined, for example, 5, 6, 7, 8 and 9 and not be fourteen, but less. Four contributions of an original nature and two of a confirmatory nature are indicated. 7 recommendations for practice were also made. In connection with the dissertation, Dr. Rafailov has a total of 4 scientific reports, two of which he participated in scientific conferences of the Faculty of veterinary medicine at LTU and another two scientific reports printed in scientific journals in which the doctoral student was in first place. In this way, it fulfills the requirements of the Law of developing of academic staff and the regulations of VMF for LTU.

I think the colleague took into account the notes of the preliminary reviewer and the other colleagues present at the previous meeting of the section.

I have the following notes and questions regarding the dissertation work: On page 9, 27 species are included in the genus *Dirofilaria*, maybe it is more than 27 species. The definition of prevalence is not precise. You present it as the total number of cases of a given disease over a period of time. The correct definition is that prevalence is the ratio of disease-affected individuals of a given population to the total number of individuals of that population at risk in a given period of time. On page 18 heartworm - not an appropriate definition. On page 24 – trimming of lungs arteries. Question: On page 55 why do you use the imaginal forms of *D.Immitis* and *D.Repens* as a positive control, and not blood from positive animals in the Knott method, or in the immune test, what is the presumption. On page 56 second paragraph there is a repeat of the positive controls for *D.Immitis* and *D.Repens*. Below you write that you measured the amount of DNA, but you do not write in what amounts it is. On page 67 fig. 17 and 18 second paragraph: The results regarding the prevalence in male dogs are presented in the next two figs. 17 and 18. However, the figures refer to the prevalence in female dogs. On page 84 fourth paragraph - Two of the positive Knott samples for microfilariae were determined to be co-infestations between *D.Immitis* and *D.Repens*, |Question: how did you determine them to be co-infestations between the two species. On page 84, the sixth paragraph repeats the sentence with the sentence of the 4th paragraph. On page 84, seventh paragraph, the sentence is unclear which one of the two or both methods tested positive for filaria. On page 84 the sentence: Ten of the antigen positive samples were negative by both the Knott method and the conventional PCR method. Question: What is the reason they are negative on these two tests? On page 84 fig. 32 it was nice to write below in the legend the size of the different filariae in base pairs e.g. *D.Immitis* -204 bp, *D.Repens*-327 bp, *Filaria*-500 bp. It is more correct to write pit, or line 1, 2, 3, etc. and not gel 1, 2, 3, because what you pour is gel, and the place where you drip the samples are slots or wells. On pages 85 and 86 when determining the diagnostic sensitivity and specificity of the Idexx Snap 4Dx and Knott methods versus conventional PCR on how many samples was probably performed on those 192 blood samples. Question: By which method is diagnostic sensitivity and specificity calculated? Which method is used as the gold standard for determining the sensitivity and specificity of tests. On page 105, how is the threshold defined, the limit of detection, why is it set so high. Where is the cut-off? On page 106 tab. 9 reflecting the Rt PCR data, initially some values are obtained from the tests, and in the re-test you have found other values. If you do a third test and get other new results, what happens then? Has a standard curve been drawn. Question: what is the reason for this difference and in your opinion until what cycle should the sample be considered positive until 38.4 or until 31.49, because it is not the same whether the sample will be found if it is positive until 38 or 31 cycle. If you assumed up to 35 cycles in fig. The 83 positive samples are not 14, they are less because you have several samples that pass the threshold after 35 cycles. On page 125 the microscopic pictures are very nice, but it would have been good to include negative controls from dogs with no known disease. On page 128 non-joint treatment - what does this mean. On page 132 The advantages of this one-step PCR is that it amplifies a common gene you might have in mind and uses specific primers. Page 133 the sentence in the first paragraph One of the samples.....was positive for filaria by which method probably Rt PCR. On the same page 133, last paragraph, the absence of microfilariae can be explained by the presence of only female imaginal forms or the use of antinematode agents. The first statement is false because PCR detects specific DNA and 12 S RNA regardless of whether the filaria are female or male. The

second assumption is more likely. Page 134 second paragraph below Low sensitivity results were obtained in co-infested animals by both methods. Question: What do you think is the reason for this? Page 147 Conclusions: Conclusion No. 2 cannot be accepted as a conclusion because there is one judgment and it does not derive from the present studies. In the contributions section, I don't accept the last two contributions because they are known things previously established. Recommendations for practice - Recommendations 3, 4, 5 and 6 could be combined into one, recommending that veterinarians pay attention to these changes when making a diagnosis. Written like that, they sound like results, not recommendations.

The notes and questions I mentioned do not reduce the value of the development, they are not of substance, but more of a technical nature and are intended to see how the author thinks and answers the questions that arose and asked by me.

Conclusion: The dissertation work presented to me for review on the topic: Studies on cardiac heartworm disease in dogs in Bulgaria for the award of a scientific and educational degree "Doctor" by Radoslav Mitkov Rafailov, is an up-to-date scientific work of great importance for veterinary parasitology. The set goals and tasks are correctly set and fulfilled, and the results obtained and the conclusions drawn are reliable. I believe that these data will be of benefit to fellow veterinary parasitologists working with pets. I believe that the dissertation fulfills the criteria of the law of developing of academic staff and the regulations of the VMF at LTU and I give it a positive review.

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