A prospective study on the efficiency of ciprofloxacin in combination with chloramphenicol and probiotics against multiple antibiotics resistant *Klebsiella pneumoniae*

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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Declaration

I hereby declare that the thesis project titled **"A prospective study on the efficiency of ciprofloxacin in combination with chloramphenicol and probiotics against multiple antibiotics resistant *Klebsiella pneumoniae*"** has been written and submitted by me, Akash Ahmed and has been carried out under the supervision of Dr M. Mahboob Hossain, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

It is further declared that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. All explanations that have been adopted literally or analogously are marked as such.

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Dedicated

TO

MY Parents &

Beloved Sumaiya Akhtar Mimi

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Abstract

Pneumonia is the single largest infectious cause of death in children worldwide. Not only children but also adult from all age groups can be suffered from pneumonia. Pneumonia is a form of acute respiratory infection that affects the lungs. One of the prime pathogens of this infection is *Klebsiella pneumoniae*. Apparently, it seems pretty uncomplicated to treat *K. pneumoniae*, however, it is more than challenging to combat because of antimicrobial resistance.

The purpose of the study was to develop new approach to treat antibiotic resistant *K. pneumoniae* infection. This study aimed in quest of a drug to combine with ciprofloxacin, a broad spectrum antibiotic frequently used to treat lung infections. A total of 23 lung infection bacterial samples were collected and studied against 14 antibiotics of different classes. After primary screening of antibiotic susceptibility, they were categorized into multidrug resistant (MDR), extensively drug resistant (XDR) and pan drug resistant (PDR) pathogens where 9 isolates were MDR, 5 were XDR and 3 isolates were PDR. Furthermore, they were trialed in combination ciprofloxacin along with other 7 drugs in disk diffusion to explore synergistic effect. The combination of ciprofloxacin and moxifloxacin, ciprofloxacin and chloramphenicol and ciprofloxacin with probiotic was found to be synergic. Then the minimum inhibitory concentration test was done for the two combination Ciprofloxacin + chloramphenicol and ciprofloxacin + probiotic. When the individual MIC result was generated, the MIC of the respective combination was analyzed. Furthermore, the fractional inhibitory concentration (FIC) was calculated and in accordance with the results of FIC index, ciprofloxacin-chloramphenicol combination has shown value 0.4510 which revealed synergistic effect against multi drug resistant *Klebsiella pneumoniae*.

Although, in vivo animal modelling is needed for further validation, this finding is indisputably a novel and great one to combat highly drug resistant *Klebsiella pneumoniae* in upcoming future.

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Abbreviation

MDR	**Multi Drug Resistant**
XDR	Extensively Drug Resistat
PDR	Pan Drug Resistant
CFU	Colony Forming Unit
et al	And Others
CDC	Center for Disease Control
mg	milligram
µg	microgram
MIC	Minimum Inhibitory Concentration
FIC	Fractional Inhibitory Concentration
WHO	World health Organization
MHA	Muller Hington Agar
NA	Nutrient Agar
BHI	Brain Heart Infusion
Fig.	Figure

Chapter 1
Introduction
&
Literature Review

1.1 Overview

Pneumonia has become the leading cause of child death since many decades in developing countries (Saha *et al.*, 2016). It is responsible for 28 % of under-five deaths in Bangladesh (The Daily Star, 8 March, 2015). Not only children but also adult from different age group to aged can be suffered from pneumonia. There are several infectious agent including virus and bacteria behind pneumonia. *Klebsiella pneumoniae* is one of the bacteria, a member of the family Enterobacteriaceae which can infect in upper respiratory tract and leads to pneumonia. In addition to that the sufferings of the patients rise several folds when the infectious agent is resistant to conventional antibiotic and modern as well.

Combating the *Klebsiella pneumoniae* is harder when it is resistant to several antibiotics. Undoubtedly, the healthcare system has been updated in each second but it was 1987 last, when a new antibiotic class was introduced. As a result, what will be the future of treating severely antibiotic resistant bacterial infection is a burning question in front of the modern science (Groopman, *2008).*

However, physicians start using timely antibiotic combination therapy to improve the patient survival (Bush &Fisher, 2011) since the antibiotic treatment for these resistant bacteria is limited. Hence, proper in vitro combinations of various class of antibiotics data is important for a sustainable guideline to the usages of antibiotics when it will fight against antibiotic resistant bacterium.

Moreover, a lot of combination trial has been reported against *Klebsiella pneumoniae* including tigecycline + gentamicin and tigecycline + colistin (Falagas *et al.,* 2014), azithromycin + chloramphenicol, levofloxacin + rifampin, polymyxin B + tigecycline (Lim *et al.,* 2016). The combination therapy attracts because of the successive outcome of the combination treatment against tuberculosis and ulceritis. This study will focus on the interactive efficiency of ciprofloxacin having combination with other drugs against drug resistant *Klebsiella pneumoniae.*

1.2 Character and Morphology

Classification of *Klebsiella pneumoniae*

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

Klebsiella pneumoniae is a Gram-negative, nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. The *Klebsiella* genus is named for German physician and bacteriologist Edwin Klebs. It is also called Friedländer's bacillus which was first described in 1882 by German microbiologist and pathologist Carl Friedländer (The Editors of Encyclopædia Britannica). It is a facultative anaerobic, Gram-negative, rod-shaped bacterium. Though some of the strain of *Klebsiella pneumoniae* is considered as normal microbiota, it is a major cause of upper respiratory tract infection when immune system is weakened (Groopman, 2008). It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions (Postgate*, 1998).*

1.3 Emergence of antibiotic resistant *Klebsiella pneumoniae*

Before the discovery of antibiotic, a single infection might be fatal. It was 1928 Alexander Fleming came up with penicillin which saved a lot of life until the infectious agent became resistant. Throughout the 20^{th} century, numerous antibiotics like vancomycin, ciprofloxacin, carbapenem, cefixime are developed which successfully treated *Klebsiella pneumoniae* infection. Nevertheless, the successive treatment management is being challenged by the development of resistant mechanism of the smart *Klebsiella pneumoniae*.

The emergence of antibiotic resistant microorganism is natural and genetically over time. Still there are certain acceleration factors yielding the development of resistant strain. According to World Health Organization (WHO) the overuse and misuse of frequent antibiotic is the culprit behind the resistant mechanism (WHO fact sheet, November 2017). Further, WHO explores Poor infection control, inadequate sanitary conditions and inappropriate food-handling encourage the spread of antimicrobial resistance.

Particularly, the developing country like Bangladesh is greatly threatened by the emergence of extremely drug resistant bacteria. In Bangladesh, it is figured out that up to 86 percent of antibiotics are consumed without the prescription (Morgan *et al.,* 2011) resulting misuse of conventional and modern antibiotic as well. Researchers are warning that antimicrobial resistance to antibiotics will be a great danger to humankind than cancer by the middle of the century unless world leaders agree international action to tackle the threat, The Guardian (2016).

Since the new antibiotic class is not generating for a long time, in the era of rising antimicrobial resistance, coupled with a continued dwindling pipeline of drugs to treat these infections (Pucci & Bush, 2013). It is evident that combination therapy will be the most suitable advantage to treat resistant *Klebsiella pneumoniae*.

1.4 Mechanism of antibiotic resistance: *Klebsiella pneumoniae*

Klebsiella pneumoniae is one of the MDR organisms claimed as an serious danger to health by the World Health Organization, the US Centers for Disease Control and Prevention and the UK Department of Health (Kidd *et al.,* 2017). Researchers point out the emergence of colistin resistance in MDR *K. pneumoniae* rising from the mutations of the *mgrB* gene, a negative regulator of the PhoPQ signalling system (Lippa & Goulian, 2009; Cannatelli *et al.,* 2013; Wright *et al.,* 2015; Zowawi *et al.,* 2015). The PhoPQ component system is a regulator of envelope remodelling, predominantly the lipopolysaccharide (LPS) lipid A section, and subsidizes to bacterial resistance to innate immune killing (Groisman, 2001; Llobet *et al.,*

2011). *K. pneumoniae* PhoPQ also manages lipid A plasticity *in vivo* and *in vitro* (Llobet *et al.,* 2015) resulting developing resistant mechanism.

To infect and survive within the host, certain bacteria have established several defenses of counter-attack and avoidance strategies. Likely, opportunistic pathogens, *K. pneumoniae* has also various virulence factors that aid the bacterium endure the host (Podschun and Ullmann, 1998). Further, the capsule of *K. pneumoniae* protects against phagocytosis, antimicrobial peptides and complement-mediated lysis which is the most protective factor.

Virulent *K. pneumoniae* strains, belonging to clonal complex 23 assimilated a large virulence plasmid encrypting different virulence factors like siderophores (Struve *et al.,* 2015). The acquirement of iron via siderophores is vital for growth and virulence within the host. Deletion of the gene encoding for the siderophore, reduced growth in serum and virulence in a lung infection model (Russo *et al.,* 2015).

However, the resistance mechanisms of *K. pneumoniae* against antibiotics are mostly release of antibiotic-inactivating enzymes, change in membrane permeability, activation of efflux pump systems, modification of antibiotic target sites, and alteration of metabolic pathways (Tenover, 2006). Compared to these mechanisms, the enzymatic degradation and efflux pump systems play an important role in the development of multidrug resistance in *K. pneumoniae* (Pages, 2006).

Admittedly, *Klebsiella pneumoniae* develop resistant mechanism against fluoroquinolones like ciprofloxacin from the efflux pumps, belonging to the resistance-nodulation-division (RND) family which can extrude amphiphilic and charged antibiotics (Zhong, *et al.,* 2013).

1.5 Global epidemiology of antibiotic resistant *Klebsiella pneumoniae*

Klebsiella pneumoniae carbapenemases (KPCs) were identified in the USA in 1996 (Yigit, *et al.,* 2001). The KPC is produced by the *Klebsiella pneumoniae* to resist the antibiotic class carbapenem. The mortality among patients infected with KPC is high, as a result of the limited

antibiotic options remaining (often colistin, tigecycline, or aminoglycosides). Triple drug combinations using colistin, tigecycline, and imipenem have recently been associated with improved survival among patients with bacteraemia (Munoz-Price *et al.,* 2013).

However, recent studies claims, *K. pneumoniae* has a flexible and diverse pangenome containing numerous accessory genes that allow the bacterium to adapt to various habitats and respond to environmental stresses such antibiotic treatment (Holt *et al.,* 2015).

Consequently, the outbreaks by KPC-producing *K. pneumoniae* have been recounted in the USA (Woodford *et al.,* 2004) and Israel (Leavitt *et al.,* 2007), recently, similar outbreaks related with patients traveling to endemic areas have also been reported in many European counties.

Moreover, throughout the world, antibiotic resistant *Klebsiella pneumoniae* causes challenging infection which is not only tough to treat but also crucial to nosocomial infection associated with hospital management.

Last but not least, the treatment of *K. pneumoniae* infections has become more challenging hence the emergence of MDR lineages of *K. pneumoniae*. These lineages transport an extensive range of antimicrobial resistance genes that confine the available options to commendably treat *K. pneumoniae* infections (Moradigaravand *et al.,* 2017).

1.6 About Ciprofloxacin

Ciprofloxacin is a manmade chemotherapeutic antibiotic from the fluoroquinolone drug class which is a second-generation fluoroquinolone antibacterial. It exterminates bacteria by interfering with the enzymes that cause DNA to rewind after being copied, which stops DNA and protein synthesis (Drlica & Zhao, 1997). The patent history for ciprofloxacin makes reference to a 1982 European Patent (patent number 0049355), as well a German patent dated 21 January 1986. Bayer introduced ciprofloxacin in 1987 and was later approved by the U.S.

FDA on 22 October 22 1987 for use in the United States to treat specific bacterial infections. In 1991, the intravenous formulation was introduced (Oxford Handbook of Infectious Diseases and Microbiology 1997).

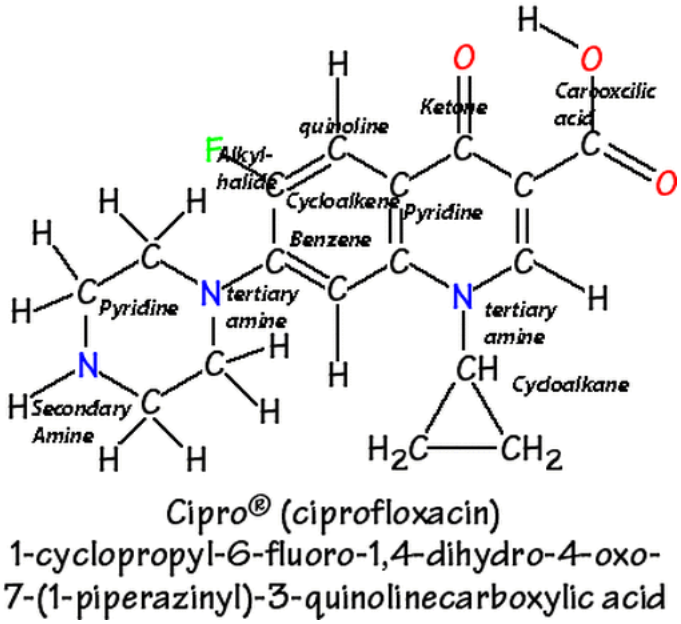


Fig. 1 The functional groups of ciprofloxacin

Ciprofloxacin is consist of 8 different functional group (shown in figure 1). It is a broad spectrum antibiotic against mostly gram negative bacteria. Ciprofloxacin combination with metronidazole is one of several first-line antibiotic regimens recommended by the Infectious Diseases Society of America for the treatment of community-acquired abdominal infections in adults (Solomkin *et al.*, 2010). Gradually, ciprofloxacin is being succeeded to combat the infection sinusitis to bone and joint infection (Osmon, 2012). The infections, ciprofloxacin can treat is listed below-

- Anthrax
- Cyclosporiasis
- Bacterial Conjunctivitis
- Food Poisoning
- Bacterial Skin Infection
- Gastroenteritis
- Cystitis

- Salmonellosis
- Dysentery
- *E. coli* Infection
- Gonorrhea
- Haemophilus Infection
- Neutropenia
- *Klebsiella* Infection
- Osteomyelitis
- Prostatitis
- *Proteus* Infection
- *Pseudomonas* Infection
- *Staphylococcus* Infection
- Sinusitis
- Typhoid Fever
- Urinary Tract Infection

Undoubtedly, ciprofloxacin has several and severe side effects to human. In pre-approval clinical trials of ciprofloxacin most of the adverse events reported were described as mild or moderate in severity, abated soon after the drug was discontinued, and required no treatment (FDA, September, 2016). Further, the fluoroquinolones rapidly cross the blood-placenta and blood-milk barriers, and are extensively distributed into the fetal tissues. For this reason, the fluoroquinolones are contraindicated during pregnancy due to the risk of spontaneous abortions and birth defects. Tendinitis (Saint, 2000), psychosis, anxiety, hallucinations, paranoia, and suicide attempts (Heidelbaugh & Holmstrom, 2013) is complained against the use of ciprofloxacin. Again, it displays high activity not only against bacterial topoisomerases but also against eukaryotic topoisomerases and are toxic to cultured mammalian cells and in vivo tumor models (Lawrence *et al.*, 1996). Although quinolones are highly toxic to mammalian cells in culture, its mechanism of cytotoxic action is not known. Quinolone induced DNA damage was first reported in 1986 (Tempel, 1987).

Still, having such diverse side effect, ciprofloxacin is a good choice to combat acute and chronic infection which is mentioned above. Particularly, the wide range of antibiotic activity of ciprofloxacin attracts to re-establish the efficiency.

1.7 Defining MDR, XDR & PDR

Different definitions for multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria are being used in the medical literature to characterize the different patterns of resistance found in healthcare-associated, antimicrobial resistant bacteria (Magiorakos *et al.,* 2012). A joint initiative by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC), some experts come together to definite MDR, XDR & PDR. Many definitions are being used in order to characterize patterns of multidrug resistance (MDR) in Gram-positive and Gram-negative organisms which sometimes become very tough to compare. The definition most frequently used for MDR Gram-positive and Gram-negative bacteria is 'resistant to three or more antimicrobial classes' (Falagas, 2006). Again, MDR is defined as resistant to one key antimicrobial agent (Hidron, 2008).

Similarly, extensively drug resistant (XDR) microorganism is defined by two sets of criteria, the first is based on the number of antimicrobials or classes or subclasses to which a bacterium is resistant, and the second on whether they are resistant to one or more key antimicrobial agents (Cohen *et al.,* 2008; Hidron *et al.,* 2008).

From the Greek prefix 'pan', meaning 'all', pandrug resistant (PDR) term is generated which states resistant to all antimicrobial agent. The definition of pandrug resistant (PDR) is greatly varied from study to study, depending on the number of antibiotics are used in that particular study. Nevertheless, the definition of PDR is not a rigid term and it should be defined as resistant to all antimicrobials routinely tested (Magiorakos *et al.,* 2012).

Moreover, MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and PDR is defined as non-susceptibility to all agents in all antimicrobial categories (Magiorakos *et al.,* 2012).

1.8 Aim of the project

- Developing new approach to manage antibiotic resistant *K. pneumoniae*
- Studying the synergic effect of several drugs in combination with ciprofloxacin against *K. pneumoniae*

Chapter 2
Materials & Methods

2.1 Methodology

The experiment was carried in the laboratory of BRAC University to study the activity of ciprofloxacin having combination with several drugs against multidrug resistant *Klebsiella pneumoniae*. The experiment proceed mainly involved in collecting the pathogenic *Klebsiella pneumoniae* and their antibiotic susceptibility testing. Then, the samples were categorized into Multidrug resistant (MDR), Extensively Drug resistant (XDR) and Pan Drug resistant (PDR) on the basis of their susceptibility to 14 different antibiotics which are commonly prescribed in day to day. The antibiotic susceptibility test was done twice to avoid fallacy. The study started with 23 different *Klebsiella pneumoniae* isolates.

Randomly 2 MDR, 2 XDR and 2 PDR pathogen are picked for the activity study of several drugs combined with ciprofloxacin. In this study, 8 different drugs of various group are combined with ciprofloxacin disc to demonstrate their combined effect. The ciprofloxacin antibiotic disc was soaked in the stock solution of these drugs before the disc diffusion test and the activity was observed after 24 hours, interpreting the zone diameter size. If the zone of inhibition of any combination is found to be greater than the zone of inhibition of ciprofloxacin alone, the combination gets the priority to explore the efficiency.

The combination of chloramphenicol and probiotic with ciprofloxacin is assumed to be efficient from exploring the zone of inhibition data result. To be clear with the assumption, the Minimum Inhibitory Concentration (MIC) of these combinations were figured out along with the MIC of individual one.

Finally, the Fractional Inhibitory Concentration (FIC) index of each combination (Except the combination of ciprofloxacin and probiotic) was calculated and compared with the standard for statistical validation.

2.2 Collection of pathogenic *Klebsiella pneumoniae*

Clinically identified *Klebsiella pneumoniae* was collected from the National Institute of Diseases of the Chest and Hospital (NIDCH) and microbiology department of Uttara Adhunik Medical College Hospital (UAMCH). The collection started on September 2017 and ended in October 2017. The isolates of *Klebsiella pneumoniae* was sub-cultured to nutrient agar slant and carried to BRAC University laboratory. The nutrient agar slant was incubated at 37^0 Celsius for 24 hours. Then the pathogen was transferred to nutrient agar plate by streaking plate method.

At the same time these samples are stored at -20^0 Celsius in glycerol media as stock.

2.3 Collection of antibiotics and drugs for combination

All the antibiotics and drugs for combination was brought from BRAC University nearby pharmacy. Ciprofloxacin was taken from the product of Square Pharmaceuticals, Ciprocin 500 mg. The other drugs for combination purpose are listed in table 1.

Table 1: The list of drugs used for combination

Trade Name	Company Name	Generic Name	Class
Fexo 120mg	Square Pharma	Fexofenadine	Antihistamine
Rifagut 200mg	Opsonin Pharma	Rifaximin	Miscellaneous Antibiotic
Indever 10 mg	ACI Limited	Propranol Hydrochloride	Calcium channel blocker
Cloram 5mg/ml	Ibn Sina Pharma.	Chloramphenicol	Antibiotic
Probio	Square Pharma.	*Lactobacillus* and *Bifidobacterium spp*	Probiotic
Tycil 500 mg	Beximco Pharma	Amoxicillin	Antibiotic
Moxibac	Popular Pharma	Moxifloxacin	Antibiotic

2.4 Preparation of media

For this experiment Nutrient Agar (NA), Muller Hington Agar (MHA), Brain Heart Infution (BHI) broth was prepared on regular basis.

2.4.1 Preparation of Muller Hinton Agar (MHA)

Muller-Hinton Agar **(**MHA) medium was mainly used to observe the antimicrobial activities of the both antibiotics and the plant extracts as Muller-Hinton Agar **(**MHA) typically used in determining antimicrobial activity of antibiotics and extracts.

The required amount of agar was first measured in electronic balance and mixed with distilled water in two conical flasks and then were heated and dissolved by heating until the agar melted. The mouth of the conical flask was covered with aluminum foil paper. After sealing the mouth it was then placed into an autoclave machine at 121^0 C and 15 psi for 15 minutes. After autoclaving the mixture was then poured into the sterile large sized petri dishes. When the agar solidified the petri dishes were then labeled and were stored at 4^0 C inside the refrigerator for further use.

2.4.2 Preparation of Brain Heart Infusion (BHI) broth

Brain Heart infusion broth were used in serial broth dilution to determine the minimum inhibitory concentration (MIC). The required amount of broth powder was first measured in electronic balance and mixed with distilled water and final volume of the solution was brought to 500 ml. Then 10 ml aliquots were added in 50 sterile 15 ml test tube. After autoclaving the tubes, they were appropriately labelled and stored in a clean beaker and refrigerator.

2.4.3 Preparation of Nutrient Agar (NA)

Nutrient Agar (NA) was used to subculture the selected pathogen for this study. Amount needed for preparing specific amount of NA was calculated and weight was taken by electronic balance. Until the powder completely dissolved in distilled water, it was boiled. Finally the media was autoclaved at 121^0 C and 15 psi for 15 minutes.

2.5 Preparation of physiological saline

Physiological saline was made to prepare bacterial suspension and it was matched with McFarland standard 1 solution. At first 0. 9 g NaCl was dissolved in 80 ml deionized or distilled water in clean conical flask. Then the water was added to bring total solution volume to 100 ml. After mixing saline was transferred to 15 ml test tube and autoclaved.

2.6 Preparation of stock solution of drugs and antibiotics

Commercially available tablet or capsule was bought and dissolved in 10 ml physiological saline shown in figure 2. Though the excipients of those drug was also inside the solution, it was considered not to be interfered with desired product since excipients are chemically inert.

Tablet or capsule was poured in physiological saline. As a result, the volume of the 10 ml saline raised very little which was negligible and excluded from the calculation.

Fig. 2 The stock of antibiotics and drugs for combination

2.7 Preparation of probiotic culture supernatant

Probio capsule was poured in physiological saline. Then 100 µl of the saline was transferred to Nutrient Agar Broth. After 48 hours incubation at 37^0 Celsius, 1 ml nutrient broth was transferred to Eppendorf tube and centrifuged for 10 minutes at 3000 rpm. The supernatant from the Eppendorf was collected for combination.

2.8 Disc diffusion method

Agar surface of Muller-Hinton Agar plate was streaked by a sterile cotton swab with the collected pathogenic *Klebsiella pneumoniae* strain from the physiological saline which was compared with McFarland standard 1 solution. McFarland standard 1 solution shows the density of 3×10^8 CFU (Colony Forming Unit) per ml. Antibiotics discs were placed on solidified agar plates at equal distance apart. The plates were kept standby for 10 min. Then the plates were incubated at 37 °C for 24 hours. The disc diffusion test was done to determine the antibiotic resistant pattern of the pathogens as well as to categorize to MDR, XDR and PDR by the guideline of Clinical and Laboratory Standards Institute (CLSI). Around 14 antibiotic discs were used in this study.

2.9 Determining the Minimum Inhibitory Concentration (MIC) of antibiotics

The study was designed to observe the efficiency of ciprofloxacin alone and with combination against highly antibiotic resistant *Klebsiella pneumoniae*. Hence, the MIC of ciprofloxacin and the MIC of other individual antibiotics were figured out with or without combination.

Determining the MIC, different concentration of antibiotic was required. For this purpose, serial dilution was carried out with the aid of Brain Heart Infusion (BHI) broth as diluent. From the $C_1V_1 = C_2V_2$ formula, desired concentration was prepared by the addition of stock solution to BHI broth.

A wide range dilution was prepared with individual antibiotics and combination as well. Throughout the study, it was practiced to keep the concentration of different compounds same. Each test tube having known concentration of antibiotic was inoculated with 100 µL of

McFarland 1 standard pathogenic suspension and kept at 37^0 Celsius for 24 hours. On the next day, each tube was critically observed to identify either turbid or clear. The lowest concentration of antibiotic gave clear tube was considered as MIC value.

The MIC test was done twice with different dilution range to get more sophisticated result through arithmetic mean calculation.

2.10 Determining the Fractional Inhibitory Concentration (FIC) Index

Fractional Inhibitory Concentration (FIC) index is a statistical tool for validation. The standard value of FIC index is 0.5 to 4. The lower value represents the synergism and higher value for antagonism.

To get FIC index, firstly, FIC was calculated by the following equation-

$$\textbf{FIC} = \textbf{MIC of the agents in combination} / \textbf{MIC of the agent alone}$$

FIC index was calculated by the formula of-

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

The average FIC index from six *Klebsiella pneumoniae* was determined and compared to standard.

Chapter 3
Results

Results

In the present study 23 *Klebsiella pneumoniae* was collected from two different hospitals and through the disc diffusion method *Klebsiella pneumoniae* were categorized to Multidrug Resistant (MDR), Extensively Drug Resistant (XDR) and Pan Drug Resistant (PDR) from using 14 different antibiotics, the result is shown in table 2. Further, ciprofloxacin is combined with 8 several drugs to find if the combination can kill the pathogen which is presented in table 3.

3.1 Categorizing the pathogenic *Klebsiella pneumoniae*

Table 2: The number of MDR, XDR and PDR *Klebsiella pneumoniae*

Total Sample	MDR*	XDR**	PDR***
23	9	5	3

* Resistant to Penicillin G, Penicillin V, Chloramphenicol, Tetracycline, Ciprofloxacin, Amoxicillin, Nalidixic Acid, Rifampicin and Moxifloxacin

** Susceptible to Cefixime, Emepenem

*** Resistant to all the 14 antibiotics

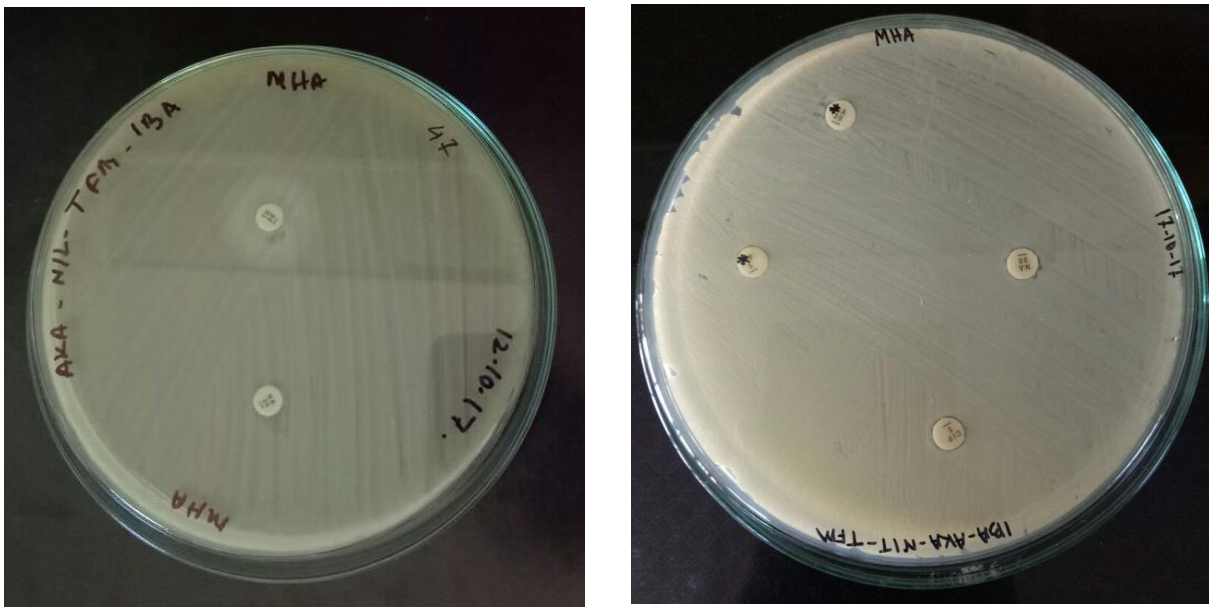


Fig. 3: The antibiotic susceptibility test result of *Klebsiella pneumoniae*

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

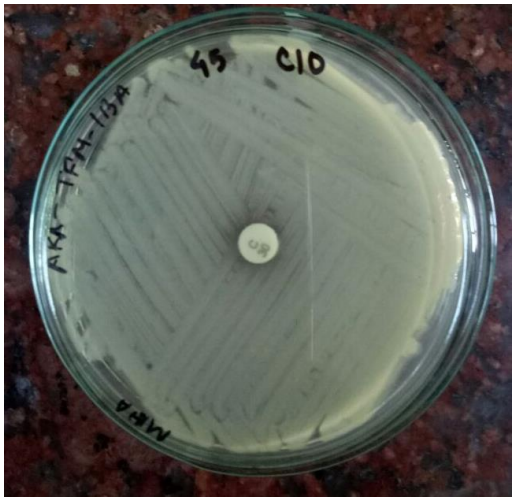
From the collected *Klebsiella pneumoniae* pathogen, randomly 2 MDR, 2 XDR and 2 PDR were selected in an effort to inhibit the growth of the pathogen by ciprofloxacin having various combinations with 8 different drugs including antihistamine, antibiotic, calcium channel blocker and probiotic which are shown in table 3.

Table 3: The combination of ciprofloxacin with several drugs and the synergy screening.

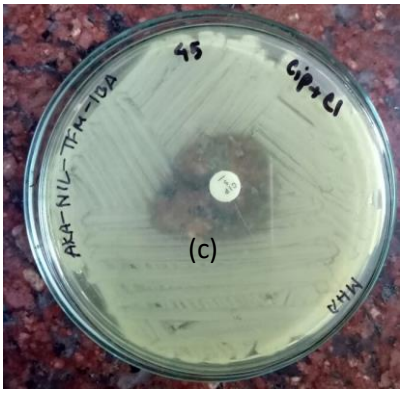
Combination of Drugs	Inhibition of Growth
Ciprofloxacin + Chloramphenicol	+
Ciprofloxacin + Amoxicillin	-
Ciprofloxacin + Rifaximin	-
Ciprofloxacin + Propranol Hydrochloride	-
Ciprofloxacin + Fexofenadine	-
Ciprofloxacin + Moxifloxacin	+
Ciprofloxacin + Probiotics	+
Ciprofloxacin + Clonazipum	-

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

(a)



(b)



(c)

Fig. 4 The zone of inhibition (a) ciprofloxacin only (b) chloramphenicol only (c) the combination (Ciprofloxacin & Chloramphenicol) which is higher than the zone of inhibition of single antibiotic.

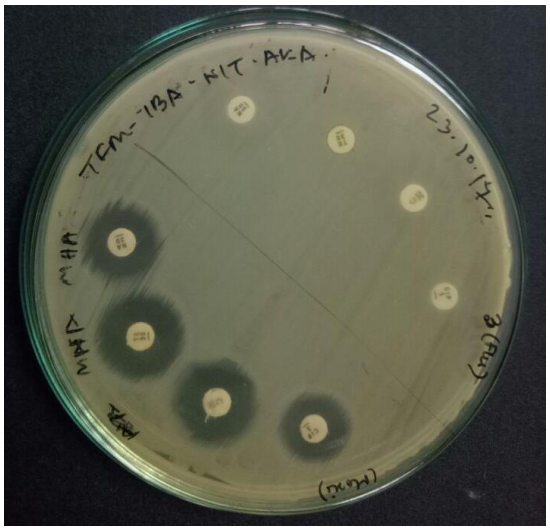


Fig. 5 The zone of inhibition is from the combination of moxifloxacin & ciprofloxacin

3.3 Determination of Minimum Inhibitory Concentration (MIC)

After the screening of 3 drugs, chloramphenicol, moxifloxacin and probiotic with combination of ciprofloxacin were further explored from their minimum inhibitory concentration (MIC). In the meantime, along with MIC, the fractional inhibitory concentration (FIC) was also calculated and compared to FIC index. It is mentionable here that FIC index is considered as statistical validation tool to determine synergistic effects (Hall *et al.*, 1983).

3.3.1.1 (a) Determination of ciprofloxacin, chloramphenicol and the combination of ciprofloxacin and chloramphenicol MIC (First Phase)

Serial dilution was done for several times to prepare desired antibiotic concentration. The results of the first phase is shown in table 4. To summarize, 6 *Klebsiella pneumoniae* was selected to determine the MIC value of the antibiotic alone and also the combination of them. The result revealed the high MIC value for the pathogens since they were supremely drug resistant. The 2 MDR & 2 XDR pathogen showed minimum inhibitory concentration of ciprofloxacin only at 250 µg/ml and 500 µg/ml respectively where 2 PDR showed 500 µg/ml.

Table 4 The MIC value of ciprofloxacin, chloramphenicol and their combination (First Phase).

Antibiotic Name	Antibiotic Concentration	Sample Number					
		MDR		XDR		PDR	
		Sample 2	Sample 27	Sample 8	Sample 42	Sample 1	Sample 3
Ciprofloxacin Only	2.5 mg/ml	C	C	C	C	C	C
	500 µg/ml	**C**	C	**C**	C	**C**	**C**
	250 µg/ml	T	**C**	T	**C**	T	T
	125 µg/ml	T	T	T	T	T	T
	62.5 µg/ml	T	T	T	T	T	T
	31.25 µg/ml	T	T	T	T	T	T
	15.625 µg/ml	T	T	T	T	T	T
Chloramphenicol Only	500 µg/ml	C	C	C	C	C	C
	250 µg/ml	**C**	C	C	**C**	**C**	**C**
	125 µg/ml	T	**C**	C	T	T	T
	62.5 µg/ml	T	T	**C**	T	T	T
	31.25 µg/ml	T	T	T	T	T	T
	15.625 µg/ml	T	T	T	T	T	T
Ciprofloxacin + Chloramphenicol	500 µg/ml	C	C	C	C	C	C
	250 µg/ml	C	C	C	C	C	C
	125 µg/ml	C	C	C	C	C	**C**
	62.5 µg/ml	**C**	C	C	**C**	**C**	T
	31.25 µg/ml	T	C	**C**	T	T	T
	15.625 µg/ml	T	**C**	T	T	T	T
	7.81 µg/ml	T	T	T	T	T	T

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

For chloramphenicol the MIC value against those pathogen was mostly 500 µg/ml. When the combination was tested the MIC value was drastically reduced even 15.625 µg/ml shown in table 3.

3.3.1.1 (b) Determination of ciprofloxacin, chloramphenicol and the combination of ciprofloxacin and chloramphenicol MIC (Second Phase)

From the first phase result, a limitation was understood that the concentration gap range of the antibiotics were high. Therefore, to get more authentic result, the same test was done twice where the dilution gap range was reduced, results shown in table 5.

Table 5 The MIC value of ciprofloxacin, chloramphenicol and their combination (Second Phase).

Antibiotic Name	Antibiotic Concentration	Sample Number					
		MDR		XDR		PDR	
		Sample 2	Sample 27	Sample 8	Sample 42	Sample 1	Sample 3
Ciprofloxacin Only	500 µg/ml	C	C	C	C	C	C
	450 µg/ml	C	C	C	C	C	C
	400 µg/ml	C	C	C	C	T	C
	350 µg/ml	C	C	T	C	T	T
	300 µg/ml	T	C	T	C	T	T
	200 µg/ml	T	C	T	C	T	T
	150 µg/ml	T	T	T	T	T	T
	100 µg/ml	T	T	T	T	T	T
	50 µg/ml	T	T	T	T	T	T
Chloramphenicol Only	250 µg/ml	C	C	C	C	C	C
	200 µg/ml	C	C	C	C	C	C
	150 µg/ml	T	C	C	T	T	T
	100 µg/ml	T	C	C	T	T	T
	50 µg/ml	T	T	C	T	T	T
	25 µg/ml	T	T	T	T	T	T
Ciprofloxacin + Chloramphenicol	100 µg/ml	C	C	C	C	C	C
	75 µg/ml	C	C	C	C	C	C
	50 µg/ml	C	C	C	T	C	T
	25 µg/ml	T	C	C	T	T	T
	20 µg/ml	T	C	T	T	T	T
	10 µg/ml	T	C	T	T	T	T
	5 µg/ml	T	T	T	T	T	T

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

3.3.1.1 (c) Determination of the arithmetic mean MIC of ciprofloxacin, chloramphenicol and the combination of ciprofloxacin and chloramphenicol

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

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

Category	Sample Number	MIC (in µm/ml)			FIC Index*
		Ciprofloxacin Only	Chloramphenicol Only	Ciprofloxacin + Chloramphenicol	
MDR	Sample 2	425	225	56.25	0.3823
	Sample 27	225	112.5	12.81	0.1708
XDR	Sample 8	450	56.25	31.25	0.5625
	Sample 42	225	250	68.75	0.5805
PDR	Sample 1	475	250	56.25	0.3434
	Sample 3	450	225	100	0.6666

[Key: MIC = Minimum Inhibitory Concentration, MDR = Multidrug Resistant, XDR = Extensively Drug Resistant, PDR = Pan Drug Resistant, FIC = Fractional Inhibitory Concentration which is determined by

MIC of the agents in combination/MIC of the agent alone]

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

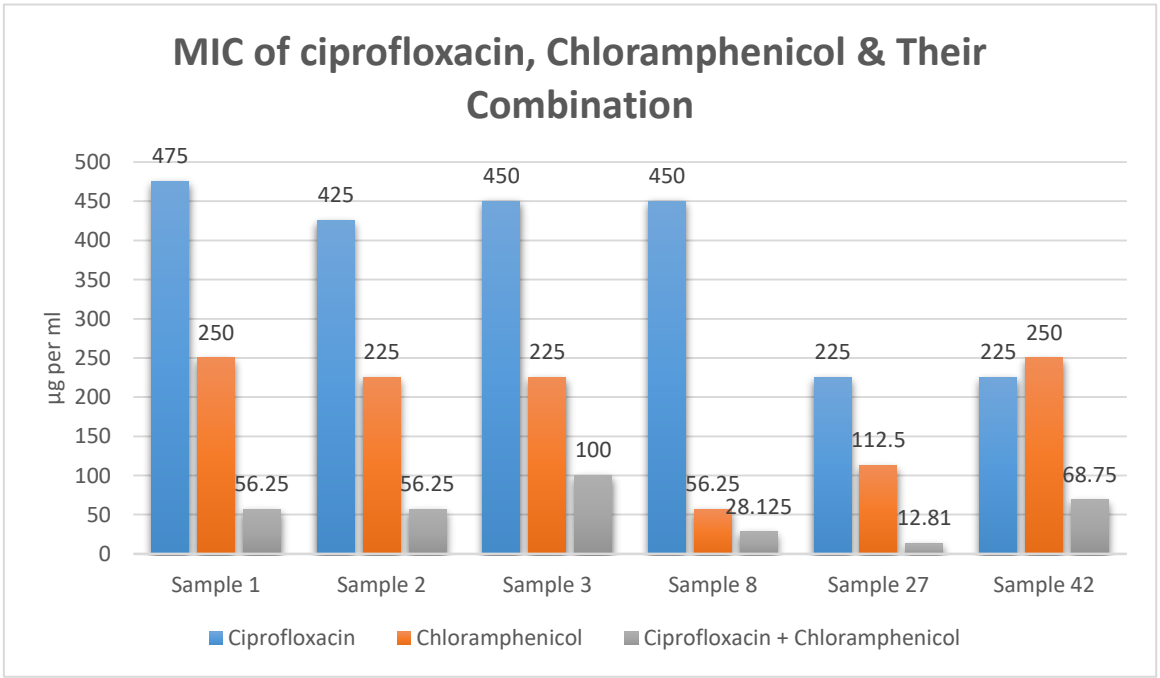


Fig. 6 MIC of ciprofloxacin, chloramphenicol & their combinations.

3.3.1.2 FIC Index Interpretation:

The fractional inhibitory concentration (FIC) index is defined by the range of 0.5 to 4 to express the result of antimicrobial agent combinations (Meletiadis *et al.*, 2010). The lower value represents the synergy and the higher value determines the antagonism which is statistically significant, shown in table 7.

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

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

3.3.1.3 The Average FIC Index (MDR, XDR, PDR) of Ciprofloxacin and Chloramphenicol

The arithmetic mean of FIC index is 0.4510 which is less than 0.5 yielding statistical significant synergistic effect of ciprofloxacin and chloramphenicol against the *Klebsiella pneumoniae.*

Fig. 7 (a) The MIC test of ciprofloxacin, chloramphenicol & their combination (First Phase).

Fig. 7 (b) The MIC test of ciprofloxacin, chloramphenicol & their combination (Second Phase).

3.4 Determination of MIC Ciprofloxacin in Combination with Probiotics

In this study, commercial probiotic (Probio from Square Pharma.) was used but the result was not satisfactory. Although from the screening of disc diffusion the study got synergistic higher activity of ciprofloxacin in combination with probiotic, in case of MIC determination the synergistic relation was rarely observed.

The combination: ciprofloxacin and probiotic was only able to kill sample 27 (MDR), 42 (XDR) at a lower concentration. Indeed, ciprofloxacin with probiotic was not able to kill any pandrug resistant (PDR) bacteria.

However, the combination showed unstable result in every trial. The experiment was done in thrice but the result was not stable. The MIC of the combination against sample 27 and 42 was more or less same during the study shown in table 8.

Table 8 The MIC of ciprofloxacin and probiotic against sample 27 and sample 42

Sample Number	Sample Type	MIC (in µg)
27	MDR	150
42	XDR	100

3.5 Qualitative toxicity analysis of ciprofloxacin and chloramphenicol combination against *Saccharomyces spp.*

Since the combination of ciprofloxacin and chloramphenicol has not been well documented, the toxicity against eukaryotic cell should be analyzed first. This qualitative analysis was done by well diffusion method and *Saccharomyces spp.* was used as a control eukaryotic cell. Result revealed no higher toxicity of the combination than the single antibiotic ciprofloxacin & chloramphenicol.

Table 8 The zone of inhibition (in mm) of ciprofloxacin, chloramphenicol and their combination against *Saccharomyces spp.*

	zone of inhibition (in mm)		
Sample	**Ciprofloxacin**	**Chloramphenicol**	**Combination of ciprofloxacin & Chloramphenicol**
Saccharomyces spp.	8	22	11

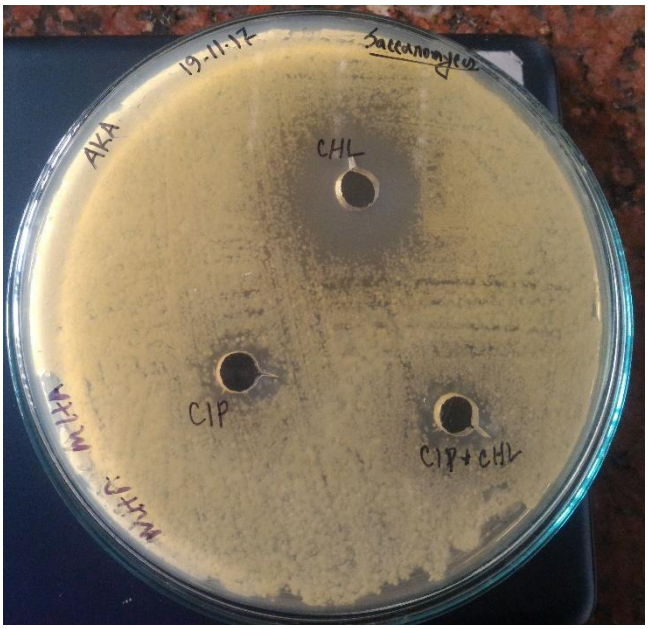


Fig. 7 Qualitative toxicity analysis of ciprofloxacin and chloramphenicol combination

Chapter 4
Discussion

Discussion

Antibiotic resistant bacterial infection is becoming the greatest threat to mankind as mentioned earlier, commercial available antibiotics are being challenged by the pathogen as well. The United States Centers for Disease Control and Prevention estimate the infections caused by antibiotic-resistant bacteria result in some two million cases of illness and 23,000 deaths in the U.S. annually. Again, the European Centre for Disease Prevention and Control produces similar numbers, estimating that antibiotic-resistant bacteria kill approximately 25,000 Europeans every year (Duke University Press, 2017).

This study clearly presents the emerging antibiotic resistant *Klebsiella pneumoniae* resistant pattern. It is noted that, out of 23 samples 17 of the bacteria were antibiotic resistant which is definitely challenging to medical science. A recent study figured out that a woman died in September, 2016 at Nevada, USA was infected with *Klebsiella* bacteria which was resistant to 26 different antibiotics. Indeed, the bacteria was resistant to all available antimicrobial drugs in the USA reported by The US Centre for Disease Control and Prevention (New Scientist magazine, 2017). This study found several *Klebsiella pneumoniae* which was resistant to all antibiotic used in this study.

Several studies have been done to find out the reasons behind emerging antibiotic resistant bacteria. The emerging of antibiotic resistant bacteria is found to be very high in developing country like Bangladesh (Morgan *et al.*, 2011). It was found that one of the major factor of increasing antibiotic resistance is the overuse and misuse of antibiotics which is common practice in the countries like India, Pakistan, Bangladesh and Sri Lanka (Bajwa, 2015). As a result, the future treatment against resistant pathogen should be highly concerned to public health department in Bangladesh.

Since the pathogen is emerging with the ability to survive within antibiotic treatment and new antibiotics are not developed, treatment procedure attracts the combination available antibiotic and herbal extracts. It has been noted that combination therapy may often be necessary for successful patient outcomes, but data in humans are still lacking and are often limited by retrospective and non-comparative study designs (Hirsch and Tam, 2010). This study only

finds the in vitro synergistic activities of ciprofloxacin and chloramphenicol against immensely drug resistant *Klebsiella pneumoniae* which should be followed up from in vivo modeling.

Further, antibiotic resistant *Klebsiella pneumoniae* has been considered as a major threat to global healthcare system. As a result, numerous antibiotic combination study has been developed against this pathogen. The combination of rifampin and colistin has been found to be bactericidal in KPC-producing *K. pneumoniae* isolates (Elemam, *et al.,* 2010). Again, the combination of rifampin, meropenem, and colistin was bactericidal against MBL-producing *K. pneumoniae* found in a study (Tängdén *et al.,* 2014). Another combination of imipenem and tobramycin has been reported bactericidal against *Klebsiella pneumoniae* (Kadar *et al.,* 2014). Tigecycline and meropenem were found to be bactericidal against XDR *Klebsiella pneumoniae* (Lim *et al.,* 2015).

On the other hand, polymyxin B and carbapenem combination has figured out to be effective against polymyxin B resistant pathogen (Cai *et al.,* 2016). Fosfomycin and aztreonam combination has been successfully combated multidrug resistant *E.coli* infection (Linda *et al.,* 2014). Tigecycline and amikacin combination scored 1.25 FIC index on a study against *Klebsiella pneumoniae* (Humphries *et al.,* 2010).

Again, the combination of doripenem and levofloxacin scored 0.5 FIC index against *Klebsiella pneumonia* infected ICU patient (Celik *et al.,* 2014) which represents the synergistic effect of these two antibiotic, the study also documented doripenem and colistin as 0.75 FIC index considering additive activity.

Furthermore, study shows that the combination of tigecycline and gentamicin can reduce up to 50% mortality than only tigecycline monotherapy (Falagas *et al.,* 2013). The combination of triple drug therapy has also been documented where the combination of rifampin, meropenem and colistin was successful to kill NDM producing *Klebsiella pneumoniae* (Tangden *et al.,* 2014).

This study reveals it's novelty for combination of ciprofloxacin and chloramphenicol which has not been documented yet. It is the first time when the synergistic activity of these two antibiotic has been found in vitro experiment. The use of antibiotics in combination is already a common hospital procedure in empirical treatment of severe infections (Lim *et al.*, 2015) but the guideline of using the combination has not well established. Several investigations have explored the use of various combination regimens for highly antibiotic resistant *Klebsiella pneumoniae*, but these investigations often lack in vivo validation. It remains unknown which combinations of antimicrobial agents/classes are most effective for the treatment of resistant pathogen.

However, the molecular mechanism for the effectiveness of ciprofloxacin and chloramphenicol is still unknown which needs to be explored. Ciprofloxacin itself is toxic to in vitro mammalian cell culture mentioned before. Though this study additionally found no greater change of toxicity to eukaryotic cell (*Saccharomyces spp.*), the molecular analysis of toxicity for this combination needs further validation. Added to this, in spite of getting synergistic relation, the MIC of the combination found in this study is still high. Therefore, in vivo animal model study needs to be performed to know whether this combination is suitable for human.

Another interesting finding is the FIC index of ciprofloxacin and chloramphenicol found in this study is 0.45; compared to literature, the combination of doripenem and levofloxacin scored 0.5, this levofloxacin is from quinolone group other than ciprofloxacin.

On the other hand, the combination of ciprofloxacin and probiotic represented confusing randomized findings. Repeating the experiment three times, probiotic combination result were not stable except one MDR and one XDR *Klebsiella pneumoniae.* It might be the manufacture quality lacking or the inability to emerge in culture media of the probiotics since sometimes it showed efficient synergistic relationship with ciprofloxacin. Still the pure probiotic fresh supernatant needs to be studied with ciprofloxacin to demonstrate either it really synergic or not. As a result, the combination of probiotics and ciprofloxacin is not recommended against highly antibiotic resistant pathogen.

Conclusion

To conclude, it should not be wrong to claim the upcoming threat of antibiotic resistant pathogen, especially *Klebsiella pneumoniae* which is emerging as a superbug and developing resistant mechanism smartly. A developing country like Bangladesh, is going to face a terrible challenge of these emerging pathogen unless and until the frequent misuse and overuse of antibiotic is abridged. Undoubtedly, the healthcare system due to antibiotic resistant bacterial infection also faces economic penalties as well. However, when the question is about life, new methods must be developed to combat these superbug and existing antibiotic combination can be a good choice. Nevertheless, ciprofloxacin is well established antibiotic having broad spectrum bactericidal activity. Hence, if the efficiency of this antibiotic can be accelerated from combination with other drugs, it might be lifesaving and cost effective as well. Moreover, developing country like ours' can grab the chance to combat antibiotic resistant *Klebsiella pneumoniae* from this combination and reduce the mortality rate from prolonged pneumonia since we are endangered floating in the sea of emerging antibiotic resistant pathogen.

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Appendix

Appendix 1

Media Composition

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Muller Hilton Agar

Component	Amount (g/L)
Beef, dehydrated infusion form	300
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH	7.3± 0.1 at 25°C

Physiological saline

Component	Amount (g/L)
Sodium Chloride	9.0

Brain-Heart Infusion Broth

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

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