



To the President of
the Scientific Jury appointed by Order
№ 581/01.12.2023 of the NCIPD

R E V I E W

by **Prof. Stefan Vacev Panaiotov, DSc**

on the dissertation for the award of the educational and scientific degree "Doctor of Philosophy" in the scientific field 01.06.12 Microbiology on the topic: "Study on the genomic features determining drug resistance (resistome) and virulence (virulome) in extensively drug resistant *Pseudomonas* spp.".

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Scientific supervisor. Associate professor **Ivan Nikolaev Ivanov**, PhD

Scientific consultant: **Prof. Stefana Sabcheva, PhD**

The documents submitted by the candidate are in accordance with the requirements of the internal regulations of NCIPD for the implementation of the Law for the Development of Academic Staff in the Republic of Bulgaria.

I declare that I have no conflict of interest according to the Act for the Development of the Academic Staff in the Republic of Bulgaria

Career development of the dissertant.

Ivan Stoykov completed his secondary education in Gabrovo. He graduated from high school in 2013. In 2019 he graduated with a Master's degree in Genetic and Cellular Engineering at the Faculty of Biology, Sofia University. Since 2020 he is a full-time PhD student in the Microbiology Department at the National Reference Laboratory "Control and Monitoring of Antibiotic Resistance" at the National Center for Infectious and Parasitic Diseases. He completed and presented his dissertation during the three years of research and training.

Relevance of the topic

The subject of this dissertation concerns the study of genomic characteristics underlying drug resistance (resistome) and virulence (virome) in extensively drug-resistant *Pseudomonas* spp.

Pseudomonas are ubiquitous bacteria that are an essential part of bacterial communities and perform essential ecological functions in the environment. The genus *Pseudomonas* contains 314 species and their number is constantly increasing. In addition to the well-known human pathogen *P. aeruginosa*, some species of *Pseudomonas* also cause disease in plants, fish, insects or other animals.

Resistance of *P. aeruginosa* and other bacterial pathogens to antimicrobial agents has become one of the most significant problems for medicine and public health, and is increasingly being identified as a global threat.

P. aeruginosa ranks sixth worldwide in terms of antimicrobial resistance-related mortality and is part of the group of high-risk pathogens. The facts described in the thesis review underline the relevance of the topic. An effective approach to limit antimicrobial resistance is the detailed study of the genetic mechanisms leading to resistance. The most advanced and comprehensive approach to solve this task is whole genome sequencing, enabling simultaneous investigation of all gene determinants associated not only with multiple resistance but also with pathoadaptive mechanisms of a microorganism.

General description of the thesis

The dissertation contains 233 pages, 31 figures and 20 tables. The bibliography includes 680 references. The dissertation is structured in a standard way and includes: Introduction - two pages, Literature Review - 63 pages, Aim and Objectives - 1 page, Materials and Methods - 39 pages, Results and Discussion - 47 pages, Conclusion, Conclusions and Contributions - 2 pages each.

The literature review, in a detailed and concise style, describes the state of research on the problem. Characterization of the genus *Pseudomonas* and a general genomic description of the species *P. aeruginosa* are given. Chromosome-encoded resistance mechanisms in *P. aeruginosa*, penicillin-binding proteins, different types of efflux systems, large deletions that confer resistance, and biofilm-dependent resistance are described in detail. Virulence factors in *P. aeruginosa* are described.

Aim and objectives. The aim of the thesis is clearly stated - To investigate the genomic characteristics underlying drug resistance (resistome) and virulence (virology) in extensively drug-resistant *Pseudomonas* spp. Five tasks were set.

Materials and methods are described in detail. The study included 100 MDR *Pseudomonas* isolates collected from all over the country. More than 20 state-of-the-art laboratory techniques, some of which were applied for the first time in the country, are described and successfully applied. The high level of laboratory competence of the candidate is evident from the description.

Results and discussion. A wide range of in-depth studies have been conducted and significant results have been obtained. A unique set of MDR, XDR and PDR strains of *Pseudomonas* spp. were selected for study by a complex of modern phenotypic and genetic methods. The strains were tested for susceptibility to latest generation antibiotics such as ceftazidime/avibactam, ceftolozane/tazobactam, meropenem/vaborbactam, imipenem/relebactam and cefiderocol. Very high rates of resistance to β -lactams and fluoroquinolones have been observed. Molecular genetic techniques have been used to identify mechanisms of resistance, to demonstrate carbapenemase carriage, to determine the effect of efflux systems to various antimicrobials, to study porin deficiency, etc. In parallel, studies on virulence factors and biofilm production ability have been conducted.

The mechanisms of resistance identified by the dissertant include a wide variety of enzymes and mutations. Different types of β -lactamases have been identified, including CARB, GES, PER, VEB and TEM. Three types of class B carbapenemases have been identified (VIM, NDM, IMP), and among them a novel variant IMP-100 has been identified and characterized.

Overexpression of various efflux systems was found among most of the isolates studied. The increased expression of various efflux systems demonstrated has a strong correlation with resistance to β -lactams and quinolones. A number of important mutations associated with antimicrobial resistance have been identified.

The selected 100 MDR isolates of *Pseudomonas* spp. were whole genome sequenced. Using different bioinformatics approaches, the PhD student identified and characterized the determinants of resistance and virulence and their potential for dissemination was investigated by in-depth analysis of mobile genetic elements such as plasmids, integrative conjugative elements, transposons, etc. Through core genome multilocus sequence typing (cgMLST), extensive phylogenetic and phylogeographic analysis of the studied isolates was performed. The sequenced genomes have been deposited in the NCBI database. As a result of the whole-

genome analysis, VIM carbapenemases localized in integrative conjugative elements were detected in some isolates. The freely available information provided is a rich and informative resource from which strategic conclusions can be drawn about the epidemic potential and prevalence of key determinants of antibiotic resistance and virulence. For the first time in the country, the resistance and virology of rare or misidentified species in the past, such as *P. soli* and *P. protegens*, whose role as hospital pathogens has been increasing in recent years, has been studied.

Conclusions and contributions of the dissertation

The dissertant formulated 6 conclusions. The most significant of them are:

- The majority of genes encoding carbapenemases in *Pseudomonas* spp. are found in chromosomal mobile genetic elements, most localized in integrative conjugative elements.
- Clinical isolates of *P. aeruginosa* were characterized by multiple and diverse virulence factors classifying into all known categories, with 85% showing moderate to strong biofilm production.
- Most of the plasmids in *Pseudomonas* spp. cannot be assigned to known plasmid families.

The following original contributions of the dissertant can be listed:

- A novel genetic variant of carbapenemase IMP-100 associated with newly discovered mobile genetic elements - integron In4886 and transposon Tn7700 localized in a new plasmid.
- Strains of *P. aeruginosa* resistant to cefiderocol have been documented for the first time in Bulgaria, and plasmid-mediated cefiderocol resistance has also been demonstrated.
- A novel PCR-based replicon typing scheme for 13 resistance-associated plasmid families in *Pseudomonas* spp. was developed.

Of the contributions of a scientific and applied nature, the following can be highlighted:

- The genetic mechanisms of resistance to β -lactams, aminoglycosides and quinolones were identified in the studied *Pseudomonas* spp.
- A large-scale comparative phylogenetic and phylogeographic analysis of the Bulgarian isolates against a collection of 2470 isolates from around the world was performed based on cgMLST including 3876 SNP loci.

On the basis of the presented results of the dissertation work it can be concluded that the PhD student Ivan Stoykov has worked thoroughly and with diligence. He has correctly interpreted the obtained results. The aim and objectives of the dissertation have been achieved. I have no remarks on the planning, the way of execution and the achieved results of the dissertation. I have no comments on the layout and text of the thesis and the abstract.

Publications and personal contribution of the PhD student

On the topic of the dissertation the PhD student Ivan Stoykov submitted four articles in journals with impact factor and impact rank. Two of the articles are in journals with impact factor and he is the lead author - Microorganisms - IF 4.1, Q2 and Biotechnology and Biotechnology Equipment - IF 1.67, Q3. The overall impact factor is 6.17. Results have been reported at 1 international and 4 national scientific congresses. The attached publications are directly relevant to the aims and results of the thesis. The results and contributions presented are original and have not been borrowed from research and publications in which the dissertant had no involvement. The credits accumulated during the PhD exceed the minimum required.

It is evident that the PhD student Ivan Stoykov made a major contribution to the development of the dissertation. He has made a special effort to master and apply innovative molecular biology methods to solve a difficult scientific problem.

Conclusion.

The dissertant Ivan Stoykov has achieved the goal and the set tasks. The dissertation is innovative and of scientific and applied importance. The dissertation, publications and other documents submitted by the candidate meet all the scientific criteria according to the Law for the Development of Academic Staff in the Republic of Bulgaria for the award of the educational and scientific degree “Doctor of Philosophy”.

I confidently give my **positive evaluation** and recommend the Scientific Jury to award the degree “Doctor of Philosophy” to Ivan Ivanov Stoykov in the field of higher education 4. "Natural Sciences, Mathematics and Informatics", field 4.3 "Biological Sciences" in the scientific specialty "Microbiology".

Sofia, 8/02/2024

Reviewer:

/Prof. Stefan Panaiotov, DSc/