**SCIENTIFIC OPINION**

from **Prof. Maria Bogomilova Angelova, DSc**, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences
on a dissertation presented to a Scientific Jury, formed by order No. I-163/26.11.2024 of the Director of the Stephan Angeloff Institute of Microbiology the award of the educational and scientific degree "Doctor" in Professional field: 4.3. Biological Sciences (Microbiology)
Doctoral candidate: **Dayana Borislavova Borisova**
Dissertation title: **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**
**Prof. Tanya Vassileva Strateva, PhD**

Cystic fibrosis (CF) is a genetic disease that severely threatens the lives of those affected by blockages and complications in the respiratory and digestive systems. Before the 1980s, many people with CF did not survive into their 20s. Today, the use of various antibiotics, together with new therapeutic interventions, is gradually increasing life expectancy and offering hope for more significant successes. Antibiotic treatment is an essential part of therapy, especially in pulmonary exacerbations. A current therapeutic strategy is the aerosol delivery of antibiotics, which significantly improves the quality of life in CF. Despite the progress made, serious problems are emerging in this area. Available therapeutic strategies are often insufficient to completely eradicate the pathogens concerned, which is a prerequisite for the emergence of antimicrobial resistance (AMR). A major cause of AMR is the use of subtherapeutic levels of antibiotics, which increases the risk of treatment failure. The problem is that the main pathogens that colonize the patient's bronchial tree, such as Pseudomonas aeruginosa, adapt to the drugs administered. This is the theoretical focus of Diana Borisova's development. The supervisor and the doctoral student are proposing a new approach to the study of the disease, which provides data on the involvement of antibiotics in the development of bacterial resistance. There is a lack of data in the literature on the effect of a specific antibiotic on the mechanism of adaptation of *P. aeruginosa* in CF cases as presented in this paper. It focuses on genotypic and phenotypic changes in pairs of strains isolated from CF patients before and after inhaled tobramycin therapy. Based on all of the above, I consider the topic to be current and theoretically significant with application prospects.

The dissertation is structured in a traditional form with relevant sections. It consists of 148 standard computer pages, including 133 pages of text and 15 pages of references. The literature review is focused and specific and covers all aspects of the research. It includes 240 references (238 in Latin) and 8 websites corresponding to each of the tasks set. The review presents the current state of the problem, taking into account historical developments and achievements. The available literature on the disease, the aetiological progression of bacterial infections in CF

patients, the role of the major pathogen *P. aeruginosa*, biofilm formation, and virulence determinants are discussed in great detail. The adaptation of P. aeruginosa to chronic lung infection and the treatment of the disease with antimicrobial drugs are also discussed. At the end of the review, there is a section entitled "Summary of the literature review", which highlights unresolved questions and the need for further research. The novelty of the development and the rationale for this evaluation are highlighted. In my opinion, this is a very good idea, which once again emphasizes the relevance of the thesis. The material is clearly presented and is easy and interesting to read, aided by the inclusion of tables and figures.

The aim of the dissertation corresponds to the topicality of the problem and emphasizes the innovative character of the development. It is clear, well formulated, and unites all directions of experimental work. To achieve this objective, 7 specific, interrelated, and logically consecutive tasks have been formulated, covering all the obligatory stages of such a study. Even here, the serious amount of work set before the doctoral student is evident.

The Materials and Methods section reflects the multidisciplinary nature of the work, using routine and modern microbiological, morphological, molecular biological, genotypic, and phenotypic methods. All are appropriate to the research and provide the basis for correct results. In addition, the methods are presented in an understandable way and complete enough to be reproducible. I would like to mention the RAPD-PCR analyses and the construction of dendrograms, the characterization of biofilm formation, the study of invasiveness and intracellular growth of P. aeruginosa in a lung adenocarcinoma cell line, and so on. All these are sufficient grounds for credibility and precision.

The Results and Discussion section contains extensive experimental material characterized by logical consistency and outlines a thorough scientific study. Comparative analysis of pairs of P. aeruginosa strains isolated from the same patient before and after treatment with tobramycin, varying the age and cycle of inhaled treatment, provided data on the different phases of infection. Molecular epidemiological typing of the isolates was performed as a starting point for pathogen characterization and selection of antibiotic treatment.

The significance of phenotypic differences between them for their development when cultured in 3 culture media was investigated, as was the effect of inhaled tobramycin treatment on the characteristics of pairs isolated from the same patient. The PhD student presents data on changes in 6 strain pairs in terms of growth phases, growth rate and generation time, lag phase duration, and biofilm formation. The static analysis carried out provides information on trends in the growth behaviour of the strains and increases the level of confidence in the results. Scanning electron microscopy experiments for morphological characterization of the strains followed in a logical sequence. This provided information on the structure of the biofilms and the qualitative and quantitative composition of the matrix. The PhD student suggested that the differences observed were a consequence of the inhaled tobramycin treatment.

In search of the relationship between antibiotics and tolerance of isolated *P. aeruginosa* strains, a morphometric study of bacterial cell size after antibiotic therapy was performed. The presence of enlarged cells, characteristic of unfavourable growth conditions, was demonstrated,

probably representing an adaptive change at the phenotypic level after antibiotic treatment. However, a similar relationship between strain motility and tobramycin treatment of patients was not found.

An important part of the research in this thesis is experiments to determine the invasiveness of *P. aeruginosa* strains in the lung adenocarcinoma cell line A549. The results showed that all strains tested were able to penetrate, survive, and replicate in cultured cells. One of them (RaT-6) was characterized by a very high multiplication rate, suggesting that invasiveness is part of the adaptation mechanism for survival under therapy. I would like to highlight the importance of the comparison of the characteristics of the strain pairs obtained in this work with literature data on other serial isolates. This approach allows the PhD student to explain the lack of a high similarity coefficient between early and later isolated strains by the occurrence of genetic diversification.

In the next sub-section, Diana Borisova examines the effects of prior therapy on the growth and development of surviving strains after treatment in the presence of the antibiotic. Similar studies are not found in the available literature, which gives the thesis an innovative focus. The original approach used and developed in this thesis provides new information on the processes of adaptation during bacterial persistence. The relationship between tobramycin therapy, in the host evolution of bacteria from paired strains, and changes in phenotypic response to treatment with sub-MPC of the same antibiotic was investigated. The results support the hypothesis that protective adaptation of bacteria involves biofilm formation.

It is a very good idea to include a subsection "Summary of results", in which the PhD student presents a synthesized reading of the achievements of the work. This facilitates the readers and enables a better perception of the results. Simultaneously with the correct reporting of the obtained data, a thorough and professional discussion based on relevant literature sources is made. The discussion is skillfully interwoven with the authors' own data, giving the impression of confidence and comparability with what has been published by other researchers.

I have an excellent impression of the layout of the dissertation, the scholarly style in which it is written, the correct presentation of the results in tables and figures, and their professional presentation.

The PhD student presents 12 conclusions that are a logical consequence of the data obtained and provides the necessary information about the value of the research conducted. I also accept the wording of the contributions and would like to stress once again the innovative nature of the topic and the approaches to developing it.

In fact, this is a dissertation of a high theoretical level with future applied significance, bearing the handwriting of leading scholars in the field - Assoc. Prof. T. Stoitsova and Prof. T. Strateva, which is a prerequisite for the relevance and significance of the research.

Data from the dissertation are included in 2 journal articles, 1 of which has an impact factor, 1 has an SJR (quartile Q4), and 1 book chapter. In two of them, the PhD student is ranked first, which is an indication of her personal contribution. The publications have been cited 17 times.

The submitted abstract has been prepared according to the relevant requirements.

In conclusion, I would like to emphasize that the presented work is dissertable, a very topical topic has been developed that offers new information on an important problem of infectious microbiology. The experiments are methodologically correctly set up, the results obtained are reliable and provide a solid basis for further scientific and applied work, and significant scientific and methodological contributions have been made. The developed dissertation fully complies with the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for the Implementation of the LDASRB, and the Regulations of the SAIM-BAS for the Implementation of the LDASRB, as well as the specific requirements and quantitative criteria in the Regulations of the SAIM-BAS.

Based on the analysis made and the proven growth of the PhD student, I give my positive assessment and strongly recommend to the Scientific Jury, to award Diana Borislavova Borisova the educational and scientific degree "Doctor" in the scientific field 4.3 Biological Sciences (Microbiology).

February 05, 2025
Sofia

Signature:……..
/Prof. Maria Angelova, DSc/