# The combined effect of Moxifloxacin and Ciprofloxacin against multidrug-resistant *Klebsiella pneumoniae*

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of B.Sc. in Biotechnology


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## Declaration:

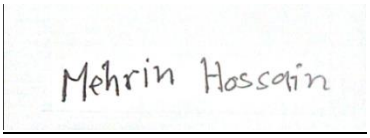
It is hereby declared that

1. The thesis submitted is our own original work while completing a 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.

4. We have acknowledged all main sources of help.



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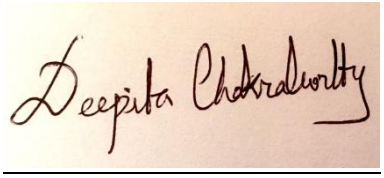

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# Dedicated To

# Family, Friends and Faculties

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## Abstract:

People, particularly children under the age of five suffer from pneumonia. One of the supreme pathogens of Pneumonia is *Klebsiella pneumoniae* which needs to be treated but it's being burdensome because organisms are getting vastly antimicrobial resistant. Antibiotic resistance is a major problem of the present time; it is currently wreaking havoc on people's lives and livelihoods around the world, and it is expected to grow exponentially in the coming decades if it's left without treatment. The goal of the research is based on originating an effective way to fight diseases caused by multidrug-resistant *Klebsiella pneumoniae* in Bangladesh.

From collected bacterial samples, 6 out of 9 were multidrug-resistant. Antibiogram was performed using 19 antibiotics of different classes (Macrolides, Polymyxins, Tetracycline, Nitroimidazole, Cephalosporin, Beta-lactam, Quinolone, Fluoroquinolones, Aminoglycosides, and Penicillin) to differentiate between resistant and sensitive isolates. Then, the research was continued by the screening of several combinations of antibiotics (azithromycin, chloramphenicol, and ciprofloxacin) with moxifloxacin. Individual antibiotics and combinations of screened antibiotics activity were measured using the Minimum Inhibitory Concentration (MIC) determination method. Among the 3 combinations which were screened, moxifloxacin in combination with ciprofloxacin exhibited the best result. Hence, this combination was chosen. This was followed by the Fractional Inhibitory Concentration (FIC) index to provide statistical substantiation of results. The lowest obtained FIC index was 0.5 which indicates a synergistic effect.

The research result showcases that a combination of antibiotics can decrease the needed amount of antibiotics on resistant organisms. These findings could have far-reaching consequences for the future of combination therapy against multidrug-resistant *Klebsiella pneumoniae*.

## Contents:

## List of Acronyms:

| Abbreviations | Full forms |
|---|---|
| MHA | Muller Hington Agar |
| BHI | Brain Heart Infusion |
| MAC | Macconkey Agar |
| WHO | World health Organization |
| MDR | Multi Drug Resistant |
| XDR | Extensively Drug Resistant |
| PDR | Pan Drug Resistant |
| MIC | Minimum Inhibitory Concentration |
| FIC | Fractional Inhibitory Concentration |
| µg | Microgram |
| mg | Milligram |
| *K.P* | *Klebsiella pneumoniae* |

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# CHAPTER 1

## Introduction and Literature review

# 1. Introduction

Pneumonia is a disease that causes inflammation of the lungs. This can be caused by a broad range of bacteria and viruses. Pneumonia is one of the prime reasons for child death under five years old causing almost 16% of child death under five years old ("Pneumonia and other respiratory diseases", 2010). In Bangladesh, the percentage of child death under five years old is around 28% ("Pneumonia and other respiratory diseases", 2010). Every year the number of child deaths caused by pneumonia in Bangladesh is 50,000 ("Pneumonia and other respiratory diseases", 2010). Therefore, the severity of pneumonia globally and especially in Bangladesh is prominent.

In research where 375 clinical samples from Mymensingh, Bangladesh were examined and ESBL gene was detected in 51.4% of *Klebsiella pneumoniae* samples (Khan et al., 2018). The ESBL gene is responsible for causing antimicrobials such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines, and chloramphenicol resistant *Klebsiella pneumoniae*. (Rawat & Nair, 2010). This fact indicates the prevalence of antibiotic-resistant *Klebsiella pneumoniae* in Bangladesh. Hence, finding ways to battle this drug-resistant *Klebsiella pneumoniae* problem has become a major concern for Bangladesh.

A way of tackling the resistant organism problem can be antibiotic combination therapy. A combination of a broad spectrum β-lactam and an aminoglycoside or a fluoroquinolone can be used against *Pseudomonas spp* (Tängdén, 2014). Research also suggested that using a carbapenem with a MIC of $\leq$4 mg/L in the combination can give better results (Tängdén, 2014).

To treat antibiotic-resistant *Klebsiella pneumoniae,* polymyxin-based antibiotic combinations can give the desired result (Jacobs et al., 2017). Some other prominent combinations to fight against antibiotic-resistant *Klebsiella pneumoniae* can be ceftazidime + avibactam combination , β-lactam/β-lactamase inhibitor combinations such as ceftolozane + tazobactam and aztreonam–avibactam (Jacobs et al., 2017).

This study will try to determine the efficiency of moxifloxacin in combination with other antibiotics against antibiotic-resistant *Klebsiella pneumoniae* found in the clinics of Bangladesh.

## 1.1: Characteristics and Morphology of Klebsiella pneumoniae

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: Klebsiella
Species: ***Klebsiella pneumoniae***

*Klebsiella pneumoniae* is a gram-negative, rod-shaped *Enterobacteriaceae*. It is non-motile, meaning the bacteria grows only in the stabbed line. It is a lactose fermenting bacteria. This bacteria has a capsule around it. This capsule protects the bacteria from the adverse environmental effects and the activity of antibiotics. This makes the bacteria more fatal (Lenchenko et al., 2020). The structure of *Klebsiella pneumoniae* consists of i) capsular and somatic antigens and ii) endotoxins. Moreover, some strains of *Klebsiella pneumoniae* can manufacture exotoxins. These characteristics make *Klebsiella pneumoniae*'s antigenic structure complex (Lenchenko et al., 2020). *Klebsiella pneumoniae* also carries mucus viscosity-associated gene A (magA) and a regulator of mucoid phenotype A (rmpA) gene. These genes add to the virulence factor of *Klebsiella pneumoniae* (Ito et al., 2015).

## 1.2: Diseases caused by *Klebsiella pneumoniae*

***Klebsiella pneumoniae*** causes-
Pneumoniae
Meningitis
Bloodstream infection

Urinary tract infection
Abscess.

*Klebsiella pneumoniae* is normally found in the intestines. It is harmless in that part of the body. However, when it is spread in other areas of the body such as the lungs, bladder, blood, wounds, livers, eyes, and brains it can infect these parts. (Nunez, 2019). The infection to other organisms occurs generally during the treatment of another disease. Generally, patients dependent on devices, for instance, respiratory machines, catheters, feeding tubes, etc, and patients consuming extensive antibiotic courses are more prone to get infected by *Klebsiella pneumoniae* ("Klebsiella pneumoniae in Healthcare Settings," 2010). This bacteria has a liking for the upper lobe so these are the most sensitive part of the body to get infected ( Corrin & Nicholson, 2011).

## 1.3: Emergence of antibiotic-resistant *Klebsiella pneumoniae*:

Antibiotic-resistant organisms are one of the biggest threats to human beings in the 21st century. The presence of antibiotic-resistant bacteria is on the rise. Among those bacteria, *Klebsiella pneumoniae* is a prominent one. *Klebsiella pneumoniae* exhibits resistance against a wide range of antibiotics. β-lactam, fluoroquinolones, and aminoglycosides are examples of some of those antibiotics. (Silva et al., 2019). This bacteria is gaining resistance against commonly used antibiotics. This fact is making the treatment of *Klebsiella pneumoniae* more and more challenging. The escalation of transmissible plasmid and emergence of resistance genes originating when the gene is transferred horizontally are some causes behind resistant characteristics of organisms (Silva et al., 2019). In research for biofilm formation in Indonesia around 167 isolates of *Klebsiella pneumoniae* were collected from hospitals and tested among which 54.49% isolates were multidrug-resistant *Klebsiella pneumoniae*.(Nirwati et al., 2019).

## 1.4: Mechanism of antibiotic-resistant *Klebsiella pneumoniae*

The main reason behind antibiotic-resistant *Klebsiella pneumoniae* is the prevalence of *Klebsiella pneumoniae* carbapenemases (KPC). Carbapenemases are an enzyme that is β-lactamases. This enzyme carries hydrolytic activity which means it can break a chemical

compound with the help of the $H_2O$ reaction (Queenan & Bush, 2007). KPC coding genes are present on the transferable plasmids (Fehlberg et al., 2012). KPC enzyme hydrolyzes penicillins, cephalosporins, monobactams, and carbapenems, and is prevented by clavulanic acid and tazobactam (Fehlberg et al., 2012). As a result penicillins, cephalosporins, monobactams, and carbapenems antibiotics do not work on KPC-producing *Klebsiella pneumoniae*. This helps *Klebsiella pneumoniae* to gain resistance against antibiotics.

Normal non-pathogenic *Klebsiella pneumoniae* which belongs to the human gut can transform into carbapenem-resistant Enterobacterales. This *Klebsiella pneumoniae* is called CRE (carbapenem-resistant Enterobacterales) which exhibits high levels of resistance against antibiotics ("Klebsiella pneumoniae in Healthcare Settings," 2010).

## 1.5: Epidemiology of antibiotic resistant *Klebsiella pneumoniae*

The emerging resistance of ***Klebsiella pneumoniae*** against a wide variety of antibiotics has changed the effectiveness of antibiotics on this organism. In a research of studying 27384 isolates of ***Klebsiella pneumoniae*** in Saudi Arabia, it was observed resistance rate -

- Amoxicillin - 72% - High resistance rate
- Ampicillin - 99.9% - High resistance rate
- Penicillin - 80.4% - High resistance rate

However, in the Penicillin class, it was shown that a combination of piperacillin and tazobactam can lower the resistance rate by 58.7% (Al-Zalabani et al., 2020).

- Levofloxacin - 57.7% - Low resistance rate
- Ciprofloxacin - 61.1% - Low resistance rate
- Amikacin - 36.3% - Low resistance rate
- Gentamicin - 52.2% - Low resistance rate

Fluoroquinolones exhibited a lower resistance rate compared to penicillin (Al-Zalabani et al., 2020).

- Cephalexin - 92% - High resistance rate

- Cephalothin - 80.8% - High resistance rate
- Cefazolin - 78% - High resistance rate

First, second and third generations of cephalosporins antibiotics exhibited similar high resistance rates ( Al-Zalabani et al., 2020).

Another research on the molecular epidemiology of resistance to antibiotics among *Klebsiella pneumoniae* isolates that took place in Iran showed that 49% of the isolates were resistant against fluoroquinolones, 52% isolates were resistant against tetracycline and 54% isolates were resistant against aminoglycosides (Kashefieh et al., 2021).

These researches prove the fact that the resistance of *Klebsiella pneumoniae* against antibiotics is increasing. Therefore, the treatment of *Klebsiella pneumoniae* is becoming more difficult day by day. The ways of treating this organism are getting narrower.

## 1.6: Overview of Moxifloxacin

Moxifloxacin belongs to the Fluoroquinolones antibiotic class. It is a fourth-generation fluoroquinolone. This antibiotic treats bacterial infection that is related to the skin, sinuses, lungs, or stomach. Moxifloxacin is given in hydrochloride form. Moxifloxacin hydrochloride is a yellow powder as a result, dissolved Moxifloxacin is a yellow color liquid. The oral form of moxifloxacin contains a form of Moxifloxacin 0.5% (5 mg/mL) and an inactive form of Boric acid, sodium chloride, and purified water.

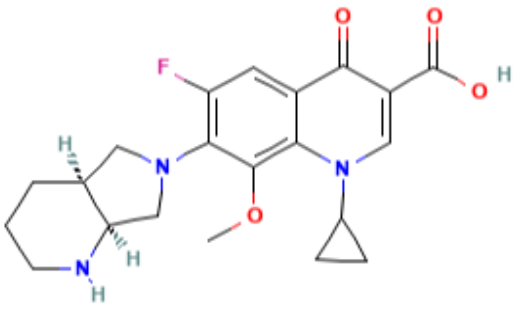



Figure 1: Structure of Moxifloxacin.

In the above picture, the structure of the moxifloxacin is displayed. The structure is composed of 4-oxo-1,4-dihydroquinoline-3-carboxylic acid holding a cyclopropyl substituent at position 1 (" PubChem Compound Summary for CID 152946, Moxifloxacin", 2022). The molecular weight of moxifloxacin is 401.4. The molecular formula is $C_{21}H_{24}FN_3O_4$ (" PubChem Compound Summary for CID 152946, Moxifloxacin", 2022).

Moxifloxacin binds on the bacterial surface and blocks the bacterial enzymes topoisomerase II and topoisomerase IV (" PubChem Compound Summary for CID 152946, Moxifloxacin", 2022). These enzymes are DNA gyrase. So when these enzymes are blocked bacterial DNA replication and DNA repair process is stopped. As a result, the bacteria dies (" PubChem Compound Summary for CID 152946, Moxifloxacin", 2022).

Moxifloxacin treats-

- Respiratory tract infection
- Chronic bronchitis
- Community-acquired pneumonia
- Acute bacterial sinusitis
- Uncomplicated skin
- Skin structure infections

These can be treated using Moxifloxacin (Scholar, 2007).

## 1.7: MDR, XDR, and PDR

MDR, XDR, and PDR are significant terms when antibiotic-resistant organisms are the topic of the discussion. In this modern era of advanced treatment and therapeutics MDR organisms are one of the biggest problems. These three are the categories of resistant organisms. These three categories help to divide the resistant organisms into groups which is helpful in the research sector.

MDR stands for multidrug resistance. If the organism is resistant to at least one key antimicrobial agent it is called MDR (Rex, 2019). As there is no specific definition of MDR to categories gram-positive and gram-negative resistant bacteria it often creates barriers in comparing the data properly. To deal with this problem, in the laboratories organisms are categorized as MDR depending on in vitro antimicrobial susceptibility test results (Magiorakos et al., 2011). The definition which is more commonly used is when an organism (gram-positive or gram-negative) is resistant to three or more antimicrobial classes it is recognized as MDR (Magiorakos et al., 2011).

XDR stands for extensive drug resistance or extremely drug-resistant or extensively drug-resistant. Initially, the XDR term was used to define extensively drug-resistant *Mycobacterium tuberculosis* which is also called XDR MTB (Magiorakos et al., 2011). Two methods are used to define XDR. The first one depends on the number of classes or subclasses an organism is resistant to. The second one depends on the number of key antimicrobial agents the organism is resistant to. The number should be more than one (Magiorakos et al., 2011).

PDR stands for pan drug-resistant. This has come from the Greek prefix “Pan” which means all (Magiorakos et al., 2011). If the organism is resistant to all the key antimicrobial agents then it will be called PDR (Rex, 2019). The organism will be resistant to almost all the antimicrobials available commercially (Magiorakos et al., 2011).

## 1.8: Objectives of the research

- Developing a new sustainable way to deal with the rising problem of antibiotic-resistant ***Klebsiella pneumoniae***.
- Studying the effectiveness of Moxifloxacin in combination with several antibiotics against multi-drug resistant (MDR) ***Klebsiella pneumoniae***.

# CHAPTER 2

# Materials and Method

## 2. Methodology

The research was performed in the laboratories of BRAC University. Experiments were performed to do a prospective study on the effectiveness of moxifloxacin against multi-drug resistant *Klebsiella pneumoniae*.

At first samples of *Escherichia coli* , *Klebsiella pneumoniae* , *Staphylococcus aureus,* and *Pseudomonas* were collected from different clinics. The samples were isolated and grown on nutrient agar. Antibiogram was then executed. Nineteen different antibiotics from 10 different classes were selected for the antibiogram on MHA. The antibiotics were commonly prescribed by doctors for the treatment of bacterial infections. In the result of the antibiogram, the samples showed resistance against 79% of the used antibiotics. This also indicated that all the used samples were both MDR and XDR. However, no PDR was observed.

From the list of resistant antibiotics, 3 antibiotics were chosen to conduct the combination trial with moxifloxacin. Initially, individual Minimum Inhibitory Concentration (MIC) for all 4 antibiotics were determined for the collected 4 organisms. After that MIC's of 3 different antibiotic combinations with moxifloxacin was conducted. According to the initial screening result, it was observed that moxifloxacin in combination with ciprofloxacin exhibited the best result. Moreover, the combination worked best on *Klebsiella pneumoniae*. As a result, *Klebsiella pneumoniae* was selected as the research organism, and moxifloxacin + ciprofloxacin was the chosen combination.

After the initial screening and determination, 9 more samples of *Klebsiella pneumoniae* were collected. Among those 9 samples 6 samples were found to be MDR in the antibiogram, the other 3 were not. Individual MIC of moxifloxacin and ciprofloxacin for each MDR *Klebsiella pneumoniae* sample were determined. From the revived data of all the 6 samples, 10 different combinations were done to run the combination MIC determination process. $C_1V_1 = C_2V_2$ formula was used in the MIC determination process.

Finally, the Fractional Inhibitory Concentration (FIC) was calculated using the individual MIC and combination MIC for all 6 samples. The FIC then was compared with the standard to identify the efficiency of the obtained result.

## 2.1: Sample collection of *Klebsiella pneumoniae*

Clinical samples of *Klebsiella pneumoniae* were collected from the National Institute of Diseases of the Chest and Hospital (NIDCH) and from the microbiology department of Birdem Hospital. The samples were collected on nutrient agar tubes. The samples were subcultured on the nutrient agar slant. It was carefully brought to the BRAC University laboratory and put in the incubator at 37℃ for 24 hours. After the 24 hours incubation, the samples were subcultured again on the nutrient agar dish using the streak plate method, and incubation was done for 24 hours at 37℃.

After the initial growth, each sample's gram staining and other biochemical testing were done to ensure the purity of the sample.

The samples were then streaked on MacConkey agar plates for further subculture. This step was done to avoid contamination.

The samples were also stored at -20℃ in $T_1N_1$ agar and the presence of paraffin oil in the vial. The stock was prepared.

## 2.2: Performed Biochemical testing

**Gram staining:** At first gram staining was conducted where under the microscope the samples exhibited pink rod-shaped bacteria. The result matched the standard.

**Citrate Utilization test:** the sample gave a positive result means the color of the media changed from green to blue.

**MIU ( Motility Indole Urease) test:** The samples grew only in the stabbed line which indicated non-motile characteristics of *K.P*.

Indole was negative so color change occurred.

Urease was weakly positive as the media turned from yellow to pink.

**Lactose fermentation:** On the MacConkey agar plate the colonies of the organism changed the color of the agar from pink to yellow.

**TSI (Triple Sugar iron):** In a TSI slant agar tube, bacteria were inoculated with the help of a needle. The color of the slant and butt changed from red to yellow. This ensured the samples were *Klebsiella pneumoniae*.

Figure 2: Biochemical testing (TSI) result.

## 2.3: Collection of antibiotics

The 4 antibiotics used in MIC determination process were:

1)Moxifloxacin

2)Ciprofloxacin

3)Azithromycin

4)Chloramphenicol

These antibiotics were used in eye drop form except moxifloxacin.

- **Moxifloxacin:** Pharmaceutical grade water-soluble moxifloxacin hydrochloride INN was used. 0.055 mg of moxifloxacin was dissolved in 10 ml distilled water. Then the solution was filtered using a syringe filter. This created a solution of 05% moxifloxacin.
- **Ciprofloxacin:** Ciprocin eye drop of Square Pharmaceuticals Ltd was used. The eye drop contained 0.3% ciprofloxacin.
- **Azithromycin:** Az eye drop of Aristopharma Ltd was used. This contained 200 mg azithromycin per 5 ml.
- **Chloramphenicol:** A-phenicol eye drop of Acme Laboratories Ltd was utilized. There were 0.5% chloramphenicol solutions.

The antibiotics used forantibiogram were selected fromdiscs of antibiotics.

**Table 1: List of used Antibiotics for Antibiogram**

| Number | Name of the Antibiotic | Class |
|---|---|---|
| 1 | Moxifloxacin | Fluoroquinolones |
| 2 | Levofloxacin | Fluoroquinolones |
| 3 | Azithromycin | Macrolides |
| 4 | Erythromycin | Macrolides |
| 5 | Colistin | Polymyxins |
| 6 | Tetracycline | Tetracycline |
| 7 | Doxycycline | Tetracycline |

| Number | Name of the Antibiotic | Class |
|---|---|---|
| 8 | Metronidazole | Nitroimidazole |
| 9 | Cefixime | Cephalosporin |
| 10 | Cefuroxime | Cephalosporin |
| 11 | Ciprofloxacin | Fluoroquinolones |
| 12 | Ceftazidime | Beta lactam |
| 13 | Nalidixic Acid | Quinolone |
| 14 | Amikacin | Aminoglycosides |
| 15 | Norfloxacin | Fluoroquinolones |
| 16 | Kanamycin | Aminoglycosides |
| 17 | Streptomycin | Aminoglycosides |
| 18 | Ampicillin | Penicillin |
| 19 | Chloramphenicol | Penicillin |

## 2.4: Preparation Of Media:

### 2.4.1: Nutrient Agar Preparation:

Nutrient agar was used to initially grow the organisms. Twenty-eight gms of nutrient agar powder was dissolved in 1 liter of distilled water by boiling it till the agar was melted. Then the dissolved agar was autoclaved at 121℃ for 15 minutes. After the autoclave was done the media cooled down. Then the media was poured into the Petri dishes and left in the laminar to solidify. Finally, the solidified dishes were stored in the fresh media fridge.

### 2.4.2: MacConkey Agar Preparation:

MacConkey agar plates were used in the research for the daily subculture process. *Klebsiella pneumoniae* samples were streaked daily to get the young culture the next day as to prepare the bacterial suspension young culture is needed. MacConkey is a selective media for *Klebsiella pneumoniae* on which K.P exhibits mucoid light pink lactose fermenter colonies. This selective media was used to avoid any contamination and ensure the purity of the sample.

49.53g of MacConkey agar powder was dissolved in 1 liter of distilled water. The powder agar was first dissolved by applying heat. Then it was autoclaved at 121℃ for 15 minutes. The autoclaved media was cooled down and poured into Petri dishes in the laminar. The media was left there to solidify and later stored in the fresh media fridge.

### 2.4.3: Mueller Hinton Agar (MHA) Preparation:

MHA was utilized in the antibiogram process. It is a non-selection media that means all kinds of organisms can grow on it. As it is a loose agar, diffusion occurs easily. This is a great advantage for the disc diffusion method.

38g of MHA powder was heated and dissolved in 1 liter of distilled water. After that, the autoclave was done at 121℃ for 15 minutes and cooled down the media for a few minutes. The media was then poured into Petri dishes in the laminar and solidified. The solidified media were then used to perform antibiograms.

### 2.4.4: Brain Heart infusion (BHI ) Broth Preparation:

BHI broth was utilized for the MIC determination process. Thirty-seven gms of BHI powder was dissolved in 1 liter of distilled water. No heat was needed to dissolve the broth. The broth was transferred in test tubes using a glass pipette. Each test tube contained 5 ml of BHI broth. The test tubes were placed in a beaker and autoclaved at 121℃ for 15 minutes. The beaker with the test tubes was then stored in the fresh media fridge for further use.

## 2.5: Physiological Saline Preparation

Physiological saline was used to prepare bacterial suspensions. 0.9 g NaCl was dissolved in 100 ml of distilled water as the saline should not contain more than 0.9% NaCl or else all the bacteria would die due to excessive alkaline conditions. Then 10 ml saline was poured into each test tube with the help of a glass pipette. The test tubes were then autoclaved at 121℃ for 15 minutes and stored at room temperature after the autoclave.

## 2.6: Bacterial Suspension Preparation

The bacterial suspension was made in physiological saline. A small number of bacteria from a single colony of young culture was taken with the help of a loop and dissolved in the saline. The suspension was then vortexed to fully dissolve the bacteria. Finally, it was compared with MacFarland standard 0.5 solutions.

## 2.7: Antoibiogram

Antibiogram is a profile of antimicrobial susceptibility testing. This is done via the disc diffusion method. On the Mueller Hinton Agar (MHA) plate at first bacterial suspension was distributed evenly with the help of sterilized cotton swabs. On each plate then 5 antibiotic disks were placed. For each sample of *Klebsiella pneumoniae* 4 MHA plates were used. The plates were then incubated in the incubator for 24 hours at 37℃. After 24 hours readings were recorded for each antibiotic disk and compared the clear zones were with the standard chart. This helped to determine which K.P. sample was sensitive to which antibiotic and resistant to which antibiotic.

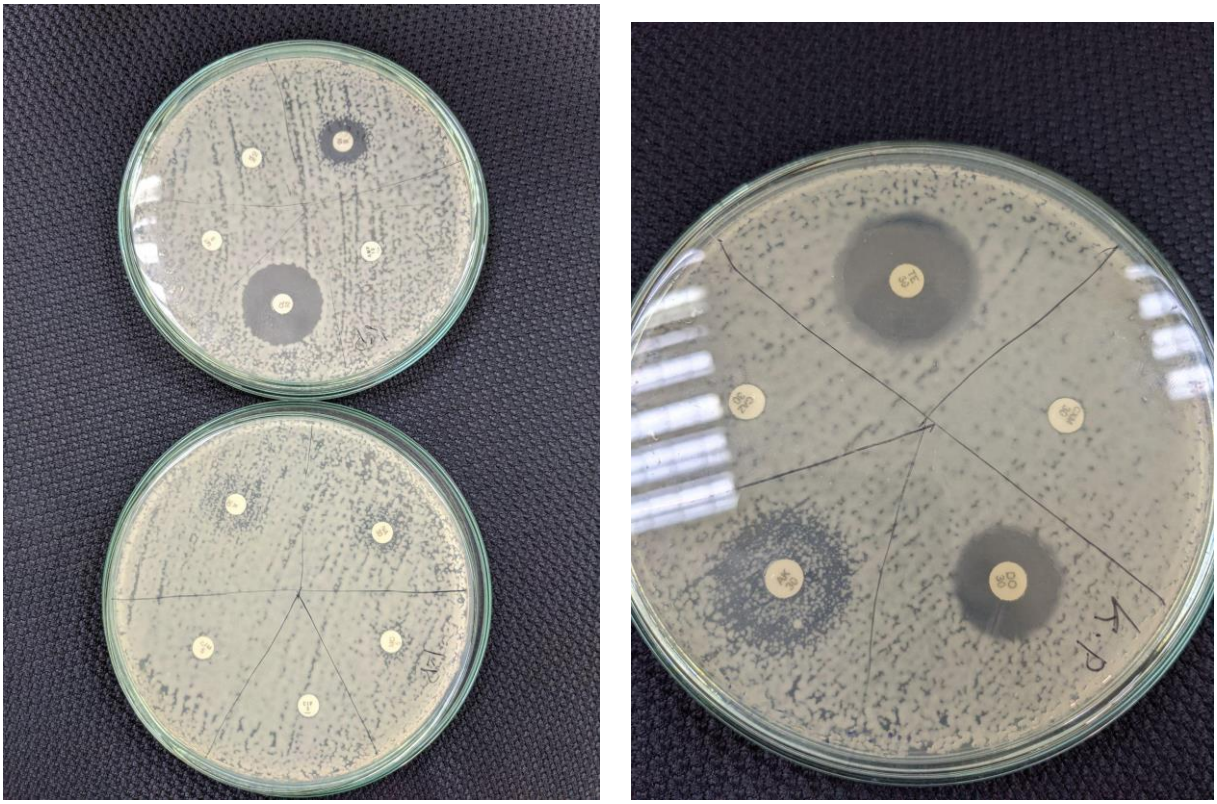


Figure 3: Antimicrobial susceptibility testing.

This process also helped us to determine which sample was MDR, XDR, and PDR.

**Table 2: Antibiotic susceptibility by disk diffusion method**

| Number | Name of the Antibiotic | Result |
|---|---|---|
| 1 | Moxifloxacin | Resistant |
| 2 | Levofloxacin | Resistant |
| 3 | Azithromycin | Resistant |
| 4 | Erythromycin | Resistant |
| 5 | Colistin | Sensitive |
| 6 | Tetracycline | Sensitive |
| 7 | Doxycycline | Sensitive |
| 8 | Metronidazole | Resistant |

| Number | Name of the Antibiotic | Result |
|---|---|---|
| 9 | Cefixime | Resistant |
| 10 | Cefuroxime | Resistant |
| 11 | Ciprofloxacin | Resistant |
| 12 | Ceftazidime | Resistant |
| 13 | Nalidixic Acid | Resistant |
| 14 | Amikacin | Sensitive |
| 15 | Norfloxacin | Resistant |
| 16 | Kanamycin | Resistant |
| 17 | Streptomycin | Resistant |
| 18 | Ampicillin | Resistant |
| 19 | Chloramphenicol | Resistant |

[* Yellow color indicated sensitivity]

## 2.8: Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) indicates the minimum concentration of an antibiotic that will completely prevent the growth of a bacteria. This helped in the study of determining the efficiency of an antibiotic against a bacteria.

Here, BHI was used for the process. At first individual MIC for each antibiotic was determined against each sample. Each test tube contained 5ml BHI. Thirteen different concentrations of antibiotics were chosen to run the individual MIC determination process of the first phase. For the second phase, 13 new concentrations were selected. The amount needed to be added for each antibiotic (Moxifloxacin and Ciprofloxacin) in the 5ml BHI tubes were calculated using the $C_1V_1 = C_2V_2$ formula. The calculated amount was first discarded from the BHI tube and then the same amount of respective antibiotic was added.

After that 100 µL bacterial suspension was added to each tube. The tubes were kept in a shaker incubator at 37°C and 80 rpm for 24 hours. After 24 hours turbidity of the tubes were observed and the lowest concentration tube with clear media was determined as the MIC of the antibiotic for the respective bacteria sample.

For the identification of the MIC of combination, the serial dilution method was used for the first phase of detection. Six combinations were run. For the second phase, 10 new combination concentrations were selected and the $C_1V_1=C_2V_2$ formula was used.

The amount of antibiotics used was determined by using the same $C_1V_1=C_2V_2$ formula to get the desired concentration in the BHI broth. The rest of the process was the same as the individual MIC determination process. The lowest total concentration of both antibiotics containing clear media was determined as the combination MIC.
Each MIC determination was done 2 times to ensure the authenticity of the result.

## 2.9: Calculation of Fractional Inhibitory Concentration (FIC) Index

FIC index is calculated to regulate the effectiveness of the result. There are 4 different levels of FIC index:

- Synergistic: < 0.5
- Additive: > 0.5 - 1
- Indifference: > 1 - 4
- Antagonism: > 4

Lower the value of FIC the result is more effective.

The formula of FIC:

**FIC = MIC of the agents in combination/MIC of the agent alone**

The formula of FIC Index:

**FIC Index = ∑ (MIC of the agents in combination/MIC of the agent alone)**

These formulas were used to calculate for all 5 samples of *Klebsiella pneumoniae* the FIC index using the previously obtained data. Then the FIC index was then compared with the standards and the effectiveness of each sample was determined.

# CHAPTER 3

Results

## 3. Results

In the present study, 9 samples of *Klebsiella pneumoniae* were collected from two different hospitals and research institutes, and through the disc diffusion method, *Klebsiella pneumoniae* were categorized to Multidrug-Resistant (MDR) and Extensively Drug-Resistant (XDR) from using 19 different antibiotics. Further, moxifloxacin was combined with 3 antibiotics to find if the combination can kill the pathogen which is presented in table 3.

### 3.1 Categorizing the pathogenic Klebsiella pneumoniae

Total collected sample: 9
MDR: 3
XDR: 3
PDR: 0

- 3 samples are MDR and resistant to more than 3 classes
- 3 samples are XDR and only susceptible to 2 antibiotics

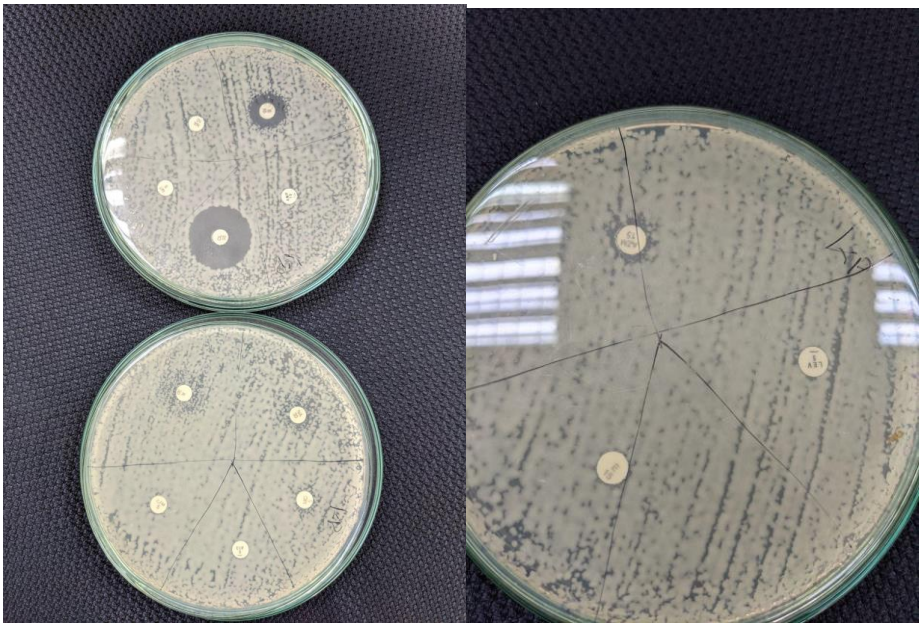


Figure 4: Antibiogram

## 3.2 Screening antibiotic combination against MDR, XDR, and PDR *Klebsiella pneumoniae*

From the collected *Klebsiella pneumoniae* pathogen, randomly 3 MDR and 3 XDR were selected to inhibit the growth of the pathogen by moxifloxacin having various combinations with 3 different drugs including different classes of antibiotics which are shown in table 3.

**Table 3:The combination of moxifloxacin with several antibiotics and the synergy screening**

| Combination of Antibiotics | Inhibition of Growth |
|---|---|
| Moxifloxacin + Ciprofloxacin | + |
| Moxifloxacin + Chloramphenicol | - |
| Moxifloxacin + Azithromycin | + |

(+ ve means combination was effective and - ve means combination was not effective)

## 3.3 Determination of Minimum Inhibitory Concentration (MIC)

After the screening of 3 drugs, Ciprofloxacin, Chloramphenicol, and Azithromycin with the combination of Moxifloxacin were further explored from their minimum inhibitory concentration (MIC). FIC index is considered as a statistical validation tool to determine synergistic effects. So, along with MIC, the fractional inhibitory concentration (FIC) was also calculated and compared to the FIC index.

### 3.3.1: Determination of moxifloxacin, ciprofloxacin, and the combination of moxifloxacin and ciprofloxacin MIC (First Phase)

To prepare the necessary antibiotic concentration, serial dilution and the $C_1V_1 = C_2V_2$ technique were used several times. Table 4 shows the outcomes of the first phase. To summarize, six *Klebsiella pneumoniae* samples were obtained to assess the MIC value of each antibiotic as well as the combination of antibiotics. The pathogens with high MIC values were revealed as a result of their extreme antibiotic resistance. Individual moxifloxacin concentrations of 200 µg/ml and

600 µg/ml were found to be the minimum inhibitory concentrations in the three MDR pathogen samples. 950 µg/ml, 750 µg/ml, and 425 µg/ml were found in case three XDR samples.

**Table 4: The MIC value of moxifloxacin, ciprofloxacin and their combination (First Phase)**

| **Antibiotic Name** | **Antibiotic Concentration** µg/ml | **Sample Number** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **MDR** | | | **XDR** | | |
| | | Sample 3 | Sample 4 | Sample 5 | Sample 1 | Sample 2 | Sample 7 |
| Moxifloxacin only | 1000 | C | C | C | C | C | C |
| | 950 | C | C | C | C | C | C |
| | 900 | C | C | C | T | C | C |
| | 850 | C | C | C | T | C | C |
| | 800 | C | C | C | T | C | C |
| | 750 | C | C | C | T | C | C |
| | 700 | C | C | C | T | T | C |
| | 600 | C | C | C | T | T | C |
| | 500 | C | C | T | T | T | C |
| | 400 | C | C | T | T | T | T |
| | 300 | C | C | T | T | T | T |
| | 200 | C | C | T | T | T | T |
| | 100 | T | T | T | T | T | T |
| Ciprofloxacin Only | 1000 | C | C | C | C | C | C |
| | 950 | C | C | C | C | C | C |
| | 900 | C | C | C | C | C | C |
| | 850 | C | C | C | C | C | C |
| | 800 | C | C | C | C | C | C |
| | 750 | C | C | C | C | C | C |

| Antibiotic Name | Antibiotic Concentration µg/ml | Sample Number | | | | | |
|---|---|---|---|---|---|---|---|
| | | MDR | | | XDR | | |
| | | Sample 3 | Sample 4 | Sample 5 | Sample 1 | Sample 2 | Sample 7 |
| Ciprofloxacin only | 700 | C | C | C | C | C | C |
| | 600 | C | C | C | C | C | C |
| | 500 | C | C | C | C | C | C |
| | 400 | C | T | C | C | T | C |
| | 300 | T | T | C | T | T | C |
| | 200 | T | T | C | T | T | C |
| | 100 | T | T | T | T | T | T |
| Moxifloxacin + Ciprofloxacin | 500 | C | C | C | C | C | C |
| | 250 | C | C | C | C | C | C |
| | 125 | T | C | T | T | T | C |
| | 62.5 | T | T | T | T | T | C |
| | 31.25 | T | T | T | T | T | T |
| | 15.625 | T | T | T | T | T | T |

[Key: C = Clear, T = Turbid, C = MIC Value]

For individual antibiotics, the MIC value against that organism was higher. When the combination was tested the MIC value was reduced that shown in table 4.

### 3.3.2: **Determination of moxifloxacin, ciprofloxacin, and the combination of moxifloxacin and ciprofloxacin MIC (Second Phase)**

The high concentration gap range of the antibiotics was recognized as a limitation from the first phase result. As a result, the same test was repeated twice with the gap range lessening, yielding the findings indicated in table 5.

**Table 5: The MIC value of moxifloxacin, ciprofloxacin and their combination (Second Phase)**

| Antibiotic Name | Antibiotic Concentration µg/ml | Sample Number | | | | | |
|---|---|---|---|---|---|---|---|
| | | MDR | | | XDR | | |
| | | Sample 3 | Sample 4 | Sample 5 | Sample 1 | Sample 2 | Sample 7 |
| Moxifloxacin only | 950 | C | C | C | C | C | C |
| | 900 | C | C | C | T | C | C |
| | 850 | C | C | C | T | C | C |
| | 800 | C | C | C | T | C | C |
| | 750 | C | C | C | T | C | C |
| | 700 | C | C | C | T | T | C |
| | 600 | C | C | C | T | T | C |
| | 500 | C | C | T | T | T | C |
| | 425 | C | C | T | T | T | C |
| | 400 | C | C | T | T | T | T |
| | 300 | C | C | T | T | T | T |
| | 200 | C | C | T | T | T | T |
| | 100 | T | T | T | T | T | T |
| Ciprofloxacin Only | 900 | C | C | C | C | C | C |
| | 850 | C | C | C | C | C | C |
| | 800 | C | C | C | C | C | C |
| | 750 | C | C | C | C | C | C |
| | 700 | C | C | C | C | C | C |
| | 600 | C | C | C | C | C | C |

| Antibiotic Name | Antibiotic Concentration µg/ml | | Sample Number | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | MDR | | | XDR | | |
| | | | Sample 3 | Sample 4 | Sample 5 | Sample 1 | Sample 2 | Sample 7 |
| Ciprofloxacin Only | 500 | | C | C | C | C | C | C |
| | 400 | | C | T | C | C | T | C |
| | 300 | | T | T | C | T | T | C |
| | 200 | | T | T | C | T | T | C |
| | 125 | | T | T | T | T | T | C |
| | 100 | | T | T | T | T | T | T |
| Moxifloxacin + Ciprofloxacin | **Mox** | **Cip** | | | | | | |
| | 31.25 | 31.25 | T | T | T | T | T | C |
| | 20 | 60 | T | C | T | T | T | C |
| | 50 | 100 | T | C | C | T | T | C |
| | 60 | 100 | T | C | C | T | T | C |
| | 70 | 100 | C | C | C | T | T | C |
| | 80 | 100 | C | C | C | T | T | C |
| | 100 | 100 | C | C | C | T | T | C |
| | 30 | 200 | C | C | C | T | C | C |
| | 50 | 200 | C | C | C | C | C | C |
| | 50 | 250 | C | C | C | C | C | C |

[Key: C = Clear, T = Turbid, C = MIC Value]

## 3.4: Determination of the arithmetic mean MIC value of moxifloxacin, ciprofloxacin and their combination

Since these pathogens were multidrug resistant, the MIC value of moxifloxacin and ciprofloxacin was pretty high and the MIC value of the combination was nearly low as shown in table 6.

**Table 6: The Average MIC value of moxifloxacin, ciprofloxacin and their combination in µg/ml & FIC Index**

| Category | Sample Number | MIC (in µg/ml) | | | | FIC Index* |
|---|---|---|---|---|---|---|
| | | Moxifloxacin Only | Ciprofloxacin Only | Moxifloxacin + Ciprofloxacin | | |
| | | | | Mox | Cip | |
| MDR | Sample 3 | 200 | 400 | 70 | 100 | 1.29 |
| | Sample 4 | 200 | 500 | 20 | 60 | 0.5 |
| | Sample 5 | 600 | 200 | 50 | 100 | 1 |
| XDR | Sample 1 | 950 | 400 | 50 | 200 | 0.88 |
| | Sample 2 | 750 | 500 | 30 | 200 | 0.78 |
| | Sample 7 | 425 | 125 | 31.25 | 31.25 | 0.64 |

[Key: MIC = Minimum Inhibitory Concentration, MDR = Multidrug Resistant, XDR = Extensively Drug Resistant, FIC = Fractional Inhibitory Concentration which is determined by MIC of the agents in combination/MIC of the agent alone]

***FIC index = ∑ (MIC of the agents in combination/MIC of the agent alone)**

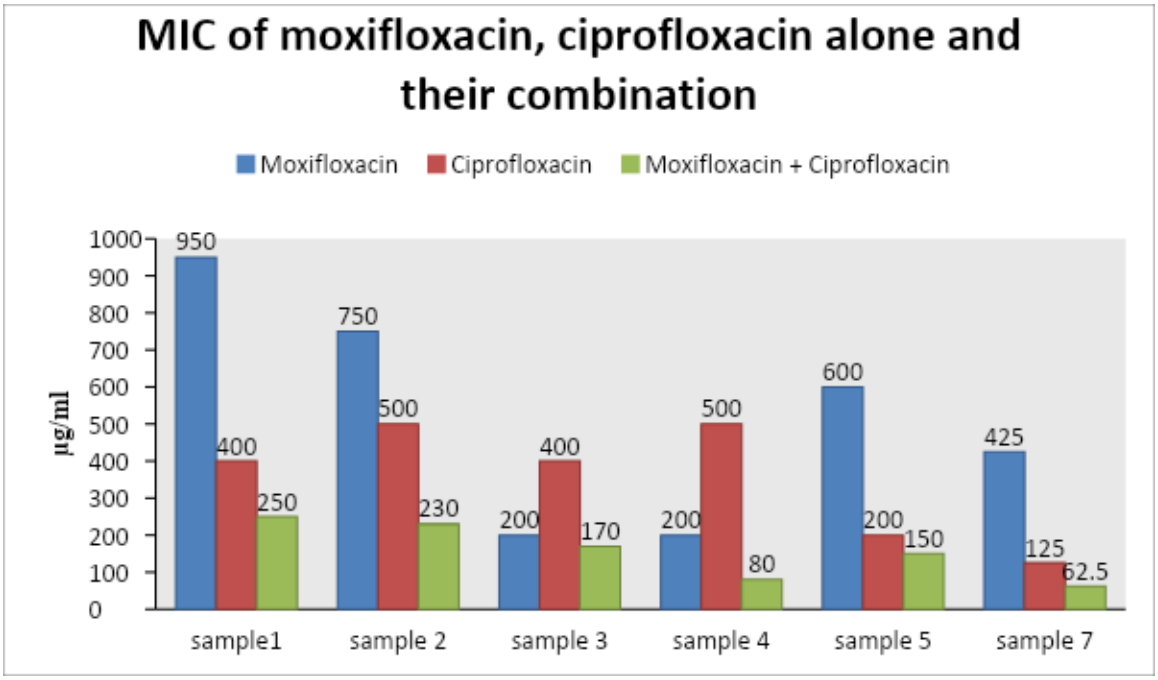
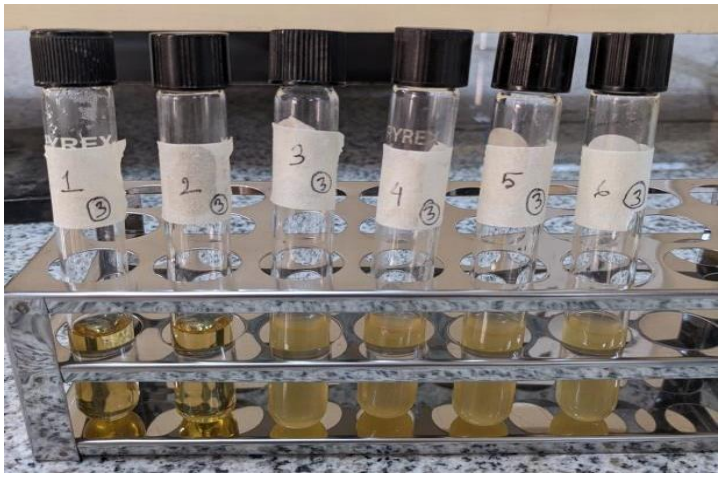



Figure 5: MIC of Moxifloxacin, Ciprofloxacin alone and their combination

## 3.5: The Average FIC Index (MDR, XDR) of Moxifloxacin and Ciprofloxacin

The arithmetic mean of FIC index is 0.5 statistical synergistic effects of moxifloxacin and ciprofloxacin against the *Klebsiella pneumoniae.*



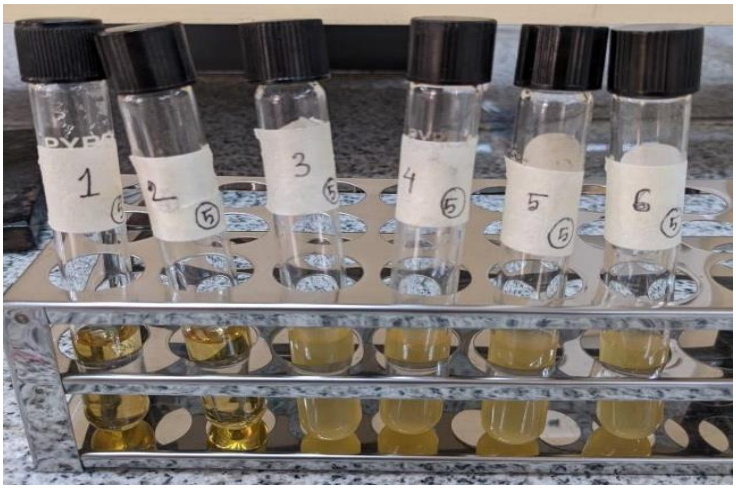
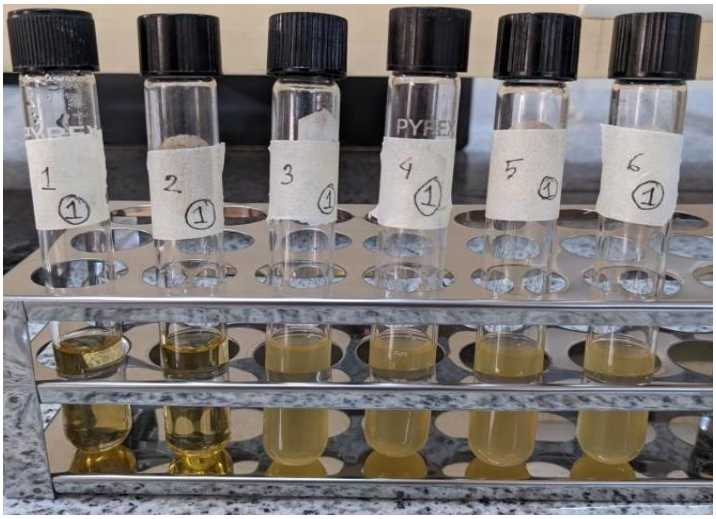


Figure 6: MIC test of Moxifloxacin + Ciprofloxacin combination - First phase (MDR Sample 3,4 and 5)

In the first phase, the MDR samples 3, 4 and 5 exhibited MIC of the moxifloxacin + ciprofloxacin combination at 200 µg/ml, 125 µg/ml, and 200 µg/ml respectively.



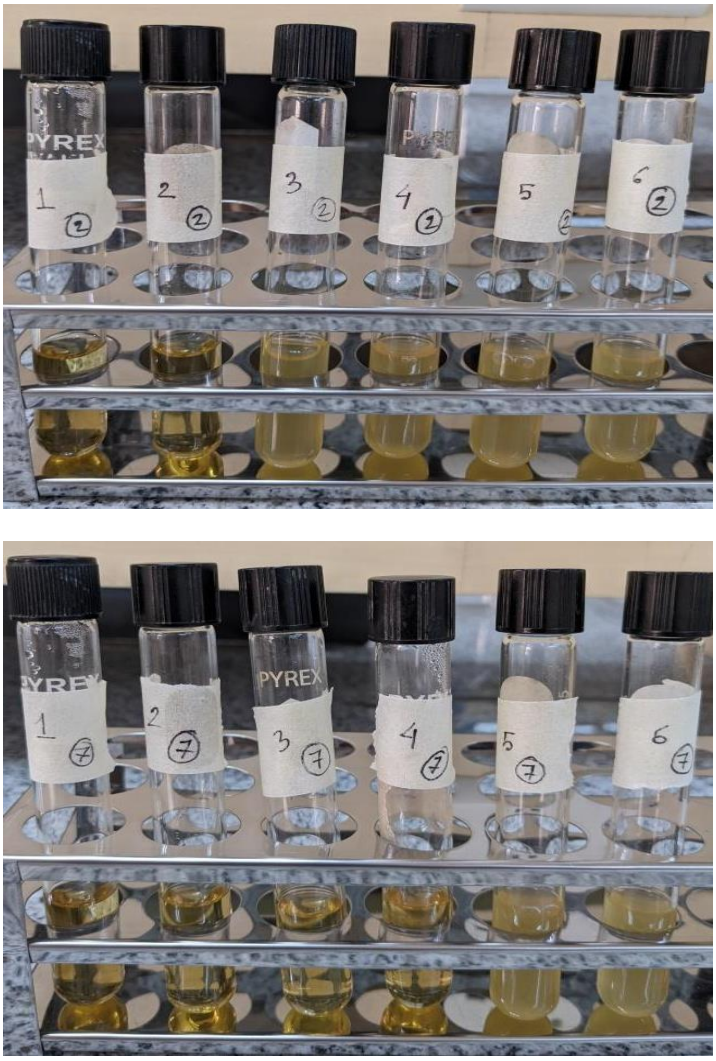



Figure 7: MIC test of Moxifloxacin + Ciprofloxacin combination - First phase (XDR Sample 1,2 and 7)

In the first phase, in the case of MDR samples 1, 2, and 7 the MIC of the combination was 200 µg/ml, 200 µg/ml, and 62.5 µg/ml respectively.

Figure 8: MIC test of moxifloxacin alone (Sample 1,2 and 3)

In the picture individual MIC of moxifloxacin for sample 1, 3 and 2 are shown. For sample 1 MIC was 950 µg/ml, for sample 3 MIC was 200 µg/ml and for sample 2 it was 750 µg/ml.

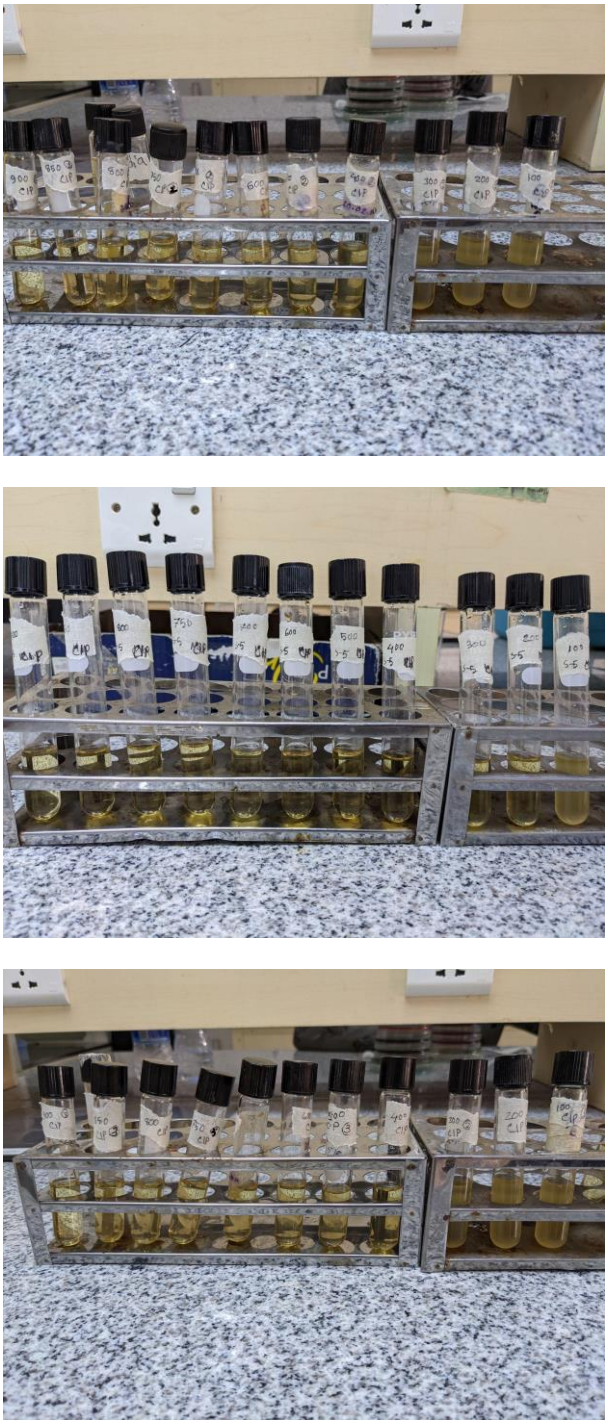


Figure 9: MIC test of Ciprofloxacin alone ( Sample 2,3 and 5)

The pictures display the individual MIC of ciprofloxacin . For sample 2 it was 500 µg/ml, for sample 3 it was 400 µg/ml and for sample 5 it was 200 µg/ml.

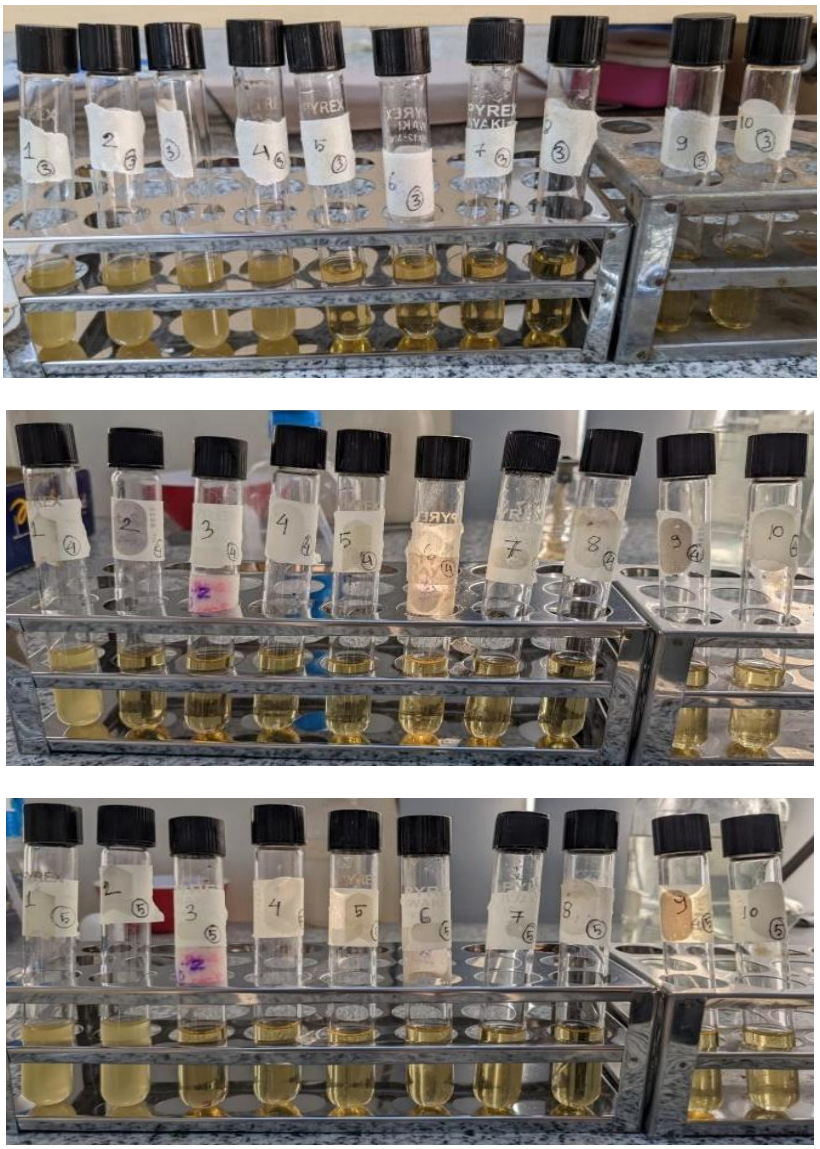


Figure 10: MIC test of Moxifloxacin + Ciprofloxacin combination - Second phase (MDR Samples)

In the pictures, the second phase MIC of the combination results for MDR samples is shown.

Figure 11: MIC test of Moxifloxacin + Ciprofloxacin combination - Second phase (XDR sample).

In the pictures, the second phase MIC of the combination for the XDR samples are exhibited.

# CHAPTER 4

# Discussion

## 4. Discussion

Gram-negative bacterial infections are particularly concerning since they are growing resistant to practically all antibiotics now available, evoking pre-antibiotic conditions. The rise of MDR gram-negative bacteria has had an impact on medical practice across the board. *Enterobacteriaceae,* mainly *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Acinetobacter,* are the most common causes of gram-negative infections in healthcare settings. Gram-negative bacteria that are MDR are also becoming more common in the community globally (Ventola, 2015). Overuse of antibiotics and deficiency of developing new antibiotics by the pharmaceutical companies are two of the major reasons behind the rise of MDR bacteria (Ventola, 2015).

In this study, the initial screening was done using three antibiotics i) Ciprofloxacin, ii) Chloramphenicol and iii) Azithromycin in combination with Moxifloxacin. The effectiveness of these three combinations was observed via the MIC determination process done in BHI , among them moxifloxacin + chloramphenicol failed to inhibit the bacterial growth even in the highest concentration bar set for the combination which was 500 µg/ml. The other two combinations exhibited inhibition. Among those two combinations, the combination of moxifloxacin + ciprofloxacin exhibited the best result. As a result, moxifloxacin + ciprofloxacin was the selected combination for further study.

For this study, 9 samples were collected. Among them, 3 of the samples were MDR, and 3 were XDR. In the individual MIC determination process, the six samples displayed MIC at very high concentrations of antibiotics which indicated strong resistance of the samples against moxifloxacin and ciprofloxacin. For moxifloxacin, the highest exhibited MIC value was 950 µg/ml and the lowest was 200 µg/ml. For ciprofloxacin, the highest value one was 500 µg/ml and the lowest one was 200 µg/ml.

The process to determine the MIC of the combination showed some hopeful results. The results indicated in the drop of MIC means the minimum needed antibiotic concentration to inhibit the bacterial growth was being able to lower down simply by combining two previously resistant

antibiotics. The lowest combination of MIC was 80 µg/ml. This spotlighted the effectiveness of using antibiotics in a combination manner.
The FIC index was synergistic for one sample, additive for four of the samples, and indifference for one sample. The lowest FIC index was 0.5. These findings give hope to the success of using combinations of antibiotics in treating MDR organisms.

In a study determining the effectiveness of ciprofloxacin in combination with chloramphenicol against MDR *Klebsiella pneumoniae* , positive results were observed where the FIC index was 0.1708 (Ahmed, 2020). This synergistic result indicates the effectiveness of the ciprofloxacin + chloramphenicol.

In another study, cefixime + azithromycin combination was used against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. For *Klebsiella pneumoniae* an observed FIC index was 0.193 which is a synergistic result (Salahuddin, 2019). For *Pseudomonas aeruginosa* the lowest observed synergistic FIC result was 0.193 (Salahuddin, 2019). Moreover, the study showed that the cefixime + azithromycin combination was more effective on *Klebsiella pneumoniae* rather than *Pseudomonas aeruginosa* as *Pseudomonas aeruginosa* has a higher FIC index than *Klebsiella pneumoniae* (Salahuddin, 2019).

Furthermore, another study reflected light on the synergistic effect of doripenem + tetracycline on *Klebsiella pneumoniae* strains collected from the patients of ICU. In the study, the FIC index was 0.25 for a strain of *Klebsiella pneumoniae* which is a synergistic result (Celik et al., 2014). In the study, other antibiotic combinations were observed. Among other combinations, doripenem + tobramycin exhibited the best results for two strains (Celik et al., 2014). The FIC index of this combination was 0.25 (Celik et al., 2014). However, the lowest FIC index, which was 0.125, was exhibited by the combination of doripenem + levofloxacin (Celik et al., 2014).

All the antibiotics mentioned above belong to different antibiotic classes. This indicates that a wide variety of antibiotic combinations can be useful in terms of treating MDR *Klebsiella pneumoniae*. This widens the path to explore more in the sector of antibiotic combination treatment.

## Conclusion

To sum up, it would be threatening to dismiss the looming threat of antibiotic-resistant pathogens, particularly *Klebsiella pneumoniae*, which has emerged as a superbug and is rapidly evolving resistance mechanisms. Antibiotics' remarkable health advantages are being jeopardized by the rapid emergence of antibiotic-resistant microorganisms. This is a global crisis, owing to the widespread overuse of antibiotics and a dearth of new antibiotic agents being developed by pharmaceutical corporations to address the problem. If the efficacy of this antibiotic can be increased by the use of other medications, it could be life-saving and cost-effective. Furthermore, developing countries like ours can take advantage of this combination to combat antibiotic-resistant *Klebsiella pneumoniae* and minimize the mortality rate from pneumonia, as we are menaced by a huge number of rising antibiotic-resistant pathogens. When it comes to life and death, however, new techniques to tackle these superbugs must be devised, and existing antibiotic combinations may be a suitable option.

The research result clearly showcases that a combination of antibiotics can decrease the needed amount of antibiotics on resistant pathogens. These findings could have far-reaching consequences for the future of combination therapy against multidrug-resistant *Klebsiella pneumoniae.*

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# Appendix

## Appendix 1:

Media Composition:

**Nutrient Agar:**

| Component | Amount (g/L) |
|---|---|
| Peptone | 5.0 |
| Sodium chloride | 5.0 |
| Beef extract | 3.0 |
| Agar | 15.0 |

Final pH: 7.0

**Muller Hilton Agar:**

| Component | Amount (g/L) |
|---|---|
| Beef, dehydrated infusion form | 300g |
| Casein hydrolysate | 17.5g |
| Starch | 1.5g |
| Agar | 17.0g |
| Distilled Water | 1 liter |

Final pH: 7.3± 0.1 at 25°C

**Brain-Heart Infusion Broth:**

| Component | Amount |
|---|---|
| Brain Heart, Infusion from (Solids) | 8.0g |
| Peptic Digest of Animal Tissue | 5.0g |
| Pancreatic Digest of Casein | 16.0g |
| Sodium Chloride | 5.0g |
| Glucose | 2.0g |
| Disodium Hydrogen Phosphate | 2.5g |
| Agar | 13.5g |
| Distilled Water | 1 liter |

**MacConkey Agar:**

| Component | Amount |
|---|---|
| Peptone (Pancreatic digest of gelatin) | 17g |
| Proteose peptone (meat and casein) | 3g |
| Lactose monohydrate | 10g |
| Bile salts | 1.5g |
| Sodium chloride | 5g |
| Neutral red | 0.03g |
| Crystal Violet | 0.001g |
| Agar | 13.5g |
| Distilled Water | 1 liter |

Final pH: 7.1 +/- 0.2 at 25°C.

# Appendix 2:

Instruments:

The important equipment used through the study are listed below:

| | |
|---|---|
| Autoclave, Model No: WAC-47 | Korea |
| Balance(Core series): Adam | UK |
| Centrifuge, Model No: Code: 5433000.011 | Eppendorf, Germany |
| Freezer (-20°C) | Siemens Germany |
| Incubator | UK |
| Laminar air flow | UK |
| Micropipettes | Eppendorf, Germany |
| Oven (Universal drying oven)<br>Model: LDO-060E | Labtech, Singapore |
| Refrigerator, Model: 0636 | Samsung |
| Vortex Mixture | VWR International |