The combined effect of Levofloxacin and Azithromycin against multidrug-resistant *Acinetobacter baumannii, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*.

By:

Progga Parmita Belal

ID: 17336013

Bachelor in Biotechnology

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 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 that 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.

Progga Parmita Belal

17336013

Approval:

The thesis titled "The combined effect of Levofloxacin and Azithromycin against multidrug-resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*." submitted by Progga Parmita Belal (17336013) of Summer, 2017 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Biotechnology on January 2022

Examining Committee:

Supervisor: ____ 

M. Mahboob Hossain

Professor, Department of Mathematics and Natural Science

BRAC University.

Co-Supervisor:

Akash Ahmed

Lecturer, Department of Mathematics and Natural Sciences

BRAC University.

Program Director:

Munima Haque, PhD

Assistant Professor, Department of Mathematics and Natural Sciences

BRAC University.

Departmental Head:

A F M Yusuf Haider

Professor and Chairperson, Department of Mathematics and Natural Sciences

BRAC University.

Dedicated To

Family, Friends, and Faculties

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Abstract

Pneumonia affects everyone, particularly young children under the age of five. *Acinetobacter baumannii, Klebsiella pneumonia,* and *Pseudomonas aeruginosa* are one of the main causes of pneumonia as they cause a wide range of diseases which includes lung infections (pneumonia), bloodstream infections, wound or urinary tract infections. *A. baumannii* has become a major public health threat because 63 percent of *Acinetobacter* strains are multidrug-resistant.

The purpose of this study is to develop a viable method for battling infections brought about by multidrug-resistant *Acinetobacter baumannii, Klebsiella pneumonia,* and *Pseudomonas aeruginosa* in Bangladesh. From gathered bacterial samples, 10 out of 18 samples were multiple antibiotic resistant which was identified by performing an antibiogram utilizing 18 different antibiotics from 13 of various classes including Macrolides, Polymyxins, Tetracycline, Nitroimidazole, Cephalosporin, Beta-lactam, Quinolone, Fluoroquinolones, Aminoglycosides, and Penicillin. Then, primary screening of antibiotic susceptibility was done which categorized the pathogens into multidrug-resistant, extensively drug-resistant (XDR), and pan-drug-resistant (PDR). Next, by using the Minimum Inhibitory Concentration (MIC) method, individual antibiotics and a combination of screened antibiotics activity were measured. Afterward, the Fractional Inhibitory Concentration (FIC) index to provide statistical substantiation of results. The highest demonstrated MIC value for Levofloxacin was 400 µg/ml, while the lowest was 50 µg/ml. The highest value for Azithromycin was 400 µg/ml and the lowest was 100 µg/ml. For *Acinetobacter baumannii, Klebsiella pneumonia,* and *Pseudomonas aeruginosa* samples, a synergic effect was found in Levofloxacin in combination with azithromycin determined by FIC Index was below 0.5 suggesting the synergistic effect of their combination.

The research result is very significant because by using a combination of antibiotics, the needed amount of antibiotics on resistant pathogens can be decreased. This study has far-reaching consequences for the future of combination therapy against multidrug-resistant bacteria.

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List of Acronyms:

Abbreviations	Full forms
BHI	Brain Heart Infusion
MHA	Muller Hington Agar
MAC	MacConkey Agar
WHO	World Health Organization
MDR	Multi-Drug Resistant
XDR	Extensively Drug-Resistant
PDR	Pan Drug Resistant
Levo	Levofloxacin
AZ	Azithromycin
µg	Microgram
mg	Milligram
MIC	Minimum Inhibitory Concentration
FIC	Fractional Inhibitory Concentration
ACB	*Acinetobacter baumannii*
KP	*Klebsiella pneumoniae*
PSU	*Pseudomonas aeruginosa*

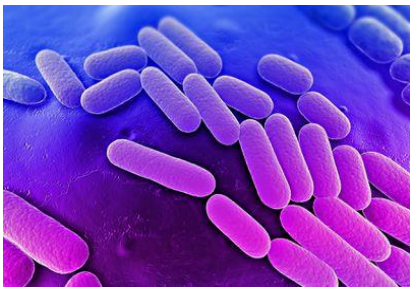
Chapter 1

Introduction and Literature review

1. Introduction

Acinetobacter baumannii, Klebsiella pneumoniae, and *Pseudomonas aeruginosa* are gram-negative bacteria that cause serious infections in the lungs and lead to pneumonia. Pneumonia is caused by bacteria, viruses, or fungi, and is life-threatening for children as they have to fight to breathe because their lungs become filled with pus and fluid. In Bangladesh, Pneumonia is the leading cause of mortality as it is responsible for 13% of under-five deaths. (UNICEF Bangladesh, 29 January 2020) According to researchers, if Bangladesh takes the necessary steps to combat pneumonia, it alone can give rise to almost 140,000 predicted under-five child deaths. They can be harmful to other parts of the body, generating the brain to swell, and interfering with blood flow. Additionally, when an infectious bacterium is resistant to both traditional and contemporary antibiotics, patient suffering increases dramatically.

Acinetobacter baumannii



Klebsiella pneumoniae

Pseudomonas aeruginosa

One of the major issues is the rising antibiotic resistance of harmful bacteria. Most of the strains of *Acinetobacter baumannii, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* are multidrug resistant. Often, just one mutation in the bacterial cell leads to the formation of a new drug resistance mechanism. (Urszula et al., 2014) The prospect of treating severely antibiotic-resistant bacterial infections is thus a concerning issue for modern science. (Groopman, 2008).

Combination antibiotic therapy is being used more frequently to boost the antibacterial properties of currently available antibiotics against multi-drug resistant pathogens. So, it is frequently used to treat severe Gram-negative infections. However, *in vitro* data of various classes of antibiotic combinations can be useful to screen for effective combinations and to support therapeutic decisions for severe infections with multidrug-resistant Gram-negative bacteria.

However, a lot of combination trials have been reported against these multidrug-resistant bacteria. such as,

A. baumannii: polymyxin B or colistin + rifampin, imipenem, or azithromycin; rifampin + azithromycin; sulbactam + rifampin, azithromycin, or a quinolone; and the triple combination of polymyxin B, imipenem, and rifampin (James, 2006).

Klebsiella pneumoniae: tigecycline + gentamicin and tigecycline + colistin (Falagas et al., 2014), azithromycin + chloramphenicol, levofloxacin + rifampin, polymyxin B + tigecycline (Lim et al., 2016).

P. aeruginosa: polymyxin B + rifampin; ceftazidime or cefepime + a quinolone; ceftazidime + colistin; clarithromycin + tobramycin; and azithromycin + tobramycin, doxycycline, trimethoprim, or rifampin. (James, 2006)

1.1 Character and Morphology

ESKAPE pathogen is a group of pathogens with a high rate of antibiotic resistance that is responsible for the majority of nosocomial infections which includes, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and Enterobacter species. (Louis, 2008)

Acinetobacter baumannii:

Acinetobacter baumannii is named after the bacteriologist Paul Baumann. (Lin et al., 2014) It is a short, rod-shaped (coccobacillus) Gram-negative, oxidase-negative bacteria with a DNA G+C content of 39% to 47%. It can be an opportunistic pathogen in humans, affecting people with compromised immune systems, and is becoming increasingly important as a hospital-derived (nosocomial) infection. Different species of *Acinetobacter* are found in soil samples, and water samples and also can be isolated from hospital environments. Severe community-acquired *A. baumanni*i infections have been reported in tropical climates of Australia and Asia.

During the COVID-19 pandemic, coinfection with *A. baumannii* secondary to SARS-CoV-2 infections has been reported multiple times in medical publications as just like covid, it also causes pneumonia in patients with critical conditions. (Ioannis et al., 2021). *Acinetobacter* has impressive genetic plasticity, rapid genetic mutations and rearrangements, and, integration of foreign determinants carried by mobile genetic elements which are considered one of the key forces for shaping bacterial genomes and ultimately evolution. (Ioannis et al., 2021)

Klebsiella pneumoniae:

Klebsiella pneumoniae is a Gram-negative, encapsulated, and nonmotile bacteria that normally live in intestines and feces. It has a high tendency to become antibiotic-resistant. These bacteria are harmless when they're in the intestines or stool but if they spread to another part of the body, such as your lungs, they can cause severe infections. It can cause bacterial meningitis, including fever, confusion, neck stiffness, and sensitivity to bright lights. *K. pneumoniae* can be resistant to antibiotics by producing enzymes such as Extended Spectrum β-Lactamase (ESBLs) and Carbapenems.

Pseudomonas aeruginosa:

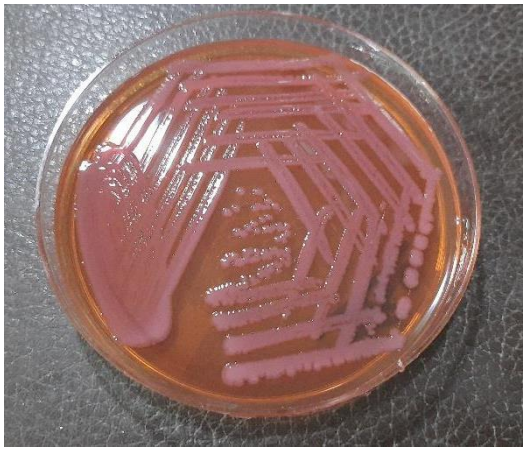
Pseudomonas aeruginosa is a very common bacterium that is found in soil, water, skin flora, and most man-made environments throughout the world and it can cause diseases in plants and animals as well as humans. It is an encapsulated, gram-negative, aerobic–facultatively anaerobic, rod-shaped bacterium. It is considered a multidrug-resistant pathogen for its intrinsically advanced antibiotic-resistance mechanisms. People can easily be infected with *P. aeruginosa* because it can grow on fruits and vegetables and by eating contaminated food, anyone can be infected. It can spread through improper hygiene besides it thrives in moist areas such as pools, hot tubs, bathrooms, and kitchens, and in the skin of some healthy persons. *P. aeruginosa* causes serious infections including malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicemia.

1.2 Emergence of antibiotic-resistant bacteria

The first multidrug-resistant bacteria were reported in Hong Kong in 1955. There are two main causes of multidrug resistance in bacteria: The development of genes that each code for resistance to a particular agent on on-resistance) plasmids or transposons, as well as the activity of multidrug efflux pumps, each of which can release more than one drug type. The World Health Organization recently alerted the public to the global emergence of bacteria that are multidrug-resistant which poses a significant threat to healthcare.

Acinetobacter baumannii: Back in the early 20th century, in 1911, a Dutch microbiologist, Beijerinck, isolates an organism named *Micrococcus Calco-ascetics* from the soil, and over the decades, At least 15 different genera and species were discovered similar to the same organism. In 1954, Acinetobacter was initially proposed by Brisou and Prévot. Since the early 1980's there has been an outbreak of multidrug-resistant *A. baumannii* in Europe, mainly in England, France, Germany, Italy, Spain, and The Netherlands. (Lin et al., 2014) Because the airline travel, the intercontinental spread of multidrug-resistant *A. baumannii* occurred in many countries across the world. From the year 1986 to 2003, many hospitals report multidrug-resistant Acinetobacter

throughout the United States, where there was a significant increase in the Acinetobacter strains resistant to amikacin (5% to 20%; $P < 0.001$), ceftazidime (25% to 68%; $P < 0.001$), and imipenem (0% to 20%; $P < 0.001$) (Gaynes et al., 2005) The emerging resistance mechanism renders all clinically significant aminoglycosides, such as gentamicin, tobramycin, and amikacin, highly resistant by impairing aminoglycoside binding to its target site.



Acinetobacter growth on MacConkey agar.

According to Fournier et al., The genome of a multidrug-resistant *A. baumannii* strain can encode a wide array of multidrug efflux systems. The persistence of *A. baumannii* in the hospital environment may be caused by three main factors:

- resistance to major antimicrobial drugs,
- resistance to desiccation, and
- resistance to disinfectants.

Klebsiella pneumonia: Friedlander discovered a capsulated bacillus in the patient's lungs who had died of pneumonia which was given the name Friedlander's bacillus in his honor. Later, these bacteria were referred to as Klebsiella. In 1983, an outbreak of ESBL-producing *pneumoniae* infections was reported in Europe, the United States, and South America. *K.pneumoniae* is normally found in the human stool or intestine. It infects those people who are hospitalized for different diseases and receive treatments through ventilators (breathing machines) or intravenous

(vein) catheters. A person must be exposed to the bacteria to get a Klebsiella infection. For instance, in order to get infected by pneumoniae or to have a bloodstream infection, Klebsiella must reach the respiratory system.

Penicillin binding proteins (PBPs), which are specialized targets of β-lactam antibiotics and enzymes that catalyze the formation of peptidoglycans, are one of the resistance strategies used by *K. pneumoniae*. Some factors for which it becomes a resistant bacterium are:

- It can accumulate antibiotic resistance genes (ARGs), by de novo mutations
- Acquisition of plasmids to encode ARGs and
- Transferable genetic elements.

K. pneumoniae growth on MacConkey agar.

Pseudomonas aeruginosa: In 1882, *Pseudomonas aeruginosa* was first isolated by Carle Gessard from green pus. According to the Centers for Disease Control and Prevention (CDC), *Pseudomonas aeruginosa* is the most common disease-causing bacteria. because of its versatile nature and high ability to endure, it can make due on dry lifeless surfaces climate from 6 hours to almost a year. It is an environmental bacterium but under stressful conditions, it can infect humans and cause pneumonia, blood infection, hemorrhagic septicemia, gill necrosis, abdominal distension, splenomegaly, friable liver, and congested kidney-6. According to the 2019 AR Threats Report, in the year 2017, multidrug-resistant *Pseudomonas aeruginosa* caused an expected 32,600 contaminations among hospitalized patients and 2,700 assessed deaths in the United States alone. The infection can spread vastly because people can easily be infected with these bacteria if they

get exposed to water or soil that is contaminated with this germ. The resistant strain can spread in the same way. That is the reason this resistant strain of bacteria can be found worldwide. The main factors are:

- Presence of chromosomally encoded antibiotic resistance genes.
- the horizontal gene transfer of ARGs or mutations.
- Different strains can asset many genetic events like mutation.

The emergence of resistant gram-negative pathogens is the most concerning issue of this decade. However, "pan-resistant" gram-negative strains have just recently begun to appear, particularly those from *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. This is because the majority of large pharmaceutical corporations have stopped researching and developing new antibacterial drugs. As a result, there are very few medications that could be utilized to treat these strains, which have a low-permeability outer membrane barrier, a variety of effective multidrug efflux pumps, and a wide variety of distinct resistance mechanisms (Annu Rev Biochem., 2009).

In Bangladesh, the emergence of bacteria that are resistant to many drugs is a concerning issue and a significant barrier to treating many infectious diseases. Antibiotic resistance is boosted by the indiscriminate, unneeded, and careless use of antibiotics, which results in the emergence of multidrug-resistant (MDR) microorganisms in the environment. The study will aid in the fight against the Bangladeshi situation in the global concern over increasing antibiotic resistance, which has become a danger to people.

1.3: Mechanism of antibiotic-resistant bacteria

Bacterial multidrug resistance is generated by one of three mechanisms.

- First, these bacteria can accumulate multiple genes in a single cell, each encoding resistance to a single drug. This accumulation usually occurs in resistant (R) plasmids.
- Inactivation of antibiotics directly by hydrolysis or modification.
- And, multidrug resistance may also result from increased expression of genes encoding multidrug efflux pumps, thereby extruding a wide range of drugs

Acinetobacter baumannii: One of the most important weapons in Acinetobacter is its impressive genetic plasticity. This not only facilitates rapid genetic mutation and rearrangement but also the integration of foreign determinants into mobile genetic elements (Ioannis et al., 2021). The mechanism of antibiotic resistance in *A. baumannii* is based on:

- transportation through membrane
- enzymatic modifications
- target site alteration

Among these, the insertion sequences are thought to be one of the key forces shaping the bacterial genome and, ultimately, evolution (Vrancianu et al., 2020). Baumani can form biofilms, which can extend survival in medical devices such as intensive care unit (ICU) ventilators. The mechanisms of antimicrobial resistance are primarily related to the modulation of antibiotic transport across bacterial membranes, changes in antibiotic target sites, and enzymatic modifications that lead to antibiotic neutralization (Ioannis et al., 2021).

K. pneumoniae: In *K. pneumoniae*, more than 100 unique acquired antimicrobial resistance genes have been found that encode proteins that confer resistance to various classes of antibiotics. The renowned Klebsiella pneumoniae Carbapenemase (KPC) gene and the recently discovered plasmid-borne RND efflux pump gene cluster, tmexCD1-toprJ1, are two examples of the numerous resistance-determining factors that were initially discovered in *K. pneumoniae*. In

addition, *K. pneumoniae* uses a variety of gene products to get beyond the host's innate immune system. There are several virulence factors that have been thoroughly studied, including:

- siderophores,
- fimbriae,
- the capsule, and
- lipopolysaccharide.

Active antibiotic therapy and sufficient source control are both necessary for the management of *K. pneumoniae* infections.

Pseudomonas aeruginosa is resistant to various antibiotics, including aminoglycosides, quinolones, and β-lactams (Hancock and Speert, 2000). In general, the main mechanisms used by *P. aeruginosa* to counter antibiotic attack can be divided into:

- intrinsic resistance,
- acquired resistance, and
- adaptive resistance.

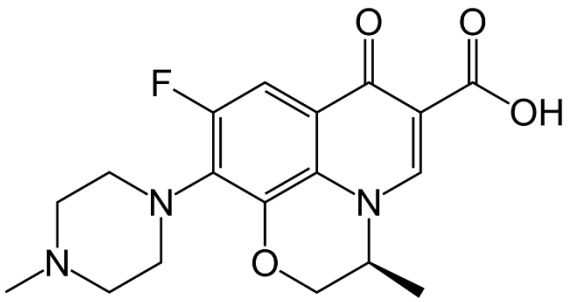
Intrinsic resistance of *P. aeruginosa* includes low outer membrane permeability, expression of efflux pumps that efflux antibiotics from the cell, and production of antibiotic-inactivating enzymes. (Zheng et al., 2019) Acquired resistance in *Pseudomonas aeruginosa* can be achieved by either horizontal transfer or mutational change of the resistance gene (Breidenstein et al., 2011). Adaptive resistance of *P. aeruginosa* includes biofilm formation in the lungs of infected patients. This biofilm acts as a diffusion barrier that limits the access of antibiotics to bacterial cells (Drenkard, 2003). In addition, multidrug-resistant persister cells that can withstand antibiotic attack may form in biofilms. (Zheng et al., 2019) These cells are responsible for persistence and recurrence.

1.4: About Levofloxacin

Levofloxacin, a third-generation fluoroquinolone and is an isomer of ofloxacin that was created and produced by researchers at Daiichi Seiyaku. (Walter, 2005) Ofloxacin is racemic, as the Daiichi scientists were aware, but they were unable to synthesize the two isomers separately. They finally succeeded in synthesizing the pure levo form in 1985 and demonstrated that it was more effective and less dangerous than the other form. Infections that can be treated with Levofloxacin are:

- respiratory tract infections,
- cellulitis,
- pneumonia
- urinary tract infections,
- prostatitis,
- anthrax,
- meningitis,
- pelvic inflammatory disease,
- traveler's diarrhea,
- tuberculosis, and
- plague

Levofloxacin is a fluorinated quinolone carboxylic acid, like all fluoroquinolones. It is a chiral molecule and the sole source of the racemic antibiotic ofloxacin's (S)-enantiomer. (Morrissey et al., 1996) Compared to its (+)-(R) counterpart, this enantiomer more strongly interacts with both topoisomerase IV and the DNA gyrase enzyme. (McGregor, 2008)



Levofloxacin structure.

In the United States, levofloxacin was given medical approval in 1996. It appears on the WHO's list of essential medications (The American Society of Health-System Pharmacists, 2016). It is accessible as a generic drug. With more than 3 million prescriptions written, it was the 182nd most popular drug in the US in 2019. ("The Top 300 of 2019", 2021)

In Bangladesh, Levofloxacin is one of the most available and cheap antibiotics. Almost every pharmacy always has levofloxacin in their stock. A developing country like Bangladesh with a huge population needs this kind of antibiotic which has multipurpose uses and is also affordable. Fighting against antibiotic-resistant bacteria with such an antibiotic will be a groundbreaking discovery.

1.5: MDR, XDR, and PDR

When discussing antibiotic-resistant organisms, the categories MDR, XDR, and PDR are important. MDR organisms are one of the main issues in today's modern era of sophisticated therapies. These three groups of species exhibit resistance. These three classes assist in classifying resistant organisms, which is beneficial for the research world.

Multidrug resistance is referred to as MDR. The organism is referred to as MDR if it is resistant to at least one important antimicrobial agent (Rex, 2019). Gram-positive and gram-negative resistant bacteria are not specifically defined as MDR, which frequently makes it difficult to compare the data accurately. To address this issue, laboratories classify organisms as MDR based on the findings of in vitro tests for antibiotic susceptibility (Magiorakos et al., 2011). When an organism experiences one of the following: (gram-positive or gram-negative) Being resistant to three or more types of antibiotics MDR is acknowledged as the cause (Magiorakos et al., 2011).

The terminology XDR refers to either excessively or extensively high drug resistance. The extensively drug-resistant Mycobacterium tuberculosis, commonly known as XDR MTB, was the

first organism for which the XDR name was used (Magiorakos et al., 2011). There are two ways to define XDR. The first one is based on how many classes or subclasses an organism is capable of surviving. The second one is based on how many important antimicrobials an organism is resistant to. There need to be more than one (Magiorakos et al., 2011).

Pan drug-resistant is the abbreviation. The prefix "pan-," which means "all" or "whole," has its roots in the ancient Greek language. According to Dorland's Illustrated Medical Dictionary, it is significant because it has been used to create a variety of combined biomedical terminology to signify the inclusion of all components or facets of an organism. (Matthew and Drosos, 2018) The word "pan resistance" or "pandrug resistance" (PDR) in this context cannot be understood in any other way than to denote resistance to all antibiotics.

1.6: Objectives of the Study

- Creating a new, long-lasting solution to the increasing issue of gram-negative antibiotic-resistant *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa.*
- Analyzing Levofloxacin's efficiency when used in combination with several medications to treat *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa.* that is multidrug-resistant (MDR).

Chapter 2

Materials and Method

2. Methodology

The experiment was carried out in the BRAC University's laboratories. Prospective research on the efficiency of Levofloxacin against multi-drug resistant gram-negative bacteria *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa* including experiments.

Therefore, *Acinetobacter baumannii, Klebsiella pneumoniae, and Pseudomonas aeruginosa* samples were first collected from several hospitals. On nutrient agar, the samples were grown after being isolated. Then, an Antibiogram was put into action. For the antibiogram on MHA, 18 different antibiotics from 13 different classes were used. Doctors frequently recommended antibiotics to treat bacterial infections. The antibiogram revealed that the samples were resistant to 96% of the common antibiotics. This demonstrated that every sample that was examined was MDR, XDR, and also PDR.

Four antibiotics including Gentamicin, Azithromycin, Levofloxacin, and Moxifloxacin were selected from the list of resistant antibiotics to test in combination with Levofloxacin. First, individual Minimum Inhibitory Concentrations (MIC) for each of the four antibiotics were calculated for the three organisms that had been collected. The next step was to conduct MIC tests with 4 different antibiotic combinations and Levofloxacin. The first test results revealed that the combination of Azithromycin and Levofloxacin produced the best results. Furthermore, all the resistant isolates responded best to the combination. As a consequence, Azithromycin + Levofloxacin was decided upon as the treatment regimen for *Acinetobacter baumannii, Klebsiella pneumoniae, and Pseudomonas aeruginosa* as the research organism.

To do that, 18 more samples of *Acinetobacter baumannii, Klebsiella pneumoniae, and Pseudomonas aeruginosa* were gathered after the first examination and determination. 10 of those 18 samples were identified as MDR, XDR, and PDR in the antibiogram, whereas the other three were not. Each multidrug-resistant *Acinetobacter baumannii, Klebsiella pneumoniae, and Pseudomonas aeruginosa* sample's unique MIC for Azithromycin and Levofloxacin was

determined. Different distinct combinations were made from the resurrected data of all 10 samples to execute the combination MIC determination method. In the MIC determination procedure, the $C_1V_1 = C_2V_2$ formula was applied.

Lastly, the combined MIC and individual MIC for each of the 10 samples were used to estimate the Fractional Inhibitory Concentration (FIC). The effectiveness of the produced result was then determined by comparing the FIC to the standard.

2.1: Sample collection

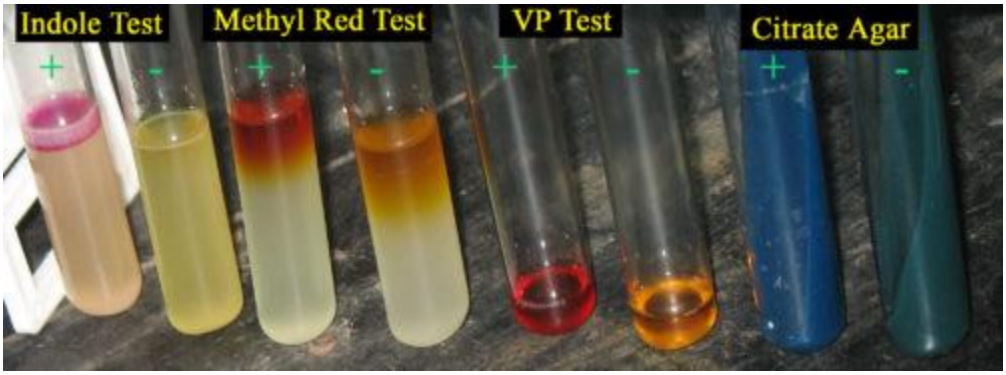
Acinetobacter baumannii, Klebsiella pneumoniae, and Pseudomonas aeruginosa clinical samples have been collected from the microbiology division of BIRDEM Hospital, Uttara Adhunik Medical College Hospital (UAMCH) and the National Institute of Diseases of the Chest and Hospital (NIDCH). The samples were taken on tubes of nutrient agar. The samples underwent nutrient agar slant subculture. It was carefully transported to the BRAC University lab and placed in the incubator for 24 hours at 37°C. The samples were subcultured once again on the nutrient agar dish using the streak plate method after the initial 24 hours of incubation, and the incubation was carried out for 24 hours at 37°C.

Furthermore, each sample was tested for purity using gram staining and other biochemical procedures after the initial growth.

Hence, for continuous culturing, the samples were then streaked on agar plates with specific media, for example, *Acinetobacter baumannii* and *Klebsiella pneumoniae* were streaked in MacConkey agar plates and *Pseudomonas aeruginosa* were streaked in Cetrimide agar plates. To prevent contamination, this action was taken. Additionally, the samples were kept at -20°C on T1N1 agar with paraffin oil in the vial. The stock had been made.

2.2: Performed Biochemical testing

- **Gram staining:** When gram staining was first used, samples showed the expected results. Like, *Acinetobacter baumannii* showed small pink coccobacilli, *Klebsiella pneumoniae* showed pink rod-shaped and *Pseudomonas aeruginosa* appeared as reddish rod-shaped bacteria under the microscope. which also proved these are gram-negative bacteria and the outcome was as expected.
- **Citrate Utilization Test:** A positive result indicates that the media's green hue changed to blue. All three bacteria tested positive and showed a deep blue color.



Biochemical Test.

- **The MIU (Motility Indole Urease) test:** It showed that *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* Indole had no non-motile properties since samples only developed in the stabbing line. As a result, a color change happened.

  Acinetobacter baumannii and *Pseudomonas aeruginosa* were urease negative so, the yellow color remains the same. But as for *Klebsiella pneumoniae*, the media changed from yellow to pink since urease was only marginally beneficial.

- **Lactose fermentation:** The colonies of *Klebsiella pneumoniae* on the MacConkey agar plate caused the agar to shift from pink to yellow. Which means these are non-lactose fermenting bacteria. On the other hand, *Acinetobacter baumannii* is a lactose fermenting bacterium so the colonies remain pink. And *Pseudomonas aeruginosa* does not ferment or produce acid from lactose.

- **TSI (Triple Sugar Iron):** Bacteria were injected into a TSI slant agar tube using a needle. For *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* the slant and butt remain red. But for *Klebsiella pneumoniae*, the slant and butt turned yellow instead of red due to the production of acid from glucose.

2.3: Collection of antibiotics

The 4 antibiotics used in MIC determination process were:

1. Gentamicin,
2. Azithromycin,
3. Levofloxacin,
4. Moxifloxacin

- **Gentamicin:** Chemically speaking, gentamicin sulfate, a white to buff powder that is soluble in water, is what is known as gentamicin sulfate, USP. In 10 ml of distilled water, 0.4 g of Gentamicin was dissolved and the solution was filtered using a syringe filter.
- **Azithromycin**: Aristopharma Ltd.'s Az eye drop was utilized. Every 5 ml of this contains 200 milligrams of azithromycin. So, a 1% Azithromycin solution was created.
- **Levofloxacin**: Levobac eye drop of Popular Pharmaceuticals Ltd was utilized. It was 0.5% Levofloxacin solutions.
- **Moxifloxacin**: Moxifloxacin hydrochloride of pharmaceutical grade was utilized (INN). 10 ml of distilled water was used to dissolve 0.055 mg of moxifloxacin. A syringe filter was then used to filter the fluid. A 0.5% moxifloxacin solution was produced as a result.

Table 1: List of used Antibiotics

Number	Name of Antibiotics	Class
1.	Amikacin	Aminoglycosides
2.	Ampicillin	Penicillin
3.	Azithromycin	Macrolides
4.	Cefalexin	Cephalosporins
5.	Ceftriaxone	Cephalosporins
6.	Ceftazidime	Beta lactam
7.	Co-trimoxazole	Sulfonamides
8.	Cefixime	Cephalosporins
9.	Colistin	Polymyxins
10.	Doxycycline	Tetracycline
11.	Gentamicin	Aminoglycosides
12.	Imipenem	Carbapenems
13.	Kanamycin	Aminoglycosides
14.	Levofloxacin	Fluoroquinolones
15.	Moxifloxacin	Fluoroquinolones
16.	Tetracycline	Tetracycline
17.	Tazobactam	β-lactamases
18.	Tigecycline	Tetracycline

2.4: Preparation of Media:

2.4.1: Nutrient Agar Preparation:

The organisms were first grown on nutrient agar. Nutrient agar was taken in distilled water as per the direction in the container (1 liter of distilled water, and 28 grams of nutrient agar powder were dissolved by heating the mixture until the agar melted). The dissolved agar was then autoclaved for 15 minutes at 121°C. The medium cooled down following the autoclave's completion. After that, the media was added to the Petri dishes and left to set in the laminar.

The fresh media fridge was used to keep the solidified dishes.

2.4.2: MacConkey Agar Preparation:

The frequent subculture process in the research was carried out on MacConkey agar plates. To get the young culture the next day, which is necessary to make the bacterial suspension, samples were streaked daily. To assure sample purity and prevent any contamination, this selective medium was utilized. MacConkey media was prepared as per the direction in the container (1 liter of distilled water, and 49.53g of MacConkey agar powder were dissolved by heating the mixture until the agar melted). It was then put into an autoclave for 15 minutes at 121°C. In the laminar, the autoclaved medium was chilled before being put onto Petri dishes. The media was kept in the refrigerator to store and solidify.

2.4.3: Mueller Hinton Agar (MHA) Preparation:

During the antibiogram procedure, MHA was used. Since it is a non-selection medium, any type of organism can grow there. Being a soft agar, diffusion happens quickly. This is a significant benefit of the disc diffusion procedure. A liter of distilled water was boiled and used to dissolve 38g of MHA powder. Following that, the medium was cooled down for a short while being

autoclaved for 15 minutes at 121°C. After that, the media were put onto Petri dishes in the laminar and allowed to solidify. Antibiograms were then run on the solidified media.

2.4.4: Brain Heart infusion (BHI) Broth Preparation:

The procedure for determining the MIC was carried out using BHI broth. One liter of distilled water was used to dissolve 37 grams of BHI powder. The broth dissolved without the need for heat. Using a glass pipette, the broth was poured into test tubes. BHI broth is made up of 5 ml of each test tube. A beaker containing the test tubes was sterilized by autoclaving at 121°C for 15 minutes. The test tube-filled beaker was then placed in the fresh media refrigerator for later use.

2.5: Physiological Saline Preparation

Biological suspensions of the bacteria were made using physiological saline. Since the saline should only contain 0.9% sodium chloride, it was dissolved in 100 ml of distilled water. Any more than that would cause the environment to become too alkaline, which would kill all the bacteria. Using a glass pipette, 10 ml of saline was then added to each test tube. After being autoclaved at 121°C for 15 minutes, the test tubes were then kept at ambient temperature.

2.6: Bacterial Suspension Preparation

Physiological saline was used to create the bacterial solution. Using a loop, a tiny number of bacteria were extracted from a single colony of a young culture and dissolved in saline. To completely dissolve the germs, the solution was then vortexed. It was then contrasted with solutions using the MacFarland standard 0.5.

2.7: Antibiogram

Antibiogram is a profile of testing for antibiotic susceptibility. The disc diffusion technique is used to accomplish this. Initially, bacterial suspension was equally spread over the Mueller Hinton Agar (MHA) plate using sterile cotton swabs. Five antibiotic disks were then put on each dish. Four MHA plates were used for each *Acinetobacter baumannii*, *Pseudomonas aeruginosa and Klebsiella pneumoniae samples*. After that, the plates underwent a 24-hour period of 37°C incubation in the incubator. Following 24 hours, measurements for each antibiotic disk were taken, and the clear zones were compared to the norm. This made it possible to identify which sample was susceptible to a particular antibiotic and which antibiotic was resistant to it.

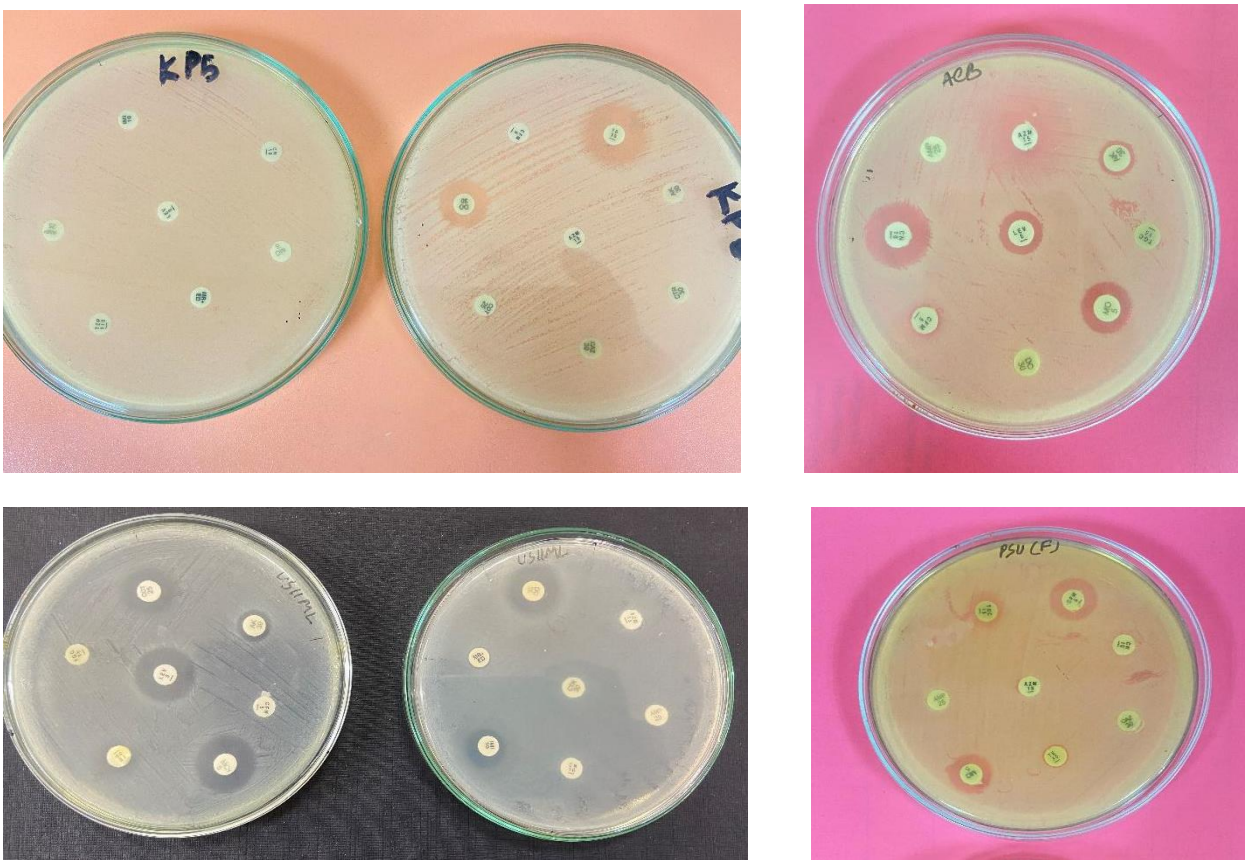


Figure : Antibiogram testing for antibiotic susceptibility *Acinetobacter baumannii, Pseudomonas aeruginosa, and Klebsiella pneumoniae* samples.

2.8: Determination of Minimum Inhibitory Concentration (MIC)

The term "minimum inhibitory concentration" (MIC) refers to the lowest concentration of an antibiotic required to totally stop bacterial growth. This aided in the study to evaluate an antibiotic's effectiveness against a bacterium.

BHI was employed in this situation. First, each antibiotic's MIC against each sample was calculated individually. There were 5 ml of BHI in each test tube. For the first phase's individual MIC determination technique, 13 different antibiotic concentrations were used. 13 additional concentrations were selected for the second phase. The $C_1V_1 = C_2V_2$ formula was used to determine how much of each antibiotic—Azithromycin, and Levofloxacin—had to be added to the 5 ml BHI tubes. Before adding the determined amount of the appropriate antibiotic, the calculated amount was first removed from the BHI tube.

Then each tube received 100 µL of the bacterial suspension (have a MacFarland standard of 0.5). For 18 to 24 hours, the tubes were incubated in a shaker incubator at 37 °C and 80 rpm. After 24 hours, the tubes' turbidity was assessed, and the MIC of the antibiotic for the particular bacterium sample was found in the tube with the lowest concentration of the clear medium.

To identify the MIC of the combination, the serial dilution approach was employed in the initial detection stage. Run six different combinations. The $C_1V_1 = C_2V_2$ formula was applied to 10 novel combination concentrations for the second phase.

The same $C_1V_1 = C_2V_2$ formula was used to calculate the amounts of antibiotics needed to achieve the target concentration in the BHI broth. The remaining steps were identical to the MIC determination steps used for each person. The combination MIC for both antibiotics with a clear medium was established as the lowest total concentration.

To make sure the results were accurate, each MIC determination was carried out twice.

2.9: Calculation of Fractional Inhibitory Concentration (FIC) Index

To control the efficacy of the outcome, the FIC index is calculated. The FIC index is divided into 4 levels:

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

The outcome is more efficient when the FIC value is lower.

- The formula of FIC:

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

- The formula of FIC Index:

$$\text{FIC Index} = \sum(\text{MIC of the agents in combination} / \text{MIC of the agent alone})$$

Using the previously gathered information, these formulae were applied to determine the FIC index for each of the ten *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* samples. The efficacy of each sample was then assessed by comparing the FIC index to the standards.

Chapter 3

Results

3. Results

In the current study, 18 total collected sample samples were gathered from three different hospitals and research institutes, and using the disc diffusion method, those were classified as being either Multidrug-Resistant (MDR) or Extensively Drug-Resistant (XDR) after having been exposed to 19 different antibiotics. Additionally, Levofloxacin was coupled with three antibiotics to see if the mixture could eradicate the pathogen shown in table 3 by itself.

3.1 Categorizing the pathogenic Bacteria

At first, all 18 samples were tested for antibiogram then later on 10 samples which were MDR, XDR, and PDR were selected for MIC determination.

Table 2: Name of the samples

	Acinetobacter baumannii	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***
Sample name	ACB1. ACB2, ACB3, ACB4, ACB5	KP1, KP2, KP3, KP4, KP5, KP6, KP7	PSU1, PSU2, PSU3, PSU4, PSU5, PSU6

Table 3: Antibiogram results of all sample.

NO.	Antibiotic Name	Sample Name																
		ACB1	ACB2	ACB3	ACB4	ACB5	KP1	KP2	KP3	KP4	KP5	KP6	KP7	PSU1	PSU2	PSU3	PSU4	PSU5	PSU6
1	Amikacin	R	R	R	R	R	R	R	R	R	S	R	S	R	S	R	R	S	S
2	Ampicillin	R	R	R	R	R	R	R	I	R	S	R	R	R	R	R	R	R	S
3	Azithromycin	R	R	I	R	S	R	R	R	R	I	R	R	R	S	R	R	R	S
4	Cefalexin	R	R	S	R	R	S	R	R	R	R	S	I	R	S	R	R	S	R
5	Ceftriaxone	R	R	R	R	S	S	S	R	R	R	R	R	R	S	S	R	R	R
6	Ceftazidime	R	R	R	R	S	R	S	I	R	R	S	S	S	R	R	S	R	R
7	Co-trimoxazole	R	S	R	R	R	S	R	R	S	S	R	R	R	R	R	R	S	S
8	Cefixime	R	R	S	S	R	R	R	R	R	R	R	S	R	R	R	S	R	R
9	Doxycycline	R	S	S	R	S	R	R	R	S	S	R	S	R	I	R	R	R	R
10	Gentamicin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
11	Imipenem	R	R	S	R	S	S	R	S	S	R	R	S	R	R	S	R	R	R
12	Levofloxacin	R	R	S	R	S	R	R	R	R	S	R	S	R	I	R	R	S	S
13	Moxifloxacin	R	R	S	R	R	R	R	S	R	S	R	R	R	R	R	R	R	S
14	Tetracycline	R	R	S	R	S	R	R	S	R	R	S	R	R	S	R	R	S	R
15	Tazobactam	S	S	R	R	R	R	S	R	R	S	R	R	S	R	R	S	R	R
16	Tigecycline	R	R	R	S	I	S	R	S	R	R	R	R	R	S	R	R	S	I

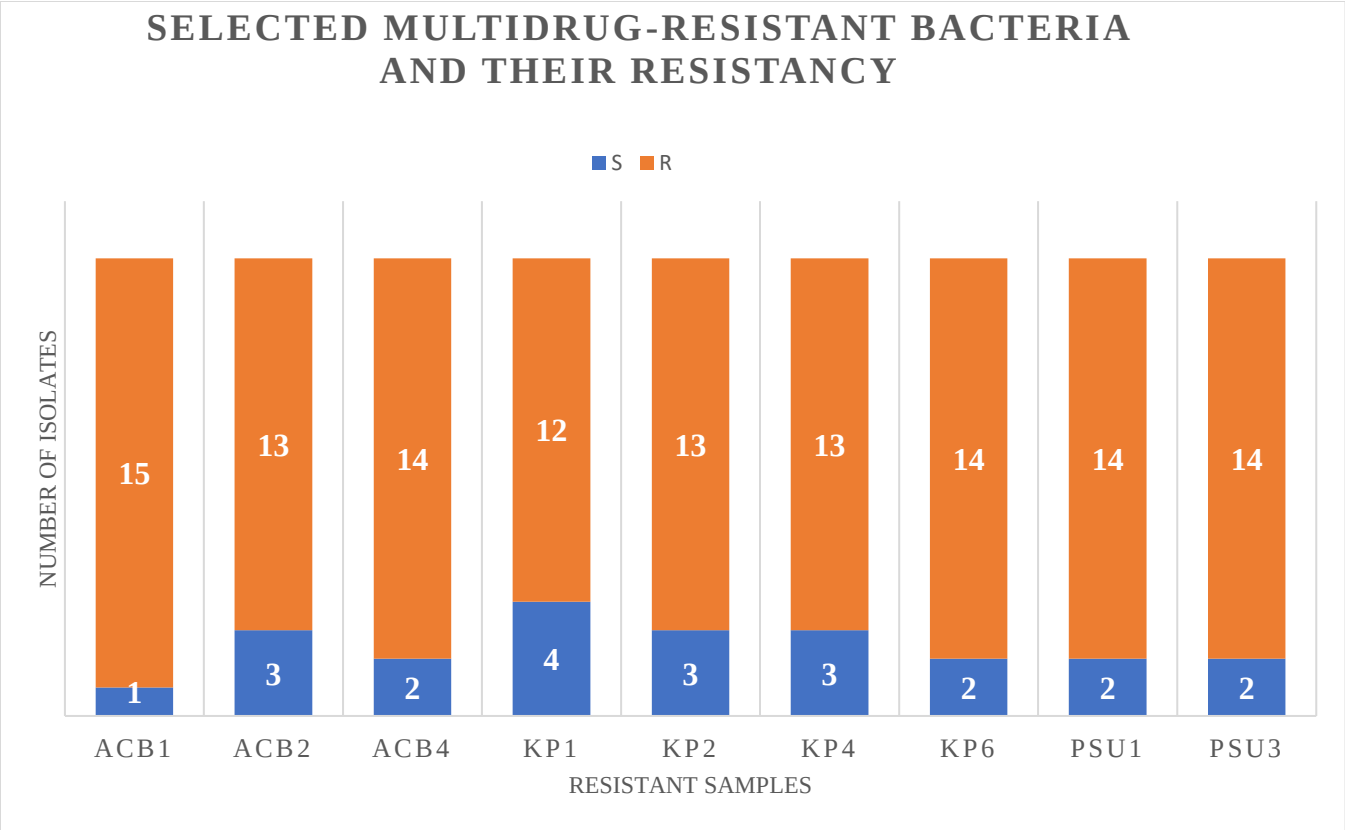


Figure 2: Selected Multidrug resistant bacteria and their resistance.

The disc diffusion technique made it possible to identify which sample was susceptible to a particular antibiotic and which antibiotic was resistant to it. At first, all 18 samples were tested for antibiogram then later on 10 samples which were MDR, XDR, and PDR were selected for MIC determination.

Table 4: Selected multidrug-resistant bacteria for MIC

Total collected sample	MDR	XDR	PDR
18	4	4	2

Table 5: Antibiotic susceptibility of the selected bacteria

Name of the Antibiotic	Samples									
	Acinetobacter baumannii			*Klebsiella pneumoniae*				*Pseudomonas aeruginosa*		
	ACB1	ACB2	ACB4	KP1	KP2	KP4	KP6	PSU1	PSU3	PSU4
Amikacin	R	R	R	R	R	R	R	R	R	R
Ampicillin	R	R	R	R	R	R	R	R	R	R
Azithromycin	R	R	R	R	R	R	R	R	R	R
Cefalexin	R	R	R	S	R	R	S	R	R	R
Ceftriaxone	R	R	R	S	S	R	R	R	S	R
Ceftazidime	R	R	R	R	S	R	S	S	R	S
Co-trimoxazole	R	S	R	S	R	S	R	R	R	R
Cefixime	R	R	S	R	R	R	R	R	R	S
Doxycycline	R	S	R	R	R	S	R	R	R	R
Gentamicin	R	R	R	R	R	R	R	R	R	R
Imipenem	R	R	R	S	R	S	R	R	S	R
Levofloxacin	R	R	R	R	R	R	R	R	R	R
Moxifloxacin	R	R	R	R	R	R	R	R	R	R
Tetracycline	R	R	R	R	R	R	S	R	R	R
Tazobactam	S	S	R	R	S	R	R	S	R	S
Tigecycline	R	R	S	S	R	R	R	R	R	R

[Key: R = Resistant, S = Sensitive]

3.2: Screening antibiotic combination against MDR, XDR, and PDR bacteria

To inhibit the infection from spreading by using Levofloxacin in various combinations with three different medications, including various antibiotic classes, as indicated in table 3, four MDR and four XDR, two PDR strains of the *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* pathogen were chosen at random.

Table 6: The combination of Levofloxacin with several antibiotics and the synergy screening

Combination of Antibiotics	Inhibition of Growth
Levofloxacin + Gentamicin	-
Levofloxacin + Azithromycin	+
Levofloxacin + Moxifloxacin	+

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

3.3: Determination of Minimum Inhibitory Concentration (MIC)

Following the screening of three medications, the effects between Levofloxacin and the minimal inhibitory concentrations of Gentamicin, Moxifloxacin, and Azithromycin were further investigated (MIC). When determining synergistic effects, the FIC index is used as a statistical validation technique. To compare the fractional inhibitory concentration (FIC) to the FIC index, the MIC and FIC were both determined.

3.3.1: Determination of Levofloxacin, Azithromycin, and the combination of Levofloxacin and Azithromycin MIC (First Phase)

Several repeats of the $C_1V_1 = C_2V_2$ procedure and serial dilution were utilized to create the required antibiotic concentration.

Table 7: The MIC value of Levofloxacin for the *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (First Phase)

Antibiotic Name	Antibiotic concentration (µg/ml)	Samples									
		Acinetobacter baumannii			*Klebsiella pneumoniae*				*Pseudomonas aeruginosa*		
		ACB1	ACB2	ACB4	KP1	KP2	KP4	KP6	PSU1	PSU3	PSU4
Levofloxacin	40	T	T	T	T	T	T	T	T	T	T
	50	T	T	T	T	T	T	T	T	C	T
	60	T	T	T	T	T	T	T	T	C	T
	70	T	T	T	T	T	T	T	T	C	T
	75	C	C	T	T	T	T	T	C	C	T
	80	C	C	C	T	T	T	T	C	C	T
	90	C	C	C	T	T	T	T	C	C	T
	100	C	C	C	T	T	T	T	C	C	T

	125	C	C	C	T	T	T	T	C	C	T
	150	C	C	C	T	T	T	C	C	C	T
	200	C	C	C	T	T	T	C	C	C	T

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

The results of the first phase for *Acinetobacter baumannii* are displayed in the Tables 7. To evaluate the MIC value of each drug as well as the combination of antibiotics, three samples were selected from which one was MDR and two were PDR. As for Levofloxacin, the MIC values were 75 µg/ml, 75 µg/ml, and 80 µg/ml.

For *Klebsiella pneumoniae*, four samples were selected for the test of which three were XDR and one was MDR. The MIC value of Levofloxacin were not found. Because KP isolates needs higher concentration.

In the case of *Pseudomonas aeruginosa*, 3 samples were selected that were antibiotic resistant from which one was XDR and two were MDR. The individual Levofloxacin concentrations for two isolates were found which was 75 µg/ml, 50 µg/ml. These were the minimum inhibitory concentrations (Table 7).

Table 8: The MIC value of Azithromycin for the *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (First Phase)

Antibiotic Name	Antibiotic concentration (µg/ml)	Samples									
		Acinetobacter baumannii			*Klebsiella pneumoniae*				*Pseudomonas aeruginosa*		
		ACB1	ACB2	ACB4	KP1	KP2	KP4	KP6	PSU1	PSU3	PSU4
Azithromycin only	50	T	T	T	T	T	T	T	T	T	T
	75	T	T	T	T	T	T	T	T	T	T
	100	C	T	T	T	T	T	T	T	C	T
	125	C	C	T	T	C	T	T	C	C	T
	150	C	C	T	C	C	C	T	C	C	T
	200	C	C	C	C	C	C	C	C	C	T
	250	C	C	C	C	C	C	C	C	C	T
	300	C	C	C	C	C	C	C	C	C	T
	350	C	C	C	C	C	C	C	C	C	T
	400	C	C	C	C	C	C	C	C	C	C

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

In the results of the second phase (Table 8), the individual Azithromycin concentrations for *Acinetobacter baumannii* were 100 µg/ml, (MDR), 125 µg/ml (PDR), 200 µg/ml (PDR) which was found to be the minimum inhibitory concentrations.

For *Klebsiella pneumoniae*, the individual Azithromycin concentrations 150 µg/ml, 125 µg/ml, 150 µg/ml, and 200 µg/ml were found to be the MIC value (Table 8).

In the case of *Pseudomonas aeruginosa*, the individual Azithromycin concentrations 125 µg/ml, 25 µg/ml, and 400 µg/ml were found to be the minimum inhibitory concentrations (Table 8).

Table 9: The MIC value of the combination of Levofloxacin and Azithromycin for the *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (First Phase)

Antibiotic Name	Concentration Antibiotic (µg/ml)		Samples									
			Acinetobacter baumannii			*Klebsiella pneumoniae*				*Pseudomonas aeruginosa*		
	Levofloxacin	Azithromycin	ACB1	ACB2	ACB4	KP1	KP2	KP4	KP6	PSU1	PSU3	PSU4
Levofloxacin + Azithromycin	5	5	T	T	T	T	T	T	T	T	T	T
	10	10	T	C	T	T	T	T	T	T	T	T
	10	15	C	C	T	T	T	T	T	C	C	T
	15	15	C	C	C	T	T	T	T	C	C	T
	20	20	C	C	C	T	T	T	C	C	C	T
	20	30	C	C	C	T	C	T	C	C	C	T
	30	30	C	C	C	C	C	C	C	C	C	T
	35	35	C	C	C	C	C	C	C	C	C	T
	40	40	C	C	C	C	C	C	C	C	C	C

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

In the first phase in table 9, the ACB1, ACB2 and ACB4 exhibited MIC of the Levofloxacin + Azithromycin combination at 25 µg/ml, 20 µg/ml, and 30 µg/ml respectively.

For the *Klebsiella pneumoniae* KP1, KP2, KP4, and KP6 exhibited MIC of the Levofloxacin + Azithromycin combination at 55 µg/ml, 55 µg/ml, 60 µg/ml, and 40 µg/ml respectively shown in table 9.

For *Pseudomonas aeruginosa*, PSU1, PSU3 and PSU4 exhibited MIC of the Levofloxacin + Azithromycin combination at 25 µg/ml, 25 µg/ml, and 80 µg/ml respectively.

3.3.2: Determination of Levofloxacin, Azithromycin, and the combination of Levofloxacin and Azithromycin MIC (Second Phase)

A limitation of the first phase result was the significant concentration gap range of the antibiotics. The results are shown in tables as a consequence of doing the same test twice with a smaller gap range.

Table 10: The MIC value of Levofloxacin for the *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Second Phase)

Antibiotic Name	Antibiotic concentration (µg/ml)	Samples									
		Acinetobacter baumannii			*Klebsiella pneumoniae*				*Pseudomonas aeruginosa*		
		ACB1	ACB2	ACB4	KP1	KP2	KP4	KP6	PSU1	PSU3	PSU4
Levofloxacin only	40	T	T	T	T	T	T	T	T	T	T
	45	T	T	T	T	T	T	T	T	T	T
	50	T	T	T	T	T	T	T	T	C	T
	55	T	T	T	T	T	T	T	T	C	T
	60	T	T	T	T	T	T	T	T	C	T
	70	T	T	T	T	T	T	T	T	C	T
	75	C	C	T	T	T	T	T	C	C	T
	80	C	C	C	T	T	T	T	C	C	T

	90	C	C	C	T	T	T	T	C	C	T
	100	C	C	C	T	T	T	T	C	C	T
	125	C	C	C	T	T	T	T	C	C	T
	150	C	C	C	T	T	T	C	C	C	T
	200	C	C	C	T	T	T	C	C	C	T
	250	C	C	C	T	T	T	C	C	C	T
	300	C	C	C	T	T	C	C	C	C	C
	350	C	C	C	T	T	C	C	C	C	C
	375	C	C	C	T	C	C	C	C	C	C
	400	C	C	C	C	C	C	C	C	C	C
	405	C	C	C	C	C	C	C	C	C	C

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

The results of the second phase for *Acinetobacter baumannii* are displayed in the Table 10. This second phase was done as the confirmatory test. As for Levofloxacin, the MIC values were 75 µg/ml, 75 µg/ml, and 80 µg/ml which was as same as the first phase. Which confirms the accurate MIC value.

For *Klebsiella pneumoniae,* four samples were selected for the test of which three were XDR and one was MDR. The MIC value of Levofloxacin were not found in the first phase. But in second phase, the individual Levofloxacin concentrations 400 µg/ml, 375 µg/ml, 300 µg/ml and 150 µg/ml (Table 10) were found to be the minimum inhibitory concentrations.

In the case of *Pseudomonas aeruginosa,* the individual Levofloxacin concentrations for all isolates were found which was 75 µg/ml, 50 µg/ml, 300 µg/ml. These were the minimum inhibitory concentrations (Table 10).

Table 11: The MIC value of Azithromycin for the *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Second Phase)

Antibiotic Name	Antibiotic concentration (µg/ml)	Samples									
		Acinetobacter baumannii			*Klebsiella pneumoniae*				*Pseudomonas aeruginosa*		
		ACB1	ACB2	ACB4	KP1	KP2	KP4	KP6	PSU1	PSU3	PSU4
Azithromycin only	90	T	T	T	T	T	T	T	T	T	T
	100	C	T	T	T	T	T	T	T	C	T
	110	C	T	T	T	T	T	T	T	C	T
	120	C	T	T	T	T	T	T	T	C	T
	125	C	C	T	T	C	T	T	C	C	T
	130	C	C	T	T	C	T	T	C	C	T
	150	C	C	T	C	C	C	T	C	C	T
	175	C	C	T	T	C	C	T	C	C	T
	200	C	C	C	C	C	C	C	C	C	T
	350	C	C	C	C	C	C	C	C	C	T
	400	C	C	C	C	C	C	C	C	C	C

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

The results of the second phase in Table 8, the individual Azithromycin concentrations for *Acinetobacter baumannii* were 100 µg/ml, (MDR), 125 µg/ml (PDR), 200 µg/ml (PDR) which was found to be the minimum inhibitory concentrations. Which was a confirmatory test.

For *Klebsiella pneumoniae*, the individual Azithromycin concentrations 150 µg/ml, 125 µg/ml, 150 µg/ml, and 200 µg/ml were found to be the MIC value (Table 8).

In the case of *Pseudomonas aeruginosa*, the individual Azithromycin concentrations 125 µg/ml, 25 µg/ml, and 400 µg/ml were found to be the minimum inhibitory concentrations (Table 8).

Table 12: The MIC value of the combination of Levofloxacin and Azithromycin for the *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Second Phase).

Antibiotic Name	Concentration Antibiotic (µg/ml)		Samples									
			Acinetobacter baumannii			*Klebsiella pneumoniae*				*Pseudomonas aeruginosa*		
	Levofloxacin	Azithromycin	ACB1	ACB2	ACB4	KP1	KP2	KP4	KP6	PSU1	PSU3	PSU4
Levofloxacin + Azithromycin	5	5	T	T	T	T	T	T	T	T	T	T
	10	10	T	C	T	T	T	T	T	T	T	T
	5	15	T	C	T	T	T	T	T	T	T	T
	10	15	C	C	T	T	T	T	T	C	C	T
	15	15	C	C	C	T	T	T	T	C	C	T
	20	20	C	C	C	T	T	T	C	C	C	T
	20	30	C	C	C	T	C	T	C	C	C	T
	25	25	C	C	C	T	C	T	C	C	C	T
	30	30	C	C	C	C	C	C	C	C	C	T
	30	25	C	C	C	C	C	C	C	C	C	T
	35	35	C	C	C	C	C	C	C	C	C	T
	40	40	C	C	C	C	C	C	C	C	C	C

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

In the second phase, the MIC value of the combination of Levofloxacin and Azithromycin for the *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* was similar to first phase. Here, the difference between the concentration was less than in the first phase. To lessen the concentrations in combination screening in second phase.

In the second phase in table 12, three samples of *Acinetobacter baumannii* were selected from which one was MDR and two were PDR. The ACB1, ACB2, and ACB4 exhibited MIC of the Levofloxacin + Azithromycin combination at 25 μg/ml, 20 μg/ml, and 30 μg/ml respectively of which is similar to first phase.

For the *Klebsiella pneumoniae,* of which three were XDR and one was MDR. In table 12, KP1, KP2, KP4, and KP6 exhibited MIC of the Levofloxacin + Azithromycin combination at 55 μg/ml, 55 μg/ml, 60 μg/ml, and 40 μg/ml respectively which consider as the confirmatory test.

For *Pseudomonas aeruginosa,* 3 samples were selected that were antibiotic resistant from which one was XDR and two were MDR. Here, PSU1, PSU3, and PSU4 exhibited MIC of the Levofloxacin + Azithromycin combination at 25 μg/ml, 25 μg/ml, and 80 μg/ml respectively.

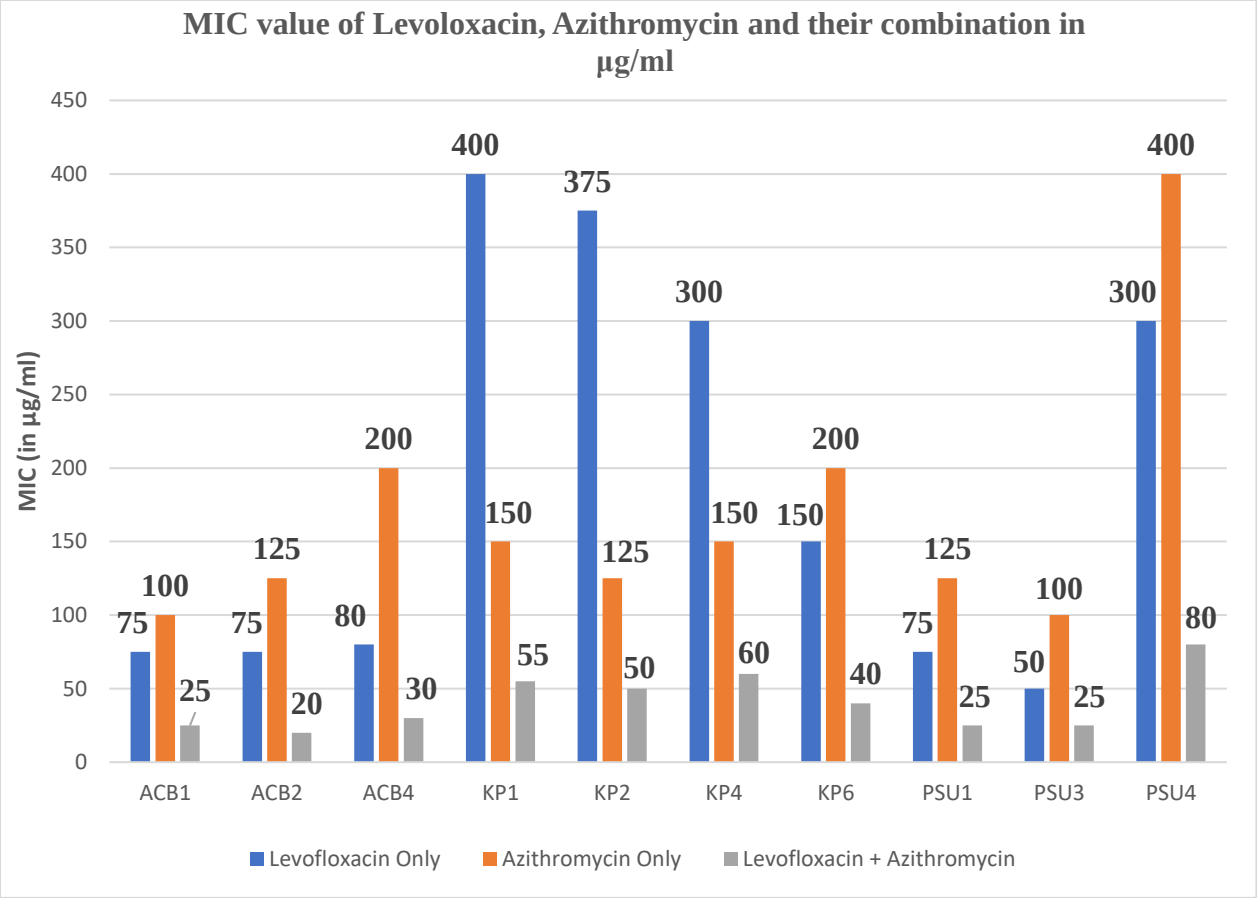


Figure 8: MIC value of Levofloxacin, Azithromycin and their combination in µg/ml.

Figure 9: MIC test of Levofloxacin + Azithromycin combination for *Acinetobacter baumannii* - First phase (ACB1, ACB2 and ACB4)

Figure 10: MIC test of Levofloxacin + Azithromycin combination for *Klebsiella pneumoniae* - First phase and second phase (KP1, KP2, KP4 and KP6)

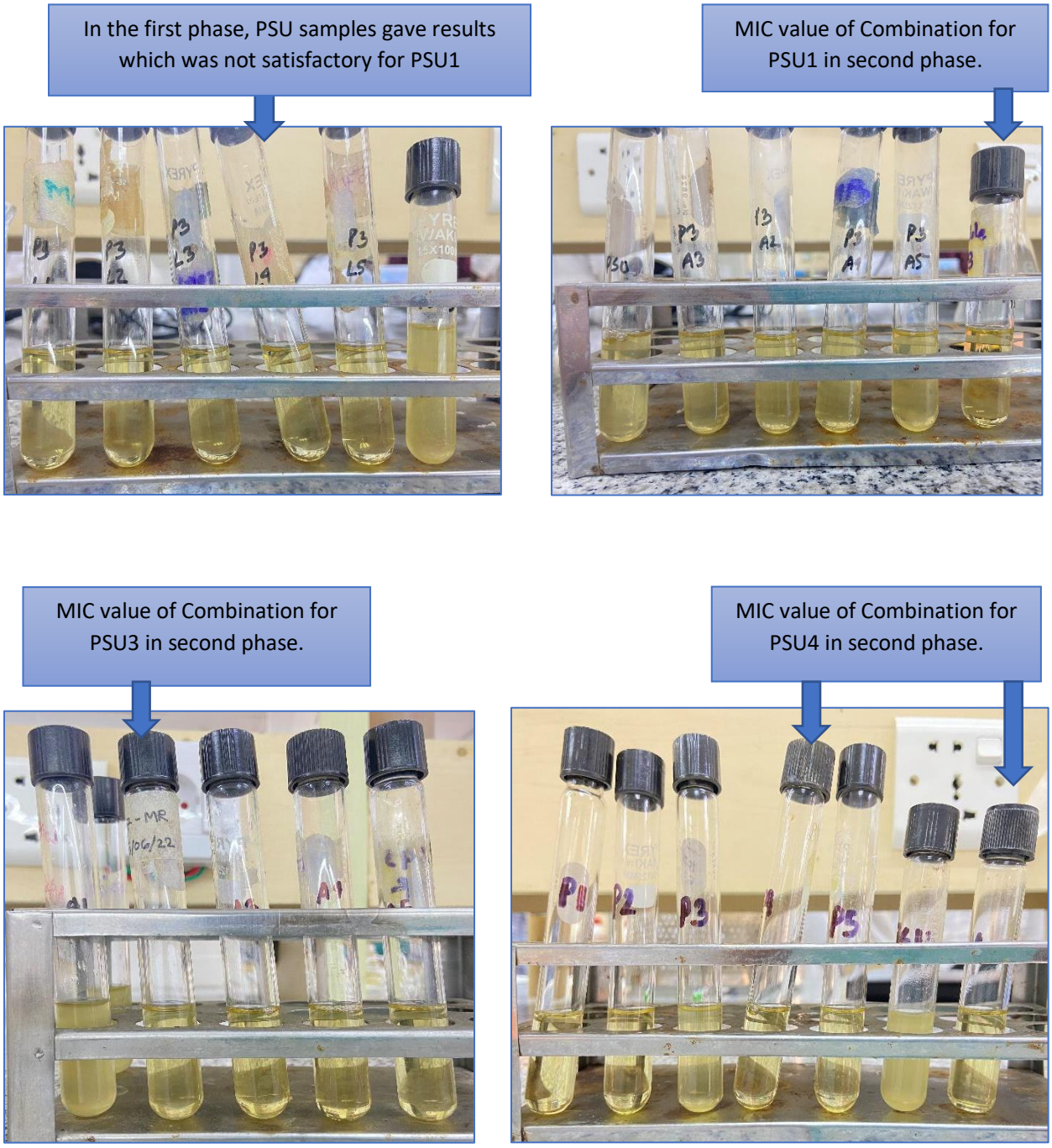


Figure 11: MIC test of Levofloxacin + Azithromycin combination for *Pseudomonas aeruginosa* - First phase and second phase (PSU1, PSU3 and PSU4)

3.4: Determination of the arithmetic mean MIC value of Levofloxacin, Azithromycin and their combination

Table 13: The Average MIC value of Levofloxacin, Azithromycin and their combination in µg/ml & FIC Index.

Samples	Sample Number	MIC (in µg/ml)				FIC Index*
		Levofloxacin Only	Azithromycin Only	Levofloxacin + Azithromycin		
				Levo	Az	
Acinetobacter baumannii	ACB1	75	100	10	15	0.55
	ACB2	75	125	10	10	0.30
	ACB4	80	200	15	15	0.52
Klebsiella pneumoniae	KP1	400	150	30	25	0.43
	KP2	375	125	20	30	0.53
	KP4	300	150	30	30	0.60
	KP6	150	200	20	20	0.40
Pseudomonas aeruginosa	PSU1	75	125	10	15	0.53
	PSU3	50	100	10	15	0.75
	PSU4	300	400	40	40	0.46

[FIC = Fractional Inhibitory Concentration which is determined by MIC of the agents in a combination of the agent alone. *FIC index = ∑ (MIC of the agents in combination/MIC of the agent alone)]

3.5: The Average FIC Index of Levofloxacin and Azithromycin

For *Acinetobacter baumannii*, the arithmetic mean of the FIC index is 0.45 which is below 0 .5, and statistical synergistic effects of Levofloxacin and Azithromycin. In the first phase, the ACB1, ACB2 and ACB4 exhibited MIC of the Levofloxacin + Azithromycin combination at 25 µg/ml, 20 µg/ml, and 30 µg/ml respectively (Table 13). In the first phase, *Acinetobacter baumannii* samples gave satisfactory results, and even in confirmatory tests in the second phase, the results were similar.

For *Klebsiella pneumoniae*, the arithmetic mean of the FIC index is 0.5 which statistical synergistic effects of Levofloxacin and Azithromycin. In the first phase, the *Klebsiella pneumoniae* KP1, KP2, KP4, and KP6 exhibited MIC of the Levofloxacin + Azithromycin combination at 55 µg/ml, 55 µg/ml, 60 µg/ml, and 40 µg/ml respectively (Table 13). In the first phase, KP samples gave results which were not satisfactory. In the MIC of Levofloxacin, the concentrations were above expected. But in the second phase, MIC of the samples was counted and in the confirmatory test, the results were similar and satisfactory.

For *Pseudomonas aeruginosa*, the arithmetic mean of FIC index is 0.5 which is statistical synergistic effects of Levofloxacin and Azithromycin. In the first phase, the Pseudomonas aeruginosa PSU1, PSU3 and PSU4 exhibited MIC of the Levofloxacin + Azithromycin combination at 25 µg/ml, 25 µg/ml, and 80 µg/ml respectively (Table 13). In the first phase, Pseudomonas aeruginosa samples gave satisfactory results and even in confirmatory test for in the second phase, the results were similar.

4. Discussion

Gram-negative bacterial infections are especially worrisome since they are becoming more and more resistant to almost every antibiotic now in use, resembling pre-antibiotic circumstances. Across the board, medical practice has been affected by the emergence of MDR gram-negative bacteria. In hospital settings, *Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter* are the most typical Enterobacteriaceae pathogens that cause gram-negative infections. Globally, the prevalence of MDR gram-negative bacteria is also rising (Ventola, 2015). The numbers of Multidrug resistant bacterial cases are rising up because of overuse of the high doses of antibiotics. Soon, the treatment for MDR will include antibiotic combination therapy, using synergies, rejuvenating outdated medications, and reducing resistance. (Anthony et al., 2020)

Previously, researchers have used different antibiotic combinations for the gram-negative bacteria such as, polymyxin B or colistin + rifampin, imipenem, or azithromycin; rifampin + azithromycin; sulbactam + rifampin, azithromycin, or a quinolone; and the triple combination of polymyxin B, imipenem, and rifampin (James, 2006), tigecycline + gentamicin and tigecycline + colistin (Falagas et al., 2014), azithromycin + chloramphenicol, levofloxacin + rifampin, polymyxin B + tigecycline (Lim et al., 2016). For this study, my goal was to find the most available and cost-effective combination as we live in a where large portion of people live in a rural area. That is the reason I have choose Levofloxacin. It appears on the WHO's list of essential medications (The American Society of Health-System Pharmacists, 2016). Levofloxacin is one of the most widely accessible and reasonably priced medicines in Bangladesh. Levofloxacin is often constantly on hand at pharmacies. This class of antibiotics, which has several applications and is also reasonably priced, is necessary for a growing nation with a large population like Bangladesh. It will be a ground-breaking finding to use such an antibiotic to combat bacteria that are resistant to other antibiotics.

In this experiment, three antibiotics — Gentamicin, Azithromycin, Moxifloxacin — were used in conjunction with Levofloxacin for the first screening. Levofloxacin + Gentamicin and

Levofloxacin + Moxifloxacin have failed to stop bacterial growth even at the maximum concentration bar established for the combination, which was above 500 g/ml, according to the results of the MIC determination method carried out in BHI. Inhibition was present in the other two pairings. The Levofloxacin + Azithromycin combination outperformed the other two combinations. As a consequence, the combination of Levofloxacin and Azithromycin was chosen for additional research as the combination of Azithromycin and Levofloxacin produced the best results for *Acinetobacter baumannii, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa.*

In this study, 18 more samples of *Acinetobacter baumannii, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* were gathered from which 10 of them were identified as MDR, XDR and PDR in the antibiogram. The 10 samples showed MIC during the individual MIC testing technique at extremely high antibiotic doses, indicating that the samples had considerable resistance to Levofloxacin and Azithromycin. The highest demonstrated MIC value for Levofloxacin was 400 μg/ml, while the lowest was 50 μg/ml. The highest value for Azithromycin was 400 μg/ml and the lowest was 100 μg/ml. Each multidrug resistant *Acinetobacter baumannii, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* sample's unique MIC for Azithromycin and Levofloxacin was determined. Different distinct combinations were made from the resurrected data of all 10 samples to execute the combination MIC determination method. In the MIC determination procedure, the $C_1V_1 = C_2V_2$ formula was applied.

For *Acinetobacter baumannii,* the arithmetic mean of the FIC index is 0.45 which is below 0 .5, and statistical synergistic effects of Levofloxacin and Azithromycin. In the first phase, the ACB1, ACB2 and ACB4 exhibited MIC of the Levofloxacin + Azithromycin combination at 25 μg/ml, 20 μg/ml, and 30 μg/ml respectively (Table 13). For *Klebsiella pneumoniae,* the arithmetic mean of the FIC index is 0.5 which statistical synergistic effects of Levofloxacin and Azithromycin. In the first phase, the *Klebsiella pneumoniae* KP1, KP2, KP4, and KP6 exhibited MIC of the Levofloxacin + Azithromycin combination at 55 μg/ml, 55 μg/ml, 60 μg/ml, and 40 μg/ml respectively (Table 13), the results were similar and satisfactory. For *Pseudomonas aeruginosa,* the arithmetic mean of FIC index is 0.5 which is statistical synergistic effects of Levofloxacin and Azithromycin. In the first phase, the Pseudomonas aeruginosa PSU1, PSU3 and PSU4 exhibited

MIC of the Levofloxacin + Azithromycin combination at 25 µg/ml, 25 µg/ml, and 80 µg/ml respectively (Table 13).

Results from the procedure to calculate the combination's MIC were encouraging. According to the data shown in the reduction in MIC, it was possible to decrease the minimum antibiotic concentration required to suppress bacterial growth by simply mixing two previously resistant medicines. The MIC with the lowest combination was 20 µg/ml. This brought attention to the value of utilizing antibiotics in combination.

This study demonstrates the previously undocumented originality of the effectiveness of the combination of Levofloxacin and Azithromycin on the multidrug-resistant *Acinetobacter baumannii, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*. This in vitro investigation has discovered the synergistic action of these two antibiotics. Combining antibiotics is currently a standard hospital practice for treating severe infections empirically (Lim et al., 2015), although the guidelines for doing so are not yet clearly defined. The utilization of various combination regimens for treating *Acinetobacter baumannii, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* that is extremely antibiotic resistant has been the subject of several studies, although these studies frequently lacked in vivo confirmation. Which antimicrobial agent/class combinations work best for treating resistant pathogens is still a mystery.

Another interesting finding is the lowest FIC index of Levofloxacin and Azithromycin for *Acinetobacter baumannii, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* were found 0.3, 0.4, and 0.46 accordingly which is a groundbreaking discovery.

5. Conclusion

In summation, it would be dangerous to minimize the threat posed by microorganisms resistant to antibiotics, especially ESKAPE pathogens such as *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, which has emerged as a superbug and is quickly developing resistance mechanisms (Louis, 2008). The extraordinary health benefits of antibiotics are at danger due to the quick spread of bacteria that are resistant to them. Due to the widespread overuse of antibiotics and the lack of new antibiotic agents being created by pharmaceutical companies to address the issue, this is a worldwide disaster. If the effectiveness of this antibiotic can be boosted by the use of other drugs, it may be both life-saving and economical.

Additionally, as there are an increasing number of antibiotic-resistant infections in developing nations like ours, we can benefit from this combination to fight *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* and reduce the pneumonia mortality rate. However, when it comes to life or death, it is necessary to develop new methods to combat these superbugs, and existing antibiotic combinations may be a good choice.

The findings of the study definitely demonstrate how combining antibiotics can reduce the quantity of antibiotics required to treat infections that are resistant to them. The future of combination treatment against multidrug-resistant *Acinetobacter baumannii, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* may be significantly impacted by these studies.

References:

1. Annu Rev Biochem. (2016) Multidrug Resistance in Bacteria. doi: 10.1146/annurev.biochem.78.082907.145923
2. Breidenstein Césarde la Fuente-Núñez Robert E.W. Hancock (2011). Pseudomonas aeruginosa: all roads lead to resistance. volume 19.
3. Corrin, B., & Nicholson, A. G. (2011). Pathology of the Lungs E-Book. Elsevier Gezondheidszorg. Ferreira, R. L., da Silva, B. C. M., Rezende, G. S., Nakamura-Silva, R., Pitondo-Silva, A., Campanini, E. B., Brito, M. C. A., da Silva, E. M. L., Freire, C. C. D. M., Cunha, A. F. D., & Pranchevicius, M. C. D. S. (2019). High Prevalence of Multidrug-Resistant Klebsiella pneumoniae Harboring Several Virulence and β-Lactamase Encoding Genes in a Brazilian Intensive Care Unit. Frontiers in Microbiology, 9. https://doi.org/10.3389/fmicb.2018.03198
4. Drenkard - Microbes and infection, 2003. Antimicrobial resistance of Pseudomonas aeruginosa biofilms.
5. Falagas ME, Lourida P, Poulikakos P, Rafailidis PI, Tansarli GS. (2014). Antibiotic treatment of infections due to carbapenem-resistant enterobacteriaceae: systematic evaluation of the available evidence. Antimicrob Agents Chemother 58:654–663. 10.1128/AAC.01222-13.
6. Fehlberg, L. C., Carvalho, A. M., Campana, E. H., Gontijo-Filho, P. P., & Gales, A. C. (2012). Emergence of Klebsiella pneumoniae-producing KPC-2 carbapenemase in Paraíba, Northeastern Brazil. The Brazilian Journal of Infectious Diseases, 16(6), 577– 580. https://doi.org/10.1016/j.bjid.2012.07.001
7. Garbati, M., & al Godhair, A. (2013). The Growing Resistance of Klebsiella pneumonia; Healthcare-Associated Infections (HAIs). Pseudomonas aeruginosa in Healthcare Settings Joseph Bennington-Castro. (2020) What Is Pseudomonas Aeruginosa? Symptoms, Causes, Diagnosis, Treatment, and Prevention. https://www.cdc.gov/hai/organisms/pseudomonas.html
8. Groopman, J (2008-08-11). "Superbugs". The New Yorker. Retrieved 2013-07-07. The new generation of resistant infections is almost impossible to treat. https://www.everydayhealth.com/pseudomonas-aeruginosa/

9. Gaynes, AJ Rush, M Trivedi, SR Wisniewsk General hospital (2005). A direct comparison of presenting characteristics of depressed outpatients from primary vs. specialty care settings: preliminary findings from the STAR* D clinical trial. 500-510
10. Hancock, DP Speert - Drug resistance updates, 2000 Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment
11. Infection. Healthline. https://www.healthline.com/health/klebsiella-pneumonia
12. Ioannis Kyriakidis 1 2, Eleni Vasileiou 1, Zoi Dorothea Pana 3, Athanasios Tragiannidis (2021) Acinetobacter baumannii Antibiotic Resistance Mechanisms. DOI: 10.3390/pathogens10030373
13. Ito, R., Shindo, Y., Kobayashi, D., Ando, M., Jin, W., Wachino, J. I., Yamada, K., Kimura, K., Yagi, T., Hasegawa, Y., & Arakawa, Y. (2015). Molecular Epidemiological Characteristics of Klebsiella pneumoniae Associated with Bacteremia among Patients with Pneumonia. Journal of Clinical Microbiology, 53(3), 879–886. https://doi.org/10.1128/jcm.03067-14
14. James, RJ Moore, MJ Perry - British Journal of Oral and Maxillofacial (2006) Impregnation of antibiotic into porous high-density polyethylene material using negative pressure. 29-28.
15. Journal of Infectious Diseases, 7(1). https://doi.org/10.4314/ajid.v7i1.2
16. Klebsiella pneumoniae: Antimicrobial Resistance, Virulence and Therapeutic Strategies (2022) https://www.frontiersin.org/research-topics/33656/klebsiella-pneumoniae-antimicrobial-resistance-virulence-and-therapeutic-strategies#overview
17. Lenchenko, E., Blumenkrants, D., Sachivkina, N., Shadrova, N., & Ibragimova, A. (2020). Morphological and adhesive properties of Klebsiella pneumoniae biofilms. January-2020, 13(1), 197–200. https://doi.org/10.14202/vetworld.2020.197-200
18. Lim TP, Cai Y, Hong Y, Chan EC, Suranthran S, Teo JQ, Lee WH, Tan TY, Hsu LY, Koh TH, Tan TT, Kwa AL. 2015. In vitro pharmacodynamics of various antibiotics in combination against extensively drug-resistant Klebsiella pneumoniae. Antimicrob agentss Chemother 59:2515–2524. 10.1128/AAC.03639-14.
19. Lim, T., Cai, Y., Hong, Y., Chan, E. C., Suranthran, S., Teo, J. Q, Kwa, A. L. (2015). In Vitro Pharmacodynamics of Various Antibiotics in Combination against Extensively

Drug-Resistant Klebsiella pneumoniae. Antimicrobial Agents and Chemotherapy, 59(5), 2515-2524.

20. Osmon, D. R.; Berbari, E. F.; Berendt, A. R.; Lew, D.; Zimmerli, W.; Steckelberg, J. M.; Rao, N.; Hanssen, A.; Wilson, W. R.; Infectious Diseases Society of America. (2012). "Diagnosis and Management of Prosthetic Joint Infection: Clinical Practice Guidelines by the Infectious Diseases Society of America". Clinical Infectious Diseases. 56 (1): e1–e25.
21. Matthew E. Falagas, Drosos E. Karageorgopoulos (2018) Pandrug Resistance (PDR), Extensive Drug Resistance (XDR), and Multidrug Resistance (MDR) among Gram-Negative Bacilli: Need for International Harmonization in Terminology. https://doi.org/10.1086/528867
22. Magiorakos, A. P., Srinivasan, A., Carey, R., Carmeli, Y., Falagas, M., Giske, C., Harbarth, S., Hindler, J., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D., Rice, L., Stelling, J., Struelens, M., Vatopoulos, A., Weber, J., & Monnet, D. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology and Infection, 18(3), 268–281.
23. Olsson, A., Wistrand-Yuen, P., Nielsen, E. I., Friberg, L. E., Sandegren, L., Lagerbäck, P., & Tängdén, T. (2020). Efficacy of Antibiotic Combinations against Multidrug-Resistant *Pseudomonas aeruginosa* in Automated Time-Lapse Microscopy and Static Time-Kill Experiments. Antimicrobial Agents and Chemotherapy, 64(6).
24. Queenan, A. M., & Bush, K. (2007). Carbapenemases: the Versatile β-Lactamases. Clinical Microbiology Reviews, 20(3), 440–458. https://doi.org/10.1128/cmr.00001-07
25. Rex, J. (2021, October 3). Categories of resistance: MDR, XDR, PDR, UDR, and (new!) DTR. AMR.Solutions. https://amr.solutions/2019/01/13/categories-of-resistance-mdr-xdr-pdr-udr-and-new-dtr/
26. Shiri Navon-Venezia, Kira Kondratyeva, Alessandra Carattoli (18 May 2017). Klebsiella pneumoniae: a major worldwide source and shuttle for antibiotic resistance. https://doi.org/10.1093/femsre/fux013
27. The Journal of Global Antimicrobial Resistance meets the World Health Organization (WHO). (2019b). Journal of Global Antimicrobial Resistance, 18, 305–308. https://doi.org/10.1016/j.jgar.2019.07.022

28. The Need to Expand Our Antibiogram: Case Report and Review of the Literature. African
29. Urszula Surel, Katarzyna Niemirowicz, Michal Marzec, Paul B. Savage, Robert Bucki. (2014) Ceragenins – a new weapon to fight multidrug resistant bacterial infections. 56-59.
30. Ventola C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. P & T : a peer-reviewed journal for formulary management, 40(4), 277–283.
31. Vrancianu, I Gheorghe, IB Czobor, MC Chifiriuc - Microorganisms, 2020. Antibiotic Resistance Profiles, Molecular Mechanisms and Innovative Treatment Strategies of Acinetobacter baumannii.
32. Walter (2005) In vitro activity of telithromycin against macrolide-susceptible and macrolide-resistant pharyngeal isolates of group A streptococci in the United States.
33. Yearul Kabir (2012) Multidrug Resistant bacteria in the hospital sewage water of Dhaka city,
34. Zheng Panga Renee Raudonisb Bernard R.Glickc Tong-JunLina Zhenyu Cheng (2019) Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. https://doi.org/10.1016/j.biotechadv.2018.11.013

Appendix

Appendix 1:

Media Composition:

Nutrient Agar:

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

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) Model: LDO-060E	Labtech, Singapore
Refrigerator, Model: 0636	Samsung
Vortex Mixture	VWR International