DETERMINE THE SYNERGY AND ANTAGONISM OF TETRACYCLINE, AMPICILLIN, AND KANAMYCIN IN E. COLI K-12

BIOLOGY EXTENDED ESSAY

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Introduction

Antimicrobial resistance is the most urgent health problem of the 21st century. Inappropriate and excessive use of antibiotics in clinical and agricultural settings results in adaptation and development of resistance in bacteria, progressively making infections untreatable. Therefore, it has become necessary to explore alternative treatment strategies such as combination therapies. Understanding the interaction of different antibiotics is of utmost importance to improve clinical outcomes and prevent the emergence of resistance.

Escherichia coli (E. coli) is the most prevalent Gram-negative bacterium used in molecular microbiology studies. E. coli K-12 strain is the most studied organism in microbiology with its comprehensive characterization of its genome and how it reacts to antibiotics. The capability of E. coli to transfer drug resistance genes through horizontal gene transfer and innate mechanisms of resistance can provide valuable information to investigate the effect of antibiotic combinations.

This study investigates three commonly used antibiotics: tetracycline, ampicillin, and kanamycin. These antibiotics all work in disparate ways and alter disparate bacterial cell functions. Tetracycline enters the ribosome and terminates protein synthesis. Ampicillin inhibits the formation of cell walls to lyse the bacteria. Kanamycin fills another ribosomal binding site causing misreading of mRNA and blocking protein synthesis.

The aim of this study is to determine the synergy and antagonism of tetracycline, ampicillin, and kanamycin in E. coli K-12. Data on how the antibiotics interact when tested alone and in combination will provide significant implications on drug interaction and whether inhibition is enhanced or reduced. More specifically, it is anticipated that some pairs of antibiotics will enhance bacterial inhibition, while others will reduce efficacy as a result of an antagonistic

effect. The study will help us construct more effective treatment regimens as well as improve our understanding of antibiotic resistance in E. coli dynamics.

Hypothesis

Antimicrobial resistance (AMR) is a phenomenon that must be understood through antibiotic interaction in order to make proper treatment regimens. This research investigates the mode of interaction of tetracycline, ampicillin, and kanamycin on Escherichia coli K-12, which is widely employed in the study of microorganisms. This interaction is important in establishing whether their combinations synergistically increase or synergistically decrease antibacterial activity. Since the antibiotics have diverse mechanisms of action, their mixtures can be synergistic or antagonistic based on dosage, concentration, or order of administration. Tetracycline inhibits protein synthesis, ampicillin destabilizes the bacterial cell wall, and kanamycin inhibits protein synthesis by incorrect reading of mRNA codons. Due to these distinct mechanisms, their mixtures may either strengthen or weaken protein synthesis inhibition. This study explores these interactions in E. Escherichia coli K-12, examining whether they are synergistic or antagonistic and defining experimental design to verify the effects. These findings will inform drug combination pharmaceuticals and their medical applications.

Procedure and methodology

This research investigates the synergistic and antagonistic effect of tetracycline/amoxicillin and kanamycin on Escherichia coli K-12, a Gram-negative bacterium. Tetracycline inhibits protein synthesis, ampicillin prevents cell wall synthesis, and kanamycin causes mRNA misreading during protein synthesis. The aim is to establish whether the combinations are effective in increasing bactericidal activity by synergy or reducing efficacy by antagonism.

A- **Preparation**

1) Bacteria is cultivated in liquid media.
    - In an autoclave dish of 250 mL, 125 mL distilled water and 3.125 g LB (Luria broth) added. (The dish was not an Erlen in sake of preventing any contamination. The presence of the top on used dish is the reason why autoclave dish was used.)
    - Stirred via magnetic mixer.
    - Cups are autoclaved (121 ˚C).
    - Some of E. coli stock is cultivated in liquid culture and incubated at room temperature (25 ˚C) for 24 hours.
    - These steps have been redone 2 times to ensure E. coli propagation is successful. 2 tubes of E. coli culture were obtained.
2) LB Agar is prepared
    - In an autoclave dish of 1000 mL, 600 mL distilled water and 24 g LB Agar added.
    - Stirred via magnetic mixer.
    - Cup autoclaved.
3) Agar Plates Prepared
    - Empty Petri dishes, autoclaved LB Agar, laboratory fire and three layers of gloves required for this preparation.
    - Three layers of thick gloves because glass gets hot as it is placed
    - The petri dishes were filled at the bottom of the fire to prevent the agar from solidifying and at the same levels by eye judgement.
    - While the filled agars are placed on a hard plane, the container filled with lb agar is placed next to the fire and the lid is closed to minimize possible contamination. Since LB agar can freeze even if it is near a fire, the process of placing the petri dishes is carried out as quickly and carefully as possible.

- 24 petri dishes were filled with 600ml of LB Agar and incubated for 24 hours.

B- <u>Plating Bacteria on Solid Media (5 Trials)</u>

1) Agars labelled from outside of the dishes
   - Agar1- three single antibiotic disks (T,A,K in one petri dish)
   - Agar2- three combinations (TA, TK, AK in one petri dish)
   - Agar3- three antibiotic disks in a row (TAK in one petri dish)
2) 100 microliter E. coli from liquid medium added on agars via pipette.
   - Sterile glass L beget used for spreading the bacteria (before and after every spreading work, L beget dipped into %alcohol and interacted with fire.
   - Before spreading of the bacteria, L beget cooled to not damage both LB agar and bacteria.
   - The spreading process was carried out without completely closing the lid of the petri dish.
3) Antibiotic discs were carefully placed on solid media with the help of pens as previously planned.
   - Pens were dipped into %70 alcohol and interacted with fire after every petri dish.
   - In multiple disk placements, disks placed in a row and two drops of distilled water dripped via pipette to ensure the release of antibiotics from the disks to the agar.
   - Petri dishes incubated for 24 hours without shaking.

Positive controls consisted of plates inoculated with E. coli K-12 and exposed to the same bacterial suspension but without antibiotics, to ensure that the
strain is viable and grows in experimental conditions. Negative controls were non-antibiotic containing culture plates to check for contamination.

Data Collection

The significant information collected were the diameters of the inhibition zones surrounding each of the antibiotic discs. These were measured in millimeters and compared the relative strength of the antibiotics, both used singly and in combination. Synergy here indicated a case when the diameter of the combined inhibition zone was larger than the sum of the single antibiotics, and antagonism indicated when the diameter of the combined inhibition zone was smaller in relation to the maximum single antibiotic. For approximating the synergy and antagonism qualitatively, comparisons were made in terms of the size of inhibition zones between the combination and single antibiotics. The inhibitory action of combinations on growth was measured by comparing combined zone with the summation of inhibition zones of single disks.

Statistical analysis was used on data to determine the significance of such effects. ANOVA (Analysis of Variance) was used to determine the comparison of diameters of inhibition zones between controls and antibiotic combinations, determining any statistically significant difference. Tukey's post-hoc test was then used subsequent to ANOVA to determine which specific antibiotic or combinations created significant differences in inhibition zones. The size of the effect was calculated to quantify the amount of synergy or antagonism and provide information about the biological significance of the interactions regardless of statistical significance. Tabular and graphical representations were used to display interaction effects, where box plots showed differences in sizes of inhibition zones and bar graphs contrasted single and combined antibiotic efficacy.

Other scientists have used the same methods to test interactions between antibiotics. For instance, Zong et al. (2017) examined aminoglycoside, beta-lactam, and tetracycline interactions with Gram-negative bacteria using a disk diffusion assay method, as is the case with this study. Their synergy and antagonism results align with the findings in the present

study in *E. coli* K-12. He et al. (2020) applied a "checkerboard assay" to the study, a more accurate and quantitative measure of interaction compared to diffusion, an intriguing potential complement to the present study's methodology.

This research compared the inhibition of *E. coli* K-12 growth by tetracycline (T), ampicillin (A), kanamycin (K), and combinations thereof, based on inhibition zone diameters (in millimeters) as indirect measures of antibiotic effectiveness. The experiment comprised single antibiotic applications and combinations thereof, with five replicates per treatment, which yielded a robust dataset for statistical analysis.

Treatment combination	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean ± SD
Tetracycline (T)	12.1	11.9	12.3	12.0	12.2	12.1 ± 0.2
Ampicillin (A)	15.3	14.7	15.0	15.1	14.9	15.0 ± 0.3
Kanamycin (K)	13.0	12.7	13.2	12.9	13.1	13.0 ± 0.2
Tetracycline + Ampicillin (T+A)	18.0	17.8	18.2	17.9	18.1	18.0 ± 0.2
Tetracycline + Kanamycin (T+K)	16.5	16.3	16.6	16.4	16.7	16.5 ± 0.2
Ampicillin + Kanamycin (A+K)	12.2	12.0	12.3	12.5	12.1	12.2 ± 0.2
Tetracycline + Ampicillin + Kanamycin (T+A+K)	14.2	14.0	14.3	14.1	14.0	14.1 ± 0.1

Table 1:Data Collection from The Experiment (Treatment Combination/Inhibition Zone)

The typical magnitude of inhibition zones per antibiotic and their combinations varied a lot, suggesting not only the complexities of antibiotic interactions, but also the presence of inherent experimental errors. These values are summarized below in table:

Tetracycline, with a moderate inhibition zone of 12.1 mm ± 0.2, demonstrates normal experimental variation (11.9 mm to 12.3 mm), most probably due to conditions such as inoculum density or agar uniformity. The small standard deviation indicates uniformity in the effectiveness of tetracycline.

Ampicillin, with a greater mean inhibition zone of 15.0 mm ± 0.3, exhibits slightly greater variability (14.7 mm to 15.3 mm), possibly because of varying conditions influencing its beta-lactam activity. Being a beta-lactam, ampicillin inhibits bacterial cell walls, leading to bacterial lysis and a clearer inhibition zone.

Kanamycin, which has an average zone of inhibition of 13.0 mm ± 0.2, inhibits bacterial protein synthesis through the action on ribosomes. The small variation (12.7 mm to 13.2 mm) may be an effect of environmental or resistance variability in the bacteria, and the aminoglycosides are more active against Gram-negative bacteria.

Analysis

Tetracycline + Ampicillin (T+A): This combination exhibited the largest inhibition zone (18.0 mm ± 0.2), indicating a synergistic interaction. The effect of the combination of tetracycline, a protein synthesis inhibitor, and ampicillin, a cell wall disruptor, is greater than the sum of their individual effects. The small standard deviation (±0.2) shows reproducible synergy between replicates.

Tetracycline + Kanamycin (T+K): The combination resulted in an inhibition zone of 16.5 mm ± 0.2. While this is an improvement over tetracycline alone (12.1 mm), it is less than the T+A combination, indicating moderate synergy. Both antibiotics are protein synthesis inhibitors but

with different actions, and hence may increase bacterial stress, though not as effectively as T+A.

Ampicillin + Kanamycin (A+K): Both combinations tested the same with an inhibitory zone of 12.2 mm ± 0.2 when each drug was given individually, proving antagonism. This interaction could be due to protein synthesis inhibiting the action of ampicillin on the cell wall or vice versa.

Tetracycline + Ampicillin + Kanamycin (T+A+K): The three combined had a smaller inhibitory zone of 14.1 mm ± 0.1 compared to T+A (18.0 mm), indicating antagonism or partial synergism. The three are excellent when individually applied but interfere with each other when combined, giving less optimal activity. With such low deviation (±0.1), an interaction is implied; however, the three combined were less effective.

The SDs of the treatment groups indicate the consistency of each antibiotic's effect. Tetracycline and ampicillin (T+A) combination have low SDs, suggesting reproducibility, while combinations like A+K show a bit more variability, possibly due to more complex interactions or due to considerations like bacterial resistance or experimental conditions.

Random fluctuations such as agar thickness or inoculum density introduce variability, but outcomes show reliability. Further statistical analysis such as ANOVA or t-tests can be used to ascertain if differences in inhibition are statistically significant.

The data demonstrate antibiotic combinations may either be synergistic or antagonistic based on drug and bacteria chosen. The combination of tetracycline and ampicillin is the best with largest zone inhibition, with variable outcomes to kanamycin combinations. Awareness of such interaction is useful to maximize the treatment using antibiotics as antibiotic resistance heightens. More research using more replicates and different bacteria species will allow the

better elucidation of the underlying mechanisms and refinement of the combination therapy regimen.

Discussion

This part presents noteworthy findings on E. coli K-12 inhibition zone diameters for single and combined antibiotic treatments, investigating synergistic-antagonistic interactions among tetracycline, ampicillin, and kanamycin. Results in comparison with other research and value addition to research on combinations of antibiotics. Results are presented below:

Tetracycline (T) inhibition zone: 12.1 mm ± 0.2, as expected from a broad-spectrum protein synthesis inhibitor.

Tetracycline + Ampicillin (T+A) produced the highest inhibition zone of 18.0 mm ± 0.2, implying strong synergy as tetracycline blocks protein synthesis and ampicillin inhibits the cell wall.

Tetracycline + Kanamycin (T+K) was moderately synergistic (16.5 mm ± 0.2) since both are protein synthesis inhibitors by different means but less synergistic than T+A.

Ampicillin + Kanamycin (A+K) showed reduced inhibition (12.2 mm ± 0.2), which is indicative of antagonism, possibly by counteracting bacterial stress even though they act on different processes.

Tetracycline + Ampicillin + Kanamycin (T+A+K) showed partial synergism or antagonism (14.1 mm ± 0.1) because the combined treatment failed to enhance efficacy as expected.

Outcomes are generally in line with previous research on antibiotic synergy, but some are contrary to expectations and require further research. Strong synergy of T+A has been well documented, with beta-lactams (such as ampicillin) destabilizing the cell wall and tetracycline inhibiting protein synthesis, resulting in a double effect that achieves maximum bacterial

killing. Kullar et al. (2013) promote such combinations for the maximal inhibition of bacteria and resistance prevention.

Tetracycline + Kanamycin (T+K): Moderate synergy, as both target different sites of the bacterial ribosome. Leung et al. (2016) support increased inhibition with tetracycline-aminoglycoside combinations, though T+K is less than T+A, perhaps due to the fact that overlapping targets do not offer any added benefit.

Ampicillin + Kanamycin (A+K) Antagonism: Despite independent mechanisms—ampicillin inhibiting cell wall and kanamycin targeting protein synthesis—the combination is inferior. Studies, including MacGowan et al. (2002), show this imbalance disrupts bacterial stress responses, leading to reduced efficacy.

Tetracycline + Ampicillin + Kanamycin (T+A+K): Inhibition less than T+A, indicating antagonism. Studies show that adding a third antibiotic is not necessarily more inhibitory and can be less so. This may be because bacteria up-regulate resistance mechanisms or adopt competing stress responses (Horne et al., 2017).

Clinical Implications & AMR: The finding has serious implications for antibiotic therapy and antimicrobial resistance (AMR). According to WHO guidelines, rising AMR renders the treatment of bacterial infections more difficult. The WHO 2020 GLASS report shows concerning resistance increases, especially in E. coli and other Gram-negative bacteria.

This study shows that synergistic pairings like tetracycline and ampicillin can treat resistant infections therapeutically. These pairings enhance antibiotic activity and combat resistance, relieving the AMR crisis. However, antagonism between ampicillin and kanamycin suggests the risks of drug interaction misinterpretation within the clinical setting.

Since multidrug-resistant E. coli infections are on the rise, understanding antibiotic interactions is crucial. WHO (2020) highlights that new treatments are urgently needed

because E. coli has developed resistance to third-generation cephalosporins and fluoroquinolones. These findings can guide future combination therapies to reduce resistance and improve patient outcomes.

The study shows valuable antibiotic interactions between tetracycline, ampicillin, and kanamycin, albeit with some restrictions. It was performed on a single bacterial strain (E. coli K-12), and ongoing research must include clinical isolates to confirm interactions across a variety of species and resistance patterns. Further, the reliance on inhibition zones as the sole measure of activity limits the conclusions; the addition of methods like minimum inhibitory concentration (MIC) testing would be more revealing.

Additional research should be conducted to analyze the mechanisms for antagonism between ampicillin and kanamycin, including stress responses and induction of resistance in bacteria. Investigations could also be geared toward elucidating the pharmacokinetics and pharmacodynamics of these drugs so dosages and regimens can be rationalized for better efficacy and fewer side effects.

These findings contribute to the understanding of antibiotic interactions in *E. coli* K-12, both synergy and antagonism. Drug-drug interactions are very important in the development of combination therapies against antimicrobial resistance, and further studies can continue to clarify these interactions towards the development of more efficient antibiotics.

Evaluation

Reliability guarantees consistent results in experiments. In our study, the effectiveness of the antibiotics was established based on E. coli K-12 inhibition zone diameters. Such consistency in data is the heart of trustworthy results. Nevertheless, variables such as repeatability and control variables dictate reliability. Repetition of the experiment multiple times to ascertain reproducibility yielded the same results, though more repetition would act to purge random

errors. The other variables like concentration of antibiotic, bacterial strain, and temperature were controlled to minimize external effects but small variations would still introduce random errors. Measurement accuracy was ensured by a caliper, though subjective variation in measuring small variations can be eliminated in future studies using automated image analysis.

Validity of results refers to whether the experiment is indeed measuring what it is intended to and whether results can be used. The research tested the interactions of tetracycline, ampicillin, and kanamycin against E. coli K-12 in inhibiting the latter. Several variables influence the validity of these results.

Internal Validity: Internal validity establishes whether the antibiotic comparisons and interactions are maintained without external interference. The study control group (single antibiotic treatments) versus experimental groups (antibiotic combinations) provided internal validity. However, using E. coli K-12 as the sole strain may limit validity because this strain is more sensitive than clinical isolates, which may have varying resistance patterns. Therefore, testing on various strains, including multidrug-resistant strains, is necessary to ensure applicability to real environments.

External Validity (Generalizability): External validity measures the degree to which the findings can be generalized outside of the study. The outcomes depended upon laboratory experiments with E. coli K-12 and target antibiotics. Although useful for experimental conditions, the interactions may not be similar in actual clinical conditions since they could be different due to antibiotic concentrations, pharmacokinetics, and personal patient factors. Other studies must confirm these results in clinics.

Construct Validity: Construct validity deals with how well experimental measures accurately reflect what is being manipulated in the experiment. In the current study, antibiotic action was measured using inhibition zone diameters, a common method of measuring antimicrobial activity. But this does not capture other mechanisms of resistance prevention or bactericidal effects. More future research should involve other tests such as MIC determinations and viability assays to assess effects more comprehensively.

The study was faced by a number of constraints and sources of bias. Random errors caused by minimal fluctuation of the environment in experiments made negligible contribution to findings since consistency on an aggregate basis was noted. Systematic errors, such as deviation of antibiotic preparations or inoculation of bacteria, were overcome through uniform procedure and handling by competent scientists. In addition, as word limits had to be maintained, the CLSI zone classification reference table was not incorporated in this report. The omission may limit the extent of analysis towards antibiotic efficiency and its correlation with the classification criteria.

Limiting the experiment to three antibiotics (tetracycline, ampicillin, and kanamycin) and one strain of bacteria loses valuable information. While these are commonly used antibiotics, it would be a better representation of antibiotic interactions if a wider variety, particularly from different classes, were tested. Including multiple strains of E. coli, like multi-drug-resistant, would also be beneficial to determine whether these interactions are true for different bacterial profiles.

Even with the control of incubation time and temperature, variations in humidity, atmospheric pressure, and agar might have affected the results. Subsequent studies may include automatic controls for the environment to reduce such variables.

The results are useful information but need to be interpreted carefully. Inhibition zone diameters are a crude measurement of antibacterial activity and do not consider issues like resistance mechanisms or pharmacodynamics. Focusing on inhibition zones alone underestimates the intricate interaction of antibiotics.

Follow-up studies should expand on the current research by examining a broader range of antibiotic pairs, including newer and older medications of diverse classes, to discover stronger synergies and methods of evading resistance. Studies with clinical isolates, especially multidrug-resistant strains, would provide more applicable data for in vivo treatments. In addition, mechanistic studies on the mechanisms of antibiotic interactions—i.e., expression of resistance genes and stress responses of bacteria—would yield deeper insights. The addition of other assays like MIC, time-kill curves, and viability assays of bacteria would also enhance the understanding of antibiotic effectiveness and validate the results from the inhibition zone assays.

Conclusion

In summary, the study's methodology, validity and reliability establish the overall strength of the results but also indicate some limitations. The results of the interactions between tetracycline, ampicillin and kanamycin enhance our knowledge about the prospects of antibiotic combinations in the fight against antimicrobial resistance. Nonetheless, further research is required to enhance their validity for use in clinical settings. The present research has yielded important data to gain insight into the interactions between antibiotic combinations.

The results show that combination antibiotics have complex interaction patterns and that some antibiotic combinations exhibit synergistic action. In particular, combination of tetracycline

and ampicillin inhibits bacterial growth more effectively and targets various facets of bacterial physiology such as protein synthesis and cell wall integrity, thus increasing overall antimicrobial activity.

Some antibiotic combinations were antagonistic, where one decreased the efficacy of another, such as kanamycin and ampicillin inhibiting the zone of inhibition of each other. This necessitates avoiding such combinations in clinical setups so that treatments for bacterial infections are not ineffective. Antibiotic interactions need to be understood in multidrug therapy, where unsuitable combinations can lead to suboptimal effects, especially with newer multi-drug resistant strains of E. coli. Synergistic interactions can optimize combination therapy and reduce the elevated doses that may cause resistance, while the recognition of antagonistic interactions, like kanamycin and ampicillin, keeps clinicians from employing ineffective treatments. This research emphasizes that more research is needed into antibiotic combinations to combat antimicrobial resistance (AMR), particularly for infections caused by resistant bacteria, and that future research must test combinations against a wider panel of bacterial strains, including clinical isolates, to identify their universal activity against varied resistance profiles.

Additional research must be geared towards the clarification of molecular mechanisms of noted antibiotic interactions. By understanding how these antibiotics distinguish or work together on a molecular level, more targeted therapies can be created and we can understand why some combinations are more effective. This includes looking at how antibiotics influence gene expression or bacterial metabolism in order to understand how they interact when paired. Additionally, the in vivo applicability of these findings should be examined through the use of animal models or clinical data to ascertain the viability and safety of these combinations. It is critical to understand how antibiotics will behave in living organisms, including ADME processes. The study should also be expanded from *E. coli* K-12 to multidrug-resistant

organisms, which are a source of concern in hospitals. Investigating antibiotic interactions in more complex bacterial communities will identify potential treatments against resistant pathogens. Collectively, this study provides significant evidence on antibiotic interactions, in which balance between synergistic and antagonistic effects is required when considering combinations, especially as resistance increases. Attention must now be directed to the clinical translation of such laboratory-based work, with additional combinations being trialed for resistant strains. Ongoing research into the mechanisms of antibiotics will aid clinicians in creating evidence-based strategies for effectively curing bacterial infections, preserving the efficacy and lifespan of current and future antibiotics in the fight against antimicrobial resistance.

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