Combination of several therapeutic drugs with tetracycline to combat highly drug resistant urinary tract infection pathogens

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

Submitted by:
ISHRAT BINTE AFTAB
Student ID: 14126010
December, 2017

Microbiology Program
Department of Mathematics and Natural Sciences
BRAC University
Dhaka, Bangladesh

Declaration

I hereby declare that the thesis project titled **"Combination of several therapeutic drugs with tetracycline to combat highly drug resistant urinary tract infection pathogens"** has been written and submitted by me, Ishrat Binte Aftab 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.

(Ishrat Binte Aftab)
Candidate

Certified by

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

Dedicated

To

My

Beloved

Mother and Father

Acknowledgement

My exquisite gratitude goes to Professor **A F M Yusuf Haider**, Chairperson of MNS Department, and to Professor **A. A. Ziauddin Ahmad**, Former Chairperson of MNS Department.

It is my radiant sentiment to place my best regard, gratitude and appreciation to my respected Supervisor **M. Mahboob Hossain**, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University for his constant supervision, constructive criticism, expert guidance, enthusiastic encouragement to pursue new ideas and never ending inspiration throughout the entire period of my research work.

I would like to extend my appreciation to the respective Lab officer Asma Binte Afzal. By the same token, I wholeheartedly acknowledge the assistance of the teaching assistant, Salman khan and Nahreen Mirza for their immense support and suggestions in laboratory.

Similarly, my heartfelt gratitude goes to my friend Akash Ahmed & Tonima Fairooz Mouly for their consistent support, kind cooperation and inspiration throughout the work.

Ishrat Binte Aftab

December, 2017

Abstract

Urinary tract infection (UTI) is one of the most frequent bacterial infections worldwide. Presently, the most crucial factor of treating UTI is antibiotic resistance. The overutilization of antibiotics has become the greatest pitfall of modern public health development process. This study was aimed to find out a combination of drug with tetracycline to combat UTI pathogens- *Klebsiella pneumoniae* & *Pseudomonas aeruginosa*. A number of 90 samples were collected, of them 47 (52.22%) were *K. pneumoniae* and 43 (47.78%) were *P. aeruginosa*. They were further categorized into multidrug resistant (MDR), extensively drug resistant (XDR) and pan drug resistant (PDR) pathogens. A total of 11 antibiotics were trialed in combination with tetracycline against these two pathogens. Antibiotic susceptibility test (disk diffusion method) was done for primary screening and minimum inhibitory concentration (MIC) test was done for more accurate results. A fractional inhibitory concentration (FIC) index was calculated to evaluate the effect of combined drugs. A value of $\leq$0.5 indicates synergistic effect and more than 4 shows antagonism. Among all the combined drug trials, tetracycline-amoxicillin with clavulanic acid and tetracycline-chloramphenicol showed great efficiency. Moreover, tetracycline-amoxicillin with clavulanic acid and tetracycline-chloramphenicol both combinations had FIC index value of 0.3278 and 0.3785 respectively, indicating that both of them had synergistic effect. A further extension of validation by in vivo animal model study needs to be performed to know whether the combination is suitable for human.

Table of content

List of Tables

List of Figures

Chapter 1

Introduction

Introduction

1.1 Overview

Urinary tract infection (UTI) is the one of the most frequent infections experienced by humans after respiratory and gastro-intestinal infections (Najar et al., 2009). It is well established that UTI is the second most common bacterial infection in community affecting 150 million people each year worldwide (Stamm et al., 2001; Akram et al., 2007). Literally, it is accounting for 25% of all infections and also, one of the most important causes of morbidity making it one of the most severe public health concern. (Yasmeen et al., 2015). Indisputably, the mortality rate shows the intensity of risk world is having right now in terms of urological diseases. According to Virginia Department of Health overview, 13,000 deaths (mortality rate 2.3%) are attributed to UTIs annually in the United States and in case of secondary bacteremia, mortality rate increases to approximately 10%. Moreover, in United States, urological diseases are responsible for 8 million office visits and 1 million emergency department visits each year, eventually resulting in approximately 100,000 hospitalizations each year (Mody & Juthani-Mehta, 2014).

Clinically, UTIs are categorized as uncomplicated and complicated. Typically, uncomplicated UTIs affect individuals who are otherwise healthy and have no structural or neurological urinary tract abnormalities (Nielubowicz et al., 2010). Then again, these infections are differentiated into lower UTIs (cystitis) and upper UTIs (pyelonephritis). On the other hand, complicated UTIs are defined as UTIs associated with factors that compromise the urinary tract or host defence, including urinary obstruction, urinary retention caused by neurological disease, immunosuppression, renal failure, renal transplantation, pregnancy and the presence of foreign bodies such as calculi, indwelling catheters or other drainage devices (Lichtenberger et al., 2008). Amongst the complicated UTI, catheter-associated UTIs (CAUTI) are the most frequent, causing 1 million cases per year in United States (Foxman, 2010). It is highly associated with increased morbidity and mortality, and are collectively the most common cause of secondary bloodstream infections (Chenoweth et al., 2014).

Having less distance between the urethra and bladder makes women more vulnerable to urinary tract infection than men. Therefore, this multi-agent syndrome annually causes millions of women to suffer frequent, painful urination, suprapubic pressure, and an urgent need to urinate. Although

men also are at risk, the frequency of UTI in women is an order of magnitude higher than that in men (Foxman, 2013).

Usually, UTIs are caused by both Gram-negative and Gram-positive bacteria, as well as by certain fungi. However, the most common causative agent for both uncomplicated and complicated UTIs is uropathogenic *Escherichia coli* (UPEC). Then again, in uncomplicated UTIs, UPEC is followed by *Klebsiella pneumoniae, Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* group B *Streptococcus*, *Candida* spp. etc. On the other hand, for complicated UTIs, causative agents include, UPEC, *Enterococcus* spp., *K. pneumoniae*, *Candida* spp., *S. aureus*, *P. mirabilis*, *P. aeruginosa* etc.

UTI treatment has long been associated with different antibiotics, howbeit, escalated resistance of uropathogens against numerous antibiotics making it a crucial problem. Usually, ampicillin, amoxicillin, amoxicillin with clavulanic acid, tetracycline, cefalexin, chloramphenicol, gentamycin are used to treat UTI. Nevertheless, the misuse and overuse of antibiotics have led to a condition where antibiotic resistance has become the greatest pitfall of modern public health development process. According to 2017 fact sheet of World Health Organization (WHO), antibiotic resistance is considered to be the biggest threat to global health, food security, and development today. Center for Disease and Control and Prevention (CDC) states, each year in the United States, at least 2 million people become infected with bacteria that are resistant to antibiotics and at least 23,000 people die each year as a direct result of these infections. Additionally, European Center for Disease and Control Prevention (ECDC) estimates, 25,000 people die each year in the EU from antibiotic-resistant bacterial infections. Despite of having no particular data for Bangladesh, the scenario in these developed countries figuratively shows how massive the antibiotic resistance problem is. Unfortunately this problem is more complicated for UTI as most of the existing antibiotics are found to be resistant when used against organisms.

As uropathogens are increasingly becoming resistant to currently available antibiotics, it is high time to explore alternative strategies for managing UTI. Vaccines and herbal medicines is two possible ways to combat the problem. However, one of the most frequent strategies is combination drug therapy. Simply put, it is the therapeutic intervention in which more than one therapy is administered to the patient. There are different combination drug trials given to combat uropathogenic *Pseudomonas aeruginosa*, such as, aminoglycoside-meropenem (Nakamura et al.,

2000), imipenem-aminoglycoside (Yadav et al., 2016) etc. On the other hand, to fight *Klebsiella pneumoniae,* tigecycline-gentamicin and tigecycline-colistin (Manousakaa et al., 2013), tigecycline-rifampin (Aliskan et al., 2009) trials are done. Then again, when uropathogens became resistant against amoxicillin, it was combined with clavulanic acid. For a certain period of time the attempt was successful (Iravani, 1982). Additionally, in 2015, FDA has approved the drug 'Zerbaxa', a combination of cephalosporin antibacterial drug (ceftolozane) and a beta-lactamase inhibitor (tazobactam) to treat complicated UTIs. Again in 2017, FDA gives approval of the drug 'Vabomere', combined of meropenem and vaborbactam. With FDA approving new drug combinations, it is evident that the need for combination drug is crucial. Having reservoir of less amount of antibiotics and versatile pattern of its resistance, it has become more than necessary to trial within different antibiotics. A systematic in vitro study of combination drugs is the crying need of time for further guideline in supporting therapeutic decisions for severe resistant uropathogens.

From the epidemiology of UTI, it is quite evident which organisms are mostly responsible for infection which is, in reality, quite a lot. Even though the prime pathogen of UTI is *E.coli*, none of the samples showed multi drug resistance. Therefore, this study will focus on two other highly multi drug resistant pathogens, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* Also, the organisms will be categorized based on their degree of resistance. According to the definitions proposed by the European Centre for Disease Prevention and Control (ECDC), MDR refers to an organism's non-susceptibility to at least one agent in three or more antimicrobial categories, XDR indicates non-susceptibility to at least one agent in all but two or fewer antimicrobial categories, and PDR suggests non-susceptibility to all agents in all antimicrobial categories.

1.2 Character and Morphology

Table 1.1:Character and morphology of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

Scientific Classification	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Domain:	Bacteria	Bacteria
Phylum:	Proteobacteria	Proteobacteria
Class:	Gammaproteobacteria	Gammaproteobacteria
Order:	Enterobacteriales	Pseudomonadales
Family	Enterobacteriales	Pseudomonadaceae
Genus:	*Klebsiella*	*Pseudomonas*
Species	*K. pneumoniae*	*P. aeruginosa*

1.2.1 About *Klebsiella pneumoniae*

Klebsiella pneumoniae is a prominent nosocomial pathogen that accounts for up to 10% of all hospital-acquired infections. It is a Gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It appears as a mucoid lactose fermenter on MacConkey agar. In 1882, Friedlander C. Uber first discovered *Klebsiella* to be a pathogen that caused pneumonia (MicrobeWiki). Many hospital cases around the world have been linked to *K. pneumoniae.* It is considered to be the second most responsible organism for UTI.

1.2.2 About *Psedomonas aeruginosa*

Pseudomonas aeruginosa has become an important cause of gram-negative infection, especially in patients with compromised host defense mechanisms. It is a common Gram-negative, rod-shaped bacterium that can cause disease in plants and animals, including humans. The organism is considered opportunistic as serious infection often occurs during existing diseases or conditions. It is a multidrug resistant pathogen recognised for its ubiquity, its intrinsically advanced antibiotic

resistance mechanisms are associated with serious illness. It is the most common pathogen isolated from patients who have been hospitalized longer than 1 week, and it is a frequent cause of nosocomial infections. Pseudomonal infections are complicated and can be life-threatening.

1.3 Resistance Mechanism of UTI pathogens

Klebsiella pneumoniae

The usual antibiotic treatments for *K. pneumoniae* infections include beta-lactams such as cephalosporins and carbapenems, aminoglycosides such as gentamycin, and quinolones (Qureshi, 2015). However these are now ineffective against certain strains of *K. pneumoniae* that contain effective resistance mechanisms. Usually, *K. pneumoniae* have two main resistance mechanisms: production of enzymes and biofilm formation (Cian, 2015). One prominent enzyme for resistance is extended spectrum beta-lactamases (ESBLs). These have the ability to hydrolyze a wide spectrum of beta-lactam drugs by conferring hydrolyzation of the beta-lactam ring in beta-lactam drugs by nucleophilic attack (Wallace et al., 2011). Then again, the plasmids that encode the ESBL genes also have been found to carry genes that express resistance for drugs other than beta-lactams, such as aminoglycosides (Vuotto et al., 2014). The second prominent enzyme to mention is metallo-beta-lactamases (MBLs). They also hydrolyze, but using cations and Zn^{+2} is the most common, in the nucleophilic attack of beta-lactam rings (Tzouvelekis et al., 2012). OXA-48, an enzyme of Oxacillinases (OXAs), which was found to be resistant due to hydrolysis relying on a rotation of a substituent of the carbapenem molecule within the enzyme's active site (Cian R, 2015). Out of all, most frequent enzyme responsible for resistance is *Klebsiella pneumoniae* Carbapenemases (KPCs). It exhibits activity against a wide spectrum of beta lactams. There has also been an emergence of distinct plasmids to encode for multiple variants of KPC enzymes, designated KPC-1 to KPC-13. In particular KPC-2 and KPC-3 have spread around the world alarmingly fast (Gasink et al., 2009). Also, resistance to macrolides is common in *K. pneumoniae* due to its production of macrolide esterases (Broberg et al., 2014). For KPC producing strains, colistin has become the last resort, but recently even resistance to colistin has been observed (Lim et al., 2010).

K. pneumoniae has been found to form biofilms, especially in hospital settings, and in particular on catheters. Thus, antimicrobial resistance of catheter associated UTI organisms are on peak. The biofilm protects the pathogen from antibiotic treatments. This protection, is thought to be a result of limited penetration of antibiotic molecules, is instead a result of slow growth of cells at the center of the biofilm (Vuotto et al., 2014)

Another mechanism would be efflux pump. A strain of *K. pneumoniae* isolated in France was found to have efflux mechanisms that increased resistance of chloramphenicol, the macrolide erythromycin, and the quinolone nalidixic acid, as well as a previously unknown efflux mechanism against beta-lactams (Pages et al., 2009).

Additonally, the inactivation or down-regulation of the mgrB gene, which is a negative regulator of the PhoPQ signalling system, is a source of colistin resistance in *K. pneumoniae* (Poirel et al., 2015)

In a nutshell, the resistance mechanisms of *K. pneumoniae* against antibiotics are mostly due to 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.).

Pseudomonas aeruginosa

P. aeruginosa carries an inducible AmpC cephalosporinase and its production is only in low basal level, which makes it susceptible to antipseudomonal penicillins, penicillin-inhibitor combinations, cephalosporins, and carbapenems. However, when the production is significantly increased, *P. aeruginosa* develops resistance to all β-lactams. Simply put, *P. aeruginosa* has an inducible AmpC β-lactamase and is inherently resistant to those β-lactams that induce this enzyme and are hydrolyzed by it (e.g., cephalothin and ampicillin) (David, 2002).

Moreover, many antibiotics are excluded from the pseudomonal cell which was mostly attributed to the cell's impermeability as *P. aeruginosa* copiously manufactures a porin (OprF) that forms

large outer membrane pores (Benz & Hancock, 1981). However, this impermeability-mediated resistance actually reflected efflux by MexAB-OprM, a pump system that removes β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim, as well as various dyes and detergents (Poole, 2001). Although efflux is the greater factor, the role of impermeability in pseudomonal resistance was confirmed (Li & Poole, 2000). Then again, up-regulation of other efflux systems—for example, MexCD-OprJ and MexEF-OprN—confers resistance to fluoroquinolones and some β-lactams; up-regulation of MexXYOprM also affects aminoglycosides (Poole, 2001).

Also, modifications in the QRDR in the genes coding for the quinolone target enzymes DNA gyrase and topoisomerase IV gives resistance against quinolone group antibiotics. This confers fluoroquinolone resistance more readily *(*Jalal & Wretlind, 1998).

Loss of OprD, a porin that forms narrow transmembrane channels that are accessible to carbapenems but not to other β-lactams, is associated with resistance to imipenem and reduced susceptibility to meropenem.

On the other hand, many acquired β-lactamases and aminoglycoside-modifying enzymes have been noted in *P. aeruginosa* which is widely prevalent worldwide responsible for antimicrobial resistance. The most frequently acquired β-lactamases are PSE-1 and PSE-4. Like classical TEM and OXA enzymes (which also occur, albeit rarely, in *P. aeruginosa*), these PSE enzymes can be circumvented with the use of carbapenems, oxyimino-aminothiazolyl cephalosporins (e.g., ceftazidime, cefepime, or cefpirome) or with monobactams (David, 2002). Other acquired resistance determinants were adenylyltransferase genes (*aadA1*, *aadA2*, and *aadB*), acetyltransferase genes [*aac*(*6′*)-*Ib*, *aac*(*6′*)-*Ib′*, *aacA7*, and *aacA8*], phosphotransferase gene (*aphA1-IAB*), β-lactamase genes, $bla_{\text{OXA-2}}$ and $bla_{\text{PSE-1}}$, etc. (Henrichfreise et al., 2007).

In a nutshell, loss of OprD, alterations in type II topoisomerases, AmpC overproduction, efflux pumps and numerous acquired genes are responsible for *Pseudomonas aeruginosa* resistance.

1.4 Global Emergence and Epidemiology

Klebsiella pneumoniae

Globally, non-susceptibility of urinary *K. pneumoniae* to commonly used oral and parenteral antimicrobial agents is rapidly increasing and shows large variation temporally and regionally. (Bouamri et al., 2015). This is not a scenario of one day. After the discovery of antibiotic, different generations and spectrums of antibiotics were generated to combat *K. pneumoniae* and the effort was pretty successful. Norfloxacin, levofloxacin, ampicillin etc antibiotics were highly effective against this organism. Ironically, the success, with time, has become the worst nightmare. Due to immense success of different generation's antibiotics, a rise of use of non-prescribed antibiotic is seen. According to the World Health Organization (WHO) fact sheet 2017, the misuse and overuse of antibiotics without professional oversight is accelerating antibacterial resistance problem.

In the developed world, multi drug resistant *K. pneumoniae* (MDR KP) has been documented to cause disease outbreaks leading to significant morbidity and mortality (Lee et al., 2010; Patricia et al., 2004). Development of bacterial resistance to Aminoglycosides has been documented to be the slowest amongst the antibiotics (Rennie et al., 1977). In Toronto, the first Gentamycin resistance was documented 7 years after first use (Curie et al., 1978). Then again, MDR KP has been found to be resistant to third generation Cephalosporins, Aminoglycosides and Quinolones but often sensitive to Carbapenems (Bradford, 2001; Hirsch et al., 2010). Carbapenem resistant *K. pneumoniae* (KPC) is considered the most resistant strain. It was first identified in the USA in 1996 (Yigit et al., 2001). Therefore, the mortality among patients infected with KPC is high, as a result of the limited antibiotic options remaining.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is the third most common cause of urinary tract infections (7 to 11%) (Lister et al., 2009). It is also a leading cause of nosocomial infections and is responsible for 10% of all hospital-acquired infections (National Nosocomial Infection Surveillance System data summary 2004). It was effectively treatable with first line antibiotics. However, according to World Health Organization (WHO), due to excessive and/or inappropriate use of antibiotics has led to this devastating scenario of antibiotic resistance. In Bangladesh, antibiotics are often over-

prescribed by health workers and by the same token, over-used by the public. Which is why, the emergence and spread of resistance is becoming worse.

According to Center for Disease Control and Prevention (CDC), an estimated 51,000 healthcare-associated *P. aeruginosa* infections occur in the United States each year. More than 6,000 (13%) of these are multidrug-resistant, with roughly 400 deaths per year attributed to these infections. By the same note, multidrug-resistant *Pseudomonas aeruginosa* was given a declared as serious threat in the CDC antimicrobial resistance threat report of 2013. Carbapenems were the most effective drugs against infections caused by multidrug resistant Gram-negative bacteria including *Pseudomonas* and *Acinetobacter* species (Wallace et al., 2011; Manenzhe et al., 2015). However, unsupervised management of this drug has led to antibacterial resistance. Minnesota Department of Health states, in 2001, an isolate producing a Verona Integron-Mediated (VIM) metallo-beta-lactamase was the first carbapenemase-producing *P. aeruginosa* reported in the United States. Since then, Reports of carbapenemase-producing *Pseudomonas aeruginosa* (CRPA) are increasing. It also showed resistance to ciprofloxacin an almost 2 decades ago. It exhibits the highest rates of resistance for the fluoroquinolones, with resistance to ciprofloxacin and levofloxacin ranging from 20 to 35% (Lister et al., 2009). In US intensive care units, the rate of *P. aeruginosa* resistance to ciprofloxacin had tripled from 11% during 1990–93 to 32% in 2000 (Donald et al., 2005).

Therefore, without urgent action, we are heading for a post-antibiotic era, in which common infections and minor injuries can once again lead to death. This is high time to take alternative ways and also, initiatives are more than necessary from individuals, health professionals and policy makers.

1.5 Overview of tetracycline

Tetracycline is a broad spectrum polyketide antibiotic produced by the Streptomyces genus of Actinobacteria. It exerts a bacteriostatic effect on bacteria by binding reversible to the bacterial 30s ribosomal subunit and blocking incoming aminoacyl tRNA from binding to the ribosome

acceptor site. It also binds to some extent to the bacterial 50s ribosomal subunit and may alter the cytoplasmic membrane causing intracellular components to leak from bacterial cells.

It is used to treat many different bacterial infections of the skin, intestines, respiratory tract, urinary tract, genitals, lymph nodes, and other body systems. It is often used in treating severe acne, or sexually transmitted diseases such as syphilis, gonorrhea, or chlamydia. Besides, it is also used to treat infections from direct contact with infected animals or contaminated food. In some cases, tetracycline is used when penicillin or another antibiotic cannot be used to treat serious infections such as Anthrax, Listeria, Clostridium, Actinomyces, and others.

Using tetracycline during pregnancy could harm the unborn baby or cause permanent tooth discoloration later in the baby's life. Tetracycline passes into breast milk and may affect bone and tooth development in a nursing baby. Another mentionable adverse effect is increased sensitivity of skin to sunlight. However, because of its broad spectrum activity, it is well used till now.

1.6 Aim and objective

The aim and objective of this work is to identify an optimal combination of therapeutic drugs to combat UTI. This is accomplished by identifying drug effect interaction (synergy, additivity, antagonism) for resistant organisms to each drug in the combination. This study solely focuses on interactive effect of tetracycline with other drugs which are also resistant to UTI organisms.

Chapter 2

Methods & Materials

2.1 Study area

The study was conducted at the BRAC University in Dhaka, Bangladesh. The laboratory processing, analysis of data and the overall experimental work were done in Microbiology Research Laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

2.2 Study duration

The study was conducted during the period September-November, 2017.

2.3 Study method

To establish the combination therapy approach, an experiment protocol was designed and done accordingly. Every now and then, part of different experiments were optimized for more accurate results. Firstly, clinical samples of UTI were collected from a hospital and antibiotic susceptibility testing was done. Among them *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* pathogens were selected for further experiment. Later, they were categorized into multi drug resistant (MDR), extensively drug resistant (XDR) and pan drug resistant (PDR) organism based on their resistance showed against 15 antibiotics prescribed most frequently in daily life. A total of 25 organisms were taken for categorization and 12 MDR, 8 XDR and 5 PDR were found. To ease the way of experiment, 8 organisms (4 *K. pneumoniae*, 4 *P. aeruginosa*) were selected finally to trial against different antibiotics in combination with tetracycline.

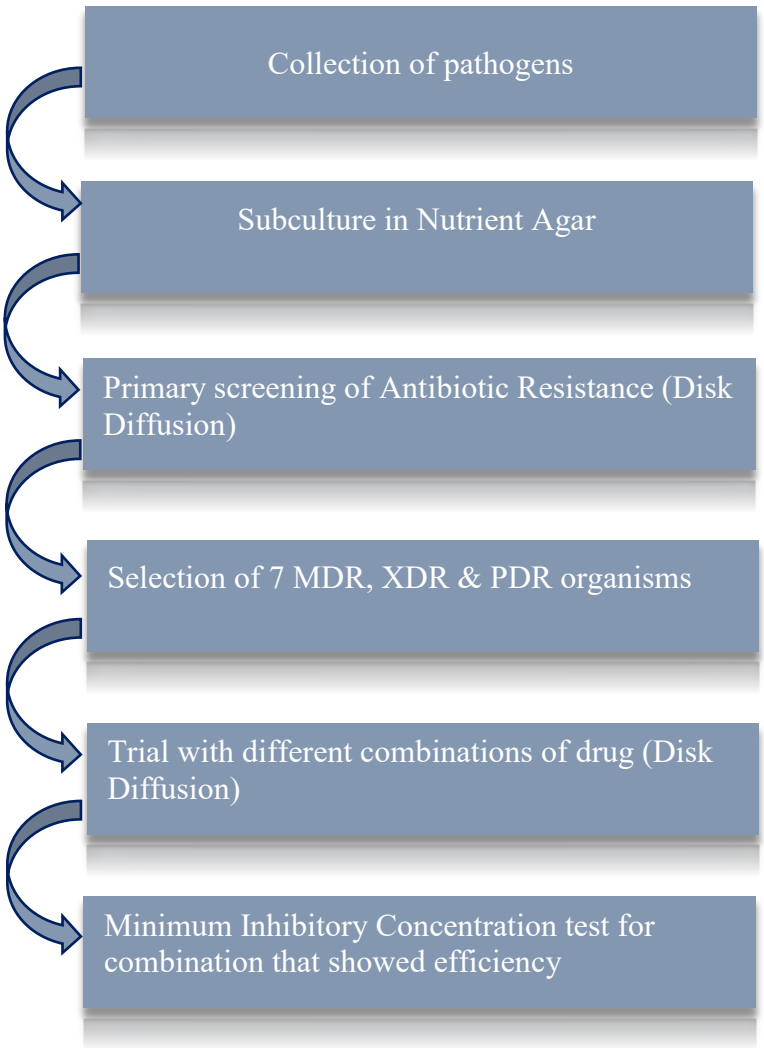
In this study, 11 different drugs of various group were combined with tetracycline disc to demonstrate their combination. All trials were done by antibiotic susceptibility testing (disk diffusion method) and observed after 24 hours with interpretation from zone diameter. Amongst them, few combinations were found that showed greater susceptibility than tetracycline and the combined drug itself, it was selected to check efficiency more accurately.

From the primary antibiotic susceptibility testing (disk diffusion), it was assumed that combination of tetracycline with amoxicillin + clavulanic acid, chloramphenicol, moxifloxacin and probiotic would be more efficient than all the antibiotics alone. Later, Minimum Inhibitory Concentration

(MIC) was done to get an extended view of assumption and clearer result. MIC was done for individual antibiotics and combinations both.

Even though combination with 4 drugs (amoxicillin with clavulanic acid, chloramphenicol, moxifloxacin and probiotic) showed more efficiency in disk diffusion method, however, after MIC, only amoxicillin with clavulanic acid and chloramphenicol showed mentionable efficiency. Thereupon, the Fractional Inhibitory Concentration (FIC) index of each successful combination was calculated and compared to the standard for statistical validation.

2.4 Flow chart of the overall study design



2.5 Sample size:

A total of about 90 urinary tract infection samples were collected from a private hospital.

2.6 Equipment

Equipment that were used in this study include:

- Laminar airflow cabinet (Model-SLF-V, vertical, SAARC group Bangladesh)
- Incubator (Model-0SI-500D, Digi system Laboratory Instruments Inc. Taiwan)
- Vortex machine (Digi system Taiwan, VM-2000)
- Autoclave machine (Model: WIS 20R Daihan Scientific Co. ltd, Korea)
- Petri-dishes, Test-tubes, Micro-pipettes, Bunsen burner, Electric balance, etc.

2.7 Collection of pathogens

Clinically identified *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was collected from the microbiology department of a private hospital. The collection started on September 2017 and ended in October 2017. The isolates of the pathogens collected 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 and used over time from subcultures. Simultaneously, these samples were stored at -20^0 Celsius in glycerol media as stock.

2.8 Collection of antibiotics and drugs for combination

All the antibiotics and drugs for combination was brought from the pharmacy nearby of BRAC University. Tetracycline used in this experiment was taken from the product of Renata Pharmaceuticals, Tetracyn 500 mg. The other drugs for combination purpose are listed in table 2.1.

Table 2.1: List of drugs used for combination drug therapy trial

Sl no	Trade Name	Company Name	Generic Name	Class
1	Indever 10mg	ACI Limited	Propranol Hydrochloride	Calcium channel blocker
2	Ciprocin 500mg	Square pharma	Ciprofloxacin	Antibiotic
3	Moxibac 400mg	Popular Pharma	Moxifloxacin	Antibiotic
4	Fimoxyclav 375 mg	Sanofi aventis Ltd	Amoxicillin + Clavulanic Acid	Antibiotic
5	Gento HC	G. A Company Ltd	Gentamicin	Antibiotic
6	Fluclox 500mg	ACI Limited	Flucloxacillin	Antibiotic
7	Tycil 500mg	Beximco Pharma	Amoxicillin	Antibiotic
8	Fenadin 120mg	Renata Ltd	Fexofenadine	Antihistamine
9	Rifagut 200mg	Opsonin Pharma	Rifaximin	Miscellaneous Antibiotic
10	Probio	Square Pharma	*Lactobacillus and Bifidobacterium spp*	Probiotic
11	Cloram 5mg/ml	Ibn Sina Pharma	Chloramphenicol	Antibiotic

2.9 Preparation of Media

Media used in this experiment-

1. Nutrient Agar (NA)
2. Muller Hington Agar (MHA)
3. Brain Heart Infution (BHI) broth

All this media were prepared and used right after plating. Fresh media was used every time and therefore, they were prepared on a regular basis.

2.9.1 Preparation of Nutrient Agar (NA)

Nutrient Agar was used for the cultivation and subculuture of microbes supporting growth of the organisms. It is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth. For this experiment, required amount of NA was calculated and weighted by electronic balance. Distil water was used to dissolve the NA. Further it was boiled and autoclaved at 121℃ and 15 psi for 40 minutes. After plating it was placed in incubator for a day to check the sterility and then used for subculture.

2.9.2 Preparation of Muller Hinton Agar (MHA)

Muller-Hinton agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing. It usually determines the antimicrobial activity of antibiotics and plant extracts. It is a non-selective, non-differential medium which makes it excellent to use antibiotic. A fixed amount of MHA needed for the test was measured and dissolved by distil water. It was boiled right after that and autoclaved for 121℃ and 15 psi for 40 minutes. Large petri dishes were used for plating of the MHA and plates were stored at 4℃ for further use.

2.9.3 Preparation of Brain Heart Infusion (BHI) broth

Brain Heart infusion broth is most useful for serial broth dilution minimum inhibitory concentration test. It is an excellent enrichment broth which supports almost all type of growths. The required amount of broth powder at first measured in electronic balance and mixed with distilled water and final volume of the solution was made 500 ml. Later, 5ml aliquots were added consecutively in 10 sterile test tubes. Likewise, 20 other sterile test tubes were used for 8ml and 9ml aliquots consecutively. The number of test tubes and aliquots were modified for the optimization of the serial dilution method. They were sent for autoclaving after proper labelling and stored in a clean beaker in the refrigerator.

2.10 Preparation of physiological saline

Physiological saline is a sterile solution of sodium chloride that is isotonic to body fluids, used to maintain living tissue temporarily and as a solvent for parenterally administered drugs. In this experiment, it was made to prepare bacterial suspension and it was matched with McFarland standard 1 solution. Also, it was used for the stock solution of drugs and antibiotics. Firstly, 0.9g

NaCl was dissolved in 80 ml deionized or distilled water in clean conical flask. Then the water was added to bring final solution volume to 100 ml. After mixing saline was transferred to 15 ml test tube and was autoclaved.

2.11 Preparation of stock solution of drugs and antibiotics

To prepare the stock solution of drugs and antibiotics, tablet/capsule was collected which were commercially available. It was then dissolved in 10 ml physiological saline shown in figure 1. Even though the excipients of those drug also got mixed into the solution, it was considered to be chemically inert, thus, not interfering with the result. Furthermore, as tablet/capsule was mixed in the physiological saline, the volume of the 10 ml saline increased a little bit. However, the amount was too little to ignore and thereupon, excluded from the overall calculation. Two eye drops were used for combination which was used as it is without mixing in physiological saline.

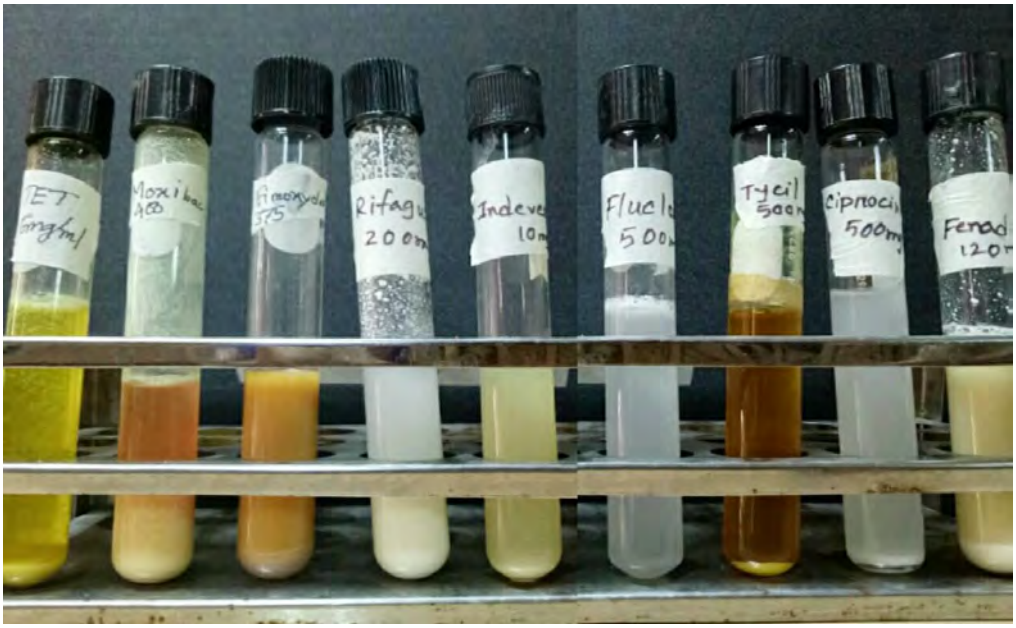


Figure 2.1: The stock of antibiotics and drugs for combination

2.12 Preparation of probiotic culture supernatant

Probiotic 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.

2.13 Disc diffusion method

In terms of convenience, efficiency and cost, disk diffusion method is one of the best methods for primary screening of antimicrobial resistance. Sterile cotton swab was used to take a certain amount of organisms from the subculture of collected pathogens and it was put into a test tube containing sterile physiological saline which was compared with McFarland standard 1 solution. A gentle mixing was done with cotton swab. Then, the agar surface of Muller-Hinton Agar plate was streaked by the swab. McFarland standard 1 solution shows the density of 3×10^8 CFU (Colony Forming Unit) per ml. Later, antibiotic discs were placed on solidified agar plates at equal distance. 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). Almost 15 antibiotic discs were used in this study. To ensure the antibiotic susceptibility, disk diffusion was repeated thrice.

2.14 Determination of the Minimum Inhibitory Concentration (MIC) of antibiotics

The aim of the study was to determine the efficiency of tetracycline with combinations against highly antibiotic resistant UTI pathogens- *Klebsiella pneumoniae* & *Pseudomonas aeruginosa.* Disk diffusion was a method of primary screening. To observe more accurate result, minimum inhibitory combination of tetracycline and other antibiotics were done with or without combination.

Minimum inhibitory concentration is that lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism. It is a significant research methodology to determine the in vitro activity of new antimicrobials and to monitor resistance to antimicrobial agents. The MIC of individual antibiotics and MIC of combined antibiotics were observed and compared for this study.

To determine the MIC, different concentrations of antibiotic was required. For this purpose, serial dilution was carried out with different amount of Brain Heart Infusion (BHI) broth as diluent. To get the desired concentration, $C_1V_1 = C_2V_2$ formula was used followed by addition of antibiotic stock solution to BHI broth. Depending on the original concentration of tablet/capsule, diverse and wide range of dilution was done customized for each individual antibiotics and combination as well. According to the method of MIC, each test tube having known concentration (decreasing to a fixed amount by each dilution) of antibiotic was inoculated with 100 µL of McFarland 1 standard pathogenic suspension. Later, the test tubes were kept at 37^0 Celsius for 24 hours and observed keenly for turbidity or clearness. The concentration of antibiotic in last clear test tube was determined as MIC value. In order to obtain efficient and accurate result, MIC test was done thrice with diverse range of dilutions and final value was figured out through the calculation of arithmetic mean.

2.15 Determination of the Fractional Inhibitory Concentration (FIC) Index

Fractional Inhibitory Concentration (FIC) index is a statistical test to estimate the interaction between two or more drugs intended to be used in combination. This is a major tool for validation of new antimicrobial agents/drugs in combination with existing for determining the synergistic effect, additive effect and antagonism.

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 8 UTI pathogens were determined and compared to standard.

Chapter 3

Results

The collected UTI samples were cultured and later, in order to see resistance, antibiotic susceptibility testing was done. Among them, 25 samples were taken which was further categorized into multidrug resistant (MDR), extensively drug resistant (XDR) and pan drug resistant bacteria. The result is shown in table 3.1.

3.1 Categorizing the pathogenic *Klebsiella pneumoniae & Pseudomonas aeruginosa*

A total of 25 organisms were taken and categorized into multidrug resistant (MDR), extensively drug resistant (XDR) and pan drug resistant (PDR).

Table 3.1: Sample categorized in terms of resistance to different antibiotics

Number of samples		
Multi drug resistant (MDR)	Extensively drug resistant (XDR)	Pan drug resistant (PDR)
12 (48%)	8 (32%)	5 (20%)

*Multidrug resistant bacteria are resistant to Tetracycline, Nalidixic acid, Azithromycin, Amoxycillin, Amoxicillin with clavulanic acid, Chloramphenicol, Ciprofloxacin. Gentamycin, Moxifloxacin

*Extensively resistant bacteria are resistant to all antibiotics except Cefixime and Rifampin

*Pan drug resistant bacteria are resistant to all the 15 drugs

3.2 Screening antibiotic combination against MDR, XDR and PDR organisms

Prior to resistance categorization, 8 pathogens were chosen for primary screening against combination of drugs. All the 8 pathogens were trialed against 11 different drugs including antibiotic, antihistaimne, probiotic, calcium channel blocker and miscellaneous antibiotics which are shown below in table 3.2.

Table 3.2: The combination of tetracycline with several drugs and the synergy screening

Sl no	Combination	Result
1	Tetracycline + Indever	-
2	Tetracycline + Ciprocin	-
3	Tetracycline + Moxibac	+
4	Tetracycline + Fimoxyclav	+
5	Tetracycline + Gento HC	-
6	Tetracycline + Fluclox	-
7	Tetracycline + Tycil	-
8	Tetracycline + Fenadin	-
9	Tetracycline + Rifagut	-
10	Tetracycline + Probio	+
11	Tetracycline + Cloram	+

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

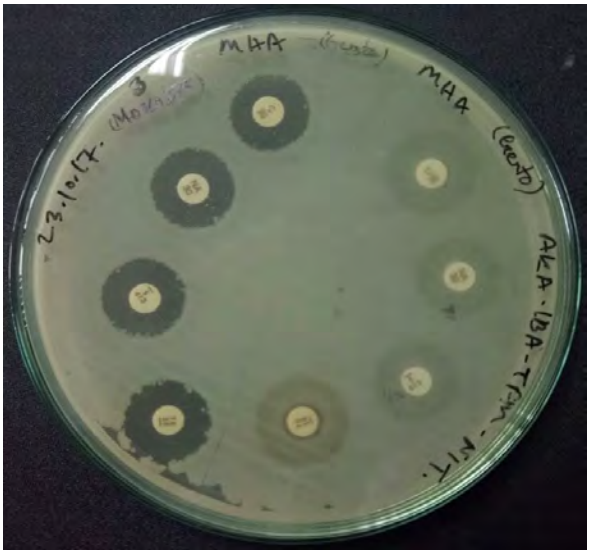


Figure 3.1: Zone of inhibition of UTI pathogens against tetracycline- amoxicillin with clavulanic acid combination

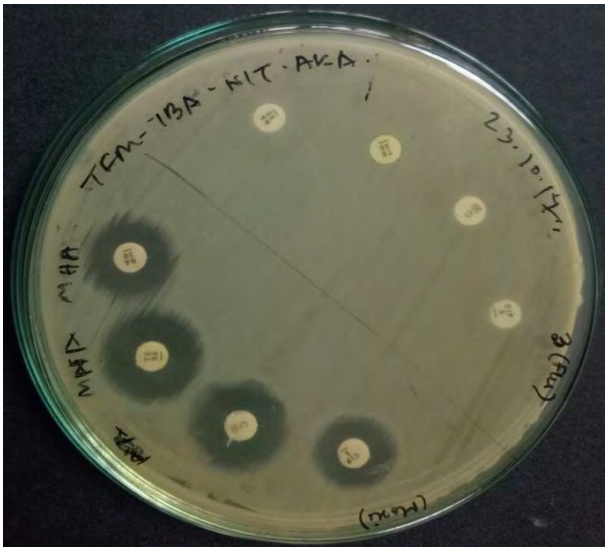


Figure 3.2: Zone of inhibition of UTI pathogens against tetracycline-moxifloxacin combination

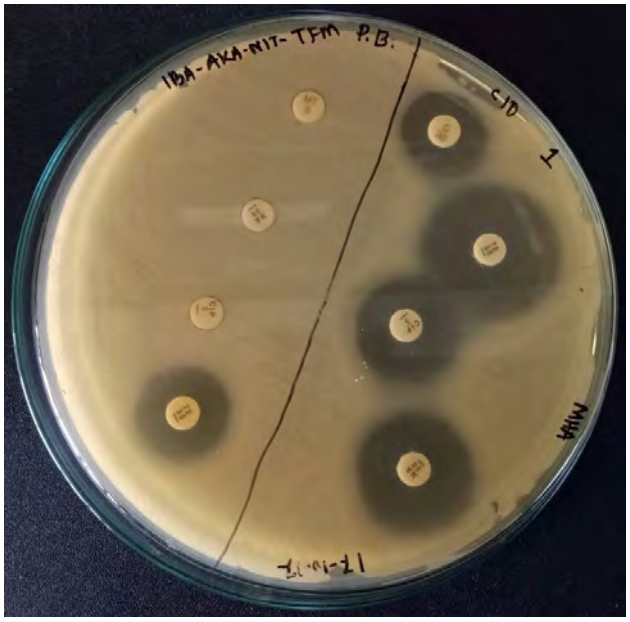


Figure 3.3: Zone of inhibition of UTI pathogens against tetracyclin-chloramphenicol and tetracycline-probiotic combination

3.3 Determination of Minimum Inhibitory Concentration (MIC)

After the screening 4 drugs showed efficiency to combine with tetracycline. Therefore, their minimum inhibitory concentration (MIC) were calculated. Among them, only tetracycline-amoxicillin with clavulanic acid and tetracycline-chloramphenicol showed efficient results.

3.3.1 Determination of minimum inhibitory concentration of Tetracycline, Amoxicillin with clavulanic acid and Chloramphenicol individually against *Klebsiella pneumoniae*.

Minimum inhibitory concentration value was determined for tetracycline, amoxicllin with clavulanic acid and chloramphenicol individually against *Klebsiella pneumoniae*. Serial dilution for MIC test was optimized for good result. In first phase the antibiotic concentration gap was wide, later in second phase the concentration gap was lessened within a specific range found from first phase. It's shown in table 3.3.

Table 3.3: MIC value of Tetracycline, Amoxicillin with clavulanic acid and Chloramphenicol against *Klebsiella pneumoniae*

Tetracycline (1st phase)							
Sample no	Antibiotic Concentration						
	700 µg/ml	600 µg/ml	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
3	C	C*	T	T	T	T	T
5	C	C	C	C*	T	T	T
25	C	C	C*	T	T	T	T
45	C	C*	T	T	T	T	T

Tetracycline (2nd phase)														
Sample no	Antibiotic Concentration													
	590 µg/ml	570 µg/ml	550 µg/ml	530 µg/ml	510 µg/ml	490 µg/ml	470 µg/ml	450 µg/ml	430 µg/ml	410 µg/ml	390 µg/ml	370 µg/ml	350 µg/ml	330 µg/ml
3	C	C*	T	T	T	T	T	T	T	T	T	T	T	T
5	C	C	C	C	C	C	C	C	C	C	C	C	C*	T
25	C	C	C	C	C	C	C*	T	T	T	T	T	T	T
45	C*	T	T	T	T	T	T	T	T	T	T	T	T	T

Amoxicillin + Clavulanic acid (1st phase)							
Sample no	Antibiotic Concentrations						
	700 µg/ml	600 µg/ml	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
3	C	C	C*	T	T	T	T
5	C	C	C*	T	T	T	T
25	C	C*	T	T	T	T	T
45	C	C	C*	T	T	T	T

Amoxicillin + Clavulanic acid (2nd phase)								
Sample no	Antibiotic Concentrations							
	570 µg/ml	550 µg/ml	530 µg/ml	510 µg/ml	490 µg/ml	470 µg/ml	450 µg/ml	430 µg/ml
3	C	C	C	C	C	C*	T	T
5	C	C	C	C	C	C*	T	T
25	C	C	C	C*	T	T	T	T
45	C	C	C	C	C	C	C*	T

Chloramphenicol (1st phase)							
Sample no	Antibiotic concentrations						
	700 µg/ml	600 µg/ml	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
3	C	C	C	C*	T	T	T
5	C	C	C	C*	T	T	T
25	C	C	C	C	C*	T	T
45	C	C	C	C*	T	T	T

Chloramphenicol (2nd phase)							
Sample no	Antibiotic concentrations						
	390 µg/ml	370 µg/ml	350 µg/ml	330 µg/ml	310 µg/ml	290 µg/ml	270 µg/ml
3	C*	T	T	T	T	T	T
5	C	C*	T	T	T	T	T
25	C	C	C	C	C	C*	T
45	C*	T	T	T	T	T	T

[C= clear, T= turbid C*=MIC value]

MIC value of tetracycline, amoxicillin with clavulanic acid and chloramphenicol against *Klebsiella pneumoniae* was shown in the table. Different dilution was done to get the accurate value. Firstly, the antibiotic concentration gap was 100 µg/ml to see the primary value. Once the range of the MIC of a specific sample was found in the first phase, it was further extended by dilution of smaller range of 20 µg/ml gap in the 2nd phase.

3.3.2 Determination of minimum inhibitory concentration of Tetracycline, Amoxicillin with clavulanic acid and Chloramphenicol individually against *Pseudomonas aeruginosa*

Minimum inhibitory concentration value was determined for tetracycline, amoxicllin with clavulanic acid and chloramphenicol individually against *Pseudomonas aeruginosa*. Serial dilution for MIC test was optimized for good result. In first phase the antibiotic concentration gap was wide, later in second phase the concentration gap was lessened within a specific range found from first phase. It's shown in table 3.4.

Table 3.4: MIC value of Tetracycline, Amoxicillin with clavulanic acid and Chloramphenicol against *Pseudomonas aeruginosa*

Tetracycline (1st phase)							
Sample no	Antibiotic Concentration						
	700 µg/ml	600 µg/ml	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
6	C	C	C*	T	T	T	T
22	C	C	C	C*	T	T	T
32	C	C	C*	T	T	T	T
42	C	C	C*	T	T	T	T
Tetracycline (2nd phase)							
Sample no	Antibiotic Concentration						
	490 µg/ml	470 µg/ml	450 µg/ml	430 µg/ml	410 µg/ml	390 µg/ml	370 µg/ml
6	C*	T	T	T	T	T	T
22	C	C	C	C	C	C*	T
32	C	C	C*	T	T	T	T
42	C	C	C*	T	T	T	T
Amoxicillin with Clavulanic acid (1st phase)							
Sample no	Antibiotic Concentrations						
	700 µg/ml	600 µg/ml	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
6	C	C	C*	T	T	T	T
22	C	C	C*	T	T	T	T
32	C	C*	T	T	T	T	T
42	C	C*	T	T	T	T	T
Amoxicillin with Clavulanic acid (2nd phase)							
Sample no	Antibiotic Concentrations						
	570 µg/ml	550 µg/ml	530 µg/ml	510 µg/ml	490 µg/ml	470 µg/ml	450 µg/ml
6	C	C	C	C	C*	T	T
22	C	C	C	C	C	C*	T
32	C	C	C	C	C	C*	T
42	C	C*	T	T	T	T	T
Chloramphenicol (1st phase)							
Sample no	Antibiotic concentrations						
	700 µg/ml	600 µg/ml	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
6	C	C	C	C	C*	T	T
22	C	C	C	C	C*	T	T
32	C	C	C	C	C*	T	T
42	C	C	C	C*	T	T	T
Chloramphenicol (2nd phase)							
Sample no	Antibiotic concentrations						
	350 µg/ml	330 µg/ml	310 µg/ml	290 µg/ml	270 µg/ml	250 µg/ml	230 µg/ml
6	C	C	C	C*	T	T	T
22	C	C	C	C	C*	T	T
32	C	C	C	C	C	C*	T
42	C	C*	T	T	T	T	T

[C= clear, T= Turbid, C*= MIC value]

MIC value of tetracycline, amoxicillin with clavulanic acid and chloramphenicol against *Pseudomonas aeruginosa*is was shown on the table. Different dilution was done to get the accurate value. Firstly, the antibiotic concentration gap was 100 µg/ml to see the primary value. Once the range of the MIC of a specific sample was found in the first phase, it was further extended by dilution of smaller range of 20 µg/ml gap in the 2nd phase.

3.3.3 Determination of minimum inhibitory concentration of Tetracycline-Amoxicillin with clavulanic acid and Tetracycline-Chloramphenicol against *Klebsiella pneumoniae*

Minimum inhibitory concentration value was determined for tetracycline-amoxicllin with clavulanic acid and tetracycline-chloramphenicol against *Klebsiella pneumoniae*. Serial dilution for MIC test was optimized for good result. In first phase the antibiotic concentration gap was wide, later in second phase the concentration gap was lessened within a specific range found from first phase. It's shown in table 3.5.

Table 3.5: MIC value of the combination of Tetracycline-Amoxicillin + clavulanic acid and Tetracycline-chloramphenicol against *Klebsiella pneumoniae*

Tetracycline-Amoxicillin with Clavulanic acid (1st phase)					
Sample no	Antibiotic Concentration				
	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
3	C	C	C	C*	C
5	C	C	C	C	C
25	C	C	C	C	C
45	C	C	C	C	C

Tetracycline-Amoxicillin with Clavulanic acid (2nd phase)								
Sample no	Antibiotic Concentrations							
	150 µg/ml	130 µg/ml	110 µg/ml	90 µg/ml	70 µg/ml	50 µg/ml	30 µg/ml	10 µg/ml
3	C	C	C*	T	T	T	T	T
5	C	C	C	C	C*	T	T	T
25	C	C	C	C	C	C*	T	T
45	C	C	C	C	C	C*	T	T

Tetracycline-Chloramphenicol (1st phase)					
Sample no	Antibiotic Concentrations				
	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
3	C	C	C	C	C
5	C	C	C	C	C*
25	C	C	C	C	C
45	C	C	C	C	C

Tetracycline-Chloramphenicol (2nd phase)								
Sample no	Antibiotic Concentrations							
	150 µg/ml	130 µg/ml	110 µg/ml	90 µg/ml	70 µg/ml	50 µg/ml	30 µg/ml	10 µg/ml
3	C	C	C*	T	T	T	T	T
5	C	C	C	C*	T	T	T	T
25	C	C	C	C*	T	T	T	T
45	C	C	C	C	C*	T	T	T

[C= clear, T= turbid, C*= MIC value]

MIC value of the combination of tetracycline-amoxicillin with clavulanic acid and tetracycline-chloramphenicol against *Klebsiella pneumoniae* was shown here. Different dilution was done to get the accurate value. Firstly, the antibiotic concentration gap was 100 µg/ml to see the primary value. Once the range of the MIC of a specific sample was found in the first phase, it was further extended by dilution of smaller range of 20 µg/ml gap in the 2nd phase.

3.3.4 Determination of minimum inhibitory concentration of Tetracycline-Amoxicillin with clavulanic acid and Tetracycline-Chloramphenicol against *Pseudomonas aeruginose*

Minimum inhibitory concentration value was determined for tetracycline-amoxicllin with clavulanic acid and tetracycline-chloramphenicol against *Pseudomonas aeruginosa*. Serial dilution for MIC test was optimized for good result. In first phase the antibiotic concentration gap was wide, later in second phase the concentration gap was lessened within a specific range found from first phase. It's shown in table 3.6.

Table 3.6: MIC value of the combination of Tetracycline-Amoxicillin with clavulanic acid and Tetracycline-Chloramphenicol against *Pseudomonas aeruginosa*

Tetracycline-Amoxicillin with Clavulanic acid (1st phase)					
Sample no	Antibiotic Concentration				
	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
6	C	C	C	C	C
22	C	C	C	C	C
32	C	C	C	C*	T
42	C	C	C	C	C

Tetracycline-Amoxicillin with Clavulanic acid (2nd phase)								
Sample no	Antibiotic Concentrations							
	150 µg/ml	130 µg/ml	110 µg/ml	90 µg/ml	70 µg/ml	50 µg/ml	30 µg/ml	10 µg/ml
6	C	C	C	C	C	C	C*	T
22	C	C	C	C	C	C	C*	T
32	C	C*	T	T	T	T	T	T
42	C	C*	T	T	T	T	T	T

Tetracycline-Chloramphenicol (1st phase)					
Sample no	Antibiotic Concentrations				
	500 µg/ml	400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml
6	C	C	C	C	C
22	C	C	C	C	C
32	C	C	C	C	C
42	C	C	C	C	C

Tetracycline-Chloramphenicol (2nd phase)								
Sample no	Antibiotic Concentrations							
	150 µg/ml	130 µg/ml	110 µg/ml	90 µg/ml	70 µg/ml	50 µg/ml	30 µg/ml	10 µg/ml
6	C	C	C	C	C	C	C*	T
22	C	C	C	C	C*	T	T	T
32	C	C	C	C	C*	T	T	T
42	C	C	C	C*	T	T	T	T

[C= clear, T= turbid, C*= MIC value]

MIC value of the combination of tetracycline-amoxicillin with clavulanic acid and tetracycline-chloramphenicol against *Pseudomonas aeruginosa* was shown in the table. Different dilutions were done to get the accurate value. Firstly, the antibiotic concentration gap was 100 µg/ml to see the primary value. Once the range of the MIC of a specific sample was found in the first phase, it was further extended by dilution of smaller range of 20 µg/ml gap in the 2nd phase.

3.4 Determination of the arithmetic mean of MIC value (individual and combination) and FIC index

Since the 2nd phase of the MIC test for individual antibiotic and combined antibiotic was done thrice, an arithmetic mean was needed to determine the final FIC index. The values are shown in table 3.7 and 3.8.

Table 3.7: The Average MIC value of Tetracycline, Amoxicillin with Clavulanic acid individual and combination & FIC Index

Category	Sample Number	Organism	MIC (in µm/ml)			FIC Index*	Avg. of FIC index
			Tetracycline Only	Amoxicillin with clavulanic acid Only	Tetracycline-Amoxicillin with Clavulanic acid		
MDR	Sample 25	*K. pneumoniae*	470	510	53.33	0.2180	0.3278
	Sample 32	*P. aeruginosa*	456.67	523.33	116.67	0.4784	
XDR	Sample 5	*K. pneumoniae*	363.33	470	76.67	0.3741	
	Sample 6	*P. aeruginosa*	483.33	463.33	43.33	0.1831	
PDR	Sample 3	*K. pneumoniae*	570	443.33	123.33	0.4945	
	Sample 22	*P. aeruginosa*	390	470	60	0.2815	
	Sample 42	*K. pneumoniae*	450	443.33	76.67	0.3433	
	Sample 45	*P. aeruginosa*	583.33	450	63.33	0.2493	

Table 3.8: The Average MIC value of Tetracycline, Chloramphenicol individual and combination & FIC Index

Category	Sample Number	Organism	MIC (in µm/ml)			FIC Index*	Avg. of FIC index
			Tetracycline Only	Chloramphenicol Only	Tetracycline-Chloramphenicol		
MDR	Sample 25	*K. pneumoniae*	470	290	86.67	0.4832	0.3785
	Sample 32	*P. aeruginosa*	456.67	260	63.33	0.3823	
XDR	Sample 5	*K. pneumoniae*	363.33	373.33	83.33	0.4526	
	Sample 6	*P. aeruginosa*	483.33	280	26.67	0.1504	
PDR	Sample 3	*K. pneumoniae*	570	390	100	0.4318	
	Sample 22	*P. aeruginosa*	390	283.33	66.67	0.4062	
	Sample 42	*K. pneumoniae*	450	343.33	76.67	0.3936	
	Sample 45	*P. aeruginosa*	583.33	390	76.67	0.3280	

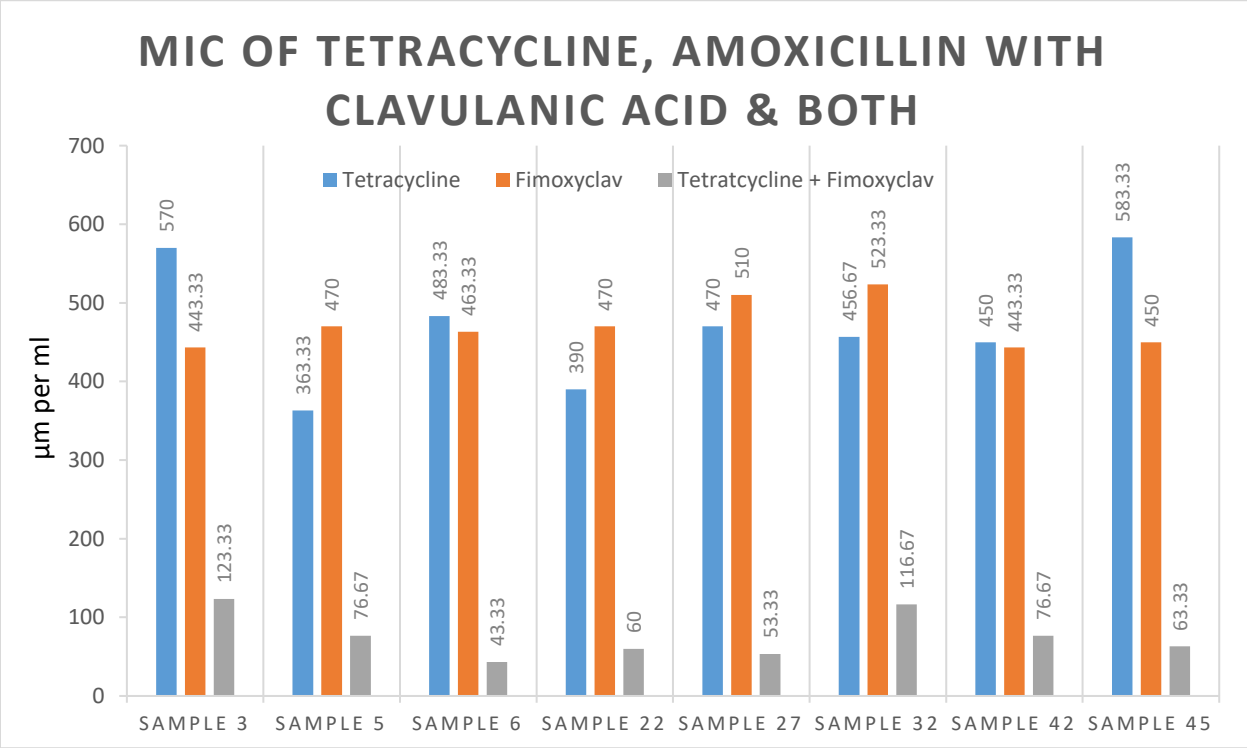


Figure 3.4: MIC of tetracyclin, amoxicillin with clavulanic acid their combinations

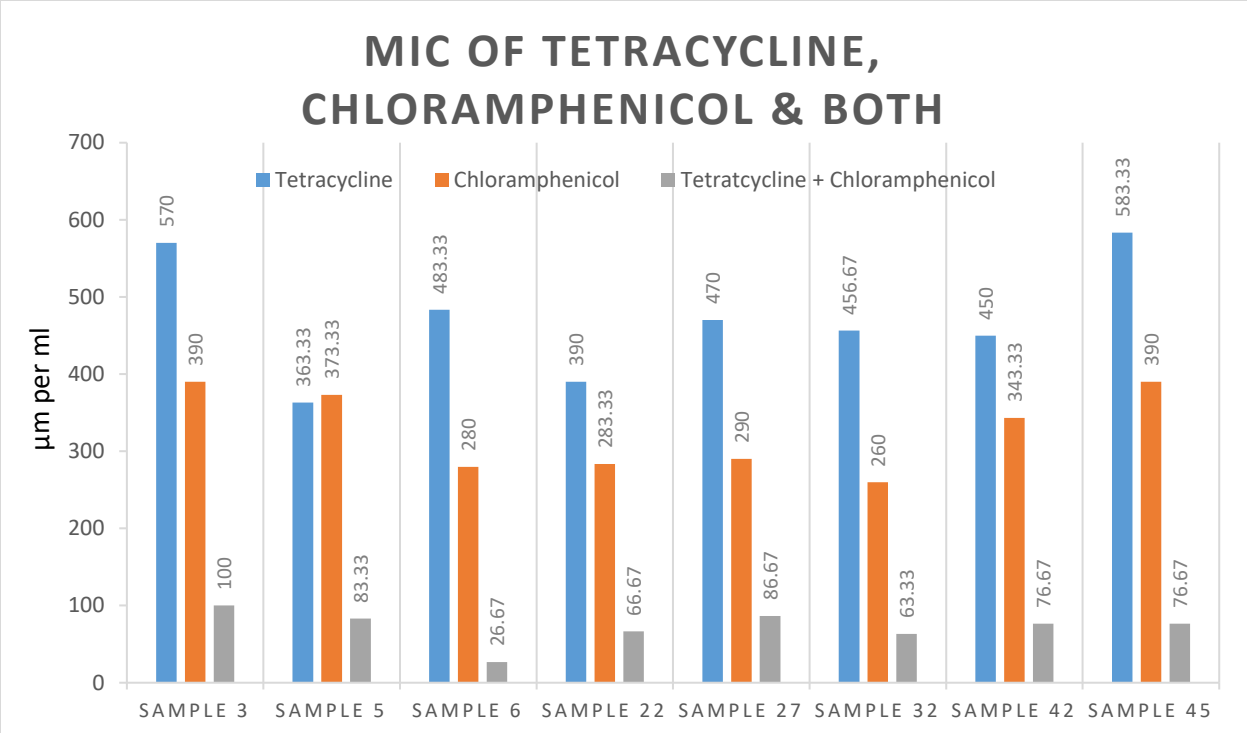


Figure 3.5: MIC of tetracyclin, chloramphenicol their combinations

3.5 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. Also, the range greater than 0.5 and lower or equal to 1 is considered as additive.

Table 3.9: Range of FIC index interpretation

Interpretation	FIC
Synergy	≤ 0.5
Additive	> 0.5 & ≤ 1.0
Indifference	> 1.0 & ≤ 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.]

3.6 The Average FIC Index of Tetracycline in combination with Amoxicillin + Clavulanic Acid & Chloramphenicol

The arithmetic mean of FIC index for tetracycline-amoxicillin with clavulanic acid is 0.3278 and for tetracycline-chloramphenicol was 0.3785, both of which was less than 0.5, indicating statically significant synergistic effect against UTI pathogens- *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

Figure 3.6: MIC of tetracycline alone against *Klebsiella pneumoniae*

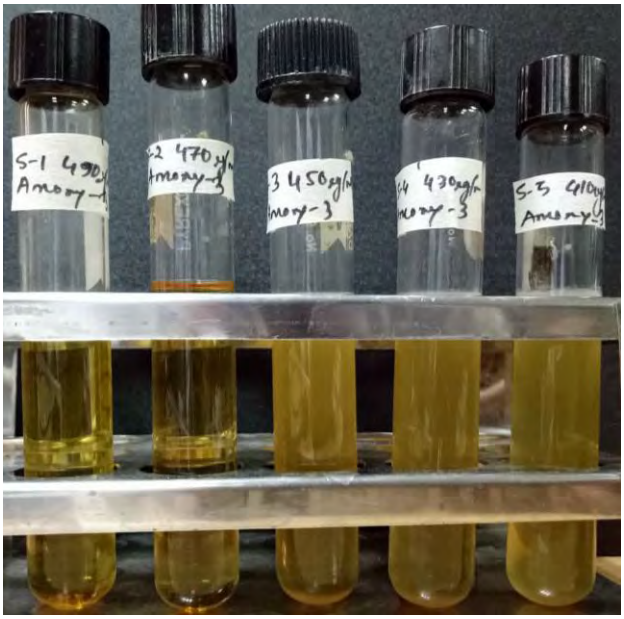


Figure 3.7: MIC of amoxicillin with clavulanic acid alone against *Klebsiella pneumoniae*

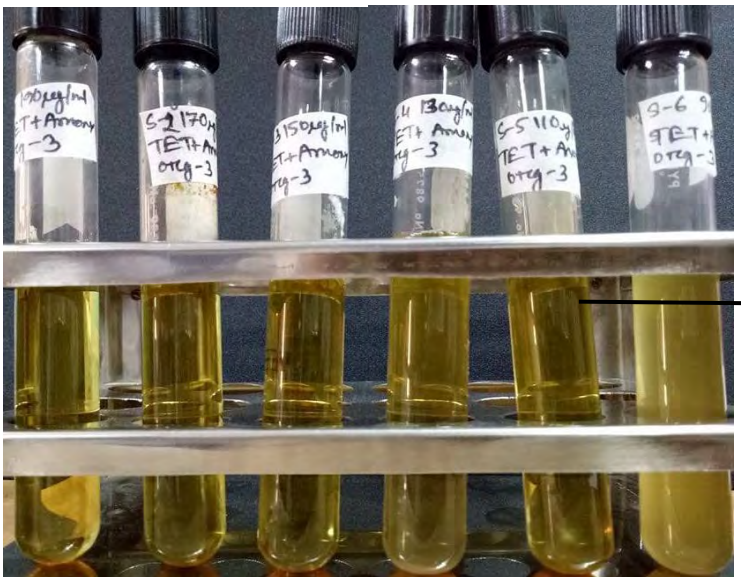


Figure 3.8: MIC of tetracycline-amoxicillin with clavulanic acid alone against *Klebsiella pneumoniae*

Figure 3.9: MIC of tetracycline alone against *Pseudomonas aeruginosa*

Figure 3.10: MIC of amoxicillin with clavulanic acid alone against *Pseudomonas aeruginosa*

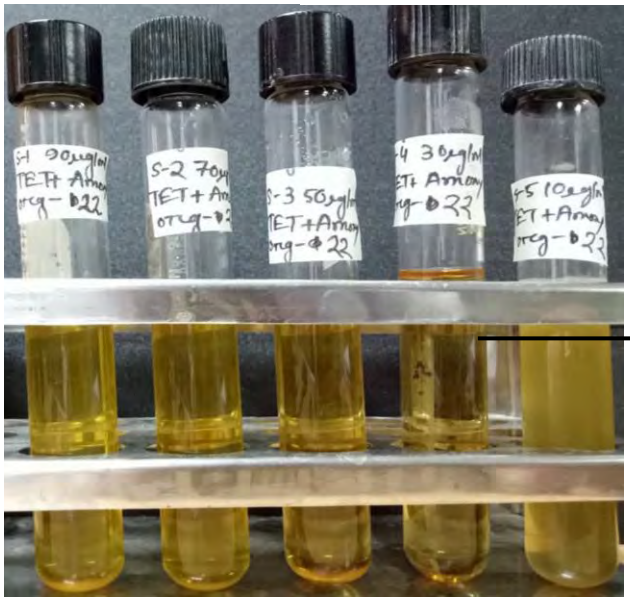


Figure 3.11: MIC of tetracycline-amoxicillin with clavulanic acid against *Pseudomonas aeruginosa*

Figure 3.12: MIC of tetracycline alone against *Klebsiella pneumoniae*

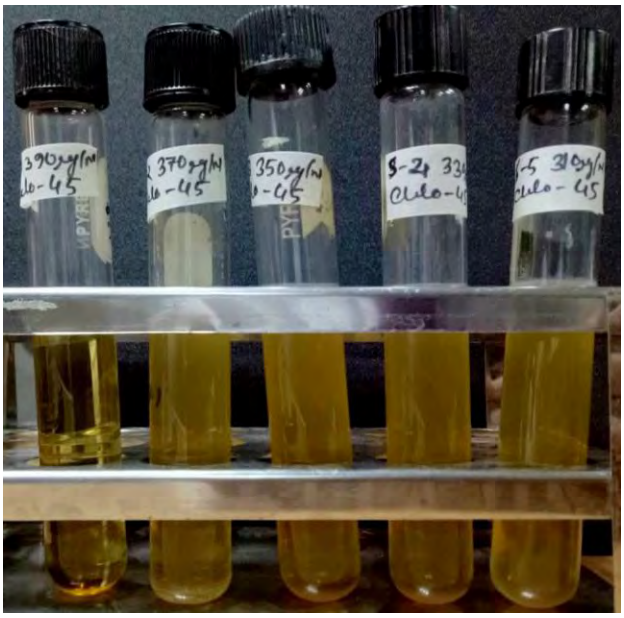


Figure 3.13: MIC of chloramphenicol alone against *Klebsiella pneumoniae*

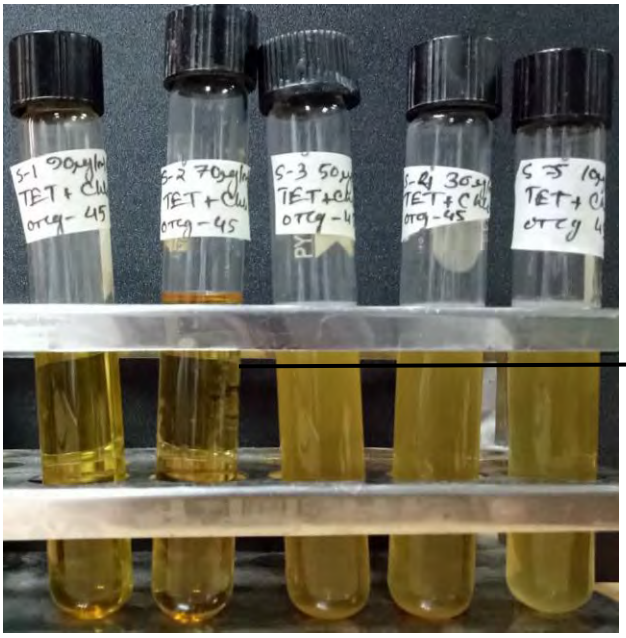


Figure 3.14: MIC of tetracycline-chloramphenicol against *Klebsiella pneumoniae*

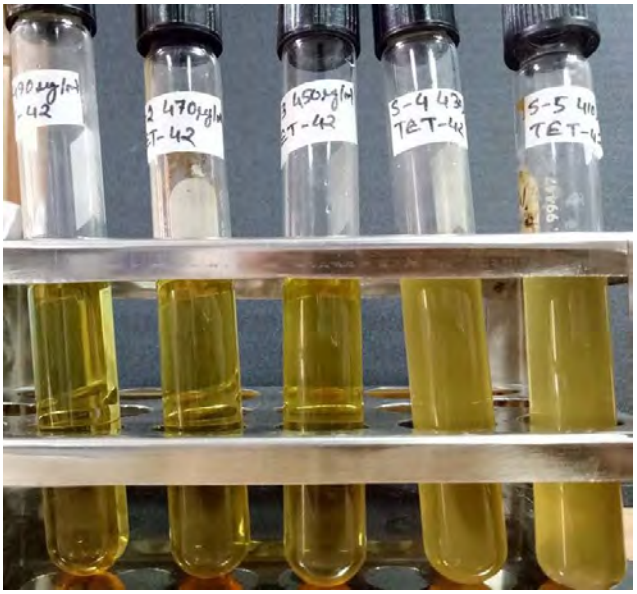


Figure 3.15: MIC of tetracycline alone against *Pseudomonas aeruginosa*

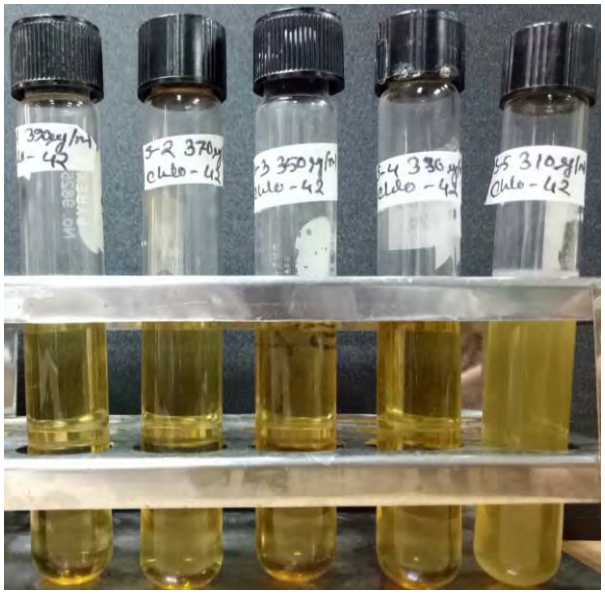


Figure 3.16: MIC of Chloramphenicol alone against *Pseudomonas aeruginosa*

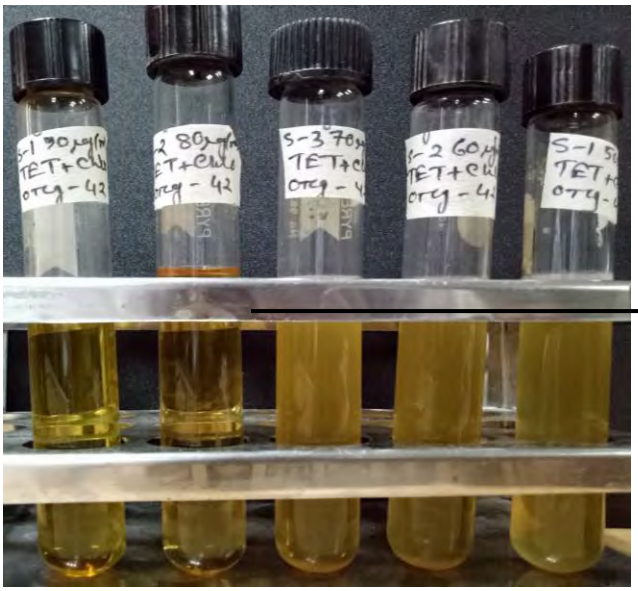


Figure 3.17: MIC of tetracycline-chloramphenicol against *Pseudomonas aeruginosa*

3.7 Toxicity analysis

A qualitative toxicity analysis was done by using the antibiotics individually and in combination both against *Saccharomyces cerevisiae.* No mentionable zone of inhibition was observed in the analysis.

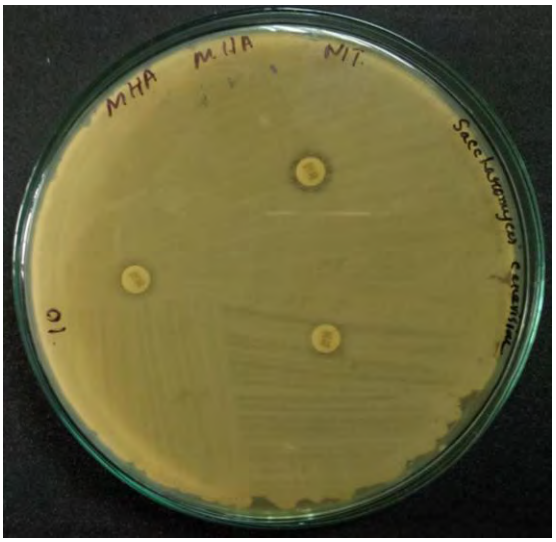


Figure 3.18: Tetracycline, Amoxicillin with Clavulanic Acid and Chloramphenicol against *Saccharomyces cerevisiae*

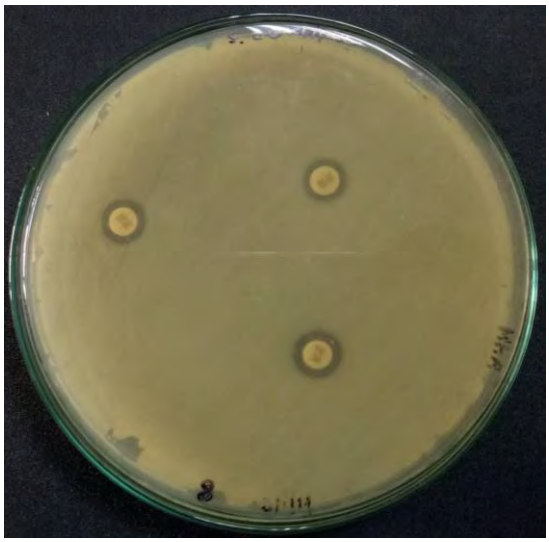


Figure 3.18: Tetracycline, Tetracycline-Amoxicillin with Clavulanic Acid and Tetracycline-Chloramphenicol against *Saccharomyces cerevisiae*

Chapter 4

Discussion

4.1 Discussion

The rapid emergence of antibiotic resistance of bacteria is occurring globally, jeopardizing the efficacy of antibiotics, which have transformed medicine and saved millions of lives (*Golkar, 2014).* A project commissioned by the British government, Review on Antimicrobial Resistance (AMR) estimates, antibiotic resistance creates 700,000 deaths per year worldwide. Even though the number is at jaw dropping level, not enough measures are taken to prevent the situation. Alternative drug therapy options are a crying need of time. By the same token, antibiotic resistance problem by UTI pathogens are on the peak. According to a report of the New Scientist, it affects an estimated 250 million people a year, most of them women. Additionally, UTI pathogens seem to be resistant against all the existing antibiotics except colistin. However, a large amount of colistin resistant bacteria is reported to be found in India and some other developed countries. Therefore, the situation indulges public health system in a position where it is almost compulsory to walk in new directions to find out a way for this life-threatening problem.

This study holds the aspiration to evaluate the eradication rate of combination drugs to combat antibiotic resistance problem. A number of 25 UTI isolates were selected and studied. Due to less number of multi-drug resistant *E.coli*, which is one of the prime pathogen of UTI, was not focused in this study. Alternatively, the aim of the study was fixed on another two highly antibiotic resistant UTI pathogens, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. After the antibiotic susceptibility testing in disk diffusion method was done, they were categorized into multidrug resistant (MDR), extensively drug resistant (XDR) and pan drug resistant (PDR) pathogen. Among them, 12 were MDR, 8 were XDR and 5 were PDR pathogens. After the primary screening for resistance, 8 isolates were selected among which 2 were MDR, 2 were XDR and 4 were PDR. Later, they were trialed against different combination of therapeutic drugs keeping tetracycline the primary one. This trial was first carried out through disk diffusion method where antibiotics were used from their stock solution. Even though there was other ingredients present, they were considered to be chemically inert, hence, creating no effect on result. In the antibiotic susceptibility testing, amoxicillin with clavulanic acid, moxifloxacin, chloramphenicol and probiotic expressed efficiency in combination with tetracycline against both- *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* However, in minimum inhibitory concentration test, moxifloxacin and probiotic did

not show any stable and effective result. On the other hand, amoxicillin with clavulanic acid and chloramphenicol showed fine result. From the statistical validation of FIC index, the value for tetracycline and amoxicillin with clavulanic acid was 0.3278 and for tetracycline and chloramphenicol was 0.3785. Both of the value of FIC index was less than 0.5, indicating statically significant synergistic effect against UTI pathogens- *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* No individual strain showed antagonism or additive effect.

Combination drug therapy is a very familiar therapeutic idea for coping up with the hideous situation of antibiotic resistant era. Till now, many studies have been carried out to find out new combination of drugs since antibiotic reservoir is becoming very insufficient in course of time.

When uropathogens became resistant against amoxicillin, it was combined with clavulanic acid. For a certain period of time the attempt was successful (Iravani, 1982). However, in recent decades, numerous studies have been done to see the efficacy of combination drug therapy to combat urinary tract infection.

Klebsiella pneumoniae and *Pseudomonas aeruginosa* both are highly drug resistant and a great threat to public health system. Therefore, as different combinations get inefficient after few years of success, continuous trials are going on, in diverse methods.

The FIC index of Meropenem were reduced to ≤1.56 mg/L in combination with the aminoglycosides tested against *Pseudomonas aeruginosa* (Nakamura, 2000). Then again, imipenem-amikacin showed supper additive effect with a FIC index value of <1 (Tasaka, 2002). It was also seen ceftazidime-avibactam is effective in combination with imipenem against *Klebsiella pneumoniae* (Gaibani, 2017). Tigecycline-amikacin combination had a score of 1.25 in FIC index against *K. pneumoniae* (Humphries, 2001). Another study shows, combination of tigecycline and gentamicin can reduce mortality up to 50% than tigecycline monotherapy (Falagas, 2013). Additionally, ceftolozane/tazobactam also demonstrated superior *in vitro* activity against *K. pneumoniae.* By the same token, in 2015, FDA has approved the drug 'Zerbaxa', a combination of cephalosporin antibacterial drug (ceftolozane) and a beta-lactamase inhibitor (tazobactam) to treat complicated UTIs. Furthermore, again in 2017, FDA gives approval of the drug 'Vabomere', combined of meropenem and vaborbactam.

Certainly, this study shows novelty as the combination of tetracycline-amoxicillin with clavulanic acid and tetracycline-chloramphenicol has not been documented yet. The synergistic effect of these two combinations found in-vitro experiment is totally new. Nevertheless, the molecular mechanism for the effectiveness of tetracycline-amoxicillin with clavulanic acid and tetracycline-chloramphenicol is unknown till now. This extension of knowledge should be explored further. Another mentionable fact is, tetracycline itself is not toxic to mammalian cell culture in vitro. Also, there is no great change of toxicity by individual and combined drugs to eukaryotic cells *(Saccharomyces* spp.*)*. Even so, a molecular analysis of toxicity for this combination needs further validation. Additionally, in vivo animal model study needs to be performed to know whether the combination is suitable for human.

On the contrary, moxifloxacin and probiotic showed great zone of inhibition in disk diffusion method even after repeating the experiment thrice. Howbeit, while doing MIC test, results were verily unstable and to some extent, inefficient even in the repeating times. There may have some contamination in the product during manufacture or inability of the pathogens to emerge in the culture media. Thereupon, the combination of tetracycline-moxifloxacin and tetracycline-probiotic should not be recommended against highly antibiotic resistant pathogen without further stable and efficient validation.

4. 2 Conclusion

The discovery of antibiotics and their widespread availability revolutionized healthcare after the Second World War. They have underpinned many of the greatest medical advances of the 20th century. Howbeit, the overutilization of antibiotics have led to an era where the immense success has become the worst nightmare. According to AMR review, the global burden of infections resistant to existing antimicrobial medicines is now growing at an alarming pace. Drug-resistant infections are already responsible for more than half a million deaths globally each year. Early research commissioned by the Review suggests that if the world fails to act to control resistance, this toll will exceed 10 million each year by 2050 and have cost the world over 100 trillion USD in lost output. This numbers evidently shows, that the world has reached a critical point and must

act now on a global scale to slow down antimicrobial resistance. Failure to act will indulge healthcare system in an almost unthinkable scenario where antibiotics no longer work and we will cast back into the dark ages of medicine. To handle the situation, combination drug therapy is considered one of the best ways. Tetracycline, an immensely prescribed broad spectrum antibiotic is now in its lowest edge of efficacy. Nevertheless, combination to amoxicillin with clavulanic acid and chloramphenicol can accelerate its effect to combat UTI pathogens- *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Even though further validation is needed, this study indisputably can contribute as a prominent finding to fight highly drug resistant urinary tract infection organisms.

Chapter 5

Reference

Akram M, Shahid M, Khan AU Ann. (2007). Etiology and antibiotic resistance patterns of community-acquired urinary tract infections in JNMC Hospital Aligarh, India. Annals of Clinical Microbiology and Antimicrobials. Vol. 6:4

Benz R, Hancock RE. (1981). Properties of the large ion-permeable pores formed from protein F of *Pseudomonas aeruginosa* in lipid bilayer membranes. Biochim Biophysica Acta; vol. 646; 298-308

Bouamri M, Arsalane L, Kamounim Y, Zouhair S. (2015). Antimicrobial susceptibility of urinary *Klebsiella pneumoniae* and the emergence of carbapenem-resistant strains: A retrospective study from a university hospital in Morocco. North Africa, In African Journal of Urology; Vol. 21:1; 36-40

Bradford PA. (2001). Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clinical Microbiology Rev; 14; 933-951.

Broberg A, Palacios M, & Miller L. (2014). *Klebsiella*: a long way to go towards understanding this enigmatic jet-setter. F1000Prime Reports; 6; 64.

Cian R, Antimicrobial Resistance in *Klebsiella pneumoniae*: Mechanisms and Clinical Impact and Developments. BIOM 250

Curie K, Speller DCE, & Simpson RA. (1978). A hospital epidemic caused by gentamycin resistant *Klebsiella aurogenes*. Journal of Hygiene, 80, 115-123.

Flores-Mireles L, Walker N, Caparon, M, Hultgren J. (2015). Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nature Reviews. Microbiology; Vol. 13:5; 269–284

Foxman B. (2010). The epidemiology of urinary tract infection. Nat Rev Urol; Vol 7:12; 65360

Gaibani P, Lewis RE, Volpe SL, Giannella M, Campoli C, Landini MP, Viale P, Re MC, Ambretti S. (2017). In vitro interaction of ceftazidime-avibactam in combination with different antimicrobials against KPC-producing *Klebsiella pneumoniae* clinical isolates. Int J Infect Dis.; Vol. 22; 65

Gasink, L. B., Edelstein, P. H., Lautenbach, E., Synnestvedt, M., & Fishman, N. O. (2009). Risk Factors and Clinical Impact of *Klebsiella pneumoniae* Carbapenemase–Producing *K. pneumoniae*. Infection Control and Hospital Epidemiology: The Official Journal of the Society of Hospital Epidemiologists of America; Vol. 30:12; 1180–1185.

George M, Maria L, Vassiliki P, Styliani M, Nikolaos S, Athanasios T, Spyros P. (2013). Activity of Tigecycline in Combination with Colistin, Meropenem, Rifampin, or Gentamicin against KPC-Producing Enterobacteriaceae in a Murine Thigh Infection Model. Antimicrobial Agents and Chemotherapy; Vol. 57:12; 6028–6033

Golkar Z, Bagazra O, Pace DG. (2014). Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. J Infect Dev Ctries; 8:2; 129–136

Henrichfreise B, Wiegand I, Pfister W, Wiedemann B. (2007). Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. Antimicrobial Agents and Chemotherapy; Vol. 51; 4062–4070

Iravani, A, & Richard A. (1982). Treatment of urinary tract infections with a combination of amoxicillin and clavulanic acid. Antimicrobial Agents and Chemotherapy; Vol. 22:4; 672–677.

Jalal S, Wretlind B. (1998) Mechanisms of quinolone resistance in clinical strains of *Pseudomonas aeruginosa*, Microb Drug Resistance; Vol. 4; 257-361

Laurent P, Aurélie J, Séverine B, Maria V, Melda O, Salih T, Patrice N. (2015). The mgrB gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. Journal of Antimicrobial Chemotherapy; Vol. 70:1; 75–80

Lee K, Lee MA, Lee CH, Lee J, Roh KH, Kim S. (2010). Increase of ceftazidime- and fluoroquinolone-resistant *Klebsiella pneumoniae* and imipenem-resistant *Acinetobacter spp*. in Korea: analysis of KONSAR study data from 2005 and 2007.Yonsei Med J; Vol 51:6; 901-911.

Li XZ, Zhang L, Poole K. Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonas aeruginosa*. (2000). Journal of Antimicrobial Chemotherapy; Vol. 45; 433-6

Lichtenberger P, Hooton TM. (2008). Complicated urinary tract infections; Curr Infect Dis Rep; 10(6); 499-504

Lim M, Ly N, Anderson D, Yang C, Macander L, Jarkowski A. Tsuji, B. T. (2010). Resurgence of Colistin: A Review of Resistance, Toxicity, Pharmacodynamics, and Dosing. Pharmacotherapy, 30:12; 1279–1291.

Lister D, Wolter J, Hanson D. (2009). Antibacterial-Resistant Pseudomonas aeruginosa: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. Clinical Microbiology Reviews; Vol. 22:4; 582–610.

Livermore D. (2002). Multiple Mechanisms of Antimicrobial Resistance in Pseudomonas aeruginosa: Our Worst Nightmare. Clinical Infectious Diseases; Vol. 34:5; 634–640

Livermore DM. β -Lactamases in laboratory and clinical resistance. (1995). Clinical Microbiology Reviews; Vol. 8; 557-84

Magiorakos A, Srinivasan, R.B. Carey, Y.Carmeli, M.E. Falagas, C.G. Giske. (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; volume 18; 268-281

Maki PG and Tambyah PA. (2001). Engineering Out the Risk of Infection with Urinary Catheters. Emerging Infectious Diseases; Vol. 7:2; 178-183.

Manenzhe RI, Zar HJ, Nicol MP, Kaba M. (2015). The spread of carbapenemase-producing bacteria in Africa: a systematic review. Journal of Antimicrobial Chemotherapy; Vol.70:1; 23–40

Meletiadis J, Pournaras, S, Roilides, E, Walsh, J. (2010). Defining Fractional Inhibitory Concentration Index Cutoffs for Additive Interactions Based on Self-Drug Additive Combinations, Monte Carlo Simulation Analysis, and In Vitro-In Vivo Correlation Data for Antifungal Drug Combinations against Aspergillus fumigatus. Antimicrobial Agents and Chemotherapy; Vol. 54:2; 602–609

Mody, L., & Juthani-Mehta M. (2014). Urinary Tract Infections in Older Women: A Clinical Review. JAMA; Vol. 311:8; 844–854.

Najar S, Saldanha L, Banday A. (2009). Approach to urinary tract infections. Indian Journal of Nephrology; Vol. 19:4; 129–139

Nakamura A, Hosoda M, Kato T, Yamada Y, Itoh M, Kanazawa K, Nouda H. (2000). Combined effects of meropenem and aminoglycosides on *Pseudomonas aeruginosa* in vitro. Journal of Antimicrobial Chemotherapy; Vol. 46:6; 901–904

Neuhauser M, Weinstein A, Rydman, R. et al. (2003). Antibiotic resistance among gram-negative bacilli in US intensive care units: implications for fluoroquinolone use. Journal of the American Medical Association; Vol. 289; 885–8.

Nielubowicz GR, Mobley HL. (2010). Host-pathogen interactions in urinary tract infection. Nat Rev Urol; Vol 7:8; 430-41

Pages M, Lavigne P, Leflon-Guibout V, Marcon, E, Bert F, Noussair L, Nicolas-Chanoine H. (2009). Efflux Pump, the Masked Side of ß-Lactam Resistance in *Klebsiella pneumoniae* Clinical Isolates. PLoS ONE; Vol. 4:3; 4817.

Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. (2011). Carbapenems: past, present, and future. Antimicrobial Agents and Chemotherapy; Vol. 55:11; 4943–4960.

Poole K. (2001). Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms, Journal of Molecular Microbiology and Biotechnology; Vol. 3; 255-64

Rennie RP, Duncan BR. (1977). Emergence of gentamycin resistant Klebsiella in a general hospital. Antimicrobial agents and chemotherapy; Vol. 11:2; 179-184

Rogers MA, Mody L, Kaufman SR, Fries BE, McMahon LF, (2008). Use of urinary collection devices in skilled nursing facilities in five states. J Am Geriatr Soc; Vol. 56:5; 854-861

Stamm WE, Norrby SR. (2001). Urinary tract infections: disease panorama and challenges. J Infect Dis; Vol. 183

Tasaka K, Ishida A, Chinzei T. (2002). Antimicrobial activity of carbapenems and the combined effect with aminoglycoside against recent clinical isolates of Pseudomonas aeruginosa. Jpn J Antibiot; Vol. 55(2); 181–186

Tzouvelekis S, Markogiannakis A, Psichogiou M, Tassios T, Daikos L. (2012). Carbapenemases in *Klebsiella pneumoniae* and Other Enterobacteriaceae: an Evolving Crisis of Global Dimensions. Clinical Microbiology Reviews; Vol. 25:4; 682–707

Ventola L. (2015). The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharmacy and Therapeutics*, Vol. 40:4; 277–283

Vuotto C, Longo F, Balice P, Donelli G, Varaldo E. (2014). Antibiotic Resistance Related to Biofilm Formation in Klebsiella pneumoniae. Pathogens; Vol. 3:3; 743–758

Weinstein JW, Mazon D, Pantelick E, et al. (1990). A decade of prevalence surveys in a tertiary-care center: trends in nosocomial infection rates, device utilization, and patient acuity. Infect Control Hosp Epidemiol; Vol. 20; 543-548.

Yadav R, Bulitta JB, Nation RL, Landersdorfer CB. (2017). Optimization of synergistic combination regimens against carbapenem- and aminoglycoside-resistant clinical *Pseudomonas aeruginosa* isolates via mechanism-based pharmacokinetic/pharmacodynamic modeling. Antimicrobial Agents Chemotherapy; Vol. 61

Appendix

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 (g/L)
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