Warsaw, 26 May, 2025

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Title of the doctoral dissertation: **Deciphering the role of antibiotic exposure at the sub-Minimum inhibitory concentration on bacterial heterogeneity at single-cell level**

Abstract

Bacterial cells can exhibit a variety of phenotypes with respect to growth, multiplication, gene expression, metabolism and more. This phenotypic diversity becomes extremely significant under stress conditions, such as exposure to an antibiotic. Within a seemingly isogenic bacterial population, the presence of diverse subpopulations is referred to as heterogeneity. A specific form of heterogeneity, known as heteroresistance, is characterized by a wide range of susceptibilities to an antibiotic within a clonal population. Importantly, this allows a subset of population to temporarily survive antibiotic exposure, potentially acting as a reservoir for the development of stable resistance. Unlike conventional resistance, heteroresistance often goes undetectable by standard susceptibility tests, leading to its underestimation during the clinical diagnostics. However, it is emerging as an underrecognized but pivotal phenomena with potential to complicate treatment strategies and diagnostics, ultimately contributing to the global threat of antimicrobial resistance.

One of the key drivers of heteroresistance is the inefficient and inappropriate use of antibiotics, including subtherapeutic dosing, incomplete treatment courses and overuse. Despite its significance, the impact of subtherapeutic dosing on individual bacterial cell responses has not been thoroughly explored. This thesis addresses an important question in this domain- how does brief exposures to sub-MIC levels of antibiotic alter the distribution of single-cell susceptibilities upon subsequent exposure to same or a different antibiotic. To investigate this, three different concentrations of two bactericidal antibiotics, ciprofloxacin and streptomycin, were used to pre-expose *Escherichia coli MG1655* and *Pseudomonas aeruginosa* PA14 cells in independent experiments. The susceptibility of individual cells was then analyzed using droplet microfluidics upon re-exposure to either of the antibiotics.

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Experimental results reveal that pre-exposures to sub-lethal doses of antibiotics can increase the MICs of individual cell growing inside microfluidic droplets, as well as alter the degree of heteroresistance. Ciprofloxacin, a DNA damaging antibiotic, was found to be a stronger inducer of heteroresistance at sub-MIC pre-exposures, both when cells were subsequently treated with ciprofloxacin and streptomycin independently. Moreover, the obtained results were also dependent on the strain, the antibiotic type and the concentration of pre-exposure.

In MG1655 cells, pre-exposures with quarter and half MIC of ciprofloxacin in separate experiments increased the heteroresistance of cells by more than 20-folds during subsequent treatment with ciprofloxacin. Not only that, half MIC ciprofloxacin pre-exposure also increased the heteroresistance of cells by 15-folds when susceptibilities were performed with streptomycin, highlighting the possibility of cross antibiotic effect.

In PA14 cells, half MIC streptomycin pre-exposure increased the heteroresistance of cells by 4-fold on subsequent streptomycin exposure and 3-fold on subsequent ciprofloxacin exposure. Surprisingly, one eighth MIC ciprofloxacin pre-exposure, was more potent in generating heteroresistance than the higher concentrations, but only for susceptibility testing with ciprofloxacin. Some pre-exposures also led to reduced heteroresistance, highlighting the complexity of these interactions.

The findings presented in this thesis offer a novel perspective on the consequences of suboptimal antibiotic use, particularly regarding how it influences heteroresistance in bacteria at the single-cell level during subsequent treatments. These insights may prove valuable for designing more effective antibiotic regimens, developing new antibacterial agents and also addressing the broader issue of antibiotic resistance.