Optimization of treatment protocols to prevent de novo development of antibiotic resistance in Pseudomonas aeruginosa

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General introduction
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**Antibiotic resistance increases the risks of infectious diseases**

Antibiotics are antimicrobials that either kill or inhibit the growth of bacteria by interacting with specific targets. Since penicillin was discovered by Alexander Fleming in 1928, a large number of antibiotics has been discovered or synthesized (1, 2). They are characterized by various modes of action, including inhibition of cell wall synthesis, interference with DNA replication or RNA synthesis, as well as disruption of protein synthesis (3). The introduction of antibiotics has revolutionized medicine and saved millions of lives by making previously lethal infectious diseases curable. Since 1930s, millions of tons of antibiotics have been produced and applied in clinical practice. These antibiotics play crucial roles in almost all aspects of modern medicine. Without antibiotics, doctors are unable to perform basic surgeries, cure cancers efficiently, or prevent common infections from becoming life-threatening (1).

The unmitigated success of antibiotics, however, did not last long as bacteria, owing to their remarkable adaptability, are able to acquire resistance towards the therapeutic drug in response to drug exposure (4, 5). This can be well illustrated by the fact that the history of antibiotic discovery is concomitant with the development and spread of resistance in pathogens. Penicillinase, being isolated a few years after penicillin was discovered, was soon prevalent in bacteria that previously did not or rarely produce it (6). Sulfonamide resistance emerged in the strains of *Streptococcus pyogenes* in hospitals at the same time that this antibiotic was used in clinics (7). More and more *Mycobacterium tuberculosis* isolates resisted the therapeutic effects of streptomycin soon after this drug was introduced to treat tuberculosis (8). Similarly, the corresponding resistance has closely followed application of other drugs in clinical practice (4).

Antibiotic resistance is defined as the inability of an antibiotic to effectively kill bacteria or control their growth. The level of resistance of a bacterium to a certain drug is quantified by the minimal inhibitory concentration (MIC) of this drug that prevents visible growth. By comparing the measured MIC with clinical susceptibility breakpoints, the suitability of a specific antibiotic considered for treatment of a pathogen can be predicted. The prevalence of antibiotic resistance has been reported in almost all clinically significant pathogenic species, including *Acinetobacter baumannii, Enterococcus faecium, Klebsiella pneumoniae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Staphylococcus aureus*, and species of *Enterobacteria, Shigella*, and *Salmonella*, to
a different extend (7, 9-18). With world globalization, resistant strains travel from locations where resistance is acquired to faraway places which were never before confronted with such infections, making the problem a worldwide issue (19). An excellent example is the ESBL <i>Klebsiella pneumoniae</i> that translocated from India to the United Kingdom and Europe in less than 24 hours (20).

The emergence of multidrug resistant (MDR) strains makes the situation even worse. It generally renders the infections more refractory and costly to treat. In the worst case, patients may lose their lives because of the MDR organisms since none of the available antibiotics can clear the infection. The globally notable MDR strains in hospitals and community are <i>Acinetobacter baumannii</i>, <i>Enterococcus faecium</i>, <i>Enterobacter cloacae</i>, <i>Klebsiella pneumoniae</i>, <i>Mycobacterium tuberculosis</i>, <i>Pseudomonas aeruginosa</i> and <i>Staphylococcus aureus</i> (21-28). The MDR bacteria especially found in developing countries include MDR <i>Salmonella enteritidis</i>, <i>Shigella flexneri</i> as well as <i>Vibrio cholera</i> (29).

The economic burden caused by antibiotic resistance is alarming. According to the Centers for Disease Control and Prevention (CDC), antibiotic resistance costs an estimated $55 billion a year and more than 8 million additional days of hospital stay in United States (30). Earlier, the EU commission reported 25,000 deaths and an extra cost of at least 1.5 billion annually as consequence of antibiotic resistance within EU (31). Unlike these economic parameters, the long-term effects of antibiotic resistance to patients and their surroundings are hard to estimate but potentially more harmful (32). The real unaffordable cost, however, is a so called post-antibiotic era when no effective drugs are available for common infectious pathogens (33). To curb the prevalence of antibiotic resistance, novel drugs, especially those targeting MDR organisms, are urgently needed. However, the pipelines for development of new antibiotics have dried up (34). Therefore, preserving the currently available antibiotics for as long as possible by prudent administration is of paramount importance. In order to do so, thoroughly understanding the mechanisms by which the bacteria become resistant towards the therapeutic drugs has been on the top of the list of current scientific research.

**Molecular mechanisms of antibiotic resistance**

Bacteria may acquire antibiotic resistance either by <i>de novo</i> evolution or by acquiring resistant genes by horizontal gene transfer from another bacterium (35, 36). The <i>de novo</i> development of resistance can be achieved through multiple
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ways including single nucleotide substitution, deletion, and insertion, as well as duplication of resistance-conferring genes. These genomic changes result in drug resistance, as shown in Figure 1, through alteration of the cell target where the drug attacks, over-activation of the production of the enzyme that hydrolyzes or inactivates antibiotics, decrease of the uptake of the antimicrobial molecules, or induction of the efflux pumps to expel antibiotics out of the cell (37, 38).

In addition to de novo evolution, bacteria can also become resistant by genetic exchange with other bacteria. Antibiotic resistance genes are able to transfer from one bacterium to the other with the aid of mobile genetic elements such as plasmids, bacteriophages and transposons (39, 40). Plasmid-mediated antibiotic resistance is commonly reported in pathogenic bacteria. It involves almost all classes of antibiotics currently used in clinics, especially the frequently applied beta-lactams, aminoglycosides, cephalosporins and fluoroquinolones (41-43). There is no doubt about the association of phages with the formation of mobile resistance elements because of the occurrence of resistant genes flanked by the phage “fingerprints” (44). Transposons also play an important role in transmission and spread of resistant genes, as exemplified by the transposons accommodating tet(M) tetracycline resistance genes (45).

Although resistance has originated from the biological process of evolution, the de novo development of drug resistance during a specific treatment of a patient is not yet well documented, as opposed to plasmid-conferred antibiotic resistance. The difficulties to accomplish this work in vivo are several. First, it is hard to acquire in vivo samples from the location inside the body (organ) where the infection occurs. Likewise, measurement of the drug concentration reaching the target pathogens in real time is also fraught with technical difficulties. Second, the previous drug exposure as well as the concurrent treatments for other symptoms irrelevant to bacterial infections, are expected to interfere with the resistance development towards the antibiotics. Third, the co-existence of multiple bacterial species in vivo complicates the analysis. Fourth, the sole role of de novo evolution in resistance development can be hardly assessed as simultaneous genetic transmission cannot be excluded for infections in a hospital environment. These difficulties can be overcome by the application of in vitro continuous culture cultivation models, e.g. chemostat, morbidostat, hollow fiber infection model, combined with the Pharmacokinetic/Pharmacodynamic (PK/PD) data obtained from the patient population (46-48). Within in vitro models, bacterial growth conditions can be strictly manipulated and factors of therapeutic regimens can be
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Figure 1. Examples of mechanisms of antibiotic resistance in a bacterial cell, being adapted from reference 37 & 38.

separately investigated. By performing high throughput sequencing across whole populations, the molecular mechanisms behind the occurrence and development of resistance can be also assessed over time (49).

Besides the evolutionary trajectory towards drug resistance, the destiny of acquired resistance after treatment has also aroused significant concerns because it determines the persistence and dissemination of resistance. It was predicted that bacteria may lose or reverse a resistant phenotype when the selective pressure exhibited by antibiotic treatments is halted, because antibiotic resistance may be accompanied by fitness cost (50, 51). Such fitness cost, in the form of reduction of growth rate, minimization of ecological range or variation of virulence, has been documented in resistant bacteria. However, absence of fitness cost might also be observed, as shown by both in vitro and in vivo data (52, 53). This is explained by suggesting that either the resistance mutations barely compromise
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bacterial fitness, or that fitness-compensating mutations occurred concomitantly. Nevertheless, long-term tracking of the fate of the resistance mutations after the removal of antibiotics across the whole genome is currently still lacking.

**Current guidelines aiming to reduce antibiotic resistance**

Making a rational drug choice is essential for preventing antibiotic resistance. There is consensus that the antibiotic should be chosen specifically according to the infection-causing pathogen since some pathogens are inherently resistant to certain antibiotics (54, 55). Among the drugs with activity against the target bacteria, the ones with narrow coverage are preferred over those having broad spectrums, with the intention to avoid selection of resistance among other microorganisms irrelevant to the infection (56). The local epidemiology of antibiotic resistance patterns and the drug consumption history of the patients would assist determination of the empirical treatment (57). If time is allowed, prescription of drugs based on the result of the drug susceptibility test is more ideal not only in terms of therapeutic effects but also from the perspective of the prevention of resistance development (58). Unfortunately, this is hard to adopt in the clinic, especially at the starting of the therapy, partly because the current methods for determining drug susceptibility are not so rapid yet.

Apart from the aforementioned principles, there are other proposed tactics on how to avoid resistance by adjusting application of the drugs during treatment. One of such tactics is alternating different drugs in a single therapy, which was proposed as the use of one drug may be capable to select against the resistance built up during the application of the other drug (59-62). This strategy, however, has not been introduced to the clinic. One of the main reasons is that the evidence supporting the potential collateral sensitivity in clinical pathogenic isolates remains equivocal compared to the cross resistance (63). Besides drug rotation, combination of multiple drugs is also expected to be effective in decreasing the development of resistance. This idea is based on the assumption that the chance of the occurrence of mutation conferring resistance towards all the drugs applied concurrently is very rare (62, 64). However, evidence in support of combination treatment is still scarce and inconclusive (65, 66).

Besides choice of antibiotic, dosage is a well known element directly influencing development of resistance in bacteria (67, 68). Based on this observation, the concept of mutation prevention concentration (MPC) has been proposed (69, 70).
This concept, as its name indicates, is used to define a dosing level that is able to block the emergence of resistant mutants. Beside the MPC, the Pharmacokinetic (PK)/Pharmacodynamic (PD) parameters such as $C_{\text{min}}$ (the minimal concentration of an antibiotic)/MIC ratio and AUC (area under the curve, indicating the total exposure of an antibiotic to an organism)/MIC ratio, are also evaluated with respect to their effects in curbing the occurrence of resistance (71, 72). However, the application of these parameters in practice is very challenging since the actual drug concentration reaching the infected compartment or location is dynamic over time and varies in individuals. In some cases, the drug exposure can become intermittent owing to the poor compliance of patients or improper dose intervals. How these dosing patterns encountered in patients are relevant to the development of resistance in bacteria is still not understood.

Treatment duration is also important. As it has been often suggested, the therapeutic course has to be as short as possible to prevent emergence of drug resistance (56, 73, 74). This seems reasonable as the shorter the bacteria interact with the antibiotics, the less they would develop resistance. However, a realistic treatment lasts normally more than a few days in order to avoid a relapse of the infection (75, 76). In the case of some infections like tuberculosis, the treatment might last several months or even years (77). Considering the remarkable genetic capabilities of bacteria, the occurrence of resistance development is very likely already during a single treatment. However, no clear cut-off of treatment period has been recommended for specific drugs aiming to prevent the pathogens from becoming resistant.

**Research questions and thesis outline**

This thesis attempts to answer the following questions: First, whether the therapeutic levels of drugs expected in patients are able to cause the de novo development of resistance? Second, how the resistance evolved in the initial treatment influences the therapeutic effects of the subsequent treatments? In the third place, how the genome of bacteria dynamically changes during and after the treatment with different types of antibiotics? Finally, what is the optimal way to administrate antibiotics to guarantee both control of infections and prevention of resistance?

This thesis consists of six chapters. **Chapter 1** serves as a general introduction. It starts with pointing out the severity of antibiotic resistance problem in
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Clinical pathogens and its heavy burden for the whole society. After that, the mechanisms by which the clinical pathogens acquire resistance are briefly described. At the same time, the lack of information about de novo acquisition of antibiotic resistance during the patient therapy, as well as the necessity of tracking the genome-wide mutations during and after the antibiotic therapy are discussed. Finally, the current recommendations for administering antibiotics in order to avoid development of antibiotic resistance are summarized. The lack or incompleteness of evidence supporting the specific recommendations is noticed as well.

Chapter 2 concentrates on examination of development of antibiotic resistance during simulated antibiotic treatment of Pseudomonas aeruginosa (P. aeruginosa) infections in vitro. Two first-line anti-P. aeruginosa antibiotics, ceftazidime and meropenem, are studied. Their concentration-time profiles resulting from clinically used treatment regimens are simulated by computer modeling. Among the profiles, the 5th percentile, called “low”, 50th, dubbed “medium” and “high” (95th percentile) profiles are mimicked in chemostats. During the simulation of treatment, drug susceptibility, cellular morphology and the number of surviving cells are followed every day. What is also included in the daily assessment is the proportion of different levels of resistant subpopulations, the mutations in the resistance-relevant genes and the fitness cost defined as reduction of growth rate.

Chapter 3 focuses on elucidating how therapy failure of an initial antibiotic treatment may influence the effects of subsequent treatments. In this study, initially is tested whether resistance would occur during the treatment with sub-lethal concentrations of amoxicillin. The influence of the amoxicillin-resistant mutants acquired in the first treatment on the subsequent amoxicillin therapy is assessed by performing the growth competition experiments with the original wild type Escherichia coli (E. coli) strain and the mildly or highly amoxicillin-resistant E. coli strain evolved from it. The influence is also examined when the second treatment is switched to a third generation cephalosporin by comparing the evolutionary speed towards cefotaxime resistance of the wild type E. coli strain and the E. coli strain made mildly amoxicillin-resistant.

Chapter 4 highlights the linkage between the increase of antibiotic resistance levels and the dynamics of mutations across the whole genome of bacteria. For this purpose, P. aeruginosa is exposed to stepwise increasing concentrations of five
medically relevant antibiotics: ceftazidime, meropenem, piperclillin/tazobactum, ciprofloxacin and tobramycin. Whole genome sequencing was applied to identify the genomic changes at multiple time points during the resistance development process. The reversibility of the acquired resistance after the treatment and its corresponding genomic variations are also explored. Since resistance towards beta-lactam antibiotics is often related to overexpression of beta-lactamases, the relationship between beta-lactamase activity and resistance levels of \textit{P. aeruginosa} towards beta-lactam drugs is documented. The fitness, as demonstrated by the maximum growth rate, the pH and salt tolerance, and the maintenance energy of \textit{P. aeruginosa} before and after the acquisition of resistance, is also investigated.

\textbf{Chapter 5} presents the comparison of a series of treatment strategies with respect to both bacteria killing efficacy and resistance preventive potential, aiming to provide better evidence for recommendations of antimicrobial administration guidelines. The tested administration protocols, including continuous infusion, fluctuating infusion, intermittent treatment, drug alteration as well as drug combination, are mimicked in chemostats. All the drug concentrations applied are those expected to occur in patients. To gain insights into the differences of evolutionary trajectories among the assessed treatment regimes, the mutation profiles of the \textit{oprD} gene are followed during each mimicked treatment due to the fact that the meropenem resistance is closely related to \textit{oprD} mutations in \textit{P. aeruginosa}.

\textbf{Chapter 6} summarizes the results achieved in this thesis. In addition, we elaborate on the discussion of several issues concluded from \textbf{chapter 2} to \textbf{chapter 5}, including \textit{de novo} development of antibiotic resistance during treatment and its consequences, the complexity of evolutionary trajectory towards antibiotic resistance, completion and improvement of the guidelines to antibiotic administration.