SCREENING OF MICROBES ISOLATED FROM ENVIRONMENTAL SAMPLES FOR ANTIBIOTIC RESISTANCE

Marium Fiaz

00000117403

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

(2015-2017)

SCREENING OF MICROBES ISOLATED FROM ENVIRONMENTAL SAMPLES FOR ANTIBIOTIC RESISTANCE

By

Marium Fiaz

00000117403

A thesis submitted in partial fulfillment 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

(2015-2017)

CERTIFICATE

Certified that the contents and form of the thesis entitled "**Screening of microbes isolated from environmental samples for antibiotic resistance**" submitted by Ms. Marium Fiaz has been found satisfactory for partial fulfillment of the requirements of the degree of Master of Science in Environmental Science.

Supervisor: ....

Dr. Muhammad Arshad

Associate Professor

IESE, SCEE, NUST

GEC Member: ....

Dr. Imran Hashmi

Professor

IESE, SCEE, NUST

GEC Member: ....

Dr. Iftikhar Ahmed

Principal Scientific Officer

NARC, Islamabad

I dedicate this thesis to my beloved parents to whom I owe all my success and fulfilments in life

ACKNOWLEDGEMENTS

This piece of work would not have been accomplished without the blessings, wisdom and power given to me by Allah Almighty.

I would like to express my sincerest gratitude to my supervisor, ***Dr. Muhammad Arshad*** for accepting me into his research group. Without his encouragement and efforts, this thesis would not have been completed. One simply could not wish for a better supervisor. I am grateful to my GEC member ***Dr. Iftikhar Ahmed*** for his guidance. I greatly benefited from his keen scientific insight and without his efforts my job would have undoubtedly been more difficult. I am thankful to ***Dr. Imran Hashmi*** in particular for his valuable suggestions and recommendations. I am thankful to Ms. Maria Manzoor for guiding me throughout my research work. I was fortunate to have the chance to work with Ms. Rabaila Riaz (NARC) and highly collaborative and supportive team at National Agriculture Research Institute, Islamabad, Pakistan. I would also like to thank Higher Education Commission Pakistan for the funding provided under project no. NPRU-4155 that allowed me to complete this research. I would like to acknowledge the efforts of Brig. (R) Arif Siddique for facilitating me at every step during this research period.

Finally, I owe a debt of gratitude to my precious parents, my beloved grandmother and my dearest uncle and aunt for their constant love and support. My friends especially Asadullah Shoaib, Zara Maqsood, Awais Javaid, Tehreem Mustansar, Rabia Akmal, Zunaira Jabeen, Maryam Sarfaraz and Amrah Qureshi for their friendship, love, and support. Last, but definitely not the least, my deepest thank to all laboratory staff at IESE and NARC for their help and cooperation.

May the Almighty Allah richly bless you all.

Marium Fiaz

TABLE OF CONTENTS

LIST OF ABBREVIATIONS..ix
LIST OF TABLES..x
LIST OF FIGURES..xi
ABSTRACT..xii
Chapter 1..1
INTRODUCTION..1
1.1 Background..1
1.1.1 Pharmaceutical industry in Pakistan..2
1.1.2 Usage of antibiotics..3
1.2 Problem statement..4
1.3 Significance of the study..4
1.4 Objectives of study..4
Chapter 2..5
LITERATURE REVIEW..5
2.1. Discovery and development of antibiotics..5
2.2 Classification of antibacterial drugs..6
2.3 Development of antibiotic resistance..7
2.4. Mechanism of antibiotic resistance..8
2.4.1. Mutational resistance..9
2.4.1.1 Alteration of antibiotic molecule..9
a) Chemical modification of the antibiotic..9
b) Destruction of the antibiotic molecule..9
2.4.1.2. Decreased penetration of antibiotic and efflux..10
a) Reduced permeability/uptake..10
b) Efflux pumps..11
2.4.1.3. Changes in target sites..11
a) Target protection..11
b) Modification of the target site..11
c) Mutations of the target site..12
d) Replacement or bypass of the target site..12
2.4.1.4. Resistance due to cell adaptations..12
2.5. Spread of antibiotic resistance in environment..12

2.6. Effects of antibiotic resistance......15
Chapter 3......17
MATERIALS AND METHODS......17
3.1. Sample collection......17
3.2. Sample processing......18
3.3. Isolation of bacterial strains......18
3.4. Characterization of isolated strains......18
3.5. Preparation of glycerol stocks......19
3.6. Identification of strains......19
3.6.1. DNA extraction & amplification......19
3.6.2 Agarose gel electrophoresis......20
3.7. Antimicrobial resistance testing......21
3.7.1 Selection of antibiotics......21
3.7.2 Preparation of Muller Hinton agar plates......22
3.7.3. Preparation of broth culture......22
3.7.4. Disc diffusion assay......23
Chapter 4......24
RESULTS AND DISCUSSION......24
4.1 Isolation of bacteria from wastewater samples......24
4.2. Frequency of bacteria isolated from each site......24
4.3. Characterization of isolated bacterial strains......24
4.4. Identification of isolates......27
4.5. Screening of antibiotic resistant bacteria......38
4.5.1. Ciprofloxacin resistance test results......38
4.5.2. Levofloxacin resistance test results......41
4.5.3. Ofloxacin resistance test results......42
4.5.4 Amoxicillin resistance test results......46
4.5.5. Ampicillin resistance test results......48
Chapter 5......54
CONCLUSIONS AND RECOMMENDATIONS......54
5.1 Conclusions......54
5.2. Recommendations......55
5.2.1. Track the resistance frequency......55
5.2.2. Regulation and introduction of new therapeutic approaches......55

5.2.3. Replacement of antibiotics..56
REFERENCES..57
APPENDICES..69

LIST OF ABBREVIATIONS

Abbreviations	Description
ARGs	Antibiotic resistant genes
CLSI	Clinical and Laboratory Standard Institute
Mm	Milli meter
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
Rpm	Revolutions per minute
TBE	Tris borate EDTA
TE	Tris EDTA
UV	Ultraviolet
WHO	World Health Organization
Mg	Micro gram
µL	Micro liter

LIST OF TABLES

Table 3.1. Location of the sampling sites……………….……………………………..…17

Table 3.2. Reagents used for PCR …………………..………………………………….……....19

Table 3.3. PCR profile for 16S rRNA gene….……….……………..………..……......…20

Table 3.4. Dosage of selected antibiotics (μg) for disc diffusion test……….……….…...22

Table 4.1. Identification of strains based on 16S rRNA gene sequencing and their accession numbers published in DNA data base………………………...……………………..……………31

Table 4.2. Susceptibility and resistance percentages of different microbial genera against tested antibiotics..…………………………………………………………..…………...52

A1. Survey questions for the most commonly prescribed antibiotics……………………......70

B1. Morphological characterization of isolates………………………..…………….…………73

B2. Biochemical characteristics of all isolated bacterial species……...……………...……....74

LIST OF FIGURES

Figure 1.1. Schematic diagram showing possible sources and pathways for occurrence of pharmaceutical residues in environment…………………….……………………......……1
Figure 1.2. Spread of antibiotic resistance……………………………..……………..….…4
Figure 2.1. Timeline of antibiotics discovery……………………………………….…...……6
Figure 2.2. Mechanism of action of antibiotics……………………………………...…...7
Figure 4.1. Frequency of bacterial isolates………………………………………........…24
Figure 4.2. Gram staining of isolates collected from environmental samples…....……...…25
Figure 4.3. Biochemical characteristics of isolated species……………………………..…..26
Figure 4.4. Molecular Phylogenetic analysis of isolates by maximum likelihood method…29
Figure 4.5. Biodiversity of isolated strains. a) Diversity pie chart of isolated strains b) Bar graph representing the percent similarity range of identified strains with the known closely related species of the respective genera……………………………………...….……..30
Figure 4.6. Bacterial isolate showing complete resistance to all 5 tested antibiotics………..38
Figure 4.7. Resistance pattern of different microbial genera against ciprofloxacin…...……40
Figure 4.8. Resistance test results of all isolated bacterial species against levofloxacin..…...43
Figure 15. Resistance test results of all bacterial isolates against ofloxacin. ………….....…45
Figure 16. Resistance test results of all bacterial isolates against amoxicillin. ………….…47
Figure17. Resistance test results of all bacterial isolates against ampicillin…………..……49
Figure 18. Antibiotic resistance test results of 109 isolates against selected antibiotics…….51
C1. Agarose gel electrophoresis of 16S rRNA amplified gene……………………..........76-78

ABSTRACT

Wide spread occurrence of antibiotics and antibiotic resistant bacteria in environmental matrices is posing a serious threat to public health worldwide. Since the significance of bacterial isolates from environmental samples as a reservoir of antibiotic resistance is not well documented in Pakistan, therefore the aim of present study was to isolate and characterize bacterial strains from wastewater channels in vicinity of pharmaceutical industries and hospitals of Islamabad, Rawalpindi and Faisalabad, to inspect the presence of antibiotic resistant microorganisms. Antibiotic susceptibility of one hundred nine isolated strains was tested according to the Kirby–Bauer disc diffusion method against five most commonly used antibiotics. Results of this study indicated that amongst all tested antibiotics, Ampicillin and Levofloxacin resistance had the highest and lowest frequency, respectively. The resistance pattern observed was; Ampicillin (92.0%)> Amoxicillin (83.5%) > Ofloxacin (67.0%) > Ciprofloxacin (28.0%) > Levofloxacin (21.1%). Out of the tested strains, 30.3% showed resistance to more than three drugs. Among frequently isolated species, maximum resistance was observed in species from the genus *Escherichia* (57.1%), *Aeromonas* (56.3%), *Acinetobacter* (41.2%), *Shewanella* (25.0%), *Proteus* (14.0%) and *Pseudomonas* (8.3%) while among the less frequently isolated strains *Citrobacter* sp., *Comamonas* sp., *Bacillus* sp. and *Alishewanella* sp. showed 100% resistance to all the tested antibiotics. Incidence of Ciprofloxacin resistance in *Acinetobacter* sp., *Escherichia* sp. and *Aeromonas* sp. was higher than that of Levofloxacin resistance, while a large number of isolates showed intermediate resistance to Levofloxacin. The results of present study confirm presence of multidrug resistant isolates in wastewater streams of Pakistan which may lead to the proliferation of antibiotic resistance in pathogens and ultimately becoming the reason of treatment failures.

Chapter 1

INTRODUCTION

1.1. Background

Since last decade, contamination of aquatic environments with emerging environmental pollutants is considered to be a major environmental concern, because of their potential to cause detrimental effects on the ecosystem and public health (Huang *et al*., 2011; Kummerer, 2009). Among these, considerable attention has been given to pharmaceuticals and their residues, as they are highly biologically active compounds. Wastewater and manufacturing industries are continuously introducing the residues from human and animal antibiotic usage into the environment, which have impacts on the quality of water, human/animal health and the ecosystem (Yuan *et al*., 2009). Besides these residues, antibiotics have a massive share of attention in the whole world (Kummerer, 2009) for their unconstrained usage.

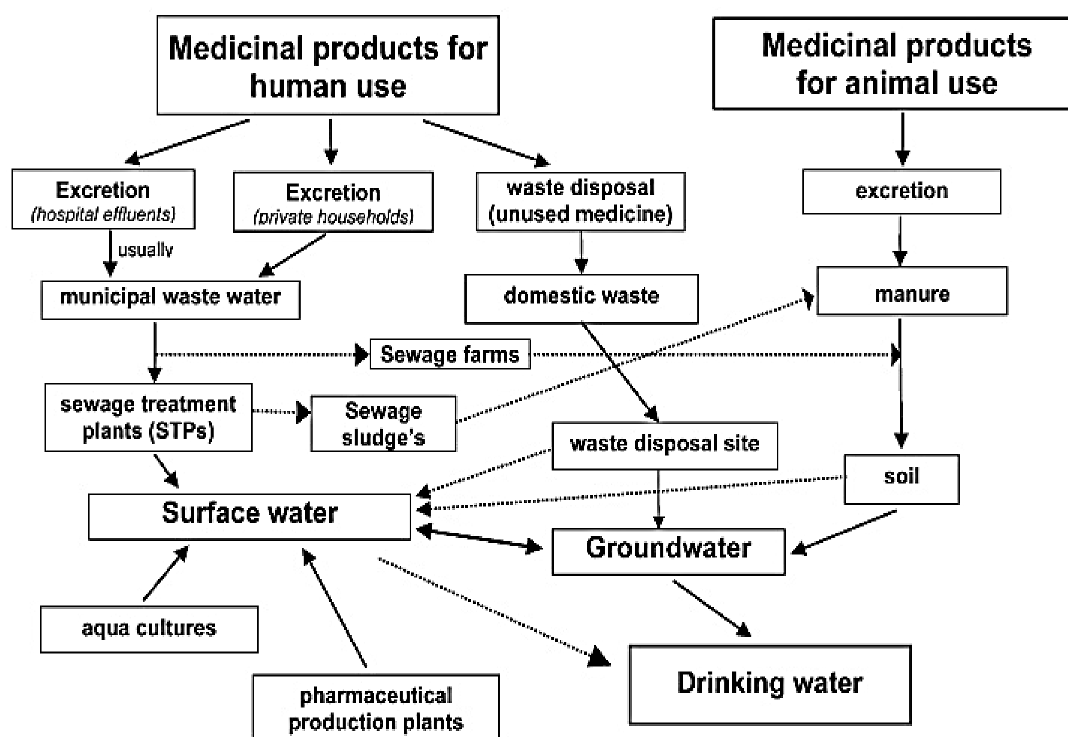


Figure 1.1. Schematic diagram showing possible sources and pathways for occurrence of pharmaceutical residues in environment.

Excessive utilization of antibiotics for the treatment of bacterial infections in humans, plants and animals are directly discharged into the aquatic environment in the form of their intact parent compounds via different pathways, including runoff from land to which agricultural or human waste has been applied, discharge of wastewater effluent and by leaching (Rehman *et al.,* 2015). It is responsible for increasing antibiotic resistance and upshot in the spread of antibiotic resistance genes in environment (Figure 1.1). The matter is intensified as most of the antibiotics are recalcitrant against different wastewater treatment procedures. They are not eliminated completely and are persistent in the environment, which can cause harmful effects on human and animal health (Wepener *et al*., 2001; Yadav *et al*., 2009).

1.1.1. Pharmaceutical industry in Pakistan

Pakistan's US$1.64 billion pharmaceutical market is the 10th largest in Asia Pacific. There are 316 manufacturing units in Pakistan and their share in the industry is 53% with production of US$1.0 billion pharmaceuticals while the share of multinational companies in the pharmaceutical industry of Pakistan is 47% (Zaman, 2011).

As the national manufacturing companies are taking over, about 80% need is fulfilled domestically while 20% are covered by imports. Raw materials are imported from China, India, Europe, North America and other countries. The pharmaceutical industry imports approximately 91% of all raw materials. The local availability of raw materials is restricted to the following active ingredients; Amoxacilin, Ampicillin, Aspirin, Ibuprofen, Ciprofloxacine, Cefixime, Cefadroxil, Cephalexin, Cefradine, Cloxacillin, Ephedrine, Ephedrine Sulphate, Flucloxacillin, Norfloxacillin, Paracetamol, Parabinez, Piperazine, Pyrazinamide and Santonin. These raw materials do not necessarily meet the entire need of the industry and a major portion of special grades is still imported (PPMA/Pharma Bureau-OCCI).

Figures of Pakistan Bureau of Statistics (PBS) showed that 5,611 tonnes of pharma products worth $103.3 million were exported in July-Dec 2015-16 (pbs.gov.pk). Increase in

exports of pharmaceuticals is 17% per year. Pakistan exports these pharmaceuticals mainly to Syria, Sri Lanka, and Singapore (European Commission–Trade related technical assistance program (TRTA) for Pakistan, 2007). Besides this growth, this industry is not complying with environmental standards, and discharging its effluent into the domestic wastewater networks.

1.1.2. Usage of antibiotics:

There are 47000 registered drugs in Pakistan (www.osec.ch) and the market growth is 14% per year by volume. The local consumption of pharmaceuticals in Pakistan is US$1 billion. Broad spectrum antibiotics like fluoroquinolones are most commonly consumed by humans (European Commission–Trade related technical assistance program (TRTA) for Pakistan, 2007).

Apart from human consumption, a large amount of antibiotics (mostly tetracyclines and fluoroquinolones) are used for livestock production to maintain health and promote growth, contributing to the spread of drug-resistant pathogens in both livestock and humans. According to an estimate in 2010, 29.8 mg per PCU (population correction unit) antimicrobials were consumed for livestock in Pakistan (Van *et al*., 2015). The use of fluoroquinolones in food producing animals mainly cattle and chicken (Moyane *et al.,* 2013) has resulted in the development of ciprofloxacin-resistant *Salmonella, Campylobacter* and *E. coli,* which have caused human infections that proved difficult to treat (WHO, 2014).

Antibiotics in wastewater effluent exert selective pressure in favor of resistant bacteria by completely eliminating or inhibiting the growth of susceptible bacteria; resistant bacteria can modify and acclimatize themselves to environmental conditions and serve as vectors for the spread of antibiotic resistance (Kruse, 1999) as depicted in Figure 1.2. The presence of antibiotic resistance in the environment can be genetically detected by the presence of antibiotic resistant genes (ARGs), which has been found in various aquatic environments (Zhang *et al*., 2009). The major probable risk is the dissemination of resistant genes from environmental

bacteria to human pathogen (Wegener *et al*., 1999). It has been proven that many antibiotics play a major part in the spread of antibiotic resistance in water ecosystem (Zhang *et al*., 2009; Pruden *et al*., 2006; Wu *et al*., 2010)

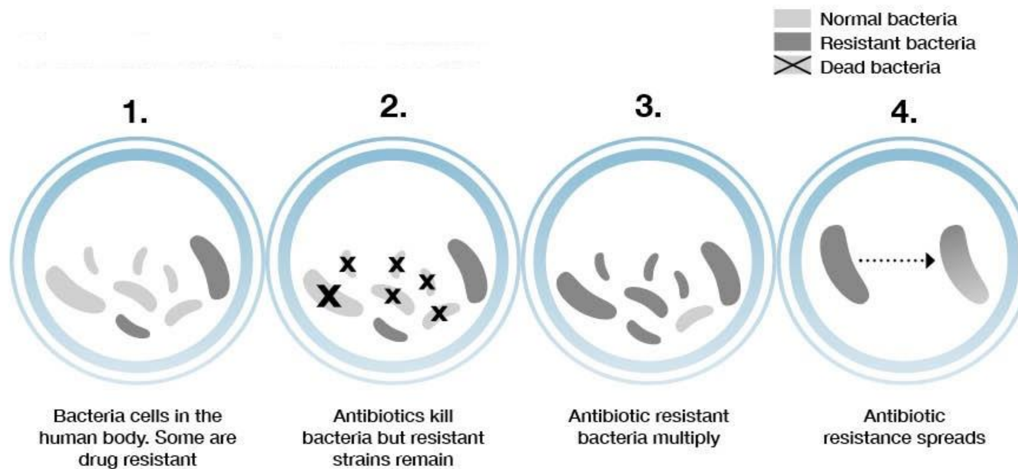


Figure 1.2. Spread of antibiotic resistance (www.gov.uk)

1.2. Problem statement

Presence of antibiotic resistant organisms in environmental samples is responsible for accelerated increase of antibiotic resistance in bacteria. This accelerated resistance results in decreased effectiveness of previously used drugs for treatment of infections thus causing a serious public health problem.

1.3. Significance of the study

A few studies from Pakistan have reported the importance of microbes isolated from wastewater samples and their role in spread of antibiotic resistance. Therefore, the aim of this study was to collect wastewater samples from different selected sites and inspect the prevalence of antibiotic resistance in microbes isolated from those samples.

1.4. Objectives of study

Keeping in view the recent literature survey and information presented above, the specific objectives of present study were;

- Characterization and identification of bacterial isolates
- Testing of isolates for resistance against five different antibiotics

Chapter 2

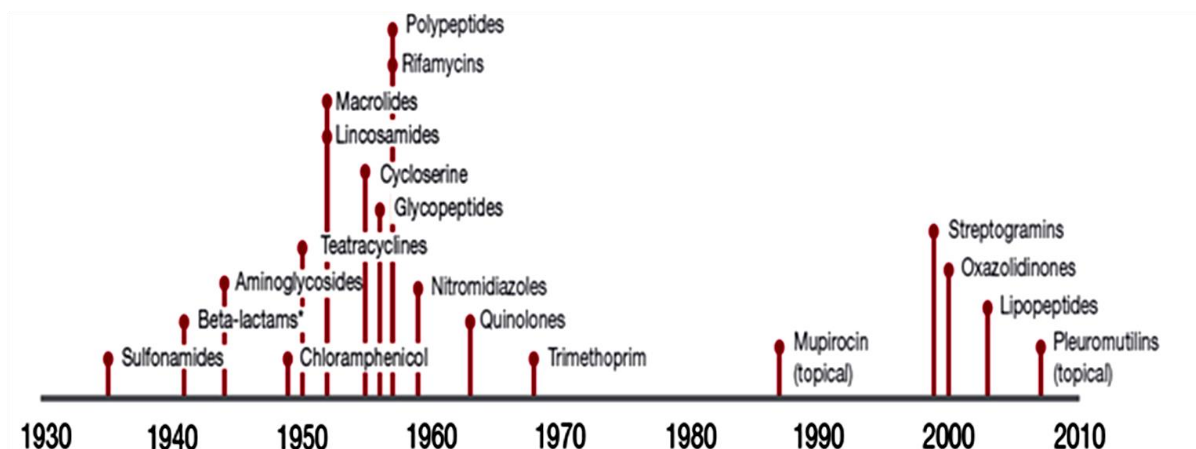
REVIEW OF LITERATURE

This chapter is structured to highlight the general background of antibiotic resistance development and how the microorganisms developed resistance against them. The resistance is mainly discussed against floroquinolones and penicillins. It also discusses the spread of resistance in the environment and environmental microorganisms and the consequent impacts on public health and the ecosystem.

2.1. Discovery and development of antibiotics

Throughout most of the human history, bacterial infections could only be treated with herbal therapies and folk medicines. These were often insufficient and led to serious illness and high mortality rates. Interest in antimicrobial therapy aroused by the revelations of Louis Pasteur and Robert Koch in the late nineteenth century about the existence of microbes and demonstration that they were responsible for most of the serious diseases including anthrax and cholera (Madigan *et al.,* 2006). This revolutionized the treatment of serious bacterial infections by providing an identified cause of disease and target for therapy. The revolution in the chemical industry with the interest to manufacture "magic bullets"–the chemicals that can selectively kill only infectious microbes (Strebhardt & Ullrich, 2008) and these don't harm the human body, led to the discoveries of first synthetic antimicrobial "sulphonamides" introduced in 1930s (Madigan *et al*., 2006). Second revolution in medicine came with the discovery of penicillin, an antimicrobial substance produced by microbes themselves, by Alexander Fleming (Ligon, 2004). The enormous use of penicillin during Second World War led to the interest in searching more natural antibiotics. Use of the whole cell anti-bacterial activity screening platform developed by Waksman (Kresge *et al.,* 2004) directed at a wide variety of fungi and bacteria, led to the "golden age" of antibiotic discovery in the 1950's when about

half of the antibiotics known today were found (Wright, 2007). Subsequently, much of the progress in drug development involved generating synthetic or semisynthetic derivatives of natural antibiotics, with better pharmacokinetic and pharmacodynamics properties, and improved spectrum of activity (Figure 2.1).



Beta-lactams include three groups sometimes identified as separate classes: penicillins, cephalosporins and carbapenems

Figure 2.1. Timeline of antibiotics discovery (Cruickshank, 2011)

2.2 Classification of antibacterial drugs

Antibiotics are generally classified into two categories. The first one includes synthetic drugs like quinolones and sulphonamides and the second category includes those derived from microorganisms such as penicillins. In the recent years the discovery of semi-synthetic drugs, has blurred the difference between natural and synthetic drugs. Antimicrobials either natural or synthetic work by targeting and disrupting the vital processes in the bacterial cells which are not present or different in mammalian cells.

Antibiotics can be grouped according to several different criteria: inhibitory effect, spectrum of activity, and molecular target. Some antimicrobial compounds are bactericidal at clinically used concentrations and thus capable of killing the infecting bacteria, whereas others are bacteriostatic, inhibiting the growth or reproduction of the bacterial cells. Some drugs are considered to have a broad spectrum of activity, and hence used against a wide range of Gram-positives and Gram-negatives, whereas others have a relatively narrow spectrum of clinical

activity. Antibacterial drugs also differ in their bacterial targets and mechanisms of action (Figure 2.2). Important targets for clinical antibacterial drugs include cell wall biosynthesis and membrane integrity, folic acid metabolism, protein synthesis, and DNA replication and transcription.

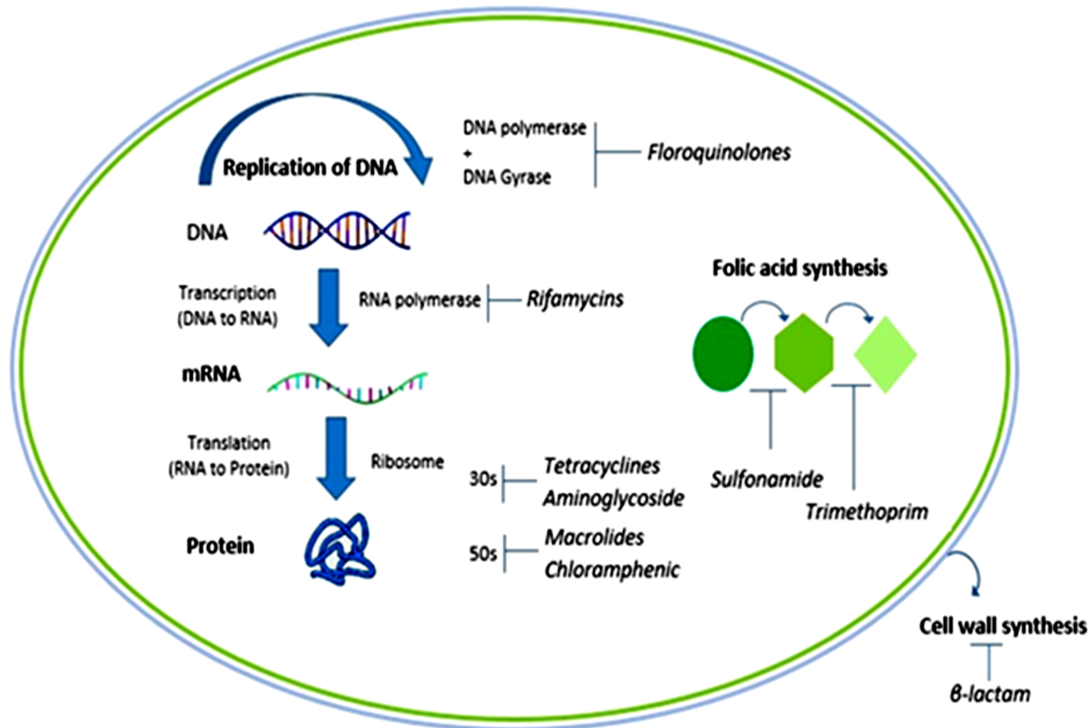


Figure 2.2. Mechanism of action of antibiotics

2.3 Development of antibiotic resistance

Antibiotics are produced by environmental bacteria and antibiotic resistance occurred long before humans started to use antibiotics for treatment of infections. The antibiotic producing bacteria and those susceptible to them co-existed in their own natural habitats without facilitating the selection of deadly resistant human pathogens so that those remained susceptible to most antibiotics found in nature (Sengupta *et al*. 2013). Once humans started to use high antibiotic concentrations in the clinical settings more than 70 years ago, led to the selection of highly resistant strains and resistance in human pathogens. With the selective pressure those bacteria were selected which had either acquired resistance from the microbiota

present in environment via horizontal gene transfer, or evolved their resistant phenotype by *de novo* mutations, which transformed them into drug resistant pathogens (Martinez, 2009).

As all major human pathogens were initially susceptible to antibiotics, the effectiveness of therapy in 1930s was remarkably successful and led to a significant decrease in human morbidity and mortality (Martinez, 2009). Even though antibiotic resistance emergence was soon recognized after the discovery of penicillin but the innovation and discovery of new drug derivatives of higher potency in 1940's to 1960's, did not let this resistance *per se* to rule out the antibiotic therapy as the development of new antibiotics kept pace with the resistance emergence. Therefore, the emergence of resistant bacteria did not raise major concerns. On the contrary, the initial efficacy of antibiotics let to the misbelief that the problem of bacterial infections has been overcomed and will be soon eradicated (Aminov, 2009). This led to the "innovation gap", a period of almost 40 years between the mid-1960s and 2000 when no new class of antibiotics was introduced. Thus the infections caused by multidrug-resistant pathogens became inevitable and the available antimicrobials started losing efficacy.

Currently multidrug resistance has spread all over the world, and makes treatment of resistant bacteria difficult, and requires multiple, sometimes six to seven different drugs to treat infections (Fair *et al*., 2014).

2.4. Mechanism of antibiotic resistance

Bacteria possess an exceptional genetic flexibility due to which they have the ability to respond to any threat to their existence in the environment including antibiotics. Bacteria that share the same ecological niche develop ability and strategies to survive in the presence of harmful antimicrobials in the environment by adopting mainly two major genetic modifications.

i) Mutations in the genes due to the action mechanism of the compound.

ii) Horizontal gene transfer (HGT) due to acquisition of foreign DNA.

2.4.1 Mutational resistance

In this case, the action of antibiotic is altered by one of the following mechanisms;

i) Reduced affinity for the antibiotic/ modifications of the drug target

ii) Reduction in the drug uptake

iii) Activation of efflux mechanism to eject the antibiotic molecule

iv) Changes in primary metabolic pathways

Therefore, resistance emerging due to acquired mutational changes varies in complexity and diversity (Munita and Arias, 2016).

2.4.1.1 Alteration of antibiotic molecule

One of the best bacterial strategies to resist antibiotic molecules is to produce enzymes that either destroy the drug or inactivate the target molecule. This will ultimately make the antibiotic unable to attack its target (Munita and Arias, 2016).

a) Chemical modification of the antibiotic

A well-known mechanism of acquired antimicrobial resistance in both Gram negative and Gram positive bacteria is by producing enzymes that are capable of introducing chemical changes in the antibiotic molecule. Interestingly, the mechanism of action behind this enzymatic modification in the antibiotics is exerted by inhibiting the synthesis of protein at the ribosome level (Wilson, 2014). One of the best example of antimicrobial resistance by inducing chemical modifications in the antibiotics is mainly found in Gram negative isolates including *Enterobacteriaceae*, *Acinetobacter* and *Pseudomonas* that affect most of the aminoglycosides including gentamicin and amikacin (Ramirez *et al.,* 2010).

b) Destruction of the antibiotic molecule

The mechanism behind β-lactam resistance majorly relies by destroying antibiotic molecules by the activity of β- lactamases enzymes which destroy the amide bond of the β-

lactam ring, making the antimicrobial ineffective. β-lactam resistance was first described in 1940s (D'Costa *et al*., 2011). In order to overcome this issue, new broad spectrum β-lactam compounds with reduced susceptibility to penicillinases (such as ampicillin) were manufactured. But two decades after that, a new plasmid encoded β-lactamase was detected among Gram negatives that was capable of hydrolyzing ampicillin (named TEM-1 after the patient in which it was first discovered) (Paterson *et al.,* 2005). From then on, with every new addition in β-lactam antibiotics, the bacteria devises some mechanisms and enzymes that are capable of destroying that novel compound. This activity is a prime example of antibiotic-driven adaptive bacterial evolution (Munita and Arias, 2016)

2.4.1.2. Decreased penetration of antibiotic and efflux

a) Reduced permeability/uptake

A lot of antibiotics used in the clinical stings have intracellular targets, therefore, the drug must penetrate the outer membrane in order to reach the target and exert effect. Bacteria have developed this mechanism by reducing the amount of antibiotic uptake ad thus preventing it reaching the target. Therefore, this mechanism is particularly important in Gram negative bacteria in which the outer cytoplasmic membrane actually acts like the first barrier against the antibiotic (Munita *et al*., 2016). Hydrophilic molecules such as tetracyclines, β-lactams and some fluoroquinolones are remarkably affected by changes in permeability of the outer cytoplasmic membrane. These antibiotic molecules often use diffusion channels filled with water known as porins to cross this barrier (Pagès *et al*., 2008). An example of this phenomenon is the intrinsic low susceptibility of *Acinetobacter* and *Pseudomonas* to β-lactam (compared to *Enterobacteriaceae*) because of the reduced number of porins or differential expression (Hancock *et al*., 2002).

b) Efflux pumps

Complex bacterial mechanisms that are able to pump antibiotic molecules from the bacterial cell is a well-known mechanism of bacterial resistance. Explanation of an efflux system able to extrude tetracycline out of the *E. coli* cytoplasm was among the first to be described in early 1980s (Tadesse *et al*., 2012). This may be substrate-specific for a particular antibiotic (like *mef* genes for macrolides and *tet* determinants for tetracycline) or with broad substrate specificity, which are usually present in multiple drug resistant bacteria (Poole, 2005). A wide range of antibiotic classes including inhibitors of protein synthesis fluoroquinolones, β-lactams and carbapenems are affected by this mechanism of resistance. A classic example of this efflux mediated resistance is in tetracyclines in which *tet* efflux pumps extrude tetracyclines using proton exchange as a source of energy (Poole, 2005; Roberts, 2005).

2.4.1.3. Changes in target sites

Bacteria have evolved several mechanisms to avoid antibiotics action which includes either protecting or modifying the target site resulting in reduced affinity of target molecule. Alterations in drug targets, interferes with the bacteriostatic/bactericidal effects of antibiotics used and thus limit its effectiveness and cause resistance (Blair *et al*., 2015).

a) Target protection

Antibiotics affected by this mechanism of resistance include fluoroquinolones, tetracycline, and fusidic acid (Munita and Arias, 2016).

e) ***Modification of the target site***

These modifications in the target may consist of

i) Point mutations in the genes encoding the target site

ii) Bypass or replacement of the original target

iii) Enzymatic modification of the binding site (for example addition of methyl groups) (Tomlinson *et al.,* 2016).

c) Mutations of the target site

The mechanism of fluoroquinolone resistance is a well-characterized example of mutational resistance. Fluoroquinolones inhibit the two crucial bacterial enzymes (DNA gyrase and topoisomerase IV), hence alter the replication of DNA and kill bacteria. Since fluoroquinolones work by inhibiting two essential enzymes (DNA gyrase and topoisomerase), that are required for the survival of bacteria, the level of resistance achieved by inducing changes in one of these enzymes will depend on the potency with which the antibiotic obstructs the unaltered target (Hooper, 2002)

d) Replacement or bypass of the target site

Bacteria have evolved another mechanism to avoid antibiotic target by over producing the target and thus bypass the metabolic pathway that the antimicrobials inhibit. An important and relevant clinical examples include methicillin resistance in *S. aureus* and vancomycin resistance in enterococci via modifications in the structure of peptidoglycan mediated by the *van* gene clusters (Hiramatsu *et al.,* 2013).

2.4.1.4. Resistance due to cell adaptations

Bacterial pathogens have evolved a very sophisticated and complex mechanism to combat hostile environments and environmental stressors and avoid disruption of crucial processes required for their survival, such as cell wall synthesis. Development of resistance against vancomycin (low-level in *S. aureus*) and daptomycin (DAP) are the most relevant examples which results due to cell adaptive response to the antibiotic attack (Munita and Arias, 2016).

2.5. Spread of antibiotic resistance in environment

Concern over emerging antimicrobial resistance was initially confined to resistance in clinically relevant microorganisms that cause disease outbreak. However, antibiotic resistant bacteria have been recently isolated from virtually every environment on earth. We have now

known that the spread of resistant genes can be far wider than once believed and is developing in non-pathogenic bacteria present in humans, animals, and the environment. The pathogenic bacteria discharged into the environment, e.g. via sewage or agricultural runoff ultimately become resistant by acquiring resistant genes from the non–pathogenic bacteria present in the environment which acts as a source of resistance proliferation. Thus, dissemination of antibiotic resistant bacteria is not only due to the resistant pathogens, but also due to the availability of resistance genes to pathogens via gene transfer (Zhang *et al*., 2009).

A study conducted by Khan *et al*. (2013) observed high level of antibiotics downstream in river Ravi near Lahore. Maximum concentrations reported were 1100, 1700 and 2700 ng L^{-1} for oxytetracycline, trimethoprim, and sulfamethoxazole, respectively. Highest detected levels i.e. 1100, 4100, 6200, 7300, 8000, 27000, 28000 and 49000 ng L^{-1} of erythromycin, lincomycin, ciprofloxacin, ofloxacin, levofloxacin, oxytetracycline, trimethoprim and sulfamethoxazole, respectively were detected in the effluent of one of the drug formulation facilities. Antibiotic resistant genes (ARGs) were also detected at the sites and the highest levels of ARGs detected, *sulI* and *dfrA1*, were directly linked with the antibiotics detected at the highest concentrations which indicate that high level of ARGs were associated with environmental levels of antibiotics.

Elevated levels of antibiotics in the environment will promote natural selection and could result in the development of antibiotic resistance genes (ARGs). This will also help in establishing the environment as a reservoir of antibiotic resistant genes and their further propagation to pathogens via water and food webs. Discharge of antibiotics from basic drug formulation facilities have shown to be a considerable point sources with levels much higher than any other route. This can be categorized as levels up to mg L^{-1} (Larsson *et al.,* 2007; Li *et al.*, 2008), >100 µg L^{-1} (Lin *et al*., 2008; Sim *et al.,* 2011) and <1 µg L^{-1} (Hoerger *et al.,* 2009) on the basis of contribution from such point sources. The highest reported levels ever from the

drug manufacturing facilities is 31 mg L^{-1} for ciprofloxacin (fluoroquinolone), present in the effluent of wastewater treatment plant that was receiving wastewater from about 90 drug manufacturers in Patancheru near Hyderabad, India (Larsson *et al.*, 2007). In addition to this, a metagenomic study of this wastewater effluent showed presence of antibiotic resistant genes belonging to several classes of antibiotics, including fluoroquinolones, sulfonamides and aminoglycosides along with class 1 and 2 integrons (Kristiansson *et al.*, 2011). Although, resistant bacteria can be found naturally in the environment, but mostly it is linked with anthropogenic impacts of some type that include agricultural impacts or direct human impact. A study conducted by Malik *et al*. (2011) to observe resistance in microbes isolated from soil irrigated with wastewater reported that 87.5% of *Pseudomonas* isolates were resistant to sulphadiazine whereas 79.1% showed resistance to both ampicillin and erythromycin. Majority of the *Pseudomonas* sp. isolated from water and soil were multiple drug resistant as they exhibited resistance to multiple antibiotics. Resistance was transferable to recipient *Escherichia coli* AB2200 strains by conjugation.

After the treatment of infection by antibiotic, the resistant strains thus left unaffected are eventually 'diluted out' by the susceptible counterparts of the organisms, and the growth of such resistant strains is suppressed by these natural competitors. This, however, does not happen in case where the whole population is being treated with the same drug, and thus the number of susceptible strains are reduced to a point where they are not enough to provide competition to the resistant ones, and the resistant strains are able to strive and acquire the dominant position in the ecological niche.

In a study conducted by Bolaji *et al.* (2011), it was observed that all the bacterial species isolated from hospital wastewater including *Proteus mirabilis, Pseudomonas aeruginosa, Pseudomonas putida., Pseudomonas fluorescens, Shigella sp., Enterobacter aerogenes, Klebsiella edwardsii,* and *Flavobacterium meningosepticum* were all 100%

resistant to the tested antibiotics. These results showed that these microorganisms have been well exposed to the tested antimicrobials and they have developed resistance mechanism against them. This imbalance creates population of microbes containing pool of antibiotic resistant genes (Alekshun *et al.,* 2007). The usage of antibiotics in terms of their density is responsible for selection of resistance strains and speeding up the collective resistance in bacteria against the drug. Thus, instead of the individual use, the use of the total population as a whole defines and shapes the resistance trends. A study in Nepal concluded that the trend in rate of resistance corresponds to the total use of antibiotics by the community and not by the individual person (Walson *et al*., 2001).

A study conducted by Ahmad *et al.* (2014) reported the concentration of ofloxacin as 0.067 μg mL^{-1} in downstream river water. Downstream to first municipal wastewater drain *S. typhi* was detected and 33 % of them were found to be resistant to ofloxacin. The bacteria isolated from river water downstream to the city were 100% resistant to the antibiotic. The co-culture results of ofloxacin resistant *S. typhi* and ofloxacin sensitive *E. coli* revealed that the resistant bacteria were able to transfer the antibiotic resistance to another species.

2.6. Effects of antibiotic resistance

As a result of increase in resistance in bacteria, various problems arise including,

- Increased morbidity and mortality due to treatment failure
- Reduced efficacy of related antibiotics
- Increased health care cost
- Increased potential for dissemination of antibiotic resistant genes
- Emergence of multi antibiotic resistance in bacteria (Friedman *et al.,* 2016).

It has been estimated that the increase in resistance of bacteria not only increases the stay of patients in the hospital but also increases the cost of infection treatment and mortality rate (Sydnor *et al.,* 2011).

Critically analyzing the literature cited, it is obvious that selective pressure caused by presence of antibiotics in environment and genetic acquisition of ARGs among bacteria is causing the resistance proliferation in the environment. Since presence of antibiotics in wastewater of Pakistan is reported in a number of studies, situation of antibiotic resistance in the water environment is not very well documented. In this context, there is a need for comprehensive study on the current situation of wastewater microbiota and the extent of resistance prevalence in the environment. The current work is a contribution to understand the present status of antibiotic resistance in the environmental samples collected from different cities of Pakistan.

Chapter 3

MATERIALS AND METHODS

Experiments were carried out at Institute of Environmental Science and Engineering (IESE), School of Civil and Environmental Engineering (SCEE), National University of Sciences and Technology (NUST), Islamabad, Pakistan to assess the antibiotic resistance pattern in the bacteria isolated from environmental samples. For identification of the isolated strains, support was also obtained from IMCCP, National Agriculture Research Council (NARC) Islamabad, Pakistan. The details of all experiments are presented in this chapter.

3.1. Sample collection

Samples were collected from four selected wastewater streams receiving hospital effluents, within a period of three months from September 2016 to November 2016. Sampling sites included 2 sites from Islamabad (near Al-Shifa hospital and PIMS hospital), one from Rawalpindi (IJP road) and one from Faisalabad. The locations of the sampling sites are given in the Table 3.1. Wastewater samples were collected from these sites in sterile 500 mL sample collecting bottles and transported to the laboratory in cool condition (within an ice box) and stored at 4°C for further analysis.

Table 3.1. Location of the sampling sites

Sr. No.	Sampling city	Latitude	Longitude
1	Faisalabad	31°22’48.39” N	73°04’20.19” E
2	Rawalpindi	33°38’09.03” N	73°02’03.06” E
3	Islamabad (Site 1)	33°40’30.75” N	73°04’05.31” E
4	Islamabad (Site 2)	33°42’10.82” N	73°02’45.34” E

3.2. Sample processing

The collected samples were processed within 24 hours of collection by standard microbiology techniques. Prior to isolation, all the glassware required for the experiment was autoclaved at 121 °C, 15 psi for 15 minutes for sterilization. Nutrient agar was used as growth medium. Agar was prepared and sterilized before pouring. The plates were poured in laminar flow-hood to minimize contamination. After the agar solidification, the plates were inverted so that the water droplets do not contaminate the agar plate. The prepared nutrient agar plates were incubated at optimum temperature (37°C) for 24 hours to check sterility.

3.3. Isolation of bacterial strains

Isolation of bacterial strains from environmental samples was done by serial dilution technique. Wastewater sample was serially diluted seven times up to 10-folds. From each dilution, 0.2 mL of the bacterial suspension was dropped onto a prepared nutrient agar plate. Then, the drop of suspension was spread uniformly on the agar plate by a sterilized glass spreader. These plates were incubated at 37 degrees Celsius for 24 hours, aerobically. After incubation, the plates showing countable colonies (30-300) were selected and colonies were counted with the help of colony counter.

Based on the morphology of bacterial colonies on the plates, representative colonies were picked with sterilized loop and sub-cultured to obtain pure colonies. Same procedure was followed for all the other samples and out of these samples one hundred and nine bacterial isolates were recovered. Each colony was subjected repeatedly (4 to 5 cycles) to streak plate technique to get different colonies separated. Each pure colony was stored in the refrigerator at 4°C for further use.

3.4. Characterization of isolated strains

Morphology of separated colony was studied by noting their form, elevation, size, margin, surface, odor, color, pigmentation, and opacity and, Gram reaction using standard

techniques (Appendix B). Gram staining was performed as per methods described in standard methods (APHA, 2005). Cultural characteristics of isolates were assessed by performing biochemical tests including catalase, oxidase, Simmons citrate and Mannitol salt agar.

3.5. Preparation of glycerol stocks

Glycerol stocks of samples were prepared by autoclaving the vials having 750 µL of glycerol (70%). The vials were then filled with 750 µL of 24-48 hours incubated nutrient broth containing dense bacterial growth. These were stored at -80°C.

3.6. Identification of strains

3.6.1. DNA extraction and amplification

Template DNA from bacteria was extracted from colony of overnight streaked plate. Heat shock of 10 minutes at 95 degrees Celsius was given to this PCR tube and then centrifuged using micro centrifuge (Eppendorf Minispin, Germany) for 3-4 minutes until pellet was formed (Ahmed *et al.,* 2007). The clear supernatant was collected in a sterile Eppendorf and stored at 4°C, which was then used as a template DNA. The template DNA was amplified by using conventional PCR, with the help of specified primers. For all primers, master mix was prepared by using same recipe in PCR tubes (Ahmed *et al*., 2007) (Table 3.2). The reaction mixture was centrifuged for thorough mixing using micro centrifuge (Eppendorf Minispin, Germany). Then PCR was performed in thermocycler (Veriti, Applied Biosystems).

Table 3.2. Reagents used for PCR of isolates

Sr. No.	Materials	Volume used (µL)
1	**Forward primer (9F)**	2
2	**Reverse primer (1510 R)**	2
3	**Template**	1
4	**Nuclease Free water**	20

5	**Pre-Mix Taq kit**	25
6	**Total volume**	50

Amplification of 16S rRNA of isolates were carried out in PCR using following primers and set of conditions:

- Forward primer (9F; 5'-GAGTTTGATCCTGGCTCAG-3') and
- Reverse primer (1510R; 5'GGCTACCTTGTTACGA-3') (Hayat et al. 2013, Katsivela *et al.,* 1999).

Each vial contained 50 µL of reaction volume. The reaction mixture was heated for 2 min at 94°C and then amplification was carried out in 29 cycles. Each cycle was comprised of 1 min at 94°C, 1 min at 50°C and 1.30 min at 72°C. The final extension was at 72°C for 5 minutes followed by storage at 4°C (Table 3.3).

Table 3.3. PCR profile for amplification of 16S rRNA gene

Time	Temperature (°C)	Definite
2min	94	Initial denaturation
1 min	94	Denaturation
1 min	50	Annealing
1:30 min	72	Extension
5 min	72	Final extension

3.6.2. Agarose gel electrophoresis

The amplification of 16S rRNA gene was confirmed with the help of gel electrophoresis. For this purpose, 0.8% agarose gel was prepared in 1X TBE and volume was adjusted to 1 L. Gel was stained by adding 5 µL of ethidium bromide (0.5 µg/mL). Mixture of 10X loading dye and amplified PCR product was run on the gel. Power supply was then switched on to allow the gel for the process of electrophoresis (Mupid, Takara Bio). The gel

was then visualized under UV trans-illuminator to see the amplified product. The image of the resulting gel was taken by Gel Documentation System (Gel Doc System FireReader V4 -Uvitec Cambridge). The PCR product was stored at 4°C for further use. To avoid contamination experiments were carried out adhering to the standard precautions, including preparation of reaction mixtures in separate place (DNA-free cabinet/hood) and the use of gloves, laboratory coats, facemasks and negative PCR (water) control. The amplified products were then sent to Macrogen (Seoul, Korea) for sequencing using universal 16S rRNA sequencing primers and checked for their homology with other reported species. The gene sequences were compared with others in the GenBank databases using Ez Taxon (Chun *et al*., 2007; Kim *et al.,* 2012). Phylogenetic analyses were performed using bioinformatics software MEGA-7 (Tamura *et al.,* 2007). CLUSTAL X and BioEdit were used for sequence alignment and comparison, respectively. The aligned sequences were used to construct a phylogenetic tree using the maximum likelihood method with 1000 bootstrap value (Ahmed *et al.,* 2014).

3.7. Antimicrobial resistance testing

Clinical and Laboratory Standard Institute (CLSI) guidelines were considered for media preparations, media selection, antibiotic discs' placement and then measurement of its zone of inhibitions.

3.7.1 Selection of antibiotics

For the selection of antibiotics, a short comprehensive survey was carried out in August, 2016. In this survey, a questionnaire (Appendix A) comprising of 10 brief questions regarding the commonly prevailing diseases and mostly prescribed antibiotics for them was prepared. These questions were asked from 5 different doctors from the cities of Faisalabad, Rawalpindi and Islamabad and the most commonly prescribed antibiotics were screened out. This selection was also cross checked with the literature. The dosage of these antibiotics to be used in the experiment was selected from literature survey and CLSI standards. The antimicrobial

susceptibility testing was performed against the selected most commonly used antibiotics i.e. ciprofloxacin, levofloxacin, ofloxacin, ampicillin and amoxicillin. The concentrations were selected on the basis of literature survey (Deak *et al*., 2015; Fuchs *et al*., 1989; Doern *et al*., 1987; Saeed *et al*., 2009) and CLSI guidelines. The dosage of the selected antibiotics is given in the Table 3.4.

Table 3.4. Dosage of selected antibiotics (µg) for disc diffusion test

Antibiotic discs	Concentration	Supplier	Action spectrum
Ampicillin	25 µg	Oxoid	Broad spectrum antibiotic
Amoxicillin	10 µg	Oxoid	Broad spectrum antibiotic
Ciprofloxacin	5 µg	Oxoid	Broad spectrum antibiotic
Levofloxacin	5 µg	Oxoid	Broad spectrum antibiotic
Ofloxacin	5 µg	Oxoid	Broad spectrum antibiotic

3.7.2 Preparation of Muller Hinton agar plates

After the isolation of bacteria from each collected sample, the standard Kirby-Bauer disk diffusion method was used to determine the presence of antibiotic resistance in the isolates. Mueller-Hinton agar was used for the preparation of plates as it consists of defined set of components and contains low quantities of some substances that may inhibit certain antibiotics. Plates were poured with sterile media to a depth of 4 mm approximately. The sterility of the plates was checked by incubating the prepared plates at 37°C for 24 hours.

3.7.3. Preparation of broth culture

The inoculum was prepared by suspending the fresh bacterial culture in 10 mL sterile nutrient broth grown for 12-24 hours to reach log phase of growth. The bacterial inoculum was prepared to a turbidity of 0.5 (1.5 x 10^8 CFU/mL) McFarland standard. The inoculum was used within 15-20 minutes of preparation.

3.7.4. Disc diffusion assay

Previously made, Muller Hinton agar plates were inoculated by a sterile cotton-tipped swab using the lawn inoculation technique. Disks (Oxoid-UK) were individually placed onto the surface of the plate with sterilized forceps. The plates were incubated at 37°C for 18–24 hours, aerobically. The diameter of the zone of 'no growth' (in mm) around a disk was measured using a ruler. Where individual colonies were present in the zone, after ensuring the culture is pure, the resistant subpopulation is sub cultured and the antimicrobial susceptibility test was repeated. The zones of inhibition were then interpreted using CLSI guidelines (Cockerill, 2011).

This experiment was performed in triplicate and the antibiotic resistance results were expressed as the mean of inhibition diameters (mm) produced by the respective drugs.

Chapter 4

RESULTS AND DISCUSSION

4.1 Isolation of bacteria from wastewater samples

A total of one hundred and nine strains were isolated from wastewater samples of Islamabad, Rawalpindi and Faisalabad. The strains were picked randomly from dilution plates and sub cultured to obtain pure colonies.

4.2. Frequency of bacteria isolated from each site

The frequency of bacterial species isolated from different cities are shown in the Figure 4.1. Among 4 selected sites 23.0% strains were collected from Rawalpindi, 24.0% from Faisalabad, 29.4% from the first site in Islamabad and 24.0% from the second site in Islamabad. Most of the strains 53.2% were isolated from Islamabad samples.

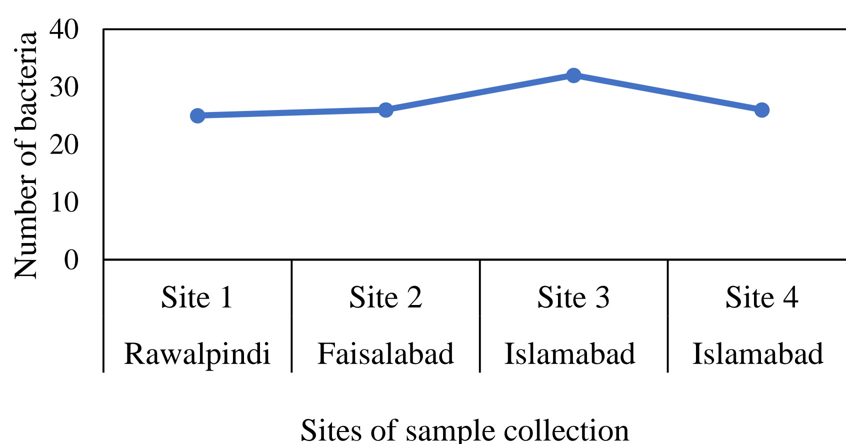


Figure 4.1. Frequency of bacterial isolates at different sampling points

4.3. Characterization of isolated bacterial strains

One hundred and nine bacterial strains varying in colony morphology in terms of shape, form, margin, pigmentation and opacity (Appendix B) were isolated from environmental samples. Details on the morphological characteristics of all bacterial strains

are given in Table 4.2. Results showed that all the isolated strains were circular in shape and margin of most strains was entire. Out of all the isolated species only two were pigmented while remaining one hundred and seven were none-pigmented.

All these morphologically characterized bacterial strains were also tested for their biochemical characteristics. Most of the isolates tested were Gram negative and all strains exhibited catalase-positive reaction. Details on the biochemical characteristics of all bacterial isolates are given in Table 8. From the results of Gram staining, it was observed that 97.5% strains showed Gram negative characteristics while 2.5% were Gram positive (Figure 7).

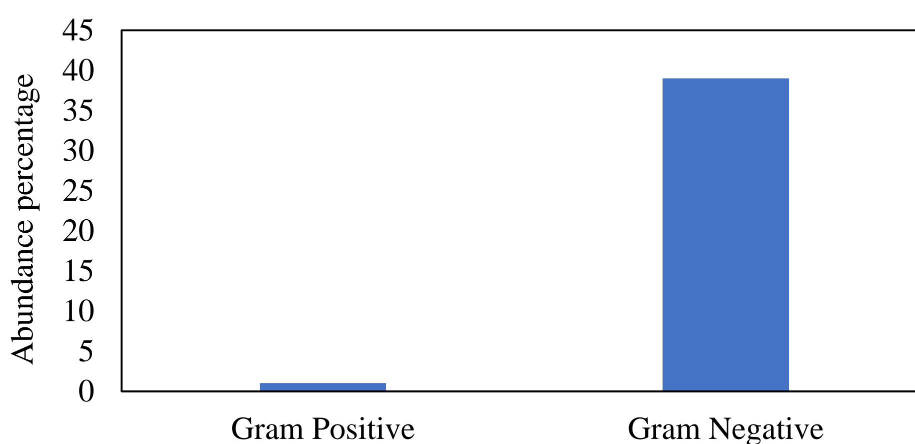


Figure 4.2. Gram staining of isolates collected from environmental samples

The biochemical test results of isolates showed that 100% strains exhibited catalase positive results. 52% strains were oxidase positive while 48% showed oxidase negative results. Differential media was also used to differentiate different microorganisms, results showed that 65% strains were citrate positive whereas 35% showed citrate negative results. Mannitol salt agar test results showed that 48% strains were positive and 52% were negative (Figure 4.3).

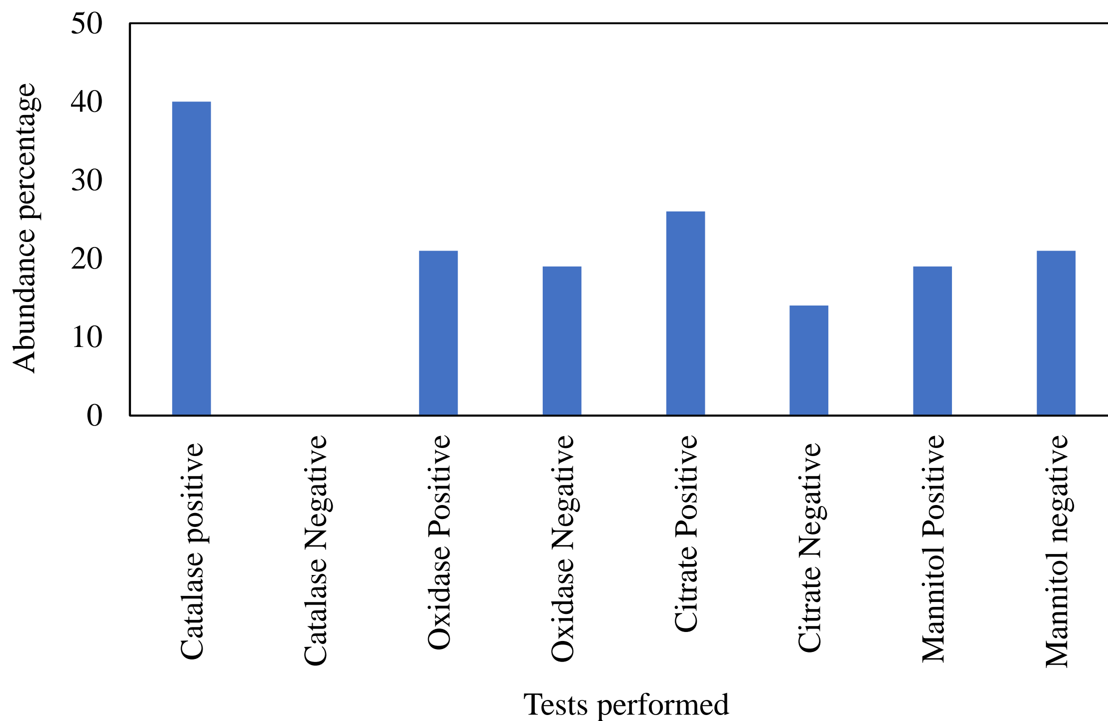


Figure 4.3. Biochemical characteristics of isolates collected from environmental samples

The biochemical tests performed helped in preliminary characterization and identification of isolates. Catalase test was performed to differentiate between catalase positive staphylococci from catalase negative streptococci. Catalase test also aided in identification of family *Enterobacteriaceae* (Taylor and Achanzar, 1972). The catalase enzyme is produced by bacteria that respire using oxygen as a terminal electron acceptor, hence, catalase-positive bacteria include strict aerobes and facultative anaerobes. Catalase-negative bacteria may be anaerobes or facultative anaerobes that ferment only and do not use oxygen as a terminal electron acceptor in respiration, like *Streptococci*. Results of oxidase test identified bacteria that may produce an enzyme of the bacterial electron transport chain (cytochrome c oxidase). Bacteria that possessed a high TMPD oxidase activity were aerobic which means that they can use oxygen as a terminal electron acceptor in respiration while those showing low TMPD oxidase activity were facultatively anaerobic (Jurtshuk *et al.,* 1976).

Two differential mediums were also used to characterize the isolated strains. The Simmons citrate agar was used to differentiate *Enterobacteriaceae* on the basis of utilization of citrate as the sole carbon source. It also differentiated Gram-negative bacteria on the basis of citrate utilization.

Results of mannitol salt agar differential media test showed tolerance for saline environments. Thus, MSA selectively isolates *Staphylococcus* sp. i.e. selective media for *Staphylococcus* sp., *Enterococcus faecalis* and *Enterococcus faecium* (most common Enterococcal species that has been isolated from human infections). They may ferment mannitol and produce lactic acid, producing yellow colored colonies on MSA. Catalase test will then differentiate between *Enterococcus* (-ve) and *Staphylococcus* (+ve).

4.4. Identification of isolates

DNA from the bacterial isolates was extracted by heat shock method. The extracted DNA was quantified with UV visible spectrophotometer. The results of amplification are shown in Appendix C.

It is accepted that culturally viable microorganisms isolated from any sample collected from an environment represents only a small segment of the total population that is actually present. In this study, one hundred and nine bacterial strains were identified on the basis of 16S rRNA gene sequence (Table 4.1), which were isolated from environmental samples. The results of 16S ribosomal RNA gene sequence showed a diverse bacterial community. The identified species belonged to two phyla including Proteobacteria and Firmicutes. These strains belonged to thirteen different genera with forty different species (Figure 4.5a). The most frequently identified species from these samples were *Proteus* sp. (26.6%) followed by *Pseudomonas* sp. (22.0%), *Acinetobacter* sp. (15.6%), *Aeromonas* sp. (14.7%), *Escherichia* sp. (6.4%), *Shewanella* sp. (3.7%), *Comamonas* sp. (2.8%) and seven other less frequently isolated species

which include *Shewanella* sp., *Comamonas* sp., *Citrobacter* sp., *Rheinheimera* sp., *Bacillus* sp., *Morganella* sp., *Stenotrophomonas* sp., *Shigella* sp. and *Alishewanella* sp. This was also indicated in studies by Sader *et al.* (2005) and Maluping *et al.* (2005) that these are the most frequently isolated species from wastewater.

Phylogenetic tree of all identified strains is shown in Figure 4.4. All the identified strains were grouped together with closely related match of those strains. Numbers at the nodes represent percentage of bootstrap value. A good value is considered up till 50. They represent the significance of the node that placement of the strains is accurate and same placement and nodes will be reproduced even with 1000 resampling. The value in front of each genus (Figure 4.5b) shows the sequence similarity percentage of 16S rRNA gene with their closely related species in the respective genera. The percent sequence similarity with the closely related species of the respective genera was 94.5% to 100%. Similarity percentage of some species in genus *Acinetobacter* (94.5%) and *Proteus* (97.9%) indicates that some of these strains may be characterized taxonomically to delineate as novel species.

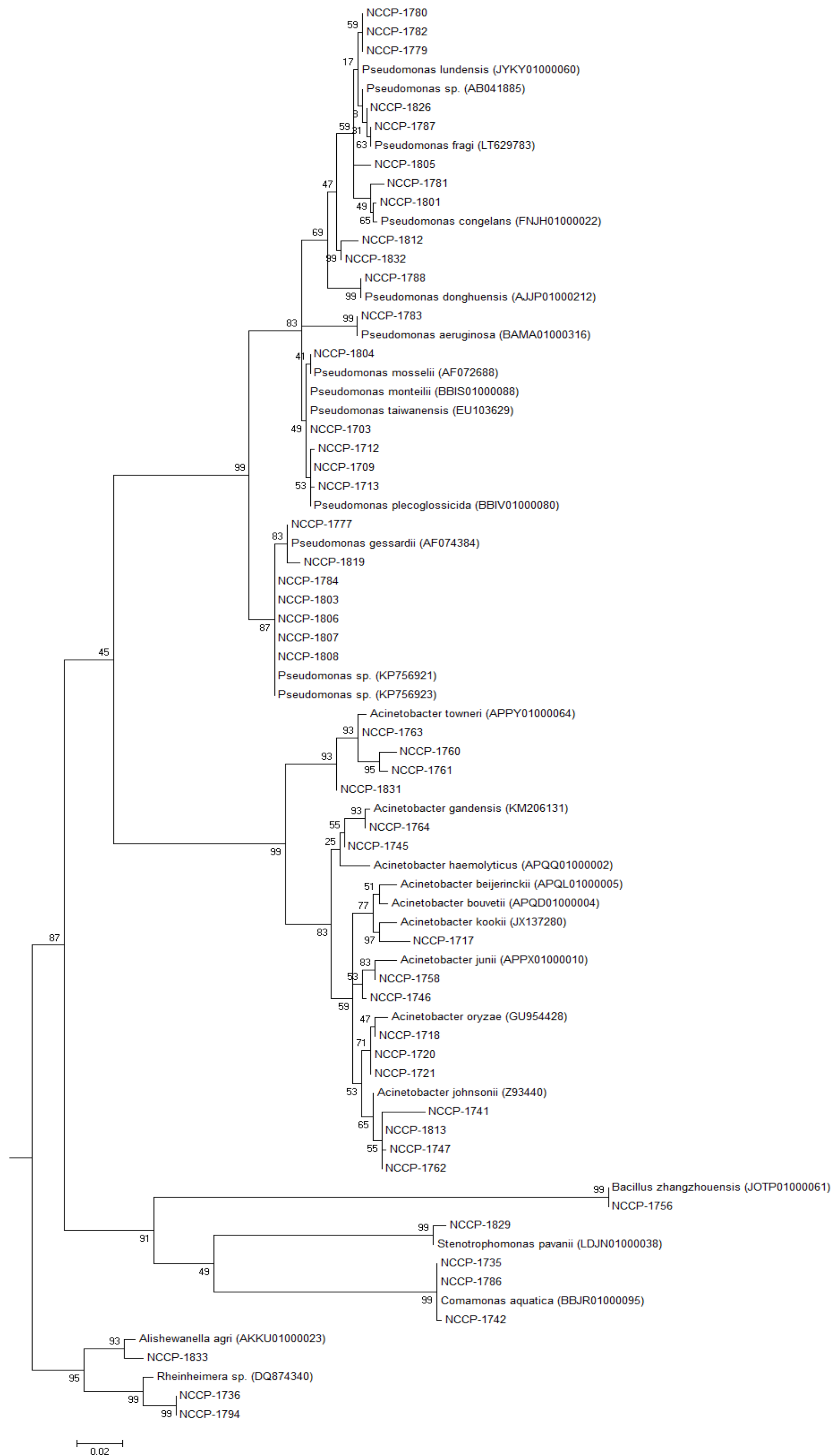
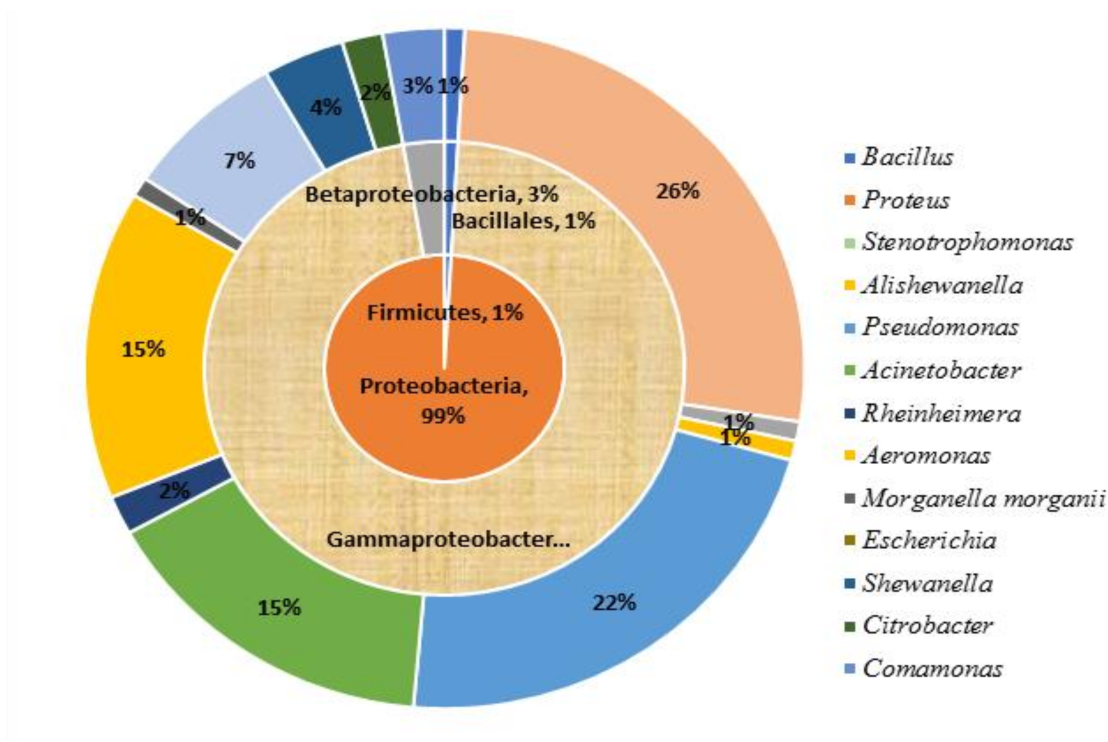


Figure 4.4. Phylogenetic analysis of isolates by maximum likelihood method

a)



b)

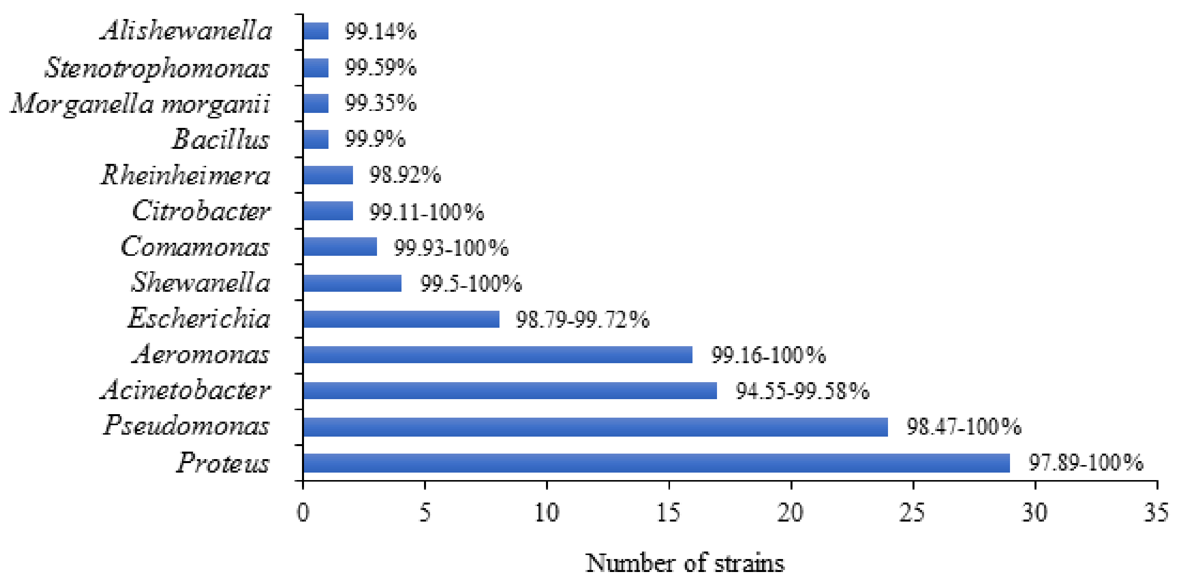


Figure 4.5. Biodiversity of isolated strains. a) Diversity pie chart of isolated strains b) Bar graph representing the percent similarity range of identified strains with the known closely related species of the respective genera.

Table 4.1. Identification of strains based on 16S rRNA gene sequencing and their accession numbers published in DNA data base

Strain ID	Number of nucleotides of 16S rRNA gene	Accession number of 16S rRNA gene	Closely related validly published taxa	Similarity %age of 16S rRNA gene sequence with closely related species	Coverage %age	No. of closely related species having >=97% (>=98%) similarity of 16S rRNA gene sequence
NCCP-1703	965	LC270154	*Pseudomonas monteilii* NBRC 103158(T)	100.0	66.6	30 (21)
NCCP-1704	931	LC270155	*Aeromonas caviae* CECT 838(T)	100.0	63.9	30 (16)
NCCP-1707	1078	LC270156	*Shewanella xiamenensis* S4 (T)	99.7	73.4	8 (5)
NCCP-1709	1403	LC270157	*Pseudomonas taiwanensis* BCRC 17751(T)	98.5	58.4	17 (9)
NCCP-1711	966	LC270158	*Shewanella xiamenensis* S4(T)	100.0	66.1	9 (5)
NCCP-1712	807	LC270159	*Pseudomonas taiwanensis* BCRC 17751(T)	99.3	55.4	30 (7)
NCCP-1713	843	LC270160	*Pseudomonas plecoglossicida* NBRC 103162(T)	99.9	81.0	> 50 (50)
NCCP-1714	935	LC270161	*Aeromonas caviae* CECT 838(T)	99.9	64.1	30 (16)
NCCP-1717	761	LC270162	*Acinetobacter kookii* ANC 4667 (T)	98.8	96.6	9 (2)
NCCP-1718	868	LC270163	*Acinetobacter oryzae* B23	99.4	59.7	17 (4)
NCCP-1720	926	LC270164	*Acinetobacter johnsonii* CIP 64.6	99.6	96.2	30 (10)
NCCP-1721	1407	LC270165	*Acinetobacter johnsonii* CIP 64.6	98.9	97.7	8 (3)
NCCP-1723	1037	LC270166	*Acinetobacter haemolyticus* CIP 64.3(T)	98.1	96.2	11 (2)

NCCP-1724	971	LC270167	*Aeromonas caviae* CECT 838	99.2	65.5	29 (8)
NCCP-1728	956	LC270168	*Escherichia coli* ATCC 11775	99.2	65.7	22 (8)
NCCP-1731	956	LC270169	*Escherichia marmotae* HT073016(T)	98.9	96.6	50 (8)
NCCP-1732	935	LC270170	*Aeromonas caviae* CECT 838	100.0	64.1	30 (16)
NCCP-1733	950	LC270171	*Shewanella xiamenensis*S4	99.6	64.7	11 (6)
NCCP-1735	943	LC270172	*Comamonas aquatica*	100.0	65.3	7 (2)
NCCP-1736	975	LC270173	*Rheinheimera tangshanensis* JA3-B52(T)	98.9	96.5	6 (2)
NCCP-1737	952	LC270174	*Aeromonas sanarellii* LMG 24682	99.9	65.3	30 (15)
NCCP-1739	974	LC270175	*Aeromonas caviae* CECT 838	99.5	65.6	30 (11)
NCCP-1741	868	LC270176	*Acinetobacter johnsonii* CIP 64.6	94.6	65.7	0 (0)
NCCP-1742	989	LC270177	*Comamonas aquatica* NBRC 14918	99.9	96.5	8 (2)
NCCP-1743	802	LC270178	*Shewanella xiamenensis* S4	99.5	54.8	8 (6)
NCCP-1744	901	LC270179	*Citrobacter amalonaticus* CECT 863(T)	99.1	61.3	22 (7)
NCCP-1745	1404	LC270180	*Acinetobacter bouvetii* DSM 14964(T)	98.2	67.1	15 (3)
NCCP-1746	1017	LC270181	*Acinetobacter beijerinckii* CIP 110307	98.7	96.6	27 (8)
NCCP-1747	1069	LC270182	*Acinetobacter johnsonii* CIP 64.6(T)	98.9	73.3	19 (4)

NCCP-1748	1001	LC270183	*Aeromonas caviae* CECT 838(T)	99.8	68.3	30 (26)
NCCP-1749	1075	LC270184	*Aeromonas sanarellii* LMG 24682(T)	99.9	73.3	31 (20)
NCCP-1750	944	LC270185	*Citrobacter amalonaticus* CECT 863(T)	100.0	64.7	34 (11)
NCCP-1831	997	LC270186	*Acinetobacter towneri* DSM 14962(T)	98.9	68.5	2 (2)
NCCP-1751	908	LC270187	*Aeromonas taiwanensis* LMG 24683(T)	99.7	62.0	30 (12)
NCCP-1752	957	LC270188	*Aeromonas sanarellii* LMG 24682(T)	100.0	65.2	30 (25)
NCCP-1753	897	LC270189	*Escherichia fergusonii* ATCC 35469(T)	99.7	96.6	50 (10)
NCCP-1754	951	LC270190	*Escherichia coli* ATCC 11775(T)	99.5	64.7	23 (8)
NCCP-1755	932	LC270191	*Escherichia coli* ATCC 11775(T)	99.2	63.1	20 (8)
NCCP-1756	969	LC270192	*Bacillus zhangzhouensis* DW5-4(T)	99.9	66.1	7 (6)
NCCP-1757	799	LC270193	*Aeromonas caviae* CECT 838(T)	99.6	54.2	29 (9)
NCCP-1758	1405	LC270194	*Acinetobacter junii* CIP 64.5(T)	98.5	69.8	3 (1)
NCCP-1833	1049	LC270195	*Alishewanella agri*	72.2	99.1	6(2)
NCCP-1760	1409	LC270196	*Acinetobacter towneri*	97.4	60.8	1(0)
NCCP-1761	1404	LC270197	*Acinetobacter towneri* DSM 14962(T)	96.8	55.8	0 (0)
NCCP-1762	1405	LC270198	*Acinetobacter johnsonii* CIP 64.6(T)	98.5	71.6	17 (2)

NCCP-1763	1404	LC270199	*Acinetobacter towneri* DSM 14962(T)	98.8	66.4	1 (1)
NCCP-1764	930	LC270200	*Acinetobacter gandensis* UG 60467(T)	99.1	63.9	4 (1)
NCCP-1765	965	LC270201	*Aeromonas sanarellii* LMG 24682(T)	99.7	65.8	30 (15)
NCCP-1767	1010	LC270202	*Aeromonas caviae* CECT 838(T)	99.9	69.1	30 (23)
NCCP-1768	956	LC270203	*Aeromonas enteropelogenes* CECT 4487(T)	99.4	65.2	30(9)
NCCP-1769	955	LC270204	*Aeromonas taiwanensis* LMG 24683(T)	100.0	65.2	30 (24)
NCCP-1771	1076	LC270205	*Proteus mirabilis*	99.8	73.4	7(5)
NCCP-1830	945	LC270206	*Shigella sonnei* GTC 781(T)	99.6	64.6	20 (8)
NCCP-1772	1038	LC270207	*Proteus mirabilis* ATCC 29906(T)	99.9	71.0	7 (6)
NCCP-1773	954	LC270208	*Proteus mirabilis* ATCC 29906(T)	99.9	65.3	7 (5)
NCCP-1774	1074	LC270209	*Proteus mirabilis* ATCC 29906(T)	99.9	73.3	7 (5)
NCCP-1775	1072	LC270210	*Proteus mirabilis* ATCC 29906(T)	99.9	73.2	7 (5)
NCCP-1776	1038	LC270211	*Proteus mirabilis* ATCC 29906(T)	99.4	70.9	7 (5)
NCCP-1777	998	LC270212	*Pseudomonas gessardii* CIP 105469(T)	99.7	67.8	30 (20)
NCCP-1778	1002	LC270213	*Proteus mirabilis* ATCC 29906(T)	100.0	68.5	7 (5)
NCCP-1827	1074	LC270214	*Escherichia coli* NCTC9001(T)	98.8	73.4	27 (20)

NCCP-1779	987	LC270215	*Pseudomonas lundensis* DSM 6252(T)	99.9	68.1	31 (18)
NCCP-1780	961	LC270216	*Pseudomonas lundensis* DSM 6252(T)	99.9	66.5	31 (18)
NCCP-1781	1079	LC270217	*Pseudomonas psychrophila* E-3(T)	99.4	74.1	30 (28)
NCCP-1782	1014	LC270218	*Pseudomonas lundensis* DSM 6252(T)	99.1	69.4	31 (5)
NCCP-1783	1080	LC270219	*Pseudomonas aeruginosa* JCM 5962(T)	100.0	74.1	3 (2)
NCCP-1784	850	LC270220	*Pseudomonas paralactis* WS4992(T)	100.0	58.4	30 (30)
NCCP-1785	1032	LC270221	*Aeromonas hydrophila subsp. hydrophila* ATCC 7966(T)	99.9	70.4	31 (25)
NCCP-1786	1013	LC270222	*Comamonas aquatica* NBRC 14918(T)	100.0	69.9	6 (2)
NCCP-1787	976	LC270223	*Pseudomonas fragi* NRRL B-727(T)	99.9	67.2	30 (28)
NCCP-1788	984	LC270224	*Pseudomonas donghuensis* HYS(T)	99.7	67.5	24 (7)
NCCP-1789	985	LC270225	*Proteus mirabilis* ATCC 29906(T)	99.6	67.1	7 (5)
NCCP-1790	1423	LC270226	*Proteus mirabilis* ATCC 29906(T)	97.9	61.7	3 (0)
NCCP-1791	941	LC270227	*Morganella morganii subsp. sibonii* DSM 14850(T)	99.4	64.4	3 (3)
NCCP-1794	1391	LC270228	*Rheinheimera tangshanensis*	98.9	77.3	4(2)
NCCP-1826	1097	LC270229	*Pseudomonas psychrophila* E-3(T)	99.7	75.4	30 (17)
NCCP-1796	1052	LC270230	*Proteus mirabilis* ATCC 29906(T)	99.5	71.7	7 (5)

NCCP-1797	822	LC270231	*Proteus mirabilis* ATCC 29906(T)	99.5	56.3	6 (5)
NCCP-1828	950	LC270232	*Proteus mirabilis* ATCC 29906(T)	99.9	65.1	7 (5)
NCCP-1798	1003	LC270233	*Proteus mirabilis* ATCC 29906(T)	99.2	68.5	6 (5)
NCCP-1829	989	LC270234	*Stenotrophomonas pavanii* DSM 25135(T)	99.6	67.4	9 (6)
NCCP-1799	1007	LC270235	*Proteus mirabilis* ATCC 29906(T)	100.0	69.0	7 (5)
NCCP-1800	1016	LC270236	*Proteus mirabilis* ATCC 29906(T)	100.0	69.5	7 (5)
NCCP-1801	1071	LC270237	*Pseudomonas congelans* DSM 14939(T)	99.5	73.2	34 (27)
NCCP-1802	909	LC270238	*Escherichia coli* NCTC9001(T)	99.3	62.3	28 (10)
NCCP-1803	904	LC270239	*Pseudomonas lactis* WS4672(T)	99.9	62.3	30 (29)
NCCP-1804	971	LC270240	*Pseudomonas mosselii* CIP 105259(T)	100.0	67.0	30 (13)
NCCP-1805	903	LC270241	*Pseudomonas fragi* NRRL B-727(T)	99.5	62.0	30 (10)
NCCP-1806	882	LC270242	*Pseudomonas lactis*	99.9	60.3	30(24)
NCCP-1807	1004	LC270243	*Pseudomonas lactis* WS4672(T)	100.0	69.1	30 (30)
NCCP-1808	998	LC270244	*Pseudomonas lactis* WS4672(T)	100.0	68.9	30 (30)
NCCP-1809	990	LC270245	*Proteus mirabilis* ATCC 29906(T)	99.9	67.7	7 (5)
NCCP-1810	966	LC270246	*Proteus mirabilis* ATCC 29906(T)	99.7	65.8	7 (5)
NCCP-1812	794	LC270247	*Pseudomonas plecoglossicida* NBRC 103162(T)	98.6	54.3	30 (5)

NCCP-1832	990	LC270248	*Pseudomonas plecoglossicida* NBRC 103162(T)	98.9	68.3	30 (18)
NCCP-1813	1033	LC270249	*Acinetobacter oryzae* B23(T)	99.0	71.1	22 (5)
NCCP-1814	1074	LC270250	*Proteus mirabilis* ATCC 29906(T)	99.4	73.3	7 (5)
NCCP-1815	966	LC270251	*Proteus mirabilis* ATCC 29906(T)	99.3	66.2	7 (5)
NCCP-1816	975	LC270252	*Proteus mirabilis* ATCC 29906(T)	99.4	66.6	7(5)
NCCP-1817	948	LC270253	*Proteus mirabilis* ATCC 29906(T)	100.0	65.1	7 (5)
NCCP-1818	961	LC270254	*Proteus mirabilis* ATCC 29906(T)	99.7	65.7	7 (5)
NCCP-1819	853	LC270255	*Pseudomonas gessardii*	58.5	99.4	30(14)
NCCP-1820	959	LC270256	*Proteus mirabilis* ATCC 29906(T)	99.9	65.7	7 (5)
NCCP-1821	1410	LC270257	*Proteus mirabilis* ATCC 29906(T)	98.5	77.6	5 (1)
NCCP-1822	959	LC270258	*Proteus mirabilis* ATCC 29906(T)	100.0	65.7	7 (5)
NCCP-1823	988	LC270259	*Proteus mirabilis* ATCC 29906(T)	99.9	67.6	7 (5)
NCCP-1824	967	LC270260	*Proteus mirabilis* ATCC 29906(T)	99.8	66.1	7 (5)
NCCP-1834	957	LC270261	*Proteus mirabilis* ATCC 29906(T)	99.8	65.3	7 (5)
NCCP-1825	916	LC270262	*Proteus mirabilis* ATCC 29906(T)	100.0	62.7	7 (5)

4.5. Screening of Antibiotic Resistant Bacteria

The antibiotic resistance testing was performed on all the isolated strains against five selected antibiotics. Variable trends for antibiotic resistance were shown by different species (Figure 4.6). The overall resistance percentage of isolates to ampicillin was 91.7%, followed by amoxicillin 83.5%, ofloxacin 66.9%, ciprofloxacin 27.5% and least resistant being levofloxacin 21.1%.

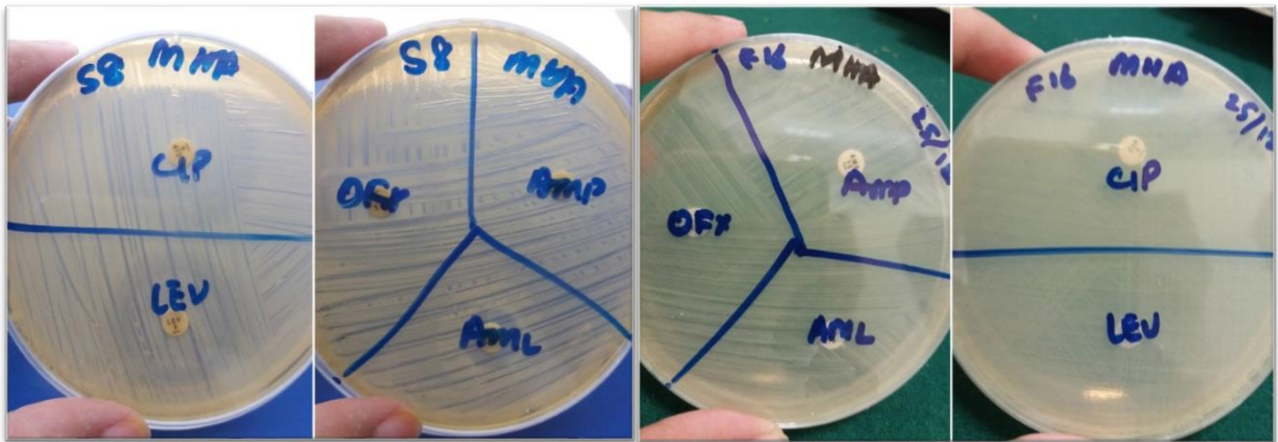


Figure 4.6. Bacterial isolates showing complete resistance to all 5 tested antibiotics

4.5.1. Ciprofloxacin resistance test results

All the isolated bacterial species were tested against ciprofloxacin. The percentage of isolates susceptible to ciprofloxacin was 60.5% while 11.9% isolates showed intermediate resistance and 27.5% isolates were completely resistant to the antibiotic. The resistance was found to be maximum in genus *Aeromonas* in which 11 out of 16 strains showed resistance (Figure 4.7). Similar trends were reported in literature in studies conducted by Cattior *et al.* (2008) and Zhang *et al.* (2009) who reported that resistance to fluoroquinolones in *Aeromonas* species, via the plasmid-mediated quinolone resistance has been reported in a number of environmental isolates from lakes and natural water sources. Among *Aeromonas* species the resistance was maximum in *Aeromonas caviae* followed by *Aeromonas sanerelli* and then *Aeromonas taiwanenses*. *Aeromonas caviae* strains were either partially resistant or

completely resistant to the antibiotic. Overall 68.8% *Aeromonas* strains showed ciprofloxacin resistance.

After *Aeromonas,* species from genus *Escherichia* showed highest resistance to ciprofloxacin. Out of seven isolates, four were resistant to ciprofloxacin. Within this genus, *Escherichia coli* showed maximum resistance followed by *Escherichia fergusonii*. The overall resistance percentage observed in this genus was 57.1%. Accelerated resistance in *E. coli* has been reported by a number of studies in literature. A study conducted by Mavroidi *et al.* (2012) reported that 21% of *E. coli* isolates collected from environmental samples were found resistant to quinolones. Consistent stepwise increase in *E. coli* resistance to ciprofloxacin was observed from 1995 (0.7%) to 2001 (2.5%) (Martínez et *al.,* 1998). The percentage of ciprofloxacin resistance observed by Cavaco and Aarestrup (2009) was 65%, which is on the high side. High resistance of *E. coli* to ciprofloxacin has also been documented by Hopkins *et al.* (2005). They observed that 24% of 189 *E. coli* isolates were resistant to ciprofloxacin.

Species from genus *Acinetobacter* also showed resistance to ciprofloxacin. *Acinetobacter jhonsonii* showed maximum resistance among the genus with 2 out of 5 species showing resistance to the drug. Some other *Acinetobacter* strains, *Acinetobacter junii, Acinetobacter kooki* and *Acinetobacter towneri* showed resistance to ciprofloxacin. Overall resistance to ciprofloxacin observed in this genus was 29.4%. Similar to our results, Hujer *et al.* (2006) reported more than 90% of *Acinetobacter* isolates resistant to ciprofloxacin.

Among the less frequently isolated species, *Citrobacter* sp. (100%), *Comamonas* sp. (66.6%) and *Bacillus* sp. (100%) showed maximum resistance to ciprofloxacin. The overall resistance pattern observed was *Aeromonas* sp.> *Escherichia* sp.> *Acinetobacter* sp.> *Comamonas* sp.> *Citrobacter* sp.> *Bacillus* sp.> *Proteus* sp.> *Pseudomonas* sp.>*Shewanella* sp.> *Alishewanella* sp. while *Rheinheimera* sp., *Morganella* sp., *Stenotrophomonas* sp., and *Shigella sonnei* showed no ciprofloxacin resistance at all.

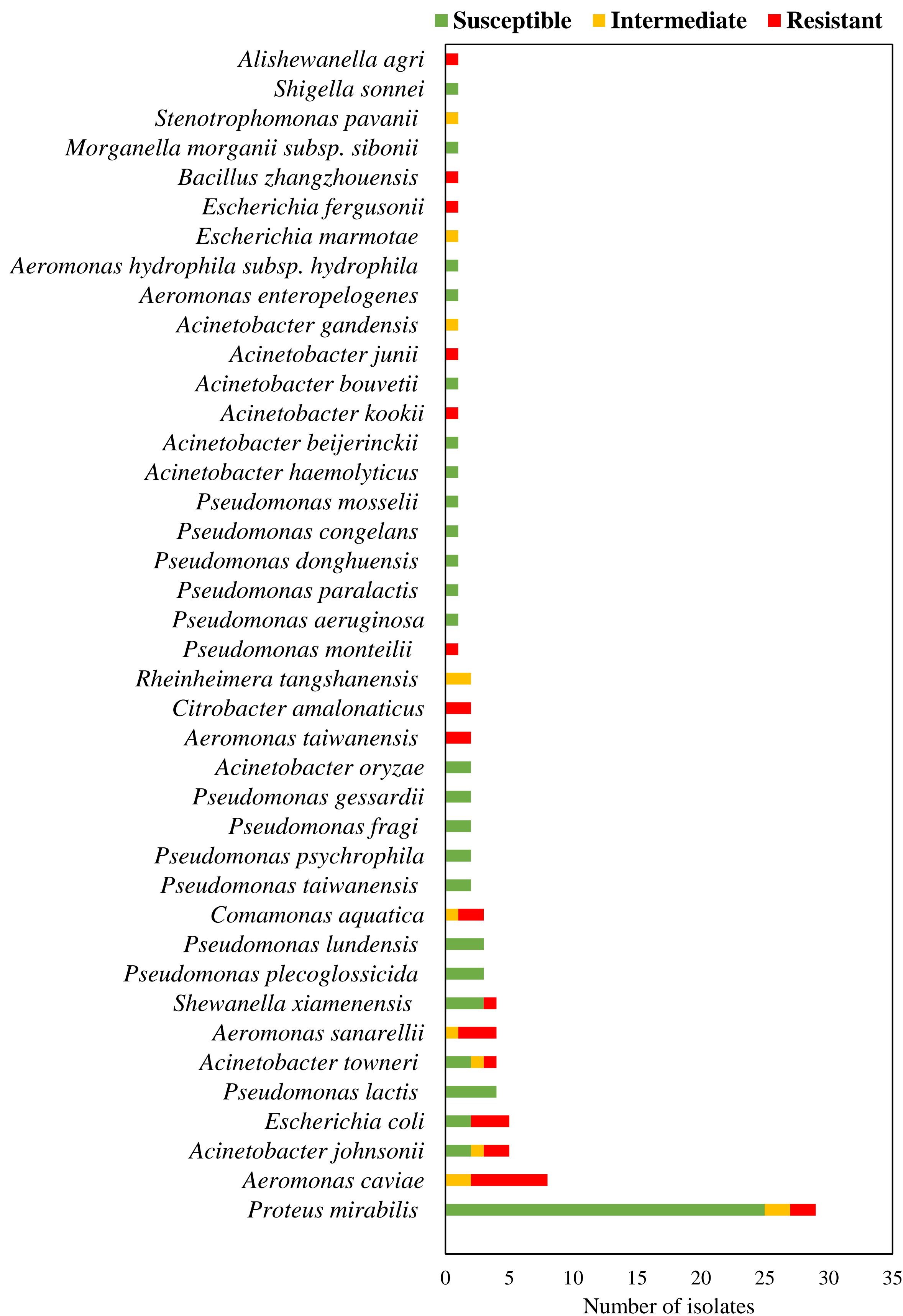


Figure 4.7. Resistance pattern of different microbial genera against ciprofloxacin

4.5.2. Levofloxacin resistance test results

All isolated bacterial species were tested against antibiotic levofloxacin. From the results of resistance test, it was observed that 56.0% isolates were susceptible to levofloxacin, 22.9% were intermediately resistant while 21.1 % isolates showed complete resistance.

The resistance was found to be maximum in genus *Escherichia* in which four out of total seven strains showed resistance. Among the genus, resistance was observed maximum in *Escherichia coli* with 3 out of 5 strains being resistant to the drug, followed by *Escherichia fergusonii*. Overall 57.1% of *Escherichia* strains showed levofloxacin resistance. Rapid increase in *E. coli* resistance has been observed since past two decades. For example, a study of the susceptibility of *E. coli* isolates recovered from hospitals during a 12-year period (1971–1982) showed no major change in resistance to any of the antimicrobial drugs tested (Atkinson *et al.,* 1984). In contrast, a retrospective analysis of *E. coli* from urine specimens collected from patients during 1997–2007 showed an increasing resistance trend for ciprofloxacin, trimethoprim/sulfamethoxazole, and amoxicillin/clavulanic acid (Blaettler *et al.,* 2009). Similar trend was observed by Rath *et al.* (2015) in India who studied prevalence of antibiotic resistance in *E. coli* by testing against 23 antibiotics and found them highly resistant to all antibiotics including ciprofloxacin and levofloxacin.

The second genus with high resistance to levofloxacin was *Aeromonas* in which *Aeromonas caviae* showed highest resistance with three out of eight strains showing resistance followed by *Aeromonas sanarelii* and *Aeromonas taiwanensis*. Overall resistance percentage observed in this genus was 37.5%. Some *Acinetobacter* species were also observed to be resistant against levofloxacin. *Acinetobacter jhonsonii* with highest resistance followed by *Acinetobacter towneri* and *Acinetobacter junii*. Overall resistance prevalence in this genus was observed to be 29.4%. Among the less frequently isolated strains, *Citrobacter* sp, *Comamonas* sp. and *Bacillus* sp. showed maximum resistance to levofloxacin. The results were consistent

with Gales *et al.* (2001) studies, showing isolates less than 30% susceptibility to third generation antibiotics including ciprofloxacin.

Apart from being completely resistant to the drug, large number of isolates showed intermediate resistance to levofloxacin. Species from genus *Aeromonas*, *Acinetobacter* and *Escherichia* sp. showed maximum intermediate resistance with the resistance percentages of 37.5%, 29.4% and 28.5% respectively. The probable explanation would be that these organisms could have acquired resistant genes from other microorganisms. West *et al.* (2011) showed that coliforms have ability to transfer and receive resistance determinants (Mubbunu *et al.,* 2014). Among the less frequently isolated strains, *Citrobacter* sp. *Rheinheimera* sp. *Stenotrophomonas* sp. *Shigella sonnei* showed maximum intermediate resistance.

The resistance pattern of all isolates against levofloxacin was *Escherichia* sp.> *Aeromonas* sp.> *Acinetobacter* sp. >*Shewanella sp.*> *Pseudomonas* sp. > *Proteus* sp. while among the less frequent isolated species maximum isolates resistant to levofloxacin were *Bacillus* sp.> *Alishewanella* sp. > *Citrobacter* sp.> *Comamonas* sp. (Figure 4.8).

4.5.3. Ofloxacin resistance test results

Among all the fluoroquinolones tested, ofloxacin resistance was observed to be the highest as 67.0% isolates showed resistance to ofloxacin. The number of isolates susceptible to the antibiotic were 16.0% while 17.4% showed intermediate resistance. Maximum resistance was observed in *Pseudomonas* isolates with 100% resistance to the antibiotic. *Acinetobacter* isolates also showed a very high resistance percentage (94.11%) to ofloxacin. Most of the *Acinetobacter* species (*Acinetobacter jhonsonii, Acinetobacter towneri, Acinetobacter kooki, Acinetobacter oryzae, Acinetobacter junii, Acinetobacter bejierinckii* and *Acinetobacter haemolyticus*) were resistant to ofloxacin. Similar trends have been reported in a study conducted by Kowalski *et al.* (2003) who observed 12 out of 25 *Pseudomonas* isolates being resistant to ciprofloxacin and ofloxacin. Similarly, multiple drug resistant species of genus *Acinetobacter* are also repeatedly reported in literature (Van *et al.,* 2004).

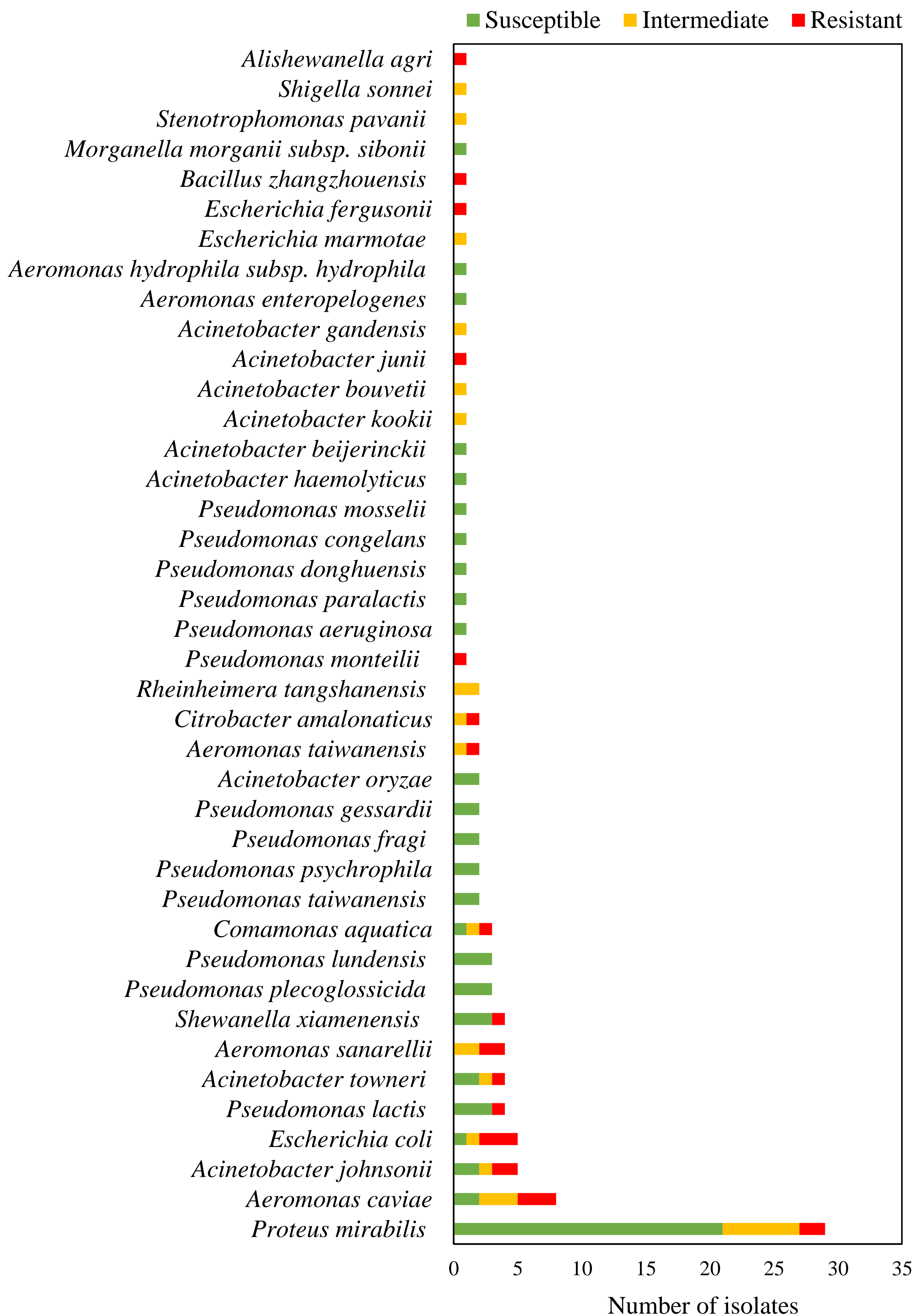


Figure 4.8. Resistance pattern of different microbial genera against levofloxacin

Acinetobacter spp. are frequently resistant to fluoroquinolones, aminoglycosides, and all β-lactams, with the exception of the carbapenems. Thecarbapenems are therefore often increasingly considered the drugs of choice for the treatment of infections due to *Acinetobacter* sp. (Giske *et al.,* 2008).

Species from genus *Escherichia* showed very high resistance to ofloxacin, mainly *Escherichia coli* with 3 out of 5 strains being resistant followed by *Escherichia fergusonii.* The overall resistance prevalence in the genus was 57.1%. Forth most resistant species to ofloxacin belonged to genus *Aeromonas*. Among them, *Aeromonas caviae* showed resistancein 5 out of 8 strains followed by *Aeromonas sanerlii* with 3 out of 4 strains resistant to ofloxacin and then *Aeromonas taiwanensis*. Overall resistance in *Aeromonas* species was observed as 56.2%. In the less frequently isolated strains *Shewanella* sp., *Comamonas* sp., *Citrobacter* sp., *Bacillus* sp., *Shigella* sp. and *Alishewanella* sp. showed 100% resistance to the antibiotic ofloxacin.

A number of isolates were observed to be intermediately resistant to ofloxacin. 31.2% isolates of genus *Aeromonas* and 28.5% isolates of genus *Escherichia* showed intermediate resistance. The trend of resistance observed was *Pseudomonas* sp. > *Escherichia* sp. > *Proteus* sp. Among the less frequent isolates, the trend was; *Shewanella* sp.> *Comamonas* sp. > *Citrobacter* sp. > *Bacillus* sp.> *Shigella sonnei* > *Alishewanella* sp. (Figure 4.9).

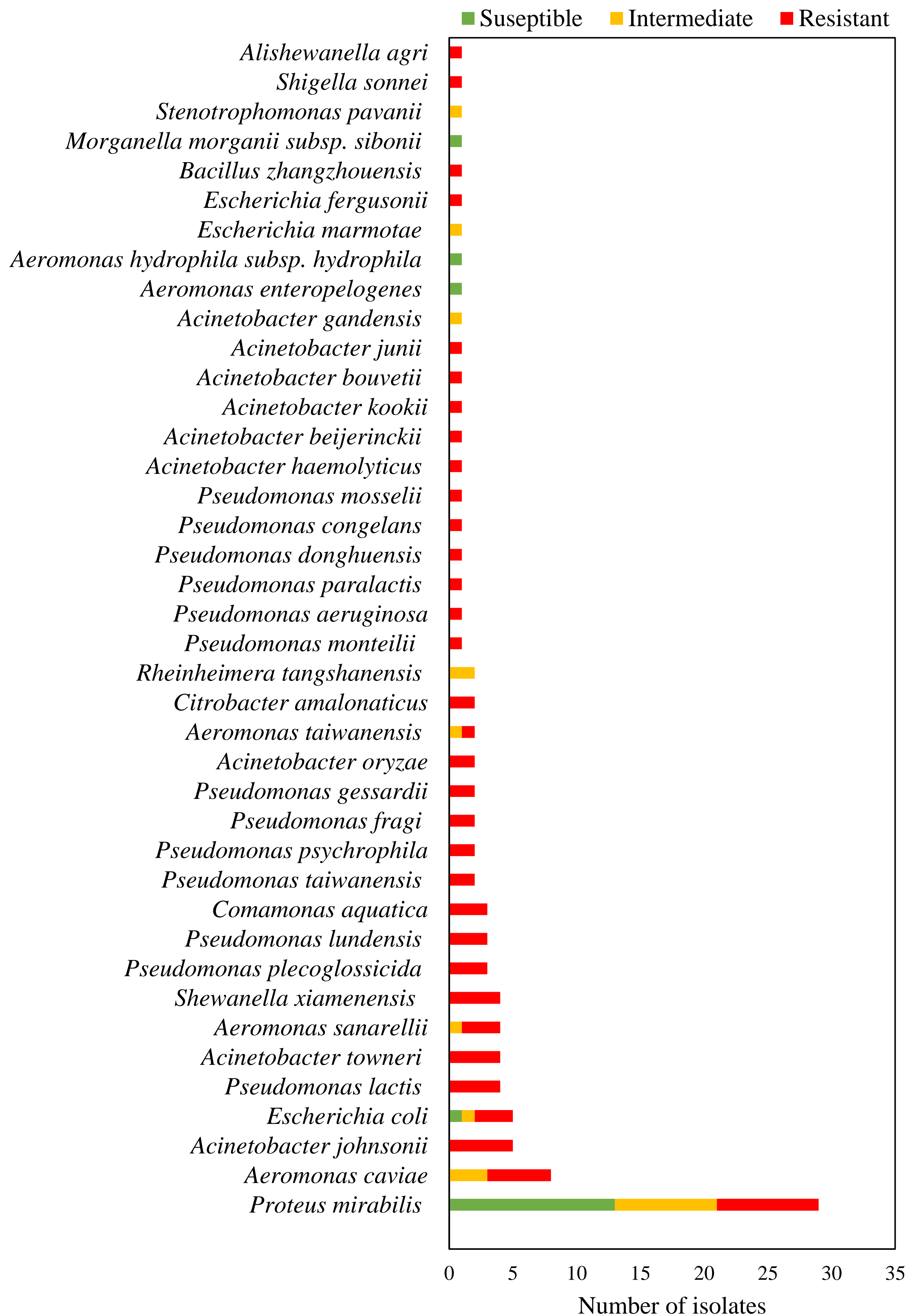


Figure 4.9. Resistance pattern of different microbial genera against ofloxacin.

4.5.4 Amoxicillin resistance test results

Antibiotic resistance test of all isolates against amoxicillin showed that the percentage of isolates susceptible to amoxicillin were 6.4% while 83.5% isolates showed resistance. Species from genus *Escherichia* (*E. coli, E. fergusonii and E. marmotae*) showed highest resistance percentage (100%) with all the strains being resistant to the drug. Previously, high resistance of *E. coli* to antimicrobial agents was observed in a study (Jafri *et al.,* 2014) which is similar to the results of this study in which very high resistance of *E. coli* isolates to ampicillin and amoxicillin was observed.

Followed by *Pseudomonas*, with most of the strains (*Pseudomonas plecoglossicida, Pseudomonas taiwanensis, Pseudomonas monteilii, Pseudomonas lundensis Pseudomonas aeruginosa, Pseudomonas paralactis, Pseudomonas fragi, Pseudomonas donghuensis, Pseudomonas congelans, Pseudomonas lactis, Pseudomonas mosselii, Pseudomonas gessardii*) showing 100% resistance to amoxicillin. Similar study conducted by Mubbunu *et al*. (2014) reported *E. coli*, *Pseudomonas* and *Streptococuss* isolates resistant to amoxicillin. Resistance prevalence in *Acinetobacter* and *Aeromonas* strains was also very high i.e. 94.0% and 93.7% strains showed resistance to the antibiotic amoxicillin, respectively. Few strains of *Acinetobacter gandensis* and *Aeromoas caviae* showed intermediate resistance while most of them were completely resistant to amoxicillin. A number of *Proteus* isolates were intermediately resistant to amoxicillin. In another study by Maluping *et al.* (2005), maximum isolates showed high MIC values for ampicillin and amoxicillin. Similarly, Gad *et al.* (2011) observed that *P. aeruginosa* isolates were 100% resistant to ampicillin and amoxicillin, highly resistant to tetracycline (95%) and amoxicillin/clavulanate (95%). Among the less frequently isolated species *Shewanella* sp., *Comamonas* sp., *Citrobacter* sp., *Rheinheimera* sp., *Bacillus* sp., *Morganella* sp., *Stenotrophomonas* sp., *Shigella* sp. and *Alishewanella* sp. showed 100% resistance to amoxicillin.

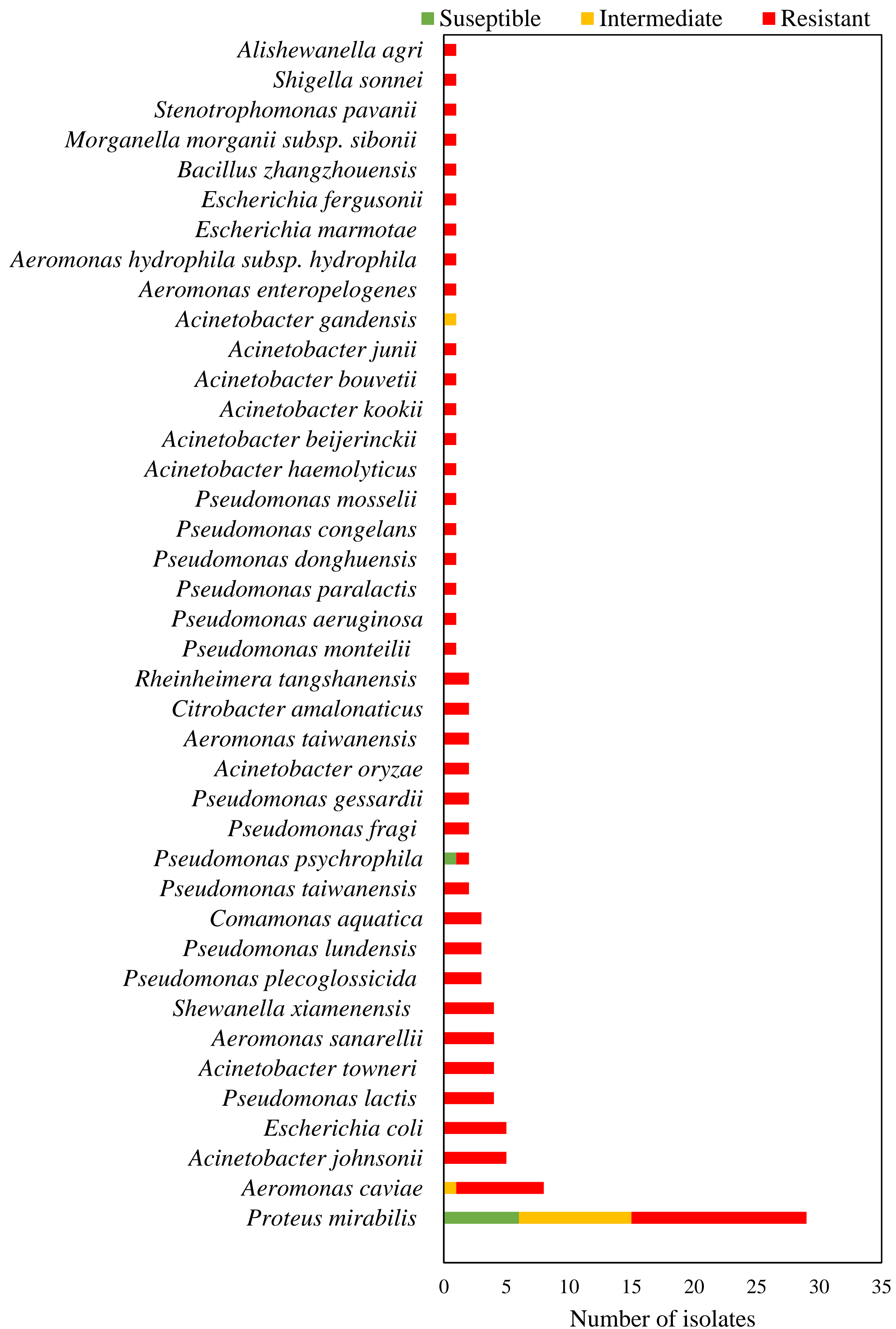


Figure 4.10. Resistance pattern of different microbial genera against amoxicillin

The trend observed in case of amoxicillin was *Escherichia* sp.> *Pseudomonas* sp.> *Acinetobacter* sp.> *Aeromonas* sp.> *Proteus* sp. while among less frequently isolated species all showed complete resistance to amoxicillin (Figure 4.10).

4.5.5. Ampicillin resistance test results

Ampicillin and amoxicillin resistance pattern was observed to be almost same in all the isolates, except *Proteus* being more resistant to ampicillin. The percentage of isolates susceptible to ampicillin were 1.8% while 91.7% isolates showed resistance. Species from the genus *Escherichia (E. coli, E. marmotae, E. fergusonii)*, *Pseudomonas* sp. including *P. plecoglossicida, P. taiwanensis, P. monteilii, P. lundensis, P. aeruginosa, P. paralactis, P. fragi, P. donghuensis, P. congelans, P. lactis, P. mosselii, P. gessardii* and *P. gandensis* and *Shewanella ziamensis* showed 100 % resistance to ampicillin, followed by genus *Acinetobacter* with species *A. haemolyticus, A. beijerinckii, A. johnsonii, A. kookii, A. oryzae, A. bouvetii, A. junii, A. towneri* showed 100% resistance while *Acinetobacter gandensis* strains showed intermediate resistance to ampicillin. The overall resistance percentage in the genus was 94%. High resistance pattern among *E. coli* strains has been reported in literature previously by Jafri *et al.* (2014) who observed that most of the *E. coli* strains were resistant to different tested antibiotics. Similarly, a 30-year (1979–2009) follow-up study on *E. coli* in Sweden showed an increasing resistance trend for ampicillin, sulfonamide, trimethoprim, and gentamicin (Kronvall *et al.,* 2010).

Aeromonas strains were also observed to be highly ampicillin resistant. Except few *Aeromonas caviae* strains, all showed 100% resistance to ampicillin. Resistance percentage observed was as high as 93% in *Aeromonas* strains. These results were consistent with a study conducted by Tadesse *et al*. (2012) in which they observed that the most common resistance phenotypes were the older drugs such as sulfonamide, streptomycin and ampicillin.

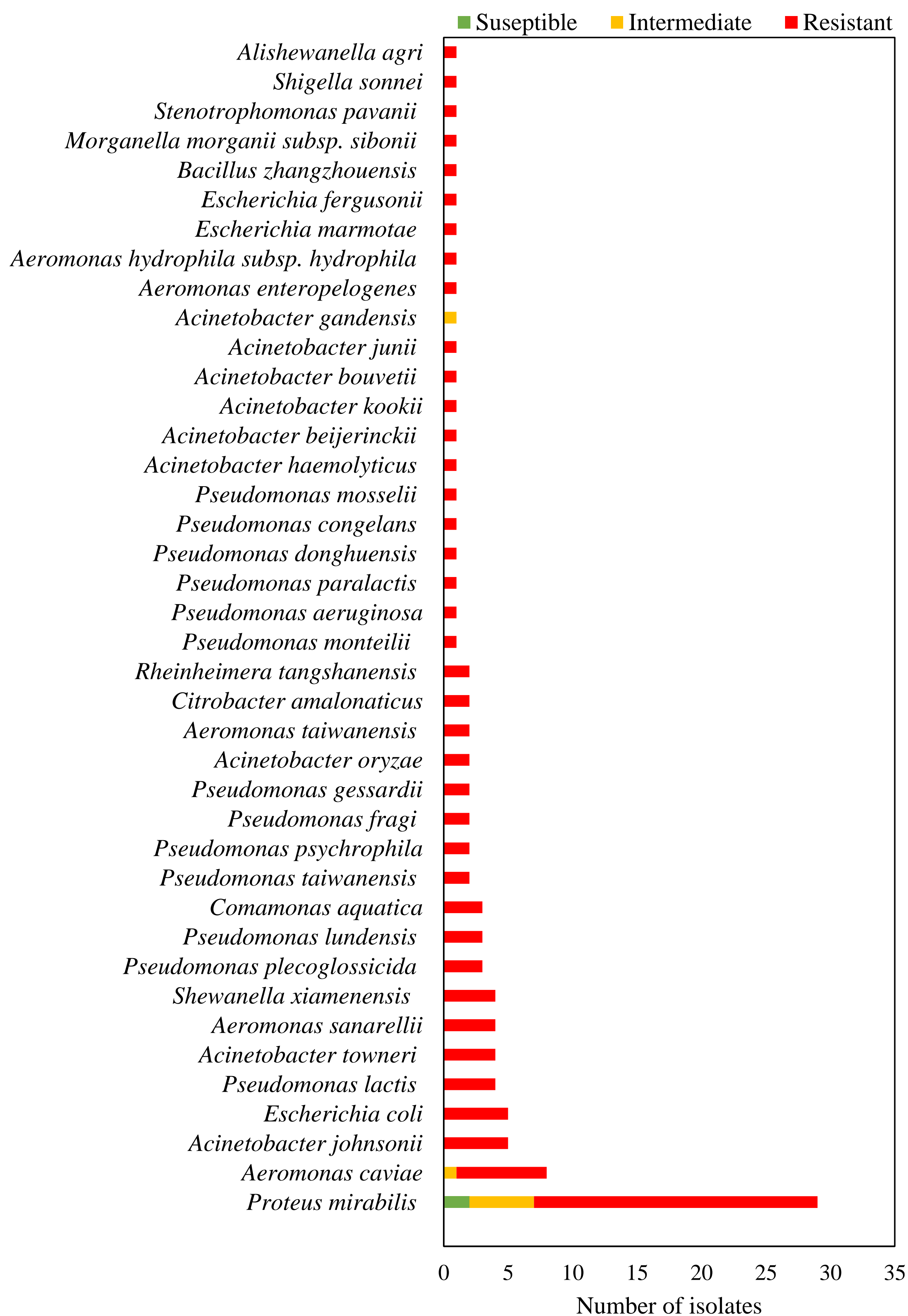


Figure 4.11. Resistance pattern of different microbial genera against amoxicillin

Proteus sp. showed more resistance to ampicillin than amoxicillin with 75% resistance percentage. More than 17% were intermediately resistant to ampicillin. Among the less frequently isolated strains, *Shewanella* sp., *Comamonas* sp., *Citrobacter* sp., *Rheinheimera* sp., *Bacillus* sp., *Morganella* sp., *Stenotrophomonas* sp., *Shigella* sp. and *Alishewanella* sp. showed 100% resistance to ampicillin. As discussed in the literature, members of genus *Shewanella* are generally susceptible to third- and fourth-generation quinolones but resistant to penicillin (Holt *et al.,* 2004; Kang *et al.,* 2016). In another study, trends similar to our results were reported (Feglo *et al.,* 2010; Newman *et al.,* 2006) in which 70–90% of *P. mirabilis* isolates exhibited resistance to ampicillin.

The trend observed for frequently isolated species was *Pseudomonas* sp.> *Escherichia* sp.> *Acinetobacter* sp.> *Aeromonas* sp.> *Proteus* sp. among less frequently isolated species *Shewanella* sp., *Comamonas* sp., *Citrobacter sp., Rheinheimera sp., Bacillus* sp. *Morganella* sp., *Stenotrophomonas* sp., *Shigella sonnei* sp., *Alishewanella* sp. all were observed to be 100% resistance to ampicillin (Figure 4.11).

Highest resistance was observed against ampicillin and lest resistant was levofloxacin. A large number of isolates showed intermediate resistance to levofloxacin. The resistance pattern observed among tested antibiotics was ampicillin (91.7%)> amoxicillin (83.5%)> ofloxacin (66.9%)> ciprofloxacin (27.5%)> levofloxacin (21.1%) (Figure 4.12). In terms of susceptibility, most of the isolates were susceptible to ciprofloxacin.

The incidence of ciprofloxacin resistance was observed higher than levofloxacin in the tested organisms. The same trend was also reported by previous studies that showed a higher number of ciprofloxacin resistant strains than levofloxacin among the clinical isolates of *Enterobacteriaceae* (Hoban *et al.,* 2010; Fu *et al.,* 2013). In the present investigation, we also

observed similar data among the isolates of *Acinetobacter, Aeromonas* and *E. coli*. The overall prevalence of multiple drug resistance (MDR) in this study was 30.3% (Table 4.2).

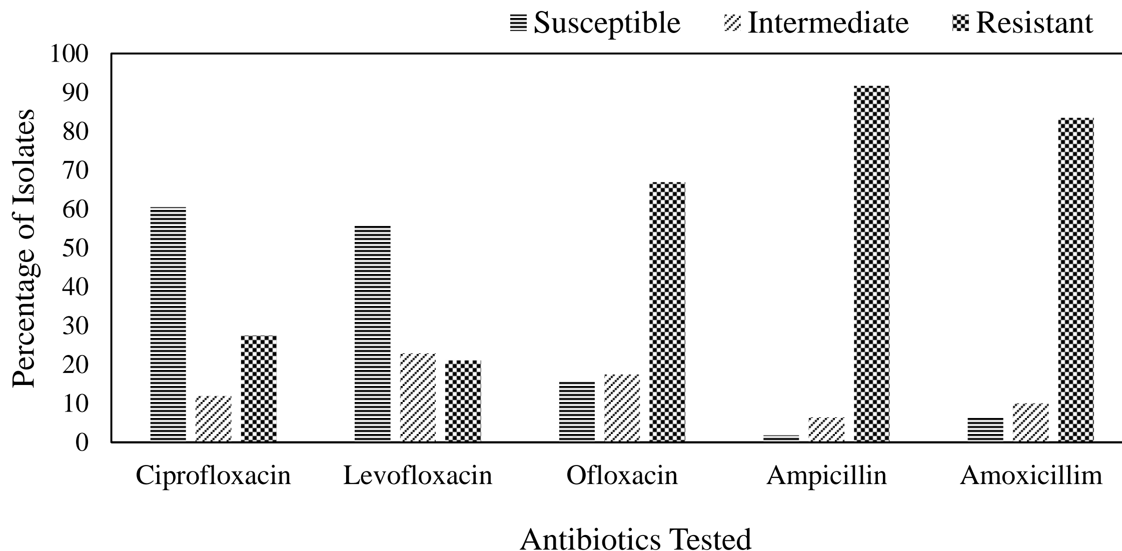


Figure 4.12. Antibiotic resistance test results of one hundred nine isolates against selected antibiotics

From the present study, presence of multi antibiotic resistance microbes in the wastewater/water channels of Pakistan is confirmed. This reflects an extensive release of these agents in the environment. As the growing increase in resistance is posing threats to public health due to delayed treatments or treatment failure, therefore this kind of studies can help in assessment of prevalence of antibiotic resistance in the environment and take appropriate measures to reduce the proliferation of resistant organisms in the environment by either controlling the antibiotic discharge into the water environments or reducing the number of resistant microbes with the help of some biological techniques.

Table 4.2. Susceptibility and resistance percentages of different microbial genera against tested antibiotics

Antibiotics	CIP			LEV			OFX			AMP			AML		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Proteus sp.	86.2	6.9	6.9	72.4	20.7	6.9	44.8	27.6	27.6	6.9	17.2	75.9	20.7	31.0	48.3
Pseudomonas sp.	95.8	0.0	4.2	91.7	0.0	8.3	0.0	0.0	100.0	0.0	0.0	100.0	4.2	0.0	95.8
Acinetobacter sp	52.9	17.6	29.4	47.1	29.4	23.5	0.0	5.9	94.1	0.0	5.9	94.1	0.0	5.9	94.1
Aeromonas sp.	12.5	18.8	68.8	25.0	37.5	37.5	12.5	31.3	56.3	0.0	6.3	93.8	0.0	6.3	93.8
Escherichia sp.	28.6	14.3	57.1	14.3	28.6	57.1	14.3	28.6	57.1	0.0	0.0	100.0	0.0	0.0	100.0
Shewanella sp.	75.0	0.0	25.0	75.0	0.0	25.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0
Comamonas sp.	0.0	33.3	66.7	33.3	33.3	33.3	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0
Citrobacter sp.	0.0	0.0	100.0	0.0	50.0	50.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0
Rheinheimera sp.	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0
Bacillus sp.	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0
Morganella sp.	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0
Stenotrophomonas sp.	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0
Shigella sonnei sp.	100.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0

There were some limitations of the study as well. Repeated samplings should be done from the sites in order to assess the prevalence of resistance more accurately. Keeping in mind the different concentration of antibiotics and environmental conditions present in the particular area the results may vary if the sample is collected from any other sites even of the same city.

The number of antibiotics considered for this study were five as it was a small scale experiment. More antibiotics, from each group and generation, should be tested for their resistance in the environmental microbiota. Then an organism can be declared as multidrug resistant if it is resistant to more than 3 antibiotic classes.

At this stage we cannot claim that the resistance in the environmental microorganisms is either due to the selective pressure due to the presence of environmental level of antibiotics in that particular area or due to the horizontal gene transfer mechanism. Therefore, more work needs to be done on the mechanism of resistance and which genes are responsible for the prevalence of resistance in these microorganisms. Environmental concentrations of antibiotics in the area should also be considered and linked with the genes that express themselves under the antimicrobial stress conditions.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

A diverse bacterial community was observed in the samples collected from 3 cities. One hundred and nine isolated strains belonged to two phyla including Proteobacteria and Firmicutes and thirteen different genera with forty different species. The most frequently identified species from these samples were *Proteus* sp. (26.6%) followed by *Pseudomonas* sp. (22.0%), *Acinetobacter* sp. (15.6%), *Aeromonas* sp. (14.7%), *Escherichia* sp. (6.4%), *Shewanella* sp. (3.7%), *Comamonas* sp. (2.8%) and seven other less frequently isolated species.

The antibiotic resistance testing was performed on all the isolated strains against five selected antibiotics i.e. ciprofloxacin, levofloxacin, ofloxacin, ampicillin and amoxicillin. Highest resistance was observed against ampicillin and least resistance against levofloxacin. A large number of isolates showed intermediate resistance to levofloxacin. The resistance pattern observed among tested antibiotics was ampicillin (91.7%)> amoxicillin (83.5%)> ofloxacin (67.0%)> ciprofloxacin (27.5%)>levofloxacin (21.1%). In terms of susceptibility, most of the isolates remained susceptible to ciprofloxacin.

The results indicate that strains of bacteria isolated were multi-resistant to majority of the tested therapeutic agents, thus making these drugs ineffective for the treatment of infections caused by these pathogens. This is clear from a considerable percentage (30.3 %) of isolates that are resistant to more than three tested antibiotics. Species from the genus *Escherichia* (57.1 %), *Aeromonas* (56.3 %), *Acinetobacter* (41.2 %), *Proteus* (13.8 %), *Pseudomonas* (8.3%), *Shewanella* (25.0%) and *Comamonas* (66.7%) showed resistance to more than three antibiotics while among the less frequently identified species, isolates from the genus *Citrobacter*, *Bacillus and Alishewanella* showed 100% resistance to all the five tested antibiotics. The incidence of ciprofloxacin resistance was observed higher than levofloxacin in the tested organisms.

5.2. Recommendations

In this study, the resistance was detected in the microbes to the most commonly used antibiotics which indicates that these organisms have been well exposed to the tested antimicrobials and have developed mechanisms to evade these antibiotics. Therefore, there is a need to control the release of antibiotics in the environment in order to control the proliferation of resistant organisms. Understanding the mechanism and exploring particular genes involved in antibiotic resistance can help to propose an effective solution. Bio-control strategies like use of bacteriophages for destruction of resistant microbes can be useful and effective in treatments where antibiotic resistant microbes are the causal organisms. Furthermore, extensive research needs to be done for better understanding and to address this issue. Future work in following dimensions can help to handle this issue.

5.2.1. Track the resistance frequency

It is important that the frequency of resistance in various types of bacteria be traced, in order to predict the trends and possible threats of resistance in the important human pathogens. National and global surveillance systems will help to shape the picture of resistance profile in the given area, and thus help the health practitioners and public health officers making more careful choices of treatment and will also warn them of possible new threats and treads in the emergence of resistance (Levy & Marshall, 2004).

5.2.2. Regulation and introduction of new therapeutic approaches

Reduced and prudent use of antibiotics is one of the most important measures needed to be taken to reduce the emergence of resistance. The pace of emergence of resistance and development of new drugs is unmatched, and it is therefore necessary that the regulation of the use of current drugs be carried out on a massive level. This will not only reduce the emergence of resistance but will also provide time for an effective drug to emerge (Levy & Marshall,

2004). In addition to this, novel drugs with newer targets or ones that block the current resistant mechanism need to be developed.

5.2.3. Replacement of antibiotics

Another approach is to inhibit the processes that are involved in infection process (Alekshun *et al.*, 2007). Early and rapid diagnostics and vaccinations may further ease the burden and alternate therapies including use of bacteriophages are also an option that needs to be deeply explored (Levy & Marshall, 2004).

REFERENCES

Ahmad, M., Khan, A.U., Wahid, A., Farhan, M., Butt, Z.A., & Ahmad, F. (2014). Urban wastewater as hotspot for antibiotic and antibiotic resistant bacteria spread into the aquatic environment. *Asian Journal of Chemistry*, 26(2), 579.

Ahmed, I., Kudo, T., Abbas, S., Ehsan, M., Iino, T., Fujiwara, T. & Ohkuma, M. (2014). *Cellulomonas pakistanensis* sp. nov., a moderately halotolerant Actinobacteria. *International Journal of Systematic and Evolutionary Microbiology*, 64(7), 2305-2311.

Ahmed, I., Yokota, A. & Fujiwara, T. (2007). A novel highly boron tolerant bacterium, *Bacillus boroniphilus* sp. nov., isolated from soil, that requires boron for its growth. *Extremophiles*, 11(2), 217-224.

Alekshun, M. N. & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell*, 128(6), 1037–1050.

Aminov, R.I. (2009). The role of antibiotics and antibiotic resistance in nature. *Environmental Microbiology*, 11(12), 2970-2988.

Atkinson, B.A. & Lorian, V. (1984). Antimicrobial agent susceptibility patterns of bacteria in hospitals from 1971 to 1982. *Journal of Clinical Microbiology*, 20(4), 791-796.

Blaettler, L., Mertz, D., Frei, R., Elzi, L., Widmer, A.F., Battegay, M. & Flückiger, U. (2009). Secular trend and risk factors for antimicrobial resistance in *Escherichia coli* isolates in Switzerland 1997–2007. *Infection*, 37(6), 534-539.

Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O. & Piddock, L.J.V. (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*, 13(1), 42-51.

Bolaji, A.S., Akande, I.O., Iromini, F.A., Adewoye, S.O. & Opasola, O.A. (2011). Antibiotic resistance pattern of bacteria sp. isolated from hospital waste water in Ede South Western, Nigeria. *European Journal of Experimental Biology*, 1(4), 66-71.

Cattoir, V., Poirel, L., Aubert, C., Soussy, C.J. & Nordmann, P. (2008). Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* sp. *Emerging Infectious Diseases*, 14(2), 231.

Cavaco, L.M. & Aarestrup, F. M. (2009). Evaluation of quinolones for use in detection of determinants of acquired quinolone resistance, including the new transmissible resistance mechanisms qnrA, qnrB, qnrS, and aac (6′) Ib-cr, in *Escherichia coli* and *Salmonella enterica* and determinations of wild-type distributions. *Journal of Clinical Microbiology*, 47(9), 2751-2758.

Chun, J., Lee, J.H., Jung, Y., Kim, M., Kim, S., Kim, B.K., & Lim, Y.W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, 57(10), 2259-2261.

Cockerill, F. R. (Ed.). (2011). Performance standards for antimicrobial susceptibility testing: twenty-first informational supplement. *Clinical and Laboratory Standards Institute (CLSI).* Retrieved from: *clsi.org*

Cruickshank, M. (2011). Antimicrobial stewardship in Australian hospitals. *Sydney: Australian Commission on Safety & Quality in Health Care*.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W., Schwarz, C. & Golding, G. B. (2011). Antibiotic resistance is ancient. *Nature*, 477(7365), 457-461.

Deak, E., Hindler, J. A., Skov, R., Sjölund-Karlsson, M., Sokovic, A. & Humphries, R. M. (2015). Performance of E-test and disk diffusion for detection of ciprofloxacin and levofloxacin resistance in *Salmonella enterica*. *Journal of Clinical Microbiology*, 53(1), 298-301.

Doern, G.V., Daum, G.S. & Tubert, T.A. (1987). Ampicillin disk diffusion susceptibility testing of *Haemophilus influenzae*. *Journal of Clinical Microbiology*, 25(9), 1675-1678.

European Comission (EC) Trade related technical assistance program (TRTA) for Pakistan: The pharmaceutical sector in Pakistan, Export performance and potential implications of WTO agreements, August 2007.

Fair, R.J. & Tor, Y. (2014). Antibiotics and bacterial resistance in the 21st century. *Perspectives in Medicinal Chemistry*, 6(1), 25.

Federation, W. E. & American Public Health Association. (2005). Standard methods for the examination of water and wastewater. *American Public Health Association (APHA): Washington, DC, USA*.

Feglo, P.K., Gbedema, S.Y., Quay, S.N.A., Adu-Sarkodie, Y. & Opoku-Okrah, C. (2010). Occurrence, species distribution and antibiotic resistance of *Proteus* isolates: A case study at the Komfo Anokye Teaching Hospital (KATH) in Ghana. *International Journal of Pharma Sciences and Research*, 1(9), 347-52.

Friedman, N.D., Temkin, E. & Carmeli, Y. (2016). The negative impact of antibiotic resistance. *Clinical Microbiology and Infection*, *22*(5), 416-422.

Fu, Y., Zhang, W., Wang, H., Zhao, S., Chen, Y., Meng, F. & Zhang, F. (2013). Specific patterns of gyr A mutations determine the resistance difference to ciprofloxacin and levofloxacin in *Klebsiella pneumoniae* and *Escherichia coli*. *BMC Infectious Diseases*, 13(1), 8-14.

Fuchs, P.C., Jones, R.N., Barry, A.L. & Gavan, T.L. (1989). Ofloxacin susceptibility testing quality control parameters for micro dilution and disk diffusion, and confirmation of disk diffusion interpretive criteria. *Journal of Clinical Microbiology*, 27(1), 49-52.

Gad, G.F., Mohamed, H.A., & Ashour, H.M. (2011). Aminoglycoside resistance rates, phenotypes, and mechanisms of Gram-negative bacteria from infected patients in upper Egypt. *PLoS One*, 6(2), e17224.

Gales, A.C., Jones, R.N., Forward, K.R., Linares, J., Sader, H.S. & Verhoef, J. (2001). Emerging importance of multidrug-resistant Acinetobacter species and *Stenotrophomonas maltophilia* as pathogens in seriously ill patients: geographic patterns, epidemiological features, and trends in the SENTRY Antimicrobial Surveillance Program (1997–1999). *Clinical Infectious Diseases*, *32*(2), 104-113.

Giske, C.G., Monnet, D.L., Cars, O., & Carmeli, Y. (2008). Clinical and economic impact of common multidrug-resistant Gram-negative bacilli. *Antimicrobial Agents and Chemotherapy*, 52(3), 813-821.

Hancock, R.E. & Brinkman, F.S. (2002). Function of *Pseudomonas* porins in uptake and efflux. *Annual Reviews in Microbiology*, 56(1), 17-38.

Hayat, R., Sheirdil, R.A., Iftikhar-ul-Hassan, M. & Ahmed, I. (2013). Characterization and identification of compost bacteria based on 16S rRNA gene sequencing. *Annals of Microbiology*, 63(3), 905-912.

Hiramatsu, K., Ito, T., Tsubakishita, S., Sasaki, T., Takeuchi, F., Morimoto, Y. & Baba, T. (2013). Genomic basis for methicillin resistance in *Staphylococcus aureus*. *Infection and Chemotherapy*, 45(2), 117-136.

Hoban, D.J., Bouchillon, S.K., Hawser, S.P., Badal, R.E., La Bombardi, V.J. & DiPersio, J. (2010). Susceptibility of Gram-negative pathogens isolated from patients with complicated intra-abdominal infections in the United States, 2007-2008: Results of the study for monitoring antimicrobial resistance trends (SMART). *Antimicrobial Agents and Chemotherapy*, 54(7), 3031-3034.

Hoerger, C.C., Dorr, B., Schlienger, C. & Straubt, J.O. (2009) Environmental risk assessment for the galenical formulation of solid medicinal products at Roche Basle, Switzerland. *Integrated Environmental Assessment and Management,* 5 (2) 331–337.

Holt, H.M., Gahrn-Hansen, B. & Bruun, B. (2004). *Shewanella* species. *Clinical Microbiology & Infection Supplement*, 10, 348-349.

Hooper, D.C. (2002). Fluoroquinolone resistance among Gram-positive cocci. *The Lancet Infectious Diseases*, 2(9), 530-538.

Hopkins, K.L., Davies, R.H., & Threlfall, E.J. (2005). Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. *International Journal of Antimicrobial Agents*, 25(5), 358-373.

Huang, C.H., Renew, J.E., Smeby, K.L., Pinkston, K., & Sedlak, D.L. (2011). Assessment of potential antibiotic contaminants in water and preliminary occurrence analysis. *Journal of Contemporary Water Research and Education*, 120(1), 4.

Hujer, K.M., Hujer, A.M., Hulten, E.A., Bajaksouzian, S., Adams, J.M., Donskey, C.J. & Thomson, J.M. (2006). Analysis of antibiotic resistance genes in multidrug-resistant *Acinetobacter* sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. *Antimicrobial Agents and Chemotherapy*, 50(12), 4114-4123.

Jafri, S.A., Qasim, M., & Masoud, M.S. (2014). Antibiotic resistance of *E. coli* isolates from urine samples of urinary tract infection (UTI) patients in Pakistan. *Bioinformation*, 10(7), 419-422.

Jurtshuk, P., & McQuitty, D. N. (1976). Use of a quantitative oxidase test for characterizing oxidative metabolism in bacteria. *Applied and Environmental Microbiology*, 31(5), 668-679.

Kang, C.H., & So, J.S. (2016). Antibiotic and heavy metal resistance in *Shewanella putrefaciens* strains isolated from shellfishes collected from West Sea, Korea. *Marine Pollution Bulletin*, 112(1), 111-116.

Katsivela, E., Bonse, D., Kruger, A., Strompl, C., Livingston, A. & Ittich R.M. (1999). An extractive membrane biofilm reactor for degradation of 1,3-dichloropropene in industrial wastewater. *Applied Microbiology Biotechnology*, 52(6), 853–862.

Khan, G.A., Berglund, B., Khan, K.M., Lindgren, P.E., & Fick, J. (2013). Occurrence and abundance of antibiotics and resistance genes in rivers, canal and near drug formulation facilities–a study in Pakistan. *PLoS One*, 8(6), e62712.

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi H., Won, S. & Chun, J. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *International Journal of Systematic and Evolutionary Microbiology,* 62(1), 716–721.

Kim, S. & Aga, D.S. (2007). Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *Journal of Toxicology and Environmental Health, Part B*, 10(8), 559-573.

Kowalski, R.P., Dhaliwal, D.K., Karenchak, L.M., Romanowski, E.G., Mah, F.S., Ritterband, D.C., & Gordon, Y.J. (2003). Gatifloxacin and moxifloxacin: an *in vitro* susceptibility comparison to levofloxacin, ciprofloxacin, and ofloxacin using bacterial keratitis isolates. *American Journal of Ophthalmology*, 136(3), 500-505.

Kresge, N., Simoni, R.D. & Hill, R.L. (2004). Selman Waksman: the father of antibiotics. *Journal of Biological Chemistry*, *279*(48), 7-7.

Kristiansson, E., Fick. J., Janzon, A., Grabic, R., Rutgersson C. (2011). Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PLoS ONE*, 6 (2), e17038.

Kronvall, G. (2010). Antimicrobial resistance 1979–2009 at Karolinska hospital, Sweden: normalized resistance interpretation during a 30-year follow-up on *Staphylococcus aureus*

and *Escherichia coli* resistance development. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica (Apmis)*, 118(9), 621-639.

Kruse, H. (1999). Indirect transfer of antibiotic resistance genes to man. *Acta Veterinaria Scandinavica. Supplement (Denmark),* 92(1), 59-65.

Kümmerer, K. (2009). Antibiotics in the aquatic environment–a review–part II. *Chemosphere*, 75(4), 435-441.

Larsson, D.G.J., de Pedro, C. & Paxeus, N. (2007). Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *Journal of Hazardous Materials*, 148(3), 751–755.

Levy, S.B., & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine*, 10,122-129.

Li, D., Yang, M., Hu, J., Ren, L., Zhang, Y. & Li, K. (2008). Determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river. *Environmental Toxicology and Chemistry*, 27(1), 80-86.

Ligon, B.L. (2004). Penicillin: its discovery and early development. *Seminars in Pediatric Infectious Diseases***,** 15(1), 52-57.

Madigan, M.T., Martinko, J. M., & Parker, J. (2006). *Brock biology of microorganisms*. Upper Saddle River, NJ: Prentice Hall.

Malik, A. & Aleem, A. (2011). Incidence of metal and antibiotic resistance in Pseudomonas spp. from the river water, agricultural soil irrigated with wastewater and groundwater. *Environmental Monitoring and Assessment*, 178(1), 293-308.

Maluping, R.P., Lavilla-Pitogo, C.R., DePaola, A., Janda, J.M., Krovacek, K., & Greko, C. (2005). Antimicrobial susceptibility of *Aeromonas* spp., *Vibrio spp*. and *Plesiomonas shigelloides* isolated in the Philippines and Thailand. *International Journal of Antimicrobial Agents*, 25(4), 348-350.

Martinez, J.L. (2009). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proceedings of the Royal Society of London B: Biological Sciences*, 276(1667), 2521-2530.

Martínez-Martínez, L., Pascual, A. & Jacoby, G.A. (1998). Quinolone resistance from a transferable plasmid. *The Lancet*, 351(9105), 797-799.

Mavroidi, A., Miriagou, V., Liakopoulos, A., Tzelepi, E., Stefos, A., Dalekos, G.N., & Petinaki, E. (2012). Ciprofloxacin-resistant *Escherichia coli* in central Greece: mechanisms of resistance and molecular identification. *BMC Infectious Diseases*, 12(1), 371.

Moyane, J.N., Jideani, A.I.O., & Aiyegoro, O.A. (2013). Antibiotics usage in food-producing animals in South Africa and impact on human: Antibiotic resistance. *African Journal of Microbiology Research*, 7(24), 2990-2997

Mubbunu, L., Siyumbi, S., Katongo, C., & Mwambungu, A. (2014). Waste water as reservoir of antibiotic resistant micro-organisms: a case of Luanshya waste water ponds. *International Journal of Research in Medical and Health Sciences*, 4(9), 15-20.

Munita, J.M. & Arias, C.A. (2016). Mechanisms of antibiotic resistance. *Microbiology Spectrum*, *4*(2).

Newman, M., Frimpong, E., Asamoah-Adu, A., & Sampene-Donkor, E. (2006). Resistance to antimicrobial drugs in Ghana. *The Ghanaian-Dutch Collaboration for Health Research and Development Project number 2001* (No. 5, pp. 8-26). GD/07. Technical Report series.

Pagès, J.M., James, C.E. & Winterhalter, M. (2008). The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nature Reviews Microbiology*, 6(12), 893-903.

Paterson, D.L. & Bonomo, R.A. (2005). Extended-spectrum β-lactamases: A clinical update. *Clinical Microbiology Reviews*, 18(4), 657-686.

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*, 56(1), 20-51.

Pruden, A., Pei, R., Storteboom, H. & Carlson, K.H. (2006). Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environmental Science & Technology*, 40(23), 7445-7450.

Ramirez, M.S. & Tolmasky, M.E. (2010). Aminoglycoside modifying enzymes. *Drug Resistance Updates*, 13(6), 151-171.

Rath, S. & Padhy, R.N. (2015). Prevalence of fluoroquinolone resistance in *Escherichia coli* in an Indian teaching hospital and adjoining communities. *Journal of Taibah University Medical Sciences*, 10(4), 504-508.

Rehman, M.S.U., Rashid, N., Ashfaq, M., Saif, A., Ahmad, N. & Han, J.I. (2015). Global risk of pharmaceutical contamination from highly populated developing countries. *Chemosphere*, 138, 1045-1055

Roberts, M.C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiology Letters*, *245*(2), 195-203.

Sader, H.S., & Jones, R.N. (2005). Antimicrobial susceptibility of uncommonly isolated non-enteric Gram-negative bacilli. *International Journal of Antimicrobial Agents*, 25(2), 95-109.

Saeed, H.A. & Ahmed, W.B. (2009). *In vitro* activity of some antimicrobial agents against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* in Khartoum, Sudan. *Research Journal of Microbiology*, 4(10), 366-369.

Sengupta, S., Chattopadhyay, M.K. & Grossart, H.P. (2013). The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in Microbiology*, 4, 47.

Sim, W.J., Lee, J.W., Lee, E.S., Shin, S.K. & Hwang, S.R. (2011). Occurrence and distribution of pharmaceuticals in wastewater from households, livestock farms, hospitals and pharmaceutical manufactures. *Chemosphere*, 82(2), 179–186.

Strebhardt, K., & Ullrich, A. (2008). Paul Ehrlich's magic bullet concept: 100 years of progress. *Nature Reviews Cancer*, 8(6), 473-480.

Sydnor, E.R., & Perl, T.M. (2011). Hospital epidemiology and infection control in acute-care settings. *Clinical Microbiology Reviews*, 24(1), 141-173.

Tadesse, D.A., Zhao, S., Tong, E., Ayers, S., Singh, A., Bartholomew, M.J. & McDermott, P.F. (2012). Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950–2002. *Emerging Infectious Diseases*, 18(5), 741.

Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24(8), 1596–1599

Taylor, W.I. & Achanzar, D. (1972). Catalase test as an aid to the identification of Enterobacteriaceae. *Applied Microbiology*, 24(1), 58-61.

Tomlinson, J.H., Thompson, G.S., Kalverda, A.P., Zhuravleva, A. & O'Neill, A.J. (2016). A target-protection mechanism of antibiotic resistance at atomic resolution: insights into FusB-type fusidic acid resistance. *Scientific Reports*, 6.

Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S. A., Robinson, T.P. & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences*, 112(18), 5649-5654.

Van Looveren, M. & Goossens, H. (2004). Antimicrobial resistance of *Acinetobacter* spp. in Europe. *Clinical Microbiology and Infection*, 10(8), 684-704.

Walson, J.L., Marshall, B., Pokhrel, B.M., Kafle, K.K. and Levy, S.B. (2001). Carriage of antibiotic-resistant fecal bacteria in Nepal reflects proximity to Kathmandu. *Journal of Infectious Diseases*, 184(9), 1163–1169.

Wegener, H.C., Aarestrup, F.M., Gerner-Smidt, P. & Bager, F. (1999). Transfer of antibiotic resistant bacteria from animals to man. *Acta Veterinaria Scandinavica. Supplementum*, 92, 51-57.

Wepener, V., Van Vuren, J.H.J. & Du Preez, H.H. (2001). Uptake and distribution of a copper, iron and zinc mixture in gill, liver and plasma of a freshwater teleost, *Tilapia sparrmanii*. *Water SA*, 27(1), 99-108.

West, B.M., Liggit, P., Clemans, D.L. & Francoeur, S.N. (2011). Antibiotic resistance, gene transfer, and water quality patterns observed in waterways near CAFO farms and wastewater treatment facilities. *Water, Air, & Soil Pollution*, 217(1-4), 473-489.

Wilson, D.N. (2014). Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nature Reviews Microbiology*, 12(1), 35-48.

World Health Organization. (2014). *Antimicrobial resistance: Global report on surveillance*. World Health Organization.

Wright, G.D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Reviews Microbiology*, 5(3), 175-186.

Wu, N., Qiao, M., Zhang, B., Cheng, W.D. & Zhu, Y.G. (2010). Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. *Environmental Science & Technology*, 44(18), 6933-6939.

Yadav, A.K., Kumar, R., Saikia, R., Bora, T.C., & Arora, D.K. (2009). Novel copper resistant and antimicrobial *Streptomyces* isolated from Bay of Bengal, India. *Journal de Mycologie Médicale/Journal of Medical Mycology*, 19(4), 234-240.

Yuan, F., Hu, C., Hu, X., Qu, J. & Yang, M. (2009). Degradation of selected pharmaceuticals in aqueous solution with UV and UV/H_2O_2. *Water Research*, *43*(6), 1766-1774.

Zaman, K. (2011). Review of Pakistan pharmaceutical industry: SWOT analysis. *International Journal of Business and Information Technology*, *1*(2).

Zhang, X.X., Zhang, T. & Fang, H.H. (2009). Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology*, 82(3), 397-414.

APPENDIX A

Table A1. Survey questions for the most commonly prescribed antibiotics

QUESTIONS	Allied Hospital, Fsd.	Civil Hospital, Fsd	Holy Family hospital, Rwp.	Private hospital, Rwp.	Children Hospital, Lhr.
What are the most common diseases prevailing in Pakistan?	Hepatitis C and TB	Diabetes mellitus and viral hepatitis	Acute respiratory infections (Viral infections, Pneumonia, Influenza-like illness), malaria, Viral Hepatitis, Cholera, Dengue, T.B.	Acute respiratory infections (For example Pneumonia, Influenza, Typhoid, Viral Hepatitis, Cardiovascular diseases Diabetes, Lung cancer.	Enteric fever (typhoid)
Is there any shift in the pattern of disease occurrence?	Not much shift. Prevalence of both have increased with time	Metabolic diseases and malignancies are more common	While treatment of diseases, increased drug resistance has been observed. This is accelerated by misuse, as well as poor infection prevention and control.	71% of the new born infections in Pakistan now are due to antibiotic resistance. Also, increased recovery time is required now due to same reason.	Yes, asymptomatic carriers are increased.
Which are the most commonly prescribed antibiotics for the above mentioned diseases?	Sofosbuvir (nucleotide analogue) with ribavirin and interferons for Hep C, Anti-tuberculous drugs include isoniazid, rifampicin, pyrazinamide, streptomycin, ethambutol.	Quinolones and penicillin are more commonly prescribed.	Amoxicillin(Penicillin) , Cephradine(1st generation cephalosporin), Ciprofloxacin (Cephalosporin) , Levofloxacin (Cephalosporin), Doxycycline (cephalosporin)	Amoxacillin (Penicillin), Metronidazole (Flaygl, Amoebicide), Ciprofloxacin Doxycycline,Clarithromycin (Macrolide), Azithromycin (Macrolide)	Ciprofloxacin, Ceftrioxone
Which medicines do you think patients find more effective?	Same as above.	Penicillin	Amoxil (Amoxicillin), Augmentin (Amoxacilline & Clavulinic acid) , Velosef (Cephradine- 1st generation cephalosporin), Cefspan (Cefixime- 3rd generation Cephalosporin) , Novidat (Ciprofloxain, Cephalosporin)	Amoxicillia (Penicillin) Cephradine (1st generation cephalosporin) Ciprofloxacin (Cephalosporin) , Clarithromycin (Mariolide)	Ceftriozone

Which medicines do people buy the most/ or in your opinion are prescribed the most by doctors?	Same as above.	Penicillin	Cap. Amoxil, Tab. Augmentin, Cap. Velosef, Cap. Cefspan	Flagyl (Metronidazole), Augmentin (penicillins, Amoxicilline, Alavuliaic acid), Novidat (ciprofloxacin)	Ciprofloxacin
Also state the dosage of the above mentioned medicines.	Sofosbuvir 400mg 1 OD with rifampicin 500mg 1 BD, For TB- isoniazid 5mg/kg body weight Rifampicin 10mg/kg Streptomycin 15mg/kg Pyrazinamide 25mg/kg	Variable in different diseases	Cap. Amoxil 250-500mg/8h, Tab. Augmentin 375-625mg/8h, Cap. Velosaf 250-500 mg/8hr-12hr Cap. Cefspan 400mg/12hr	Tab. Flagyl 200-400mg/8hr, Tab. Augmentin 375-625 mg/8hr, Novidat 250mg/12hr, Tab. Abozole 400mg/8hr, Azomax 500mg OD	Cipro 500mg bd, ceftriaxone IV bd
State any change in prescribed antibiotic for that particular disease?	For Hep. C now Daclatasavir replacing sofosbuvir And for TB, second line antituberculous replacing the first line due to resistance	Yes, resistance is becoming common	While treatment of pneumonia e.g MDR Stephalococcus Pneumonia isolates have been identified.	In acute respiratory infections the bacteria have developed resistance. Multiple newborn infections are a result of bacterial resistance.	No change
Which antibiotic proved to be the most effective one?	Same as 3	Colistin	Cephradine (1 st generation cephalosporin), Cefixime (3rd generation cephalosporin) , Ciprofloxacin (Cephalosporin), Levofloxacin (Cephalosporin)	Amoxacillin (penicillin), Clarithromycin (Macrolide), Ciprofloxacin (Cephalosporin), Doxycycline (Tetracycline)	Combination of above 2
Is there any change in pattern of prescription? Like if there was some other dosage given years ago but now it is different?	Same as 7	Yes, Dosage is increased because of resistance	Penicillin was prescribed more a few years ago but now cephalosporins are prescribed more.	Penicillin resistance was rare a few years back. However now multiple drug resistance isolates are identified.	No
Are you observing any change in the prescription of antibiotics by doctors in terms of dosage?	Same as 7		Dosage of antibiotics has increased in patients because of the emerging ineffectiveness of the antibiotics.	Increased recovery time. Therefore longer durations of treatment hence the prolonged exposure to antibiotic consumption.	No

APPENDIX B

Table B1. Colony morphology of isolated bacterial species

STRAINS	Morphological Characteristics						
	Form/ shape	Color	Elevation	Margin	Texture	Opacity	Pigment
Proteus sp.NCCP 1771	Circular	Creamy White	Flat	Entire	Smooth	Translucent	Non Pigmented
Pseudomonas sp.NCCP 1713	Punctiform	Cream	Flat	Entire	Dry	Translucent	Non Pigmented
Pseudomonas sp.NCCP 1709	Punctiform	Cream	Flat	Entire	Smooth	Translucent	Non Pigmented
Pseudomonas sp.NCCP 1703	Circular	Cream	Raised	Entire	Smooth	Opaque	Non Pigmented
Pseudomonas sp.NCCP 1779	Punctiform	Cream	Raised	Undulate	Smooth	Translucent	Non Pigmented
Pseudomonas sp.NCCP 1781	Circular	Cream	Raised	Curled	Smooth	Translucent	Non Pigmented
Pseudomonas sp.NCCP 1783	Circular	Cream	Convex	Entire	Smooth	Translucent	Non Pigmented
Pseudomonas sp.NCCP 1784	Punctiform	Cream	Flat	Undulate	Smooth	Translucent	Non Pigmented
Pseudomonas sp.NCCP 1787	Circular	Glisteing	Convex	Entire	Smooth	Translucent	Non Pigmented
Pseudomonas sp.NCCP 1788	Circular	Cream	Raised	Entire	Smooth	Translucent	Non Pigmented
Pseudomonas sp.NCCP 1801	Circular	Yellowish white	Raised	Entire	Smooth	Opaque	Non Pigmented
Pseudomonas sp.NCCP 1807	Circular	Cream	Raised	Entire	Smooth	Opaque	Non Pigmented
Pseudomonas sp.NCCP 1804	Circular	Creamy yellow	Raised	Entire	Smooth	Opaque	Non Pigmented
Pseudomonas sp.NCCP 1777	Circular	Cream	Pulvinate	Entire	Smooth	Opaque	Non Pigmented
Acinetobacter sp.NCCP 1723	Punctiform	White	Convex	Entire	Smooth	Slightly Opaque	Non Pigmented
Acinetobacter sp.NCCP 1746	Circular	Creamy white	Convex	Entire	Smooth	Opaque	Non Pigmented
Acinetobacter sp.NCCP 1747	Circular	Cream	Raised	Entire	Smooth	Translucent	Non Pigmented
Acinetobacter sp.NCCP 1717	Circular	Cream	Raised	Entire	Smooth	Opaque	Non Pigmented
Acinetobacter sp.NCCP 1718	Punctiform	White	Raised	Entire	Smooth	Translucent	Non Pigmented
Acinetobacter sp.NCCP 1745	Circlular	Cream	Convex	Entire	Smooth	Slight Opaque	Non Pigmented
Acinetobacter sp.NCCP 1758	Circular	Yellow	Convex	Entire	Smooth	Slight Opaque	Non Pigmented
Acinetobacter sp.NCCP 1831	Circular	Cream white	Convex	Entire	Smooth	Translucent	Non Pigmented
Acinetobacter sp.NCCP 1764	Circular	Cream	Raised	Entire	Smooth	Opaque	Non Pigmented
Aeromonas sp.NCCP 1767	Circular	Yellow/ Red	Convex	Entire	Smooth	Opaque	Pigmented
Aeromonas sp.NCCP 1765	Circular	Beige	Convex	Entire	Smooth	Opaque	Non Pigmented
Aeromonas sp.NCCP 1769	Circular	Beige	Convex	Entire	Smooth	Opaque	Non Pigmented
Aeromonas sp.NCCP 1768	Circular	Yellow	Convex	Entire	Smooth	Translucent	Non Pigmented
Aeromonas sp.NCCP 1785	Circular	Cream	Convex	Entire	Smooth	Translucent	Non Pigmented
Escherichia sp.NCCP 1731	Circular	Creamy	Raised	Entire	Smooth	Opaque	Non Pigmented
Escherichia sp.NCCP 1753	Circular	Shiny Creamy	Convex	Entire	Smooth	Translucent	Non Pigmented
Escherichia sp.NCCP 1755	Circular	Shiny Creamy	Raised	Entire	Smooth	Opaque	Non Pigmented
Shewanella sp.NCCP 1743	Circular	Creamy Yellow	Raised	Entire	Smooth	Translucent	Non Pigmented
Comamonas sp.NCCP 1742	Punctiform	Creamy	Raised	Entire	Smooth	Translucent	Non Pigmented
Citrobacter sp.NCCP 1750	Round	Shiny Creamy	Raised	entire	Smooth	Translucent	Non Pigmented
Rheinheimera sp.NCCP 1736	Circular	Creamy	Raised	Entire	Smooth	Translucent	Non Pigmented
Bacillus sp.NCCP 1756	Circular	Cream Yellow	Convex	Entire	Smooth	Translucent	Non Pigmented
Morganella sp.NCCP 1791	Punctiform	Cream	Raised	Curled	Smooth	Translucent	Non Pigmented
Stenotrophomonas sp.NCCP 1829	Circular	Yellow	Raised	Entire	Smooth	Translucent	Non Pigmented
Shigella sp.NCCP 1830	Circular	Creamy White	Raised	Entire	Smooth	Translucent	Non Pigmented
Alishewanella sp.NCCP 1833	Circular	Cream	Pulvinate	Entire	Smooth	Translucent	Non Pigmented

Table B2. Biochemical characteristics of all isolated bacterial species

Strains	Gram Reaction	Catalase	Oxidase	Simmon Citrate	Mannitol Salt
Proteus sp.NCCP 1771	Negative, straight rod	+	-	+	-
Pseudomonas sp.NCCP 1713	Negative /rods	+	+	+	-
Pseudomonas sp.NCCP 1709	Negative /rods	+	+	+	-
Pseudomonas sp.NCCP 1703	Negative/ motile rods	+	+	+	-
Pseudomonas sp.NCCP 1779	Negative / rods	+	+	+	-
Pseudomonas sp.NCCP 1781	Negative /rods	+	+	+	+
Pseudomonas sp.NCCP 1783	Negative /rods	+	+	+	+
Pseudomonas sp.NCCP 1784	Negative /rods	+	+	+	-
Pseudomonas sp.NCCP 1787	Negative /rods	+	+	+	-
Pseudomonas sp.NCCP 1788	Negative /rods	+	+	+	+
Pseudomonas sp.NCCP 1801	Negative /rods	+	-	+	+
Pseudomonas sp.NCCP 1807	Negative /rods	+	+	+	-
Pseudomonas sp.NCCP 1804	Negative /rods	+	+	+	-
Pseudomonas sp.NCCP 1777	Negative /rods	+	+	+	+
Acinetobacter sp.NCCP 1723	Negative /rod	+	-	+	-
Acinetobacter sp.NCCP 1746	Negative cocobacilli	+	-	+	-
Acinetobacter sp.NCCP 1747	Negative /rod	+	-	+	-
Acinetobacter sp.NCCP 1717	Negative /rod	+	-	-	+
Acinetobacter sp.NCCP 1718	Negative /rod	+	-	+	-
Acinetobacter sp.NCCP 1745	Negative /cocobacilli	+	-	-	-
Acinetobacter sp.NCCP 1758	Negative /rod	+	-	+	-
Acinetobacter sp.NCCP 1831	Negative /cocobacilli	+	-	-	-
Acinetobacter sp.NCCP 1764	Negative /rod	+	-	-	-
Aeromonas sp.NCCP 1767	Negative/ rod	+	+	+	+
Aeromonas sp.NCCP 1765	Negative /motile rods	+	+	-	+
Aeromonas sp.NCCP 1769	Negative /motile rods	+	+	+	+
Aeromonas sp.NCCP 1768	Negative /straight rods	+	+	Variable	Variable
Aeromonas sp.NCCP 1785	Negative /straight rods	+	+	Variable	+
Escherichia sp.NCCP 1731	Negative /rod	+	-	-	+
Escherichia sp.NCCP 1753	Negative /bacilli	+	-	-	+
Escherichia sp.NCCP 1755	Negative / coco bacilli	+	-	-	+
Shewanella sp.NCCP 1743	Negative /rod	+	+	-	+
Comamonas sp.NCCP 1742	Negative /rod	+	+	+	-
Citrobacter sp.NCCP 1750	Negative /rod	+	-	+	+
Rheinheimera sp.NCCP 1736	Negative/rod	+	-	-	+
Bacillus sp.NCCP 1756	Positive/rod	+	-	-	+
Morganella sp.NCCP 1791	Negative/rod	+	-	-	-
Stenotrophomonas sp.NCCP 1829	Negative/rod	+	+	+	-
Shigella sp.NCCP 1830	Negative/rods	+	-	-	+
Alishewanella sp.NCCP 1833	Negative/rods	+	+	-	+

"-" corresponds to negative results and "+" corresponds to positive results

APPENDIX C

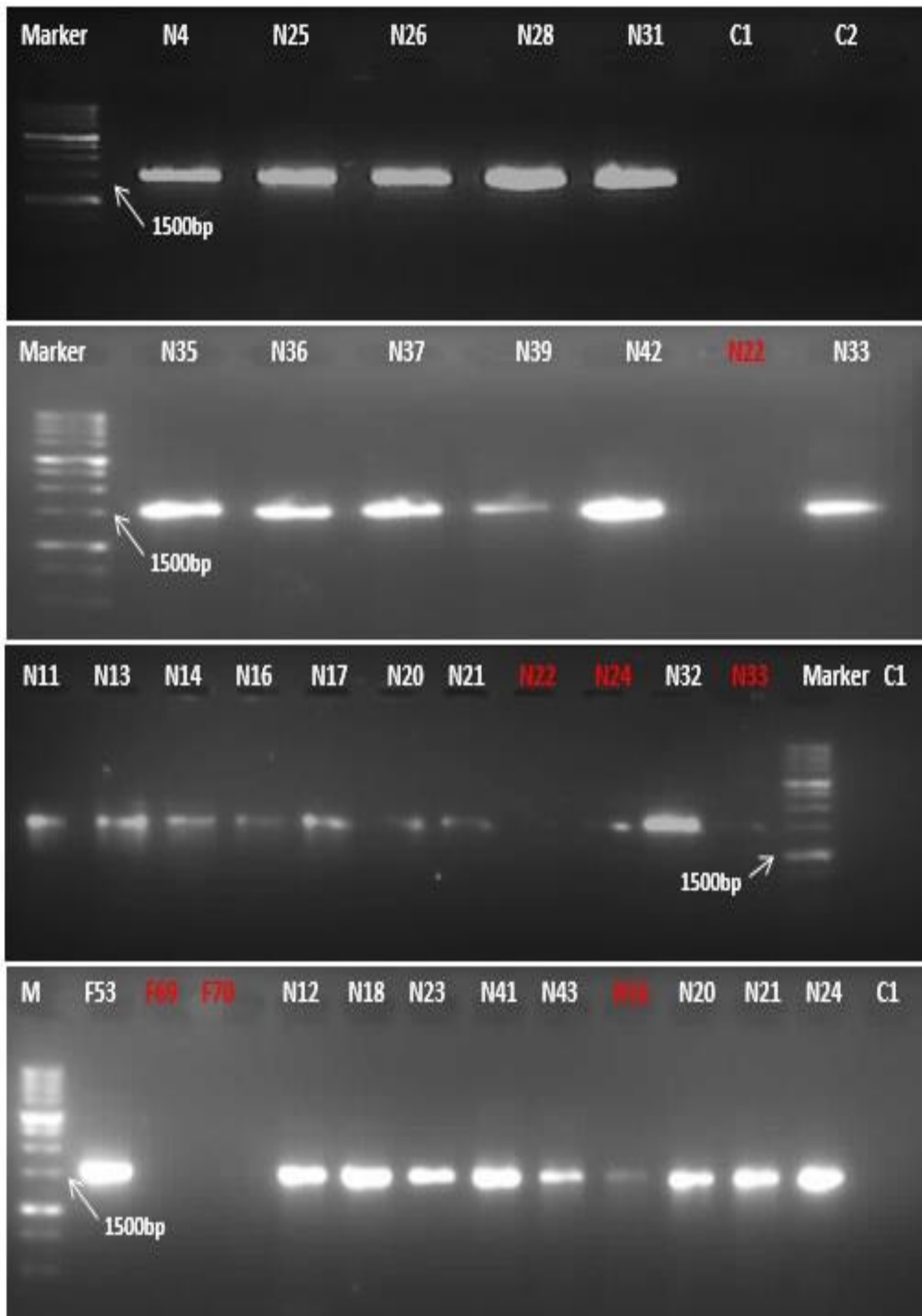


Figure C1. Agarose gel electrophoresis of 16S rRNA amplified gene, M: 1 kb Ladder, C: Control

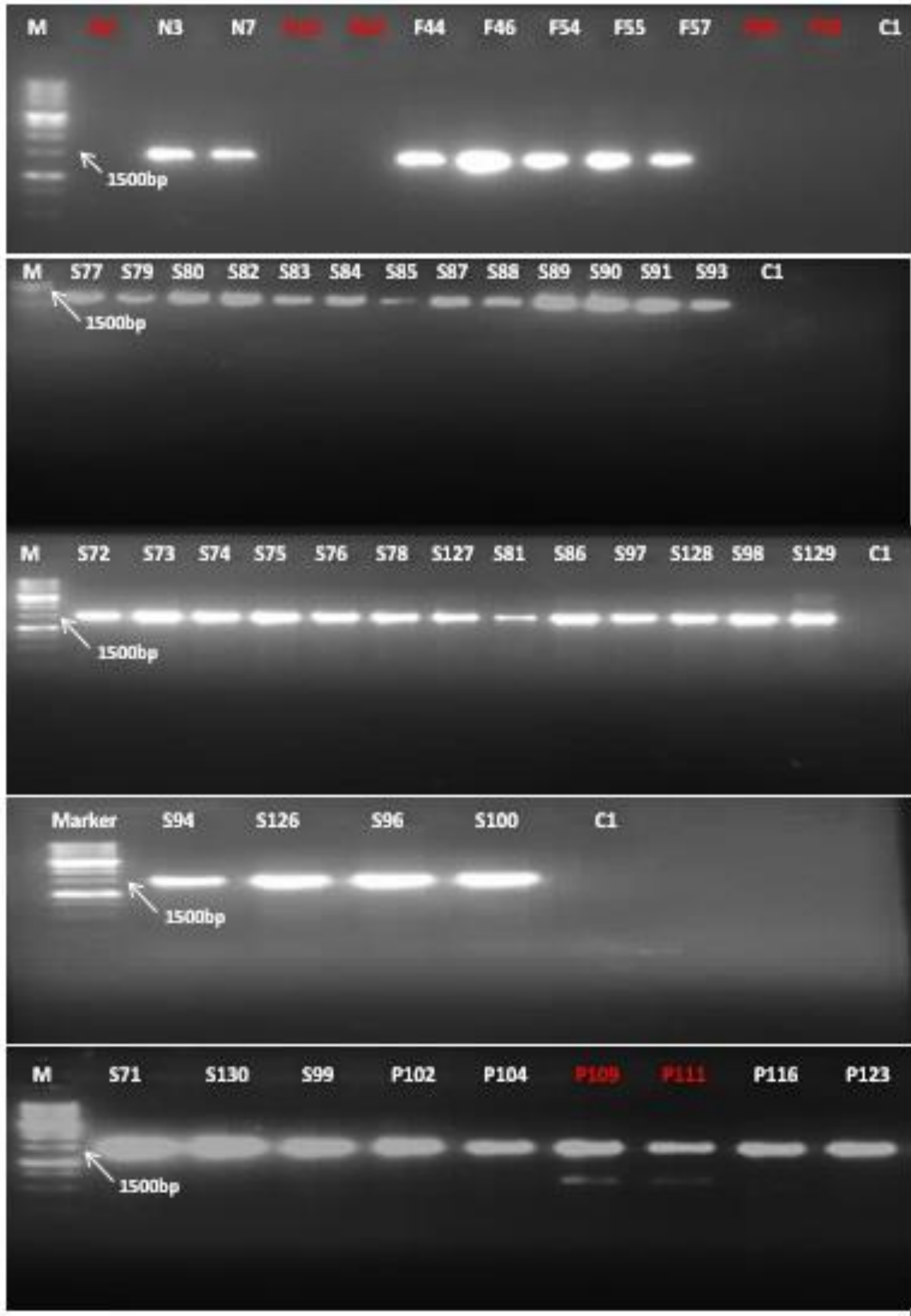


Figure C1. Agarose gel electrophoresis of 16S rRNA amplified gene, M: 1 kb Ladder, C: Control

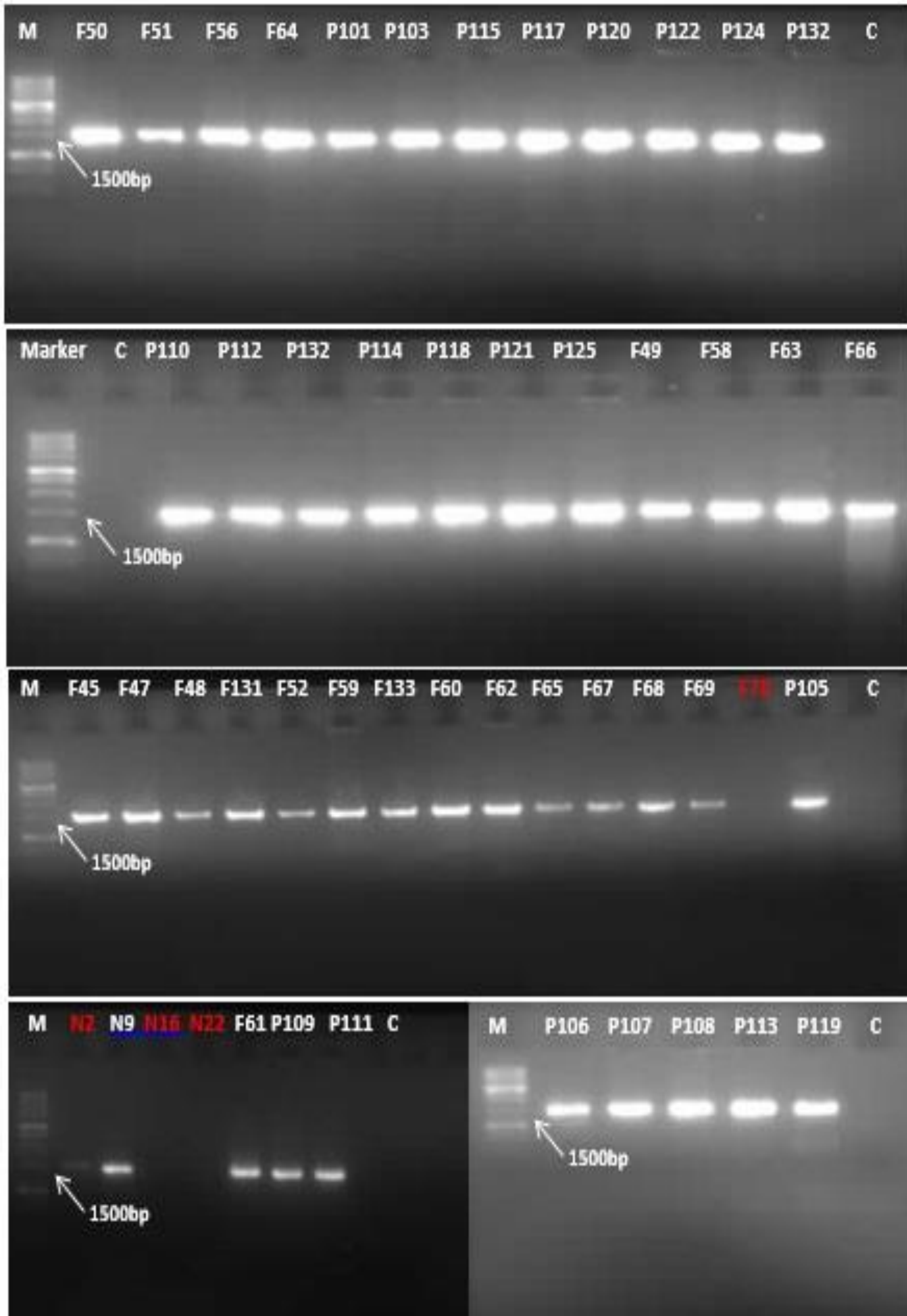


Figure C1. Agarose gel electrophoresis of 16S rRNA amplified gene, M: 1 kb Ladder, C: Control