



REVIEW

by Professor Georgi Georgiev Beev, PhD, Trakia University, Stara Zagora

on the **PhD thesis** submitted for the acquisition of the educational and scientific degree "Doctor"

Field of Higher Education: 4. Natural Sciences, Mathematics, and Informatics

Professional Field: 4.3. Biological Sciences

Specialty: Microbiology

Author: Deyan Valentinov Donchev

Title: "Metagenomic Studies on Determinants of Antibiotic Resistance in Model Environmental Samples"

Scientific Advisor: Assoc. Prof. Ivan Ivanov, MD, National Centre of Infectious and Parasitic Diseases, Sofia

1. General Description of the Submitted Materials

By order No. 439 dated 20.12.2024 from the Director of the National Centre of Infectious and Parasitic Diseases (NCIPD), I have been appointed as a member of the scientific jury for the defense of the PhD thesis titled "*Metagenomic Studies on Determinants of Antibiotic Resistance in Model Environmental Samples*", submitted for the acquisition of the educational and scientific degree "Doctor" in the field of Higher Education 4. Natural Sciences, Mathematics, and Informatics, professional field 4.3. Biological Sciences, specialty "Microbiology".

The author of the PhD thesis is Deyan Valentinov Donchev, a full-time PhD student at the National Reference Laboratory for Control and Monitoring of Antibiotic Resistance (NRL CMAR), NCIPD, Sofia, under the supervision of Assoc. Prof. Ivan Ivanov, MD.

The materials submitted by Deyan Valentinov Donchev comply with the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (ZRASRB) and the internal regulations of NCIPD.

2. Brief Biographical Data of the PhD Student

Deyan Valentinov Donchev holds a Bachelor's degree in Molecular Biology and a Master's degree in Microbiology and Microbiological Control, both obtained from the Faculty of Biology at Sofia University "St. Kliment Ohridski". He is currently employed as a biologist-specialist in laboratory microbiology at NRL CMAR, NCIPD, Sofia.

3. Relevance of the Topic

The PhD thesis addresses an issue of exceptional relevance, considering the growing global threat of antimicrobial resistance (AMR). The World Health Organization (WHO) identifies AMR as one of the most pressing public health challenges, necessitating urgent monitoring and control measures.

This research approaches the problem from an environmental perspective, recognizing the environment as a reservoir and intermediary in the spread of resistant microorganisms and antibiotic resistance genes (ARGs). Given that no systematic studies on AMR in the environment have been conducted in Bulgaria to date, this work is both innovative and significant.

The application of metagenomic methods to analyze microbial communities and ARGs provides a modern, highly sensitive approach that enables a detailed characterization of bacterial composition and resistance mechanisms.

The study's practical significance is considerable, as its findings could inform future monitoring programs and regulatory policies in public health and environmental protection. This is the first comprehensive study of its kind in Bulgaria, laying the groundwork for systematic ecological surveillance of AMR.

In conclusion, the PhD thesis is timely, scientifically sound, and of high public importance, addressing a critical issue with both public health and environmental implications.

4. Understanding of the Problem

The PhD student demonstrates an in-depth understanding of AMR in the environment, clearly outlining its global significance and the ecological factors contributing to the spread of resistant bacteria (RMB) and ARGs.

The PhD thesis exhibits broad awareness of advanced analytical methods, including metagenomic approaches and bioinformatics tools, highlighting the technical competence of the researcher.

The research objectives and tasks are well-formulated, covering a broad spectrum of scientific questions—from methodological optimization of DNA extraction to analyzing bacterial diversity and determinants of resistance in environmental samples. The research tasks are diverse and ambitious, yet they are effectively integrated into the overall research logic, maintaining a clear focus on the main scientific hypothesis.

The Materials and Methods section is particularly well-developed, demonstrating a deep understanding of modern microbiological, molecular-genetic, and bioinformatics techniques. Notably, the chosen methods are carefully selected and well-justified, with the PhD student not only applying established approaches but also successfully adapting and optimizing laboratory protocols, particularly in DNA isolation and bacterial biomass concentration. Special attention is given to metagenomic and bioinformatics analyses, which significantly enhance the scope of conventional culture-based methods and provide a detailed perspective on the distribution of resistant bacteria and ARGs in the environment.

The Results and Discussion section is logically structured, with findings categorized according to different environmental sample types (wastewater, river water, groundwater), enabling a clear organization of results and their interpretation within a relevant ecological context. The discussion is conducted competently, based on reliable and quantitatively processed data, allowing the identification of key trends and patterns.

The analysis of the scientific and applied contributions of the PhD thesis reveals progress in several key areas:

- Development and validation of new methodological approaches, including an optimized in-house DNA extraction method, which outperforms commercial kits in terms of yield and DNA quality.
- Evaluation of different bacterial biomass concentration techniques, highlighting the advantages of milk-based flocculation over traditional vacuum filtration.
- Comprehensive metagenomic analysis of samples from ecologically significant locations, including wastewater treatment plants (WWTPs), rivers, and groundwater.
- Identification of high-risk zones for the spread of antimicrobial resistance, emphasizing the role of wastewater treatment plants as major reservoirs and sources of ARGs.

The PhD student not only presents experimental results but also analyzes them critically, comparing them with international studies, demonstrating a strong ability to interpret data within a broader scientific context.

The conclusions are well-supported and practically applicable, laying a foundation for future monitoring programs. This confirms the PhD student's high level of theoretical preparation and experimental expertise.

5. Research Methodology

The PhD thesis employs a comprehensive, well-documented, and scientifically sound methodology, integrating modern molecular, microbiological, and bioinformatics approaches to analyze AMR in the environment. Key methodological aspects include:

- Collection and processing of samples from surface, groundwater, and wastewater, utilizing various bacterial biomass concentration techniques, including vacuum filtration and milk-based flocculation.
- DNA extraction using an optimized in-house protocol, proven to be superior to commercial kits.
- Metagenomic sequencing (16S rRNA and shotgun sequencing) for taxonomic and functional characterization of microbial communities.
- Bioinformatics analysis, including taxonomic identification, ARG assessment, and mobile genetic element (MGE) analysis.

The methods are carefully selected and validated, and the experimental design is well-structured, ensuring high reliability of results. The integration of molecular-biological and bioinformatics techniques enables a comprehensive assessment of AMR distribution and its impact on the environment.

6. Evaluation of the PhD Thesis and Its Contributions

The PhD thesis follows the classical structure of a scientific study, beginning with a well-organized introduction to the research problem, the definition of the main objective, and the formulation of specific research tasks. The literature review is thorough and encompasses key global studies, clearly highlighting the significance of antimicrobial resistance (AMR) as a global health and environmental challenge.

The findings on horizontal gene transfer and public health risks could support strategies for controlling resistant pathogens. Furthermore, the validated methodologies hold potential applications in healthcare and pharmaceuticals, contributing to the development of new antibacterial strategies.

These insights could also be integrated into academic curricula and support participation in international research collaborations focused on environmental AMR monitoring and control.

Final Conclusion

The PhD thesis meets all legal and academic requirements for awarding the Doctor degree. Given its significant scientific contributions, I strongly recommend conferring the degree of PhD in Microbiology upon Deyan Valentinov Donchev.

Reviewer:

Prof. Georgi Georgiev Beev, PhD

- Investigation of horizontal gene transfer (HGT) potential in natural environments, raising significant concerns about the ecological and health implications of resistant bacteria in water resources.

Among the most significant applied contributions are specific recommendations for AMR monitoring and control, which could serve as the foundation for future regulatory measures in Bulgaria. The PhD thesis not only provides fundamental scientific insights but also presents practically applicable solutions for monitoring ARGs and assessing the risk of contamination with resistant bacteria in the environment.

In some aspects, the study could be further expanded, particularly regarding the experimental validation of certain detected resistance genes and their functional role in natural conditions. For instance, presenting experimental evidence of gene expression in isolated bacterial strains would add further depth. However, this does not diminish the scientific value of the research but rather outlines prospects for future investigations in this field.

Undoubtedly, the achieved results and conclusions have broad applications, both in academic research and in the development of environmental and health policies. The PhD thesis makes a valuable contribution to both science and practice, offering an innovative approach to studying antimicrobial resistance in environmental samples.

7. Evaluation of Publications and the PhD Student's Contribution

The PhD thesis is based on two scientific publications in international journals indexed in WoS and/or Scopus. In both publications, the PhD student is listed as first author, confirming their substantial individual contribution.

According to the regulations of the Bulgarian Academic Development Act (ZRASRB), the PhD student fully meets the required 30 points from publications, as the articles collectively amount to 45 points. The research findings have also been presented at two scientific conferences, one of which is international.

8. Recommendations for Future Use of the PhD Thesis Contributions and Results

The PhD thesis provides significant scientific and practical contributions. It can serve as the foundation for developing a national AMR monitoring system, aiding early detection and prevention of resistant microorganisms in high-risk ecosystems.