Escherichia Coli Adaptive Resistance to Clinical Antibiotics

Twinkle Patel¹ and Anastasia Levitin²*

¹Genomics Department, University of California, Riverside, USA
²School of Applied Life Sciences, Keck Graduate Institute, USA

Abstract

Escherichia coli are beneficial bacteria found in human intestinal tracts. However, certain E. coli strains are pathogenic and can cause serious infections, such as neonatal sepsis and meningitis. The goal of this work is to create an awareness of the phenomenon of bacterial adaptation to antibiotics. We demonstrate that the pre-exposure of E. coli to low levels of antimicrobial agents (inducers), including broad-spectrum agents such as chloramphenicol and piperacillin, results in a decreased sensitivity of E. coli to lethal doses of the same or another antibiotic (testers), including ceftriaxone and vancomycin. We suggest that the treatment options considered for a particular E. coli infection should be based upon a review of the patient’s treatment history. Antibiotic treatments for prior infections might result in residual drug accumulation by the body acting as inducers and therefore, influence treatment effectiveness for a current infection. In this work, nearly six hundred combinations of two antibiotics were tested, and the combinations that resulted in an increase in the half maximal inhibitory concentration (IC₅₀) to the tester by an average of 1.5 folds or more were selected. The results of this work show that E. coli can transiently adapt to antibiotics and consequently become more resistant to lethal doses of treatment antibiotics.

INTRODUCTION

Antimicrobial resistance is a growing global concern. Overuse and misuse of antibiotics have resulted in an increasing number of resistant microorganisms that fail to respond to standard treatment measures. As naturally occurring antibiotics used for the treatment of infections become unavailable, more expensive and longer treatments must be used, which greatly increase health-care costs [1]. There are various types of antibiotic resistance, most broadly categorized as inherited and non-inherited. Inherited antibiotic resistance refers to a type of resistance that is passed genetically from one generation of bacteria to the next, or through horizontal gene transfer [2]. Conversely, non-inherited antibiotic resistance describes a form of transient resistance that occurs due to the organism’s ability to adapt and phenotypically modify itself in order to develop resistance to certain stressors [3], such as heat [8], etc. where pre-exposure to antibiotics remains a major cause of future resistance [4]. This paper specifically focuses on a particular type of non-inherited antibiotic resistance, i.e. adaptive antibiotic resistance. Adaptive resistance is an ephemeral type of resistance that is characterized by a decreased bacterial sensitivity to inhibitory or lethal doses of one antibiotic (tester) due to a short pre-exposure to residual levels of the same or another antibiotic (inducer). Pre-exposure occurs when bacteria is introduced to a sub-inhibitory concentration of an antibiotic which does not hinder bacterial growth, but is capable of activating certain mechanisms resulting in increased resistance to same or different antimicrobial agents. Although, this phenomenon is transitory and the removal of an inducer restores normal sensitivity [Levitin, unpublished results], a longer pre-exposure to sub-inhibitory levels of antibiotics can result in the emergence of permanent mutations. Although the study of the mechanism of adaptive resistance lags behind, it has been shown that it involves down-regulation of bacterial resistance gene expression [5]. This work, nearly six hundred combinations of two antibiotics were tested, and the combinations that resulted in an increase in the half maximal inhibitory concentration (IC₅₀) to the tester by an average of 1.5 folds or more were selected. The results of this work show that E. coli can transiently adapt to antibiotics and consequently become more resistant to lethal doses of treatment antibiotics.

positive and gram-negative organisms, including *P. aeruginosa*, *S. aureus*, and *E. coli* [6, 7, 12]. A number of studies discuss adaptive resistance between antibiotics belonging to the aminoglycosides group [4, 9]. It has also been shown that adaptive resistance lasts temporarily after an initial exposure to sub-inhibitors and increases greatly with constant exposure [10]. This knowledge is vital to patient care establishments when designing the most effective regimen to fight infections [3]. Recently, it has been shown that the dosage of antibiotics is also of eminent importance [11]. Currently, there are a number of combinations known to result in adaptation in *E. coli*. These include triclosan, a synthetic antimicrobial chemical, whose pre-incubation results in an adaptation to imipenem, tetracycline, trimethoprim, etc. [12]. Pre-exposure to kanamycin has also been shown to cause adaptation to ampicillin, streptomycin, tetracycline, etc. [13].

One of the proposed mechanisms of adaptive resistance is the divergent expression of the genes encoding efflux pumps, which are responsible for expelling antibiotics from the cell [Levitin, unpublished results, 14].

This paper focuses on the identification of combinations of antibiotics resulting in *E. coli* adaptive resistance. *E. coli* is a human pathogen that is responsible for a wide array of bacterial infections in humans, most commonly intestinal infections and urinary tract infections [15,16]. Both antibiotics that are prescribed to treat broad spectrum infections and antibiotics that are very specific to *E. coli* infections are tested in this work. Although other treatment methods exist, antibiotics still remain significant players in the regulation of *E. coli* infections [17]. While mild *E. coli* infections do not require any treatment, more serious infections such as neonatal pneumonia might entail dialysis, blood transfusions, and the replacement of bodily fluids [17,18]. In this work, we have tested an assortment of commonly used clinical antibiotics, including vancomycin, amoxicillin, and ciprofloxacin. We have found that many combinations of the tested antibiotics result in *E. coli* adaptation and an increase in resistance to the lethal doses of certain antibiotics. Therefore, we suggest that a review of a patient’s antibiotic history be a part of the prescription practice to allow the best treatment outcome for patients with *E. coli* infections.

We postulate that residual amounts of antimicrobial agents accumulated by the body from previous treatment regimens might act as inducers and consequently result in bacterial adaptation and an increased resistance of *E. coli* to the same or different antibiotics, thus abolishing the effectiveness of the treatment. We believe that providing the healthcare practitioners with a database of antibiotic combinations that result in adaptive resistance, in conjunction with the knowledge of a patient’s previous treatment history, will allow them to make educated choices when prescribing an efficient treatment for patients with *E. coli* infections.

**MATERIALS AND METHODS**

**Bacteria and media**

All experiments were done on an *E. coli* bacterial strain C600 (F- tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1 λ-, CGSC# 3004) and used lysogeny broth (LB) media. The *E. coli* was initially grown on a LB agar plate at 37°C. Subsequently, the liquid bacterial culture was grown from a single colony by shaking at 200 RPM at 37°C for a period of 16 hours prior to the beginning of the experiment.

**Antibiotics**

A total of twenty-six antibiotics were used in this project, and were tested in combinations of two. The antibiotics were dissolved in either water or dimethyl sulfoxide (DMSO) and diluted in LB medium to initial concentrations ranging from 2.5 µg/ml to 200 mg/ml.

**Initial Kill-Curve Experiments**

This set of experiments was aimed at determining the optimal sub-inhibitory, inhibitory and lethal concentrations (no visible change in bacterial growth, visible adverse effects on growth, and no proliferation of *E. coli* observed at optical density of 600nm (OD600) as compared to control cultures, respectively). A set of twelve different concentrations at a two-fold concentration gradient was used during testing. The overnight grown culture was diluted in fresh LB medium to 0.05 OD600 and aliquoted in 100µl into a 96-well plate, followed by the addition of an array of tester antibiotic concentrations in volumes of 5µl. The same volumes of LB media were added to control wells. The plates were grown with constant shaking at 37°C and the absorbance was measured by a SpectraMax Plus Microplate Reader (Molecular Devices) every 1000 seconds for a period of 6 hours. The concentrations of antibiotics that did not result in a detrimental change in the visible growth of the bacterial culture were chosen as sub-inhibitory concentrations. The concentrations that reduced bacterial growth, as well as the concentrations that inhibited growth, were used as tester concentrations in subsequent experiments.

**Combination Kill-Curve Experiments**

The antibiotics were tested in combinations of two with one antibiotic added at a sub-inhibitory concentration (inducer) and another added as a gradient of inhibitory/lethal concentrations (tester) to study whether such combinations resulted in adaptive resistance. The overnight bacterial culture was diluted in fresh LB media to OD600 of 0.05 and grown at 37°C for one hour to acclimatize the cells to specific growth conditions. The culture was aliquoted in 100µl into a 96-well plate, followed by the addition of an inducer in a volume of 5µl. The same volumes of LB media with corresponding concentration of the solvent used to prepare the inducer solution (water or DMSO (< 0.1%)) was added to control wells. The plate was incubated for 1 hr at 37°C with constant shaking. Next, a gradient of five different inhibitory and lethal concentrations of the tester antibiotic were added in volumes of 5µl in order to create a widely spread kill-curve with a control well containing the same amount of LB media with corresponding concentration of the solvent used to prepare the inducer solution. The effect of the sub-inhibitory level of the inducer on bacterial growth was compared to the growth rate of un-induced cells exposed to the same concentrations of the tester antibiotics.

**RESULTS**

**Initial Kill-Curve Experiments**

The antibiotics used in this paper are representative of broad-
spectrum antibiotics commonly prescribed as part of empiric therapy (e.g. chloramphenicol, amoxicillin, piperacillin, etc.), as well as specific antibiotics routinely prescribed to treat E. coli infections (e.g. ceftriaxone, vancomycin, ciprofloxacin, etc.). Each of the antibiotics used in this work was tested as both an inducer and a tester. The E. coli strain C600 proved to be insensitive to the tested concentrations of three out of the twenty-six antibiotics used in this work. Therefore, these antibiotics could not be used as testers, but were assessed as inducers. Those antibiotics were novobiocin, bacitracin, and oleandomycin. As a result, twenty-six inducers and twenty-three testers and a total of 598 possible combinations were tested.

**Combination Kill-Curve Experiments**

An E. coli strain C600 was pre-incubated with a sub-inhibitory level of an inducer antibiotic and subsequently grown in the presence of lethal doses of tester antibiotics. IC50 values of tester antibiotics in control cultures with no pre-incubation with an inducer are indicated in the Solvents section. IC50 values of tester antibiotics in cultures pre-exposed to sub-inhibitory levels of inducer antibiotics are listed in the Inducers section. Antibiotics were diluted in either water (highlighted in gray), or in DMSO (indicated in white). The IC50 values shown are averages of 3 independent experiments.

Out of 598 total antibiotic combination kill-curve experiments performed, 30 antibiotic combinations displayed the phenomenon of adaptive resistance. The results of the combinations are shown in (Table 1), with inducers listed horizontally and testers listed vertically with the half maximal inhibitor concentration (IC50) shown for each combination resulting in adaptive resistance.

**DISCUSSION**

Adaptive resistance is a phenomenon whereby pre-exposure of an organism to sub-inhibitory levels of one antibiotic results in transient [Levitin, unpublished results] resistance of bacteria to lethal doses of the same or other antibiotics to which the organism was sensitive prior to the pre-exposure. The mechanisms underlying such adaptation were shown to involve efflux pumps, and components involved in control of the anaerobic respiratory pathway, thickening of the cell wall, etc. [Levitin, unpublished results, 6, 7, 14].

**Table 1:** Inducer-tester combinations that give rise to a reduced sensitivity of E. coli to lethal levels of tester antibiotics as a result of pre-exposure to sub-inhibitory doses of inducer antibiotics.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>H2O</th>
<th>DMSO</th>
<th>QC</th>
<th>CQ</th>
<th>FM</th>
<th>NB</th>
<th>CX</th>
<th>CC</th>
<th>PP</th>
<th>OF</th>
<th>BT</th>
<th>CF</th>
<th>SF</th>
<th>CA</th>
<th>CT</th>
<th>VM</th>
<th>OM</th>
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<tbody>
<tr>
<td>NA</td>
<td>31 ± 1.8</td>
<td>30 ± 1.6</td>
<td>50 ± 3.6</td>
<td>55 ± 5.1</td>
<td>62 ± 3.8</td>
<td>61 ± 29</td>
<td>44 ± 1.4</td>
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<tr>
<td>QC</td>
<td>608 ± 33</td>
<td>615 ± 37</td>
<td>1954 ± 59</td>
<td>1841 ± 25</td>
<td>1319 ± 51</td>
<td>2189 ± 37</td>
<td>1644 ± 73</td>
<td>2767 ± 136</td>
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<td>NM</td>
<td>8.3 ± 0.6</td>
<td>7.9 ± 0.7</td>
<td>12 ± 1.2</td>
<td>0.1 ± 0.02</td>
<td>0.15 ± 0.04</td>
<td>0.15 ± 0.02</td>
<td>0.17 ± 0.01</td>
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<tr>
<td>FM</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.003</td>
<td>0.17 ± 0.03</td>
<td>0.01 ± 0.003</td>
<td>0.04 ± 0.003</td>
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<td>CC</td>
<td>87 ± 7.4</td>
<td>91 ± 6.3</td>
<td>154 ± 21</td>
<td>22 ± 1.4</td>
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<tr>
<td>AM</td>
<td>7.2 ± 0.5</td>
<td>7.3 ± 0.9</td>
<td>1.8 ± 0.4</td>
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<tr>
<td>AC</td>
<td>0.8 ± 0.1</td>
<td>3.1 ± 0.3</td>
<td>25 ± 0.6</td>
<td>14 ± 0.6</td>
<td>25 ± 1.6</td>
<td>0.1 ± 0.03</td>
<td>12 ± 1.1</td>
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<tr>
<td>CS</td>
<td>1.2 ± 0.6</td>
<td>14 ± 0.6</td>
<td>8.1 ± 0.9</td>
<td>0.32 ± 0.01</td>
<td>0.36 ± 0.03</td>
<td>0.65 ± 0.07</td>
<td>1.2 ± 0.09</td>
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<td>0.36 ± 0.03</td>
<td>0.65 ± 0.07</td>
<td>1.2 ± 0.09</td>
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<td>0.65 ± 0.07</td>
<td>1.2 ± 0.09</td>
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<tr>
<td>CA</td>
<td>8.2 ± 1.2</td>
<td>8.1 ± 0.9</td>
<td>12 ± 1.1</td>
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<tr>
<td>CT</td>
<td>0.32 ± 0.01</td>
<td>0.36 ± 0.03</td>
<td>0.65 ± 0.07</td>
<td>1.2 ± 0.09</td>
<td>8.1 ± 1.3</td>
<td>0.69 ± 0.04</td>
<td>5.4 ± 1.1</td>
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<tr>
<td>VM</td>
<td>174 ± 14</td>
<td>179 ± 12</td>
<td>1096 ± 38</td>
<td>362 ± 18</td>
<td>3.3 ± 0.2</td>
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| RM       | 1.3 ± 0.3 | 1.4 ± 0.3 | 3/5

**Abbreviations:** Nalidixic acid, NA; Quinacrine, QC; Neomycin, NM; Chloroquine, CQ; Phosphomycin, FM; Novobiocin, NB; Ceftriaxone, CX; Cloxacillin, CC; Azithromycin, AM; Piperacillin, PP; Amoxicillin, AC; Ofloxacin, OF; Bacitracin, BT; Ciprofloxacin, CF; Cefsulodin, CS; Sparfloxacin, SF; Chloramphenicol, CA; Cefotaxime, CT; Vancomycin, VM; Oleandomycin, OM.
From the 598 combinations of clinically relevant antibiotics, 5% of all combinations tested expressed adaptive resistance. Each combination that showed evidence of adaptive resistance by displaying an increase in IC50 was recorded in Table 1 and represents the averages of the IC50 values obtained from three independent experiments. For example, pre-incubation with a low dose of ofloxacin commonly used to treat chronic bronchitis, pneumonia, and urinary tract infections results in an increase in IC50 to cefotaxime, an antibiotic used to treat numerous infections including bacteremia/septicemia, lower respiratory tract, and gynecologic infections caused by E. coli. 

Based on our observations done on gram-positive bacteria, the degree of adaptive resistance is dependent on the length of pre-incubation with the inducer. We found that the increase in the length of pre-treatment from zero minutes to 1 hour is directly proportional to the increase in the magnitude of adaptive resistance to the tester antibiotic. However, pre-incubation for periods longer than 1 hour resulted in only a modest further change in adaptive resistance. Concentration of an inducer was also observed to directly affect adaptation to a tester antibiotic [Levitin, unpublished results]. 

Although adaptive resistance is both transient and reversible, the occurrence of this phenomenon can result in the inability to control the infection and the emergence of antibiotic resistant strains. Many of combinations with a potential to result in adaptive resistance consist of broad-spectrum antibiotics as inducers and narrow-spectrum antibiotics as testers, such as phosphomycin (inducer) and vancomycin (tester), a combination which might be prescribed to treat endocarditis and E. coli infections. Certain inducers in combinations resulting in adaptive resistance are broad-spectrum antibiotics that treat a number of bacterial infections, such as ciprofloxacin, ceftriaxone, and vancomycin. 

The prescription of broad-spectrum antibiotics prior to the identification of the cause of an infection might give rise to bacterial adaptation to E. coli-specific antibiotics, resulting in an ineffective treatment or an exacerbated infection. Various organs of the body accumulate residual levels of treatment antibiotics, and depending on antibiotic half-life, might serve as an inducer and affect subsequent treatment regimens. As a consequence, treatment for E. coli infections initiated with broad-spectrum antibiotics (inducers) as part of empiric therapy, and followed by treatment with narrow-spectrum antibiotics (testers), such as amoxicillin and chloramphenicol, would be inefficient due to the phenomenon of adaptive resistance in E. coli.

**CONCLUSION**

This work offers a record of antibiotic combinations that are capable of exhibiting adaptive resistance. More testing of various antibiotics combinations should be done to create an extensive database of antibiotic combinations that give rise to adaptive resistance. This database would allow healthcare professionals to make wise and well thought-out decisions when prescribing medication in order to quickly and effectively treat patients. 

The mechanism for adaptive resistance is still largely unknown; however, the results of this screening may assist in further discovering what cellular components play a part in this phenomenon.

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