

R E V I E W

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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

1. Biographical data

Diana Borisova was born in Sofia. In 2013 she graduated with a Bachelor's degree in Molecular Biology, and in 2015 - a Master's degree in "Microbiology and Microbiological Control" at the Faculty of Biology of Sofia University "St. Kliment Ohridski".

Immediately after completing her bachelor's degree, Diana joined the Institute of Microbiology, in 2013 as a microbiologist, and since 2016 - as an assistant-Prof. in the Laboratory "Cellular Microbiology" of the Institute of Microbiology. Since 2022 she has been an "Assistant-Prof." at the New Bulgarian University. Since 2018 she has been enrolled as a doctoral student in independent training with scientific supervisors Assoc. Prof. Dr. Stoyanka Stoitsova and Prof. Dr. Tanya Strateva.

2. Relevance of the topic of the dissertation

Cystic fibrosis, also known as cystic fibrosis, is a genetic disease affecting the lungs. It is due to an autosomal recessive mutation, with patients inheriting two copies of the defective CFTR gene. So far, over 1,300 mutations in the CFTR gene have been proven in the world, divided into 6 large groups, and different gene mutations lead to different severity of cystic fibrosis, with the amount of functioning CFTR produced being related to the clinical status. The symptoms of this incurable disease are mucus secretion in the respiratory tract, which leads to frequent infections. Cystic fibrosis is most common among people of Northern European origin, where approximately 1 in 2,500 to 3,500 newborns are diagnosed with the condition. The frequency of carrying mutations in the CFTR gene in Bulgaria is estimated at 1/3,400, with 20 children born with clinical manifestations each year. The disease is accompanied by persistent infection with typical

cystic fibrosis pathogens such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia* and mucoid and non-mucoid *Pseudomonas aeruginosa*. Treatment always involves the use of anti-inflammatory drugs and antibiotics, either orally, inhaled or intravenously. Inhaled drugs are crucial for overcoming bacterial infection, as they help to open the airways, reduce the viscosity of mucus and facilitate its clearance. The dangers from a microbiological and health point of view are associated with the development of antibiotic-resistant bacterial strains, in many cases of the *Pseudomonas aeruginosa* species. The topic of inhaled therapy with the antibiotic tobramycin on *Pseudomonas aeruginosa* strains for the treatment of patients with cystic fibrosis is topical and of great public importance, especially since the disease is becoming more common and it is expected to be removed from the list of rare diseases due to its increasing prevalence.

3. Analysis of the thesis

The dissertation is written on 148 pages with the following sections: 37 pages of literature review with a summary of the literature review, 1 page "Aim and tasks", 14 pages "Materials and methods", 69 pages "Results and discussion", 2 pages "Conclusions" and 1 page "Contributions". The cited sources are about 240, most of which are from the last ten years, a third - from the last five years. The material is illustrated with 15 tables and 48 figures, half of which are composite.

The literature review shows the excellent awareness of the doctoral student on the topic and an analytical approach to the information read. Modern statistical data on the prevalence of cystic fibrosis in Europe and in our country are extracted from both scientific articles and medical websites. The largest part of the review is devoted to the description of the leading cause of bronchopulmonary infections in patients with cystic fibrosis - *Pseudomonas aeruginosa*, with a general characterization of the species as a pathogen, the structure and characteristics of biofilms, secreted virulence factors, organelles for motility and adhesion, and the adaptation of the microorganism to chronic infection of the lungs of patients. Studies of the metabolic spectrum of clinical isolates of *P. aeruginosa* reveal heterogeneity in metabolic pathways, emphasizing the role of spatial and chemical diversity in the environment during the evolutionary process in the lungs, consistent with the extensive phenotypic and genotypic heterogeneity. The section concludes with a description of the treatment of cystic fibrosis with antimicrobial drugs in patients with bronchopulmonary infections caused by *P. aeruginosa*. The literature review is illustrated with very good quality author's diagrams, and some of the cited sources related to inhaled treatment with tobramycin are publications by one of the scientific supervisors - Prof. Strateva - and show the Bulgarian contribution to global research on the topic.

The section "Aim and tasks" clearly outlines the goal of the dissertation: through comparative genotypic and phenotypic analysis of pairs of *P. aeruginosa* strains isolated from patients with cystic fibrosis before and after inhaled therapy with tobramycin, to

study the adaptations of the pathogen to the effects of the antibiotic. The main hypotheses and stages in the work process. The formulated 7 tasks, the last of which consists of 4 subtasks.

The "Materials and Methods" section describes the pairs of *P. aeruginosa* strains (provided by the Department of Medical Microbiology of the Faculty of Medicine, MU-Sofia), isolated before and after inhalation treatment with tobramycin, as well as a number of reference strains. This is followed by descriptions of classical methods for cultivation and growth characteristics of the studied strains (determination of minimal and subminimum inhibitory concentrations of tobramycin) and a detailed section on genetic typing of the isolates with molecular approaches. Scanning electron microscopy was used in the study of bacterial cells. The data were processed statistically reliably.

The "Results and Discussion" section clearly and systematically describes the steps to achieve the goal of the dissertation. The strains were genetically typed using the RAPD method and the phylogenetic diversity of *P. aeruginosa* from different geographical areas among Bulgarian patients with cystic fibrosis was established. The strains isolated after chronic infections have a reduced growth rate, an extended generation time and a longer lag phase compared to the strains before treatment with tobramycin. The analyses of growth, biofilm formation and vitality of the strains isolated after treatment with the antibiotic show, unfortunately, a better adaptability of the pathogen to the drug.

The dissertation skilfully combines results and discussion. Analogies are made with other persistent bacterial infections, e.g. the author notes that the longer lag phase often contributes to the ability of bacteria to evade the host immune response. In her work, Diana Borisova reveals that under the pressure of inhalation therapy with the antibiotic tobramycin, in most pairs of strains, an evolution of pathogenic bacteria is observed in the host itself, in the pulmonary tracts of patients with cystic fibrosis. One of the impressive chapters concerns the characterization of the structure of biofilms by scanning electron microscopy, making a connection between the morphology of *P. aeruginosa* and the synthesis of three different types of exopolysaccharides. In addition, cell size and variations in colony size were studied, as well as the various mechanisms by which bacteria achieve mobility and greater invasiveness in order to avoid the effects of antibiotic action. A very important result is the observed trend towards increasing tolerance to tobramycin in strains isolated after treatment with the antibiotic, as well as the stimulation of biofilm formation by sublethal doses of the antibiotic. The dissertation ends with 12 conclusions and 4 contributions, which are based on a huge amount of experimental material.

4. Publications on the topic of the dissertation

The total number of publications of Diana Borisova to date is 12, six of which are in refereed journals, there are 17 citations.

The results of the dissertation have been published in two scientific articles in refereed journals (in "Reports of the Bulgarian Academy of Sciences" and *Acta Microbiologica Bulgarica*) and one chapter of a book. Diana Borisova is the first author of two of the articles on the dissertation, and to her good performance as a doctoral student should be added the participation in congresses and conferences: 3 participations in international scientific forums.

Diana Borisova is a participant in 6 scientific projects, she is the leader of two.

5. Notes and questions:

Although it is written in an engaging and good style, there are some passages in the dissertation that sound popular, e.g. on p. 74 states that "the longer the generation time, the slower the growth of bacteria" – a fact that is obvious to microbiologists. As a presentation, Table 12 is of poorer quality and stands out from the rest of the text, and Fig. 48 has very small characters and is not readable. By mentioning these minor remarks on the layout of the dissertation, I in no way want to belittle the otherwise excellent presentation of the results and the extensive, detailed and interesting discussion.

I have the following question for the doctoral student:

Since you mention that there are many unexplained facts about the adaptive mechanisms of *P. aeruginosa* as a persistent pathogen, do you foresee a continuation of the topic with future proteomic or transcriptomic studies? In what conditions would you put the pairs of *P. aeruginosa* strains in order to observe significant differences in the transcriptomic response?

6. CONCLUSION

The dissertation work of Diana Borisova is dedicated to a current health topic related to the rare disease cystic fibrosis and the complications of bacterial infection by *P. aeruginosa*, treated by inhalation with the antibiotic tobramycin. The results obtained are new, significant and have a contributing character to clarifying the mechanisms of adaptation of persistent pathogens such as *P. aeruginosa*. It is clearly evident that the dissertation is the personal work of the doctoral student, supported by ideas, strains, materials and funding from the scientific supervisors.

The dissertation work and all provided materials related to the scientific and educational program of the doctoral student (publications and participation in congresses and conferences), are in full compliance with the requirements of the Law on the Prevention and Control of Diseases and Diseases of the Republic of Bulgaria and the Regulations for its implementation in the Republic of Bulgaria, the Bulgarian Academy of

Sciences and the Stefan Angelov Institute of Microbiology. I express my entirely POSITIVE opinion and recommend that the other members of the Scientific Jury support the awarding of the ONS "Doctor" to Diana Borislavova Borisova.

09.02.2025

Signature:

/Prof. Penka Petrova, DSc/