Combined effect of Cefixime and Azithromycin against multiple antibiotics resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

By

Fariba Salahuddin
16136033

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

B.Sc. in Biotechnology

Department of Mathematics and Natural Sciences
BRAC University

Declaration

It is hereby declared that

1. The thesis submitted is my 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. I have acknowledged all of the main sources of help.

Student's Full Name & Signature:

Fariba Salahuddin
16136033

Approval

The thesis/project titled "Establishment of combination therapy of Cefixime and Azithromycin against multiple antibiotics resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*" submitted by

1. Fariba Salahuddin (16136033)

of Spring 2013, has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Biotechnology on 24th December 2019

Examining Committee:

Supervisor:
(Member)

_____
M. Mahboob Hossain
Professor, Department of Mathematics and Natural Sciences
BRAC University

_____
Akash Ahmed
Lecturer, Department of Mathematics and Natural Sciences
BRAC University

Co-Supervisor:
(Member)

Program Coordinator:
(Member)

_____
Iftekhar Bin Naser
Assistant Professor
BRAC University

Departmental Head:
(Chair)

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

Abstract

Pneumonia is a lung infection with a variety of possible causes. It can be a severe disease with an infection of bacteria, virus or fungus. The lungs are inflamed and the tiny air sacs become filled with fluid inside. Infants, young children and people over the age of 65 suffer most because they have a vulnerable immune system. Two major pathogens causing pneumonia that were studied are *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

The objective of the present study was to design a new method to manage antibiotic resistance against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. A total of 30 bacterial samples of lung infection were studied against 10 antibiotics of different classes. The primary screening was accomplished with the combination of cefixime with other antibiotics to understand the synergistic effect. Cefixime disc combined with azithromycin antibiotic solution gave the synergistic effect. Furthermore, the minimum inhibitory concentration (MIC) was performed individually with cefixime and azithromycin. A high number of multidrug-resistant (MDR) *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showed more susceptibility to azithromycin compared to that of cefixime. Then the minimum inhibitory concentration (MIC) was generated combining both cefixime and azithromycin. Furthermore, the results were interpreted with FIC index after calculating the FIC (Fractional inhibitory concentration).

Keywords: antibiotic resistant; antimicrobial agent; lung infection; synergistic effect; combination; primary screening

Dedication

To My Family & Friends

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Table of Contents

List of Tables

List of Figures

List of Acronyms

MDR	Multi Drug Resistant
XDR	Extensively Drug Resistant
PDR	Pan Drug Resistant
CFU	Colony Forming Unit
et al	And others
NIDCH	National Institute of Diseases of the Chest and Hospital
Fig	Figure
mg	milligram
µg	microgram
MIC	Minimum Inhibitory Concentration
FIC	Fractional Inhibitory Concentration
NA	Nutrient Agar
MHA	Muler Hinton Agar
BHI	Brain Heart Infusion

Chapter 1

INTRODUCTION

Chapter 1

Introduction and Literature Review

1.1 Overview

Pneumonia is a lung infection with a variety of possible causes. It can be a serious disease that is life-threatening. It usually starts with an infection of bacteria, virus, or fungus. According to the germs type, Pneumonia has been classified into- (i) Community-acquired pneumonia which occurs outside of hospitals or other health care facilities. (ii) Hospital-acquired pneumonia, in this case, people catch pneumonia during a hospital stay. (iii) Health care-acquired pneumonia where the patient receives care in outpatient clinics like kidney dialysis centers. (iv) Aspiration pneumonia is the state where pneumonia develops from brain damage, swallowing problems, or excessive addiction for alcohol or drugs. Numerous germs can be responsible for pneumonia. Bacteria and viruses in the air that we breathe are the most harmful and lead to cause pneumonia among children and adults from different ages. Bacterial pneumonia, viral pneumonia, and mycoplasma pneumonia are the main types of pneumonia. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are two common bacteria that are capable to infect the upper respiratory tract and infect the air sacs with pus and other liquid.

Antibiotics are medicines that are used to cure and prevent bacterial infections. The discovery of penicillin by Sir Alexander Fleming in 1928 paved a new way for antibiotics. Since then, the modern era of antibiotics was initiated and saved millions of lives. During World War II, penicillin was successful to cure bacterial infections among the soldiers (Sengupta *et al.*, 2013). Antibiotics have played a key role in making significant progress in medicine and surgery. Patients with diabetes, renal disease, rheumatoid arthritis or have gone through organ transplants, joint replacements, or cardiac are possible to treat their infections successfully with antibiotics (Gould *et al.,* 2013). But during recent decades, the chromosomal change or the

exchange of genetic material via plasmids and transposons have made the bacteria to become resistant to antimicrobial agents (Neu, 1992). Acquisition of foreign DNA material through Horizontal Gene Transfer (HGT) is another cause of bacterial evolution and responsible for the development of antimicrobial resistance (Munita *et al.,* 2016).

Klebsiella pneumoniae is experiencing resistant to quite a lot of antibiotics **(**Ashurst *et al.,* 2019) So combating *Klebsiella* has been much challenging **s**ince it causes nosocomial infections and gives rise to substantial morbidity and mortality. In the future for treating *Klebsiella* would be hard since it has almost become an antibiotic-resistant bacterium.

Pseudomonas aeruginosa is known to be an opportunistic pathogen because it attacks a host when that particular person has compromised the immune system (Moradali *et al.,* 2017). The strains of *Pseudomonas aeruginosa* causes life-threatening infections because it has a high range of adaptive mechanism to a high number of different antibiotics.

1.2 Characteristics of *Klebsiella pneumoniae*

Klebsiella pneumoniae is categorized as a Gram-negative, non-motile with encapsulated lactose fermenting bacterium. It is a rod-shaped bacterium of the Enterobacteriaceae family (Boone et al., 2001). The genus is named after German physician and bacteriologist Edwin Klebs. It is also known Friedländer's bacillus because it was first described by German microbiologist Carl Friedlände during 1882. (Ashurst *et al.,* 2019).

1.3 Characteristics of *Pseudomonas aeruginosa*

This bacterium is aerobic Gram-negative with rod-shaped. The presence of polar flagellum allows its mobility and thus helps in locomotion as well. The microscopic view ranges from

0.5-0.8 micrometers by 1.5-3.0 micrometers approximately (Iglewski *et al.,* 1996). *Pseudomonas* produces a blue-green pigment named as pyocyanin and a yellow-green pigment named as fluorescein (Lamont *et al.,* 2003). Pharmacist Carle Gessard first isolated *Pseudomonas aeruginosa* from green pus in1882 (Abdelraouf *et a*l., 2017). When the immune system is compromised this opportunistic bacterium breaks down the skin and enters the body of the individuals.

1.4 Emergence of antibiotic-resistant *Klebsiella pneumoniae*

Klebsiella pneumoniae is resulting in high resistance to a mass amount of antibiotics including beta-lactam antibiotics such as aminoglycosides and fluoroquinolones, **(**Fair *et al., 2014*). This growing tendency of resistance towards antibiotics is a worldwide problem (Davies *et al.,* 2010). Antibiotics of the β-lactam group such as penicillins, cephalosporins, monobactams, and carbapenems are the most commonly prescribed antibiotics for the treatment of hospital-acquired infections (Samaha *et al.,* 2003). The rising of carbapenemase in *Klebsiella pneumoniae* has caused considerable challenges that led to significant morbidity and mortality **(**Patel *et al.,* 2008) Antimicrobial resistance is commonly associated with the spread of transmissible plasmids and the acquisition of resistance genes which normally occur via horizontal transfer of genes that can also bear virulence determinants (Derakhshan *et al.,* 2016). The acquisition of tolerance and virulent features is essential for pathogen survival.

Bangladesh is significantly exposed by the emergence of extremely drug-resistant bacteria. A study shows that around 86% of antibiotics are consumed without a properly prescribed prescription (Morgan *et al*, 2011). This is leading to the misuse of antibiotics.

1.5 Emergence of antibiotic-resistant *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is another multidrug-resistant organism. Infections caused by *Pseudomonas aeruginosa* organisms are difficult to treat because it includes challenges related to diagnosis and treatment and causes increased morbidity and mortality (Boucher *et al.*, 2019). Moreover, *Pseudomonas aeruginosa* is one of the leading causes of nosocomial infections. Beta-lactam antibiotics inhibit the synthesis of peptidoglycan layer of the bacterial cell wall. This class of antibiotics includes- penicillin, cephalosporin, carbapenem, and monobactam. Among them, piperacillin and ticarcillin, ceftazidime, cefepime imipenem and meropenem (Köck *et al.*, 2010).

These are the most effective beta-lactam that is used for the treatment of *Pseudomonas aeruginosa*. The ineffectiveness of β-lactam is hindered by β-lactamases enzymes destroying the amide bond of the β-lactam ring and further makes the antimicrobial ineffective.

1.6 About Cefixime

Cefixime is a third-generation antibiotic It is a broad-spectrum cephalosporin antibiotic. In the presence of beta-lactamase enzymes, cefixime is highly stable. The presence of beta-lactamase**s** results in the organisms to be resistant to some cephalosporins and penicillin but may be susceptible to cefixime.

Cefixime inhibits bacterial cell wall synthesis by disrupting the peptidoglycan layer and thus results in cell lysis. Further cell lysis is mediated by an autolytic enzyme such as autolysins.

Cefixime has vinyl and (2Z)-2-(2-amino-1,3-thiazol-4-yl)-2-[(carboxymethoxy)imino] acetamidogroups at positions 3 and 7, respectively, of the cephemskeleton It is prescribed for the treatment of urinary tract infections, gonorrhea, pharyngitis, tonsilitis, and bronchitis.

About 40-50% absorbed orally administrated with food. Approximately 50% of the absorbed drug is excreted in 24 hours. Overdose can cause blood in the urine, diarrhea, nausea, upper abdominal pain, and vomiting (Matsumoto *et al.*, 2001).

1.7 Aim of the project:

1. Designing a new strategy to manage antibiotic-resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.
2. Developing knowledge about the synergic effect of various antibiotics with cefixime against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

Chapter 2

MATERIALS & METHOD

Chapter 2

Methods & Materials

2.1 Methodology

The entire experiment was done in the laboratory of BRAC University with the aim of studying Cefixime having combination with Azithromycin, Chloramphenicol, Levofloxacin and Tetracyclin against multidrug-resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The samples were divided into Multidrug-resistant (MDR), Extremely Drug-resistant (XDR) and Pan Drug-resistant (PDR). According to the susceptibility to 10 different antibiotics, the susceptibility test was conducted. The experiment started with different samples of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and each sample went through twice susceptibility test to avoid fallacy.

Furthermore, 4 different drugs of various groups were combined with cefixime disc to demonstrate their combined effect. The cefixime antibiotic disc was soaked in the stock solution of the rest of the antibiotics before the disc diffusion test and the activity was observed after 24 hours. The zone of inhibition of any combination is greater than the zone of cefixime alone, the combination will further go for efficacy.

For clear assumption, the Minimum Inhibitory Concentration (MIC) of cefixime and azithromycin was done along with the MIC of an individual one.

Finally, the Fractional Inhibitory Concentration (FIC) index was calculated and compared with the standard for statistical validation.

2.2 Collection of Pathogens:

The pathogens *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were collected from the microbiology laboratory of the National Institute of Diseases of the Chest and Hospital (NIDCH). The collection period was from June 2019 to October 2019. The samples were taken into nutrient agar slant tubes and further carried to BRAC University and were then incubated at 37 °C for 24 hours. The samples were further subcultured to the nutrient agar plate by streaking plate method.

2.3 Collection of antibiotics and drugs for combination

The antibiotics were available in powdered crude form without excipients. The antibiotics were provided by Aristopharma Ltd. Chloramphenicol was the only antibiotic (solution form) that was bought from the nearby pharmacy of BRAC University.

Table 1: The list of drugs for the screening of antibiotics for a combination effect

Trade Name	Company Name	Generic Name
Zithromax 500mg	Aristopharma Ltd	Azithromycin
Levaquin 500mg	Aristopharma Ltd	Levofloxacin
Sumycin 500mg	Aristopharma Ltd	Tetracyclin
Cloram 5mg/ml	Ibn Sina Pharma	Chloramphenicol

2.4 Preparation of Media

For conducting the entire experiment Nutrient Agar (NA), Muller Hinton Agar (MHA), Brain Heart Infusion (BHI) broth was prepared on a regular basis.

2.4.1 Preparation of Nutrient Agar (NA)

Nutrient agar medium is basically used for the cultivation of the growth of a wide range of non-fastidious organisms. The accurate amount of media was calculated and weighed by using an electronic balance and then was boiled until the powder got dissolved completely. Finally, the media was autoclaved at 121°C for 15 minutes.

2.4.2 Preparation of Muller Hinton Agar (MHA)

Muller Hinton agar (MHA) was used for testing the susceptibility of microorganisms. This media permits for better diffusion of the antibiotics than most other plates. Better diffusion leads to a clearer zone of inhibition. It is the standard medium for the Bauer Kirby method.

The required amount of media was weighed by electronic balance and dissolved into the desired amount of distilled water by heating until the powder melted well.

The media was heated in a conical flask and then autoclaved for 121 °C. After the completion of autoclaving, the media was poured into large sterile petri dishes. The whole plating phase took place inside the laminar hood. Once the media got solidified, the plates were labeled with dates and names of the media. For further avoidance of contamination, the plates were incubated at 37°C for 24 hours. Contaminated plates were discarded, and the fresh plates were stored at 4°C inside the fresh media refrigerator.

2.4.3 Preparation of Brain Heart Infusion (BHI) broth.

Brain Heart Infusion broth was used for serial dilution to determine the minimum inhibitory concentration (MIU). The desired amount of broth powder was weighed with the help of an electronic balance. The broth powder was mixed well with distilled water and then transferred into 10 ml test tubes and given for autoclave at 121°C. After that, they were properly labeled and stored at 4°C inside the fresh media refrigerator.

2.5 Preparation of physiological saline:

Physiological saline was prepared for bacterial suspension. To make saline first, 0.9 g NaCl was dissolved in 100 ml distilled water in a clean conical flask and then 5 ml of saline was transferred into each test tubes. After that, the test tubes were autoclaved at 121°C for 15 minutes.

Fig 1: Physiological saline containing bacterial strain

2.6 Preparation of stock solution of antibiotics

The powdered antibiotics were dissolved in 10 ml autoclaved distilled water. The solution was dissolved well with the help of a vortex machine.

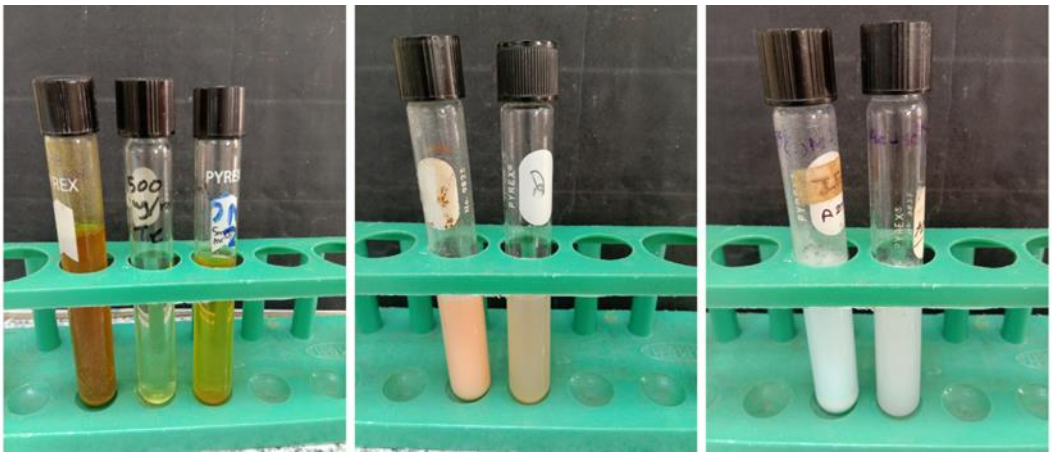

Fig 2: Stock of antibiotic solution

2.7 Disc diffusion method

The disc diffusion method is further done for the susceptibility of microorganisms. To perform disk diffusion, Muller Hinton Agar (MHA) plates were used. Sterile and pre autoclaved cotton swab was used to collect bacterial strain from nutrient agar (NA) and was inoculated inside the physiological saline. The tubes having physiological saline with bacterial strain went through vortexing. The physiological saline which was compared with McFarland standard 1 solution which shows the density of 3×10^{-8} CFU (Colony Forming Unit) per ml. Muller Hinton Agar (MHA) plates were streaked from the pathogenic strain from the saline. After that disc diffusion was done and the plates were incubated at 37 °C for 24 hours. For this experiment, 10 antibiotic discs were used.

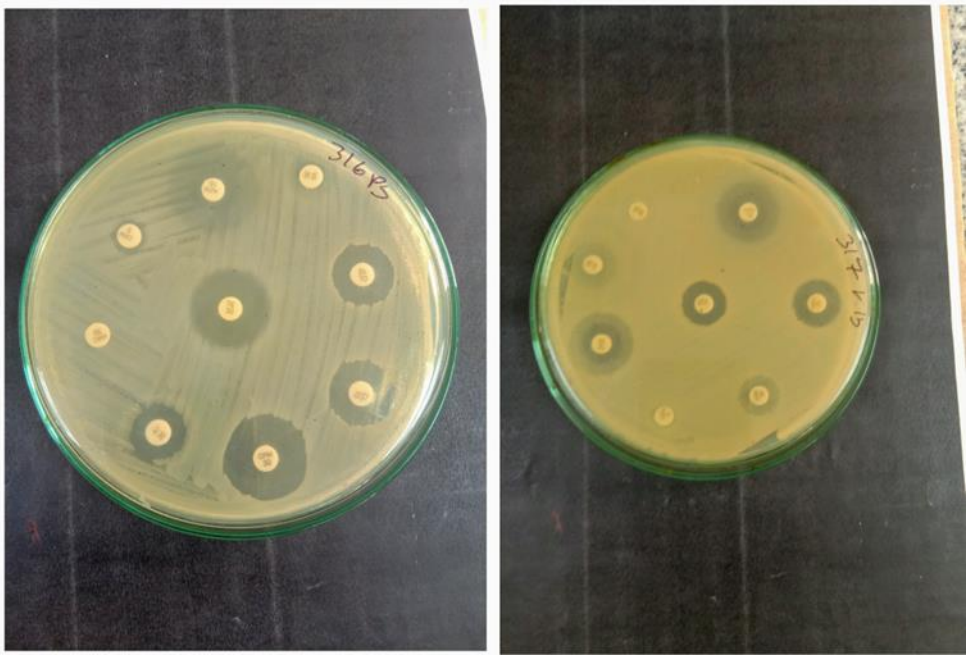

Fig 3: Antibiotic susceptibility tests of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

2.8 Determining the Minimum Inhibitory Concentration (MIC) of antibiotics

The aim of doing MIC was to observe the efficiency of observing the cefixime alone against *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. The MIC of azithromycin was observed as well.

To run the MIC, different concentration of antibiotic was required. Serial dilution was carried out with the aid of Brain Heart Infusion (BHI) broth. Dilution has been performed at various ranges starting from 5000 µg/ml to 7.81 µg/ml with individual antibiotic solution and combined antibiotic solution as well. After that, 100 µL of bacterial suspension was inoculated inside the tubes and was kept at shaker incubator 37° Celsius for 24 hours. After 24 hours, the turbidity of each tube was observed. The tube having the lowest concentration gave clear sight was considered as MIC value.

Chapter 3

RESULTS

Chapter 3

Results

The study was done with 30 samples of *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* that were collected from the microbiology laboratory of the National Institute of Diseases of the Chest and Hospital (NIDCH). Then disc diffusion was done with ten antibiotic discs and the next four antibiotics were combined with cefixime for screening purposes.

3.1 Screening antibiotic combination against MDR *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

For screening tests, 5 samples of *Klebsiella pneumoniae* and 5 samples of *Pseudomonas aeruginosa* were chosen and the combination was done. The combination was done with cefixime to 4 different antibiotics including tetracycline, azithromycin, chloramphenicol, and levofloxacin.

Table 2: The combination of cefixime with several drugs to study the synergistic effect

Combination of drugs	Inhibition of growth
Cefixime + Azithromycin	+
Cefixime + Tetracyclin	_
Cefixime + Chloramphenicol	_
Cefixime + Levofloxacin	_

[Key: + = synergic, - = No change]

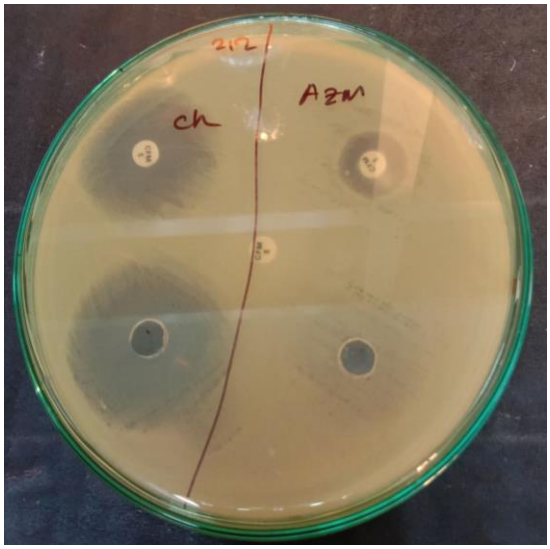

Fig 4*: (a- Klebsiella pneumoniae)*

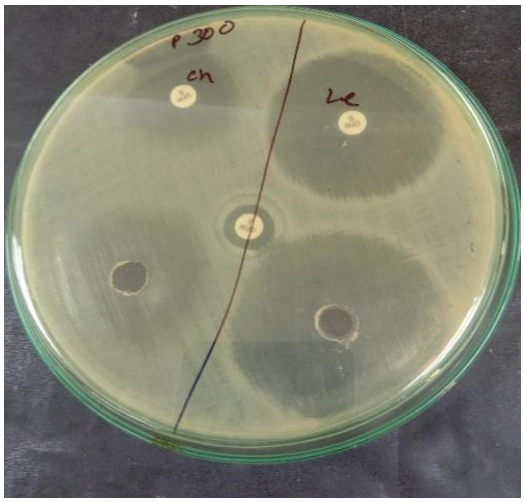

Fig 5: *(b- Pseudomonas aeruginosa)*

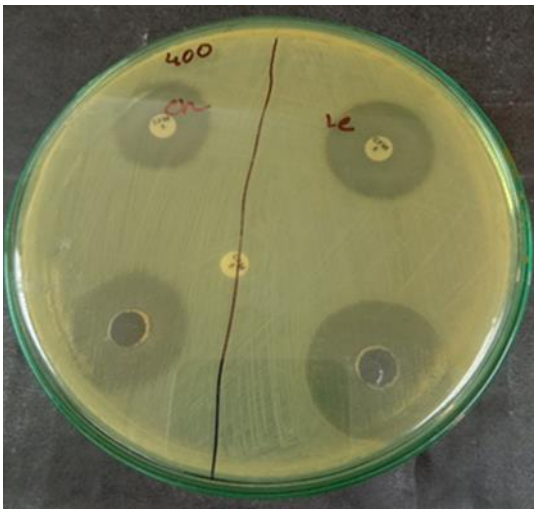

Fig *6:* *(c- Pseudomonas aeruginosa)*

Fig 4- (a) The zone of inhibition of combination of cefixime with chloramphenicol, cefixime with azithromycin and cefixime as control (b) & (c) combination of cefixime with chloramphenicol, cefixime with levofloxacin and cefixime as control.

3.2 Determination of Minimum Inhibitory Concentration (MIC)

After completion of screening, minimum inhibitory concentration (MIC) was done with cefixime and azithromycin. The fractional inhibitory concentration (FIC) was also calculated.

3.2.1 (a) Determination of cefixime, azithromycin and the combination of cefixime and azithromycin of *Klebsiella pneumoniae*

Serial dilution was prepared according to the desired concentration of antibiotics. The results of the MIC value of *Klebsiella pneumoniae* of single antibiotic and their combination with cefixime is shown in table 3.

Table 3: The MIC value of cefixime and azithromycin and their combination for *Klebsiella pneumoniae*

Antibiotic Name	Antibiotic Concentration	Sample Number											
		MDR				XDR				PDR			
		KP 1	KP 2	KP 3	KP 4	KP 5	KP 6	KP 7	KP 8	KP 9	KP 10	KP 11	KP 12
Cefixime	5000 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	700 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	600 µg/ml	C	C	T	C	T	C	T	C	C	T	C	C
	500 µg/ml	C	T	T	T	T	C	T	T	T	T	T	C
	400 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	300 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	250 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	125 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	62.5 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	31.25 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	15.625 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	7.81 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
Azithromycin	5000 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	700 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	600 µg/ml	C	C	C	T	C	C	C	C	T	T	C	C
	500 µg/ml	C	C	C	T	C	C	T	C	T	T	T	C
	400 µg/ml	C	T	T	T	T	C	T	T	T	T	T	T
	300 µg/ml	C	T	T	T	T	T	T	T	T	T	T	T
	250 µg/ml	C	T	T	T	T	T	T	T	T	T	T	T
	125 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	62.5 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	31.25 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	15.625 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	7.81 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
Cefixime + Azithromycin	5000 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	700 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	600 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	500 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	400 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	300 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	250 µg/ml	C	T	C	T	C	C	C	C	C	C	C	C
	125 µg/ml	C	T	C	T	T	C	C	C	T	T	T	T
	62.5 µg/ml	C	T	T	T	T	T	C	T	T	T	T	T
	31.25 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	15.625 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T

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

3.2.2 (b) Determination of cefixime, azithromycin and the combination of cefixime and azithromycin of *Pseudomonas aeruginosa*

For obtaining MIC value, several serial dilutions was performed starting from 5000 µg/ml to 7.8125 µg/ml. The MIC value of cefixime and azithromycin and their combination for *Pseudomonas aeruginosa* is shown in table 4.

Table 4: The MIC value of cefixime and azithromycin and their combination for *Pseudomonas aeruginosa*

Antibiotic Name	Antibiotic Concentration	Sample Number											
		MDR				XDR				PDR			
		PS 1	PS 2	PS 3	PS 4	PS 5	PS 6	PS 7	PS 8	PS 9	PS 10	PS 11	PS 12
	5000 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	700 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	600 µg/ml	C	T	C	C	C	C	T	T	C	C	C	T
	500 µg/ml	T	T	T	C	T	T	T	T	C	T	T	T
	400 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	300 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	250 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	125 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	62.5 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	31.25 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	15.625 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	7.81 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
Azithromycin	5000 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	700 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	600 µg/ml	C	C	T	C	T	C	C	C	T	C	T	T
	500 µg/ml	C	C	T	T	T	T	C	C	T	T	T	T
	400 µg/ml	C	T	T	T	T	T	T	T	T	T	T	T
	300 µg/ml	C	T	T	T	T	T	T	T	T	T	T	T
	250 µg/ml	C	T	T	T	T	T	T	T	T	T	T	T
	125 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	62.5 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	31.25 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	15.625 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	7.81 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
Cefixime + Azithromycin	5000 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	700 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	600 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	500 µg/ml	C	C	C	C	C	C	C	C	C	C	C	C
	400 µg/ml	C	C	C	T	C	C	C	C	C	T	C	C
	300 µg/ml	C	C	C	T	C	C	C	C	T	T	C	T
	250 µg/ml	C	C	C	T	C	T	C	C	T	T	C	T
	125 µg/ml	C	C	T	T	C	T	C	T	T	T	T	T
	62.5 µg/ml	C	C	T	T	C	T	T	T	T	T	T	T
	31.25 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T
	15.625 µg/ml	T	T	T	T	T	T	T	T	T	T	T	T

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

3.2.3 (c) Determination of the arithmetic mean MIC of cefixime, azithromycin and the combination of cefixime and azithromycin for *Klebsiella pneumoniae*

The MIC value of cefixime and azithromycin for multidrug resistant bacteria *Klebsiella pneumoniae* was high and their combination was comparatively low. The arithmetic mean MIC of cefixime, azithromycin and their combination is mentioned in the table below.

Table 5: The Average MIC value of cefixime, azithromycin and their combination in µg/ml & FIC Index for *Klebsiella pneumoniae*

Category	Sample Number	MIC (in µm/ml)			FIC Index
		Cefixime only	Azithromycin only	Cefixime+ Azithromycin	
MDR	KP 1	500	250	62.5	0.375
	KP 2	600	500	300	1.1
	KP 3	700	500	125	0.428
	KP 4	600	700	300	0.928
XDR	KP 5	700	500	250	0.757
	KP 6	500	400	125	0.5625
	KP 7	700	600	62.5	0.193
	KP 8	600	500	125	0.458
PDR	KP 9	600	700	250	0.773
	KP 10	700	700	250	0.714
	KP 11	600	600	250	0.833
	KP 12	600	500	125	0.458

3.2.4 (d) Determination of the arithmetic mean MIC of cefixime, azithromycin and the combination of cefixime and azithromycin for *Pseudomonas aeruginosa*

The arithmetic mean MIC of cefixime, azithromycin and their combination is mentioned in the table below:

Table 6: The Average MIC value of cefixime, azithromycin and their combination in µg/ml & FIC Index for *Pseudomonas aeruginosa*

Category	Sample Number	MIC			FIC Index
		Cefixime only	Azithromycin only	Cefixime+ Azithromycin	
MDR	PA 1	600	250	62.5	0.354
	PA 2	700	500	62.5	0.214
	PA 3	600	700	250	0.773
	PA 4	500	600	500	1.833
XDR	PA 5	600	700	62.5	0.193
	PA 6	600	500	300	1.1
	PA 7	700	500	125	0.428
	PA 8	700	500	250	0.857
PDR	PA 9	500	700	400	1.371
	PA 10	500	600	500	1.833
	PA 11	600	700	250	0.773
	PA 12	700	700	400	1.142

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

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

3.3 FIC Index Interpretation

Table 7 The reference value scale for FIC interpretation

Interpretation	FIC
Synergy	≤ 0.5
Additive	> 0.5 and ≤ 1.0
Indifference	> 1 and ≤ 4.0
Antagonism	> 4.0

Fig 7: FIC Index Interpretation

[Courtesy: CLSI. Performance Standards for Antimicrobial Susceptibility Testing; 26th ed. CLSI Supplement M100S. Wayne, PA: Clinical and Laboratory Standards Institute; 2016.]

The average FIC index is 0.61975 which is more than 0.90475 yielding the statistical additive effect of cefixime and azithromycin against the *Klebsiella pneumonia* and *Pseudomonas aeruginosa* respectively.

Fig 8: The MIC test of cefixime, azithromycin & their combination for both *Klebsiella pneumonia* and *Pseudomonas aeruginosa.*

Chapter 4

DISCUSSION

Chapter 4

Discussion:

A significant amount of awareness is needed among the public about antibiotic-resistant bacteria and antibiotics as well. The general public must be made aware of the facts concerning the important roles that bacteria have in their lives and well-being, the precious nature of antibiotics and the concomitant importance of using them prudently (Bush *et al.,* 2011).

Moreover, the features related to resistant bacteria are the reasons for deprived hospital hygiene, overcrowding of patients, lack of available resources for controlling infection and lack of trained personnel in hospitals (Faiz *et al.,* 2011).

The health care systems and the pharmaceutical industry have been trying to battle against antibiotic strains of bacteria for a long period. Novel structural classes of antibiotics that are not affected by known or existing mechanisms of resistance should be introduced. New approaches for the alternatives of antibiotics should be encouraged that might include the use of antibacterial vaccines, phage therapy, immunostimulants, adjuvants, antivirulence therapies, probiotics and their combinations (Alekshun *et al.,* 2004).

Before performing MIC, screening tests were done for tetracycline, levofloxacin, chloramphenicol, and azithromycin with cefixime antibiotic disc to know the combinations which give synergistic effect. The antibiotics were in powdered form which was diluted in autoclaved distilled water. After performing screening tests for both strains of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, azithromycin gave a synergistic effect. The positive result means, the zone of inhibition was much bigger when azithromycin was added to the cefixime disc. There was no zone for cefixime alone and smaller when azithromycin was given alone in the well. Cefixime disc was there as control (which did not show any zone of

inhibition). The other antibiotics solution along with cefixime disc gave zone of inhibitions but the zone was increased suggesting no synergistic effect. Cefixime shows excellent activity with cephalosporines against *Staphylococcus aureus* where the zone size was 27 mm (Ghatage *et al.,* 2014).

From this study, it was found that most of the samples were antibiotic-resistant which can be an alarming challenge for the future medical aspects. During the study, the average combined MIC (cefixime+azithromycin) value for *Klebsiella pneumoniae* was 185.41 µm/ml (185.41 mg/L) whereas *Pseudomonas aeruginosa* had average MIC value 242.70 µm/ml (242.7 mg/L), which indicates *Pseudomonas aeruginosa* is more antibiotic-resistant than *Klebsiella pneumoniae* and hard to treat with single antibiotic.

Of the four MDR strains of *Klebsiella pneumoniae* the lowest MIC of cefixime and azithromycin were 500 µm/ml and 250 µm/ml respectively and the maximum MIC of cefixime and azithromycin were 700 µm/ml and 500 µm/ml respectively. For XDR strains, the lowest MIC value was 500 µm/ml and 400 µm/ml respectively where the highest MIC of cefixime and azithromycin were 700 of µm/ml and 600 µm/ml respectively. In the case of PDR strains, the lowest MIC for cefixime and azithromycin were 600 µm/ml and 500 µm/ml respectively where the maximum value was both 700 µm/ml (Table -5). All the MIC values were very high.

The MDR strains of *Pseudomonas aeruginosa* having the lowest MIC of cefixime and azithromycin were 500 µm/ml and 250 µm/ml respectively and the maximum MIC of cefixime and azithromycin were 700 µm/ml and 500 µm/ml respectively. For XDR strains, the lowest value was 600 µm/ml and 500 µm/ml respectively where the highest MIC of both cefixime and azithromycin were 700 of µm/ml. In the case of PDR strains, the lowest MIC was 500 µm/ml 600 µm/ml respectively and the maximum value was both 700 µm/ml in both cases. All the MIC values were very high compared to the findings of other authors (Andrews, 2001).

Four strains of each MDR, XDR, and PDR of *Klebsiella pneumoniae* were chosen for the study. The lowest combined MIC (cefixime+azithromycin) value for MDR strains was 62.5 µm/ml and the highest was 300 µm/ml. The XDR strains showed the lowest combined MIC (cefixime+azithromycin) of 62.5 µm/ml where the highest value was 250 µm/ml. On the other hand, the combined MIC (cefixime+azithromycin) value of PDR strains was 125 µm/ml and the highest value was 250 µm/ml. The combined MIC (cefixime+azithromycin) value widely varied from strain to strain (Table-5). The data suggest that the combination of the drug has a synergistic effect. However, the MIC value is still very high. Another study reveals that combining several agents under the lone captions of beta-lactam agents or aminoglycosides is void because *Klebsiella* spp. have grown susceptibilities to the novel antibiotics (Korvick *et al.*, 1992).

In the same way, four strains of each MDR, XDR, and PDR of *Pseudomonas aeruginosa* were studied. The lowest combined MIC (cefixime+azithromycin) for MDR strains was 62.5 µm/ml where the highest value was 500 µm/ml. For XDR, the lowest value was 62.5 µm/ml and the maximum value was 400 µm/ml. PDR strains to have the minimum combined MIC (cefixime+azithromycin) value of 250 and the maximum value was 500 µm/ml. These MIC values are much higher than that of other studies.

Among all the strains of both *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, the results indicate that *Pseudomonas aeruginosa* has a higher combined MIC (cefixime+azithromycin) value than *Klebsiella pneumoniae*. Gatifloxacin has synergic effects in vitro while combining with β-lactam antibiotics (Gradelski *et al*., 2001). A combination of fluoroquinolones and ciprofloxacin shows a good synergic activity (Fish *et al.*, 2002). Colistin–tigecycline can be combined with aminoglycoside, carbapenem, colistin, fosfomycin, rifampin, or tigecycline for treating carbapenemase-producing Enterobacteriaceae (Hirsch *et al.*, 2010). According to the retrospective analysis, it has been suggested to practice combinations including a carbapenem

for these Enterobacteriaceae class bacteria if the carbapenem minimum inhibitory concentration (MIC) is ≤4 mg/L (Daikos *et al.,* 2011).

During the study, it was observed that azithromycin having a MIC value of 500 μg/ml and cefixime having MIC value of 700 μg/ml, and while they were combined with each other, the MIC value became 5000 μg/ml. That means for few bacterial strains, azithromycin and cefixime may work more effectively in , form rather than combining them.

The fractional inhibitory concentration (FIC) index range of 0.5 to 4 defining additivity results. The arithmetic mean of FIC index is 0.61975 which is more than 0.90475 yielding the statistical additive effect of cefixime and azithromycin against the *Klebsiella pneumonia* and *Pseudomonas aeruginosa* respectively. The additivity results show that no interactions have occurred in combination studies of antibacterial agents. However, the results may vary from in vivo results. Furthermore, from a study it was observed that for *Pseudomonas aeruginosa* the MIC value of azithromycin showed quite high which was considered as a negative value and for cefixime the value was 16 μg/ml. But the study did not show the combined MIC of cefixime and azithromycin (Andrews, 2001). From another study, it was suggested to include an aminoglycoside, ampicillin/sulbactam, a carbapenem, colistin, or rifampin for combination purposes since they were successful against multidrug-resistant Acinetobacter spp. (Kuo *et al.,* 2007).

Synergistic action was performed between β-lactams and aminoglycosides for *Pseudomonas aeruginosa* which gave no major correlation between in vitro synergy testing (Hilf *et al.,* 1989).

Another study revealed that, for both colistin-susceptible reference strain and colistin - susceptible clinical isolate, the MIC value was 0.5 μg/ml (Algaba *et al.,* 2018). In another study, Zoliflodacin was introduced as a dual antibiotic therapy to treat *Neisseria gonorrhoeae* where

the FIC index of zoliflodacin and cefixime was 2.50 defining additivity result (Foerster *et al.*, 2019).

This study only reflects the findings of the in vitro synergistic activities of cefixime and azithromycin against antibiotic-resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The use of combined antibiotics is a common hospital procedure for treating severe infections but the guideline of using the combination has not well established yet. Since in vivo tests were not done, investigations may vary with in vitro results.

A further experiment could have done by using natural herbal medicines. Instead of the antibiotic disc and antibiotic powder, the extract of different parts of the plants and products can be used as microbial agents. Not only that, the suggested combinations found from other studies can bring a change in the results part as well.

Conclusion

To sum up, unregulated dispensing and production of antibiotics, shortened antimicrobial therapy, insufficient antimicrobial remedy, poor access to effective antibiotics and poverty are likely to be causative to antimicrobial resistance in Bangladesh. In the present study, it was found that MIC of several antibiotics against multiple-antibiotic resistant *Klebsiella pneumonia* and *Pseudomonas aeruginosa* collected from clinical samples were very high. The combined effect of cefixime and azithromycin reduced the MIC value but still, it was very high suggesting the urgency of controlled use antibiotics.

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Appendix A.

Media Composition

Table 7: Nurtient Agar

Component	Amount per Litre solution
Beef extract	3.0 g
Peptone	5.0 g
Agar	20.0 g
pH	7.0-7.2

Table 8: Muller Hilton Agar

Component	Amount per Litre solution
Beef extracts powder	20 g
Acid digest of Casein	17.5 g
Starch	1.5 g
Agar	1.7 g
pH	7.3 ±1 at 25°C

Table 9: Physiological saline

Component	Amount per Litre solution
Sodium Chloride	9.0 g

Table 10: Brain-Heart Infusion Broth

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