REVIEW
of a dissertation on the topic:

"Comparative investigations on *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients prior- and post- inhalatory tobramycin therapy"

Professional field 4.3. Biological Sciences (Microbiology)

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Reviewer: Assoc. Prof. Dr. Zlatka Alexieva

Dayana Borisova graduated with a Bachelor's degree in Molecular Biology and a Master's degree in Microbiology and Microbiological Control from the Faculty of Biology of Sofia University. Since 2013, she has been working at the Institute of Microbiology of the Bulgarian Academy of Sciences, and since 2016 she has been appointed as an assistant professor, and on 01.04.2018 she was enrolled as a doctoral student in free preparation.

The topic of the presented work is dedicated to the study of the influence of inhalation therapy with the antibiotic tobramycin on *Pseudomonas aeruginosa* strains in the process of treating patients with cystic fibrosis.

The presented dissertation project is traditionally structured, within 150 standard pages, 15 of which are occupied by the cited literature. The list of cited literature includes 239 literary sources and 9 Web pages. The material is excellently illustrated with 14 tables and 51 figures. 41 of the figures illustrate the results and can be defined as author's and 10 are illustrations to the literature review and the methodological part, 9 of which are correctly cited, and one is author's.

The introduction provides general information about the nature and spread of the disease cystic fibrosis and the leading role of the pathogenic bacterium *Pseudomonas aeruginosa* in various stages of the disease. Without a doubt, the topic and planned research concerning the mechanisms for controlling the manifestations of CF are relevant, in view of the significant number of people affected by this "rare", severe disease, both around the world and in our country. The World Health Organization (WHO) includes *P. aeruginosa* in the ESKAPE group of pathogens, which pose a global risk due to their increasing resistance to treatment. The relevance of the presented dissertation work is also confirmed by the fact that over 30% /73/ of the cited literature are from the last 5 years.

To date, there is no information about the possible involvement of a particular antibiotic in the development of adaptive changes in *Pseudomonas aeruginosa*, which determines the originality of the presented study.

The literature review begins with a brief description of the cause and characteristics of the disease Cystic Fibrosis. The bacteria that colonize the affected lung are presented. The bacteria *Pseudomonas aeruginosa* are considered as the main

causative agent of bronchopulmonary infections. The role of strains with a mucoid morphotype, which are practically irreversible, is emphasized.

The mechanisms, mainly the synthesis of a number of exopolysaccharides, by which bacteria of the *P. aeruginosa* species manage to reduce or completely avoid the action of antimicrobial agents, especially when they have formed a biofilm, have been explained in detail.

Significant attention has been paid to the multiple virulence factors of *P. aeruginosa*, as well as the ability of these strains to adapt to different stages of the disease course, by forming resistant biofilms, increased mutation frequency, and morphological and biochemical changes.

Of particular interest are also strains of *P. aeruginosa* capable of entering and multiplying in eukaryotic cells - an invasive phenotype. Examples are presented regarding the unclear role of a number of exoenzymes in the invasiveness of the strains. The role of the type III secretion system is also unclear. Some bacterial regulatory mechanisms play an important role in invasiveness.

The author has paid serious attention to molecular disorders in DNA repair, changes in the expression of mucoidity genes, the formation of persistent cells of strains in the bacterial populations of *P. aeruginosa*. High heterogeneity of cellular metabolic pathways is observed. As a result, complex and diverse bacterial communities are formed, which change their characteristics at the beginning and subsequent stages of the disease, both at the genetic and phenotypic levels.

A large part of the literature review is devoted to the treatment of CF patients with bronchopulmonary infections caused by P. aeruginosa with antimicrobial drugs, bacterial resistance to their widespread use, and the formation of biofilms.

Overall, the literature review is written competently, with very good knowledge of older and modern scientific literature, of solved and unsolved problems in the study of the role of *Pseudomonas aeruginosa* in patients with CF, and creates a favorable information environment for conducting the research planned in the dissertation.

The goal clearly reflects the essence of the dissertation: To study the adaptation of *P. aeruginosa* strains to the effects of tobramycin, before and after inhalation therapy with the antibiotic, through comparative genotypic and phenotypic analysis. For its implementation, 7 main research tasks have been defined, the last of which includes 4 sub-items. The formulated tasks outline the specific course of the research.

The chapter "Materials and Methods" describes in detail the classical and state-of-the-art analysis approaches used in the scientific research. The approaches cover a number of microbiological methods for cultivation, determination of morphological characteristics and motility of microbial cells, determination of invasiveness and intracellular survival. Methods for determining the effects of sub-MIC tobramycin on microbial cells and biofilms, molecular biological methods (PCR, RAPD)–PCR), electron microscopic (CEM), for morphometric and statistical data processing, etc. are described.

At the beginning of the chapter “Results and Discussion” data obtained as a result of RAPD-PCR and subsequent UPGMA analyses are presented and illustrate the relationship between 12 *P. aeruginosa* isolates from Bulgarian patients with CF and 14 reference strains of *P. aeruginosa* from an international reference panel. Using the Dice similarity coefficient, a phylogenetic tree was created. A high similarity of the

strains isolated from Bulgarian patients and strains from an international reference panel was found.

The growth curves of the different 6 pairs of *P. aeruginosa* strains from serial isolates from white slave of patients with cystic fibrosis were studied in detail and in parallel. The pairs consisted of strains isolated before and after exposure to tobramycin, respectively. The influence of different nutrient media, including minimal salt media, on the growth, growth rate and generation time of strains isolated before and after treatment with the antibiotic was established. The study confirms that for MB - strains of *P. aeruginosa*, auxotrophy is not a mandatory adaptive element.

A significant increase in the duration of the lag phase of growth was found in almost all strains isolated after inhalation therapy with tobramycin compared with their paired strains before inhalation. Such an observation of a relationship between prolongation of lag phases and *in vivo* administration of an antibacterial agent in the host is reported for the first time in in-host evolution of MV strains and has not been described in the literature.

An important contribution has been made by studies showing the capabilities of persistent cells in adapting to microbial culture and their ability to resist antibiotics through a mechanism different from the standard genetically determined resistance.

As a result of a comparative analysis of biofilm formation, differences in the dynamics of their development were identified, depending on the composition of the culture medium. The study identified a wide range of strain-specific reactions and no general trend in biofilm formation was observed in view of the time of their isolation or treatment with tobramycin.

Practically the same conclusion can be made after conducting a statistical analysis to compare the effect of the three media on the growth of the strains in liquid medium and as a biofilm. The only constant trend is observed when cultivating the strains in minimal mineral medium M63. All studied strains have the weakest growth in this liquid medium and the best growth as a biofilm, and this is not affected by the antibiotic therapy performed.

Of particular interest are the results obtained through electron microscopic analyses (SEM) of the morphology of the biofilms. The majority of cystic fibrosis strains of *P. aeruginosa* have mucoid colony morphology, due to a high level of secretion of exopolysaccharides. This phenotype is characteristic of the deteriorated condition of CF patients. The analysis revealed a clear difference between the strains from the different pairs under the influence of tobramycin administration. In most of them, it is observed that the cells are not so compactly packed in the extracellular matrix and the biofilm is less consistent, but in other strain pairs the mucous matrix grows and thickens. The identified structural differences provide a perspective for studies to establish differences in the composition of the extracellular matrix of biofilms formed by different strains of *P. aeruginosa*, which will be essential in the approach to treatment with inhaled tobramycin.

A very good discussion of the results of the morphometric analysis of bacterial cell size was made, in which an increase in their size was noted in four of the pairs after exposure to an antibiotic. This response is poorly reported in the available literature and differs from the standard one, when cells become smaller under the influence of adverse factors.

In the following studies, the appearance of variant small colonies (VSCs) in two of the strain pairs was monitored, the appearance of which is often a sign of increased resistance to antibiotics. The studied *P. aeruginosa* strains exhibited different, relatively good degrees of cell motility, which is due to the presence of flagella. Relatively weak movement by "swarming" was found in all strains, but with a morphology different from that described so far in the literature for other strains. Practically no motility by oscillation was observed, with the exception of 1 pair of strains, in which it was weaker than that reported in the reference strain *P. aeruginosa* PAO1.

In the next stage, the invasiveness of *P. aeruginosa* strains in the lung carcinoma cell line A549 was investigated. It was proven that all the studied strains are invasive and can successfully multiply intracellularly in cultured lung epithelial cells. A strain (PaT-6) was identified, whose growth characteristics characterize it as the strain with the best developed ability to invade and multiply in host cells.

Original information is provided by experiments to establish the effects of previous therapy on the growth and development of strains surviving treatment in the presence of sub-MIC (sub-minimum inhibitory concentration) tobramycin. Of great importance are studies aimed at establishing phenotypic tolerance to the presence of antibiotics, regardless of genetically determined resistance. In this regard, the influence of ½ MIC and ¼ MIC of tobramycin on the growth and formation of biofilms, vitality and motility of cells in rich nutrient medium and in mineral medium was monitored. Despite some heterogeneity in the reactions of individual strains, in general, a better adaptation of all studied strains to the presence of sub-MIC of the antibiotic was found compared to the reference strain *P. aeruginosa* PAO1. It should be noted that the strains isolated after *in vivo* treatment were more tolerant to tobramycin. In mineral medium, the values of these parameters were similar to the data for the reference strain.

As a result of the data obtained, Dayana Borisova formulated 12 conclusions and 5 contributions, adequately reflecting the results obtained during the development of the dissertation. The tasks set have been fully completed.

Here it is necessary to emphasize the competence of the scientific supervisors, as well as the team of the Cellular Microbiology Laboratory, who for many years under the leadership of Assoc. Prof. Stoitsova before and currently with Assoc. Prof. Paunova, have been successfully working on topics closely related to the mechanisms of formation and regulation of the functioning of microbial biofilms.

I have no comments on the dissertation presented for defense, since after the internal defense, my recommendations were reflected.

CONCLUSIONS

During the development of this dissertation, doctoral student Dayana Borisova has gained 437 credits, which is a significantly higher score than the required minimum of 250. Part of the results included in the doctoral thesis have been published in 2 articles, one of which in Reports of the Bulgarian Academy of Sciences, with an IF – 0.321 and the second in Acta Microbiologica Bulgarica. A chapter of a book published by an international publishing house has also been written on the topic of the dissertation. The publications have been cited 17 times. Three reports have been

presented at 3 international scientific forums. Diana Borisova is a participant in 6 scientific projects, 2 of which she is the leader. The total number of publications in which she is a co-author, including those presented for the defense, is 12, 6 of which are indexed and referenced in international databases.

The problems posed in the presented work are original, current and significant, mainly for the field of fundamental microbiological and medical research. The results obtained, the conclusions formulated and the contributions are respectively original and of high scientific value.

Doctoral student Dayana Borisova has used a rich arsenal of classical and modern analytical, biochemical, microbiological and molecular genetic methods, which has established her as a highly qualified, methodologically prepared specialist.

The dissertation submitted for official defense on the topic "Comparative investigations on *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients prior- and post- inhalatory tobramycin therapy", by doctoral student Diana Borisova, fully complies with all the basic and additional criteria reflected in the ZRASB and the Regulations for its application at the Bulgarian Academy of Sciences and at the Institute of Microbiology at the Bulgarian Academy of Sciences. Based on everything mentioned so far, I confidently propose to my colleagues on the Scientific Jury to vote for awarding the scientific and educational degree "PhD" to Diana Borislavova Borisova in the professional field 4.3. Biological Sciences.

05.02.2025 Reviewer:

/Assoc. Prof. Dr. Zlatka Alexieva/