Assessment of *In vitro* Synergy among Kanamycin, Ampicillin, and Tetracycline Combinations against Escherichia coli K12

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ABSTRACT

Given the severe impact of COVID-19 in recent years, it is evident that a world with overwhelming antibiotic resistance can have a substantially greater toll on life than the world has ever experienced before. The purpose of this study was to discover synergistic antibiotic combinations that inhibit the growth of a gram-negative bacterium: Escherichia coli. In this study, tetracycline, ampicillin, and kanamycin were tested individually and in combination against the E. coli K12 strain at varying concentrations using the broth dilution method. The MICs of tetracycline, ampicillin, and kanamycin were 16µg/mL, 32µg/mL, and 2µg/mL respectively. However, a combination of tetracycline and ampicillin only required 2µg/mL of each antibiotic with a FIC index of 0.1875, indicating a synergistic effect. These results suggest that the combination of tetracycline and ampicillin could improve the fight against antibiotic resistant E. coli.

INTRODUCTION

The prevalence of antibiotic resistance is often overlooked by healthcare systems worldwide. Disease-inducing bacteria are increasingly becoming resistant to antibiotics at a faster rate than we can produce them (Bartlett et al. 2013). Without proper intervention, this global crisis can lead to 10 million deaths annually by 2050, with 3 million of those fatalities expected to be caused by one bacterial infection: drug-resistant E. coli (O'Neill 2014). This would also account for more than 40 percent of the cumulative 100 trillion USD lost from world production over the next 35 years (63).

But even today, antibiotic resistance poses a huge threat and is one of the greatest worldwide challenges to modern medicine and society at large. In 2019, antimicrobial resistance was responsible for at least 1.2 million deaths, with antibiotic resistance alone accounting for 700,000 deaths (Tarín-Pelló et al. 2022). The World Health Organization (WHO) has now classified this crisis as a "serious threat [that] is no longer a prediction for the future."

To prevent us from further heading into a post-antibiotic era where common infections can be lethal, coordinated efforts must be made to combat this global crisis. This includes further implementing policies such as the Antibiotic Stewardship Pledge (Dellit et al. 2007) or renewing/ investing more into research.

The use of combination antibiotics with synergistic activities have been shown to optimize great results during treatments for bacterial infections (Allen et al. 2002). Synergistic antibiotic combinations are two or more antibiotics combined to form an effect greater than the sum of their predicted individual effects (Kolmer 1948). These combinations allow for lower doses of the constituents followed by considerably enhanced effects (Tallarida 2011). This is achieved by each antibiotic targeting a different aspect of the bacterium, providing a more comprehensive approach to treating the infection (Worthington 2013).

Tetracycline (TET) is a broad-spectrum polyketide antibiotic that binds reversibly to the 30S ribosomal subunit, preventing amino-acyl tRNA from binding to the A-site of the ribosome, thereby inhibiting the bacterium's protein synthesis (Chopra and Roberts 2001). Comparably, kanamycin (KAN) is an aminoglycoside antibiotic that irreversibly binds to 16S ribosomal RNA of 30s ribosomal subunit, causing interruptions in t-RNA readings that result in inability to synthesize proteins (Franklin and Snow 2005). Ampicillin (AMP) is a β-lactam antibiotic that is used to treat a wide range of infections by interrupting the construction of the bacteria's cell wall, ultimately leading to the lysis of the bacteria. It achieves this by binding to the bacterium's primary receptors called membrane-associated penicillin-binding proteins (PBPs) (Tipper 1985).

This study aimed to assess *in vitro* antibiotic affects of the combinations among tetracycline, kanamycin, and ampicillin against Escherichia coli K12 with the intent of discovering synergistic activity. Considering that ampicillin targets the cell wall for destruction and that both tetracycline and kanamycin target the ribosome to inhibit protein synthesis, it was hypothesized that the combination of ampicillin with either tetracycline or kanamycin would exhibit synergistic antibiotic effects. On the other hand, the combination of tetracycline and kanamycin is hypothesized to have an indifference in antibiotic effects as compared to their individual effects since they both target the ribosome to inhibit protein synthesis.

METHODOLOGY

Antibiotics, bacterial strain, and culture media used in antibiotic assays

Tetracycline HCL (TET) (BioShop, Burlington, Canada), ampicillin (AMP), and kanamycin (KAN) (Merlan Scientific, Toronto, Canada) were used individually as reference antibiotics and in combination against gram-negative Escherichia coli K12. The antibacterial assays were prepared using lysogeny broth (LB) for determining minimum inhibitory concentrations (MIC) and LB agar for determining minimum bactericidal concentrations (MBC).

Determination of minimum inhibitory concentration (MIC)

0.444g of tetracycline HCL powder with a potency of 900µg/mg was diluted with 4mL of LB broth to produce a stock solution of 100mg/mL. 10.24µL of this solution was then diluted with 3989.76µL of LB broth to produce 4mL of tetracycline 256µg/mL working solution. 102.4µL of ampicillin stock solution with a concentration of 10mg/mL was diluted with 3897.6µL of LB broth to produce 4mL of ampicillin 256µg/mL working solution. Similarly, 341.3µL of kanamycin stock solution with a concentration of 3mg/mL was diluted with 3658.7mL of LB broth to produce 4mL of kanamycin 256µg/mL working solution. Combination antibiotics were then prepared by combining equal amounts of volume to form 1:1 ratios. The wells were filled with 1000µL of individual reference antibiotics and combination antibiotics which were serial diluted with LB broth at concentrations ranging from 0.25µg/mL to 128µg/mL and 0.125:0.125µg/mL to 64:64µg/mL, respectively.

E. coli K12 isolates were diluted in LB broth and adjusted to spectrophotometer readings of 0.188 at OD_{600} to reach a 0.5 McFarland Standard ($1.5x10^8$CFU/mL). 1mL of the prepared inoculum was then diluted with 99mL of LB broth to give an inoculum density of $1x10^6$CFU/mL. Then, 1000µL of the inoculum was added to each well plate (except negative control) to give a final inoculum density of $5x10^5$CFU/mL. Blank LB broth was used as the negative control.

The well plates were incubated at 25˚C for 30h then analyzed using Epson® Perfection® V370 Photo to measure assay colour. The MICs of each reference antibiotic and combination antibiotic were defined as the lowest concentrations that showed no growth in the LB broth assay as represented as indifference of colour and turbidity from negative control.

Determination of minimum bactericidal concentration (MBC)

After collecting MIC data, 10µL from both the MIC and the concentration twofold higher, were sampled and inoculated onto LB agar plates which were then incubated at 25˚C for 50h. The MBCs of each reference antibiotic and combination antibiotic were defined as the lowest concentrations that showed no growth on agar.

Statistical analysis for synergism

Antibiotic interactions were assessed algebraically by determining the fractional inhibitory concentration (FIC) index and the fractional bactericidal concentration (FBC) of each combination.

$$FIC_A = \frac{MIC_{A\ in\ presence\ of\ B}}{MIC_A} \qquad FIC_B = \frac{MIC_{B\ in\ presence\ of\ A}}{MIC_B}$$

$$FIC\ index = FIC_A + FIC_B$$

A FIC index of ≤ 0.5 indicates synergism, 0.5 to 4 indicates indifference, and > 4 indicates antagonism where drugs interfere with each other's mechanism of action, resulting in a weaker effect when used together (Johansen et al. 2000; Hall et al. 1983). The same applies to FBC index.

RESULTS

The results of the MIC and FIC index against E. coli K12 for single reference antibiotics and combination antibiotics were summarized in Table 1., respectively.

In the broth dilution assay, antibiotic combinations against E. coli K12 showed signs of synergism and indifference

Table 1. MIC and FIC index from single reference antibiotics (TET, AMP, KAN) and combination antibiotics against E.coli K12

Minimum Inhibitory Concentration (µg/mL)			Fractional Inhibitory Concentration Index			
AMP	**KAN**	**AMP:KAN**	**FIC_{AMP}**	**FIC_{KAN}**	**$FICI_{AMP+KAN}$**	**Remarks**
32	2.0	4.0:4.0	0.1250	2.000	2.125	Indifference
TET	**AMP**	**TET:AMP**	**FIC_{TET}**	**FIC_{AMP}**	**$FICI_{TET+AMP}$**	**Remarks**
16	32	2.0:2.0	0.1250	0.06250	0.1875	Synergistic
TET	**KAN**	**TET:KAN**	**FIC_{TET}**	**FIC_{KAN}**	**$FICI_{TET+KAN}$**	**Remarks**
16	2.0	2.0:2.0	0.1250	1.000	1.125	Indifference

from MIC data. The combination of tetracycline and ampicillin were predominantly synergistic with an FIC index of 0.1875. Tetracycline and kanamycin presented an FIC index of 1.125, showing indifference of the combination. Lastly, ampicillin and kanamycin presented an FIC index of 2.125, also indicating indifference.

However, most of the MBC and all FBC index against E. coli K12 for single reference antibiotics and combination antibiotics were inconclusive due to lack of MBC data. All concentrations except 2 single antibiotics showed bacteria growth: tetracycline (32µg/mL) and ampicillin (32µg/mL).

DISCUSSION

The purpose of combination therapy is to enhance antibiotic activity through synergistic interactions of single antibiotics. Utilizing these combinations can minimize antibiotic resistance with lower doses of constituents, potentially saving the healthcare industry trillions of dollars (O'Neill 2014).

In this study, the FIC index for the combination of tetracycline and ampicillin against E. coli K12 was found to be 0.1875, thereby indicating the synergistic effect of the combination, and partially complying with the initial hypothesis. The mechanism involved ampicillin's inhibition of cell wall peptidoglycan synthesis, increasing the permeability of the bacterium to tetracycline which binds reversibly to the 30S ribosomal subunit and prevents amino-acyl tRNA from binding to the A-site of the ribosome. This comprehensive mechanism synergistically enhances tetracycline's inhibition of the bacterium's protein synthesis.

The combination of tetracycline and kanamycin had an FIC index of 1.125, indicating an indifference in antibiotic activity level. This is consistent with part of the initial hypothesis where it was predicted that the antibiotics' similar mechanism of action may not induce enhanced effects. On the other hand, the combination of ampicillin and kanamycin exhibited an FIC index of 2.125, also indicating indifference and rejecting the initial hypothesis.

It was also observed that the β-lactam antibiotic, ampicillin, was least active when put against E. coli K12 alone. This can be explained by the bacteria's production of enzymes called β-lactamases (Jacoby et al. 1988), which also creates the possibility for some degree of resistance to have already existed (Jarlier et al 1988). In addition, it is important to note that the synergistic effects of the antibiotic combinations observed may differ per bacterial strain because of potential differences in the efflux pumps present (Pathania 2019). Some efflux pumps are specific to particular antibiotics or are influenced by the presence of certain substrates/ environmental conditions (Elkins and Mullis 2007). Therefore, this study can benefit from using different strains of E. coli, including antibiotic resistant strains to further investigate the potential for the combination of tetracycline and ampicillin to reduce the advancement of antibiotic resistance of E. coli.

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