Warszawa, 25.08.2025 r.

Review of PhD Thesis by Shakeel Ahmad

Title: *"Deciphering the role of antibiotic exposure at the sub-Minimum inhibitory concentration on bacterial heterogeneity at single-cell level"*
Institution: Institute of Physical Chemistry PAS (Warsaw-4-PhD Doctoral School)
Supervisor: prof. dr hab. Piotr Garstecki
Auxiliary supervisor: dr Ilona Foik

This review concerns the doctoral dissertation of Mr. Shakeel Ahmad, entitled *"Deciphering the role of antibiotic exposure at the sub-Minimum inhibitory concentration on bacterial heterogeneity at single-cell level."* The thesis, submitted to the Institute of Physical Chemistry PAS, fulfills all formal requirements. The work addresses an important problem at the intersection of microbiology and biophysical chemistry. It focuses on heteroresistance represented by the occurrence of subpopulations within a clonal bacterial culture that differ in their susceptibility to antibiotics and examines how short-term exposure to sub-inhibitory antibiotic levels (below the *Minimum Inhibitory Concentration, MIC*) shapes this heterogeneity at the single-cell level. The candidate approaches this challenge in an interdisciplinary manner, combining advanced microfluidic single-cell analysis with microbiological studies of antibiotic response. In this review, I evaluate the thesis with respect to its scientific value, research methodology, validity of results, quality of writing, and its broader relevance to microbiology and antimicrobial resistance.

Scientific Value and Originality

This dissertation addresses an underexplored question of how exposure to sub-minimal antibiotic concentrations (sub-MIC) influences the susceptibility of individual bacterial cells in a population? The results show that pre-exposure can markedly reshape single-cell susceptibility distributions, increasing heteroresistance in both *E. coli* and *P. aeruginosa*. Ciprofloxacin proved a stronger inducer of heterogeneity than streptomycin, likely through DNA damage, SOS response activation, and oxidative stress, while efflux activity and metabolic adjustments played a role in *P. aeruginosa*. The study also revealed cross-antibiotic effects whereby low-dose exposure to one drug sometimes enhanced tolerance to another, although in some cases heteroresistance was unexpectedly reduced, illustrating the complexity of the phenomenon. While the thesis discusses genetic and stress-response mechanisms, future research could explain the underlaying mechanisms. From a translational perspective, the findings highlight the limitations of standard antibiotic susceptibility testing, which often overlooks heteroresistant subpopulations. This supports the case for single-cell diagnostics, or other simplified microfluidic adaptations suitable for clinical settings. The work also underscores the risks of subtherapeutic dosing and incomplete antibiotic courses, carrying important implications for stewardship. Overall, the thesis advances our understanding of how sub-MIC exposures shape bacterial heterogeneity while also pointing to new biological, diagnostic, and clinical questions for future investigation.

Research Methodology

Mr. Ahmad employed droplet microfluidics for single-cell antibiotic susceptibility testing, an advanced technique that allows phenotypic responses of individual bacteria to be quantified in high throughput manner. Traditional bulk assays measure only the average response of a bacterial population, in contrast, the single-cell droplet platform can reveal heterogeneity that would otherwise remain hidden. The thesis describes in detail the development and use of a microfluidic droplet system in which individual

bacterial cells (either *Escherichia coli* MG1655 or *Pseudomonas aeruginosa* PA14) are encapsulated in nL droplets together with growth medium and a range of antibiotic concentrations. This approach enabled antibiotic susceptibility tests on thousands of single cells in parallel, both with and without prior sub-MIC exposure. The choice of two antibiotics with distinct mechanisms of action – ciprofloxacin (a DNA synthesis inhibitor) and streptomycin (a protein synthesis inhibitor) – further strengthens the methodology, as it allows comparison of effects across different antibiotic classes. The design included exposing cells to three sub-MIC levels (0.125×, 0.25×, 0.5× MIC) of each antibiotic, then measuring the single-cell MIC distributions upon re-exposure to the same or the alternate antibiotic. This comprehensive experimental matrix is a strong aspect of the methodology, capturing a wide scope of conditions. The dissertation details the fabrication of microfluidic chips, the droplet generation protocol, and the image-based analysis used to determine growth in each droplet, indicating a high level of technical competency. Overall, the methodology is robust and *state-of-the-art*. It not only was appropriate for testing the hypotheses but also pushes the frontier of how microbiological experiments can be conducted. The candidate has demonstrated experimental skills and independence in mastering this complex toolkit, reflecting the ability to conduct research independently which is a key requirement for a PhD.

Validity and Significance of Findings

The study found that brief pre-exposure to sub-inhibitory antibiotic concentrations can alter the subsequent antibiotic susceptibility of individual cells, in some cases making the population more heteroresistant. For example, in *E. coli* (strain MG1655), a pre-treatment with ciprofloxacin at 0.25× or 0.5× MIC led to a dramatic increase in heteroresistance: upon later treatment with ciprofloxacin, the heteroresistant subpopulation fraction was over 20-fold higher compared to control (no pre-exposure). Even when those ciprofloxacin-pre-exposed cells were subsequently treated with a different antibiotic (streptomycin), their heteroresistance was about 15-fold higher than unexposed cells, indicating a remarkable cross-protection effect. These quantitative changes underscore the biological significance of sub-MIC exposure, even a transient, low-level antibiotic encounter can prime bacteria for increased survival against future doses. In the case of *P. aeruginosa* PA14, the effects were more complex and variable: for instance, a half-MIC pre-exposure to streptomycin raised the heteroresistant fraction by roughly 4-fold upon streptomycin re-challenge (and by ~3-fold upon ciprofloxacin challenge). Interestingly, some low-dose pre-exposures in *P. aeruginosa* did not always increase heteroresistance and even reduced it in certain cases, highlighting that the outcome depends on a nuanced interplay of factors (antibiotic class, concentration, and species-specific responses). These findings were addressed exploring mechanistic reasons; for example, why ciprofloxacin (a DNA-damaging, SOS-response inducing drug) might be a stronger inducer of heteroresistance than streptomycin. The validity of the results is supported by controls and systematic repetition.

The work uses well-characterised bacterial species, and the experimental design compares multiple conditions side-by-side, lending confidence that the observed differences are due to the pre-exposure treatment. Moreover, the data analysis (including distribution of single-cell MICs, calculations of heteroresistance indices, etc.) appears rigorous. The dissertation also compares the findings with existing literature, noting consistencies (e.g., ciprofloxacin's effect correlating with its known ability to induce stress responses) and explaining divergences where relevant.

The candidate prepared these results for publication which has been written up and submitted to a peer-reviewed journal. This attests to the significance and originality of the results. The candidate also presented his findings at multiple international conference, both as a poster and oral presentation.

The research demonstrates the power of modern single-cell techniques in studying microbial behavior; this is relevant to microbiologists seeking better diagnostic tools or new ways to screen antibiotics. In a broader sense, the thesis contributes to our understanding of bacterial population dynamics under stress, bridging a gap between microbiology and biophysics. The fact that the work involves *E. coli* and *P. aeruginosa* increases the applicability of the findings to real-world problems. In summary, the dissertation's topic and findings align closely with current priorities in microbiology and AMR research.

It might also inspire further studies on single-cell resistance mechanisms or the development of diagnostic methods to detect heteroresistance.

Quality of Writing and Thesis Structure

Mr. Ahmad's dissertation is written in English, and the quality of language is generally very good. Each experimental chapter is organised logically: it begins by outlining the specific question being addressed, then details the methods and presents the results (with well-chosen figures), and finally provides a summary that ties the findings back to the broader context. The writing throughout is clear and engaging. The level of detail provided would allow other researchers to reproduce the experiments, reflecting good scientific practice. The data are illustrated with numerous figures and tables, all of which are labeled and referenced in the text. The figures are of good quality and aid in understanding the results, for instance, bar graphs showing changes in distributions of single-cell MICs, effectively convey the key trends. The candidate demonstrates the ability to explain complex concepts in a coherent way. The thesis also includes a summary in Polish, satisfying formal requirements for language accessibility. Overall, the writing is clear. I noted minor linguistic errors (e.g., a few word choices that could be refined), but they do not hinder comprehension.

Below, I list a few questions that I invite Mr. Ahmad to discuss during the defense. The questions are relevant to the thesis, but not explicitly discussed in the thesis text. They address mechanistic gaps, bridge to diagnostic translation and extend methodological thinking.

Question 1: Single-cell variation could be linked to pre-existing physiological states, not only induced changes. This wasn't analysed but could explain some variability. Did you consider whether cell cycle stage or growth rate differences at the time of sub-MIC exposure contributed to heteroresistance outcomes?
Question 2: Sub-MIC antibiotics are known to promote HGT. The thesis doesn't cover this, but it is highly relevant to long-term resistance development. Could sub-MIC exposure increase the frequency of plasmid transfer or uptake of resistance determinants in bacterial populations?
Question 3: The thesis highlights the problem of underdiagnosis but doesn't quantify detection limits needed in practice. How sensitive would a clinical diagnostic test need to be to detect a heteroresistant fraction of, say, 1 in 10,000 cells?
Question 4: The thesis shows cross-effects but doesn't explore whether rational antibiotic pairing (combination therapy) could offset them. Based on your findings, could drug combinations (e.g., ciprofloxacin + aminoglycosides) reduce the risk of heteroresistance by preventing cross-protection?
Question 5: The thesis mentions promise but not implementation pathways. What modifications would be needed to adapt your droplet microfluidic platform for real hospital labs (automation, turnaround time, robustness)?
Question 6: Could you propose alternative single-cell methods which would be feasible beyond their chosen method. If droplet microfluidics is too complex for diagnostics, could flow cytometry or microcolony imaging provide simpler single-cell AST approaches?
Question 7: How would you integrate single-cell genomics with your platform to distinguish phenotypic heteroresistance from genetic resistance emergence?

Compliance with Article 187 of the Higher Education and Science Act (2018)

Polish law (Article 187 of the Act of 20 July 2018 – Law on Higher Education and Science) sets specific criteria for a doctoral dissertation. The dissertation should demonstrate the candidate's general theoretical knowledge of the discipline and ability to conduct independent research, and it must present an original solution to a scientific problem. Having reviewed Mr. Ahmad's thesis, I confirm that these requirements are satisfied. In line with Article 187, the thesis is also presented as a written monograph

with an English summary and a Polish summary attached, meeting all formal structural requirements. Therefore, the dissertation fulfills the legal and substantive criteria for a doctoral thesis as specified by the Polish Act on Higher Education and Science.

Recommendation

In conclusion, I give this dissertation a positive evaluation. Mr. Shakeel Ahmad's thesis entitled "*Deciphering the role of antibiotic exposure at the sub-Minimum inhibitory concentration on bacterial heterogeneity at single-cell level*" is a valuable and original piece of research. The work advances our understanding of how low-level antibiotic exposure can influence bacterial population heterogeneity and resistance development. The candidate has demonstrated scientific knowledge, methodological skills, and the ability to interpret and discuss results. Taken together, these qualities mean that the thesis meets the standards expected for the doctoral degree. I am confident that Mr. Ahmad's findings will be of interest to the broader scientific community and that he has shown himself capable of independent, high-quality research.

I hereby recommend that Mr. Shakeel Ahmad be awarded the degree of Doctor of Philosophy (Ph.D.).

Ocena końcowa

Ja, niżej podpisana, stwierdzam, że recenzowana rozprawa doktorska Pana Shakeel Ahmad spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r. poz. 1668 z późn. zm.) i wnioskuję do Rady Naukowej Instytutu Chemii Fizycznej PAN o dopuszczenie Pana Shakeel Ahmada do dalszych etapów postępowania ws. nadania stopnia doktora.

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25 sierpień 2025 r.

review date/ data sporządzenia recenzji

reviewer's signature/ podpis recenzenta

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