Synergistic Combinational Effect of Antibiotics on Multi Drug Resistant *P. aeruginosa* Clinical Isolate

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I certify that this research work titled "**Synergistic Combinational Effect of Antibiotics on Multi Drug Resistant *P. aeruginosa* Clinical Isolate**" is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

Muhammad Sufyan Vohra

Dedicated to

My beloved Family

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List of Acronyms

ACRONYMS	GENERIC NAME
MDR	Multidrug resistance
FICI	Fraction Inhibitory Concentration Index
MIC	Minimal Inhibitory Concentration
CDC	Center for Disease Control and prevention
ESBL	Extended spectrum β-lactamase
TK	Time kill assay
FDA	Food and Drug Administration
CLSI	Clinical and Laboratory Standards Institute
CFU	Colony Forming Unit
RAD	Cephradine
CXM	Cefuroxime
CAZ	Ceftazidime
CTX	Cefotaxime
CRO	Ceftriaxone
FEP	Cefepime
AK	Amikacin
CN	Gentamycin
TOB	Tobramycin
TGC	Tigecyline
FOS	Fosfomycin
OFX	Ofloxacin

LVX	Levofloxacin
SPX	Sparfloxacin
MOX	Moxifloxacin
IPM	Imipenem
MEM	Meropenem
AUF	Amoxicillin/clavulanic acid
TZP	Piperacillin/tazobactum

Chapter 1: ABSTRACT

Pseudomonas aeruginosa is equipped with serious health problems that cause high morbidity and high mortality rates. Amongst them, the incidence rate of multidrug resistant (MDR) *P. aeruginosa* has been increasing worldwide. The treatment of MDR *P. aeruginosa* has particularly become more challenging due to its inherent and acquired resistance to many existing antibiotic drugs. There is a demand for effective therapies, but the development of new antibiotics is in the pipeline, since an alternative such as the phage therapy has not been promising, however, strategies like combination therapy are more effective as compared to others.

In this study, we identified synergistic combinations that showed effective against MDR *P. aeruginosa*. A total 84 combinations of 20 commercially available FDA cleared antibiotics were made, which include 50 double and 34 triple combinations. Out of 84, 25 combinations have high synergism, lower antibiotic generation and high MIC value individually. Imipenem-Levofloxacin and Amoxicillin-Clavulanic acid-Ofloxacin were found to be the most effective combinations against MDR *P. aeruginosa* isolates. These combinations were validated by time kill kinetics, and efficacy of combinations were checked on other isolates, whereby, IMP-LVX showed bactericidal killing against 80%, and AMX-CLA-OFX showed 60% synergistic killing of other MDR *P. aeruginosa* clinical isolates.

Keywords Antibiotic combinations, Multi drug resistance *Pseudomonas aeruginosa*, synergy.

Chapter 2: INTRODRUCTION

P. aeruginosa is a serious cause of nosocomial infections amongst hospital and community acquired patients (Foca *et al.*, 2000) (Cryz Jr, 1984) (Young and Amstrong, 1972) (Deretic, 2000). They carry more multiple resistant plasmids than *Klebsiella pneumoniae, Stenotrophomonas maltophilia* and Enterobacter species (Livermore, 2002). The first human infection caused by *P. aeruginosa* was reported in 1882 (Gessard, 1882). *P. aeruginosa* properties make itself unique by already containing chromosomal resistance, in addition to the ability to acquire resistance to many antimicrobial agents that are contributing a serious and frequent role in *P. aeruginosa* infections (Obritsch *et al.*, 2004). *P. aeruginosa* has the ability to colonize in chronic cystic fibrosis (CF) patients which cause high morbidity and high mortality rates worldwide (Valerius *et al.*, 1991). It has the ability to produce secondary metabolites i.e. bluish redox active compound Pyocyanin that has a virulence role in pathogenesis (Lau *et al.*, 2004). Antibiotic resistant in *P. aeruginosa* has been increasing in recent years that include carbapenem resistance and multi drug resistance (MDR) *P. aeruginosa* (Driscoll *et al.*, 2007). According to Center for Disease Control and prevention (CDC), about 5.1 million pseudomonas infections were reported per year. Amongst them, the death rate was 440 per year, and multi drug resistance (MDR) *P. aeruginosa* rate was about 6700 cases per year (Control and Prevention, 1987) (Sievert *et al.*, 2013). *P. aeruginosa* has the capability to adhere it selves in a hydrated polymeric matrix of polysaccharide to form a slim layer i.e. biofilm, which also involves a resistance mechanism of antibiotics that may help in persistent and chronic infections (Stewart and Costerton, 2001) (Mah *et al.*, 2003) (Costerton *et al.*, 1999).

There are many published reports that claim the existence of Extensively-Drug Resistant (XDR) and carbapenem resistant *P. aeruginosa* in health care settings (Perez *et al.*, 2014) (Cabot *et al.*, 2012) (Willmann *et al.*, 2015). Recently, clinical *P. aeruginosa* XDR was reported with the co-expression of Metallo β-Lactamase (MBLs), OXA β-lactamase and the Extended spectrum β-lactamase (ESBL) genes that conferred resistance to many classes of β-lactam drugs (Ríos *et al.*, 2018). Whilst Pakistan recently reported the emergence of XDR *P. aeruginosa* from patients admitted in the surgical ICU room (Uddin *et al.*, 2018).

First report on carbapenem resistance arised from Japan during 1992-94 which confirmed that MBLs gene is responsible for carbapenem resistance (Senda *et al.*, 1996). Another report from Kidney Center of Rawalpindi, Pakistan confirmed the existence of carbapenem resistance due to bla-$_{NDM}$ and bla-$_{OXA}$ gene in immunocompromised patients (Braun *et al.*, 2018). This concludes that the overexpression of different efflux systems and the loss of Opr-D protein are responsible for carbapemen resistance in *P. aeruginosa* (Rodríguez-Martínez *et al.*, 2009).

Widespread use of drugs in MDR *P. aeruginosa* infections has created resistance to most of the commercially available antibiotics (Paterson, 2006). The occurrence of MDR *P. aeruginosa* has been reported in many countries (Hota *et al.*, 2009) (Obritsch *et al.*, 2005). According to one study, a patient infected with *P. aeruginosa*, resistant to almost all antimicrobial agents that include aminoglycosides, cephalosporin, quinolones and penicillin, was treated with last resort of drugs like Colistin. (Levin *et al.*, 1999). The only alternative drug available is colistin/polymyxin B antibiotic, however it causes adverse effects like nephrotoxicity and neurotoxicity in immunocompromised patients (Balkan *et al.*, 2014) (John *et al.*, 2015) (Javan *et al.*, 2015).

Due to this alarming condition, immediate control measures are required to limit the spread of highly resistant clones as they become a major health challenge to treat MDR *P. aeruginosa.* Moreover, whilst the new antibiotics are in the production phase, alternative options like phage therapy and vaccine development are being used, however they are not showing promising results (Wright *et al.*, 2009) (Mesaros *et al.*, 2007). To overcome this problem, the development of alternative therapeutic strategies like combination therapy is urgently needed. Recent studies of meta-analyses reveal that multi-resistant *P. aeruginosa* has found more benefit from combination therapy as in comparison to monotherapy (Hilf *et al.*, 1989). Effective combination therapy show numerous advantages including increased bacterial broader spectrum, increased antibiotic activity (synergy), preventing emergence of resistance, bactericidal activity and reliable regimens to combat poly-microbial infections (Giamarellou *et al.*, 1984) (Milatovic and Braveny, 1987) (Pizzo, 1995) (Shlaes and Bass, 1983). Our study objective was to determine effective synergistic combinations from FDA approved antibiotics that would be immediately available for patients as well as the removal of MDR *P. aeruginosa* infections from their root cause.

In this study, we explored effective combinations that could be used to eradicate MDR *P. aeruginosa*. We targeted those *P. aeruginosa* isolates that were resistant to many antibiotics. To begin with, the different classes of antibiotics were combined in double and triple combinations to find their synergistic, partially synergistic, antagonistic or indifferent effects. We used 19 clinically available antibiotics and made a total of 84 antibiotic combinations, among them 50 were double, and 34 were triple combinations. After determining the synergistic antibiotic combinations, the effective combinations were validated by Time Kill (TK) Assay. Subsequently, we then tested the combinations for anti-biofilm capability. Lastly, the effective combinations were tested on a series of other clinical *P. aeruginosa* isolates for efficacy of synergistic combinations.

RESEARCH AIM

To test significant antibiotic combinations against locally prevalent Multidrug Resistant *Pseudomonas aeruginosa* to treat its infections.

RESEARCH OBJECTIVES

1. To test effective combinations of commercially available antibiotics revealing *in vitro* antimicrobial synergy against MDR *P. aeruginosa.*
2. Time-kill Kinetics/analysis of effective antibiotic combinations.
3. To test significant combinations for eradication of biofilm formation.
4. Validation of the efficacy of synergistic combinations on randomly selected *P. aeruginosa* isolates.

Chapter 3: LITERATURE REVIEW

3.1 Antibiotics

3.1.1 Invention of Antibiotics:

The invention of antibiotics into clinical practices has modernized the treatment and control of infectious diseases, which were previously tough to treat (Aminov, 2017). Sulfonamides was first discovered in 1937. It was the first ever effective antimicrobial agent that was used as a therapeutic medicine at that time. The emergence of sulfonamides resistance was reported at the end of the 1930s and remained existent for over 70 years (Davies and Davies, 2010). Until 1940, when penicillinase was identified as the first antibiotic resistance encounter to penicillin, which is known as the world's most powerful medicine, pioneered by Fleming in 1928. Since then, a sufficient amount of classed antibiotics has been developed and industrially produced. The use of antibiotic simultaneously increased with the resistance of antibiotics (Figure 1) (Abraham and Chain, 1988).

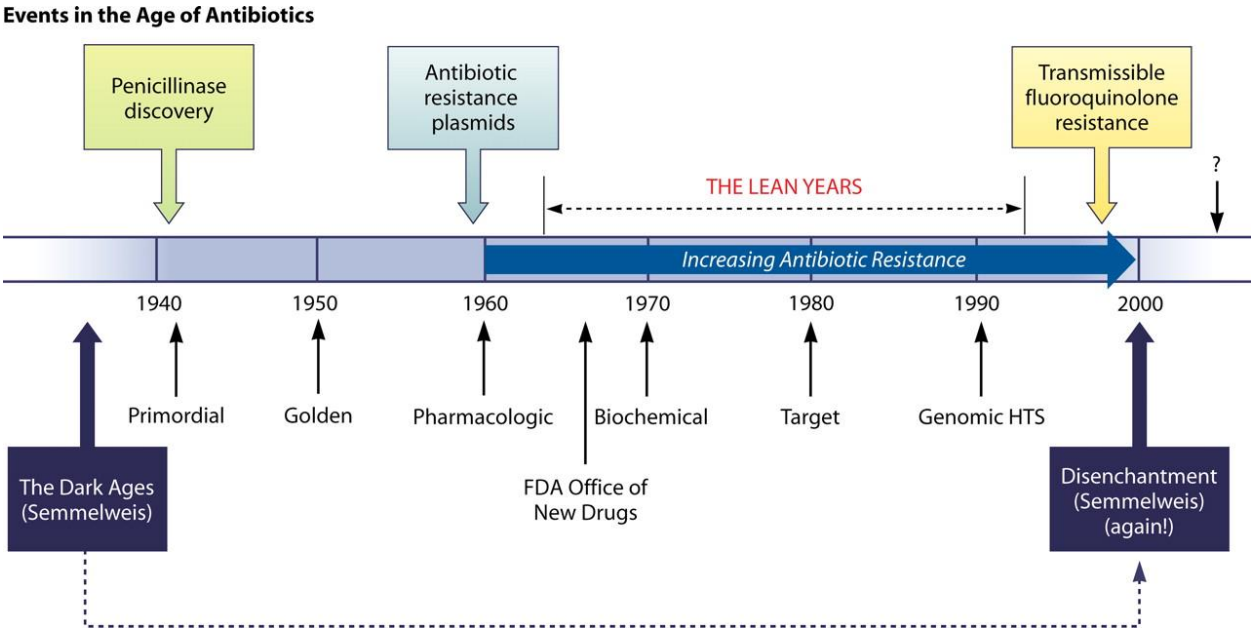

Figure 1: Here is one figure depicting the important events occur in the Age of Antibiotics:

3.1.2 Classification of Antibiotics:

Antibiotics could be classified using a number of methods, the most common are based on their chemical structure, mode of action and broad/narrow spectrum activity (Adzitey, 2015) (Waksman, 1961).

The common classes of antibiotics based on their chemical classification;

i. β-lactams classes (act on bacterial cell wall)
ii. Macrolide antibiotics (inhibit bacterial 50s ribosomes)
iii. Tetracycline antibiotics (inhibit bacterial 30s ribosomes)
iv. Fluoroquinolone antibiotics (DNA synthesis inhibitors)
v. Aminoglycosides antibiotics (inhibit bacterial 30s ribosomes)
vi. Glycopeptide antibiotics
vii. Oxazolidinone antibiotics (Van Hoek *et al.*, 2011) (Frank and Tacconelli, 2009).

I. β-lactams:

β-lactam antibiotics are one of the oldest and most used antibiotic class from the previous eras (Page, 2012)(Figure 2). They are known for their bactericidal mode of action, as they interact with the synthesis of the bacterial cell-wall (Donowitz and Mandell, 1988). This class of antibiotics interrupts with the trans-peptidation process of the bacteria which ties each peptidoglycan component of bacteria and causes disruption of the cell-wall (Morin and Gorman, 2014). This class includes Penicillin, Cephalosporin, Carbapenems and Monobactams group: that are based on their functional group in the β-lactam ring (Page, 2012) (Figure 3).

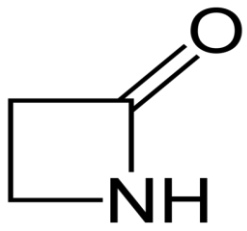

Figure 2: Chemical structure of β-lactam antibiotic: β-lactam ring in the center with nitrogen attached to the carbonyl ring.

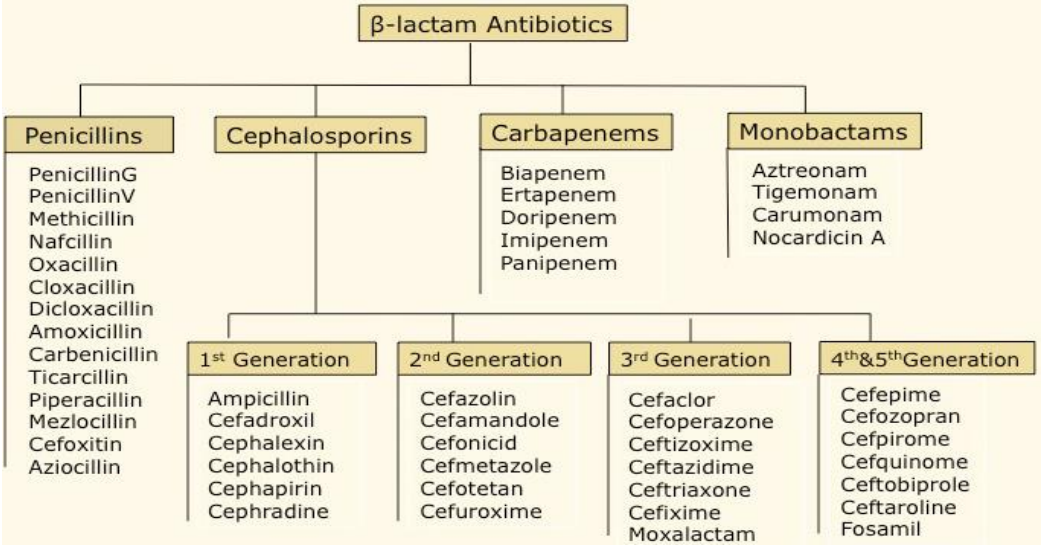

Figure 3: list of different β-lactams antibiotic classification with generations that include Penicillin, Cephalosporin, Carbapenems and Monobactams β-lactam antibiotics.

II. Macrolide:

Macrolides are clinically useful antibiotics; they are all attached to the bacterial large ribosomes subunit i.e. 50s, nearby the peptidyl transferase center to inhibit the growth (Porse and Garrett, 1999).(Figure 4) This center is made up of RNA and catalyzes formation of peptide bonds during protein elongation (Nissen *et al.*, 2000).

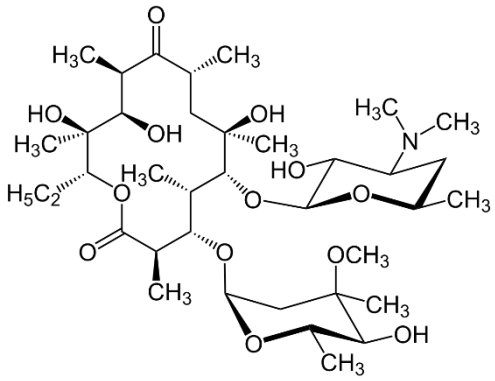

Figure 4: Chemical structure of Macrolides antibiotic: a macrocyclic lactam ring in the center of structurpe.

III. Tetracycline:

Tetracycline penetrates into the bacterial cells by a route of passive diffusion. They have the capability to hinder the bacterial protein synthesis process and demolish the bacterial cell-membrane (Schnappinger and Hillen, 1996). They have access to the cell via passive-flow through hydrophilic pores in the outer membrane of the cell wall, which then pass across the interior cytoplasmic membrane by an energy driven active transport force (Roberts, 1996). (Figure 5).

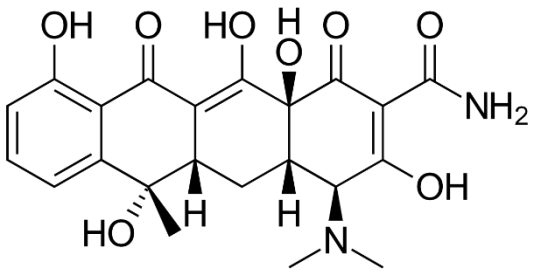

Figure 5: Chemical structure of Tetracycline antibiotics: Chlortetracycline, doxycycline, minocycline, oxytetracycline, and tetracycline belong to this group.

IV. Fluoroquinolone:

The nalidixic acid and oxolinic acid are the first generation of fluoroquinolones, as they target bacterial DNA gyrase (topoisomerase II) and DNA topoisomerase IV (Cozzarelli, 1980). Their mode of action is based on the enzyme which kills the bacteria. Regardless, the exact mechanism of killing is unknown; however, it may involve cleavage of bacterial chromosomal DNA by DNA gyrase (Aldred *et al.*, 2014) (Figure 6).

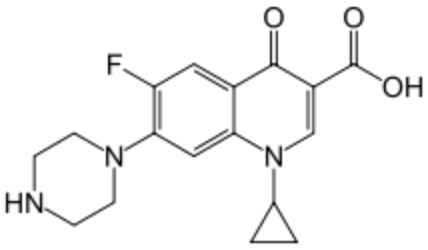

Figure 6: Chemical framework of quinolones structure: a bicyclic core structure linked to the 4-quinolone compound.

V. Aminoglycoside:

Aminoglycosides are multifunctional hydrophilic sugars which possess various amino and hydroxy functionalities (Figrure 7). The amine moieties are largely protonated in biological media; thus, these antibiotics can be considered poly-cationic species to assist in understanding their biological interactions. As they are poly-cationic, they display a binding affinity for nucleic acids. Aminoglycosides in particular, possess high affinities for certain portions of RNAs, especially the prokaryotic rRNA (Fourmy *et al.*, 1998).

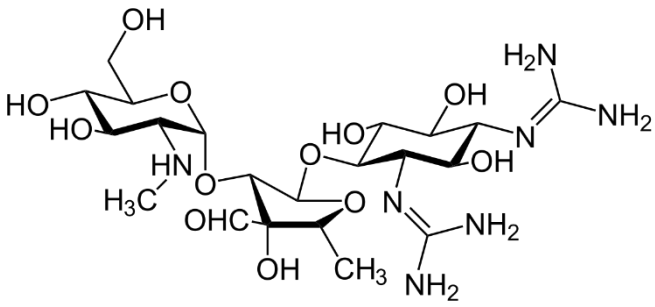

Figure 7: Chemical Structure of aminoglycoside antibiotic: contain more than two amino sugars connected with glyosidic linkage to an hexose nucleus.

VI. Glycopeptides:

Glycopeptide class of antibiotics consist of glycosylated and poly-cyclic peptides that interfere in the bacterial cell-wall synthesis (Figure 8). Their mode of action involves the late stage of assembling in the bacterial cell-wall, above all, they bind to the amino acids i.e. Acyl-D-alanyl-D-alanine in peptidoglycan (Reynolds, 1989).

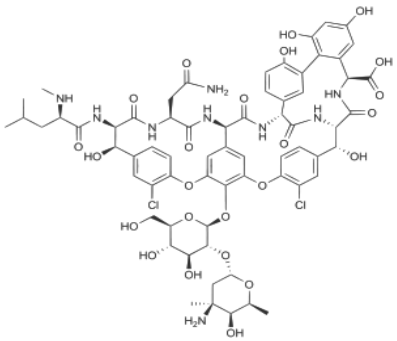

Figure 8: Chemical structure of Glycopeptide antibiotic: made up of glycosylated cyclic or poly-cyclic peptides.

VII. Oxazolidinone:

Oxazolidinone antibiotic represents a new class of synthetic antibacterial agents that are active against multi-resistant Gram-positive pathogens (Shinabarger, 1999) (Figure 9). Oxazolidinones are protein synthesis inhibitors which target an early step involving the binding of N-formylmethionyl-tRNA to the bacterial ribosomes (Vardakas *et al.*, 2007).

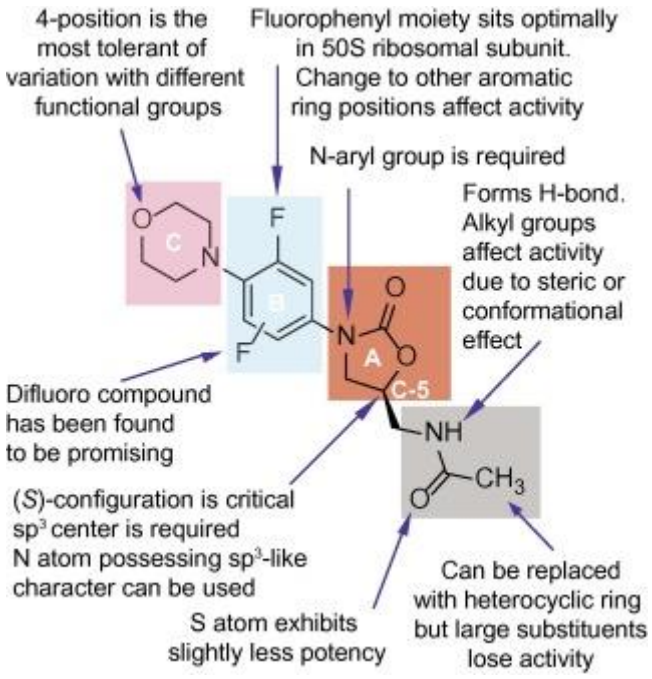

Figure 9: Chemical structure of Oxazolidinone antibiotic: highlighting the important parts of the structure.

3.1.3 Antibiotic Resistance:

The Discovery of the magic bullet "Antibiotics" was not only to tackle bacteria, however as soon as the new antibiotics were discovered, its resistance also emerged. Initially, it was assumed that the different frequency of mutations in bacteria made them super-bugs to many antimicrobial agents (Sood *et al.*, 2006). Unfortunately, at first, nobody anticipated that microbes would create resistance to antibiotics, but due to the changing environment and bacterial nature, they were acknowledged using a wide variety of mechanisms. Surprisingly, the ability of interchanging genes between bacteria by horizontal gene transfer (HGT) was also discovered. It was later found that resistance was developed even before the first antibiotic, penicillin, was discovered.

β-lactam drugs were first identified in *E.coli,* before the distribution of penicillin for use as therapeutic medicine (Sood *et al.*, 2006). Apart from β-lactam, the aminoglycoside antibiotics was one of the effective antibiotics that encountered resistance to many infections (Ramirez and Tolmasky, 2010). Over time, it became more clear that the increase and miss-use of antibiotics contributed to the rise of antibiotic resistance. (Van Duijkeren *et al.*, 2008).

Bacteria have developed resistance through a number of mechanisms which include:

- Change of permeability in the bacterial cell wall.
- Mutation in the efflux pumps.
- Modification of antibiotic by enzymes.
- Inactivation of the drugs through degradation.
- Acquisition of resistance genes.
- Antibiotic target modifications.
- Over-production of enzyme involved in targeting.

3.2 Prevalence of *Pseudomonas aeruginosa* Infections:

The incidence rate of drug resistant *P. aeruginosa* has been increasing globally and has become a major problem in health care settings (El-Shouny *et al.*, 2018). The majority of multi-resistance *P. aeruginosa* were involved in hospital and community acquired infections, which causes high mortality and high morbidity rates (Palavutitotai *et al.*, 2018). The emergence of Carbapenem resistant *P. aeruginosa* has also increased in recent years, contributing to long hospital stay which further increase costs (Jabalameli *et al.*, 2018). ESBL *P. aeruginosa* was reported over 20 years ago, it included *bla* (i.e. *CTX-M, SHV, TEM, BEL, PER, VEB* and *OXA-10 gene*) and MBL genes *(IMP, VIM and NDM gene*) (Croughs *et al.*, 2018) (Weldhagen *et al.*, 2003) (Dubois *et al.*, 2002) (Potron *et al.*, 2015). Recently, MDR *P. aeruginosa* was also reported in the North West of Pakistan, in immunocompromised patients (Gill *et al.*, 2011).

3.3 Antibiotics prescribed for *Pseudomonas aeruginosa* Infection:

The first human infection cause by *P. aeruginosa* was reported in 1862 (Lyczak *et al.*, 2000). When anti-pseudomonas antibiotics was introduced in health care centers which proved effective for treatment against *P. aeruginosa* infections (Kang *et al.*, 2003). From 1971 to 1975, combination of anti- pseudomonas (i.e. Carbenicillin or other β- lactams) drugs were prescribed for cystic fibrosis patients that were involved in chronic infections with *P. aeruginosa* (Szaff *et al.*, 1983).

In one study from 1997 to 2000, which monitored the consumption of drugs against patients infected with *P. aeruginosa* conferred that the used of imipenem antibiotic was responsible for the carbapenem and β-lactam resistance in *P. aeruginosa* (Lepper *et al.*, 2002). Surveillance report from 2006 to 2008 was carried out to identify the correlation of antibiotics prescription for Gram-negative resistance infections which revealed that prescription of broad-spectrum antimicrobial agents was high. It became one of the factor that caused an increase in antibiotic resistance rates for gram-negative bacteria (Hsu *et al.*, 2010).

According to one comparison study of appropriate versus inappropriate antimicrobial therapy, patients infected with the resistant bacteria revealed that inappropriate therapy was responsible for high mortality rates worldwide (Ibrahim *et al.*, 2000) (Micek *et al.*, 2005). Therefore, the appropriate choice of antibiotic is vital for effective treatment of infections. For this, many scientific societies are working together to guide health-care organizations give the right prescription of antibiotics for the treatment of infectious diseases, societies like the American and British-Thoracic Society, the Canadian Infectious Diseases Society, the Infectious Diseases Society of America (IDSA), the Spanish Society of Pulmonology and Thoracic Surgery (SEPAR) (Mandell *et al.*, 2003). Hence, suitable prescription of antibiotic practices should be followed regularly with accordance to surveillance and research data, not from observation (Singh and Victor, 2000). By optimizing and reducing antibiotic usage, there is a decrease in the emergence of MDR gram-negative pathogens (Lemmen *et al.*, 2000).

One clinical study on Colistin inhalation therapy vs placebo inhalations of isotonic saline showed successful results against cystic fibrosis (CF) patients (Jensen *et al.*, 1987). Recently updated, carbapenem (cell wall synthesis), fluoroquinolone (DNA replication) and aminoglycoside

(protein synthesis) are considered to be the best resorts for treatment of MDR *P. aeruginosa* (Hancock and Speert, 2000).

3.4 Antibiotic Resistant Trend against *Pseudomonas aeruginosa*:

MDR *P. aeruginosa*, when treated with a research based antibiotic (that may be single/combination), proved successful in eradication of bacteria from the root level (Hawkey *et al.*, 2018). According to Mageto, the definition of MDR bacteria is resistance to at least three classes of antibiotic, not more than that (Mageto *et al.*, 2018). There are many reports that show the emergence of resistant *P. aeruginosa* to different antibiotic drugs.

i. Cephalosporin Resistance:

CTX-M enzymes are majorly responsible for the resistance in cephalosporin that vary across regions, and they are mostly transmitted through mobile element i.e. plasmid (Bonnet, 2004). The variation of different Cephalosporin *CTX-M* resistance enzymes has changed over time (Bevan *et al.*, 2017). *CTX-M* enzymes majorly contribute in ESBLs resistance and they are usually sensitive to Cefoxitin, but recently, emergence of cefoxitin resistance was also reported in patients (Livermore *et al.*, 2007).

3.4.1 β-lactams Resistance:

Resistance to β- lactams drug has begun since the old centuries, production of β-lactamase enzymes inhibit the β-lactam antibiotics (Tenover, 2006). Many acquired β-lactamase enzyme had been reported in *P. aeruginosa* (Livermore, 2002). In *P. aeruginosa*, *PER-1* is frequently involved and cause resistance to β-lactam antibiotics (Livermore, 1995). *PER-1* and *OXA*-ESBL, as well as the *OXA-15* mutant of *OXA-2* gene are reported in *P. aeruginosa* from Turkey (Naas and Nordmann, 1999). IMP (i.e. *IMP-2, -3, -4, -5, -6,* and *-8* gene) and VIM (*VIM-1* and *VIM-2* gene) are Metallo β-lactamases gene that was also involved in β-lactam resistance (Livermore and Woodford, 2000).

3.4.2 Carbapenem Resistance:

Globally, about 20% of Carbapenem resistance *P. aeruginosa* was reported worldwide and still resistance is increasing with time (Giske, 2007). There are two Carbapenem antibiotics that are

FDA approved, i.e. Imipenem and Meropenem that are marketed all over the world. Most major mechanism which underlie the resistance is low-regulation of Opr-D porin and high expression of different efflux pumps i.e. Mex-AB-Opr-M (Perez and Bonomo, 2018). The imipenem resistance in *P. aeruginosa* was due to loss of OprD-2 genes which are primarily responsible for up-take carbapenem antibiotic (Zhishan *et al.*, 1994). Apart from low Opr-D regulation and high expression of efflux pumps, another mechanism i.e. mutation in penicillin-binding protein (PBP) has also reported for carbapenem resistance (Pinho *et al.*, 2001).

3.4.3 Fluoroquinolone Resistance:

DNA gyrase (*gyr-A* and *gyr-B*), Topoisomerases II (*par-E*) and IV (*par-C*), mutations in Efflux systems i.e. *mex-R* and *nfx-B* are responsible for the resistance in fluoroquinolone (Livermore, 2002). Over expression of efflux pumps can also result in MDR *P. aeruginosa* (Kriengkauykiat *et al.*, 2005). Resistance to fluoroquinolone is about 20-23% globally, and is increasing day by day (Lister *et al.*, 2009). Point mutations that are present in *gyr-A, gyr-B, par-C* or *par-E* genes also reported resistance to quinolone drug (Poole *et al.*, 1996). It was also recently reported that resistance to all fluoroquinolones i.e. orbifloxacin, difloxacin, enrofloxacin, marbofloxacin, gatifloxacin, levofloxacin, and ciprofloxacin was observed (Rubin *et al.*, 2008).

3.4.4 Aminoglycosides Resistance:

Usually, these class of antibiotics block protein making by attachment to bacterial 30S ribosomal subunit (Fourmy *et al.*, 1996). Resistance to aminoglycosides has been reported in *P. aeruginosa* due to up-regulation of bacterial outer membrane protein i.e. opr-H, that actually prevents the binding to cell envelope phospholipids from antibiotic (Gilleland *et al.*, 1989). According to a reviewed report, there are three class of enzymes that inactivate drugs that lead to aminoglycosides resistance (Shaw *et al.*, 1993). More importantly, aminoglycoside resistance is adaptive resistance that is reversible after post antibiotic effects, but the mechanism of adaptive resistance is still not fully understood (Karlowsky *et al.*, 1997).

3.5 Combination therapy:

Basically, antibiotic combination therapy is the use of two or more antibiotic classes in combined form to treat severe infections, that may contain different targets inside the bacterial cell and

simultaneously create underlie pressure of collateral sensitivity (Beabout *et al.*, 2017; Gonzales *et al.*, 2015). Combination therapy has revealed a number of treatment advantages. Firstly, it may have increased broader spectrum of antibiotic against infections. Secondly, it may increase the antibiotics potential (synergy) because of using more than one antibiotic as compare to use of a single antibiotic (Giamarellou *et al.*, 1984). Thirdly, it may help in suppression of emergence of resistance to antibiotics (Milatovic and Braveny, 1987). Fourthly, provide serum bactericidal concentration for killing of bacteria (Pizzo, 1995). Fifthly, provide reliable regimens for treatment of poly-microbial infections (Shlaes and Bass, 1983). A study which reviewed prescribed antibiotics data, revealed that the development of antibiotics resistance was due to using different antibiotic therapies (single/combination). The resistance rates was 9.2 % for broad spectrum penicillins, 8.6 % for 2nd and 3rd generation cephalosporin, 4.7 % for carbapenem, 11.8 % for ciprofloxacin and 13.4% for aminoglycosides (Milatovic and Braveny, 1987).

540 patients were compared with the efficacy and safety of ciprofloxacin alone with ciprofloxacin combinations therapy that found to be effective for treatment of a broad range of severe infections (Krumpe *et al.*, 1999).

However, the disadvantages of combination therapy include: increased drug cost, risk of drug related toxic effects, antagonism effects between drug in combination, and the possibility to cause super-infections (Shlaes and Bass, 1983). One prospective study revealed that monotherapy had a high cure rate as compared to combination therapy for treatment of trauma nosocomial patients (Croce *et al.*, 1993).

3.6 Successful combination therapies against other infection:

Use of improper antibiotic monotherapy causes emergence of resistance which ultimately offers consideration for combination therapy in the treatment of many infectious diseases (Safdar *et al.*, 2004). Combination therapy should be recommended for the treatment of serious Gram-negative infections caused by *Enterobacter cloacae, Pseudomonas aeruginosa* and *Serratia marcescens,* and certain Gram-positive infections caused by Enterococcus spp. and Staphylococcus spp. (Shlaes and Bass, 1983). β-lactam with β-lactamase inhibitor combinations i.e.

ticarcillin/clavulanic acid, ampicillin/ sulbactam and piperacillin/tazobactam was tested with the mice model which was infected with β-lactamase-positive *E. coli, K. pneumoniae, Proteus mirabilis*, and *Staphylococcus aureus*. It proved an increased spectrum and potential efficacy in clinical practices (Kuck *et al.*, 1989). Recently, Methicillin-resistant *Staphylococcus aureus* (MRSA) isolate was resistant to all tested β-lactam antibiotics, but triple antibiotics combination of meropenem, piperacillin and tazobactam showed bactericidal against resistant the MRSA isolate (Gonzales *et al.*, 2015). Ciprofloxacin with rifampin, and vancomycin, were compared in the rabbit model of *Staphylococcus aureus* endocarditis which demonstrates the addition of rifampicin to ciprofloxacin that may decrease the frequency of Ciprofloxacin resistance in *S. aureus* (Kaatz *et al.*, 1989). *Streptococcus pneumonia* infection indicated improved survival rates by combination therapy vs monotherapy among critically ill patients with severe pneumococcal illness (Baddour *et al.*, 2004). Penicillin with gentamicin therapy produced bactericidal effect to all strains of enterococci (Moellering Jr *et al.*, 1971). Whilst, aminoglycoside with 3rd generation cephalosporin has been proposed for the treatment of *Enterobacter aerogenes* and *E. cloacae* infections (Ehrhardt and Sanders, 1993).

Table 1: Guidelines for empirical treatment of infection in adult patients.

Site	Combination regimen
Abdominal infections	Ampicillin + clindamycin or metronidazole + gentamicin
Nosocomial pneumonia	Antipseudomonal penicillin + aminoglycoside
Febrile neutropenia	Antipseudomonal penicillin + aminoglycoside ± vancomycin
Endocarditis	Vancomycin + gentamicin
Meningitis	Third generation cephalosporin + ampicillin or vancomycin
Gynaecological infections	Ampicillin + clindamycin + gentamicin
Systemic inflammatory response syndrome	Ampicillin + clindamycin + aminoglycoside

3.7 Successful combination therapies against *P. aeruginosa*:

Traditionally**,** more than one antibiotic was used through observation (not researched based) to treat infections with *P. aeruginosa.* However, it is now important to identify effective antibiotic combinations that are based on research data to not only eradicate infections from root level but also to diminish emergence of resistance and provide a system level understanding of the infectious process(Walsh, 2000) (Krumpe *et al.*, 1999).

P. aeruginosa causes nosocomial and life threatening infections, especially in critically ill and immunocompromised patients. Several studies suggested that appropriate combination therapy leads to lower mortality rates in *P. aeruginosa* bacteremia (Kumar *et al.*, 2010). One study concluded that combination therapy was substantially superior ($p<0{\cdot}02$) as compare to aminoglycosides alone (Leibovici *et al.*, 1997).

Classically, *P. aeruginosa* infections treated by aminoglycoside with an antipseudomonal β-lactam combination was recommended (Strateva and Yordanov, 2009). Notably, antipseudomonal antibiotics include β-lactams, aminoglycosides and fluoroquinolones (Carmeli *et al.*, 1999). Ciprofloxacin and azlocillin therapy tested in vitro model of *P. aeruginosa* for adjusted dose regimen by using in-vitro kinetic model of infection (Dudley *et al.*, 1991). Urinary tract infections by *P. aeruginosa* should be treated with β-lactam (antipseudomonal) with an aminoglycoside combination which has a high success rate (Rybak *et al.*, 1986). Ceftazidime plus ciprofloxacin reported a bactericidal effect for the treatment of Pseudomonas infected orthopedic prostheses (Brouqui *et al.*, 1995). Recently, a study proposed that the potential role of ceftazidime plus avibactam, with inhaled amikacin combination therapy, involved successful suppression of ceftazidime resistance in *P. aeruginosa* that harbored bla $_{kpc3}$ gene which was observed during ceftazidime monotherapy (Abuhussain *et al.*, 2018).

Chapter 4: MATERIAL AND METHODS

4.1 Sample collection and processing:

50 samples were obtained from the clinical patients of Pakistan Institute of Medical Sciences (PIMS) hospital in Islamabad, Pakistan. Samples were in the form of antibiotic susceptibility plates exhibits contamination. Isolates were purified by inoculating in Nutrient Broth (NB) for enrichment at 37 °C for 24 hours and then were cultured in Pseudomonas Citrate Agar media (PCA) at 37 °C for 24 hours where PCA is a selective media for *P. aeruginosa*. Further confirmation was done by biochemical testing.

4.2 Antimicrobial Susceptibility Assay:

After characterization of *P. aeruginosa*, antibiotic susceptibility profile was identified by using antibiotic discs (OXIDE, UK) according to the Clinical Laboratory Standard Institute (CLSI [2018]) guidelines to obtain an anti-biogram of the highly resistance Isolates.

4.2.1 Disk Diffusion Assay:

Antibiotic susceptibility pattern was determined by the Kirby Bauer (KB) method, which was adapted from a protocol published by (Hudzicki, 2009) (Table 2).

Table 2: Following antibiotic disks were used for *P. aeruginosa* isolates according to CLSI[2018] standards.

S.no.	***Code***	***Antibiotic Disks***	***CLSI [2018]***		
	Penicillin		**S(mm)**	**I(mm)**	**R(mm)**
1	PRL	Piperacillin	≥21	15-20	≤14
2	TZP	Piperacillin/ tazobactam	≥21	15-20	≤14
	Cephalosporin				
3	FEP	Cefepime	≥18	15-17	≤14
4	CAZ	Ceftazidime	≥18	15-17	≤14

5	SCF	Cefoperazone/ sulbactam 2:1	≥21	16-20	≤15
6	CRO	Ceftriaxone			
		Carbapenem			
7	IPM	Imipenem	≥19	16-18	≤15
8	MEM	Meropenem	≥19	16-18	≤15
		Monobactem			
9	ATM	Aztreonam	≥22	16-21	≤21
		Fluoroquinolones			
10	CIP	Ciprofloxacin	≥21	16-20	≤21
11	LEV	Levofloxacin	≥17	14-16	≤13
12	OFX	Ofloxacin	≥16	13-15	≤12
		Aminoglycosides			
13	CN	Gentamicin	≥15	13-14	≤12
14	TOB	Tobramycin	≥15	13-14	≤12
15	AK	Amikacin	≥17	15-16	≤14
		Lipopepetides			
16	PB	Polymyxin B	-	-	-
17	CT	Colistin	-	-	-

4.2.2 Preparation of Antibiotic Solutions:

10 mg stock solution of each antibiotic was weighed aseptically and dissolved in 1 mL of solvent i.e. Dimethyl Sulfoxide (DMSO) (DIEGEN KOREA) (Table 3). 2-fold dilution was made from 1024 µg/mL to 0.5 µg/mL in Muller Hilton Broth (MHB) (OXIDE UK) media in a 96-well plate. 102.4 µL was picked and added in 897.6 µL MHB to make a final concentration of 1024 µg/mL and was serially diluted to 0.5 µg/mL in 96 micro-titer plate.

Table 3: Following commercial antibiotics were used for individual MIC and Antibiotic Combination assay.

S.no.	*Antibiotics*	*Generations*	*Antibiotic Conc. mg/mL*	*Conc. of stock*	*Solvent*
	Penicillin				
1	Piperacillin-Tazobactam		10mg	10.8 mg/mL	DMSO
2	Amoxicillin-Clavunate-Acid		10mg	16 mg/mL	DMSO
3	Ampicilin		10mg	10 mg/mL	DMSO
	Carbapenem				
4	Imipenem		10mg	21.4 mg/mL	DMSO
5	Meropenem		10mg	10 mg/mL	DMSO
	Cephalosporin				
6	Cephradine	1st	10mg	16.1 mg/mL	DMSO
7	Cefuroxime	2nd	10mg	10 mg/mL	DMSO
8	Ceftazidime	2nd	10mg	13.04 mg/mL	DMSO
9	Cefotaxime	3rd	10mg	10 mg/mL	DMSO
10	Ceftriaxone	3rd	10mg	10 mg/mL	DMSO
11	Cefepime	4th	10mg	18 mg/mLL	DMSO
	Aminoglycosides				
12	Amikacin		10mg	40 µl/mL	DMSO
13	Gentamicin		10mg	250 µl/mL	DMSO
14	Tobramycin		10mg	250 µl/mL	DMSO
	Fluoroquinolones				
15	Ofloxacin	1st	10mg	23.55 mg/mL	DMSO

16	Levofloxacin	2nd	10mg	15.74 mg/mL	DMSO
17	Sparfloxacin	3rd	10mg	14.8 mg/mL	DMSO
18	Moxifloxacin	4th	10mg	16.25 mg/mL	DMSO
19	Fosfomycin		10mg	24.6 mg/mL	DMSO
20	Tigecyclin/Tetracyclin		10mg	10 mg/mL	DMSO

4.3 Minimum Inhibitory Concentration (MIC) using Broth Micro dilution method:

Minimum Inhibitory Concentration (MIC) of antibiotics (Table 3) were identified through broth micro-dilution method in a 96 well micro-titer plate by the published protocol (Wiegand *et al.*, 2008).

4.3.1 Preparation of inoculum:

Pure colony of isolates was obtained from PCA media. 4 or 5 colonies were picked with pipette tips and dropped in 2 mL of MHB in an autoclaved test tube. The culture was grown for 2-3 hours at 225 rpm at 37 °C in a shaking incubator to obtain turbidity equal to that of McFarland 0.5 standard.

Meanwhile, 50 µL sterilized MHB were added in all wells, then the antibiotic stock solution was prepared as shown in Table 3, which was then serially diluted from 1024 µL to 0.5 µL from 1st to 10th well of micro-titer plate. Finally, 50 µL of inoculum was inoculated in all wells except 11th well of plate. The 11th well had a negative control, which contained MHB only. Whereas, the 12th well had positive control, as it contained bacterial culture and MHB. Thereafter, the plates were sealed with a lid, and wet tissue paper was placed on the top of the plate for drying, which was then incubated at 37°C for 18-20 hrs. (Figure 10).

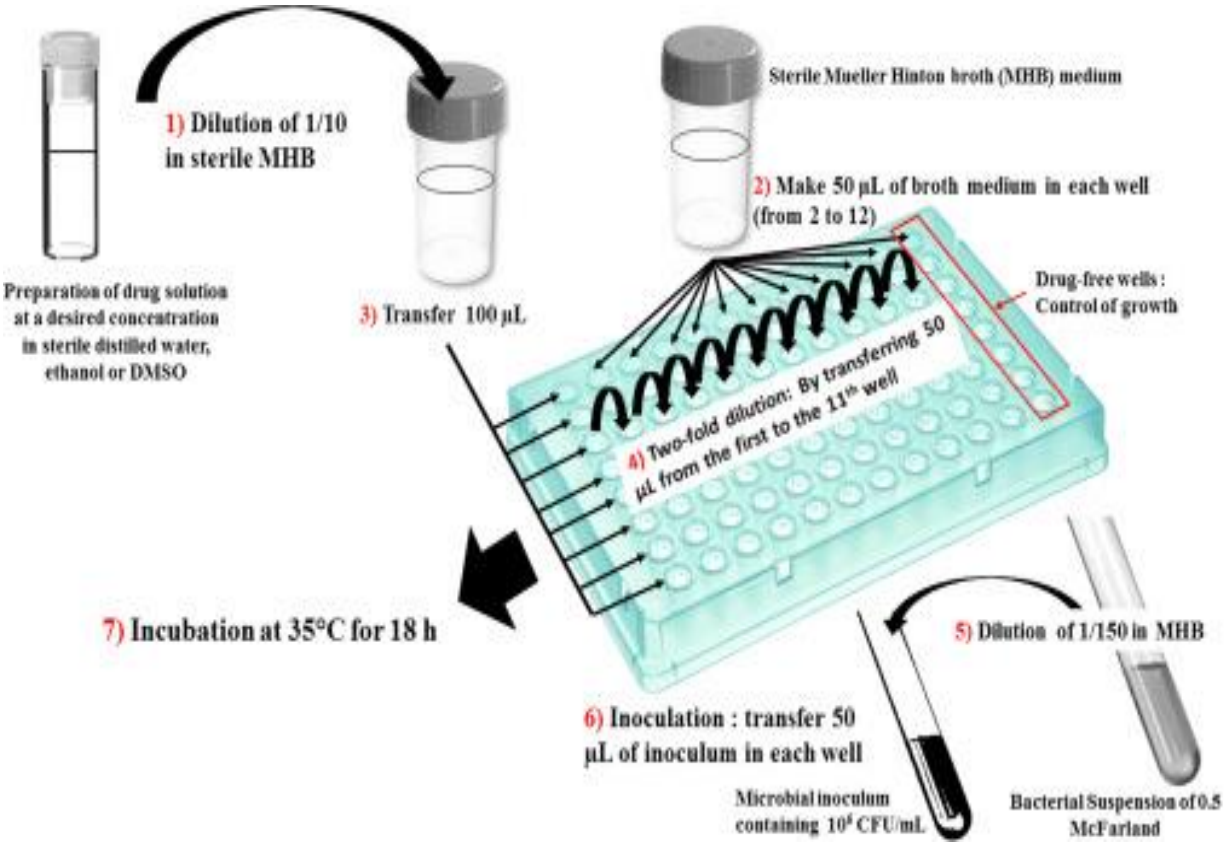

Figure 10: Broth micro-dilution method for antibacterial testing, as recommended by CLSI[2018] protocol.

4.3.2 Interpretation:

Naked eye was used to carefully observe the bacterial growth in each well of micro-titer plate from a downward direction in dark/light background. Additionally, the micro-titer plate reader was also used to determine optical density (O.D) at 620 nm of wavelength. Followed by calculating the O.D, which was used to identify the corresponding MIC values.

4.4 Minimal Inhibitory Concentration (MIC) for Drug synergy and Combination Assay:

The procedure was then repeated for each of the double and triple antibiotic combinations, using the following methods (Table 4).

50 µL of sterilized MHB was added in all wells of micro-titer plate except the first one. 50 µL of each antibiotic stock solution along with 50 µL of stock solution of secondary antibiotic was combined and was added in the 1st well. Then, the combined antibiotic was serially diluted (1st to 10th well) in micro-titer plate. Each well was further inoculated with 50 µL of 0.5 McFarland standard of bacterial cultures. The final volume of each well was 100 µl. Whereby, the 11th well had a negative control, which contained MHB only. Whereas, the 12th well had positive control, as it contained bacterial culture and MHB. The experiment of Combination antibiotics was triplicated to ensure reproducible results.

Table 4: Following double and triple antibiotic combinations were made for the Combination Assay.

S.No.	***DOULBE AND TRIPLE ANTIBIOTIC COMBINATIONS***
	AMOXICILLIN-CLAVULANIC-ACID-CEPHELOSPORIN COMBINATION
1	Amoxicillin-Clavulanic-Acid- Cephradine
2	Amoxicillin- Clavulanic-Acid- Cefuroxime
3	Amoxicillin- Clavulanic-Acid- Ceftazidime
4	Amoxicillin- Clavulanic-Acid- Cefotaxime
5	Amoxicillin- Clavulanic-Acid- Cefriazone
6	Amoxicillin- Clavulanic-Acid- Cefepime
	AMOXICILLIN-CLAVUNATE-ACID-CARBAPENEM COMBINATION
7	Amoxicillin-Clavulanic-Acid- Meropenem
8	Amoxicillin-Clavulanic-Acid- Imipenem
	PIPERACILLIN-TAZOBACTAM-CEPHELOSPORIN COMBINATION
9	Piperacillin-Tazobactam – Cephradine

10	Piperacillin-Tazobactam- Cefuroxime
11	Piperacillin-Tazobactam –Ceftazidime
12	Piperacillin-Tazobactam – Cefotaxime
13	Piperacillin-Tazobactam – Cefriazone
14	Piperacillin-Tazobactam – Cefepime
	PEPERACILLIN-TAZOBACTUM-CARBAPENEM COMBINATION
15	Piperacillin-Tazobactam – Meropenem
16	Piperacillin-Tazobactam – Imipenem
	AMOXICILLIN-CLAVUNATE-ACID-FLUOROQUINOLONES COMBINATION
17	Amoxicillin-Clavulanic-Acid – Ofloxacin
18	Amoxicillin-Clavulanic-Acid - Levofloxacin
19	Amoxicillin-Clavulanic-Acid – Sparfloxacin
20	Amoxicillin-Clavulanic-Acid-Moxifloxacin
	AMOXICILLIN-CLAVUNATE-ACID-AMINOGLYCOSIDES COMBINATION
21	Amoxicillin-Clavulanic-Acid –Amikacin
22	Amoxicillin-Clavulanic-Acid –Gentamycin
23	Amoxicillin-Clavulanic-Acid –Tobramycin
	PEPERACILLIN-TAZOBACTUM-TETRACYLINE COMBINATION
24	Piperacillin-Tazobactam - Tigecycline
	AMOXICILLIN-CLAVUNATE-ACID-TETRACYLINE COMBINATION
25	Amoxicillin-Clavulanic-Acid –Tigecycline
	PEPERACILLIN-TAZOBACTUM-MISCELLANEOUS AGENT COMBINATION
26	Piperacillin-Tazobactam- Fosfomycin
	AMOXICILLIN-CLAVUNATE-ACID-MISCELLANEOUS AGENT COMBINATION
27	Amoxicillin-Clavulanic-Acid – Fosfomycin
	PEPERACILLIN-TAZOBACTUM-FLUOROQUINOLONES COMBINATION

28	Piperacillin-Tazobactam – Ofloxacin
29	Piperacillin-Tazobactam – Levofloxacin
30	Piperacillin-Tazobactam – Sparfloxacin
31	Piperacillin-Tazobactam – Moxifloxacin
	PEPERACILLIN-TAZOBACTUM-AMINOGLYCOSIDES COMBINATION
32	Piperacillin-Tazobactam –Amikacin
33	Piperacillin-Tazobactam – Gentamycin
34	Piperacillin-Tazobactam – Tobramycin
	IMIPENEM-AMINOGYCOSIDES COMBINATION
35	Imipenem – Amikacin
36	Imipenem – Gentamycin
37	Imipenem- Tobramycin
	MEROPENEM-AMINOGLYCOSIDES COMBINATION
38	Meropenem – Amikacin
39	Meropenem –Gentamycin
40	Meropenem –Tobramycin
	OFLOXACIN-CEPHELOSPRIN COMBINATION
41	Ofloxacin – Cephradine
42	Ofloxacin – Cefuroxime
43	Ofloxacin – Ceftazidime
44	Ofloxacin – Cefotaxime
45	Ofloxacin – Cefriazone
46	Ofloxacin –Cefepime
	SPARFLOXACIN-CEPHELOSPRIN COMBINATION
47	Sparfloxacin – Cephradine
48	Sparfloxacin – Cefuroxime
49	Sparfloxacin – Ceftazidime
50	Sparfloxacin – Cefotaxime
51	Sparfloxacin – Cefriazone
52	Sparfloxacin – Cefepime

	LEVOFLOXACIN-CEPHELOSPRIN COMBINATION
53	Levofloxacin – Cephradine
54	Levofloxacin – Cefuroxime
55	Levofloxacin – Ceftazidime
56	Levofloxacin – Cefotaxime
57	Levofloxacin – Cefriazone
58	Levofloxacin –Cefepime
	MEROPENEM-CEPHELOSPRIN COMBINATION
59	Meropenem – Cephradine
60	Meropenem – Cefuroxime
61	Meropenem – Ceftazidime
62	Meropenem – Cefotaxime
63	Meropenem – Cefriazone
64	Meropenem –Cefepime
	IMIPENEM-FLUOROQUINOLONE COMBINATION
65	Imipenem- Ofloxacin
66	Imipenem – Levofloxacin
67	Imipenem – Sparfloxacin
68	Imipenem – Moxifloxacin
	MEROPENEM-FLUOROQUINOLONE COMBINATION
69	Meropenem-Ofloxacin
70	Meropenem –Levofloxacin
71	Meropenem –Sparfloxacin
72	Meropenem – Moxifloxacin
	MOXIFLOXACIN-CEPHALOSPORIN COMBINATION
73	Moxifloxacin- Cephradine
74	Moxifloxacin – Cefuroxime
75	Moxifloxacin – Ceftazidime
76	Moxifloxacin – Cefotaxime
77	Moxifloxacin – Cefriazone

78	Moxifloxacin –Cefepime
	IMIPENEM-CEPHALOSPORIN COMBINATION
79	Imipenem-Cephradine
80	Imipenem-Cefuroxime
81	Imipenem-Ceftazidime
82	Imipenem-Cefotaxime
84	Imipenem-Cefriazone

4.5 Fractional Inhibitory Concentration Index (FICI) analysis:

Fractional Inhibitory Concentration Index (FICI) was piloted to evaluate the synergistic relationship between drugs when they were given in combination. The outcomes of FICI analysis was synergy, indifferent and antagonism.

4.5.1 Fractional Inhibitory Concentration Index:

The results of FICI analysis were interpreted as synergy, partial synergy, additivity, indifferent and antagonism, depending upon the FICI value (Table 5). P

Table 5: Criteria of FICI index.

FICI value	***Interpretation***
Less than 0.5	Synergistic effect
Between 0.5 to 1	Partial synergistic effect
Equal to 1	Additive effect
Between 1 to 4	Indifferent effect
Greater than 4	Antagonism effect

4.5.2 FICI formulae:

The FICI value was determined by combining two antibiotic concentrations against each individual antibiotic concentration. The FICI was scored and calculated using the formula given below (Equation 1).

4.5.2.1 Equation 1: FICI formula for drug synergy

$$\text{FICI} = \frac{(\text{MIC drug A+B})}{\text{MIC agent A}} + \frac{(\text{MIC drug A+ B})}{\text{MIC agent B}}$$

Where, MIC drug A + B was two antibiotics concentration (MIC) which are required in combination and MIC agent A or MIC agent B was MIC value of drug A and drug B only.

4.6 Biofilm assay:

Synergistic combinations were checked with the biofilm activity from the protocol published by Stepanović (Stepanović *et al.*, 2007).

Bacterial culture was prepared in MHB containing antibiotic alone and combinations were grow in 96 well micro-titer plate. The plates were covered with a lid and incubated for 24 hours at 37 ºC. After 24 hour of incubation, loosely attached cells were removed and washed with saline. The plate was then air dried and fixed with methanol. The tightly bound cells were then stained with crystal violet.

To quantify the biofilm, glacial acetic acid was added and 100µL was transferred to another micro-titer plate and O.D was taken at 620 nm in spectrophotometer reader (Figure 11).

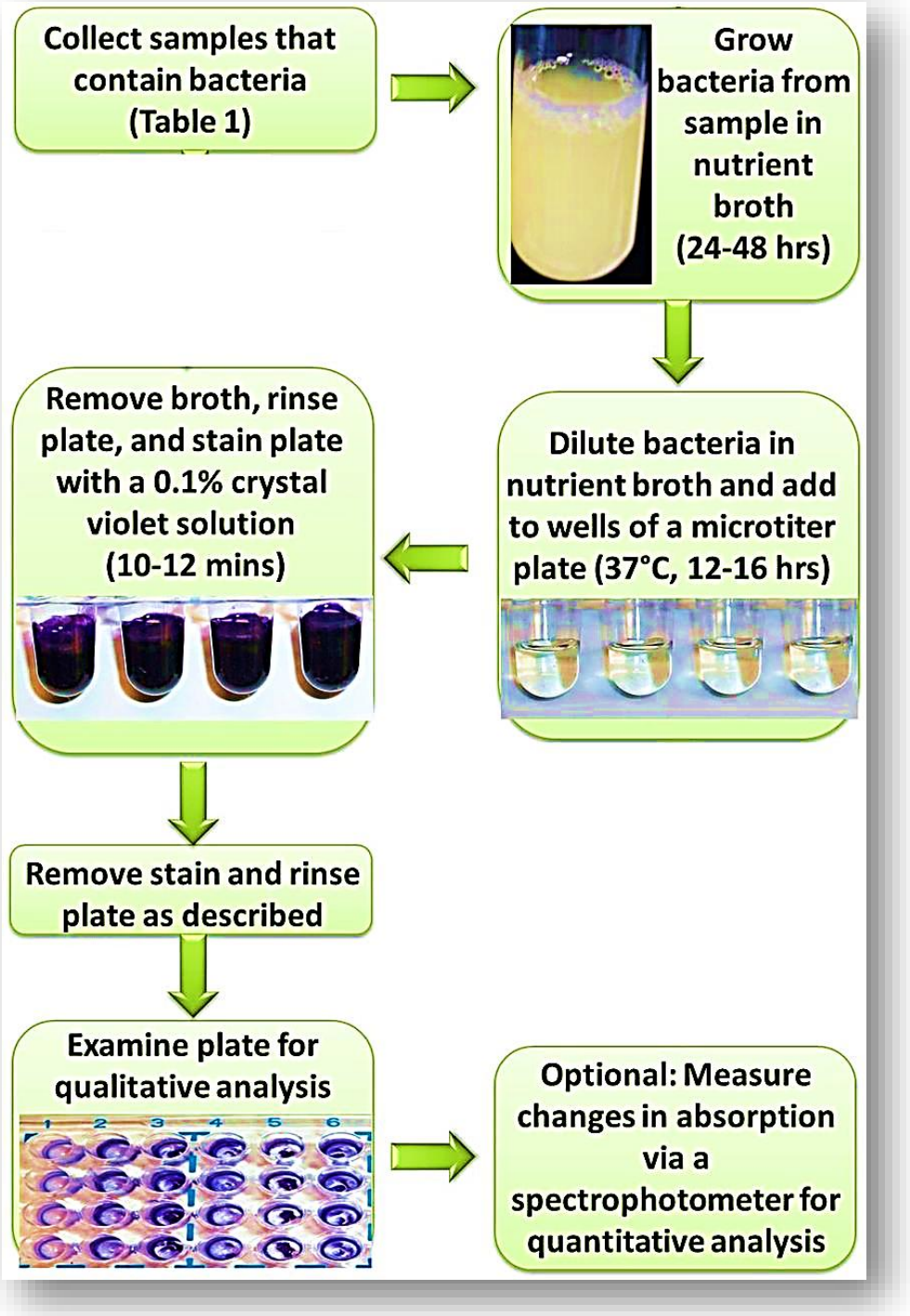

Figure 11: Biofilm Assay for quantification.

4.6.1 Solutions for Micro-Titer Assay:

Crystal violet and sodium chloride solutions were made and used for quantification of biofilm, which is stated below (Table 6 & 7).

Table 6: 0.1% Crystal Violet Solution.

S.No.	*Components*	*Quantity (g/100 mL)*
1	Crystal Violet	0.1
2	Distilled water	100.0

Table 7**:** 0.85% Sodium Chloride.

S.No.	*Components*	*Quantity (g/100 mL)*
1	NaCl	0.85
2	Distilled water	100.0

4.7 Validation of Drug synergy by Time kill assay:

Selected antibiotic combinations that showed synergy were validated by Time Kill (TK) experiment. TK analysis defined “Synergy” that showed a ≥ 2 - $\log^{10}$ reduction in Colony forming Unit (CFU). Kill kinetics were performed by a protocol previously described with some modifications (Ruppen and Sendi, 2015).

We used the following time points to read out results: 0, 2, 4, 6 and 8h. 50 µL sterilized MHB was added from the 1st well to the 5th well of the micro-titer plate. 50 µL selected antibiotic combinations, 50 µL antibiotic A, and 50 µL antibiotic B were added in three rows, from 1st well to 5th well at their respective concentration, and finally 50 µL bacteria was inoculated from 1st well to 5th of all three rows (total volume = 100 µL) of 96 well plate. Positive control was in 12th well that contained 50 µL MHB, 50 µL bacteria and no antibiotics (total volume = 100 µL)

whereas negative control was in 11th well, that contained 100 µL MHB, no bacteria and no antibiotics (total volume = 100 µL) in 96 well plate. After that, every 2 hours, inoculum was taken and diluted from 10^{-5} to 10^{-7} and spread out on Nutrient Agar (NA), which was then incubated at 37º C for 24 h. On the following day, the Colony Forming Unit (CFU) was calculated and scored (Figure 12).

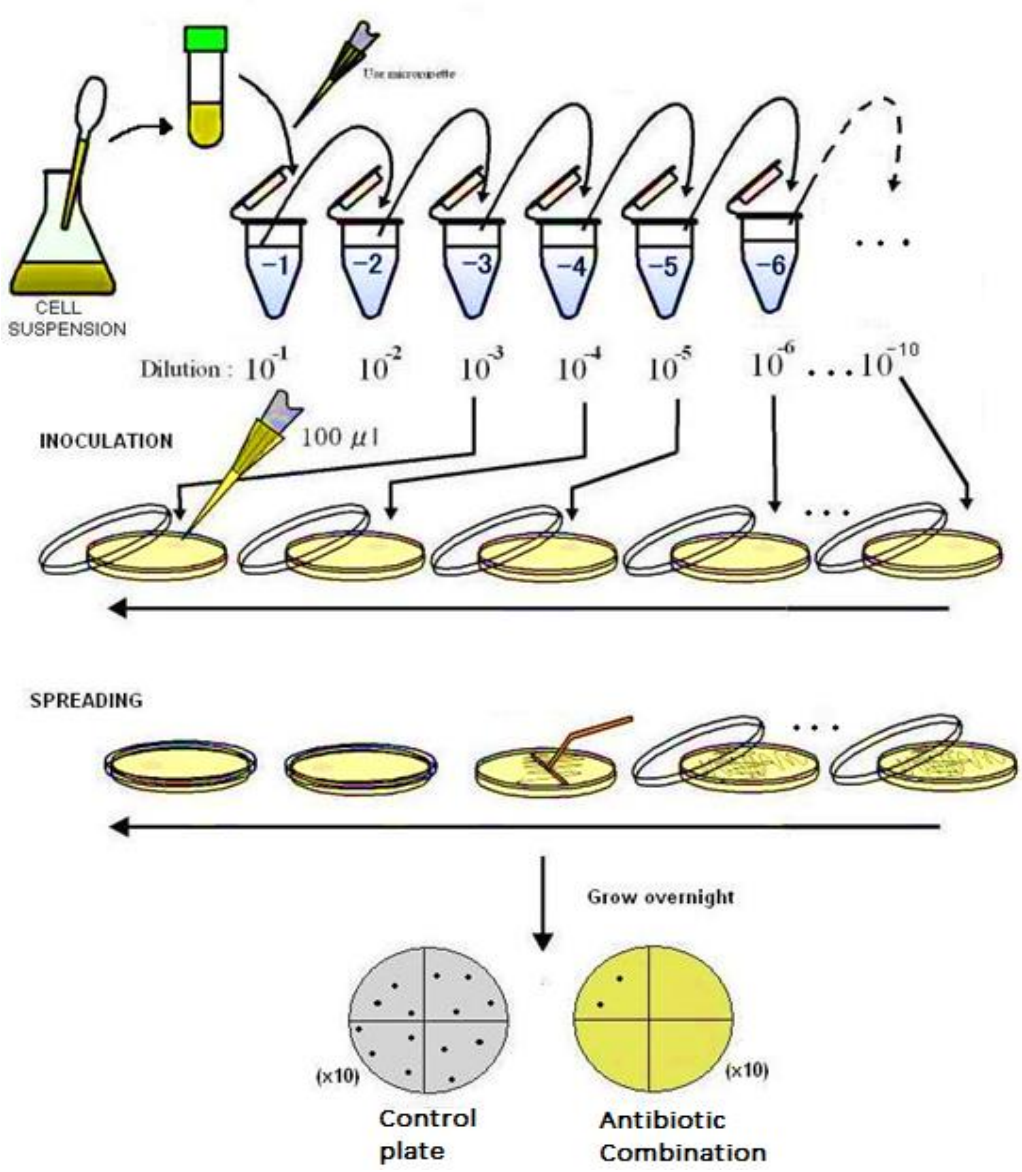

Figure 12: Time Kill assay protocol.

4.8 Validation of Efficacy of Synergistic Combinations by Killing Assay:

Significant combinations were then checked on 20 other clinical *P. aeruginosa* isolates. 100µL of the effective synergistic combinations, and antibiotic alone were added with 100 µL other clinical *P. aeruginosa* isolates in micro-titer plate. Then the plates were covered with a lid, and wet tissue paper was placed on top of the plate and incubated at 37°C for 24 hrs.

All Experiments were performed in triplicates to get reproducible results.

Chapter 5: RESULTS

5.1 Identification of *P. aeruginosa*:

Characterization of *P. aeruginosa* was done by selective media i.e. PCA. After 24 hours of incubation at 37°C, the colonies grew opalescent, with a white precipitate, and revealed a light amber color, as shown in (Figure 13). Further confirmation was done by following biochemical testing (Table 8).

Figure 13**:** Light amber colored colonies with white precipitate in PC Agar media.

Table 8: Biochemical tests of *P. aeruginosa* Isolate 1278.

Test	***Results***
Gram Staining	-Ve
Catalase	+Ve
H2S	-Ve
Oxidase	+Ve

5.2 Antimicrobial Susceptibility Profile:

5.2.1 Kirby Bauer Disk Diffusion Assay:

On the basis of antimicrobial susceptibility patterns, we focused on those strains that were resistance to many antibiotics. We selected *P. aeruginosa* 1278 which was classified as a Multidrug Resistant (MDR) that exhibits resistance to Penicillin, Carbapenem, and Fluoroquinolones, but was sensitive to 4th generation cephalosporin (Cefepime) and aminoglycoside (Gentamicin and Tobramycin). The zone of inhibition diameters is listed in (Table 9).

Table 9: Antibiotics Susceptibility results used for MDR *P. aeruginosa* isolate 1278.

S.No.	*Code*	*Antibiotics*	*Zone size (mm)*	*Results*
		PENECILINS		
1	PRL	Piperacillin	10	R
2	TZP	Piperacillin/ tazobactam	8	R
		CEPHOLOSPORINS		
3	FEP	Cefepime	24	S
4	CAZ	Ceftazidime	12	R
5	SCF	Cefoperazone/ sulbactam 2:1	10	R
6	CRO	Ceftriaxone	12	R
		CARBAPENAM		
7	IPM	Imipenem	10	R
8	MEM	Meropenem	12	R
		MONOBACTEM		
9	ATM	Aztreonam	10	R
		FLUOROQUINOLONES		
10	CIP	Ciprofloxacin	12	R
11	LEV	Levofloxacin	9	R
12	OFX	Ofloxacin	12	R
		AIMINOGLYCOSIDES		

13	CN	Gentamicin	18	S
14	TOB	Tobramycin	21	S
15	AK	Amikacin	12	R
	LEPOPEPTIDES			
16	PB	Polymyxin B	12	S
17	CT	Colistin	10	S

5.3 Minimum Inhibitory Concentration (MIC) using Broth Micro dilution method:

MIC results showed complete resistance to penicillin, carbapenem, cephalosporin and were sensitive to new generations of aminoglycosides/fluoroquinolones against MDR *P. aeruginosa* 1278, as shown in (Table 10).

Table 10: Minimal Inhibitory Concentration (MIC) results for MDR *P. aeruginosa* 1278.

S.No.	***Antibiotics***	***CLSI [2018]***			***MIC Results***	***S/I/R***
		S (µg/mL)	**I (µg/mL)**	**R (µg/mL)**	**µg/mL**	**µg/mL**
	Penicillin					
1	Piperacillin Tazobactam	<16/4	32/4-64/4	>128/4	256	R
2	Augmentin				256	R
3	Ampicilin				256	R
	Carbapenem					
4	Imipenem	<2	4	>8	256	R
5	Meropenem	<2	4	>8	128	R
	Cephalosporin					
6	Cephradine				256	R
7	Cefuroxime				256	R

8	Ceftazidime	<8	16	>32	64	R
9	Cefotaxime				256	R
10	Ceftriaxone				256	R
11	Cefepime	<8	16	>32	16	I
	Aminoglycosides					
12	Amikacin	<16	32	>64	128	R
13	Gentamicin	<4	8	>16	4	S
14	Tobramycin	<4	8	>16	1	S
	Fluoroquinolones					
15	Ofloxacin	<2	4	>8	8	R
16	Levofloxacin	<2	4	>8	8	R
17	Sparfloxacin				1	S
18	Moxifloxacin				4	R
19	Fosfomycin				8	S
20	Tigecyclin/Tetracyclin				64	R

5.4 Antibiotic Combination Assay by FICI method:

A total 84 antibiotic combinations were made. Out of 84, 50 combinations were double, and 34 were triple antibiotic combinations (Table 3).

Among all 84 antibiotic combinations tested, 25 antibiotic combinations showed a synergistic relationship i.e. FICI < 0.5 (Figure 14), 12 showed partial synergism i.e. 0.5 < FICI < 1 (Figure 15), 19 showed additive activity i.e. FICI=1 (Figure 16), 22 were indifferent effect i.e. 1 < FICI < 4 (Figure 17) and 6 showed an antagonistic activity i.e. FICI > 4 (Figure 17).

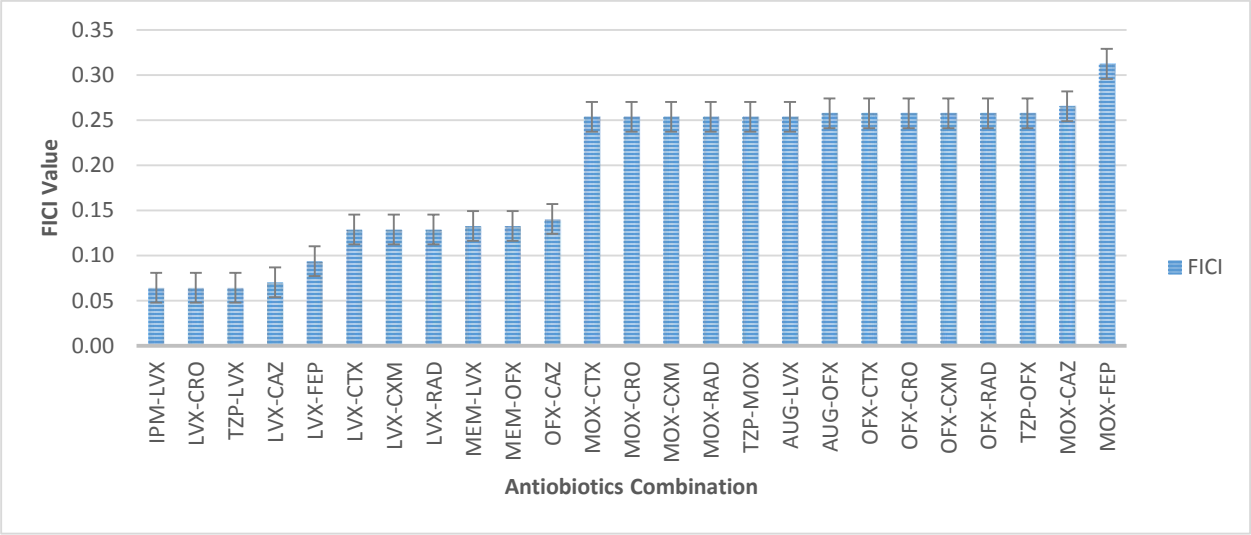

Figure 14**:** 25 antibiotic combinations showed synergy i.e. FICI < 0.5.

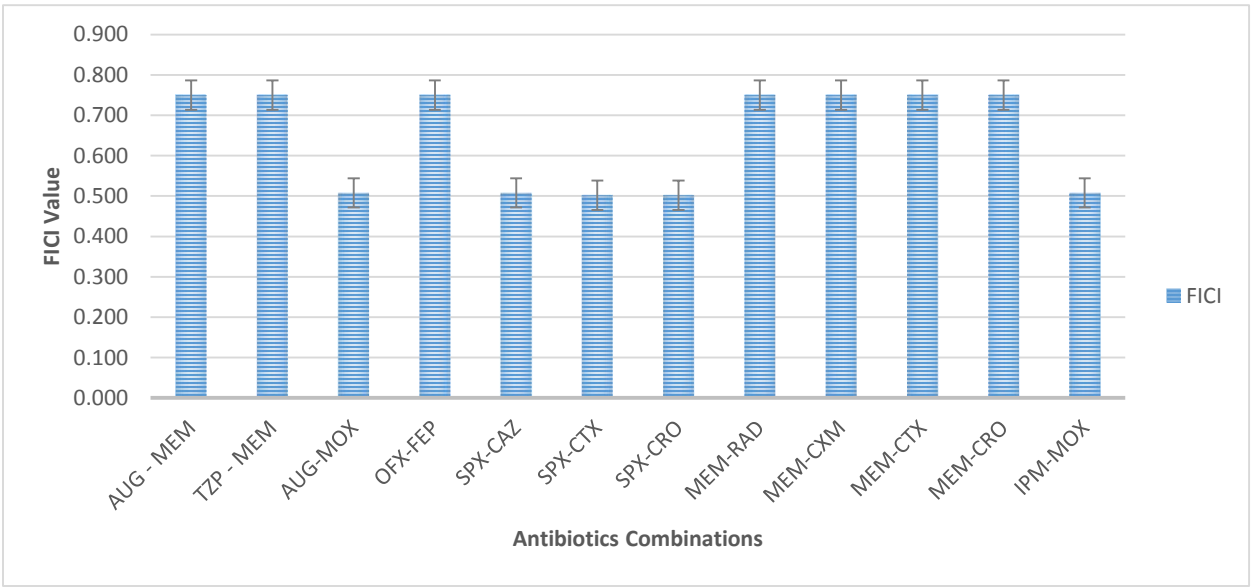

Figure 15**:** 12 combinations showed partial synergy i.e. 0.5 < FICI < 1.

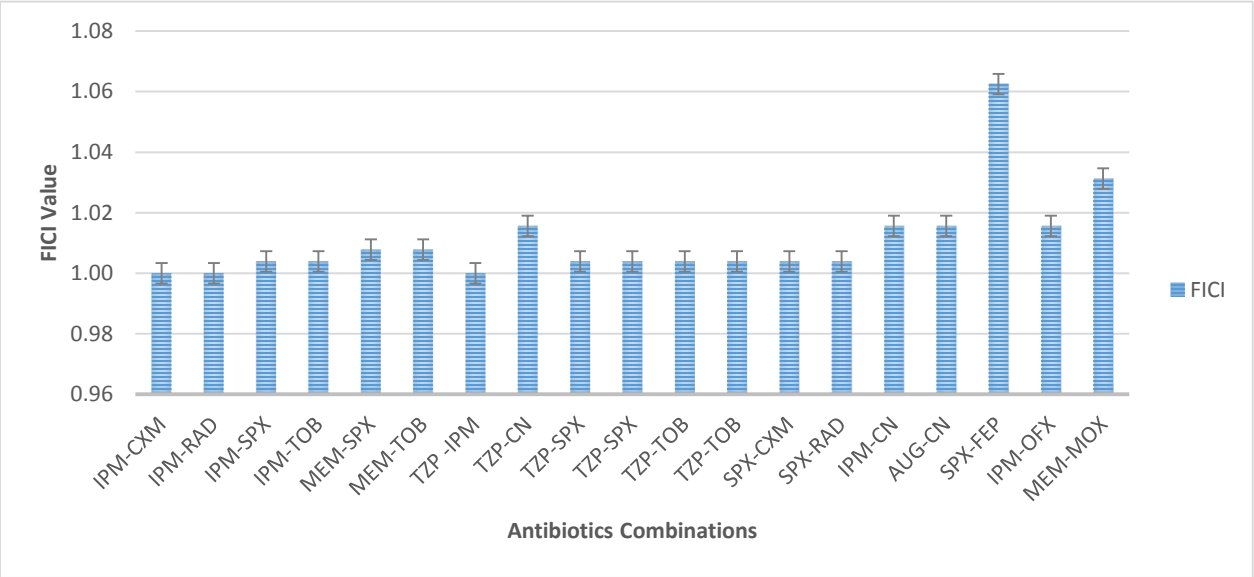

Figure 16**:** 19 combinations showed additive activity i.e. FICI=1.

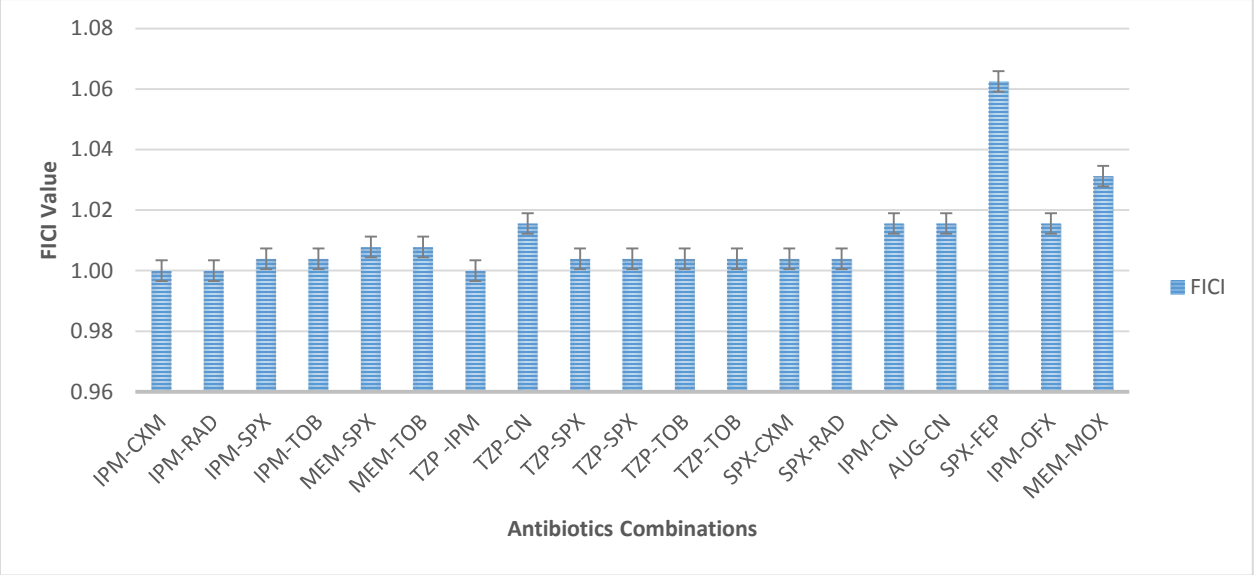

Figure 17**:** 22 were indifferent effect i.e. 1 < FICI < 4.

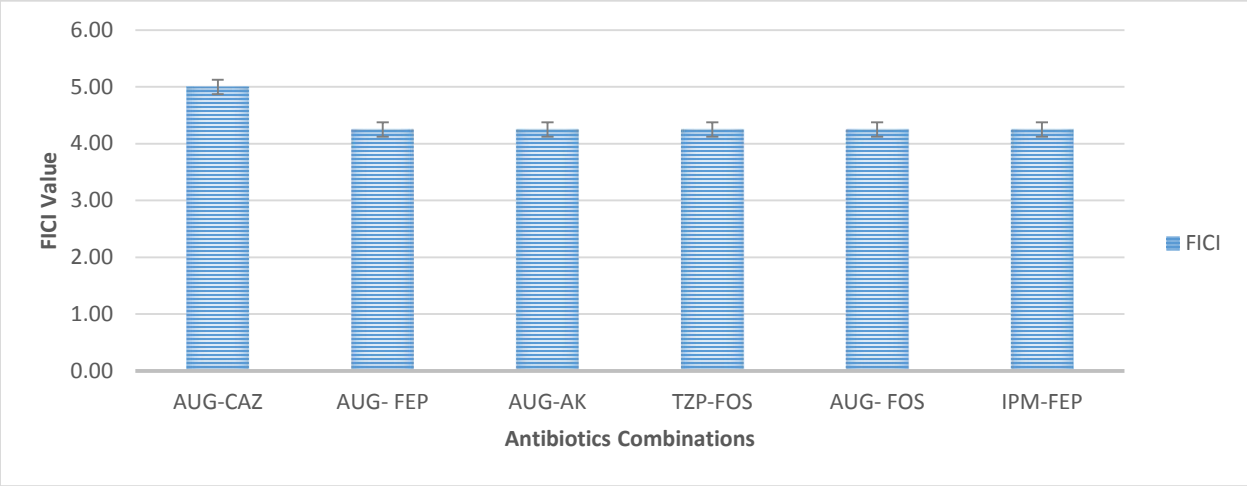

Figure 18**:** 6 showed an antagonistic activity i.e. FICI > 4.

5.5 Validation of Drug Synergy:

Out of 25 synergy combinations, four antibiotic combinations were selected for validation of drug synergy. On the basics of low FICI value, high MIC value individually, and lower generation of antibiotics with simple mechanism of action (Figure 19). Selected antibiotic combinations included two double and two triple combinations, which are given below:

1. Piperacillin-Tazobactam-Levofloxacin.
2. Amoxicillin-Clavulanic acid-Ofloxacin.
3. Imipenem-Levofloxacin.
4. Ceftazidime-Levofloxacin.

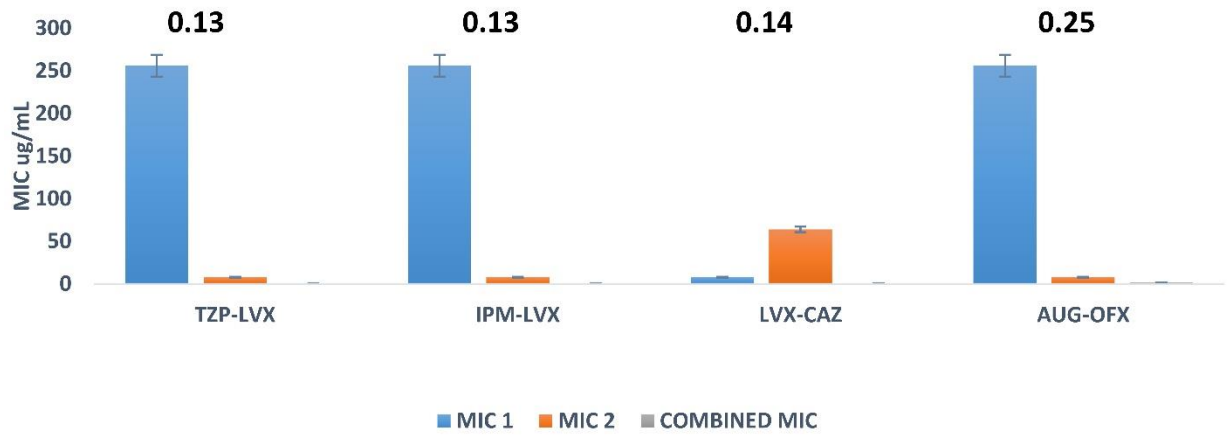

Figure 19**:** Antibiotic combinations selected for validation of drug synergy and upper values indicate the FICI values.

5.6 Biofilm assay:

The micro-titer plate method was used to check bacterial anti-biofilm ability on selected antibiotic combinations. We found that all selected antibiotic combinations showed complete biofilm inhibition in 96 well plates, at their effective concentration, as shown in (Figure 20).

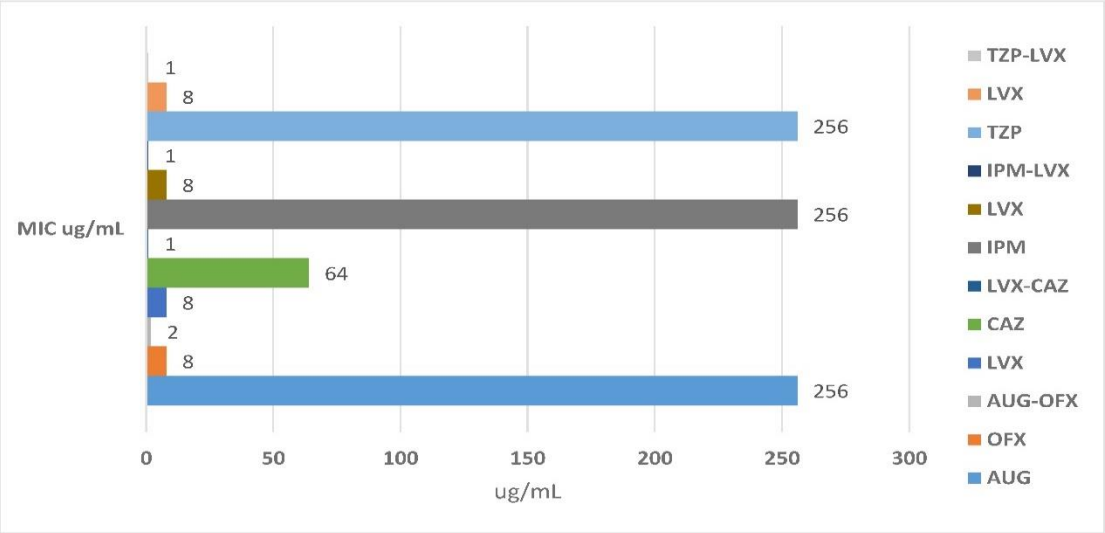

Figure 20**:** Biofilm formation assay of individual and combinations antibiotics.

5.7 Time Kill Assay:

Time kill kinetics determined the bacterial killing rate in respect to time. Synergy was defined, when a ≥ 2-log ^{10} reduction in Colony Forming Unit (CFU) was observed. Results from time kinetics showed TZP-LVX with 1.5-log^{10} CFU reduction, AUG-OFX with 2.1-log^{10} CFU reduction, IMP-LVX with 2-log^{10} CFU reduction, and CAZ-LVX with 1.2-log^{10} CFU reduction in time interval of 0 h, 2 h, 4 h, 6 h, 8 h.

Triple combination of Piperacillin-Tazobactam-Levofloxacin showed no killing in the initial 4 hours, however, killing was observed in 6 to 8 hours (Figure 21). AUG-OFX showed killing in 6 to 8 hours (figure 22). Imipinem-Levofloxacin showed 2-fold reduction from 4 to 8 hours, no killing was observed in initial hours (figure 23). CAZ-LVX with 1.2 log^{10} CFU reduction in 4 to 6 hour. (figure 24).

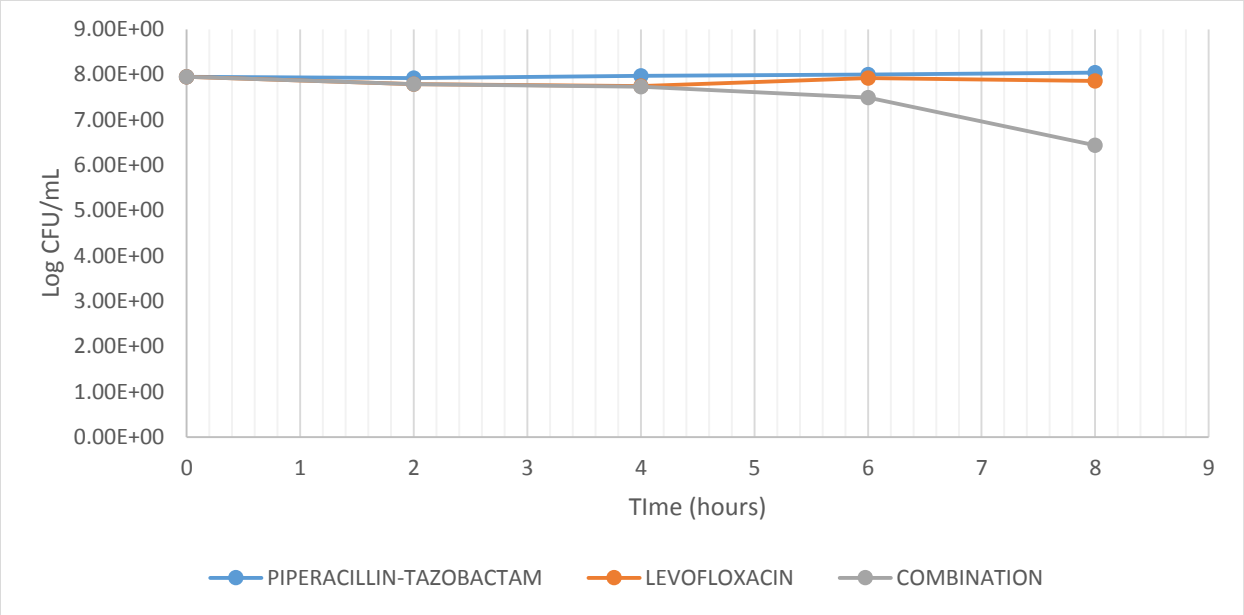

Figure 21: Time Kill Assay of Piperacillin-Tazobactam with Levofloxacin combination showed 1.5-log^{10} reduction in CFU.

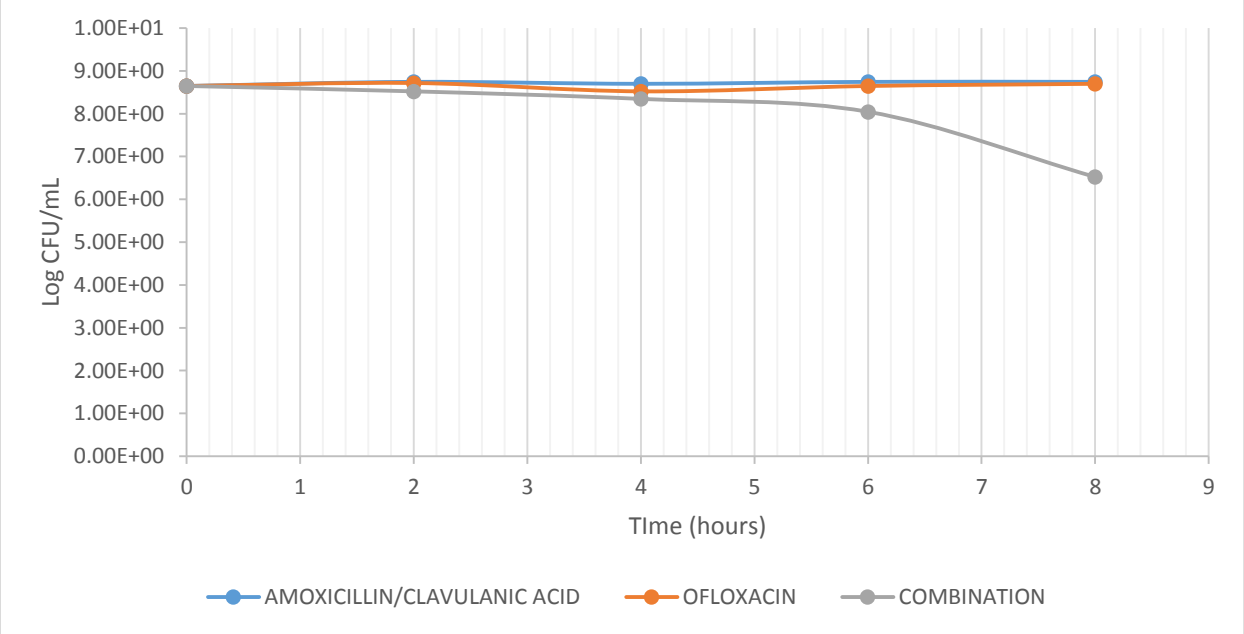

Figure 22: Time Kill Assay of AUG-OFX showed synergy, as 2.1-log^{10} reduction in CFU.

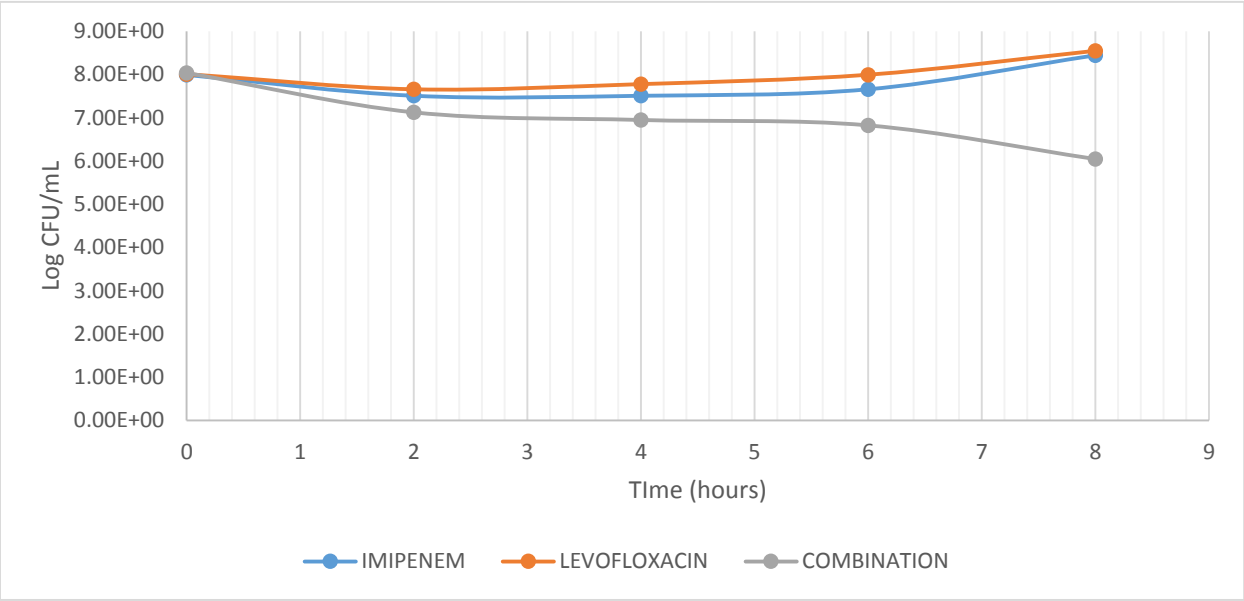

Figure 23: Time Kill Assay of IMP-LVX showed 2-fold reduction from 4 to 8 hours.

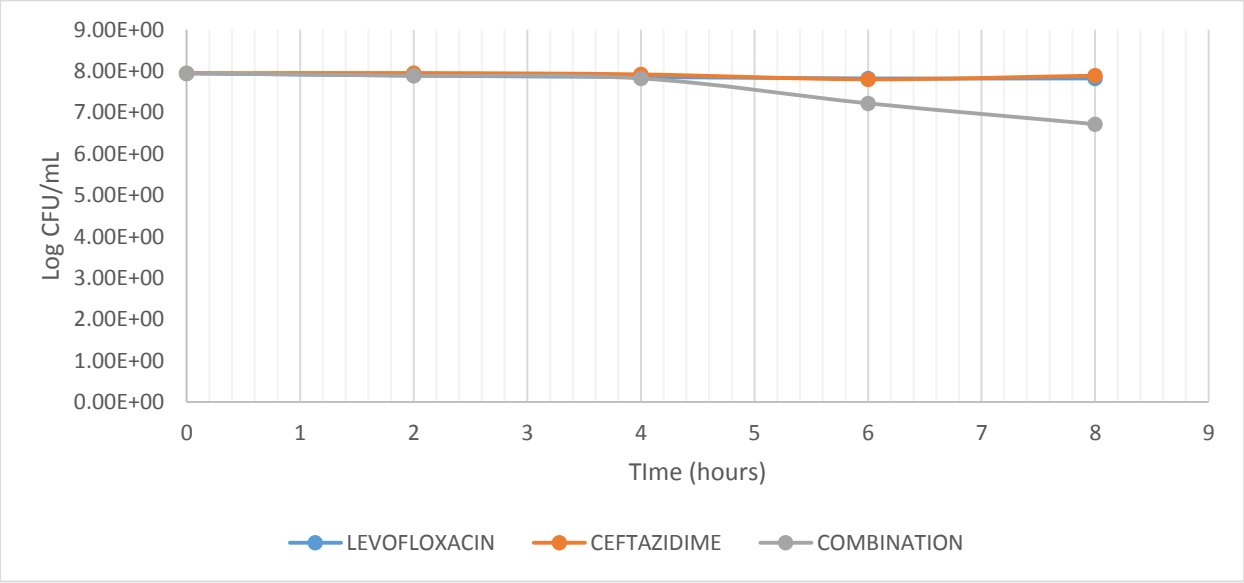

Figure 24: CAZ-LVX showed ~1.2-log10 CFU reduction.

5.8 Killing Assay for validation of Synergistic Efficacy:

The combinations which showed 2-log^{10} CFU reduction in time kill assay and complete biofilm inhibition was AUG-OFX (Figure 25) and IMP-LVX (Figure 26), which were further assayed on the series of clinical MDR *P. aeruginosa* isolates. Results of AUG-OFX showed 80 % of complete killing, and IMP-LVX showed 60 % of killing of other *P. aeruginosa* isolates.

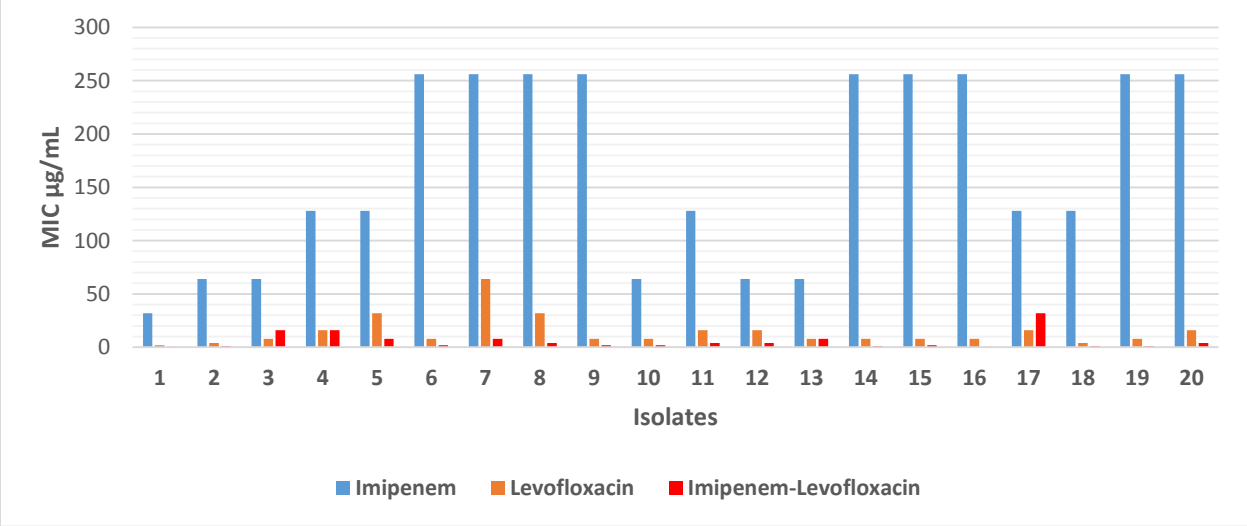

Figure 25: Killing assay of Imipenem-Levofloxacin combination showed synergy in 16 out 20 isolates of *P. aeroginosa.*

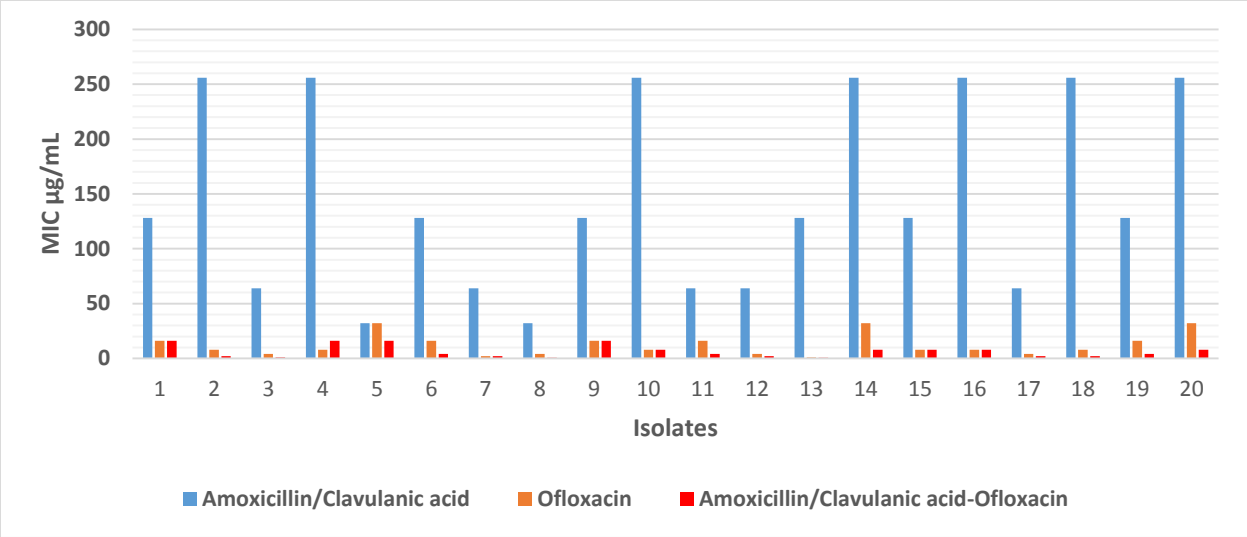

Figure 26: Amoxicillin-Clavulanic acid-Ofloxacin combination showed synergy in 12 out 20 isolates *P. aeroginosa.*

Chapter 6: DISCUSSION

Our study aimed to identify those synergistic combinations (i.e. available in commercial and FDA approved) against prevalent MDR *P. aeruginosa*. Here, we suggested two antibiotic combinations i.e. one double and one triple antibiotic combination that could be used for the treatment of *P. aeruginosa* infection in emergency situations. These combinations were validated by time kill kinetics i.e. reduction of bacterial loads in Colony Forming Unit (CFU) was determined to confirm the killing actions. Additionally, the efficient combinations were then tested for eradication of biofilm formation. Lastly, efficacy of synergistic combinations was checked on randomly selected clinical *P. aeruginosa* isolates.

Pairwise combination of Imipenem-Levofloxacin showed synergistic action of calculated FICI = 0.13 (i.e. synergy defined when FICI < 0.5) and confirmed 2-$\log^{10}$ CFU reduction in killing assay. Moreover, efficacy of combination was validated by showing synergistic killing of 80% on other selected clinical *P. aeruginosa* isolates. (Notably, Imipenem and Levofloxacin showed complete resistance in individual MIC i.e. 256µg/mL and 8µg/mL respectively). In one study, IMP-LVX combination facilitated in preventing emergence of resistance and considered an effective against *P. aeruginosa* infection even when they were resistant to both Imipenem and Levofloxacin (Lister and Wolter, 2005). Thus, this study further establishes strong evidence and proved that the combination of Imipenem with Levofloxacin should be used in clinical practice.

Secondly, triple combination of Amoxicillin-Clavulanic acid-Ofloxacin showed synergism i.e. FICI= 0.2578 and showed 2.1-$\log^{10}$ CFU reduction in 6 to 8 hours in kill assay. Furthermore, AMX-CLA-OFX combination showed bactericidal killing in 60% of other selected clinical *P. aeruginosa* isolates. In one study, Amoxicillin with Ofloxacin proved as an effective combination which help decrease dose level and also prevent resistance of antibiotics. Similarly, this triple combination showed both bacteriostatic, as well as bactericidal effect in comparison to a study reported earlier (Omoya and Ajayi, 2016).

However, Ceftazidime-Levofloxacin and Piperacillin-Tazobactam-Levofloxacin combination results were not promising as they showed no killing in initial 5 hours, but a 1.2-$\log^{10}$ and 1.5-

log^{10} CFU reduction respectively in time kill assays. As synergy is defined when a > 2 log^{10} reduction is observed in CFU during time kill experiment.

Remarkably, among all synergistic results, fluoroquinolones were actively involved in most of the synergistic combinations. One renown mechanism of fluoroquinolones is to inhibit the bacterial growth by associating it with the cell wall (Diggle *et al.*, 2007). But complete synergistic mechanism is not understood well. However, our findings proved that fluoroquinolones have effective combinations and are recommended for treatment of *P. aeruginosa infections* (Pesci *et al.*, 1999).

On the other hand, we noted that most antagonistic results of combinations, Amoxicillin-Clavulanic acid was involved. As previously reported, Amoxicillin and Clavulanic acid showed antagonized activity in *P. aeruginosa*, as compared to piperacillin-tazobactam (Lister, 2000).

Biofilm assay demonstrated that IMP-LVX and AUG-OFX combinations showed complete biofilm inhibition at their effective concentration (i.e. IMP-LVX; 1µg/mL and AUG-OFX; 1µg/mL). As previously acknowledge, biofilm contributed a major role in antibiotic resistance mechanism (Stewart and Costerton, 2001). Biofilm formation involved a decrease permeation and low penetration of antibiotics which ultimately develop persistent infections caused by *P. aeruginosa* (Whiteley *et al.*, 2001) (Mah *et al.*, 2003) (Walters *et al.*, 2003). A study on penetration rates in biofilm showed that Imipenem, Levofloxacin and Oflaxacin has high penetration rates in *P. aeruginosa* (Shigeta *et al.*, 1997). Thus, these combinations further proved the eradication of biofilm formation as they have a general trait of enhanced antimicrobial resistance and persistence of infections (Davies, 2003).

In 1983, triple antibiotic combination of ticarcillin-tobramycin-rifampin demonstrated significantly lower mortality in clinical isolates of *P. aeruginosa* (Zuravleff *et al.*, 1983). Previously, β-lactams with an aminoglycoside combinations were considered effective for preventing sepsis (Paul *et al.*, 2014). But soon this combination showed significant nephrotoxicity risk in immunocompromised patients (Lincopan *et al.*, 2005). One study showed that the combination of tobramycin with ceftazidime may help decrease antibiotic dose below the individual MIC, and resulted in a complete killing of resistant Pseudomonas strains (Den Hollander *et al.*, 1997). Similarly, fosfomycin-tobramycin combination demonstrated synergistic

and bactericidal killing in multidrug resistance (MDR) *P. aeruginosa* (Díez-Aguilar *et al.*, 2015). Recently, the use of Colistin with Tobramycin combinations was considered effective as compared to monotherapy, for the treatment of *P. aeruginosa* infection (Herrmann *et al.*, 2010) (Rahal, 2006). since the last resort of drugs like carbapenem and polymyxins were used for treatment of gram negative infections, it has made us alert, as bacteria is becoming resistant to all drugs (McKenna, 2013) (Michalopoulos *et al.*, 2005).Therefore, the search for more synergistic combinations must be primacies.

However, due to time, all possible synergistic combinations could not be tested with broth micro-dilution assay for the time kill kinetics. Imipenem with levofloxacin was found to be the most effective combination against prevalent MDR *P. aeruginosa* because its showed complete killing of other pseudomonas isolates. We would suggest effective combinations need further in vivo assessment like mice model to provide reliable regimens to combat multidrug resistant infections. It is also necessary to understand the molecular mechanisms of synergistic drug interactions to discover their safe therapeutic prospective and produce more consequential synergistic combinations in clinical practice.

Chapter 7: CONCLUSION

We identified two antibiotic combinations that could be used for treatment of MDR *P. aeruginosa* in emergent situations, i.e. Imipenem-Levofloxacin and Amoxicillin-Clavulanic acid-Ofloxacin. These combinations are the first line of antibiotics which are FDA approved for human use, and have the ability to inhibit biofilm formation, in addition, they have bactericidal effects that extend the lifespan of existing antibiotics. Although, the resistance to antibiotics is inevitable, from evidence based on synergistic studies which suggest the diminishing of resistance. For future prospective, these combinations deserve further in vivo assessment to provide reliable regimens to combat multidrug resistant infections. It is also necessary to understand the molecular mechanisms of synergistic drug interactions to discover their safe therapeutic prospective and produce more consequential synergistic combinations in clinical practice.

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