



To
The Chairperson of the Scientific Jury,
Appointed by order No. 581 – 01/12/2023

of the Director of the National Center of Infectious and Parasitic Diseases, Sofia

Statement of Opinion

By Assoc. Prof. Ivanka Gergova, MD, PhD,

Head of the Microbiology Laboratory, Military Medical Academy - Sofia

Subject: procedure for the defense of the dissertation work for awarding an educational and scientific degree "Doctor".

Field of higher education: 4. Natural Sciences, Mathematics, and Informatics.

Professional field: 4.3. Biological Sciences.

Scientific speciality: 'Microbiology'.

PhD program 'Microbiology'.

Author: Ivan Ivanov Stoikov.

Thesis title: 'Study on the genomic features associated with drug resistance (resistome) and virulence (virulome) of extensively-resistant *Pseudomonas* spp.'.

Scientific super adviser: Assoc. Prof. Ivan N. Ivanov, PhD.

Scientific adviser: Prof. Stefana Sabcheva, MD, PhD.

Chairperson of the Scientific Jury: Assoc. Prof. Viktoriya Levterova, PhD.

I. GENERAL PRESENTATION OF THE PROCEDURE AND THE AUTHOR OF THE DISSERTATION

1. Procedure

This opinion was prepared in accordance with Order No. 581/01.12.2023 of the Director of the National Center of Infectious and Parasitic Diseases, Sofia and with the decisions of a meeting of the Scientific jury appointed for the purpose, at which I was chosen as an external member of the Scientific jury.

I declare I have no conflict of interest within the meaning of Art 4, Para 5 of the Law on the Development of the Academic Staff of the Republic of Bulgaria (LDASRB). I have no common publications with Ivan Stoikov.

The presented set of documents for the procedure is in accordance with the requirements of the LDASRB and its regulations at the NCIPD, Sofia.

2. Brief biographical and professional data for the author of the dissertation

Ivan Ivanov Stoikov completed his secondary education in "Acad. Ivan Gyuzelev", Gabrovo (2013). He graduated the Sofia University 'St. Kliment Ohridski' as Bachelor of Biotechnology (2017) and as Master of Gene and Cell Engineering (2019).

Since 01/11/2019 he has been working at the National Reference Laboratory "Control and Monitoring of Antibiotic Resistance", NCIPD, as a biologist. He is fluent in English.

II. RELEVANCE OF THE TOPIC

The steady global trend towards an increase in antimicrobial resistance observed in recent decades is a major reason for severely limiting the possibilities for adequate therapeutic intervention, especially in healthcare-associated infections.

P. aeruginosa is enlisted in the ESKAPE and ESCAPE groups summarizing the leading problematic hospital pathogens, and is ranked sixth in the world in the rate of mortality associated with antimicrobial resistance. In recent years, there has been a widespread increase in the prevalence of infections caused by *P. aeruginosa*, including in Bulgaria, where they are among the common causative pathogens in all the major healthcare facilities.

The intrinsic resistance of *P. aeruginosa* to a wide spectrum of antimicrobial groups commonly used for treatment, as well as the ability to acquire resistance to strategic carbapenems, is a major problem in the delivery of effective antimicrobial therapy, particularly in immunocompromised, comorbid and intensive care patients.

A core element in solving these problems is the detailed study of the genetic mechanisms of antimicrobial resistance and virulence factors. The most advanced and comprehensive approach to address this issue is the whole-genome sequencing, which provides an opportunity to simultaneously examine all of the gene determinants associated not only with multiple resistance but also with the pathoadaptive mechanisms of a particular microbial pathogen.

In this aspect, I consider that the topic developed by Ivan Stoikov is especially relevant, both in theoretical and applied aspects for the healthcare system in the country.

III. STRUCTURE OF THE DISSERTATION WORK

The doctoral thesis contains 233 standard pages (including the appendices) and is optimally illustrated with 31 figures (35 including subfigures) and 20 tables (36 including subtables).

The title accurately represents the essence of the developed theme. The dissertation is structured properly and includes a title page, acknowledgements, table of contents, abbreviations

used, introduction, literature review, clearly stated purpose and objectives, materials and methods, results and discussion, conclusion, well-defined conclusions and scientific contributions, list of publications and participation in scientific events related to the thesis, and bibliography. The bibliography contains 680 titles (all in Latin), and 95 of the sources (14%) are from the last 5 years and 205 of the sources (30%) from the last 10 years.

The Abstract is presented in a synthesized form, in 128 pages and corresponds adequately to the content of the dissertation work, and all the requirements of LDASRB were observed during its preparation.

IV. ADEQUACY OF FORMULATED PURPOSE AND OBJECTIVES

Stoikov aims to examine the genomic characteristics underlying drug resistance (resistome) and virulence (virome) in extensively drug-resistant *Pseudomonas* spp. The purpose is precisely and clearly formulated and the five objectives set logically result from it and fully represent the thesis topic.

V. GENERAL CHARACTERISTICS AND EVALUATION OF THE DISSERTATION

1. Literature review

The literature review is presented in a volume of 63 pages, and is well-structured, balanced, and informative, with definitions and facts. Based on an impressive literature reference, the review is written competently and at a high scientific level.

It presents a comprehensive overview of the taxonomy, discussed in historical perspective. The determinants and mechanisms of resistance to different groups of antimicrobials are described in great detail. Virulence factors are discussed at length, including genetic determinants and their role in the pathogenesis of the infections they cause.

The literature review is comprehensive and summarizes the contemporary status of the problem.

2. Material and methods

The materials and methods are presented in detail in 39 pages.

The research on the theme is based on the data from the studies conducted on 100 clinical isolates of *Pseudomonas* (96 *P. aeruginosa*, 2 *P. soli*, 1 *P. kurunegalensis* and 1 *P. protegens*), from the collection of the NRL on 'Control and Monitoring of Antibiotic Resistance' at the NCIPD. All selected isolates have the characteristics of MDR, XDR or PDR, and were collected in the last 14 years.

All applied methods (identification, antimicrobial susceptibility testing, molecular biological methods, biofilm formation ability testing, and bioinformatics analysis) were selected

precisely, in accordance with the set objectives and are presented very comprehensively, with pedantic descriptions of the used equipment and techniques. A method for the rapid extraction of high-quality RNA from *P. aeruginosa* has been developed, and its advantages and limitations of application have been critically evaluated.

The manner of presenting the techniques demonstrates the doctoral student's excellent methodological preparation.

3. Results and discussion

The results obtained from the conducted own research are reported and discussed within 61 pages and are well-illustrated with relevant tables and figures. They are highly informative and are presented in sections according to the logical sequence of the assigned objectives.

The discussion provides an in-depth analysis of the results obtained in experiments performed to demonstrate carbapenemases, expression of efflux systems and genes associated with antimicrobial resistance (RT-qPCR), biofilm formation ability analysis, MLVA9 and MLST genotyping, plasmid replicon typing by PCR, conjugation and plasmid profile analysis of all strains by plasmid linearization and pulsed field gel electrophoresis (PFGE).

A particular focus of the thesis is bioinformatics analysis, including analysis of the quality of the short and long genomic reads obtained, with assessment of the quality of the genomes obtained and their deposition in NCBI GenBank; isolate identification; determination of determinants of antimicrobial resistance; and *oprD* and plasmid analysis. Results of MLST- and cgMLST-typing of *P. aeruginosa* isolates and determination of virulence determinants are discussed in detail. The characteristics of IMP-producing *P. aeruginosa* and the emergence of IMP-100, a novel plasmid variant existing in combination with chromosomal VIM-4, are specified. Antimicrobial susceptibility to all drugs available for treatment and screening for determinants of resistance were investigated, and plasmid analysis, cloning, transformation and conjugation, as well as phylogeny and genetic environment analysis of *blaIMP-100* were performed.

The accumulation of information in this regard has significant theoretical and practical implications in the solution of problems concerning both the therapy of infections caused by *P. aeruginosa* and the control of their dissemination in the hospital environment.

I consider that one of the significant contributions of this thesis is the identification of mechanisms underlying resistance involving a wide variety of enzymes and mutations. Different types of β -lactamases including CARB, GES, PER, VEB and TEM have been detected, and 3 types of class B carbapenemases (VIM, NDM, IMP) have been identified, among which a newly discovered variant IMP-100 has been detected and characterized.

The intimate mechanisms of resistance to the major groups of antimicrobial agents used in the therapy of infections caused by *P. aeruginosa* are elucidated, and virulence factors are studied and discussed in detail.

The dissertation has a completed form and the purpose and objectives posed have been fulfilled.

4. Conclusions and contributions.

On the basis of the literature review and the own research and experience, six conclusions were properly and logically drawn, which directly correspond to the stated purpose and objectives. The conclusions reached have theoretical and practical value and represent the core of the thesis.

Substantial contributions of original (6 contributions) and scientifically applied character (5 contributions) were obtained. I accept the contributions indicated by Stoikov, which are adequately and realistically derived from the developed thesis.

5. Scientific publications and participation in scientific forums, related to the dissertation

The significance of the achieved results is demonstrated by the attached list with a total of four publications in reputable scientific journals, two of them with an Impact Factor (total IF: 6.17). In three of these publications, Stoikov is the first author, and in the fourth - the second, which unequivocally proves his leading role in the research conducted and described in the dissertation.

The results of the research conducted were presented at five scientific forums, one of them international, and in three of them, Stoikov was the first author.

VI. ABSTRACT

The presented abstract corresponds to the requirements, completely representing the content of the thesis.

VII. CONCLUSION

The dissertation is a massive study on a very relevant issue, with a solid theoretical basis, performed with a variety of modern molecular genetics methods.

The scientific value of the dissertation work originates from the in-depth research, conclusions and recommendations, and from the formulated contributions. I consider the doctoral thesis to be complete, useful, analytical and precise in its conclusions. All this is a reason to consider it positive and to recommend to the respective members of the Scientific Jury to unanimously grant Ivan Ivanov Stoikov the educational and scientific degree 'Doctor' in the scientific specialty 'Microbiology'.

26/01/2024

Reviewer:

Sofia

Assoc. Prof. Ivanka Gergova, MD, PhD