



Review

of a PhD thesis on the topic:

"Metagenomic studies on the determinants of antibiotic resistance in model environmental samples"

for the award of the educational and scientific degree "Doctor of Philosophy" in the scientific field 4.3. "Biological Sciences"

PhD student: *Deyan Valentinov Donchev* from the National Reference Laboratory "Control and Monitoring of Antimicrobial Resistance" (NRL "CMAR") of the Department of Microbiology, National Center of Infectious and Parasitic Diseases **with scientific supervisor Assoc. Prof. Ivan Ivanov**

Prepared by: *Assoc. Prof. Svetoslav Dimov*, chairman of the Dept. of Genetics, Faculty of Biology, Sofia University "St. Kliment Ohridski", appointed as a member of the Scientific jury by Order No. 439/20.12.2004 of the Director of the National Center of Infectious and Parasitic Diseases

1. Professional career of the PhD student

Deyan Donchev began his professional career by graduating with a Bachelor's degree in "Molecular Biology" at the Faculty of Biology - Sofia University "St. Kliment Ohridski". He continued his education with a Master's degree program in "Microbiology and Microbiological Control" at the same institution. Since 2021, he has been a full-time doctoral student at the National Center for Infectious and Parasitic Diseases. He worked as a biologist in the Laboratory of Microbiology and Virology at the "Lozenets" University Hospital and as a biologist-specialist in the Laboratory of Microbiology at the NRL "CMAR" at the National Center for Infectious and Parasitic Diseases, where he currently continues to work.

2. Scientific relevance of the PhD thesis.

The PhD Thesis submitted to me for evaluation is focused on a severe problem on a global scale, namely the study of the presence in the environment of genetic determinants that determine resistance to antimicrobial agents. The reason lies in the fact

that microorganisms, on the one hand, possess a large set of mechanisms for horizontal gene transfer, including interspecific ones, and on the other hand, their high mutability, which gives them enormous ecological plasticity, including in the form of acquiring resistance to antimicrobial agents. Thanks to this, it is possible for pathogenic strains of bacteria that possess genetic determinants that determine resistance to antimicrobials to enter the environment through sewage and wastewater, where even if they cannot survive for an extended period of time, they can transfer them to other bacteria. The extreme seriousness of this problem on a global scale is also evidenced by the fact that a few years ago, genetic determinants for antibiotic resistance to antimicrobials used exclusively in human medicine, but not in agriculture and aquaculture, were found in fecal samples of warm-blooded animals in Antarctica, which at the time was considered protected from such contamination. Another extremely worrying fact is that statistics show that the time required for the first resistant strains of microorganisms to a fundamentally new and different class of antibiotics to appear is, as a rule, much shorter than the time required for its development and testing for use in human medicine. A particular merit of the dissertation work is the chosen methodology – whole-metagenomic next-generation sequencing, which allows for avoiding the shortcomings of classical cultivation microbiological techniques. Therefore, I can confidently state that the topic of Deyan Donchev's PhD thesis is highly relevant worldwide and has not been worked on in Bulgaria so far, except for a limited pilot study in the vicinity of the Bulgarian Antarctic Base on Livingston Island in Antarctica.

3. General structure of the dissertation.

The presented PhD thesis is developed within 202 pages, including the bibliography. It is structured in a standard way, consisting of the following main sections: "literature review", "materials and methods", and "results and discussion" (presented together). "Introduction", "Aim and tasks", "Assumptions", "Conclusion", "Contributions" and "Supplementary materials" are presented separately.

3.1. Introduction

The PhD thesis begins with a brief introduction of two and a half pages, which indicates the specific and currently unexplored scientific problem - environmental pollution with bacteria possessing genes that confer resistance to various antibiotics and the associated risks. It justifies the need for the research conducted within the PhD.

3.2. Literature review

The literature review is developed within 66 pages. It is divided into two subsections - the first examines the essence of metagenomics, while the second focuses on antimicrobial resistance. In terms of content, the first part of this section reviews the essence and principles on which metagenomic studies are based, including the methods for bioinformatic analysis of the obtained data. It begins with definitions of basic concepts such as genomics, transcriptomics, proteomics and metabolomics, which is a prerequisite for examining in detail the essence of metagenomics, which in turn is the primary research approach in the present study. The applications of metagenomic studies are examined in depth, as well as the two main approaches –amplicon-based metagenomics and whole-metagenomic sequencing, indicating their advantages and disadvantages. Extensive attention has also been paid to the bioinformatics processing of data from next-generation metagenomic sequencing. My impression is that the doctoral student has deep knowledge and skills in the field of metagenomic research.

The second part of the literature review focuses on bacterial resistance to antibiotics. This part begins again with definitions of some basic concepts, followed by a detailed review of the mechanisms for acquiring and spreading genes that cause antibiotic resistance, such as mutations of cellular genes, transformation, transduction and conjugation. The current status of AMR and the occurrence and spread of genes that cause AMR in the environment in general and in water bodies, in particular, are reviewed. Finally, the section logically concludes with a review of the methods for studying AMR in the environment - a review of cultivation methods, methods based on polymerase chain reaction and those based on metagenomic studies (with an indication of the principles, advantages and disadvantages).

The section contains three tables and four figures of excellent graphic quality, and the sources on which they were based are correctly indicated. Overall, from the presentation of this part of the PhD thesis, I can be convinced that the PhD student has extensive scientific knowledge on the topic of its thesis and is well acquainted with the latest research worldwide in this field. It also indicates that he can very skillfully interpret scientific literature from various sources.

3.3. Aim and tasks

One goal has been clearly formulated for implementing the PhD thesis: to study the distribution and type of antimicrobial resistance determinants, mobile genetic elements and bacterial biodiversity in environmental samples and to assess the potential risk to human health. For its implementation, eight experimental tasks have been set, which are entirely sufficient for its achievement and, at the same time, cover a wide range of experimental work and data interpretation, the mastery of which is necessary for acquiring the PhD degree.

3.4. Materials and methods.

The section is developed within 19 pages. In a precise and reproducible manner, detailed methodologies for isolating total DNA from various water sources are described in detail to carry out the subsequent amplicon-based and comprehensive metagenomic studies. The set of bioinformatics methods used also makes a good impression. All this indicates that in the process of preparing his thesis, the PhD student has acquired a vast arsenal of practical skills corresponding to the acquisition of the PhD degree. The fact that the PhD student has not been tempted to discuss and interpret his results within this section is impressive. The section is illustrated with two figures and nine tables. In my opinion, the chosen approach to present the methodological steps in the form of tables is appropriate from the point of view of their more straightforward perception.

3.5. Results and Discussion

The section is developed concisely and with the help of rich illustrative material (5 tables and 24 figures with good graphic quality) within 62 pages. It presents the PhD student's own experimental results obtained. It makes a good impression that the PhD student does not rush to present experimental data from the study of water samples but begins with an "introductory" part, which aims to justify the choice of methodology for the study. It begins with the presentation and discussion of the results for biomass isolation using the created in-house protocol for flocculation in skim milk and its comparison with vacuum filtration, then proceeds to a comparative review of different kits and protocols for DNA isolation. At the end of this "introductory" part, the results of the applied optimizations for generating 16S sequence libraries follow.

The second part of the section logically presents the results from the metagenomic studies of water samples from the Iskar River in the area of the villages of Dragushinovo and Mechkata and the area of the city of Stara Zagora - groundwater and both wastewater treatment plants included in the study. This subsection presents the data from the metagenomic sequencing, alpha-diversity analyses, MAG assemblies and analysis of the presence of genes determining antimicrobial resistance. Concerning this part of the research work, of particular merit is not only the reporting of their presence but also the analysis of their genetic "environments" - plasmids, mobile genetic elements, etc. This is particularly important, as it allows for formulating hypotheses regarding the mechanisms of spread. Also of particular importance is the data obtained, indicating that wastewater treatment plants are ineffective in removing bacteria possessing genes determining antimicrobial resistance.

My overall impression of this section is that the PhD student possesses the necessary knowledge and skills to carry out a complete scientific study and correctly interpret his experimental results, analyzing and comparing them, where possible, with other similar scientific studies. This prerequisite is necessary for obtaining the "Doctor of Philosophy" degree. I have only a few minor technical remarks about this section, such as separating the legends of some figures on different pages.

3.6. Assumptions

Seven assumptions have been formulated based on the experimental work carried out and the scientific-theoretical and scientific-applied results obtained. It makes a good impression that the PhD student has not been tempted to unnecessarily increase the number of assumptions, and that there are no contributions formulated as conclusions.

3.7. Conclusion

Within three and a half pages, the justification for conducting this study, the results obtained, and the most important conclusions made are presented concisely.

3.8. Contributions

A total of 8 contributions are presented, of which one is formulated as fundamental, five as scientific and two as methodological. The obtained experimental results fully justify them, so I accept them without substantive remarks. A good impression is also made here because no conclusions are formulated as contributions are presented. My only comment is technical since the numbering of the conclusions is inconsistent.

4. Abstract.

The abstract is developed within 78 pages. Its content repeats the PhD thesis itself in an abbreviated form. It contains the main sections "Introduction", "Materials and Methods" and "Results and Discussion". Additionally, "Aim and Tasks", "Assumptions", and "Contributions" are presented. The illustrative material includes 22 figures and 13 tables. In my opinion, the abstract's volume exceeds the generally accepted norms, as it would be pretty sufficient only to list the methods but not to present them in detail. As a technical comment, I can point out the incorrect numbering of the "Aim and Tasks" section and the separation of some figures on separate pages from their legends. An abstract in English is not presented; instead, within two pages, a brief summary in English is presented at the end of the abstract in Bulgarian.

5. General remarks on the dissertation project.

Overall, the work presented for consideration gives a good impression of a completed comprehensive scientific research on a current problem carried out with some of the most modern methods in the field of molecular genetics. The general impression that I am left with is that the PhD student is a complete and promising young scientist who is capable of carrying out comprehensive scientific research on a specific topic, skillfully using a large set of laboratory techniques and methods, as well as adequately and reasonably interpret his own experimental and bioinformatics results.

6. Acquired competencies and compliance with the requirements of the educational and scientific degree "Doctor of Philosophy"

In carrying out the scientific research laboratory, theoretical and bioinformatics work, as well as in shaping the PhD thesis work itself, Deyan Donchev has acquired competencies, expressed in knowledge in the specific scientific field, skills for setting and solving specific scientific research tasks, skills for applying modern research methods in the specific field, as well as skills for evaluating the results obtained. They are fully adequate for acquiring the educational and scientific degree "Doctor of Philosophy".

7. Eligibility for disclosure of a PhD thesis defense procedure

According to the information presented to me, Deyan Donchev is a co-author of one scientific publication with a quartile Q1 and one with a quartile Q2. According to the Regulations for the implementation of the Law for the Development of Academic Staff in the Republic of Bulgaria for Professional Field 4.3. "Biological Sciences", he collects the required 50 points under indicator 1 by writing a PhD thesis. Under the group of indicators "G" (indicator 7), he collects 45 points with a required minimum of 30 points, with which he covers and even exceeds the requirements by the Law minimum.

8. CONCLUSION.

In terms of content, the PhD thesis work presented to me represents a comprehensive and adequate study in terms of the volume necessary for the acquisition of the educational and scientific degree "Doctor of Philosophy" in Professional Field 4.3. "Biological Sciences". Deyan Donchev is an accomplished young researcher who has acquired significant scientific knowledge and skills, as evidenced by his co-authorship in 18 scientific publications and a Scopus h-index of 3. He also collects the necessary points according to the Regulations for the Implementation of the Law for the Development of Academic Staff in the Republic of Bulgaria, which is why I will vote positively for the acquisition of the educational and scientific degree "Doctor of Philosophy" in Professional Field 4.3. "Biological Sciences", and I will encourage my colleagues from the Scientific Jury to do the same.

Sofia, February 09, 2025

Assoc. Prof. Svetoslav Dimov

**Svetoslav
Gueorgui
ev Dimov**

Digitally signed by
Svetoslav
Gueorguiev
Dimov
Date: 2025.02.10
20:09:45 +02'00'