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REVIEW

from associate professor Veselin Nanev Nanev PhD. IEMPAM – BAS,

member of a scientific jury appointed by Order of the Rector of University of Forestry ЗПС-567/14.11.2023г.

CONCERNING: My submitted dissertation on the topic "Studies on cardiac heartworm disease in dogs in Bulgaria" for the acquisition of the educational and scientific degree "Doctor" in the field of higher education 6. Agricultural sciences and veterinary medicine, professional direction 6.4. Veterinary medicine, scientific specialty "Parasitology and invasive diseases of animals and humans", developed by master Radoslav Mitkov Rafailov with scientific consultant

Assoc. professor Kostadin Pavlov Kanchev dvm

As a member of the Scientific Jury, according to the Order of the Rector of University of Forestry ЗПС-567/14.11.2023г. i got acquainted with the materials provided for the procedure for the defense of the dissertation work of master veterinarian doctor Radoslav Mitkov Rafailov. He is a doctoral student of an independent form of study with scientific consultant Assoc. professor Kostadin Pavlov Kanchev dvm and has been awarded with the right to defense, having passed all the legal indicators for the defense before a scientific jury in the field of higher education 6. Agricultural sciences and veterinary medicine, professional direction 6.4 Veterinary medicine, scientific specialty "Parasitology and invasive diseases of animals and humans" .

Master veterinarian Radoslav Mitkov Rafailov has successfully passed exams for his doctoral program in the following disciplines: "Methods for the diagnosis of parasitosis", "Parasitis in companion animals" and "Parasitosis in productive animals". The doctoral student's work as a veterinarian at COOP DOVERIE LESIDREN OOD, clinic "ST GEORGI" ESTRELA VET OOD and teaching assistant Forestry University gives me reason to believe that the personal qualities listed in the candidate's CV (communicativeness, adaptability, intransigence, loyalty, responsibility and teamwork) are well expressed in the Ph.D. candidate. To this we must add his participation in conferences and seminars - 10 pcs. , projects -4 pcs. , publications - 8 pcs. in total, with 2 of them being on the topic of the dissertation. Also 3 pcs. citations from publications related to the topic of the dissertation. The honors and awards received by the doctoral student - 5 pcs. from participation in scientific forums, speaks of his high activity and competence not only in the subject of the dissertation, but also in the field of veterinary medicine.

The dissertation work is structured in the generally accepted way, with the individual components being logically connected. The important role of cardiac dirofilariasis and its spread

in dogs in Bulgaria is emphasized. The dissertation is presented on 187 pages and contains an impressive number of 393 literary sources.

The literature review shows a comprehensive and comprehensive overview of the available literature and authors. The doctoral student is familiar with the state and directions of research on the topic. According to his literature review, the question of exactly which mosquito species are the potential carriers of invasive larvae and are responsible for the spread of the disease and the invasion of the final hosts in our country remains unclear. In this regard, there is a lack of sufficient information regarding the invasion of mosquitoes with invasive larvae and their summer dynamics such as number and distribution, and to what extent climatic features are the basis of the wider spread of the disease in recent years.

The introduction and the literature review provide a rationale for the set goals and objectives. The goal of the doctoral student is to conduct a study of the prevalence of heart heartworm disease in dogs in different regions of Bulgaria, as well as to specify the possibilities for diagnosis of the disease, to deepen the knowledge about the pathological changes in the final hosts, about the species composition of the intermediate hosts, the seasonal dynamics of mosquitoes in different areas of the country and to make a correlation between their number and their carriage of *D. immitis* microfilariae.

To achieve the goal, the doctoral student sets himself the following tasks:

1. Study of the distribution of *D. immitis* in dogs in different regions in Bulgaria by using rapid antigen tests.
2. Comparison of the results obtained by the Knott method with those of rapid antigen tests and the conventional PCR method in the studied dogs.
3. To determine the species composition of mosquitoes known to carry *D. immitis* in regions of Bulgaria with different climatic and geographical conditions.
4. To establish the beginning and end of the period of effective infection of mosquitoes;
5. To make a comparison between the seasonal monthly summer dynamics of mosquitoes in different cities of the country, investigated for the carriage of D. immitis microfilariae, by Real time PCR.
6. To make a comparison between the seasonal monthly summer dynamics of mosquitoes in different cities of the country, investigated for the carriage of *D. immitis* microfilariae, by Real time PCR.

Material and methods

A large number of final hosts were studied - 2626, more than 3000 were caught and studied. female mosquitoes, 192 blood samples were also examined by Knott's method and conventional PCR test.

The results are correctly presented based on the applied methods. They are illustrated with 13 tables and 105 figures. They are original and emphasize the most significant of the purpose and

tasks. I was particularly impressed by the discussion chapter, in which the doctoral student made a comparative analysis of the results obtained by the Knott method with those of rapid antigen tests and conventional PCR in the studied dogs.

From the research carried out, the doctoral student formulated the following more important conclusions:

1. The complex study conducted on the spread of cardiac heartworm disease showed that the disease occurs in all regions of the country, dominating in the southern, warmer parts of Bulgaria.

2. The territorial ubiquity of cardiac heartworm disease is likely due to climate change and global warming, with temperature being the main abiotic factor in the spread of established mosquitoes.

3. The screening study for *D. immitis* antigens showed a prevalence of 11.12% in dogs in our country.

4. The widest distribution of positive dogs for the parasite is found in the designated Central South region.

5. The main reservoir of the parasite is the stray dogs, where the extent of invasion is 24.75%, probably due to the lack of application of effective means for the prevention of the disease.

6. No correlation was found between the sex of the dogs and the prevalence of infestation.

7. The percentage of infested dogs from the breeds Caucasian Shepherd Dog (36.84%), Akita Inu (33.33%), Beagle (23.53%), Drathaart (23.08%), Kurzhar (20.00%) and Bulgarian Shepherd Dog (20.00%) was higher.

8. The number of established infestations in "Shepherd and herding dogs" and "Hunting dogs" is greater.

9. Animals between 2 and 7 years of age, mainly large breeds, are at the highest risk of disease.

10. A precise differentiation of *D. immitis* larvae from *D. repens* larvae is achieved by conventional PCR.

11. The sensitivity and specificity of the IDEXx SNAP 4Dx Plus rapid antigen test compared to conventional PCR for D. immitis was 90.0 and 94.5%, respectively, and for co-invasion between *D. immitis* and *D. repens* was 75.0 and 91.5%. The sensitivity and specificity of the Knott method compared to conventional PCR for *D. immitis* was 80.0 and 100.0%, respectively, and for co-invasion between *D. immitis* and *D. repens* was 50.0 and 100.0%.

12. The following mosquito species have been identified in our country: *C. pipiens*, *Ae. albopictus*, *Ae. cinereus*, *Ae. vexans*, *Ae. caspius*, *Anopheles maculipennis* complex and *Culiseta* sp. The species *C. pipiens* is found with the highest frequency.

13. The highest numbers of mosquito catches are achieved on days with high temperature and little precipitation.

14. A directly proportional correlation is established between the number of mosquitoes caught and the aggregate Real time PCR samples tested positive by them during the summer months of the year.

Data is analyzed precisely, and contributions formulated clearly and concretely. I fully accept the presented reference for the 6 scientific contributions of the study. The doctoral student's complex study of the spread of cardiac dirofilariasis in dogs in some regions of Bulgaria has established the types of mosquito vectors and the dependence between their spread and temperature and seasonal characteristics. He also diagnosed *D. immitis* microfilariae in mosquitoes from our country by Real time PCR. The presence of microfilariae from the parasite *D. immitis* was also found in the following mosquito species: *Ae. albopictus, C. pipiens, Ae. cinereus, Ae. vexans* and *Anopheles maculipennis complex*.

I would like to emphasize that I was particularly impressed by the comparative analysis of the results obtained by the Knott method with those of rapid antigen tests and conventional PCR in the studied dogs. According to the PhD student, the main drawback of all microscopic methods, including Knott's, is the low sensitivity they have, especially in animals with low microfilariemia. In recent years, the trend towards the application of molecular methods for the differentiation and diagnosis of the types of dirofilariosis has become more and more important. The PhD student used an adapted methodology of Giola et al. (2010). which provides a highly sensitive and specific protocol for the detection and speciation of microfilariae, based on the detection of the mitochondrial gene 12S rDNA, which is conserved for all canine filariae. Extrachromosomal mitochondrial DNA is known to be relatively less fragile than nuclear DNA and is present in high copy numbers in each organism, providing high sensitivity of the method (Mishra et al., 2007). The advantage of this one-step PCR is that it amplifies a specific common gene for canine filaria and specific primers for D. immitis and D. repens. In this way, double testing of the samples for the presence of both filarids is avoided, which ensures speed and less financial costs. It also allows the detection of coinfections in a single blood sample. The conclusion reached by the doctoral student is that for an accurate diagnosis there is a need for complex studies with the aim of minimal chance of wrong results. This rapid, sensitive and specific one-step PCR assay he applied is capable of distinguishing between the two most common filarial parasites in dogs and represents an additional tool for epidemiological studies and routine disease assessment in co-endemic areas for both species.

The PhD student presents a report on his participation in conferences and seminars - 10 pcs. , projects 4- pcs. , publications - 8 pcs. in total, with 2 of them being on the topic of the dissertation. Also 3 pcs. citations from publications related to the topic of the dissertation. The honors and awards he received - 5 nos. from participation in scientific forums, speaks of his high activity and competence as a veterinarian doctor

The recommendations for practice section is also very impressive.. Based on the results obtained, he formulates 3 recommendations:

1. To achieve an objective result in the diagnosis of heart heartworm disease can be achieved with the combined application of conventional PCR, IDEXx SNAP 4Dx test and Knott's method.

2. It is necessary that the imaging diagnostics and the patho-anatomical examination be focused primarily on the pulmonary arteries and the right side of the heart, where the largest number of specimens of the parasite are found.

3. The characteristic macroscopic changes in cardiac dirofilariasis are dilatation and thickening of the vascular wall of the pulmonary arteries.

The dissertation is a timely original work on a large volume of material (ultimate hosts - 2,626, more than 3,000 female mosquitoes were caught and examined, 192 blood samples were also examined using the Knott method and conventional PCR test). The results are correctly presented based on the applied methods. They are illustrated with 13 tables and 105 figures, are original and emphasize the most significant of the purpose and tasks. Conclusions and contributions are well structured and articulated. This shows that Master Radoslav Mitkov Rafailov is a well-rounded specialist-veterinarian with serious knowledge in the field of epizootology, pathomorphology, pathology, parasitology, biochemistry and molecular biology.

General evaluation of the candidate's compliance with the minimum mandatory requirements according to the regulations for the development of the academic staff at University of Forestry

The dissertation project presented by the PhD student meets the requirements of the Regulations for the Development of the Academic Staff at the Forestry University, and can be submitted for printing.

Conclusion:

As a member of a scientific jury in the field of higher education 6. Agricultural sciences and veterinary medicine, professional direction 6.4 Veterinary medicine, scientific specialty "Parasitology and invasive diseases of animals and humans", I consider that the candidacy of PhD student Radoslav Mitkov Rafailov in the competition announced for ONS "Doctor" complies with the regulations of University of Forestry based on the ZRASRB. Therefore, I vote positively and wish success to the PhD student.

City of Sofia

16.02.2024

Member of a scientific jury:

Assoc. prof, Veselin Nanev Ph.D.