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Determining the Rate of Development of Antibiotic Resistance to Streptomycin and Doxycycline in *Escherichia coli*

ARTICLE INFORMATION

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ABSTRACT

Antibiotic resistance is a pressing issue in the medical field today. It is important to understand the development of bacterial resistance to implement effective preventative measures against antibiotic resistant bacteria. This study investigated the rate at which *Escherichia coli* (*E. coli*), a common pathogen, developed resistance to streptomycin and doxycycline, as Oz et al. (2014) showed differing levels of resistance in *E. coli* to these two antibiotics. The development of antibiotic resistance was measured by adding *E. coli* to 96-well plates in the presence of increasing doses of doxycycline, streptomycin, or a combination treatment. Successive generations were added to the same treatments to see whether they would grow at higher concentrations of antibiotic. The change in minimum inhibitory concentration for streptomycin and doxycycline was determined as the bacteria became increasingly resistant to each antibiotic. The fastest rate of antibiotic resistance was observed for streptomycin, with doxycycline resistance exhibiting a slower rate of development. The rate of resistance development for the combination treatment was the slowest, potentially due to small differences in target domains. Some cross-resistance was also observed. This study provides a small-scale methodological basis and preliminary insight on antibiotic resistance trends for two antibiotic classes and a combination treatment.

Keywords: Antibiotic resistance, *E. coli*, selective pressures, cross-resistance

INTRODUCTION

Antibiotic resistance is becoming more prevalent, resulting in diminished effectiveness of many antibiotics.[1] Antibiotic resistance occurs when bacteria evolve to survive in the presence of antibiotics designed to eliminate them. This occurs for several reasons, including the overuse of antibiotics both for treatment in humans and livestock.[2] Antibiotics are becoming less effective against bacterial infections as bacteria evolve and become resistant to antibiotic treatments.[2] In order to combat this issue and create new antibiotics that prevent the development of antibiotic resistance, it is important to understand how the rates of antibiotic resistance in bacteria differ between antibiotics and how this relates to the mechanisms of antibiotic resistance. This study attempted to provide a small-scale methodical basis, adapted from Oz et al. (2014), to investigate the development of antibiotic resistance by comparing the rate of resistance development for two antibiotics in *Escherichia coli* (*E. coli*).

Oz et al. analyzed the levels of resistance of *E. coli* under various selective pressures to 22 antibiotics by measuring the minimum inhibitory concentration (MIC) of the antibiotics daily for 21 days.[3] MIC is the lowest effective dose of a drug. Knowing the MIC is vital to appropriately prescribe treatment doses so that all bacteria are eliminated. Breakpoints are also crucial to investigate, and they are correlated with MIC values. Breakpoints represent the concentration of antibiotic at which the bacteria are no longer susceptible to the antibiotic.[4] If the MIC is greater than or equal to

the breakpoint value, the bacteria are considered resistant to the antibiotic. Therefore, it is essential to ensure that the MIC of the antibiotic remains below the breakpoint value to ensure effective treatments.

For this study, two antibiotics, streptomycin and doxycycline, were chosen as Oz et al. showed that *E. coli* displayed differing levels of resistance to these antibiotics after 21 days.[3] The antimicrobial targets of these antibiotics are involved in protein synthesis.[5] Specifically, tetracyclines, including doxycycline, are thought to prevent binding of tRNA to the A site of the ribosome; and aminoglycosides, including streptomycin, are thought to cause misreading and premature termination of mRNA translation, thereby acting against the bacteria to reduce its effects on the host.[5] Doxycycline is commonly used for the treatment of acne,[6] and can also be used to prevent malaria.[7] Streptomycin is used in the treatment of tuberculosis,[8] as well as other serious infections like the plague.[9] The aim was to replicate the results of Oz et al. through an adapted method and gain insight on the relationship between antibiotic class and the rate of development of antibiotic resistance.

There are several ways by which antibiotic resistance can occur in bacteria. One such mechanism is mutational resistance, where bacteria develop genetic mutations that overcome the activity of the drug, thereby leading to predominance of resistant bacteria.[10] These genetic mutations can lead to the decrease in drug uptake in the cell; activation of pumps or efflux mechanisms to remove the drug from the cell; alteration of metabolic pathways; or the modification of the antimicrobial target to decrease its affinity for the drug.[10] Another way bacteria can become resistant is by acquiring foreign DNA that contain antibiotic resistance genes through horizontal gene transfer, which can be accomplished through transformation, transduction or conjugation.[10] Transformation is the uptake of naked DNA from the environment. Transduction occurs when part of a host bacterium's genome is passed to another bacterium by a bacteriophage. Conjugation involves transfer of genetic material between bacteria via direct contact.[10] Bacteria are known to become resistant to streptomycin by using aminoglycoside modifying enzymes that alter the hydroxyl or amino groups of the antibiotic.[10] Doxycycline is possibly susceptible to a different mechanism of resistance where bacteria develop resistance through efflux pumps.[10]

The resistance mechanism that bacteria develop to one antibiotic may allow them to become resistant to another antibiotic at the same time through a process known as cross-resistance.[3,11] The opposite can also occur, whereby bacteria become more susceptible to other antibiotics upon developing resistance to one antibiotic, which is a phenomenon known as collateral sensitivity.[12] In this study, cross-resistance was determined using antibiotic disks and agar plates. This is known as the disk diffusion method, used in both hospitals and laboratories, to determine the susceptibility of bacteria to the effects of antibiotics.[13] Cross-resistance is problematic because it can cause bacteria to become resistant to antibiotics they have never been exposed to, which hastens the process of antibiotic resistance as a whole. In order to understand the phenomenon of cross-resistance, chemogenomic profiling has been used.[11] This method uses drug-mutant interactions and gene fitness to determine the mechanism of action of drugs. Similarities between chemogenomic profiles may be a strong predicting factor for cross-resistance.[11]

This study focused on comparing the rate of development of antibiotic resistance in *E. coli* to streptomycin, doxycycline, and a combination treatment. In addition to measuring rate of resistance, cross-resistance was also measured since understanding cross-resistance poses implications for the design of new antibiotics.

METHODS

Antibiotic Resistance Study

E. coli was exposed to one of three treatments: streptomycin (STR), doxycycline hyclate (DOX), or a combination of the two antibiotics (both obtained from Sigma-Aldrich). Minimum inhibitory concentration (MIC) values were determined in 96-well plates with each antibiotic added in duplicate (n=2) (Figure 1, Table 1).

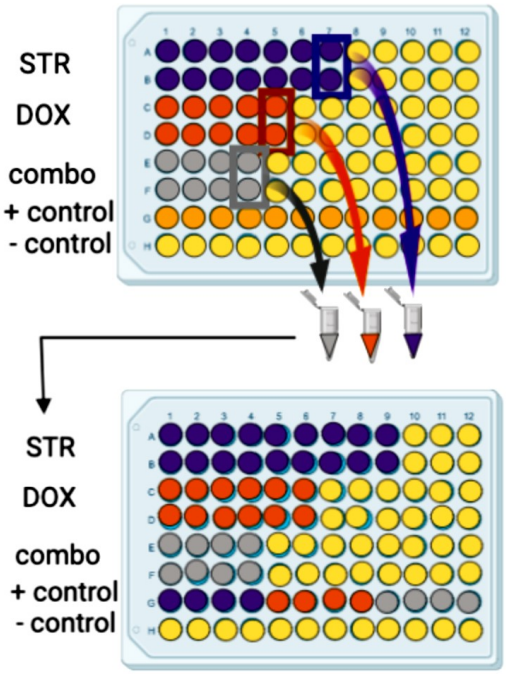

Figure 1. Visual representation of the plating procedure for generations zero and one of the antibiotic resistance study. Schematic diagram of the process of isolating bacteria from MIC/2 (mg/L)

wells which were then grown and added to a new 96-well plate. The respective treatment colours in this diagram refer to bacterial growth after a 24-hour incubation period. All bacteria were added to wells at an optical density at 610 nm between 0.08 and 0.10 to ensure an equal starting point so that growth could accurately be determined. Yellow wells represent no bacterial growth. This process was repeated for eight generations. Figure created using BioRender.

Difco™ Nutrient Broth was added to all wells: 50 µL in rows A-G and 100 µL in row H. Row H acted as a negative control, as it contained a solution of nutrient broth only, so no bacterial growth was expected. 50 µL of STR at a concentration of 80 mg/L was added to well 12 of the first two rows. This concentration was changed to 5120 mg/L as the *E. coli* continued to grow at higher concentrations, likely due to their development of a resistance mechanism. 50 µL of DOX at a concentration of 512 mg/L was added to well 12 of the next two rows. For the combination treatment, the highest concentration consisted of 40 mg/L of STR and 256 mg/L of DOX. For this treatment, 25 µL of the specified STR concentration and 25 µL of the specified DOX concentration were added into well 12 of the next two rows. Two-fold serial dilutions were performed from well 12 to well 1 of rows A to E so that each well would have a concentration half of that in the well to their right. For the initial well set-up, 50 µL of wild type *E. coli* with an optical density at 610 nm between 0.08 and 0.10 was added to all wells in rows A to G (Figure 1). Row G acted as a positive control, as it contained 50 µL nutrient broth and 50 µL wild type *E. coli* and would represent optimal bacterial growth used as a comparison for growth in treatment wells. After all components were added, the plate was incubated at 37˚C for 24 hours, at which point it was read to determine the MIC of each antibiotic.

A scale was created from zero to four, where zero referred to no growth and four referred to maximum growth seen in the positive control well. The MIC was defined as the lowest concentration where no bacterial growth was observed. Bacteria were isolated from the MIC/2 wells, or the highest antibiotic concentration with bacterial growth. This is represented as the well the farthest to the right that had bacterial growth (see Figure 1 where growth is represented by colour). Bacteria isolated from the same treatment were combined and centrifuged, the supernatant was removed, and the pellet was resuspended in Difco™ Nutrient Broth and grown overnight in a water bath at 37˚C. These bacteria were then used to repeat the plating procedure outlined above. Bacteria were added to the same treatment from which they had been isolated. For example, bacteria isolated from the wells treated with STR were used for the next generation of STR treatment. Isolated bacteria from each treatment were also added to four wells in the positive control row. This procedure was repeated for eight generations.

Statistical Methods

Percent difference comparing the final generation MIC and initial generation MIC for both replicates of each treatment were determined. A one-way ANOVA was performed in order to test whether at least one pair of treatments was statistically different. If the ANOVA yielded significant results, a post hoc Tukey test was performed to determine which treatments were significantly different from each other. The statistical analysis was performed using R software.

Cross-Resistance Study

Cross-resistance was tested by plating 100 µL of the final generation of each bacteria treatment and the control (initial wild type *E. coli*) on four different petri dishes containing Difco™ Nutrient Broth agar. Each plate was split into quadrants, and an antibiotic disk was placed in each quadrant. The disks used were 10 µg streptomycin (S10), 10 µg penicillin (P10), 30 µg chloramphenicol (C30), and 5 µg tetracycline (Te5) (Figure 3A). Penicillin and chloramphenicol were used in addition to streptomycin and doxycycline as they belong to different classes of antibiotics, β-lactams and chloramphenicols respectively.[5] If the antibiotic was effective against the bacteria, a ring of inhibition would be seen where there was no bacterial growth. A larger ring suggests a more effective antibiotic and a smaller ring or lack of a ring suggests antibiotic resistance. These plates were incubated for 24 hours at 37°C and diameter of inhibition was manually measured in centimetres.

RESULTS

Table 1. Recorded MIC (mg/L) for the first and last generations of the three antibiotic treatments. For the combination treatment, the volume of each antibiotic was half the volume of the independent treatments.

Treatment	Initial MIC (mg/L)	Final MIC (mg/L)
Streptomycin (STR)	0.94	160
Doxycycline (DOX)	8	64
Combination (STR/DOX)	0.32/2	1.25/8

Bacteria treated with STR became resistant at a faster rate than the bacteria treated with DOX (Figure 2A). This was determined by comparing the change in MIC of each generation to the initial MIC. When analyzing the percent difference (comparing the final and first generations' MIC) for each treatment, the difference in MIC was statistically significant ($P<0.05$) between STR and DOX, STR and combination, and DOX and

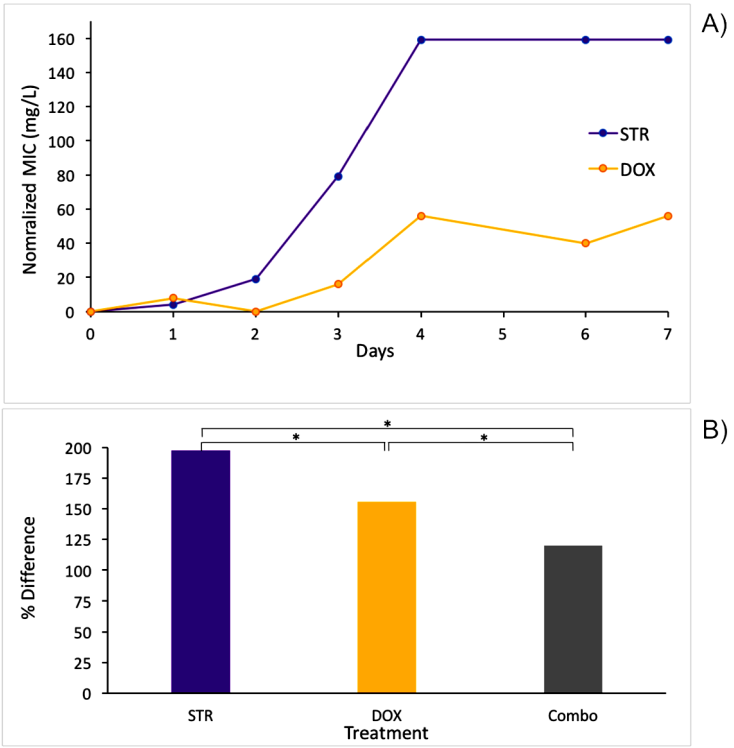

Figure 2. Change in MIC for STR, DOX and combination treatments. A) Normalized MIC of STR and DOX treatments over eight generations. STR treated bacteria developed resistance at a faster rate than DOX treated bacteria. **B)** Percent difference of final and initial MIC of all three treatments (n = 2) over eight generations (* = P < 0.05). The percent difference was statistically significant between all treatments indicating that STR treated bacteria become resistant the fastest, followed by DOX treated bacteria and the combination treated bacteria respectively.

combination (Table 1, Figure 2B). This further confirms that STR-treated bacteria become resistant at a faster rate than DOX-treated bacteria. It also shows that the combination-treated bacteria become resistant at an overall slower rate compared to STR and DOX-treated bacteria.

The resistance of these bacteria was further confirmed in the cross-resistance test as STR-treated bacteria were resistant to the S10 antibiotic disk and DOX-treated bacteria were resistant to the Te5 antibiotic disk (Figure 3B, Table 2). This means that STR-treated bacteria thrived in the presence of streptomycin and DOX-treated bacteria thrived in the presence of tetracycline, the class of antibiotics to which doxycycline belongs. This test also showed some incidence of cross-resistance; DOX-treated bacteria become resistant to streptomycin and penicillin. This can be seen by comparing the diameter of inhibition of the control to the diameter of inhibition of DOX-treated bacteria (Table 2).

Bacteria from all treatments became more susceptible to C30 in comparison to the control. There was no inhibition by this antibiotic disk on the control, but all other treatments were inhibited to some degree by C30 (Table 2). P10 and Te5 were more effective against STR-treated bacteria than the control. The combination treatment exhibited almost no changes from the control, indicating that very little resistance occurred in these bacteria.

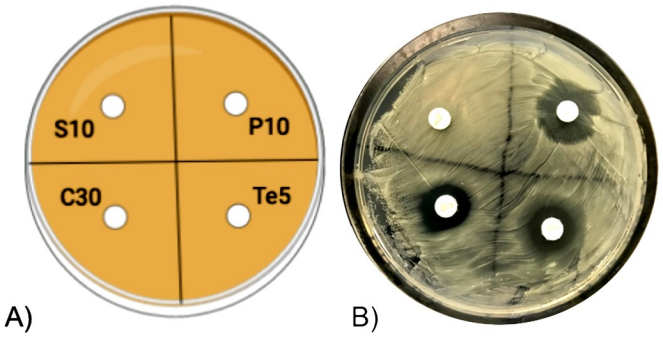

Figure 3. Set-up and results of the cross-resistance study. A) Schematic diagram of the set-up of the cross-resistance test plate with four different antibiotic disks: 10 µg streptomycin (S10), 10 µg penicillin (P10), 30 µg chloramphenicol (C30) and 5 µg tetracycline (Te5). Four of these plates were prepared, each for a different treatment. **B)** Image of STR-treated bacteria plate with the same orientation as in A. No ring of inhibition is seen around the S10 antibiotic disk, but all other antibiotics had an effect. Figure created using BioRender.

Table 2. Results of the cross-resistance study. Diameter of inhibition is measured as the diameter of the area around the antibiotic disk where no bacterial growth was observed. A higher diameter of inhibition indicates a higher efficacy of the antibiotic or a lower level of resistance of the bacteria.

	Diameter of Inhibition for each Antibiotic (cm)			
Treatment	**S10**	**P10**	**C30**	**Te5**
Control	1.0	0.8	0	0
STR-treated bacteria	0	1.9	1.6	1.3
DOX-treated bacteria	0	0	2.3	0
Combination-treated bacteria	1.2	0.8	2.3	0

Overall, these results showed bacteria that become resistant to DOX are most likely to become resistant to other antibiotics, and bacteria treated with a combination treatment are least likely to become resistant to other antibiotics, regardless of class.

DISCUSSION

It was observed that bacteria become resistant to STR at a faster rate than they become resistant to DOX. Trends similar to those observed in this study were seen by Oz et al., demonstrating that this specific small-scale method was able to replicate their results.[3]

The faster rate of resistance of STR-treated bacteria is possibly due to a difference in mechanism or amplification of resistance between these two antibiotics.[14] DOX and STR belong to different classes of antibiotics: tetracyclines and aminoglycosides, respectively.[5] Both of these antibiotics are known to have a mechanism of action involving the 30S subunit of the ribosome, which impacts protein synthesis.[5] Although they have similar antimicrobial targets, their rates of resistance may differ based on distinct mechanisms of antibiotic resistance, such as the development of efflux pumps or modification of the 30S ribosomal subunit.[14] It is also important to note that this study only looked at the rates of antibiotic resistance using *E. coli;* other species of bacteria may show different trends.

The results of this study showed that the combination treatment using both STR and DOX had the slowest rate of development of antibiotic resistance. A possible explanation for this is that it may be more difficult to become resistant to two different antibiotics at the same time as the bacteria could require development of multiple mechanisms of resistance. Other studies have also found that combination treatments are effective in reducing antibiotic resistance in bacteria.[11] However, research about the effectiveness of combination antibiotic treatments in comparison to monotherapies remains inconclusive.[15] Additionally, there is an increased risk of adverse side effects and a higher cost associated with combination treatments, making them a less attractive option.[16]

While the rate of resistance is important to consider, breakpoint values are also essential. Breakpoints are universal values defined as the concentration at which bacteria become resistant to an antibiotic based on their MIC.[4] For STR, this is 32 mg/L,[17] which was surpassed by day 3. For DOX, a tetracycline, it is 16 mg/L,[17] which was reached on day 4. The combination treatment never surpassed either of these breakpoint values. This supports the notion that a combination treatment of these two antibiotics will lead to a slower rate of resistance than a treatment with a single antibiotic.

As mentioned previously, in some cases the bacteria developed cross-resistance and in other cases the bacteria became more susceptible to antibiotic treatments. These results can potentially be explained by the differences in mechanism and/or amplitude of resistance between each treatment.[5] Specifically, the results illustrated that DOX-treated bacteria become resistant to streptomycin (S10) and penicillin (P10) disks. Streptomycin and doxycycline target protein synthesis as their mode of action, while penicillins target the cell wall of the bacteria.[5] Cross-resistance to streptomycin by the DOX-treated bacteria can be explained by general mechanism similarities. However, penicillins have a different mechanism of action. These conflicting results suggest it is not merely similarities in chemical structure and the general mode of action that can be used to predict cross resistance. Lázár et al. (2014) found that chemogenomic profiling is the strongest predicting factor of cross-resistance.[11] Chemogenomic profiles of these antibiotics in *E. coli* may provide further insights on the drug's mechanism of action, which may allow for a more in depth analysis of the cross-resistance results. However, further replications of these studies are necessary to confirm the validity of results.

This research provides a simple method to study antibiotic resistance; however, it is important to recognize the limitations of this study. Although trends were observed, there is no clear explanation for why these trends were observed. In order to dissect their meaning, whole genome sequencing of the *E. coli* at multiple generations would be required to determine mutations that may have developed to cause antibiotic resistance.[18,19] This would help confirm if there is a relationship between the rate at which resistance is acquired, antibiotic class and underlying mechanisms of resistance. Understanding these relationships would allow for the creation and implementation of antibiotic treatments that prevent rapid development of antibiotic resistance. Future steps would also include chemogenomic profiling to determine if the observation of cross-resistance could be due to similarities in chemogenomic profiles. Despite a low statistical power due to the small sample size (n=2) for each treatment, this analysis provides a methodological basis for future studies with larger sample sizes. Therefore, this study should be replicated in order to produce results with increased power. These results may also differ if replicated *in vivo* rather than *in vitro,* so further investigation is required to determine how these interactions might change when other factors, such as varied metabolism of the antibiotic,[20] are present. If these trends were to be analyzed based on data collected in a clinical setting, it would be of interest to determine the degree to which differing mechanisms of resistance are responsible for the development of resistance, as compared to other factors such as prescription doses and frequency. Other strains of bacteria may develop resistance at different rates from *E. coli* so further testing would be required to assess the trends of antibiotic resistance in other bacteria.

CONCLUSION

This study showed that *E. coli* developed resistance to streptomycin at the fastest rate, followed by doxycycline and then the combination treatment. In some cases, cross-resistance was also observed, however further replications and analyses are required to draw conclusions about the cross-resistance results. Although this study only investigated these two antibiotics, it would be beneficial to know the rates of resistance of bacteria to other antibiotics as well to allow

for an in depth comparison between antibiotic class and antibiotic resistance. There are also many implications for this study that can be applied to a clinical setting. This study demonstrated that combination treatments can reduce the prevalence of antibiotic resistant bacteria. Research comparing monotherapies and combination antibiotic treatments currently remain inconclusive and controversial.[15] The question that remains unanswered is the underlying cause of varying resistance rates. Understanding the mechanisms of resistance and how this relates to the structure and mechanism of each antibiotic can be achieved through whole genome sequencing. Determining which structures, classes and chemogenomic profiles of antibiotics lead to slower rates of resistance in bacteria could allow for the creation of new antibiotics that would induce slower rates of resistance.

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APPENDIX A

Analysis of Variance Table

Response: percent difference

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Antibiotic	2	0.60472	0.30236	7606.4	2.768e-06	***
Residuals	3	0.00012	0.00004			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Tukey multiple comparisons of means

95% family-wise confidence level

	diff	lwr	upr	p adj
DOX-COMBO	35.55556	32.92093	38.19018	0
STR-COMBO	77.67140	75.03677	80.30603	0
STR-DOX	42.11585	39.48122	44.75047	0

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1