In Vitro Collateral Sensitivity: A Potential Approach Against Multiple Antibiotic Resistant Bacteria

By

Nasrat Ahmed Yousha
ID: 19136020
Sadia Islam Badhan
ID: 19136030
Shatabdi Deb Dina
ID: 19136009
MD Jahid Ahsan
ID: 19326021

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology and Bachelor of Science in Microbiology.

Biotechnology & Microbiology Program
Department of Mathematics and Natural Sciences
Brac University
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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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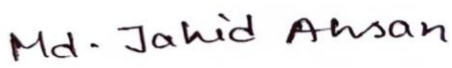
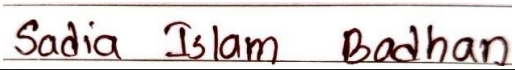
Student's Full Name & Signature:

Shatabdi Deb Dina
Student ID: 19136009



Md. Jahid Ahsan
Student ID: 19326021

Nasrat Ahmed Yousha
Student ID: 19136020



Sadia Islam Badhan
Student ID:19136030

Approval

The thesis titled 'In Vitro Collateral Sensitivity: A Potential Approach Against Multiple Antibiotic Resistant Bacteria' submitted by

1. Nasrat Ahmed Yousha (19136020)
2. Sadia Islam Badhan (19136030)
3. Shatabdi Deb Dina (19136009)
4. MD Jahid Ahsan (19326021)

has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science of Biotechnology and Bachelor of Science of Microbiology.

Examining Committee:

Supervisor:

_____

Dr M. Mahboob Hossain
Professor, Microbiology Program, Department of Mathematics and Natural Sciences
BRAC University

Co-Supervisor:

_____

Akash Ahmed
Senior Lecturer, Microbiology Program, Department of Mathematics & Natural Sciences
BRAC University

Program Director:

_____

Dr. Munima Haque
Associate Professor, Department of Mathematics & Natural Sciences
BRAC University

Departmental Head:

_____

Professor A F M Yusuf Haider
Professor and Chairperson, Department of Mathematics & Natural Sciences
BRAC University

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Abstract

Antimicrobial Resistance (AMR) has long been recognized as a major issue. With the emergence and rapid spread of antibiotic-resistant bacteria, the world's stock of viable antibiotics is rapidly depleting. In this regard, Collateral Sensitivity (CS) is a promising and alternative approach with the potential to treat bacterial infections. The project aimed to study the collateral sensitivity of gram-negative resistant bacteria by exposing clinical isolates to antibiotics in individual and combination antibiotic trials. The results were promising for both *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The efficiency of Linezolid against gram-negative bacteria was one of novel findings of this study. Exposure to Linezolid in combination with other antibiotics produced encouraging results. 50% of the *P. aureginosa* strains showed susceptibility to Ceftriaxone, Levofloxacin, Tetracycline, Amikacin and Meropenem when exposed to Linezolid itself whereas 75% of the *K. pneumonia* strains showed susceptibility to Tetracycline. Although the clinical implementation is still under study, the collateral sensitivity treatment could prove be an adequate addition to the current treatment to lower the antibiotic resistance ratio. Further research at the molecular level is suggested to better understand the effect of collateral sensitivity.

Keywords: Antibiotic resistance; Antibiotic Susceptibility testing; Antibiotic exposure; Collateral sensitivity

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List of Acronyms

Abbreviation	**Elaboration**
CS	Collateral Sensitivity
AMR	Antimicrobial Resistance
MDR	Multi-Drug Resistant
XDR	Extensively drug resistant
PDR	Pandrug-resistant
ABR	Antibiotic Resistance
WHO	World Health Organization
CDC	Center for Disease Control and prevention
P. aeruginosa	*Pseudomonas aeruginosa*
E. coli	*Escherichia coli*
K. pneumoniae	*Klebsiella pneumoniae*
FDA	Food and Drug Administration
UTI	Urinary Tract Infection
LB	Luria-Bertani
AST	Antibiotic Susceptibility Test
LZ	Linezolid
AK	Amikacin
MRP	Meropenem
TE	Tetracycline
COT	Co-trimethoprim
AMX	Amoxicillin
E	Erythromycin

Chapter 1
Introduction and Literature Review

1.1. Antimicrobial Resistance (AMR)

One of the most terrifying and earnest public health issues in this century is the emergence of Antimicrobial Resistance or AMR. According to WHO, AMR happens when microbes like a virus, bacteria, parasites, etc. develop resistance towards the medicines that were designed to treat infections. It has become a growing concern in this era because as pathogens are becoming AMR, the treatment procedure for an infection is becoming more and more difficult and, in some cases, it is almost impossible to treat a patient. According to a report by CDC (n.d.), around 1.27 million people died due to infections by AMR pathogens all around the world in 2019. So, it is safe to say that AMR has become a global threat. Again, it has been reported that around 2.8 million antibiotic-resistant infections have been reported in the United States alone each year, accounting for more than 35,000 deaths due to the lack of proper treatment. (Keeton V. Department of Health and Human Services, 21 F. 3d 1064 - Court of Appeals, 11th Circuit 1994 - Google Scholar, n.d.).

With the rapid emergence of AMR, Bangladesh is also facing the consequences of it along with all the other countries around the world. One of the main reasons for AMR in pathogens is the extensive use and misuse of antibiotics, unnecessary use of antibiotics, and acquiring resistant genes by bacterial R plasmid help a bacterium to become resistant to antimicrobial agents. People often take antibiotics for a viral infection instead of anti-viral medications or even for a normal fever without a proper prescription. According to Hoque et al. (2020), around 18% of all isolates of bacteria from children suffering from pneumonia were found to be resistant to almost all commonly used antibiotics in a study done at a hospital in Dhaka, Bangladesh. Again, in another study done by Safain et al. (2020), it was noted that among 430 preserved bacterial strains collected from patients admitted to different hospitals in Dhaka, Bangladesh, 53% were Multidrug-Resistant bacteria. It was a yearlong study where they found that the

MDR isolates gradually increased over the year from 2015 to 2018, and by 2019, the growth was about two times more than in 2015 (Safain et al., 2020).

1.2. Types of Resistant Bacteria (MDR, XDR, PDR)

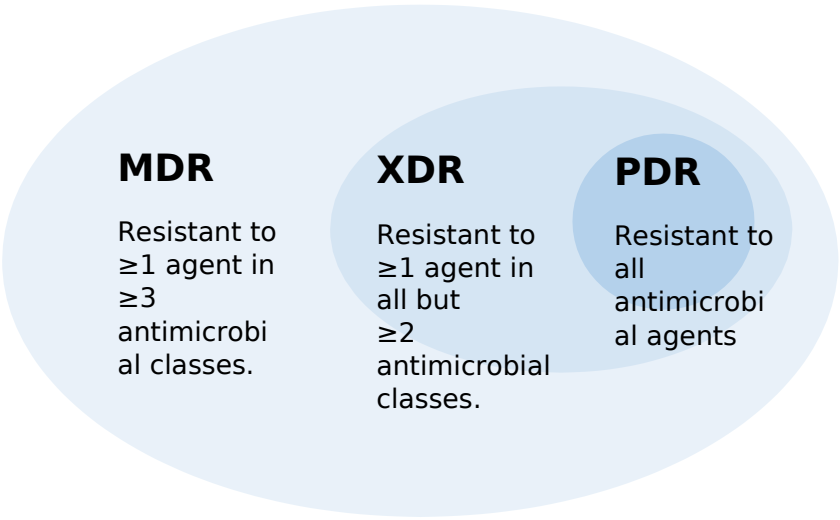
All Resistant bacteria can be divided into 3 categories. These are:

Multi-Drug Resistant Bacteria (MDR): Multidrug-resistant bacteria mean when a bacterium or bacterial species is resistant to an antibiotic among three or more classes of antibiotics. Multidrug-resistant bacteria or MDR are becoming untreatable with the existing antibiotics and the quantity of this type of bacteria is increasing day by day. According to a study done by the University of OXFORD, almost 1.2 million people died in 2019 due to infections caused by MDR bacteria worldwide and this number could increase to 10 million by the year 2050.

Extensively Drug-Resistant (XDR): Extensively drug-resistant bacteria, often known as XDR bacteria, are a class of organisms with multiple drug resistance that are immune to every type or nearly every type of antimicrobial substances that have received FDA approval (Magiorakos et al., 2012). *Mycobacterium tuberculosis* was one of the very first bacteria to be referred to as XDR. Only one or two antimicrobial groups continue to be effective against XDR bacterial isolates meaning that XDR bacteria are non-susceptible to almost all antibiotics. This is the main difference between MDR and XDR bacteria (Basak et al., 2016).

Pandrug-Resistant (PDR): Pandrug-resistant or PDR bacteria are non-susceptible or are resistant to all antibiotics in all antimicrobial categories (Basak et al., 2016). The increasing rate of “pan-resistant” gram-negative strains like *Pseudomonas aeruginosa* and *Acinetobacter baumanii*, has been found most recently and as they are resistant to all antibiotics, there are no antibiotics that can be used against these strains.

These organisms can easily acquire resistance mechanisms and can make a low-permeability outer membrane barrier (Nikaido, 2009).



(Source: Magiorakos et al., 2012)

Figure 1: Types of antibiotic-resistant bacteria (MDR, XDR, and PDR)

1.3. The Emergence of Antibiotic Resistant Bacteria:

Taking unnecessary antibiotics which are not needed for curing the infection, using antibiotics for treating animal infection, and taking antibiotics randomly are the causes of creating multidrug resistance bacteria. Moreover, with the help of transposons (genes that move from one bacterium to another) and integrons (DNA pieces that can accumulate new types of genes) bacteria can acquire multiple resistance genes. With the help of these transposons and integrons, a bacterium can build up its whole gene resistance against antimicrobial agents. Furthermore, with the help horizontal gene transfer process (transformation, conjugation, and transduction) a bacterium can acquire resistant genes into it. Then, making biofilms a bacterium can be resistant to antimicrobial agents because bacteria create LPS channels and make biofilm for their shelter or survival. There are also some causes like assembles of resistance genes in R

plasmids, maintenance of R plasmid in host cells, cell-to-cell transfer of R plasmid, multidrug efflux pumps, and altered physiological state cause for multidrug resistance (Nikaido, 2009).

1.4. Antibiotic Resistant Infections:

When an infection is occurred by bacteria, antibiotics or antimicrobial agents are used to treat or inhibit that bacterium. The issue is widespread among both Gram-positive bacteria, which includes *S. aureus, S. pneumoniae, E. faecium,* and *E. faecalis* and Gram-negative bacteria, which include *A. baumannii, E. coli, P. aeruginosa*, and *K. pneumoniae* (Bharadwaj et al., 2022). Gram-negative bacteria are often responsible for diseases like Urinary Tract Infection or UTI (*E. coli*), pneumonia, meningitis, infection in the blood and lung, and so on. These diseases can often be deadly if left untreated and so, proper and immediate treatment is needed. One of the common gram-negative bacteria is *K. pneumoniae*, which is often found living inside the human intestine. However, this bacterium can trigger diseases including pneumonia, meningitis, bloodstream infections, UTI, and more if it enters any other parts of the body (Li et al., 2022). One of the most hazardous infectious pathogens found in the usual intestinal flora is *P. aeruginosa*. This bacterium is difficult to treat with antibiotics because of its ability to run innate resistance mechanisms, such as overexpression of the efflux pump, and attaining resistance mechanisms by taking resistance genes and mutated genes that encode for proteins called porins and other proteins as well. (Bharadwaj et al., 2022). Around 2.8 million antibiotic- resistant infections have been reported in the United States alone each year, accounting for more than 35,000 deaths due to the lack of proper treatment. (Keeton V. Department of Health and Human Services, 21 F. 3d 1064 - Court of Appeals, 11th Circuit 1994 - Google Scholar, n.d.).

1.5. Problem Associated with Antibiotic-Resistant Bacteria:

Antibiotic-Resistant strains were limited earlier, but now, they are becoming more and more usual. These resistant bacteria reduced the effectiveness of treatment people take against infection. This problem creates serious complications in the case of children, pregnant women, and old people. A treatment process takes more time to treat an infection and, in this case, sometimes, there is no treatment left for this type of infection.

So, we can say that bacterial resistance toward antimicrobial agents is one of the greatest threats to public health worldwide (Tanwar et al., 2014).

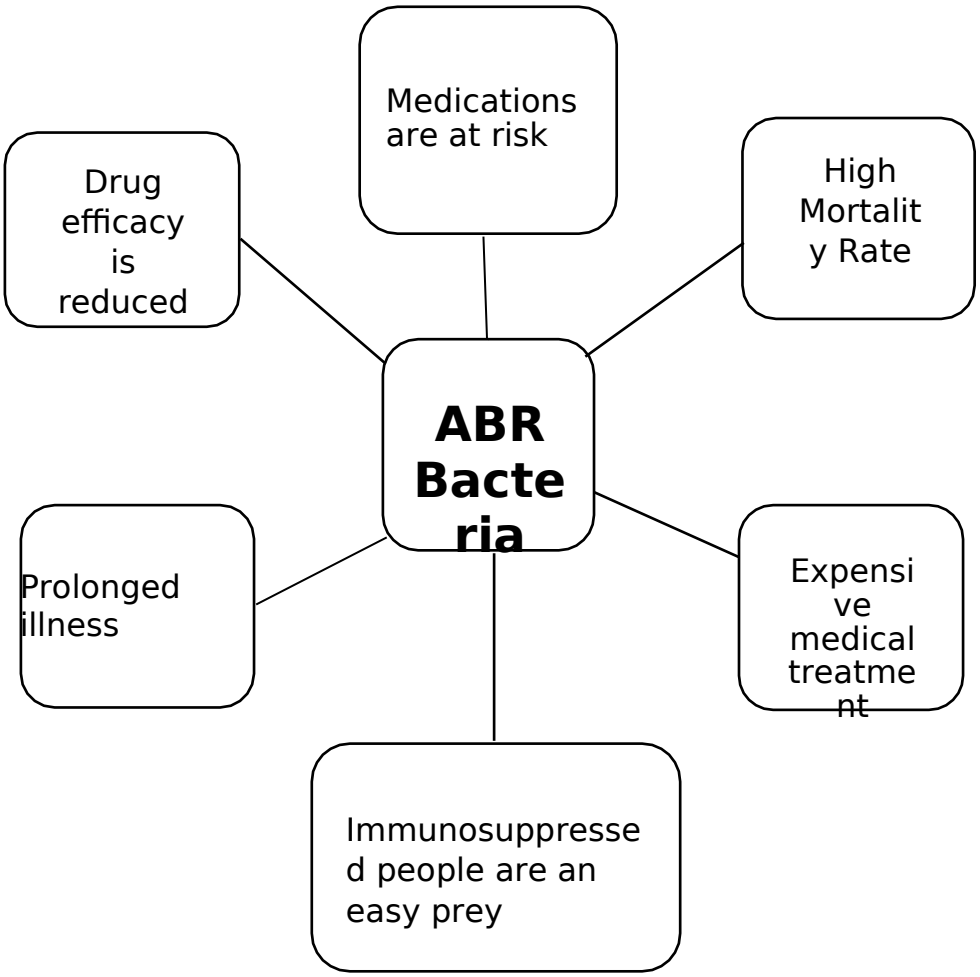


Figure 2: Some typical problems brought on by ABR Bacteria

1.6. Antibiotics:

1.6.1. The History of Antibiotics:

The very first antibiotic, penicillin was discovered back in 1928 which was a breakthrough in the history of medical science, and from then, up until the mid-1950s, the discovery of new families/ groups of antibiotics reached its peak (Hutchings et al., 2019). In the last few decades, there have been well over hundreds of antibiotics discovered most of which fall under the 6 main families of antibiotics ("Antibiotics", 2023). However, the late 1980s was the year when the final, completely novel class of antibiotics was found and after that, till now, no new classes of antibiotics have reached the human trial phase one of the main reason behind this is that discovering and bringing new antibiotics to the market is not financially rewarding to the pharmaceutical companies (Plackett, 2020). There is a rapid decline in the discovery of antibiotics.

According to WHO, till 2021, there are 43 antibiotics and antibiotic conjugation drugs currently in clinical development but 26 of these 43 antibiotics are mostly derivates of already existing classes of antibiotics and only 7 out of these 43 antibiotics might be of new classes with new mode of actions ("WHO Report Highlights Shortage of New Antibiotics," 2021). Global attempts to tackle drug-resistant diseases are threatened by the shortage of new medicines. As a result, there is an increasing demand for new treatment strategies with existing antimicrobial agents.

1.6.2. Common Classes/families of Antibiotics:

Antibiotics are often used to treat any infection caused by bacteria. Antibiotics are chemical compounds generally produced by living microorganisms or are sometimes synthetically made

in vitro (The Editors of Encyclopaedia Britannica, 2023a). Different antibiotics have different mechanism but the main function of them is to inhibit the growth of bacteria and destroy the existing bacteria in a certain environment (Felman. 2023). In today's world, there are more than hundreds of types of antibiotics available and most of them can be divided into some selective classes or groups based on their mode of action ("Antibiotics", 2023). Some of the most commonly used classes of antibiotics are:

Table 1: Common classes of antibiotics

Classes	Mechanism	Example
Penicillins	Works by inhibiting the cell wall synthesis of bacteria and thus hinders both growth and multiplications of bacteria. Mostly Gram-positive bacteria are affected by Penicillins as they work best by inhibiting the peptidoglycan production of a bacterium (Ghooi & Thatte, 1995).	Amoxicillin, Penicillin G, Ampicillin, etc.
Cephalosporins	Works by inhibiting the bacterial cell wall and is usually used instead of the penicillin group (The Editors of Encyclopaedia Britannica, 2023b).	Cefixime, ceftriaxone, cefuroxime.
Fluoroquinolones	Works by hindering bacterial DNA synthesis by inhibiting the two main enzymes necessary for the synthesis process (Blondeau, 2004).	Levofloxacin, ciprofloxacin, norfloxacin.
Oxazolidinones	Function by blocking the synthesis of bacterial proteins. These are mainly synthetic antibiotics and are effective against gram-positive bacteria (Shinabarger, 1999).	Linezolid, Tedizolid, Sutezolid.
Tetracyclines	Works by inhibiting bacterial protein synthesis and thus blocking the translation process of bacteria. (Schnappinger & Hillen, 1996).	Tetracycline, doxycycline, tigecycline.
Aminoglycosides	Inhibits protein production by firmly attaching to the 30S ribosome's A-site on the 16S ribosomal RNA.	Amikacin, streptomycin, tobramycin.

Macrolides	Targets the bacterial ribosome to prevent the production of protein (Vázquez-Laslop & Mankin, 2018).	Erythromycin, azithromycin, telithromycin.
Carbapenems	Bacterial cell walls are penetrated by carbapenems, which then attach to penicillin-binding proteins (PBPs) and cause the deactivation of intracellular autolytic inhibitor enzymes, killing the bacterial cell (Aslam et al., 2020)**.**	Meropenem, doripenem, imipenem.

There are many other antibiotic groups like Chloramphenicol and Lincosamides all of which inhibits the protein synthesis of a bacterium, and many more. (The Editors of Encyclopaedia Britannica, 2023)

1.7. An Alternative Treatment Approach:

Collateral Sensitivity:

To fight against the rapidly growing antibiotic-resistant bacteria with the already existing antibiotics, a treatment procedure can be made using the collateral sensitivity (CS) of bacteria to antibiotics. It is an effective new strategy to combat the growing issue of antibiotic resistance (ABR). According to Roemhild and Andersson (2021), a situation where the development of resistance to one antibiotic results in greater sensitivity to a different antibiotic is described as CS. The research on finding a new and better treatment approach for the MDR bacteria has been going on for years and among many experiments, the CS of bacteria to antibiotics has been proven to be an effective alternative treatment approach and may be the potential to limit the emergence of ABR bacteria.

For a number of clinically important pathogens, such as *Enterococcus faecalis, Escherichia coli (E. coli), Streptococcus pneumoniae, Pseudomonas aeruginosa* (*P. aeruginosa*), and *Staphylococcus aureus*, CS effects have been reported (Aulin et al., 2021). For example, in an

article written by Parmanik et al. (2022), it was shown that a combination of meropenem and amikacin was established to increase the effectiveness against Carbapenem-Resistant Enterobacteriaceae (CRE) and promising results were found. Again, for MDR *P. aeruginosa*, a combination of a cephalosporin with tazobactam was found successful for treatment (Parmanik et al., 2022).

Up until 2021, according to Roemhild and Andersson (2021), there has been no known clinical testing of CS but there have been many successful *in vitro* researches which were of mostly *P. aeruginosa*. One such example can be seen in the paper written by Imamovic et al. (2018), where they found that *P. aeruginosa* develops resistance to therapeutically important antibiotics, which causes phenotypic shift toward different states and it has been linked to collateral sensitivity to certain antibiotic classes. They have reported that around 75% of their isolated resistant *P. aeruginosa* strains were collaterally sensitive to at least one or more antibiotics and have proposed a collateral sensitivity network cycle that will be effective towards cystic fibrosis (CF) caused by MDR *P. aeruginosa* (Imamovic et al., 2018). Furthermore, another study was done on the CS of *E. coli* by Lázár et al. (2013) which should a possible treatment strategy for treating MDR *E. coli*-induced infections.

Mechanism of Collateral Sensitivity:

The CS of a resistance mechanism can be brought on by a rise in the amount of active antibiotic present at the target structure, which is reached either by a greater penetration of the cell membrane or a decreased antibiotic efflux (Roemhild and Andersson, 2021). Genetic alterations brought on by antibiotic resistance can occasionally make bacteria more resistant to other drugs (cross-resistance) or less resistant to them (collateral sensitivity). When a bacterium acquire resistance to any antibiotic, it often involves alteration or mutation to the gene responsible for producing the protein that is targeted by that specific antibiotic.

However, these mutations can accidentally affect other cellular function of the bacterium which makes the bacterium more sensitive towards other antibiotics. Using this side-effect, when a second antibiotic is introduced, it can target the already compromised cellular components by the previous resistant mechanism. This can result in an enhanced sensitivity to the second antibiotic. This increased susceptibility is known as collateral sensitivity.

The main idea of using CS of a bacterium as a treatment approach is to try and find a combination of antibiotics that will work together to first decrease the resistance toward other antibiotics and then using other antibiotics to treat the infection (Allen et al., 2021).

Objective:

The main purpose of this project is to

- Re-purpose the already existing antibiotics to reduce antibiotic resistance.
- Identification of Collateral Sensitivity of isolated bacteria
- Individual trials of antibiotics for antibiotic exposure
- Looking for effective chronological treatment with antibiotics

Chapter 2

Materials and Methods

The spread of antibiotic-resistant bacteria and the decreasing potency of antibiotic drugs indicate an enormous threat to medical treatment. In this study, qualitative research was held and primary data was generated. Based on the Antibiotic Susceptibility Testing of the clinical isolates, highly antibiotic-resistant bacteria were selected. Then the selected isolates were exposed to various commercially available antibiotics to study the change in antibiotic-resistant pattern. For this, individual, as well as combination trials of antibiotics, were tested during the research procedure.

2.1. Working Laboratory

The study was conducted in the Microbiology and Biotechnology Laboratory of the Department of Mathematics and Natural Sciences at Brac University.

2.2. Sample Collection

Clinical isolates of gram-negative bacteria were collected from a tertiary care hospital, Dhaka. A total of 25 isolates were collected from burn-injured patients. To elaborate, reference or clinical strains of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli* were used for the study. The samples were taken to the laboratory for further analysis.

2.3. Culture Media Preparation

Different types of media were used throughout the study.

Table 02: Types of media used

Media	Purpose
T1N1 agar	Used for bacterial stock preparation
Nutrient Agar (NA)	Used for bacterial cultivation and sub-culture purposes
Mueller Hinton agar (MHA)	Used for the determination of susceptibility of bacteria to antibiotic agents.
Luria Bertani broth (LB)	Used for liquid cultivation and maintenance of bacterial isolates. It is also used for the short-term preservation of the isolates
Brain Heart Infusion broth (BHI)	Used as a substitute for LB broth for bacterial cultivation

2.3.1. Preparation of T1N1 Agar (Tryptone Salt Agar)

- For 1000 ml of T1N1 agar, 10 gm of tryptone, 10 gm of NaCl, and 20 gm of agar were needed to add in distilled water.
- The ingredients were suspended and boiled to dissolve properly.
- Two milliliters of T1N1 agar was poured into each three milliliter sterile vial.
- The vials containing T1N1 agar were autoclaved at 121° C for 15 minutes.

2.3.2. Preparation of Nutrient Agar (NA)

- The standard form for preparing NA was 28.0g for 1000 ml of distilled water. This was used as a standard measurement and later media was prepared depending on the amount needed.
- The media was boiled to dissolve properly.
- Then the media was autoclaved at 121° C for 15 minutes.
- After that, the media was poured into Petri dishes until solidification.

2.3.3. Preparation of Mueller Hinton Agar (MHA)

- The standard form for preparing MHA agar was 38.0 g for 1000 ml of distilled water. This was used as a standard measurement and later media was prepared depending on the amount needed.
- The media was boiled to dissolve properly.
- Then the media was autoclaved at 121° C for 15 minutes.
- After that, the media was poured into petri dishes until solidification.

2.3.4. Preparation of LB Broth

- The standard form for preparing LB broth was 40.0 g for 1000 ml of distilled water. It was used as a standard measurement and later media was prepared depending on the amount needed.
- The media was boiled to dissolve properly.
- Then the media was autoclaved at 121° C for 15 minutes.
- After that, the media was poured into test tubes or micro-centrifuge tubes based on the requirement.

2.3.5. Preparation of BHI Broth

- The standard form for preparing BHI broth was 37.0 g for 1000 ml of distilled water. It was used as a standard measurement and later media was prepared depending on the amount needed.
- Finally, the media was autoclaved at 121° C for 15 minutes.
- After that, the media was poured into test tubes or micro-centrifuge tubes based on the requirement.

2.3.6. Preparation of Saline Solution

- For 100 ml saline solution, 0. 9 g Sodium Chloride (NaCl) was mixed with 100 ml distilled water.
- After mixing, the solution was autoclaved at 121° C for 15 minutes.

2.4. Bacterial Stock Preparation

For long-term preservation of the clinical isolates, T1N1 agar was used. Bacteria were inoculated by stabbing (3 times) into the agar media with the help of a sterile inoculating needle. Then, the vials were incubated overnight at 37°C. After incubation, the surface of the medium was covered with 150 µl sterile paraffin oil. The vials were stored at room temperature with appropriate labeling.

For short-term preservation, LB broth was used. In a 2 ml micro-centrifuge tube, 1 ml of LB media was poured. An isolated colony was picked from a nutrient culture plate and suspended in the LB medium. Then the tubes were incubated overnight at 37°C. After incubation, the surface of the medium was covered with 150 µl sterile 40 % glycerol solution. Later, the isolates were preserved at -20°C.

2.5. Sub-culture

For the sub-culture of the clinical isolates, freshly prepared NA plates were used. A single colony was picked with a sterile loop and spread over the media using the streak plate method (Quadrant Streaking). Later, the plates were incubated at 37°C for 24 hours to obtain pure cultures.

2.6. Antibiotic Susceptibility Testing:

All the samples were tested for the identification of antibiotic-resistant patterns. Our target was to search for highly resistant strains from the clinical isolates. For Antibiotic Susceptibility Testing (AST), the Kirby Bauer Disk Diffusion Method was employed and CLSI guidelines were followed throughout the process (CLSI guidelines, 2019). A set of 11 antibiotic agents were used to perform the initial AST.

Table 03: Information about the susceptibility test discs for Enterobacterales

Antibiotic Class	**Antibiotic Agent (Himedia®)**	**Symbol**	**Interpretative Criteria (mm)**			**Disc Content** (mcg/disc)
			S	**I**	**R**	
Penicillin	Penicillin	P	-	-	-	10
	Amoxicillin	AMX	≥ 18	14-17	≤ 13	30
Cephalosporin	Cefixime	CFM	≥ 19	16-18	≤ 15	5
	Ceftriaxone	CTR	≥ 23	20-22	≤ 19	30
Fluoroquinolone	Levofloxacin	LE	≥ 21	17-20	≤ 16	5
Oxazolidinone	Linezolid	LZ	-	-	-	30
Tetracycline	Tetracycline	TE	≥ 15	12-14	≤ 11	30
Sulfonamide	Trimethoprim	COT	≥ 16	11-15	≤ 10	25
Aminoglycoside	Amikacin	AK	≥ 17	15-16	≤ 14	30
Marcolide	Erythromycin	E	-	-	-	15
Carbapenem	Meropenem	MRP	≥ 23	20-22	≤ 19	10

[S= Sensitive, I= Intermediate, R= Resistant]

The Kirby-Bauer method of disk diffusion is a standardized approach that is widely used to determine the sensitivity or resistance of bacteria to various antimicrobial compounds. The results of this procedure are used to determine the most appropriate antibiotic drugs for the treatment of infections.

2.6.1. Inoculum Preparation:

For the preparation of the inoculum, 24 hours of fresh culture of the isolates were used. Using a sterile inoculating loop, an isolated colony was collected and suspended in a saline solution. The saline tube was vortexed to create a smooth suspension. The turbidity of this suspension was adjusted to a 0.5 McFarland standard if needed.

2.6.2. Inoculation of MHA Plates:

A sterile swab was dipped into the inoculum tube. The excess fluid was removed from the swab by rotating it against the side of the tube. After that, bacterial suspensions were spread over MH agar plates. The surface of an MHA plate was inoculated by streaking the swab three times over the entire agar surface. To ensure an even distribution of the inoculum, the plates were rotated approximately 60 degrees each time. The plates were rimmed with the swab to pick up any excess liquid. After that, the swab was discarded in an appropriate container.

2.6.3. Placing Antibiotic Discs on MHA Plates:

At first, forceps were sterilized by immersing them in alcohol and then igniting them. Using the forceps, one antibiotic disc was removed carefully from the cartridge. The discs were gently pressed with forceps to ensure complete contact with the agar surface. Six different discs were placed on the surface of one MH agar plate. To prevent any measurement issues, discs should not be placed near the edge of the plates and close to each together. Once all discs were in place, the plates were inverted, and placed in a 37°C incubator for 24 hours.

2.6.4. Measuring Zone Size:

With the aid of a ruler, the zone of inhibition was measured to the nearest millimeter. A ruler was placed against the plate's back where the zone was visible enough to measure the diameter with ease. Moreover, the presence or lack of a clear zone around the antibiotic disc determined the antibacterial pattern. The outcomes of antibiotic susceptibility tests fall into one of three categories:

● Susceptible or Sensitive (S) - It means a moderate size of the zone which indicates that this organism can be treated with this antibiotic at the recommended level.

● Intermediate (I) - It applies to those organisms that are “moderately susceptible” to an antibiotic.

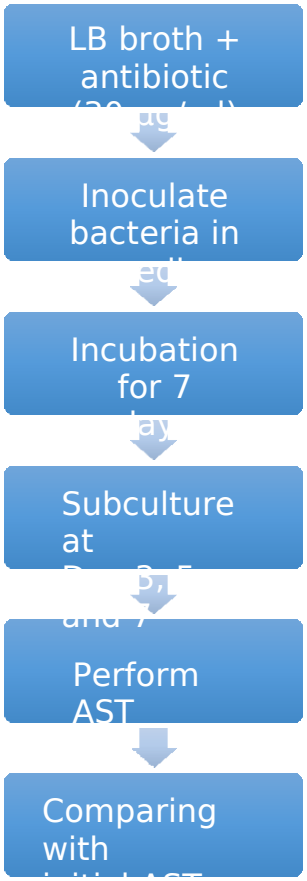
● Resistant (R)- It means that there will be no clear zone around the antibiotic disc also it can be said that the organism won't give any response to that specific antibiotic.

After the primary screening, the highly resistant isolates were chosen for the antibiotic exposure procedure. To elaborate, several isolates (7 to 10) were selected for individual trials and combination trials. Later, the AST procedure was repeated several times depending on the period of antibiotic exposure.

2.7. Methodology for Antibiotic Exposure:

To study the collateral effect of resistant bacteria, a methodology was established. The resistant bacterial strains were exposed to antibiotics in culture media. The initial concentration of an antibiotic was determined 30 µg/ml in the liquid media. The detailed procedure for antibiotic exposure is given below.

a. Individual trials



b. Combination trials

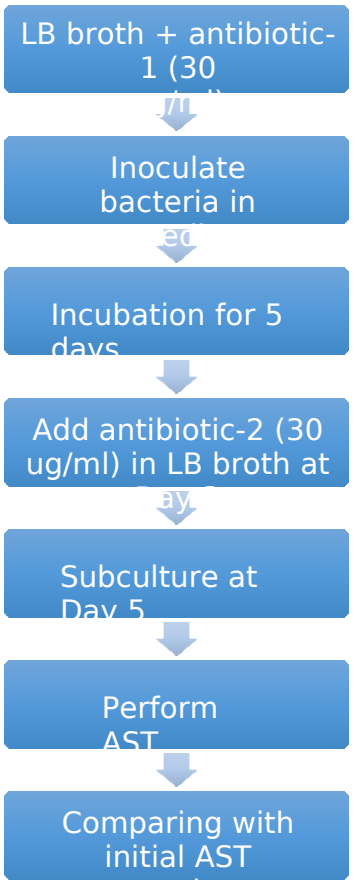


Figure 03: Workflow of the antibiotic exposure methodology

2.7.1. Selection of Antibiotics:

Commercially available antibiotics can be taken in different ways like orally, topically as well as through an injection or intravenously (IV). For the study, commercially available IV antibiotics were used.

Table 04: Commercial antibiotics used for antibiotic exposure

No.	Antibiotic	Commercial name	Company	Description
1	Ceftriaxone	Ceftron®	Square	Each 250 mg vial contains a dry substance equivalent to 250 mg Ceftriaxone (as sterile Ceftriaxone Sodium USP) for IV injections.
2	Penicillin	Benzapen 12 lac	Square	Benzathine Penicillin 12 Lac Units/Vial for IM injection.
3	Cefixime	Cef-3®	Square	Each capsule contains Cexime Trihydrate USP equivalent to Cexime 200 mg
4	Linezolid	Linzolid® 600 IV	Incepta	Each bottle contains Linezolid INN 600 mg in 300 ml (2mg/ml)
5	Tigecycline	Widebac	Incepta	Each vial contains Tigecycline INN 50 mg as lyophilized powder for IV infusion.

Table 05: Types of Trial

Type	**Trial No**	**Antibiotic name**
Individual	1	Ceftriaxone
	2	Penicillin
	3	Cefixime
	4	Linezolid
Combination	5	Linezolid + Ceftriaxone
	6	Linezolid + Tigecycline

2.7.2. Antibiotic Solution Preparation:

Intravenous antibiotics come in different forms like powder or suspension in a liquid. Depending on the solubility of the antibiotic, the antibiotic solution was prepared. The procedure was different depending on the available form of the antibiotic. Antibiotic solutions

were prepared as per the given instructions of the product. The solutions were prepared successfully for all the antibiotics except for Cefixime. Due to the unavailability of the injection form of this antibiotic, tablets were used. However, the tablets did not dissolve properly to make a uniform antibiotic solution.

The formula $C_1V_1 = C_2V_2$ was used to get our desired antibiotic concentration (C_2= 30 µg/ml) in liquid LB media. This formula helps to determine the volume of solvent V_1 that we need to add to prepare a new concentration of C_2 from the original concentration of C_1 of the stock solution.

2.7.3. Procedure for Individual Trials

2.7.3.1. Inoculum Preparation:

For the preparation of the inoculum, 24 hours of fresh culture of the selected isolates were used. LB media was prepared and autoclaved for bacterial inoculation.

2.7.3.2. Exposure to the Antibiotic:

The antibiotic solution was added to the LB media in the required amount. The LB media containing antibiotic solution (30 µg/ml) was transferred into test tubes. Bacteria were inoculated in each test tube with proper labeling. The tubes were incubated in a shaker incubator at 37°C and 120 rpm for 7 days.

2.7.3.2. Subculture and AST:

The samples were sub-cultured on NA plates on Days 3, 5, and 7. At first, the test tubes containing cultural broth were vortexed to create a homogenous solution. Then the bacteria were transferred from the broth into the NA plates with the help of a sterile loop. The bacteria was spread over the media using the streak plate technique. The NA plates were incubated at 37°C for 24 hours to perform Antibiotic Susceptibility Testing the next day. AST was

performed the next day for each sample following the Kirby Bauer Disk Diffusion method. Later, the AST results of Days 3, 5, and 7 were compared with the initial AST reading.

2.7.4. Procedure for Combination Trials

2.7.4.1. Inoculum preparation:

For the preparation of the inoculum, 24 hours of fresh culture of the selected isolates were used. LB media was prepared and autoclaved for bacterial inoculation.

2.7.4.2. Exposure to Antibiotic 1:

The first antibiotic (Antibiotic-1) solution was added to the LB media in the required amount (30 µg/ml). The bacterial samples were exposed to Antibiotic-1 for the first 3 days.

2.7.4.3. Exposure to Antibiotic 2:

On Day 3, the second antibiotic (Antibiotic-2) solution was added to the same bacterial broth in the required amount (30 µg/ml). The bacterial samples were exposed to a combination of antibiotics (Antibiotic-1+ Antibiotic-2) till Day 5.

2.7.4.4. Subculture and AST:

The samples were sub-cultured on Day 5 and AST was performed the next day. At first, the bacteria were transferred from the LB broth into the NA plates with the help of a sterile loop. The bacteria was spread over the media using the streak plate technique. The NA plates were incubated at 37°C for 24 hours to perform Antibiotic susceptibility testing the next day. AST was performed the next day for each sample following the Kirby Bauer Disk Diffusion method. The AST results of Day 5 were compared with the initial AST reading.

Chapter 2 has presented the methodology for the collection of data for this study. The next chapter demonstrated the results of the research.

Chapter

3 Results

In the present study, Antibiotic Susceptibility Testing was performed to identify the antibiotic- resistant pattern of the clinical isolates. Five antibiotic trials (individual and combination) were tested successfully. The changes in the zone of inhibition after antibiotic exposure was compared with the Initial AST result to observe any changes in the resistant pattern.

3.1. The Result of Initial Antibiotic Susceptibility Testing

For the primary screening, Antibiotic Susceptibility Testing of a total of twenty five gram-negative clinical isolates was performed. To elaborate, the resistant pattern of ten *K. pneumoniae*, fourteen *P. aeruginosa*, and one *E. coli* strains was examined for 11 antibiotic agents.

Table 06: Initial AST Result for *K. pneumoniae*

sample	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
5674-k	R	R	R	I	R	R	R	R	R	R	R
5442-k	R	R	R	R	R	R	R	R	R	R	R
6001-k	R	R	R	R	R	S	R	R	R	R	R
5944-k	R	R	R	R	R	R	S	S	R	R	R
5599-k	35	I	S	S	34	S	S	S	S	33	S
4481-k	R	R	R	R	R	R	R	S	R	R	S
5974-k	R	R	R	S	R	S	R	R	R	R	R
4787-k	R	R	R	R	R	R	R	S	R	R	R
4482-k	R	S	S	R	R	R	R	S	R	R	S
5972-k	R	R	R	R	R	R	R	R	R	R	S

[R= Resistant, I= Intermediate, S=Susceptible]

Among the *K. pneumoniae* strains, 20% of the isolates were MDR, 60% of the isolates were XDR and 20% of the isolates were PDR. To elaborate, two of the strains (5674-k and 5442-k) were non-susceptible to all the antibiotics.

Table 07: Initial AST Result for *P. aeruginosa*

sample	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
5432-p	R	R	R	I	R	S	R	R	R	R	R
5663-p	R	R	R	R	R	S	R	S	R	R	S
5961-p	R	R	R	R	R	R	R	R	R	R	R
5945-p	R	R	R	R	R	R	R	I	R	R	R
6002-p	R	R	R	R	R	R	S	S	R	R	R
688-p	R	R	R	R	R	S	R	R	R	R	R
5965-p	R	R	R	R	R	R	R	S	R	R	R
4372-p	R	R	R	R	R	S	R	S	R	R	R
5433-p	R	R	R	R	R	R	R	I	R	R	R
4472-p	R	R	R	R	R	S	R	S	R	13	S
4345-p	R	R	R	R	R	R	R	S	R	R	R
4804-p	R	R	R	R	R	I	R	S	R	R	S
4360-p	R	R	R	R	R	R	R	R	R	R	S
5973-p	R	R	R	R	R	I	R	S	R	R	S

[R= Resistant, I= Intermediate, S=Susceptible]

Among the *P. aeruginosa* strains, 14.3% of the isolates were MDR, 64.3% of the isolates were XDR and 21.4%of the isolates were PDR. To elaborate, three of the strains (5961-p, 5945-p and 5433-p) were non-susceptible to all the antibiotics.

Table 08: Initial AST Result for *E. coli*

sample	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
4368-e	R	R	R	S	R	R	R	S	R	14	S

[R= Resistant, I= Intermediate, S=Susceptible]

There was only one *E. coli* strain which was multi-drug resistant (MDR).

3.2. Categories of Resistance

There are three main types of antimicrobial resistance. All the clinical isolates were categorized accordingly.

Table 09: Categories of Resistance of the clinical isolates

Total isolates	Category	Number of isolates	Percentage
25	MDR	4	16%
	XDR	15	60%
	PDR	5	20%

The majority of the samples were XDR. Besides, 4% or only one bacterial isolate showed sensitivity to more than 3 antibiotic classes.

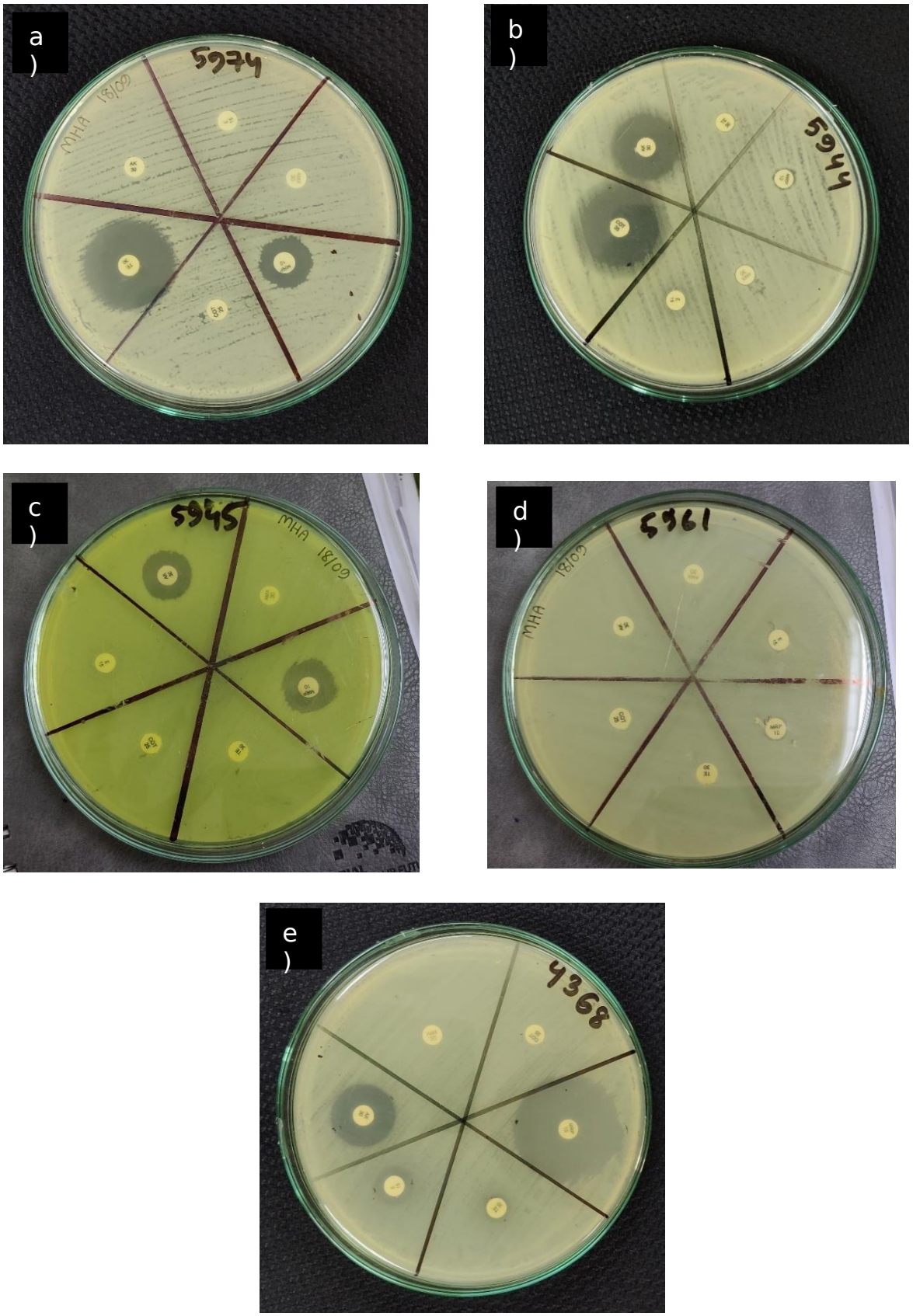


Figure 4: Antibiotic susceptibility testing (Kirby Bauer Disk Diffusion Method) of the gram negative isolates; a) 5974- *K. pneumoniae*, b) 5944- *K. pneumoniae,* c) 5945- *P. aeriginosa*, d) 5961- *P. aeriginosa,* e) 4368-*E. coli* .

3.3. Individual trials

Here, the bacterial samples were exposed to one specific antibiotic. Seven bacterial isolates (4 *K. pneumoniae*, 2 *P. aeruginosa* and 1 *E. coli*) were selected for each individual trials. Any changes in the resistant pattern were observed after the course of antibiotic exposure. This was achieved by the comparison of the initial AST result with 3rd, 5th and 7th Day AST result.

3.3.1. Trial 1- Exposure to Ceftriaxone

For the first antibiotic trial, Ceftriaxone was chosen as an antibiotic. All the Antibiotic susceptibility test results were organized in the tables mentioned below.

Table 10: Comparative analysis of antibiotic resistance pattern of ***K. pneumoniae*** strains after Ceftriaxone exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (Initial)											
Zone of Inhibition (mm)											
4481-k	0	0	0	11	0	8	0	18	0	0	35
5974-k	0	0	0	22	0	18	0	0	0	0	15
5674-k	0	0	0	18	0	0	0	0	0	0	12
4787-k	0	0	0	0	0	0	0	21	0	0	9
AST (3rd Day)											
4481-k	0	0	8	14	0	9	0	22	0	0	33
5974-k	0	0	0	28	0	27	0	0	0	0	30
5674-k	0	0	0	20	0	0	0	0	0	9	11
4787-k	0	0	0	0	0	0	0	22	0	0	8
AST (5th Day)											
4481-k	0	10	0	13	0	9	0	21	0	0	32
5974-k	0	0	0	28	0	25	0	0	0	0	32
5674-k	0	0	0	20	0	0	0	0	0	9	14
4787-k	0	0	0	0	0	0	0	24	0	0	8
AST (7th Day)											
4481-k	0	0	0	9	0	0	0	20	0	0	32
5974-k	0	0	0	25	0	21	0	0	0	0	30
5674-k	0	0	0	16	0	0	0	0	0	0	13
4787-k	0	0	0	0	0	0	0	23	0	0	9

In Trial 1, 25% of the *K. pneumoniae* strains showed susceptibility to Meropenem. The susceptibility was maintained during the course of exposure (Day 3 to Day 7).

Table 11: Comparative analysis of antibiotic resistance pattern of ***P. aureginosa*** strains after Ceftriaxone exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (Initial)											
Zone of Inhibition (mm)											
5961-p	0	0	0	0	0	0	0	0	0	0	0
5973-p	0	0	0	0	0	0	0	22	0	0	24
AST (3rd Day)											
5961-p	0	0	0	17	0	23	0	15	0	0	9
5973-p	0	0	0	9	0	8	0	17	0	0	30
AST (5th Day)											
5961-p	0	0	0	17	0	19	0	15	0	0	11
5973-p	0	0	0	13	0	8	0	21	0	0	32
AST (7th Day)											
5961-p	0	0	0	17	0	20	0	16	0	0	10
5973-p	0	0	0	10	0	0	0	20	0	0	32

In Trial 1, 50% of the *P. aeruginosa* strains showed susceptibility to Tetracycline. Also, 50% of the strains were intermediate for Levofloxacin and Amikacin.

Table 12: Comparative analysis of antibiotic resistance pattern of ***E. coli*** strain after Ceftriaxone exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (initial)											
Zone of Inhibition (mm)											
4368-e	0	0	0	23	0	0	0	19	0	0	33
AST (3rd Day)											
4368-e	0	0	0	22	0	14	0	20	0	12	35
AST (5th Day)											
4368-e	0	0	0	26	0	12	0	22	0	10	35
AST (7th Day)											
4368-e	0	0	0	20	0	7	0	22	0	10	32

In Trial 1, the resistant *E. coli* strain was intermediate to Tetracycline after Day 3 and Day 5. However, the strain showed resistance at Day 7.

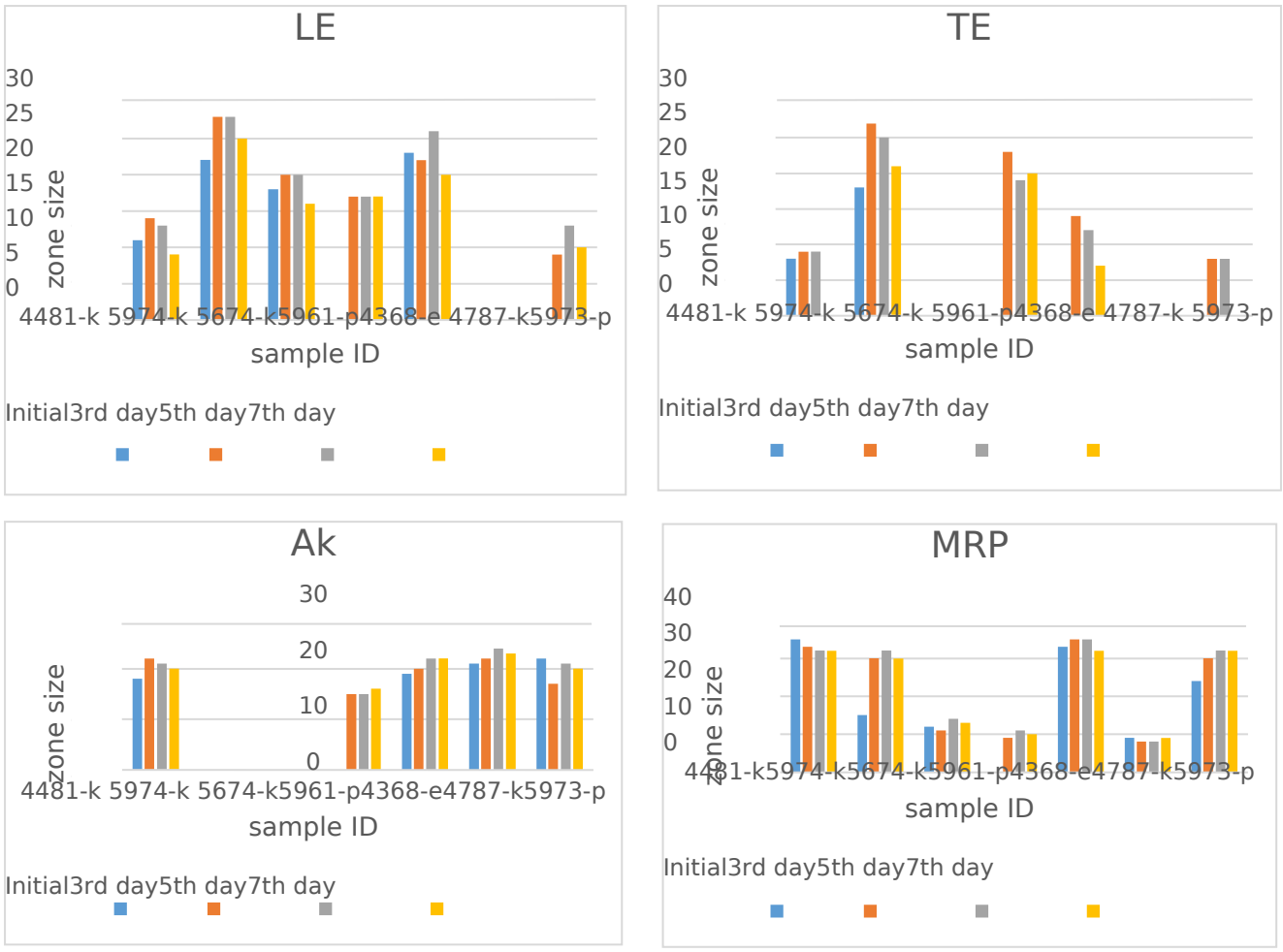


Figure 05: Changes in zone size over time in various antibiotic agents (Exposure to Ceftriaxone)

3.3.2. Trial 2- Exposure to Penicillin

For the second antibiotic trial, Penicillin was chosen as an antibiotic. All the Antibiotic susceptibility test results were organized in the tables mentioned below.

Table 13: Comparative analysis of antibiotic resistance pattern of ***K. pneumoniae*** strains after Penicillin exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (Initial)											
Zone of Inhibition (mm)											
4481-k	0	0	0	11	0	8	0	18	0	0	35
5974-k	0	0	0	22	0	18	0	0	0	0	15
5674-k	0	0	0	18	0	0	0	0	0	0	12
4787-k	0	0	0	0	0	0	0	21	0	0	10
AST (3rd Day)											
4481-k	0	0	0	11	0	8	0	16	0	0	30
5974-k	0	0	0	31	0	25	0	0	0	0	31
5674-k	0	0	0	19	0	0	0	0	0	9	14
4787-k	0	0	0	0	0	7	0	18	0	0	9
AST (5th Day)											
4481-k	0	0	0	11	0	8	0	17	0	0	30
5974-k	0	0	0	29	0	26	0	0	0	0	30
5674-k	0	0	0	17	0	0	0	0	0	9	12
4787-k	0	0	0	0	0	0	0	18	0	0	9
AST (7th Day)											
4481-k	0	0	8	15	0	10	0	19	0	0	32
5974-k	0	0	0	29	0	23	0	0	0	0	31
5674-k	0	0	0	20	0	0	0	0	0	9	15
4787-k	0	0	0	0	0	9	0	21	0	0	11

In Trial 2, 25% of the resistant *K. pneumoniae* strains showed susceptibility against Meropenem.

Table 14: Comparative analysis of antibiotic resistance pattern of ***P. aeruginosa*** strains after Penicillin exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST(initial)											
Zone of Inhibition (mm)											
5961-p	0	0	0	0	0	0	0	0	0	0	0
5973-p	0	0	0	0	0	0	0	22	0	0	24
AST (3rd Day)											
5961-p	0	0	0	18	0	23	0	10	0	0	11
5973-p	0	0	0	22	0	10	0	23	0	0	31
AST (5th Day)											
5961-p	0	0	0	17	0	23	0	10	0	0	9
5973-p	0	0	0	22	0	10	0	22	0	0	35
AST (7th Day)											
5961-p	0	0	0	13	0	23	0	14	0	0	11
5973-p	20	0	0	22	0	10	0	23	0	0	35

In Trial 2, 50% of the *P. aeruginosa* strains showed susceptibility to Levofloxacin, Tetracycline, and Amoxicillin. For Levofloxacin, one resistant strain (5961-p) was intermediate at Day 3 and Day 5. However, the strain showed resistance at Day 7.

Table 15: Comparative analysis of antibiotic resistance pattern of ***E. coli*** strain after Penicillin exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST(initial)											
Zone of Inhibition (mm)											
4368-e	0	0	0	23	0	0	0	19	0	0	33
AST (3rd Day)											
4368-e	0	0	0	24	0	11	0	15	0	12	34
AST (5th Day)											
4368-e	0	0	0	24	0	10	0	20	0	11	31
AST (7th Day)											
4368-e	0	0	0	21	0	12	0	20	0	12	33

In Trial 2, the *E. coli* strain showed no significant outcome to the antibiotic agents.

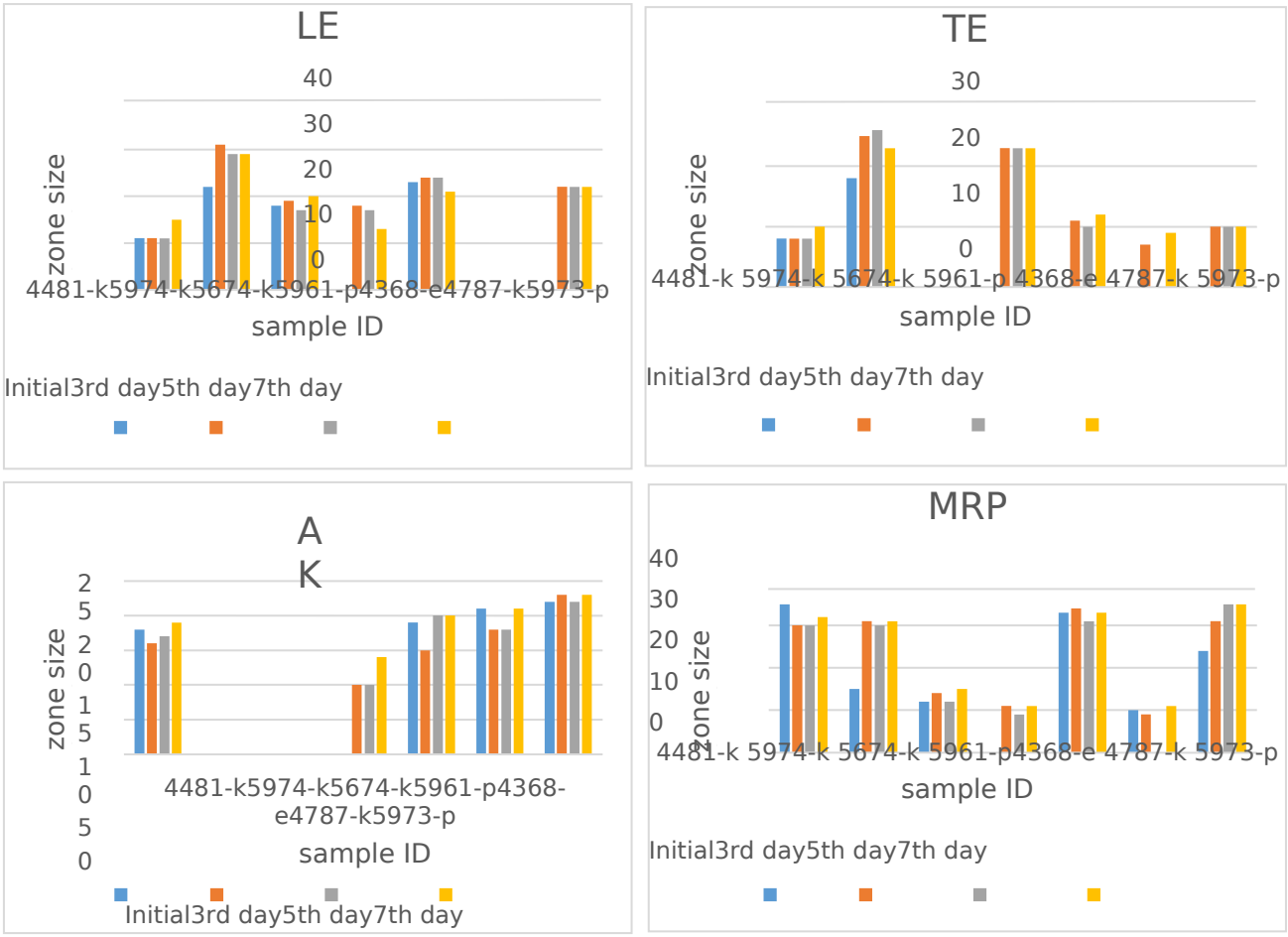


Figure 06: Changes in zone size over time in various antibiotic agents (Exposure to Penicillin)

3.3.3. Trial 3- Exposure to Cefixime

For the third antibiotic trial, Cefixime was chosen as an antibiotic. Unfortunately, no data was generated in this trial.

3.3.4. Trial 4- Exposure to Linezolid

Trial 4 was the last individual experimental trial. For this, Linezolid was chosen as an antibiotic. **Table 16:** Comparative analysis of antibiotic resistance pattern of ***K. pneumoniae*** after Linezolid exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST(initial)											
4481-k	0	0	0	11	0	8	0	18	0	0	35
5974-k	0	0	0	22	0	18	0	0	0	0	15
5674-k	0	0	0	18	0	0	0	0	0	0	12
4787-k	0	0	0	0	0	0	0	21	0	0	10
AST (3rd Day)											
4481-k	0	0	0	10	0	15	0	-	0	0	30
5974-k	0	0	0	22	0	23	0	-	0	0	32
5674-k	0	0	0	17	0	22	0	-	0		13
4787-k	0	0	0	0	0	14	0	-	0	0	9
AST (5th Day)											
4481-k	0	0	0	9	0	15	0	20	0	0	33
5974-k	0	0	0	19	0	19	0	0	0	0	31
5674-k	0	0	0	16	0	19	0	0	0	10	13
4787-k	0	0	0	0	0	14	0	0	0	0	10
AST (7th Day)											
4481-k	0	0	0	9	0	14	0	0	0	0	30
5974-k	0	0	0	20	0	16	0	0	0	0	32
5674-k	0	0	0	16	0	21	0	0	0	11	13
4787-k	0	0	0	15	0	19	0	0	0	9	12

In Trail 4, 75% of the *K. pneumonia* strains showed susceptibility to Tetracycline. To elaborate, one specific strain (4481-k) showed susceptibility at Day 3 and Day 5. But was intermediate on Day 7. Another stain (4787-k) went from intermediate at Day 3 and Day 5 to susceptible at Day 7. Besides, 25% of the strains showed susceptibility to Meropenem.

Table 17: Comparative analysis of antibiotic resistance pattern of ***P. aureginosa*** after Linezolid exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST(initial)											
Zone of Inhibition (mm)											
5961-p	0	0	0	0	0	0	0	0	0	0	0
5973-p	0	0	0	0	0	0	0	22	0	0	24
AST (3rd Day)											
5961-p	0	17	30	22	0	23	0	22	0	0	32
5973-p	0	0	0	18	0	0	0	23	0	0	34
AST (5th Day)											
5961-p	0	15	25	22	0	22	0	22	0	0	32
5973-p	0	0	0	15	0	0	0	14	0	0	10
AST (7th Day)											
5961-p	0	15	25	27	0	21	0	19	0	9	30
5973-p	0	0	0	14	0	0	0	13	0	0	10

In Trial 4, 50% of the *P. aureginosa* strains showed susceptibility to Ceftriaxone, Levofloxacin, Tetracycline, Amikacin and Meropenem.

Table 18: Comparative analysis of antibiotic resistance pattern of ***E. coli*** after Linezolid exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST(initial)											
4368-e	0	0	0	23	0	0	0	19	0	0	33
AST (3rd Day)											
4368-e	0	0	0	22	0	21	0	-	0	10	33
AST (5th Day)											
4368-e	0	0	0	11	0	0	0	0	0	10	34
AST (7th Day)											
4368-e	0	0	0	10	0	0	0	0	0	0	14

In Trial 4, the *E. coli* strain showed susceptibility after Day 3. However, it went back to resistant at Day 5 and 7.

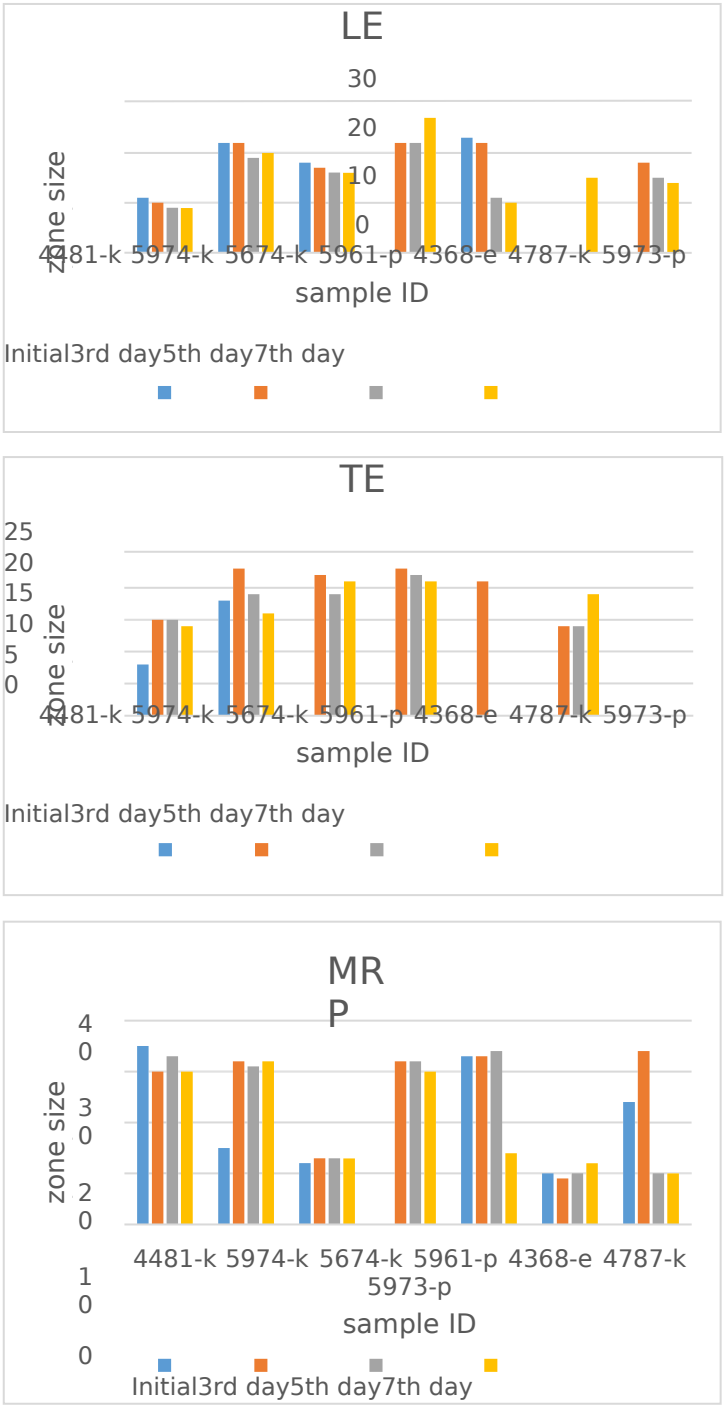


Figure 07: Changes in zone size over time in various antibiotic agents (Exposure to Linezolid)

3.4. Combination Trials

Here, the bacterial samples were exposed to two antibiotics. 2 combination trials were examined in the study. Any changes in the resistant pattern were observed after antibiotic exposure. This was achieved by the comparison of the initial AST result with 5th Day AST result.

3.4.1. Trial 5- Exposure to Linezolid with Ceftriaxone

For the first combination trial, Linezolid was used with Ceftriaxone. 10 bacterial isolates (5 *K. pneumoniae*, 4 *P. aeruginosa*, and 1 *E. coli*) were selected for this trial.

Table 19: Comparative analysis of antibiotic resistance pattern of ***K. pneumoniae*** after (Linezolid+ Ceftriaxone) exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (initial)											
Zone of Inhibition (mm)											
4481-k	0	0	0	11	0	8	0	18	0	0	35
5974-k	0	0	0	22	0	26	0	0	0	0	15
5674-k	0	0	0	18	0	0	0	0	0	0	12
4787-k	0	0	0	0	0	0	0	21	0	0	10
5944-k	0	0	0	0	0	0	27	22	0	0	0
AST 5th day											
4481-k	0	0	0	10	0	0	0	19	0	0	33
5974-k	0	0	0	11	0	0	0	21	0	0	34
5674-k	0	0	0	0	0	0	0	0	0	0	19
4787-k	0	0	0	0	0	0	0	21	0	0	10
5944-k	0	0	0	10	0	0	0	19	0	0	32

In Trial 5, 20% of the *K.pneumonia* strains showed susceptibility to Amikacin. And 40% of the strains showed susceptibility to Meropenem.

Table 20: Comparative analysis of antibiotic resistance pattern of ***P. aeruginosa*** after (Linezolid+ Ceftriaxone) exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (initial)											
Zone of Inhibition (mm)											
5961-p	0	0	0	0	0	0	0	0	0	0	0
5945-p	0	0	0	0	0	0	0	15	0	0	16
5965-p	0	0	0	0	0	0	0	21	0	0	9
5973-p	0	0	0	0	0	0	0	22	0	0	24
AST 5th day											
5961-p	0	0	0	17	0	11	18	14	0	0	12
5945-p	0	0	0	0	0	0	0	0	0	0	17
5965-p	0	0	0	0	0	0	0	20	0	0	11
5973-p	0	0	0	20	0	0	0	0	0	0	16

In Trial 5, 25% of the *P. aeruginosa* strains showed susceptibility to Trimethoprim. 2 strains were intermediate to Levofloxacin.

Table 21: Comparative analysis of antibiotic resistance pattern of the ***E. coli*** strain after (Linezolid+ Ceftriaxone) exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (Initial)											
Zone of Inhibition (mm)											
4368-e	0	0	0	23	0	0	0	19	0	0	33
AST 5th day											
4368-e	0	0	0	23	0	0	0	21	0	0	35

In trial 5, the *E. coli* strain showed no significant outcome to the antibiotic agents.

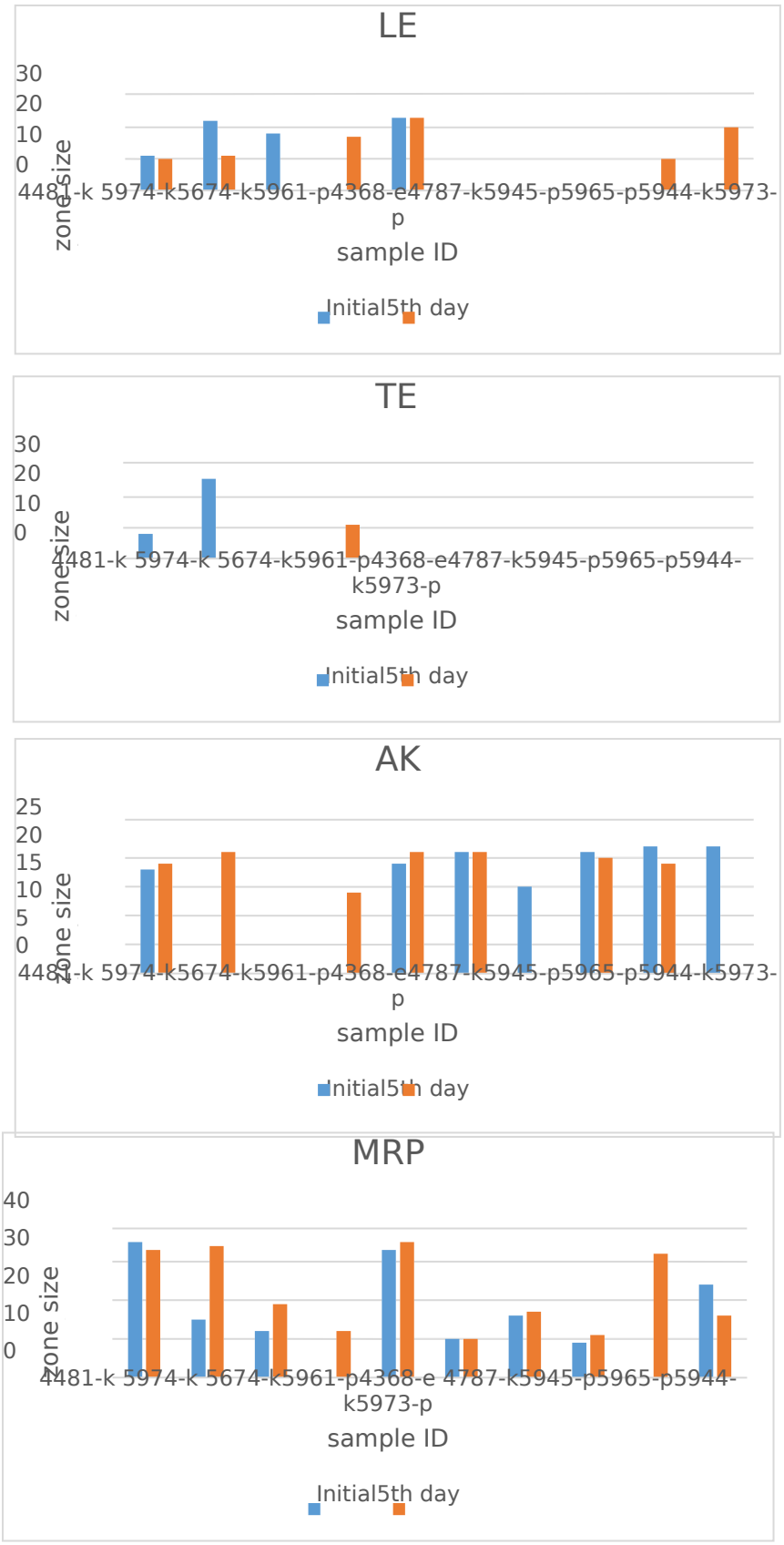


Figure 08: Changes in zone size over time in various antibiotic agents (Exposure to Linezolid with Ceftriaxone)

3.4.2. Trial 6- Exposure to Linezolid with Tigecycline

For the last combination trial, Linezolid was used with Tigecycline. 8 bacterial isolates (3 *K. pneumoniae*, 4 *P. aeruginosa* and 1 *E. coli*) were selected for this trial.

Table 22: Comparative analysis of antibiotic resistance pattern of ***K. pneumoniae*** after (Linezolid + Tigecycline) exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (initial)											
Zone of Inhibition (mm)											
4481-k	0	0	0	11	0	8	0	18	0	0	35
4787-k	0	0	0	0	0	0	0	21	0	0	10
5674-k	0	0	0	18	0	0	0	0	0	0	12
AST 5th day											
4481-k	0	0	14	12	0	0	0	22	0	0	30
4787-k	0	0	0	0	0	21	28	21	0	0	28
5674-k	0	0	0	18	0	0	0	0	0	0	19

In Trail 6, 33% of the *K. pneumonia* strains showed susceptibility to Tetracycline, Trimethoprim and Meropenem.

Table 23: Comparative analysis of antibiotic resistance pattern of ***P.aeruginosa*** after (Linezolid + Tigecycline) exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (initial)											
Zone of Inhibition (mm)											
5961-p	0	0	0	0	0	0	0	0	0	0	0
5945-p	0	0	0	0	0	0	0	15	0	0	16
5965-p	0	0	0	0	0	0	0	21	0	0	9
5973-p	0	0	0	0	0	0	0	22	0	0	24
AST 5th day											
5961-p	0	13	22	13	0	0	21	23	0	0	30
5945-p	0	0	10	0	0	0	0	28	0	0	31
5965-p	0	0	0	0	0	0	0	20	0	0	12
5973-p	0	0	0	0	0	0	0	23	0	0	22

In Trial 6, 50% of the *P. aeruginosa* strains showed susceptibility to Meropenem and Amikacin. 25% of the strains showed susceptibility to Ceftriaxone and Trimethoprim.

Table 24: Comparative analysis of antibiotic resistance pattern of ***E. coli*** after (Linezolid + Tigecycline) exposure

Sample ID	P	CFM	CTR	LE	LZ	TE	COT	AK	AMX	E	MRP
AST (initial)											
4368-e	0	0	0	23	0	0	0	19	0	0	33
AST 5th day											
4368	0	0	9	20	0	10	0	19	0	0	31

In Trial 6, no significant change was observed for the *E. coli* strain.

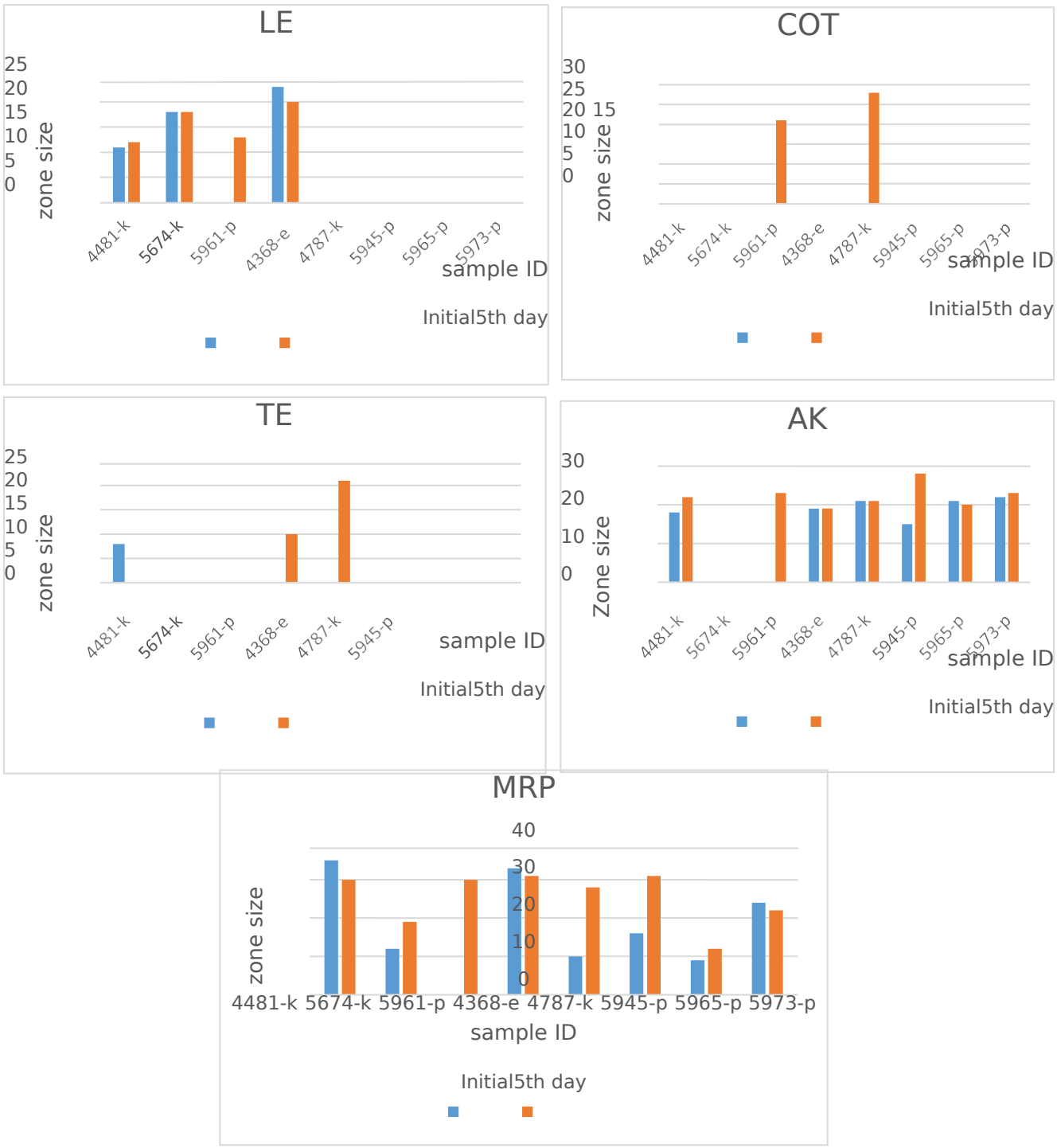


Figure 09: Changes in zone size over time in various antibiotic agents (Exposure to Linezolid with Tigecycline)

3.5. Comparison of the Individual Antibiotic Trials

The individual antibiotic trials were compared to observe the effectiveness of the trials.

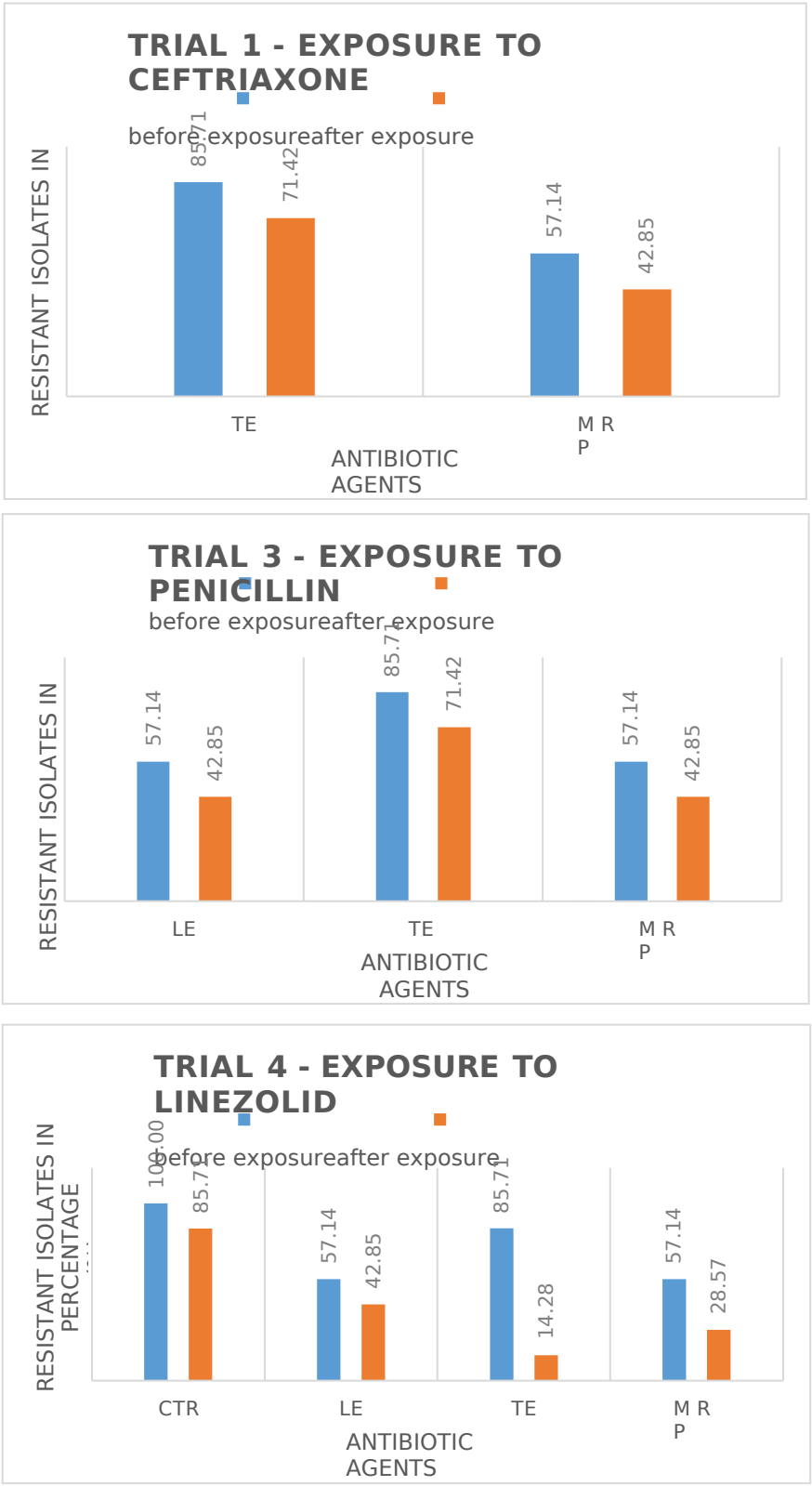


Figure 10: Comparative analysis of the individual trials

3.6. Comparison of the Combination Antibiotic Trials

The combination antibiotic trials were compared to observe the effectiveness of the trials.

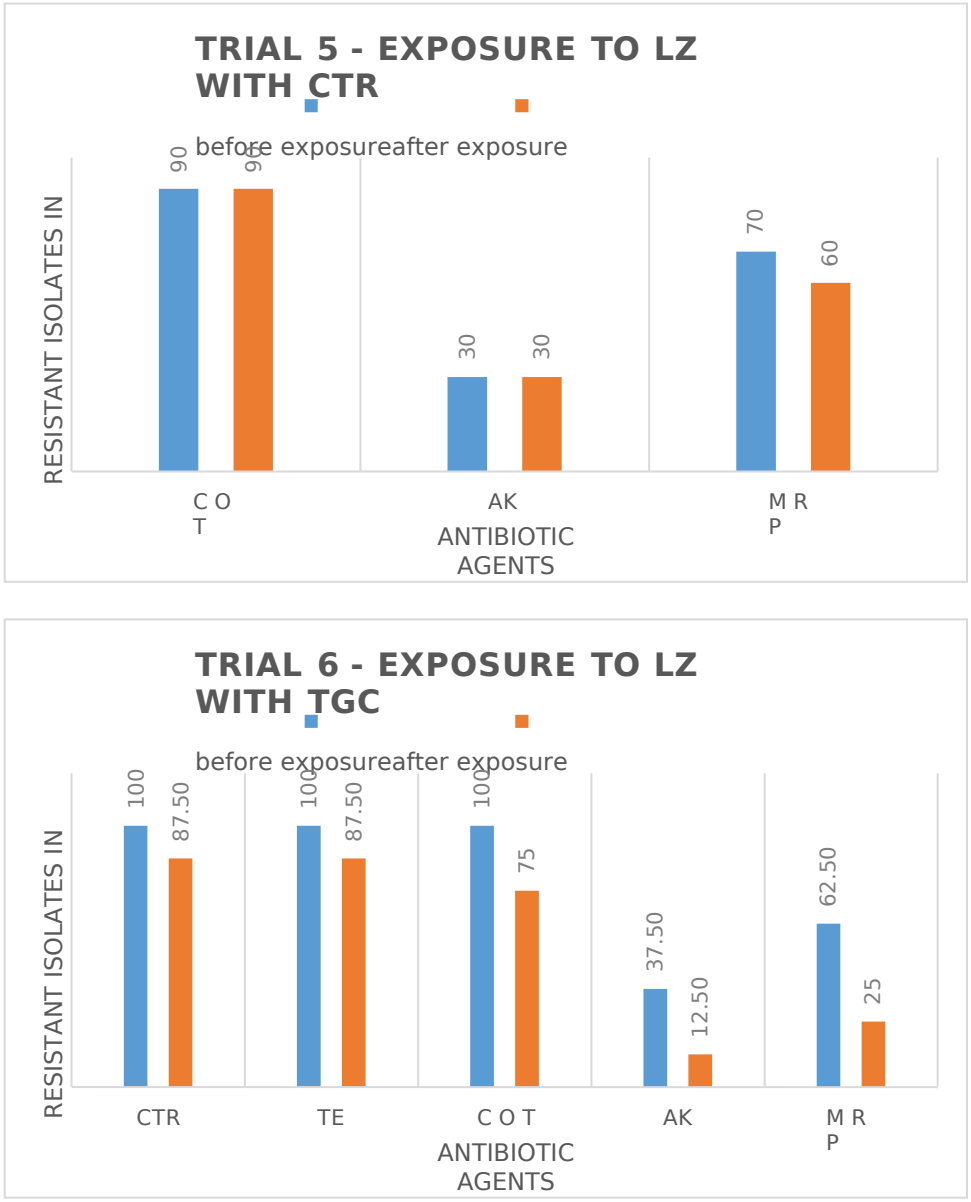


Figure 11: Comparative analysis of the combination trials

Chapter 4

Discussion

In this research, both *K. pneumoniae* and *P. aeruginos*a showed effective outcomes. *Klebsiella* strains showed susceptibility to Meropenem in both individuals as well as combination trials. Though Carbapenem family, specifically Meropenem usually works for gram-negative bacteria but in the case of resistant *Klebsiella* strains, Meropenem itself didn't show any result. In a study, 45.5% *Klebsiella* isolates were resistant to Meropenem. (Elmanakhly et al 2022). In our experiment, when exposed to other antibiotics such as Ceftriaxone, Linezolid, and Penicillin (Trail 1, 2, and 3), Meropenem showed promising results towards the resistant *Klebsiella*. On the other hand, *Pseudomonas* strains showed susceptibility to Tetracycline in all the individual trials. Currently, a few strains of multidrug-resistant *Pseudomonas aeruginosa* are resistant to many antibiotics including Tetracycline. In our trials, our findings say that resistant *P. aeruginosa* strains were susceptible to Tetracycline after exposure to other antibiotics such as Ceftraixone, Penicillin, and Linezolid.

During antibiotic trials, the selected bacterial strains showed various changes in zone size which include an increase in zone size, a decrease in zone size, and also consistent change in zone size. An increase in zone size was mostly noticed on Day 3 and sometimes Day 5. On the contrary, one common phenomenon was observed that showed a decrease in zone size on Day

7. According to the findings of recent studies, longer courses of antibiotics are not necessarily advantageous. Shorter-course antibiotics are similarly effective, and in some circumstances, they may even be favored in specific disease processes. Moreover, with a shorter course of antibiotic exposure, it lowers the risk of antibiotic resistance (Leimbach, 2016).

Among the 11 antibiotic agents used in Antibiotic susceptibility testing, LE, AK, TE, and MRP showed effective and consistent results for both the individual and combination trials. On the other hand, P, CFM, CTR, LZ, COT, AMX, and E showed insignificant change overall.

In case of the individual antibiotic trials, Trial 4 (Linezolid) showed its most effectiveness compared to Trial 1 (Ceftriaxone) and Trial 2 (Penicillin). In the exposure of Linezolid, both *klebsiella,* and *pseudomonas* showed effective susceptibility to Tetracycline. Mentionable, no data of Trial 3 (Cefixime) was collected due to the unavailability of the injectable form of antibiotic.

Based on the outcomes of the individual trials, Linezolid was chosen to perform more trials for antibiotic combinations. Two antibiotics were combined with Linezolid to examine two separate trials, Trail 5 namely (Linezolid with Ceftriaxone) and Trail 6 (Linezolid with Tigecycline). Among these, Trail 6 demonstrated effective results. In the case of *Pseudomonas* and *Klebsiella,* along with this combination antibiotics such as ceftriaxone, co-trimoxazole, Amikacin or Meropenem can be prescribed depending on the requirements. (Trail 6).

Linezolid is antibiotic from the Oxazolidinones class which is mostly used to treat infections caused by aerobic gram-positive bacteria. Approved in the year 2000, Linezolid has been the first member of the oxazolidines class which is widely used to treat skin and tissue infections. However, according to the data of our studies, we have come up with an aspect that Linezolid can be also used to treat gram-negative bacteria that cause skin and tissue infection. From Trail 4 (Linezolid) and Trail 6 (Linezolid+ Tigecycline), we can observe that both in cases of *K.pneumoniae* and *P.aeruginosa* these antibiotic has effective changes in the zone side. Linezolid and Tigecycline can be prescribed up to day five because there is a noticeable decline in zone size by day seven. we can assume that Linezolid along with the combinations can be another effective drug till 5^{th} day of the antibacterial dose in case of skin infections caused by gram-negative *P.aeruginosa*.

Previous studies showed that the combination of Linezolid along with ε-poly-l-lysine on the silica xerogel can trigger antibacterial activity not on in gram-positive bacteria but also in

gram-

negative bacteria making it suitable for clinical use (Guzul et al. 2020). A recently conducted study shows that Linezolid and polymyxin B worked together to significantly decrease bacterial growth and activity against *Klebsiella* infection in vitro and in vivo. Those studies showed that polymyxin B enhances the linezolid activity resulting in to decline in the growth of the organism (Huang et al.,2022). Another study also showed that Eravacycline with Aztreonam or Ceftazidime repressed the resistance development of *Klebsiella* (Xu et al. 2022). Although, a few cases might observe that Linezolid along with other combination trials doesn't have any adverse effect on *Klebsiella*, our conducted study states that Klebsiella worked with Meropenem when trailed with Linezolid. *Klebsiella* also developed sensitivity with the exposure of Linezolid with tetracycline in a few of the strains. (Trail 4). An alike case shows that Carbapenem resistance *Klebsiella* showed sensitivity in tetracycline and aminoglycosides. This comes to the result that, Tetracycline with exposure to another antibiotic such as Linezolid can be a potent combination of treatment.

To our knowledge, the concept of collateral sensitivity is yet to be widely used in the field of medical science. A similar study had been conducted where this therapeutical treatment has already been applied in cystic fibrosis (CF) patients associated with chronic *Pseudomonas aeruginosa* infections. As the experiment shows the infection under observation featured several resistant subpopulations, whose relative frequency increased significantly after treatment and, notably, as predicted by laboratory-evolved CS in the PAO1 strain and numerous clinical strains (Roemhild, 2021). This result can be an exceptional example of the CS study in clinical field.

According to studies conducted, new aminoglycosides (amikacin, gentamicin, and plazomicin), carbapenems (doripenem, imipenem, and meropenem), and Fosfomycin works well with linezolid are resistant towards MDR bacteria (Valderrama et al., 2020). Moreover,

Linezolid combines with a couple of Amikacin combinations to combat micrococcus organisms in addition to gram-negative bacteria (Valderrama et al. 2020).

In our study, we have experimented with a smaller number of samples. The study could have been more extensive if there had been access to a large number of resistant sample strains specifically *Pseudomonas* and *Klebsiella*. Besides, more antibiotic trials and combinations could have been tested throughout our research period. To elaborate, both individual and combination trials will give more data to work with.

CS treatment becomes more challenging as the efficiency of prolonging antibiotics cannot be predicted while ongoing treatment on a patient. The lack of predictability also could result from sampling a single colony from each population, which could happen if populations become significantly more diverse over time. The patterns of collateral effects often varying even between mutants evolved to the same drug bringing more difficulty in collateral sensitivity treatment. As previous studies showed the experiments conducted work accurately with a few families of antibiotics example tetracycline and trimethoprim, depending on the mutated organism, the in vitro and in vivo results become quite indifferent when applied clinically. It is predicted that due to the small population, the experiment works but when exposed to a larger bacterial sample the antibiotics combination failed to act. (Ardell and Kryazhimskiy, 2021). This is one of the current factors contributing to the clinical execution of the CS treatment failing.

The most crucial thing is to follow the doctor's instructions. Antibiotic misuse can result in increased antibiotic exposure and a higher likelihood of resistance. Apart from that, to develop a more long-lasting treatment for resistant bacteria, the genomic study of resistant mechanisms is required in the future. The molecular level study is important as a mechanistic understanding of CS will help to develop drug switches that combat resistance. Sequential multidrug

treatments that alternate between antibiotics with the concept of collateral sensitivity might slow the evolution of resistance. Moreover, a genetic-level study where the genotypic and phenotypic changes of an organism can be observed needs to be conducted to observe the changes in mutants before and after a collateral combination of antibiotics. For example, in a recent assessment, a system was built to help find out the changes in mutants of *Escherisia coli* to build a therapeutical treatment. Similarly, changes in the genome, transcriptome, metabolome, and so on due to environmental stress can be analyzed to target a specific region that allows susceptible development. An experiment used genome-wide analysis of the strains constructed by high-throughput laboratory evolution to find out the changes due to environmental stress which can be very useful for future clinical aspects (Horinouchi, 2017).

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