Investigation of Antibiotic Resistance Genes (ARGs) in Bacteria Isolated from Environmental Samples

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(2016-2019)

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By

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A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Science

in

Environmental Science

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

(2016-2019)

CERTIFICATE

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ACKNOWLEDGMENTS

All praises to Al-mighty ***Allah***, who is the ultimate source of all knowledge and wisdom gifted to mankind.

I would like to acknowledge my supervisor ***Dr. Muhammad Arshad***, for his motivation, support and patience through-out my research work. I could not have successfully completed my work without his guidance.

I would like appreciate my co-supervisor ***Dr. Iftikhar Ahmed,*** from NARC, Islamabad for his immensely helpful suggestions and feedback on my work and my GEC member, ***Dr. Imran Hashmi,*** for his thoughtful suggestions and constructive feedback.

I would also like to thank my friends and lab fellows(fakhria, phyzia, farhat, anum, fariha)and friends (ayesha, fatma) fromoutside the lab for providing a healthy and friendly environment to work in and for making me smile in the more stressful lab work days.

And I wouldn't be here, had it not been for the support and selflessness of my parents and siblings, may Allah bless all of them greatly.

-Saima

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Stands for
ARGs	Antibiotic Resistance Genes
ARB	Antibiotic Resistant Bacteria
ESBL	Extended Spectrum Beta-lactamases
MBL	Metallo Beta-lactamases
AmpC	Ampicillinases
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
PBP	Penicillin Binding Proteins
WHO	World Health Organization
DDD	Daily Defined Dose
HGT	Horizontal Gene Transfer
CLSI	Clinical Laboratory Standard Institute
μg	Micro gram
μL	Micro litre
μM	Micro molar
NCBI	National Centre for Biotechnology Information
MDR	Multi-drug Resistant
XDR	Extensively Drug Resistant
PDR	Pan-drug Resistant
OHE	Office of Health and Economics
EDTA	Ethylene Diamine Tetra Acetic Acid

ABSTRACT

Antibiotic resistance is a global health issue and is more daunting in developing countries. Antibiotic resistance genes (ARGs) found in clinical and environmental bacteria are investigated to understand the resistance mechanisms. There is paucity of data about spread of ARGs in environmental bacteria in Pakistan. Therefore, the aim of the study was to assess previously isolated bacteria from environmental samples for prevalence of ARGs encoding resistance to the most frequently used classes of antibiotics in Pakistan which are penicillins and fluoroquinolones. The study was designed to determine MICs of five antibiotics and to screen and confirm the previously isolated 60 bacterial strains for production of class A (ESBL), B (MBL) and C (AmpC) beta-lactamases. PCR was also optimized for detection of two ARGs, *blaTEM* and *qnrS*. Results showed that MICs were higher for penicillins than for fluoroquinolones (AMP>AMX>CIP>OFX>LEV). Only five strains (8.3 %) were ESBL producers among which four belonged to the genus *Aeromonas* and one to *Escherichia*. Three isolates (5 %) proved to be MBL producers among which two belonged to the genus *Citrobacter* and one to *Stenotrophomonas*. Four isolates (6.6 %) were AmpC producers among which three belonged to the genus *Pseudomonas* and one to *Morganella*. The optimized PCR was able to detect the ARGs blaTEM-1 and qnrS2 in *Aeromonas* spp. and *Escherichia* sp. The obtained sequences shared high homology with the previously identified ARGS. The environmental bacteria in the aquatic environment of Pakistan carry ARGs of clinical relevance which is an indication of anthropogenic antibiotic resistance contamination.

Keywords: Antibiotic resistance, Antibiotic resistance genes, ESBLs, MBLs, AmpCs, Environmental bacterial isolates

CHAPTER 1

INTRODUCTION

1.1 Background

Antibiotic resistance is a well-known global health issue now and it's defined as the ability of a bacteria to fight against the effects of the antibiotic designed to treat an infection. It is considered as one of the three major threats to human health in 21st century and that global effort is required to contain it (WHO, 2000).

Antibiotics were first discovered in 1928 as products of fungi and bacteria. Later antibiotic resistant bacteria started appearing but the problem was dismissed as of little importance but then in 1950s multidrug resistant bacteria (bacteria able to resist more than two classes of antibiotics) started creating problems (Burgland, 2015). Diseases that were once considered completely curable have now made a comeback because of antibiotic (Pruden et al., 2006).

Soon after, ARGs were discovered and it was thought that they emerged to protect target bacteria from antibiotics (Allen et al., 2010). But the recent evidence suggests that they emerged due to the sub-inhibitory concentration of antibiotics in our environment (Sengupta et al., 2013). These sub-inhibitory concentrations are 200 times below the MIC and acceptable by environmental quality standards but they can still cause selection of antibiotic resistance and may even cause development of multidrug resistant opportunistic pathogens (Andersson and Hughes, 2012). Development of resistance against antibiotics has been strongly linked to antibiotic over-use (Burgland, 2015). According to a study by Klein et al., (2018) antibiotic consumption rate in Pakistan has increased by 21% (0.5 billion DDD per thousand persons) from 2000-2015 making it the third largest consumer among the lower middle income countries

with India and China standing first and second respectively. Most of the antibiotics released by both humans and animals (up to 95%) remain in unaltered form and persist and hence wastewater treatment plants are not designed to remove micro-pollutants, only little of it is removed (Pruden et al., 2006).

Major factors playing a role in development and spread of antibiotic resistance are over-use and misuse of antibiotics such as non-compliance of patient to full prescription of antibiotics and prescription of drugs by physicians without first establishing the infection to be bacterial (Wright, 2010; Allen et al., 2010). The problem is further exacerbated in developing countries where self-medication is common (Wellington et al., 2013). A part of the problem is also the large-scale prophylactic use of antibiotics in fishery and farming industries (Phillips et al., 2004). Antibiotics exert pressure for selection of antibiotic resistance on the microflora (Pruden et al., 2006). Animal husbandry, aquaculture facilities, pharmaceutical manufacturing effluents and municipal wastewater systems are considered the hotspots for emergence of antibiotic resistance not just the medical settings. These hotspots are characterized by very high loads of bacterial contaminants and antibiotics that result in emergence of antibiotic resistant bacteria and antibiotic resistance genes that are later released in the environment.

There is increasing evidence that continuous exposure of anthropogenically generated ARGs to environmental bacteria causes development and proliferation of resistance in environmental bacteria (Berendonk et al., 2015). On the other hand, environmental bacteria are considered to have a gene pool that has not yet been explored fully. Many of the genes they harbour may have the ability to be used as ARGs if they find their way into a pathogenic bacteria (Burgland, 2015).

The increased dissemination of antibiotic resistance is considered to be because of three major mechanisms which are; genetic mutation and recombination, horizontal gene transfer and spread of ARB (Antibiotic Resistant Bacteria) owing to the selective pressure of antibiotics and micro-pollutants like biocides and heavy metals. The concentration of chemical contaminants decreases due to degradation, dilution or sorption but ARGs are able to persist in environment and are able to multiply inside a host and can spread among the bacterial population (Berendonk et al., 2015).

When it comes to ARG transfer from environment to a pathogenic bacteria, HGT (horizontal gene transfer) is of high importance. It may occur via; conjugation, transduction and transformation. The phenomenon of conjugation occurs more often and facilitates HGT with transfer of plasmids and integrative conjugative elements (Smilie et al., 2010). It has been observed in soil, marine sediments, wastewater, activated sludge, and seawater (Davison, 1999).

During genetic transfer, the element of importance is integron, capable of catching and expressing gene cassettes which can encode antibiotic resistant genes. Integrons can carry genes like intI 1 which are responsible for enzymes such as integrases, they help to integrate and excise gene cassettes into the integrons and may also shuffle the order of gene cassettes which affects their expression (Cambray et al., 2010; Mazel, 2006).

Several studies have reported increase in resistance to commonly used antibiotics in clinical isolates across Pakistan. There are reports of detection of MDR (Multidrug-resistant), XDR (Extensively drug-resistant) and PDR (Pan-drug resistant) bacteria isolated from various clinical settings in Pakistan (Kaleem et al., 2010; Kumarasamy, 2010; Tanvir et al., 2012; Hasan et al., 2014; Klemn et al., 2018). But there are only a

few reports of detection of antibiotic resistant bacteria and their determinants isolated from environmental samples (Shah et al., 2012; Nasreen et al., 2015).

1.2 Scope of the Study

This study was designed to investigate the presence of antibiotic resistance determinants that commonly occurring bacteria in our aquatic environment may harbour. There is data on detection of ARGs in clinical isolates and settings in Pakistan but there is paucity of data regarding antibiotic resistance and resistance determinants in our environment. There is a need to investigate and explore the occurrence of ARGs in our aquatic environment as water not only provides a way for spread of antibiotic resistance from one compartment to another but could also cause exposure of ARBs and ARGs to humans and animals.

1.3 Objectives

Keeping in view the literature reviewed the study was designed to achieve the following objectives:

1. To determine the minimum inhibitory concentrations (MIC) of five antibiotics in the resistant bacterial isolates.
2. To detect production of the beta-lactamase A, B and C in resistant bacteria.
3. To setup and optimize PCR protocol for detection of antibiotic resistant genes (ARGs).

CHAPTER 2

LITERATURE RVIEW

2.1 Antibiotics History

The discovery of infectious bacteria in the late 19th century compelled scientists for the search of antibiotics. Alexander Flemming was the first one to have identified the naturally produced organic compound as antibiotic which was known as “Penicillin”. This discovery was a great one in the field of medicine and a turning point in human history. In 1937, after introduction of first proper antibiotic “Sulfonamide” resistant strains had started to develop, but it was disregarded as a problem of meagre importance (Abraham and Chain, 1940; Gould, 2016).

The 20 years that followed after discovery of sulphonamides, were considered the golden age in history of antibiotics. By 1958, resistance to antibiotics had become apparent and search was started for solutions. Scientists have been able to develop newer antibiotics that are able to inactivate enzymes like beta-lactamases which hydrolyse beta-lactams. But resistance in clinically relevant strains is spreading and growing faster and is a worldwide problem so much so that it is feared that we might be headed towards the pre-antibiotic time where even a simple infection could become deadly (Gould, 2016).

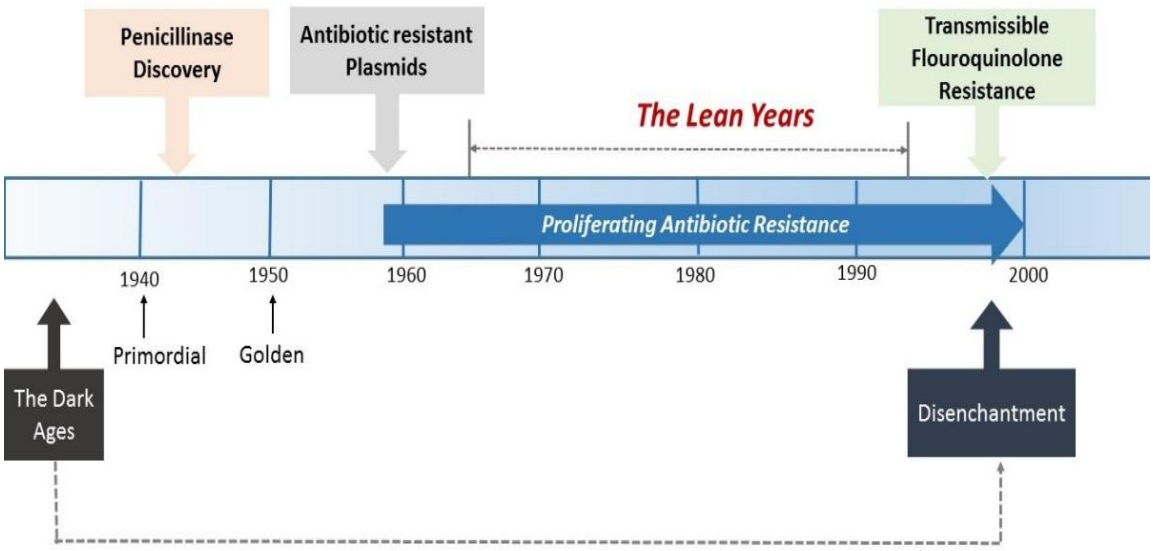


Figure 2.1 Events in the history of antibiotics (Adopted from Davies and Davies, 2010)

2.2 Beta-lactams and Fluoroquinolones

2.2.1 Beta-Lactams

Beta-lactam antibiotics were used in world war-II and proved to be very useful, they are one of the most widely used antibiotics. All beta-lactam antibiotics have a beta-lactam ring structure common in them. Naturally, bacteria have a peptidoglycan layer (cell wall) around them which gives them protection and structure. The cell wall is made with the help of an enzyme called transpeptidase which catalyses the cycle of cross-linking of amino acids for making the protein layer.

These antibiotics work by inactivating this enzyme. The transpeptidase forms a covalent penicilloyl-enzyme complex because of the stereochemical alikeness of the beta-lactam ring to the amino acid, it was supposed to bind to. This is the reason the transpeptidase is called penicillin binding protein (PBP). The enzyme-complex of transpeptidase with antibiotic stops the enzymatic activity and results in a weak cross-linked wall which ultimately causes cell lysis (Wilke et al., 2005).

2.2.2 Cephalosporins

Cephalosporins were produced from a fungi called *Cephalosporium* when it was observed that the fungus produced antibiotics which resembled penicillin. Later, cephalosporin C was produced but never marketed but then 7-aminocephalosporanic acid was derived from it and produced in large amounts. From 7-aminocephalosporanic acid cephalosporins with different properties were produced. Their mode of action is similar to penicillins. But the extra atom in the ring allowed for further semi-synthetic modifications (Katzung, 1995; Walsh, 2003; Greenwood, 2000).

2.2.2.1 Third Generation Cephalosporins, Cephamycins and Carbapenems

The third generation cephalosporins are broad spectrum antibiotics (Walsh, 2003). These compounds persist against many beta-lactamases and have improved activity against many gram-negative bacteria. They are different from earlier generations because they have a capability to reach central nervous system. The examples of these antibiotics are, Cefpodoxime, ceftazidime, cefotaxime, etc (Greenwood, 2000; Katzung, 1995). With each generation of cephalosporins, their range of activity is increased and it is attributed to better permeation of the drugs to the bacterial cell, increased affinity towards PBP and decreased affinity towards hydrolysis by beta-lactamases (Greenwood, 2000).

Cephamycins were first derived from *Streptomyces* spp. in 1972 and were found to be similar to cephalosporins as they shared the same cephem nucleus. Some of the examples are, cefoxitin, letamoxef and flomoxef, etc (Greenwood, 2000; Stapley et al., 1972).

Carbapenems are also beta-lactam ring containing antibiotics and are very effective against beta-lactamases. Due to broader range of activity, they are very effective

against gram negative and positive bacteria. Their examples include meropenem, imipenem, ertepam and others (Greenwood, 200; Walsh, 2003, and Katzung, 1995).

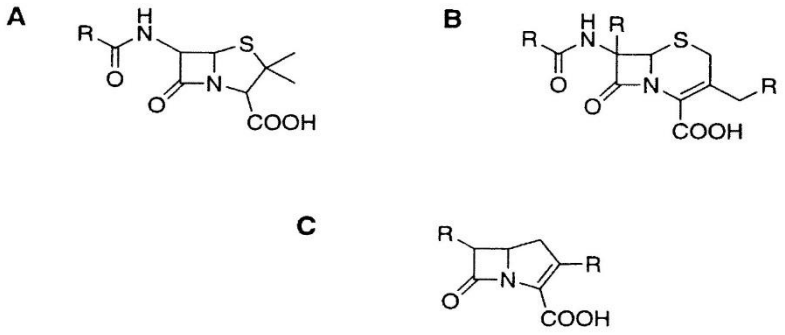


Figure 2.2 Structures of B-lactam antibiotics. A – Penicillin, B – Cephalosporin, C – Carbapenem (Walsh et al., 2003)

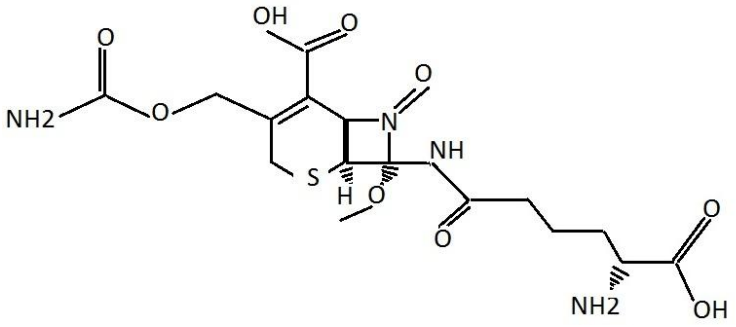


Figure 2.3 Structure of Cephamycin (Adopted from Pubchem)

2.2.3 Fluoroquinolones

Fluoroquinolones are also broad spectrum antibiotics that were developed by modifying the first generation quinolone called nalidixic acid, a narrow spectrum quinolone. Many infections caused in both humans and animals by gram positive or negative bacteria are treated with flouroquinolones. Ciprofloxacin is one of the mostly used fluoroquinolone and was available in market in 1987 (Fabrega et al., 2009). DNA replication in a bacterial cell occurs with the help of enzymes, topoisomerase and DNA gyrase. Negative supercoils are added in the bacterial DNA because of gyrase and it also helps to maintain the coiling density of the DNA during replication. It also relieves the torsional stress of the coiled DNA and catalyses the cleavage reaction by

ATP binding and hydrolyses (Levine et al., 1998). While the main function of topoisomerase IV is to remove the knots and tangles in DNA and decatenate the daughter DNA after replication (Zechiedrich et al., 2000).

The main mode of action of quinolones is to increase DNA-cleavage complexes by more production of topoisomerases. The cleavage complexes of DNA are formed when double stranded breaks are introduced in the DNA strands that are four base pairs apart and so to maintain genomic integrity, topoisomerases bind by the tyrosine residues to the 5' end termini of the cleaved DNA. The integrity of the DNA is compromised as permanent breaks are introduced and relegation is prevented, the cell then is unable to survive and hence dies (Hooper, 1999). The antibiotic also acts by decreasing the amount of DNA-cleavage complexes as it attaches to the DNA and enzyme complex and prevents the enzyme from untangling and decatenating the replicating DNA, hence causing mitotic failure and ultimately cell death (Aldred et al., 2014).

2.3 Antibiotic Resistance

When the supposed antibiotic looses its effect and the bacteria can still propagate in its presence, the bacteria is thought to have gained antibiotic resistance. It arises because of some changes in bacteria that decrease the effectiveness of the drugs. This allows them to multiply and grow continuously and in case of a pathogen the infection to spread (Hassan et al., 2014). A time ago, focus was more on resistant gram positive bacteria but then the scientists revealed that multi-drug resistant gram negative bacteria are of much bigger concern and the fact that resistance is spreading much faster in gram negative bacteria than gram positive just makes the situation even worse (Cornaglia et al., 2009). Multidrug resistant *Salmonella enterica* started making problems in 1950s (Levy and Marshall, 2004). Scientists and health related organizations have

many times stressed over the fact that antibiotic resistance poses threat to global health and that it could have grave consequences (Klein et al., 2018).

2.3.1 Situation in Pakistan

Over the time there have been reports of increasing drug resistance to commonly used antibiotics in bacteria from various clinical settings in different areas of Pakistan (Kaleem et al., 2010). The fact that consumption of antibiotic is unnecessarily high doesn't help the situation either (Afzal, 2017). In fact, it has been reported that from 2000-2015 there has been 65% increase in drug consumption in Pakistan. There is a strong correlation between antibiotic use and resistance emergence, as most of the used antibiotics from different sectors go to the environment and cause selection of resistant bacteria. A few studies have been conducted evaluating resistance of various infection causing strains. There have been reports of high resistance against b-lactams and quinolones in *Acinetobacter* spp. and non-typhoidal *Salmonella* (NTS) in strains isolated from patients in hospitals (Hassan et al., 2014, Walsh et al., 2010). About 77.5% of screened isolates in a study showed resistance to three or more than three antibiotics (Bashir et al., 2011).

A study highlighted that the predominant bacteria which were *Klebsiella* spp. and *E.coli* isolated from water samples taken from five rivers including River Soan, Chinab, Jhelum, Indus and Ravi exhibited resistance to multiple antibiotics with high level of resistance to ampicillin (45%), chloramphenicol (37%) and streptomycin (34%) (Sair and Khan, 2017). Another study was carried out in which bacteria were isolated from different types of fishes common in fish farms in Pakistan and Tanzania to see the pattern of antibiotic resistance. About 10% of the isolates were resistant to all of the nine tested antibiotics (Shah et al., 2012). It has been established that exposure of environmental microflora to antibiotics put selective pressure for better sur-

vival of competitive strains and this exposure is caused by anthropogenic contamination of aquatic environment with undigested antibiotics released by humans and animals likewise and from various other sectors.

2.3.2 Studies in Pakistan

Despite the evidence of increasing antibiotic resistance in the medicine field in Pakistan, little information is available about resistance spread in the aquatic environment. Aquatic environments are important reservoirs of resistance determinants. The following few studies have been reported from Pakistan:

Table 2.1 Studies carried out in Pakistan reporting antibiotic resistance from various aquatic environments (NI-not investigated)

ARBs reported	**Resistance to antibiotics**	**Sample**	**Location**	**ARGs reported**	**Study**
V. cholera, K. pneumoniae, S. typhi, P. aeruguinosa	Ciprofloxacin, tobramycin, amikacin, piperacillin/tazobactam, gentamicin, chloramphenicol, aztreonam	Tap water, hand pumps, tube wells	Peshawar (KPK)	NI	Ahmad et al., 2014
E. coli	Ciprofloxacin, erythromycin, ceftazidime, nalidixic acid, vancomycin, cefaclor,	Drinking water samples from inlet and outlet of reservoir tank, distribution system	Gilgit Baltistan	NI	Ahmed & Shah, 2007
Shigella spp., *Staphylococcus* spp., *Salmonella* spp., *Streptococcus*	Kanamycin, ampicillin	Public drinking water supply	Lahore metropolitan	*Kan, amp*	Samra et al., 2009

spp., *E. coli*					
NI	NI	Soil sediments	Islamabad, Lahore, Multan	*Sul1, dfrA*	Khan et al., 2013
A. baumanii, A. hydrophilla, Alcaligenes sp., *E. cloacae, P. aeruginosa, P. fluorescens, E. casseliflavus*	Amoxicillin, streptomycin, trimethoprim, chloramphenicol, erythromycin, sulphonamide, florfenicol,	Water, sediment from fish ponds	Multan, Lahore	*dfrA, sul1, sul2, blaTEM, strA-B, cat-1*	Shah et al., 2012
E. coli	Meropenem, augmentin, Amoxil, penicillin, lancomycin, ceclor, cephalexin, cephradime, streptomycin	Water from municipal reservoir, distribution line, consumer tap	Khairpur, Sindh	NI	Shar et al., 2009

2.4 Causes of Antibiotic Resistance

2.4.1 Overuse

Studies have claimed that there is a strong relationship between antibiotic consumption and emergence and spread of antibiotic resistance among pathogenic strains (McKenna, 2013). Antibiotics remove the competing strains that are sensitive to them and leave behind the resistant strains (Read and Woods, 2014).

2.4.1.1 Incorrect Prescription

Incorrect prescription of antibiotics boosts emergence of resistance in bacteria. Studies have shown that the antibiotic prescribed and or duration of treatment is wrong 30-50% of the time (CDC, Office of Infectious Disease, 2013). Studies also report that

30-60% drugs prescribed in ICUs (Intensive Care Unit) are not necessary (Luyt et al., 2014).

2.4.1.2 Use in Other Sectors

The use of antibiotic as prophylactic is very common in livestock, poultry and fish farmingindustries for growth promotion. Humans are exposed to these antibiotics when the products from these industries are consumed (Bartlett et al., 2013). It first came in observation that resistant bacteria can transfer from farm animals to humans when higher resistant rates were observed in intestinal bacteria of both farm animals and farmers about 35 years ago. Also when the livestock excretes, 95% of the antibiotic is released in unchanged form and goes in natural environment (Elmund et al., 1971).

2.4.1.3 Newer Antibiotics

Since antibiotics are cheap and are used for short period of time, it's deemed unwise to spend too much money on research and labs to develop new ones. Instead investors focus more on making drugs which treat chronic diseases like diabetes, asthma or psychiatric disorders. According to OHE (Office of Health and Economics) in London, a new antibiotic will have a net worth of 50 million while a drug that treats a neuromuscular disease will have a net worth up to 1 billion. There are reports that 15 of the 18 leading pharmaceutical industries have abandoned antibiotic fields (Barlett et al., 2013). Also microbiologists advise restrain in use of antibiotics and to preserve their use for when it's really needed. Because even if new antibiotics are developed, the bacteria will eventually find a way to resist them (Piddock, 2012). Because of lack of newer effective antibiotics, it's getting harder to control the rising multi-drug resistant strains with the older but less effective antibiotics.

2.5 Resistance Mechanism

2.5.1 Resistance Mechanism against B-lactams

There are three main resistance mechanisms which bacteria have adopted to resist the detrimental effects of b-lactams. The primary resistance mechanism in gram negative bacteria is production of enzymes that hydrolyse beta-lactams. Second one is the alteration of target site of antibiotic. The third one is the prevention of drugs to enter the cell by various mechanisms.

2.5.2 B-lactamases

There are two major schemes of classification of beta-lactamases and are known as Ambler classification based on molecular structure of the enzyme and Bush-Jacoby classification based on function of the enzyme. The one most commonly used is ambler classification.

2.5.2.1 Ambler Molecular Classification

In this scheme of classification, protein homology in particular amino acid similarity is used as the basis for dividing the groups of enzymes (Ambler, 1980). There are four classes in this scheme of classification categorized as A, C, D and B. The classes C, A and D are serine group enzymes as they use serine for hydrolysis of the target antibiotic. Class B beta-lactamases are called metallo beta-lactamases as they use one or two zinc ions for hydrolysis of beta-lactams (Medeiros, 1997; Paterson and Bonomo, 2005).

The largest group of enzymes among these four is class A. The enzymes in this group have highly similar sequence which is considered to have derived from a single ancestral gene. Despite having high similarities, they have different enzymatic properties and substrate profiles. Class A enzymes prefer penicillin for their substrate. These en-

zymes can be periplasmic or cell bound and plasmid or chromosomal gene mediated. They can be produced by gram positive and gram negative bacteria equally (Ambler, 1980; Ambler et al., 1991).

The class B of metallo beta-lactamases are a smaller class of enzymes and require zinc as a metal co-factor. They are inhibited by metal chelators only and not by beta-lactamase inhibitors like clavulanic acid (Bush, 1998; Wang et al., 1999). They can deactivate the entire spectrum of beta-lactams (penicillins, carbapenems and cephalosporins (Leonard et al., 2013)).

Class C beta-lactamases were first discovered in 1981 in *E. coil* K-12 strain. These enzymes are similar to class A enzymes except that their binding site is more open and can accept bulkier molecules of penicillins, oxyaminocephalosporins, cephamycins and monobactams (Chen et al., 2006). Unlike class A enzymes they are not inhibited by clavulanic acid (Thomson, 2010; Philippon et al., 2002). Carbapenem resistance has been related with AmpC production coupled with loss of porin (Stürenburg et al., 2002; Phillipon et al., 2002). They are both chromosome and plasmid mediated (Philippon et al., 2002; Barlow and Hall, 2002).

Class D enzymes were discovered in 1981, they don't share much homology with class A and C enzymes (Massova & Mobashery, 1998). Their subfamily are named using OXA nomenclature because it was observed that they have a strong hydrolytic activity against semi synthetic penicillins such as oxacillin. It was later discovered that they can hydrolyse cephalosporins, beta-lactam/beta-lactamase inhibitors combinations and carbapenems as well (Hujer et al., 2005).

2.5.2.2 Extended Spectrum Beta-lactamases (ESBLs)

They are capable of hydrolysing penicillins, 1st, 2nd, 3rd cephalosporins and aztreonam. They are not able to hydrolyse cephamycins (cefoxitin) and carbapenems (imipenem). They are inhibited by clavulanic acid (Paterson and Bonomo, 2005; Philippon et al., 1989).

2.5.2.3 ESBL Diversity

There are three main types of ESBLs namely TEM, SHV and CTX-M. There are others as well but they are relatively rare.

2.5.2.4 TEM

TEM-1 was first detected in a clinical isolate of Escherichia coli (Paterson and Bonomo, 2005; Heritage et al., 1999). Point mutations in TEM enzymes are clustered in five points in the enzymes and are adjacent to seven evolutionary conserved elements. These conserved elements are located near the active site and increase the size of enzymes to accommodate the oxyamino- components of cephalosporins to allow for braod spectrum resistance (Joris et al., 1991; Stürenburg and Mack, 2003; Bois et al., 1995).

2.5.2.5 SHV

In 1972, a sulphydryl variable beta-lactamase was identified and then SHV was derived from it as it is the description of the biochemical property of the enzyme. In the beginning it was chromosomally mediated and had narrow range activity. But later due to mutations its activity range increased. Currently, there are 141 SHV enzymes each varying in number and amino acid mutation. These enzymes are now mobilized as they are mediated by plasmid (Heritage et al., 1999; Joris et al., 1991).

2.5.2.6 CTX-M

The word CTX-M is derived from the name of the cephalosporin, cefotaxime indicating its ability to hydrolysing ability. They were detected on the chromosome of environmental bacteria, *Klyvera,* and are not related to TEM or SHV (Peirano and Pitout et al., 2010). These enzymes are mediated on plasmid as well and can be found in association with SHV, TEM and OXA beta-lactamases. Presently, there are 120 CTX-M enzymes and are divided into 5 phylogenetic groups. They are less effective at hydrolysis of penicillins but show better activity against cephalosporins (Bonnet, 2004).

2.5.3 Target Site Alteration

Penicillin binding proteins (PBPs) also called transpeptidases, are enzymes that catalyse the making of peptidoglycan layer around bacterial cell, have been reported to have undergone mutations so that they are less likely to bind to the beta-lactam antibiotic. This happens by mutations in the genes responsible for encoding PBPs. Mutations in PBPs have been reported in pathogenic strains such as, *N. gonorrhoeae,H. influenzae, and N. meningitides* (Dabernat et al., 2002, Ropp et al., 2002, Antignac et al., 2001).

2.5.4 Reduced Permeability

Hydrophilic compounds or b-lactam antibiotics are allowed inside the bacterial cell by proteins called porins. They make channels in the outer membrane of the cell wall and make way for substances to enter. Due to mutations in the genes responsible for their encoding their loss or structural change could occur, which makes the bacteria gain resistance against the antibiotic (Zgurskaya & Nakaido, 2000). Resistance due to porin change or loss has been reported in *E. coli, P. aeruginosa, N. gonorrhoeae, S.*

dysentariae, Proteus spp., *A. baumannii,and S. marcescens* (Kwon et al., 2003, Clarke et al., 2003, Oliver et al., 2002, Weindorf et al., 1998).

2.5.5 Efflux pump

Efflux pumps are proteins of transport, responsible for expulsion of potentially harmful substances including antibiotics. Most bacteria with overly expressed efflux pump are multidrug resistant (MDR) (Lomovskaya et al., 2001). Their increased expression could be caused by mutations in the genes responsible for coding them (Adewoye e t al., 2002). There are five systems of efflux pumps among gram negative bacteria. Efflux pump encoding genes could be found on plasmids and chromosomes alike. It is reported that resistant as well as susceptible bacteria carry genes for different efflux pumps and that they are overly expressed once the bacteria is exposed to more than one class of antibiotics as well as other toxic substances because of the extended substrate range of the efflux pump system (Lomovskaya et al., 2001). Over expression of efflux pump has been reported in *Campylobacter jejuni*, *E. coli*, *Pseudomonas aeruginosa*and *Salmonella typhimurium*(Lin et al., 2002; Poole, 2001; Nikaido et al., 2008).

2.5.6 Resistance Mechanism against Fluoroquinolones

The bacterial resistance to fluoroquinolones is either target mediated mutations, plasmid mediated or chromosome mediated.

2.5.7 Mutations in Target

Resistance to fluoroquinolones is often target mediated. To reduce the affinity of antibiotic for the target, mutations occur in gyrase and topoisomerase IV encoding genes. About ≤10 fold resistance confers to one mutation occurring in the topoisomerases (Drlica et al., 2009). About 90% of the mutations occurring are in the amino acid ser-

ine. The rest of the mutations occur in the acidic residue that hold the metal-water bridge for antibiotic (Price et al., 2003). Mutations in serine are more common because with the latter, the catalytic activity of the cell is reduced about 5-10 times (Aldred et al., 2013). Mutations have been reported in *parC*, *parE*, *gyrA* and *gyrB*, genes of *E.coli* (Komp et al., 2003).

2.5.8 Plasmid Mediated Resistance

Plasmid mediated quinolone resistance confers to low level of resistance but is an emerging problem because of ease of transfer. Unlike target mediated quinolone resistance, it can be transferred to others by horizontal gene transfer. Plasmid mediated resistance to quinolones is by three gene families.

The first are qnr genes, they encode pentapeptide repeat proteins that decrease the binding of topoisomerases to DNA and can also attach to them by themselves so that there are lesser enzyme complexes for the antibiotic to attack. There are about 100 variants of qnr divided in at least five families. qnrB gene was reported in environmental *Aeromonas* spp. isolates in France by Cattoir et al., (2007).

Another gene, aac(6′)-Ib-cr, encodes for a protein that acetylates the piperazinyl amine of the antibiotic and decreases its activity. The gene aac(6′)-Ib-cr was reported to be widespread in clinical Enterobacteriaceae isolates in US by Park et al., (2006). The third protein is efflux pump, so far only three have been identified which confer resistance to fluoroquinolones. QepA, an efflux pump which was plasmid mediated was reported in a clinical *E.coli* isolate by Yamane et al., (2007).

2.5.8.1 Chromosome mediated Resistance

Chromosome mediated quinolone resistance is achieved by downregulation of porin expression or by up regulation of efflux pump that are chromosome encoded. By these mechanisms the cellular concentration of antibiotic is decreased. It confers to low

level of resistance and is of little clinical importance (Mitscher et al., 2005). Jaffe et al., (1982) reported that down regulation of porin (OmpC and OmpF) by mutation altered the permeability of cefoxitin in *E.coli* and decreased its effectiveness.

2.6 Antibiotic resistance Consequences

How antibiotic resistance affects us depends upon the level of resistance, type and place of infection and availability of effective treatment alternatives (Rice, 2009). As resistance develops in the infectious strains, there are risks of increase in severity of diseases, length of disease time period, mortality rate and health care costs (Llor and Bjerrum, 2014).

Most of the currently available antibiotics have lost their effectiveness with only 15 out of 44 showing some activity and only 5 of them pass the phase 3 against some infection causing gram negative bacteria and the absence of newer antibiotics doesn't help the situation much (CDC, 2015; Antibiotics currently in clinical development, 2017). According to WHO (2014), antimicrobial resistance is not only a problem in developing countries but it is spread worldwide. Resistance has been reported to have increased in strains responsible for causing community and hospital associated infections which is a matter of concern because community based infections could be transferred around even by normal contact (Wise and Piddock, 2010). According to Infectious Disease Society of America, 2018, more people in America have been victims of methicillin resistant *Staphylococcus aureus* over a year than Parkinson's disease, homicide, emphysema and HIV/AIDS altogether. One bacteria may have different resistance mechanisms (Shaikh et al., 2015). Furthermore, a study claims that in European Union, United states and Thailand infections caused by resistant bacteria takes up to 25,000, 23,000 and 38,000 lives each year, respectively (WHO, 2014).

2.7 Antibiotic Resistance Genes

Resistant genes in bacteria encode to resistance mechanisms against drugs which are detrimental to their survival. But that is not considered their initial function, ARGs used to have regulatory functions but with time, due to external pressure, they adopted their roles of providing protection to its host. Environmental bacteria may carry a pool of genes that may have the ability to be used as resistance genes if ensnared by pathogenic bacteria (Bhullar et al., 2012). These genes may encode a novel resistance mechanism to antibiotics that are our last-resort (Berendonk et al., 2015). In the beginning, it was believed that ARGs evolved due to contaminated environment to shield bacteria from harsh conditions but then it was revealed that they existed even prior to the use of antibiotics and we only found out them recently (D'costa et al., 2011). Recently, it is suggested that ARGs might be ''emerging environmental pollutants'' because they are ubiquitous in various environmental compartments (Pruden et al., 2006; Rysz and Alvarez, 2004).

2.7.1 Dissemination of Antibiotic Resistance Genes in Environment

For other bacteria to ensnare ARGs, the mechanism most important is HGT. It may occur through one of the following three processes.

1. Conjugation
2. Transduction
3. Transformation (Berglund, 2015)

2.7.1.1 Conjugation

It is the exchange of genetic material by cell to cell connection via pilus formation. It was first discovered in the 1950s and has been proven to have occurred in water, soil, seawater sediments, activated sludge and sewage wastewater (Davies and Davies,

2010; Smillie et al., 2010). There have been reports of DNA transfer among vast range of hosts, even to eukaryotes (Bates and Wilkins, 1998; Davison, 1999). The important genetic material commonly getting transferred include; integrative conjugative elements (ICEs) and plasmids (Smillie et al., 2010).

2.7.1.1.1 Integrons

They are mobile genetic elements that provide a platform for capture and expression of gene cassettes that have ARGs. Its basic feature is a gene, intI which codes for an enzyme called tyrosine recombinase which excises and integrates genes into the cassette. It can also reshuffle the order of genes influencing the expression of gene cassette. The commonly found integrons are class 1 integrons in most clinical strains (Cambray et al., 2010; Mazel, 2006). Expression of intI gene is induced by SOS response. SOS response is triggered by b-lactam, fluoroquinolone and trimethoprim antibiotics. So this means that a bacteria carrying an integron in a population if exposed to these antibiotics will have high chances of expression of relevant ARGs (Guerin et al., 2009). Integrons could be mobile or chromosomal. Mobile integrons can be transferred to other bacteria via plasmids (Dominigues et al., 2012). A study conducted proved the presence of at least 1000 integrons in sea sediments showing their ubiquity (Koenig et al., 2008). The presence of class 1 integron has proved to be in connection with anthropogenic activities. Their high concentration was reported in River Ravi, Lahore (Khan et al., 2013).

2.7.1.2 Transduction

It is the transfer of genetic material to bacteria through bacteriophages. It is easier for phage particles to survive environmental degradation than naked DNA, and also due to their small size they are well suited for DNA dissemination (Davison, 1999). It is common in marine environment (Jiang and Paul, 1999). According to various studies,

bacteriophages in wastewater treatment plant, receiving water, urban sewage and activated sludge carried methicillin resistant and b-lactamase genes, proven by metagenome analysis of viruses (Rolain et al., 2012; Colomer-Lluch et al., 2011).

2.7.1.3 Transformation

It occurs when bacteria takes up genetic material from environment released by another bacteria after its decease. Successful transformation can occur if degradation and dilution of bacterial DNA is prevented by its adhesion to particles of sediment or soil. It can also occur in biofilms where the DNA of lysed bacteria is captured by other nearby bacterial cells. Studies have proven that natural transformation occurs in sea water, river bodies, ground water and soil (Davison, 1999). Transformation has been considered as a source of spread of penicillin resistance genes (Johnsborg and Havarstein, 2009).

2.7.2 Pathways for Dissemination of ARGs in Environment

Antibiotics can enter our environment through various pathways, and it is established that because of the selective pressure that antibiotics exert on the microflora wherever antibiotics go, resistant bacteria and ARGs follow. So there are points where ARGs, resistant bacteria and environmental microflora can mix. These points are called hotspots where ARGs increase in number and resistant strains develop and further evolve. There are various ways in which humans are exposed to these ARGs, so they can become a part of their microbiome (Wellington et al., 2013).

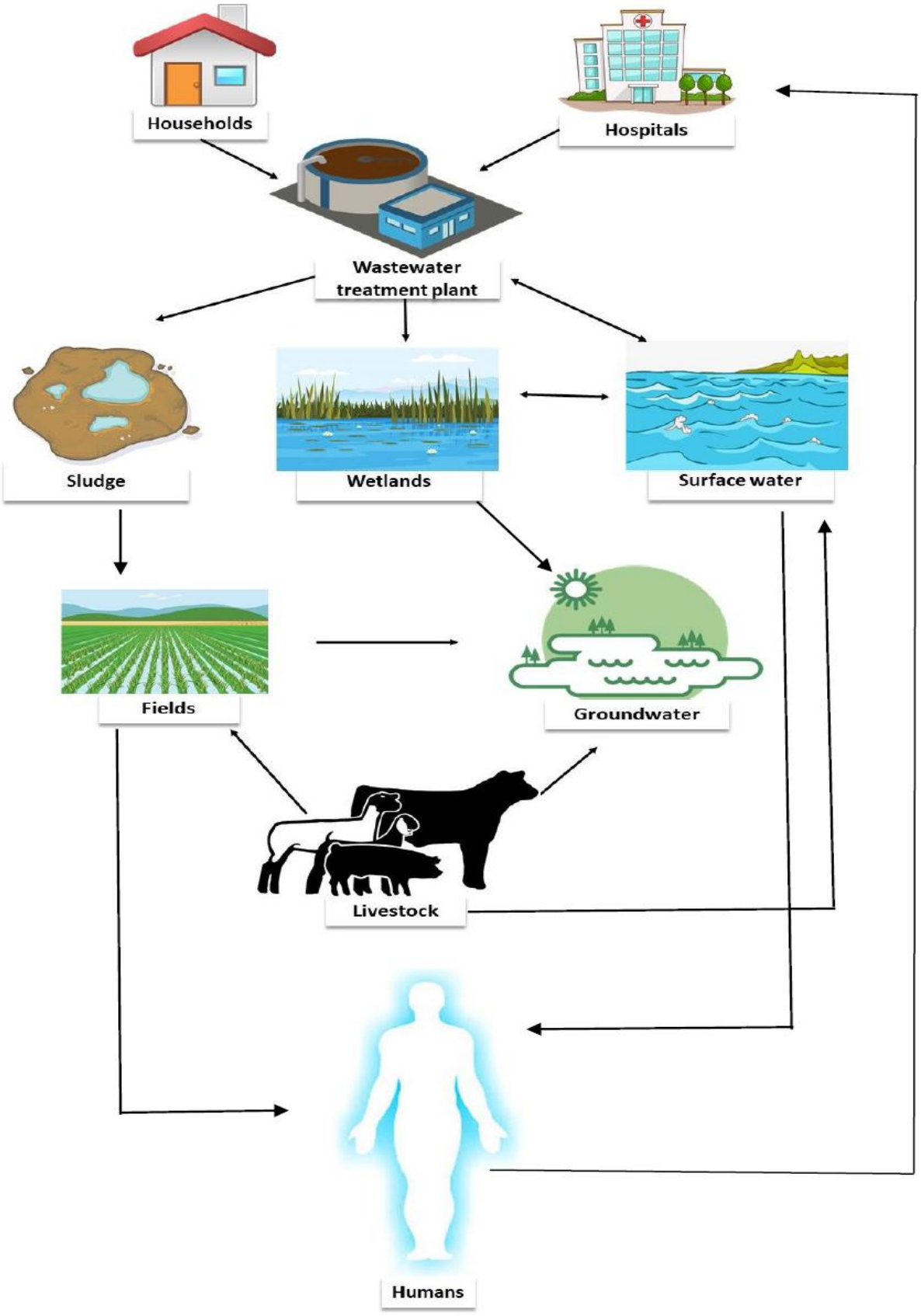


Figure 2.4 Dissemination pathways of ARGs in environment (Adopted from Berglund, 2015).

CHAPTER 3

MATERIALS AND METHODS

All of the experiments were carried out at Institute of Environmental Sciences and Engineering (IESE),National University of Science and Technology (NUST), Islamabad, Pakistan to investigate the enzymes produced and the ARGs (Antibiotic resistance genes) harboured by the most prevalent bacteria in our aquatic environment. The experiments for achieving the designed objectives have been discussed in detail in this chapter.

3.1 Revival of the Strains from Glycerol Stocks

The bacterial strains isolated by a previously in the year 2017 were stored in 70% glycerol at -70°C. The strains were revived on tryptic soy agar by scraping off the surface of the stock and streaking it on the agar plate without letting the stock thaw. The plates were then incubated at 35°C for 24 hours.

3.2 MIC (Minimum Inhibitory Concentration)

Minimum inhibitory concentration of the resistant strains was determined to ciprofloxacin, ofloxacin, levofloxacin, amoxicillin and ampicillin (Sigma Aldrich) by agar dilution method (CLSI, 2017). A total of 10 concentrations (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 μg/mL)were tested on mueller hinton agar. To the tested antibiotics prior screening for resistance was done (Marium Fiaz, 2017). Direct suspension method was used for making the test strain suspension of 0.5 Mcfarland standard, from the suspension 0.2 μL was dispensed to transfer up to 10^4 CFU/mL on the plate and incubated for 20 hours at 35 °C.

3.3 Combination Disc Test

3.3.1 Screening

The bacterial isolates were screened for production of ESBLs (Extended Spectrum Beta-lactamases), MBLs (Metallo Beta-lactamases) and AmpCs (class C Beta-lactamases). For ESBLs, the guidelines given by CLSI (2017) M100 were followed. Screening was done with three third-generation Cephalosporins, namely; Cefpodoxime (10 μg), Cefotaxime (30 μg) and Ceftazidime (30 μg). For MBLs, screening was done with Imipenem (10 μg). For AmpCs screening was done with Cefoxitin (30 μg). The test was done in triplicates and the mean was used in interpretation of the breakpoints according to the criteria set by CLSI, 2017. The strains that did not have breakpoints in the guidelines the breakpoints given for other strains were used in their interpretation.

3.3.1.1 Preparation of Plates and Inoculum

Mueller Hinton agar (MHA) was prepared in sterile plates and incubated at 35°C for 24 hours to check for sterility. Inoculum (4-5ml) was prepared by picking up 5-6 pure colonies from a 24 hour plate and suspending them in 0.85% saline. The turbidity of the inoculum was adjusted to a 0.5 McFarland standard (1.5×10^8 CFU/ml) prepared in lab following the CLSI (2017) guidelines.

3.3.1.2 Colony Count of Inoculum

To make sure that inoculum was of right amount of CFU/mL, colony count test was done by first diluting the inoculum up to 10^{-6}, and then the 100 μL from last three tubes was spread on agar and incubated overnight. If the plate with 10^{-6} dilution had up to 1-2colonies per 100 μL the standard used was considered of correct turbidity.

3.3.1.3 Use of Adjusted Inoculum

The adjusted inoculum was used within 15 minutes of preparation and swabbed on sterile MHA plate with a sterile swab. The plates were then inverted and incubated at 35°C for 16-18-hours.

3.3.1.4 Measurement of inhibition zones

The inhibition zones were measured with the help of a scale. The diameter of the disc was included in the measurement.

3.3.1.5 Confirmation Test of Combination Disk Assay (ESBLs)

The antibiotic discs used and their concentrations were selected on the basis of CLSI (2017) guidelines. The same method was followed as was used for screening of the strains. A strain having an inhibition zone difference equal to or more than 5mm between the indicator Cephalosprin and indicator + ESBL inhibitor was considered to be an ESBL producer. The following table summarises the discs used and their concentrations in both screening and confirmation. The test was repeated in triplicates.

Table 3.1 Antibiotics used in confirmation test of ESBLs.

Confirmation			
Antibiotic		**Concentration**	
Indicator cephalosporin	Indicator + ESBL Inhibitor	Indicator cephalosporin	Indicator + ESBL Inhibitor
CTX	CTX + Clavulanate	30 µg	30 + 10 µg
CAZ	CAZ + Clavulanate	30 µg	30 + 10 µg

3.4 Confirmation Test of Double Disk Synergy Assay (ESBLs)

This method by Jarlier et al., (1988) was followed to detect the ESBLs production in the test strains. For this purpose, cefotaxime (30 µg) and ceftazidime (30 µg) were placed on an already swabbed agar plate on both sides of amoxicillin+clavulanate

(20+10 μg) disk. The plates were incubated for 16-18 hours at 35 °C and observed for a synergistic zone between the cephalosporins and amoxicillin+clavulanate.

3.5 Confirmation Test of Inhibitor Based Assay (MBLs)

MBLs production by the resistant strain was confirmed with inhibitor based method (Yong et al., 2002). Stock solution of EDTA (Ethylene Diamine Tetra Acetic Acid) of 0.5M was prepared and autoclaved. After swabbing the plate two imipenem disks were placed on the plate 20 mm apart from each other centre to centre. On one disk 4 μL of the EDTA solution was dispensed to get a concentration of 750 μg. The plates were incubated for 16-18 hours at 35 °C. The strain with inhibition zone difference of 6 or more than 6 mm was considered to be a producer.

3.6 Confirmation Test (AmpCs)

To confirm the strains for production of AmpCs the method described by Gupta et al., (2014) was followed. Imipenem (10 μg), ceftazidime (30 μg), cefoxitin (30 μg) and amoxicillin+clavulanate (20+10 μg) were placed on agar plate with ceftazidime in the middle and the others surrounding it and incubated at 35 °C for 16-18 hours. The plates were observed for obvious blunting of the ceftazidime inhibition zone towards any of the substrates (imipenem, cefoxitin, amoxicillin+clavulanate).

3.7 Polymerase Chain Reaction (PCR)

3.7.1 DNA Extraction

DNA was extracted by suspending a single colony in 100 μL nuclease free water in PCR tube. The colony was boiled at 97 °C for 10 minutes and then centrifuged at 10,000 RPM for 3-4 minutes. The supernatant was collected and stored at -20 °C and the pellet was discarded. The presence of DNA was checked first by running it on gel stained with ethidium bromide.

3.7.2 DNA Quality and Quantity

To determine the quality and quantity of the extracted DNA spectrophotometer was used (Specord, 200). The DNA was diluted 100 times in sterile distilled water and then readings of absorbance were taken at 260 and 280 nm simultaneously. The samples with A260/280 value less than 1.6 were repeatedly extracted. Quantity of DNA was determined from the formula:

$$\text{DNA concentration (ug/ml)} = \frac{\text{OD260} \times 100 \text{ (dilution factor)} \times 50 \text{ ug/ml}}{1000}$$

3.7.3 Primer Stocks

Stock solutions of primers (blaTEMR, blaTEMF, QnrSR, QnrSR) were made at a concentration of 100 μM by adding nuclease free water to the tube of lyophilized primers. The solution was then gently vortexed for about a minute. Aliquots of the stock were made at a concentration of 1 and 10 μM and stored at -20 °C.

3.7.4 Magnesium Chloride Stock

Stock solution of 0.25 M magnesium chloride was prepared and autoclaved. The stock solution was diluted to get a working solution of 5 mM and stored at -20 °C.

3.7.5 Primers

The primers used in this study were previously published, their details are as under.

Table 3.2 Primers used in the study

Primer	Sequence (5′ → 3′)	Length (bases)	Reference
qnrS-F	GCAAGTTCATTGAACAGGGT	20	Dallene et al., 2010
qnrS-R	TCTAAACCGTCGAGTTCGGCG	21	
bla_{TEM}-F	CATTTCCGTGTCGCCCTTATTC	22	Cattoir et al., 2007
bla_{TEM}-R	CGTTCATCCATAGTTGCCTGAC	22	

3.8 Optimization of Primer Concentration

To optimize primers concentration stocks of two concentrations (1 and 10 µM) were prepared. Dilutions of those stocks were prepared and used in master mix. The concentrations of the rest of the components were kept the same, only the primers volume varied along the NF water. The rest of the details are given in the table.

Table 3.3 Concentration and amount of reaction components for primer concentration optimization

Reaction Component		R1	R2	R3	R4	R5
Primers	Concentration	0.1 µM	0.2 µM	0.3 µM	0.4 µM	0.5 µM
	Forward	5 µL from S1*	10 µL from S1	1.5 µL from S2**	2 µL from S2	2.5 µL from S2
	Reverse	5 µL from S1	10 µL from S1	1.5 µL from S1	2 µL from S2	2.5 µL from S2
PCR Mix (Thermo Fisher Scientific)		25 µL	25 µL	25 µL	25 µL	25 µL
$MgCl_2$ (sigma)		2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL
NF Water		13 µL	3 µL	20 µL	19 µL	18 µL
Template		2 µL	2 µL	2 µL	2 µL	2 µL
Total volume		50 µL	50 µL	50 µL	50 µL	50 µL

***S1 (stock 1 = 1 µM) **S2 (Stock 2 = 10 µM)**

3.9 Optimization of Annealing Temperature

To optimize annealing temperature, the melting temperature given on the stock tube by the manufacturer was varied. Five different annealing temperatures were tested for both of the primer sets. In this test only the annealing temperatures were varied the rest of the parameters were kept the same throughout the runs. The details are given as under.

Table 3.4 Procedure followed to optimize annealing temperature (* R1, R2, R3, R4 and R5 from table 1.)

Reaction No.	T1	T1	T2	T2	T3	T3	T4	T4	T5	T5
Primer	blaTEM	QnrS	blaTEM	QnrS	blaTEM	QnrS	blaTEM	QnrS	blaTEM	QnrS
R1	56°C	60°C	57°C	61°C	58°C	62°C	59°C	64°C	60°C	65°C
R2	56°C	60°C	57°C	61°C	58°C	62°C	59°C	64°C	60°C	65°C
R3	56°C	60°C	57°C	61°C	58°C	62°C	59°C	64°C	60°C	65°C
R4	56°C	60°C	57°C	61°C	58°C	62°C	59°C	64°C	60°C	65°C
R5	56°C	60°C	57°C	61°C	58°C	62°C	59°C	64°C	60°C	65°C

3.10 Titration of $MgCl_2$

To optimize the $MgCl_2$ concentration a stock of 5mM was prepared and varying volumes were dispensed in the PCR tube to achieve the final concentration given under. In this test only the concentration of $MgCl_2$was varied the rest were kept the same in all the runs.

Table 3.5 Procedure followed to optimize concentration of magnesium chloride ($Mgcl_2$)

Reaction No.	$Mgcl_2$ Concentration	R1	R2	R3	R4	R5
M1	0.25 mM	2 µL	2 µL	2 uL	2 µL	2 µL
M2	0.5 mM	5 µL	5 µL	5 µL	5 uL	5 µL
M3	1 mM	10 µL	10 µL	10 µL	10 µL	10 µL

3.11 Final Optimized Conditions

After completing the runs for optimization of all of the three components (Primers, $Mgcl_2$ and annealing temperature), we were able to get our results at the following running conditions.

3.12 Gel Electrophoresis

3.12.1 Preparation of TBE (Tris Boric EDTA) buffer (1X)

To make TBE buffer, 0.5 M EDTA was prepared and the PH was adjusted to 8 with NaOH pellets. To make 1X TBE of 1 litre, 4 ml of EDTA was combined with 10.8 g of tris-base and 5.5 g of boric acid. And then 996 ml of water was added to make a 1 litre solution. The solution was autoclaved before use.

3.12.2 Gel Electrophoresis

The concentration of agarose gel used was 1%, in 50 ml buffer. The gel was stained with 0.05ug/ml of ethidium bromide. To load wells 5 µL of the product was mixed with 1 µL of 6X loading dye (Thermo Fisher Scientific) and 6 µL was loaded in wells. Gel was run at 100 volts for 1 hour and then viewed via UV transilluminator. 1 kb ladder (Thermo Fisher Scientific) was used as a size marker.

The samples were sent for sequencing to BGI (Beijing Genomics Institute), Hong Kong. The obtained sequences were aligned with BioEdit and phylogenetic analyses were done with Mega-X and Muscle alignment tool. The sequences were run on blastN algorithm of Nucleotide blast, NCBI (National Centre for Biotechnology Information) for confirmation and submitted in Genbank.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Minimum Inhibitory Concentration (MIC)

MICs of five antibiotics were determined as described in chapter 3. The results showed that MICs were higher for penicillins followed by fluoroquinolones. The results are given here in tabular form and each antibiotic is discussed in detail.

4.1.1 MICs of Ampicillin

Among the antibiotics tested the strains had high MICs for the broad spectrum penicillin antibiotic, ampicillin.The table 4.1 shows that about 32% of the strains had the highest tested MIC which was 256 μg/mL.

Table 4.1MICs of test strains to Ampicillin (0.125-256 μg/mL) + (denotes visible growth) – (denotes no visible growth)

Bacterial Strain	μg/mL											
	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	-	-	-	-	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Comamonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Comamonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	-	-	-

Acinetobacter sp.	+	+	+	+	+	+	+	+	+	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Shigella sp.	+	+	+	+	+	+	+	+	+	-	-	-
Rheinheimera sp.	+	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Proteus sp.	+	+	+	+	+	+	+	+	+	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	+	-	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	-	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	-	-
Alishewanella sp.	+	+	+	+	+	+	+	+	+	+	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	-	-
Comamonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Citrobacter sp.	+	+	+	+	+	+	+	+	+	+	+	-
Morganella sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Stenotrophomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	+	+	-
Shewanella sp.	+	+	+	+	+	+	+	+	+	+	+	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	+	-
Citrobacter sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-

The strains included species from the genus *Aeromonas, Citrobacter, Pseudomonas, Escherichia, Shewanella, Acinetobacter, Alishewanella, Stenotrophomonas, Comma-monas and Morganella.* Further, about 46.2% of the strains had MIC of 64-32 μg/mL which included species from the genus *Acinetobacter, Pseudomonas, Comamonas, Rheinheimera*, *Shigella, Proteus* and *Escherichia*. About 12.9% of the strains had

MIC in the range of 0.125-2 μg/mL which included species from the genus *Acinetobacter, Aeromonas* and *Pseudomonas*.Resistant strains with high MICs were also reported by Huang et al., (2012) which were isolated from Municipal wastewater treatment plants in China. Species from the prevalent genus like *Pseudomonas, Acinetobacter* and *Aeromonas* and some from the less prevalent genus like *Escherichia, Stenotrophomonas, Citrobacter, Shewanella, Morganella* and had the highest tested MICs. Comparable results were also reported by Xin et al., (2019) for bacteria isolated from coastal waters. High MICs of ampicillin in various bacterial species have been attributed to the production of ampCs and mutations in ampC genes (Reisman et al., 2017; Schwartz et al., 2003). Ampicillin being a broad spectrum antibiotic is very frequently used for treatment of various infections in hospitals which can lead to selection of ampicillin resistance (Bergeron et al., 2017).

4.1.2 MICs of Amoxicillin

The results showed that about 33.3% of the strains had the highest tested MIC of amoxicillin. About 12.9% of the strains had MICs in the range of 64-32 μg/mL, while 64.8% of the strains had MICs in the range of 2-0.125 μg/mL.The strains were from the genus *Aeromonas, Pseudomonas, Escherichia, Rheinheimera, Shewanella, Citrobacter* and *Acinetobacter*.

Table 4.2 MICs of test strains for Amoxicillin (256-0.125 μg/mL**).**+ (denotes visible growth) – (denotes no visible growth)

Bacterial strain	**μg/mL**											
	0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Shewanella sp.	+	+	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	-	-	-	-	-	-	-	-	-	-
Escherichia sp.	+	+	+	-	-	-	-	-	-	-	-	-
Escherichia sp.	+	+	+	-	-	-	-	-	-	-	-	-

Pseudomonas sp.	+	+	+	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	-	-	-	-	-	-	-	-	-
Shigella sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Morganella sp.	+	+	+	+	+	+	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	-	-	-	-	-	-
Proteus sp.	+	+	+	+	+	+	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	-	-	-	-	-	-
Comamonas sp.	+	+	+	+	+	+	+	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	+	+	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	-	-	-	-	-
Alishewanella sp.	+	+	+	+	+	+	+	+	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	-	-	-	-
Escherichia sp.	+	+	+	+	+	+	+	+	-	-	-	-
Comamonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	+	-	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	-	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	+	+	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	+	+	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Shewanella sp.	+	+	+	+	+	+	+	+	+	+	+	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Stenotrophomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Rheinheimera sp.	+	+	+	+	+	+	+	+	+	+	+	-
Citrobacter sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	+	-

Citrobacter sp.	+	+	+	+	+	+	+	+	+	+	+	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-

A trend similar like for ampicillin was also observed for amoxicillin as well with slightly lower MICs recorded for *Acinetobacter* spp. High MICs were observed for some species from the prevalent genus like *Acinetobacter, Pseudomonas* and *Aeromonas* and also some less prevalent genus like *Citrobacter, Escherichia, Rheinheimera, Stenotrophomonas* and *Shewanella.* Comparable results were reported by Meng et al., (2017) and Vaz-Moreira et al., (2017). It was stated that CTX-M, TEM-1 and OXA type of beta-lactamases were responsible for resistance to the antibiotic. Resistance to amoxicillin can also be intrinsic such as reduced expression of porins and mutations in PBPs. In our study 20% of the strains were beta-lactamase producers, although not all of them were confirmed with PCR.

4.1.3 Ofloxacin

The results from the table 4.3 indicated that *Aeromonas*spp., and *Escherichia* spp. had the highest MICs of ofloxacin which made up to 12.9% of the total strains.

Table 4.3MICs of test strains to Ofloxacin (256-0.125 µg/mL). + (denotes visible growth) – (denotes no visible growth)

Bacterial strain	**µg/mL**											
	0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
Morganella sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-

Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Comamonas sp.	+	+	-	-	-	-	-	-	-	-	-	-
Aeromonas sp.	+	+	-	-	-	-	-	-	-	-	-	-
Escherichia sp.	+	+	-	-	-	-	-	-	-	-	-	-
Escherichia sp.	+	+	-	-	-	-	-	-	-	-	-	-
Shewanella sp.	+	+	-	-	-	-	-	-	-	-	-	-
Escherichia sp.	+	+	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	-	-	-	-	-	-	-	-	-	-
Shewanella sp.	+	+	+	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	-	-	-	-	-	-	-	-	-
Stenotrophomonas sp.	+	+	+	-	-	-	-	-	-	-	-	-
Aeromonas sp.	+	+	+	+	-	-	-	-	-	-	-	-
Proteus sp.	+	+	+	+	-	-	-	-	-	-	-	-
Rheinheimera sp.	+	+	+	+	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Alishewanella sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Comamonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	+	+	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	+	+	+	-	-	-	-
Citrobacter sp.	+	+	+	+	+	+	+	+	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	-	-	-	-
Aeromonas sp.	+	+	+	+	+	+	+	+	-	-	-	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	-	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	+	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	+	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	+	-
Citrobacter sp.	+	+	+	+	+	+	+	+	+	+	+	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	+	-

One *Acintetobacter* sp., *Pseudomonas* spp. and*Citrobacter* sp.also had high MICs of ofloxacin. About 3.7% of the strains had MIC of 64 μg/mL, which included a specie

each from the genus *Aeromonas* and *Acinetobacter*. About 64.8% of the strains had MICs in the range of 32-0.125 µg/mLwhich included species from the prevalent genera like *Aeromonas, Pseudomonas, Acinetobacter*, and some from the less prevalent genera like *Alishewanella, Shewanella, Escherichia*, *Rheinheimera*, *Comamonas, Stenotrophomonas, Proteus* and *Morganella.*Resistance to fluoroquinolones in environmental bacteria has been largely attributed to mutations in the genes encoding DNA gyrase and topoisomeras IV subunits. Two mutations can cause the bacteria to inhibit as high concentration of FQs as 256 mg/L. Environmental *Escherichia* spp.having similar MICs were reported by AmÃ¡bile-Cuevas et al., (2010). Environmental *Aeromonas* spp.having high MICs were reported by Cattoir et al., (2008). Pendland et al., (2002) reported high MICs of clinical *Pseudomonas* spp.

4.1.4 Ciprofloxacin

The table 4.4 shows that *Aeromonas* spp., *Escherichia* spp., *Acinetobacter* sp., *Citrobacter* sp., *and Pseudomonas* spp. could resist as high as 256 and 128 mg/L of ciprofloxacin which made about 14.8% of the total strains. While about 7.4% of the strains had MICs in the range of 64-32 µg/mL.

Table 4.4MICs of test strains for Ciprofloxacin (256-0.125 µg/mL). + (denotes visible growth) – (denotes no visible growth)

Bacterial strain	**µg/mL**											
	0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
Escherichia sp.	+	+	-	-	-	-	-	-	-	-	-	-
Alishewanella sp.	+	+	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	-	-	-	-	-	-	-	-	-	-
Shewanella sp.	+	+	-	-	-	-	-	-	-	-	-	-
Comamonas sp.	+	+	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	-	-	-	-	-	-	-	-	-	-
Aeromonas sp.	+	+	-	-	-	-	-	-	-	-	-	-

Acinetobacter sp.	+	+	-	-	-	-	-	-	-	-	-	-
Escherichia sp.	+	+	-	-	-	-	-	-	-	-	-	-
Proteus sp.	+	+	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	-	-	-	-	-	-	-	-
Escherichia sp.	+	+	+	+	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	-	-	-	-	-	-	-	-
Shigella sp.	+	+	+	+	-	-	-	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	-	-	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	-	-	-	-	-	-	-
Comamonas sp.	+	+	+	+	+	-	-	-	-	-	-	-
Escherichia sp.	+	+	+	+	+	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	-	-	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	-	-	-	-	-	-	-
Rheinheimera sp.	+	+	+	+	+	+	+	-	-	-	-	-
Morganella sp.	+	+	+	+	+	+	+	+	-	-	-	-
Pseudomonas sp.	+	+	+	+	+	+	+	+	+	-	-	-
Shewanella sp.	+	+	+	+	+	+	+	+	+	-	-	-
Stenotrophomonas sp.	+	+	+	+	+	+	+	+	+	+	-	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	-	-
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Comamonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Aeromonas sp.	+	+	+	+	+	+	+	+	+	+	+	-
Acinetobacter sp.	+	+	+	+	+	+	+	+	+	+	+	-
Citrobacter sp.	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia sp.	+	+	+	+	+	+	+	+	+	+	+	+
Citrobacter sp.	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia sp.	+	+	-	-	-	-	-	-	-	-	-	-

High MICs to ciprofloxacin can be attributed to mutations in housekeeping genes. Acetylation of ciprofloxacin by thetransmittable –cr variant of aac (6')-lb- gene also causes low level of resistance (Park et al., 2006). Reguation in efflux pumps can also contribute to high MICs of fluoroquinolones (Kriengkauykiat et al., 2005). About 77.8% of the strains had MICs in the range of 16-0.125 μg/mL.Transmittable qnr determinants could also cause low level of resistance to fluoroquinolones (Hooper, 1999). Cattoir et al., (2007) isolated ciprofloxacin resistant Aeromonas spp. with MICs comparable to our results and attributed this resistance to the presence of qnr determinants and *gyrA* mutations. Shakir et al., (2012) isolated ciprofloxacin resistant *Aeromonas* spp.and attributed their high MICs to mutations in *gyrA* and *parC. Escherichia* spp.with high MICs were reported by Lindgren et al., (2003) and it was determined that multiple mutations in *gyr* and *par* genes. Mutations in efflux pump systems were also identified. *Citrobacter* sp.with high MIC to fluoroquinolones was reported by Azargun et al., (2018). Resistance to fluoroquinolones was attributed to the presence of transferrable qnr determinants and efflux pumps.

4.1.5 Levofloxacin

According to the table 4.5, MICs determined for levofloxacin were low except for one *Escherichia* sp. *and Acinetobacter* sp.About 3.7% of the strains had an MIC of 8 μg/mL and 11.1% of the strains had an MIC of 2 μg/mL, while the rest of the strains had MIC ranging from 2-0.125 μg/mL.

Table 4.5MICs of test strains for Levofloxacin (256-0.125 μg/mL). + (denotes visible growth) – (denotes no visible growth)

Bacterial strain	μg/mL											
	0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
Escherichia sp.	+	-	-	-	-	-	-	-	-	-	-	-

Alishewanella sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Shewanella sp.	+	-	-	-	-	-	-	-	-	-	-	-
Comamonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Aeromonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Escherichia sp.	+	-	-	-	-	-	-	-	-	-	-	-
Proteus sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Escherichia sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Shigella sp.	+	-	-	-	-	-	-	-	-	-	-	-
Aeromonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Aeromonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Comamonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Escherichia sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Aeromonas sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	+	+	-	-	-	-	-	-	-	-	-	-
Rheinheimera sp.	+	+	-	-	-	-	-	-	-	-	-	-
Morganella sp.	+	+	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	+	+	-	-	-	-	-	-	-	-	-	-
Shewanella sp.	+	+	+	+	+	-	-	-	-	-	-	-
Stenotrophomonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Escherichia sp.	+	+	+	+	+	+	-	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	+	-	-	-	-	-	-

Comamonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Aeromonas sp.	+	+	+	+	+	+	-	-	-	-	-	-
Acinetobacter sp.	+	+	+	+	+	+	-	-	-	-	-	-
Citrobacter sp.	+	+	+	+	+	+	-	-	-	-	-	-
Escherichia sp.	+	+	+	+	+	+	-	-	-	-	-	-
Citrobacter sp.	+	+	+	+	+	+	+	-	-	-	-	-
Escherichia sp.	+	+	+	+	+	+	+	-	-	-	-	-

Escherichia sp. and *Acinetobacter* sp.with high MIC for levofloxacin were reported by Fu et al., (2013) and Goic-Barisic et al., (2016) from environmental samples. Similar to the other two fluroquinolones high MICs to levofloxacin can also be attributed to various mutations in the *gyr* and *par* subunit encoding genes. Untreated hospital and municipal wastewater could cause development of clinically originated resistant strains (Goic-Barisic et al., 2016). Other resistance mechanisms may include the qnr genes, overexpressed efflux pumps and reduced porin expression. Diwan et al., (2010) also reported low rates of resistance of environmental bacteria to levofloxacin. Despite having high MICs to the other two fluoroquinolones, MICs to levofloxacin were low because the antibiotics slightly vary in structure and the same mutation causing to inhibit high concentration of ciprofloxacin might not play the same role for levofloxacin. Also, levofloxacin is a new fluoroquinolone from the class and hence not used that often but if it is overused in prophylaxis and treatment of infections like other quinolones and fluoroquinolones, it will follow the same fate as the other quinolones and fluoroquinolones (Fabiana et al., 2004).

4.2 ESBL AmpC and MBL Screening

The screening results showed that the bacterial isolates were highly resistant to the antibiotics used in the tests. The data presented is the mean of tests repeated three times.

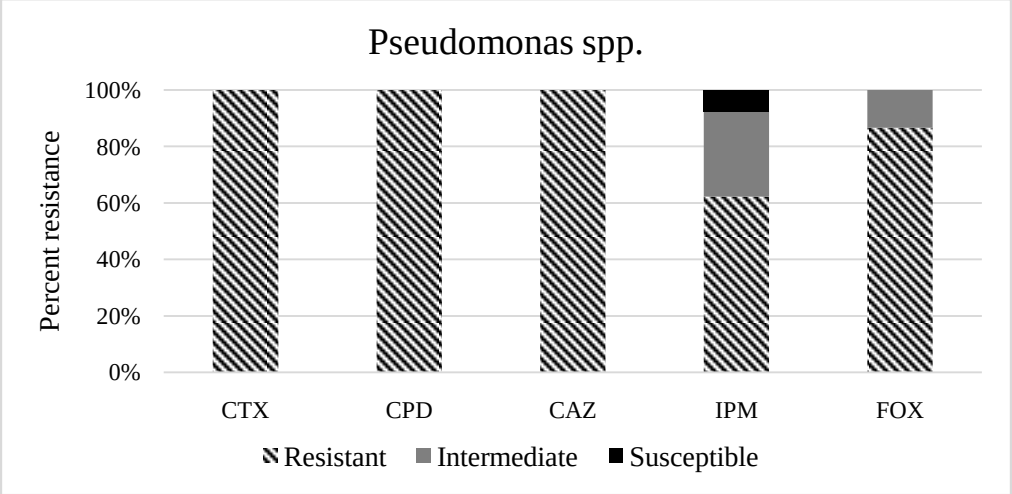


Figure 4.1 Screening results of *Pseudomonas* spp. for beta-lactamase A, B and C

The 15 *Pseudomonas* spp. strains showed 100% resistance to Cefpodoxime (CPD), cefotaxime (CTX), ceftazidime (CAZ). Chickwendu et al., (2011), Lin et al., (2017) and Knothe et al., (1991) also reported similar high rates of resistance by *pseudomonas* spp. Over-production of AmpC beta-lactamases and production of ESBLs (extended spectrum beta-lactamases) could cause *pseudomonas* spp. to resist third generation cephalosporins (Chika et al., 2016, Chikwendu et al., 2011). To imipenem (IPM) 68.75% of the species were resistant. Djenadi et al., (2018) reported similar results of resistance to imipenem and attributed it to production of plasmid-borne carbapenemases, reduced porin expression, over-active efflux pump and/or increased production of cephalosporinases. To cefoxitin (FOX) 86% of the species were resistant. Haller et al., (2018) and Chika et al.,(2016) reported similar rates of resistance to cefoxitin. *Pseudomonas* spp. are able to resist a multitude of antibiotics due to their self-induced physiological changes under stress of antibiotic overuse. They may even pick-up resistance determinants from environment with little selective pressure. They can also acquire resistance due to vulnerability to development of cross resistance (Wong et al., 2015).

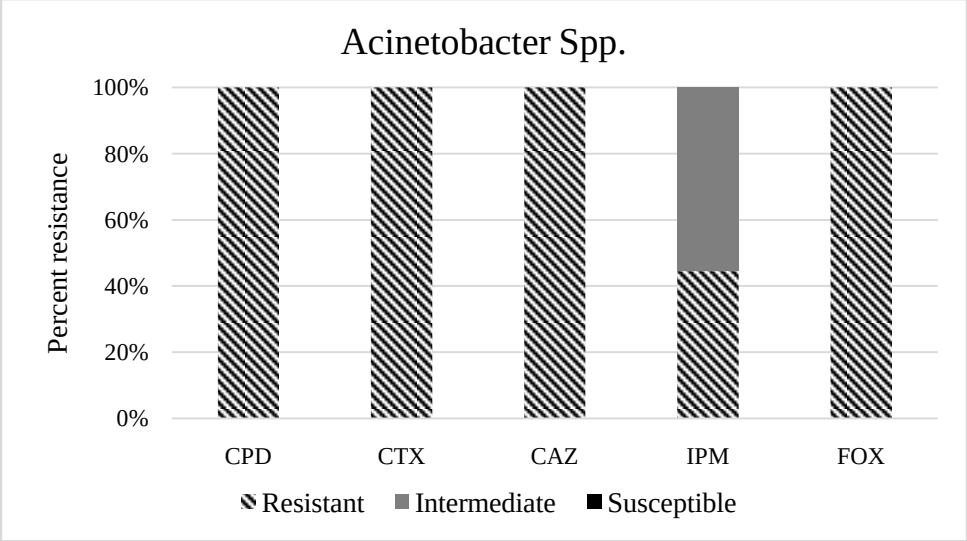


Figure 4.2 Screening results of *Acinetobacte*r spp. for beta-lactamase A, B and C

The fig. 4.2 shows that that genus which included 14 *Acinetobacter* spp.had 100% resistance to all three of the cephalosporins (CPD, CTX and CAZ). Knothe et al., (1991), Sohail et al., (2016) and Shakibaie et al., (2012) reported similar high rates of resistance by *Acinetobacter* spp. Production of ESBLs has been reported by *Acinetobacter* spp. which confers resistance to beta-lactams (Shakibaie et al., 2012). To imipenem, 44.4% of the strains were resistant. Kumari et al., (2013) reported similar results of *Acinetobacter* spp. isolated from a hospital environment. Apart from production of plasmid mediated carbapenemases intrinsic resistance to carbapenems, over-expressed efflux pump and reduced membrane permeability could confer resistance to antibiotic in this genus (Soudeiha et al., 2018). To cefoxitin, 100% of the isolates were resistant. Similar high rates of resistance have been reported by Soudeiha et al., (2018). Co-acquisition of plasmid mediated resistance determinants could cause resistance to multiple classes of antibiotics including cefoxitin (Soudeiha et al., 2018).

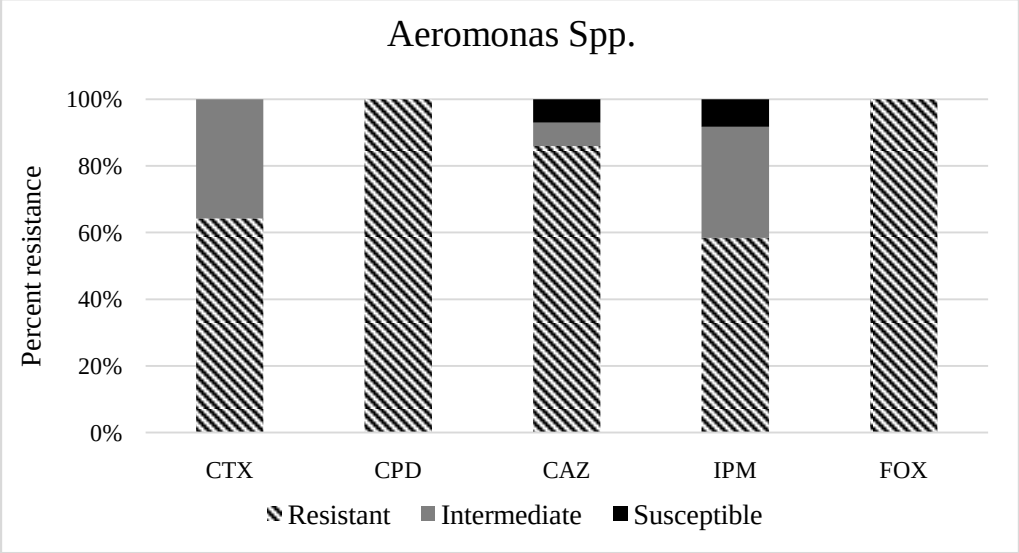


Figure 4.3 Screening results of *Aeromonas* spp. for beta-lactamase A, B and C

The fig. 4.3 shows that 64.2%, 100 % and 85.7% of the isolates which included 14 *Aeromonas* spp. were resistant to CTX, CPD and CAZ respectively. Harnisz & Korzeniewska, (2018), Lu et al., (2010) and Amsaveni et al., (2015) also reported similar results. Antibiotic resistance has increased in *Aeromonas* spp. because it's not only been recently reported in clinical isolates but also in environmental, food and vegetable samples and has been attributed to increased use of antimicrobial drugs (Alcaide et al., 2010). Resistance to B-lactams has been widely reported and is considered to be because of production of ESBLs which is normally a characteristic of *Enterobacteriaceae* (Korzeniewska and Harnisz, 2013a). It is also attributed to production of class-B and –C beta lactamases. Reduced permeability of the cell wall and over expression of efflux pump has also been seen in *Aeromonas* spp. resistant to third generation cephalosporins (Bhaskar et al., 2015). To imipenem and cefoxitin, 58.3% and 100% of the strains were resistant respectively, as was reported by Esteve et al., (2012) and Goñi-Urriza et al., (2000). The genus is known to resist carbapenems (imipenem) by producing chromosomally mediated metallo beta-lactamases and derepressed beta-lactamases (Guerra et al., 2007; Goñi-Urriza et al., 2000). *Aeromonas* spp. gain resis-

tance against cephalosporins (cefoxitin) by production of several inducible chromosome mediated beta-lactamases (Schmidt et al., 2001; Ko et al., 1998).

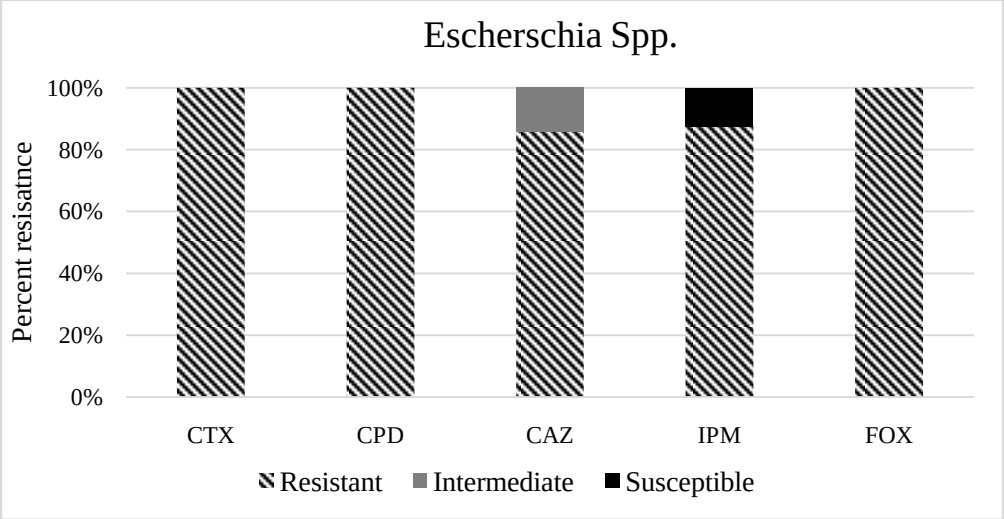


Figure 4.4 Screening results of *Escherschia* spp. for beta-lactamase A, B and C

The fig. 4.4. shows that 100% of the strains which included 7 *Escherichia* spp.were resistant to cefpodoxime and cefotaxime while 85.7% of the strains were resistant to ceftazidime. Comparable high rates of resistance by *E. coli* isolates from hospital wastewater were reported by Korzeniewska et al., (2013) and Tansawai et al., (2018). Resistance to cephalosporins in the genus is considered to be because of production of ESBLs and other beta-lactamases (Koreniezewska et al., 2013). To imipenem 87.5% of the test strains were resistant. Imipenem resistance is rare in *Escherichia spp*. but Gajamer et al., (2018) isolated *E. coli* strains from a clinical environment and reported similar high rates of resistance as this study. Production of various beta-lactamases and loss of porins can contribute to reduced susceptibility to carbapenems (Oteo et al., 2008). To cefoxitin 100% of the strains were resistant, similar results were reported by Mataseje et al., (2009) for *E. coli* isolates from recreational beaches and private drinking water in Canada. This high rate of resistance has been attributed to production of plasmid mediated AmpCs, AmpCs with mutation in promoter region and porin

deficiency (Martinez et al., 2000). Imipenem, cefotaxime and ceftazidime resistant *E. fergusonii* were reported by Glover et al., (2017). Cefoxitin resistant *E. fergusonii* was reported by Forgetta et al., (2012) from broiler chicken which had clusters of multidrug efflux system and AmpC encoding genes as a resistance mechanism.

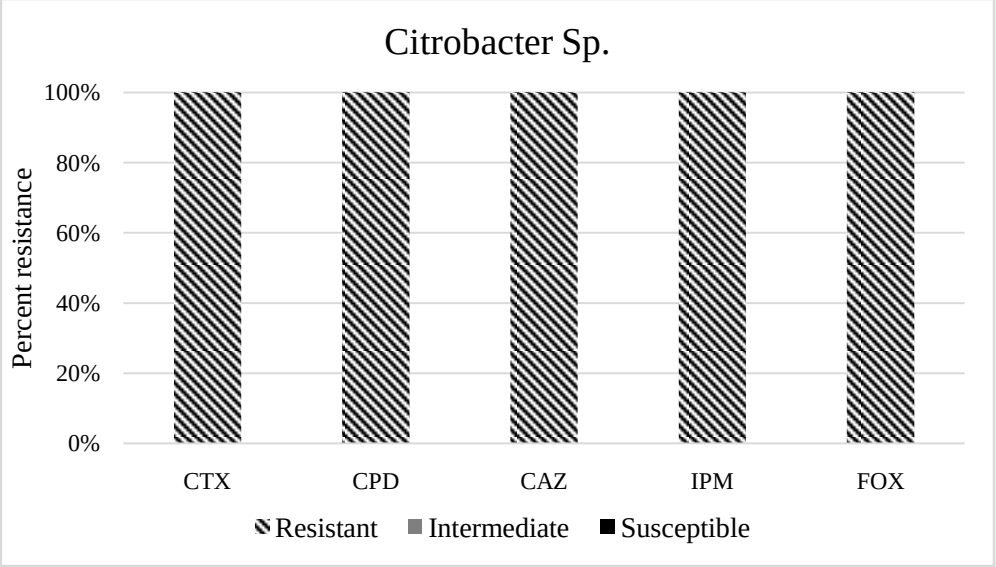


Figure 4.5 Screening results of *Citrobacter* spp. for beta-lactamase A, B and C

The two citrobacter isolates were resistant to cefotaxime, cefpodoxime, ceftazidime, imipenem and cefoxitin. Strains of *Citrobacter* spp. resistant to cefotaxime, ceftazidime (Mobashshera & Aruna, 2015), cefpodoxime, cefoxitin (Pepperell et al., 2002), imipenem (Ho et al., 2012) were isolated from various clinical and environmental samples. Reduced susceptibility to cephalosporins in *citrobacter* spp. can be explained by co-expression of chromosomal AmpC betalactamases and ESBLs (Pepperell et al., 2002). It can also be caused by reduced porin expression or their loss (Thomson et al 2009). Resistance to imipenem is attributed to production of metallo-beta lactamases, carbapenemase production, reduced porin expression or efflux pump over expression (Papp-wallace et al., 2011; Jacoby, 2005).

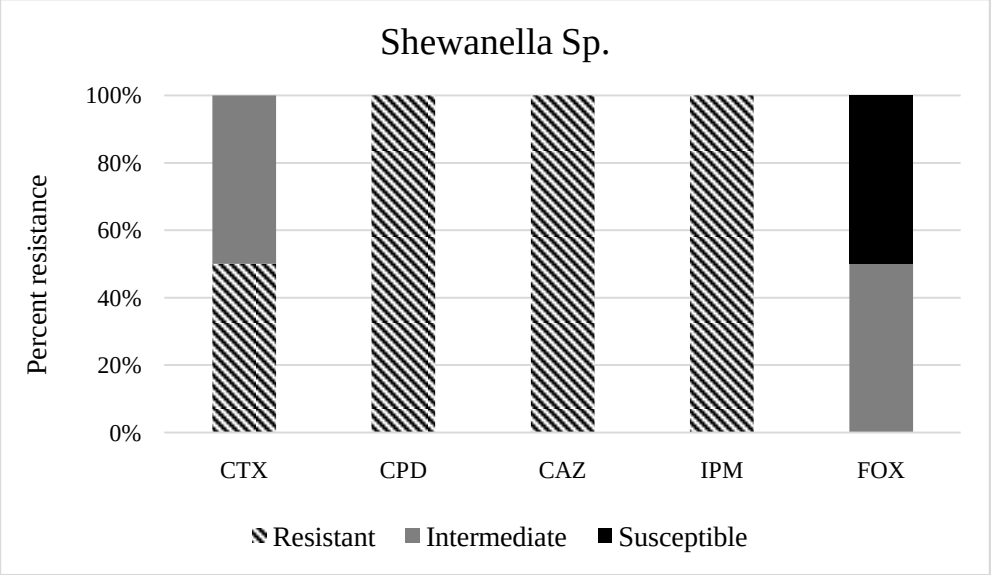


Figure 4.6 Screening results of *Shewanella* spp. for beta-lactamase A, B and C

The two *Shewanella* spp.isolates were resistant to ceftazidime, cefpodoxime and imipenem. Only one was resistant to cefotaxime and none were resistant cefoxitin. Imipnem resistant strains of *Shewanella* spp. were isolated from environmental samples by Tacão et al., (2013). Lloyd et al., (2013) reported ceftazidime resistant *Shewanella* spp. isolated from coastal environment. *Shewanella* spp.strains resistant to cefotaxime and of intermediate resistance to cefoxitin were isolated from river and estuarine water by Zhao et al., (2015). The genus has been identified to resist the cephalosporins and carbapenems by producing beta-lactamases (4 classes) and over expressed RND efflux pump system (Lloyd et al., 2018).

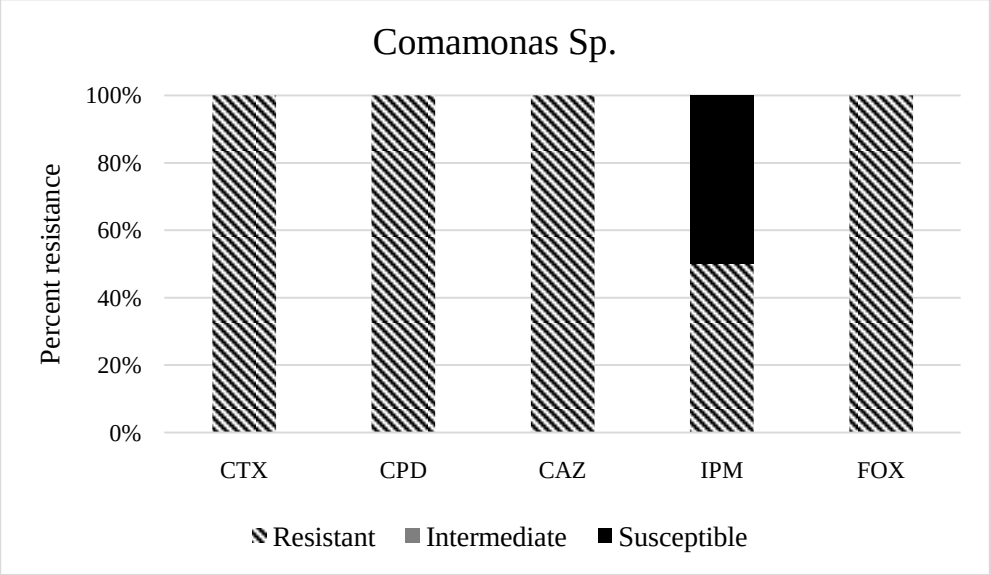


Figure 4.7 Screening results of *Comamonas* spp. for beta-lactamase A, B and C

The figure shows antibiogram of *Comamonas* spp.(2 isolates). All of the isolates were resistant to cefotaxime, cefpodoxime, ceftazidime and cefoxitin. Only one isolate was resistant to imipenem. Novovic et al., (2015) reported ceftazidime resistant *Comamonas* spp.isolated from different water samples. Cefotaxime (Day et al., 2013) and imipenem (Rui et al., 2018; Day et al., 2013) resistant *Comamonas* spp. were reported from clinical samples in Pakistan and China. Cefoxitin resistant *Comamonas* spp. were isolated from various samples taken from hospital sewage by Haller et al., (2018). The species of this genus resist cephalosporins by producing various beta-lactamases (ESBLs, carbapenemases and MBLs) (Day et al., 2013; Novovic et al., 2015). Intrinsic resistance could also contribute to reduced susceptibility to the antibiotics.

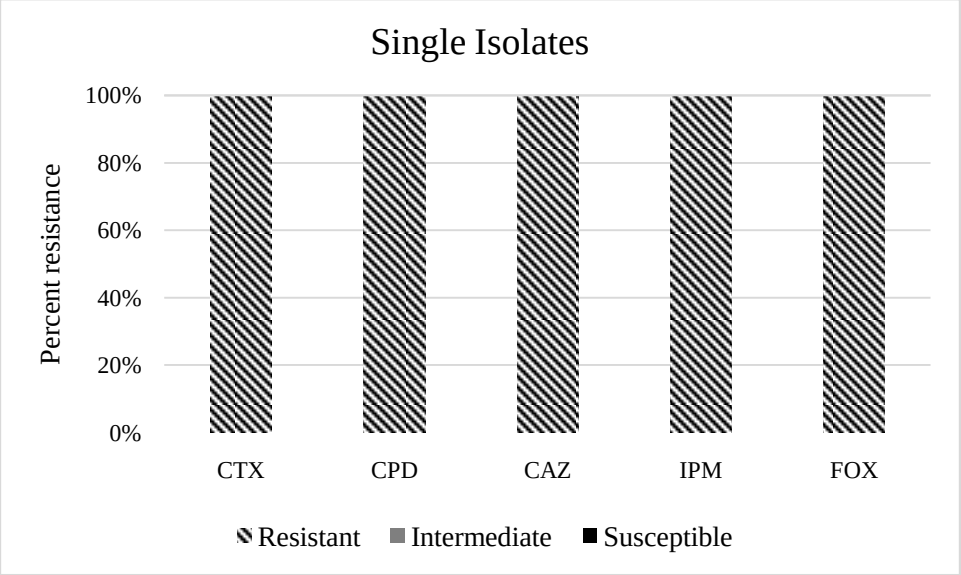


Figure 4.8 Screening results of all single isolates

The figure shows antibiogram of all single isolates of *Bacillus* sp., *Proteus* sp., *Stenotrophomonas* sp., *Morganella* sp., *Rheinheimera* sp. and *Shigella* sp. To cefotaxime,cefpodoxime and ceftazidime all of the strains were resistant. To imipenem and cefoxitin, *Bacillus* sp. was of intermediate resistance and *Rheinheimera* sp. was susceptible. To cefoxitin only *Rheinheimera* sp. was susceptible.

Third generation cephalosporins (CTX, CAZ), imipenem and cefoxitin resistant *Shigella* sp. was reported by Prabhuranjeshwar et al., (2015) and Tajbakhsh et al., (2012) isolated from clinical settings. The genus is not very often reported to be an ESBL producer(O'Hara et al., 1998; Ahamed and Kandu, 1999 and Pai et al., 2001). Gram negative bacteria often resist b-lactams by reducing porin expression or adopting other intrinsic resistance mechanism (Cohen et al., 1992).

B. zhanghouensis was the only gram positive specie among the tested strains. There was lack of CLSI inhibition zone criteria, therefore inhibition zone criteria of *Staphylococcus aureus* (gram positive)was used for its interpretation. The specie was first isolated from water and sediment in China by Liu et al., (2016). There was lack of

literature on antibiotic resistance of *B. zhanghouensis*, but the genus is known to resist b-lactams by undergoing mutations in penicillin binding proteins, a type of intrinsic resistance in both gram positive and gram negative bacteria (Giles and Reynolds, 1979; Buchanan and Strominger, 1976).

P. mirabilis was found to be resistant to all of the tested antibiotics. Kanayama et al., (2015) reported cefotaxime, ceftazidime and cefoxitin resistant strains of *P. mirabilis* from various hospitals in Japan. The genus has widely been reported to have reduced susceptibility to cephalosporins by generation of beta-lactamases (Kanayama et al., 2015; Pitout et al., 2009; Perez-perez and Hanson, 2002; Cheng et al., 2009; El-Hady and Adel, 2015). Imipenem resistance in *P. mirabilis* has been reported by Neuwirth et al., (1995) from clinical isolates in France. The genus has been identified to resist the carbapenems due to alteration in outer membrane/porins and mutations in penicillin binding proteins (Mehtar et al., 1991; Neuwirth et al., 1995). There are also reports of carbapaenem resistant *P. mirabilis* producing chromosome mediated carbapenemases (Bonnet et al., 2002).

The single isolate of *S. pavanii* was found to be resistant to all of the tested antibiotics. *S. pavanii* is not normally found in aquatic environment (Harmon et al., 2018). *S. pavanii* was first isolated from sugarcane stem. Its ecology and pathogenicity is not yet properly understood (Ramos et al., 2011). Le et al., (2016) was able to isolate ceftazidime resistant *S. pavanii* from hospital wastewater. Cefotaxime and imipenem resistant *S. pavanii* were also isolated from water bodies in Los Angeles, by Harmon et al., (2018). Resistance to b-lactams in the specie can be attributed to beta-lactamase (class A and B) production, and efflux pump systems (Kenzaka et al., 2018). No reports of cefoxitin *S. pavanii* were found but other species of the genus have been re-

ported to produce cephalosporinases that hydrolyse cefoxitin plus other cephalosporins (Walsh et al., 1997).

*M. morganii sub*sp. *Sibonii* showed resistance to all of the tested antibiotics. Kumarasamy et al., (2010) identified a *M. morganii* strain among clinical test strains which was resistant to all the above antibiotics. The strain is naturally an AmpC producer, the gene responsible for it lies on chromosome of the specie (Poirel et al., 1999). AmpC producer strains are able to hydrolyse third generation cephalosporins. Resistance to cephalosporins, especially third generation cephalosporins is known to be because of the production of beta-lactamases. Resistance to third generation cephalosporins antibiotics is directly linked with their wider use (Jones et al., 1998). *M. morganii* resistant to cefoxitin and imipenem could be carbapenemase producer which hydrolyses most of the b-lactams (Kumarasamy et al., 2010). Other than that gram negative bacteria resist b-lactams by losing or reducing porin expression and via mutations in penicillin binding protein (Jones, 1998).

R. tangshanensis was resistant to cefotaxime, cefpodoxime and ceftazidime and sensitive to imipenm and cefoxitin. The strain was isolated from rice roots in China (Zhang et al., 2008). Draft genome sequencing of the specie isolated from fresh water lake in Canada revealed that it had determinants for b-lactam resistance and carried a multidrug RND efflux system as a resistance mechanism (O'connor et al., 2015).

4.3 ESBL confirmation (combination disk test)

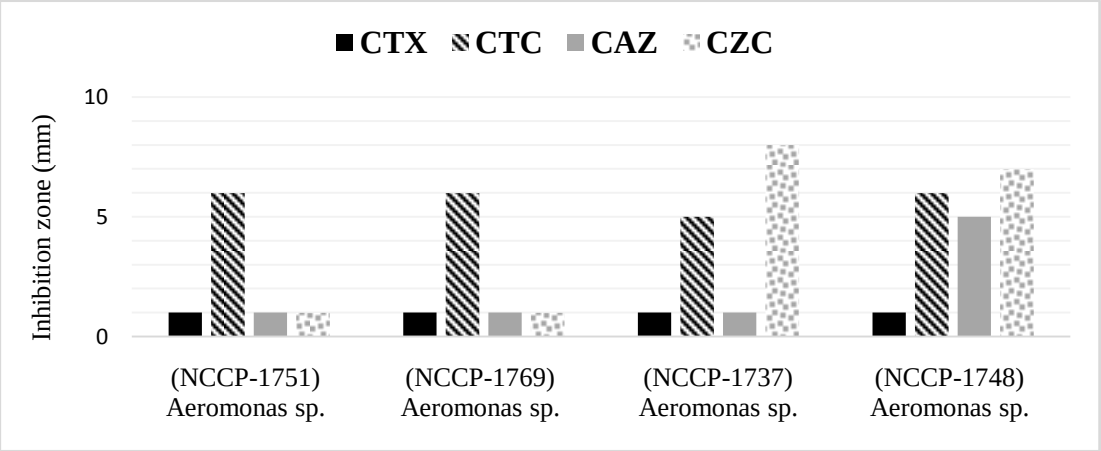


Figure 4.9 Confirmation of species for ESBL production

The figure shows results of the confirmation test of combination disk test for ESBL production among the screened strains. The strains had a difference equal to or more than 5 mm between the antibiotic (CTX, CAZ) and antibiotic plus clavulanic acid (CTC, CZC). According to this confirmation test out of 60 strains only 6.6% were ESBL producers. All of the producer strains were from genus *Aeromonas.* Among these strains three were isolated from the samples taken from Faisalabad and one was isolated from samples taken from Nullahlai. The method used here is suggested for detection of ESBLs in *Enterobacteriaceae* by CLSI but it is widely used by researchers for testing other species as well (Wu et al., 2011). Genes encoding ESBLs are mobile and hence can be transferred via conjugation to other pathogenic or environmental bacteria (Maravic et al., 2015). ESBL producing bacteria can only hydrolyse broad spectrum 3rd generation antibiotics like ceftazidime, cefpodozime and cefotaxime (Poirel et al., 2012). Production of ESBL is not an intrinsic character of *Aeromonas* spp. and is not only reported in clinical but environmental *Aeromonas* spp. isolates as well and are reported worldwide (Lu et al., 2010; Harnisz and Tucholski, 2010; Figueira et al., 2011; Harnisz and Korzeniewska, 2018). All of the ESBL pro-

ducers were highly resistant to the 3rdgeneration cephalosporins as was expected of them. The strains were also resistant to imipenem and cefoxitin which indicates towards more than one resistance mechanism. This could be explained by if they have an intrinsic resistance mechanism as *Aeromonas* spp. are known to intrinsically resist b-lactams by expression of chromosomally encoded beta-lactamases and efflux pump (Hernould et al., 2008).

4.4 ESBL confirmation (Double disk synergy test)

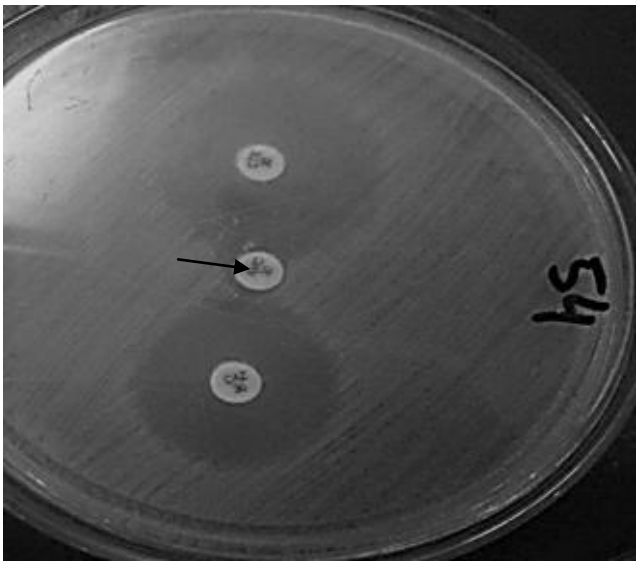


Figure 4.10 Confirmation of species for ESBL production

The figure shows results of double disk synergy test. The isolates were confirmed for ESBL production by two methods. Only one strain, (NCCP-1754) *Escherichia* sp.that tested negative by combination disk test proved to be a false negative and turned out to be an ESBL producer as shown in the picture by production of synergistic zone between Cephalosporins (3rd generation) and AMC. The strain was isolated from samples taken from Faisalabad. ESBL producing *E. coli* were detected by Korzeniewska et al., (2013) from hospital and municipal sewage and by Maravic´ et al., (2014) from marine beach water samples. ESBL producing *E. coli* are a major threat to environ-

ment. These species play their role as opportunistic pathogens and are known to cause intestinal and extraintestinal tract related infections (Donnenberg, 2002) and even if they are non-pathogenic, they are commensal bacteria and may come in contact with humans and animals via various routes (Blaak et al., 2014; Korzeniewska et al., 2013).The development of ESBL producing *Escherichia* sp. is linked with the vast use of cephalosporin antibiotics (Hu et al., 2013).

4.5 AmpC confirmation

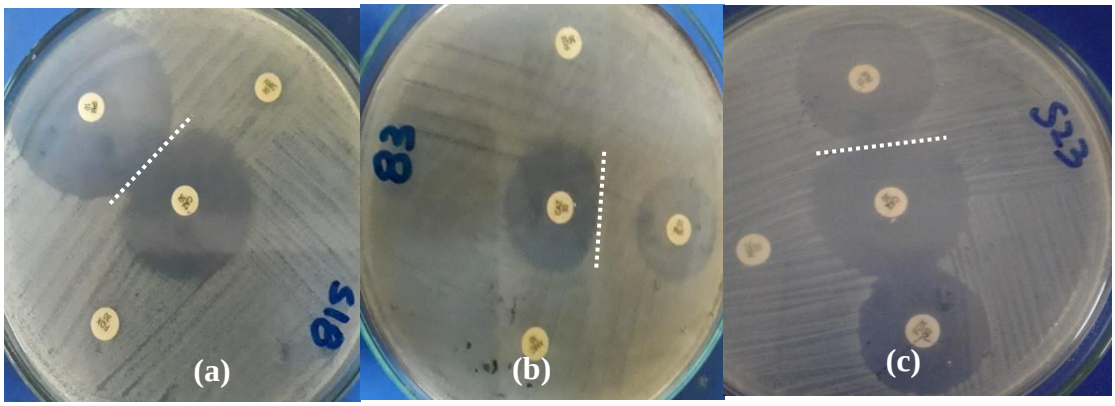


Figure 4.11 Confirmation of (a) (NCP-1791) *Morganella* sp.(b) (NCCP-1783) *Pseus-domnas* sp.and (c)(NCCP-1788) *Pseudomonas* sp. for production of AmpC.

The figure shows that the strains are AmpC (class C beta-lactamases) producers which is indicated by the flattening of inhibition zone of ceftazidime (black dotted line) towards the inhibition zone of imipenem. *Morganella* sp. are commensal gut bacteria of humans and are also opportunistic pathogens and may for example cause urinary tract infections (Dworkin and falkow, 2006). It has been established that the genes responsible for AmpC production are chromosomally mediated in *Morganella* sp.but there are reports of plasmid mediated AmpC genes in this specie. Their derep-ression causes *M. morganii* to resist third generation cephalosporins as well. Bernaud et al., (1997) detected AmpC enzyme in cefoxitin resistant clinical isolate.

Some of the *Pseudomonas* spp. known to be a nosocomial pathogen and can have both acquired and intrinsic resistance mechanisms. Clinical isolates of *P. aeruguinosa* from New York, USA, proved to be hyper-producers of AmpCs. Its resistance to a multitude of antibiotics was explained to be because of loss of porins, over-expression of efflux pump and AmpC production (Quale et al., 2006). *P. aeruguinosa* strains were isolated from river water and reported to be AmpC producers (Sreeshma et al., 2015). The genus Pseudomonas is known to be prevalent in water and soil environment and hence under the influence of antibiotic stress it can acquire various resistance determinants. Clinical *Pseudomonas* spp. strains have been reported to be AmpC producers (Manchanda and Singh, 2003).

4.6 MBL Confirmation

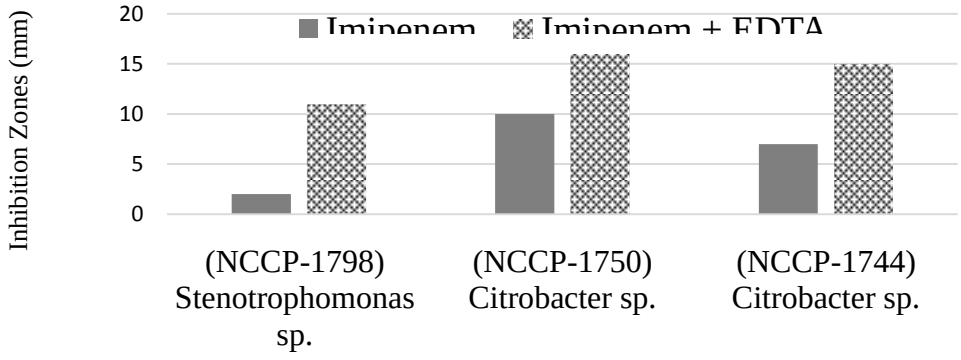


Figure 4.12 Confirmation of *Stenotrophomonas* sp.and *Citrobacter* spp.

The figure shows the inhibition zone difference for imipenem and imipenem plus EDTA of producer strains. The strains with inhibition zone difference of 6 or more than 6 mm were considered to be MBL producers. Only three producer strains were identified in this confirmation test, which were; one *Stenotrophomonas*sp.and two *Citrobacter* sp.An MBL producer *S. pavanii* was also isolated by Kenzaka and Tani, (2018)from feces of a migratory bird. It is thought that there is a greater risk of chro-

mosome mediated MBL production in *Stenotrophomonas maltophilia*, and that they are universally resistant to imipenem (Rasmussen & Bush, 1997).

MBL producer clinical isolates of *Citrobacter* spp.were also reported by Ho et al., (2012); Al-bahry et al., (2011) and Rizvi et al., (2009). Although Citrobacter spp. are not nosocomial pathogens but they still cause a variety of infections which include, meningitis, brain abscess, urinary tract infections and others (Pepperlle et al., 2002). The presence of MBL producers in aquatic environment is concerning as they cause resistance to carbapenems which are our last resort antibiotics especially in ICUs (intensive care units) and high risk wards (Gupta et al., 2005). Also, some of the genes encoding MBL are plasmid borne and hence are mobile, so this way they can be transferred to other bacteria (Carnaglia et al., 2011).

4.7 Antibiotic Resistance Genes

Among sixty test strains only three were positive for the ARGs investigated. The obtained sequences of the amplicons after sequencing were run on NCBI, Nucleotide Blast, BlastN algorithm and were found to be 100% and 99% match for *qnrS2* and *blaTEM-1* respectively.

The gene *qnrS2* was carried by only one strain; (NCCP-1737) *Aeromonas* sp.which was previously isolated from the water samples taken from Nullah Lai. Nullah Lai is a catchment basin for Marglla Hills, Islmabad and its various tributaries pass through Rawalpindi city and Islamabad. The stream is more than often subject to anthropogenic influence and hence is heavily polluted (Mustafa, 2005). There are reports of *qnrS2* gene presence in *Aeromonas* spp. isolated from environmental samples. (Cattoir et al., 2008; Picao et al., 2008; Marti & Balcazar, 2012; Varela et al., 2016 & Kim et al., 2017). Studies suggest that *qnrS*, specifically *qnrS2* allel is more commonly

found in environmental settings and is more than often carried by *Aeromonas* spp.(Poirel et al., 2012; Picao et al., 2008).Quinolones are known to persist in environment even more than beta-lactams and force selection of quinolone resistance determinants (Cattoir et al., 2008). The strain of *Aeromonas* sp.in this study had reduced susceptibility to flouroquinolones, although *qnrS2* is known to confer only low level of resistance but it does favour and complement selection of other resistance mechanisms and provide a favourable background for greater resistance at quinolone concentrations that would be fatal in its absence through other resistance mechanisms (Jacoby, 2005).

Mutations in the housekeeping genes, gyrA, and ParC are primarily known to cause resistance to these antibiotics. Other resistance mechanisms reported in *Aeromonadaceae* for flouroquinolones are; mutations in porins and over expressed efflux pumps to decrease the intracellular concentration of antibiotics (Ruiz, 2003). The progenitor of *qnrS2* gene is known to be an environmental bacteria, *Vibrio splendidus.* Their selection is driven by the presence of quinolones in water environment. The *qnrS* carrier strain in this study had ESBL phenotype, although the encoding gene couldn't be identified, the fact is worrisome because it means that they could be transferred simultaneously. If the genes are located on single plasmid selection pressure from one antibiotic could transfer resistance to another antibiotic as well (Henriques et al., 2006). Zurfluh et al., (2014) reported *Aeromonas* spp. with ESBL phenotype to be carriers of *qnrS*. In a study carried out by Winokur et al., (2001) with clinical strains obtained from worldwide, it was observed that ESBL phenotypes were commonly co-resistant to fluoroquinolones, aminoglycosides, tetracyclines and sulfamethoxazole/trimethoprime combination. According to Carattoli, (2011) and Coque et al., (2008), plasmid carrying genes are capable of conjugation and often catch resistance

determinants to other non-beta-lactam antibiotics like fluoroquinolones, aminoglycosides and tetracyclines. However, since we investigated for only two resistance genes when there are various other resistance mechanisms and variants of the encoding genes which could confer resistance to the tested antibiotics we can't establish a relationship between qnr- positive and ESBL positive strains (Henriques et al., 2006). Further, the presence of *qnrS2* in *Aeromonas* spp. and other water borne bacteria strengthens their role in dissemination of ARGs in aquatic environment (Cattoir et al., 2008).

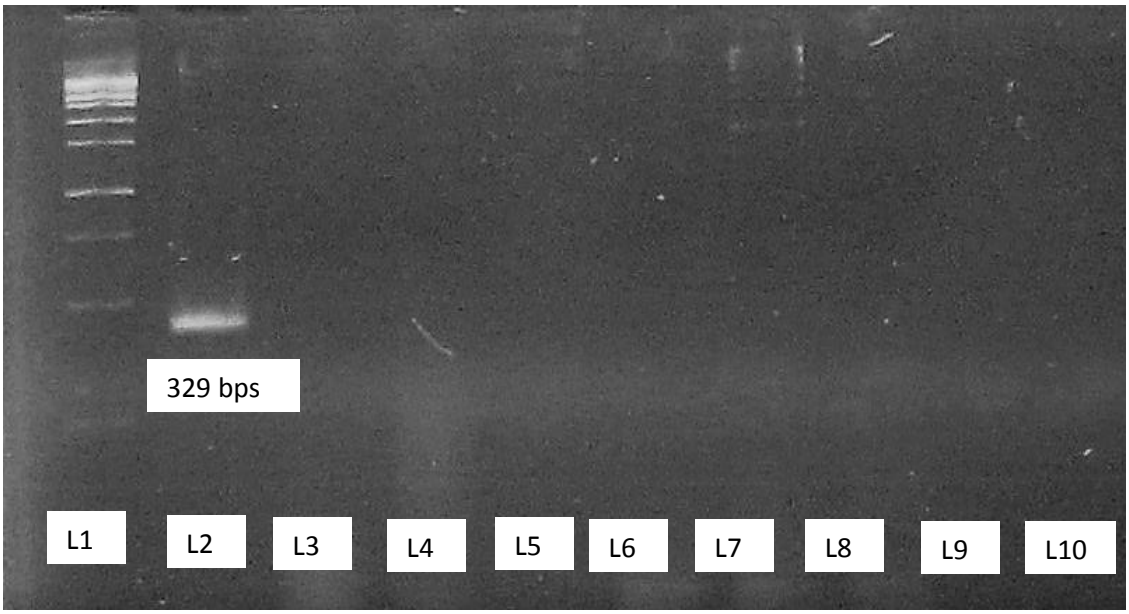
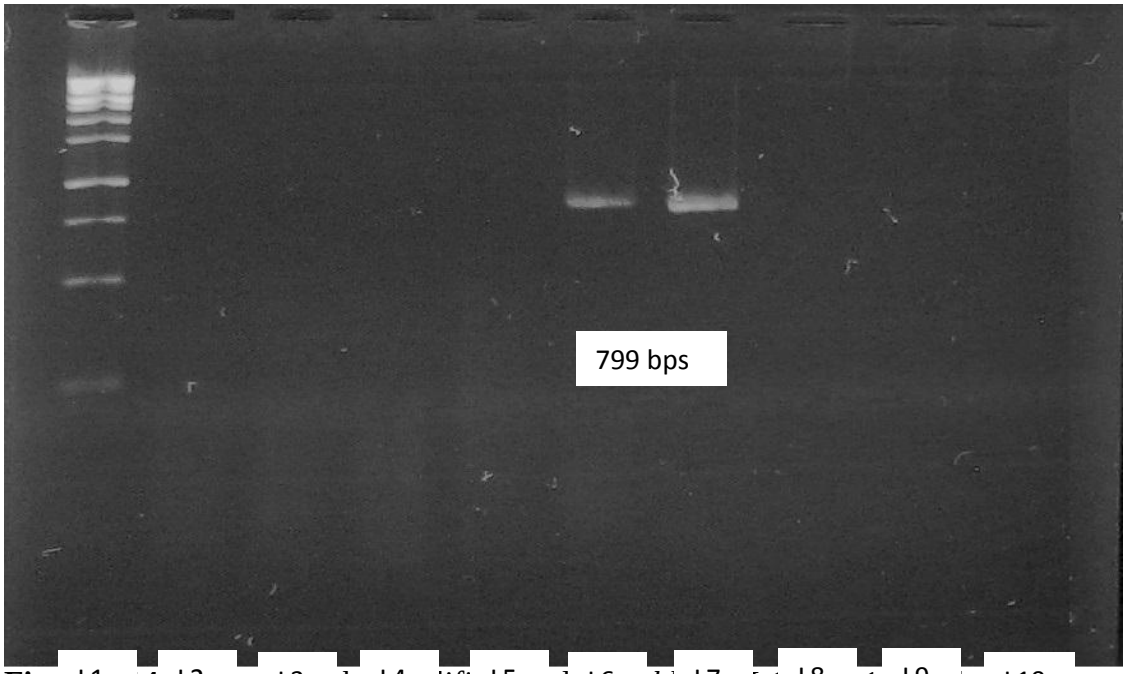


Figure 4.13 Gel image of the amplified product of *qnrS2*. Lane 1 (Ladder, 1kb), Lane 2 (*Aeromonas sanarelli*), Lane 3, 4, 5, 6, 7, 8 and 9 (other test strains), Lane 10 (negative control)

The gene *blaTEM-1* was identified in two strains with an ESBL phenotype; (NCCP-69) *Aeromonas* sp. and (NCCP-1754) *Escherichia* sp.Both of these strains were isolated from the water samples taken from Faisalabad. Specifically, the samples were taken from a wastewater stream in a living compound (Peoples Colony) subject to heavy anthropogenic influence. The presence of bacteria harbouring *blaTEM-1* is not

surprising as penicillins/cephalosporins are commonly prescribed and their residual concentration in the natural environment could cause selection of the ARG. The gene *blaTEM-1* is more of clinical relevance and its presence indicates anthropogenic antibiotic resistance contamination (Lachmayr et al., 2009; Waldhagen et al., 2003). On the other hand, natural environments are discussed for their potential to be reservoirs of ARGs (Ash et al., 2008). The ARG has been reported in various*Aeromonas* species isolated from clinical settings, environmental samples and fresh water animals (Marchandin et al., 2002; Henriques et al., 2006; Deng et al., 2014; Roch et al., 2014). Although the *Aeromonas* spp. may carry *blaTEM* genes but their common hosts are *Enterobacteriaceae* (Rocha et al., 2014). The gene *blaTEM-1* is known to cause resistance to penicillin and narrow spectrum cephalosporins but point mutations in it when subjected antibiotic stress could cause to provide resistance against broad spectrum cephalosporins as well (Waldhagen et al., 2003). Although *blaTEM-1* is a non-ESBL enzyme it's not uncommon for the ESBL producers to carry a *blaTEM-1* gene as they have previously been reported to have ESBL properties (Diwan et al., 2010). There are reports of the *Escherichia* sp.isolated from environmental samples and poultry/cattle animals to harbour *blaTEM-1* gene (Singh et al., 2018; Afsharnia et al., 2018; Hemeg, 2018; Feng et al., 2018; Kar et al., 2105). *Escherichia coli* are the most common hosts of ESBL encoding genes, among them TEM, CTX-M and SHV type ESBLs are highly prevalent and are also clinically more relevant (Valverde et al., 2004).



Figu L1 **.4** L2 ıa L3 th L4 lifi L5 ɔd L6 *bl* L7 *-1* L8 :1 L9 le1 L10

Lane 2, 3, 4 and 5 (other test strains), Lane 6 (*Aeromonas taiwanensis*), Lane 7 (*Escherichia coli*), Lane 8, 9 (other test strains) Lane 10 (negative control).

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CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Keeping in mind the objectives and results of the study it can be concluded that:

- That the average MICs were higher for penicllins than flluoroquinolones. AMP>AMX>CIP>OFX>LEV had the highest tested MICs.
- And that most of the strains were resistant to third generation cephalosporins which included; cefpodoxime (100%), cefotaxime (88.4%) and ceftazidime (91.6%).
- To imipenem and cefoxitin, 61.6% and 78.3% of the strains were resistant respectively.
- Among the 60 strains, five were (8.3%) were ESBL producers in which three were *Aeromonas* spp. and one *Escherichia* sp.
- Three strains (5%) proved to be MBL producers which included one *Stenotrophomonas* sp.and two *Citrobacter* spp.
- There were three (5%) AmpC producers which includedone *Pseudomonas aeruginosa*, one *Pseudomonas* sp. and one *Morganella* sp.
- It was also found out via PCR that two of the ESBL producers (*Escherichia* sp.and *Aeromonas* sp.) carried a *blaTEM-1* gene and only one ESBL phenotype (*Aeromonas* sp.) carried the gene *qnrS2*.

The bacterial community included in this study proved to be highly resistant to the tested five antibiotics and had high MICs for some other antibiotics. Among all of the genera *Aeromonas* stood out,as most of the ESBL producers were from this genus and two of the ARG carriers also were detected among them. *Aero-*

*monas*species in the aquatic environment are considered to be aiding in the spread of ARGs to potential pathogens.

5.2 Recommendations

- The study gave an insight into the molecular structure of antibiotic resistance in our aquatic environment but to conclude something solid it needs to be carried out on a large scale to have a better understanding of the situation.
- The environmental bacterial flora should be monitored for novel resistance mechanisms or resistance genes that could become clinically significant in the future.
- There is a need to study the acquisition potential of the ARGs at a molecular level in various sectors of our environment which would help in quantifying the dissemination risk of these ARGs.
- Quantification of the ARGs in aquatic environment would shed light on it as a reservoir of the resistance determinants.
- The strains in which the resistance determinants were detected should be investigated for pathogenic genes, as *E. coli* and *Aeromonas* spp.are associated with various community acquired and nosocomial infections.
- Novel tools such as metagenomic analysis may be used which would give much more information than the typical molecular and phenotypic tests.

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