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A STUDY ON DRUG RESISTANCE IN *E. COLI* KL96 STRAIN: EFFECTS OF COMBINATIONAL DOSES OF AMIKACIN, AMPICILLIN AND GENTAMICIN

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Abstract: The increasing antibiotic resistance of the pathogenic bacterial strains is a concerning global threat. It has potentially challenged the entire public health sector. Additionally, this rising issue has the potential to jeopardize various fields of medicine. The development of novel drugs to combat antibiotic resistance remains a significant challenge for the scientific community due to its slow progress. However, one of the promising approaches to counteract drug resistance is the use of antibiotics in combination. The current preliminary study exploits the aforementioned strategy to analyze the resistance pattern of the *E. coli* KL96 strain against three antibiotics– amikacin, ampicillin and gentamicin. Remarkably, it was observed that the combination of the three antibiotics demonstrated more efficient inhibition of the strain at comparatively lower doses, in contrast to individual antibiotic doses which displayed dose-dependent inhibition.

Keywords: **Antibiotic resistance, multi-drug resistance, ampicillin, amikacin, gentamicin.**

I. INTRODUCTION

Bacteria are sophisticated single-celled prokaryotic organisms. They lack nuclear membrane and multiply quickly via binary fission. There are both parasitic and free-living forms of bacteria. Bacteria are highly adaptable to changing environments which is facilitated by the selection of the spontaneous mutants. The discipline of bacteriology emerged due to the necessity for physicians to investigate and understand various diseases, as well as the economic concerns surrounding the spoilage of food and wine. Scientists realized the importance of understanding these microbes in relation to the human health. This prompted the need for further research and investigation into the field of bacteriology. The meticulous research in the field lead to various advancements. The two major advancements in the field of bacteriology are the development of the vaccines and antibiotics discovery. Although bacterial diseases are not completely eliminated by antimicrobial substances, they act as potent therapeutic tool *(Medical Microbiology;4th edition)*.

Bacteria are highly prevalent and are found in diverse habitats throughout the environment. Their ubiquitous nature highlights their significance in maintaining the ecosystem. Although, the majority of them are harmless and even beneficial to humans, a relatively small percentage of them have significant implications on human health. These pathogenic bacteria are responsible for causing infections which can range from mild to severe, even life-threatening illnesses. In developing nations, bacteria are responsible for 90 percent of the hospitalized cases. These cases represent just a fraction of the total number of infections in a wider population *(Medical Microbiology;4th edition)*. These infections, up to some extents are manageable due to the presence of various antimicrobials but the development of resistance against these antimicrobials poses an alarming issue *(Doron S, Gorbach SL; 2016)*. Although antibiotic resistance genes are of greater concern to the public

health, ironically, they are indispensable markers in the field of genetic engineering. The recombinant bacteria, produced via genetic engineering, are tremendously important in bacteriological research. They are used widely for the synthesis of rare biomolecules for both research and therapeutic purposes *(Medical Microbiology;4th edition)*.

Antibiotics are chemical substances that have the potential to kill the sensitive microorganisms or inhibit their growth. They can be naturally produced by microbes *(Prescott Microbiology; 7th edition)* or can be synthetically derived partly or wholly in the laboratory. The term antibiotics means "against life" in literal sense *(Etebu et al.; 2016)*. These antibiotics have played a pivotal role in not only treating the infectious diseases but also opened the way for numerous modern medical applications. Their use has been instrumental in expanding the range of various advanced procedures like cancer treatment, organ transplantation and open-heart surgery *(Hutching M. I. et al.,2019)*. Antibiotic compounds are classified based on their ability to withstand a specific group of microbes. Antibacterial antibiotics targets the bacteria, antiviral deals with the viruses and antifungal agents are used to combat fungal infections *(Brooks et al., 2004; Russell, 2004)*.

Antibiotics can be categorized based on their range of species destruction or spectrum against different microorganisms. The broad-spectrum antibiotics are effective against wide range of microorganisms as compared to the narrow-range antibiotics. The number of organisms killed or inhibited is in direct proportion with the spectrum of activity of the antibiotic (*van Saene, Rick et al.; 1998)*. Broad-spectrum antibiotics have indispensable role in in treating a range of bacterial infection without identifying the causative agent. However, they do have certain significant disadvantage of spreading antibiotic resistance *(Melander et al.; 2017)*.

Antibiotic resistant is a severe public health issue, largely caused by the misuse of drugs. Overuse of antibiotics has been a significant problem, with more than 50% of antibiotic prescriptions in hospitals lacking clear evidence of infection or medical need. Antibacterial drugs have been administered to patients with viral illnesses like cold, influenza and viral pneumonia, despite their ineffectiveness against viruses. Studies have revealed that antibiotics are prescribed without pathogen identification or bacterial sensitivity testing, and broad-spectrum antibiotics are sometimes used instead of narrow-spectrum drugs, leading to the risk of side effects, opportunistic infections and the selection of drug-resistant strains. Additionally, patients often fail to complete their full course of medication, allowing drug-resistant mutants to survive. The availability of antibiotics without a prescription also contributes to the proliferation of drug-resistant strains as individuals self-administer medications *(Prescott Microbiology; 7th edition)*.

The antimicrobial drug industry is a lucrative business, with millions of pounds of antibiotics worth billions of dollars being produced annually in United States. A significant portion, up to 70%, of these antibiotics are added to livestock feed. The inclusion of antibiotics in animal feed is undeniably a significant factor in the rise of drug resistant. Adding antibiotics at low
levels to the diets of livestock, such as cattle, pigs and chickens, enhances their growth rates and efficiency, partly by controlling infections in crowded animal populations. However, this practice also amplifies the presence of drug-resistant bacteria in the intestinal tracts of these animals. However, the widespread and extensive use of antibiotics has led to concerning rise in drug resistance, as more and more diseases are becoming resistant to treatment. A notable example is *Neisseria gonorrhoeae*, the bacterium responsible for causing gonorrhea. Initially, sulfonamides were successfully used to treat gonorrhea in 1936. However, by 1942, a majority of strains had developed resistance, prompting physicians to turn to penicillin. Within 16 years, a penicillin resistant strain emerged in Asia. In 1976, a penicillinase-producing strain of the gonococcus reached the United States and continues to spread throughout the country. As a result, penicillin is no longer effective for treating gonorrhea *(Prescott Microbiology; 7th edition)*.

The ongoing research in advancing diagnostic capabilities will have a substantial role in combating the rise of antibiotic resistance and treatment of various infections by better identification of the bacterial strains causing those infections. This approach will minimize the unnecessary use of broad-spectrum antibiotics *(Melander et al.; 2017)*. Also, the identification of new chemical compounds with the necessary characteristics for antibiotic development is a major challenge in the scientific community to combat the issue of rising drug-resistance *(Hutchings M. I. et al.; 2019)*. However, the search and screening for the new antimicrobials is relatively slow. The utilization of the existing antibiotics in combination to combat this global threat of antimicrobial resistance in pathogenic bacteria is an emerging filed and hence, can provide promising results. The strategy is being extensively explored by the scientific community all over *(Si Zhangyong et al.; 2023)*.

1.1 Objectives:

Following were the objectives of the study:

- To establish and maintain the laboratory culture of *E. coli* KL96 strain.
- To evaluate the effect of Amikacin, Ampicillin and Gentamicin on bacterial culture using individual and combinational doses.

II. MATERIALS & METHOD

S.No.	MATERIALS	SOURCE
1.	Nutrient Broth	Ready MED
2.	Agar	Ready MED
Antibiotics		
3.	Amikacin	ALKEM
4.	Ampicillin	GERMED
5.	Gentamicin	TROIKAA

2.1 Culture Medium Preparation

3.25g of Nutrient Broth and 3.75g of Agar were dissolved in 250 ml of distilled water. The solution was then autoclaved and cooled at room temperature for further use.

Fig 1: Culture Medium (Nutrient Broth+ Agar)

Composition of Nutrient Broth

S.No.	Ingredients	Quantity (g/L)
1.	Special Peptone	5.0
2.	Special Meat Extract	1.5
3.	Yeast Extract	1.5
4.	Sodium chloride	5.0
5.	pH @ 25°C	7.4±0.2

2.2 Growth Medium Preparation

3.25g of Nutrient Broth was dissolved in 250 ml of distilled water. The solution was then autoclaved and cooled at room temperature for further use.

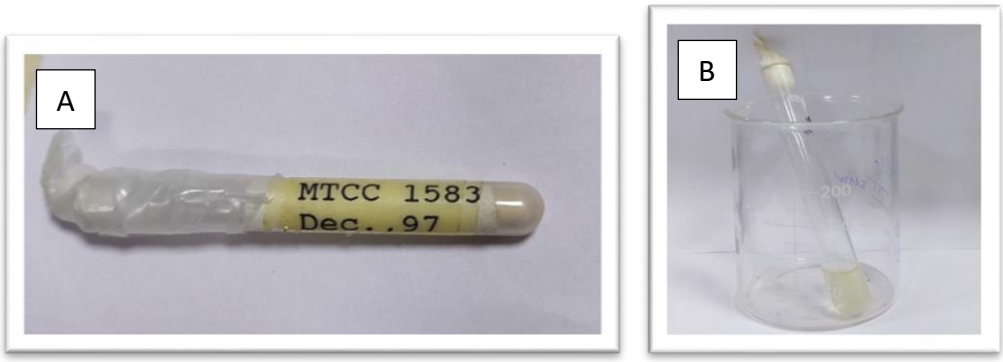

Fig 2: Growth Medium

NOTE: All the procedures were carried out in the Laminar Flow Hood to maintain the sterilized condition throughout.

2.3 Preparation of Inoculum:

E. coli KL96 strain was introduced into the test tube using a sterile loop wire containing the growth medium (Nutrient Broth). The cotton plug was then inserted and the parafilm was wrapped around the mouth of the test tube. The test tube was then kept in the incubator at 37°C for bacterial growth.

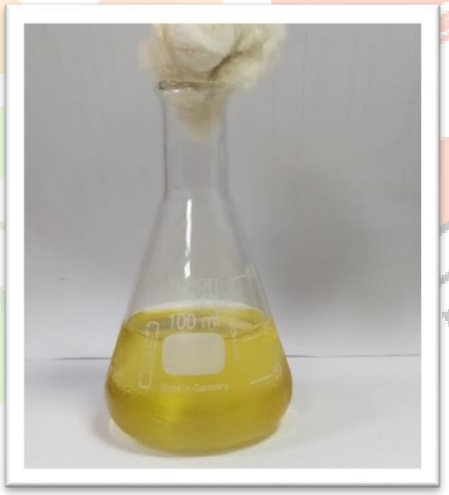

Fig 3: E. coli KL96 strain (A) and Inoculum of *E. coli* KL96 strain in Nutrient Media (B).

2.4 Observation of the *E. coli* KL96 strain growth pattern:

Optical density (OD) was observed every 2 hours for a complete duration of 24 hours using a spectrophotometer at 420 nm. Each reading was measured against the blank. The graph, OD v/s Incubation time was plotted to observe the growth pattern.

2.5 Subculturing:

2µl of the inoculum was added to the growth medium (Nutrient Broth) in a test tube. The test tube was then incubated at 37°C overnight. The subculturing can be carried out regularly throughout the duration of the experiment.

2.6 Plating:

The autoclaved glass petri dishes were poured with the culture medium (up to half volume) carefully to avoid air bubbles formation and were left to solidify. Small volume of inoculum was added to the semi-solid culture medium and was spread uniformly using a sterile glass spreader. The petri dishes were then kept in the incubator at 37°C overnight for incubation after proper labelling and wrapping using a parafilm.

2.7 Antibiotics Preparation:

The following three antibiotics were used in the current study:

ANTIBIOTICS	SYMBOL	CONCENTRATION
Amikacin	Amik	500mg/ 2ml
Ampicillin	Amp	500mg/ 5ml
Gentamicin	Gen	80mg/ 2ml

2.8 Evaluation of Drug Resistance:

A. Individual doses-

The spread plate was made as per the aforementioned procedure to obtain a uniform lawn of *E. coli* colonies. Discs were punched out using a Whatman Filter Paper#1 and were loaded with drugs in varying concentration of 10µl, 20µl and 50µl as given below:

- **Culture Plate A:** Amikacin
- **Culture Plate B:** Ampicillin
- **Culture Plate C:** Gentamicin

All the culture plates were incubated at 37°C overnight for bacterial growth to observe the effect of drugs.

B. Combinational Doses-

The combination of antibiotics were used to assess the drug resistance response after analysing the individual responses of each antibiotic. The spread plate was made as per the aforementioned procedure to obtain a uniform lawn of *E. coli* colonies. 5µl of each antibiotic was loaded on the filter paper and kept in the centre of the petri dish in following combinations:

- **Culture Plate A:** Ampicillin and Amikacin
- **Culture Plate B:** Amikacin and Gentamicin
- **Culture Plate C:** Ampicillin and Gentamicin
- **Culture Plate C:** Amikacin, Ampicillin and Gentamicin

All the culture plates were incubated at 37°C overnight for bacterial growth to observe the effect of drugs.

III. RESULT

The current study examines the antibiotic resistance in virulent *E. coli* strain KL96. This particular strain was received from R. Jayaraman, Madurai Kamraj University (MKU), Madurai, India. The strain was sourced from Microbial Type Culture Collection (MTCC) in lyophilized form. The study focuses on analysing the resistance pattern of *E. coli* KL96 against three antibiotics in individual as well as combinational doses. Throughout the duration of the study, meticulous protocol was followed to ensure a sterile environment thereby ensuring the accuracy and reliability of the experimental results.

The inoculation was done by introducing the lyophilized bacterial culture into the autoclaved liquid nutrient broth. After an appropriate overnight incubation at 37°C, the growth was visible as cloudy mass in the test tube. The turbidity of the culture was increased as the bacteria continued to multiply. In order to sustain the availability of actively propagating and healthy bacteria, subculturing was done at regular intervals throughout the study since the logarithmic phase is preferred and selected for various assessments.

The growth pattern of the bacteria was assessed to evaluate the suitability of nutrient media and the environmental conditions. The growth pattern will also assist in assessing the viability of the strain. The optical density (OD) of culture was read at regular interval for a complete duration of 24 hours at 420nm.

After gathering all the data, the information was analysed and compiled in the form of a graph between absorbance and the incubation time. The dataset revealed a characteristic sigmoid curve with different phases typical of bacterial growth curves. Based on the graph obtained, it can be concluded that the nutrient medium and the environmental conditions provided were indeed conductive to the growth of the strain.

Further, the *E. coli* strain was evaluated for drug resistance using three antibiotics, namely Amikacin, Ampicillin and Gentamicin. The method employed for the aforementioned study is Disc-Diffusion Antibiotic Sensitivity Test, also known as Kirby-Bauer Test. The principle behind this test is to load the discs with specific antibiotic concentration which diffuses on the surface of the agar. The concentration of the antibiotic is highest near the disc and decreases with the increase in distance from the disc. If the bacteria is sensitive towards the particular antibiotic at certain concentration, no growth is observed in area where the concentration is more than or equal to the effective concentration. This is demonstrated by the formation of a zone of inhibition on the agar plate, where there is complete absence of bacterial colonies. This zone is absent on the agar plate when the bacterial colonies are resistant to a particular antibiotic at a specific concentration.

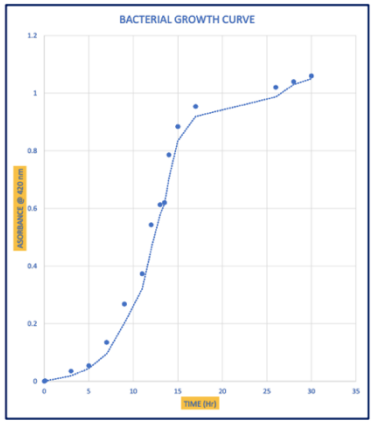

Fig 4: A typical growth curve of E. coli KL96 strain showing three phases of growth, i.e., Lag Phase, Exponential Phase and Stationary Phase.

Initially, we observed the effect of Amikacin on the growth of bacterial colonies. The antibiotic was administered in varying concentration of 2.5mg/10µl, 5mg/20µl and 12.5mg/50µl, which were loaded on separate discs. As a result, it was deduced that the bacterial colonies were susceptible to Amikacin indicated by the presence of clear zone of inhibition around the discs. Moreover, the test was extended for the other two antibiotics, Ampicillin (1mg/10µl, 2mg/20µl, 5mg/50µl) and Gentamicin (0.4mg/10µl, 0.8mg/20µl, 2mg/50µl), as well. The consistent outcomes were observed for both of them. The bacterial colonies indicated susceptibility to both Ampicillin and Gentamicin, also evidenced by the well-marked zone of inhibition around the respective antibiotic impregnated discs. Therefore, the strain can be referred to as $Amik^s$, Amp^s and Gen^s. Also, the antibiotic sensitivity of the three antibiotics showed dose-dependent relationship. In other words, the dose of antibiotic used is in direct proportion with the size of the zone of inhibition.

On comparing the zone of inhibition of the three antibiotics, Gentamicin showed the highest percentage of inhibition zone of 8.3% at the concentration of 5µl followed by Amikacin (6.3%) and Ampicillin (3.8%). The results, therefore indicate that Gentamicin had the strongest inhibitory effect on the growth of bacterial colonies.

Furthermore, the study was expanded to observe the impact of combinational doses of the three antibiotics. A total of four possible combinations were formulated. Notably, the combinational dose of Ampicillin-Gentamicin, with 5µl of each antibiotic, proved to be effective in inhibiting the growth of bacterial colonies by 22.03%. This result was closely followed by the combinational dose of Amikacin-Gentamicin (15.03%) and Ampicillin-Amikacin (12.03%) at the same dose.

Remarkably, the combination of all the three antibiotics at the used concentration i.e.; Amikacin (1.25mg/5µl), Ampicillin (0.5mg/5µl) and Gentamicin 0.2mg/5µl) at 5µl each demonstrated the most significant percentage of inhibition zone of 30.36%.

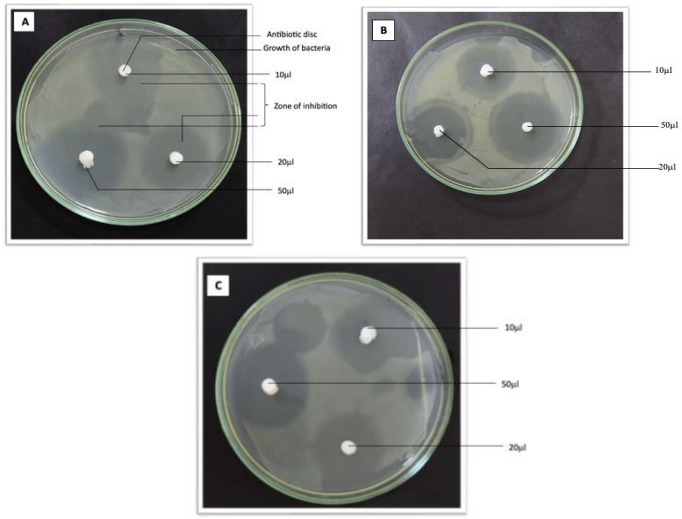

Fig 5: Treatment of *E. coli* KL96 strain with Amikacin (A), Ampicillin (B) and Gentamicin (C) at different concentrations. The strain came out to be $Amik^S$, Amp^S and Gen^S.

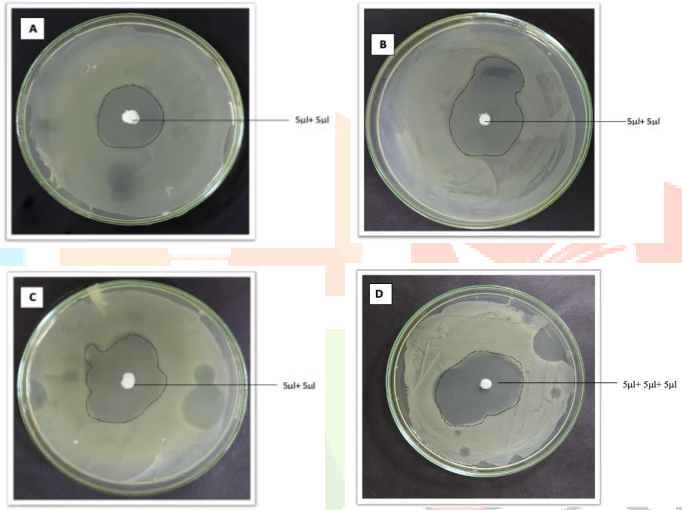

Fig 6: Combinational dose of Ampicillin (5µl) and Amikacin (5µl) [A], Amikacin (5µl) and Gentamicin (5µl) [B], Ampicillin (5µl) and Gentamicin (5µl) [C] and Ampicillin (5µl), Amikacin (5µl) & Gentamicin (5µl) [D].

IV. DISCUSSION

The primary objectives of the current study were twofold. Firstly, it aimed to establish and sustain a laboratory culture of E. coli KL96 strain and then evaluate its susceptibility to three antibiotics — Amikacin, Ampicillin and Gentamicin, both individually and in combination.

During the experiment, a growth curve was generated to assess the viability of strain, environmental conditions and nutrient medium. Over time, a significant body of literature on growth dynamics of the bacteria emerged, encompassing both imaginative and precise studies. Among these, Henrici's renowned publication stands out for its lucid analysis of how bacteria undergo changes in size during their growth cycle *(Schaechter M.; 2015).* The growth pattern of the *E. coli* KL96 strain obtained during the study was a typical sigmoid curve which remains in conformity with the already published results *(Wang L. et al.; 2015)*. The significance of observing a growth curve lies in understanding the dynamics of microbial growth and its relationship to the specific conditions provided. Studying the growth pattern of bacteria carries several implications and provides valuable insights into their biology and behaviour. It helps us in understanding the physiological changes that occurs during different phases of bacterial growth. It provides information about the metabolic activity, nutrient utilization and energy production of bacteria *(Reddy C. A. et al.; 2007)*. The growth curve is equally valuable for evaluating the effectiveness of antimicrobial agents against bacterial productions. It

allows researchers to determine the optimal timing for drug administration to target bacteria during their most vulnerable growth phase *(Karaiskos I. et al.; 2017)*. The microbial growth studies help in predicting the spread of infectious diseases and designing strategies for disease control *(Anderson and May; 1991)*.

Throughout the study, subculturing was performed at regular intervals. The procedure involves the transferring of a small portion of the culture to a fresh growth medium to ensure the continued growth of the microbe and maintain its viability. For the growth of microbes, various essential factors such as food, oxygen, moisture and space are pre-requisites. Their growth is impeded when deprived of necessary resources. Hence, subculturing helped prevent nutrient depletion and accumulation of waste products. It is an essential technique in microbiology to sustain and propagate microbial cultures for various research and practical applications *(Jain A. et al.; 2020)*.

The increasing prevalence of antimicrobial resistance in pathogenic bacteria poses a major global public health challenge. Drug-resistant bacterial infections lead to significant morbidity and mortality among patients, jeopardizing the progress achieved by antibiotics in the last several decades. This growing threat of multi-drug resistant (MDR) pathogens will have impacts on various fields of medicine. Without viable antibiotic therapy, crucial medical practices such as surgery, premature infant care, cancer chemotherapy, critical care and transplantation medicine will be greatly compromised. One promising approach to address these issues is the use of drug combinations where the drug can inhibit targets in different pathway, inhibit different targets in same pathway or inhibit same target in different pathways. Combining different drugs can enhance their efficacy against resistant bacteria, making it more difficult for the pathogens to develop resistance. Also, by using drug combinations, the effectiveness of individual drug can be synergistically enhanced, providing a more potent and comprehensive treatment strategy against MDR pathogens *(Worthington R. J. & Melander C.; 2013)*.

In conclusion, this preliminary study aimed to investigate the growth of *E. coli* KL96 strain in the presence of various antibiotics. Our findings indicated that the combination of antibiotics showed more effective inhibition of strain growth compared to individual antibiotic doses at higher concentrations. Remarkably, the combination therapy demonstrated equivalent efficacy at lower doses, which suggests the potential for reduced side effects and improved treatment outcomes. While these findings are promising, further research on larger scale is warranted to validate and expand upon our preliminary results. Scaling up the study would provide a more comprehensive understanding of the effectiveness of these antibiotic combinations against this strain infection. Additionally, conducting clinical trials would be essential to evaluate the safety and efficacy of the identified combination therapy before considering its implementation in clinical practice. Hence, further exploration into the finding has the potential to contribute to the development of optimized treatment strategies and combat the challenges posed by antibiotic resistance.

V. ACKNOWLEDGEMENT

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