MOLECULAR ANALYSIS OF β-LACTAMASE GENE IN MULTIDRUG RESISTANT CLINICAL ISOLATES OF *Pseudomonas aeruginosa*

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LIST OF ABBREVIATIONS

µg	: microgram
µl	: microliter
bla	: beta lactamase
mg	: milligram
ml	: milliliter
3GCS	: Third Generation Cephalosporins
Ab	: Antibiotic
AK	: Amikacin
*bla*IMP	: gene encoding IMP type enzyme
*bla*NDM	: gene encoding NDM type enzyme
CAZ	: Ceftazidime
CDDT	: Combined Disk Diffusion Test
CFS	: Cefoperazone/ Sulbactum
CIP	: Ciprofloxacin
CL	: Colistin
CLSI	: Clinical Laboratory Standard Institute
CPM	: Cefepime
CTX	: Cefotaxime
DDST	: Double Disk Synergy Test
DNA	: Deoxyribonucleic Acid
EDTA	: Ethylene diamine tetra acetic acid
ESBL	: Extended Spectrum Beta-Lactamase
E-test	: Epsilometer test
GIM	: Germany Imipenemase
ICU	: Intensive Care Unit
IMI	: Imipenem
LB	: Laurial Broth
LE	: Levofloxacin
MBL	: Metallo-Beta-Lactamase
MDRPA	: Multi Drug Resistant *Pseudomonas aeruginosa*
MER	: Meropenem

MHA	: Muller Hinton Agar
NA	: Nutrient Agar
NCBI	: National Center for Biotechnology Information
NDM	: New Delhi Metallo-β-lactamase
PB	: Polymyxin-B
PBP	: Penicillin Binding Protein
PCR	: Polymerase Chain Reaction
PDR	: Pan-drug Resistant
PI	: Piperacillin
PIT	: Piperacillin/Tazobactam
SHV	: Sulphydryl variable
SPM	: SauPoulo Imipenemase
spp	: Species
TEM	: Temoniera
VIM	: Verona Integron Metallo-beta-lactamase
WHO	: World Health Organization
XDR	: Extensively Drug Resistant
Zn	: Zinc

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ABSTRACT

The multi-drug resistant *Pseudomonas aeruginosa* which is one of the most prevalent opportunistic nosocomial pathogen is on the rise. Its major defense against β-lactam antibiotics is production of metallo-β-lactamases (MBLs) which degrade this group of antibiotics including carbapenems. Carbapenems are the drugs of choice for the treatment of *P. aeruginosa* infection but carbapenemases (MBLs) have emerged and have spread from this bacterium to Enterobacteriaceae. The finding of carbapenem resistance is a menacing development that challenges this "last resort antibiotic" and organisms harboring the enzyme may lead to therapeutic dead ends. The aim of the study is to determine the β-lactamase gene responsible for causing antimicrobial resistance and its prevalence in clinical isolates of *P. aeruginosa* in the context of Nepal.

The clinical isolates of *P. aeruginosa* were collected from different hospitals of Kathmandu valley. The isolates were subjected to the biochemical test for confirmation and then to the antibiotic susceptibility test. ESBL production was tested by double disk synergy test. For MBL production, the carbapenem resistant isolates were screened out and then tested for the presence of MBL enzyme and its detection was done by EDTA combined disk test. Molecular identification of the responsible gene *bla*IMP, *bla*VIM and *bla*NDM causing MDR in those MBL producers was done by Polymerase Chain Reaction (PCR) using gene specific primers. Sequencing was then carried out. Of total 67 consecutive isolates of *P. aeruginosa*, 52 (77.61%) were MDR *P. aeruginosa*, none of them were ESBL producers and 30 (75%) of total 40 carbapenem resistant isolates showed positive result for MBL phenotypically. Additionally, the results of PCR method showed that 15 strains (37.5%) of carbapenem resistant isolates contained *bla*NDM (New Delhi Metallo-β-lactamase) gene. No other gene was found in the examined samples. Further sequence analysis showed the presence of *bla*NDM-1 in majority of sequenced isolates and 3 novel variants of this gene was also detected.

The prevalence of the MBLs has been increasing worldwide, particularly among *P. aeruginosa*, leading to severe limitations in the therapeutic options. Thus, antimicrobial stewardship should be implemented to minimize the emergence of this β-lactamase producing pathogens. Molecular surveillance on a regular basis and proper resistance screening measures needs to be adopted to prevent spreading of these superbugs.

Keywords: *bla*NDM, Carbapenem, Metallo-β-lactamases, *Pseudomonas aeruginosa*, Sequencing.

CHAPTER 2
INTRODUCTION

2.1 Background

Pseudomonas aeruginosa is a leading cause of nosocomial infections. They are emerging worldwide as a threat in community and hospital settings (Lister et al., 2009). These are versatile Gram's negative bacterium that may be present in many diverse environmental surroundings as well and can be isolated from various living sources, including plants and animals. The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed this organism to persist in both community and hospital settings. Despite the cosmopolitan distribution of *P. aeruginosa* in nature and the potential for community-acquired infections, serious infections with *P. aeruginosa* are predominantly hospital acquired. The frequency of infections caused by them is increasing, that includes pneumonia, urinary tract infections, burn infection, meningitis and bacteremia (Pollack, 2000). In the past decade, acquired multidrug resistance, relating to selective antibiotic pressure, has emerged in several countries and in some cases, infections caused by multidrug resistant (MDR) *P. aeruginosa* have been untreatable and are often difficult to eradicate due to its resistant drug profile (Neu, 1983). Multidrug resistance (MDR) may be defined as resistance to at least three classes of antibiotics used during treatment of the infections: third-generation cephalosporins, fluoroquinolones, aminoglycosides, and carbapenems. Outbreaks caused by MDR *P. aeruginosa* may follow an increased use of third-generation cephalosporins or carbapenems for therapy of infections caused by these resistant bacteria (Wroblewska, 2006).

Among the wide array of antibiotics, β-lactams are the most varied and widely used agents accounting for over 50% of all systemic antibiotics in use (Agrawal et al*.,* 2008). The β-lactam group of antibiotics that include penicillins, cephalosporins, monobactams and carbapenems form the main stay of therapy. *P. aeruginosa* is naturally resistant to many antimicrobial agents; it can also acquire the resistance against available antibiotics either through the acquisition of resistance genes on mobile genetic elements (i.e., plasmids) or through mutational processes that alter the expression and/or function of chromosomally encoded mechanisms. In other words, the resistance is mediated by lack of drug penetration (i.e. porin mutations and efflux pumps) and/or carbapenem – hydrolyzing β-lactamases among which the production of β-lactamases is the major defense mechanism against these β-lactam antibiotics. β-lactamases are enzymes

produced by bacteria that provide multi-resistance to β-lactam antibiotics. Many of the second and third generation penicillins and cephalosporins were specifically designed to resist the hydrolytic action of major β-lactamases. However, new β-lactamases emerged against each of the new classes of β-lactams that were introduced and caused resistance. The latest in the arsenal of these enzymes has been the evolution of Extended Spectrum β-Lactamases (ESBLs) (Kumar et al., 2006). These ESBLs are commonly produced by many members of Enterobacteriaceae (Bhattacharya, 2006), and efficiently hydrolyze oxyimino cephalosporins conferring resistance to third generation cephalosporins such as cefotaxime, ceftazidime and ceftriaxone and to monobactams such as aztreonam. Being plasmid mediated, they are easily transmitted among members of Enterobacteriaceae thus facilitating the dissemination of resistance to β-lactam. They also carry resistant genes to quinolones and aminoglycosides. The chromosomally mediated β-lactamase production is mainly through expression of Amp C gene which is either constitutive or inducible (Rodrigues et al*.,* 2004). The Amp C beta lactamases are cephalosporinases that are resistant to oxyimino group and 7-α-methoxy group cephalosporins (Manchanda et al., 2006).

Metallo-β-lactamase (MBL) has recently emerged as one of the most worrisome resistance mechanisms owing to their capacity to hydrolyze with the exception of aztreonam, all β-lactams including carbapenems. Normally, carbapenems are highly resistant to most β-lactamases with the exception of the carbapenemases. The carbapenemases have been organized based on amino acid homology in the Ambler molecular classification system. Class A, C, and D β-lactamases all share a serine residue in the active site, while Class B enzymes require the presence of zinc for activity (and hence are referred to as metallo-β-lactamases). MBL producing *P. aeruginosa* isolates has been responsible for several nosocomial outbreaks in different parts of the world, illustrating the need for proper infection control practices. Hence the metallo-β-lactamases (MBLs) also called class B carbapenemases, belong to group B and are enzymes requiring divalent cations as cofactors for enzyme activity, being inhibited by the action of a metal ion chelator (Ambler, 1980) like EDTA and mercaptoacetate. The genes responsible for the production of MBLs are typically part of an integron structure and are carried on transferable plasmids but can also be part of the chromosome (Poirel and Nordman, 2002). Because the genes usually are carried on highly mobile elements, there is easy dissemination. The VIM, IMP, SPM and NDM types are the most clinically significant carbapenemases which are encoded by *bla*VIM, *bla*IMP, *bla*SPM and *bla*NDM genes respectively (Liakopoulos et al*.,* 2013). MBL genes were first detected in *P. aeruginosa*, which in recent years has been spread to members of Enterobacteriaceae (Nordmann and Poirel, 2002).

2.2 Current Studies

Research studies has been carried out in different parts of the world regarding prevalence of multi drug resistant *P. aeruginosa* showing increasing frequency of this bacterium causing nosocomial infections which are often severe, life threatening and are difficult to treat because of the limited susceptibility to antimicrobial agents. Recently WHO has published a list of antibiotic-resistant "priority pathogens" – a catalogue of 12 families of bacteria that pose the greatest risk to human health. The list highlights in particular the threat of gram-negative bacteria that are resistant to multiple antibiotics (States News Service, February 27, 2017). These bacteria have built-in abilities to find new ways to resist treatment and can pass along genetic material that allows other bacteria to become drug-resistant as well. The list is divided into three categories according to the urgency of need for new antibiotics: critical, high and medium priority. The most critical group of all includes multidrug resistant bacteria that pose a particular threat in hospitals, nursing homes, and among patients whose care requires devices such as ventilators and blood catheters which includes *P. aeruginosa* (States News Service, February 27, 2017). They can cause severe and often deadly infections such as bloodstream infections and pneumonia. Data from recent studies show *P. aeruginosa* as the second most common cause of nosocomial pneumonia, health care-associated pneumonia, and ventilator-associated pneumonia (Gaynes and Edwards, 2005).

Several studies concerning the surveillance of MDR *P. aeruginosa* have shown the heightened level of drug resistance which is a result of de novo emergence of resistance in an organism after exposure to antimicrobials as well as of patient-to-patient spread of resistant organisms. The resistance to the β-lactams involves the production of inactivating β-lactamases, for which several families have been identified among clinical isolates of *P. aeruginosa.* The variety, prevalence, and clinical significance of the β-lactamases in *P. aeruginosa* have been addressed in several reviews over the last decade (Lister et al., 2009). However, prevalence of different classes of carbapenem-hydrolyzing enzymes has been increasing globally. The first class B metallo-β-lactamases in *P. aeruginosa* were identified in 1991 in Japan (Watanabe et al*.,* 1991). Since that initial report, metallo-β-lactamases have been reported for *P. aeruginosa* isolates from nearly all regions of the globe (Jones et al*.,* 2005). Other chromosomally encoded resistance mechanisms in *P. aeruginosa* have been intensely studied that involves AmpC cephalosporinase, the OprD outer membrane porin, and the multidrug efflux pumps. The ability of *P. aeruginosa* to coregulate different resistance mechanisms makes this pathogen a constantly moving target that continues to challenge therapeutic strategies. According to some recent reports, infection with MBLs producing *P. aeruginosa* strains

has increased mortality. Several types of MBLs including IMP, VIM, SPM – 1 and GIM – 1 have been identified (Walsh, 2005). Of these, the IMP – type MBLs is the most common and exhibits a worldwide distribution. As result of variations in their amino acid sequences, IMP and VIM enzymes are classified in a still growing number of subgroups. At least 14 different VIMs and 23 different IMP MBLs have been identified so far. Generally MBLs are divided into several families as follows: IMP, VIM, SPM, GIM, SIM, DIM, AIM, KHM, NDM and KPC. Most of them, if not all, genes encoding IMP, VIM and SPM types as well as GIM are found as gene cassettes in class 1 integrons, although IMP MBL genes are also found on class 3 integrons (Liakopoulos et al*.,* 2013). New-Delhi-metallo-β-lactamase (NDM-1), a new type of MBL, was first detected in two of the Enterobacteriaceae strains isolated from a Swedish patient who was admitted to a hospital in New Delhi, India (Yong et al*.,* 2009). In recent years, the emergence and dissemination of NDM-1 producing isolates have been reported in several countries (Fallah et al*.,* 2011). In our context, *bla*NDM-1 gene have been identified in *P. aeruginosa* and *bla*NDM-8 in *E. coli*, which were the samples isolated from sputum and pus respectively from Tribhuvan University Teaching Hospital in Kathmandu, Nepal (Tada et al., 2013). So this study is also carried out for the similar objective, to find the responsible antibiotic resistant gene in *P. aeruginosa,* more specifically, the metallo-β-lactamase gene.

Despite the occurrence of carbapenemases, various other mechanisms are being studied like loss of porin protein and efflux pump systems are emerging as extremely important causes of multidrug resistance in *P. aeruginosa* (Livermore, 2002). Resistance to polymyxins by *P. aeruginosa* has also been studied recently. Resistance to colistin in *P. aeruginosa* is rare but has been found (Ho et al., 2002) which may be due to structural modifications of the outer cell membrane. Among all the mechanisms for drug resistance, production of MBLs is of great concern. MBL enzymes are a cause of concern because they are able to hydrolyze most β-lactams, including imipenem and meropenem, drugs considered as reserve for the treatment of Gram's negative MDR strains. In addition, MBLs are encoded on genes linked to mobile elements, a condition that facilitates their spread among different bacterial species and genera (Bennett 1999).

Recently, pandrug-resistant (PDR) bacterial strains, which are resistant to all antibacterial agents except the polymyxins and tigecycline, and extensively drug-resistant (XDR) bacterial strains, which are resistant to all antibacterial agents, were

isolated from hospital-acquired infections. There is a huge risk of these "superbugs" extending into the community and threatening public health (Tan et al., 2013).

For the proper management of the infections caused by *P. aeruginosa* so as to prevent the nosocomial outbreaks, timely appropriate treatment as suggested by sensitivity report is essential. So efficient surveillance program, proper study for the prevalence of MDR strains have to be conducted. Recently some studies on current antimicrobial resistance profiles of *P. aeruginosa* have been carried out to find out the susceptibilities of this pathogen against commonly prescribed antibiotics in health care facility. However, the detection for MBL production or other resistant enzyme production is not performed routinely in clinical laboratories due to lack of resources. Studies on the prevalence of MDR bacteria have been carried out frequently in different parts of Nepal but only a limited molecular studies regarding identification of responsible resistant gene and mutation study have been done (Awasthi et al., 2015). So our study will also intend to figure out the prevalence of the MDR strains of *P. aeruginosa,* their resistance pattern and the type of β-lactamase gene in them.

Several phenotypic methods for the detection of MBL – producing bacteria have been applied and studied. All these methods are based on the ability of metal chelators, such as EDTA and thiol – based compounds, to inhibit the activity of MBLs. These tests include the double disk synergy tests using EDTA with imipenem (IPM) or ceftazidime, 2 – mercaptopropionic acid with CAZ or IPM, the Hodge test, a combined disk test using EDTA with CAZ or IPM, the MBL E-test and a microdilution method using EDTA and 1,10 –phenanthroline with IPM. Most of the current phenotypic methods described for the detection of MBLs are especially the double disk synergy test and the Hodge test. The most common test for ESBL detection is the double disk synergy test using amoxyclav disk with ceftazidime or cefotaxime disk alone. Molecular methods are more sensitive and specific for the detection of β-lactamase genes but they are still technically demanding. In many parts of Nepal, the facilities even for the susceptibility testing is still not available leading to the irrational antibiotic treatment for the infection caused by *P. aeruginosa* resulting in treatment failure and development of more drug resistance among the bacteria. The updated knowledge and situation of this prevailing bacterial pathogen that are MDR is of prime importance for the proper use of antimicrobial drugs. Since the molecular studies are lacking in our context, the data obtained from this study will be helpful to understand the current situation of the drug resistance in pathogens and resistance mechanisms in them.

2.3 Hypothesis

The research aims to analyze "whether *P. aeruginosa* isolates from Kathmandu valley contains β-lactamase genes (*bla*IMP, *bla*VIM or *bla*NDM) which are responsible for carbapenem resistance."

Null Hypothesis: The collected isolates of *P. aeruginosa* doesn't contain β-lactamase gene.

Alternate Hypothesis: There is high prevalence of β-lactamase gene among the *P. aeruginosa* isolates.

2.4 Objectives

2.4.1 General Objective:

Molecular analysis of β-Lactamase gene in multidrug resistant clinical isolates of *Pseudomonas aeruginosa.*

2.4.2 Specific Objective:

- Determination of antibiogram for *Pseudomonas aeruginosa* isolates.
- Delineation of the prevalence of ESBL and MBL producing MDR *Pseudomonas aeruginosa* in hospitalized patients.
- Isolate DNA and to detect some of the metallo-β-lactamase genes in *Pseudomonas aeruginosa* using PCR.
- Confirmation of the responsible gene through sequencing.

2.5 Rationale and Scope

The frequency of infections caused by *Pseudomonas aeruginosa* is increasing and MDR strains, resistant to almost all available antimicrobials, are emerging in hospitalized patients. The emergence and dissemination of MDR *P. aeruginosa* is a global concern threatening not only immune compromised patients, but also healthy members of the community. The increasing prevalence of nosocomial infections produced by MDR *P. aeruginosa* strains severely compromises the selection of appropriate treatments and is therefore associated with significant morbidity and mortality. Hence it is an important bacterium due to the antimicrobial resistance characteristics as well as its pathogenicity. An increasing uncontrolled and inappropriate use of antibiotics these days in our

country may put the selective pressure on bacteria which could develop new mechanisms to escape the lethal action caused by antibiotics leading to emergence of new type of resistance genes in them that should be investigated. *P. aeruginosa* contains multiple antimicrobial resistance genes and has the potential to transfer these to other strains, which is a major clinical problem because of limited treatment options for patients infected with MDR *P. aeruginosa* and which eventually may be responsible for notable epidemics in the hospital setting.

In our country where there is least data available regarding resistant genes and very high chance of spread of these among different species within hospital settings, it is more important to understand and learn about these genes. Urgent examination of the status of resistant *P. aeruginosa* has to be done so that a rational approach to therapy may be adopted.

CHAPTER 3
LITERATURE REVIEW

Pseudomonas aeruginosa, a gradually prevailing opportunistic human pathogen, is one of the most common Gram's negative bacterium which belongs to Pseudomonadaceae family. They are obligate aerobic, motile with single polar flagellum, non-fermenting glucose, non-sporulated and oxidase positive bacilli; usually found in intestinal tract, water, soil and sewage. Its identification in the laboratory is simple, because they grow easily in a wide variety of media and requirements for its identification are limited. It secretes a variety of pigments including pyocyanin (blue – green), fluorescein (yellow-green and fluorescent also known as pyoverdin), and pyorubin (red-brown); it is useful for identification of the bacterium, and because of the characteristic color of copper oxide, the bacteria is named *aeruginosa* (Pollack, 2002). *P. aeruginosa* is often preliminarily identified by its typical odor, a grape-like odor, in vitro (Bisen et al., 2012). *P. aeruginosa* has been characterized as one of the most versatile microbial organisms, with wide habitats including soil, disinfectant solution and jet plane fuel. These bacteria can remain for long period in soap, sponges, skins, oral thermometers, inhalator equipment, taps and clothes. They are extremely resistant to disinfectants and can contaminate certain compounds and solutions. Due to its pervasive nature, ability to survive in moist environments, and innate resistance to many antibiotics and antiseptics, *P. aeruginosa* is a common pathogen in hospitals and particularly in intensive care units (ICUs) (Loureiro et al., 2002).

In the past few decades *P. aeruginosa* has been increasingly recognized as a pathogen in variety of serious infections in hospitalized patients especially with impaired immune defenses (Neu, 1983). According to data from the National Nosocomial Infections Surveillance system, *P. aeruginosa* has been reported as the second most common cause of pneumonia (18.1%), the third most common cause of urinary tract infection (16.3%) and the eighth most frequently isolated pathogen from the bloodstream (3.4%) (Gaynes and Edwards, 2005). While the overall proportion of infections caused by *P. aeruginosa* has remained stable for some years, the proportion of resistant isolates has alarming increases in recent years (NNIS). A national surveillance study in intensive care unit (ICU) patients has reported a significant increase in multidrug-resistant (MDR) *P. aeruginosa* isolates (Obritsch et al., 2004). *P. aeruginosa* is a major opportunistic pathogen causing high mortality, particularly in patients with suppressed immunity, traumatic wound, burns, cystic fibrosis, metabolic disorders and malignancies (Hancock

RE, 1998). Mortality rates ranging from 40% to more than 60% have been reported in bacteraemic nosocomial pneumonia and in ventilator-associated pneumonia (Mayhall, 2001). Due to its intrinsic and acquired antimicrobial resistance, only limited classes of antibiotics are effective for the treatment of *P. aeruginosa* infections.

3.1 Multidrug Resistance in *Pseudomonas aeruginosa*

MDR *Pseudomonas aeruginosa* has been described mainly in patients who are immune-compromised, or patients with cystic fibrosis or neoplastic diseases, or patients in Intensive Care Units (Hsueh et al., 1998). The incidence rate of infections produced by MDR *P. aeruginosa* strains may vary from 5.5 to 14 cases per 10,000 patients admitted per year (Aloush et al., 2005). The overall mortality rate due to *P. aeruginosa* infection is greater than 20%, which increases when the infection is due to MDR *P. aeruginosa* strains (Tacconelli, 2002). Multidrug resistance (MDR) is a heterogeneous phenotype, which could result from different resistance mechanisms or a combination of them. A review of studies reporting on MDR and 'pan-drug resistant' *P. aeruginosa* infections revealed considerably different definitions used in the literature, ranging from resistance to a single antibiotic agent/class to resistance to all tested antibiotics (Falagas et al., 2006). In the majority of the published studies, multidrug resistance was defined as resistance to at least three drugs from a variety of antibiotic classes, mainly aminoglycosides, antipseudomonal penicillins, cephalosporins, carbapenems and fluoroquinolones. Although there have been attempts to establish a precise definition for multidrug resistance, there is currently no international consensus. Second, there is no international surveillance system specifically designed to track MDR organisms. However, the SENTRY antimicrobial surveillance program, MYSTIC programme and Intensive Care Antimicrobial Resistance Epidemiology (ICARE) project (Fridkin et al., 1999), as well as a large number of smaller scale surveys are conducted to track antimicrobial resistance trends nationally and internationally. However, annual variations in geographic regions and participating centers limit the ability to track the true prevalence of MDR *P. aeruginosa* (Gales et al., 2001). Concerning resistance trends, data from large-scale surveillance studies indicate, overall, an increasing trend during the past few years, although with notable differences for different drugs and geographical areas (Karlowsky et al., 2003).

P. aeruginosa is a particularly difficult organism to control with antibiotics or disinfectants (Hancock, 1998). Extensive use of antibiotics to treat *P. aeruginosa*

infection has generated the selective pressure to encourage resistance development. Its general resistance is due to a combination of factors:

• It is intrinsically resistant to antimicrobial agents due to low permeability of its cell wall

• It has the genetic capacity to express a wide range of resistance mechanisms.

• It can become resistant through mutation in chromosomal genes which regulate resistance genes.

• It can acquire additional resistance genes from other organisms via plasmids, transposons and bacteriophages.

The genome of this microorganism is among the largest in the bacterial world allowing for greater genetic capacity and high adaptability to environmental changes. It has a genome containing 6.26Mbp (encoding 5567 genes), which is very large compared to 4.64 Mbp (4279 genes) in *Eschericia coli* K12, 2.81 Mbp (2594 genes) in *Staphylococcus aureus* N315 and 1.83 Mbp (1714 genes) in *Haemophilus influenzae* (Lambert, 2002). *P. aeruginosa* therefore has considerable additional genetic capacity compared with other organisms. This explains its highly adaptable nature, including the ability to develop resistance where antibiotics are used extensively (Lambert, 2002). This large genetic armamentarium that can be further enriched with the addition of genes acquired by transferable genetic elements via horizontal gene transfer is a major contributing factor to its challenging ability to develop resistance against all known antibiotics.

3.1.1 Resistance Mechanism of *Pseudomonas aeruginosa*

Antimicrobial resistance mechanisms of *P. aeruginosa* can be divided into intrinsic and acquired mechanism of resistance. Intrinsic refers to resistance that is a result of large selection of genetically – encoded mechanisms and acquired refers to resistance that is achieved via the acquisition of additional mechanisms or is a consequence of mutational events under selective pressure.

Intrinsic resistance of *P. aeruginosa*

P. aeruginosa shows inherent resistance to antimicrobial agents through a variety of mechanisms: (Moore and Flaws, 2011)

1. Decreased permeability of the outer membrane

β-lactams and quinolones need to diffuse through certain porin channels. Most bacteria possess lots of general porins and relatively few specific ones; *P. aeruginosa* expresses mainly specific porins (Hancock & Brinkman, 2002). The loss of these porin channels causes resistance to β-lactams.

2. Efflux systems which actively pump antibiotics out of the cell

P. aeruginosa expresses several efflux pumps that expel drugs together with other substances out of the bacterial cell. These pumps consist of three proteins: (1) a protein transporter of the cytoplasmatic membrane that uses energy in the form of proton motive force, (2) a periplasmic connective protein, and (3) an outer membrane porin (Lambert, 2002).

3. Production of antibiotic – inactivating enzymes

These bacteria have the ability to produce chromosomal – encoded and inducible AmpC β – lactamases. These are cephalosporinases that hydrolyze most β – lactams and are not inhibited by the β – lactamase inhibitors.

Despite the intrinsic resistance of *P. aeruginosa* to many antimicrobials, some antibiotics are active against this bacterium (Moore and Flaws, 2011). Those used more frequently belong to three antibiotic classes: β – lactams, Quinolones and Aminoglycosides. Quinolones are synthetic antimicrobials that block DNA replication by inhibiting the activity of DNA gyrase and topoisomerase IV (Hooper, 1993). The fluorquinolones with anti – pseudomonal activity are ciprofloxacin, levofloxacin and ofloxacin. Aminoglycosides inhibit protein synthesis by binding to the 30S or 50S ribosomal subunit (Dozzo and Moser, 2010). Drugs of this antibiotic class that can be used against *P. aeruginosa* are tobramycin, amikacin and gentamicin.

Isolates that exhibit resistance to virtually all available anti – pseudomonal agents (polymyxins are rarely tested in the clinical laboratory) are increasingly being reported (Sardelic et al., 2003). The appearance of similar 'panresistant' isolates is one of the most worrisome developments in the context of microbial drug resistance, recreating conditions typical of the pre – antibiotic era, and has resulted in the search for anti – pseudomonal agents with alternative mechanisms of action and in the use of polymyxins despite their toxicity (Giamarellou, 2002). Continuous surveillance of susceptibility data in nosocomial institutions is of utmost importance, not only for the determination of

guidelines for empirical routines, but also for early administration of infection control measures. The appearance of multiple *P. aeruginosa* isolates with an unusual susceptibility pattern should immediately alert those responsible for the infection control system to the possibility of a nosocomial outbreak due to an MDR strain, and should thus lead to specific control measures (Luzzaro et al., 2001).

Table 3.1: Commonly used anti-pseudomonal drugs

Antibiotic class	Mechanism of action	Drug
Penicillins	Bacterial cell wall synthesis inhibition	Ticarcillin
Penicillin / β-lactamase inhibitor	Bacterial cell wall synthesis inhibition	Ticarcillin/Clavulanic acid
		Piperacillin/Tazobactam
Cephalosporins	Bacterial cell wall synthesis inhibition	Ceftazidime
		Cefepime
Monobactams	Bacterial cell wall synthesis inhibition	Aztreonam
Carbapenems	Bacterial cell wall synthesis inhibition	Imipenem
		Meropenem
		Doripenem
Fluoroquinolones	Block of DNA synthesis	Ciprofloxacin
		Levofloxacin
		Ofloxacin
Aminoglycosides	Protein synthesis inhibition	Gentamycin
		Tobramycin
		Amikacin

Source: MLA 8th Edition

Acquired resistance of *P. aeruginosa*

Despite of being resistant to a variety of antimicrobial agents, *P. aeruginosa* develops resistance to anti – pseudomonal drugs (β – lactams, Quinolones and Aminoglycosides) as well. This acquired resistance is an outcome of mutational changes or the acquisition of resistance mechanisms via horizontal gene transfer. Mutational events may result to

over – expression of endogenous β – lactamases or efflux pumps, reduced expression of specific porins and target site modifications while gaining of resistance genes mainly refers to transferable β – lactamases and aminoglycoside – modifying enzymes.

Table 3.2: Resistance mechanisms of *P. aeruginosa* to anti – pseudomonal drugs

Resistance to	Resistance mechanism
β – lactams	Endogenous β – lactamases
	Acquired β – lactamases
	Efflux
	Diminished permeability
Fluoroquinolones	Target site mutations
	Efflux
Aminoglycosides	Aminoglycoside – modifying enzymes
	Efflux
Polymyxins	LPS modification

Source: cdn.intechopen.com

Emergence of resistance to β – lactam antibiotics began even before the first β-lactam penicillin was developed (Mandell et al., 2005).

3.2 β – Lactam Antibiotics

Penicillin, the first β – lactam was discovered by Alexander Fleming in 1928. Since then various additional antibiotics were discovered. β – lactams class comprises the largest group. The group of β – lactam antibiotics includes penicillins, cepholosporins, monobactams and carbapenems. The β – lactams that are most active against *P. aeruginosa* are: Piperacillin and ticarcillin (penicillins), ceftazidime (3rd generation cephalosporin), cefepime (4th generation cephalosporin), aztreonam (monobactam), imipenem, meropenem and doripenem (carbapenems). The popularity of these agent results from their bactericidal action and lack of toxicity to humans, and also their molecular structures can be manipulated to achieve greater activity for wider therapeutic applications (Winn et al., 2006). β – lactams bind to and inactivate penicillin – binding proteins (PBPs) that are transpeptidases involved in bacterial cell wall synthesis (Tipper, 1985).

3.2.1 Structure of β – Lactams

β – lactams belong to a family of antibiotics which is characterized by a β – lactam ring. β – lactam antibiotics contain four membered, nitrogen containing, β – lactam rings at the core of their structure (Forbes et al., 2007). The integrity of the β – lactam ring is necessary for the activity which results in the inactivation of a set of transpeptidases that catalyze the final cross – linking reactions of peptidoglycan synthesis.

Various β-lactam classes are as follow:

Penicillins –

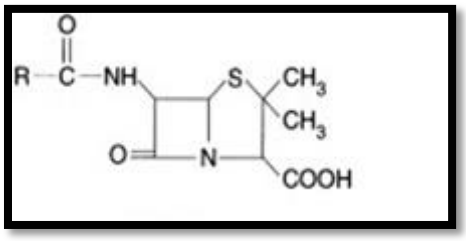


Fig. 3.1 Structure of Penicillin

Example: Penicillin, Ampicillin, Piperacillin, mezlocillin

Cephalosporins-

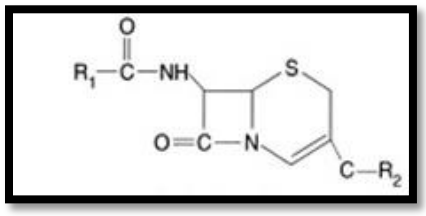


Fig. 3.2 Structure of Cephalosporin

Example: Cefazolin, Cefuroxime, Cefotetan, Cefotaxime, Ceftriaxone, Ceftazidime, Cefepime

Monobactams –

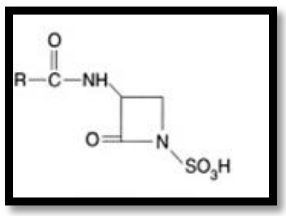


Fig. 3.3 Structure of Monobactams

Example: Aztreonam

Carbapenems –

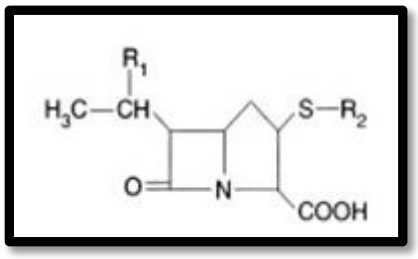


Fig. 3.4 Structure of Carbapenem

Example: Imipenem, Meropenem

3.2.2 Mechanism of Action of β-lactams

β – lactam antibiotics exert their antimicrobial effects by interfering with the formation of peptidoglycan. β – lactam antibiotics target the penicillin – binding proteins or PBPs - a group of enzymes found anchored in the cell membrane, which is involved in the cross – linking of the bacterial cell wall. The β – lactam ring portion of this group of antibiotics binds to these different PBPs, rendering them unable to perform their role in cell wall synthesis. It was recognized that a structural similarity between the Penicillin molecule and the D – alanine – D – alanine terminus of the peptidoglycan chain was integral to the antibacterial action of the compound (Forbes et al., 2007). Once penicillin inserted to the penicillin binding protein (PBP), the penicillin molecule abrogates further elongation of the peptidoglycan by blocking transpeptidation (Winn et al., 2006). Because of differences among bacteria in their PBP content, natural structural characteristics and their common antimicrobial resistance mechanisms, the effectiveness of β – lactams against different types of bacteria can vary widely (Mandell et al., 2005).

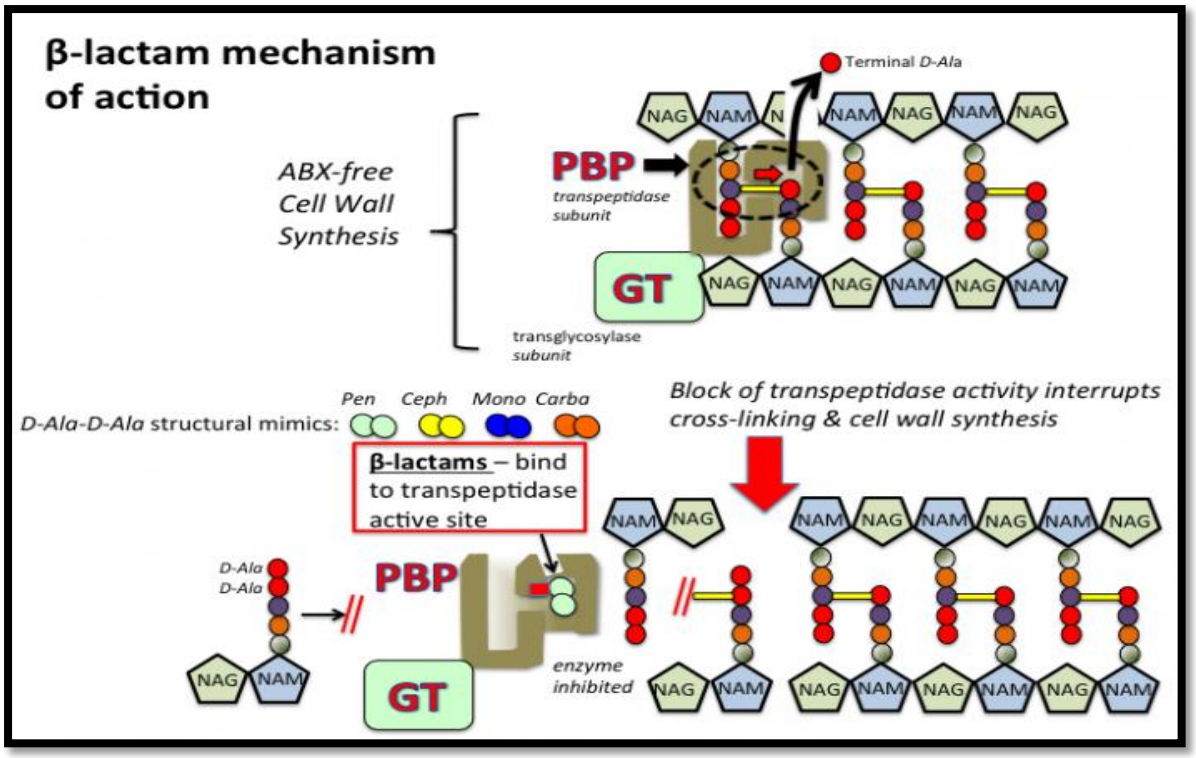


Fig 3.5: Mechanism of action of β-lactam antibiotics

Source- TUSOM/Pharmawiki

3.2.3 Mechanism of resistance to β – lactams

Common mechanism of bacterial resistance to β – lactams is production of enzymes i.e., β – lactamases that bind and hydrolyze these drugs (Rodrigues et al., 2004). Resistance to β – lactams in clinical isolates is primarily due to the hydrolysis of the antibiotic by a β – lactamase. Mutational events resulting in the modification of PBPs (penicillin binding proteins) or cellular permeability can also lead to β – lactam resistance. Among all the mechanisms, β – lactamases production is of great concern. This study deals with the genes responsible for the production of β-lactamases.

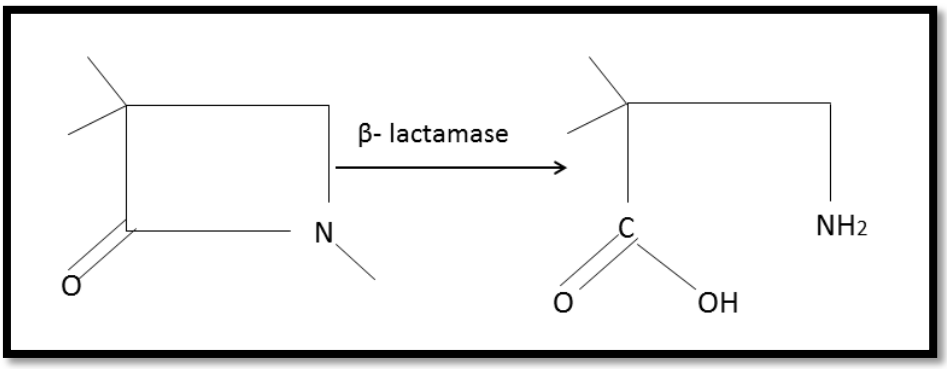
3.3 β – lactamases

β – lactamases are a family of enzymes that range in importance from the almost exclusive mechanism of *Staphylococcal* resistance to penicillin at one extreme to clinically insignificant constituents of the cell wall in some enteric bacteria. These enzymes cleave the amide bond of the β – lactam ring causing antibiotic inactivation. The physiologic role of β – lactamases is to restructure the peptidoglycan during bacterial cell growth. Any β – lactam antibiotic or group of antibiotics may be inactivated by these enzymes (Winn et al., 2006). The first β – lactamase was identified in

Escherichia coli prior to the release of penicillin for use in clinical practice (Bradford, 2001). The number of enzymes now exceeds 340 and the growth spurt shows no signs of slowing (Chaudhary and Aggarwal, 2004). Many genera possess naturally occurring, chromosomally mediated β – lactamases. These enzymes are thought to have evolved from penicillin binding proteins, with which they show some sequence homology (Knox, 1995). β – lactamases encoded by plasmids are of special concern because, the possibility of spread among different strains and different species of pathogens increases (Forbes et al., 2007).

3.3.1 Action of β – Lactamases

β – lactamases attack the amide bond in the β – lactam ring of penicillins and cephalosporins, with subsequent production of Penicillinoic acid and cephalosporic acid, respectively, ultimately rendering the compounds inactive (Chaudhary and Aggarwal, 2004). A few β – lactamases utilize zinc ions to disrupt the β – lactam ring, but a far greater number operate via the serine ester mechanism.



β-lactam β-lactam not able to bind PBPs

Fig 3.6: Action of β-lactamase in β-lactam ring

3.3.2 Contribution of β – lactamases to β – lactam antibiotic resistance (Forbes et al., 2007)

The level of antibiotic resistance mediated by particular β – lactamases in a population of bacteria is determined by several variables.

1. The efficiency of the β-lactamases in hydrolyzing the antibiotic.
2. Its affinity for the antibiotic.

3. Amount of β-lactamases produced by the bacterial cell.
4. The susceptibility of the target protein (PBP) to the antibiotic.
5. Rate of diffusion of the antibiotic into the periplasm of the cell.

3.3.3 Classification of β – lactamases

Various classification schemes have been proposed by many researchers. Classification of Sawai et al in 1968 was based on responses to antisera, Richmond and Sykes scheme by Sykes and Mathew in 1976 was based on differentiation by Isoelectric focusing, the groupings proposed by Bush in 1989 were based on correlation of substrate and inhibitory properties with molecular structure (Chaudhary and Aggarwal, 2004). However, the number and variety of enzymes have proliferated beyond the boundaries of the scheme. A more modern scheme based on molecular structure classification was proposed by Ambler includes, of necessity, only those enzymes that have been characterized.

3.3.3.1 Ambler Classification (Mandell et al., 2005) and (Winn et al., 2006)

This classification scheme includes four evolutionary distinct classes of β – lactamases. The enzymes fall into four classes on the basis of their sequence homology.

a) Class A β – lactamases

These have a molecular weight around 29000, possess a serine residue at their active site and preferentially hydrolyze penicillins. Example: TEM (Temoniera), SHV (sulphydral variable), GES, CTX and KPC (*K. pneumoniae* carbapenemase).

b) Class B β – lactamases

These enzymes are metallic enzymes that have zinc binding thiol group required for β – lactamase activity. These enzyme producers are resistant to Carbapenems, Oxyimino– Cephalosporins, β – lactamase inhibitors except Aztreonam. Example: IMP (Imipenemase), VIM (Verona integron-encoded metallo-β-lactamase), SPM (Sao Paulo metallo – β – lactamase), GIM (Germany Imipenemase), NDM (New Delhi Metallo β-lactamases), etc.

c) Class C β – lactamases

These enzymes are large proteins (molecular weight 39000) with mainly cephalosporinase activity. They also have serine at the active site. The tertiary structure of class C β – lactamases show striking similarities to penicillin binding proteins from which they might have evolved. Example: AmpC – type.

d) Class D β-lactamases

These β-lactamases are oxacillin hydrolyzing enzymes. Example: OXA (oxacillinase)

In this classification, class A, C and D enzymes employ serine as the reactive site to attack the β – lactam bond of penicillins, cephalosporins and carbapenems while class B (metallo – β – lactamases) requiring zinc ions for their activity.

3.3.3.2 Bush – Jacoby – Medeiros Classification (Winn et al., 2006)

The enzymes are categorized on the basis of their substrate spectrum and responses to inhibitors into a larger number of functional groups. The Bush Jacoby – Medeiros scheme puts 178 β – lactamases from naturally occurring bacterial isolates into four groups based on substrate and inhibitor profiles.

3.3.4 Expression of endogenous β – lactamases

Resistance to β – lactams in clinical isolates is commonly due to the presence of AmpC β – lactamases. AmpC enzymes are not carbapenemases, they possess however a low potential of carbapenem hydrolysis and their overproduction combined with efflux pumps over – expression and/or diminished outer membrane permeability has been proven to lead also to carbapenem resistance in *P. aeruginosa* (Arora and Bal, 2005).

3.3.4.1 AmpC β – lactamases

Amp C β – lactamases are Group 1 cephalosporinases that confer resistance to a wide variety of β – lactam antibiotics including alpha methoxy β – lactams such as cefoxitin, narrow and broad spectrum cephalosporins, aztreonam, and are poorly inhibited by β – lactamase inhibitors such as clavulanic acid (Subha et al., 2003). Genes for Amp C β – lactamases are commonly found on the chromosome of the several members of the family Enterobacteriaceae.

Plasmid mediated Amp C β – lactamases were first reported in 1988. These enzymes have arisen through the transfer of chromosomal genes for the inducible Amp C β-lactamase onto plasmids (Thomson, 2001). This transfer has resulted in plasmid mediated Amp C β – lactamases in isolates of *E. coli*, *Klebsiella pneumoniae*, *Salmonella* spp*, Citrobacter freundii, Enterobacter aerogenes* and *Proteus mirabilis*. These enzymes differ from chromosomal Amp C, in being uninducible and are typically associated with broad multidrug resistance.

Plasmid mediated Amp C β – lactamases represent a new threat and this resistance mechanism found around the world, can cause nosocomial outbreaks. These enzymes are also known as imported, transmissible, foreign (Thomson, 2001) or mobile Amp C β – lactamases.

3.3.5 Acquired β – lactamases

Acquired β – lactamases are typically encoded by genes which are located in transferable genetic elements such as plasmids or transposons (Giedraitiene et al., 2011) often on integrons (Poirel and Nordmann, 2002). Integrons are genetic elements that capture and mobilize genes (Cambray et al., 2010). Other genetic elements associated with transferable resistance in *P. aeruginosa* are the mobile insertion sequences called ISCR elements (Poirel et al., 2004). Different types of transferable β – lactamases have been found in clinical *P. aeruginosa* isolates around the world. Among them, carbapenemases are of major clinical importance because they inactivate carbapenems together with other β – lactams. Ambler class A ESBLs hydrolyzes penicillins, narrow – and broad – spectrum cephalosporins and aztreonam (Paterson and Bonomo, 2005). Some TEM and SHV enzymes do not possess broad – spectrum cephalosporinase activity and are called restricted – spectrum β – lactamases. Class D OXA β – lactamases are a heterogenous group of enzymes and not all share the same properties. Generally, most of them show a preference for cloxacillin over benzylpenicillin. They confer resistance to amino - and carboxypenicillins and narrow – spectrum cephalosporins even though some of them are ESBLs and a few members of the class present carbapenemase activity (Poole, 2011).

The first plasmid mediated β – lactamase TEM – 1 was originally isolated from blood culture of a patient named Temoniera in Greece in the early 1960s, hence the designation TEM (Bradford, 2001). Being plasmid and transposon mediated, it has facilitated the spread of TEM – 1 to other species of bacteria (Sharma et al., 2008). Within a few years after its first isolation, the TEM – 1 β – lactamase spread worldwide and is now found in many different species of members of the family Enterobacteriaceae, *Pseudomonas aeruginosa*, *Haemophilus influenza* and *Neisseria gonorrhea* (Bradford, 2001).

Over last 20 years, many new β – lactam antibiotics have been developed that were specifically designed to be resistant to the hydrolytic action of β – lactamases. However, with each new class that has been used to treat patients, new β – lactamases emerged that caused resistance to that class of drug (Bradford, 2001). The selective pressure due to the use and overuse of new antibiotics in the treatment of patients has selected for new variants of β – lactamases. One of these new classes was the oxyimino –

cephalosporins, which became widely used for the treatment of serious infections due to gram negative bacteria in the 1980s. Resistance to these expanded spectrum β – lactam antibiotics due to β – lactamases emerged quickly. The first of these enzymes capable of hydrolyzing the newer β – lactams, SHV-2, was found in a single strain of *Klebsiella ozaenae* isolated in Germany. Because of their increased spectrum of activity, especially against the oxyimino cephalosporins, these enzymes were called extended spectrum β-lactamases. Today, over 180 different ESBLs have been described. Amp C class β – lactamases can be differentiated from other ESBLs by their ability to hydrolyze cephamycins as well as other extended spectrum cephalosporins. Metallo – β – lactamases will hydrolyze virtually all classes of β – lactams including carbapenems (Walsh, 2005).

3.3.6 Metallo β – lactamases (MBLs)

Metallo β – lactamases (MBLs), also called class – B carbapenemases or the Ambler class B β – lactamases has the capacity to hydrolyze with the exception of aztreonam, all β – lactams including carbapenems (Mathur et al., 2008). The class B carbapenemases that bear Zn^{2+} in their active center are the most frequent around the world in *P. aeruginosa* isolates and are called metallo – β – lactamases (MBLs). MBLs were first formally categorized from serine β – lactamases in 1980 (Ambler, 1980). In recent years MBL genes have spread from *P. aeruginosa to* Enterobacteriaceae. These enzymes require divalent cations, usually zinc, as metal cofactors for enzyme activity (Umadevi et al., 2011)

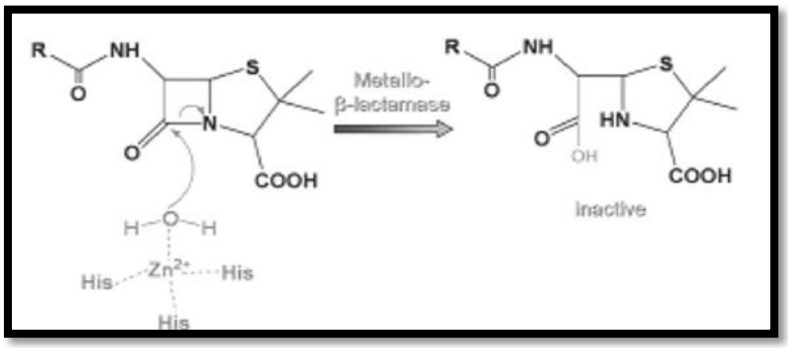


Fig 3.7: Mechanism of the hydrolysis of β-lactam antibiotics through metallo-β-lactamases.

Strains with MBL are not susceptible to therapeutic serine – β – lactamase inhibitors such as clavulanate and sulfones (Tan et al., 2008). MBLs, like all β – lactamases, can be divided into those that are normally chromosomally mediated and those that are

encoded by transferable genes. At the molecular level, attempts have been made to subdivide class B enzymes based on sequence identity and other structural features into 3 subgroups (Walsh et al., 2005)

Class B 1:

The rationale is that the enzymes possess the key zinc coordinating residues of three histidines and one cysteine and accommodates the transferable MBLs, IMP, VIM, GIM and SPM – 1.

Class B 2:

Include those that possess an asparagine instead of the histidine at the first position of the principal zinc – binding motif, NXHXD, and derive from *Aeromonas* spp, the *Serratia fonticola*. Example: enzyme SFH – 1.

Class B 3:

MBL L1 (from *Stenotrophomonas maltophilia)* the sole occupant, as it is singularly unique among all β-lactamases in being functionally represented as a tetramer.

3.3.6.1 Biochemistry of MBLs

MBLs possess a distinct set of amino acids that define the finite architecture of the active site which coordinates the zinc ions. The zinc ions in turn usually coordinate two water molecules necessary for hydrolysis. The principal zinc – binding motif is histidine – X – aspartic acid (HXHXD) which is common to most MBLs. The proposed mechanism of hydrolysis suggests that the active site orients and polarizes the β – lactam bond to facilitate nucleophilic attack by zinc – bound water or hydroxides (Walsh, 2005).

3.3.6.2 Metallo β – lactamases (MBLs) in *Pseudomonas aeruginosa*

The most notable of the acquired MBLs, the IMP – and VIM – type enzymes were first detected in the early 1990s (Watanabe et al., 1991). Thereafter, many additional types of acquired MBLs have been reported, including the SPM-, GIM-, SIM-, KHM-, NDM-, AIM-, DIM-, SMB-, TMB-, and FIM-type enzymes (Wachino et al., 2011). Since the discovery of IMP-1, which was the first MBL identified in *P. aeruginosa*, IMP-, VIM-, SPM-, GIM-, NDM- and FIM- type variants in *P. aeruginosa* have been reported steadily.

3.3.7 Transferable MBLs

3.3.7.1 IMP – type MBLs (Imipenemase)

The first indication of mobile MBLs was with the discovery of *P. aeruginosa* strain GN 17203 in Japan in 1988 (Watanabe et al., 1991). The resistance allele was found on a transferable conjugative plasmid that could be readily mobilized to other *Pseudomonas* strains. Thereafter, it was identified in many other species suggesting horizontal gene transfer of *bla*IMP – 1 between unrelated Gram-negative species (Queenan and Bush, 2007). Currently 33 of the 51 known IMP variants have been identified from *P. aeruginosa*, including the recent detection of IMP-8 producing strains in Germany (Potron et al., 2015).

3.3.7.2 VIM – type MBLs (Veronese imipenemase)

The second dominant group of acquired MBLs is the VIM – type enzymes. VIM – 1 was described first in Verona, Italy, from a *P. aeruginosa* isolate (Lauretti et al., 1999). This clinical isolate, recovered in 1997, was resistant to a series of β – lactams, including piperacillin, ceftazidime, imipenem and aztreonam. Analysis of the strain revealed a carbapenem hydrolyzing activity that was inhibited by EDTA and restored upon addition of Zn. There are total about 13 VIM – type MBLs. Currently, VIM – 2 is the most widespread MBL in *P. aeruginosa* and has been the source of multiple outbreaks (Walsh, 2005).

3.3.7.3 SPM (Sao Paulo metallo – β – lactamase)

Found in *P. aeruginosa* isolated from a blood stream infection in a 4 year old leukemic girl in 1997 from Sao Paulo (Gales, 2003). The sequence of SPM – 1 differs significantly from that of both IMP and VIM. The *bla*SPM-1 gene has been reported in *Pseudomonas spp*. isolated from various hospitals in Brazil. The *bla*SPM-1 gene is either chromosomal or plasmid-encoded (Hong et al., 2015).

3.3.7.4 GIM (German imipenemase)

It was first identified in Germany in 2002 from *P. aeruginosa* isolates (Hong et al., 2015). GIM – 1 possesses the major consensus features of the MBL class B1 family, such as the principal zinc – binding motif (HXHXD), and has been shown to contain two zincs at its active site. GIM – 1 demonstrates a hydrolytic profile similar to that of IMP – 1 but is arguably a weaker enzyme (Walsh, 2005). To date, there have been no reports of GIM – 1 producing strains outside of Germany.

3.3.7.5 NDM (New Delhi Metallo β-lactamases)

Enterobacteriaceae with NDM – 1 carbapenemases are highly resistant to many antibiotic classes and potentially herald the end of treatment with β – lactams, fluoroquinolones and aminoglycosides (Kumaraswamy et al., 2010). The new type of carbapenem resistance gene is designated *bla* carried on plasmids. NDM – 1 was first isolated in *Klebsiella pneumoniae* and *Escherichia coli* recovered from a patient who was previously admitted to a hospital in New Delhi, India, in 2009 (Yong et al., 2009). NDM – 1 producing *P. aeruginosa* strains were first reported in 2011, with two isolates recovered from Serbia (Jovcic et al., 2011). In 2012, NDM-1-producing *P. aeruginosa* sequence type 235 (ST235) strain was isolated in France from a patient previously hospitalized in Serbia (Janvier et al., 2013). Since then, NDM – 1 – positive *P. aeruginosa* isolates have been recovered throughout the world including India, Italy, Egypt, and Slovakia (Hong et al., 2015). NDM – 1 positive isolates are susceptible to colistin and tigecycline (Kumaraswamy et al., 2010).

3.3.7.6 FIM (Florence imipenemase)

In 2012, a novel FIM-1 MBL was isolated from a MDR *P. aeruginosa* in Florence (Italy). This isolate belonged to the ST235 epidemic clonal lineage (Pollini et al., 2012). Anaylsis of the kinetic parameters shows that FIM – 1 has broad substrate specificity, especially with penicillins and carbapenems (Hong et al., 2015).

3.3.8 Genetic apparatus of transferable MBLs

The genes encoding β – lactamases can be located on the bacterial chromosome, on plasmids, or transposons. The genetic environment of β – lactamase (*bla*) gene dictates whether the β – lactamases are produced in constitutive or inducible manner. Some enzymes of subclass B1 (metallo – β – lactamases) have been found on plasmids and part of transmissible genetic elements called integrons. Many studies characterizing of MBLs *bla* genes have found them inserted into common class I integron (Brizio et al., 2006). These integrons are responsible for transfer of *bla* gene among divergent species of Gram – negative bacteria. Recently, an increasing number of *bla* genes are being discovered on integrons (Weldhagen, 2004). Integrons are not mobile but their location in mobile genetic elements (plasmids, transposons) enables their movement. The transferable metallo – β – lactamases are commonly encoded by genes carried by type 1 or type 3 integrons. These integrons could be carried by large plasmids or be located on the chromosome (Walsh, 2005).

The majority of MBL genes (IMP – type or VIM – type) are mobilized by integrons and/or transposons, a minority appear to be mobilized with mobile common regions (CR) that have also been associated with other mobile elements called SXT regions. Genes encoding IMP – and VIM – type as well as GIM – 1 are found as gene cassettes in class I integrons. IMP MBL genes are also found in class 3 integrons. The majority of MBL genes are found on plasmids usually between 120 and 180 kb (Walsh, 2005). The gene encoding SPM – 1 enzyme is associated with two different types of CR (ISCR - Insertion Sequence Common Region) element (Toleman et al., 2006). The gene *bla*SPM-1 is not part of a gene cassette, nor is it found in the vicinity of class 1 integron as found other metallo – β – lactamases genes. ISCR, a new type of genetic element, was recently identified as being closely associated with spread of many antibiotic resistance genes. Toleman et al. has detected ISCR elements in several strains of *P. aeruginosa* (Toleman et al., 2006). ISCR2 was discovered in *P. aeruginosa* isolate that harbored *bla*IMP – 1 and ISCR3 was discovered in two of *P. aeruginosa* strains that have *bla*VIM – 1 (Khan, 2009). Different types of transferable β – lactamases have been found in clinical *P. aeruginosa* isolates around the world.

3.4 Epidemiology of carbapenem – resistant *P. aeruginosa*

With the spread of resistant strains in hospitals and community all over the world and the incidence of organisms producing ESBLs, Amp C, MBLs is also being continuously increasing throughout the world. With limited treatment alternatives, it becomes necessary to know the prevalence of these organisms, responsible resistance mechanisms and to formulate treatment policy (Agrawal et al., 2008). Since carbapenem is also called the last resort antibiotic, resistance to carbapenems is particularly challenging in clinical settings.

Risk factors for colonize/infection with β-lactamase producing organisms (Chaudhary, 2004).

1. Long term antibiotic exposure/therapy
2. Prolonged ICU stay
3. Nursing home residency
4. Severe illness.
5. Residence in an institution with high rates of Ceftazidime and other third generation cephalosporin use.
6. Instrumentation or Catheterization.

Among the various antimicrobial resistance mechanisms, the production of carbapenemase is one of the most important mechanisms by which *P.*

aeruginosa acquires carbapenem resistance. Many carbapenemases have been identified in *P. aeruginosa*, including KPC and GES variants of Ambler class A, IMP-, VIM-, SPM-, GIM-, NDM-, and FIM-type metallo – β – lactamases (MBLs) of Ambler class B, and OXA variant enzymes of Ambler class D, among which production of IMP-, VIM- and NDM- type MBLs is of great concern. According to the recent study done to analyze the global epidemiology of carbapenem-resistant *P. aeruginosa*, the data showed the carbapenem resistance rates in Canada (carbapenem 3.3%) and the Dominican Republic (imipenem and meropenem, both 8%) were the lowest of all countries, with ratios lower than 10%. On the other hand, ratios in Brazil, Peru, Costa Rica, Russia, Greece, Poland, Iran, and Saudi Arabia were higher than 50% in all drugs of the carbapenem class (imipenem, meropenem, doripenem, ertapenem) ranging from 50% to 75.3% (Hong et al., 2015). In some countries, *P. aeruginosa* possessing MBLs constitute nearly 20% of all nosocomial isolates, where as in other countries the number is comparatively low (Walsh, 2005). Various published reports indicate the prevalence of MBLs to range from 7%-65% (Manoharan et al., 2010). Pitout et al., in 2005 studied 241 clinical strains of 49 IMP-nonsusceptible *P. aeruginosa* from the Calgary Health Region isolated from 2002 to 2004. Of these 241 isolates, 110(46%) were MBL positive by phenotypic methods and 107(45%) were PCR positive for MBL genes. They also recommend that all IMP – nonsusceptible *P.aeruginosa* isolates be routinely screened for MBL production using the EDTA disk screen test and that PCR confirmation be performed at a regional laboratory (Pitout et al., 2005). Franklin et al., in 2006 made a study on phenotypic detection of carbapenem susceptible metallo-β-lactamase producing gram negative bacilli in the clinical laboratory by using double disk synergy test and combined disk test. Out of 134 isolates screened 84(62.7%) were MBL producers. Of these 27.2% *Serratia marcescens* , 14.7% *P. aeruginosa*, 7.35% *K. pneumoniae*, 4.41% *E. coli*, 4.41% *Enterobacter cloacae*, 0.73% *K. oxytoca,* 0.73% *Citrobacter koseri*, 0.73% *Acinetobacter junii* and 0.73% *Acinetobacter baumanii* were metallo – β – lactamase producers (Franklin et al., 2006). They concluded that early detection of MBL carrying organisms, including those with susceptibility to carbapenems is of paramount clinical importance, as it allows rapid initiation of strict infection control practices as well as therapeutic guidance for confirmed infection. Irfan et al., in 2008 evaluated frequency of MBLs producing strains among multidrug resistant (MDR) *Acinetobacter* species and *Pseudomonas aeruginosa* in critical care patients using imipenem – EDTA disk method. Of the 90 imipenem resistant isolates of *Acinetobacter* species, 83(96.6%) were MBL producer and of the 25 imipenem resistant *P. aeruginosa* isolates, all the 25(100%) showed metallo-β-lactamases production. Majority of the isolates also showed resistance to aminoglycosides and quinolones, which is a characteristic feature of metallo – β – lactamases producers (Irfan et al., 2008). They concluded that carbapenem

resistance in MDR *Acinetobacter* spp and *Pseudomonas aeruginosa* isolates in this study was due to MBLs, this call for strict infection control measures to prevent further dissemination. The worldwide emergence of carbapenem – resistant strains with similar mobile genetic elements indicates the dissemination of genes encoding carbapenemases through horizontal gene transfer.

3.4.1 Epidemiology of carbapenemase gene in *P. aeruginosa*

Carbapenem resistant *P. aeruginosa* emerged in Japan many years ago. The first 'mobile' MBL of *P. aeruginosa* characterized was IMP-1, discovered in Japan in 1988 (Hirakata et al., 2011). Japan has become a major reservoir for IMP – type metallo – β – lactamases, which now include many variants of enzymes, and these have spread to a number of strains *Pseudomonas* spp., *Acinetobacter* spp., and Enterobacteriaceae. In the recent years, new transferable MBLs have spread rapidly (Pitout et al., 2005). Most of metallo – β – lactamases (class B1) were found in *P. aeruginosa* strains. VIM family of metallo – β – lactamases is the second dominant group. VIM type enzymes demonstrate little amino acid similarity to IMP. VIM – 1 (Verona Imipenemase) was first characterized from a *P. aeruginosa* strain isolated in 1997 in Italy (Lauretti et al., 1999). Since 1995, *bla*VIM positive *P. aeruginosa* strains were isolated in many countries of the world (Walsh, 2005). Similarly, *bla*SPM – 1, *bla*GIM – 1 have been recovered from *P. aeruginosa* from Brazil and Germany respectively. And these genes are more geographically restricted. And then the report for NDM – 1 in *P. aeruginosa* was made from Serbia (Jovcic et al., 2011). Since then, NDM – 1 – producing members of the Enterobacteriaceae have been isolated in various parts of the world, including Australia, Bangladesh, Belgium, Canada, France, India, Japan, Kenya, the Netherlands, New Zealand, Pakistan, Singapore, Taiwan, and the United States (Cornaglia et al., 2011). In addition, isolates producing six NDM variants have been reported, including NDM – 2 – producing *Acinetobacter baumannii* strains from Egypt, Israel, Germany, and the United Arab Emirates, an NDM – 3 – producing *E. coli* strain from Australia (accession no. JQ734687), an NDM – 4 – producing *E. coli* strain from India, an NDM-5-producing *E. coli* strain from the United Kingdom, an NDM-6-producing *E. coli* strain from New Zealand and an NDM-7-producing *E. coli* strain from Canada (Tada et al., 2013). One of the recent study carried in Ethiopia has shown first report on blaNDM-1 producing *Acinetobacter baumannii*, no other bacterial species were found to harbor the gene cassette in that place (Pritsch et al., 2017). Ambler class A carbapenemase KPC was first reported in *P. aeruginosa* isolates in Colombia (Villegas et al., 2007). KPCs present high rates of carbapenem hydrolysis and inactivate all other β – lactams including aztreonam. Enzymes GES belong to the same enzymatic class but their carbapenemase activity are not as high as that of the KPCs. It may become

important however if combined with diminished outer membrane permeability or efflux over – expression. For *P. aeruginosa*, GES-2 has been reported in South Africa (Poirel et al*.,* 2001). Class D carbapenemases like OXA – 198 have been found in *P. aeruginosa* isolates although such findings are rather rare for this species. The most clinically important carbapenemases are summarized in the table 3.3.

Table 3.3: Clinically important carbapenemases found in *P. aeruginosa* isolates

Ambler molecular class	Bush-Jacoby-Medeiros group	Carbapenemases
A	2f	KPC
B	3	IMP enzymes, VIM enzymes SPM-1, GIM-1, AIM-1, NDM-1

Source: cdn.intechopen.com

Among the entire MBLs gene, *bla*NDM-1 gene have been recently detected with many variants in our context (Shrestha B et al., 2015). In the last few years, 17 new variants of NDM-1 have been evolved by changing one or two residues at different positions (Khan et al., 2017) and are shown in table 3.4.

Table 3.4: Genetic variations among the NDM-1 and its variants

NDM-1 variants	Amino acid(s) substitution
NDM-2	Proline 28 to Alanine
NDM-3	Aspartate 95 to Asparagine
NDM-4	Methionine 154 to Leucine
NDM-5	Valine 88 to Leucine Methionine 154 to Leucine
NDM-6	Alanine 233 to Valine
NDM-7	Aspartate 130 to Asparagine Methionine 154 to Leucine
NDM-8	Aspartate 130 to Glycine Methionine 154 to Leucine
NDM-9	Glutamic Acid 152 to Lysine

NDM-10	Arginine 32 to Serine Glycine 36 to Aspartic acid Glycine 69 to serine Alanine 74 to threonine Glycine 200 to Arginine
NDM-11	NA
NDM-12	Glycine 222 to Aspartic acid Methionine 154 to Leucine
NDM-13	Aspartic acid 95 to Asparagine Methionine 154 to Leucine
NDM-14	Aspartic acid 130 to Glycine
NDM-15	Alanine 233 to valine Methionine 154 to Leucine
NDM-16	Arginine 264 to Histidine
NDM-17	Valine 88 to Leucine Methionine 154 to Leucine Glutamic acid 170 to Lysine

Source: BMC Microbiology

The possible reason for growing trends of new markers is mutations, which may cause delaying in the discovery of new antibiotics for treatments and hence became a great public threat (Gould and Bal, 2013).

NEPALESE SCENARIO

The study on the prevalence of Multi Drug Resistant bacteria have been done in many parts of Nepal. Data on antimicrobial susceptibility profile of *P. aeruginosa* is limited in Nepal. One of the studies conducted in Kathmandu Medical College Teaching Hospital, Sinamangal / Duwakot, Kathmandu, Nepal, showed a majority of isolates of *P.aeruginosa* from specimens of pus, sputum, urine and tracheal aspirates. Resistance rates to Cotrimoxazole, piperacillin, ceftriaxone and chloramphenicol varied from 51.00% to 73.00% (Chander and Raza, 2013). Similar study done in Seti Zonal Hospital, Dhangadi, Nepal showed *E. coli* to be the most predominant organisms in hospital

setting causing urinary tract infection followed by *K. pneumonia*, *P. aeruginosa, Proteus vulgaris, Staphylococcus aureus* and *Proteus mirabilis*. In this study, out of 98 isolates, 42 (42.86%) isolates were found to be MDR, 48.08% of the *E. coli*, 19.05% of the *K. pneumonia,* 50% of the *P. aeruginosa*, were found to be MDR (Awasthi et al., 2015). So the data shows the increasing frequency in MDR strains of bacteria which is the serious issue and cannot be neglected. Another research regarding study of ESBLs done in Kathmandu Model Hospital during 2012 showed among 219 bacterial isolates, 41.1% isolates were MDR and in this study, ESBL production was detected in 55.2% of a subset of MDR *E. coli* isolates. Among the 29 MDR *E. coli* isolates, plasmids of size ranging 2-51 kb were obtained with different 15 profiles (Baral, et al., 2012). There are very limited data for the molecular detection of MBL genes in our context. So, molecular level analysis has to be done to know the exact mechanism or cause for the drug resistance. Recently some of the molecular studies regarding the MBL genes have been carried out. A novel metallo-β-lactamase, NDM-8, was identified in a multidrug-resistant *Escherichia coli* isolate, IOMTU11 (NCGM37), obtained from the respiratory tract of a patient in Nepal. The isolates *E. coli* IOMTU11 (NCGM37) and *Pseudomonas aeruginosa* IOMTU9 (NCGM1841) were positive for *bla*NDM. Sequence analysis showed that the *bla*NDM of *E. coli* IOMTU11 was a novel variant, and it was designated *bla*NDM-8 and *P. aeruginosa* IOMTU9 had *bla*NDM-1 (Tada et al., 2013). This was the first report describing NDM-1- and NDM-8-producing Gram-negative pathogens in Nepal. Later again the new variant *bla*NDM-12 in *E. coli* IOMTU388.1 was also identified by them (Tada et al., 2014). Similar type of study conducted in Kathmandu Medical College and Teaching Hospital, Nepal, showed the co-existence of *bla*CTX-M, *bla*SHV, *bla*TEM, *bla*IMP, *bla*VIM and *bla*NDM-1 β-lactamases in one ESBL *E. coli* isolate (Pokhrel et al., 2014). For the first time in Nepal, this study described high prevalence of *bla*CTX-M-type ESBL and co-existence of ESBLs and carbapenemases. Later again, from the sample of TUTH, new variant of NDM, *bla*NDM-13 was identified in *E. coli* IOMTU558. And no other genes tested (*bla*DIM, *bla*GIM, *bla*IMP, *bla*SIM, *bla*SPM, and *bla*VIM) were amplified (Shrestha et al., 2015). Another recent study carried in Thailand, showed the co-existence of *bla*OXA-23 and *bla*NDM-1 genes in *Acinetobacter baumannii* isolated from Nepal. Of total 44 isolates analyzed, 43 were carbapenem resistant isolates with presence of *bla*OXA-23 gene in all of them while *bla*NDM-1 was detected in 6 isolates concluding the high prevalence of *bla*OXA-23 gene in tertiary care hospital in Nepal (Joshi et al., 2017).

Majority of findings showed the MBL production in a considerable number of *P. aeruginosa*, *A. baumannii* and other strains of Enterobacteriaceae isolates with MDR phenotype. This type of studies and surveillance programs have to be conducted timely

for establishing the effective antibiotic policies and infection control strategies in the hospital setting. Various phenotypic and genotypic detection methods are available for the detection of the ESBLs and MBLs. Combined disc method can provide a sensible choice for phenotypic detection of MBL production in clinical microbiology laboratories. And PCR method is the most for the detection and confirmation of the responsible gene causing multidrug resistance.

3.5 Methods of Detection

Since the prevalence of *P. aeruginosa* and other Enterobacteriaceae producing ESBLs and MBL is increasing, it is mandatory that the routine clinical microbiology laboratory must employ detection methods for these enzymes, which are sensitive enough to recognize the level of resistance that would be achieved by the situation given in vivo. In the year since these enzymes were first described, a number of different testing methods have been suggested.

3.5.1 ESBL detection methods

The phenotypic tests for ESBL are done to the organisms showing reduced susceptibility to cefotaxime, ceftriaxone, ceftazidime or aztreonam. The test employ a β-lactamase inhibitor usually clavulanate, in combination with an oxyimino-cephalosporin such as ceftazidime or cefotaxime. In these tests, the clavulanate inhibits the ESBL, thereby reducing the level of resistance to the cephalosporin. Several tests that have been proposed are based on the Kirby-Bauer disk diffusion test methodology (Bradford, 2001).

3.5.1.1 Combined disk method

This method depends on comparing the inhibition zone given by disks containing an indicator cephalosporin with and without clavulanic acid. As explained in CLSI guideline, 10 µg of clavulanic acid added to cefotaxime (30 µg) and/or to ceftazidime (30 µg) disk. If an ESBL is produced, the zone diameter given by the disk with clavulanic acid are ≥ 5mm larger than those without the inhibitor at least for one of the combinations (Bradford, 2001).

3.5.1.2 Double disk approximation test/ Double disk synergy test

This was the first detection method described by Jarlier et al. in 1988 (Jarlier et al., 1988). In this test the organism is swabbed on a MHA plate. A susceptibility disk

containing amoxicillin-clavulanate is placed in the center of the plate, and disk containing one of the oxyimino-lactam antibiotics are placed center to center from amoxicillin-clavulanate disk. Enhancement on zone of inhibition of the oxyimino β-lactam caused by the synergy of the clavulanate in the amoxicillin-clavulanate disk is the positive test result.

3.5.1.3 ESBL E-test

The ESBL E-test strip carries two gradients, on the one end ceftazidime and on the opposite end ceftazidime plus clavulanic acid. MIC is interpreted as the point of intersection of the inhibition ellipse with the E-test strip edge. Ratio of ceftazidime MIC and ceftazidime clavulanic acid MIC equal to or greater than 8 indicates the presence of ESBL (Rawat & Nair, 2010).

Of the test that has been developed to date, the double disk approximation test is the easiest and the most cost effective methods for use by many clinical laboratories. However none of the detection test that is based on the phenotype of beta-lactamase produced is 100% sensitive or specific for the accurate detection of ESBLs among clinical isolates (Bradford, 2001). The need for improved detection of ESBLs in clinical isolates is essential.

3.5.2 MBL detection methods

Currently there are no CLSI guidelines for screening bacterial isolates for acquired MBL production. And also there is no standardized method for MBL detection, and despite PCR being highly accurate and reliable, its accessibility is often limited to reference laboratories (Walsh, 2005).

Several phenotypic tests have been developed for MBL detection and all these are based upon the ability of chelating agents, EDTA and thiol based compounds, to inhibit MBL activity (Andrade et al., 2007).

Screening of MBL production is done by following methods:

3.5.2.1 Imipenem (IMP) - EDTA combined disk test

In this test, test organisms are inoculated on to plates with MHA as recommended by CLSI. Two 10µg imipenem disks are placed on the plate. And appropriate amounts of 10µl of EDTA solution are added to one of them to obtain the desired concentration (750µg) and incubated for 16 to 18 hrs at 35° C. If the increase in inhibition zone with

the imipenem and EDTA disk was ≥ 7mm than the imipenem disk alone, it was considered as MBL positive (Mathur et al., 2008).

3.5.2.2 Imipenem – EDTA double disk synergy test (DDST)

In this test, the test organisms are inoculated on to plates with MHA as recommended by CLSI. An imipenem (10µg) disk was placed 20mm center to center from a blank disk containing 10µl of 0.5M EDTA (750µg). Enhancement in the zone of inhibition in the area between imipenem and the EDTA disk in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result (Mathur et al., 2008).

3.5.2.3 EDTA disk potentiation using ceftazidime, cefepime and cefotaxime

Test organisms are inoculated on the plates with MHA. A filter paper blank disk is placed and the ceftazidime (30µg), cefotaxime (30µg) and cefepime (30µg) disks are placed 25mm center to center from the blank disk. Ten microliter of 0.5M EDTA solution is added to the blank disk and the plate incubated overnight at 35° C. Enhancement of the zone of inhibition in the area between the EDTA disk and any one of the four cephalosporin disks in comparison with the zone of inhibition of the far side of the drug is interpreted as a positive result (Mathur et al., 2008).

3.5.2.4 MBL E – test

The E test MBL strip containing a double sided seven dilution range of IMP (4 to 256µg/ml) and IMP (1 to 64µg/ml) in combination with a fixed concentration of EDTA has been reported to be the most sensitive format for MBL detection. MIC ratio of imipenem/ imipenem-EDTA of >8 or >3 log 2 dilutions indicates MBL production (Mathur et al., 2008).

3.5.2.5 Hodge test (Lee et al., 2003)

In this test, the indicator organism, *E. coli* ATCC 25922, at a turbidity of 0.5 MacFarland standards, is used to swab the surface of MHA plate, and the test strain is heavily streaked from the center to the plate periphery. After that the plate is allowed to stand for 15 min at room temperature, a 10µg IMP disk is placed at the center, and the plate is incubated overnight. The presence of a distorted inhibition zone is interpreted as a positive result for MBL production. To determine the effect of the zinc ion on the test, a 50 mM zinc sulfate solution ($ZnSO_4 7H_2O$) is added in amounts of 5 to 20 µl to an IMP disk or in amounts 0.5 to 50 µl/ml to the Muller-Hinton agar.

IMP – EDTA combined disk test is frequently used phenotypic detection method for MBL production by *P. aeruginosa* (Mathur et al., 2008). Since the MBL producing strains are increasing frequently, early detection of MBL producing isolates has become crucial for clinical microbiology laboratories. The combined disc test provides a sensible choice for phenotypic detection of MBL production and can be implemented in clinical laboratory on a daily basis. In addition, routine surveillance of MBL producing bacteria is essential for establishing appropriate empirical antimicrobial therapy and limiting their spread in hospital environment.

3.5.3 Molecular detection methods

The phenotypic method described above only presumptively identifies the presence of an ESBL or MBL. Identifying which specific ESBL or MBL is present in clinical isolates is more complicated. There are different molecular methods for identification of specific gene responsible for production of ESBLs and MBLs.

Some of the different molecular methods are:

1. **DNA Probes**

   Early detection of beta-lactamase genes was performed using DNA probes that are specific for some enzymes like TEM and SHV. However using DNA probes are labor intensive.

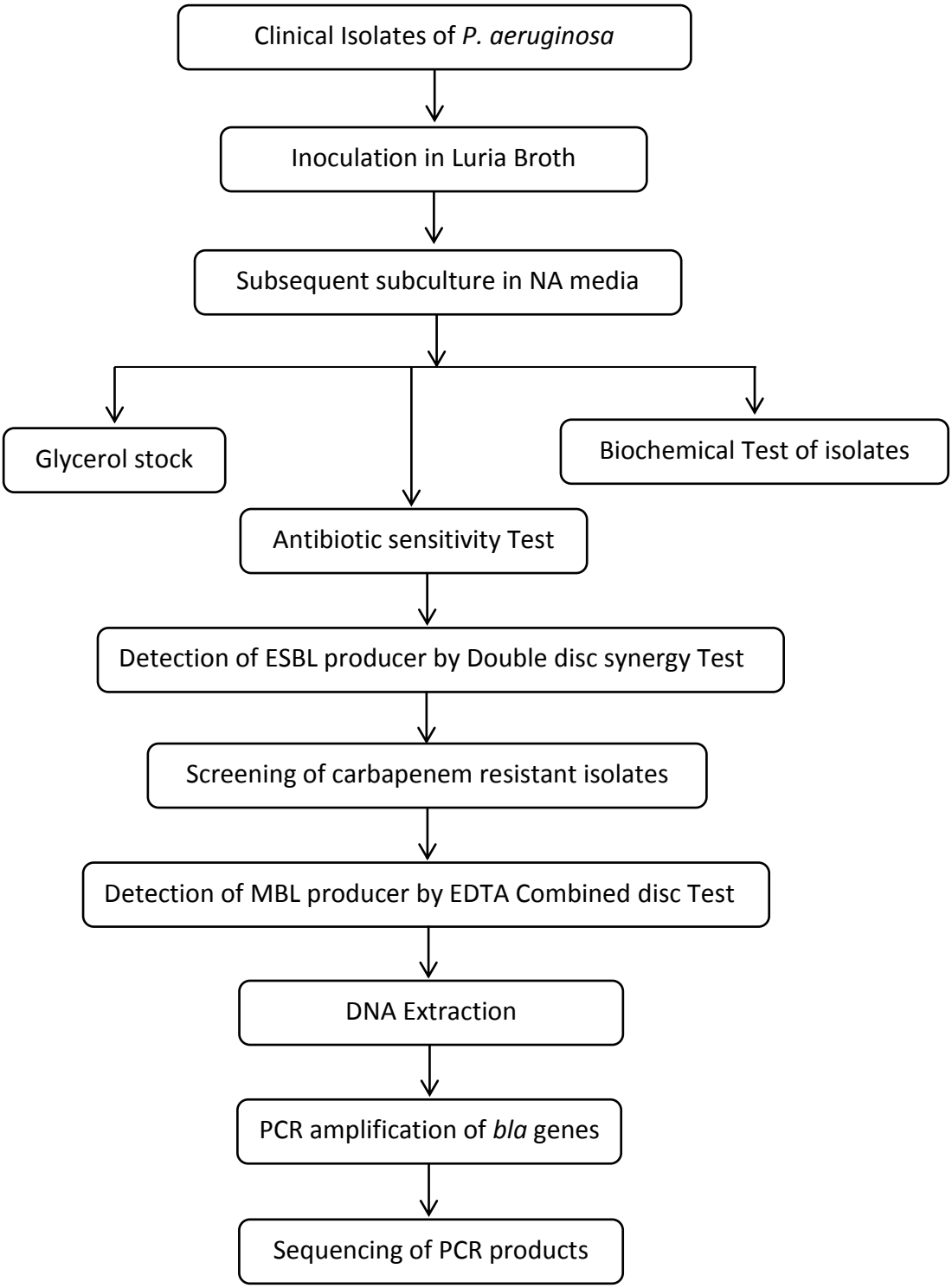
2. **PCR (Polymerase Chain Reaction)**

   The most common molecular method used to detect the presence of beta-lactamase is PCR with oligonucleotide primers that are specific for a beta-lactamase gene.

3. **Nucleotide Sequencing**

   It remains the standard for determination of the specific beta-lactamase gene present in a strain.

CHAPTER 4
MATERIALS AND METHODS



Plan of Work (Indicative methodology)

The present study of "Molecular Analysis of β-Lactamase Gene in Multidrug Resistant Clinical Isolates of *Pseudomonas aeruginosa*" was carried out in the Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, NEPAL from 19th Jan 2016 to 31st Jan 2017.

4.1 MATERIALS

Clinical isolates received for research work from Department of Microbiology, Tribhuvan University Teaching Hospital, Maharajgunj, Kathmandu and Nepal Public Health Laboratory, Teku, Kathmandu, formed the study group.

4.1.1 INCLUSION CRITERIA

Sixty-seven *Pseudomonas aeruginosa* isolated from various clinical samples like pus, blood, urine and sputum were included in the study.

4.1.2 EXCLUSION CRITERIA

All the clinical isolates other than *P. aeruginosa* were excluded from the study.

(All other materials used to accomplish this study are given in the Appendices)

4.2 METHODS

4.2.1 Transportation and Preservation of Isolates

Immediately after collection, clinical isolates were transported to the laboratory held in a cold container. Each isolates were labeled separately and subjected to culture for further study. In some cases when immediate inoculation was not possible, the isolates were preserved at 4° C.

4.2.2 Confirmation of the isolates

The collected isolates were subjected to standard bacteriological techniques to confirm that the isolates were *Pseudomonas aeruginosa*. The bacteria were inoculated in Laurial broth. After 24 hrs of incubation in 37°C, turbid bacterial growth was subjected to streaking on Nutrient Agar (NA). After incubation the bacterial colonies appeared on the plate and these typical isolated colonies were subjected to Gram-staining and conventional biochemical testing for confirmation of *P. aeruginosa* species.

4.2.2.1 Gram's staining

Gram's Staining is one of the basic tools for identification of bacteria strain by distinguishing the difference in the composition of the bacterial cell wall. Confirmation was done based on the colony and morphological characteristic, pigmentation and biochemical properties on respective media according to Bergy's Manual of Systematic Bacteriology, 1986. Biochemical test are based on the ability of microorganisms to produce enzymes responsible to utilize different substrate. The isolated colonies were inoculated into different biochemical media for different tests which are as follows:

4.2.2.2 Biochemical tests performed for confirmation

Table 4.1: Biochemical tests performed

S.N.	Tests	Biochemical Media
1.	Catalase	3% H_2O_2
2.	Oxidase	1% Tetramethyl-p-phenyl diamine dihydrochloride
3.	Indole Production	Sulfide- Indole- Motility medium (SIM)
4.	Methyl Red test	Glucose phosphate peptone water or MR-VP medium
5.	Voges- Proskauer test	Glucose phosphate peptone water or MR-VP medium
6.	Citrate utilization test	Simmon's citrate agar
7.	Fermentation of glucose, lactose, sucrose, H2S and gas production	Triple sugar iron agar (TSIA)
8.	Nitrate Reduction	Nitrate Broth
9.	Urease Production	Urea base agar

4.2.2.3 Preservation of the isolates

The confirmed *P. aeruginosa* isolates were subcultured on Laurial broth and were allowed to incubate at 37°C for 24 hrs. After the turbid growth of bacteria, the bacterial cell covered with Glycerol and was placed in deep freeze (-20°C) for preservation and further use.

4.2.3 Study of antibiotic susceptibility of isolates

Antibiotic susceptibility of isolates was assayed using a modified Kirby-Bauer disc diffusion method (Winn et al., 2006). Cells were grown at 37°C in 5 ml of nutrient broth for about 4 hours. The turbidity developed was compared with that of 0.5 McFarland standards. A sterile cotton swab was dipped into the properly prepared inoculums and firmly rotated against the upper inside wall of the tube to expel excess fluid, and then swabbed onto Muller-Hinton agar. During swabbing the plate was streaked with the swab three times turning the plate 60° between each streaking to achieve a lawn of confluent bacterial growth. The plate was kept at room temperature for 30 minutes for diffusion. Antibiotic disc from their respective vials were carefully placed in the plate with the help of a flamed forceps, at equal distance and sufficiently separated from each other to avoid the overlapping of the inhibition. The discs were lightly pressed with the forceps to make complete contact with the surface of the medium. The plate was allowed to stand at room temperature for 30 minutes for pre diffusion and then incubated at 37°C for 24 hrs. The diameter of the zone of inhibition was measured at the end of the incubation period.

The strength of antibiotic discs used (Discs obtained from Himedia Laboratories Pvt. Ltd, Mumbai)

Amikacin (AK) - 30 µg; Piperacillin (PI) - 100 µg; Ciprofloxacin (CIP) - 5 µg; Levofloxacin (LE) - 5 µg; Ceftazidime (CAZ) - 30 µg; Cefotaxime (CTX) - 30 µg; Meropenem (Mr) - 10 µg; Imipenem (I) - 10 µg; Cefepime (CPM) - 30 µg; Cefoperazone/ Sulbactum (CFS) – 75/10 µg; Piperacillin/Tazobactam –100/10 µg; Polymyxin-B (PB) – 300 Units; Colistin (CL) – 10 µg.

Organisms were classified as sensitive or resistant to an antibiotic according to the diameter of the inhibition zone surrounding each antibiotic disc as listed by manufacturer. The resistance of isolates to multi-drug (resistance showed to at least 3 classes of antibiotic used) was investigated and was grouped under MDR isolates of *P. aeruginosa*. Also, all the isolates were screened for meropenem and imipenem resistance.

Preparation of swab

Cotton wool swab on wooden applicator sticks were prepared. They were sterilized in tubes in the autoclave.

Preparation of 0.5 McFarland standards:

a) Preparation of 1% v/v H_2SO_4
   1ml of conc. H_2SO_4 was added to 99ml of distilled water and mixed well.
b) Preparation of 1% w/v $BaCl_2.2H_2O$
   0.5 gram of $BaCl_2.2H_2O$ was dissolved in 50ml of distilled water.
c) 0.6 ml of $BaCl_2.2H_2O$ was mixed with 99.4 ml of H_2SO_4 prepared were dispensed in capped tube
d) It was stored in well-sealed container in dark at room temperature.

4.2.4 Detection of Extended Spectrum Beta-lactamase (ESBL) production

The initial phenotypic screening for ESBL production was done in the isolates that were resistant to Ceftazidime (CAZ) -30µg and Cefotaxime (CTX) -30µg. These extended spectrum cephalosporin (ESCs) resistant isolates are considered as a potential ESBL producer as recommended by CLSI and can be detected by the following method:

4.2.4.1 Double Disc Synergy Test

ESBL production in extended spectrum cephalosporins resistant isolates was detected by double disc synergy test as described by Jarlier (Jarlier et al., 1988). Synergy was determined between a disc of amoxyclav (20 µg amoxycillin and 10 µg clavulanic acid) and a 30-µg disc of cefotaxime antibiotic placed 15 mm apart on a lawn culture of the isolate under test on Mueller Hinton agar (MHA). The test organism was considered to produce ESBL if the zone size around the antibiotic disc increased towards the amoxyclav disc. This criterion also fulfills the CLSI guidelines. This increase occurs because the clavulanic acid present in the amoxyclav disc inactivates the ESBL produced by the test organism.

4.2.5 Detection of Metallo-β-lactamase (MBL) production

The initial phenotypic screening for MBL production was done in imipenem and meropenem resistant isolates by the following method:

4.2.5.1 EDTA Combined Disc Test

IMP-EDTA combined disc test was done with simultaneous testing of two β-lactams (meropenem and/or imipenem), for detection of MBL in the meropenem and/or imipenem resistant isolates according to Andrade et al. (Andrade et al., 2007). A 0.5 M EDTA solution was prepared by dissolving 186.1 gram of disodium EDTA $2H_2o$ in 1000 ml of distilled water. The pH was adjusted to 8.0 by using NaOH and was sterilized by autoclaving. An overnight liquid culture of the test isolate was adjusted to a turbidity of 0.5 McFarland standard and spread on the surface of MHA plate. A 10µg meropenem/imipenem disc was placed on the agar. Then the combined disc was prepared in the inner surface of the lid of plate, by adding 10 µl of 0.5 M EDTA to 10µg meropenem/imipenem so as to obtain the desired concentration (750 µg) of EDTA. This combined EDTA disc was then transferred to the surface of the agar and was kept 10mm edge to edge apart from the meropenem/imipenem disc. After incubating at 37°C, the presence of an expanded growth of inhibition zone (≥ 7 mm) in combined disc compared to that of meropenem/imipenem disc alone was interpreted as positive for MBL production.

4.2.6 Preparation of Genomic DNA

Bacteria from a saturated liquid culture were lysed and proteins removed by digestion with proteinase K. Cell wall debris, polysaccharides, and remaining proteins were removed by selective precipitation with CTAB, and high-molecular-weight DNA was recovered from the resulting supernatant by using isopropanol precipitation.

I. 5 ml Lauryl broth was inoculated with the bacterial strain of interest and incubated at 37℃ until the culture was saturated.
II. 1.5 ml of the culture was centrifuged for 2 min, or until a compact pellet forms and supernatant was discarded.
III. 567 µL of TE buffer was added to the pelleted cells and resuspended by repeated pipetting.
IV. Then 30 µL of 10% SDS and 3 µL of a 20 mg/mL solution of proteinase K. was added, mixed and incubate for 1 hour at 37°C.
V. After incubation, 100 µL of 5 M NaCl was added and mixed.
VI. Afterwards 80 µL of a CTAB/NaC1 solution (0.7 M NaC1, 10% CTAB) was added and incubated this solution at 65°C for 10 min.
VII. After incubation, equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed.
VIII. The tube was centrifuged for 5 min, and the aqueous solution transferred to a new tube leaving the interface behind.

IX. Another equal volume of PCI was added and mixed well then centrifuged at 14,000 rpm for 5 min and supernatant transferred to a new tube.

X. This first extraction was repeated again using chloroform: isoamyl alcohol alone.

XI. Then 0.6 volumes of isopropanol was added and mixed gently until the DNA precipitated.

XII. The tube was centrifuged to remove isopropanol. And 1 mL of 70% ethanol was added to wash the salt away from the DNA.

XIII. The tube was centrifuged, and the ethanol was discarded, drying on the benchtop at room temperature.

XIV. The pellet was resuspended in 50µL of TE buffer and kept at 4°C.

4.2.7 Agarose Gel Electrophoresis for Genomic DNA

After the extraction of the genomic DNA from each of the isolate, the DNA was subjected to the agarose gel electrophoresis. Agarose gel electrophoresis is an efficient technique to separate DNA molecules according to their molecular weights. 0.8% agarose gel was prepared by dissolving 0.8 gram of agarose (HI Media) in 100 ml 1x TAE buffer and was boiled to dissolve completely. After a bit of cooling, 1.5 µl (0.15 µg/ml) of ethidium bromide was added to it. Gel was poured onto a gel tray and was allowed to set. Then DNA samples were loaded in this gel cassette along with loading dye. Voltage (50/100 Volts) was supplied for about an hour for the electrophoresis. After electrophoresis gel was observed under UV trans illuminator.

4.2.8 Primer Design

The primers were designed manually for MBL gene using gene tool software. The accession no. of the gene used for designing the primer is KF951466.1. They were then tested for various parameters like length of primer, GC content, Tm, self-complementarities, hairpin formation, etc.

Table 4.2: List of Primers used

Target	Primer sequence	Amplicon size (bp)
blaNDM (internal)	F5'-GGCCAGCAAATGGAAACTGG-3' R5'-AATACCTTGAGCGGGCCAAA-3'	460
blaNDM (full length)	F5'-AATGCTGAATAAAAGGAAAACT-3' R5-'GGCAGATTGGGGGTGA-3'	869

4.2.9 PCR Amplification of MBL gene

After the extraction of genomic DNA, the amplification of MBL gene was carried out by polymerase chain reaction (PCR) using gene specific primer. PCR is a scientific technique in molecular biology to amplify a single copy of a piece of DNA to millions of copies of a particular DNA sequence. One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand.

Steps in PCR:

Denaturation: The DNA is denatured at high temperatures (from 90°- 97° Celsius) and double stranded DNA gets separated.

Annealing: Primers attaches to the DNA template to prime extension.

Extension: Extension occurs at the end of the annealed primers to create a complimentary copy strand of DNA.

The molecular size of PCR product was calculated by running them along with ladder by agarose gel electrophoresis and by observing under UV transilluminator.

Table 4.3: Reaction component of PCR for MBL gene, Master Mix (2X) (NewEngland BioLabs):

S.N.	Reagents	Volume (µl)
1.	Template (100ng)	1.0
2.	Forward Primer	1.0
3.	Reverse Primer	1.0
4.	Master Mix	12.5
5.	Nuclease Free Water	9.5

Table 4.4: Reaction component of PCR for MBL gene, Q5'High-Fidelity DNA Polymerase (BioLabs): 25 µL Reaction (for sequencing)

S.N.	Components	Final Conc.	Volume/reaction
1.	5X Q5 Reaction Buffer	1X	5 µl
2.	10 mM dNTPs	200 µM	0.5 µl
3.	10 µM Forward primer	0.5 µM	1.25 µl
4.	10 µM Reverse primer	0.5 µM	1.25 µl
5.	Template DNA (100ng)	<1,000 ng	1.0 µl
6.	Q5 High- Fidelity DNA Polymerase	0.02 U/ µl	0.25 µl
7.	5XQ5 High GC Enhancer(Optional)	1X	5 µl
8.	Nuclease Free Water		To **25 µl**

Table 4.5: Thermal Cycler Condition for different gene

Gene	Initial Denaturation	Denaturation	Annealing	Extension	Final extension	Hold
***bla*NDM (internal)**	95° C, 3 min	95° C, 1 min	50° C, 30 sec	73 ° C, 1 min	72 ° C, 5 min	4° C
		29 cycles				
***bla*NDM (full length)**	95° C, 2 min	95° C, 30 sec	56.7° C, decrease 0.5°C per cycle, 30 sec	72° C, 90 sec	72° C, 5 min	4° C
		14 cycles				
		95 ° C, 30 sec	49.7° C, 30 sec	72° C, 90.0 sec		
		19 cycles				

4.2.10 Sequencing

After performing the polymerase chain reaction for detection of *blaNDM* gene, the PCR products showing the positive result were sent for sequencing. In Sanger sequencing, the target DNA is copied many times, making fragments of different lengths. Fluorescent "chain terminator" nucleotides mark the ends of the fragments and allow the sequence to be determined. In our study, the positive PCR products for sequencing were sent to Xcelris Labs Ltd., Ahmedabad, Gujarat, India.

Sanger sequencing used the principles of DNA replication in the development of the process now known as Sanger dideoxy sequencing. This process takes advantage of the ability of DNA polymerase to incorporate 3'-dideoxynucleotides, nucleotide base analogs that lack the 3'-hydroxyl group essential in phosphodiester bond formation. Sanger dideoxy sequencing requires a DNA template, a sequencing primer, DNA polymerase, nucleotides (dNTPs), dideoxynucleotides (ddNTPs), and reaction buffer. The Sanger method chain termination reactions are still used, but pouring, running, & reading polyacrylamide gels has been replaced by automated methods. Instead of labeling the products of all 4 sequencing reactions the same (with a radioactive deoxynucleotide), each dideoxynucleotide is labeled with a different fluorescent marker. During capillary electrophoresis the fluorescently labeled DNA fragments, separated by size, move across

the path of a laser beam. The laser beam causes the dyes on the fragments to fluoresce. An optical detection device on Applied Bio systems genetic analyzers detects the fluorescence. The Data Collection Software converts the fluorescence signal to digital data, and then records the data in a file. Because each dye emits light at a different wavelength when excited by the laser, all four colors, and therefore all four bases, can be detected and distinguished in one capillary injection (https://www.thermofisher.com/np/en/home/life-science/sequencing).

4.2.11 Sequence Analysis

Sequence analysis is the process of subjecting a DNA sequence to any of a wide range of analytical methods to understand its features, function, structure, or evolution. Methodologies used include searches against biological databases and sequence alignment. Since the development of methods of high-throughput production of gene sequences, the rate of addition of new sequences to the databases has increased exponentially. Bacterial genes typically comprise uninterrupted stretches of DNA between a start codon (usually ATG, but in a minority of genes, GTG, TTG, or CTG) and a stop codon (TAA, TGA, or TAG). After receiving the sequence data, preliminary analysis was done using Chromas Lite 2.1.1 followed by sequence editing in Codoncode Aligner. Sequence alignment was done using the software MEGA 6.06 by Clustal W. Final sequence was subjected for BLAST to compare with existing sequences in NCBI database.

CHAPTER 5

RESULTS

Primary aim of this study was to detect the presence of the genes, if any, that confer carbapenem resistance among the collected isolates of *Pseudomonas aeruginosa* from different samples from hospitals. These isolates were further analyzed simultaneously for various characteristics.

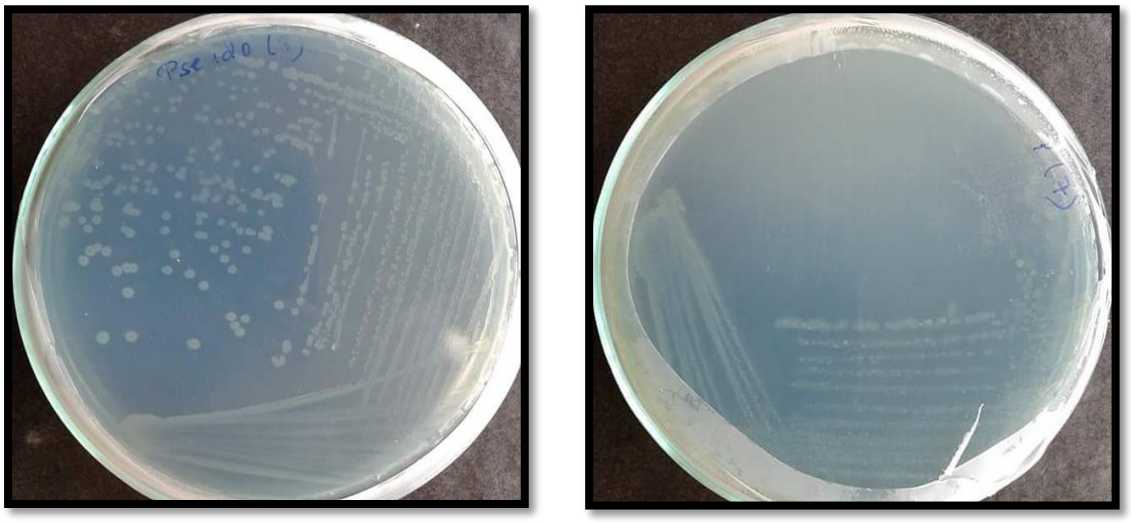
5.1 Microbiological Analysis

After obtaining the bacterial isolates, each isolates were subjected to inoculation and incubation in Nutrient agar plate in order to carry out further studies.

5.1.1 Colony characteristics

Table 5.1: Colony morphology of the *Pseudomonas* isolates

Shape	Color	Odor	Elevation	Consistency	Opacity
Round	Greenish	Grape-like	Convex	Mucoid	Opaque

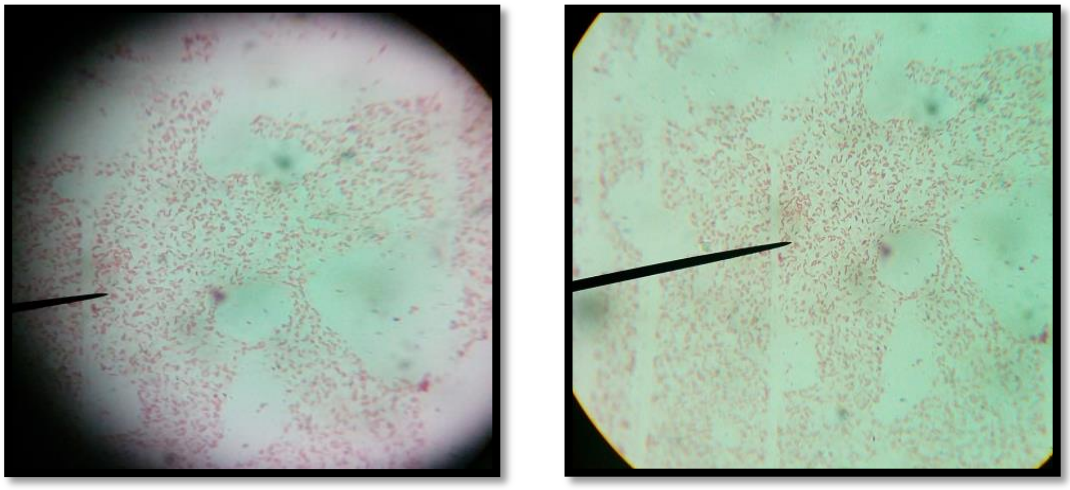


A **B**

Fig 5.1: *Pseudomonas aeruginosa* in NA plates

5.1.2 Gram's characteristics

All the isolates were subjected to the Gram's staining in order to confirm that they were Gram's negative. During this analysis, 67 isolates were found to be Gram's negative rod.



A **B**

Fig 5.2: Gram staining of isolates, showing Gram's negative, rods

5.1.3 Biochemical Tests

All the isolates were confirmed to be *Pseudomonas aeruginosa* by conventional biochemical test method which is shown in the table according to Bergy's Manual of Systematic Bacteriology, 1986. The confirmed isolates were named BT.1, BT.2, BT.3, BT.4 and so on up to BT.67.

Table 5.2: Biochemical Test result of isolates

Cat	Ox	I	MR	VP	CT	N	Ur	TSI			
								S	B	H2S	Gas
+	+	-	-	-	+	+	-	R	R	-	-

KEY: Cat-Catalase; Ox-Oxidase; I-Indole; MR-Methyl red; VP-Voges Proskauer; CT-citrate utilization; N-Nitrate; Ur-Urease; TSI-Triple Sugar iron; S-Slope; B-Butt; R-red; + positive reaction; - negative reaction.

Fig 5.3: Biochemical Test of the isolate

5.2 Various specimens of the isolates

Table 5.3: Various specimens of the isolates

Specimen	No. of cases	Percentage
Pus	21	31.34
Blood	5	7.46
Sputum	33	49.25
Urine	8	11.94

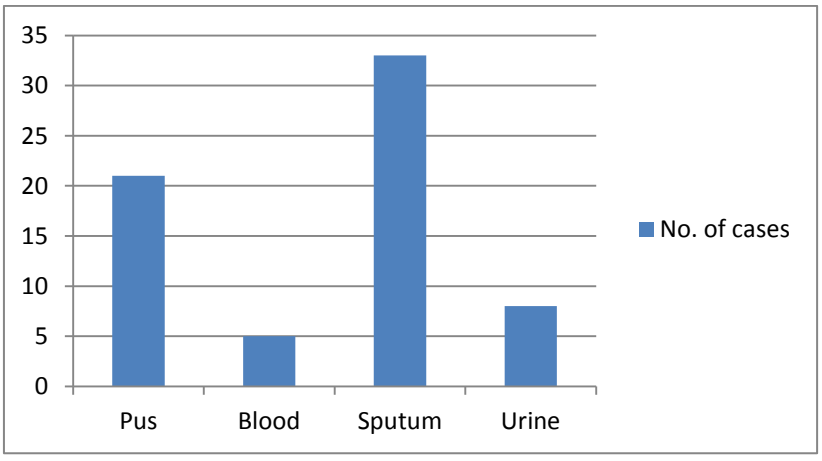


Fig 5.4: Various Specimens of the isolates

Sputum samples constituted majority of isolates accounting 49.25 %. Pus, blood and urine samples accounted for 31.34 %, 7.46 % and 11.94 % respectively.

5.3 Antibiotic Susceptibility Test

Sixty-seven isolates of *Pseudomonas aeruginosa* were tested accordingly for different antibiotics using Kirby-Bauer disc diffusion method. The antibiotics used were Amikacin (AK), Piperacillin (Pi), Ciprofloxacin (CIP), Levofloxacin (LE), Ceftazidime (CAZ), Cefotaxime (CTX), Meropenem (Mr), Imipenem (I), Cefepime (CPM), Cefoperazone/Sulbactum, Piperacillin/Tazobactam (PIT), Polymyxin-B (PB), and Colistin (CL). Out of total 67 isolates, 52 showed resistances to at least 3 classes of antibiotic, so they were grouped under Multi-Drug resistant isolates. i.e. 52 isolates were MDR strains.

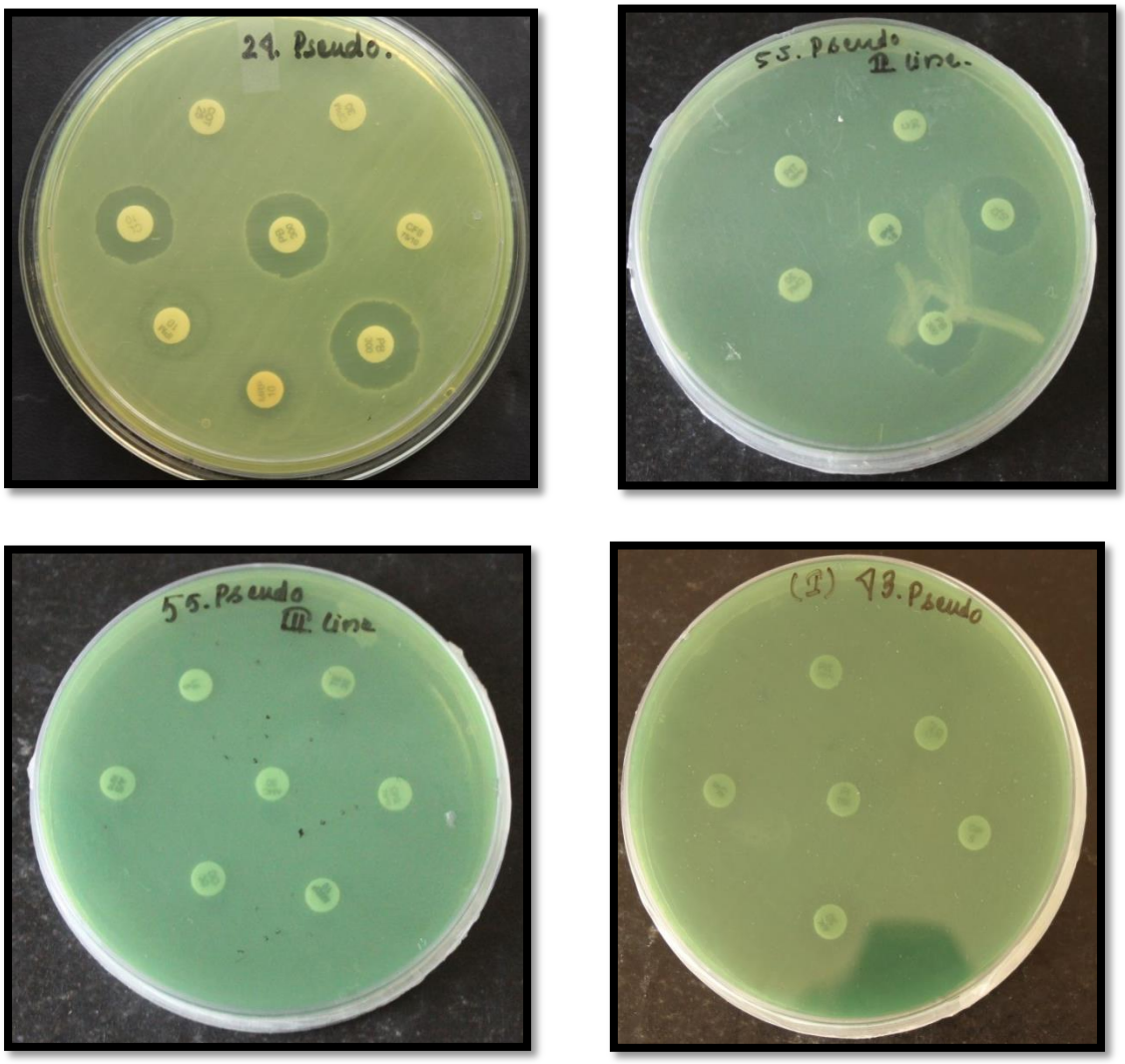


Fig 5.5: Antibiotic Susceptibility Test

After measuring the diameter of the zone of inhibition, response of isolates to each antibiotic was interpreted. Interpretation was done on the basis of the standard chart of

each of the antibiotics which is given in the Appendix. After comparative analysis and interpretation the result of antibiotic susceptibility test was as follow (Table 5.4).

Table 5.4: Interpretation of Antibiotic susceptibility pattern of *Pseudomonas* isolate:

Organism	Antibiotics	Sensitive Percentage	Resistance Percentage
Pseudomonas aeruginosa	Ceftazidime	52.83	47.17
	Cefotaxime	41.18	58.82
	Ciprofloxacin	38.00	62.00
	Levofloxacin	43.14	56.86
	Amikacin	56.06	43.94
	Cefepime	51.11	48.89
	Cefoperazone/ Sulbactum	50.98	49.01
	Piperacillin/Tazobactam	46.29	53.70
	Piperacillin	45.45	54.54
	Colistin	100	0
	Polymixin B	100	0
	Imipenem	59.37	40.63
	Meropenem	38.46	61.54

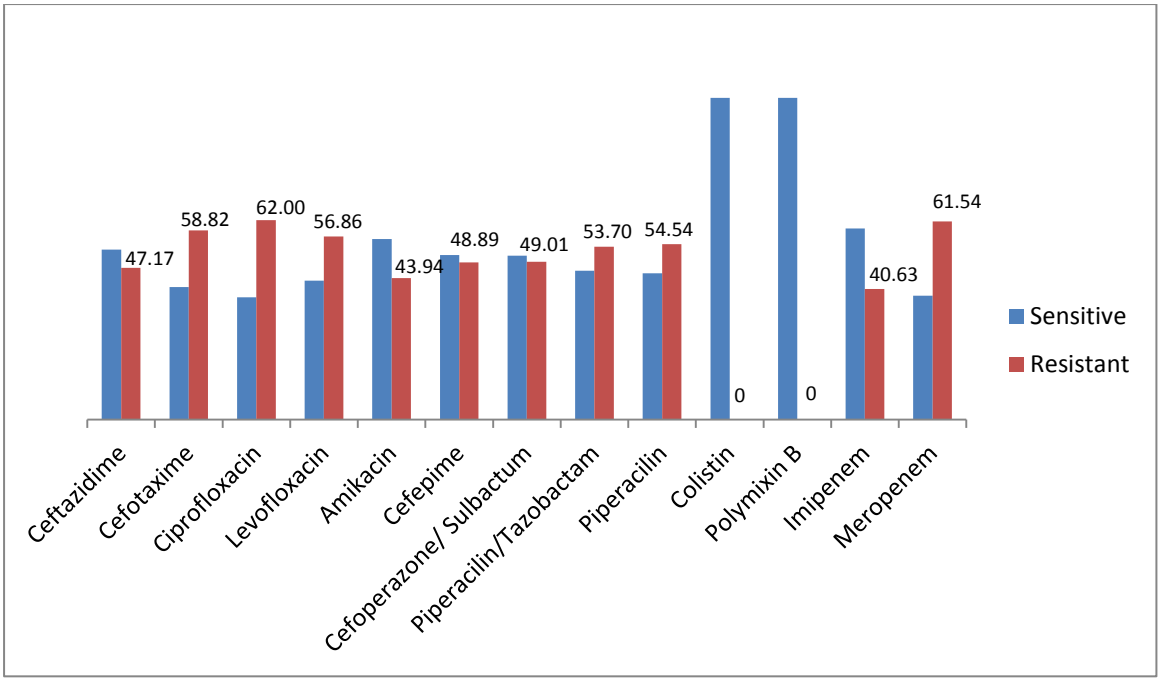


Fig 5.6: Antibiotic resistance pattern shown by the isolates

Analyzing the data shows that most of the MDR strains of *Pseudomonas aeruginosa* exhibit the resistance to majority of commercially available drugs. Though it shows resistance to most of the conventional drugs it seems to be susceptible to the modern last resort drugs like colistin and Polymyxin B. Out of the total isolates, 52 isolates showed resistant to at least 3 classes of antibiotics and hence were grouped under MDR isolates.

5.3.1 Distribution of Carbapenem Resistant *P. aeruginosa*

After performing the antibiotic susceptibility test, carbapenem resistant isolates were screened out to investigate further. The isolates that showed resistance to two antibiotics belonging to the carbapenem group- Imipenem (IMP-10µg) and Meropenem (MEM-10µg) were screened out and the data interpreted as in Table 5.5. All the carbapenem resistant isolates were MDR.

Table 5.5: Distribution of Carbapenem resistant isolates

Organism	No. of MDR isolates	No. of Carbapenem resistant isolates	Percentage
P. aeruginosa	52	40	76.92

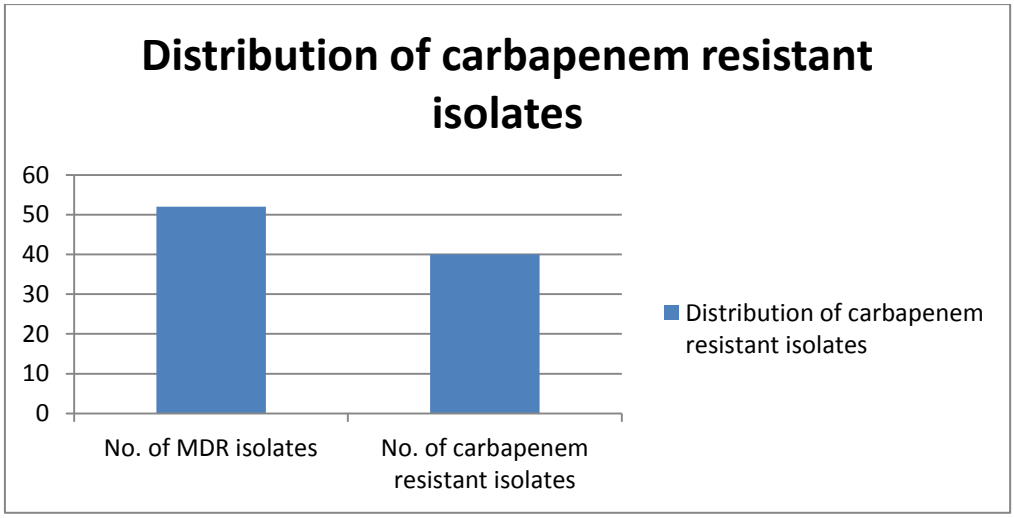


Fig 5.7: Graph showing distribution of carbapenem resistant isolate among MDR *P. aeruginosa*

Out of these 52 MDR strains, 26 were resistant to Imipenem (I) and 40 showed resistance to Meropenem (Mr) and all the isolates that were resistant to imipenem also showed resistance to meropenem. The 40 isolates that were resistant to carbapenem group of antibiotic i.e. imi and/or mero were then screened out for further processing.

5.4 Detection of ESBL Producer

The extended spectrum beta lactamase production was tested for 30 isolates that were resistant to ceftazidime (CAZ) and cefotaxime (CTX) tested. The test used a disc of amoxyclav (20 µg amoxycillin and 10 µg clavulanic acid) and a 30 µg disc of cefotaxime antibiotic placed 15-20mm apart. An increase in the zone size around the antibiotic disc towards the amoxyclav disc was considered to be positive test for ESBL.

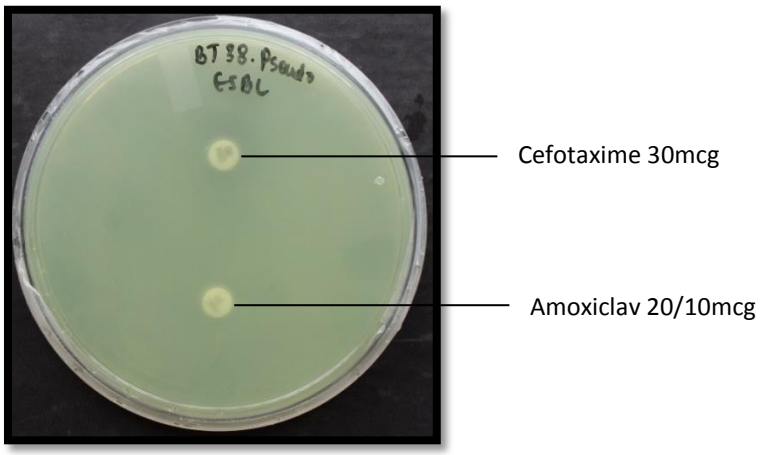


Fig 5.8: Double disc synergy test showing negative result for ESBL

Table 5.6: Distribution of ESBL producers

Organism	Ceftazidime and Cefotaxime resistant isolates	No. of ESBL producers	Percentage
P. aeruginosa	30	0	0

No any isolates resistant to extended spectrum cephalosporins showed the positive result for ESBL by this phenotypic test.

5.5 Detection of MBL Producer

The metallo-beta-lactamase production was tested for all of the 40 carbapenem resistant isolates phenotypically by EDTA Combined Disc Test. The test used imipenem and meropenem discs alone and in combination with 750 µg of EDTA. An increase of 7 mm in zone diameter in the presence of 750 µg EDTA compared to those with both IPM and MEM tested alone was considered to be a positive test for the presence of an MBL.

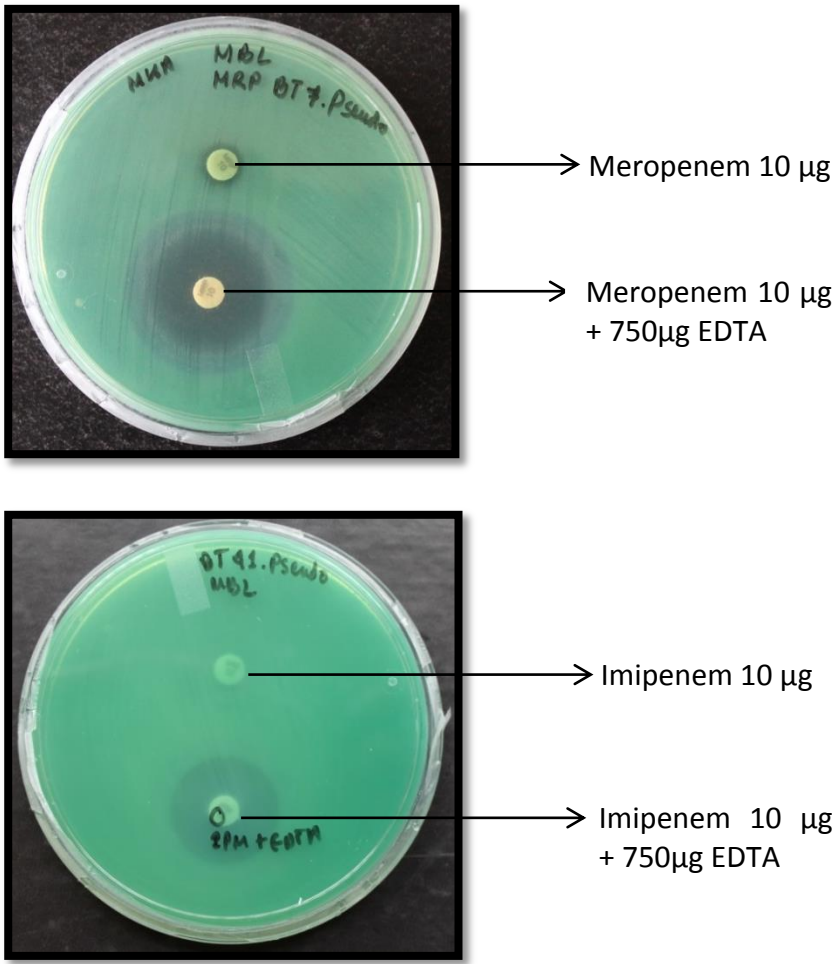


Fig 5.9: EDTA Combined Disc Test showing MBL Producer

After measuring the differences in inhibition zones for IPM and MEM discs alone and in combination with EDTA, response of carbapenem resistant isolates was interpreted and the result is as follows.

Table 5.7: Distribution of MBL producers

Organism	IMP and/or MEM resistant isolates	No. of MBL producers	Percentage
P. aeruginosa	40	30	75

The number of isolates resistant to imipenem and/or meropenem antibiotic disc was 40, which was screened out after performing AST. Majority of the isolates out of these carbapenem resistant isolates showed positive test for MBL.

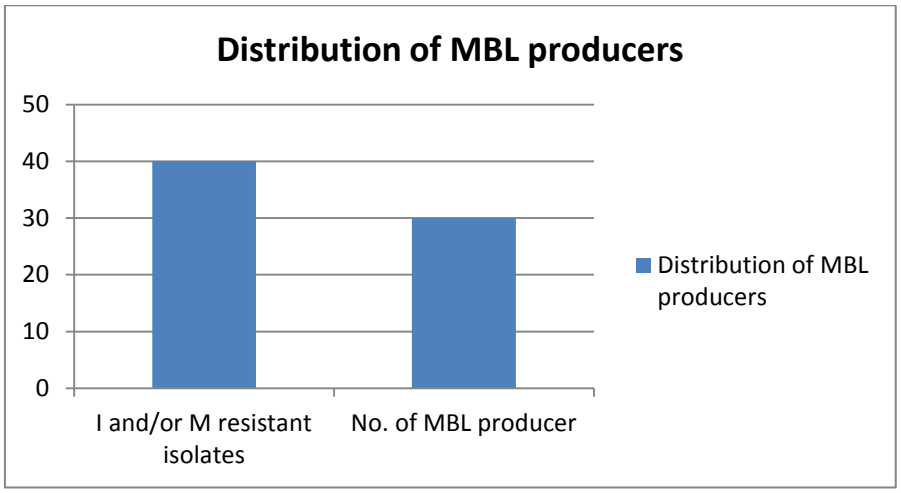


Fig 5.10: Graph showing distribution of MBL producers among Carbapenem resistant isolates of *P. aeruginosa*

Result shows that, of the 40 carbapenem resistant *P. aeruginosa* isolates, 30 (75 %) were MBL positive using phenotypic method.

5.6 Preparation of Genomic DNA

Following the standard protocol of DNA Isolation procedure by Michele K. Nishiguchi, Phaedra Doukakis, Mary Egan et al., (Nishiguchi MK et al., 2002) genomic DNA was extracted from all the 30 MBL positive isolates of *P. aeruginosa* using CTAB method. After the extraction, the genomic DNA were run in 0.8% agarose gel incorporated with

Ethidium bromide (EtBr) at 50V for about an hour and visualized under UV Transilluminator.

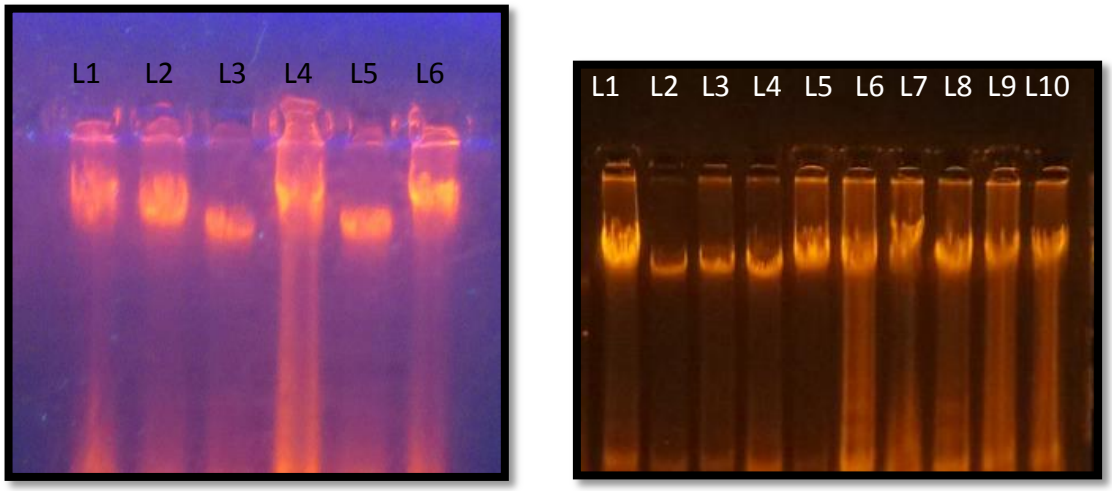


Fig 5.11: Electrophoresis of Genomic DNA

5.7 PCR amplification of MBL Gene

First of all, the presence of possible genes for *bla*IMP, *bla*VIM and *bla*NDM was tested using the primers for internal gene amplification. Only one gene *bla*NDM of 460bp was amplified using this primer and then later PCR amplification for full length gene using gene specific primer was performed.

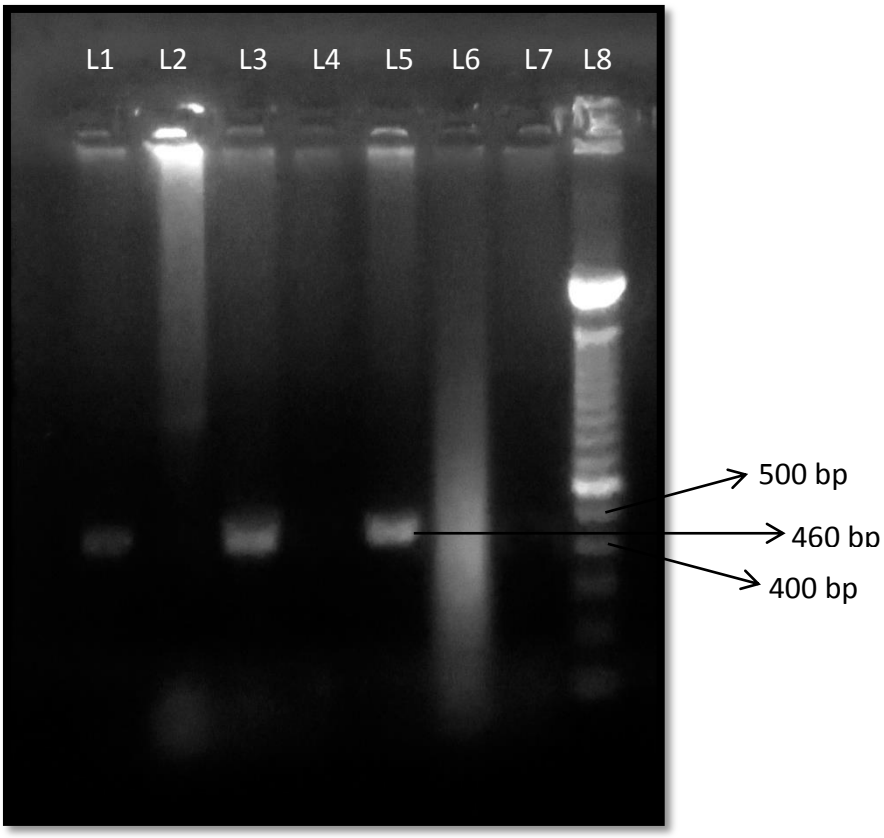


Fig 5.12: PCR amplification of internal *bla*NDM gene. L1, L3 and L5- PCR product showing positive result for int *bla*NDM, L2, L4, L6 and L7- Negative result for int *bla*NDM, L8- 100 bp ladder, Thermofisher (15628019)

The result for PCR amplification for full length genes, *bla*IMP, *bla*VIM and *bla*NDM showed presence of only one gene- *bla*NDM. Full length *bla*NDM of 869 bp was amplified using appropriate condition and master mix solution from New England Biolabs, which was then subjected to the agarose gel electrophoresis at 50V for about an hour. After electrophoresis gel was visualized in UV transilluminator in order to see the amplified band of *bla*NDM gene. These PCR products showing the positive result for *bla*NDM gene were later subjected to sequencing.

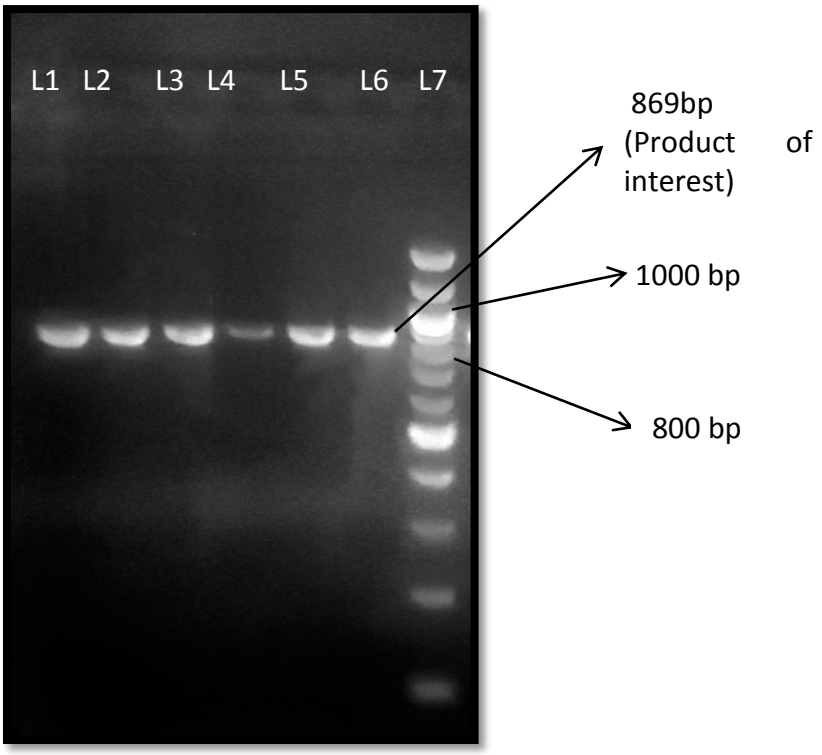


Fig 5.13: PCR amplification of full-length *bla*NDM gene. L1-BT.25, L2-BT.26, L3-BT.32, L4-BT.33, L5-BT.34, L6-BT.35 and L7- 100 bp Ladder, NEB (N32131S)

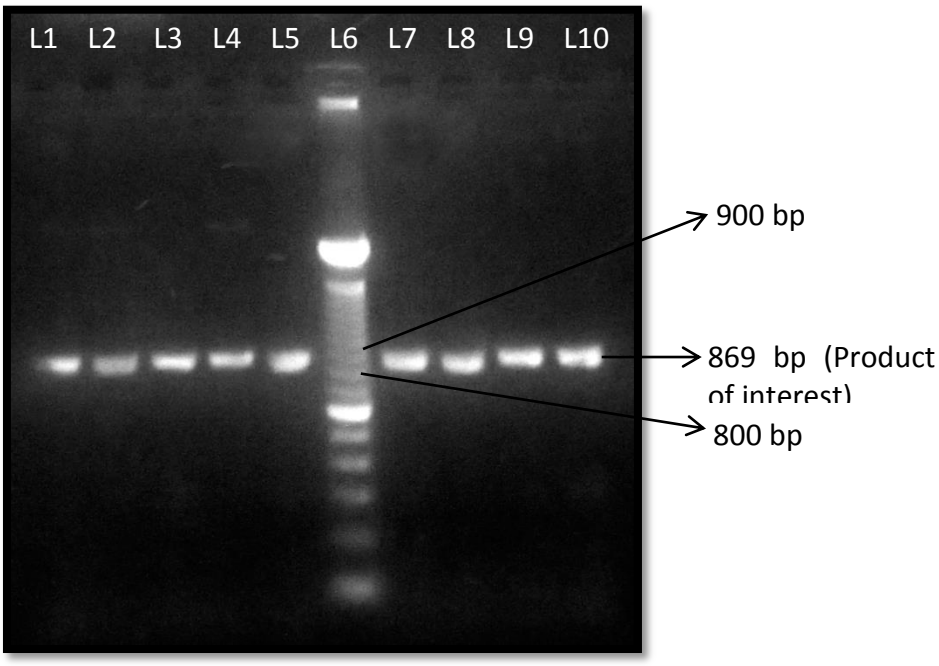


Fig 5.14: PCR amplification of full-length *bla*NDM gene. L1-BT.22, L2-BT.23, L3-BT.24, L4-BT.27, L5-BT.28, L6-100 bp ladder, Thermofisher (15628019), L7-BT.29, L8-BT.30, L9-BT31 and L10-BT.36

5.7.1 Distribution of *bla*NDM gene in *P. aeruginosa*

Fifteen isolates out of 30 MBL producers *P. aeruginosa* showed the presence of *bla*NDM gene in them. No other genes were identified.

Table 5.8: Distribution of *bla*NDM gene

No. of *P. aeruginosa* isolates	No. of MDR strains	% of MDR strains	Isolate with *bla* NDM gene	% (Resistance mediated by *bla*NDM)
67	52	77.61	15	28.85

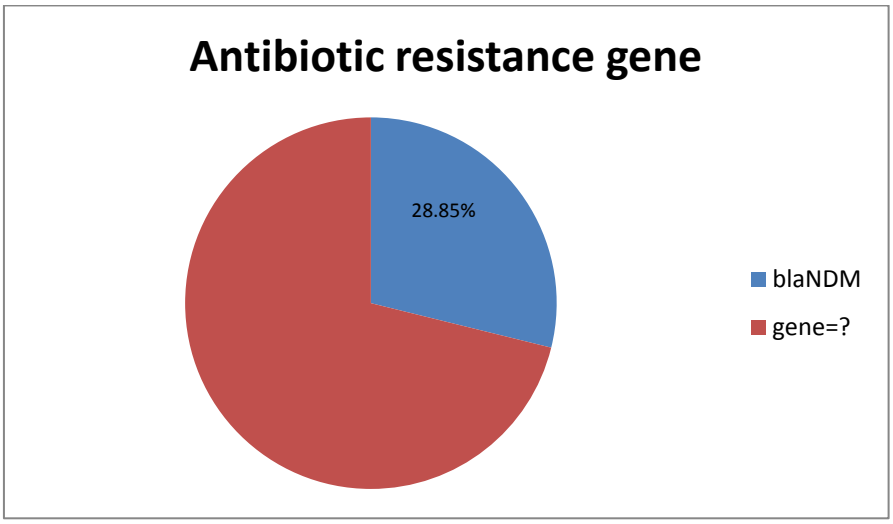


Fig 5.15: Chart showing distribution of *bla*NDM gene among collected isolates of MDR *P. aeruginosa*

Out of total 67 isolates of *P. aeruginosa,* 52 isolates were MDR (resistant to at least 3 different classes of antibiotic). Out of which 15 isolates (BT.1, BT.2, BT.4, BT.7, BT.11, BT.12, BT.23, BT.25, BT.33, BT.35, BT.39, BT.41, BT.43, BT.46 and BT.53) were found to have *bla*NDM gene in them which was responsible for causing carbapenem resistance. Therefore, 28.85% of total MDR isolates collected had *bla*NDM gene in them whereas none of them were positive for other possible genes, *bla*VIM and *bla*IMP.

5.8 Sequencing of PCR Product and Sequence Analysis

Out of total 15 samples sent for sequencing, quality data could be obtained for only 11 samples. PCR analysis and sequencing identified MBL NDM-1 gene in 11 isolates of *P. aeruginosa.* (The nucleotide sequence of all the 11 isolates is listed in the appendices).

The Sequence analysis was done using Nucleotide blast in NCBI and then the multiple sequence alignment by using the software Clustal Omega. The sequence analyzed matched with the *bla*NDM-1 gene of *P. aeruginosa* previously detected (accession no. KU510373.1) and it was taken as a reference sequence for the alignment process.

5.8.1 Multiple Sequence Alignment of ten different sample with the reference sequence (accession no. KU510373.1)

```
CLUSTAL Omega (1.2.4) multiple sequence alignment


BT.35           ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
BT.23           ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
KU510373.1      ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
BT.2            ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
BT.7            ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
BT.12           ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
BT.25           ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
BT.33           ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
BT.41           ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
BT.53           ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
BT.1            ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
                ************************************************************


BT.35           TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
BT.23           TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
KU510373.1      TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
BT.2            TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
BT.7            TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
BT.12           TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
BT.25           TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
BT.33           TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
BT.41           TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
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BT.53           TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
BT.1            TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
                ************************************************************

BT.35           ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
BT.23           ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTTTGGCAG 180
KU510373.1      ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
BT.2            ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
BT.7            ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
BT.12           ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
BT.25           ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
BT.33           ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
BT.41           ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
BT.53           ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
BT.1            ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
                ************************************************** ******

BT.35           CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
BT.23           CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
KU510373.1      CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
BT.2            CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
BT.7            CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
BT.12           CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
BT.25           CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
BT.33           CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
BT.41           CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
BT.53           CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
BT.1            CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
                ************************************************************

BT.35           AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
BT.23           AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
KU510373.1      AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
BT.2            AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
BT.7            AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
BT.12           AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
BT.25           AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
BT.33           AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
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BT.41           AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
BT.53           AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
BT.1            AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
                ************************************************************

BT.35           ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
BT.23           ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
KU510373.1      ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
BT.2            ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
BT.7            ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
BT.12           ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
BT.25           ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
BT.33           ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
BT.41           ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
BT.53           ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
BT.1            ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
                ************************************************************

BT.35           GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
BT.23           GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
KU510373.1      GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
BT.2            GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
BT.7            GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
BT.12           GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
BT.25           GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
BT.33           GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
BT.41           GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
BT.53           GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
BT.1            GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
                ************************************************************

BT.35           GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
BT.23           GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
KU510373.1      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
BT.2            GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
BT.7            GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
BT.12           GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
BT.25           GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
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BT.33           GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
BT.41           GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
BT.53           GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
BT.1            GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
                ************************************************************

BT.35           CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
BT.23           CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
KU510373.1      CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
BT.2            CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
BT.7            CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
BT.12           CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
BT.25           CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
BT.33           CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
BT.41           CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
BT.53           CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
BT.1            CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
                ************************************************************

BT.35           AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
BT.23           AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
KU510373.1      AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
BT.2            AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
BT.7            AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
BT.12           AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
BT.25           AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
BT.33           AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
BT.41           AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
BT.53           AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
BT.1            AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
                ************************************************************

BT.35           ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
BT.23           ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
KU510373.1      ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
BT.2            ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
BT.7            ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
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BT.12           ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
BT.25           ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
BT.33           ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
BT.41           ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
BT.53           ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
BT.1            ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
                ************************************************************

BT.35           CTCGGAGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
BT.23           CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
KU510373.1      CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
BT.2            CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
BT.7            CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
BT.12           CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
BT.25           CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
BT.33           CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
BT.41           CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
BT.53           CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
BT.1            CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
                ***** ******************************************************

BT.35           CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
BT.23           CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
KU510373.1      CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
BT.2            CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
BT.7            CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
BT.12           CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
BT.25           CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
BT.33           CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
BT.41           CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
BT.53           CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
BT.1            CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
                ************************************************************
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BT.35           CATACGGTCCGCATGGCCCACAAGCTGCGCTGA 813
BT.23           CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
KU510373.1      CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
BT.2            CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
BT.7            CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
BT.12           CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
BT.25           CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
BT.33           CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
BT.41           CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
BT.53           CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
BT.1            CATACGGCCCGCATGGCCCACAAGCTGCGCTGA 813
                ******* ********** **************
```

Multiple sequence alignment of genes from 10 different samples showed that among them 7 were 100% identical with the reference sequence (accession no. KU510373.1) and there was substitution of nucleotide in 3 of them. At position 799 of BT.1, the “G” nucleotide is changed to “C” and at position 174 of BT.23, “C” nucleotide is changed to “T”. In BT.35, the substitution of nucleotide was seen in 3 different positions, at position 666, “T” changed to “A”, at 789, “C” changed to “T” and at 799, “G” changed to “C”.

5.8.1.1 Phylogenetic Tree

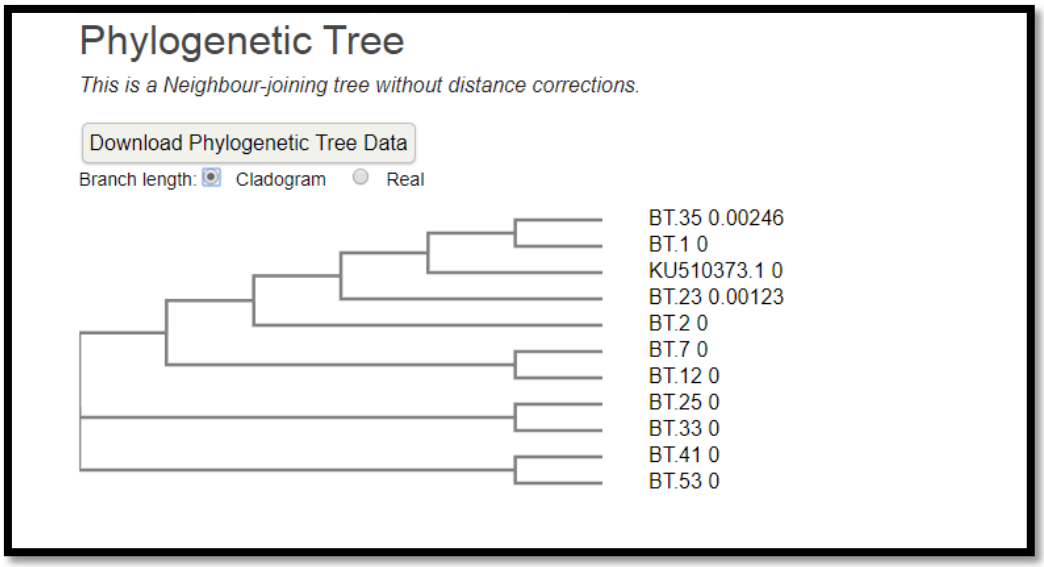

Fig 5.16: Tree diagram showing alignment between different samples with the reference sequence

5.8.1.2 Multiple Peptide Sequence Alignment of ten different sample with the reference sequence (accession no. KU510373.1)

```
CLUSTAL O(1.2.4) multiple sequence alignment

BT.35             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
KU510373.1_1      MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
BT.2              MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
BT.7              MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
BT.12             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
BT.23             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
BT.25             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
BT.33             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
BT.41             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
BT.53             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
BT.1              MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
                  ************************************************************

BT.35             HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
KU510373.1_1      HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
BT.2              HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
BT.7              HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
BT.12             HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
BT.23             HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
BT.25             HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
BT.33             HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
BT.41             HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
BT.53             HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
BT.1              HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
                  ************************************************************

BT.35             AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
KU510373.1_1      AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
BT.2              AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
BT.7              AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
BT.12             AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
BT.23             AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
BT.25             AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
```

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BT.33             AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
BT.41             AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
BT.53             AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
BT.1              AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
                  ************************************************************

BT.35             KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
KU510373.1_1      KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
BT.2              KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
BT.7              KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
BT.12             KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
BT.23             KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
BT.25             KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
BT.33             KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
BT.41             KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
BT.53             KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
BT.1              KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
                  ************************************************************

BT.35             PKASMIVMSHSAPDSRAAITHTVRMAHKLR*
KU510373.1_1      PKASMIVMSHSAPDSRAAITHTARMADKLR*
BT.2              PKASMIVMSHSAPDSRAAITHTARMADKLR*
BT.7              PKASMIVMSHSAPDSRAAITHTARMADKLR*
BT.12             PKASMIVMSHSAPDSRAAITHTARMADKLR*
BT.23             PKASMIVMSHSAPDSRAAITHTARMADKLR*
BT.25             PKASMIVMSHSAPDSRAAITHTARMADKLR*
BT.33             PKASMIVMSHSAPDSRAAITHTARMADKLR*
BT.41             PKASMIVMSHSAPDSRAAITHTARMADKLR*
BT.53             PKASMIVMSHSAPDSRAAITHTARMADKLR*
BT.1              PKASMIVMSHSAPDSRAAITHTARMAHKLR*
                  **********************.***.****
```

Multiple peptide sequence alignment of genes from 10 different samples showed that in BT.1, amino acid aspartate has been substituted by histidine causing missense mutation. Similarly, in BT.35, at position 789, amino acid alanine is substituted by valine and at position 799, aspartate substituted by histidine.

5.8.2 Pairwise Sequence Alignment of one sample (BT.11) with the reference sequence (accession no. KU510373.1)

```
KU510373.1          1 ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATT     50
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11               1 ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATT     50

KU510373.1         51 AGCCGCTGCATTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGA    100
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11              51 AGCCGCTGCATTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGA    100

KU510373.1        101 CGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTT    150
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11             101 CGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTT    150

KU510373.1        151 TTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACAT    200
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11             151 TTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACAT    200

KU510373.1        201 GCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTCAGGGATGGCG    250
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11             201 GCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTCAGGGATGGCG    250
KU510373.1        251 GCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG    300
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11             251 GCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG    300

KU510373.1        301 ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGT    350
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11             301 ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGT    350

KU510373.1        351 GGTGACTCACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATG    400
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11             351 GGTGACTCACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATG    400

KU510373.1        401 CGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCG    450
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11             401 CGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCG    450
```

```
KU510373.1      451 CAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGG    500
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11           451 CAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGG    500

KU510373.1      501 CTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTT    550
                    ||||||||||||||||||||.||||||||||||||||||||||||||||||
BT.11           501 CTGGGTCGAACCAGCAACCGGGCCCAACTTTGGCCCGCTCAAGGTATTTT    550

KU510373.1      551 ACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC    600
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11           551 ACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC    600

KU510373.1      601 ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTC    650
                    ||||||||||||||||||||||||||||||||||||.|||||.||||||
BT.11           601 ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGGAAGGACAAGTC    650

KU510373.1      651 GCTCGGCAATCTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGC    700
                    |||||||||.|||||.|||||.|||||||||
BT.11           651 GCTCGGCAAACTCGGAGATGCAGACACTGAG--------------------    681

KU510373.1      701 GCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGCCAT    750

BT.11           682 --------------------------------------------------    681

KU510373.1      751 TCCGCCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGA    800

BT.11           682 --------------------------------------------------    681

KU510373.1      801 CAAGCTGCGCTGA    813

BT.11           682 -------------    681
```

This pairwise sequence alignment of gene from BT.11 and reference gene (accession no. KU510373.1) showed nucleotide substitution in 6 different positions. At 521, “C” nucleotide is replaced by “G”, at 639, “C” replaced by “G”, at 644, “C” replaced by “A”, at 660, “T” replaced by “A”, at 666, again “T” replaced by “A” and at 672, “C” replaced by “A”.

5.8.2.1 Pairwise Peptide Sequence Alignment of one sample (BT.11) with the reference sequence (accession no. KU510373.1)

```
KU510373.1_1       1 MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLV     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11              1 MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLV     50

KU510373.1_1      51 FRQLAPNVWQHTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQ    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11             51 FRQLAPNVWQHTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQ    100

KU510373.1_1     101 ILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAAGIATYANALSNQLAP    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
BT.11            101 ILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAAGIATYANALSNQLAP    150

KU510373.1_1     151 QEGMVAAQHSLTFAANGWVEPATAPNFGPLKVFYPGPGHTSDNITVGIDG    200
                     ||||||||||||||||||||||.||||||||||||||||||||||||||
BT.11            151 QEGMVAAQHSLTFAANGWVEPATGPNFGPLKVFYPGPGHTSDNITVGIDG    200

KU510373.1_1     201 TDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAFPKASMIVMSH    250
                     ||||||||||||.|.||||.|||||||
BT.11            201 TDIAFGGCLIKDRKDKSLGKLGDADTE----------------------    227

KU510373.1_1     251 SAPDSRAAITHTARMADKLR*    271

BT.11            228 ----------------------    227
```

The peptide sequence alignment of BT.11 showed that alanine is substituted by glycine, serine by arginine, alanine by aspartic acid and asparagine by lysine.

5.8.3 Genbank accession number

All the nucleotide sequence of the genes has been deposited in Genbank and it has provided Genbank accession numbers for nucleotide sequences:

S.N	Sequence ID	Accession no.	Sample no.
1.	BankIt2027118 Seq1	MF379682	BT.1 (novel)
2.	BankIt2027118 Seq2	MF379683	BT.35 (novel)
3.	BankIt2027118 Seq3	MF379684	BT.11 (novel)
4.	BankIt2027153 Seq1	MF379685	BT.2
5.	BankIt2027153 Seq2	MF379686	BT.7
6.	BankIt2027153 Seq3	MF379687	BT.12
7.	BankIt2027153 Seq4	MF379688	BT.23
8.	BankIt2027153 Seq5	MF379689	BT.25
9.	BankIt2027153 Seq6	MF379690	BT.33
10.	BankIt2027153 Seq7	MF379691	BT.41

5.8.4 Phylogenetic relationship between protein sequences of identified genes with the NDM variants

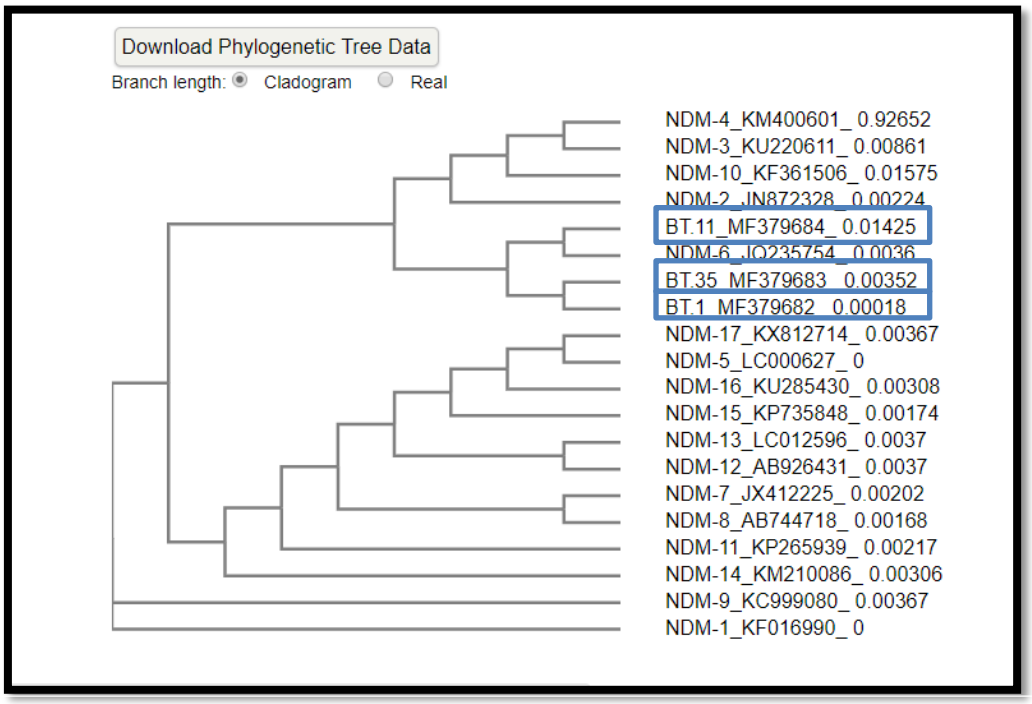

Fig 5.17: The phylogenetic relationship between protein sequences of three novel identified genes (MF379682, MF379684, MF379683) and the NDM variants

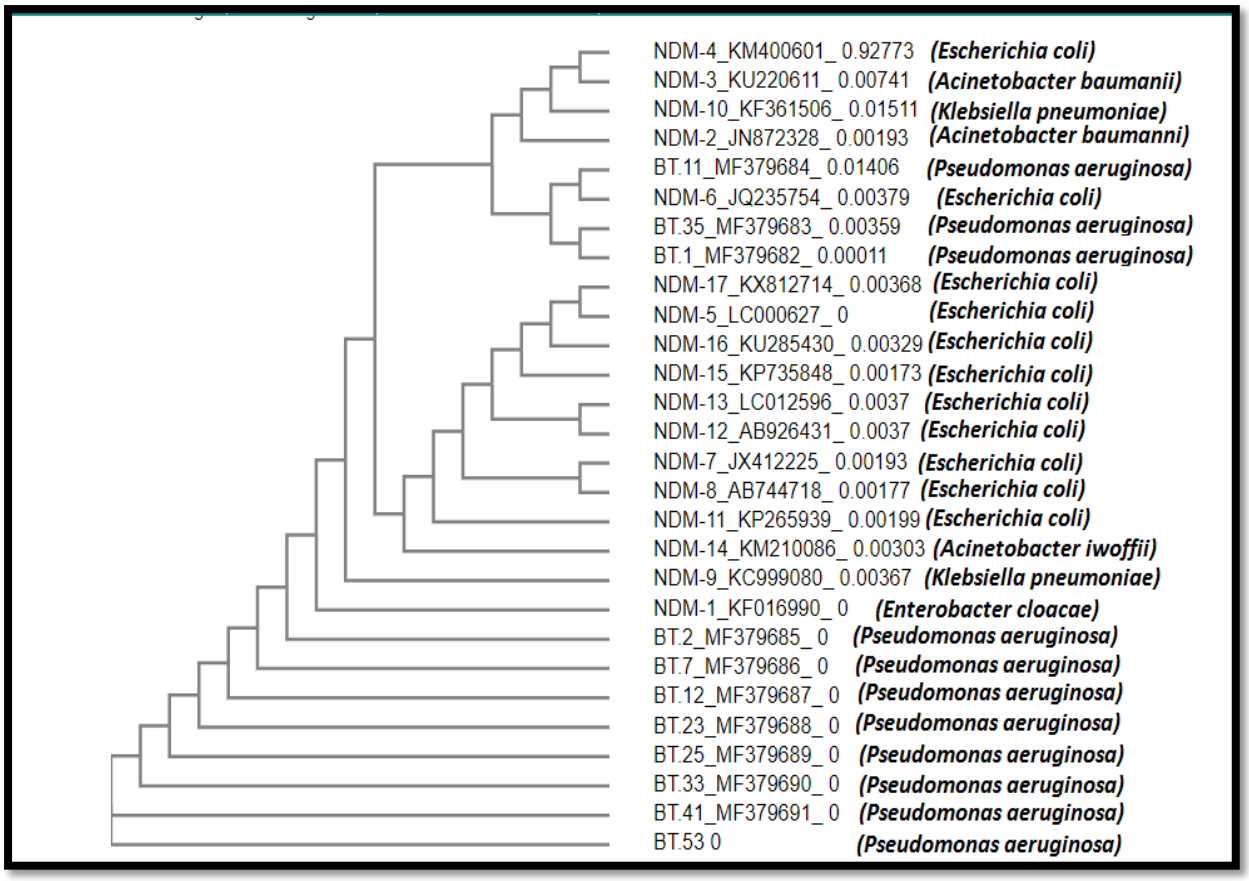

Fig 5.18: The phylogenetic relationship between protein sequences of identified genes and the NDM variants

The above figures show the phylogenetic relationship between protein sequences of NDM variants, identified in this study with previously identified variants. The tree construct has been generated using Clustal Omega.

CHAPTER 6
DISCUSSION

The most common cause of bacterial resistance to β-lactam antibiotic is the production of beta-lactamases. ESBL enzymes that mediate resistance to extended spectrum antibiotics like ceftazidime (CAZ) and cefotaxime (CTX) are not commonly found in *P. aeruginosa* but have been recently detected at low frequency. But in comparison, in recent years, MBL genes have spread from *P. aeruginosa* to members of Enterobacteriaceae. MBL enzymes are generally plasmid mediated and multidrug resistance is a characteristic feature of strains producing these enzymes. The overall prevalence of MBL producers are found to be variably increasing in different geographical areas. For many years these MBL producing isolates were restricted to Japan, but now it has disseminated worldwide. In Nepal, MBL producing *P. aeruginosa* was first reported in 2012, *Pseudomonas aeruginosa* IOMTU9 (NCGM1841) (Tada et al., 2013).

In the present study, an attempt has been made to know the prevalence of MBL in *Pseudomonas aeruginosa* and their antibiotic susceptibility pattern. Out of 67 isolates screened, 44.8% were MBL producers and none of them were ESBL producers.

The result were compared with other studies and discussed as follows.

6.1 Distribution of *P. aeruginosa* in various specimens

The collected isolates of *P. aeruginosa* were from various specimens, which were regarded to be a preliminary data. The distribution of specimens of *P. aeruginosa* may vary with each hospital as each hospital facility has a different environment associated with it. Our study included, 49.3% of the *P. aeruginosa* isolates obtained from sputum, followed by pus sample, which was 31.3%. Urine and blood sample accounted for 11.94% and 7.46% respectively. Therefore, sputum and pus/wound were the predominant sources of specimens of *P. aeruginosa.* One of the study done in Kathmandu, Nepal in 2013 showed more than 80% of the *P. aeruginosa* isolates were obtained from wound / pus, sputum, urine and tracheal aspirates. Similar results had been obtained in different studies in India reported by Arora et al. (Arora et al., 2011).

6.2 Antibiotic susceptibility pattern shown by the *P. aeruginosa* isolates

Increasing resistance to different anti-pseudomonal drugs particularly among hospital strains has been reported world-wide and this is a serious therapeutic problem in the management of disease due to these organisms. The resistance profiles of *P. aeruginosa* to the thirteen anti-microbial agents tested varied among the isolates investigated. The present study showed 77.61% of the isolate as MDR, the majority of isolates being resistant to fluoroquinolones, cephalosporins as well as to piperacillin-tazobactam and even to carbapenem group of antibiotics while Polymyxin B and colistin showed excellent effect against all MDR isolates. A MDR rate of 19.6%, 89.4% and 100% among *P. aeruginosa* isolates had been reported from studies conducted in Malaysia (Pathmanathan et al., 2008), Nepal (Bhandari et al., 2013), and Iran (Moazami-Goudarzi & Eftekhar, 2013) respectively. In our study, *P. aeruginosa* was highly susceptible to polymixin B and colistin followed by imipenem (59.37%). Similar antibiotic susceptibility pattern study by Sharma and Pant, 2017, showed that the highest rate of susceptibility of *P. aeruginosa* was found toward imipenem, after colistin and Polymyxin B (Sharma and Pant, 2017). One striking feature in this study was that majority of *P. aeruginosa* isolates were found to be resistant to ciprofloxacin (62.0%) followed by meropenem (61.54%) suggesting that these drugs should no longer be included in the treatment regimen for MDR *P. aeruginosa* infections in this population group. The resistance to meropenem, which is a carbapenem group of antibiotic, may be due to the production of metallo-β-lactamases (MBL), which can be chromosomally encoded or plasmid mediated. This poor effect of even carbapenem group of antibiotic among the isolates shows high antibiotic resistance rate against commonly used antibiotics which is a disadvantage for health care system in countries like Nepal as it can greatly effect patient management. The development of antibiotic resistance is associated with high morbidity and mortality, particularly in the intensive care unit (ICU) setting. Further, increasing rates of isolation of multidrug resistant *Pseudomonas* have compelled clinicians to use polymyxins for treatment of the infections caused by such bacteria despite their nephrotoxicity.

6.3 Distribution of carbapenem resistant *Pseudomonas aeruginosa*

The common antibiotic of carbapenem group which is highly active against *P. aeruginosa* is imipenem and meropenem. In recent years, there has been an alarming increase in the *P. aeruginosa* resistant to imipenem and meropenem. In our study, notable resistance (76.92%) to MDR *P. aeruginosa* was observed against carbapenems. The resistance to carbapenems may be due to production of metallo-beta-lactamases (MBLs). Nosocomial outbreaks of carbapenem resistant *P. aeruginosa* due to MBLs

production have been reported from different regions (Peleg et al., 2005, Oh et al., 2003). In the SENTRY antimicrobial surveillance program (SASP), 10-30% of *P. aeruginosa* strains from various countries have been found to be resistant to imipenem (Gales et al., 2001). And this resistance rate is increasing throughout the year. Our study showed an increasing rate of imipenem resistance to be 50%. Similar to this result, higher rate of resistance was seen in study done by Chaudhary, (66.5%) (Chaudhary, 2012). The isolates that were meropenem resistant was even higher, 76.92%. All the isolates resistant to imipenem were also resistant to meropenem. Therefore, among the carbapenems used in our study, imipenem tested showed the high level of efficacy than meropenem although effectiveness of meropenem has been noted in other studies (Prakash and Saxena, 2013).

6.4 Distribution of ESBL producers

ESBL enzymes mediate resistance to extended-spectrum cephalosporins, such as cefotaxime (CTX), ceftriaxone, and ceftazidime (CAZ), and the monobactam aztreonam. In our study, no ESBL producer among isolates of *P. aeruginosa* was detected phenotypically by Double disk synergy test. According to the previous studies, such enzymes are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* and have been recently detected in *P. aeruginosa* at very low frequency (Poirel et al., 2004). Also the current ESBL detection methods for *P. aeruginosa* are unreliable, and the reported numbers of ESBL producers are generally low because of up regulation of efflux systems, and decreased outer membrane permeability (Livermore, 1995). Therefore, there is a need for a reliable test to detect ESBLs in clinical isolates of *P. aeruginosa.* The test also needs to be practical for routine use in the clinical laboratory.

6.5 Distribution of MBL producers

There are a large number and variety of new resistance mechanisms that have emerged and their preliminary detection is important for infection control and adequate therapeutic guidance. Among the resistance mechanisms to carbapenems described for *P. aeruginosa,* major one can be highlighted, β-lactamases production. β-lactamases have been grouped into four molecular classes A, B, C and D, based on the amino acids sequence homology according to Ambler classification (1980). The ones that belong to A, C and D classes are called serine β-lactamases, and the others in B class are called metallo-β-lactamases (MBL). These enzymes have the property of hydrolyzing; at least partially, imipenem or meropenem, besides they hydrolyze other penicillins and cephalosporins. Since last 15 years, acquired resistance to carbapenems has been

increasingly reported and is mainly mediated by MBLs. In this study, among 40 carbapenem resistant isolates of MDRPA, 30 showed MBL positive phenotypically by EDTA Combined Disk test. Therefore we can say that 57.69% of total MDR isolates of *P. aeruginosa* have MBLs mediated resistance, which is much higher than the previous studies carried out by several authors from Nepal (18.2% by Khanal et al. in 2013 and 30.9% by Ansari et al., 2016). And comparison to the similar study in India shows quite higher data in our study. A study by Jayakumar et al in 2007 reported 54.5% MBL producers (Jayakumar and Appalaraju, 2007). Morten et al in 2001 have reported 47% of MBL producers in *P. aeruginosa* (Hentzer et al., 2001). Sen et al in 2010 reported 46.6% of MBL production among MDRPA strains (Sen et al., 2010). Poirel et al in 2000 reported 36% of MBL producers among the MDRPA (Poirel et al., 2000). A total of 75% of isolates were MBL producers among carbapenem resistant isolate. Among which 35% MBL producers was only meropenem resistant and 40% of the MBL positive isolates was both imipenem and meropenem resistant. Consequently, in the Nepalese scenario production of MBL may play a major role in carbapenem resistance among *P. aeruginosa,* which was detected phenotypically by EDTA combined disk test in our study. Many studies done for detection of MBL are carried by EDTA combined disk test and are found to be a better method than other methods like modified Hodge test (John and Balagurunathan, 2011). Although a global increase in the prevalence of MBL producing *P. aeruginosa* has been reported, limited data is available from our region.

6.6 PCR amplification of MBL gene

This study illustrates screening for MBL producing *P. aeruginosa* isolates by EDTA combined disk test and molecular approach. Results showed that 30/52 (57.69%) of isolates were MBL positive, but PCR results confirmed presence of MBL genes only in 15/52 (28.85%) of isolates. The differences between phenotypic and genotypic detection of MBL producing *P. aeruginosa* isolates have been reported in previous investigations done in different part of the world (Yatsuyanagi et al., 2004, Yousefi et al., 2010). In the present study, we found a high frequency of *P. aeruginosa* strains carrying *bla*NDM gene (28.85%) that significantly was different from other parts of our country. It seems that this is the first report of NDM- type MBL producing *P. aeruginosa* strains with a high frequency from Kathmandu, Nepal. The previous study regarding MBL genes from the clinical isolates from Kathmandu have shown to carry *bla*NDM-1 in *P. aeruginosa* IOMTU9, *bla*NDM-8 in *E. coli* IOMTU11 and recently, *bla*NDM-12 in *E. coli* IOMTU388.1, followed by *bla*NDM-13 again from *E. coli* IOMTU558 and the gene was in a plasmid (Shrestha B, 2015). It was demonstrated by several previous reports that the genes of NDM- type MBL are often encoded on mobile genetic elements which can

easily spread the resistance encoding genes among these isolates. While many underlying mechanisms may account for carbapenem resistance, the possession of MBL genes is of particular concern because they are able to hydrolyze most beta-lactams, including imipenem and meropenem, drugs considered of reserve for the treatment of Gram-negative pathogens especially in *P. aeruginosa* multidrug-resistant strains. The occurrence of these new variants of *bla*NDM in Nepal as shown by the studies indicates NDMs to evolve rapidly, therefore careful monitoring of NDM-producing pathogens is mandatory. In our study, other possible genes *bla*IMP and *bla*VIM tested was negative. This shows that most prevalently, there is a *bla*NDM gene in the hospital isolates of *P. aeruginosa.*

6.7 Sequencing of PCR products

After the sequencing of the PCR product, the gene was confirmed to be *bla*NDM-1 type variant. So in our isolates, presence of *bla*NDM-1 was responsible for causing carbapenem resistance in MDR strains of *P. aeruginosa*. The multiple sequence alignment of 11 genes with the reference sequence (accession no. KU510373.1) was done using clustal omega and 3 out of these 11 genes were a novel variant. Sequencing revealed the presence of identical *bla*NDM-1 genes (100% identity) on BT.2, BT.7, BT.12, BT.25, BT.33, BT.41 and BT.53.

The sequence of this new type *bla*NDM gene of BT.1 showed mutation corresponding to one nucleotide substitutions at position 799 compared with *bla*NDM-1 (accession number KU510373.1). This is a type of missense mutation where a change in one DNA base pair resulted in the substitution of one amino acid, aspartate for another, histidine in the protein made by a gene. Similar analysis of BT.23 showed silent mutation with one nucleotide substitution at position 174. Here no substitution of amino acid took place even after the change in one DNA base pair, so the mutation in BT.23 was a silent mutation. This type of mutation may not vary the enzyme activity compared with that of the reference gene since they are coding the same amino acid. In our analysis, amino acid valine was stable even after the gene mutation. In BT.35, the nucleotide substitution was observed in 3 different positions of the gene after sequence analysis. First at position 666, silent mutation was observed with no substitution in amino acid even the nucleotide substitution from “T” to “A” was analyzed. At 789, the nucleotide substitution resulted to the substitution in amino acid, i.e. from alanine to valine, so the mutation was of missense type. Again at position 799, nucleotide substitution resulted in substitution of amino acid resulting to missense mutation. Here aspartate was substituted by histidine. So, there were three nucleotide substitutions in BT.35 resulting

to the substitution of two amino acids so the gene may vary in enzyme activity compared to that of the original gene.

As observed from the phylogenetic tree, it can be concluded that the *bla*NDM which has been isolated and identified from the hospitals of Kathmandu bear close resemblance to those distributed worldwide as well as those previously reported from the valley (Shrestha et al., 2015). Similarly, the distribution of *bla*NDM genes across various genera signifies the high cross over event for the gene. Depending upon the type of mutation, these disparities found in the gene sequences have variable effect upon its hydrolyzing capacity. Hence, it can be concluded that the *bla*NDM genes identified in this study could have arrived either through various sources including but not limited to humans and animals; the novel genes could be the adaptation taken in by the organism so as to adapt to the existing medical practices as well as climactic variation.

Since this may be the first report for the occurrence of *bla*NDM-1 in *P. aeruginosa* in Nepal, the sequence data has been submitted to Genbank and Genbank accession number for all 10 submitted nucleotide sequences was assigned. The novel variants of *bla*NDM-1 gene was found in three of the isolates, BT.1 (accession no. MF379682), BT.35 (accession no. MF379683) and BT.11 (accession no. MF379684).

Our study showed the MBL producer *P. aeruginosa* is more prevalent than ESBL producers. The worldwide increased occurrence of carbapenem-resistant *P. aeruginosa* infections in healthcare settings has led to a greater alertness of the threat of hospital acquired infections. In the current study, there was high frequency of carbapenem-resistant *P. aeruginosa* strains that may be attributed to the extensive misuse of carbapenems. Resistance to carbapenem in clinical isolates of *P. aeruginosa* has been reported worldwide. At this time, there are limited selections of treatment options for carbapenem resistant. The high frequency of MBL producers detected in the study is not common, since previous studies show quite low rate of occurrence of MBL *P. aeruginosa* strains. Resistance due to MBL production has a potential for rapid dissemination, since it is often plasmid-mediated. Because of its ability to spread, carbapenem resistance related to NDM β-lactamase production has become a serious concern.

Regarding the molecular analysis, *bla*NDM gene was found to be more prevalent that other possible MBL genes like *bla*IMP and *bla*VIM in our context. Moreover sequence analysis has revealed that the NDM variant to cause carbapenem resistance in hospital

isolates was *bla*NDM-1. Majority of the genes identified showed 100% identical with *bla*NDM-1 (accession no. KU510373.1) and 3 novel variants were also identified after sequence analysis. The recent study done in Kathmandu, Nepal in *E. coli* isolates has found a new variant of *bla*NDM, i.e. *bla*NDM-8, *bla*NDM-12 and *bla*NDM-13. This occurrence of the new variant with each passing year suggests that the genes are being rapidly spread among the hospital isolates and may get transferred even between different isolates. This calls for the rapid initiation of the control practices in hospital setting to minimize the dissemination of the genes among the isolates within hospital environment.

CHAPTER 7
SUMMARY

Infections by *Pseudomonas aeruginosa* has spread worldwide and metallo-beta-lactamases (MBL) are being reported with increasing frequency. One of the major clinical problems regarding *P. aeruginosa* is attributed to the production of metallo-beta-lactamase (MBL) enzymes. The rapid spread of MBLs among major gram-negative pathogens, particularly *P. aeruginosa*, is an emerging threat and is a matter of concern worldwide. These organisms are resistant to almost all commonly available antibiotics with limited treatment options. MBLs usually confer reduced susceptibility to carbapenems, cephalosporins, and penicillins but not monobactams and are produced by many Gram-negative bacteria. MBLs are categorized by their amino acid sequences into various types, among which the most prevalent type being IMP-, VIM-, and NDM-type enzymes. Thus, this study was conducted to know the prevalence of MBL producing multidrug resistant *P. aeruginosa* and the molecular characterization of prevalent genes present in them.

In this study, clinical isolates of *P. aeruginosa* were collected from various hospitals of Kathmandu valley, Tribhuvan University Teaching Hospital (TUTH) and Nepal Public Health Laboratory (NPHL). Then the collected isolates were again confirmed to be *P. aeruginosa* in laboratory by conventional methods, Gram's staining and biochemical tests were performed. Then these isolates were subjected to antibiotic susceptibility test by Kirby Bauer Disk Diffusion method and multi drug resistant isolates were screened out. Majority of the collected isolates were MDR strains. Detection of ESBL producers among ceftazidime and cefotaxime resistant isolates was performed by double disk diffusion method but none of the isolates showed positive result for ESBL by this phenotypic test. There was a significant prevalence of carbapenem (Imipenem and/or meropenem) resistant isolates among the collected *P. aeruginosa* isolates and thus were subjected to MBLs testing since MBL production is the major factor contributing to the carbapenem resistance. The MBL production was confirmed phenotypically by EDTA combined disk test which showed a high number of MBL producer to be present among the carbapenem resistant isolates. And for the molecular analysis, three possible genes responsible for MBL production (*bla*IMP, *bla*VIM and *bla*NDM) were tested by PCR using gene specific primers. And further sequencing of the PCR product was done for the confirmation of the gene, for which the samples were sent to Xcelris Labs Ltd. India. After receiving the sequence data, analysis was done using various bioinformatics tools.

Our study showed there was a significant number of MBL producing multi drug resistant *P. aeruginosa* present among the isolates. And among three possible genes tested, only one gene was amplified using gene specific primer by the technique PCR. The *bla*NDM gene was only amplified which is of 869bp. The presence of this gene was found in majority of the isolates that were MBL positive. Hence it was inferred that the majority of the MDR *P. aeruginosa* had *bla*NDM gene in them, which was responsible for causing multi drug resistance. In addition, *bla*NDM nucleotide sequence analysis was performed. And sequence analysis showed the presence of *bla*NDM-1 type variant in majority of the isolates. Also 3 novel variants of *bla*NDM were identified after sequence analysis. Therefore, our study shows the increasing frequency of the MBL producers among hospital isolates and even the occurrence of novel variants of resistant gene in them. So the result directs for the active surveillance in the coming years to detect and prevent the dissemination of MDR *P. aeruginosa,* particularly those carrying integron- or plasmid- borne MBL determinants.

CHAPTER 8
CONCLUSION

The results shows that the MBL producer isolates of *P. aeruginosa* were prevalent in this study than ESBL producers which suggests that the risk of emergence of MBL producers that are resistant to carbapenem need to be minimized by continuous surveillance and proper antibiotic stewardship. The resistance of *P. aeruginosa* to carbapenem agents in the present study is due to MBL production. Among the possible resistant genes tested in the study, only *bla*NDM was found in the collected isolates. So the remaining MDR strains of *P. aeruginosa* may carry some other mechanisms to be multi drug resistant.

In conclusion, our results showed the prevalence of MBL-producing *P. aeruginosa* isolates with occurrence of NDM- type MBL. Most predominantly, *bla*NDM-1 type variant was found in the hospital isolates of *P. aeruginosa* in Kathmandu, Nepal in our study and even the novel *bla*NDM variant are present among the isolates. So this occurrence of NDM-1 and its variants producing bacteria calls for the rapid detection of these genes among different clinical isolates and its detection has become even more urgent since the spread of resistance against carbapenem has ended the hopes to control infections. Thus, this study argues for the urgent implementation of strict control measures to prevent the spread of resistance genes and that it warrants the need for constant surveillance. It is crucial to highlight the need for health care facilities to assess the antimicrobial effectiveness of antibiotics periodically to overcome dissemination of MBL-producing *P. aeruginosa* with reduced sensitivity to antibiotics. So, it's time to think prudently the ways to check infections from hospital settings and to coordinate globally for surveillance of such resistant markers producing bacteria.

Regarding to horizontal transmission of these transferable MBL genes, detecting MBL positive strains is necessary. Moreover, by using new methods for rapid identification of MBL positive bacteria in the patients, we could prevent spreading of metallo-beta lactamase strains to other patients. Proper infection control guidelines need to be implemented worldwide. Also the surveillance should be carried out to identify undetected asymptomatic carriers of carbapenem-resistant bacteria. In the absence of novel agents for the treatment of infections caused by multidrug-resistant gram-negative bacteria in the near future, the uncontrolled spread of MBL producers may lead to treatment failures with increased morbidity and mortality. The early detection of MBL-producing *P. aeruginosa* may avoid the future spread of these multidrug-resistant isolates.

Recommendations:

Though this type of study has already been carried out in many countries, the significance of reporting the presence of such type of resistance genes, *bla*NDM and even a novel variant in *P. aeruginosa* isolates from Kathmandu Valley is to indicate the level of antibiotic resistance that may cause an outbreak of the infections in hospital settings in near future. It is a small part of the molecular study which may contribute to the further studies that have to be performed like:

- Study on the multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa.*
- Study of resistance mechanisms in all Grams' negative bacteria.

It has to be noted that the clinicians, health care workers and the governing body who manage the medicine practices in Nepal also needs to reconsider their method of prescription of antibiotics in order to implement antimicrobial stewardship. Overall policies have to be created with regard to antibiotics for their appropriate use in country which is currently lacking. Active surveillance programs need to be conducted in regular basis to detect and prevent dissemination of MDR *P. aeruginosa* thus contributing for the limitation of hospital acquired infections and commencement of the One Health Initiative is the most.

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APPENDIX I

List of Materials

Equipment

- Autoclave; Electric balance; Hot air oven; Incubators; Microscope; pH meter; Refrigerator; Laminar air flow chamber; Bunsen burner; Micropipette; Centrifuge; Micro centrifuge tubes; Electrophoresis chamber; PCR machine; UV transilluminator

Glassware

- Beakers; Conical flasks; Petri dishes; Glass rods; Graduated cylinder; Microscopic-slides; Pipettes; Reagent bottles; Screw capped test tubes

Miscellaneous

- Aluminum foil; Blotting paper; Cotton roll; Cotton swab; Dropper; Forceps; Funnel; Immersion oil; Inoculating loop; Measuring scale; Sampling bottles ; Detergent; Labelling tape; Transport tray; Gloves

Chemicals/Reagents

- Lysol; α-Naphthol; Methyl red; Kovac's reagent; Paraffin; Barium chloride; Potassium hydroxide; Crystal violet; Safranin; Ethanol; Gram's iodine; Conc. Sulfuric acid; Hydrogen peroxide

Microbiological Media

- Nutrient Agar (Hi-media); Muller Hinton Agar (Hi-media); Nutrient Broth (Hi-media); Laurial Broth (Hi-media)

Biochemical Media

- Sulphide Indole Motility Medium (Hi-media); MR-VP Broth (Hi-media); Simmon Citrate agar (Hi-media); Triple Sugar Iron Agar (Hi-media); Urease Agar (Hi-media)

APPENDIX II
Composition and Preparation of Bacteriological Media

Nutrient Agar (NA)

Composition	(Gram/Litre)
Peptone	5.0
Sodium Chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Final pH (at 25°C)	7.4±0.2

Procedure

28 gms of media was dissolved in 1000 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121°C for 15 minutes.

Nutrient Broth (NB)

Composition	(Gram/Litre)
Peptone	5.0
Sodium Chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25°C)	7.4±0.2

Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Mueller Hinton Agar (MHA)

Composition	(Gram/Litre)
Beef Infusion Broth	300.0
Casein Acid Hydrolysate	17.0
Starch	1.0
Agar	17.0
Final pH (at 25°C)	7.0±0.2

Preparation:

3.8 gm of media was suspended in 100 ml distilled water, boiled to dissolve and sterilized by autoclaving at 121°C for 15 minutes. It was poured while at 45-55°C in sterile 9 cm diameter plates in 25 ml quantities. To ensure the uniformity in depth of medium, the plates were placed over level surface and the medium was poured into it.

APPENDIX III
Gram's Staining

Composition of stains and reagents

i. Crystal violet

Solution A	
Crystal violet	2.0 gm
95% ethyl alcohol	20.0 ml
Solution B	
Ammonium oxalate	0.8 gm
Distilled water	30.0 ml

Crystal violet was dissolved in ethyl alcohol and ammonium oxalate in distilled water. Then solution A and B are mixed.

ii. Gram's iodine

Iodine	1.0 gm
Potassium iodide	2.0 ml
Distilled water	300.0 ml

Iodine and potassium iodide were dissolved in distilled water.

iii. Ethyl Alcohol (95%)

Absolute alcohol	95.0 ml
Distilled water	5.0 ml

iv. Safranin

Safranin	10.0 ml
(2.5% solution in 95% ethyl alcohol)	
Distilled water	100.0 ml

Procedure:

a. Diluted suspensions of the bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2 to 3 times.
b. The slides were flooded with crystal violet solution for one minute, washed with water.
c. It was then flooded with Gram's iodine for one minute.
d. The slides were washed with water and decolorized with 95% ethyl alcohol dropped from drooping bottle until no violet colour was visible from drain off solution.
e. The slides were washed with water and counter stained with safranin stain for about 30 seconds and washed with water.

f. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter.
g. Cells were then comfirmed on the basis of colour observed, pink or red for Gram negative cells. Cell morphology was studied using Gram's reaction under oil immersion.

APPENDIX IV
Biochemical Tests

1. Catalase Test

Catalase test is done to test the presence of enzyme catalase. The enzyme catalase splits hydrogen peroxide to water and oxygen.

Reagents: (3% Hydrogen peroxides).

Composition	
Concentrated Hydrogen peroxide	3 ml
Distilled water	97 ml

Procedure

3 ml of 3% hydrogen peroxide was taken in a test tube and colony of bacteria to be tested was picked up from nutrient agar with the help of glass rod and inserted into the tube-containing reagent. The production of gas bubbles immediately indicates positive catalase test.

2. Oxidase Test

Oxidase test is done to determine the presence of the oxidase enzyme. Oxidase reaction is due to the presence of a cytochrome oxidase system.

Oxidase reagent

Whatman No.1 filter paper was cut into strips of 6-8 cm in diameter. It was soaked in the reagent till saturation. The paper strips were drained and freeze dried and stored in a dark tightly sealed bottle.

Procedure

The oxidase test paper was moistened with distilled water. A colony was picked using glass rod and rubbed to the paper. Development of violet colour within 10 seconds is an indicative of positive test.

3. Sulfide-Indole-Motility Medium (SIM)

Sulfide-Indole-Motility is a semi solid medium used for the determination of sulfide production, Indole formation and motility of enteric bacteria.

Composition	(Gram/Litre)
Beef extract	3.0
Peptone	30.0
Peptonized iron	0.2
Sodium thiosulfate	0.025
Agar	3.0
Final pH (at 25°C)	7.3±0.2

Preparation

Thirty-six grams was suspended in 100 ml distilled water. It was heated to boil to dissolve the medium completely. It was dispensed in tubes and sterilized by autoclaving for 15 minutes at 15 lbs pressure (121°C). The medium was allowed to solidify in vertical position.

Reagent: Kovac's reagent

Composition	gm/litre
P-Dim ethyl aminobenzaldehyde	5.0
Iso amyl alcohol	75.0 ml
Conc. Hydrochloric acid	25.0 ml

Procedure

The test organisms was stabbed into the medium and incubated at 37°C for 24 hrs. Motile organism show diffuse growth or turbidity away from the line of inoculation.
0.2 ml of Kovac's reagent was added to the tube and allowed to stand for 10 minutes. No dark red color in the reagent indicates a negative indole test.

4. Methyl Red test

The methyl red test is done to test the ability of an organism to produce and maintain stable acid products from glucose fermentation and to overcome the buffering capacity of the system.

MR-VP medium (glucose-phosphate broth)

Composition	(Gram/Litre)
Buffered peptone	7.0
Dextrose	5.0
Tripotassium phosphate	5.0
Final pH (at 25°C)	6.9±0.2

Preparation

Seventeen grams was dissolved in 1000 ml distilled water. It was distributed in test tubes in 10 ml amount and sterilized by autoclaving at 15 lbs pressure for 15 minutes.
Reagent - Methyl Red

Composition	(Gram/Litre)
Mehyl red	0.04 gm
Ethyl alcohol	40.0 ml
Distilled water	60.0 ml

Methyl red was dissolved in ethyl alcohol and water was added.

Procedure

The glucose phosphate broth was inoculated with culture to be tested and incubated at 37°C for 48 hrs. Methyl red indicator was added to the culture and development of red color indicates positive test while yellow color indicates negative test.

Voges- Proskauer Test

Voges- Proskauer test determine the ability of organism to produce a neutral end product, acetylmethylcarbinol from glucose formation.

Medium- MR-VP medium (glucose-phosphate broth)

Solution A

α-naphthol	5.0 gm
Ethyl alcohol (95%)	100.0ml

Solution B

Potassium hydroxide	40.0gm
Distilled water	100.0 ml

Procedure

Sterile broth was inoculated with fresh culture medium and incubated at 37°C for 48 hrs. Development of pink-red color within 30 minutes after adding α-naphthol and 40% potassium hydroxide in 1:3 proportions was recorded as positive result, no color development indicates negative test.

5. Citrate Utilization Test

Citrate utilization test is performed to determine if an organism is capable of utilizing citrate as the sole source of carbon for metabolism with resulting alkalinity.

Medium- Simmon's Citrate Agar

Composition	(gram/litre)
Monoammonium phosphate	1.0
Dipotassium phosphate	1.0
Sodium Chloride	5.0
Sodium Citrate	2.0
Magnesium Sulphate	0.2
Bromothymol blue	0.08
Agar	15.0
Final pH (at 25°C)	6.8±0.2

Preparation

24.2 grams was suspended in 1000 ml distilled water. It was heated to boil to dissolve the medium completely. It was distributed in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The mediums in tubes were solidified in slanted position.

6. Triple Sugar Iron Agar Test

The test is done to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium, with or without the production of gas, along with determination of possible hydrogen sulfide production.

Composition	(gm/litre)
Peptone	10.0
Tryptone	10.0
Yeast extract	3.0

Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.2
Sodium Chloride	5.0
Sodium Thiosulfate	0.3
Phenol – red	0.024
Agar	12
Final pH(at 25°C)	7.4±0.2

Preparation

6.5 grams was suspended in 1000 ml distilled water. It was boiled to dissolve completely. It was distributed in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The medium was allowed to set in slopped to form a butt about 1 inch long.

Procedure

The test organism was stabbed in the butt and streaked on the slant. The tubes were incubated at 37°C for 24 hrs. The change in color of butt, slant and gas formation was noted and recorded. No any change is indicative of negative test.

7. Urease Test

Urease test demonstrate the ability of an organism to split forming two molecules of ammonia by the action of the enzyme urease.

Medium- Urea agar base

Composition

Peptone	(gm/litre)
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Disodium phosphate	1.2
Monopotassium phosphate	0.8
Phenol red	0.012
Agar	15.0
Final pH (at 25°C)	6.8±0.2

Preparation

24 grams urea agar base was suspended in 950 ml of distilled water. It was boiled to dissolve completely ans sterilized by autoclaving at 10 lbs pressure for 20 minutes. It was cooled down to 55°C and aseptically introduced 50 ml of sterile 40% area solution and mixed well. It was distributed in sterile test tubes and allowed to solidify in slanted position.

Procedure

Fresh culture of test organism was streaked heavily on the slant and incubated at 37°C for overnight. No change in color of medium to pink indicates negative test.

APPENDIX V

Zone Size Interpretative Chart of Antibiotic Susceptibility Testing

Antibiotic used	Symbol	Disc content (mcg)	Diameter of zone of inhibition in mm		
			Resistant	Intermediate	Sensitive
Amikacin	AK	30	≤14	15-16	≥17
Cefepime	CPM	30	≤14	15-17	≥18
Cefoperazone/sulbactam	CFS	75/10			
Ceftazidime	CAZ	30	≤17	18-20	≥21
Cefotaxime	CTX	30	≤14	15-22	≥23
Ciprofloxacin	CIP	5	≤15	16-20	≥21
Colistin	CL	10	≤10	-	≥11
Imipenem	IPM	10	≤15	16-18	≥19
Levofloxacin	LE	5	≤13	14-16	≥17
Meropenem	MRP	10	≤15	16-18	≥19
Piperacillin	PI	100	≤14	15-20	≥21
Piperacillin/Tazobactam	PIT	100/10	≤14	15-20	≥21
Polymixin- B	PB	300Units	≤11	-	≥12

(Source: Product Information Guide, Hi-Media Laboratories Pvt. Limited, Mumbai, India)

APPENDIX VI
Nucleotide Sequences

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APPENDIX VII
Peptide Sequences

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Genbank Submission

Showing *bla*NDM gene

Pseudomonas aeruginosa (BT.1), complete sequence

LOCUS Seq1 813 bp DNA linear BCT 23-JUN-2017
DEFINITION Pseudomonas aeruginosa isolate Pseudomonas aeruginosa extrachromosomal.
ACCESSION Seq1
VERSION
KEYWORDS .
SOURCE Pseudomonas aeruginosa
ORGANISM Pseudomonas aeruginosa
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
REFERENCE 1 (bases 1 to 813)
AUTHORS Sthapit,K., Shrestha,M. and Malla,R.
TITLE Molecular Analysis of beta-lactamase gene in Multidrug Resistant Clinical Isolates of Pseudomonas aeruginosa
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 813)
AUTHORS Sthapit,K., Shrestha,M. and Malla,R.
TITLE Direct Submission
JOURNAL Submitted (23-JUN-2017) Central Department of Biotechnology, Tribhuvan University, TU Rd, Kirtipur 44618, Kirtipur, Bagmati 44600, Nepal
COMMENT Bankit Comment: ALT EMAIL:krishasthapit@hotmail.com.
Bankit Comment: TOTAL # OF SEQS:3.
Bankit Comment: TOTAL # OF SETS:1.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
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781 catacggccc gcatggccca caagctgcgc tga
//

EXTRA THESIS ACTIVITIES

Achievements:

1. Kathmandu Center for Education and Research, CAS & TU Thesis Grant for M.Sc. students – 2015 grant.

Seminar / Conference Presentations:

1. **Poster Presentation** – 7th National Conference on Science and Technology , March 29-31, 2016
2. **Poster Presentation** – International Seminar on "Interdisciplinary Approaches to Biological Science", 1st July, 2016
3. **Poster Presentation** – International Conference on "Emerging Trends in Biological, Chemical and Physical Sciences", December 9-11, 2016
4. **Poster Presentation** – World DNA Day, 2017
5. **Oral Presentation** – 3rd International South Asian Biotechnology Conference, March 16-18, 2017

Publications:

1. **Sthapit K**., (2016). Metallo beta lactamases in *Pseudomonas aeruginosa*, a cause for antibiotic resistance: Is there a need to worry? *The Transcript*. Vol. 2(1). ISSN 2505-1083. Nepalese Society of Biotechnology pp. 64-65