SCIENTIFIC OPINION

From Assoc. Prof. Mariana Ivanova Marhova-Koseva, PhD
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Member of the Scientific Jury, formed by Order No. I-163/26.06.2024 of the Director of the Stefan Angelov Institute of Microbiology, Bulgarian Academy of Sciences

Regarding: Dissertation for awarding the educational and scientific degree "Doctor"
Field of higher education: 4. Natural Sciences, Mathematics, and Informatics
Professional field: 4.3. Biological Sciences
Scientific specialty: Microbiology
Author: Dayana Borislavova Borisova
Topic: Comparative investigations on *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients prior- and post- inhalatory tobramycin therapy
Scientific supervisors: Assoc. Prof. Stoyanka Rangelova Stoitsova, PhD – Institute of Microbiology, BAS, and Prof. Dr. Tanya Vasileva Strateva, MD – Medical University – Sofia

1. Relevance and Importance of the Dissertation Topic

The topic of the presented dissertation by Dayana Borisova is related to tracking the effects of inhalation therapy with tobramycin in patients with cystic fibrosis on the morphology and physiology of isolated pairs of *Pseudomonas aeruginosa* strains at different stages of treatment. The theme intersects with several current and rapidly developing scientific fields related to the cosmopolitan nature and impressive metabolic, regulatory, and genetic adaptability of *P. aeruginosa*. In recent years, *P. aeruginosa* has become a model organism in the study of virulence and pathogenicity in opportunistic infections, systems of multidrug resistance, biofilm formation, quorum sensing, etc. The inclusion of *P. aeruginosa* in the ESKAPE group by the World Health Organization, due to its growing multidrug resistance, emphasizes the need for prolonged and extensive research on the behavior, characteristics, and adaptation of the species in different types of infections and the therapies applied. Data accumulated from studies on cystic fibrosis (CF), a relatively rare genetic disease, indicate the direct involvement of *P. aeruginosa* as an opportunistic pathogen in lung infections of CF patients. High resistance to drugs, as well as the ability to form biofilms, have been cited as major reasons for the difficulties in therapy. Considering the above, the topic of Dayana Borisova's dissertation is relevant and contributes to filling the gaps regarding the effects of inhalation antibiotic therapy as an approach to treating bacterial infections in CF patients.

2. Structure of the Dissertation and Evaluation of the Results and Contributions

The dissertation is written on 148 standard A4 pages following the commonly accepted structure: Introduction, Literature Review, Aim and Objectives, Materials and Methods, Results and Discussion, Conclusions, Contributions, References. The recommended proportions between the sections have been observed. The dissertation leaves a good impression due to its

concise scientific style and technical formatting. The work is richly illustrated with 15 tables and 51 figures summarizing the obtained results.

In the literature review, the doctoral student justifies the selected topic by briefly presenting data on the characteristics of cystic fibrosis as a genetic disease. A key element of the discussion is the information about the biology of *P. aeruginosa* as the main etiological agent in bronchopulmonary infections in CF patients. Updated information on the ability of *P. aeruginosa* to form biofilms and the impressive array of virulent traits that make it successful in opportunistic infections is systematically presented. The doctoral student has compiled a clear informational picture regarding the adaptive strategies of this successful pathogen in the onset and chronicity of infections in CF patients. The section also includes information on current therapeutic protocols for such conditions, as well as the reported dangers of the effect of inadequately selected sub-inhibitory therapeutic concentrations.

In light of the literature review, the stated aim clearly defines the direction of work in the dissertation. To achieve this, seven tasks were formulated, which logically outline the course of the conducted research.

In the Materials and Methods section, the scientific methods used, both modern and classical, from the fields of microbiology, molecular biology, and cell biology, are described in detail. The appropriate selection of strains and adaptation of in vitro protocols for a maximally realistic assessment of "in-host" evolution and adaptation of the strains in infections in CF patients are impressive. The methods applied include cultivation in various media and conditions; determination of the minimum inhibitory concentration (MIC) of tobramycin for the studied pairs of strains, isolated before and after several cycles of inhalation therapy; assessment of the effects of its subinhibitory concentration on the bacterial phenotype; biofilm formation; random amplification of polymorphic DNA (RAPD-PCR); scanning electron microscopy (SEM); and investigation of invasiveness and intracellular survival. The doctoral student has described the methods used for morphometric and statistical analysis of the obtained results and data.

In the Results and Discussion section, original data from molecular epidemic typing using RAPD-PCR of selected *P. aeruginosa* strains from CF patients in Bulgaria are presented. The applied UPGMA analysis revealed a high percentage of similarity with a reference panel of strains from different geographic regions. Such data certainly contribute to the establishment of RAPD-PCR as a method for molecular typing of *P. aeruginosa* strains and enhancing the effectiveness of infection control in CF patients.

The results of the investigation into the phenotypic characteristics of the selected strains are presented in detail. These include the tracking and determination of generation time, growth curves, planktonic growth, biofilm formation, morphological characteristics, and motility during cultivation in different growth media and conditions. The data from these original studies provide the first evidence of an extended lag phase in the "in host" evolution of CF *P. aeruginosa* strains in relation to inhalation therapy with tobramycin. To date, no connection has been reported between antibiotic therapy in patients and changes in the lag phase. The highest values obtained for biofilm formation in the minimal M63 medium, regardless of the stage of isolation of the strains, provide significant evidence supporting the likelihood that persister cells play a role in the adaptation and persistence of *P. aeruginosa* in the lungs of patients with CF.

The observations on the morphology of the biofilms formed by the studied *P.aeruginosa* strains, conducted using scanning electron microscopy (SEM), reveal a significant difference between the strain pairs as a result of the tobramycin therapy applied. The doctoral student has performed a very good morphometric analysis of the results from the determination of cell sizes. Unlike the commonly reported reduction in cell size under unfavorable conditions in the literature, the strains of *P. aeruginosa* from cystic fibrosis patients examined here showed a tendency for an increase in cell size after antibiotic therapy. These results provide a strong foundation for future research on the composition and changes in the biofilm matrix during inhalation therapy with tobramycin. The data from such studies could contribute to improving the effectiveness of this treatment approach for infections in CF patients.

To assess changes in invasiveness, the doctoral student applied co-cultivation of the selected *P. aeruginosa* strains and a human lung adenocarcinoma cell line A549, used as a model for type II alveolar cells. The choice of this model is commendable as it allows for maximal approximation and reproduction of the cross-interaction between *P. aeruginosa* and the human host, accounting for the heterogeneity in the adaptive response of strains in lung infections in CF patients before and after therapy. The assessment of invasiveness of the selected strains is related to one of the remarkable "tricks" of pathogenic bacteria to evade antibiotic therapy and participate in the chronicity of infections – penetration, survival, and replication in eukaryotic cells. Particularly indicative in this regard is the strong invasiveness of the strain (PaT-6), isolated after multiple cycles of inhalation therapy with tobramycin. Future research on the genetics of such invasiveness is of interest in optimizing current and developing new therapeutic approaches.

A significant portion of the dissertation is devoted to investigating the effects of sub-therapeutic concentrations of tobramycin – ½ MIC (minimum inhibitory concentration) and ¼ MIC – on growth, biofilm formation, motility, and viability of selected *P. aeruginosa* strains. A comprehensive evaluation of the results shows an increase in tolerance to the antibiotic in strains isolated after therapy compared to their paired strains, confirming the importance of sub-therapeutic concentrations as a risk factor. Given the reported increasing drug resistance of pathogenic microorganisms worldwide and the absence of studies on the effects of sub-therapeutic antibiotic concentrations in strains associated with infections in CF patients, the systematic data presented in Dayana Borisova's dissertation are original and have potential for contributing to optimizing therapeutic choices and procedures in treating CF patients.

As a result of the conducted research and obtained data, the doctoral student formulated 12 conclusions and 4 contributions, which fully correspond to the stated aim and objectives. The topic and development of the dissertation are original and significant, with potential for further development and exchange of results in both fundamental microbiological and clinical directions. The *P. aeruginosa* strains used in this study can be provided to enrich the existing international reference panel of strains isolated from CF patients in various geographic regions.

3. Evaluations, Opinions, Recommendations, and Notes

Doctoral student Dayana Borisova has applied a rich set of modern and classical microbiological, biochemical, and molecular-genetic methods in the development of her

dissertation, which has made her a highly qualified scientific specialist with great potential for participation in research teams for projects with both fundamental and practical orientations.

The relevance of the research and the results presented in the dissertation by Dayana Borisova provide a solid foundation for future studies using transcriptomic analysis in order to obtain in-depth data on the molecular mechanisms through which *P. aeruginosa* adapts its metabolism and behavior under conditions of inhalation antibiotic therapy with tobramycin. Transcriptomic analysis would allow for tracking changes in gene expression in response to antibiotic therapy, thereby revealing key biological processes related to resistance, biofilm formation, and invasiveness. Such data would contribute to the understanding of bacterial adaptation mechanisms and the optimization of treatment for infections caused by *P.aeruginosa* in patients with cystic fibrosis..

Dayana Borisova has exceeded nearly twice the minimum requirements for credits in the field of natural sciences according to the Bulgarian Law on the Development of Academic Staff and the rules of the Bulgarian Academy of Sciences (BAS), as well as the additional criteria for academic development at the Institute of Microbiology – BAS. The results obtained have been partially published in two papers, one of which is in a journal with an impact factor. Their significance is highlighted by the 17 citations reported so far. Additionally, the work on the dissertation is connected to the publication of a book chapter with an international publisher. The doctoral candidate has participated with 5 oral presentations and 4 posters at scientific forums both in Bulgaria and abroad. During the dissertation work, she has been a principal investigator in two and a participant in six scientific projects.

4. Conclusion

The dissertation presented by Dayana Borisova contains scientific and applied scientific results that represent an original contribution to science and meet all the requirements of the Law on the Development of Academic Staff in the Republic of Bulgaria (LDASRB) and the Regulations for the Conditions and Procedure for Acquiring Scientific Degrees and Academic Titles at the Institute of Microbiology "Stefan Angelov" – BAS. The presented materials and dissertation results, in terms of relevance, research scope, scientific contributions, and publication activity, fully comply with the specific requirements adopted in connection with the Regulations of BAS and the Institute of Microbiology – BAS for the application of LDASRB.

Based on the above, I confidently give my positive assessment of the conducted research and recommend to the esteemed scientific jury to award Dayana Borisova the educational and scientific degree of "Doctor" in the field of 4.3. Biological Sciences (Microbiology).

February 4, 2025

Signature: .....

Assoc. Prof. Mariana Ivanova Marhova-Koseva