Detection of extended spectrum β-lactamase ($ESBL_A$), screening of AmpC β-lactamase and detection of CTX-M and aacA-aphD genes among the multidrug resistant bacteria found in two tertiary hospitals of Dhaka city

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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DECLARATION

I, hereby declare that the thesis work entitled "**Detection of extended spectrum β-lactamase (ESBL$_A$), screening of AmpC β-Lactamase and detection of CTX-M and aacA-aphD genes among the multidrug resistant bacteria found in two tertiary hospitals of Dhaka city",** submitted to the Department of Mathematics and Natural Sciences, BRAC University in partial fulfilment of the requirement for the degree of Bachelor of Science in Biotechnology, is a record of work carried out by me under supervision and able guidance of my supervisor Dr. Mahboob Hossain, professor and coordinator of Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University. It is further declared that the research work presented here is original and the contents of this report in full or parts have not been submitted to any other university and institution for any degree or diploma.

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Dedicated to

my parents, all my loved ones

and

my supervisor,

Dr. Mahboob Hossain

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ABBREVIATIONS

ATP – Adenosine triphosphate

bp – Base pairs

CLSI - Clinical & Laboratory Standards Institute

CoA – Coenzyme A

CTX-M – Cefotaximase-Munich

dH_2O – Distilled water

DMCH – Dhaka Medical College and Hospital

DNA – Deoxyribonucleic acid

dNTP - Deoxynucleoside triphosphate

EDTA – Ethylenediaminetetraacetic acid

EMB – Eosin methylene blue

ESBL – Extended spectrum β-lactamase

$ESBL_A$ - 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

NICRH – National Institute of Cancer Research and Hospital

PBP – Penicillin binding protein

PCR – Polymerase chain reaction

pH – Power of hydrogen; measures the concentration of hydrogen ions to determine the acidity and alkalinity of solutions

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

rpm – Revolutions per minute

SDS – Sodium dodecyl sulfate

SHV - Sulfhydryl variable

TBE – Tris-borate-EDTA

TE – Tris-EDTA

TEM - Temoneira

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ABSTRACT

Klebsiella spp. and *Pseudomonas* spp. are opportunistic pathogens that are responsible for severe infections. These infections can be treated by antibiotics of different classes individually or in combination. However, lately, these pathogens have become resistant to antibiotics through its increased exposure or conjugal transfer of antibiotic resistant genes, making treatment difficult. Production of β-lactamases like extended spectrum β-lactamases (ESBL) or AmpC β-lactamases by pathogens could be one of the reasons for the occurrence of multidrug resistant (MDR) bacteria. The objective of this study was to molecularly detect the presence of CTX-M and aacA-aphD genes in the samples responsible for multidrug resistance. Isolates of sputum and pus origin were collected from two tertiary hospitals and antimicrobial susceptibility test was performed to detect the multidrug resistant strains. The $ESBL_A$ producers were detected by the double disk synergy test. The AmpC β-lactamase producers were also screened simultaneously by checking its susceptibility to cefoxitin. Polymerase chain reaction was then performed to detect CTX-M gene for the samples resistant to both third-generation cephalosporins and monobactams and to detect aacA-aphD for the samples resistant to aminoglycosides. All the samples were observed to be completely resistant to cloxacillin (100%) followed by kanamycin (61.5%) whereas, the samples showed least resistance to imipenem (7.7%). Of the total samples, 76.9% were screened to be AmpC β-lactamase positive and were the most prevalent (96%) among *Pseudomonas* spp. However, 65.4% of the samples were phenotypically detected to be $ESBL_A$ positive and were the most prevalent among *Klebsiella* spp. (74.1%). When PCR was carried out, 2 out of 28 samples, that were found to be resistant to both third-generation cephalosporins and monobactams, were confirmed to have the CTX-M gene of size 800bp, whereas none of the samples, that were resistant to aminoglycosides, were found positive for aacA-aphD gene.

From the results, it can be concluded that the percentage of $ESBL_A$ and AmpC β-lactamase producers were high among pathogens found in the tertiary hospitals. Hence, ESBL and AmpC β-lactamase detection methods of high sensitivity and specificity, as well as its molecular detection, should be made compulsory so that appropriate antibiotics can be used for the treatment of infection.

CHAPTER 1:

INTRODUCTION

1. INTRODUCTION

1.1 Background

Resistance to antibiotics has become a medical concern now and is causing various complications. The prolonged exposure to antibiotics increases the chances of bacteria to mutate to their resistant nature. Efficacy of conventional treatments has reduced or failed to work altogether against the infectious diseases making them irrepressible. It increases the risk of fatality in patients as well as poses a great danger to the community with the risk of spreading to others. Failure to respond to antibiotics forces doctors to turn to more complicated and expensive treatments increasing the health-care expenses which only adds to the financial load of families. Excessive use of antibiotics, to fight against infection-causing bacteria, also affects the normal flora of the body as it harms both the pathogens as well as the microbes that are responsible for keeping the body healthy and functional (Odonkor and Addo, 2011).

1.2.1 Bacteria

Bacteria are prokaryotic in nature. They have no definite nucleus, no membrane-bound organelles and two membranes – an outer cell wall and an inner cell membrane. They also have an extrachromosomal circular DNA called plasmid that replicates independently and is responsible for unique features of the bacteria that are usually not present in the genomic DNA. Bacteria is one of the two domains of prokaryotes. This domain is divided into phyla and the phylum is divided into classes and so on (Tortora *et al*., 2010).

One of the phyla of bacteria is proteobacteria which are the most diverse and the largest taxonomic group of bacteria. They can make use of the elements from the nutrient cycle or use bacteriochlorophylls to absorb a different spectrum of light to make their own food. (Starr *et al*., 2006) They are gram negative in nature their phylogenetic relationship is based on the study of their rRNA. The phylum proteobacteria are divided into five classes – alphaproteobacteria, betaproteobacteria, gammaproteobacteria, deltaproteobacteria and epsilonproteobacteria. (Tortora *et al*., 2010)

This thesis focused on proteobacteria of two different orders, that is, *Pseudomonas* of the order *Pseudomonales* and *Klebsiella* of the order E*nterobacteriales*.

1.2.2 *Klebsiella* spp.

Klebsiella spp. are aerobic, gram-negative rods of the family *Enterobacteriaceae*. They are found abundantly in the environment, that is, soil, water, sewage and on plants and also on the mucosal surfaces of animals and humans. For example, *K. pnuemoniae* grows on the mucosal surfaces of the nasopharynx and the intestinal tract of the humans. They are also opportunistic in nature and cause nosocomial infections like septicemia, urinary tract infections, pneumonia and wound infections in hospitalized patients with a weak immune system. Patients with diseases like diabetes mellitus and chronic pulmonary obstruction are at the greatest risk of being infected. *K.pneumoniae* and *K.oxytoca* predominantly cause most of the nosocomial and community-acquired infections. The increased rate of the infections caused by *Klebsiella* spp. is due to the constant and unrestrained use of antibiotics in the treatment of the diseases which has consequently led to the emergence of the multi-drug resistant strains in the environment. They colonize and spread from the hospital staff, the gastrointestinal tracts of the patients, medical equipment and blood products (Podschun and Ullmann, 1998). The mechanisms responsible for antibiotic resistance include efflux pump system combined with the increased administration of the antibiotics (Du *et al*., 2014). Several factors are involved in the pathogenesis of *Klebsiella*.spp. Firstly, the capsules, made of complex acid polysaccharides, cover the outer membrane and protect them from phagocytosis by making them unrecognizable to the macrophages and other phagocytes. It also protects them from a cascade of complement proteins that are responsible for the bactericidal serum activity. Secondly, pili found on the bacterial surfaces help them to adhere to the mucosal surfaces of the animals they infect and colonize there. Lastly, they secrete molecules called siderophores which are chelators with high affinity for iron. Iron is vital for bacterial growth and pathogens are able to take up the iron bound to the host proteins with the help of siderophores (Podschun and Ullmann, 1998). *Klebsiella* spp. is resistant to quinolones, tetracyclines and

chloramphenicol and susceptible to cephalosporins and carbapenems and hence, are used to treat infections they cause (Wasfi *et al*., 2016).

1.2.3 *Pseudomonas* spp.

Pseudomonas spp. is a heterogenous, motile and aerobic gram-negative bacteria that is ubiquitous in nature and inhabits the soil, fresh water, oceans, etc. This ubiquity is due to their versatile metabolism allowing them to break down different kinds of substrates by a complex enzyme system (Franzetti and Scarpeluni, 2007; Tortora *et al*., 2010). Some species produce fluorescent pigments that illuminate under the ultraviolet light. *Pseudomonas aeruginosa* secretes a blue-green pigmentation that diffuses into the media (Tortora *et al*., 2010). Since they are resistant antimicrobial agents, they are commonly found in hospitals, growing on medical instruments, cosmetics, disinfectants, medical products, etc. apart from the natural habitats (Franzetti and Scarpeluni, 2007; Porras-Gómez *et al*., 2012). They usually do not cause diseases but being an opportunistic pathogen, they are the root of severe nosocomial infections mostly pneumonia, bacteremia, abscesses, meningitis and urinary tract infections in critically ill patients, especially in the burn units. (Tortora, 2010; Porras-Gómez *et al*., 2012; Gales *et al*., 2001) They pose a great threat to the patients suffering from severe burns or disorders that include cystic fibrosis and neutropenia as they are immunocompromised (Yayan *et al*., 2015). A large number of genes in *Pseudomonas* spp. play an active role in conferring resistance to antimicrobial agents through various resistance mechanisms. The porins in the cell wall contribute to its low permeability, impairing the penetration of the antibiotic molecules present in the external environment. It also has an efflux pump system that pumps out antibiotics from the cell, before it can have an effect on the cell functions. In addition to this, lipopolysaccharides and elastase, produced as an ex-product by *Pseudomonas* spp., can begin the pathological process by tissue destruction. The presence of the chromosomal β-lactamase and penicillin-binding proteins also play a contributing role in the resistance to antibiotics (Tortora *et al*., 2010; Porras-Gómez *et al*., 2012). At present, *Pseudomonas* spp. are resistant to the following antibiotics – penicillin C, cephalosporins like ceftazidime and cefepime, aminoglycosides like gentamicin and kanamycin, quinolones, carbapenems,

colistin, fosfomycin, piperacillin and piperacillin/tazobactam. Administration of an increased number of antibiotics against pseudomonas have caused them to acquire resistance to it and this cross-resistance has led to the appearance of multi-drug resistant (MDR) strains of *Pseudomonas* spp. which is now a medical concern (Yayan *et al*., 2015).

1.2.4 Antibiotics

Antibiotics, the by-products of a variety of microorganisms and fungi, are chemical substances that are bacteriostatic or bactericidal in nature and are used to treat bacterial infections as well as infections caused by other microorganisms. They originate from microorganisms and in the past, they were made directly from microorganisms; however, synthetic antibiotics are currently being produced that are similar to the chemical structure of the natural antibiotics with a slight difference in the action mechanism and the target it acts on (Rayamajhi *et al*., 2010). It started with the discovery of penicillin from *Penicillin notatum* by Alexander Fleming in 1928, that repressed the growth of *Staphylococcus aureus*. The sulfa drugs, produced synthetically, were then discovered when it was observed that a compound named Protonsil Red, containing the sulfanilamide component, was able to treat streptococcal infections in mice; the first sulfa drug being protonsil (Tortora *et al*., 2010; Rayamajhi *et al*., 2010). These were widely administered in the 1940s to treat the war injuries of the soldiers during the World War II to prevent bacterial infections (Tortora *et al*., 2010; Saga and Yamaguchi, 2009). However, the onset of penicillin resistance led to the discovery of many more antibiotics with time. In the 1940s, the discovery of Streptomycin from *Streptomyces griseus,* a soil bacterium, led to the production of aminoglycosides, chloramphenicol, macrolides and tetracycline, that were isolated from soil bacteria and were bactericidal to both gram-positive and gram negative bacteria (Rayamajhi *et al*., 2010; Saga and Yamaguchi, 2009). Nalidixic acid, a quinolone, was synthesized in 1962. Its first clinical administration was in 1967 and was used to treat urinary tract infections caused by gram-negative bacteria. The first generation of cephems (cephalosporins) was synthesized in 1967 that subsequently led to the development of the second and third generation cephems. The development of the monobactams, carbapenems in the 1980s and the administration of the first, second and third generation of

cephalosporins in the 1990s has widened the choice of antibiotics administered to treat most infections that were fatal in the past (Rayamajhi *et al*., 2010). However, the increased use of antibiotics over time has instigated the bacteria to evolve and grow resistant to the antibiotics, hence leading to the emergence of multidrug-resistant bacteria. This has, once again, made bacteria a grave threat to humans, due to the reduced effectiveness of antibiotics and a dearth of development of new drugs against the MDR strains (Ventola, 2015).

The antibiotics act on the microorganisms through the following modes of action. Firstly, they interfere with the cell wall synthesis by either interfering with the enzymes responsible for the peptidoglycan synthesis or binding to the peptidoglycan layer or by targeting transglycosylation to prevent the synthesis of the peptidoglycan layer. Secondly, the antibiotics can also bind to the 50S and 30S ribosomal subunits, thus, interfering with the protein synthesis. Thirdly, antibiotics can interfere with the activity of DNA-directed RNA polymerase and type II and type IV *topoisomerases responsible for DNA synthesis. Fourthly, they disrupt the metabolic pathway of folate synthesis important for the synthesis of the fundamental components of the DNA and RNA,* that is, nucleotides. Lastly, antibiotics disrupt the integrity of the cell membrane by changing its permeability leading to the discharge of the cell contents or efflux of a large amount potassium ions and ultimately, cell death (Dzidic *et al*., 2008).

1.2.5 β - lactam Antibiotics

β - lactam antibiotics are a class of antibiotics that have an unstable β – lactam ring in their molecular structure with different side chains in different antibiotics. There are four types of β - lactam antibiotics, that is, penicillin derivatives (penem), carbapenems, cephalosporins and monobactams (Moyen *et al*., 2014). Some examples are amoxycillin, ceftazidime, ceftriaxone, imipenem, ampicillin, etc. These antibiotics work against the target microorganisms by interfering with the biosynthesis of the cell wall and disintegrating its structure. They bind to the transpeptidases, also known as, Penicillin Binding Protein (PBP) as they have a CO-N bond in the β – lactam ring that lies in the same exact position as the CO-N bond in D-alanyl-D-alanine, This CO-N bond in D-alanyl-

D-alanine is the target of transpeptidation, the process of transfer of amino acid from one peptide chain to another which result in cross-linking of peptidoglycan. Due to this similarity, the antibiotics bind to PBP and stop the cross-linking altogether, thus, inhibiting cell wall synthesis (Kong *et al*., 2010). The β – lactam ring, which is common in all the β – lactam antibiotics, is a four-member ring; a cyclic amide, that is labile and can be easily hydrolyzed by enzymes or chemicals. Once the ring is broken, the antibacterial activity of the antibiotic is compromised as it becomes inactive. Chemically, it can be hydrolyzed by acids, alkalis or weak nucleophiles and enzymatically, it can be hydrolyzed by β – lactamase, an enzyme produced by bacteria as a resistance mechanism (Odonkor and Addo, 2011).

The first β-lactam antibiotic was penicillin. However, with the increased resistance of bacteria towards penicillin, the need to produce different antibiotics rose. Pharmaceutical companies continued to produce antibiotics similar to penicillin with minor differences in their chemical structure and were managed to tackle the problem of antibiotic resistance. Some of the earliest antibiotics were methicillin and ampicillin, with methicillin being a key breakthrough against bacterial infections and their acquired resistance to antibiotics as it was β-lactamase stable. With a surge in the variety of antibiotics in the market, the companies stopped further research to develop newer antibiotics and instead turned their attention to developing drugs for more grave problems related to diabetes and cancer. In the meantime, bacteria caught up with the newer types of antibiotics, evolving and emerging resistance to them, rendering the antibiotics harmless and unusable. The growing concern to fight against the multidrug-resistant bacteria has resulted in the organization of conferences and workshops to bring this pressing matter into the light and take measures to prevent the epidemic caused by them in addition to the possibility of the reoccurrence of the pre-antibiotic era (Kong *et al*., 2010).

1.2.6 β-lactamases

β-lactamases are serine enzymes or metalloenzymes produced by bacteria as a defense mechanism against the activity of β-lactam antibiotics. They hydrolyze the β-lactam ring rendering them ineffective against bacteria; being the most predominant method of

antibiotic resistance. The increase in the production and continued use of different types of β-lactam antibiotics triggered the bacteria to undergo continuous mutations and produce new and different classes of β-lactamases (Kong *et al*., 2010; Diagbouga *et al*., 2016; Jeong *et al*., 2005). Due to the sequence similarity, it has been hypothesized that PBPs and β-lactamases come from a common ancestor (Zeng and Lin, 2013).

β-lactamases can be categorized based on two classification systems – the Ambler classification system and the Bush-Jacob-Medieros classification system. The Ambler classification system sorts the enzymes into class A, C and D, the serine enzymes and class B, the metalloenzyme, based on the amino acid sequences of the enzymes. The Bush-Jacob-Medieros classification system classifies the enzymes into four groups and various subgroups and is based on the biochemical and functional characteristics of the enzymes and takes the substrates and inhibitors into consideration (Paterson and Bonomo, 2005).

β-lactamases can be plasmid-mediated or chromosome-mediated. The chromosomally encoded β-lactamases are two types, that is, constitutive and inducible. The constitutive expression of β-lactamases occurs in bacteria at all times and does not require any specific conditions. However, in bacteria showing inducible expression, β-lactamase production is induced by β-lactam antibiotics due to the presence of a special transcriptional regulatory system (Kong *et al*., 2010).

1.2.7 AmpC β-lactamases

AmpC class of β-lactamases are cephalosporinases that are able to hydrolyze penicillins, cephalosporins of first and third generation, monobactams and cephamycins and are poorly inhibited by β-lactamases inhibitors like sulbactam, clavulanic acid and tazobactam (Tanushree *et al*., 2013; Manchanda and Singh, 2003). They are chromosome-mediated enzymes and belong to Class C of the Ambler classification scheme (Kong *et al*., 2010; Rupp and Fey, 2003). AmpC β-lactamases are inducible and their production increases with the increase in the concentration of antibiotics and the time of exposure. Strong inducers include penicillin, ampicillin, cefoxitin and first generation cephalosporins (Thenmozhi *et al*., 2014). The classes of antibiotics that have been able to successfully

inactivate AmpC β-lactamases are the fourth-generation cephalosporins like cefepime and the carbapenems (Paterson and Bonomo, 2005; Khari *et al*., 2016). They are predominant in species like *Pseudomonas aeruginosa.* and *Citrobacter* spp which have chromosomal AmpC β-lactamases (Khari *et al*., 2016). However, they have become more common in *Klebsiella* spp. and *E. coli* with an increased frequency (Tanushree *et al*., 2013). Lately, plasmid-mediated AmpC genes, derived from chromosomal AmpC genes, have also become prevalent and show high levels of expression (Manchanda and Singh, 2003; Khari *et al*., 2016). Although an increasing emergence of AmpC producers has been reported, the accurate prevalence is still not known. This is due to the dearth of simple, precise detection methods as the existing methods are non-specific and can give false-positive results. However, microorganisms can be screened for AmpC enzyme by checking their susceptibility for cefoxitin. If resistance is observed, they are further analyzed by performing confirmatory tests. These confirmatory tests are yet to be perfected and made exact to give true rates of prevalence (Tanushree *et al*., 2013; Khari *et al*., 2016).

1.2.8 Extended Spectrum β-lactamases (ESBL)

Extended spectrum β-lactamases (ESBL) are a group of plasmid-mediated enzymes that have the ability to hydrolyze the oxyimino-cephalosporins (third generation cephalosporins) like ceftazidime and cefoxitin and monobactams like aztreonam but are inhibited by the β-lactamase inhibitors which include clavulanic acid, sulbactam and tazobactam (Diagbouga *et al*., 2016; d'Azevedo *et al*., 2004). Third generation cephalosporins were introduced in the 1980s and had gained widespread popularity. However, in 1983, a new resistance pattern was identified which led to the discovery of ESBLs that hydrolyzed the extended spectrum of cephalosporins. They emerged from a single nucleotide mutation in the genes coding for the SHV and TEM genes. Lately, CTX-M ESBLs have also become a common threat worldwide, in addition to various other ESBL types. The variants of CTX-M group are rapidly growing in number day by day and have become a serious concern medically (Diagbouga *et al*., 2016; Taha *et al*., 2016). The predominant ESBL-producing bacteria are the *Klebsiella* spp. (particularly *Klebsiella pneumoniae*) and *Escherichia coli*. However, other members of *Enterobacteriaceae* which

include *Klebsiella oxytoca*, *Enterobacter aerogenes* and *Enterobacter cloacae*, non-glucose fermenters like *Pseudomonas aeruginosa* and *Acinetobacter* spp. and some respiratory pathogens like *Haemophilus influenzae* and *Moraxella catarrhalis* have also been identified to produce ESBL (Numanovic *et al*., 2013; Thenmozhi *et al*., 2014; Rupp and Fey, 2003).

According to the Bush-Jacob-Medieros classification system, ESBLs belong to group 2 and the subgroup 2be. Group 2b contains the class of β-lactamases encoded by the SHV and TEM genes. Hence, in the subgroup 2be, 'e' stands for the enzymes with an extended spectrum, having the ability to hydrolyze the extended spectrum cephalosporins and monobactams. According to the Ambler classification system, all ESBLs belong to Class A except for those that are derived from OXA genes (Class D) (Paterson and Bonomo, 2005). They differ from AmpC β-lactamases in the fact that these two classes of β-lactamases have no sequence similarity. Extended spectrum β-lactamases can be inhibited by β-lactamase inhibitors whereas, it has no effect on AmpC β-lactamases. Also, the effectiveness of the fourth generation of cephalosporins is greater on AmpC β-lactamases that on ESBLs (Kong *et al*., 2010; Paterson and Bonomo, 2005).

1.2.9 Extended Spectrum β-lactamase (ESBL) Detection

A number of techniques for phenotypic detection of ESBL producing bacteria have been recommended by Clinical and Laboratory Standards Institute (CLSI), European Committee on Antimicrobial Susceptibility Testing (EUCAST) and National Committee for Clinical Laboratory Standards (NCCLS). Besides these, several methods are also present that can be used to detect ESBL producers (Numanovic *et al*., 2013; Rupp and Fey, 2003). A few of the methods for ESBL detection have been briefly described.

1.2.9a Double Disk Synergy Test (DDST)

In this method, two disks, that is, a third-generation cephalosporin (ceftazidime, ceftriaxone or cefotaxime) or a monobactam (aztreonam) and a β-lactamase inhibitor (amoxycillin/clavulanic acid or piperacillin/tazobactam) along with cefoxitin are placed 20 mm apart on a plate of Mueller-Hinton agar spread with the sample bacteria. This is incubated for 24 hours. Positive results show that there is an increase in the zone of

inhibition of the β-lactamase inhibitor towards the cephalosporin or the monobactam disk and susceptibility to cefoxitin. This is due to the inhibitory action of the clavulanic acid or tazobactam on the ESBL producers which also helped augment the effectiveness of the cephalosporin disk, against which it was previously resistant (Numanovic *et al*., 2013; Rupp and Fey, 2003; Dhara *et al*., 2012). This method is simple and easy to perform. However, this technique is disadvantageous as its understanding is particular to the person that carries out the test, thus there could be errors in interpreting the results. Also, in microorganisms with low ESBL activity, observation of large zones of inhibition can be interpreted as negative, thus giving erroneous results (Paterson and Bonomo, 2005).

1.2.9b VITEK

VITEK is an automated method for the detection of the ESBL producers. In this test, bacteria are tested with ceftazidime and cefotaxime alone and in combination with clavulanic acid. If there is a significant increase in the bacteriostatic activity by the clavulanic acid when compared to the one without it, it is considered to be ESBL positive (Numanovic *et al*., 2013; Rupp and Fey, 2003).

Once an organism is tested to be positive for ESBL, it is said to be resistant to all penicillins, all cephalosporins except cefoxitin and cefotetan and aztreonam irrespective of the previously conducted antimicrobial susceptibility test (Rupp and Fey, 2003).

1.2.9c Three-Dimensional Test

This method requires the organism to be first spread on the Mueller-Hinton agar plate, after which a slit is created on the agar across the plate. The organism is inoculated into the slit and a third-generation cephalosporin is placed at a distance 3 mm from the slit. It is then incubated for 24 hours. It is said to be positive for ESBL if a distorted zone is formed on the side of the slit (Rupp and Fey, 2003).

Detection of ESBLs through molecular techniques like multiplex PCR, sequencing or pyrosequencing gives accurate results. These methods are fast, sensitive and consistent due to the use of highly specific DNA markers to detect the different families of TEM, SHV and CTX-M as well as distinguish between the ESBL and non-ESBL producers (Numanovic *et al*., 2013).

1.2.10 CTX-M gene

Cefotaximase-Munich (CTX-M) is a relatively newer class of ESBL, belonging to the class A of the Ambler classification system. It has the capacity to hydrolyze cefotaxime and hence, is resistant to it (Farshadzadeh *et al*., 2014). However, some CTX-M types can also hydrolyze ceftazidime with a greater intensity than cefotaxime (Thenmozhi *et al*., 2014). Another characteristic of these enzymes is that they have a higher susceptibility towards tazobactam than to sulbactam and clavulanic acid. The plasmid-mediated CTX-M β-lactamases have said to be acquired, via horizontal gene transfer, from the chromosomal ESBL genes of *Kluyvera* species which are of the family *Enterobacteriaceae* and are opportunistic in nature (Shaikh *et al*., 2015). They are not closely related to SHV and TEM groups as only 40% similarity was found between them and the CTX-M β-lactamases (Thenmozhi *et al*., 2014). The CTX-M enzymes were first discovered in 1989 and since then, it has spread worldwide and the variants have been growing rapidly. At present, they are classified into five groups - group 1, 2, 8, 9 and 25/26, based on their amino acid sequence homology (Farshadzadeh *et al*., 2014; Xu *et al*., 2005). Since ceftazidime is often used instead of cefotaxime as one of the extended spectrum cephalosporins in ESBL detection, phenotypic methods cannot accurately tell us the class of ESBL present. Nevertheless, molecular diagnosis, due to their high specificity, can give definite results and can help to identify the ESBL class as well as the variant correctly (Xu *et al*., 2005).

1.2.11 Aminoglycosides

Aminoglycosides are broad-spectrum antibiotics, that are highly potent and have a bactericidal effect on both gram-positive and gram-negative bacteria to treat serious infections (Odonkor and Addo, 2011; Gad *et al*., 2011; Huth *et al*., 2011). The aminoglycoside era began with the isolation of streptomycin from *Streptomyces griseus* in 1944 which led to the successive development of other aminoglycosides like neomycin, kanamycin, gentamicin, etc. (Huth *et al*., 2011; Davies, 2006). In the 1970s, semi-synthetic aminoglycosides (amikacin and netilmicin) were introduced in response to the emergence of bacteria that had conferred resistance to the older aminoglycosides (Gad *et al*., 2011). The main component in the aminoglycosides that is responsible for the antibacterial activity is a dibasic cyclitol formed by aminated sugars joined by glycoside bonds, which

include 2-deoxystreptamine (most of the clinically used aminoglycosides), streptidine in streptomycin and its derivatives and fortamine in fortimicin and its derivatives (Mingeot-Leclercq *et al*., 1999). They have the ability to cross the bacterial outer membrane by breaking the Mg^{2+} bridges between the lipopolysaccharide molecules. However, the size of aminoglycosides does not allow it penetrate through the porin channels and move across the cell membrane. This is done with the help of electron transport and requires energy (Mingeot-Leclercq *et al*., 1999). All aminoglycosides act on the target by interfering with its protein synthesis. They bind to the 16S ribosomal RNA of the 30S subunit of the prokaryotic ribosome and block the interaction of the transfer RNA and messenger RNA during translation (Davies, 2006).

Bacteria have become resistant to aminoglycosides through a number of resistant mechanisms. Some common, non-specific mechanisms result in the decreased uptake of the antibiotic and include the alteration of the outer membrane permeability and the cytoplasmic membrane transport, efflux pump systems that eject antibiotics out of the cell, alteration of the binding site in the 30S ribosome subunit and methylation of its binding site by 16S ribosomal RNA methylases to prevent aminoglycosides from binding to the ribosome (Gad *et al*., 2011; Davies, 2006).

The major mechanism for acquiring resistance is the enzymatic medication of the aminoglycosides by the production of aminoglycoside-modifying enzymes (AMEs). These can be plasmid-mediated or chromosome-mediated and are associated with transposable elements (Rouch *et al*., 1987). There are three types of AMEs – aminoglycoside-N-acetyltransferase (AAC), aminoglycoside-O-adenyltransferase (AAD) and aminoglycoside-O-phosphotransferase (APH) and these inactivate the antibiotics by acetyl CoA-dependent acetylation of the amino group, ATP-dependent adenylation of the hydroxyl group and ATP-dependent phosphorylation of the hydroxyl group respectively (Kim *et al*., 2012).

Recently, due to the increased occurrence of aminoglycoside resistance, older antibiotics are being used to treat the severe infections. It is hypothesized that the antibacterial activity of the early antibiotics could have been conserved since they were used less when compared to the newer drugs, thus, rendering them effective against the resistant bacteria (Gad *et al*., 2011).

Each AME is responsible for one kind of modification, excluding a bifunctional modification enzyme encoded by aacA-aphD, showing combined resistance to kanamycin, tobramycin and gentamicin and is mainly present in *Staphylococcus aureus, Staphylococcus epidermidis*
and *Streptococcus faecalis* (Rouch *et al*., 1987). It is a part of Tn4001, a non-conjugative, composite transposon with a size of 4568 bp and codes for an enzyme consisting of 479 amino acids, which is responsible for both acetyltransferase activity [AAC(6')] present in the amino-terminal domain and phosphotransferase activity [APH(2")] present in the carboxy-terminal domain (Rouch *et al*., 1987; Lange *et al*., 2003).

1.3 Objectives of the Study

The main aim of this project is to determine the prevalence of multidrug resistant (MDR) *Klebsiella* spp. and *Pseudomonas* spp. isolated from two tertiary hospitals followed by the molecular detection 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 MDR strains.
2. Phenotypic detection of $ESBL_A$ producing isolates by performing double disk synergy test and AmpC β-lactamase screening by performing antimicrobial susceptibility test.
3. Detection of CTX-M and aacA-aphD genes by PCR.

CHAPTER 2:

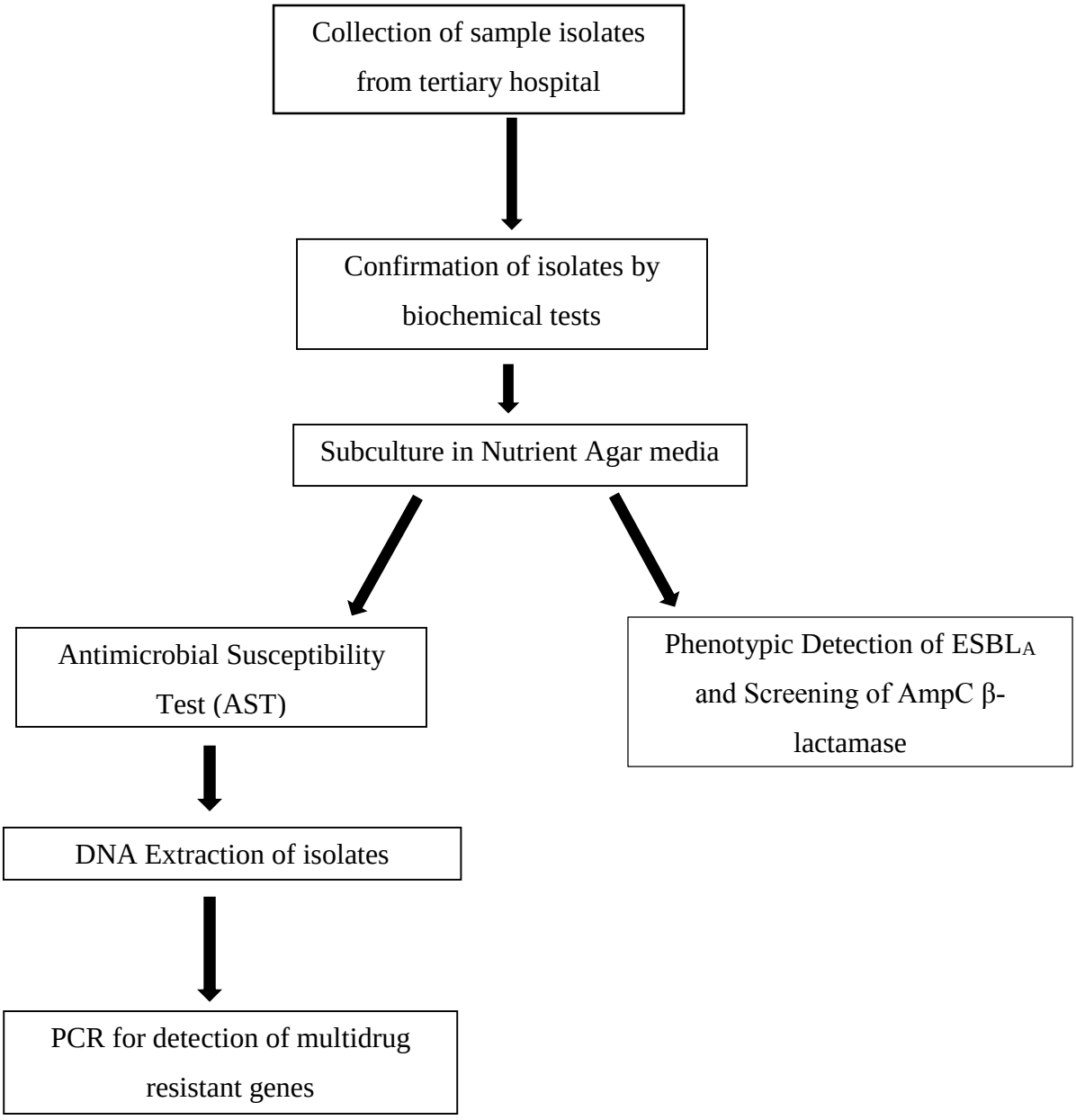
MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Study Place

All the laboratory work related to this project was carried out in the Microbiology, Biotechnology and Molecular Biology Laboratory of the Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

2.2 Flow Diagram of the Study Design



2.3 Materials

2.3.1 Equipment

- Autoclave
- Hot-air oven
- Refrigerator
- Incubator
- Laminar airflow cabinet
- Vortex machine
- Centrifuge machine
- Shaker incubator
- PCR machine
- Agarose Gel Electrophoresis apparatus
- Glass wares (conical flasks, beakers, petri-dishes, test tubes and vials), micropipette, glass pipette, electric weighing balance, Bunsen burner and pH meter

2.3.2 Culture Media

Different types of culture media were used for different purposes that include cultivation of bacteria, selective and differential growth of bacteria, antibiotic disk diffusion and stock culture. The following media were prepared and autoclaved according to the standard laboratory protocol:

- Nutrient Agar
- MacConkey Agar
- Cetrimide Agar
- Eosin Methylene Blue (EMB)
- Mueller-Hinton Agar
- Tryptone Salt (T_1N_1) Agar
- Luria-Bertani (LB) broth

2.3.3 Buffers, Chemicals and Solutions

Following chemicals, buffers and solutions were used for antibiotic susceptibility test, DNA extraction, PCR and Agarose Gel Electrophoresis.

- Saline solution
- 1x TE Buffer
- 1x TBE Buffer
- 10% SDS
- Lysis Buffer
- Phenol:Chloroform:Isoamyl (25:24:1)
- Chloroform
- 70% ethanol
- Absolute ethanol
- Proteinase K
- 32% HCl
- Ethidium Bromide (EtBr)

2.3.4 Antibiotic Disks

2.3.4a Antimicrobial Susceptibility Test (AST)

A total of 8 antibiotic disks of different classes were used for the antimicrobial susceptibility test to create a resistance profile and in turn determine the multidrug resistant (MDR) bacteria. Out of 12 antibiotics, 1 belonged to the class penicillin (cloxacillin), 3 were aminoglycosides (amikacin, gentamicin and kanamycin) and the rest were β-lactam antibiotics belonging to classes carbapenem (imipenem and meropenem), cephalosporin (ceftazidime) and monobactam (aztreonam). The antibiotic disks used in the procedure are given in Table 2.1.

Table 2.1: List of antibiotics for antimicrobial susceptibility test and their zone diameters

No.	Antibiotic	Disk code	Disk potency (µg)	Zone Diameter (According to CLSI)		
				Resistance (mm)	Intermediate (mm)	Susceptible (mm)
1.	Amikacin	AK	30	≤14	15-16	≥17
2.	Aztreonam	ATM	30			
	Klebsiella spp.			≤17	18-20	≥21
	Pseudomonas spp.			≤15	16-21	≥22
3.	Ceftazidime	CAZ	30			
	Klebsiella spp.			≤17	18-20	≥21
	Pseudomonas spp.			≤14	15-17	≥18
4.	Cloxacillin	OB	5	NA	NA	NA
5.	Gentamicin	CN	10	≤12	13-14	≥15
6.	Imipenem	IMP	10			
	Klebsiella spp.			≤19	20-22	≥23
	Pseudomonas spp.			≤15	16-18	≥19
7.	Kanamycin	K	30	≤13	14-17	≥18
8.	Meropenem	MEM	10			
	Klebsiella spp.			≤19	20-22	≥23
	Pseudomonas spp.			≤15	16-18	≥19

*NA – Not available

2.3.4b Extended Spectrum β-Lactamase ($ESBL_A$) Detection and AmpC β-Lactamase Screening

Six antibiotics were used for the double disk synergy test (DDST) to detect the production of extended spectrum β-lactamase of class A ($ESBL_A$) and simultaneously screen for AmpC β-lactamase in bacteria. Among the antibiotics, two were third-generation cephalosporins (ceftazidime and ceftriaxone), one was a monobactam (aztreonam), two were β-lactam/β-lactamase inhibitors (amoxycillin/clavulanic acid and piperacillin/tazobactam) and one was a second-generation cephamycin (cefoxitin). The list of antibiotics and their zone ranges are given Table 2.2.

Table 2.2: List of antibiotics for $ESBL_A$ detection and AmpC β-lactamase and their zone diameters

No.	Antibiotic	Disk code	Disk potency (µg)	Zone Diameter (According to CLSI)		
				Resistance (mm)	Intermediate (mm)	Susceptible (mm)
1.	Amoxycillin/Clavulanic acid	AMC	20/10	≤13	14-17	≥18
2.	Aztreonam *Klebsiella* spp. *Pseudomonas* spp.	ATM	30	 ≤17 ≤15	 18-20 16-21	 ≥21 ≥22
3.	Cefoxitin	FOX	30	≤14	15-17	≥18
4.	Ceftazidime *Klebsiella* spp. *Pseudomonas* spp.	CAZ	30	 ≤17 ≤14	 18-20 15-17	 ≥21 ≥18
5.	Ceftriaxone	CRO	30	≤19	20-22	≥23
6.	Piperacillin/tazobactam *Klebsiella* spp. *Pseudomonas* spp.	TPZ	100/10	 ≤17 ≤14	 18-20 15-20	 ≥21 ≥21

2.4 Methods

2.4.1 Collection of Sample Isolates

Fifty-two isolates were collected from National Institute of Diseases of the Chest and Hospital (NIDCH) and Dhaka Medical College and Hospital (DMCH), which are tertiary hospitals. Out of 52, 13 samples were of pus origin and 39 samples were of sputum origin. Samples, previously isolated, were aseptically streaked onto nutrient agar slants and immediately brought to the laboratory of BRAC University where it was incubated at 37^{o}C for 24 hours.

2.4.2 Growth of Isolates on Selective and Differential Media

The samples, collected from NIDCH and DMCH, were already isolated and their genus was determined. However, they were again streaked onto different selective and differential media to confirm the genus of the samples.

2.4.2a MacConkey Agar

MacConkey Agar was used as it is a selective as well as differential media that selectively inhibits the growth of gram-positive bacteria and differentiates between lactose fermenting and non-lactose fermenting bacteria. It was used to differentiate between *Klebsiella* spp., a lactose fermenter and *Pseudomonas* spp., a non-lactose fermenter. Fresh sample isolates, 24 hours old, were aseptically streaked onto plates containing autoclaved MacConkey agar and incubated at 37^{o}C for 24 hours.

2.4.2b Eosin Methylene Blue (EMB) Agar

Eosin Methylene Blue agar, a differential and selective media, was also used to differentiate between the gram-negative bacteria, in case of mixed culture and selectively isolate gram-negative bacteria from gram-positive bacteria. Overnight cultures were aseptically transferred to plates containing autoclaved EMB agar via the streak plate method and incubated for 24 hours at 37^{o}C.

2.4.2c Cetrimide Agar

Cetrimide agar was used as it is a selective media containing cetrimide that promotes the growth of *Pseudomonas* spp., turning the media blue-green due to the increased production of pyocyanin and fluorescein, the pigments present in *Pseudomonas* spp. It was used to selectively isolate *Pseudomonas* spp., in case of mixed cultures. A loopful of 24 hours old sample isolates were aseptically streaked onto autoclaved Cetrimide agar and observed after 24 hours of incubation at 37^{o}C.

2.4.3 Maintenance of Sample Isolates in Nutrient Agar

Nutrient agar, consisting of peptone, beef extract and NaCl, is a media used for the growth and maintenance of a large variety of microorganisms. Once the genus of the sample isolates was confirmed, they were aseptically transferred to the autoclaved nutrient agar plates by streak plate method, incubated at 37^{o}C for 24 hours and stored in the refrigerator for further use.

2.4.4 Antimicrobial Susceptibility Test (AST)

Antimicrobial susceptibility test was done by the Kirby-Bauer disk diffusion method to check the susceptibility of the samples to antibiotics of different classes to determine if the samples are multidrug resistant. The antibiotic diffuses into the media inoculated with bacterial culture and hence its effectiveness is determined by measuring the diameter of the zone of inhibition that it creates around the disk preventing bacterial growth.

2.4.4a Bacterial Suspension Preparation

For the disk diffusion method, firstly, bacterial suspension of the standard 0.5 McFarland was prepared. The bacterial colonies from fresh cultures, grown overnight for 24 hours, were aseptically inoculated into test tubes containing 0.9% saline solution that was prepared and autoclaved earlier. The test tubes were then vortexed to create a suspension.

2.4.4b Disk Diffusion

Kirby-Bauer disk diffusion was performed for the antimicrobial susceptibility test by evenly spreading the sample inoculum onto an autoclaved Mueller-Hinton agar to make a bacterial lawn, using a sterile cotton swab dipped into the bacterial suspension. Using forceps, the antibiotic disks were aseptically placed on the inoculated plates at appropriate places evenly spaced out to allow each antibiotic to act effectively against bacteria. They were slightly pressed onto the media by forceps to prevent their displacement and were then covered by the lid of the petri-dish. Incubated overnight at 37ºC, the diameters of the zone of inhibition were measured and recorded the next day. The zone diameters were compared to the zone ranges provided by Clinical and Laboratory Standards Institute (CLSI) guidelines and were reported as sensitive, intermediate or resistant accordingly.

2.4.5 Detection of Extended Spectrum β-Lactamases ($ESBL_A$) and Screening of AmpC β-lactamase

Double disk diffusion combinedly detected the $ESBL_A$ producers phenotypically along with the screening of AmpC β-lactamase producers as antimicrobial susceptibility test for cefoxitin was done. Inhibition zone of β-lactam/ β-lactamase inhibitor towards the cephalosporins and the monobactam along with the susceptibility of cefoxitin was interpreted as $ESBL_A$ positive. Whereas, no inhibition zones, as well as resistance to cefoxitin, was interpreted as AmpC β-lactamase positive. In this method, amoxycillin/clavulanic acid was used for *Klebsiella* spp. and piperacillin/tazobactam was used for *Pseudomonas* spp. as β-lactam/ β-lactamase inhibitors, according to the CLSI guidelines. In addition to these, ceftazidime and ceftriaxone (cephalosporins) and aztreonam (monobactam) were used for the test. A bacterial lawn was prepared by spreading the inoculum on an autoclaved Mueller-Hinton agar plate using a sterile cotton swab dipped into bacterial suspension of 0.5 McFarland turbidity, which was followed by placing amoxycillin/clavulanic acid in the center of the plate. Ceftazidime, ceftriaxone and aztreonam were placed 20 mm apart on either side of amoxicillin/clavulanic acid (as shown in Fig. 2.1). Cefoxitin was placed anywhere on the plate to check its susceptibility as its inhibitory action is not affected by the presence or absence of a β-lactam/ β-lactamase

inhibitor. The plates were incubated at 37°C and observed after 24 hours and the results were recorded.

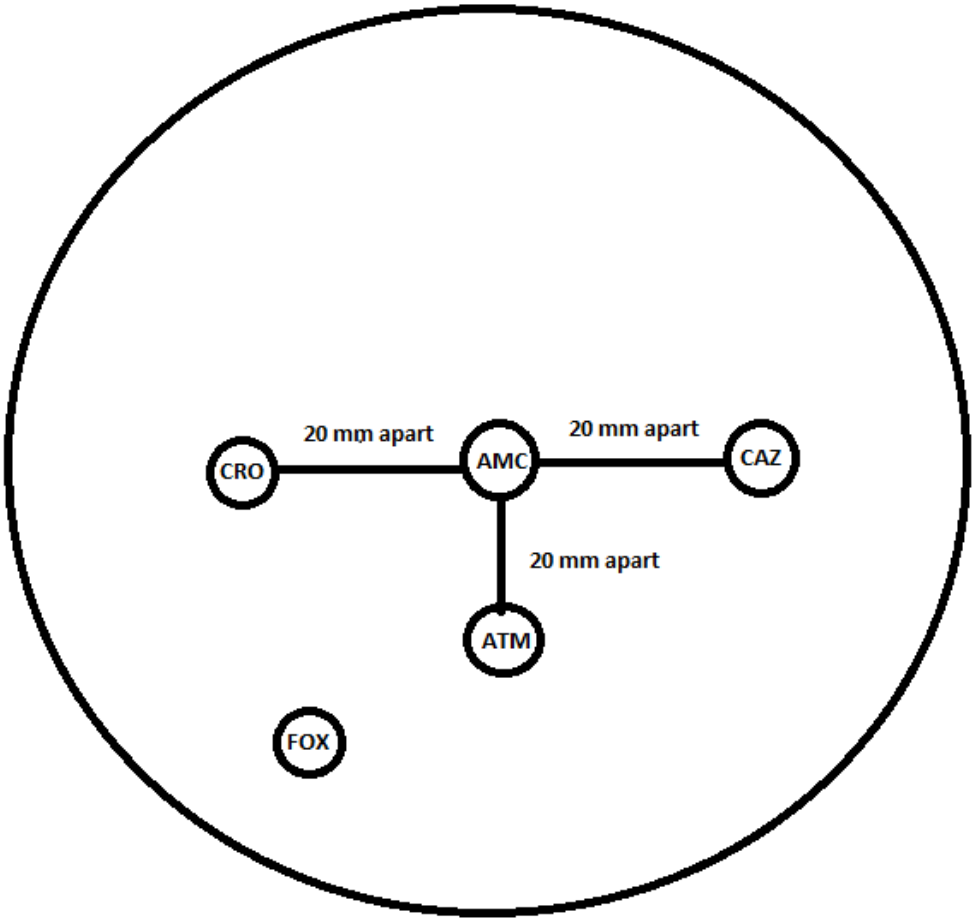


Fig. 2.1 Arrangement of antibiotic disks for ESBL detection

2.4.6 Molecular Detection of CTX-M and aacA-aphD genes

2.4.6a DNA Extraction

The DNA of samples, that showed resistance to amikacin, gentamicin and kanamycin and the samples that showed resistance to ceftazidime and aztreonam, were isolated by the phenol-chloroform DNA extraction method. Firstly, bacterial culture, grown aseptically overnight in LB broth for 18-24 hours at 37°C, were transferred to an autoclaved Eppendorf

tube and centrifuged at 13,500 revolutions per minute (rpm) to precipitate the bacterial cells to form a cell pellet. The broth was discarded, 750 µL of lysis buffer (consisting of TE buffer, 10% SDS and proteinase K) was added to it, vortexed and incubated at 37ºC for an hour. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at maximum speed to separate the cell debris and proteins from DNA. The aqueous part, containing DNA, was transferred to another autoclaved Eppendorf tube, an equal volume of chloroform was added to it and centrifuged at maximum speed, separating the phenol from DNA. The aqueous part with the DNA was once again transferred to another autoclaved Eppendorf tube and 2.5 times the volume of chilled absolute ethanol was added to it and incubated at -20ºC for 30 minutes. The samples were then centrifuged at 13,500 rpm to precipitate the DNA which is observed as a white pellet at the bottom of the tube. Absolute alcohol was discarded, the DNA was washed with 70% ethanol and centrifuged at maximum speed again. The ethanol was discarded and the DNA was suspended in TE buffer of appropriate volume. It was stored at -20ºC for further use.

2.4.6b PCR of CTX-M and aacA-aphD genes

For the detection of CTX-M gene responsible for ceftazidime and aztreonam resistance and the aacA-aphD gene responsible for resistance to amikacin, gentamicin and kanamycin, PCR was carried out for the DNA samples using primers specific for CTX-M and aacA-aphD respectively. CTX-M has a sequence 5'- ACG CTG TTG TTA GGA AGT G -3' as the forward primer and 5'- TTG AGG CTG GGT GAA GT- 3' as the reverse primer. The aacA-aphD gene has a specific forward primer of the sequence 5'- CCA AGA GCA ATA AGG GCA TAC C -3' and a specific reverse primer of the sequence 5'- CAC ACT ATC ATA ACC ACT ACC G -3'. The total reaction volume with the required components and the PCR amplification conditions are given in Table 2.3, Table 2.4 and Table 2.5.

Table 2.3: PCR components for CTX-m and aacA-aphD genes and their volumes

Components	Volume (µL)
10x Reaction buffer	5
DNTPs	1
Nuclease -free water	39.75
Forward primer	1
Reverse primer	1
Taq polymerase	0.25
Template DNA	2
Total	**50 µL**

Table 2.4: PCR conditions for CTX-M gene amplification

Step		Temperature	Duration
Initial denaturation		94ºC	5 minutes
30 CYCLES	Denaturation	94ºC	25 seconds
	Annealing	52ºC	40 seconds
	Extension	72ºC	50 seconds
Final Extension		72ºC	6 minutes

Table 2.5: PCR conditions for aacA-aphD gene amplification

Step		Temperature	Duration
Initial denaturation		94ºC	10 minutes
30 CYCLES	Denaturation	94ºC	20 seconds
	Annealing	55ºC	1 minute
	Extension	72ºC	50 seconds
Final Extension		72ºC	10 minutes

2.4.6c Agarose Gel Electrophoresis

The PCR products were separated by agarose gel electrophoresis. Firstly, 1.5% agarose gel was prepared by dissolving 0.6g of agarose in 40 mL of TE buffer. To this, 2 µL of EtBr was added which is an intercalating agent and a dye used to visualize DNA under UV light. While the agarose solution was still warm, it was poured into the chamber with a comb inserted into it. This allowed it to solidify as a gel slab containing wells in which the PCR products were loaded. Once the gel solidified, it was transferred to the gel electrophoresis apparatus containing TBE buffer. Three microlitres of DNA ladder (100-1000 bp) and 5 µL of each PCR product was added to 2 µL of loading dye, mixed well using a micropipette and loaded into the wells. It was run at 100 V for approximately 45 minutes when the DNA had run almost till the end of the slab. The gel slab was then visualized under UV light to observe the bands and record its size, if any were present.

CHAPTER 3: RESULTS

3. RESULTS

3.1 Sample Collection and Isolate Characterization

Fifty-two samples were collected from National Institute of Diseases of the Chest and Hospital (NIDCH) and Dhaka Medical College and Hospital (DMCH). Since the samples were already isolated and their genus was characterized, they were streaked on different selective and differential media like MacConkey agar, Eosin Methylene Blue (EMB) agar, cetrimide agar, etc. to confirm their genus.

3.1.1 MacConkey Agar

MacConkey agar differentiated between lactose fermenting and non-lactose fermenting bacteria. If pink colonies were observed, the culture was lactose fermenting bacteria, that is, *Klebsiella* spp. and is colourless colonies were observed, it was non-lactose fermenting, that is, *Pseudomonas* spp.

3.1.2 Eosin Methylene Blue (EMB) Agar

Eosin Methylene Blue agar was also used to distinguish between the lactose fermenting and non-lactose fermenting bacteria. It was also used to differentiate between *E. coli* and other members of *Enterobacteriaceae* as *E. coli* shows green metallic sheen, however, other members of *Enterobacteriaceae* show purple-black colonies. The sample isolates claimed as *Klebsiella* spp. gave purple-black colonies, thus confirming their species.

3.1.3 Cetrimide Agar

Cetrimide agar was used to selectively isolate *Pseudomonas* spp. If the growth of the isolates was observed, they were confirmed as *Pseudomonas* spp. All the samples collected as *Pseudomonas* spp. showed growth in cetrimide agar.

There was a total of 27 *Klebsiella* spp. out of which 1 was collected from pus and 26 were collected from sputum. 25 *Pseudomonas* spp. were collected; 12 originating from pus and

13 from sputum. The isolates and their genus are listed in Table 3.1 and the sample names with their genus are given in Table 3.2.

Table 3.1 List of isolates with their source and genus

	Pus (%)	**Sputum (%)**	**Total**
***Klebsiella* spp.**	1 (7.7%)	26 (66.7%)	**27**
***Pseudomonas* spp.**	12 (92.3%)	13 (33.3%)	**25**
Total	**13**	**39**	**52**

Table 3.2 List of sample names and their genus

	***Klebsiella* spp.**	***Pseudomonas* spp.**
Sample names	PKle1, SKle1, SKle2, SKle3, SKle4, SKle5, SKle6, SKle7, SKle8, SKle9, SKle10, SKle11, SKle12, SKle13, SKle14, SKle15, SKle16, SKle17, SKle18, SKle19, SKle20, SKle21, SKle22, SKle23, SKle24, SKle25, SKle26	PPse1, PPse2, PPse3, PPse4, PPse5, PPse6, PPse7, PPse8, PPse9, PPse10, PPse11, PPse12, SPse1, SPse2, SPse3, SPse4, SPse5, SPse6, SPse7, SPse8, SPse9, SPse10, SPse11, SPse12, SPse13

3.2 Antimicrobial Susceptibility Test (AST)

Antimicrobial susceptibility test was performed for all the isolates. A total of 8 antibiotics were used for each isolate. If clear zones were observed, the zone diameters were measured and interpreted using the zone diameters given in Clinical and Laboratory Standards Institute (CLSI) guidelines (as mentioned in Table 2.1) and reported as resistant (R), intermediate (I) or susceptible (S) accordingly. If no clear zone was observed, it was reported as resistant. The antibiotic sensitivity pattern of *Klebsiella* spp. and *Pseudomonas* spp. are given in Table 3.3 and 3.4 respectively.

Table 3.3 Antibiotic sensitivity pattern of *Klebsiella* spp. developed in response to various antibiotics

NO.	SAMPLE NAME	AK		ATM		CAZ		OB		CN		IMP		K		MEM	
		ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT
1	PKle1	0	R	0	R	0	R	0	R	0	R	24	S	0	R	27.5	S
2	SKle1	20	S	31.5	S	29	S	0	R	18.5	S	32.5	S	19.5	S	32	S
3	SKle2	0	R	0	R	0	R	0	R	0	R	29	S	0	R	23	S
4	SKle3	19	S	33 I	S	27.5	S	0	R	17	S	29	S	19 I	S	29.5	S
5	SKle4	15	I	12	R	0	R	0	R	16	S	25.5	S	11.5I	R	27.5	S
6	SKle5	19	S	0	R	0	R	0	R	15	S	25	S	18	S	26	S
7	SKle6	17.5	S	10.5I	R	10.5	R	0	R	16	S	21	I	17.5I	I	25.5	S
8	SKle7	16	I	19	I	14	R	0	R	17	S	31	S	15.5I	I	28	S
9	SKle8	18	S	21I	S	19	I	0	R	13.5	I	26	S	0	R	33	S
10	SKle9	16	I	8.5	R	0	R	0	R	0	R	20	I	11	R	17.5	R
11	SKle10	18.5	S	26	S	19	I	0	R	15	S	21	I	16.5	I	27.5	S
12	SKle11	19.5	S	30.5	S	27	S	0	R	16	S	24.5	S	19.5	S	28.5	S
13	SKle12	19	S	30	S	26.5	S	0	R	17	S	23	S	19	S	27	S
14	SKle13	20.5	S	30	S	34.5	S	0	R	15.5	S	30	S	19.5	S	28	S

NOTE: NO.=Number; ZS=Zone Size; INT=Interpretation; S=Susceptibility; I=Intermediate; R=Resistant; AK=Amikacin; ATM=Aztreonam; CAZ=Ceftazidime; OB=Cloxacillin; CN= Gentamicin; IMP= Imipenem; K=Kanamycin; MEM=Meropenem

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Table 3.3 Antibiotic sensitivity pattern of *Klebsiella* spp. developed in response to various antibiotics (Continued)

NO.	SAMPLE NAME	AK		ATM		CAZ		OB		CN		IMP		K		MEM	
		ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT
15	SKle14	20.5	S	33	S	29	S	0	R	16	S	28.5	S	20 I	S	27	S
16	SKle15	21	S	28	S	23	S	0	R	14	I	23	S	16.5	I	26	S
17	SKle16	0	R	13	R	23I	S	0	R	0	R	22	I	0	R	19.5	I
18	SKle17	21	S	10.5	R	20	I	0	R	20	S	29	S	21	S	23	S
19	SKle18	0	R	19	I	18.5	I	0	R	12	R	29	S	0	R	28	S
20	SKle19	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
21	SKle20	20	S	30	S	0	R	0	R	0	R	22	I	13	R	20	I
22	SKle21	20	S	9.5	R	14.5	R	0	R	18.5	S	29.5	S	20	S	30	S
23	SKle22	21	S	13.5	R	16	R	0	R	18	S	30	S	15 I	I	30	S
24	SKle23	21.5	S	0	R	9.5	R	0	R	18.5	S	31	S	21	S	30	S
25	SKle24	18	S	26I	S	20	I	0	R	15.5	S	23	S	22I	S	34.5	S
26	SKle25	18	S	26	S	20	I	0	R	16	S	27.5	S	18	S	29	S
27	SKle26	20.5	S	11	R	13	R	0	R	15.5	S	28.5	S	20	S	19	R

NOTE: NO.=Number; ZS=Zone Size; INT=Interpretation; S=Susceptibility; I=Intermediate; R=Resistant; AK=Amikacin; ATM=Aztreonam; CAZ=Ceftazidime; OB=Cloxacillin; CN= Gentamicin; IMP= Imipenem; K=Kanamycin; MEM=Meropenem

Table 3.4 Antibiotic sensitivity pattern of *Pseudomonas* spp. developed in response to various antibiotics

NO.	SAMPLE NAME	AK		ATM		CAZ		OB		CN		IMP		K		MEM	
		ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT
1	PPse1	0	R	0	R	0	R	0	R	0	R	22	S	0	R	13	R
2	PPse2	0	R	0	R	0	R	0	R	0	R	29	S	0	R	25	S
3	PPse3	0	R	0	R	0	R	0	R	0	R	12	R	0	R	10.5I	R
4	PPse4	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
5	PPse5	0	R	0	R	0	R	0	R	0	R	23	S	0	R	26.5	S
6	PPse6	0	R	0	R	0	R	0	R	0	R	27	S	0	R	20.5I	S
7	PPse7	0	R	0	R	0	R	0	R	0	R	26.5	S	0	R	29	S
8	PPse8	0	R	0	R	0	R	0	R	0	R	30	S	0	R	30I	S
9	PPse9	0	R	0	R	0	R	0	R	0	R	28	S	0	R	25	S
10	PPse10	0	R	0	R	0	R	0	R	0	R	28	S	0	R	30I	S
11	PPse11	0	R	0	R	0	R	0	R	0	R	25	S	0	R	25	S
12	PPse12	0	R	0	R	0	R	0	R	0	R	24	S	0	R	25	S
13	SPse1	21.5	S	27.5	S	32	S	0	R	14.5	I	27.5	S	0	R	26	S

NOTE: NO.=Number; ZS=Zone Size; INT=Interpretation; S=Susceptibility; I=Intermediate; R=Resistant; AK=Amikacin; ATM=Aztreonam; CAZ=Ceftazidime; OB=Cloxacillin; CN= Gentamicin; IMP= Imipenem; K=Kanamycin; MEM=Meropenem

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Table 3.4 Antibiotic sensitivity pattern of *Pseudomonas* spp. developed in response to various antibiotics (Continued)

NO.	SAMPLE NAME	AK		ATM		CAZ		OB		CN		IMP		K		MEM	
		ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT
14	SPse2	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
15	SPse3	23	S	28	S	31	S	0	R	13.5	I	25	S	0	R	26	S
16	SPse4	27	S	30	S	27	S	0	R	17	S	29	S	10	R	32	S
17	SPse5	10.5	R	0	R	34	S	0	R	0	R	21.5	S	0	R	26	S
18	SPse6	18.5	S	24	S	22.5	S	0	R	15	S	21	S	0	R	28	S
19	SPse7	28.5	S	24I	S	26	S	0	R	22.5	S	22	S	13.5	R	29	S
20	SPse8	22.5	S	24I	S	19	S	0	R	24.5	S	23	S	20	S	32.5	S
21	SPse9	20	S	21.5I	I	20I	S	0	R	14I	I	17.5	I	20I	S	28	S
22	SPse10	0	R	0	R	0	R	0	R	0	R	25	S	0	R	30	S
23	SPse11	19	S	28I	S	22	S	0	R	17	S	21	S	0	R	28	S
24	SPse12	19	S	23.5I	S	16.5	I	0	R	15	S	24.5	S	11	R	32.5	S
25	SPse13	18.5	S	23.5	S	21	S	0	R	15.5	S	21	S	0	R	28.5	S

NOTE: NO.=Number; ZS=Zone Size; INT=Interpretation; S=Susceptibility; I=Intermediate; R=Resistant; AK=Amikacin; ATM=Aztreonam; CAZ=Ceftazidime; OB=Cloxacillin; CN= Gentamicin; IMP= Imipenem; K=Kanamycin; MEM=Meropenem

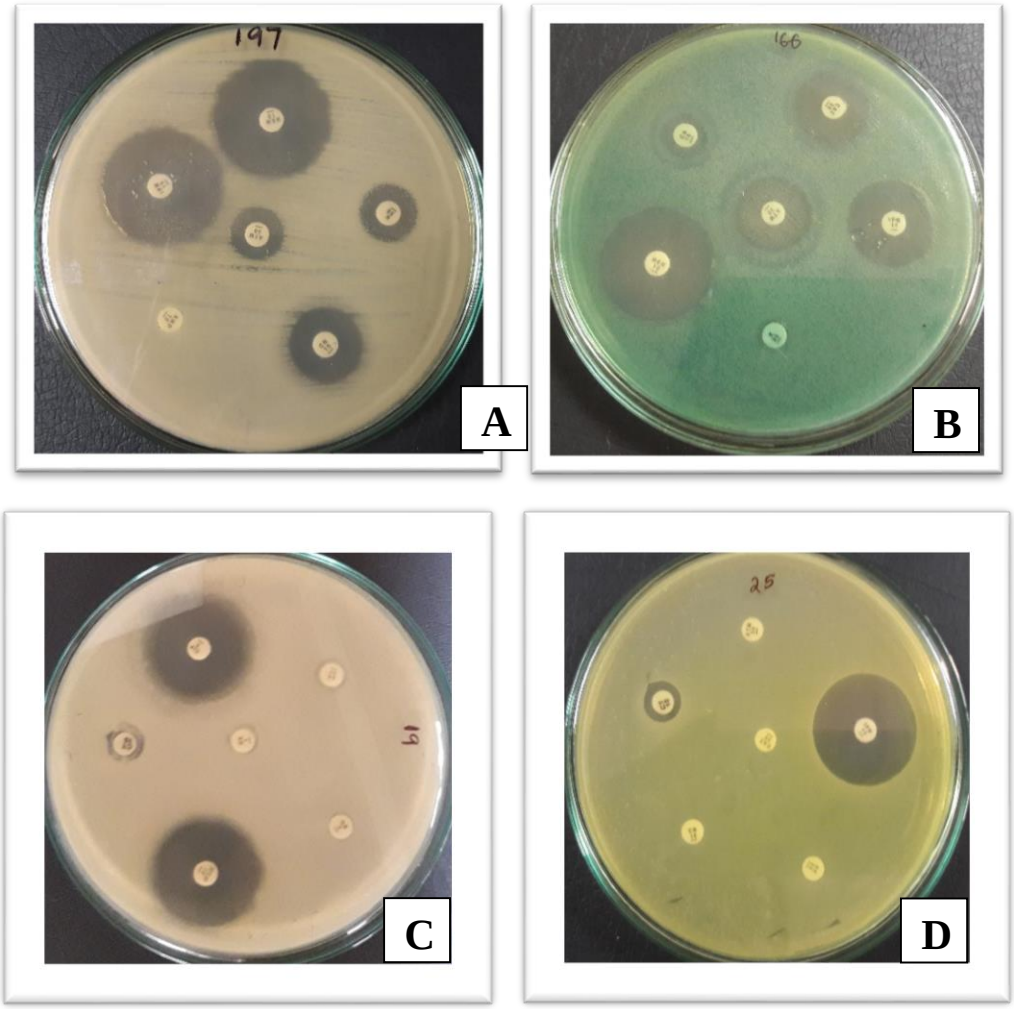

Fig. 3.1 Kirby-Bauer Disk Diffusion performed for pus and sputum samples

Figure 3.1 A shows the sample SKle22 (197) which belongs to the genus *Klebsiella* and was isolated from sputum. It shows resistance to ampicillin and aztreonam but susceptible to gentamicin, imipenem and meropenem; Figure 3.1 B shows the sample SPse5 (166) which belongs to the genus *Pseudomonas* and was isolated from sputum. It is resistant to aztreonam, gentamicin and kanamycin and susceptible to imipenem, meropenem and ceftazidime; Figure 3.1 C shows the sample number PKle1 (19) which belongs to the genus *Klebsiella* and was isolated from pus. It is resistant to amikacin and cloxacillin and shows susceptibility to meropenem; Figure 3.1 D shows the sample PPse1 (15) which belongs to the genus *Pseudomonas* and was isolated from pus. It is resistant to amikacin, aztreonam, ceftazidime and gentamicin but susceptible to imipenem.

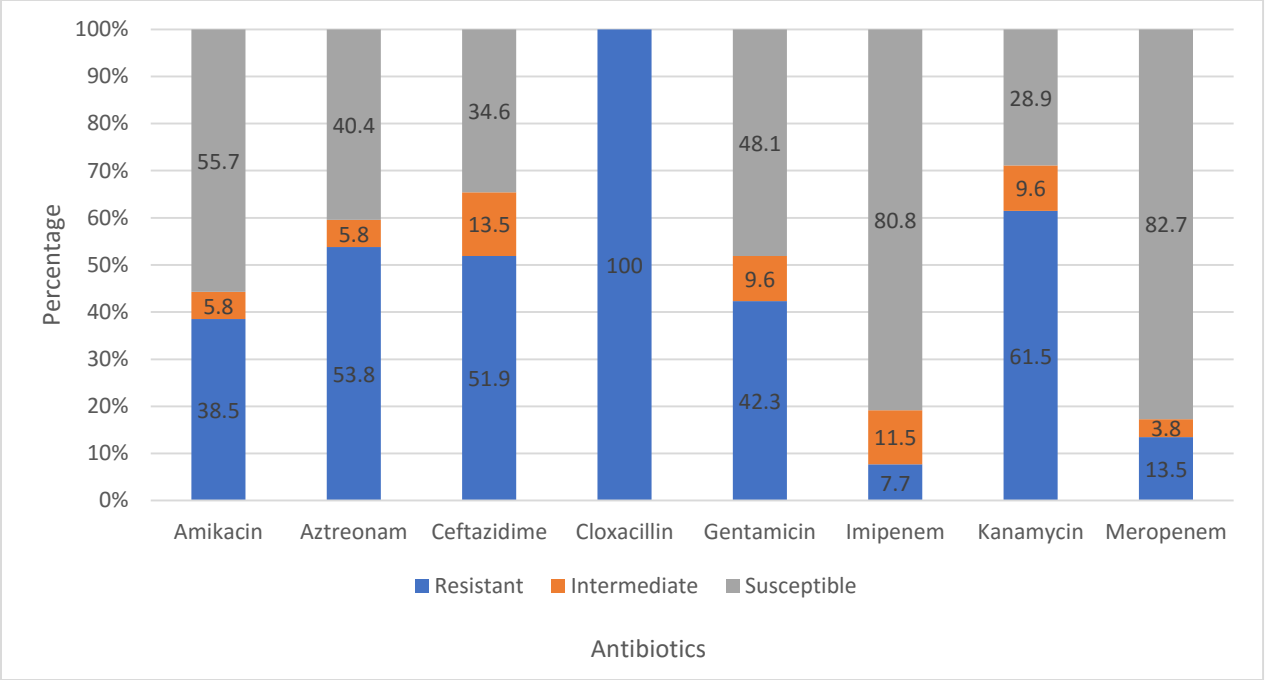

Fig. 3.2 Antimicrobial sensitivity pattern of all the isolates combined

Fig 3.2 shows the antimicrobial sensitivity patterns of all the sample isolates. The resistance percentage was the highest for cloxacillin (100%). Whereas, the highest susceptibility rate was observed for meropenem (82.7%) followed by imipenem (80.8%). The microorganisms also showed the lowest resistance to imipenem (7.7%). For other antibiotics (amikacin, aztreonam, ceftazidime, gentamicin, kanamycin and meropenem), the isolates had a resistance rate that fell within the range 13.5% -61.5%.

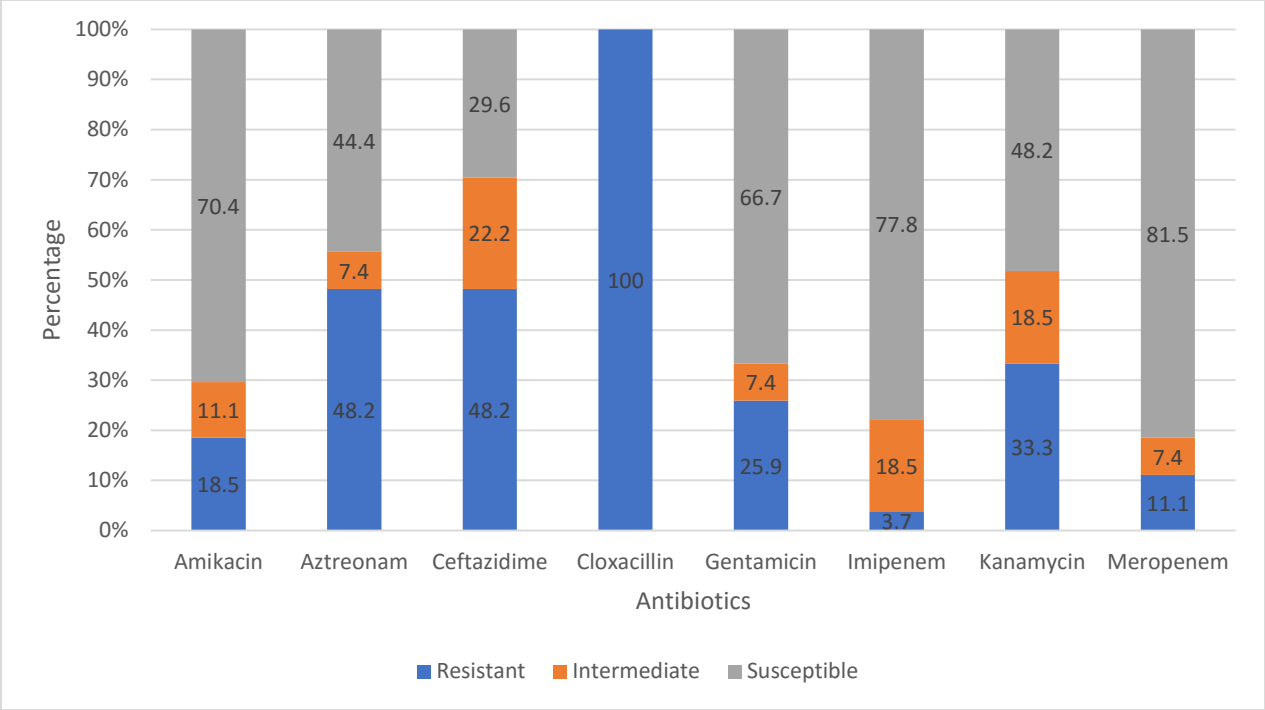

Fig. 3.3 Antimicrobial sensitivity pattern of *Klebsiella* spp.

Figure 3.3 represents the antimicrobial sensitivity pattern for *Klebsiella* spp. of both sputum and pus source. The highest resistance percentage was observed for cloxacillin (100%) as all the samples were found to be resistant. The lowest resistance rate was observed for imipenem (3.7%). The microorganisms showed the highest sensitivity to meropenem (81.5%) and the second highest sensitivity to imipenem (77.8%). The resistance to the other antibiotics was less than 50%. Also, the samples had a low intermediate percentage for all the antibiotics.

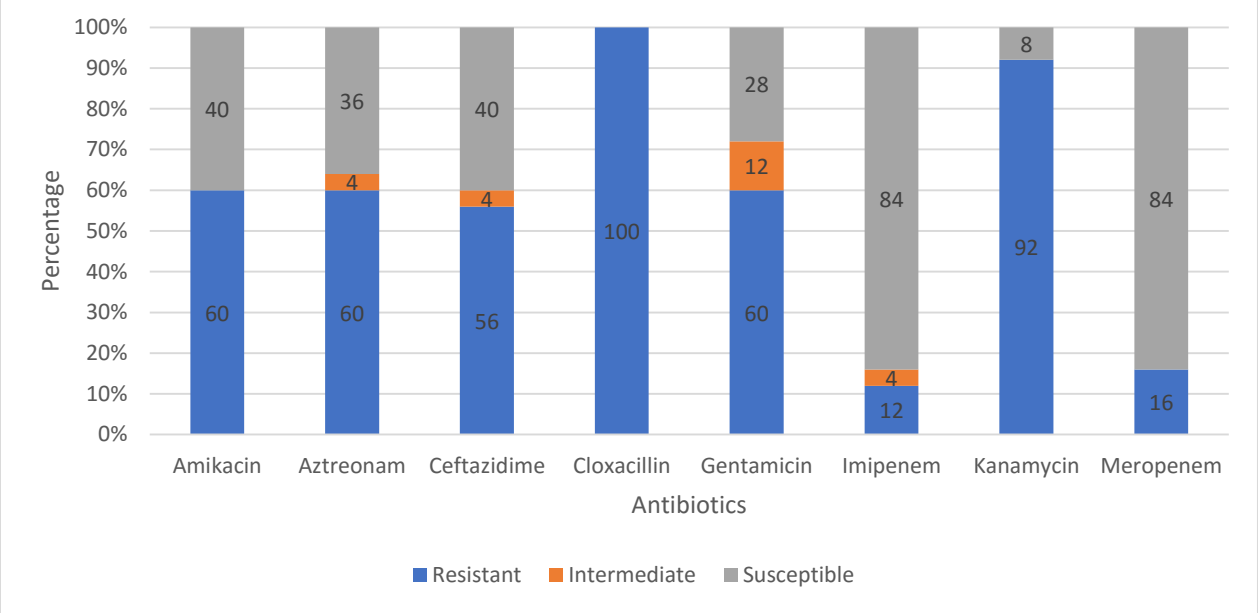

Fig. 3.4 Antimicrobial sensitivity pattern of *Pseudomonas* spp.

Figure 3.4 shows the antimicrobial sensitivity pattern for *Pseudomonas* spp. The *Pseudomonas* isolates showed the highest susceptibility to imipenem (84%) and meropenem (84%). In contrast to this, the isolates showed the highest resistance to cloxacillin (100%) followed by kanamycin (92%). The resistance percentages of the isolates for amikacin, aztreonam, ceftazidime and gentamicin were 60%, 60%, 56% and 60% respectively.

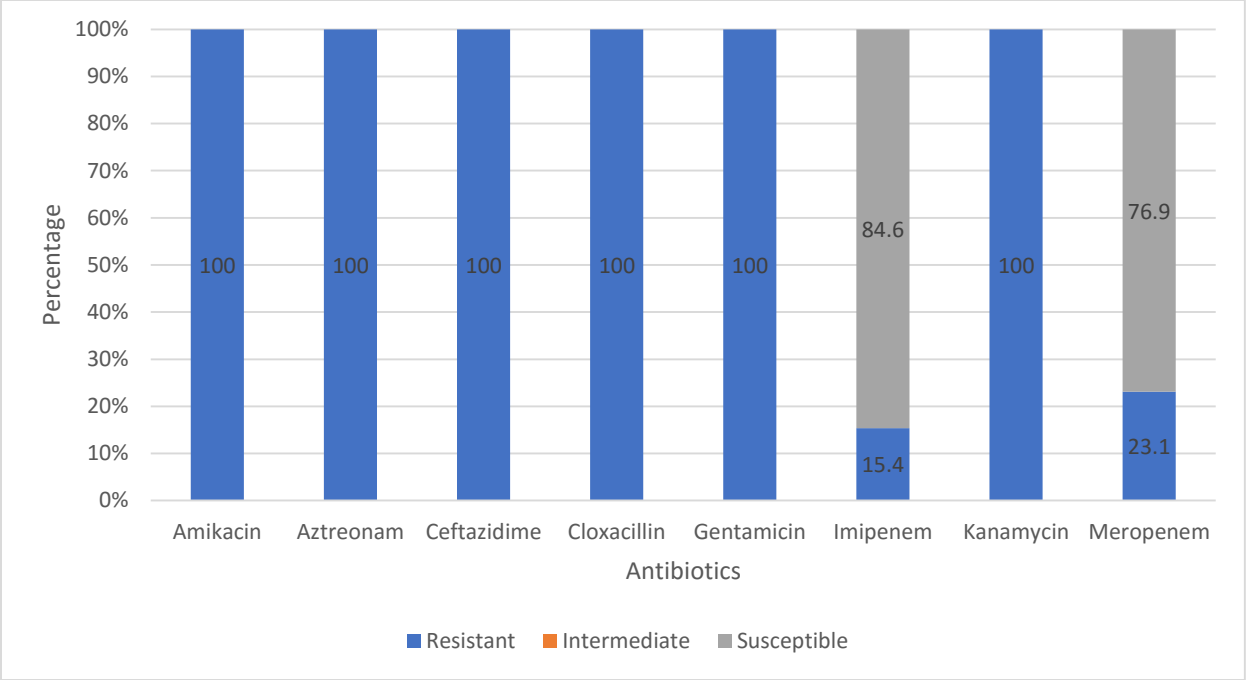

Fig. 3.5 Comparative study of antibiotic susceptibility pattern for all the isolates based on source (pus)

Figure 3.5 represents the antibiotic susceptibility pattern of all the isolates collected from pus. It is observed that the isolates showed the highest resistance to amikacin, aztreonam, ceftazidime, cloxacillin, gentamicin and kanamycin (100%) and showed the lowest resistance to imipenem (15.4%) followed by meropenem (23.1%).

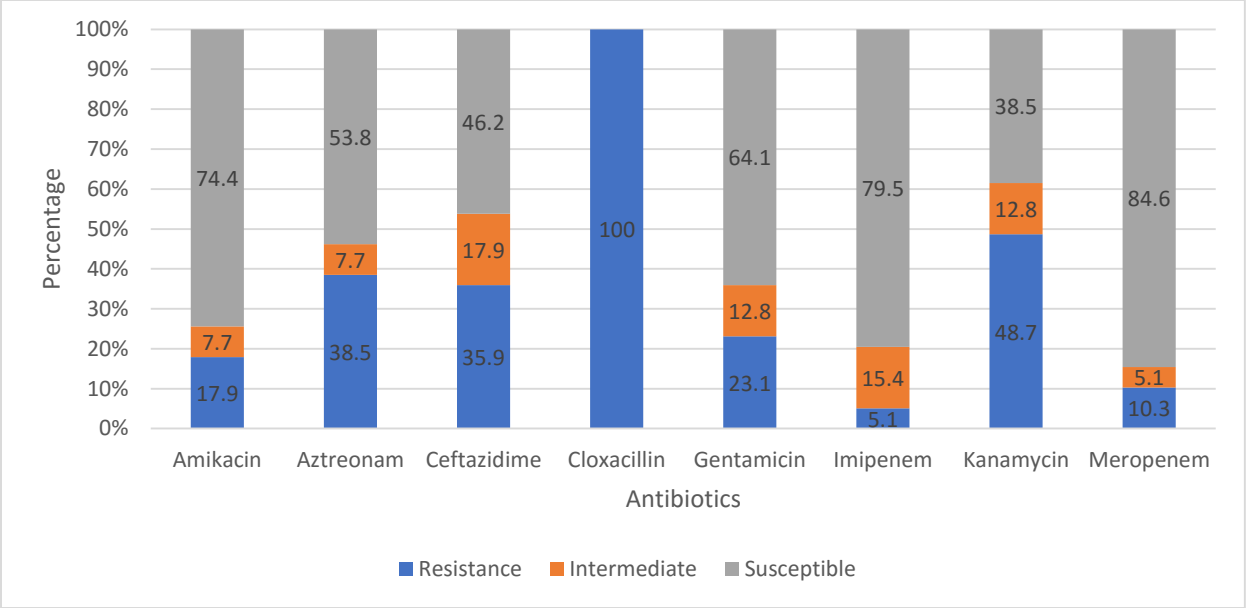

Fig. 3.6 Comparative study of antibiotic susceptibility pattern of all the isolates based on source (sputum)

Figure 3.6 represents the antibiotic susceptibility pattern for all the samples isolated from sputum. The highest susceptibility was observed for meropenem (84.6%) followed by imipenem (79.5%) and amikacin (74.4%). The samples show 100% resistance to cloxacillin. For other antibiotics, the resistance percentages were less than 50% and among them, the samples showed the highest resistance to kanamycin (48.7%) followed by aztreonam (38.5%).

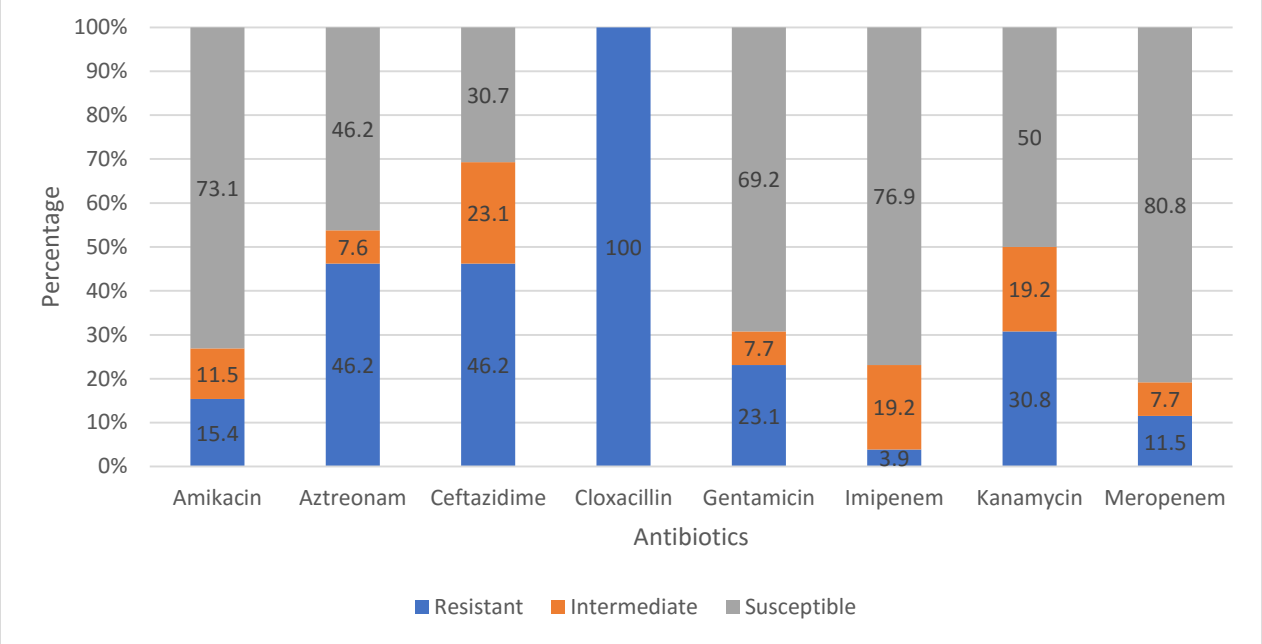

Fig. 3.7 Comparative study of antibiotic susceptibility pattern of *Klebsiella* spp. of sputum source

Figure 3.7 represents the resistance pattern of *Klebsiella* spp. collected from sputum. All the isolates showed less than 50% resistance to all the antibiotics except for cloxacillin (100% resistance). The microorganisms showed resistance to amikacin, aztreonam, ceftazidime, gentamicin and kanamycin that fell within the range 15.4% to 46.2% and the lowest resistance to imipenem (3.9%). They showed the highest susceptibility to meropenem (80.8%) followed by imipenem (76.9%).

3.3 Detection of Extended Spectrum β-lactamase (ESBLA) and Screening of AmpC β-Lactamase

In the phenotypic detection of extended spectrum β-lactamase (ESBL) producers done by double disk synergy test, six antibiotics were used. A sample was said to be positive for ESBL of class A ($ESBL_A$) if a zone of inhibition was observed for the β-lactam/ β-lactamase inhibitor towards the cephalosporins and monobactams. The $ESBL_A$ producers were also sensitive to cefoxitin. Whereas, it was said to be AmpC β-lactamase positive if it was resistant to cefoxitin. If the isolates were sensitive to all cefoxitin, ceftazidime and aztreonam, it was interpreted as both $ESBL_A$ and AmpC β-lactamase negative.

Table 3.5 Antibiotic sensitivity pattern of *Klebsiella* spp. samples in response to various antibiotics for ESBL$_A$ detection and AmpC β-lactamase screening

NO.	SAMPLE NAME	Is there a zone of inhibition of amoxycillin/clavulanic acid towards ceftazidime, ceftriaxone or aztreonam?	Sensitivity pattern for ceftazidime, ceftriaxone, aztreonam and cefoxitin								ESBL$_A$ or AmpC β-lactamase production
			FOX		CRO		CAZ		ATM		
			ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	
1	PKle1	YES	13I	R	11I	R	0	R	0	R	AmpC and ESBL$_A$
2	SKle1	YES	26	S	30	S	27.5	S	32	S	ESBL$_A$
3	SKle2	YES	0	R	0	R	0	R	0	R	AmpC and ESBL$_A$
4	SKle3	YES	26.5	S	29I	S	29	S	34.5	S	ESBL$_A$
5	SKle4	YES	10	R	0	R	0	R	0	R	AmpC and ESBL$_A$
6	SKle5	YES	21	S	13	R	0	R	0	R	ESBL$_A$
7	SKle6	YES	20.5I	S	0	R	11	R	14	R	ESBL$_A$
8	SKle7	YES	15	I	18.5	R	18	I	18	I	AmpC and ESBL$_A$
9	SKle8	NO	0	R	17I	R	20.5	S	24.5	S	AmpC
10	SKle9	YES	0	R	0	R	0	R	0	R	AmpC and ESBL$_A$
11	SKle10	YES	0	R	28	S	26.5	S	30	S	AmpC and ESBL$_A$
12	SKle11	NO	0	R	25.5	S	25	S	30	S	Negative
13	SKle12	NO	0	R	25	S	25.5	S	27.5	S	Negative
14	SKle13	YES	23.5	S	30	S	27	S	34	S	ESBL$_A$
15	SKle14	YES	24	S	21	I	14	R	0	R	ESBL$_A$
16	SKle15	NO	0	R	29	S	24.5	S	30	S	Negative
17	SKle16	YES	0	R	0	R	0	R	14	R	AmpC and ESBL$_A$
18	SKle17	YES	0	R	13	R	20	I	9.5	R	AmpC and ESBL$_A$
19	SKle18	YES	12	R	19.5	I	22I	I	20.5	S	AmpC and ESBL$_A$
20	SKle19	YES	0	R	0	R	0	R	0	R	AmpC and ESBL$_A$
21	SKle20	YES	0	R	0	R	0	R	27	S	AmpC and ESBL$_A$
22	SKle21	YES	22	S	0	R	15	R	11.5	R	ESBL$_A$
23	SKle22	YES	24	S	0	R	16	R	15	R	ESBL$_A$
24	SKle23	YES	15	I	0	R	10.5	R	0	R	AmpC and ESBL$_A$
25	SKle24	NO	0	R	15	I	20	I	24.5	S	AmpC
26	SKle25	NO	16	I	23	S	19	I	25	S	AmpC
27	SKle26	NO	0	R	17	R	20	I	14	R	AmpC
NOTE: S=Susceptible; I= Intermediate; R=Resistant; ZS=Zone Size; INT=Interpretation											

Table 3.6 Antibiotic sensitivity pattern of *Pseudomonas* spp. samples in response to various antibiotics for ESBL$_A$ detection and AmpC β-lactamase screening

NO.	SAMPLE NAME	Is there a zone of inhibition of piperacillin/tazobactam towards ceftazidime, ceftriaxone and/or aztreonam?	Sensitivity pattern for ceftazidime, ceftriaxone, aztreonam and cefoxitin								ESBL$_A$ or AmpC β-lactamase production
			FOX		CRO		CAZ		ATM		
			ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	ZS (mm)	INT	
1	PPse1	NO	0	R	0	R	0	R	0	R	AmpC
2	PPse2	NO	0	R	0	R	0	R	0	R	AmpC
3	PPse3	YES	0	R	0	R	0	R	0	R	AmpC and ESBL$_A$
4	PPse4	NO	0	R	0	R	0	R	0	R	AmpC
5	PPse5	YES	10.5I	R	0	R	0	R	0	R	AmpC and ESBL$_A$
6	PPse6	NO	0	R	0	R	0	R	0	R	AmpC
7	PPse7	YES	0	R	0	R	0	R	0	R	AmpC and ESBL$_A$
8	PPse8	NO	0	R	0	R	0	R	0	R	AmpC
9	PPse9	NO	0	R	0	R	0	R	0	R	AmpC
10	PPse10	NO	0	R	0	R	0	R	0	R	AmpC
11	PPse11	NO	0	R	0	R	0	R	0	R	AmpC
12	PPse12	NO	0	R	0	R	0	R	0	R	AmpC
13	SPse1	YES	0	R	18	I	29.5	S	26.5	S	AmpC and ESBL$_A$
14	SPse2	NO	0	R	0	R	0	R	0	R	AmpC
15	SPse3	YES	0	R	21.5	S	28	S	28	S	AmpC and ESBL$_A$
16	SPse4	YES	0	R	21	S	26.5	S	25.5	S	AmpC and ESBL$_A$
17	SPse5	YES	0	R	9.5I	R	20.5	S	0	R	AmpC and ESBL$_A$
18	SPse6	YES	0	R	20	I	27	S	26	S	AmpC and ESBL$_A$
19	SPse7	YES	0	R	23	S	30	S	28	S	AmpC and ESBL$_A$
20	SPse8	YES	0	R	20	I	30	S	29	S	AmpC and ESBL$_A$
21	SPse9	YES	0	R	19	I	28	S	25.5	S	AmpC and ESBL$_A$
22	SPse10	NO	0	R	0	R	0	R	0	R	AmpC
23	SPse11	YES	31	S	26	S	20	S	0	S	ESBL$_A$
24	SPse12	YES	0	R	20.5	S	31	S	25.5	S	AmpC and ESBL$_A$
25	SPse13	YES	0	R	21	S	28	S	27.5	S	AmpC and ESBL$_A$
NOTE: S=Susceptible; I= Intermediate; R=Resistant; ZS=Zone Size; INT=Interpretation											

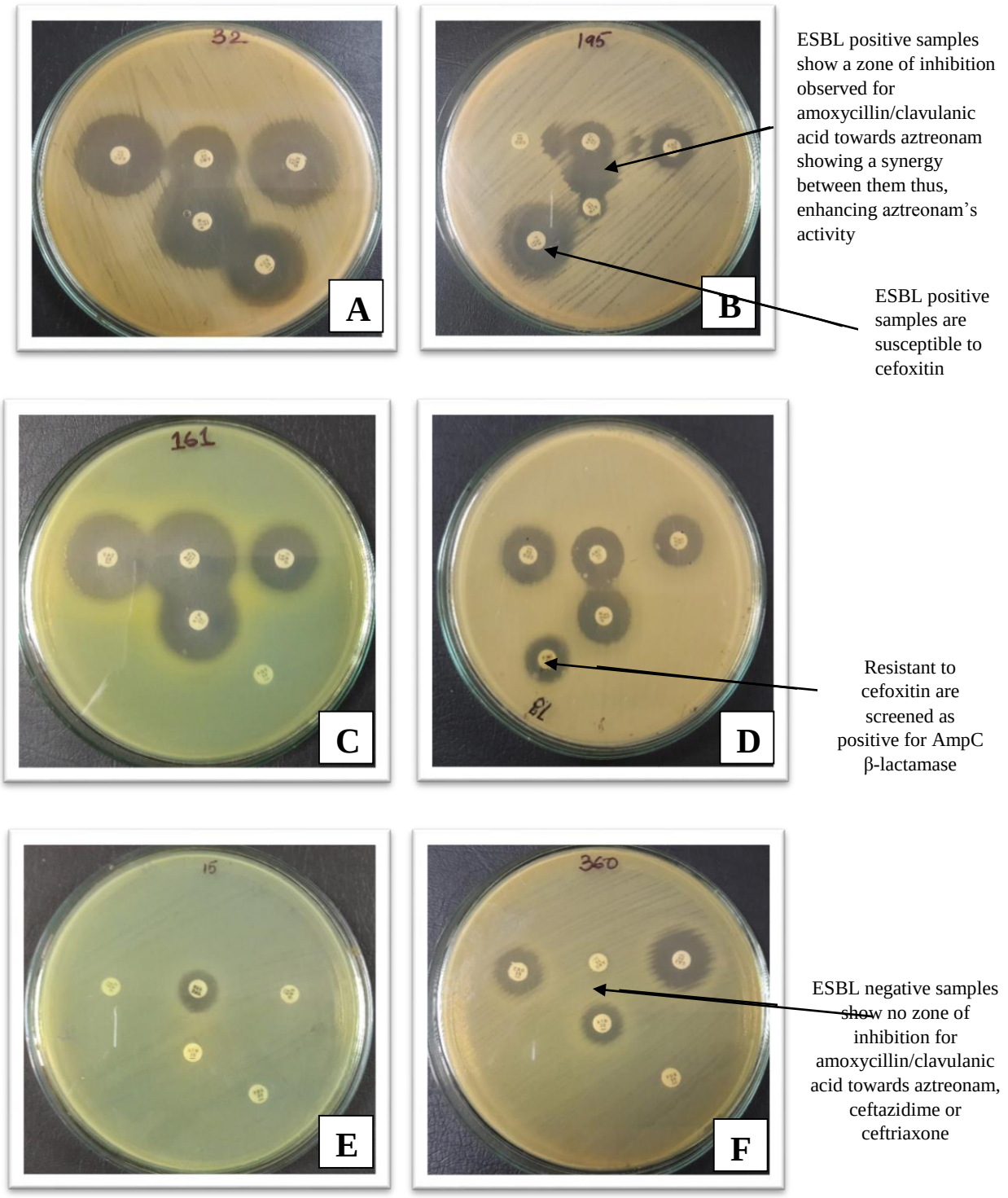

Fig. 3.8 ESBL Detection and AmpC β-lactamase Screening; Figure 3.8 A is sample SKle1 (32) and figure 3.8 B is sample SKle22 (195) and are $ESBL_A$ positive; figure 3.8 C is sample SPse4 (161) and figure 3.8 D is sample SKle7 (73) and are $ESBL_A$ and AmpC β-lactamase positive; Figure 3.8 E is sample PPse1 (15) and figure 3.8 F is sample SKle26 (360) and are AmpC β-lactamase positive

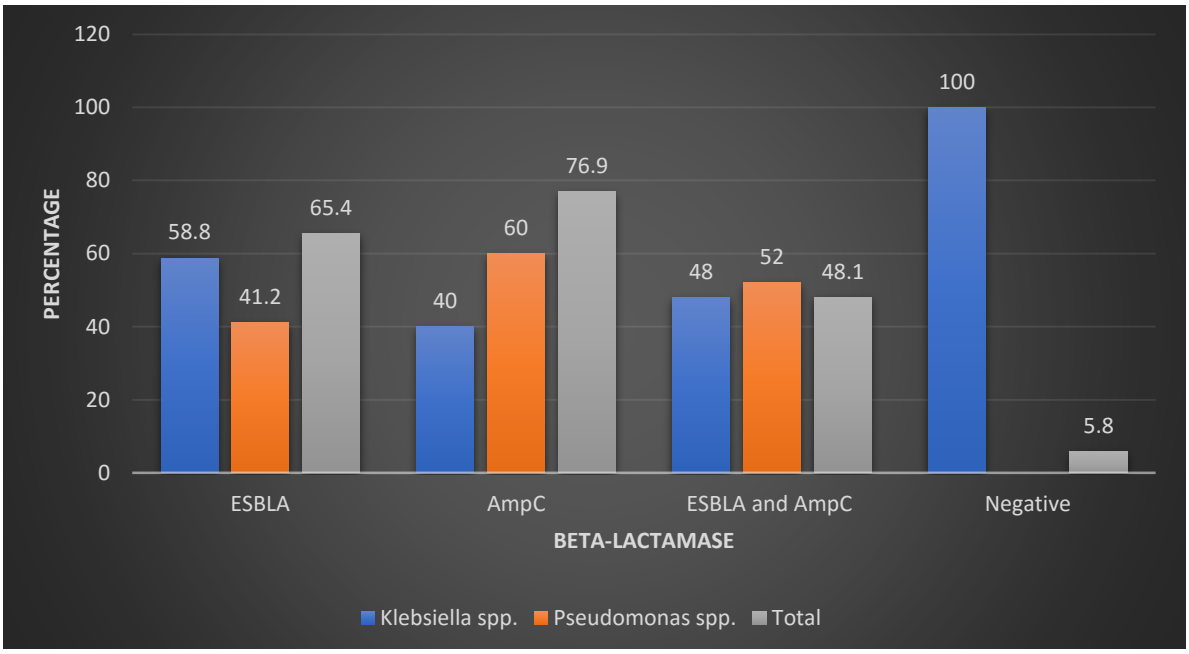

Fig. 3.9 Prevalence of ESBL$_A$ and AmpC β-lactamase producers among all the isolates combined

Figure 3.9 represents the prevalence of ESBL$_A$ and AmpC in 52 sample isolates. A higher percentage of samples produced AmpC β-lactamase (76.9%) than ESBL$_A$ (65.4%). Of all the ESBL$_A$ producers, *Klebsiella* spp. (58.8%) were more in number when compared to *Pseudomonas* spp. (41.2%). However, the opposite was observed in case of AmpC enzyme producers. Sixty percent of the isolates producing AmpC enzyme were *Pseudomonas* spp., whereas 40% were *Klebsiella* spp. Of the total isolates, 48.1% were found to be both ESBL$_A$ and AmpC β-lactamase producers, in which 52% were *Pseudomonas* spp. and 48% were *Klebsiella* spp. The sample isolates that were negative for both ESBL$_A$ and AmpC β-lactamase comprised only 5.8% of the total and were only observed in *Klebsiella* spp.

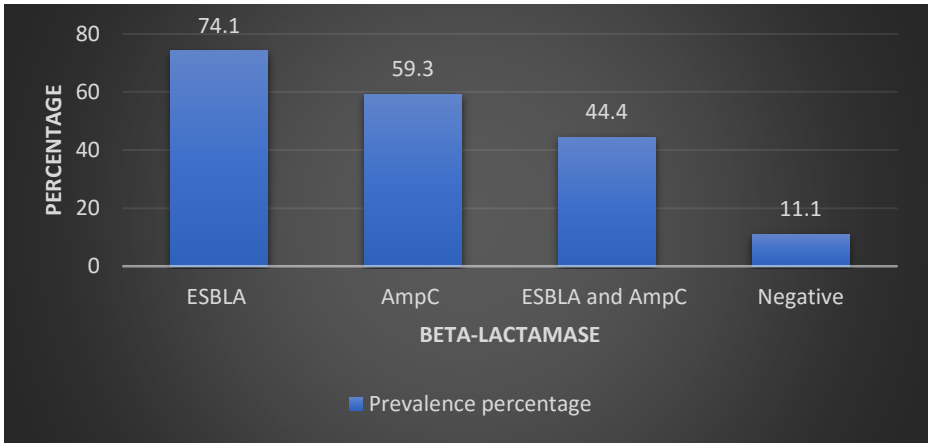

Fig. 3.10 Prevalence of ESBL$_A$ and AmpC β-lactamase positive in *Klebsiella* spp.

Figure 3.10 shows the prevalence of ESBL$_A$ and AmpC β-lactamase positive in *Klebsiella* spp. samples. Most of the samples belonged to ESBL$_A$ positive category (74.1%) and 59.3% of the *Klebsiella* spp samples produce AmpC enzyme. Of all the *Klebsiella* spp. isolates, 44.4% were observed to be positive for both ESBL$_A$ and AmpC enzyme. Only 11.1% of the total were confirmed to be negative for ESBL$_A$ and AmpC enzyme.

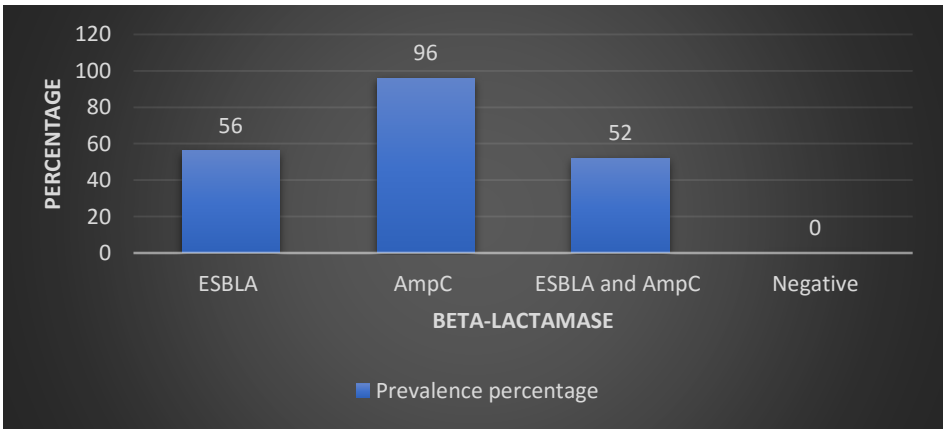

Fig. 3.11 Prevalence of ESBL$_A$ and AmpC β-lactamase positive in *Pseudomonas* spp.

Figure 3.11 shows the prevalence of ESBL$_A$ positive and AmpC β-lactamase positive in *Pseudomonas* spp. samples. Out of all the *Pseudomonas* spp. isolates, 96% were found to be AmpC β-lactamase positive, however, only 56% of the samples were ESBL$_A$ positive. Fifty-two percent were observed to be both ESBL$_A$ and AmpC enzyme producers, whereas none of the samples were tested negative for ESBL$_A$ and AmpC β-lactamase.

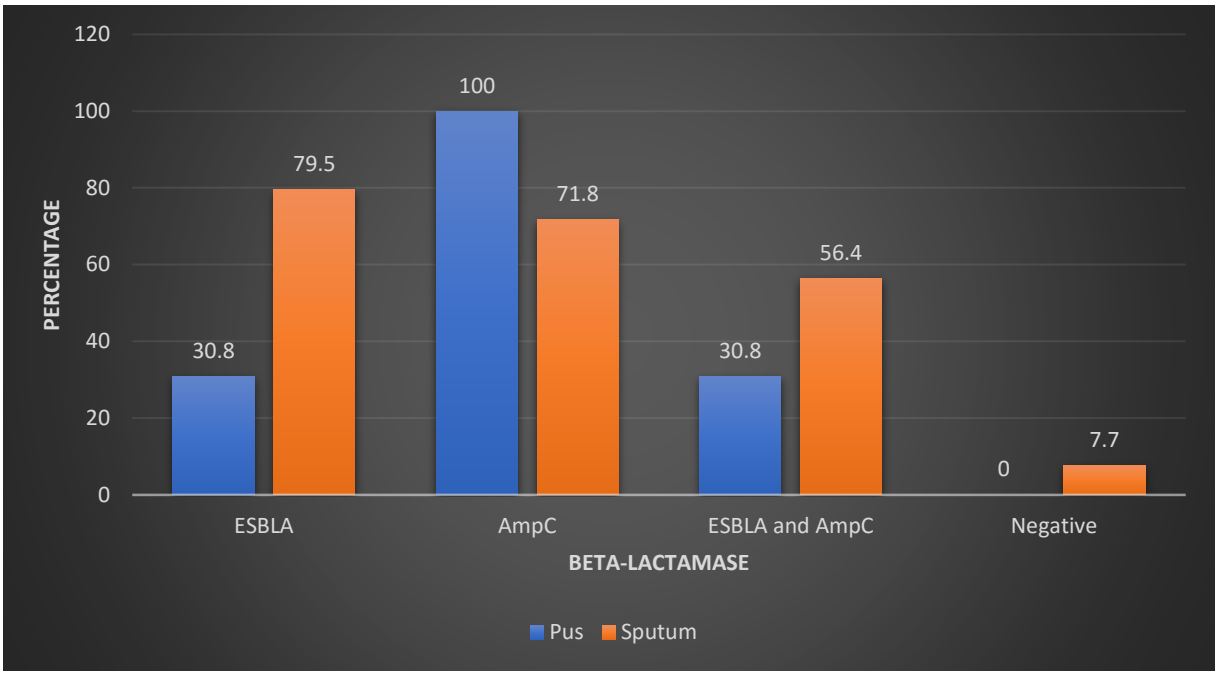

Fig. 3.12 Prevalence of ESBL$_A$ and AmpC β-lactamase positive in samples collected from pus and sputum

Figure 3.12 represents the prevalence of ESBL$_A$ and AmpC β-lactamase positive samples based on their source. A higher percentage of sputum samples were detected as ESBL$_A$ positive (79.5%) when compared to pus samples (30.8%). In contrast to this, all the pus samples were screened to be AmpC β-lactamase producers, whereas 71.8% pf the sputum samples were screened to be AmpC β-lactamase positive. Of all the samples isolated from pus, 30.8% were found to be both ESBL$_A$ and AmpC β-lactamase positive which was less when compared to the sputum samples (56.4%). Only 7.7% of the sputum samples were tested as negative for ESBL$_A$ and AmpC enzyme. However, none of the samples of pus origin were found to be ESBL$_A$ and AmpC β-lactamase negative.

3.4Molecular Detection of CTX-M and aacA-aphD genes

The molecular detection of CTX-M and aacA-aphD genes was carried out for the samples that were resistant to ceftazidime and aztreonam and resistant to amikacin, gentamicin and kanamycin respectively. For the detection of CTX-M gene, 9 out of 28 samples, that were resistant or intermediate to ceftazidime and aztreonam, were chosen for PCR, using specific forward and reverse primers. The samples, that were subject to PCR, were PPse3,

PPse8, SPse2, SKle4, SKle5, SKle9, SKle19, SPse10 and SKle26. Out of 9 samples, 2, that is SKle4 and SKle9, were confirmed to contain the CTX-M gene based on the bands visualized for these samples (as shown in Fig. 3.13).

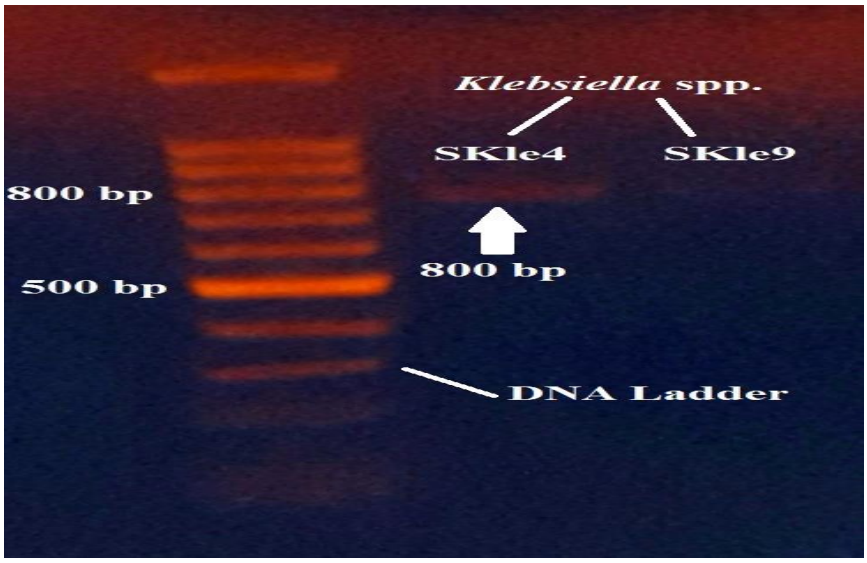

Fig. 3.13 Gel electrophoresis results of DNA bands visible for the CTX-M positive samples of *Klebsiella* spp. and *Pseudomonas* spp.

For the detection of aacA-aphD gene, out of 37 samples, that were either resistant or intermediate to either amikacin, gentamicin, kanamycin or all three antibiotics, 12 samples were chosen for PCR. The samples were PPse2, PPse3, PPse7, PPse6, PPse8, SKle2, SPse2, SKle8, SKle9, SKle16, SKle19 and SPse10. None of the samples showed positive result for the aacA-aphD gene as no bands were observed (as shown in Fig. 3.14).

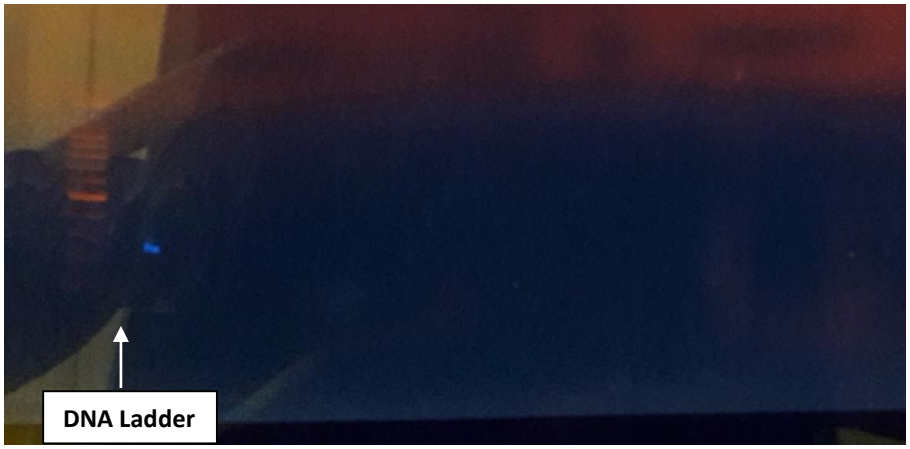

Fig. 3.14 Gel electrophoresis results did not show any bands for the aacA-aphD gene

CHAPTER 4:

DISCUSSION

4. DISCUSSION

With the emergence of multidrug resistant (MDR) strains of pathogens in the recent years, it has become important to look into the causes of this global problem and take actions accordingly. A number of studies prove the alarming increase in the number of antibiotic resistant pathogens. According to a review paper, it was found that 55% of the antibiotics prescribed were cephalosporins with the highest number of prescriptions for ceftriaxone, cefixime and cefuroxime. It also reported that *Pseudomonas aeruginosa*, isolated from wound, throat, urine, etc., had a resistance percentage above 50% for ciprofloxacin, gentamicin, ceftriaxone, cefixime and azithromycin which are commonly prescribed by doctors in Bangladesh (Faiz and Ariful, 2011). Appropriate use of antibiotics, vigilant surveillance of its extensive use and control measures are the ways by which the spread of antibiotic resistance can be minimized (Saga and Yamaguchi, 2009; Dzidic et al., 2008).

In this study, 52 sample isolates were collected from National Institute of Diseases of the Chest and Hospital (NIDCH) and Dhaka Medical College and Hospital (DMCH) and further tests were performed on them over a period of five months, from October 2016 to March 2017. Out of the total, 13 were collected from the pus of burn unit patients and 39 were collected from sputum. After being cultured in different selective and differential media (MacConkey agar, cetrimide agar and eosin methylene blue agar) to confirm the genera of the samples, most of the pus samples contained *Pseudomonas* spp. (92.3%) and *Klebsiella* spp. comprised only 7.7% of the total. Among the sputum samples, two-third of the total were *Klebsiella* spp. (66.7%) and one-third of them were *Pseudomonas* spp. (33.3%).

4.1 Antimicrobial Susceptibility Test

For the antimicrobial susceptibility test, antibiotics of different classes were used to determine if the sample isolates were of MDR type. From the result, it has been revealed that among the antibiotics, all the samples were completely resistant to the penicillin antibiotics (100%). Relatively more number of isolates were resistant to kanamycin (61.5%), aztreonam (53.8%), ceftazidime (51.9%) and gentamicin (42.3%) when

compared to amikacin (38.5%), meropenem (13.5%) and imipenem (7.7%). Meropenem was observed to be the most effective antibiotic against *Klebsiella* spp. and *Pseudomonas* spp. with the highest susceptibility rate of 82.7%. Observation of the antibiotic susceptibility pattern of pus samples revealed that the samples were 100% resistant to all the antibiotics except meropenem (23.1%) and imipenem (15.4%). This data suggests that the samples were highly resistant pathogens, restraining drug options as only one-fourth of the total antibiotics were bacteriostatic or bactericidal, imipenem being the most efficient. Unlike this, sputum samples showed different outcomes. They had lower resistance to all antibiotics except cloxacillin (100% resistant). Most samples were sensitive to meropenem followed by imipenem and amikacin. The resistant pattern for amikacin, aztreonam, ceftazidime, gentamicin, imipenem, kanamycin and meropenem were 17.9%, 38.5%, 35.9%, 23.1%, 5.1%, 48.7% and 10.3% respectively.

When the antibiotic susceptibility pattern for *Klebsiella* spp. alone was examined (isolated from pus and sputum), except for 0% susceptibility for cloxacillin, it was found that meropenem was the most efficient drug for hindering the growth of pathogens. More samples were susceptible to meropenem, imipenem, amikacin and gentamicin with a low resistance rate of 11.1%, 3.7%, 18.5% and 25.9% respectively, when compared to cloxacillin (100%), ceftazidime and aztreonam (both 48.2%) and kanamycin (33.3%). In Bangladesh, similar results to this data were obtained by Chakraborty et al. (2016), where 45% and 25% resistance was reported for ceftriaxone (cephalosporin) and gentamicin in *Klebsiella* spp. isolated from wound swab, urine, pus and sputum. In contrast to this, a research done in Iran by Alipourfard and Nili (2010) showed partially different results where *Klebsiella pneumoniae* was isolated from blood, fluid, urine, swabs, tracheal and aspirates/sputum.

Among all the samples of *Klebsiella* spp., some isolates were observed to be in the intermediate state, the rate falling in the range of 7.4% - 22.2%, for all antibiotics except cloxacillin, with the highest being ceftazidime (22.2%). *Klebsiella* spp. of sputum origin, when observed separately, also gave similar results with low resistance for imipenem, meropenem, amikacin and gentamicin and comparatively higher resistance for aztreonam and ceftazidime. This is due to the fact that only one sample of *Klebsiella* spp. was isolated from pus which was sensitive to both meropenem and imipenem and resistant to amikacin,

aztreonam, ceftazidime, cloxacillin, gentamicin and kanamycin. Therefore, a noticeable change in the resistance percentages wasn't observed for *Klebsiella* spp.

After the antibiotic susceptibility pattern of the *Pseudomonas* spp. samples of pus and sputum origin were studied together and separately, it was found that they had a relatively higher resistance rates for the antibiotics when compared to the resistance rates of *Klebsiella* spp. Imipenem and meropenem were equally effective against the pathogen with a low resistance rate of 12% and 16%, respectively. The samples showed the highest resistance to cloxacillin(100%) followed by kanamycin (92%), gentamicin (60%), aztreonam (60%), amikacin (60%) and ceftazidime (56%). This high rate of resistance is due to the highly resistant pus samples. Significantly different results were observed in studies done by Begum et al. (2013) and Hoque et al. (2015). The study by Begum et al. (2013) showed that samples had relatively higher levels resistance for the antibiotics whereas Hoque et al. (2015) mentioned higher levels of susceptibility compared to the data in this research. When observed separately for pus samples, all the antibiotics except imipenem and meropenem were found completely ineffective with no observable zones of inhibitions. Sample isolates showed low resistance to meropenem (25%) and imipenem (16.7%). Hence, imipenem was concluded to be the drug of the highest potential for treatment of infections caused by *Pseudomonas* spp. found in the pus. Similarly, imipenem was found to be the second most effective against sputum samples, meropenem being the most efficient. Expect for resistance to cloxacillin (100%) and kanamycin (84.6%), samples showed low resistance to the other antibiotics (below 25%). However, strict monitoring and controlled use of antibiotics can help prevent the rise of MDR strains keeping the existing therapeutic options open.

From the data, it can be inferred that cloxacillin, belonging to the class penicillin has completely lost efficiency with no visible inhibition zones. Once developed against the antibiotic resistant pathogens with great potential, the third-generation cephalosporins and monobactams are now losing their effectiveness as the pathogens continue to gain resistance at a fast pace. Among aminoglycosides, kanamycin and gentamicin had a high sensitivity for *Klebsiella* spp. rather than for *Pseudomonas* spp. However, it can be seen that amikacin is still a likely choice for treatment against both *Klebsiella* spp. and *Pseudomonas* spp. When the patterns were examined based on source, it was gathered that

the pus samples had no or low susceptibility when compared to the sputum samples that show higher susceptibility. The carbapenems are the drugs of high efficacy and can be used for the treatment of pathogens regardless of their origin. However, if these antibiotics are extensively, there will soon come a time, when resistance patterns similar to penicillins and third-generation cephalosporins will be observed.

4.2 Extended Spectrum β-lactamase ($ESBL_A$) Detection and Screening of AmpC β-Lactamase

To detect extended spectrum β-lactamases of class A ($ESBL_A$), amoxycillin/clavulanic acid (β-lactam/ β-lactamase inhibitor), ceftazidime and ceftriaxone (third-generation cephalosporins) and aztreonam (monobactam) were used. Zones of inhibition of amoxycillin/clavulanic acid towards cephalosporins were detected positive for $ESBL_A$. Cefoxitin (cephamycin) was used to detect $ESBL_A$ and screen for AmpC β-lactamase. Samples susceptible to cefoxitin were reported as $ESBL_A$ positive and samples resistant to cefoxitin were screened as AmpC β-lactamase producers. Among all the samples, 65.4% were $ESBL_A$ positive and among the $ESBL_A$ positive samples, more samples belonged to the *Klebsiella* spp. (58.8%). Contrary to this, a majority of the AmpC β-lactamase producers (76.9%) were *Pseudomonas* spp. (60%). Out of all the samples, 48.1% were observed to be both $ESBL_A$ and AmpC β-lactamase positive, among which, 48% was *Klebsiella* spp. and 52% was *Pseudomonas* spp. Comparison based on genera revealed that a greater percentage of *Klebsiella* spp. were $ESBL_A$ positive (74.1%), whereas more number of *Pseudomonas* spp. were AmpC β-lactamase positive (96%). In the study done by Biswas et al. (2015) in Dhaka, the prevalence rate of $ESBL_A$ positive in *Klebsiella* spp. was 71.42% which shows similarity to the current study. However, in the study by Chakraborty et al., 2016 which was carried out in Sylhet, a lower percentage was observed (45%). This suggests that the prevalence of $ESBL_A$ producing strains is based on geography and changes from place to place. This is also evident from the fact that France has a 40% prevalence and Pakistan has 17% prevalence (Chakraborty et al., 2016). Biswas et al. (2015) also mentioned a study about India having a high percentage of $ESBL_A$ producers in *Klebsiella* spp. (71.23%) showing resemblance to this study. In this study, 56% of *Pseudomonas* spp. were $ESBL_A$ producers. Begum et al. (2013) have mentioned, in their

study, that 37.8% of *Pseudomonas* spp. (collected from Dhaka and Rajshahi) were $ESBL_A$ positive. On the contrary, only 25% of *Pseudomonas* spp. (collected from Dhaka) were found to be positive in the study by Biswas et al. (2015). This is not in accordance with this study, the reason being that the samples were collected from different regions. However, Biswas et al. (2015) mentioned that the study done in India found 56.75% of the *Pseudomonas* spp. samples to be $ESBL_A$ positive. When the results were studied based on source, all the pus samples were screened to be positive for AmpC enzyme, however, a considerably smaller number of pus samples were detected as $ESBL_A$ positive (30.8%). For all the samples collected from sputum, similar results were observed for $ESBL_A$ positive (79.5%) as well as AmpC β-lactamase positive (71.5%) samples. A greater number of sputum samples (56.4%) were both $ESBL_A$ and AmpC β-lactamase positive when compared to pus samples (30.8%).

The data, collected from the performance of double disk synergy method and the antimicrobial test of cefoxitin, reveals that the $ESBL_A$ and AmpC β-lactamase prevalence rates of the samples are very high and have become common among the opportunistic pathogens. It is an indication of our failure to treat infections efficiently (Chakraborty et al., 2016). Multidrug resistant pathogens are so because of their ability to produce $ESBL_A$, AmpC β-lactamase or other kinds of β-lactamases that allow them to confer resistance to a wide variety of antibiotics. Clavulanic acid, in case of *Klebsiella* spp., and tazobactam, in case of *Pseudomonas* spp., were used to detect the presence of $ESBL_A$ as it is a β-lactamase inhibitor. It effectively inhibits their growth and consequently enhances the action of other cephalosporins that are placed close to them. When the zone diameters of ceftazidime and aztreonam, tested alone and with amoxycillin/clavulanic acid or piperacillin/tazobactam, were compared, an increase in diameter was observed when used in combination with amoxycillin/clavulanic acid or piperacillin/tazobactam. The samples were only screened for AmpC enzyme; hence this study does not give an accurate rate of prevalence. This issue could be resolved by following up with screening tests with confirmatory tests. However, it could not be performed due to limited time and unavailability of required materials.

4.3 Molecular Detection of CTX-M and aacA-aphD genes

CTX-M and aacA-aphD genes were detected by carrying out PCR using specific primers and subsequently gel electrophoresis. For CTX-M, DNA bands of 800 bp were observed for 2 samples (SKle4 and SKle9) out of the 9 samples that were amplified and belonged to *Klebsiella* spp. of sputum source. Compared to the number of samples, the number of samples positive for CTX-M gene was very low. The occurrence of a very low number could be due to various reasons. CTX-M β-lactamase genes could be plasmid-mediated or chromosome-mediated. Since only bacterial genomic DNA was isolated, only the samples with containing chromosomal CTX-M genes were visualized. For observing plasmid-mediated CTX-M gene, plasmid isolation of bacterial samples needs to be carried out. There are also several types of CTX-M genes. Since only one CTX-M primer was used, there is a possibility that the samples might contain other types of CTX-M genes. Carrying out PCR using specific primers for variants of CTX-M gene could give results with higher percentage of samples with CTX-M gene responsible for resistance to ceftazidime and aztreonam. Lastly, it is probable that some samples had given false positive results leading to an inaccurate interpretation. This can be solved by performing more sensitive and specific detection methods.

aacA-aphD gene was not found in any of the samples as no bands were visible when viewed under UV light. This gene is usually found in *Staphylococcus aureus* chromosomally. However, it can be acquired by other species of bacteria by conjugal transfer, thus being present as a plasmid-mediated gene. Genomic DNA extraction of the samples resistant to aminoglycosides gave no positive results. Though some samples might have given false-positive results, it cannot be concluded that this gene was absent in all the samples. Plasmid DNA extraction and subsequently PCR and gel electrophoresis could be carried out to further analyze the samples.

4.4 Conclusion

With the onset of antibiotic development of different classes, bacterial pathogens are also gaining resistance to these antibiotics. This has led to research and progress in designing newer antibiotics. But, pathogens still continue to mutate and evolve to become resistant to these. This has become a public health concern as it has made infection treatment challenging endangering the lives of many. This study was performed with a focus on *Klebsiella* spp. and *Pseudomonas* spp. collected from different sources (sputum and pus) and its results of antimicrobial susceptibility test, $ESBL_A$ detection and AmpC β-lactamase screening were compared. From the results, it can be concluded that the multidrug resistant bacteria have become prevalent now. The pus samples of both *Klebsiella* spp. and *Pseudomonas* spp. showed higher resistance to the antibiotics than the samples of sputum source. In addition to this, it was also observed that $ESBL_A$ positive were predominant among *Klebsiella* spp. of sputum source. However, AmpC β-lactamase producers were predominant among *Pseudomonas* spp. of pus source. MDR strains and consequently ESBL and AmpC β-lactamase positive have become globally prevalent now and the number of antibiotics, that the bacteria is conferring resistance against, is increasing day by day. Since prevalence varies regionally, hospitals in different places should make these methods mandatory, in addition to molecular techniques as they give accurate results. Hence, ESBL and AmpC β-lactamase detection tests should be carried out regularly to keep the increasing number in check. The policy of continuous monitoring of ESBL and Amp β-lactamase producers, strict usage of drugs and development of appropriate treatment of infections should be implemented and carried out stringently, in addition to an adequate supply of the required laboratory facilities.

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APPENDICES

APPENDIX I

Media Composition

1. Nutrient Agar

Component	Amount (g/L)
Peptone	5.00
Sodium chloride	5.00
Beef extract	3.00
Agar	15.00
Final pH	7.00

2. MacConkey Agar

Component	Amount (g/L)
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

3. EMB Agar

Component	Amount (g/L)
Peptone	10.00
Dipotassium phosphate	2.00
Lactose	5.00
Sucrose	5.00
Eosin yellow	0.14
Methylene blue	0.065
Agar	13.50

4. Cetrimide Agar

Component	Amount (g/L)
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

5. Mueller-Hinton Agar

Component	Amount (g/L)
Beef infusion	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH	7.3±0.1

6. T_1N_1 Agar

Component	Amount (g/L)
Tryptone	1.00
Sodium chloride	1.00
Agar	0.60-0.75

7. Luria-Bertani broth

Component	Amount (g/L)
Tryptone	10.00
NaCl	10.00
Yeast extract	5.00

APPENDIX II

Composition of buffers and solutions

1. Physiological saline

Component	Amount (in 100 mL solution)
NaCl	0.90 g

2. 10% SDS

Component	Amount (in 100 mL solution)
SDS	10.00 g

3. 1 M Tris-HCl

Component	Amount (g/L)
Tris	121.14
HCl	As required to adjust the pH
Final pH	8.00

4. 0.5 M EDTA

Component	Amount (g/L)
EDTA	186.00
NaOH	As required to adjust pH
pH	8.00

5. 1x TE buffer

Component	Amount (mL/L)
1 M Tris-HCl	10.00
0.5 M EDTA	2.00
pH	8.00

6. 1x TBE buffer

Component	Amount (mL/L or g/L)
Tris base	10.80 g
Boric acid	5.50 g
0.5 M EDTA	4.00 Ml
pH	8.00

7. Lysis buffer

Component	Amount (for 10 mL)
1x TE buffer	9.34 Ml
10% SDS	600 µL
Proteinase K	60 µL

APPENDIX III

List of equipment used in the study and the manufacturer

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
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Refrigerator (4^{O}C) Model: 0636	Samsung