Prevalence and Molecular Characterization of Extended Spectrum Beta-Lactamase Producing Multidrug-Resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* Isolates

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF SCIENCE IN BIOTECHNOLOGY

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DECLARATION

This is to declare that the research work embodying the results reported in this thesis entitled "**Prevalence and Molecular Characterization of Extended Spectrum Beta-Lactamase producing multidrug-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* isolates**" submitted by Sadia Afsana, has been carried out under the supervision and guidance of Dr. Mahboob Hossain, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University in partial fulfilment of MS. in Biotechnology, at BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

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DEDICATION

To

My Beloved Family

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ABBREVIATIONS

ABBREVIATION	ELABORATION
bp	Base pairs
CLSI	Clinical & Laboratory Standards Institute
CoA	Coenzyme A
CTX-M	Cefotaximase-Munich
dH2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMB	Eosin methylene blue
ESBL	Extended spectrum β-lactamase
ESBLA	Extended spectrum β-lactamase of Ambler class A
et. al	and others
EtBr	Ethidium bromide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MDR	Multidrug-resistant
mm	Millimeter
NDM	New-Delhi Metallo Beta- Lactamase
NICRH	National Institute of Cancer Research and Hospital
OXA	Oxacillinase
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
pH	Power of hydrogen
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SHV	Sulfhydryl variable
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEM	Temoneira

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ABSTRACT

The discovery of antibiotics has revolutionized health care worldwide. However, the life-saving role of antibiotics is threatened by the emergence of antibiotic- resistant 'superbugs'. With the emergence of antibiotic resistance and rise of 'superbug' bacteria has begun to produce Extended Spectrum Beta Lactamase (ESBL).

In this study, twenty-five *Klebsiella pneumoniae* isolates were collected from patients with respiratory tract infection whereas twenty-five *Pseudomonas aeruginosa* isolates were collected from both respiratory tracts infected patients and burn patients. The study was also performed to estimate the prevalence of ESBL producing Multidrug Drug Resistant *K. pneumoniae* and *P. aeruginosa* isolates. The ESBL producing isolates were further investigated by six primers (blaIMP, blaVIM-2, blaNDM-1, blaTEM, blaCTX-M and blaCLR) to confirm the presence of specific antibiotic resistant genes.

In this study, isolates of *P. aeruginosa* from wound were found more multidrug-resistant than those from sputum samples. The percentage of resistance to oxacillin, aztreonam, ceftazidime, amikacin, and ampicillin was 100% for pus samples. Regarding the *P. aeruginosa* isolates from the sputum samples were 100% resistant to all of the aminoglycosides (ampicillin and oxacillin) but resistance percentage was less for all the other antibiotics. *P. aeruginosa* (n=25) and *K. pneumoniae* (n=25), both the organisms were highly resistant to oxacillin and ampicillin (100%) whereas least resistance was found for imipenem and meropenem. This study showed that 88% of isolates of *K pneumonia* and 100% of isolates of *P. aeruginosa* were ESBL producers. Out of the 44 ESBL producer organisms, five had blaNDM-1 gene, one had the blaMCR gene and one had CTX-M gene. One of the samples showed resistant gene for both blaNDM-1 and blaCLR whereas one sample showed resistant gene for both blaNDM-1 and blaCTX-M. All the seven organisms were *P. aeruginosa* isolates from pus of burn patients. Only one sample showed resistance against imipenem. This proves that carbapenems are effective antibiotics for ESBL treatment.

This study helps us to understand the need for more precaution in use of antibiotics and the alarming rate of resistance seen in ICU burn patients and respiratory tract infected patients. Future studies can be focused on sequencing of NDM-1, MCR and CTX-M gene.

CHAPTER 1

INTRODUCTION

&

LITERATURE

REVIEW

1. INTRODUCTION

1.1 BACKGROUND

The discovery of antibiotics has revolutionized health care worldwide. For example, penicillin has saved millions of lives since its discovery (Bud, 2007). However, the life-saving role of antibiotics is threatened by the emergence of antibiotic- resistant 'superbugs' (Wise et al., 1998, Levy and Marshall, 2004). The use of antibiotics in raising food animals has contributed significantly to the global pool of antibiotic-resistant organisms. There is no doubt that use of antibiotics provides selective pressure that results in their resistance. While some resistant bacteria are found naturally in the environment, pathogens and non-pathogens are released into the environment in several ways, contributing to a web of resistance that involves humans, animals and the environment.

With the emergence of antibiotic resistance and rise of 'superbug' bacteria has begun to produce Extended Spectrum Beta Lactamase (ESBL). Extended-Spectrum Beta-Lactamases (ESBLs) produced by Gram-negative bacteria are considered on the largest and rapidly evolving group of plasmid-mediated enzymes that confer resistance to oxyimino-cephalosporins and monobactams (Pitout, 2010). *Escherichia coli* and *Klebsiella pneumoniae*, being the major source of community and hospital acquired infections are mostly ESBL producers (Pitout and Laupland, 2008). On the other hand, *Pseudomonas aeruginosa* has become an important hospital pathogen (Vizujė et al., 2007). Production of beta-lactamases or carbapenemase such as Metallo-beta-lactamases (MBLs) in both *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* has increased worldwide (Chakraborty et al., 2010, Kouda et al., 2007). A variety of ESBLs have been reported in *Enterobacteriaceae*, being mostly of the CTX- M, TEM and SHV types (Bush and Jacoby, 2010, Poirel et al., 2008). A strong relationship exists between third-generation cephalosporin use and acquisition of an ESBL- producing strain (Lautenbach et al., 2001). Other antibiotic classes have been found to be associated with subsequent infections due to ESBL-producing organisms include quinolones, trimethoprim- sulphamethoxazole, aminoglycosides, and metronidazole (Paterson and Bonomo, 2005).

1.2 LITERATURE REVIEW

1.2.1 Antibiotics and their Classification

Antibiotics or Antimicrobial agents are of natural or synthetic origin, generally work by inhibiting or disrupting vital processes within the bacterial cell, targeting structures or pathways sufficiently different or absent in mammalian cells. Antibiotics can be grouped according to several different criteria: inhibitory effect, the 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.

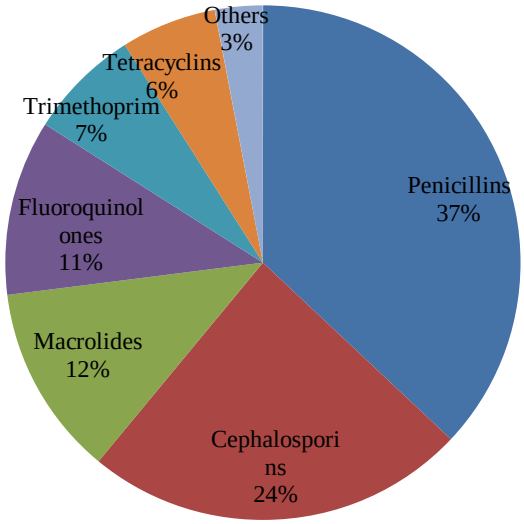

Fig. 1.1: Distribution of worldwide antibiotic consumption by class in 2010 (Van Boeckel et al. 2014)

Fig. 1.1 show that most consumed worldwide antibiotic is Penicillin (37%). Cephalosporins (ceftazidime, cefoxitin, cefotaxime, cefpodoxime or aztreonam) are a second most consumed antibiotic. These antibiotics are followed by macrolides (12%), fluoroquinolones (11%), trimethoprim (7%) and tetracycline (6%).

Table 1.1: Classification of antibiotics according to their mechanism

Cell Wall Synthesis	**Beta- lactams**	**Glycopeptides**			
	Penicillins	Vancomycin			
	Cephalosporins	Avoparcin			
	Carbapenems				
Protein Synthesis	**Aminoglycosides**	**Chloramphenicol**	**Tetracyclines**	**Macrolides**	**Streptogramins**
	Streptomycin			Erythromycin	Virginiamycin
	Neomycin			Azithromycin	Pristinamycin
	Kanamycin			Clarithromycin	Quinupristin-Dalfopristin
	Gentamycin				
Nucleic Acid Synthesis	**Sulfonamides (diaminopyrimidine)**	**Quinolones**			
	Sulfamethoxazole-trimethoprim	Ciprofloxacin			
		Norfloxacin			

Table 1.1 shows the classification of antibiotics according to their mechanism of action. The three major processes are via cell wall synthesis, protein synthesis, and nucleic acid synthesis. Cell wall synthesis class includes beta-lactams and glycopeptides. Protein synthesis class includes aminoglycosides, chloramphenicol, tetracyclines, macrolides, and streptogramins. The nucleic acid synthesis class has sulfonamides and quinolones.

1.2.1.1 Inhibitor of Cell Wall Synthesis

Beta-lactams

The beta-lactams include antibiotics like penicillins, cephalosporins, and carbapenems. These agents bind to the penicillin binding proteins that cross-link strands of peptidoglycan in the cell wall. The mechanism of beta-lactam resistance is via the action of the beta-lactamases. These enzymes catalyze the hydrolysis of beta-lactam ring and, thereby, inactivate these antibiotics. Many bacteria contain chromosomally encoded beta-lactamases necessary for cell wall production and it is only through over-production of these enzymes that resistance occurs. Beta-lactamases encoded on plasmids or other transmissible elements can lead to such over-production and, therefore, to resistance (Normark and Normark, 2002).

1.2.1.2 Inhibitor of Protein Synthesis

Aminoglycosides

The three major groups of aminoglycosides are the streptomycins, neomycins, and kanamycins. These drugs enter bacterial cells by an active transport that involves quinones that are absent in anaerobes and streptococci, thus excluding these organisms from the spectrum of action. Streptomycins act by binding to the 30S ribosomal subunit. Kanamycins and neomycins bind to both the 50S subunit and to a site on the 30S subunit different from the streptomycin (Greenwood (ed.), 2000). Activity involving initiation complexes and cell membrane proteins that contribute to cell death plays a role in the action of these antibiotics, but this is poorly understood. There are three mechanisms of aminoglycoside resistance. Since streptomycin binds to one particular protein on the ribosome, alteration of this protein, even by a single amino acid in its structure, confers high-level resistance to the drug. The other mechanisms involve decreased uptake of the antibiotic and in one of these, the cell membrane is altered, preventing active transport of the drug. In the other, one of many enzymes alters the antibiotic as it enters the cell, causing a block in further active transport.

1.2.1.3 Inhibitor of Nucleic Acid Synthesis

Quinolones

The quinolones class include nalidixic acid, norfloxacin, and ciprofloxacin. Quinolones inhibit bacterial growth by acting on DNA gyrase and topoisomerase IV, which are necessary for correct functioning of supercoiled DNA. Although quinolones target both enzymes, in gram-negative organisms the primary target is DNA gyrase and, in gram-positive organisms, the primary target is topoisomerase IV (Ruiz, 2003). There are three main mechanisms of resistance to quinolones. Resistance to some quinolones occurs with decreased expression of membrane porins. Cross-resistance to other drugs requiring these porins for activity also results from these changes. The second mechanism of resistance is an expression of efflux pumps in both gram negative and gram positive organisms (Normark and Normark, 2002) and the third is an alteration of the target enzymes.

1.2.2 Origin and Scope of Antibiotic Resistance

Antibiotics are of natural origin and a wide range of fungi and bacteria are natural producers of these biologically active molecules, which they release into their surroundings. The origin of antibiotics is ancient and antibiotic biosynthetic genes and resistance conferring genes has begun to evolve millions of years ago (Wright & Poinar, 2012). At the very low concentrations in which antibiotics are present in nature, they can act as signaling molecules and as such trigger transcription responses important for environmental survival (Yim et al., 2006). The accumulation of manufactured antibiotics in the environment creates the conditions for the proliferation of resistant bacteria. Resistance arises and persists in places which are regularly exposed to antibiotics and has poor sanitation. The reason behind resistance differs from country to country. Once resistance is present it can be passed between various inter-related species of bacteria and quickly disseminates around the globe (Ruiz, 2003).

1.2.3 Mechanism of Antibiotic Resistance

For drug discovery, development and for optimizing drug therapy it is important to have standard and agreed assays and definition for the susceptibility and resistance to antimicrobial drugs. Bacteria has the ability to acquire resistance by both intrinsic and acquired manner.

The intrinsic antibiotic resistance can be gained by both outer membrane permeability and efflux pump. Decreasing the permeability of the cytoplasmic membrane will decrease the fluidity and will have a detrimental effect on the structure and activity on the membrane proteins present within the bilayer. It has been estimated that the peptidoglycan of Gram-positive bacteria has a large permeability threshold and is accessible to molecules of up to 30-57 kDa (Scherrer and Gerhardt, 1971). On the other hand, Gram-negative bacteria are intrinsically insusceptible to many of these antibacterial agents owing to the presence of a much finer molecular sieve, dubbed the outer membrane, which surrounds a thinner peptidoglycan layer. The intrinsic resistance contributed by the outer membrane is particularly apparent in the opportunistic pathogen like *P. aeruginosa*. Despite the low permeability of the *P. aeruginosa* outer membrane it has been shown to possess a large exclusion limit and allows the uptake of molecules of approximately 3000 Da. This reduction of permeability of the outer membrane is advantageous for the inducible, periplasmically located beta-lactamase innately present within the pathogen. The

enzyme inactivates beta-lactams at an optimum rate as the molecules pass into the periplasm. This mechanism plays a key role in the intrinsic resistance of *P. aeruginosa* to beta-lactam antibiotics. Efflux pumps are widespread and present in the chromosome of all organisms (Van Bambeke et al, 2000). Efflux pumps can either be substrate specific and only export one molecule, or they can be more broad-spectrum and export structurally distinct classes of molecules. It was discovered that the intrinsic resistance of *P. aeruginosa* to different classes of antibiotics is due to the presence of chromosomally encoded efflux pumps (Li et al, 1994). In prokaryotes, there are five main divisions of efflux proteins that span the bacterial membrane: the ATP-binding cassette (ABC), the major facilitator (MF), the multidrug and toxic compound efflux (MATE), the small multidrug resistance (SMR) and the resistance- nodulation- Division family (RND). Efflux of antibiotics is an active process which means that they require energy to move compounds up a concentration gradient. Apart from ABC family which hydrolyzes ATP providing the energy for efflux of molecules, the remaining families of efflux proteins use the proton motive force to provide the energy for substrate export (Paulsen et al., 1996).

The acquired antibiotic resistance can be gained by gene transfer and integrons. In order to acquire an antibiotic resistant gene from the environmental gene pool, the pathogenic bacteria must transfer the gene by conjugation, transformation or transduction. On the other hand, Integrons are genetic assembly platforms capable of capturing and expressing gene cassettes, which can encode antibiotic resistance determinants. Integrons are typically divided into mobile integrons and chromosomal integrons. Chromosomal integrons are usually stationary in the bacteria, whereas mobile integrons are readily disseminated between bacteria. Mobile integrons cannot mobilize and transfer themselves, they are often associated with genetic elements which can mobilize, such as plasmids (Mazel, 2006).

1.2.4 Extended Spectrum Beta-Lactamases

1.2.4.1 Beta-Lactam Antibiotics

One of the most commonly used types of antibiotics is the beta-lactams. The beta-lactam antibiotics have the beta-lactam ring in common, while side chains are variable. There are four groups of beta-lactam antibiotics: penicillins, monobactams, cephalosporins and carbapenems. Beta-lactams inhibit cell wall synthesis by binding to penicillin binding proteins (PBPs), which

are crucial enzymes in the cross-linking of peptidoglycan. Additionally, beta-lactams have a different spectrum of activity, as some are broad-spectrum antibiotics with activity against both Gram-positive and Gram–negative bacteria.

1.2.4.2 Beta-Lactamases

The beta-lactamases are a class of enzymes that hydrolyze the beta-lactam antibiotics (Medeiros, 1984) which inactivate the drug, giving it a slightly different structure. This structural difference makes it unable to bind to the PBP active site. Beta-lactamases are classified into narrow-spectrum (penicillinases), broad-spectrum, extended-spectrum beta- lactamases (ESBLs), and carbapenemases (Lohr, 2014), based on a range of activity.

1.2.4.3 Extended Spectrum Beta-Lactamases

Extended-spectrum beta-lactamases (ESBL) are a group of enzymes which have the capability of hydrolyzing third-generation cephalosporins and aztreonam (but not cephamycins and carbapenems) and which are sensitive to inhibitors such as clavulanic acid, sulbactam, and tazobactam. They developed from point mutation of genes which code production of primordial TEM-1, TEM-2 or SHV-1 beta-lactamases with the replacement of the configuration of amino acids at an active site for these enzymes (Paterson and Bonomo, 2005). Due to their activity, they can hydrolyze a wide range of beta-lactam antibiotics, such as penicillins and cephalosporins. Besides *K. pneumoniae* and *E. coli*, which represent the most important pathogens producing ESBLs, other ESBL producers such as *P. aeruginosa* have been registered lately. These bacteria have not only caused outbreaks but have become endemic in many hospitals throughout the world (Perez et al., 2007). When a significant proportion of Gram-negative isolates in a particular unit is ESBL producers, empirical therapy may change towards the use of imipenem, quinolones, or beta-lactam/ beta-lactamase inhibitor combinations. Control of endemic ESBL producers is difficult, and may only be possible after significant nursing and medical reorganization, at substantial financial cost. Patients at high risk for developing colonization or infection with ESBL producing organisms are often seriously ill patients with prolonged hospital stays and in whom invasive medical devices are present for a prolonged duration. To date, 890 various ESBLs have been discovered worldwide.

1.2.4.3 Dissemination of ESBL

Dissemination of ESBL can occur either by clone or plasmid. Some bacteria may be more successful than others, depending on many different factors, including pathogenicity, virulence, and antibiotic resistance. These are called successful clones and may be present in the environment for a very long time (Lohr, 2014). They spread through normal cell division and may cause infections or even outbreaks. In the case of plasmid-mediated dissemination, they harbor accessory genes (encoding virulence or antibiotic resistance traits) which may result in increased bacterial survival in a given environment. Some plasmids are present in high numbers in the host cell, while others may have only a few copies present (Madigan et al., 2012). Plasmids may harbor ESBL-encoding genes. Along with these, the plasmids may also carry genes that confer resistance to other antibiotics, making treatment options even narrower.

1.2.5 Enterobacteriaceae

1.2.5.1 Klebsiella pneumoniae

Klebsiella pneumoniae is a Gram-negative, non-motile, lactose fermenting, rod-shaped in the Enterobacteriaceae family. *K. pneumoniae* is a home- grown microorganism in that it resides in the microbiota of human. The bacterium is found indigenously in soil and water, but also on mucosal surfaces in mammals, including humans (Podschun and Ullmann, 1998). *K. pneumoniae* is part of the gut microflora in humans and usually does not cause disease. It can cause infections when present elsewhere in the body, causing urinary tract infection, respiratory tract infection, and bloodstream infection (Forbes et al., 2007). *K. pneumoniae* is often associated with nosocomial infections, which are infections acquired during hospitalization. Virulence in *K. pneumoniae* is based on many factors, but the capsule is regarded as the most important (Brisse et al., 2009). Lipopolysaccharide and capsular polysaccharide are two of the most important virulence factor of *K. pneumoniae* in causing sepsis. *K. pneumoniae* use capsular antigens composed of complex acidic polysaccharides, which are essential to the virulence to this pathogen. As a mechanism of protection, the bacterium evades phagocytosis by utilizing thick bundles of fibrillous structures by polymorphonuclear granulocytes. This mechanism also prevents killing by bactericidal serum factors and inhibits complement constituents.

1.2.5.2 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative, rod-shaped bacterium that is motile by means of a single polar flagellum and known to be highly antibiotic resistant and able to grow in a variety of generally in hospital environments, often through its ability to form resilient biofilms (Textbook of Bacteriology. Todar's online textbook of Bacteriology). *P. aeruginosa* is one of the common causes of infection in burn patients and an important agent for hospital-acquired infections and death in immunocompromised such as cystic fibrosis and cancer patients (Neu, 1983). The pathogenesis of *P. aeruginosa* is multifactorial and complex because they are both invasive and toxigenic. The three stages are (1) bacterial attachment and colonization, (2) local infection and (3)bloodstream dissemination and systemic disease.
P. aeruginosa has a variety of virulence factors that contribute to its ability to grow in various host environments and cause many different types of infections. These virulence factors include adhesions, secreted toxins, proteases, effector proteins, and pigments, which disrupt or control host cellular pathways and target the extracellular matrix.

This bacterium is often resistant to many antimicrobial agents. The cause of resistance can be efflux pumps, decreased outer membrane permeability, and secretion of beta-lactamase enzymes (Arakawa et al., 2000). These enzymes were initially seen in Gram-negative bacteria which were detected in periplasmic space. Carbapenems are effective antibiotics against Pseudomonas infections. But because the genes for MBLs are often carried on plasmids and class I integron, they can rapidly spread among different species of this bacterium and other bacteria. MBLs can potently hydrolyze all beta-lactam antibiotics except aztreonam. hence, their activity is inhibited by chelators like ethylenediaminetetraacetic acid (EDTA), sodium mercaptoacetic acid (SMA), 2-mercaptopropionic acid (MPA), and dipicolinicacid (DPA). Sulbactam, tazobactam and clavulanic acid which are often used to inhibit beta-lactamase enzymes are not effective against MBLs.

1.2.6 Sources of Antibiotic Resistance in the Environment

Originally the concern was only about the acquisition of resistance by microorganisms which caused epidemic diseases. But, in the recent times, antibiotic-resistant bacteria have been found from every possible environment on earth. Antibiotic resistance is not restricted to pathogenic or commensal microbes but also ubiquitous in the environment. The environmental microbiome is believed as the natural reservoir of antibiotic resistance genes and but has diversity and novelty than expected ever (Wright, 2010, Lin et al, 2015). Also, a pool of resistance is developing in non-pathogenic organisms found in human, animals and also the environment.

There are two reasons for the increase of antibiotic resistance in the environment (Purden et al., 2013, Kummerer, 2004, Martinez, 2008).

- Resistance is present within bacteria in the environment through exposure to naturally occurring antibiotics. However, the proportion of resistant bacteria is increased through the use of manufactured antibiotics.
- Antibiotics and resistant bacteria from human and animal origin enter into the terrestrial and aquatic environments, such as soil, marine areas, and surface water directly.

Although resistant organisms are found in the environment, most resistance is associated with agriculture and human impacts.

1.2.6.1 Agricultural Impact

The poultry industry has become one of the largest livestock industries throughout the world, with a 44% production increase in the U.S. between 1982 and 1994 (Williams et al., 1999). Antibiotics are used heavily throughout the poultry production process. Although antibiotic use statistics are difficult to obtain for the poultry production industry, one early study reported that 80% of poultry raised in the U.S. have been fed antibiotics (Dupont and Steele, 1987). Though many classes of antibiotics are used, fluoroquinolones, avoparcin, and virginiamycin are of particular concern, since resistance to these drugs is associated with resistance the human therapeutic drugs like ciprofloxacin, vancomycin, and quinupristin/dalfopristin, respectively. The use of antibiotics in poultry leads to resistant organisms within the chickens themselves and throughout the production environment. Resistant strains of many organisms, including

Staphylococcus, Streptococcus, Clostridium, Pseudomonas, and *Aeromonas,* have been isolated from these sources (Kelley et al., 1998). One of the most recent threats is resistance in *Enterococcus sp.* isolated from poultry. With the increase in poultry production, management of poultry waste is a serious problem. Heavy metals, such as arsenic, copper, iron, selenium, and zinc, are often added to poultry feed to improve weight gain and to prevent disease. Since most of the metals are not absorbed, they pass through the bird and are excreted with the waste. Fecal coliforms from poultry manure are antibiotic and heavy metal resistant. The most common method of utilization of poultry waste is land application as manure. Runoff from manure application is increasingly being recognized as a serious environmental problem.

Antibiotics are used in cattle production in much the same way as in poultry. The chief difference is in the concentration of waste from a pasture versus a chicken house but the same issues with resistance arise. Cattle production includes beef, veal, and dairy cows with beef cows generally shipped to feedlots and kept in large groups. As in poultry, many species of resistant bacteria can be isolated from cattle and the pathogens of particular concern include *E. coli, Salmonella, Enterococcus, and Campylobacter sp*. Antibiotic-resistant bacteria are also capable of spreading through the cattle farm environment, even without antibiotic use. Dairy calves have been shown to be colonized with sensitive E. coli as long as they were kept with their dams, but become colonized with multiple drugs resistant E. coli shortly after being moved to other pens (Marshall et al., 1990).

Aquaculture is the fastest growing food industry in the world and will continue to grow in the future (Cabello, 2006, Miranda et al., 2013). It has become a large agricultural business worldwide. Both fresh and saltwater farms are common and as with other agricultural endeavors, antibiotics are generously applied. The high fish densities and increased stress weaken the fish immune system and further promote the need for antibiotics. Antibiotic treatment of fish in the aquaculture is done by the administration of medicated feed. This process has a direct effect on the aquatic environment as the food added to the water and uneaten food settles to the sediment or is dispersed. Antibiotics that are not metabolized by the fish move directly into the environment (Kerry et al., 1996). Increased use of antibiotics selects for resistance among environmental, commensal and pathogenic fish bacteria making the resistance problem even worse (Cabello, 2006). Development of antibiotic resistant reservoirs from where the resistance

can emerge and transfer to pathogenic bacteria is a major concern. So the prevalence of resistance in fish pathogens and the gene transfer routes and food chain on global scale remain largely unknown and require further studies.

1.2.6.2 Human Impact

Wastewater has vastly increased multidrug-resistant organisms. Antibiotics consumed by humans can act to select for resistant organisms in the human gut. Both the antibiotics residues and resistant microorganisms are excreted which enter into the sewage system. In all the stages of sewage treatment antibiotics and resistant bacteria from human sources have been detected. This includes treated water that is released into the environment (Davidson et al., 2002, Zhang et al., 2001, Schluter et al., 2007) and sludge applied to the farmland (Gaze, W, et al, 2008). Although it is believed that the environment is safe from untreated contaminated sewage, breaches occur frequently where leakage or overflow into groundwater or natural water occurs (Harwood et al., 2001).

Biocides are substances which are used to control micro-organisms like fungi, viruses, and algae whereas antibiotics are medication which is designed to kill bacteria. They are usually used as antiseptics, disinfectants, and preservatives. They are present in a wide range of consumer, healthcare, food and industrial products. There has been evidence that antibiotic resistance can rise due to biocide exposure (Maillard, 2013, Gaze et al., 2011).

Hospital effluent is another major reason for multidrug resistance in microorganism as a result of human impact. Antibiotics used in medicine for the treatment of infections are mainly released non-metabolized into the aquatic environment via waste water. This can only have an effect if an active compound is present. Unused therapeutic drugs are sometimes disposed of down drains. An important source of the resistance in hospital effluents is also the input of bacteria already resistant because of the use of antibiotics in medical treatment. There have been reports that the widespread use of biocides such as triclosan and quaternary ammonium compounds used in hospitals and homes could select for antibiotic-resistant bacteria (McMurry et al., 1998).

1.2.7 Spread of Antibiotic Resistance

Historically, researchers have assumed that the danger posed by resistant organisms in the environment would be minimal since bacteria introduced to an environment were generally believed to survive only for a relatively short time. This drastically increases the probability that humans will come into contact with resistant pathogens from runoff or sewage. The environmental spread of resistance is primarily determined by two factors:

1. The release of substances into the environment that promotes resistance
2. The release of antibiotic resistant bacteria directly into the environment

1.2.8 Transfer in the Environment

The evidence for transfer in various environments is ample, though indirect, involving the comparison of gene sequences from different species or the use of microcosms or plasmid analysis. Though this is not proof of horizontal gene transfer, it is strongly suggestive that it can occur especially in the presence of selective pressure.

Studies focusing on the dangers of releasing genetically modified bacteria into the environment have been performed to examine the transfer of genes in the rhizosphere. Antibiotic resistance markers were used and demonstrate not only horizontal gene transfer in the rhizosphere but also the horizontal transfer of resistance genes. The transfer has been found to occur between several species under various environmental conditions. In the wheat rhizosphere, *Pseudomonas* has been shown to transfer plasmids to indigenous bacteria (van Elsas et al., 1988). Disturbingly, fecal bacteria are able to transfer genes to natural soil bacteria, as in the case of transfer from E. coli to *Rhizobium sp.* (Richaume et al., 1989).

As with studies of other environments, the evidence for horizontal gene transfer in marine and aquatic environments is largely circumstantial, relying on microcosm studies, in vitro transfer between aquatic organisms, and the presence of plasmids. Antibiotic resistant organisms have been isolated from seawater samples collected from both sewage affected sites and control sites of the Spanish and U.S. coasts. Plasmids were found in many of these isolates and strains from polluted areas had both more plasmids and higher levels of resistance (Baya et al., 1986). In another investigation, 34% of environmental *Vibrio, Aeromonas, E. coli* and *Pseudomonas*

isolates from the Chesapeake Bay and Bangladesh were found to contain plasmids (McNicol et al., 1982). Plasmids have also been found in bacteria isolated from sediments and seawater from Antarctica. Forty-two percent of sediment isolates and 20% of water isolates carried plasmids, some of which were associated with antibiotic resistance (Klare et al., 1999). Transformation and transduction are theorized to occur in the marine environment, but few studies have been done explicitly to demonstrate the phenomenon. However, the presence of a large number of the virus makes it likely that transduction is an important means of gene transfer in the environment. As stated above, transduction provides a means of gene transfer in freshwater environments.

Horizontal gene transfer occurs throughout animal environments, including farms and in the farm animals themselves. Animals in the wild and insects also contribute to the spread of antibiotic resistance genes. In a study of enteric bacteria isolated from horses, cattle, poultry, and reptiles, scientists found Class 1 integrases in 46% of the isolates tested (Acar and Goldstein, 1997). *E. coli* carrying transferable trimethoprim and ampicillin resistance plasmids from chickens carried the plasmids, even in the absence of selective pressure (Chaslus-Dancla et al., 1987). Several studies of gene transfer and antibiotic resistance in wild animals both from areas heavily impacted by humans and in remote regions have been done.

1.2.9 Emergence of Antibiotic Resistance and Rise of Superbug

Antibiotics have surely revolutionized health care worldwide. However, the antibiotics have given rise to a serious threat. There is a strong correlation between antibiotic use and antibiotic resistance. The role of the life-saving antibiotic is threatened by the emergence of antibiotic resistance pathogens known as ‘superbugs’ (Wise, R et al., 1998, Levy et al., 2004). The resistance can arise against a large number antibiotics which include fully synthetic antibiotics like broad-spectrum fluoroquinolones. Major role in the evolution and transmission of the resistance genes is played by horizontal gene transfer which has given rise of extended spectrum beta lactamase (ESBL) enterococci in both community and hospital environments (Davies and Davies, 2010). Superbugs are pathogenic bacteria that have acquired multiple resistance genes. Some strains also acquire increased virulence and also enhance transmissibility. Due to massive use of antibiotics, multiple drug resistant pathogens have become associated with human disease epidemics.

1.2.10 Challenges in Detecting and Quantifying Antibiotic Resistance in the Environment

Databases of clinical breakpoints, such as EUCAST help in monitoring antibiotic resistance worldwide. Molecular techniques like whole genome sequencing combined with data from susceptibility testing can be used to figure out previously unknown resistant determinants acquired through mutation or horizontal gene transfer. Sequencing of whole genome sequencing gives an insight of the genetic environment of the antibiotic resistance genes of the bacteria. Horizontal transfer genes present on mobile genetic elements poses a bigger threat of resistance spread in the environment (Martinez et al., 2014). PCR and quantitative PCR (qPCR) methods can be used in detecting genes from environmental DNA. Especially when using qPCR array for hundreds of genes the culturing method is not at all required (Zhu et al., 2013, Vizcaíno, et al., 2014, Looft et al., 2012). It is also difficult to predict the functionality of the target gene with molecular methods.

Metagenomics, the sequencing of the whole community DNA, has been used to detect antibiotic resistance in diverse environment and is not restricted to a few chose genes (Chen et al., 2013, Hu et al., 2013, Li et al., 2015, Nesme et al., 2014, Yang et al., 2013, Zhang et al., 2011). The annotation of antibiotic resistance genes relies on known genes in public antibiotic resistance gene databases. Metagenomic sequencing platforms produce short reads which give only limited information about the sequenced genes. After assembling short reads into longer overlapping DNA segments information can be acquired about the phylogeny and genetic context of the genes. Functional metagenomics, the cloning and expression of DNA in a laboratory host, can overcome the limits in detecting mostly known resistance genes. Sub-cloning, mutagenesis analysis is performed for antibiotic resistance in clones with resistance phenotype. These laboratory techniques are laborious and time-consuming. Disadvantages of functional metagenomics can be solved to some extent by using other laboratory hosts. Proteomics combined with functional metagenomics is a promising new way for screening of potential clones. Using proteomic tools the expressed proteins can be detected in a high-throughput manner and by comparing to a strain without the cloned DNA, the putative new resistance determinants can be identified ((Fouhy et al., 2015).

1.3 OBJECTIVE OF THE STUDY

The main objective of this study is to determine the prevalence of Extended Spectrum Beta-Lactamase production in multidrug resistant (MDR) *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated collected from sputum and pus. This detection is further investigated by the molecular characterization of the gene responsible for the antibiotic resistance. The specific objectives of this study are as follows:

1. Screening of collected isolates by disk diffusion to detect the multidrug resistant strains.

2. ESBL detection of the multidrug resistant isolates by performing double disk synergy test.

3. Detection of IMP, TEM, CTX-M, VIM-2, NDM-1 and CLR genes by PCR.

CHAPTER 2

MATERIALS

&

METHODS

2. MATERIALS AND METHODS

2.1 Bacterial Isolate Collection

Fifty clinical isolates of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were collected for the study. Twenty-five *K. pneumoniae* isolates were collected from National Institute of Diseases of the Chest and Hospital (NIDCH). Among the twenty-five, *P. aeruginosa* isolates thirteen were collected from National Institute of Diseases of the Chest and Hospital and twelve were collected from Dhaka Medical College Hospital. The isolates from National Institute of Diseases of the Chest and Hospital were obtained from sputum whereas isolates from Dhaka Medical College Hospital were obtained from pus of burn patients. The isolates were obtained over a period of one year from January 2016 to December 2016. The *K. pneumoniae* isolates were cultured in Mac Conkey agar and *P. aeruginosa* isolates were cultured in Cetrimide agar for further confirmation.

2.2 Antimicrobial Susceptibility Test

With the introduction of a variety of antimicrobials, it has become necessary to perform the antimicrobial susceptibility test as a routine. The isolates were tested for their antimicrobial susceptibility by the disc diffusion method according to the CLSI guidelines (Tzelepi et al., 2000). The following antibiotics were used: Imipenem (10 μg), Meropenem (10 μg), Colistin sulfate (10 μg), Oxacillin (1 μg), Cloxacillin (5 μg), Kanamycin (30 μg), Gentamycin (10 μg), Aztreonam (30 μg), Ceftazidime (30 μg), Amikacin (30 μg), Ampicillin (10 μg) and Ciprofloxacin (5 μg).

2.3 Extended Spectrum Beta-Lactamase Test

Extended Spectrum Beta-Lactamases are beta-lactamases that hydrolyze extended- spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime as well as the oxyimino-monobactam aztreonam. Thus ESBL confers multidrug resistance to these antibiotics and related oxyimino-beta lactams. A broader set of beta-lactam antibiotics is susceptible to hydrolysis by TEM, SHV, and CTX-M enzymes. The ESBLs are frequently plasmid encoded. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes. This may be detected by testing

the suspected organism to a 3rd generation cephalosporin alone and in combination with a clavulanic acid or piperacillin- tazobactam. If the combination results in an expanded zone of inhibition compared to that of the 3rd generation cephalosporin alone, it is indicative of the presence of an ESBL.

The protocol used is given below:

1. A bacterial suspension of the organism to be tested that has a turbidity equivalent to that of a 0.5 McFarland standard was prepared and was inoculated to a Mueller-Hinton agar plate
2. The Amoxicillin-clavulanic acid (20 μg/ 10 μg) or Piperacillin-tazobactam (100 μg/10 μg) disc was placed towards the center of the plate.
3. The plate was carefully measured 20 mm out from the edge of that disc at 90° angles.
4. A Ceftazidime (30 μg) disk was placed on the plate so that its inner edge is 20 mm from the Amoxicillin-clavulanic acid or Piperacillin-tazobactam base. Same was done with Ceftriaxone (30 μg) and Aztreonam (30 μg) discs.
5. A Cefoxitin (30 μg) disc was placed in any available space remaining on the plate.
6. The plate was incubated at 35°C in the presence of Oxygen for 18-24 hours and the zone diameters was recorded for all the cephalosporins as per NCCLS guidelines.

Interpretation Note: The following applies to *E. coli, Klebsiella* species and *Proteus* species and *Pseudomonas* species.

1. Zone size for all antibiotics was documented.
2. Potentiation of the inhibition zone (i.e. increase in the inhibition zone) of any one of ceftazidime, ceftriaxone or aztreonam when combined with a clavulanic acid (Klebsiella sp.) or piperacillin-tazobactam (Pseudomonas sp.) was observed.
3. If a reduction of the zone of inhibition of any one of ceftazidime, ceftriaxone or aztreonam when combined with clavulanic acid for *Klebsiella* species and piperacillin-tazobactam for *Pseudomonas* species was observed, it was kept to recheck the identification of the isolate and testing was repeated later.

2.4 DNA Extraction

Bacterial DNA was extracted by the phenol-chloroform method. A phenol-chloroform extraction is a liquid-liquid extraction. A liquid-liquid extraction is a method that separates mixtures of molecules based on the differential solubility of the individual molecules in two different immiscible liquids (Stenesh, 1989).

The protocol used is presented below:

1. 1.5 ml of the overnight bacterial culture (grown in LB medium) was transferred to a 1.5 ml Eppendorf tube and was centrifuged at max speed for 1 min to pellet cells.
2. The supernatant was discarded.
3. The cell pellet was resuspended in 600 µl lysis buffer and was vortexed to completely resuspend cell pellet.
4. Incubation was done for 1 hour at 37 °C.
5. An equal volume of phenol/ chloroform/ isoamyl alcohol (25: 24: 1) was added and mixed well by inverting the tube until the phases are completely mixed.
6. Spinning was done at max speed for 5 min at room temperature. There was a white layer (protein layer) in the aqueous phenol/chloroform/isoamyl alcohol interface.
7. The upper aqueous phase was carefully transferred to a new tube.
8. To remove phenol, an equal volume of chloroform was added to the aqueous layer. Again, it was mixed well by inverting the tube.
9. Spinning was done at max speed for 5 min.
10. Aqueous layer was remved to a new tube.
11. To precipitate the DNA, 2.5 or 3 volume of cold ethanol (store ethanol at -20°C freezer) was added and mixed gently.
12. The tube was incubated at -20 °C for 30 min or more.
13. The tube was spinned at max speed for 15 min at 4 °C.
14. The supernatant was discarded and the DNA pellet was washed with 1 ml 70% ethanol.
15. The tube was spinned at max speed for 3 min. The supernatant was carefully discarded and the DNA pellet was air-dried.
16. DNA was resuspended in TE buffer.
17. Isolated DNA was stored at -20°C for further use.

2.5 Conventional PCR

Polymerase Chain Reaction is a technique used to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands o millions of copies of a particular DNA sequence in molecular biology. The majority of PCR methods rely on thermal cycling that involves exposing the reactants to cycles of repeated heating and cooling, permitting different temperature dependent reactions- specifically, DNA melting and enzyme-driven DNA replication- to quickly proceed many times in sequence.

Table 2.1: The oligonucleotide primers set as forward and reverse

PRIMERS	AMPLICON SIZE	SEQUENCE	REFERENCE
IMP	587 bp	blaIMP-F: 5′- GAAGGCGTTTATGTTCATAC-3′ blaIMP-R: 5′- GTATGTTTCAAGAGTGATGC-3′	Pitout et al., 2005
TEM	768 bp	bla TEM F : 5’ AAAATTCTTGAAGACG-3’ bla TEM R : 5’ TTACCAATGCTTAATCA-3’	Sharma et al. 2010
CTX-M	759 bp	blaCTX-M F: 5’-ACGCTGTTGTTAGGAAGTG-3’ blaCTX-M R: 5’-TTGAGGCTGGGTGAAGT-3’	Sharma et al. 2010
NDM 1	200 bp	blaNDM-1 F: 5’-ACCGCCTGGACCGATGACCA-3’ blaNDM-1 R: 5’-GCCAAAGTTGGGCGCGGTTG-3’	Farzana et al., 2012
VIM 2	864 bp	blaVIM-2 F: 5’-AAAGTTATGCCGCACTCACC-3’ blaVIM-2 R: 5’-TGCAACTTCATGTTATGCCG-3’	Farzana et al., 2012
CLR	309 bp	blaCLR F: 5'-CGGTCAGTCCGTTTGTTC-3’ blaCLR R: 5'-CTTGGTCGGTCTGTAGGG-3’	Liu et al., 2015

Preparation of Master Mix

For PCR, reagents used along with the primers are Taq polymerase 5µl/ml (Thermofisher), 10x PCR buffer (Invitrogen, India), deoxynucleoside triphosphates (dNTPs) 2.5mM, $MgCl_2$ 50mM and nuclease free water as required.

Table 2.2: PCR components for IMP and TEM genes and their volumes

PCR COMPONENTS	VOLUME
10X Reaction Buffer	2.5 µL
dNTPs	0.5 µL
Nuclease-free water	18.375 µL
Forward Primer (10 µM)	0.5 µL
Reverse Primer (10 µM)	0.5 µL
Taq Polymeerase (1U/ml)	0.125 µL
Template DNA	2.5 µL
Total Volume	25 µL

Table 2.2.1: Thermal cycle condition for IMP

Steps	Temperature	Time
Initial Denaturation	95	5 min
30 cycles	95	1 min
Annealing	54	1 min
Extension	72	1 min 30 sec
Final Extension	72	10 min

Table 2.2.2: Thermal cycle condition for TEM

Steps	Temperature	Time
Initial Denaturation	95	2 min
30 cycles	95	45 sec
Annealing	62	45 sec
Extension	72	1 min
Final Extension	72	5 min

Table 2.3: PCR components for NDM-1 and VIM-2 genes and their volumes

PCR COMPONENTS	VOLUME
10X Reaction Buffer	5 µL
dNTPS	1 µL
Nuclease-free water	33.8 µL
Forward Primer (10 µM)	2.5 µL
Reverse Primer (10 µM)	2.5 µL
Taq Polymeerase (3U/ml)	0.2 µL
Template DNA	5 µL
Total Volume	50 µL

Table 2.3.1: Thermal cycle condition for NDM- 1

Steps	Temperature	Time
Initial Denaturation	95	10 min
30 cycles	95	1 min
Annealing	63	45 sec
Extension	72	1 min 30 sec
Final Extension	72	10 min

Table 2.3.2: Thermal cycle condition for VIM- 2

Steps	Temperature	Time
Initial Denaturation	95	10 min
30 cycles	95	1 min
Annealing	59	45 sec
Extension	72	1 min 30 sec
Final Extension	72	10 min

Table 2.4: PCR components for CTX-M gene and their volumes

PCR COMPONENTS	VOLUME
10X Reaction Buffer	5 µL
dNTPS	1 µL
Nuclease-free water	39.75 µL
Forward Primer (10 µM)	1 µL
Reverse Primer (10 µM)	1 µL
Taq Polymerase (1U/ml)	0.25 µL
Template DNA	2 µL
Total Volume	50 µL

Table 2.4.1: Thermal cycle condition for CTX- M

Steps	Temperature	Time
Initial Denaturation	94	5 min
30 cycles	94	45 sec
Annealing	58	45 sec
Extension	72	1 min
Final Extension	72	7 min

Table 2.5: PCR components for CLR gene and their volumes

PCR COMPONENTS	VOLUME
10X Reaction Buffer	3.25 µL
dNTPS	0.5 µL
Nuclease-free water	17.6 µL
Forward Primer (10 µM)	0.5 µL
Reverse Primer (10 µM)	0.5 µL
Taq Polymeerase (5U/ml)	0.15 µL
Template DNA	2.5 µL
Total Volume	25 µL

Table 2.5.1: Thermal cycle condition for CLR

Steps	Temperature	Time
Initial Denaturation	94	5 min
30 cycles	94	30 sec
Annealing	45	40 sec
Extension	72	30 sec
Final Extension	72	10 min

2.6 Gel Electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA, and proteins) and their fragments, based on their size and charge. The PCR products were further investigated through agarose gel electrophoresis. The PCR products were loaded into a gel with 6x loading dye. 100 bp ladder was used to estimate the size of the template DNA. The gel was run for 50 minutes at 80V. DNA was visualized using ethidium bromide under ultraviolet transilluminator.

CHAPTER 3

RESULTS

3.1 Antibiotic Susceptibility Test

To check the antimicrobial susceptibility pattern of *P. aeruginosa* collected from both sputum and pus and *K. pneumoniae* collected from sputum antibiotic disk diffusion method was performed. To compare the collected result CLSI guideline was followed.

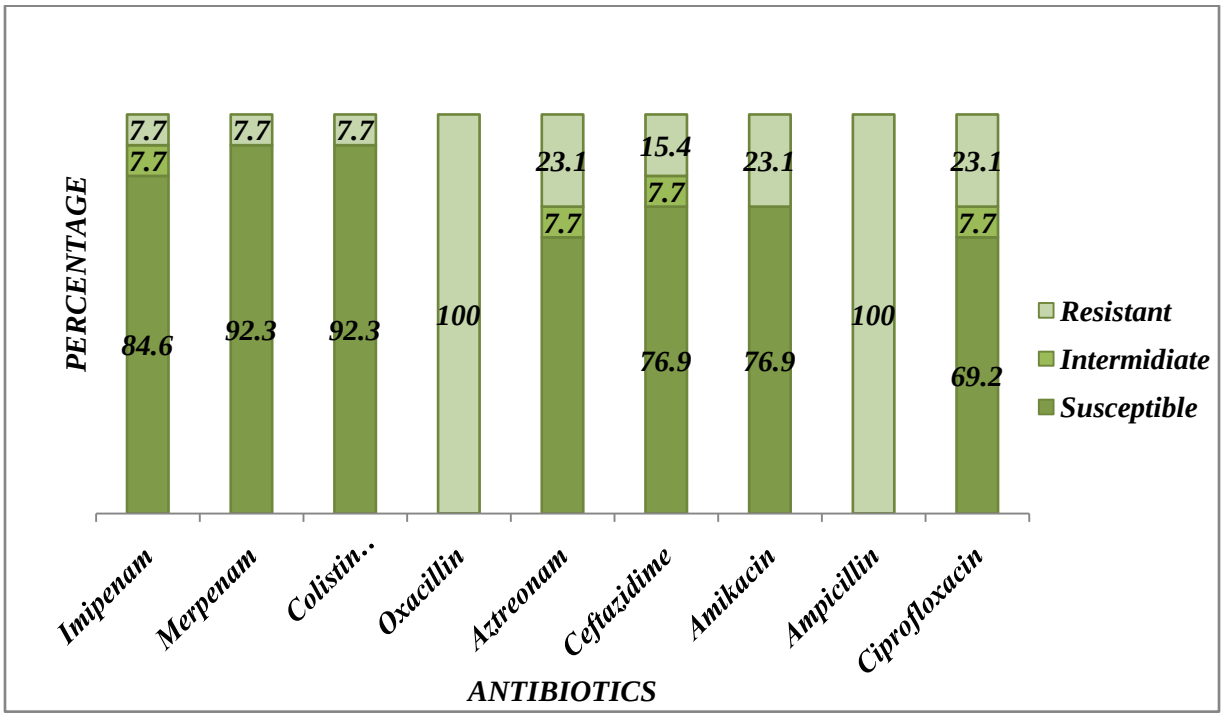

Fig. 3.1: Antimicrobial Susceptibility Pattern of *P.aeruginosa* isolates collected from sputum

Fig. 3.1 shows antimicrobial susceptibility pattern of *P. aeruginosa* isolates collected from sputum. The percentage of resistance to oxacillin and ampicillin was 100%. Resistance percentage was 23.1% to ciprofloxacin and ceftazidime and the resistance percentage was 15.4% to aztreonam. Carbapenems such as imipenem and meropenem and polymyxin E antibiotic colistin showed least resistance percentage of 7.7%.

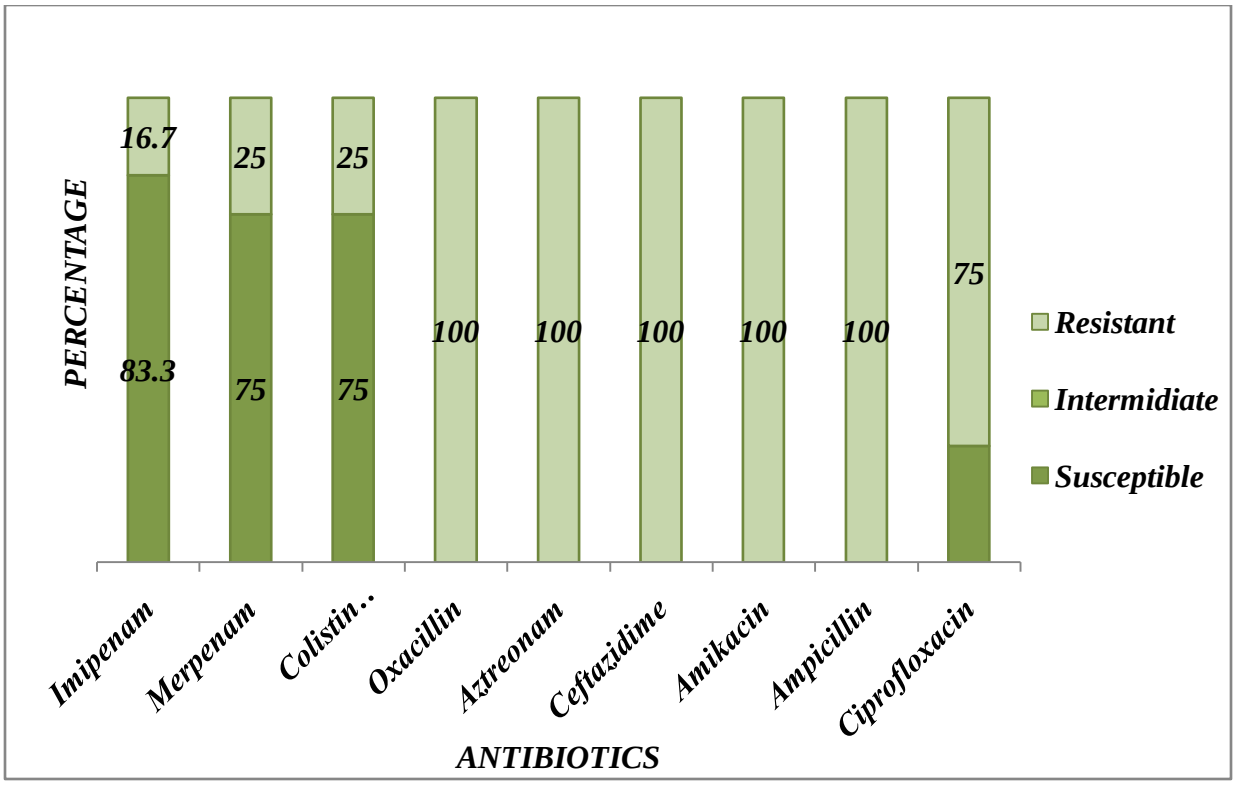

Fig. 3.2: Antimicrobial Susceptibility Pattern of *P.aeruginosa* isolates collected from pus

Fig. 3.2 shows the antimicrobial susceptibility pattern of *P.aeruginosa* isolates collected from pus. The percentage of resistance to oxacillin, aztreonam, ceftazidime, amikacin, and ampicillin was 100%. Resistance percentage of *P.aeruginosa* isolates was 75% to ciprofloxacin, 33.3% to meropenem, 25% to colistin and 16.7% to imipenem.

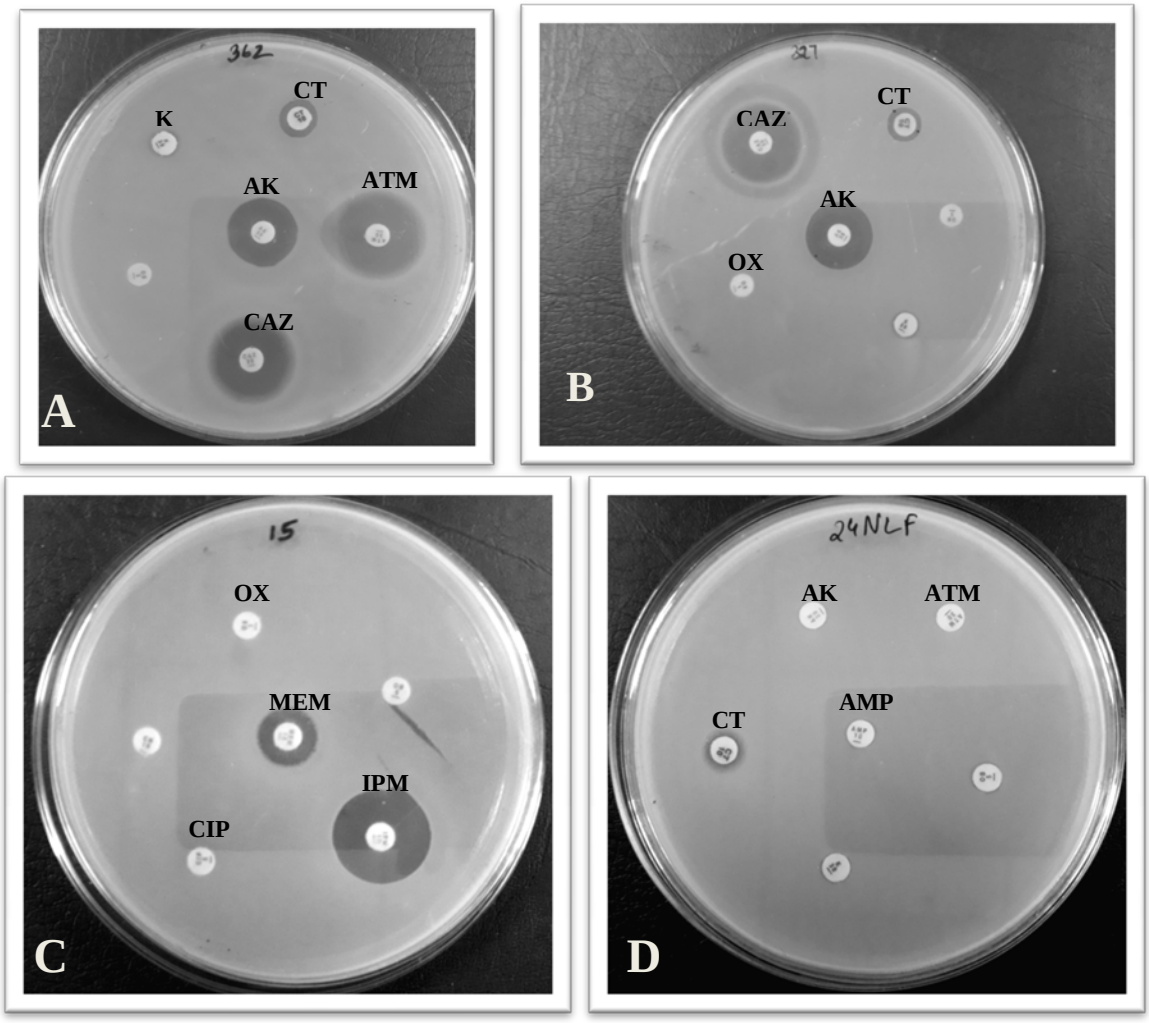

Fig. 3.3: Disk Diffusion method performed to check antimicrobial susceptibility pattern in *P. aeruginosa* isolates collected from sputum and pus

Fig. 3.3 A: shows the antimicrobial susceptibility pattern of the isolate of 362 *P. aeruginosa* collected from sputum. The isolate shows sensitivity to colistin sulfate, aztreonam, amikacin, and ceftazidime. Fig. 3.3 B: shows the antimicrobial susceptibility pattern of the isolate of 327 *P. aeruginosa* collected from sputum. The isolate shows resistance to oxacillin, and colistin sulfate and shows sensitivity to ceftazidime and amikacin. Fig. 3.3 C: shows antimicrobial susceptibility pattern of the isolate of 15 *P. aeruginosa* collected from pus. The isolate shows resistance to oxacillin and ciprofloxacin and shows sensitivity to imipenem and meropenem. Fig. 3.3 D: shows the antimicrobial susceptibility pattern of the isolate of 24NLF *P. aeruginosa* collected from pus. The isolate shows resistance to aztreonam, colistin sulfate, and ampicillin.

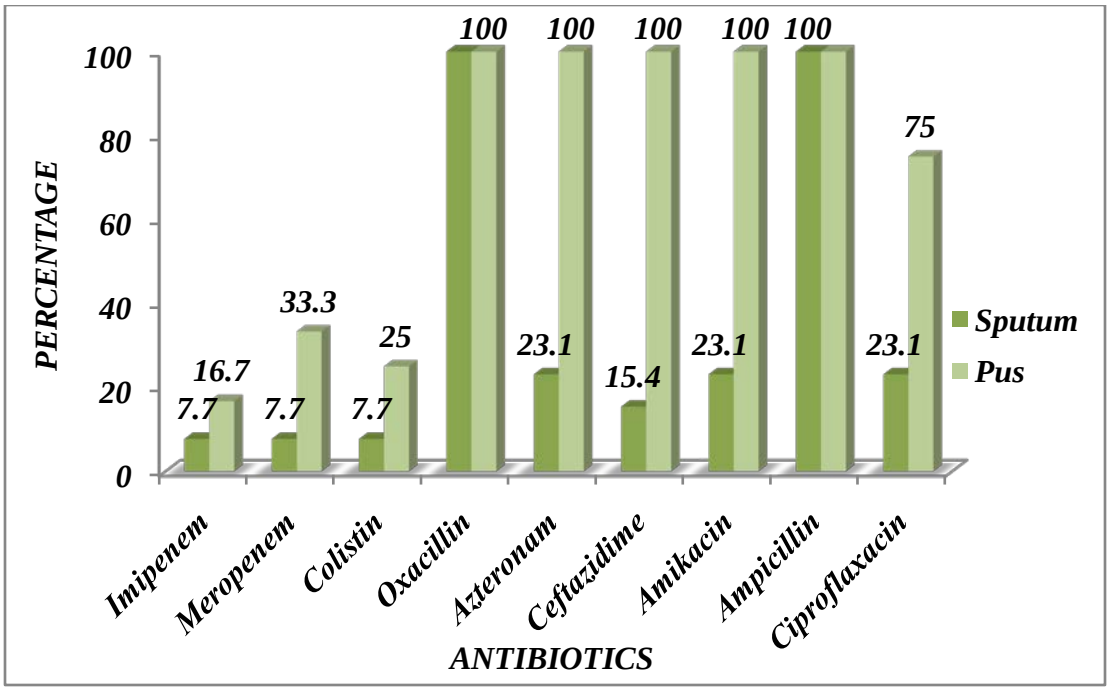

Fig. 3.4: Comparison of antibiotic resistance pattern in *P.aeruginosa* isolates collected from sputum and pus

Fig. 3.4 shows the comparison of antibiotic resistance pattern of *P.aeruginosa* isolates collected from sputum and pus. The percentage of resistance to oxacillin, aztreonam, ceftazidime, amikacin, and ampicillin was 100% in case of pus samples whereas sputum samples showed resistance percentage of 100% only to ampicillin and oxacillin. The resistance percentage to aztreonam, amikacin and ciprofloxacin were 23.1% in *P.aeruginosa* collected from sputum samples. The least resistance percentage was to imipenem, meropenem and colistin sulfate (7.7%) collected from sputum samples. The rate varied in pus samples but least resistance rate was found for same antibiotics as sputum samples.

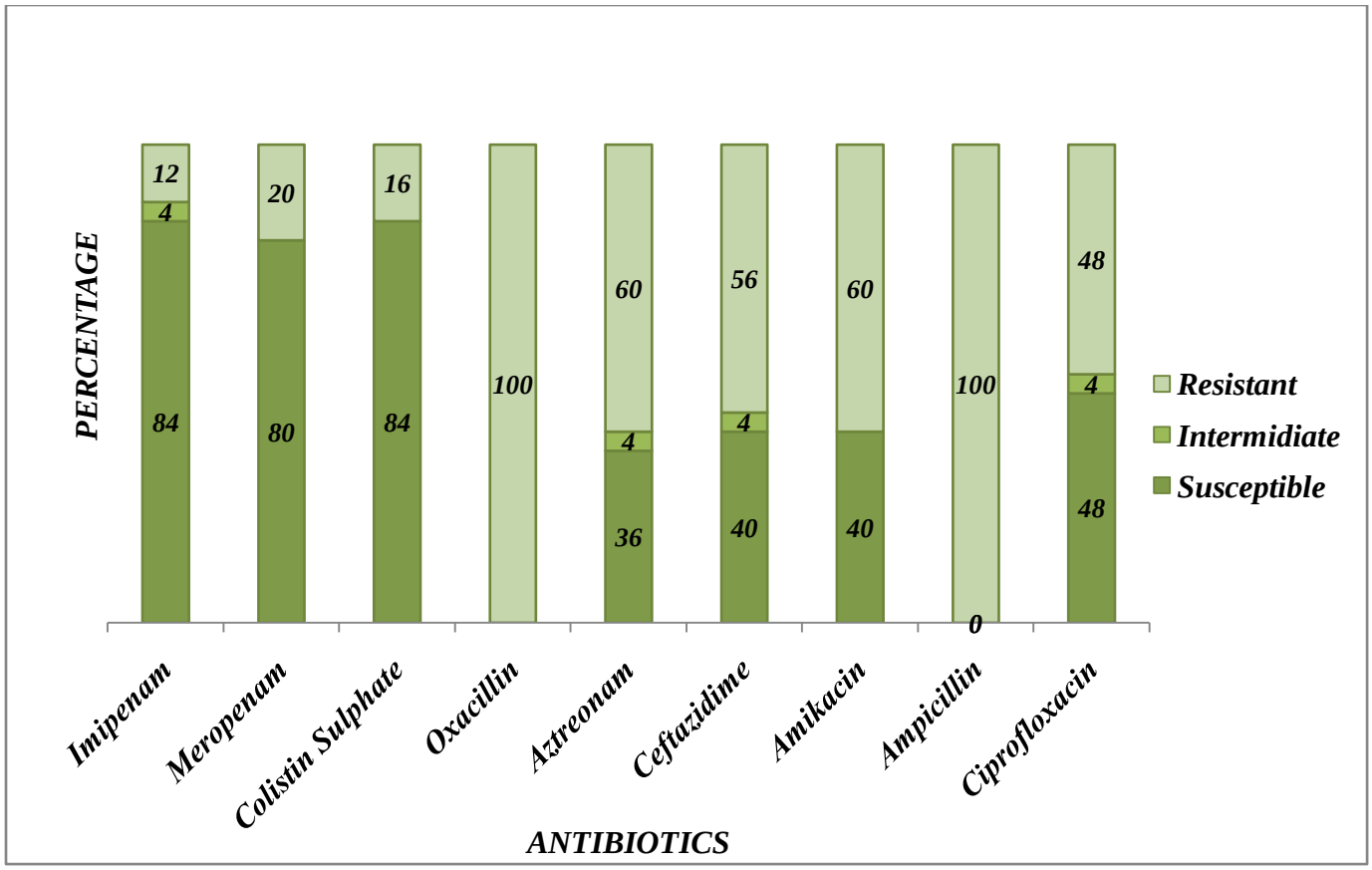

Fig. 3.5: The antimicrobial susceptibility pattern of all *P. aeruginosa* isolates

Fig. 3.5 shows the antimicrobial susceptibility pattern of *P. aeruginosa* isolates collected from both sputum and pus. All the isolates showed maximum resistance percentage to ampicillin and oxacillin (100%). The isolates showed the least resistance to imipenem (12%), colistin sulfate (16%) and meropenem (20%). The resistance percentage was 56% and 48% to ceftazidime and ciprofloxacin respectively. The *P.aeruginosa* isolates showed resistance percentage of 60% to both ceftazidime and amikacin.

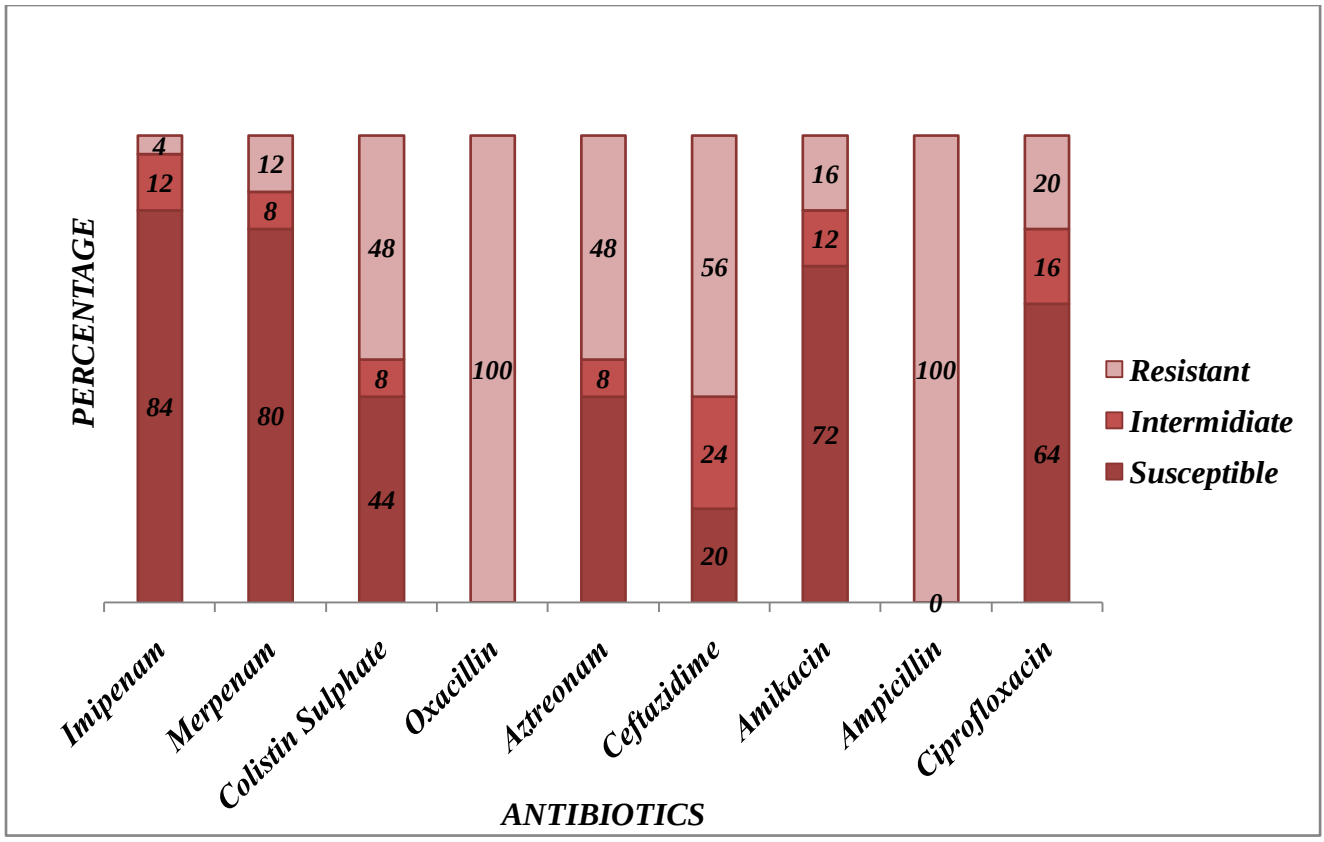

Fig. 3.6: The antimicrobial susceptibility pattern of *K. pneumoniae* isolates

Fig. 3.6 shows the antimicrobial susceptibility pattern of *K. pneumoniae* isolates collected from sputum. All the isolates show maximum resistance percentage to ampicillin and oxacillin. The isolates show the least resistance to both meropenem (12%) and imipenem (4%). The *K. pneumoniae* isolates show resistance percentage of 56% to ceftazidime and 48% to colistin sulfate and aztreonam. The resistance percentage is 20% to ciprofloxacin and 36% to amikacin.

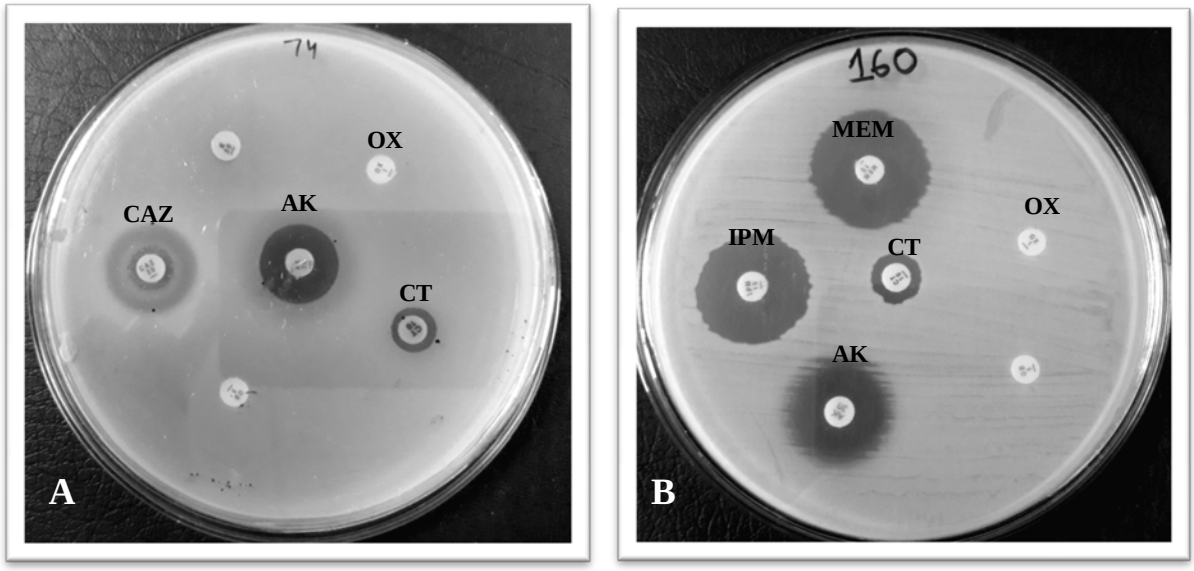

Fig. 3.7: Disk Diffusion method performed to check antimicrobial susceptibility pattern in *K. pneumoniae* isolates collected from sputum

Fig. 3.7 A: shows antimicrobial susceptibility pattern of the isolate of 74 *K. pneumoniae* collected from sputum. The isolate shows resistance to oxacillin and colistin sulfate and shows sensitivity to amikacin. Fig. 3.7 B: shows the antimicrobial susceptibility pattern of the of isolate 160 *K. pneumoniae* collected from sputum. The isolate shows resistance to oxacillin and shows sensitivity to imipenem, meropenem, amikacin and colistin sulfate.

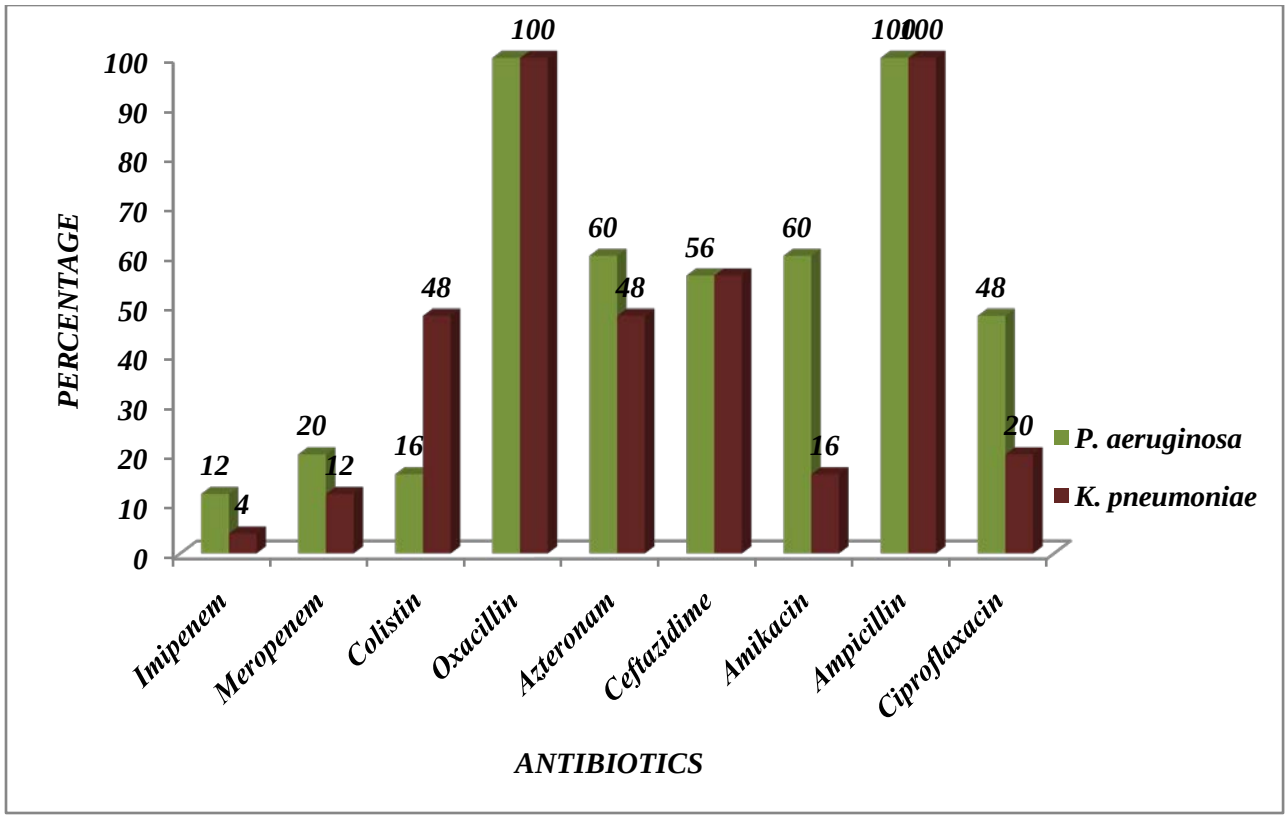

Fig. 3.8: Comparison of antibiotic resistance pattern of *P. aeruginosa* and *K. pneumoniae* isolates

Fig. 3.8 shows the comparison of antibiotic resistance of *P. aeruginosa* (n=25) and *K. pneumoniae* (n=25). Both the organisms were highly resistant to oxacillin and ampicillin (100%) whereas least resistance was found to imipenem and meropenem. *P. aeruginosa* showed more resistance percentage to ciprofloxacin, amikacin, and aztreonam which are 48%, 60%, and 60% respectively. On the other hand, *K.pneumoniae* showed less resistance percentage to ciprofloxacin, amikacin, and aztreonam which are 20%, 16%, and 56% respectively. *K. pneumoniae* showed more resistance to colistin (48%) than *P. aruginosa* (16%).

3.2 ESBL Detection Test

Table 3.1: Interpretation criteria of ESBL classes

Reporting Comment	**Potentiation of the inhibition zone of any one of ceftazidime, ceftriaxone or aztreonam when combined with clavulanic acid/ piperacillin- tazobactam**	**Cefoxitin**	**Ceftazidime, Ceftriaxone or aztreonam**
The susceptibility pattern suggests that this organism contains a class An extended spectrum beta- lactamase (ESBL)	Yes	S	S/R
The susceptibility pattern suggests that this organism contains a class A and C extended spectrum beta- lactamase (ESBL)	Yes	I/R	S/R
The susceptibility pattern suggests that this organism contains a class C extended spectrum beta- lactamase (ESBL)	No	I/R	R
The susceptibility pattern suggests that this organism contains a class extended spectrum beta- lactamase (ESBL) other than class A and C.	No	S	R
Not ESBL- no reporting comment	No	S/R	S

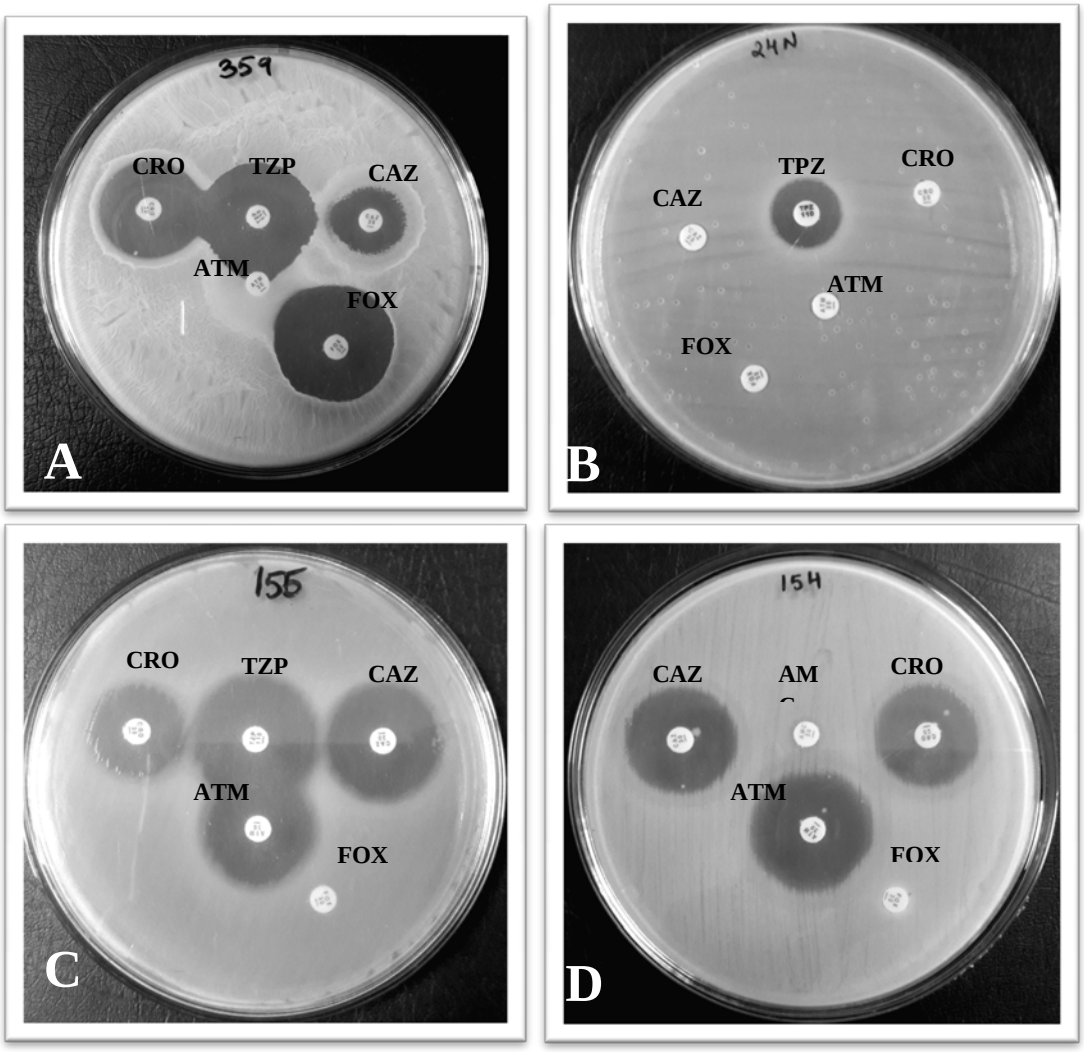

Fig. 3.9: ESBL detection by disk diffusion method

Fig. 3.9 A: shows ESBL class A producing *P.aeruginosa*. The isolate showed potentiation of the inhibition zone towards ceftriaxone and aztreonam when combined with piperacillin-tazobactam and is susceptible to cefoxitin. Fig. 3.9 B: shows ESBL class C producing *P. aeruginosa.* The organism showed no potentiation with piperacillin-tazobactam and is resistance to cefoxitin, ceftazidime, ceftriaxone, and aztreonam. Fig. 3.9 C: shows ESBL class A and C producing *P. aeruginosa.* The organism showed potentiation of the inhibition zone towards aztreonam when combined with piperacillin-tazobactam and is resistant to cefoxitin and susceptible to ceftazidime and ceftriaxone. Fig. 3.9 D: shows ESBL negative *K. pneumoniae.* The organism shows no potentiation with clavulanic acid and is resistant to cefoxitin and susceptible to all of the ceftazidime, ceftriaxone, and aztreonam.

Table 3.2: ESBL class detection of *P. aeruginosa* isolates

NO.	SAMPLE	POTENTIATION OF INHIBITION ZONE			FOX	R/S FOR CAZ, CRO, ATM			ESBL +/-
	SPUTUM	**CRO**	**CAZ**	**ATM**		**CRO**	**CAZ**	**ATM**	
1.	**327**	NO	NO	YES	R	I	S	S	A & C
2.	**328**	YES	YES	YES	R	S	S	S	A & C
3.	**350**	NO	YES	YES	R	I	S	S	A & C
4.	**352**	NO	YES	YES	R	I	S	S	A & C
5.	**358**	NO	NO	NO	R	R	R	R	C
6.	**359**	YES	NO	YES	S	S	S	S	A
7.	**361**	NO	YES	YES	R	S	S	S	A & C
8.	**362**	NO	YES	YES	R	S	S	S	A & C
9.	**155**	NO	NO	YES	R	S	S	S	A & C
10.	**161**	NO	YES	YES	R	S	S	S	A & C
11.	**166**	NO	YES	YES	R	R	S	R	A & C
12.	**48**	NO	YES	YES	R	I	S	S	A & C
13.	**49**	NO	NO	NO	R	R	R	R	C
	PUS								
14	**15**	NO	NO	NO	R	R	R	R	C
15.	**20**	NO	NO	NO	R	R	R	R	C
16.	**22**	NO	NO	C	R	R	R	R	C
17.	**23**	NO	NO	NO	R	R	R	R	C
18.	**24**	NO	NO	C	R	R	R	R	C
19.	**24LF**	NO	NO	C	R	R	R	R	C
20.	**24NLF**	NO	NO	NO	R	R	R	R	C
21.	**25**	NO	NO	NO	R	R	R	R	C
22.	**31**	NO	NO	NO	R	R	R	R	C
23.	**33**	NO	NO	NO	R	R	R	R	C
24.	**35**	NO	NO	NO	R	R	R	R	C
25.	**37**	NO	NO	NO	R	R	R	R	C

Continued

Table 3.2 shows the ESBL class detection of all the *P. aeruginosa* isolates. Among the thirteen *P.aeruginosa* isolates collected from sputum ten isolates were both ESBL class A and C producers. Two of the *P. aeruginosa* isolates were ESBL class C producer and only one isolate was ESBL class A producer. In the case of the twelve *P. aeruginosa* isolates collected from pus all were ESBL class C producers.

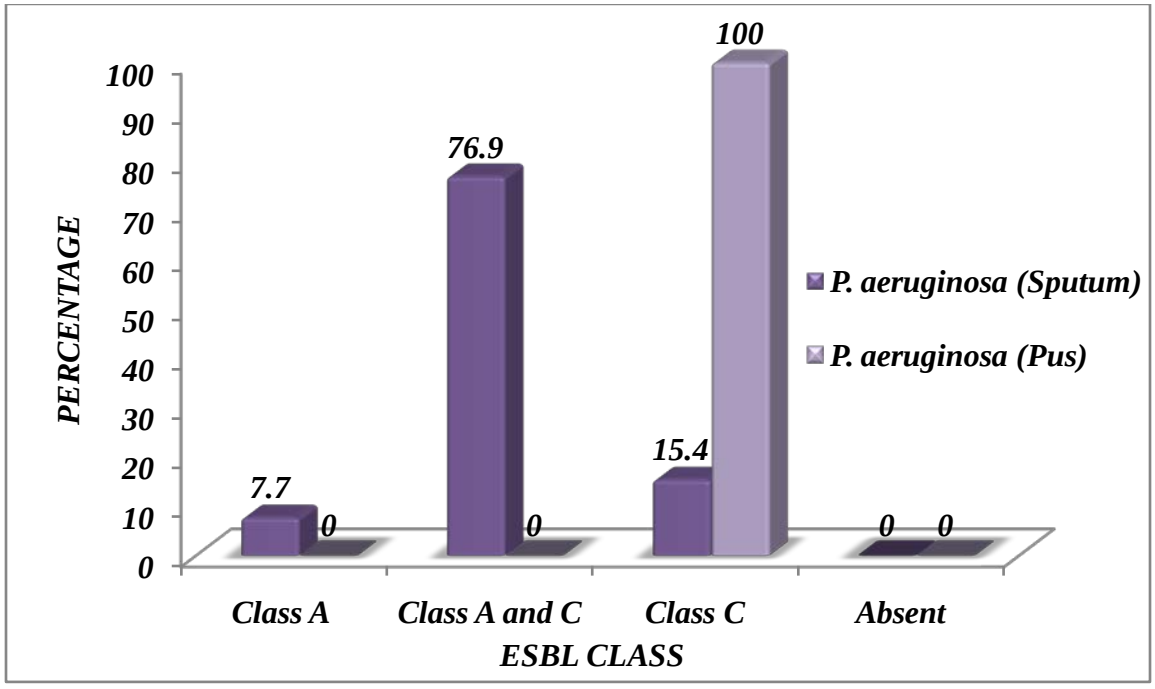

Fig. 3.10: ESBL class assay of *P. aeruginosa* isolates collected from sputum and pus

Fig. 3.10 shows ESBL class assay of P. aeruginosa collected from both sputum and pus. It shows a high prevalence of ESBL class C positive *P. aeruginosa* collected from pus (100%). Out of all the *P. aeruginosa* isolates collected from sputum highest prevalence was of ESBL class A and C (76.9%). Class A producing *P. aeruginosa* was least in number and no isolate was ESBL negative.

Table 3.3: ESBL class detection of *K. pneumoniae* isolates

NO.	SAMPLE	POTENTIATION OF INHIBITION ZONE			FOX	R/S FOR CAZ, CRO, ATM			ESBL +/-
		CRO	CAZ	ATM		CRO	CAZ	ATM	
1.	**61**	NO	NO	NO	R	R	R	R	C
2.	**62**	NO	NO	YES	S	R	R	R	A
3.	**63**	NO	NO	YES	S	R	R	R	A
4.	**73**	NO	NO	YES	I	R	I	I	A & C
5.	**74**	NO	NO	NO	R	R	S	S	NEG
6.	**75**	NO	NO	YES	R	R	R	R	A & C
7.	**77**	NO	NO	YES	R	S	S	S	A & C
8.	**341**	NO	NO	NO	R	I	I	S	NEG
9.	**348**	NO	NO	NO	I	S	I	S	C
10.	**360**	NO	NO	NO	R	R	I	R	C
11.	**154**	NO	NO	NO	R	S	S	S	NEG
12.	**156**	NO	NO	NO	R	S	S	S	NEG
13.	**159**	NO	YES	YES	S	I	R	R	A
14.	**160**	NO	NO	NO	R	S	S	S	NEG
15.	**162**	NO	NO	YES	R	R	R	R	A & C
16.	**167**	NO	NO	NO	R	R	I	R	C
17.	**168**	NO	NO	YES	R	I	I	S	A & C
18.	**32**	NO	YES	YES	S	S	S	S	A
19.	**34**	NO	NO	NO	R	R	R	R	C
20.	**47**	NO	NO	YES	S	S	S	S	A
21.	**190**	NO	NO	YES	R	R	R	R	A & C
22.	**192**	NO	NO	YES	R	R	R	S	A & C
23.	**195**	NO	NO	YES	S	R	R	R	A
24.	**197**	NO	NO	YES	S	R	R	R	A
25.	**199**	NO	NO	YES	I	R	R	R	A & C

Continued

Table 3.3 shows the ESBL class detection of all the *K. pneumoniae* isolates. Among the twenty-five, *K. pneumoniae* isolates collected from sputum eight of the isolates were both ESBL class A and C producers. Five *K. pneumoniae* isolates were ESBL class C producer and seven of the isolates were ESBL class A producer. Out of the twenty-five, *K. pneumonia* isolates five of them were ESBL non-producers.

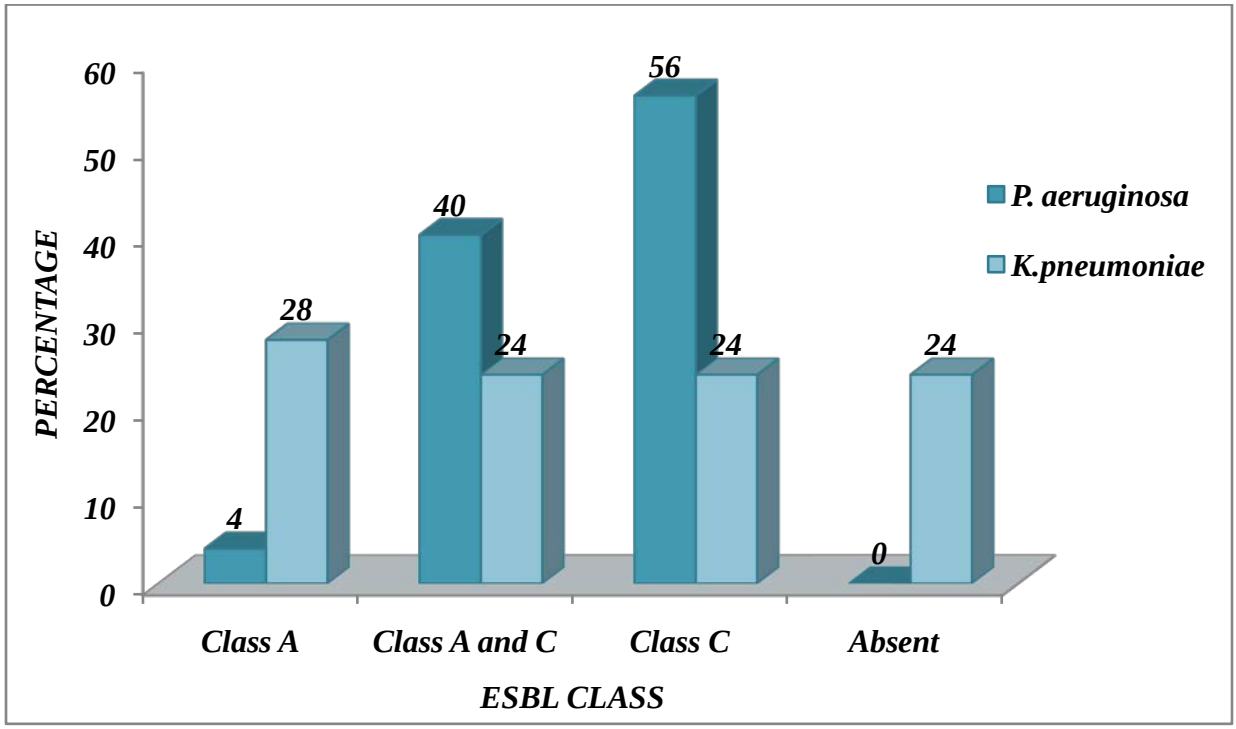

Fig. 3.11: ESBL class assay of *P. aeruginosa* and *K. pneumoniae* isolates

Fig. 3.11 shows ESBL class assay of *P. aeruginosa* and *K. pneumoniae*. It shows a high prevalence of ESBL class C positive *P. aeruginosa* (56%). The 40% of the *P. aeruginosa* isolates were ESBL class A and C producing whereas only 4% were ESBL class A producing. None of the *P. aeruginosa* isolates was ESBL negative. On the other hand, 28% of *K. pneumoniae* isolates were ESBL class A producing which is the highest prevalence. *K. pneumoniae* showed the equal percentage of prevalence for ESBL class A and C, ESBL class C and ESBL negative (24%).

3.3 Identification of Antibiotic Resistant Gene through PCR

The template DNAs were amplified with antibiotic resistant gene specific primers through PCR. Six primers blaIMP (587 bp), blaVIM-2 (864 bp), blaNDM-1 (200 bp), blaMCR (309bp), blaCTX-M (759 bp) and blaTEM (768bp) were used to confirm the presence of the antibiotic resistant gene. Agarose gel electrophoresis was used to detect the gene specific band with the help of UV transilluminator.

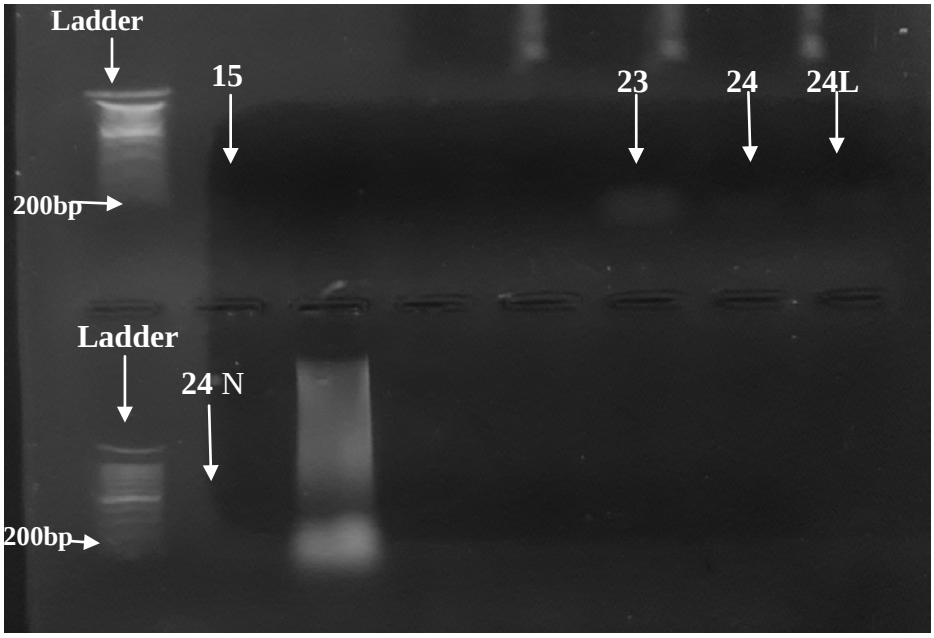

Fig. 3.12: Gel electrophoresis of 100bp ladder and PCR product of blaNDM-1

Fig. 3.12 shows the gel electrophoresis result of blaNDM-1 gene. The gel shows five bands of which all the isolates were *P. aeruginosa* collected from pus. The samples were 15, 23, 24, 24L and 24N. The isolates showed the band at 200 bp which confirms the presence of the blaNDM gene.

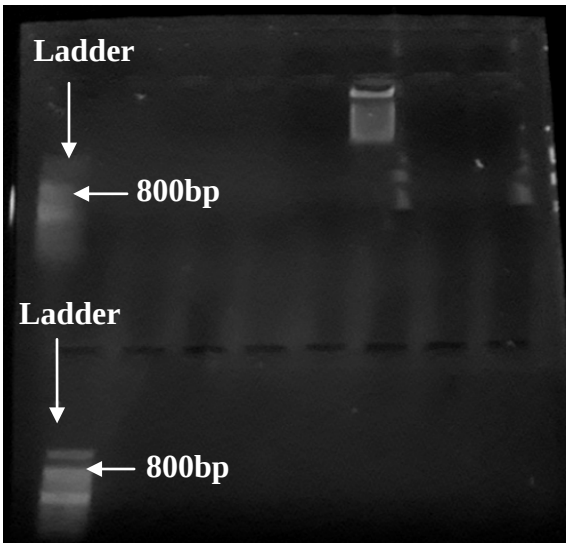

Fig. 3.13: Gel electrophoresis of 100bp ladder and PCR product of blaVIM-2

Fig. 3.13 shows the gel electrophoresis result of blaVIM-2 gene. The gel shows no band for any of the samples. All the samples were either resistant or intermediate to meropenem. The isolates should show the band at around 864 bp but no band was observed in the gel.

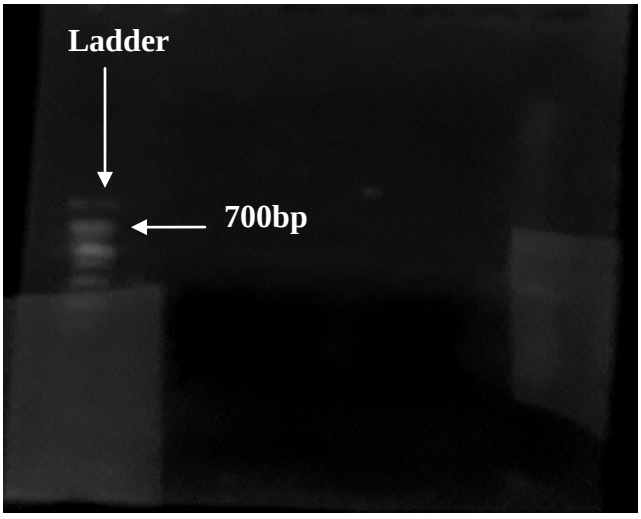

Fig. 3.14: Gel electrophoresis of 100bp ladder and PCR product of blaTEM

Fig. 3.14 shows the gel electrophoresis result of the blaTEM gene. The gel shows no band for any of the samples although the isolates were resistant to amikacin, ampicillin, and ciprofloxacin. The isolates should show the band at around 768 bp but no band was observed in the gel.

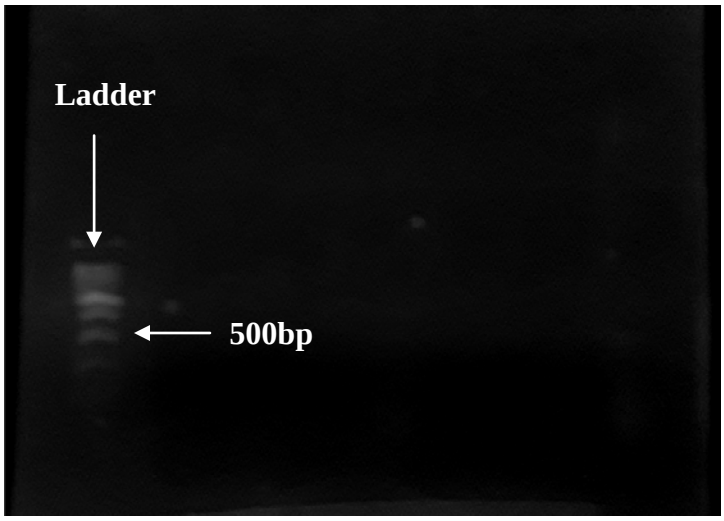

Fig. 3.15: Gel electrophoresis of 100bp ladder and PCR product of blaIMP

Fig. 3.15 shows the gel electrophoresis result of the blaIMP gene. The gel shows no band for any of the samples although the isolates were resistant to imipenem. The isolate should show the band at around 587 bp but no band was observed in the gel.

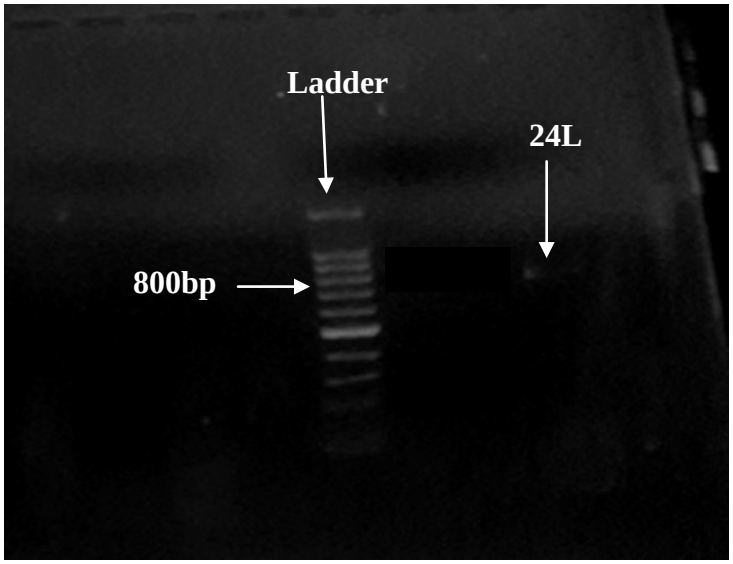

Fig. 3.16: Gel electrophoresis of 100bp ladder and PCR product of blaCTX-M

Fig. 3.16 shows the gel electrophoresis result of blaCTX-M genes. The gel shows band for one sample. The 24L was *P. aeruginosa* isolate from pus. The isolate confirms the presence of CTX-M genes as the length of the DNA is 759 bp which is close to 800bp.

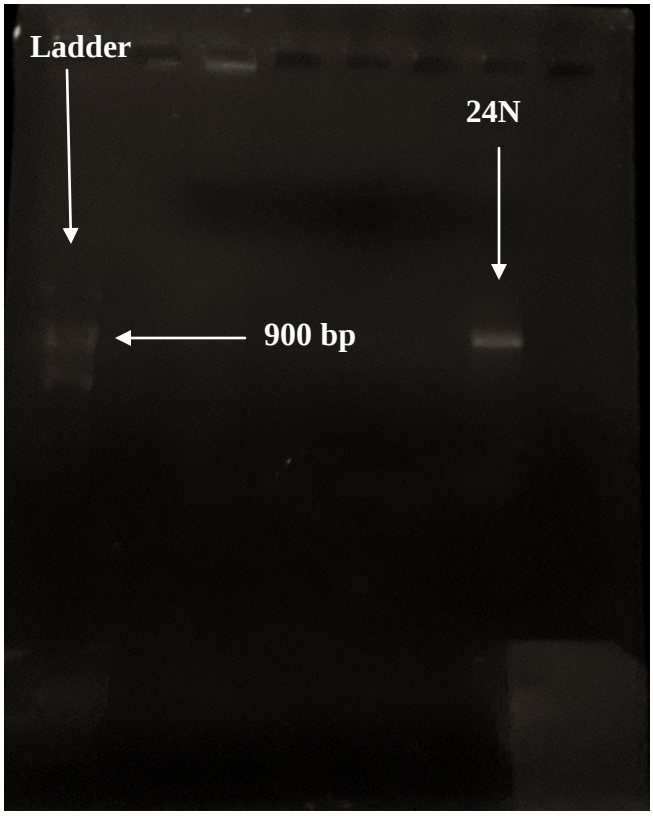

Fig. 3.17: Gel electrophoresis of 100bp ladder and PCR product of blaCLR

Fig. 3.17 shows the gel electrophoresis result of blaCLR genes. The gel shows band for one sample. The sample 24N was *P. aeruginosa* isolate from pus. The isolate shows band at around 900bp. But according to the primer the length of the DNA should be 300 bp. This isolate needs further confirmation to detect the presence of the CLR gene.

CHAPTER 4

DISCUSSION

4.1 Antibiotic Susceptibility Test

There is mounting evidence that proportion of resistance among *P. aeruginosa* and *K. pneumoniae* are increasing steadily. In addition to being intrinsically resistant, it can acquire resistant traits during therapy through an array of mechanisms. In this study, isolates of *P. aeruginosa* from wound were found relatively more resistant than those from sputum samples. The frequency of resistance to oxacillin, aztreonam, ceftazidime, amikacin, and ampicillin was 100%. Resistance percentage was 75% to ciprofloxacin, 33.3% to meropenem, 25% to colistin and 16.7% to imipenem.

A previous study (Rashid et al., 2016), in Bangladesh, showed that resistance percentage of *P. aeruginosa* to co-trimoxazole was 92%, ciprofloxacin 62.5%, cephalexin 100%, ceftriaxone 75% and ceftazidime 37%. The number of multi-drug resistant strains has increased in recent years. An Iranian study in 2003 had their resistance pattern worse than this study, where the percentage of resistance to gentamycin was 93.7%, ceftazidime 96%, amikacin 93% and ciprofloxacin 86%. A five-year retrospective Indian study, from 1997-2002, found resistance pattern of *P. aeruginosa* as amikacin 52%, gentamycin 69%, ciprofloxacin 89%, and ceftazidime 62%, which shows that the trend of resistance was also increasing. Taneja in 2004 found the pattern of resistance about 90% each for ceftazidime and amikacin while 45% to ciprofloxacin. The previous study in 2016, (Rashid et al., 2016), in Bangladesh, showed that percentage of resistance of *P. aeruginosa* to co-trimoxazole was 92%, ciprofloxacin 62.5%, cephalexin 100%, ceftriaxone 75% and ceftazidime 37%. However, in another study, the resistance to amikacin was 2%, ceftriaxone 43%, ceftazidime 25% and ciprofloxacin 50%.9 The number of multi-drug resistant strains has increased in recent years. An Iranian study in 2003 had their resistance pattern worse than this study, where the percentage of resistance to ceftazidime 96%, amikacin 93%, and ciprofloxacin 86% (Singh et al., 2003). Taneja in 2004 found the pattern of resistance about 90% each for ceftazidime and amikacin while 45 % tociprofloxacin (Taneja et al., 2004).

Regarding the *P. aeruginosa* isolates collected from the sputum samples were 100% resistant to all of the aminoglycosides (ampicillin and oxacillin). Resistance percentage was 23.1% to ciprofloxacin and ceftazidime. Carbapenems such as imipenem and meropenem and polymyxin E antibiotic colistin show least resistance percentage of 7.7%. In vitro data showed a wide range

of beta-lactams, aminoglycosides, quinolones and other antibiotics which are useful for the treatment of *Klebsiella* infections (Weisenberg et al., 2009, Chan et al., 2009, Adams-haduch et al., 2009). Both Gram-positive and Gram-negative bacteria have cell walls which are composed of heavily cross-linked peptidoglycan layers which are catalyzed by cell-wall transpeptidases also known as penicillin binding protein(PBP). Beta-lactam antibiotics disturb peptide bond formation by acting as competitive inhibitors to these PBPs. These result in the formation of irreversible covalent bonded penicilloyl-enzyme complexes with weak cross-linked peptidoglycans, thus ease bacteria lyses and death (Wilke et al., 2005).

This study shows the high resistance of *K. pneumoniae* to the first line of antibiotics that are commonly prescribed such as ampicillin and oxacillin. The resistance percentage was 56% to ciprofloxacin and 48% to both aztreonam and colistin. The least resistance percentage was seen 20%, 16%, 12% and 4% to ciprofloxacin, amikacin, meropenem and imipenem respectively. Another study shows *K.pneumoniae* strains from clinical cases were found highly susceptible to quinolones and aminoglycoside, amikacin, and ciprofloxacin. 56% of them were resistant to cephalosporins. Cephalosporins have been widely used as monotherapy and in combination with aminoglycosides for the treatment of *Klebsiella* infection. Plasmid-encoded resistance to broad-spectrum cephalosporins is becoming a widespread phenomenon in clinical medicine. These antibiotics are inactivated by an array of differently extended-spectrum beta-lactamases (ESBLs) which have evolved by stepwise mutation of TEM/SHV type beta-lactamases. Plasmid encoding these enzymes has been encountered in several members of the family Enterobacteriaceae, but are, for unknown reasons, most often harboured by *K. pneumoniae.* Epidemic and endemic nosocomial infections caused by ESBL producing *K. pneumoniae* represent a persistent problem in many parts of the world, especially in ICUs (Arlet et al., 1990, Meyer et al., 1993).

In comparison with *P. aeruginosa* (n=25) and *K. pneumoniae* (n=25), this study proved that both the organisms were highly resistant to oxacillin and ampicillin (100%) whereas least resistance was found in imipenem and meropenem. *P. aeruginosa* showed more resistance percentage to ciprofloxacin, amikacin, and aztreonam, which is 48%, 60%, and 60% respectively. On the other hand, *K.pneumoniae* showed less resistance percentage to ciprofloxacin, amikacin, and

aztreonam which is 20%, 16%, and 56% respectively. *K.pneumoniae* showed more resistance to colistin (48%) than *P. aeruginosa* (16%).

4.2 Extended Spectrum Beta-Lactamase Test

ESBLs are the main cause of resistance to beta-lactam antibiotics which are among the safest and most frequently prescribed antimicrobial agents all over the world. As their occurrence has been increasing, it becomes essential to evaluate their occurrence in *E. coli* and *K. pneumoniae* which are mostly ESBL producers (Pitout and Laupland, 2008, Sahu et al., 2011). The incidence of ESBL-producing *K. pneumoniae* varies from one country to another depending upon various factors, like the antibiotic policy, the carriage rate among hospital personnel, and the type of disinfection used especially in the ICU (Sarojamma and Ramakrishna, 2011). It is recognized that Egypt has an extremely high rate of ESBL producers, with up to 70% of isolates producing the enzyme (Borg et al., 2006). In the present study, 88% (n= 44) of the 50 tested strains were ESBL producers and 12% (n=6) were non- ESBL producers. This could be attributed to the empirical usage of 3rd generation cephalosporins in the treatment of nosocomial infections in hospitals. Although molecular methods brought speed and accuracy, they are costly and not suitable for low-income developing countries (Gazin et al., 2012).

This study showed that 84% of isolates of *K. pneumoniae* were ESBL producer whereas 100% of isolates of *P. aeruginosa* were ESBL producers. In another study, 7.6% and 15.3% of isolates of *E. coli* and *K. pneumoniae*, respectively, were identified as ESBL producers (Singh et al., 2011). The incidence of ESBL organisms varies significantly all over the world. The prevalence of ESBL producers is 88% in this study. Findings from other studies in Nepal have shown ESBL production ranging from 18% to 62.7% (Thakur et al., 2013, Shrestha et al., 2011, Poudyal et al., 2011). A variation might have occurred due to a low number of samples studied. During a six years period (1997-2002), the prevalence of ESBL producing *Klebsiella sp* was reported from Latin America (42.7%), Europe (21.7%) and North America (5.8%) (Biedenbach et al., 2004). Strict antibiotic policies might be the reason for a lower rate of ESBL organisms in Europe and America. As these microorganisms are prevalent in the hospital environment, the patients are more at risk of acquiring these enzymes. Such isolates could be responsible for outbreaks of infections in hospitalized patients resulting in higher morbidity and mortality. As the third-generation cephalosporins are predominantly used in several hospitals, resistance to these

antibiotics can lead to treatment failures. Hence, detection of isolates expressing ESBLs is very important so as to limit the spread of ESBL-producing isolates.

4.3 Molecular Characterization of blaIMP, blaVIM-2, blaNDM-1, blaCLR, blaCTX-M and blaTEM genes

Six primers blaIMP, blaVIM-2, blaNDM-1, blaCLR, blaCTX-M, and blaTEM were used to confirm the presence of the resistant gene. The organisms which were resistant by disc diffusion test were subjected to PCR. Out of the 44 ESBL producer organisms, five had blaNDM-1 gene, one had the blaCLR gene and one had CTX-M gene. Among the seven samples, all were resistant to ceftazidime, aztreonam, ceftriaxone, cefoxitin, oxacillin, and ampicillin. One of the samples showed resistant gene for both blaNDM-1 and blaCLR whereas one sample showed resistant gene for both blaNDM-1 and blaCTX-M. All the seven samples were *P. aeruginosa* isolates from pus of burn patients. Only one sample showed resistant against imipenem. This proves that carbapenems are effective antibiotics for ESBL treatment.

The present investigation reveals an alarming percentage of ESBL producing strains within the *Enterobacteriaceae* strain. The ESBL producing genes are mostly found to be coded within the plasmid and hence readily transferred to other organisms, which could be the reason for a high percentage of ESBL genes harbouring organisms. In an earlier investigation regarding an outbreak in Chicago by ESBL producing *K. pneumoniae* and *E. coli*, it was concluded that a common plasmid harbouring TEM-10 was isolated from patients admitted to several hospitals and nursing homes and subsequently plasmid expressing TEM-10 was transferred to the normal flora of other patients (Bradford et al., 1994).

Until now, colistin resistance has occurred via chromosomal mutations and, although clonal outbreaks have been reported, the resistance is often unstable, imposes a fitness cost upon the bacterium and is incapable of spreading to other bacteria (Falagas et al., 2011). The rapid dissemination of previous resistance mechanisms (eg, NDM-1) indicates that, with the advent of transmissible colistin resistance, the progression of Enterobacteriaceae from extensive drug resistance to pan-drug resistance is inevitable and will ultimately become global (Kumarasamy et al., 2010). Also, one single organism containing two resistant genes is an alarming incidence. In this context, the emergence of transmissible, plasmid-mediated colistin resistance in the form of MCR-1 with NDM-1 is a finding of global significance.

There are enough gaps in the existing body of scientific evidence to make it a risky response to increasing public concern to deny that there is a problem with respect to a resistance in the environment. On the basis of our present knowledge, an increased direct impact of antibiotics on bacteria in the aquatic environment and in soils is questionable. According to present knowledge, the prudent use of antibiotics in all areas seems to be the key to coping successfully with resistance in the environment. Therefore, the proper use of antibiotics and disinfectants in human medicine and livestock farming will significantly reduce the risk for the general public and for the environment. It also includes controlling the dissemination of antibiotics being used.

Knowledge about how antibiotic resistance arises, how resistant strains and resistance genes spread in nature and the significance of this for humans and nature is far from complete. There are not enough data available to draw a final conclusion especially with respect to the input of already resistant bacteria into the environment. This topic needs further consideration and investigation. An assessment of the different pathways also has to take into consideration the ingestion of resistant bacteria with food.

The importance of the different sources of resistance has been found in the environment. For this purpose, it is important to make a more detailed assessment of the significance of culture-dependent and laboratory-based methods in relation to conditions found in the environment. This also applies to the concentrations of antibiotics applied in the identification of resistant bacteria in laboratory testing compared to the concentrations of antibiotics in the environment. Thresholds favoring selection and transfer of resistance genes between different species under environmental conditions should be established. For this purpose, the significance of the availability and activity of the antibiotics in the environment, i.e. the extent and importance of their sorption to sludge, particles in surface water, sediment, and soil should be determined. The conditions and time scales under which antibiotics and resistance are lost in the environment are also of importance in relation to the input of antibiotics and resistant bacteria.

4.4 Conclusion

ESBL producing isolates have emerged as a major challenge which is due to overuse of extended-spectrum cephalosporins in the hospitals and nursing homes. In this study, significant numbers of clinical isolates were ESBL producers. Out of the fifty isolates forty-four isolates were ESBL producers. The *P. aeruginosa* isolates collected from severe burn patients showed resistance to almost all the antibiotics which were tested. ESBL class A & C producers were predominant in the collected isolates. Out of the forty-four ESBL producer organisms, five had blaNDM-1 gene, one had the blaCLR gene and one had CTX-M gene. Among the seven samples, one showed resistant gene for both blaNDM-1 and blaCLR whereas one sample showed resistant gene for both blaNDM-1 and blaCTX-M. All the seven samples were *P. aeruginosa* isolates were collected from pus of burn patients. Only one sample showed resistant against imipenem. This proves that carbapenems are effective antibiotics for ESBL treatment.

The discovery of highly potent “second generation” beta-lactamase inhibitors are eagerly awaited. Considering the grave scenario of antibiotic resistance in our country, it is high time that all clinical laboratories start detecting the ESBLs routinely and accurately. This study helps us to understand the need for more precaution in use of antibiotics and the alarming rate of resistance seen in ICU burn patients and respiratory tract infected patients. Future studies will be focused on sequencing of NDM-1, MCR and CTX-M gene.

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APPENDICES

APPENDIX I

MEDIA COMPOSITION

Name of the Media	Composition	
	Name of the ingredients	**Amount (g/L)**
Nutrient Agar	Peptone	5.00
	Sodium Chloride	5.00
	Beef extract	3.00
	Agar	15.00
	Final pH	7
MacConkey Agar	Peptic digest of animal tissue	1.50
	Casein enzymic hydrolysate	1.50
	Pancreatic digest of gelatin	17.00
	Lactose	10.00
	Bile salts	1.50
	Crystal violate	0.001
	Neutral red	0.03
	Agar	15.00
EMB Agar	Peptone	10.00
	Lactose	5.00
	Dipotassium phosphate	2.00
	Sucrose	5.00
	Eosin Yellow	0.14
	Methylene blue	0.065
	Agar	13.50
Cetrimide Agar	Pancreatic digest of gelatin	20.00
	Magnesium chloride	1.40
	Potassium sulphate	10.00
	Cetrimide	0.30
	Agar	15.00
	Final pH	7.2±0.2

Name of the Media	Composition	
	Name of the ingredients	**Amount (g/L)**
Muller-Hinton Agar	Beef infusion	300.00
	Casein acid hydrolysate	17.50
	Starch	1.50
	Agar	17.00
	Final pH	7.3±0.1
T1N1 Agar	Tryptone	1.00
	Sodium chloride	1.00
	Agar	0.60-0.75
Luria-Bertani Broth	Tryptone	10.00
	NaCl	10.00
	Yeast extract	5.00

APPENDIX-II

BUFFERS AND REAGENTS

Name of the Buffer or reagents	Component	Amount (g/L)
1 M Tris-HCl	Tris	121.14
	HCl	As required to adjust the pH
	Final ph	8.00
0.5 M EDTA	EDTA	186.00
	NaOH	As required to adjust pH
	pH	8.00
1X TE Buffer	1 M Tris-HCl	10.00
	0.5 M EDTA	2.00
	pH	8.00
1x TBE Buffer	Tris base	10.80
	Boric acid	5.50
	0.5 M EDTA	4.00
	pH	8.00
Lysis Buffer	1X TE Buffer	9.34mL/10mL
	10% SDS	600 µL
	Proteinase K	60 µL

APPENDIX-III

LIST OF EQUIPMENT

INSTRUMENT	MANUFACTURER
Weighing Machine	Adam Equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator	Model: WIS-20R Daihan Scientific Companies, Korea
UV Transilluminator	Model: MD-20 Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Magnetic Stirrer	Model: JSHS-180 JSR, Korea
Vortex Machine	VWR International
Microwave Oven	Model: MH6548SR LG, China
pH Meter	pHep Tester Hanna Instruments, Romania
Eppendorf	Germany, Ireland
Refrigerator	(4OC) Model: 0636 Samsung