

Enhanced Combination Therapy of Aminoglycosides Against Resistant *Staphylococcus aureus*

Olivia Liu

Hong Kong International School, 1 Red Hill Rd, Tai Tam, Hong Kong; liuliviaa@gmail.com

ABSTRACT: *Staphylococcus aureus* is a fatal bacterial infection that commonly colonizes the skin and mucous membranes in humans. Each year, *S. aureus* is associated with an estimated 50,000 deaths, and this number is only rising with the increased prevalence of the pathogen's resistance against many antibiotics. In particular, the emergence of Methicillin-resistant *Staphylococcus aureus* (MRSA) has made it difficult to find suitable treatments for patients without the use of common clinical antibiotics. Combination therapy, which administers two or more antibiotics at once, is an effective approach to increase antibiotic efficacy against these resistant pathogens. This study investigates the efficacy of five antibiotics against the *S. aureus* Newman strain and finds that the Newman strain had significant bacterial resistance to kanamycin, chloramphenicol, and gentamicin. Contrarily, cefotaxime and erythromycin were highly effective even at lower doses. Eight combination therapy groups were tested with the more resistant antibiotics to find that the combination of gentamicin (10 µg/mL) and kanamycin (50 µg/mL) yielded the most effective results; the enhanced result of the two aminoglycosides working in combination led to a 91.18% reduction in bacterial growth compared to the untreated control group.

KEYWORDS: Medical and Health Sciences, Microbiology, *Staphylococcus aureus*, Antibiotic Combination Therapy, Aminoglycoside Synergy.

■ Introduction

Staphylococcus aureus is a bacterial human pathogen that colonizes the skin and mucous membranes, identifiable by its “grape-like” clusters and golden pigmentation at the microbiological level. This widespread bacterium infects approximately 20 to 50 individuals in a population of 100,000 annually, with an estimated 10 to 30 percent of these cases leading to fatalities.¹ In most healthy individuals, *S. aureus* can appear asymptotically on the skin and nasal cavities. In fact, a report by the European Centre for Disease Prevention and Control (ECDC) confirms that about 30 percent of the human population persistently carries *S. aureus* in their nasal mucous membranes without showing significant symptoms.² However, when the *S. aureus* bacterium gains access to internal organs or the bloodstream, it can cause severe infections, including sepsis and pneumonia.³ In the United States alone, over 119,000 cases of *S. aureus* entering the bloodstream were recorded in 2017, leading to an estimated 20,000 deaths from *S. aureus* just that year.⁴

Considering the nature of *S. aureus* as a bacterial pathogen, the treatment of *S. aureus* infections typically relies on antibiotic therapies, including commonly used agents such as cefazolin, nafcillin, oxacillin, and vancomycin.⁵ Yet, despite the prevalence of *S. aureus*, an antibiotic treatment specifically targeting all *S. aureus* infections is still elusive, particularly due to rising antibiotic resistance across strains of *S. aureus*. To test the efficacy of certain antibiotics on a human strain of *S. aureus*, we used five different antibiotics in varying concentrations. Among the antibiotic treatments we explored in this study, kanamycin is an aminoglycoside antibiotic with resistance rates exceeding 90 percent in many strains of *S. aureus*. Its mechanism of action

involves its irreversible binding to the 16S rRNA receptor in the bacterial 30S ribosomal subunit, causing a change in the translation process.⁵ Because of the faulty readings of codon and anticodon pairing, the resultant polypeptide becomes deformed, and the accumulation of these abnormal proteins inside the bacteria causes the cell to undergo apoptosis due to its high toxicity.⁵ With a similar mechanism of action, gentamicin exhibits its bactericidal function by binding to the 30S subunit, leading to functionally impaired proteins that ultimately result in cell death.⁶ The third antibiotic tested is cefotaxime sodium, which shows a high affinity for penicillin-binding proteins (PBPs), essential for bacterial wall biogenesis.⁷ When cefotaxime binds to the PBPs, it inactivates these proteins, hence weakening the bacterial cell wall and ultimately leading to bacterial lysis.⁸ The fourth antibiotic tested is chloramphenicol, and it binds to the 50S ribosomal subunit to interrupt the peptidyl transferase enzyme necessary for protein synthesis.¹⁰ By doing so, it halts the elongation of the growing polypeptide chain and prevents bacterial growth. Lastly, erythromycin also targets the 50S subunit, leading to slowed bacterial growth and consequently achieving a bacteriostatic effect.¹¹

Despite using antibiotics as medication to treat *S. aureus*, the emergence of drug-resistant strains like the Methicillin-resistant *Staphylococcus aureus* (MRSA) makes it difficult to treat the bacterial infection with single antimicrobials. MRSA is resistant to beta-lactam antibiotics, including the widely prescribed penicillins and cephalosporins, and it is directly associated with rising *S. aureus* death rates and lengthened hospital stays.¹² Due to overexposure to common antibiotics, bacterial strains can develop resistance mechanisms, including enzymatic inactivation of the antibiotic, efflux pumps, and decreased binding

affinity for the antibiotic, hence undermining the function of the antibiotics.¹³ Indeed, infections caused by antibiotic-resistant bacteria led to more than 33,000 deaths and 2.5 million additional days of hospitalization every year in the European Union, further resulting in an economic impact of nearly 900 million Euros.¹⁴ Standard antibiotics typically used to treat a wide range of bacterial pathogens are no longer as effective at treating many *S. aureus* strains; therefore, independent antimicrobial treatments may necessitate higher dosages to achieve substantial therapeutic effects. For this reason, using independent antibiotics to treat multidrug-resistant bacterial strains like *S. aureus* can lead to higher and quicker rates of bacterial resistance.

To combat this issue, combination therapy is a new approach that is being explored in treating *S. aureus* infections and other bacterial pathogens. It involves combining multiple antibiotics with varying mechanisms of action to achieve a synergetic effect, enhancing the therapeutic effectiveness compared to individual antibiotic treatment.¹⁵ The combination of antibiotics expands the treatment efficacy of the medication against bacteria, as multiple recombinant strains often cause pathogens. For example, beta-lactam antibiotics are commonly used against *Staphylococcus* and *Streptococcus* infections; however, if these antibiotics are administered in combination with Aminoglycosides, the efficacy of the treatment can expand to treating bacteria present in the intestines as well.¹⁶ Moreover, combination therapy often involves administering lower dosages of individual antibiotics for a significantly more effective pharmacological effect, leading to a wider range of possibilities for treatment. In many cases, these combinations decrease the time needed for recovery and treatment of bacterial infections like *S. aureus*. Further, research has shown that these multi-drug combinations can eradicate biofilm-forming pathogenic infections.¹⁷ Specifically, it has been shown that anprocide, a type of steroidal amine, is effective against the biofilms formed by *S. aureus* and *S. epidermidis*.¹⁸

To evaluate the effect of specific antibiotic combinations on *S. aureus* infections, we utilized the Newman strain of *S. aureus* and grew colonies exposed to varying concentrations of five different antibiotics. After analyzing the effects of the independent antibiotics on the Newman strain, we selected two of the most resistant antibiotics to use in combination treatment. We aimed to determine if the combination treatment would yield statistically significant results in inhibiting bacterial growth compared to individual antibiotic treatment. While previous studies have tackled the efficacy of independent antibiotics on strains of *S. aureus*, this paper aims to determine the effect of specific combinations on the Newman strain that have not been tested in detail in the past. Overall, this study aspires to contribute to the growing field of combination therapies for *S. aureus* and similar resistant strains.

■ Methods

1. Data and Methodology:

1.1. Recovery of *S. aureus* and Preparation of Antibiotics:

1.1.1. Recovery of Newman Bacteria:

In this study, we utilized the Newman strain of *S. aureus*, previously stored in a 4°C refrigerated environment. Initially, we added 2 mL of Tryptic Soy Broth (TSB) into a 14 mL polypropylene round-bottom tube. Using a sterile pipette, three clones of the Newman's strain were then swiped and carefully placed into the tube containing the 2 mL of TSB. The contents in the tube were swirled to ensure thorough mixing. We then incubated the tube in a 37°C warm culture room with shaking overnight to maximize aerobic bacterial growth and recovery.

1.1.2. Preparation of Antibiotic Solutions:

We used five antibiotics in this study, namely kanamycin, erythromycin, chloramphenicol, gentamicin, and cefotaxime. We measured out 100 mg each of solid erythromycin and cefotaxime sodium salt. We dissolved the 100 mg solid erythromycin in 2 mL of ethanol, while we dissolved 100 mg cefotaxime sodium salt in 2 mL of water, both prepared in separate 15 mL conical tubes over an open flame until the antibiotic was fully dissolved in the solution. The remaining three antibiotics were already in aqueous form and were used directly without any additional preparation. All five prepared antibiotic solutions were stored in a refrigerator at 4°C overnight before use. Before any dilutions, the kanamycin solution had an initial concentration of 40 mg/mL, while the other four antibiotic solutions (erythromycin, chloramphenicol, gentamicin, and cefotaxime) had initial concentrations of 50 mg/mL.

1.1.3. Bacterial Dilution and Counting:

Newman's bacterial density was measured using a Microplate Reader in terms of its Optical Density at 600 nanometers (OD600). We prepared a control cuvette by adding 1 mL of TSB over an open flame, while we added 1 mL of the recovered Newman bacteria into an experimental cuvette. The Microplate Reader was calibrated to the TSB control cuvette, which was set to zero. The starting overnight bacterial density showed expected day-to-day variation. Before the individual antibiotic experiment, the bacterial culture OD600 measured 2.094; before the combination antibiotic experiment, the OD600 measured 1.880.

An inoculum of approximately 10^8 CFU/mL was used, as this is a standard density for antibiotic susceptibility screening. This density provides a reliable OD600 reading and stays within the Microplate Reader's linear range once diluted in the 200 μ L wells. To achieve the standard concentration of 10^8 CFU/mL for the experiment, the bacterial sample needed to be diluted with Phosphate-Buffered Saline (PBS). We used the established conversion factor of $1 \text{ OD} = 5 \times 10^8 \text{ CFU}$. By rearranging terms, we found the dilution factors to be 10.47 and 9.40 for the individual antibiotic experiment and the combination antibiotic experiment, respectively.

We used OD600 as a unit of measurement in later experiments to determine the efficacy of either individual antibiotic

treatments or a combination of two antibiotics. Each experimental group contained three wells for the purpose of accuracy, all of which were read by the Microplate Reader to obtain OD600 values. All tables below detailing OD600 of experimental groups, such as Table 3, describe the average OD600 value among the three wells.

1.1.4. Dilution of Antibiotics.

To test the effect of different concentrations of antibiotics on the Newman strain of *S. aureus*, we diluted the antibiotics to concentrations of 1 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, and 500 µg/mL. The preparation of the dilutions was calculated, and the volumes of antibiotic and water are detailed in Tables 1 and 2, as shown below.

Table 1: Volume of kanamycin solution and water needed to create the respective diluted antibiotic concentrations.

Diluted Concentration (µg/mL)	Volume of Antibiotic (µL)	Volume of Water (µL)
1	5.00	995.00
2.5	12.50	987.50
5	2.50	975.00
10	5.00	95.00
25	12.50	87.50
50	25.00	75.00
100	50.00	50.00
500	2.50	N/A

Table 2: Volume of antibiotic (gentamicin, erythromycin, chloramphenicol, cefotaxime) solutions and water needed to create respective diluted antibiotic concentrations.

Diluted Concentration (µg/mL)	Volume of Antibiotic (µL)	Volume of Water (µL)
1	4.00	996.00
2.5	10.00	990.00
5	2.00	98.00
10	4.00	96.00
25	10.00	90.00
50	20.00	80.00
100	40.00	60.00
500	2.00	N/A

1.2. Broth Dilution Method:

1.2.1. Independent Antibiotic Treatment:

We added 200 µL of TSB over an open flame to 45 wells in a 96-well cell culture plate. In each well, 1 µL of the diluted Newman bacteria was added, followed by 1 µL of the diluted antibiotic concentration specific to each well. However, we did not need to dilute the antibiotics for the 500 µg/mL tests; instead, we added 2.5 µL of the initial kanamycin solution (40 mg/mL) and 2 µL for the other four antibiotics (50 mg/mL). We mixed the solutions in each well thoroughly with a pipette, and tips were changed after each well to prevent contamination. The culture plate was stored in a warm room at 37°C overnight for ample bacterial growth.

1.2.2. Combination Antibiotic Treatment:

Minimum Inhibitory Concentration (MIC) testing was performed to determine the antimicrobial susceptibility of *S. aureus* Newman when treated with the various individual antibiotic groups. MIC testing is performed when a standardized

amount of bacteria is exposed to serial dilutions of an antibiotic treatment group to establish the lowest concentration of the treatment that completely prevents visible growth of the bacteria. In our tests, we determined the MIC of each antibiotic treatment group to be the lowest treatment concentration without any bacterial growth.

After the MIC test with the independent antibiotic treatment was retrieved and evaluated with respective OD600 values, we determined the Newman strain to have the most resistance against kanamycin (at 25 µg/mL and 50 µg/mL), chloramphenicol (at 5 µg/mL and 10 µg/mL), and gentamicin (at 5 µg/mL and 10 µg/mL). In the procedure and results sections, we will use the initial letter of the antibiotic and the concentration value to represent the tested group. For instance, K25 refers to a treatment using 25 µg/mL concentrated kanamycin; similarly, K50/C5 refers to a treatment of 50 µg/mL concentrated kanamycin in combination with 5 µg/mL concentrated chloramphenicol.

Our combination therapy experiment consisted of 15 groups, with three wells allocated per group. This part of the experiment consisted of eight combination groups (K25/C5, K25/C10, K25/G5, K25/G10, K50/C5, K50/C10, K50/G5, K50/G10), six single antibiotic groups (K25, C5, G5, K50, C10, G10), and one negative control group with no antibiotic treatment. We repeated the dilution of the Newman bacterial sample, where the OD600 measurement showed a value of 1.880. We diluted the sample to 10^8 bacteria/mL, then added 1 µL of the diluted bacteria and 200 µL of TSB into each well. Finally, 1 µL of the respective antibiotics was added to the appropriate wells. The culture plate was stored at 37°C overnight for bacterial growth, and OD600 values were measured the following day to determine the efficacy of the treatments.

1.2.3. Experimental Controls:

Several control conditions were included in the experiments to ensure the results are valid, specifically through using sterility controls, positive controls for each antibiotic treatment group, and a sterile TSB control for the Microplate Reader calibration.

To ensure that the TSB growth medium was not contaminated with bacteria or other unwanted substances, we included three sterility control wells on each 96-well culture plate. Each sterility control well contained 200 µL TSB and 1 µL PBS with no bacteria present. After overnight incubation, any visible bacterial growth in these sterility control wells would suggest contamination of the growth medium and/or the pipettes.

For both the individual antibiotic and combination antibiotic treatments, each antibiotic row had positive control wells containing 200 µL TSB and 1 µL of the 108 CFU/mL diluted bacteria, with no antibiotic added to the wells. Positive control OD600 values were taken from the Microplate Reader to calculate percent reductions for the respective antibiotic treatments in varying concentrations.

We used cuvettes containing 200 µL of sterile TSB with no bacteria or antibiotic treatments to calibrate the Micro-

plate Reader. This sets the OD600 readings against a reference frame for more accurate readings.

■ Results and Discussion

1. Individual Antibiotic Treatment Results:

To identify independent antibiotic treatments resistant to some degree when treated to Newman *S. aureus*, we determined the Minimum Inhibitory Concentration (MIC) using the Broth Dilution Method, indicated by the lowest concentration of the antibiotic that leaves no visible trace of the bacteria following the incubation stage. The visibility of *S. aureus* growth was further confirmed with OD600 readings following the overnight incubation.

Results from the Microplate Reader determined that cefotaxime and erythromycin were both nearly immediately effective on Newman. With reference to Table 3, at just 1.0 µg/mL of aqueous cefotaxime administered, the OD600 reading dropped from 0.809 to 0.054. Similarly, 1.0 µg/mL of aqueous erythromycin administered led to an OD600 decrease from 0.688 to 0.067. Analysis of this data shows percentage decreases of 93.33% and 90.26% for cefotaxime and erythromycin after the initial dose, respectively. It can therefore be determined that Newman *S. aureus* exhibits little resistance to cefotaxime and erythromycin.

Comparatively, Table 3 indicates that the administration of chloramphenicol, kanamycin, and gentamicin was not effective at the initial doses. The MIC for chloramphenicol is between 5.0 µg/mL and 10.0 µg/mL: at a concentration of 5.0 µg/mL, we recorded an OD600 reading of 0.411, only a 37.92% decrease from the positive control; whereas, at a concentration of 10.0 µg/mL, we recorded an OD600 reading of 0.083, pointing to an 87.42% decrease from the positive control. Gentamicin exemplified similar trends, with an MIC between 5.0 µg/mL and 10.0 µg/mL: at a concentration of 5.0 µg/mL, we recorded an OD600 reading of 0.579, only a 24.21% decrease from positive control; conversely, at a concentration of 10 µg/mL, we found the OD600 reading to be 0.062, leading to a 91.88% decrease from the positive control group. Yet, out of all three antibiotics, Newman seemed to show the most resistance to kanamycin, as the MIC was indicated to be between 25.0 µg/mL and 50 µg/mL. The OD600 readings and percentage changes from the positive controls are calculated to be 0.401 and -39.33% for 25.0 µg/mL kanamycin, and 0.073 and -92.70% for 50 µg/mL kanamycin.

Table 3: Average OD600 results from individual antibiotic MIC treatment. Results found that individual administration of chloramphenicol, kanamycin, and gentamicin was not effective at initial doses; therefore, Newman showed a degree of resistance to these three antibiotics.

Antibiotic Type	Administered Concentration (µg/mL)								
	Control	1.0	2.5	5.0	10.0	25.0	50.0	100.0	500.0
Cefotaxime	0.809	0.054	0.055	0.053	0.051	0.051	0.050	0.050	0.054
Erythromycin	0.688	0.067	0.071	0.070	0.065	0.059	0.057	0.056	0.059
Chloramphenicol	0.662	0.517	0.466	0.411	0.083	0.050	0.051	0.051	0.053
Kanamycin	0.661	0.592	0.555	0.525	0.431	0.401	0.073	0.060	0.053
Gentamicin	0.764	0.605	0.578	0.579	0.062	0.057	0.054	0.055	0.053

Interpretation of the percent reduction values requires consideration of the variation in the positive control groups. Even within the same experiment, positive control OD600 values varied across different antibiotics (ranging from 0.661 to 0.809), which likely reflects minuscule differences in the inoculum while setting up separate rows of the 96-well plate. However, because each antibiotic was tested with its own positive control group, we calculated the OD600 percent reductions using the control group specific to the antibiotic rather than using a single baseline control. Between the individual antibiotic and combination antibiotic experiments, positive controls also differed slightly, likely due to normal day-to-day variation. Despite this, all wells—meaning the positive control groups, individual antibiotic treatments, and combination antibiotic treatments—all received the same starting bacterial density, so comparisons with the data remain valid.

2. Combination Antibiotic Treatment Results:

To determine the effect of combination antibiotic therapy on resistant Newman *S. aureus*, we combined kanamycin (25 µg/mL and 50 µg/mL), gentamicin (5 µg/mL and 10 µg/mL), and chloramphenicol (5 µg/mL and 10 µg/mL) to create eight experimental groups. OD600 readings for the combination antibiotic treatment using the Microplate Reader were collected and graphed using GraphPad Prism. The OD600 readings for all eight experimental groups were graphed alongside the positive control and their respective independent antibiotics. For statistical analysis, we ran unpaired two-tailed t-tests with 95% confidence intervals for each comparison (combination vs. two individual antibiotics, combination vs. control). The error bars in the graphs show the standard deviation (SD) of the three repeated wells per antibiotic treatment. The statistical significance in the analysis was defined with p-value thresholds (*p < 0.05, **p < 0.01, ***p < 0.001). Only Figures 1 and 2 are provided in this research paper to demonstrate the most remarkable combination treatment results.

Following statistical analysis, we determined the treatment administering gentamicin at 10 µg/mL combined with kanamycin at 50 µg/mL (G10/K50) to be most effective. Figure 1 indicates the combination group showed no statistical significance (p > 0.05) compared to the individual antibiotic treatments of gentamicin at 10 µg/mL and kanamycin at 50 µg/mL. However, the combination group compared to the positive control showed three asterisks (***p < 0.001), while both the individual antibiotic treatments showed only one asterisk (p < 0.05) compared to the positive control group. The statistical significance of the G10/K50 combination group can be supported with the data from the OD600 readings. The means of three tested wells for each antibiotic treatment are as follows: 0.3220 for gentamicin (10 µg/mL) only, 0.2907 for kanamycin (50 µg/mL) only, 0.05700 for combination treatment, and 0.6460 for positive control. Compared to the positive control group, gentamicin at 10 µg/mL showed a 50.15% decrease, kanamycin at 50 µg/mL showed a similar 55.00% decrease, while the combination treatment showed a drastic 91.18% decrease. Compared to the independent treatments, the change in percentage comparing the combination group to the indepen-

dent antibiotic treatments was calculated to be -82.30% and -80.39% for gentamicin (10 µg/mL) and kanamycin (50 µg/mL), respectively.

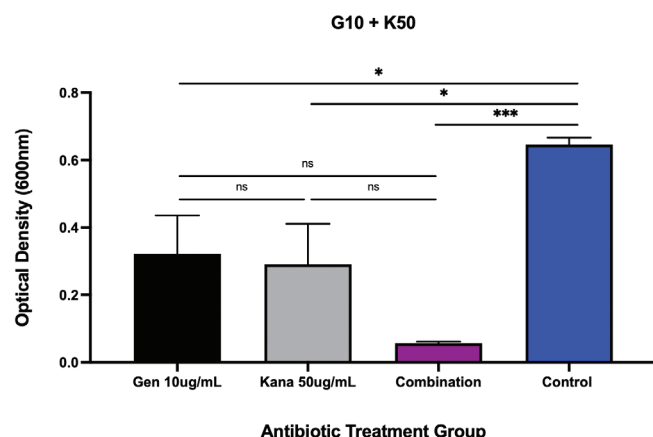


Figure 1: Results from combination therapy (gentamicin 10 µg/mL and kanamycin 50 µg/mL). Significance levels showed that this combination is the most effective treatment group in all eight combination groups.

From the OD600 readings and the analysis from the graph, we determined the second most effective combination treatment to be gentamicin (10 µg/mL) working with kanamycin (25 µg/mL). Figure 2 indicates that the combination group showed no significance ($p > 0.05$) compared to the individual 10 µg/mL gentamicin treatment, while it showed a significance of one asterisk ($p < 0.05$) compared to the individual 25 µg/mL kanamycin treatment. In comparison to the positive control group with no antibiotic treatment, the combination treatment showed a significance value of three asterisks ($p < 0.001$), while the individual kanamycin and gentamicin treatments showed no significance ($p > 0.05$) and one asterisk ($p < 0.05$), respectively. Based on the OD600 readings collected, the percent differences between the combination groups, the individual antibiotic treatment groups, and the positive control support the relationship from the graph. The OD600 mean values are as follows for the G10/K25 combination group: 0.3200 for gentamicin (10 µg/mL) only, 0.4800 for kanamycin (25 µg/mL) only, 0.08400 for combination treatment, and 0.6460 for the positive control group. Compared to the positive control group, gentamicin at 10 µg/mL showed a 50.15% decrease, kanamycin at 25 µg/mL showed a lower 25.70% decrease, while the combination treatment showed a notable 87.00% decrease, albeit being less than the 91.18% decrease from the positive control recorded from the G10/K50 combination group. Compared to the independent treatments, the change in percentage comparing the combination group to the independent antibiotic treatments was calculated to be -73.91% and -82.50% for gentamicin (10 µg/mL) and kanamycin (25 µg/mL), respectively. As aforementioned, while the results from the G10/K25 group are significant, the G10/K50 combination treatment shows statistically more significant results.

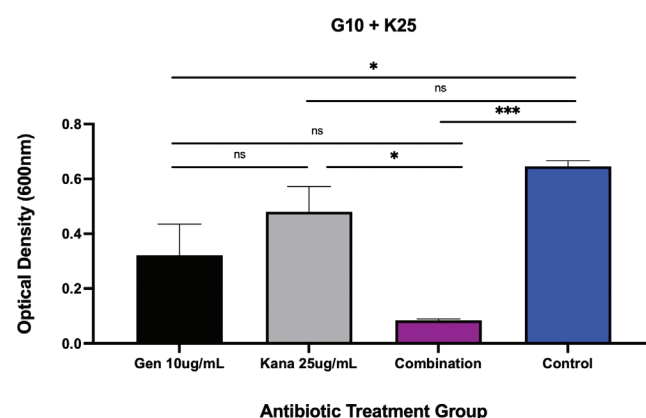


Figure 2: Results from combination therapy (gentamicin 10 µg/mL and kanamycin 25 µg/mL). Significance levels showed that this combination is the second most effective treatment group in all eight combination groups.

3. Discussion:

Our study demonstrated that, among eight combination therapies tested against *S. aureus* Newman, the combination of gentamicin (10 µg/mL) and kanamycin (50 µg/mL) exhibited the most significant antibacterial result. This was determined by analyzing the highest percentage of growth inhibition of the combination group compared to the positive control group and the two individual antibiotic groups.

The significant effect demonstrated by the G10/K50 combination therapy group could be attributed to a number of reasons. Both gentamicin and kanamycin are aminoglycoside antibiotics; their primary mechanism of action is to bind to the bacterial 30S ribosomal subunit, causing a misreading of the mRNA and further inhibition of protein translocation. Ultimately, this leads to bacterial cell death. Aminoglycoside antibiotics are also concentration-dependent, meaning higher peak concentrations of the doses lead to greater efficacy. It is therefore made clear that combining a high MIC of gentamicin and kanamycin, two drugs from the same class, led to an enhanced effect, meaning the results from combination therapy are significantly stronger compared to the results from individual therapy. We did not calculate a Fractional Inhibitory Concentration Index (FICI), which is the standard for defining formal synergy. Thus, we describe the effect we observed as “enhanced” rather than true synergy.

Since aminoglycosides are positively charged and cannot easily penetrate through the negatively charged cell membrane of *S. aureus*, their uptake into the cell is through a proton motive force generated by the bacterial electron transport chain. By creating a strong electrochemical gradient, the electron transport chain transports the aminoglycoside molecules into the cell. This process can help explain why the combination of gentamicin (10 µg/mL) with kanamycin (50 µg/mL) worked best against the formerly resistant *S. aureus* Newman. Understanding the relationship and mechanism of action for aminoglycosides, we propose that the combination of the two drugs could have created a positive feedback loop to enhance the overall antibacterial effect. The first drug, such as gentamicin at 10 µg/mL, could have initially entered a few bacterial cells. Once inside the cell, the gentamicin will begin to take

effect and create mistranslated proteins, some of which will insert themselves into the cell membrane. Having faulty proteins in the cell membrane could lead to lower permeability, making it much easier for the kanamycin at 50 µg/mL to enter the bacterial cells and cause even more rapid protein disruptions, therefore leading to more notable cell death compared to one individual antibiotic working alone. The dose of the antibiotics also plays an integral role; the concentration of the gentamicin at 10 µg/mL was enough to initiate the membrane-damaging process without being too high to work entirely well on its own, and likewise, the concentration of kanamycin at 50 µg/mL was enough to work well with the gentamicin to speed up the process of protein disruptions and bacterial cell death.

It is worthwhile to consider whether these antibiotic concentrations are clinically realistic and relevant. Gentamicin at 10 µg/mL is clinically possible, but it rests at the upper end of peak concentrations in humans, which typically ranges from 5 µg/mL to 12 µg/mL, depending on the dosing. However, kanamycin at 50 µg/mL exceeds its typical peak concentrations, ranging from 20 µg/mL to 30 µg/mL. The two antibiotics, gentamicin and kanamycin, are both aminoglycosides, a class of bactericidal antibiotics known to cause drug-induced nephrotoxicity.⁹ Put differently, aminoglycosides risk causing kidney damage and hearing loss at high doses. Hence, while the G10/K50 combination worked well *in vitro*, clinical use would need lower doses of the combination therapy to prevent drug-induced nephrotoxicity.

While the gentamicin at 10 µg/mL combined with kanamycin at 50 µg/mL showed the most significant antibacterial effects, all other experimental combination therapy groups also led to decreases in OD600 readings compared to individual therapies, albeit less notable. For the gentamicin and kanamycin pairs, data collected from the experiments suggest that the doses of either antibiotic below the critical threshold would lead to a less desired outcome. From the G10/K50 therapy group, in contrast to the G10/K25 group, it becomes clearer that most of the differences in the outcome can be highly attributed to the lower concentration of kanamycin. Most notably, all combinations involving chloramphenicol were significantly less effective. We propose that this could be due to a conflict in the mechanism of action, where the bacteriostatic action of chloramphenicol inhibits the metabolic activity needed for the uptake of aminoglycosides like kanamycin into the bacterial cell. It can therefore be made clearer why the G10/K50 combination seemingly worked most successfully among the eight combination therapy groups.

4. Limitations of the Study:

While this study provides evidence of an enhanced effect occurring between gentamicin and kanamycin against *S. aureus* Newman, several limitations should be considered when interpreting the results from the experiments.

Firstly, the experiments from this study were conducted utilizing a single strain of *S. aureus*, namely the Newman strain. The strain we utilized was extracted from patients with *S. aureus* and recovered before use in this study. The experiments with antibiotic treatment were administered *in vitro*, meaning

the effects of the combination therapy may not translate completely when the same treatments are applied *in vivo*. There are many factors, including host immune responses, that are important in affecting antibiotic efficacy yet difficult to replicate outside of the patient's body. Therefore, it is made more difficult to determine whether the results from this lab still stand valid under *in vivo* conditions.

Moreover, while the Newman strain is widely used in biomedical research to test for *S. aureus* trends, testing on only the Newman strain cannot be representative of all the different isolates and strains under the generalized term *Staphylococcus aureus*. Thus, it can be difficult to ascertain whether these results are merely due to variance in this one strain or if they remain true under different circumstances. In addition, each antibiotic treatment group was tested in only one independent experiment with three replicate wells. We did not repeat the entire experiment on different days, which limits our ability to assess whether the results would remain valid on different days with new bacterial cultures. Assuming that our proposed mechanism is true for G10/K50, the entry into the bacterial cell of the first aminoglycoside (either gentamicin or kanamycin) is a process that can be affected by factors including the presence of specific protein transporters, variance in the electron transport chain, and membrane permeability. Without testing the combination therapy across a variety of strains, including MRSA and strains with known aminoglycoside resistance, the results from this study are limited.

Lastly, our proposed mechanism of action for the G10/K50 combination group is merely an inference based on current research and the observed results. We predicted that the success in the G10/K50 combination could be due to gentamicin enhancing the effect and uptake of kanamycin through the initial protein disruption in the membrane; however, our study did not directly test this hypothesis. Without confirmation of this mechanism, it is difficult to conclusively explain the results from this study, regardless of how plausible this explanation may be.

5. Future Directions:

Future studies can build on these findings in a number of ways. To begin, checkerboard assays can be run to determine the Fractional Inhibitory Concentration Index (FICI) for the G10/K50 and G10/K25 antibiotic combinations, which would confirm whether the enhanced bactericidal effect can be deemed synergistic using its formal definition. Additionally, the G10/K50 combination should be tested on other *S. aureus* strains, including MRSA, to see if the strong bactericidal effect works beyond the Newman strain of *S. aureus*. Membrane permeability assays, such as propidium iodide staining, can also directly test our proposed mechanism that one aminoglycoside influences and amplifies the absorption of the other aminoglycoside in the combination treatment. These future experiments would confirm some of the mechanisms we proposed and minimize any uncertainties.

■ Conclusion

In conclusion, this study identified the combination of gentamicin at 10 µg/mL and kanamycin at 50 µg/mL to be the most effective treatment against the Newman strain of *Staphylococcus aureus* in the laboratory *in vitro*, showing an enhanced effect that ultimately leads to notable bacterial cell death. In the initial stage, *S. aureus* Newman showed some resistance to gentamicin, chloramphenicol, and kanamycin until the dose reached a high enough threshold; contrarily, Newman showed little to no resistance to cefotaxime and erythromycin. The combination therapy is shown to have a stronger effect in comparison to individual antibiotic treatments, and even more notable when compared to the *S. aureus* group exposed to no antibiotic treatment. We propose that the combination therapy of gentamicin at 10 µg/mL and kanamycin at 50 µg/mL is most successful due to a mechanism that allows for increased antibiotic uptake in the bacterial cell, leading to more rapid malfunctions inside the cell over a shorter period of time. Despite clinical relevance being limited by this study from its *in vitro* nature and use of only one strain of *S. aureus*, the findings from this study still highlight notable results about the efficacy of certain combination therapy groups over individual treatment for the five original individual antibiotics.

■ Acknowledgments

I would like to give my sincere appreciation to PhD student Ms. Zhao for her relentless guidance, feedback, and inspiration throughout this entire research process. I would also like to give my immense thanks to Prof. Huang for providing the lab materials and for his genuine support in this project, and to my family for their utmost encouragement throughout this entire process.

■ References

- van Hal, S. J.; Jensen, S. O.; Vaska, V. L.; Espedido, B. A.; Paterson, D. L.; Gosbell, I. B. Predictors of Mortality in *Staphylococcus Aureus* Bacteremia. *Clinical Microbiology Reviews* **2012**, *25* (2), 362–386. <https://doi.org/10.1128/cmr.05022-11>.
- Etter, D.; Corti, S.; Spirig, S.; Cernela, N.; Stephan, R.; Johler, S. *Staphylococcus Aureus* Population Structure and Genomic Profiles in Asymptomatic Carriers in Switzerland. *Frontiers in Microbiology* **2020**, *11*. <https://doi.org/10.3389/fmicb.2020.01289>.
- Taylor, T. A.; Unakal, C. G. *Staphylococcus aureus* infection. *National Library of Medicine*. <https://www.ncbi.nlm.nih.gov/books/NBK441868/>.
- Centers for Disease Control and Prevention. *Staph infections can kill*. Centers for Disease Control and Prevention. <https://www.cdc.gov/vitalsigns/staph/index.html>.
- Bruno, A. Identifying Patterns on the Development of Kanamycin Resistance in *Staphylococcus epidermidis* with Four-Welled Plates. <https://doi.org/10.13140/RG.2.2.16783.20649>.
- Chaves, B. J.; Tadi, P. *Gentamicin*. PubMed. <https://www.ncbi.nlm.nih.gov/books/NBK557550/>.
- Drugbank. *Cefotaxime*. [go.drugbank.com](https://go.drugbank.com/drugs/DB00493). <https://go.drugbank.com/drugs/DB00493>.
- Macheboeuf, P.; Fischer, D. S.; Brown, T.; Zervosen, A.; Luxen, A.; Joris, B.; Dessen, A.; Schofield, C. J. Structural and Mechanistic Basis of Penicillin-Binding Protein Inhibition by Lactvicins. *Nature Chemical Biology* **2007**, *3* (9), 565–569. <https://doi.org/10.1038/nchembio.2007.21>.

- Mingeot-Leclercq, M. P.; Tulkens, P. M. Aminoglycosides: Nephrotoxicity. *Antimicrobial Agents and Chemotherapy* **1999**, *43* (5), 1003–1012. <https://doi.org/10.1128/AAC.43.5.1003>.
- What is the mechanism of Chloramphenicol? Patsnap.com. <https://synapse.patsnap.com/article/what-is-the-mechanism-of-chloramphenicol>.
- What is the mechanism of Erythromycin? Patsnap.com. <https://synapse.patsnap.com/article/what-is-the-mechanism-of-erythromycin>.
- Methicillin-Resistant *Staphylococcus Aureus* (MRSA) – How Can People Become Infected? 2025. <https://www.bfr.bund.de/cm/349/questions-and-answers-about-methicillin-resistant-staphylococcus-aureus.pdf> (accessed 2026-01-05).
- Pantosti, A.; Sanchini, A.; Monaco, M. Mechanisms of Antibiotic Resistance In *Staphylococcus Aureus*. *Future Microbiology* **2007**, *2* (3), 323–334. <https://doi.org/10.2217/17460913.2.3.323>.
- Ratia, C.; Soengas, R. G.; Soto, S. M. Gold-Derived Molecules as New Antimicrobial Agents. *Frontiers in Microbiology* **2022**, *13*. <https://doi.org/10.3389/fmicb.2022.846959>.
- Kamble, E.; Sanghvi, P.; Paredes, K. Synergistic Effect of Antibiotic Combinations on *Staphylococcus Aureus* Biofilms and Their Persister Cell Populations. *Biofilm* **2022**, *4*, 100068. <https://doi.org/10.1016/j.biofm.2022.100068>.
- When is it necessary to combine antibiotics? Vinmec International Hospital. <https://www.vinmec.com/eng/blog/when-to-combine-antibiotics-en>.
- Bognár, B.; Spohn, R.; Lázár, V. Drug Combinations Targeting Antibiotic Resistance. *npj Antimicrobials and Resistance* **2024**, *2* (1). <https://doi.org/10.1038/s44259-024-00047-2>.
- Pettit, R. K.; Weber, C. A.; Lawrence, S. B.; Pettit, G. R.; Kean, M. J.; Cage, G. D. *In Vivo* Activity of Anprocide Alone, and *In Vitro* Activity in Combination with Conventional Antibiotics against *Staphylococcus Aureus* and *Staphylococcus Epidermidis* Biofilms. *Journal of Medical Microbiology* **2009**, *58* (9), 1203–1206. <https://doi.org/10.1099/jmm.0.008268-0>.

■ Author

Olivia Liu is a senior at Hong Kong International School. Passionate about the intersection of biomedical research and healthcare to improve public health for all, she hopes to continue conducting meaningful research and contributing to medicine, with plans to pursue a pre-med track in her future collegiate studies..