REVIEW

on PhD Dissertation titled: "Study on the genomic features determining drug resistance (resistome) and virulence (virulence) in extensively resistant *Pseudomonas* spp.",
developed by **full-time doctoral student Ivan Stoykov**
with supervisor: Assoc. Professor Ivan Ivanov; Scientific advisor: Prof. S. Sabcheva, USBALO
REVIEWER: Prof. S. Danova, PhD, Institute of Microbiology "Stefan Angelov", BAS

1. Regarding the procedure for official protection and the requirements of the Low-ZRASRB with a brief presentation of the PhD student:

The PhD student **Ivan Stoykov** is enrolled in a regular doctoral Program at the National Center Infections and Parasitic Diseases (NCIPD), by order **N 255/ 01.10.2020** of for the period from 01.10.2020 to 01.10.2023. Within the regular period, he has successfully completed the curriculum and the scientific research program, in full compliance with the requirements of the Low-ZRASRB and the Rules for its implementation. The current procedure for the acquisition of Educational and Scientific degree "Doctor" is conducted on the basis of Art. 31 of the Regulations for the implementation of the NCIPD, in connection with Art. 9 of the Low- ZRASRB and on the basis of the Decision of the Scientific counsil of the NCIPD, Minutes No. 04/27.11.23

Pursuant to Order No. 581/01.12.2023 of the Director of the NCIPD, I was elected as a member of the Scientific Jury under the above-mentioned procedure and was designated as a reviewer at its first meeting. In my capacity as such, I declare that there is no conflict of interest within the meaning of §1, item 2a of the additional provisions of the RSARB between me and the candidate under the procedure for the ONS "Doctor" and I am not subject to the restrictions under Art. 33 of ZRASRB. The set of documents and materials presented to me on an electronic medium meets the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria and the Regulations on the Terms and Procedures for Acquiring Scientific Degrees and Holding Academic Positions. The absence of plagiarism in the candidate's scientific works has been proven according to the law. There is a Declaration of originality and authenticity of the presented results.

2. Relevance of the problem developed in the dissertation:

The work submitted to me for review represents an innovative and in-depth study on the genetic basis of antibiotic resistance targeting a problematic pathogen for effective treatment, which is the widespread *Pseudomonas aeruginosa.* The PhD student and his supervisor have targeted one of the most frequently isolated nosocomial pathogens with a very wide spectrum of pathogenesis: a proven main cause of nosocomial pneumonia, urinary tract infections and a pathogen in surgical wounds. The species is among the multi-resistant disease-causing agents. Antibiotic resistance is currently the most serious global threat to the effective treatment of bacterial infections. Antibiotic resistance seems inevitable and there is a growing need for new

research. *Pseudomonas spp.* demonstrates its remarkable adaptability in the adverse host environment by exploiting a wide array of virulence factors playing a key role in establishing successful infections and in accelerating disease processes. The work reveals the genetic basis of these processes, which will facilitate new therapeutic approaches and help limit antibiotic resistance.

Last but not least, the production of resistant biofilms represents a huge challenge in the field of medicine, as it favors the persistence of chronic infections. All this gives me reason to evaluate the dissertation being developed as very relevant and significant.

3. Evaluation of the structure and content of the dissertation work

The dissertation is set out in 235 standard pages of text. The generally accepted scheme and the recommended ratios between the separate parts of the work were followed, as follows: *Introduction - 1 pages, Literature review - 63 p., Aim and tasks - 1 p., Materials and methods - 39 p., Results and discussion - 61 p., Conclusions - 2 p., Contributions - 1 p.; Bibliography - 50 pages.* The bibliographic reference includes an impressive 680 titles, even for a large doctoral thesis. All sources are in Latin and mostly from the last 15 years, which shows an excellent theoretical awareness of the problem developed by the doctoral student.

Technically, the work is very well designed, with rich illustrative material and excellently presented results incl. from molecular genetic and bioinformatic analyses. They are summarized in 19 tables and 28 figures. The PhD student skillfully applies various modern software programs in the genetic analyzes of the obtained data, as can be seen from the presented figures.

3.1. Literature review

The literature review is very well-structured and examines in a logical sequence the theoretical knowledge and new information key to the work regarding:

1) Characteristics of the genus *Pseudomonas* - correctly noting the latest data as of 24.11.2022 for the species representation of 314 validly named species according to the List of Prokaryotic Names with Standing in Nomenclature (Parte, 2018)

2) General genomic characterization, with special attention to accessory elements, with impact in understanding the mechanisms of antibiotic resistance in the species

3) Resistome and mechanisms determining resistance factors and their genetic basis discussed in detail

4) The author has also paid special attention to the virulence - correctly appreciating the importance, the remarkable adaptability of *Pseudomonas aeruginosa* in the adverse environment of the host, and the wide range of virulence factors playing a key role in the establishment of successful infections and in the acceleration of disease, processes are discussed.

Each of the above points has been examined very analytically in the light of the latest scientific evidence and data. The citation of the Bulgarian experience and publications makes a very good impression. This shows an excellent theoretical preparation and search for new knowledge to solve the problem of growing antibiotic resistance.

3.2. Aim and tasks

The objective: "Studies on genomic features underlying drug resistance (resistome) and virulence (virulence) in extensively resistant *Pseudomonas* spp." is clearly and precisely stated. It is very ambitious, but it responds to the urgent need for knowledge in the fight against increasing

antibiotic resistance on a global scale, the lack of new preparations, as well as the problem of misuse of available funds.

For the achievement and in the logical sequence and very good structuring, 5 experimental tasks are set, one of which has 3 subtasks, as follows:

1. Selection and phenotypic characterization of multi-resistant strains of Pseudomonas spp.

2. Preliminary analyzes to form a representative sample of isolates for further whole-genome analysis.

2.1. Evidence of genetic mechanisms of resistance to beta-lactams by molecular genetic techniques (carbapenemases, efflux, porin deficiency, etc.).

2.2. Demonstration of virulence factors by PCR and determination of biofilm production.

2.3. Genotyping (MLVA, MLST) and strain selection for whole-genome sequencing (WGS).

3. Detailed genomic characterization of a newly discovered variant of plasmid-mediated imipenemase IMP-100 as well as other IMP-producers in *P. aeruginosa*.

4. Bioinformatic genomic analysis of resistance and virulence determinants.

5. Publishing/depositing the sequenced genomes in global databases.

3.3. Materials and Methods:

The PhD thesis was developed on the basis of a vast array of modern molecular genetic methods up to whole-genome sequencing with the necessary bioinformatics analyses. They were applied in the study of an impressive number of clinical isolates – 100 MDR (extensively resistant) *Pseudomonas* isolates, of which 96 - *Pseudomonas aeruginosa*, 2-*Pseudomonas soli*, 1-*Pseudomonas protegenes* and 1- *Pseudomonas kurunegalensis (P. putida complex)*. They are part of the collection of the National Reference Laboratory for "Control and Monitoring of Antibiotic Resistance" (CMAR) at the National Center for Infectious and Parasitic Diseases. The collected panel of clinical isolates is very large, but the PhD student correctly appreciates the need to characterize the genetic determinants in different representatives of the species. The PhD student uses the routine techniques of DNA and RNA isolation, adapting the protocols, skillfully using the experience and developments of the scientific supervisor and the team of the KMAR Laboratory. The number of PCR-based methods is impressive, such as Detection of class A, B and D carbapenemases by multiplex EVAGREEN Real Time PCR; PCR for detection of virulence factors; Multilocus variable number of tandem repeat analysis (MLVA9) for typing P. aerugnosa; Multilocus sequence typing; Plasmid replicon typing; RT-qPCR for analysis of efflux systems and other genes associated with antibiotic resistance, etc. The steps and technical details for Cloning - PCR for amplification of inserts and linearization of vectors are presented in detail. Whole genome sequencing, Bioinformatics analyses. This impressive set of methods proves that Ivan Stoykov is a built molecular biologist with excellent theoretical preparation and practical skills to apply them in working with pathogenic microorganisms.

3.4. Results and Discussion

- The PhD thesis is a modern molecular genetic study based on 100 strains of clinical isolates, dominantly of the P. aeruginosa species, originating from a total of 14 cities in Bulgaria. They are included based on differences in their spectrum of antibiotic susceptibility, ranging from multidrug-resistant (MDR), through extensively drug-resistant (XDR) to pan-resistant (PDR). The collected panel of clinical isolates is very large, but the PhD student correctly appreciates the need to characterize the genetic determinants in different clinical representatives of the species. All of

them were collected over a period of 14 years - from 2010 to 2023. I highly appreciate the choice of the PhD student and his supervisor, for multivariate genetic analysis of these clinical strains.

The logical sequence of the experimental stages is impressive, starting from a general characterization of the isolates used and their species identification, followed by the determination of antimicrobial resistance. Regardless of the large number of tested strains, the doctoral student determined their resistance using 19 different antimicrobials, including new generation ones such as Meropenem/Vaborbactam, Ceftazidime/Avibactam, Ceftolozane/Tazobactam, Imipenem-relebactam and Cefiderocol. Applies both disk diffusion and broth dilution methods using commercial panels. Results are correctly interpreted according to EUCAST standards (www.eucast.org).

I highly appreciate the doctoral student's work on optimizing part of the molecular genetic methods needed to solve the experimental tasks on:

- Evidence of genetic mechanisms of resistance to beta-lactams through molecular genetic techniques (carbapenemases, efflux, porin deficiency, etc.);
- Demonstration of virulence factors by PCR;
- Genotyping (MLVA, MLST) and strain selection for whole-genome sequencing (WGS)

The work on RT-qPCR to prove the expression of efflux systems and genes related to antimicrobial resistance is original and contributing.

At various stages of the work, the PhD student has successfully implemented protocol optimization and validation, e.g. by typing by the highly discriminatory methods MLVA9 (nine loci) and MLST, selecting the most informative published primers and locus schemes. In this regard, I would like to highlight the new scheme developed for Plasmid Replicon Typing PCR (PBRT). Based first on in silico bioinformatic analysis, on which the development and design of new original primers covering some of the rarer and/or newly discovered plasmid replicons in *Pseudomonas* such as IncG/U/P6, IncP-2, IncP-10, pMOS94 is based -like, pKLC102-like, followed by PCR-based plasmid replicon typing (PBRT). This scheme was implemented correctly in all isolates with primers for 10 of the more common replicons (IncP-1abyde, IncP-4, IncP-7, IncP-9, IncW, IncQ, IncA/C, IncN, etc.), as well as for the recently described pMOS94 and pKLC102-like plasmid families.

The excellent preparation of the PhD student is evident not only from the experimental part, but also from the part in which modern bioinformatics analyzes are implemented. It starts with an analysis of the quality of the obtained short and long reads and an assessment of the quality of the resulting genomes.

The PhD student critically commented on the resulting short reads from the sequencing performed using Illumina and provided valuable information on data quality. Building on these studies is third-generation long-chain sequencing of 17 genomes. In combination with short-chain sequencing (or so-called hybrid assembly), this technology provides an opportunity to perform additional scaffolding of the obtained genomes, as well as to circularize the chromosome and available plasmids. The selection of the isolates was made according to different criteria: 1) presumed presence of plasmids; 2) potential resistance to cefiderocol; 3). potential presence of new genes/alleles; 4) extensively resistant phenotype. After extensive analysis, the best-quality assembled genomes from short- and long-chain sequencing were selected and deposited in the NCBI genome bank (Genbank), which was set as a task. This experimental algorithm enabled the identification of 96 of the P. aeruginosa isolates, including 4 isolates originally identified as P. *putida* complex by MALDI-Tof. The exact species was precisely determined as two of them

(Pput3333 and Pput3334) were identified as *Pseudomonas soli* and *P. protegenes*, and strain number 3229 was proved to belong to the species *Pseudomonas kurunegalensis*. Importantly, three of these isolates appeared to carry VIM carbapenemases, confirming the increasing role of species other than P. aeruginosa as clinically relevant pathogens.

Based on knowledge and experience, Ivan Stoykov emphasizes the need to combine different methods for precise identification of bacterial isolates, the most reliable being the results obtained by whole-genome sequencing. This is actually the final stage and successful completion of the task.

Characterization of IMP-producing *P. aeruginosa* and emergence of IMP-100, a novel plasmid variant co-existing with chromosomal VIM-4. After a complete phenotypic and genomic analysis of three *P. aeruginosa* clinical isolates, the presence of 3 different variants of IMP carbapenemases was found. Until now, carbapenemases of this type have not been reported in Bulgaria for P. aeruginosa. The three strains (Paer3541, Paer3796A and Paer4782MK) were isolated in Sofia, Bulgaria, between 2018 and 2022. The new carbapenemase IMP-100 was localized to previously undescribed mobile genetic elements integron In4886 and transposon Tn7700, which is a contribution to epidemiological data not only for the country.

The correct methodological approach and professionally selected, optimized and deduced molecular analyzes are the basis of many new scientific data and information. They have also been evaluated by the reviewers of refereed Bulgarian and international scientific publications. The PhD student has published his results in a journal published by MDPI -Microorganisms 2023, with a high IF 4.5, Q2; in Biotechnol & Biotechnol Eq. (2023, with F 1.67, Q3), in Probl Inf Parasit Dis., 2023, Q4, H-INDEX 6, and in Acta Microbiol. Bulgaria 39, 2023, with Q4, H - INDEX 3. Moreover, Ivan Stoykov is the first author, which proves his personal involvement. I highly appreciate this publication activity, as a guarantor of the level of scientific research that is summarized in the dissertation work.

The conclusion of the Results and discussion section makes a very good impression, as in 4 pages the doctoral student has summarized what has been achieved, outlining the contribution to a wide range of specialists, not only as scientific achievements, but also as a scientific basis for clinicians in the fight against antibiotic resistance and mutability of pathogens. In this regard, I would like to ask the PhD student: *"How the results of this modern molecular study demonstrating the expression of efflux systems and genes associated with antimicrobial resistance, as well as the new data on the axillary genome, can contribute to positive changes in clinical practice and in what time range?'*

The indisputable results of the work are summarized in 6 well and precisely formulated conclusions. The PhD student has very concisely summarized the many data obtained in the form of conclusions, which is another proof of his professionalism and knowledge.

I give a very high rating to the first genomic study conducted in the country on a collection of clinical multidrug resistant strains of *Pseudomonas aeruginosa* (n=100) over a period of 14 years (2010-2023), which is an original scientific contribution, relevant to clinical practice, as formulated other such:

1) A new genetic variant of the carbapenemase IMP-100 was discovered, associated with the newly discovered mobile genetic elements - integron In4886 and transposon Tn7700 located in a new plasmid p4782-IMP from the pMOS-94 family

2) For the first time in Bulgaria, P. aeruginosa strains resistant to cefiderocol were proven and plasmid-mediated resistance was proven

3) A new scheme was developed for PCR-based replicon typing of 13 resistance-associated plasmid families in *Pseudomonas* spp.

4) 100 genomes of multidrug resistant strains of *Pseudomonas* spp. have been sequenced and 96 have been deposited in the NCBI GeneBank. A new method for the extraction of high-quality intact RNA from *Pseudomonas* and other bacteria has been developed

They have both a scientific and a scientific-applied nature and confirm the value of the dissertation work.

4. CONCLUSION

Ivan Stoykov's dissertation summarizes the obtained scientific and applied results by demonstrating both in-depth theoretical knowledge and the ability for independent, logically constructed and in-depth molecular genetic research. The set tasks were performed at a modern level and the goal was very successfully realized. The experiments and methodological approaches used are at a level and volume that can be assessed as a challenge even for a large research team. The work is very current and multidisciplinary, combining complex preparation and skills. Based on the presented arguments for the topicality of the issue, the above-mentioned evidence of obtained excellent scientific results and the original contributions reflected in the dissertation work, I give my high evaluation and strongly recommend to the members of the respected Scientific Jury to award full-time **doctoral student Ivan Stoykov** to receive the educational and scientific degree "Doctor" in professional direction 4.3. Biological Sciences, specialty Microbiology.

02/02/2024

Reviewer: .....

Prof. Svetla Danova, DSc.