MOLECULAR INVESTIGATON OF CARBAPENEM RESISTANT *Pseudomonas aeruginosa* ISOLATED FROM TERTIARY CARE HOSPITAL OF NEPAL

M.Sc. Thesis
(2018)

Submitted to:
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Tribhuvan University
Kirtipur, Kathmandu, Nepal

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Declaration

I hereby declare that the thesis entitled **"MOLECULAR INVESTIGATION OF CARBAPENEM RESISTANT *Pseudomonas aeruginosa* ISOLATED FROM TERTIARY CARE HOSPITAL OF NEPAL**" is based on work carried out by me and the work has not been submitted in candidature for any other degree. The research work has been carried out at Central Department of Biotechnology under the guidance of Prof. Rajani MallA and Dr. Suresh Subedi. I will have no objection for availability of the thesis for photocopy and inter- library loan for the purpose of scholarly research.

Samikshya Kafle

Acknowledgements

It is my immense pleasure to express my deep sense of thanks and gratitude to my thesis supervisors Prof. Dr. Rajani Malla, former Head of Department and Dr. Suresh Subedi, Assistant Professor, Central Department of Biotechnology, Tribhuvan University. Without their support and devoted contribution in every step throughout the process, this thesis would have never been accomplished. I would like to thank both of you very much for your support and understanding over these past years.

I owe a deep sense of gratitude to Prof. Dr Krishna Das Manandhar, Head of Central Department of Biotechnology, Tribhuvan University, Kirtipur for his support to complete my thesis in the department. I would like to give special thanks to my senior Mr Mitesh Shrestha on this project for who has provided excellent support and expert knowledge to all work and experimental procedures during this project.

My sincere thanks to Dr Tilak shrestha, Dr Tribikram Bhattarai, Dr Ganga Kharel, Mrs Jarina Joshi, Dr Smita Shrestha, Dr Pramod Aryal, Ms Pragati Pradhan, Mrs Preeti Regmi and Mrs Alina Sapkota and all the teachers of Central Department of Biotechnology for supporting me by providing their valuable suggestions. I am equally thankful to all the technical and administrative staff of CDBT for their kind help and cooperation throughout my study period.

I want to thank Mr. Hari P Kattel, Lab In charge, Tribhuvan University Teaching Hospital and Mr. Santosh Yadav for providing me with the samples for the thesis work.

I am extremely grateful to University Grant and Commissions, Nepal for providing the 'Master thesis Support Grant' as financial support.

Furthermore I would like to thank my friends namely Apshara Parajuli, Anju Tamang, Deepesh Thapa, Manju Maharjan, Mahesh Lamsal, Milan Mainali, Navin Adhikari Pradip Chaudhary, Pratikshya Mainali, Rashmi thapa, Rajani Singh, Rocky Maharjhan, Sumeena karki, Sudhiksha Chaulagain, Sapna Sakya, Suprim Tha, Sila Mahatara, Saraswati Adhikari and all my seniors, juniors of CDBT for their support to complete my work.

Finally, I wish to thank my parents and my husband for their constant support and encouragement to accomplish this work on time.

Glossary acronyms

ATCC : American type culture collection

bla : Beta lactamase

CA : Clavulanic acid

CAZ : Ceftazidime

CDDT : Combined disk diffusion test

CDC : Center for Disease Control

CLSI : Clinical and Laboratory Standards Institute

CRPA : Carbapenem resistant *P. aeruginosa*

CTX : Cefotaxime

CX : Cefoxitin

DDST : Double disk synergy test

DNA : Deoxyribonucleic Acid

EDTA : Ethylene Diamine Tetra Acetic acid

ESBL : Extended Spectrum Beta Lactamase

Fig : Figure

IMP : Imipenem

LB : Luria broth

MBL : Metallo beta lactamase

MDR : Multi drug resistance

MHA Muller Hinton Agar

MRP : Meropenem

MLST : Multi Locus Sequence Typing

NA : Nutrient Agar

NCBI : National Center for Biotechnology Information

OMPs : Outer Membrane Proteins

PBPs : Penicillin binding proteins

PCR : Polymerase chain reaction

RNA : Ribonucleic Acid

WHO : World Health Organization

ZOI : Zone of Inhibition

KPC : *Klebsiella pneumoniae* Carbapenemase

NDM : New Delhi metallo beta lactamase

OXA : Oxacillinase

ASM : American Society of Microbiology

Table of Contents

List of Figures

List of Tables

ABSTRACT

Background: *P. aeruginosa* are categorized as second most critical group of pathogen by WHO. These opportunistic pathogens mainly affect patients with compromised host defense mechanisms. Infections caused by *P. aeruginosa* can be life threatening. Several resistance mechanisms make them able to resist multiple classes of antibiotics including *β*-lactams. Carbapenem are potent beta lactam antibiotics and last resort of drug. However, resistance to this drug also has been reported lately. Loss of OprD porin have significant role against carbapenem. Similarly, β – lactamase (MBL and ESBL) have potential to hydrolyze carbapenem of which gene bla_{NDM} has great concern. In addition, AmpC beta lactamase with combination to other mechanism also showed resistance to carbapenem. Due to such mechanism attributed by *P. aeruginosa* against carbapenem it has become very difficult to combat them, leaving fewer options for antibiotic therapy.

Aims: This study aims to provide knowledge on carbapenem resistance mechanism including loss of porin, overexpression of AmpC beta -lactamase and carbapenemase at molecular level.

Methods: In this study, 95 isolates of *P.aeruginosa* were collected. Antimicrobial susceptibility profile based on the disk-diffusion tests was performed to detect multidrug resistance isolates. Carbapenem resistance isolates were selected and classified into IMP^R, $MRP^{R,}$, IMP^RMRP^R types. Phenotypic detection for MBL-producer, ESBL-producer and AmpC producer were performed. Molecular identification of carbapenem resistant gene of *P. aeruginosa* was carried out by PCR and followed by sequencing.

Results and Conclusion: Among 95 isolates, total 73 (76.84%) were found to be Multidrug resistance *P. aeruginosa* and out of 73 MDR, 61 (83.56%) were carbapenem resistant isolates. Phenotypic test revealed that 55 (90.1%) MBL-producer and 45(61.64%)were ESBL producer according to the standard microbiological method CLSI. The PCR analysis result showed that most of the carbapenem resistant isolates were found to have (42.62%) *ampC* gene while (31.14%) were found to lack OprD gene. Among 61 carbapenem resistant isolates, 7 (11.47%) and 6 (9.8%) were found to have detection to bla_{NDM} gene. The sequence of these genes showed mutation that could potentially lead to stronger carbapenemase activity.

Our results confirmed that multiple resistance mechanism such as OprD loss, carbapenemase and AmpC beta lactamase production conferred resistance to the carbapenem in *P. aeruginosa,* isolated from hospital settings.

Keywords: Antibiotics resistance, Carbapenem, *P. aeruginosa,* Outer membrane porins.

CHAPTER 1
INTRODUCTION

1.1 Background

P. aeruginosa is one of the most common, Gram-negative rod shaped bacteria that can be found in soil, water, natural environment, hospitals and also present as the normal flora of human body parts. It is an important pathogen involved in healthcare-associated infections. *P. aeruginosa* is associated with numerous acute and chronic human infections, such as respiratory tract infection, urinary tract infection, pneumonia and bacteremia etc. It can opportunistically infect people, particularly who are immune-compromised, patients with burn wounds, cystic fibrosis and cancers (Gellatly & Hancock, 2013). Infections caused by *P. aeruginosa* can even be life threatening since they have potential to resist multiple classes of drugs. Prevalence of MDR *P. aeruginosa* is increasing worldwide due to inappropriate use of drugs and improper dosage of antibiotics. They can resist multiple classes of antibiotics, hence are referred as multi drug resistance and the eradication of such multidrug resistance pathogens have become more difficult in clinical settings (Hirsch & Tam, 2010). This may result in high level of morbidity and mortality rates in infected patients. According to data reported at centers for disease control in 2013, *P. aeruginosa* is categorized as critical group of multi drug resistant bacteria that has serious threat to public health (CDC, 2013).

P. aeruginosa is resistant to various classes of antibiotics including beta-lactam antibiotics such as penicillin and carbapenem, tetracyclines, aminoglycosides such as gentamicin, tobramycin, and amikacin, and fluoroquinolones such as ciprofloxacin (Paterson et al., 2006). There are several resistance mechanisms exhibited by *P. aeruginosa* such as intrinsic resistance mechanism that includes beta-lactamase production, efflux-mediated and porin-related resistance, and target site modification. In Addition, they also possess acquired resistance mechanism through the acquisition of different mutations and transferable resistant mechanisms. Intrinsic and acquired resistance mechanisms confer resistance to antibiotics, to which they were initially susceptible, making them difficult to kill due to short of treatment options (Livermore et al, 2002).

Carbapenems are the most powerful drugs of all time. Meropenem, entrapenem and imipenem are the member of β- lactam family which are frequently used to trea infections caused by *P. aeruginosa*. They possess broad spectrum of activities and great potency against pathogens. Numerous gram negative bacterial cells are killed by carbapenem by binding to penicillin-binding proteins and inhibiting cell wall synthesis. They are stable in the presence of almost all beta-lactamases including AmpC beta-lactamases and extended-spectrum beta-lactamases (ESBLs).

Carbapenems that include imipenem and deripenem etc are potent antibiotics for gram positive bacteria while carbapenems like meropenem, etrapenem are slightly effective to gram negative bacteria. Because of their unique molecular structure, they are not

easily diffusible through the cell wall and bind to critical penicillin binding proteins of the bacterial cell wall, thereby disrupting growth and structural integrity. They are last resort drugs used against serious infections caused by multidrug resistant *P. aeruginosa* including those producing, AmpC beta lactamase and extended spectrum beta lactamase (ESBL). However, expanded and unjustified use of drugs has resulted in an increased resistance of pathogens against Carbapenem antibiotics, throughout the world, leaving even fewer therapy options (Buehrle et al., 2017)

In *P. aeruginosa*, multiple mechanisms are involved in carbapenem resistance including plasmid mediated production of carbapenem hydrolyzing enzyme -carbapenemase such as metallo beta-lactamase (MBLs), extended spectrum beta lactamase (ESBL) and chromosomal mediated AmpC overproducer, mutation in porin protein OprD gene and over-expression of efflux-pumps (Buehrle et al., 2017).

Carbapenemases are beta lactamase enzymes that are capable of hydrolyzing all beta-lactam antibiotics, including monobactams, extended spectrum cephalosporins and carbapenem. According to the Ambler molecular classification beta lactamases are classified into four classes (A to D) (Silveira et al, 2018).

Classes A, C and D utilize serine at their active site for beta lactam hydrolysis, whereas molecular class B comprises all metalloenzymes which utilize active-site zinc for substrate hydrolysis (Ambler, 1980).

Molecular class A beta lactamase is further categorized into families - SME, NMC, IMI and KPC, which can hydrolyze all the antibiotics including Penicillin, extended spectrum cephalosporins, and even carbapenems. Recently GES are also placed in class A family. These enzymes can be inhibited by clavulanic acid but not by EDTA. Among these, SME, NMC and IMI are chromosomal mediated while KPC and GES are plasmid mediated enzymes. Similarly, Molecular class B includes five major families such as IMP, VIM, GIM, SPM, NDM which can hydrolyze all antibiotics but are inhibited by aztreonam and metal ion chelators (EDTA). Most of the MBL are plasmid mediated and the resistance mechanism gainst carbapenem is different for different MBLs. Ambler molecular D class includes OXA enzymes, which can hydrolyze cloxacillin or oxacillin and are plasmid mediated. They are second largest family of beta lactamase.

Ambler molecular class C beta lactamase includes AmpC type beta lactamase which can hydrolyze extended spectrum cephalosporin. In recent studies of *P. aeruginosa*, they confer resistance to carbapenems through the overexpression of AmpC type's enzyme. However, over production of AmpC enzyme alone does not confer resistance to carbapenems, additional resistance mechanisms like overproduction of AmpC enzymes and others are responsible for resistance. Different enzymes such as FOX, CMY etc are clinically significant AmpC enzymes present in *P. aeruginosa* which can be both chromosomal mediated as well as plasmid mediated. They are less common types of beta lactamases than ESBL.

Plasmid mediated extended spectrum beta lactamases (ESBL) are Ambler A class beta lactamase that can hydrolyze oxyimino-cephalosporins and monobactams but not carbapenems, being inhibited by combinations of amoxicillin-clavulanate and piperacillintazobactam. Since they are easily transferable among one another, the rapid spread of ESBL producing *P. aeruginonsa* is one of the most worrisome resistanc e mechanisms around the world. Among different families of ESBL enzymes SEV, CTX-M and TEM are now the most prevalent while PER, VEB types are less prevalent in carbapenem resistance *P.aeruginosa* (Fowler et al., 2014).

In *P. aeruginosa* with loss/mutation of outer membrane protein (OprD) and reduced accumulation of drugs due to active efflux pump plays significant role in chromosomal mediated carbapenem resistance. Recent studies have reported that the broad spectrum resistance mechanism typically involves porin loss i.e loss of outer membrane protein OprD reduces the entry of carbapenem, especially imipenem resistance and if combined with over expression of efflux systems leads to high level of carbapenem resistance in bacteria for both imipenem and meropenem. OprD is a substrate-specific outer membrane porin of *P. aeruginosa*, which allows the diffusion of basic amino acids, small peptides and imipenem into the cell. Mutations, in the specific porin OprD/loss of OprD2 protein in absence of carbapenemase reduce the uptake of imipenem. Sequence analysis of the *OprD* gene usually reveals various routes of inactivation, including single nucleotide change resulting in premature stop codon, insertion or deletion resulting in frameshift, and disruption of protein by insertion sequences.

Although the development of bacterial resistance to carbapenems largely parallels its use, the rate of emergence of resistance has been relatively low (Kattan et al, 2008).

1.2 Current studies

P. aeruginosa cause healthcare associated infections (HAI), mostly in case of immune compromised or critically ill patients (Chatzinikolaou et al., 2000). Concerning the ability to cause infections of several types, National nosocomial infections surveillance system reported, based on the data from 1986 to 2003, that *P. aeruginosa* was the second most common cause of pneumonia(18.1%), third most responsible for urinary tract infections (16.3%) and the eighth most organism isolated from the blood stream (Gaynes & Edwards, 2005) .

Burgeoning nosocomial infections caused by multidrug resistant *P. aeruginosa* compromises the choice of suitable antibiotics which is directly related to morbidity and mortality (Pena et al., 2012**).** The threat posed by this organism arises from its extraordinary ability to develop resistance nearly against all available antibiotics by the selection of mutations in genes, transferrable resistance determinants, mainly encoding carbapenemases (MBLs) or ESBLs, which are frequently co-transferred with genes encoding aminoglycoside- modifying enzymes. Further, concerns have been raised by emergence of MDR/XDR clones emerging at hospitals worldwide.

As the evolutionary courses of antimicrobial resistance in *P. aeruginosa* remains unexplored, whole genome sequencing performed in the wild-type and mutated strains subjected to relevant antimicrobials in increasing concentrations revealed the accumulation of random mutations, mostly transitions. Further, error-polymerase DNA activity due to induction of SOS system was also seen which led to frameshift mutagenic signature.

Along with other antimicrobials, a striking finding was seen in the evolutionary perspective of meropenem resistance. An extremely large deletion of size (> 250kb) was observed, providing growth advantage in presence of the antibiotic (Cabot et al., 2016)

Similar to the global issue, the rise in antimicrobial - resistance has also been a serious threat to Nepal. Few research and published documents are not enough to track the use, trends of antibiotic resistance and underlying mechanisms (Dahal & Chaudhary, 2018)

The study conducted by Tada et. al, reported that ST664 *P. aeruginosa* spread in Nepal in medical settings and gave the first report describing carbapenemases and 16S Rrna methyl transferase co-producing *P. aeruginosa* in case of Nepal. And, suggested further necessities of survey of *P. aeruginosa* in Nepal in clinical settings.

In attempt to further explore the molecular mechanisms, this study focused on the presence of genes – Oprd (proved to confer resistance against carbapenems), NDM (found to hydrolyze carbapenems), and AmpC (found to confer resistance in co-ordination with Oprd and other carbapenemaase) in *P. aeruginosa* found in hospital settings of Nepal, responsible for conferring resistance.

1.3 Hypothesis

Null Hypothesis:

A single resistant mechanism solely confers resistance against carbapenems in *P. aeruginosa,* prevalent in Nepal.

Alternative Hypothesis: Multiple mechanisms exist to confer resistance against carbapenems in *P. aeruginosa*prevalent in Nepal, with one method more common than other.

1.4 Objectives

1.4.1 General objective

The objective of the research was to understand the molecular mechanism of carbapenem resistance in *P. aeruginosa.*

1.4.2Specific objectives

I. To isolate and identify carbapenem-resistant *P. aeruginosa* by the phenotypic method.
II. To detect ESBL, MBL, and AmpC producing *P. aeruginosa*
III. To perform PCR amplification of the gene encoding the outer membrane protein, Metallo beta-lactamase gene and AmpC encoding gene in *P. aeruginosa.*
IV. To confirm the gene responsible for resistance through sequencing.
V. To identify novel mutation

1.5 Rationale and scope of the study

Multidrug resistance bacteria are emerging across the globe and Nepal is not an exception. *P. aeruginosa* is considered as the most critical group of multidrug-resistant bacteria and are reasons behind the mortality and morbidity of the patient with a various infectious disease. They have been major health concern since they have shown resistance to last resort of drug carbapenem. They are evolving resistance to most of the antibiotics because of inadequate dosage of antibiotics, improper use of antibiotics etc. Different mechanisms such as intrinsic mechanism including a low outer membrane permeability, the production of an AmpC β-lactamase, and the presence of numerous genes coding for different multidrug resistance efflux pumps and β-lactamases, MBL and ESBL etc are found in *P. aeruginosa*. They can develop other new mechanisms in their genome to escape the lethal action caused by antibiotics by selection pressure and even can transfer those genes responsible for resistance to other strain which is a major problem in clinical settings. Therefore, it is very crucial to detect antimicrobial resistant strain and resistant gene involved at early state before they cause outbreaks in the hospitals. In developing countries like Nepal, usually phenotypic and presumptive detections are carried out for multidrug resistance. However, the interpretations with this method are not sufficient and uncommon resistance pattern is often complicated by false-positive or false negative results. This can lead to inappropriate antibiotic prescriptions and therapy failure. Furthermore, a phenotypic test does not allow a detailed molecular identification of antimicrobial resistance which is needed for epidemiological and infection control purposes. Recent advanced molecular methods are essential for an accurate, fast and sensitive determination of resistance status among such pathogens. Hence, this study aims to provide a view to understand the molecular spectrum of carbapenem-resistant *P. aeruginosa* for optimal treatment of patients and to control the dissemination of resistance.

CHAPTER 2
LITERATURE REVIEW

2.1 *P. aeruginosa*

P. aeruginosa is a gram negative, rod shaped bacteria measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. They are obligate aerobes, motile having single polar flagella, non-sporeforming, glucose and lactose non fermenting microorganisms. *P. aeruginosa* have polysaccharide capsule and are citrate, oxidase and catalase positive. They belong to one of the major families*, Pseudomonadaceae.* They have multiple colony morphologies (rough, smooth and mucoid). Smooth and mucoid colonies are presumed to have role in colonization and virulence. They produce varieties of pigments including pyocyanin (blue), pyoverdine (yellow and fluorescent) also known as florescent pigment, pyorubin (red), and pyomelanin (brown).

Only *P. aeruginosa* is capable of producing water soluble pigment; pyocyanin (blue-green) which distinguish them from other *Pseudomonas* species. They have distinctive grapelike odor due to production of amino acetophenone and have ability to grow up to 42ºC. Colorization, odor and colonies morphologies help in the preliminary identification of *P. aeruginosa*. They are ubiquitous microorganisms, typically found in soil, water as well as in living source such as plant and animals. They can survive in minimum nutritional requirement and variety of physical conditions; they can also be isolated from the sewage and hospital settings. *P. aeruoginosa* are commonly present in the surface of the skin and moist part of the healthy human body without causing harm, yet are responsible for several infections once immune system is compromised (Slekovec et al., 2012). They are associated in most of the hospital acquired infections and nosocomial infections such as Pneumonia, urinary tract infection, respiratory tract infection, dermatitis and bacteremia. *P.aeruginosa* is often referred as opportunistic pathogen because of its ability to invade in immunocompromised state with diseases like cystic fibrosis and burns (Mulcahy et al, 2014).

Scientific classification of ***P. aeruginosa***

Kingdom: Bacteria

Subkingdom: Negibacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Geneus: *Pseudomonas*

Species: *P. aeruginosa*

P. aeruginosa is the most common hospital pathogen, causing approximately 10% of the 2 million hospital infections in the United States annually. According to the data from the National health care safety network at Center for disease control and prevention, *P. aeruoginosa* is the sixth most common nosocomial pathogen that cause health care associated infections from 2011 to 2014 and second most common pathogen in ventilator- associated pneumonia (VAP) in U.S hospitals (Weiner et al., 2016).

It is the second most common cause of Pneumonia (18.1%), the third most common cause of urinary tract infection (16.3%) and the eight most frequently isolated pathogen from blood stream (3.4%) (Gaynes & Edwards, 2005).

The prevalence of *P. aeruginosa* infections is 11.5% in Europe and 17% in developing countries. Infections caused by these organisms have always been difficult to treat. Antibiotics including gentamicin, tobramycin, colistin, carbapenem and amikacin are relatively effective at treating *P. aeruginosa*-related infections. Even these antibiotics are not effective against most of the strains, due to high intrinsic and acquired resistance in them. Controlling antimicrobial resistant *P. aeruginosa* infections is very challenging in the hospital settings. And their increment at the alarming rate, leaves few options for antibiotic therapy.

2.2 Genomic structure of *P. aeruginosa*

The complete genome sequence reveals, *P. aeruginos*a has the genome size of about 5.2 to 7 million base pairs (Mbp) which is largest bacterial genome that has been sequenced to this time. It consistsof 65% Guanine + Cytosine and 5,570 predicted open reading frames (ORFs). *P. aeruginosa* genome also contains large number of genes to encode outer membrane proteins involved in adhesion, motility, antibiotic efflux, virulence factor export, and environmental sensing by two-component systems. It also contains gene that are involved in nutritional uptake, metabolism and transport system. 8.4% of the regulatory genes are predicted which is the highest percent that has been predicted among all bacteria. Genome of the *P. aeruginosa* consist variable and core region. The core genome constitutes 90% of the total genome that encode most of the metabolic and pathogenic factor in which variable region are distributed. The core genome is conserved in all strain whereas accessory gene is present only in some of the strain of *P.aeruginosa*.The variable accessory genome is characterized by a set of genomic islands and islets from a primeval tRNA-integrated island type (Kung et al, 2010).

Pseudomonas aeruginosa has a single and super-coiled chromosome but large number of plasmids that carries several mobile genes. These plasmids encoded genes are responsible for production of beta lactamase etc. Such plasmid mediated and chromosomal mediated genes are responsible for causing various virulenceand pathogenicityin *P. aeruginosa*.

2.3 Carbapenems

Carbapenems are the potent series of beta lactams antimicrobial agents that possess broad spectrum of activity and greatest potency against Gram negative and Gram positive bacteria. Their use has been increased due to the rise in the resistance to cephalosporin after the bacteria started producing beta lactamse against the antibiotic (Hawkey & Livermore, 2012). Carbapenem has been used in clinical trials for past 20 years.

Carbapenems have unique nuclear structure that are different from the nucleus of the penicillin having a carbon atom replacing Sulphur at position 1 and also have an unsaturated bond between carbon atoms 2 and 3 in the 5-membered ring.

Thienamycin and olivanic acids are the members of carbapenem which were first discovered at the same year 1976, from species -*Streptomycescattleya* and *Streptomycesolivaceus* respectively. Likewise, several natural carbapenems have been discovered in past decades and several semi synthetic carbapenems are been synthesized. Some of them are asparenomycins, carpetimycins, and Pluracidomycin etc (Moellerin et al, 1989).

According to recent classification system, carbapenem are classified into different types. They are:

Imipenem: It is the oldest carbapenem that has broad spectrum activity against several bacteria. However, its drawback was its unstable chemical structure.It was not approved by the US Food and Drug Administration (FDA) for several diseases like meningitis, and should be avoided in the treatment of central nervous system infections because of its propensity to cause seizures in patients with elevated risk factors, e.g. renal failure or structural brain disease (Chambers, 2005). *Pseudomonas* exhibit resistance to imipenem due the downregulation of the carbapenem-specific OprD porin in the cell wall.

Panipenem: It is the second approved carbapenem, introduced in Japan, 1993. It requires co administration of inhibitor called betamipron for successful inhibition.

Meropenem: It is approved by the US FDA for the clinical trials. As compared to the imipenem it is more potent antibiotics for several gram negative bacterial infections. P. aeruginosa confers resistance to the Meropenem is due to the active efflux system.

Entrapenem: It was developed in 2001 having more resistance to DPH-I inactivation without addition of DPH-I inhibitor such as cilastatinor betamipron. It is often used for the empirical treatment of complicated community-acquired bacterial infections, where a mixed flora of anaerobes and aerobes is likely to be found, e.g. community-acquired pneumonia, complicated skin and skin structure infection, complicated urinary tract infection, or community-acquired complicated intra-abdominal infection, in both

children and adults. Now use of entrpenem is recommended for the treatment of nosocomial infections caused by pathogens, less in *P. aeruginosa*.

Doripenem: It is a parenteral 1-β-methyl carbapenem that has completed phase 3 trials for nosocomial infection including including ventilator-associated pneumonia, complicated intra-abdominal infection, and complicated urinary tract infection. The doripenem has lower MIC valve for *P. aeruginosa* than are those of other anti pseudomonal agents, and it inhibits a great proportion of carbapenem-resistant *P. aeruginosa*.

Despite having ability to inhibit beta lactamase they are chemically unstable; thus they have not been deployed clinically. Later, the N-formimidoyl derivative of thienamycin (now known as imipenem) proved much more stable, thus are preferred for clinical treatment. Imipenem at the beginning showed broad spectrum activity against gram negative bacteria including *P. aeruginosa* and others. The reason was: Imipenem has ability to penetrate well through the outer cell envelope of Gram-negative bacilli and its high affinity for certain penicillin-binding protein (PBP) targets. Development of small spheres or elipsoid forms which proceed to lysis, without the production of long filamentous form as seen in penicillin and cephalosporin leads to high affinity binding to PBP2 and low affinity for PBP3 (Kahan et al, 1983).

Other mithicillin-resistant *Staphylococcus aureus*, and methicillin-resistant coagulasenegative *staphylococci* etc are generally resistant to imipenem. Imipenem can be hydrolyzed by dehydropeptidase I (DHP-I) therefore, some pathogens were insensitive to imipenem while there is no evidence of emergence of resistance to imipenem in Enterobacteriaceae. However, resistance to imipenem has developed up to 25% in *P. aeruginosa* infections which were treated with imipenem alone, probably as a result of permeability changes in these organisms, and not due to hydrolysis by beta lactamases. Moreover, extensive use of imipenem causes significant increase in resistant *P. aeruginosa* (Nicolau, 2008). More stable carbapenems with broader spectrum have been developed such as meropenem, biapenem, etrapenem, doripenem. Meropenem is stable carbapenem which is different from imipenem with a 6-a-hydroxyethyl group, which is the addition of a methyl group to the 1-β position. This modification protected meropenem from DHP-I hydrolysis.

Meropenems have high affinity for PBPs 1, 2, and 4 and low affinity for PBP3. It differs from imipenem in its high affinity for both PBP2 and 3 in *P. aeruginosa*. They are regarded to be more potential than imipenem i.e.8-32 times more active than imipenem against H. influenza, more active against *Enterobacteriaceae*, *Haemophilus influenzae*, *gonococci and P. aeruginosa.*Hence, meropenem are more potential candidate for antibiotics therapeutics developed under carbapenem family (Papp-Wallace et al, 2011).

2.3.1 Mode of action

Carbapenems are bactericidal that penetrates the bacterial cell through the outer membrane proteins which function to access essential nutrients. Once, carbapenems

enter the periplasmic space they permanently acylate the PBP and inhibit peptide cross-linking as well as other peptidase reactions. Therefore, it disturbs the formation of the cell wall and autolysis occurs at the same time. Eventually the peptidoglycan weakens, and the cell bursts due to osmotic pressure. Carbapenems have ability to bind to multiple different PBPs at same time and inhibit their functions i.e formation of peptidoglycan in the cell wall of bacteria. Affinity of the various carbapenem varies for different PBPs which account for resistance to a broad range of beta lactamases, such as: Imipenem binds preferentially to PBP2, followed by PBP1a and 1b, but has weak affinity for PBP3. They bind to PBP5 more strongly. Also, imipenem has two to four times more affinity than others. Meropenem binds to PBPs 2, 3 and most strongly to PBP6. Ertapenem has the strongest binding affinity for PBPs 2 and 3 of *Escherichia coli*, although it can also bind to PBPs 1a, 1b, 4 and 5, similar to imipenem. Preliminary studies showed that doripenem strongly binds to PBP2 of E. coli and PBPs 2 and 3 of *P. aeruginosa*, similar to meropenem. High affinities to PBP2 and PBP3 in *P. aeruginosa* explain why the meropenem and doripenem are more active than imipenem (Breilh et al, 2013).

Subsequently, the efficacy of carbapenems and other β -lactams is time-dependent (i.e., directly related to the percentage of the dosing interval during which free-drug concentration exceeds minimum inhibitory concentration [%T > MIC]). Carbapenem requires lower [%T > MIC] than other beta lactams for bacterial stasis which is 20% as compared to 40% cephalosporin while for maximum killing it is $\sim$ 40% for carbapenems, 50% for penicillins and 60 – 70% for cephalosporins. Similarly, for more efficacies prolonged infusion of carbapenem are required (Nicolau, 2008).

Therefore, with proven efficacy in severe infections caused by harmful pathogens and their broad spectrum activity carbapenem are referred as last line of defense in antibiotic therapeutics (Meletis, 2016).

2.4 Antibiotic resistance in *P. aeruginosa*

In the past few decades, *P. aeruginosa* are recognized as one of the most critical pathogens which are responsible for most of the community and hospital acquired infections. Such infections are difficult to treat because severalclasses of antibiotics are ineffective for them. A major reason for the antibiotics insensitive is due to antibiotic resistance mechanism. High level intrinsic and acquired resistance mechanism in microorganism can withstand antimicrobials including antibiotics and disinfectants (Henrichfreise et al, 2007).

To make the matter worst, *P. aeruginosa* have turned into MDR where some classic as well as some newly available drugs are no longer effective. It is due to an excessive use of antibiotics leading to accumulation of resistance to antibiotics and cross-resistance between antibiotics have result in multidrug-resistant (MDR) *P. aeruginosa* (Yayan et al, 2015)

Unfortunately rate of multidrug resistance *P. aeruginosa* are increasing worldwide. It was estimated that approximately 51000 heathcare associated *P. aeruginosa* infections occur in the United States each year and more than 13% of these are multidrug resistance, with roughly 400 deaths per year are due to these infections (Bassetti et al, 2018).

There are several definitions used by many researchers and literatures to define MDR ranging from, resistance to a single antibiotic agent to resistance to all tested antibiotics. In majority of the published studies, MDR is defined as resistance to at least three drugs from a variety of antibiotic classes, mainly aminoglycosides, antipseudomonal penicillins, cephalosporins, carbapenems and fluoroquinolones (Saderi & Owlia, 2015)**.**

Although several attempts were made to establish a specific definition for multidrug *P. aeruginosa*, there still is no international accord. In addition, there is no international surveillance system specifically to know the true prevalence of MDR organisms. However, the SENTRY antimicrobial surveillance program, MYSTIC program and Intensive Care Antimicrobial Resistance Epidemiology (ICARE) project as well as other numerous surveys are conducted to tract antimicrobial resistance trends nationally and internationally. Annual variations in geographical regions and participating centers limit the ability to track the MDR *P.aeruginosa* (Andrade et al, 2003).

The emergence of multidrug-resistant (MDR) *P. aeruginosa* has enhanced morbidity and mortality rate and are notorious in clinical settings. Therefore, they are referred as "super bugs" (Skalweit Helfand, 2008).

2.5 Mechanism in antibiotic resistance

Resistance occurs through various mechanisms such i) intrinsic resistance ii) acquired and acquisitions of additional resistance mechanisms from other bacteria in the form of plasmid encoded gene.

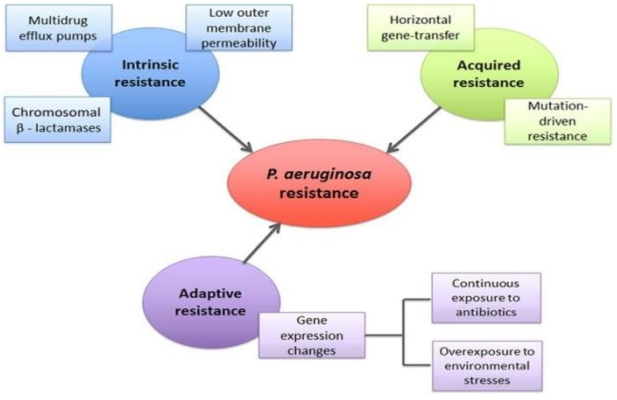

Figure 1: Mechanisms of antibiotic resistancein *P. aeruginosa*, Source (Pires, Vilas Boas, Sillankorva, & Azeredo, 2015)

2.5.1 Intrinsic resistance

P. aeruginosa possess remarkable genetic makeup which they have achieved through evolution which helps them to withstand wide array of environmental threats, including the presence of harmful antibiotics. Consequently, their intrinsic resistance permits them to thrive in its presence. Intrinsic resistance (innate) is ability of the bacteria to resist the activities of the antibiotics through inherent structural and functional characteristics. Intrinsic antibiotic resistance occurs in three different ways which are as follows:

I. Decreased permeability of the outer cell membrane
II. The use of mechanical efflux pumps which actively pump the antimicrobial agent out of the cell
III. The production of chromosomally encode beta lactamase

2.5.2 Acquired resistance

Another main factor of carbapenem resistance is related to dissemination of acquired resistance mechanisms, which involves horizontal gene transfer and mutational resistance. DNA elements carried on plasmid, transposons, integrons, prophage, and resistance islands etc. harbors antibiotic resistance gene which can be acquired by neighboring bacteria through horizontanal gene transfer mechanism: Conjugation, transformation or transduction. Transformation is the process of uptake of naked DNA from the natural environment by the bacteria. Conjugation is the process which involves transfer of DNA via sexual pilus and requires cell to cell contact and transduction involves the transfer of DNA from donor bacteria to other recipients via bacteriophages.

Many acquired genes cause deleterious effects in the chromosome of the bacterial recipient leading to loss of bacteria from the population over time while some acquired genetic elements confer a selective advantage to the host, and are able to perform their functions. They have the potential to spread rapidly within a bacterial population (Thomas & Nielsen, 2005).

Increase in resistance to several classes of antibiotics but are mainly observed in beta lactams and aminoglycosides in *P. aeruginosa* due to horizontal gene transfer. For example, when aminoglycoside modifying enzyme located on the mobile elements get transferred from donor to recipients it can inactivate aminoglycoside leading to chemical modification which in turns leads to reduced affinity for the 30S ribosome subunit which is main targets for aminoglycosides. In addition, acquired beta lactamase in *P. aeruginosa* includes extended spectrum beta lactamse (ESBL), carbapenemase (MBL), AmpC beta lactamase which are typically encoded by plasmid- or transposon-bone genes, often on integrons (Poole, 2011).

2.5.3 Adaptive resistance

P. aeruginosa can also exhibit adaptive resistance, in which certain modulation of gene expression, which results in phenotypic changes. Unlike intrinsic and acquired resistance, they are unstable and usually revert at the liminal of the inducing status. Changes in environmental conditions, for example: metabolic changes that are sensed by the microorganisms or due to continuous exposure to the antibiotics, would result in antibiotic resistant bacteria.

2.6 Outermemberne mediated carbapenem resistance

Drugs that are typically used to treat infections caused by *P. aeruginosa* have different modes of action. However outer membrane of cell walls of bacteria act as first line of defense. Therefore, in order to reach their intracellular target to exert their effect, they all have to penetrate through it. Outer membrane of the P. aeruginosa naturally prevents the large hydrophilic molecule from passing through it. Decrease in the permeability of hydrophilic molecules such as β-lactams, tetracyclines and some fluoroquinolones are particularly affected since they often use porins, which are protein channels that span the outer membrane to cross this barrier.

While some antibiotics such as colistin and aminoglycosides interact with the lipopolyssacrides of the outer memberane thereby changes the permeability and enter into the cell. There are several types of porins based on their structure, their selectivity and their regulation of expression. General porin permits almost any hydrophobic molecule to pass through it whereas specific porins have binding sites where the molecule binds and change their orientations so that they can enter through it. Most of the bacteria have general porins in them but *P. aeruginosa* have mainly specific porins (Tamber et al, 2006)**.**

One of the specific porins which contribute intrinsic resistance in outer membrane protein/porin in P. aeruginosa is OprD. OprD porins normally uptake imipenem- a type of carbapemen antibiotics but mutations in OprD in outer memebrane has shown resistance to carbapenem in *P. aeruginosa* (Fournier et al., 2013).

2.6.1 Outer membrane porin (OprD)

Several studies conferring the fact that imipenem readily select resistant mutant of *P. aeruginosa* those having decreased transcriptional expression of *OprD* and/or loss of function, and mutations that disrupt protein activity. Specific mechanisms resulting in decreased transcriptional expression of *OprD* include (i) disruption of the *OprD* promoter, (ii) premature termination of *OprD* transcription, (iii) co-regulation with trace metal resistance mechanisms, (iv) salicylate-mediated reduction, and (v) decreased transcriptional expression via co-regulation with the multidrug efflux pump encoded by mexEF-oprN (Agah Terzi et al, 2015).

OprD was first studied as protein having molecular weight 45-49 kilodalton, that were missing in imipenem resistance *P. aeruginosa*. The OprD encoding genes are present in

between 71 and 75 min on the *P. aeruginosa* PAO1 chromosome consisting 443 aminoacids. The OprD protein is 1332-bp nucleotides in length (Yoneyama et al, 1992).

OprD protein is substrate-specific outer-membrane channel in *P. aeruginosa* which facilitates the diffusion of amino acid, small peptides, and carbapenem antibiotics, such as imipenem and meropenem (Epp et al., 2001).

OprD consists of 16 strands transmembrane beta barrel and 8 loops on the external surface (Ochs et al, 2000). OprD are closest porin protein to OmpF of E.coli except that they have narrower channel which cause 12–100 fold lower outer membrane permeability (Taylor et al., 2014).

Similarly, any substitution or deletion within loop 2 and 3 changes the conformations and loses its ability to bind imipenem. Also, deletion of loop 5, loop 7, loop 8 increases the suspectibilty of *P. aeruginosa* to β-lactams, quinolones, chloramphenicol, and tetracycline indicating that these three loops reduce the nonspecific movement of molecules through OprD channel. Amino acid substitutions within loop 7 result in increased meropenemsusceptibility with a 4-fold decrease in MIC**.** OprD porin of *P. aeruginosa* also works as protease with the catalytic activity established in residues His156, Asp208, and Ser296 and such protease activity can be inhibited by serine protease inhibitor (Epp et al., 2001).

2.7 Efflux system mediated carbapenem resistance

The second mechanism by which *P. aeruginosa* exerts resistance to the antibiotics is by pumping the molecules out of the bacterial cell via efflux pumps. Antimicrobial efflux systems have been grouped into 5 families on the basis of structure, energy source, and range of substrates they are able to pump out and in the type of bacterial organisms in which they are distributed. They are: i) major facilitator superfamily (MFS), ii) the small multidrug resistance family (SMR), iii) the resistance-nodulation-celldivision family (RND), iv) the ATP-binding cassette family (ABC), and v) the multidrug and toxic compound extrusion family (MATE). *P. aeruginosa* express RND type multidrug efflux pumps. Among several RND pumps: MexAB OprM and MexXY, etc play important roles in contributing antibiotic resistance (X.-Z. Li et al., 2016).

Efflux pumps are tripartite systems that are composed by i) a protein transporter of the cytoplasmic membrane ii) a periplasmic connective protein and iii) an outer membrane protein component with a barrel configuration that are known to contribute meropenem resistant. They are not only known to increase MIC but also reduce the antibiotic concentration from inside the cell (Meletis et al, 2012).

Among several efflux pump MexAB-OprM (Multidrug efflux system AB- Outer membrane protein M), are largest multi drug resistant efflux pump with high level of expression have shown significant role in meropenem resistant(Pan et al, 2016).

Expression of MexAB-OprM and MexCD-OprJ, mexXY-oprM are regulated by mexR, nfxB, and mexz respectively.Mutation in repressor gene, *mexR* (*nalB* mutant) located

upstream of the MexAB-OprM operon attributed the intrinsic resistance by the overexpression of MexABOrpM (Choudhury et al., 2015).

Overexpression is Due to mutation Mex R repressor protein loses its capacity to bind with Mex mexA intergenic region as fails to repress mexAB- OprM. Likewise, nalD and nalC type of mutants have also been identified which occurs in response to mutation in nalC and nalD gene respectively.

The efflux pumps that remove antimicrobial agents along with some of their substrates are:

--MexAB-OprM:	Beta-lactams	fluoroquinolones
--MexXY-OprM:	Fluoroquinolones,beta-lactams	aminoglycosides
--MexCD-OrpJ :	Beta-lactams	fluoroquinolones
--MexEF-OprN :		Fluoroquinolones
--MexJK-OprM :	Tetracycline	erythromycin
--MexPQ-OpmE:		Fluoroquinolones
--MexGHI-OpmD:		Fluoroquinolones

Most antimicrobial agents are pumped out by these efflux pumps. Only the polymyxins are not removed from the cell via efflux pumps (Lister et al, 2009)**.**

2.8 Beta lactamase mediated carbapenem resistance

All *P. aeruginosa* strains have most common method to resist the inhibitory effects of antimicrobial agents by producing the enzymes such as β-lactamases, aminoglycosidemodifying enzymes, or chloramphenicol acetyltransferases that either irreversibly modify or inactivate the antibiotics. Beta lactamase like ESBL (GES-2), carbapenemase (metallo beta lactamase) and AmpC bata-lactamase are well characterized enzymes that usually confer resistance to carbapenem. They can hydrolyze the amide bond of the beta lactam ring of beta lactams antibiotic including penicillins, cephalosporins, monobactams, and carbapenems.

Beta lactamases are classified into different classes by 2 schemes. The simplest classification is Ambler molecular classification where beta-lactamases are classified into four molecular classes, A, B, C, and D based on conserved and distinguishing amino acid sequence.

In 1995, scientists classify the enzymes into three major groups depending on their ability to hydrolyse of specific beta lactam classes and on the inactivation properties of the beta lactamase inhibitor clavulanic acid sulbactam, and tazobactam (Bush & Jacoby, 2010).

Group 1 cephalosporinas: class C

Group 2 serine beta-lactamases: (Class A and Class D)

Group 3 MBLs: class B

Ambler class beta lactamase A, C and D have the active-site serine for hydrolysis activity whereas Ambler class beta lactamse B are metallo-β-lactamases that require a bivalent metal ion, usually Zn^{2+} for their hydrolyzing activity. These major ambler classes of enzymes are further divided into many sub groups (Ozturk et al, 2015).

2.8.1 Ambler class A beta lactamase:

Molecular class A beta lactamase has been increasing worldwide. *P. aeruginosa* produce enzymes that have active serine site activity at position 70 and presence of a disulfide bond between Cys69 and Cys238 (changes the overall shape of the active site). Structural change decreases the steric hindrance causedby the C-6 hydroxyethyl side chain of carbapenems, confers resistance to imipenem. There are five main types of classes of Ambler class A beta lactamse: SME (for Serratia marcescens), IMI (for imipenem hydrolyzing β-lactamase), NMC (for not metalloenzyme carbapenemase) which are chromosomally encoded enzymes whereas KPC (for Klebsiella pneumoniae carbapenemase), GES (for Guiana extended spectrum) are plasmid mediated enzymes. These enzymes are inhibited by clavunalic acid and are sub grouped in 2f functional subgroup of beta–lactamases.

SME enzymes have three varients (SME1, SME2, SME3) and differ by one or two amino acids. Geographically, SME producing isolates are found in United States and UK but they are not identical (Deshpande et al, 2006).

NMC-A and IMI enzymes have 97% amino acid homology. IMI have two variant (IMI-1, IMI2). Similarly, Plasmid mediated GES have the ability to hydrolyze carbapenem but are weak carbapenemases. They have nine variants identified in P. aeruginosa and differ from each other by 1-4 amino acid substitutions. Initially, they belonged to ESBL family,later GES-2 involved in hydrolysis of imipenem, was reported in a clinical isolate of *P. aeruginosa* (Queenan & Bush, 2007).

In year 2006, plasmid mediated KPC enzyme in *P. aeruginosa* was first reported in columbia. It can hydrolyze all penicillins, cephalosporins, and carbapenems. Recent reports on KPC gene whichis located in Tn440 of the plasmids, make these elements highly transferableand shows the potential rapid dissemination of this mechanism of resistance to the world (Cuzon et al., 2011). There are 22 variants of KPC enzyme of which KPC-2 are most frequently reported during outbreaks caused by such bacteria (Carrara-Marroni et al., 2015). Among various carbapemenase, KPC enzyme has greatest potential for causing outbreaksdue to its location in the plasmid and mobile characteristic (Walther-Rasmussen & Høiby, 2007). Unlike GES, they do not need other additional mechanism such as outer membrane premeabilty or multiple efflux system to hydrolyze Carbapenem. Although they are able to hydrolyze carbapenem but the resistance is undetectable (Shridhar Rao P.N, 2012).

2.8.1.1 Extended spectrum beta lactamase

ESBL belongs to molecular ambler class A beta lactamase that have emerged as critical cause of resistance to the antibiotics including oxyimino-βlactams (the third- and fourth-generation cephalosporins). ESBLs that do not readily hydrolyze carbapenems on their own but can confer carbapenem resistance when combined with chromosomal porin mutations that prevent accumulation of β-lactam agents in the bacteria. They are plasmid mediated and are inhibited by β-lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam (Paterson & Bonomo, 2005). Characterization of ESBL producer revealed that they are the mutants of TEM and SHV types broad spectrum beta lactamase. In TEM type ESBL mutations extension of the substrate spectrum is achieved by single or few amino acid substitutions that have effect on broadening the active site of native enzyme and same in case of SHV types ESBL that affects the confirmation of the binding site and permit to bulkier extended spectrum cephalosporins. Despite having similar in structure in TEM and SHV types that exhibit similar ESBL activity, they follow different pathway for the modulation of substrate specificity by mutation. For instance, in the ω loop the preferred hotspot for the mutations extending the substrate specificity is at position 164 in TEM type enzymes and 179 in SHV type enzymes which is a major mechanism of ESBL. Several classes of ESBL have been described which are usually non TEM type non SHV type including CTX-M, PER, VEB, GES, TLA, SFO, and BES. Among them, predominant are the CTX-M types (Rossolini & Docquier, 2006).TheCTX-M got its name from cefotaximase activity which was isolated from Munich. There are five groups of CTX-M classified on the basis of amino acid sequence similarities where member of the same group shares 94% similarity and 90% similarity between the members of different groups. Including five groups are CTX—M 1, 2, 8, 9 and 25 are total 128 different CTX-M enzymes that have been characterized. Amino acids located at 240 and 167 appear to be involved in evolution of CTX-M enzyme. Initially CTX showed its hydrolytic activity against cefotaxime, later mutation around the active side of the enzymes made capable of hydrolyzing Ceftazidime by CTX-15, 16, 19, 25, 27 and 32.

VEB type ESBL: (Vietnamese extended spectrum beta lactamase) was first reported in *E.coli* later also found in *P. aeruginosa* and other pathogens.VEB-1 ESBL is widespread in Southeast Asia but reported in India, UK, France, Iran and many other countries. They are present on plasmid which is located on gene cassette class 1 intregon. They are responsible for various hospital based outbreaks.

PER type ESBL: (for *Pseudomonas* extended resistance) type beta lactamase first reported in

P. aeruginosa in France ,1991 from the patients transferred from Turkey. A total of seven PER that have been discovered. PER enzymes exhibit high resistance to ceftazidime but are found to show lower resistance to other penicilin and cephalosporin. However, PER enzymes are found ineffective against carbapenem and are inhibited by clavulanic acid.

Other minor ESBL types Beta lactamses are SFO-1 and TLA-1, BES-1, BEL-1 and GES-1etc having different spectrum of hydrolyzing activity (P.N, 2015)

2.8.2 Ambler class B beta lactamase:

Class B beta lactamases are the metallo beta lactamase enzymes that distinctly differ from the rest of molecular ambler classes as they utilize one or two Zn2+ cations for their hydrolysis activity. MBLs are inhibited by aztreonam and metal ion chelators (EDTA) and the inhibition can be reversed by adding Znions. Among all carbapenemase, MBLs (IMP, VIM, SPM, GIM types) are considered as most clinically important and has been detected in *P. aeruginosa* all around the world. IMP (active on imipenem) were identified in Japan. There are currently known 37 types of IMP. VIM (Verona integron-encoded metallo-β-lactamase) were identified Italy and there are total 34 types of VIM among which VIM-2 are predominant, SPM-1 (Sao Paolo metallo-β-lactamase) caused serious outbreak in Brazil and GIM-1 (German imipenemase) were spread in Germany, whereas NDM-1 (New Delhi Metallo-β-lactamase) in P. aeruginosa was identified first in Serbia (Meletis et al., 2012).

Metallo beta lactamase are subgrouped into three groups (B1, B2, and B3) on the basis of sequence alignments. B2 consist one zinc ions on their active site whereas active site of B1 and B3 contains two zinc ions. Zinc binding site of B1 is 1 composed of three His residues (His116, His-118, His196) and zinc binding site 2, composed of one His263, one Cys-221 and one Asp-120. Similarly, in sub group B2 the zinc ligands on site 2 are conserved whereas His-116 in site 1 is replaced by Asp which attributes efficient hydrolysis of carbapenem. in subgroup B3 it has same ligand on zinc binding site as B1, but the cysteine ligand of subclasses B1 and B2 is binding site 2 is replaced by histidine.Such zinc ions coordinate water molecules that are necessary for hydrolysis (Sacha et al., 2008).

The genes encoding metalloβ-lactamases are located on the bacterial chromosome, on plasmids, or transposons. Most MBL genes (including VIM, IPM) are found as gene cassettes on class 1 integrons while IMP genes are located on class 3 integrons. However, SPM-1 genes are not located on integrons or transposons but are associated with new types of transposable structure. GIM-1 was found on class 1 integron in a 22-kb nontransferable plasmid and did not hydrolyze serine beta lactamase inhibitor (Shridhar Rao P.N, 2012).

NDM-1 (New Delhi metallo beta lactamase) was first reported in 2009 which was isolated from the Swedish patient having urinary tract infection caused by Klebsiella Pneumoniae who was hospitalized in New Delhi (Yong et al., 2009).

NDM has ability to hydrolyze a wide variety of beta lactams including the penicillins, cephalosporins, and carbapenems, but not the monobactams (i.e., aztreonam). There are 17 variants of NDM among which NDM-1 has become clinically significant carbapenemase. The location of the site mutation present on the gene encoding beta lactamse predicts the rates of hydrolyzing activing among the variants. NDM-2 and NDM-3 has similar hydrolytic activity with NDM-1, as the mutations are not located in the active site of the enzyme (Zmarlicka et al., 2015).

NDM-2 and NDM-3, proline is substituted t o alanine at position 28 and aspartate to asparagines at postion 95 respectively.

Similarly, NDM-4(substitution on 154th Methionine to Leucine), NDM-5(substitution of Valine by Leucine at position 88 and Methionine by Leucine at position 154)**,**NDM-6 (substitution of Alanine to Valine at 233 position) and NDM-7(Substitutions of Aspartate to Asparagine at position 130 and Methionine to Leucine at position 154) variants have acquired increase hydrolytic activity towards carbapenem as they have different alteration on their active site.NDM-8 variant having substitutions at positions 130th (Aspartic acid to Glycine) and 154th (Methionine to Leucine) resulted in enzymatic activities against β-lactams similar to those shown by NDM-1.NDM-9 has single substitution. Similarly, NDM-10 has the maximum number of substitution mutation (Arginine 32 to Serine, Glycine 36 to Aspartic acid, Glycine 69 to Serine, Alanine 74 to Threonine and Glycine 200 to Arginine). NDM-12 has two amino acid substitutions at 154th (Methionine to Leucine) and 222th (Glycine to Aspartic acid)**.** NDM-13, a novel New Delhi Metallo-β-lactamase was identified in Nepal (substitutions of Asparagine in place of Aspartic acid at position 95 and Leucine in place of Methionine at position 154). Likewise, substitution of Aspartic acidat 130th position to Glycine present in NDM-14**.** NDM-15 showed substitution of Alanine to valine at 233th position and Methionine to Leucine at 154th position. NDM-16 variant showed substitution at 264th position of Arginine to Histidine and lastly, NDM-17 showed amino acid substitution of valine 88 to leucine, methionine 154 to leucine and glutamic acid 170 to lysine (Khan et al., 2017).

NDM-1 encoding gene are carried by plasmid which are transposable and its wide spread has prompted worldwide concern.NDM-1 gene has been isolated in more than 11 bacterial species from natural environment**.**NDM–1 is a single-chain protein, which N-terminal has a putative signal peptide domain of 18 amino acids, and the core region of the enzyme composed of 270 amino acids (Shen et al., 2013).

NDM-1producing *P. aeruginosa* strains were first reported in 2011 with two isolates recovered from Serbia.Again, NDM-1 was isolated from Pseudomans aeruginosa in France. The novel NDM-1 which have novel amino acid on active site have, very little identity with other MBLs, with the most similar MBLs being VIM-1/VIM-2, with which it has only 32.4% identity. As compared toVIM-2, NDM-1 displays tighter binding to most cephalosporins where as does not bind to carbapenem as much strong thanIMP-1 or VIM-2(Jovcic et al., 2011).

2.8.3 Ambler class D beta lactamase

These carbapenemase are the OXA (oxacillin hydrolyzing) enzyme type, which are serine Beta lactamase and poorly inhibited by clavunalic acid or EDTA. They can hydrolyze oxacillin and cloxacillin but are weak in hydrolyzing carbapenem, they are plasmid encoded and are commonly found in *P. aeruginosa*. These enzymes are commonly associated with integrons, insertion sequences and transposons, they can be transferred between specie. Among all four molecular classes enzymes,class D β-lactamases are the most diverse enzymes i.e.there on amino acid homologies, OXA carbapenemases are sub-divided into nine major subgroups (Sridhar Rao P.N, 2012**).**

According to the bush functional classification they are classified as subgroup 2df (Naas & Nordmann, 1999).

In OXA type enzymes Serine 67 are the active sites for their catalytic activity. Serine adds to the carbonyl of the β-lactam antibiotic to initiate turnover after whicha water molecule is itself activated as a nucleophile to add to the carbonyl carbon of the acyl-enzyme intermediate, and subsequent collapse of this intermediate to restore the free serine and the hydrolyzed (inactivated) β-lactam antibiotic.

Enzymes belonging to the OXA-23, OXA-24/40, OXA-48, OXA-51, OXA-58, and OXA-143 subgroups are of major clinical importance, among where OXA 2 and OXA 10 are predominantly occur in *P. aeruginosa* (Antunes et al., 2014).

2.8.4 Ambler Class C beta lactamase

AmpC beta lactamase are ambler class C beta lactamase typically encoded on the chromosome found in many clinically important pathogens like Pseduomonas aeruginosa and other gram negative bacteria**.** AmpC are usually inducible and in some are not inducible, although it can be hyper expressed. They are group 1 cephalosporinase and serine beta lactamase 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. In *P. aeruginosa* chromosomally encoded Ampc beta lactamase possess analanine residue at position 105 that has a slight carbapenemase activity that is sufficient to compromise the efficacy of carbapenems when the enzyme is overexpressedThere are extended-spectrum cephalosporinases (ESACs) of with broadened hydrolytic activity toward imipenem that has been reported in P. aeruginosa(Rodriguez-Martinez et al., 2009).

Over production of AmpC beta lactamase is due to the mutation in the regulatory gene*. P. aeruginosa* has three AmpD genes and successive inactivation of each *ampD* gene causes stepwise upregulation of AmpC production. Other, regulatory gene that are involved in AmpC beta lactamse areAmpR mutations result in high-constitutive or hyperinducible phenotypes and mutations in AmpG result in constitutive low-level expression. AmpR belongs to the LysR family of transcriptional regulators that typically autorepress their own expression (Balasubramanian et al., 2012).

During disruption of murein biosynthesis by a beta-lactam agent leads to an accumulation of *N*-acetylglucosamine-1,6-anhydro-*N*-acetylmuramic acid oligopeptides. Which are removed to produce a series of 1,6-anhydro-*N*-acetylmuramic acid tri-, tetra-, and pentapeptides. These oligopeptides compete with oligopeptides of UDP-*N*-acetylmuramic acid for a binding site on AmpR. Displacement of the UDP-*N*-acetylmuramicacid peptides signals a conformational change in AmpR, which activates the transcriptionof *ampC.* In addition, the cell has an enzyme, AmpD, a cytoplasmic *N*-acetyl-muramyl-L-alanine amidase, that removes stem peptides from the 1,6-anhydro-*N*acetylmuramic acid and *N*-acetylglucosamine-1,6anhydro-*N*acetylmuramic acid oligopeptide derivatives, thus reducingtheir concentrations and preventing the overexpression of AmpC (Jacoby, 2009).

Similarly, *ampE* (coding for an inner membrane-bound sensory transducer), *dacB* (coding for a D-alanyl-D-alanine carboxypeptidase also known as PBP4), and *nagZ* (coding for an *N*acetyl-β-D-glucosaminidase) have been shown to play an important role in the regulatory network of *ampC* in *P. aeruginosa.*

Plasmid mediated AmpC beta lactamase cephamycins have sub grouped into (CMY), cefoxitin (FOX), and moxalactam (MOX) or latamoxef (LAT). Over expression Ampc beta lactamse attritubing resistance to several classes of antibiotics have been found in Africa (Algeria, Tunisia), Asia (India, Japan, Pakistan, South Korea), Europe (France, Germany, Greece, Italy, Sweden, United Kingdom), the Middle East (Saudi Arabia), North America (United States), and South and Central America (Argentina, Guatemala). Plasmid mediated AmpC beta lactamse that are transfer from chromosome was first reported in 1988. Case of (CMY-2) from Pakistan to the United Kingdom,several CMY types (CMY-2, CMY-6, CMY-7) from Punjab (India) to London (unpublished results), CMY-2 from Algeria to France, CMY-4 from India to Sweden,FOX-2 from Guatemala to Germany,ACC-1 from Tunisia to France and and MOX-2 from Greece to France have been reported in various publications(Philippon et al., 2002).

Resistance due to plasmid mediated inducible AmpC beta lactamese is less common. Therefore combination of AmpC beta lactamase with other resistance mechanism such outermembrane protein ans effux pump system and other beta lactams has induced resistance to many antibiotics (Mohamudha Parveen et al, 2010).

Also there are no Clinical laboratory standard institute or approved guidelines for the detection of AmpC Beta lactamase production in organisms. AmpC producing organism gives positive result for the ESBl Screening but gives while fails to give confimatory result by increasing sensitivity against clavulanic acid. AmpC producer can occur with certain complex TEM mutants, OXA type ESBL and Carbapenemase.Thus needing other confimatory test (ElHady & Adel, 2015).

2.9 Epidemiology of *P. aeruginosa*

With the spread of hospitals and community based infection, emergence of the antibiotic resistance *P. aeruginosa* is on rise. Antibiotic resistance is acquired due to several mechanisms they have developed against antibiotics which includes the production of various enzymes such as Mello beta lactamase, Amp C beta lactamase, extended spectrum beta lactamse and alteration of loss of outermembrane protein responsible for changes in their permeability and activation of Efflux pump system. Carbapenem are used as last resort of drug, used for treating severe types of infection caused by *P. aeruginosa*. While, resistance to carbapenem has limited treatment alternatives. Some factors which are responsible for antibiotic resistance in *P. aeruginosa* are long term antibiotic exposure, misuse of antibiotics, improper doses of antibiotics etc. *P. aeruginosa* is most frequently associated with human infectionsincluding ventilator-associated pneumonia, catheter-associated urinary tract infections, wound infections in severe burn patients and septicaemia

According to National Healthcare Safety Network (NHSN), from 2009 to 2010, *P. aeruginosa* (16.6%) ranked second in the USA among the pathogens (Moradali et al, 2017)

Center for Disease control reported that the average *P. aeruginosa* infections in US hospitals approximates to 0.4% (4 per 1000 discharges), and is listed as the fourth most commonlyisolated nosocomial pathogen contributing 10.1% of all hospital-acquired infections (HAI) ("*Pseudomonas aeruginosa* in Healthcare Settings," 2018).

Hospital acquired Pneumonia (HAP) is encountered at the rate of 5–10 cases in every 48 hours after hospitalizations and the ventilator associated Pneumonia (VAP) is found to occur in the range of 8% to 28% (Bassetti et al, 2012). Together they have contributed to mortality, ranging from 24% to 76 (Masterton et al., 2008) whereas in the Europe and North America, the annual occurrence of community associated pneumonia CAP was found to be 34–40 cases per 1000 children and 500,000 hospital admitted CAP patients were reported. CAP was found to be much lower compared to HAP(Ostapchuk et al, 2004).

P. aeruginosa was identified as the most abundant, among pathogens found in the lower respiratory tract, 13.37% from 2005 to 2007, 12.31% from 2002 to 2004, and 12.82% from 1999 to 2001 according to data from National Nosocomial Infection Surveillance System (NNISS), China (Ding et al., 2016).

Likewise, from the data reported by the European Centre for Disease Prevention and Control 2011-2012 Point-Prevalence Survey for health-care associated infections (HCAIs), it was found that *P.aeruginosa* caused 9% of total infections and was also the fourth most prevalent pathogen in European Hospitals (Carl Suetens, 2013).And, 13% prevalent infections were found to be from *P. aeruginosa* according to the Spanish Society of Intensive Care Medicine in Spain in 2016 whileIn 2017,, *P. aeruginosa* was the second cause of hospital-acquired infections, and accounted 10.5% of all these infections with higher prevalence among ICU patients (Ruiz-Garbajosa & Cantón, 2017).

P.aeruginosa is one of the leading causes of mortality and morbidity in Cystic Fibrosis (CF)and is recognized as one of the major pulmonary pathogens (Bhagirath et al., 2016).

However, fraction of individuals with positive culture of *P. aeruginosa* seemed to have declined over time, with highest decrease observed in individuals younger than 18 years of age (In 1996, 49.8 % had a positive culture while only 29.1 % were positive in 2016). Many patients of CF now switched to adult care with respiratory tract free of *P. aeruginosa*.

Leeds criteria which classify individuals on the basis of infection status caused by *P.aeruginosa* into 3 groups: never having a positive *P. aeruginosa* culture called free of a positive *P. aeruginosa* in the past 12 months, intermittent infection which has less than 50 percent of their cultures in the past year were positive for *P. aeruginosa*, and chronic infection which has more than 50 percent of their cultures in the past year were positive for *P.aeruginosa* which accounts, 19.7 % of individuals having cystic fibrosis had never had a positive culture for *P. aeruginosa*. Another 30.0 percent of individuals had cultures that were negative for *P. aeruginosa* during the entire calendar year, but had a positive culture in a previous year.

According to the classification of leeds criteria of individual's infection status is categorized into three groups: free of a positive *P. aeruginosa* culture in the past 12 months - never having a positive *P. aeruginosa* culture, intermittent infection- less than 50%of their cultures positive in the past year, chronic infections- more than 50% of their cultures positive in the past year. Culture of *P. aeruginosa* from 19.7% of the cystic fibrosis individuals never had a positive result, the other 30% of the cultures were negative for *P. aeruginosa* in the year but showed positive cultures in the previous year. 46.9 % Some 46.9 percent of individuals had at least one positive culture, of which 29.1 percent were categorized as having chronic infection, and 17.8 percent as having intermittent infection in 2016 (Bruce Marshall & Alexander Elbert, 2017).

There are several pathways that are responsible for raising MDR *P. aeruginosa*.

Literally, MDR means, the strain is resistant to more than one antimicrobial agent, but the medical community has not yet given the standard definitions for MDR. Falagas et al has given a comprehensive review on the variability of the definition (Falagas et al., 2006) where the authors have mentioned that substantial number of studies have not proposed any definitions for MDR, but most of them have defined MDR as resistant to three or more antimicrobial classes (Magiorakos et al., 2012).

About 13% of multi drug resistance *P. aeruginosa* are found to be responsible for roughly 400 deaths per year (CDC, 2013).

In Asia several survey was carried out, to which they found that *P. aeruginosa* have signifant proportion of isolates are MDR.They found *P. aeruginosa* contribute (15.6%) in total isolated bacteria where they are frequently isolated. In case of VAP, *P. aeruginosa* showed (25.9%), HAP inside *P. aeruginosa* (18.4%) and HAP outside ICU showed (14.4%) respectively. *P. aeruginosa* isolates showed a quite high MDR rate (42.8%), and imipenem resistance was also high (27.2%) (Chung et al., 2011).

In carbapenem resistant *P. aeruginosa* (CRPA), early reports have emphasized the role of outer membrane protein (OprD) inactivation (Kohler et al, 1999; Margaret et al, 1989; xfc et al., 1987)

Furthermore, the overexpression of the efflux pump system has been linked with the meropenem resistance but not the imipenem (Kohler et al., 1999; Poole et al., 1993).

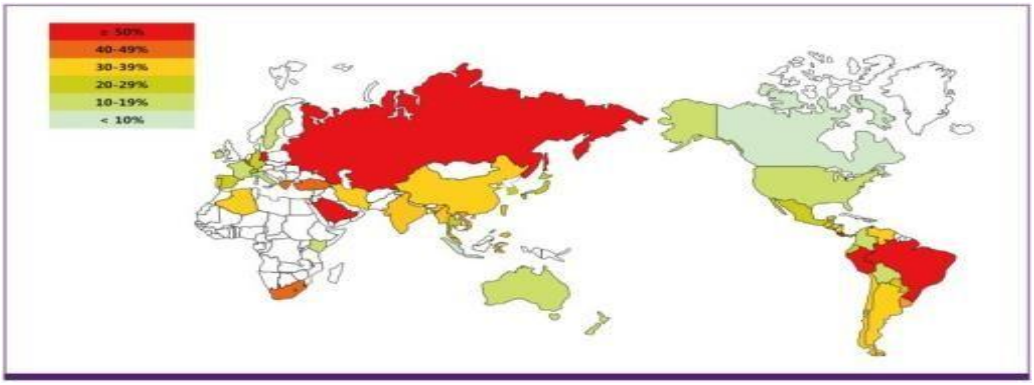

Figure 2 Geographical distribution of carbapenem resistance –*P. aeruginosa*, source: (Hong et al., 2015)

According to the studies carried to analyze the global epidemiology of carbapenem resistance P. aeruginosa, the data showed 3.3% carbapenem resistance in Canada and the Dominican Republic (imipenem and meropenem 8%) were the lowest of all countries with ratio lower than 10%. The rate of carbapenem resistance P. aeruginosa and MDR are very high in Asia (Kang & Song, 2013).

Retrospective study conducted on 917 Nepalese patients in departments of Nepalgunj Medical College and teaching Hospital, Banke, Nepal, 2014 showed that in 917 samples occurance of multidrug resistance was found 24.74% which was relevant to the context of Nepal and 15 of them were resistance to meropenem while none of them were found to be resistant to imipenem (Salman Khan et al, 2014)

Similar case was reported in 2012 that most of the isolates were found to be sensitive to imipenem, it may be due to less use of imipenem antibiotics in antibiotic therapeutics (Chander & Raza, 2013).

Another significant finding in this study was the rate of multi-drug resistance to be 2017 found out of 98 *P. aeruginosa* isolates, 68.8% were MBL-producing including 75% isolated detected with blaVIM and 25% isolates with blaIMP gene (Acharya et al., 2017).

Further study carriedout from 2009–11 in 14 European and Mediterranean countries reported that carbapenem resistance *P. aeruginosa* are highly genetically diverse and have more intrinsic resistance mechanisms, the loss of OprD being the most common cause of elevated carbapenem MIC values and was detected among 94.9% of the *P. aeruginosa* followed by AmpC (44.4%)(Castanheira et al., 2014).

Similarly, the China National Antimicrobial Resistance Investigation Net annual report of 2011, 96.5% of the 141 CRPA strains analyzed showed a loss or insertion of the $OprD_2$ encoding gene.

Resistance to carbapenem is not confined by OprD inactivating mutations in clinical strains of *P. aeruginosa.* They can also be present in susceptible strains with MICs of imipenem or meropenem (Ocampo-Sosa et al., 2012).

According to the studies conducted by European Centre for disease Prevention and Control reported that CMY-2 is most common and widely spread AmpC plasmid mediated gene (Voets et al., 2013).

In 2015, *E.coli* strain collected from human sample showed that in 1033 samples, 8 isolates (0.77%) samples have chromosomally encoded AmpC indicating their low prevalence rate. those 8 isolates have different sites for mutations in the AmpC chromosomal promoter region such as 4 isolates had -42 mutation, 3 isolates had a -32 mutation and one isolate had a nucleotide insertion at position -14 (van Hoek et al., 2015).

Similarly, different class of carbapenemases such as VIM, IMP, AIM, SPM, GIM, SIM, DIM and NDM as well as KPC and OXA-type enzymes also have played important role in carbapenem resistance (Nascimento et al., 2016). IMP-1 was first detected in Japan in 1994 (Senda et al., 1996) then IMP-7 in Canada in 1995 (Gibb et al., 2002), followed by the detection of VIM-1 in Italy in 1997 (Lauretti et al., 1999), and IMP-9 in china in 2000

(Xiong et al., 2006). Currently NDM is a great concern and considered the predominant type of MBL along with IMP, VIM and SPM (Partridge et al, 2009; Walsh et al, 2005)

Around 58.15% of the NDM-1 variants has been distributed in Asia, mostly China and India being them as major reservoir of NDM producer (Woodford & Ellington, 2007).

16.8 % of the total producers are found to be in Europe, with highest spread of NDM-1 variant in France, Italy, Turkey, Germany Greece, London, Ireland Bulgaria, Croatia, Poland, Ukraine, Azerbaijan, Serbia, Italy, Romania while NDM-4 being reported in Italy and NDM-5 and NDM7 in Denmark and France.

Similarly, American and African continent shows around 10.8% of the total NDM-1 producer whereas Australia shows 1.6% of the total NDM -1 producer (Khan et al., 2017).

First report of NDM -8 in E.coli and NDM-1 in *P.aeruginosa* was obtained in 2013, Nepal (Tada et al., 2013). Later in the year 2014-2015, NDM-12 and NDM-13 were obtained from E.coli isolates (B. Shrestha et al., 2015; Tada et al., 2014).

Similarly, in 2017 another research from Nepal revealed *P. aeruginosa* harboring gene encoding one or more carbapenemase such as NDM-1, DIM-1 and VIM-2 and 16s rRNA methyl tranferase gene responsible for resistance (Tada et al., 2017) .

KPC gene is also considered as major contributor of Carbapenem resistance. This gene has been reported frequently in most of the Enterobacteriaceae family while few been reported in *P. aeruginosa*. The International Center of Medical Research and Education in Columbia, during year 2006 reported the first case of P. aeruginosa harboring KPC gene (Almeida et al., 2012). Subsequently, a study from Puerto Rico reported on two types of KPC (KPC-2 and KPC-5) in *P. aeruginosa* (Jacome et al, 2012).

According to the report of Antimicrobial Surveillance Program, 3.3 % of Enterobacteriaceae isolates collected during 2010 in five Latin American countries were found to be KPC-2producing, with a dramatic increase in carbapenem resistance (Castanheira et al, 2012)

CHAPTER 3
MATERIALS AND METHODS

3.1 Methods

3.1.1 Ethical consideration

Ethical approval was taken from Nepal Health Research Council (reg no:253/2017) and Central Department of Biotechnology.

3.1.2 Sampling location

A total of 97 clinical isolates of *P. aeruginosa* were collected from Microbiology Department, Teaching Hospital, Maharajganj from April - July 2017 and further research activity were carried out at Central Department of Biotechnology, Tribhuvan university, Kirtipur.

3.1.3 Transportation and preservation of isolates

P. aeruginosa were preserved in semi-solid media at temperature of 2-8ºC at Teaching hospital and strains were transported in cold container to Central Department of Biotechnology, Laboratory. Each isolates were labeled separately and preserved at 4ºC.

3.1.4 Selection and identification of strain

In order to identify the isolates were subjected to standard bacteriological technique. The bacteria were inoculated in Luria-Bertani (LB) broth. After overnight incubation at 37ºC, turbid bacterial growth was inoculated on nutrient agar plates. After incubations the bacterial colonies appeared on the plates were used for gram staining Gram staining.

3.1.5 Gram's staining

Gram staining is one of the most common method to classify and identify bacterial strain as Gram positive and Gram negative. Based on their structural and chemical composition of the bacterial cell wall, they have difference in ability to retain color of the stain during staining reaction. Once stained, the morphological characteristic, arrangement of the bacteria is identified under microscope.

3.1.6 Biochemical test for identification of *P. aeruginosa.*

Different biochemical tests were performed in order to identify the *P. aeruginosa* isolates.

3.1.6.1 Citrate utilization test

Inoculum was streaked over the slant of Simmon's citrate agar in a tube and incubated for 24 hr – 48 hr. Positive result shows growth on the slant and change in color to blue medium.

3.1.6.2 SIM test:

Bacteria are transferred to the tube containing SIM medium by the inoculating needle straight down into the tube going about 2/3 of the way down and then pulling the needle straight out. It is Incubated at 35-37 C for 1-2 days.

3.1.6.3 Methyl Red/Voges-Proskauer (MR/VP)

3.1.6.3.1 MR- test

The MR/VP broth was inoculated with the pure culture of the test organism and incubated at 35°C for 48 to 72 hrs. 5 drops of MR reagent was added to the broth.

Positive result: Red- indicate pH below 6
Negative result: yellow –indicate no acid Production

3.1.6.3.2 VP test

Pure culture of the test organism was inoculated in MR/VP broth and incubated for 24 hrs at 37°C. 1 ml of the broth was aliquoted to a sterile test tube 0.6ml of VP (A) was added followed by VP (B) shaking the tube gently to expose the medium to atmosporic oxygen and allowed the tube to remain undisturbed for 10-15 min.

Positive result: Pinkish red color at the surface of the medium
Negative result: Yellow color to the surface of the medium

3.1.6.4 Triple sugar iron test

Isolated colony was inoculatd with a sterile straight wire by first stabbing the center of the medium to the bottom of the tube and then streaking the surface of the slant and incubated at 37°C for 18-24 hrs.

3.1.6.5 Oxidase test

Filter paper soaked in a freshly prepared 1% solution of tertramethyl-p-phenylene-diamine dihydrochloride was used and laid in a petri dish and moistened with distilled water. Then a colony was picked with loop and smeared over the moist area.

A positive result shows intense deep-purple , appearing within few seconds, and a negative result shows the absence of colouration or by colouration later than 60 seconds.

3.1.7 Antibiotic susesptibility test

Different classes of antibiotics like Ceftazidime-30 mcg (CAZ), cefotaxime-30mcg (CTX), imipenem-10 mcg(IMP), meropenem-10 mcg (MEM), ciprofloxacin- 5 mcg (CIP), gentamicin-10 mcg (GEN), were used to perform antibiotic susceptibility testing according to the Clinical Laboratory Standard Institute (CLSI) guidelines by Kirby-Bauer disc diffusion method using Mueller Hinton agar (MHA). The diameter of each zone of inhibition (in mm) was measured and results were interpreted with the help of zone size interpretive chart.

3.1.8 Detection of Carbapenem resistant *P. aeruginosa*

Susceptibility of isolates to carbapenem antibiotics were tested using the Kirby-Bauer disk diffusion method using Muller Hinton agar against selected antibiotics, namely imipenem (IMP)- 10µg and meropenem (MEM)- 10µg. The sensitivity test was standardized using ATCC strain of *P. aeruginosa* (27853). Inhibition zone size was interpreted using standard recommendation of the Clinical Laboratory Standards Institute.

3.1.9 Detection of Metallo-beta lactamase (MBL) producer

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

3.1.9.1Screening of MBL-producing isolates

It was performed using a combined disk diffusion method. The isolates were evaluated phenotypically for the presence of a metallo-β-lactamase (MBL), using the metal chelating agent: EDTA. Identification of MBL activity was performed by carbapenem-EDTA combined disk method. Two imipenem or meropenem [IMP (10 µg) or MEM (10 µg)] discs were applied to a Muller Hinton agar plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards, and 10 µl of a sterile 0.5 M EDTA (pH 8.0) solution was applied to one disk. The plates were incubated at 37°C under ambient air for 18 h. The zones of inhibition around the MEM and MEM-EDTA discs were measured for all carabapenem resistant isolates. Zone increases of ≥7 mm in the presence of EDTA were noted and interpreted as indicative of an MBL phenotype on the basis of criteria described previously.

3.1.10. Detection of extended spectrum beta-lactamase (ESBL) producer

3.1.10.1Screening of ESBL producing isolates

Phenotypic screening for the ESBL producing bacteria were performed by using Ceftazidime (CAZ)-30µg and Cefotaxime (CTX) -30 µg disks. Inoculums with 0.5 McFarland standard turbidity was prepared from culture plates of *P. aeruginosa*. MHA plates were then inoculated by lawn culture using a sterile cotton swab and antibiotics were placed firmly. If the zone of inhibition were $\leq$ 17 mm for ceftazidime and $\leq$ 22 mm for cefotaxime, the bacteria was considered as a potential ESBL producer as recommended by CLSI guidelines .

3.1.10.2 Confirmation of ESBL producing isolates

A Muller Hinton agar plate was taken and a lawn culture of potential ESBL producing *P. aeruginosa* was made. Then ceftazidime (30µg) disc alone and with clavulanic acid (10µg) were placed at an appropriate distance from each other on the plate and incubated aerobically at 37°C overnight. A ≥ 5mm increase in zone diameter for antimicrobial Ceftazidime tested in combination with clavulanic acid in comparison to the zone diameter when tested alone confirmed the organisms to be an ESBL producer by Double Disc Test.

3.1.11 Detection of AmpC- beta lactamase

3.1.11.1 Screening of AmpC

Screening test was performed by cefoxitin (30 µg) disk. Isolates that yielded a zone diameter less than 18 mm (screen positive) were further subjected to confirmatory testing by disc antagonism test.

3.1.11.2 Confirmation of AmpC producer

Test isolate with a turbidity equivalent to that of 0.5 McFarland standards was spread over a Mueller Hinton agar plate. Cefotaxime (30 µg) and cefoxitin (30 µg) disks were placed 20 mm apart from centre to centre. Isolates showing blunting of cefotaxime zone of inhibition adjacent to the cefoxitin disc were screened as positive for AmpC betalactamase.

3.1.12 Preparation of genomic DNA

1. For the extraction of genomic DNA, bacteria were grown in 5ml of Luria-Bertani (*LB*) broth media and incubated at 37ºC until the culture was saturated.
2. From the primary culture,3 ml of culture was centrifuge at 12000 rpm for 2 min.
3. The supernatant was discarded and the pellet were resuspended well in 567µl (TE) Tris EDTA buffer by repeated pipetting

4. Then 30 µl of 10% Sodium dodecyl sulfate and 3 µl of 20 mg/ml Proteinase K was added, mixed and incubated for 1 hour at 37 ºC
5. After incubation, 100 µl of 5M NaCl was added and mixed thoroughly
6. Afterward s 80 µl of CTAB/NaCl solution (0.7 M NaCl, 10% CTAB) was added, then incubated at 65ºC for 10 min.
7. After incubation, equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12000 rpm for 5 min.
8. Aqueous supernatant was transferred to a fresh tube, leaving the interface and organic solution behind
9. To the supernatant, 0.6 times volume of isopropanol was added and mixed gently until the DNA precipited.
10. Then the tube was centrifuged at 13500 rpm to remove isopropanol.
11. 1ml of 70% ethanol was added and centrifuged at 13500 rpm to wash the salt away from the DNA.
12. The ethanol was pipette out without disturbing the DNA pellet and the excess of ethanol were allowed to dry.
13. Finally, the pellet was resuspended in 25µl of TE buffer and stored at 4ºC.

3.1.13 Agarose gel electrophoresis

Agarose gel electrophoresis is an efficient technique to separate DNA molecule according to their molecular weight. 0.8% the agarose gel was prepared by dissolving 0.8 gram of agarose in 100 ml of 1X TAE buffer and boiled to dissolve completely. After cooling, 5µl EtBr (Ethidium Bromide) was added from the stock of 10mg/ ml. The gel was poured onto gel casting tray and allowed to set. The extracted DNA was mixed with loading dye and was loaded in the well of gel. Electrophoresis was carried out at constant volt (100V) for 1 hr. After completion, the gel was observed under UV transilluminator.

3.1.14 Polymerase chain reaction:

After DNA extraction polymerase chain reaction was carried out. It is a molecular technique, which is used to amplify the targeted sequences of a DNA into millions copiesof that particular sequence in short time. It can analyze a short sequence of DNA) even in samples containing only minute quantities of DNA. PCR relies on (i) DNA templates that has targeted gene of interest. At first, double stranded DNA are separated to two single strands of DNA template. (ii) Primers: they are short pieces of single-stranded DNA that are complementary to the target sequence. They bind to 3'-OH of each single strand and helps to construct the new strand. (iii) dNTPs are DNA building blocks i.e. the nucleotides consist of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). (iv) DNA polymerases, enzymes that string together individual nucleotides to form a long nucleotide strand. The DNA polymerase typically used in PCR is called ***Taq* polymerase**, after the heat-tolerant bacterium from which it was isolated (***T****hermus* ***aq****uaticus*)

Steps in PCR:

Denaturation: the DNA is denatured at high temperature (from 90º-97ºC) and double stranded DNA gets separated.
Annealing: Primers attached 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 the PCR product was calculated by running them along with ladder by agarose gel electrophoresis and by observed under UV transilluminator.

Table 1 Reaction components of PCR, Master mix (2X) Promega

S.N	Reagent	Final concentration	Volume(µl)
1	Template	100ng/25µl	2.5
2	10µl forward primer	1µM	2.5
3	10µl reverse primer	1µM	2.5
4	2X master mix	2x	12.5
5	Nuclease free water		5
Total			25

3.1.14.1 PCR amplification of OprD gene

Forward primer sequence: ATGAAAGTGATGAAGTGGAGCG (Tm = 55.1ºC)

Reverse primer sequence: TTACAGGATCGACAGCGGATAG (Tm = 56.9ºC)

PCR product length: 1332 bp

Table 2 PCR codition for OprD gene

gene	Initial Denaturation	Denaturation	Annealing	Extension	Final extension	Hold
OprD gene	95ºC, 2 min.	95ºC, 30 sec	59.0ºC, 30 sec	72ºC, 140.0 sec	72ºC, 5 min	4ºC, forever
		Repeat steps 2-4 29 more times				

3.1.14.2 PCR amplification of BlaAmpC gene

Forward primer sequence: ATGCAGCCAACGACAAAGG (Tm = 53.7ºC)

Reverse primer sequence: CGCCCTCGCGAGCGCGCTTC (Tm = 66.5ºC)

PCR product length: 1243 bp

Table 3 PCR codition for AmpC gene

<table>
<tr><th>gene</th><th>Initial denaturation</th><th>Denaturation</th><th>Annealing</th><th>Extension</th><th>Final Extension</th><th>Hold</th></tr>
<tr><td rowspan="5">blaAmpC gene</td><td rowspan="5">95ºC, 2 min</td><td>95ºC, 30 sec</td><td>62.7ºC, 30 sec</td><td>72ºC, 130.0 sec</td><td rowspan="5">72ºC, 5 min</td><td rowspan="5">4ºC, forever</td></tr>
<tr><td colspan="3">decrease 0.5ºC per cycle
14 more times</td></tr>
<tr><td>95ºC, 30 sec</td><td>55.7ºC, 30 sec</td><td>72ºC, 130. sec</td></tr>
<tr><td colspan="3">Repeat steps 6-8 19 more times</td></tr>
</table>

3.1.14.3 PCR amplification of BlaNDM gene

Forward primer sequence: AATGCTGAATAAAAGGAAAACT (Tm = 47.6ºC)

Reverse primer sequence: GGCAGATTGGGGGTGA (Tm = 51.8ºC)

PCR product length: 869 bp

Table 4 PCR condition for BlaNDM gene

<table>
<tr><th>Gene</th><th>Initial Denaturation</th><th>Denaturation</th><th>Annealing</th><th>Extension</th><th>Final Extension</th><th>Hold</th></tr>
<tr><td rowspan="5"></td><td rowspan="5">95ºC, 2 min</td><td>95ºC, 30 sec</td><td>56.7ºC , 30 sec</td><td>72ºC, 90.0 sec</td><td rowspan="5">72ºC, 5 min</td><td rowspan="5">4ºC, forever</td></tr>
<tr><td colspan="3">decrease 0.5ºC per cycle, 14 more times</td></tr>
<tr><td>95ºC, 30 sec</td><td>49.7ºC, 30 sec</td><td>72ºC, 90.0 sec</td></tr>
<tr><td colspan="3">19 more times</td></tr>
</table>

3.2.15 DNA sequencing by Sanger sequence method and analysis

After positive results of Polymerase chain reaction, PCR product of amplified (14) OprD gene, (5) blaAmpC, (1) blaNDM were sent for sequencing. In our study, the positive PCR product for sequencing was sent to Xcelris labs Ltd., Ahmedabad, Gujarat, India.

DNA sequencing is a method of determining the sequence of nucleotide bases present in a DNA strand. Sanger sequence is a standard DNA sequencing technique which can sequence DNA up to about 900 base pairs. This method requires DNA template, sequencing primers, DNA polymerase enzyme, the four nucleotides (dATP, dTTP, dCTP, dGTP), dideoxynucleotide (ddNTPs) and reaction buffers. At first, DNA strands are denatured into single strands andthe primer binds to their 3’OH. In the presence of the 4 nucleotides, the polymerase extends the primer by adding on the complementary nucleotide from the template DNA. As the DNA is synthesized, dideoxynucleotides appears in the fragments of DNA being sequenced which then terminates the process. This is because the ddNTP lacks a 3'OH, which is required to form a link with the next nucleotide in the chain. Since the ddNTPs are randomly incorporated, synthesis terminates at many different positions for each reaction. Synthesized DNA are denatured once again and each labeled synthesized DNA are separated by electrophoresis, which separates them by their size. The colors of bands on the gel are detected, and then the sequence data is read from the fluorescence peak appeared on the chromatogram. Due to the use of dideoxynucleotides in the reactions, Sanger sequencing is also called "dideoxy" sequencing.

The chromatogram obtained after sequencing was subjected to base calling in Chromas and the DNA sequence analysis was done using Sequencher software 4.1.4 version and

BioEdit Softwere for editing. Various bioinformatics tools such as nucleotide Blast in NCBI and multiple sequence alignment using T-Coffee multiple sequence alignment Programs were used. The sequence analyzed compared with the OprD gene (NCBI Reference Sequence: (NC_002516.2), BlaAmpC gene, blaNDM-1 gene (MF379684) of *P. aeruginosa* strain and therefore taken them as reference strains.

CHAPHTER 4
RESULTS

4.1 Microbiological analysis

The clinical isolates were collected from the Tribhuvan University Teaching Hospitals, Maharajganj, Kathmandu. During this period total ninety-seven samples were collected from which only ninety-five of the isolates were recovered from overnight culture in lauria broth while two of the isolated samples were excluded from this study. These clinical isolates were subjected to inoculation and incubation in nutrient agar plates in order to carry out various characteristic studies.

4.1.1 Colony characteristics

Table 5 Colony morphology of the P. aeruginosa

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

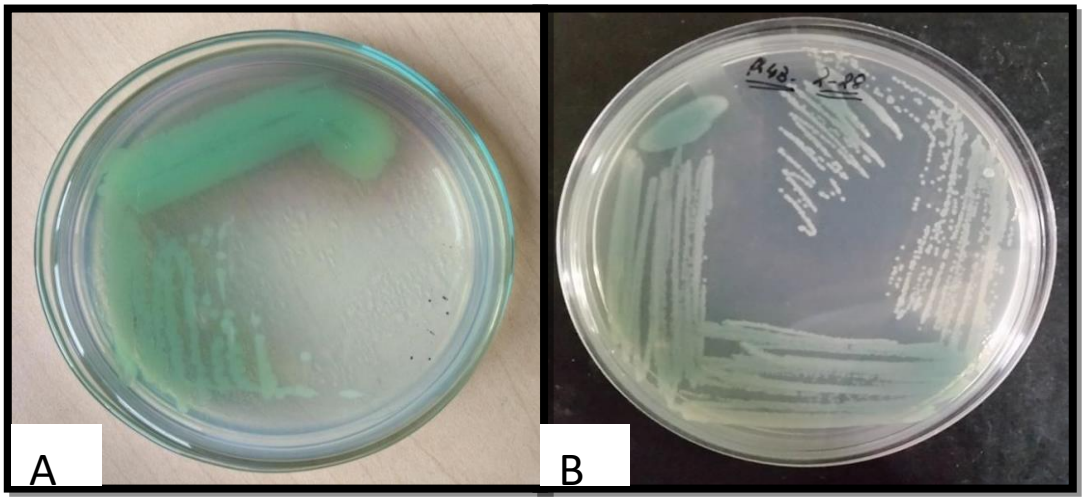

Figure 3 *P. aeruginosa* in nutrient agar Plate A and B

4.1.2 Gram's staining

Isolates were subjected for gram's staining in order to confirm that they were gram negative bacteria.

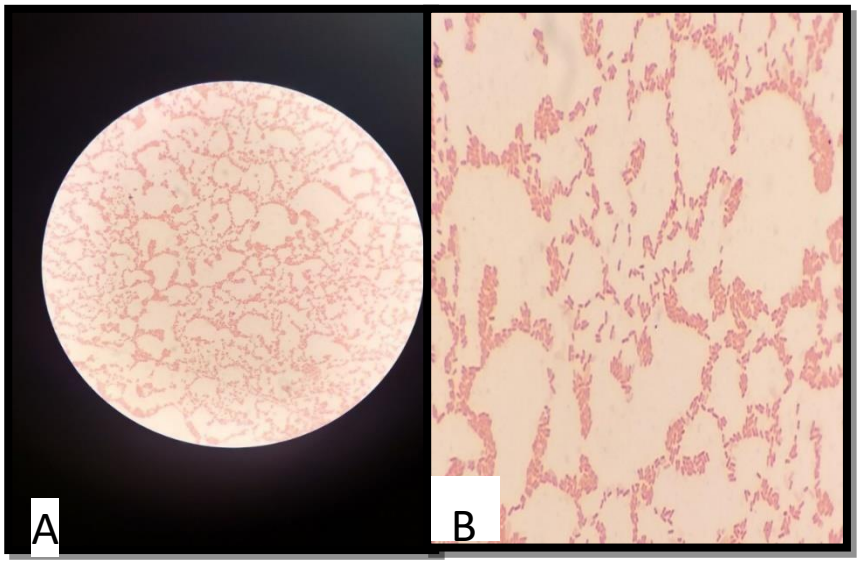

Figure 4 G ram's staining of isolates showing (A) Gram negative, (B) rods

4.1.3 Biochemcial test for identification of *P. aeruginosa*

Table 6 Biochemical test result for the identification of *P.aeruginosa*

Biochemical Test	Result
Citrate Test	Positive
Oxidase Test	Positive
SIM (sulfide, Indole, Motilty) test	H2S negative, Indole negative, Motility Positive
Methyl red (MR) test	Negative
Voges–Proskauer (VP) Test	Negative
Triple Sugar Iron (TSI)Test	R/R, alk/alk, H2S Negative

1 tube: TSI, 2 tube: CITRATE, 3 tube: SIM, 4 tube: MR, 5 tube: VP

4.2 Distribution of *P. aeruginosa* isolates in various specimens

Majority of the isolates were from sputum i.e 36 (37.89%) followed by pus 31(32.63%), swab 16 (16.84%), urine 8 (8.42%), and blood accounted for 4 (4.21%).

Table 7 Distribution of isolates in various specimens

Specimen	No of cases	Percent
Pus	**31**	**32.63%**
Blood	**4**	**4.21%**
Sputum	**36**	**37.89%**
Urine	**8**	**8.42%**
Swab	**16**	**16.84%**

4.3 Antibiotic Susceptibility test

Antibiotic susceptibility test was performed according to the Clinical Laboratory Standard Institute (CLSI) guidelines by Kirby-Bauer disc diffusion method. *P. aeruginosa* accounted resistance to cefotaxime with 84.21%, followed by ciprofloxacin, ceftazidime, gentamicin, meropenem and imipenem with 76.84%, 73.68%, 61.5%, 60%, 36.84% resistance respectively.

Table 8 Resistance pattern of *P. aeruginosa* to various antibiotic

Antibiotics	Sensitive (%)	Resistant(%)
Imipenem(IMP)	63.16	36.84
Meropenem(MEP)	40	60
Ceftazidime(CAZ)	26.32	73.68
Cefotaxime(CTX)	15.79	84.21
Ciprofloxacin (OF)	23.16	76.84
Gentamicin (GEN)	38.95	61.05

4.3.1 Prevalence of MDR *P. aeruginosa*

Out of 95 isolates 73(76.84%) showed resistance to at least 3 classes of antibiotics and grouped under multidrug resistant isolates and 22 (23.16%) are non MDR strain.

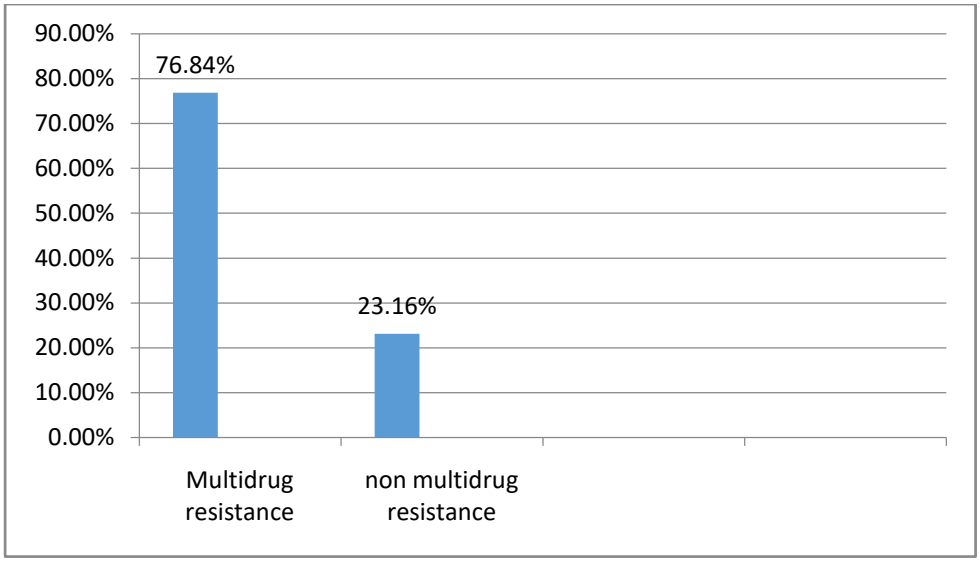

Figure 6 Prevalence of Multi drug resistance *P. aeruginosa*

4.4 Detection of carbapenem resistance *P. aeruginosa*

After performing the antimicrobial susceptibility test, carbapenem resistance isolates were screened out for further investigation. Among 73 MDR strains, 61 were found to be carbapenem resistant Pseudomonas aeroginosa. These isolates were grouped into three Phenotypic types: (30) IMP^{R} MRP^{R}, (4) $IMP^{R}MRP^{S}$,(27) $IMP^{S}MRP^{R}$

Table 9 Distribution of carbapenem resistance

Organism	Total isolates	No of MDR isolates	No of carbapenem resistant
P. aeruginosa	95	73 (76.84%)	61 (64.21%)

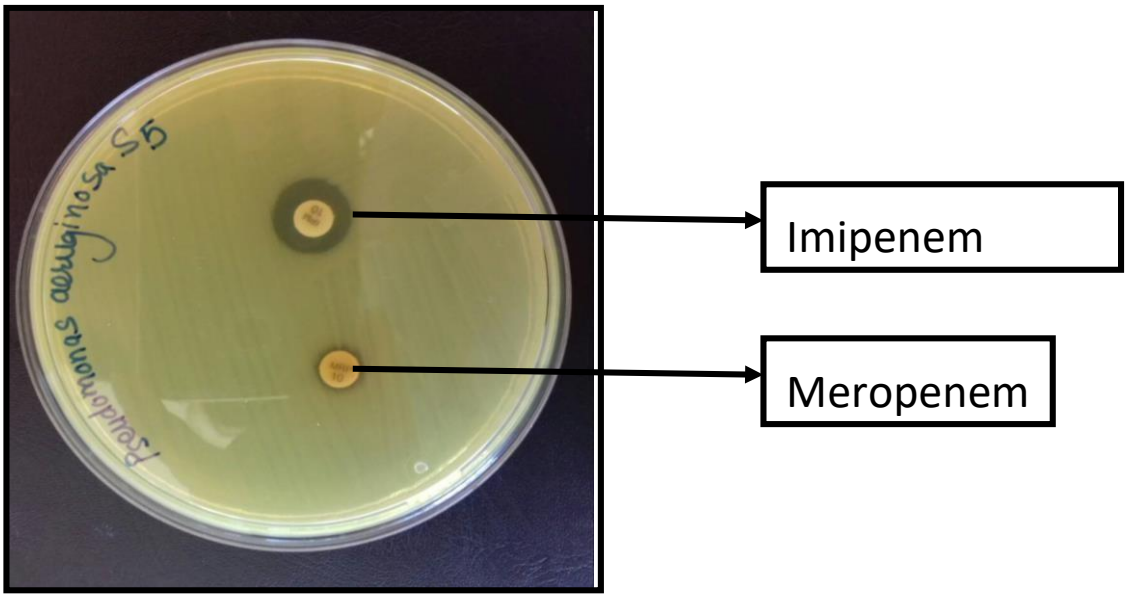

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Figure 7 detection of carbapenem resistant isolates

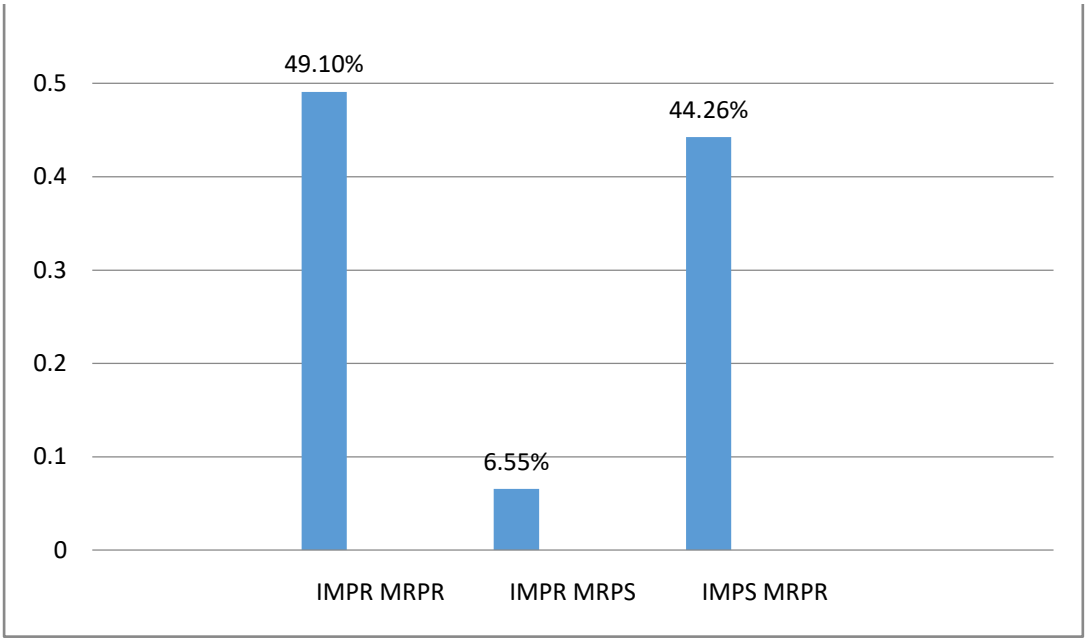

Figure 8 Types of carbapenem resistant isolates

4.5 Detection of MBL producer

Sixty-one isolates of *P. aeruginosa* were screened for the MBL production by using imipenem and meropenem antibiotics out of which 55 samples of *P. aeruginosa* were confirmed positive for metallo beta lactamase production. The *P. aeruginosa* demonstrating a zone diameter ≥ 7 mm around the meropenem/EDTA disc compared to that of meropenem disc alone was considered to be positive for the presence of MBLs.

Table 10 Detection of MBL producer *P. aeruginosa*

Total	IMP and/orMEP resistant	MBL producer
95	61	55

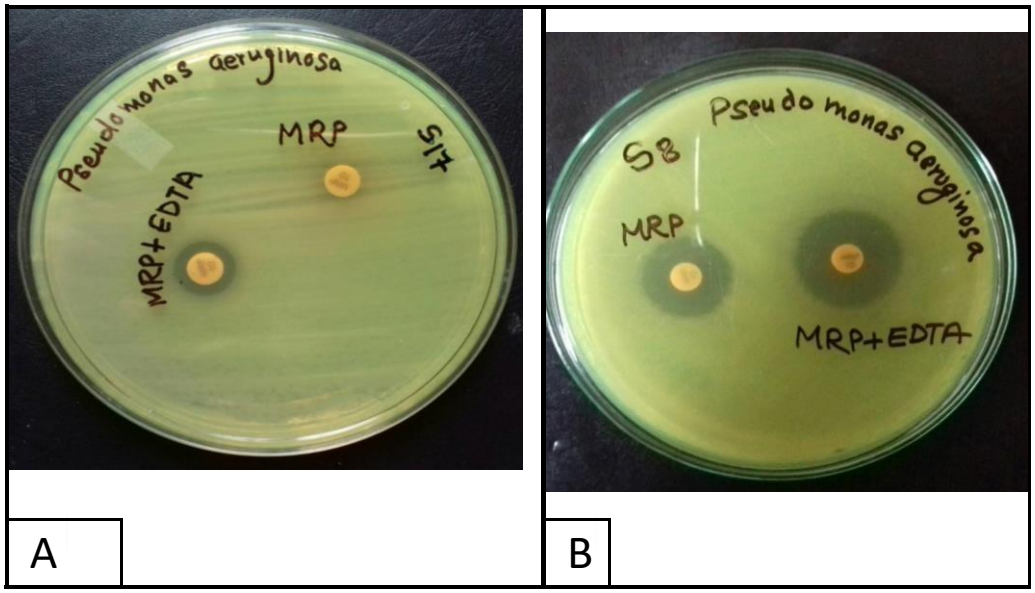

Figure 9 EDTA combined Disc test showing MBL producer

4.6 Detection of ESBL producer

45 isolates out of 73 were found to be positive for ESBL producer *P. aeruginosa* by this phenotypic test. Isolates were considered a potential ESBL producer if the zone of inhibition for ceftazidime was observed to be <22mm.

Table 11 distribution of ESBL producer in *P.aeruginosa*

Total isolates	Screening of potential ESBL producer using Ceftazidime 30 mcg	Confirmation of ESBL producer by using Ceftazidime alone and ceftazidime–clavulanic acid in MDR isolates
95	79	45

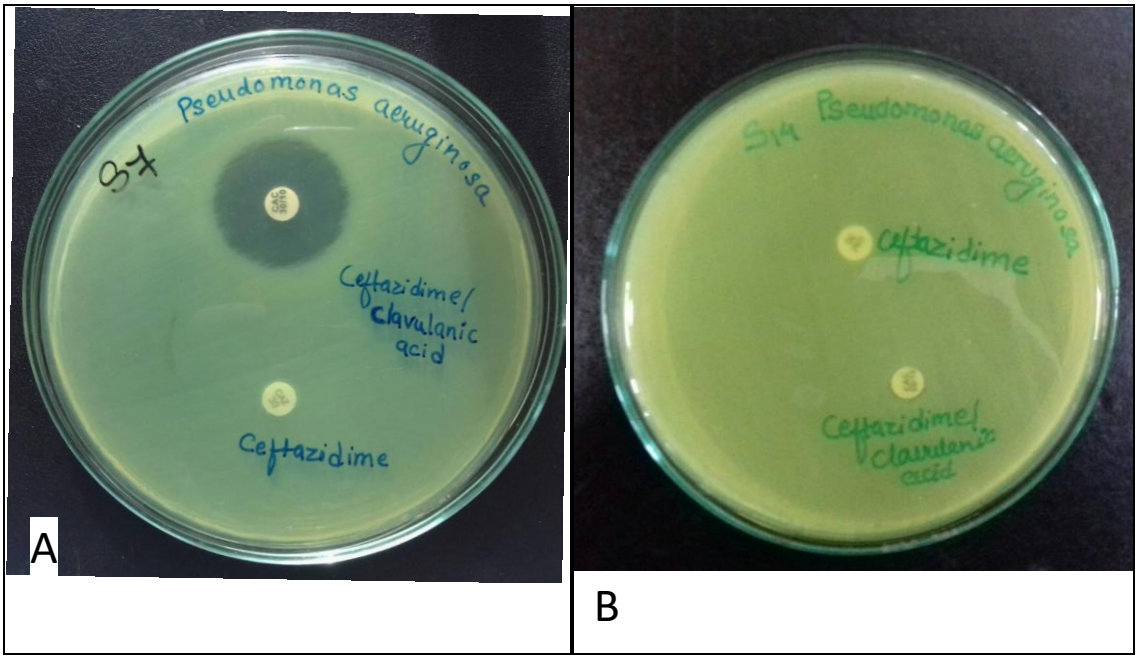

Figure 10 Double disk synergy test showing positive result for ESBL

4.7 Detection of AmpC producer

Among, all of the 73 isolates of them that have less than 18 mm zone diameter are considered as cefoxitin resistant. No any isolates show blunting of the cefotaxime zone of inhibition adjacent to the cefoxitin disk. Therefore, they were considered as cefoxitin resistant AmpC β-lactamase producer.

Table 12 Distribution of AmpC producer

Total MDR	Number of isolates resistant to cefoxitin	Number of isolates producing cefoxitin resistant AmpC beta lactamase.
73	73	0

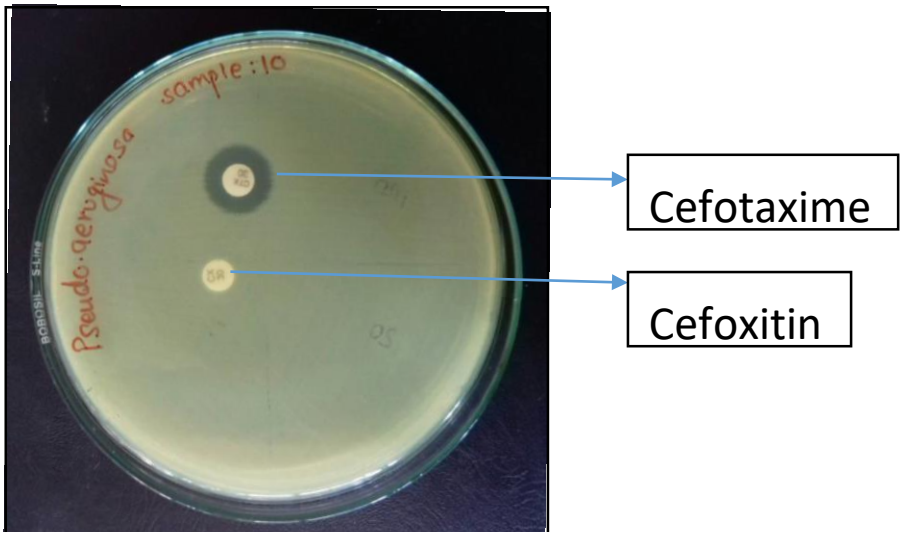

Figure 11 Disc antagonism test showing non inducible for Ampc producer *P. aeruginosa*

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4.8 Preparation of genomic DNA

Genomic DNA of 73 MDR *P. aeruginosa*were extracted using standard protocol of DNA isolation method by Michele K Nishiguchi, Phaedra Doukakis, Maery Egan et al. In this method CTAB is used.

4.9 Agarose gel electrophoresis

After the extraction, the genomic DNA were run in 0.8% agarose gel electrophoresis at 80V for 45 min and the bands were visualized under UV transilluminator. L1, L3, L4, L5, L6, L7, L8, L9, L10, L13, L14 showing positive band for genomic DNA while L2, L11, L12 showing negative band for genomic DNA.

Figure 12 0.8% agarose gel electrophoresis of genomic DNA

4.10 PCR amplification of OprD gene

PCR was performed for all 61 carbapenem resistant isolates for the detection of OprD gene. 19 (31.14%) of the carbapenem isolates were negative result in PCR amplification which indicates that there was loss of OprD gene and 42 (68.86%) of them show positive result in pcr amplification having PCR product size 1332 bp which indicates that there was no loss of OprD gene. L1, L2, L3, L4, L5, L6, L7, L8, L9, L11 shows presence of OprD gene. L10 shows absence of OprD gene, L12 -1kb ladder

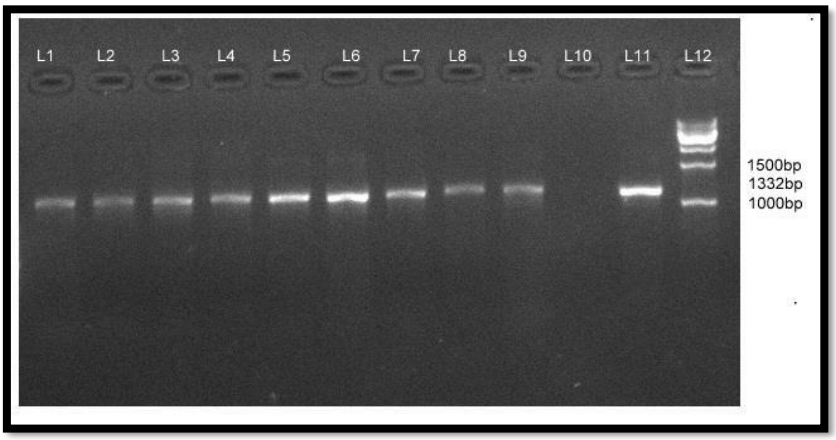

Figure 132% agarose gel electrophoresis showing Oprd gene

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4.11 PCR amplification of AmpC gene

Phenotypic detection was unable to detect the AmpC producer, therefore presence of AmpC gene in the isolates of Carbapenem resistant MDR isolates were detected by PCR amplification of blaAmpC gene. The resulting amplification demonstrated that 26 out of 61 (42.62%) isolates carring blaAmpC gene of 1243 bp product length.

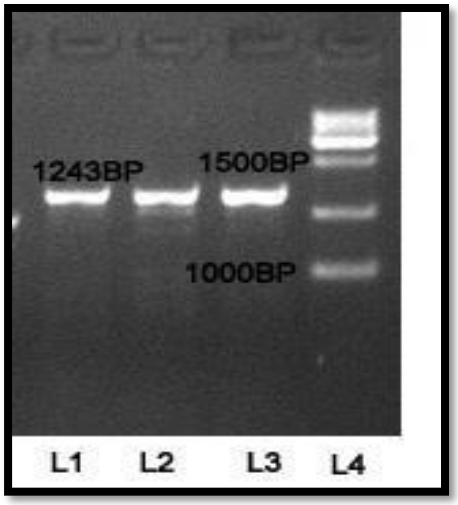

Figure 14 % agarose gel electrophoresis showing AmpC gene

4.12 Pcr amplification of BlaNDM

After PCR amplification of NDM gene in 61 Carbapenem resistance *P. aeruginosa*, 7(11.47%) of them were found to be NDM positive having product size of 862bp. L1, L2, L3, L4, L5 shows positive result of NDM gene.

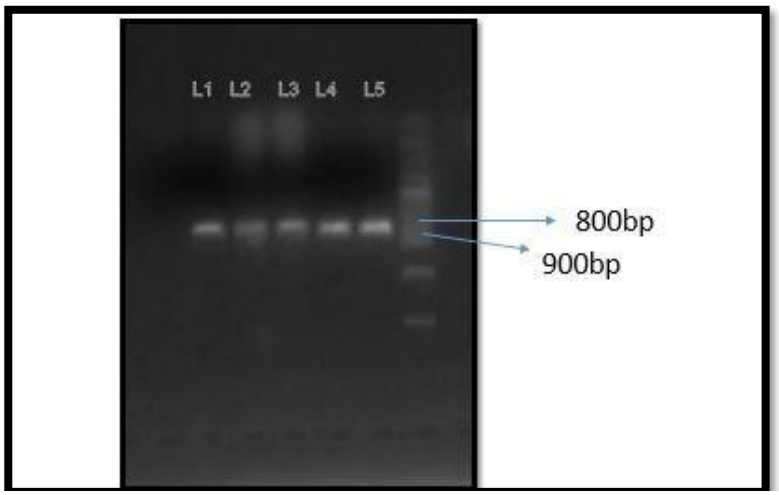

Figure 15 1% agarose gel electrophoresis shows positive result of NDM gene

4.13 Seqencing of PCR product and sequence analysis

4.13.1 Multiple sequence alignment of OprD gene

Total 14 positive PCR product were sent for sequencing from which seven of them gave quality data those seven sequence result were further analysed by using Sequencher softwere and Nucleotide Blast in NCBI. Bioedit tools was used to edit the sequence and for multiple sequence alignment. The sequence matched with the OprD gene of PA01 and hence used as reference sequence for alignment process. In this study, the $OprD_2$-encoding gene sequence analysis revealed the mutation in the coding region causes Frameshifts and substitutions due to the lack of small fragments or multi-point mutation or insertions of 1 or more base pairs, in different locations. we observed a large fragment deletion in case of (ProjectSOP42) from 1057-1139.

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          ....|....| ....|....| ....|....| ....|....| ....|....|

                   10         20         30         40         50

NC_002516.  ATGAAAGTGA TGAAGTGGAG CGCCATTGCA CTGGCGGTTT CCGCAGGTAG

project so  ---------- --------AC AATGGACAGT GGGGGGTTTC TGCAGGGTAG

OprD 18 co  ---------- ---------- CCAGGTCCCG GGGGGGGGTT TCGCAGGTAG

OprD9 reco  ---------- ---------- ---------- ------CGTC AGGCGTGCTG
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 OprD36 rec  ---------- ---------- ---------- ---------- ----------
 OPRD37      ---------- ---------- ---------- ---------- ---------G
 OprD85 85   ---------- ---------- ---------- ---------- ----------
 OprD92 rec  ---------- ---------- ---------- ---------- ----GGTCAA

             ....|....| ....|....| ....|....| ....|....| ....|....|
                    60         70         80         90        100
 NC_002516. CACTCAGTTC GCC--GTGGC CGACGCATT- CGTCAGCGAT CAG--GCCGA
 project so CACTCAGTTC --GCCGTGGC CGACGCATT- CGTCAGCGAT CAA--GCCGA
 OprD 18 co CACTCAGTTC GCCG--TGGC CGACGCATT- CGTCAGCGAT CAG--GCCGA
OprD9 reco  ATCCGTTCTC GTCA-ATGCC CTACGCGTTG CGTGCAAAGA AAG--GGCGA
OprD36 rec  ---TGAGC-C GCAATATAAT TGCTTGCAC- GCATATCAGT CAA-----GA
OPRD37      ATCCGAGCTC GTTAAATGAC CGAC-GATC- CCATTCAAGT AAAAAGCCGA
OprD85 85   --ATCAGCAC -CTAAGTAAT ATCCTGGTA- AAATACAGAG CCG-----AA
OprD92 rec  CGTACACCTT CGAACGTGGC CGACGCATT- CGTCAGCGAT CAG--GCCGA
             ....|....| ....|....| ....|....| ....|....| ....|....|
                   110        120        130        140        150
NC_002516.  AGCGAAGGGG TTCATCGAAG ACAGCAGCCT CGACCTG-CT GCTCCGCAAC
project so  AGCGAAGGGG TTCATCGAAG ACAGCAGCCT CGACCTG-CT GCTCCGCAAC
OprD 18 co  AGCGAAGGGG TTCATCGAAG ACAGCAGCCT GAACCTG-CT GCTCCGCAAC
9 reco      AGCGAAGGGG TTCATCGAAG ACAGCAGCCT CGACCTG-CT GCTCCGCAAC
36 rec      AGCGAAGGGG TTCATCGAAG ACAGCAGCCT CGACCTGGCT GCTCCGCAAC
OPRD37      AGCGAAGGGG TTCATCGAAG ACCGCAGCCT CGACCTG-CT GCTCCGCAAC
OprD85 85   GGCGAAGGGG TTCATCGAAG ACAGCAGCCT CGACCTG-CT GCTCCGCAAC
OprD92 rec  AGCGAAGGGG TTCATCGAAG ACAGCAGCCT CGACCTG-CT GCTCCGCAAC
             ....|....| ....|....| ....|....| ....|....| ....|....|
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                  160        170        180        190        200
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project so    TACTATTTCA ACCGTGACGG CAAGAGCGGC AGCGGGGACC GCGTCGACTG
OprD 18 co    TACTATTTCA ACCGTGACGG CAAGGGAGGT CGGGGTGATC GCGTCGATTG
OprD9 reco    TACTATTTCA ACCGTGACGG CAAGAGAGGT CGGGGGGACC GCGTCGACTG
OprD36 rec    TACTATTTCA ACCGTGACGG CAAGAGCGGC AGCGGGGACC GCGTCGACTG
OPRD37        TACTATTTCA ACCGTGACGG CAAGAGCGGC AGCGGGGACC GCGTCGACTG
OprD85 85     TACTATTTCA ACCGTGACGG CAAGAGCGGC AGCGGGGACC GCGTCGACTG
OprD92 rec    TACTATTTCA ACCGTGACGG CAAGAGCGGC AGCGGGGACC GCGTCGACTG

              ....|....| ....|....| ....|....| ....|....| ....|....|
                  210        220        230        240        250
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project so    GACCCAAGGC TTCCTCACCA CCTATGAATC CGGCTTCACC CAAGGCACCG
OprD 18 co    GACCCAGGGC TTCCTCACCA CCTACGAATC CGGCTTCACT CAAGGCACCG
OprD9 reco    GACCCAGGGC TTCCTCACCA CCTACGAATC CGGCTTCACC CAAGGCACCG
OprD36 rec    GACCCAAGGC TTCCTCACCA CCTATGAATC CGGCTTCACC CAAGGCACTG
 OPRD37       GACCCAAGGC TTCCTCACCA CCTATGAATC CGGCTTCACC CAAGGCACCG
 OprD85 85    GACCCAAGGC TTCCTCACCA CCTATGAATC CGGCTTCACC CAAGGCACTG
 OprD92 rec   GACCCAAGGC TTCCTCACCA CCTATGAATC CGGCTTCACC CAAGGCACCG

              ....|....| ....|....| ....|....| ....|....| ....|....|
                  260        270        280        290        300
 NC_002516    TGGGCTTCGG CGTCGATGCC TTCGGCTACC TGGGCCTGAA GCTCGACGGC
 project so   TCGGCTTCGG CGTCGATGCC TTCGGCTACC TCGGTCTGAA GCTCGACGGC
OprD 18 co    TGGGCTTCGG CGTCGATGCC TTCGGCTACC TGGGCCTGAA GCTCGACGGC
OprD9 reco    TGGGCTTCGG CGTCGATGCC TTCGGCTACC TGGGCCTGAA GCTCGACGGT
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OprD36 rec   TGGGCTTCGG CGTCGATGCC TTCGGCTACC TGGGCCTGAA GCTCGACGGC
OPRD37       TCGGCTTCGG CGTCGATGCC TTCGGCTACC TGGGTCTGAA GCTCGACGGC
OprD85 85    TGGGCTTCGG CGTCGATGCC TTCGGCTACC TGGGCCTGAA GCTCGACGGC
OprD92 rec   TGGGCTTCGG CGTCGATGCC TTCGGCTACC TGGGCCTGAA GCTCGACGGC
             ....|....| ....|....| ....|....| ....|....| ....|....|
                   310        320        330        340        350
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project so   ACCTCCGACA AGAGCGGTAC CGGCAACCTG CCAGTGATGA ACGACGGCAC
OprD 18 co   ACCTCCGACA AGACCGGCAC CGGCA-CCTG CCGGTGATGA ACGACGGCAA
OprD9 reco   ACCTCCGACA AGACCGGCAC CGGCAACCTG CCGGTGATGA ACGACGGCAA
OprD36 rec   ACCTCCGACA AGACCGGTAC CGGCAACCTG CCGGTGATGA ACGACGGCAA
OPRD37       ACCTCCGACA AGAGCGGTAC CGGCAACCTG CCAGTGATGA ACGACGGCAC
 OprD85 85    ACCTCCGACA AGACCGGCAC CGGCAACCTG CCGGTGATGA ACGACGGCAA
OprD92 rec   ACCTCCGACA AGAGCGGTAC CGGCAACCTG CCGGTGATGA ACGACGGCAC
             ....|....| ....|....| ....|....| ....|....| ....|....|
                     360        370        380        390        400
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 project so  GCCCCGTGAC GACTAC-GCC GCGCCGGTGG CGCCGTGAAG GTACGCATCT
 OprD 18 co  GCCGCGCGAT GACTAC-GCC GCGCCGGCGG CGCCGTGAAG GTGCGCATCT
OprD9 reco   GCCGCGCGAC GACTACAGCC GCGCCGGCGG CGCCGTGAAG GTGCGCATTT
OprD36 rec   GCCCCGCGAC GACTACGGCC GCGCCGGCGG CGCCGTGAAG GTGCGCATCT
 OPRD37      GCCCCGTGAC GACTACGGCC GCGCCGGTGG CGCCGTGAAG GTACGCATCT
OprD85       GCCGCGCGAT GACTACAGCC GCGCCGGCGG CGCCGTGAAG GTGCGCATCT
  OprD92 rec   GCCCCGTGAC GACTACAGCC GCGCCGGCGG CGCCGTGAAG GTACGCAT
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                  410        420        430        440        450
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project so   CCAAGACCAT GTTGAAGTGG GGCGAGATGC AGCCGACCGC TCCGGTCTTC
OprD 18 co   CCAAGACCAT GCTGAAGTGG GGCGAGATGC AACCGACCGC CCCGGTCTTC
OprD9 reco   CCAAGACCAT GCTGAAGTGG GGCGAGATGC AACCGACCGC GCCGGTCTTC
OprD36 rec   CCAAGACCAT GTTGAAGTGG GGCGAGATGC AACCGACCGC TCCGGTCTTC
OPRD37       CCAAGACCAT GCTGAAGTGG GGCGAGATGC AACCGACCGC TCCGGTCTTC
OprD85       CCAAGACCAT GCTGAAGTGG GGCGAGATGC AACCGACCGC CCCGGTCTTC
OprD92 rec   CCAAGACCAT GTTGAAGTGG GGCGAGATGC AACCGACCGC TCCGGTCTTC

               ....|....| ....|....| ....|....| ....|....| ....|....|
                     460        470        480        490        500
NC_002516.    GCCGCTGGCG GCAGCCGCCT GTTCCCGCAG ACCGCGACCG GCTTCCAGCT
project so    GCCGCCGGCG GCAGCCGCCT GTTCCCGCAG ACCGCGACCG GCTTCCAATT
 OprD 18 co    GCCGCTGGCG GCAGCCGCCT GTTCCCGCAG ACCGCGACCG GCTTCCAGCT
OprD9 reco    GCCGCCGGCG GCAGCCGCCT GTTCCCGCAG ACCGCGACCG GCTTCCAACT
OprD36 rec    GCCGCTGGCG GCAGCCGCCT GTTCCCGCAG ACCGCGACCG GCTTCCAGTT
OPRD37        GCCGCTGGCG GCAGCCGCCT GTTCCCGCAG ACCGCGACCG GCTTCCAGCT
 OprD85        CCCGCTGGCG GCAGCCGCCT GTTCCCGCAG ACCGCGACCG GCTTCCAGCT
OprD92 rec    GCCGCCGGCG GCAGCCGCCT GTTCCCGCAG ACCGCGACCG GCTTCCAACT

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   510        520      530        540      550
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project so   GCAGAGCAGC GAACTCGA-- AGGGCTCGAT CTC-GAAGCG GGCCACTTC-
OprD 18 co   GCAGAGCAGC GAATTCGA-- AGGGCTCGAC C-TCGAGGCA GGCCACTTC-
OprD9 reco   GCAGAGCAGC GAATTCGA-- AGGGCTCGAT CGTCGAGGCG GGCCACTTC-
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OprD36 rec   GCAGAGCAGC GAATTCGA-- AGGGCTCGAC CTC-GAGGCG GGCCACTTCC
OPRD37       GCAGAGCAGC GAATTCGACC AGGGCTCAAT CTCCGAGGCG GGCCACTTC-
OprD85       GCAGAGCAGC GAATTCGA-- AGGGCTCGAC CTC-GAGGCA GGCCACTTC-
OprD92 rec   GCAGAGCAGC GAACTCGA-- AGGGCTCGAT CTC-GAAGCG GGCCACTTC-
             ....|....| ....|....| ....|....| ....|....| ....|....|
                  560        570        580        590        600
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project so   ACCGAAGGCA AGCAGGGC-A CCACCACCAA GTCGCGCGGC GAACTCTACG
OprD 18 co   ACCGAGGGCA AGGA-GCCGA CCACCGTCAA ATCGCGCGGT GAGCTCTACG
OprD9 reco   ACCGAGGGCA AGGA-GGCGA CCACCGTCAA GTCGCGCGGC GAACTCTATG
OprD36 rec   ACCGAAGGCA AGGAGGCC-A CCACCGTCAA ATCGCGCGGC GAACTCTACG
OPRD37       ACCGAAGGCA AGGAGGGC-A CCACCGTCAA ATCGCGTGGC GAACTCTATG
OprD85 85    ACCGAGGGCG AAGGAGCCGA CCACCGTCAA ATCGCGTGGC GAACTCTATG
OprD92 rec   ACCGAAGGCA AGGAGGGC-A CCACCATCAA ATCGCGCGGC GAACTCTACG

              ....|....| ....|....| ....|....| ....|....| ....|....|
                     610        620        630        640        650
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 project so   CAACCT-ATG CAGGCGAGAC CGCCAAGA-- GCGCCGATTT CATTGGGGGC
 OprD 18 co   CCACCT-ACG CCGGCCAGAC AGCCAAGA-- GCGCCGACTT CGCTGGGGGC
 OprD9 reco   CCACCT-ATG CAGGCGAGAC CGCCGAGA-- GCGCGGATTT CGCCGGCGGC
OprD36 rec    CAACCT-ATG CAGGCGAGAC CGCCAAGA-- GCGCCGATTT CATTGGGGGC
 OPRD37       CACCCTCATG CAGGCGAGAC CGCCAAGATC GCGCCGTTTT CATTGGGGGC
 OprD85       CCACCT-ACG CAGGCGAGAC CGCCAAGA-- GCGCCGATTT CATTGGGGGC
OprD92 rec    CAACCT-ATG CAGGCGAGAC CGCCAAGA-- GCGCCAATTT CATTGGGGGC
              ....|....| ....|....| ....|....| ....|....| ....|....|
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                660        670        680        690        700
NC_002516.   CGCTACGCAA --TCACCGAT AACCTCAGCG CCTCCC-TGT ACGGCGCCGA
project so   CGCTACGCAA --TCACCGAT AACCTCAGCG CCTCCC-TGT ACGGTGCTGA
OprD 18 co   CGCTACGCGA --TCACCGAC AACCTCAGCG CCTCCC-TCT ATGGCGCAGA
OprD9 reco   CGCTACGCAA --TCACCGAC AACCTCAGCG CCTCCC-TGT ATGGCGCCGA
OprD36 rec   CGCTACGCAA --TCACCGAT AACCTCAGCG CCTCCC-TGT ACGGTGCTGA
OPRD37       CGCTACGCAA ATTCACCGAT AACCTCACCG CCCCCCGTGT ACGGTGCGGA
OprD85 85    CGCTACGCAA --TCACCGAT AACCTCAGCG CCTCCC-TGT ACGGCGCCGA
OprD92 rec   CGCTACGCAA --TCACCGAT AACCTCAGCG CCTCCC-TGT ACGGTGCTGA

             ....|....| ....|....| ....|....| ....|....| ....|....|
                  710        720        730        740        750
NC_002516.   ACTCGA--AG ACATCTATCG CCAGTATTAC CTGAACAGCA ACTACACCAT
project so   ACTCGA--AG ACATCTATCG TCAGTATTAC CTGAACAGCA ACTACACCAT
 OprD 18 co   GCTGAA--AG ACATCTATCG CCAGTACTAC CTGAACACCA ACTACACCAT
OprD9 reco   ACTGAA--AG ACATCTATCG CCAGTATTAC CTGAACACCA ACTACACCAT
 oprd 36     ACTCGA--AG ACATCTATCG CCAGTATTAC CTGAACAGCA ACTACACCAT
OprD 37      ATTCGAGAAG ACATCTAGCG CCAGTATTAC CTGAACAGCA ACTACACCAT
             ACTCGAGGAG ACATCTATCG CCAGTATTAC CTGAACAGCA ACTACACCAT
            ACTCGA--AG ACATCTATCG TCAGTATTAC CTGAACAGCA ACTACACCAT

             ....|....| ....|....| ....|....| ....|....| ....|....|
               760        770        780        790        800
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 project so  CCCACTGGCA TCCGACCAAT CGCTGGGCTT CGATTTCAAC ATCTACCGCA
OprD 18 co   CCCGCTGGCC TCCGATCAAT CGCTGGGCTT CGACTTCAAC ATCTACCGCA
OprD9 reco   CCCGCTGGCA TCCGACCAAT CGCTGGGCTT CGACTTCAAC ATCTACCGCA
```

OprD36 rec CCCACTGGCA TCCGACCAAT CGCTGGGCTT CGATTTCAAC ATCTACCGCA

OPRD37 CCCACTGGCA TCCGACCAAT CGCTGGGCTT CGATTTCAAC ATCTACCGCA

OprD85 85 CCCACTGGCA TCCGACCAAT CGCTGGGCTT CGATTTCAAC ATCTACCGCA

OprD92 rec CCCACTGGCA TCCGACCAAT CGCTGGGCTT CGATTTCAAC ATCTACCGCA

....|....| ....|....| ....|....| ....|....| ....|....|

810 820 830 840 850

NC_002516. CAAACGATGA AGGCAAGGCC AAGGCCGGCG ACATCAGCAA CACCACTTGG

project so CAAACGATGA AGGCAAGGCC AAGGCCGGCG ACATCAGCAA CACCACTTGG

OprD 18 co CCACCGACGA AGGCAAGTCC AAGGCTGGCG ACATCAGCAA CACCACCTGG

OprD9 reco CCACCGATGA AGGCAAGGCC AAGGCCGGCG ACATCAGCAA CACCACTTGG

OprD36 rec CAAACGATGA AGGCAAGGCC AAGGCCGGCG ACATCAGCAA CACCACTTGG

OPRD37 CAAACGATGA AGGCAAGGCC AAGGCCGGCG ACATCAGCAA CACCACTTGG

OprD85 85 CAAACGATGA AGGCAAGGCC AAGGCCGGCG ACATCAGCAA CACCACTTGG

OprD92 rec CAAACGATGA AGGCAAGGCC AAGGCCGGCG ACATCAGCAA CACCACTTGG

....|....| ....|....| ....|....| ....|....| ....|....|

860 870 880 890 900

NC_002516 TCCCTGGCGG CAGCCTACAC TCTGGATGCG CACACTTTCA CCTTGGCCTA

project so TCCCTGGCGG CAGCCTACAC TCTGGATGCG CACACTTTCA CCTTGGCCTA

OprD 18 co TCCCTGGCGG GCGCGTATAC CCTGGACGCC CACACCTTCA CCCTGGCCTA

OprD9 reco TCCCTGGCGG CCGCGTACAC CCTGGACGCG CACACCTTCA CCCTGGCCTA

OprD36 rec TCCCTGGCGG CAGCCTACAC TCTGGATGCG CACACTTTCA CCTTGGCCTA

OPRD37 TCCCTGGCGG CAGCCTACAC TCTGGATGCG CACACTTTCA CCTTGGCCTA

OprD85 TCCCTGGCGG CATCCTACAC TCTGGATGCG CACACTTTCA CCTTGGCCTA

OprD92 rec TCCCTGGCGG CAGCCTACAC TCTGGATGCG CACACTTTCA CCTTGGCCTA

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          ....|....| ....|....| ....|....| ....|....| ....|....|

                910        920        930        940        950

NC_002516.  CCAGAAGGTC CATGGCGATC AGCCGTTTGA TTATATCGGC TTCGG-CCGC

project so  CCAGAAGGTC CATGGCGATC AGCCGTTTGA TTATATCGGC TTCGG-CGAG

OprD 18 co  CCAGCAGGTG CATGGCGACG AGCCGTTCGA CTACATCGGC TTCGG-CGGC

OprD9 reco  CCAGAAGGTG CATGGCGACG AGCCGTTTGA CTACATCGGC TTCGG-CGAG

OprD36 rec  CCAGAAGGTC CATGGCGATC AGCCGTTTGA TTATATCGGC TTCGG-CGAG

OPRD37      CCAGAAGGTC CATGGCGATC AGCCGTTTGA TTATATCGGC TTCGG-CGAG

OprD85 85   CCAGAAGGTC CATGGCGATC AGCCGTTTGA TTATATCGGC TTCGG-CCGC

OprD92 rec  CCAGAAGGTC CATGGCGATC AGCCGTTTGA TTATATCGGC TTCGGGCGAG


          ....|....| ....|....| ....|....| ....|....| ....|....|

               960        970        980        990        1000

NC_002516.  AACGGCTCTG GCG----CAG GTGGCGACTC GATTTTCCTC GCCAACTCTG

project so  AACGGTTCCG GCG---GC-G GCGGTGACTC GATTTTCCTC GCCAACTCCG

OprD 18 co  AACGGTTCCG GCG----CCG GCGGCGACTC GATCTTCCTC GCCAACTCCG

OprD9 reco  AACGGTTCTG GCG----GCG GCGGTGACTC GATTTTCCTC GCCAACTCCG

OprD36 rec  AACGGCTCCG GCG---GC-G GCGGCGACTC GATTTTCCTC GCCAACTCCG

 OPRD37     AACGGCTCCG GCG---GC-G GCGGTGACTC GATTTTCCTC GCCAACTCCG

  OprD85 85  AACGGCTCTG GCGCACGCAG GTGGCGACTC GATTTTCCTC GCCAACTCTG

OprD92 rec  AACGGTTCCG GCG---GC-G GCGGTGACTC GATTTTCCTC GCCAACTCCG
```

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1010       1020       1030       1040       1050
NC_002516.      TCCAGTACTC CGACTTCAAC GGCCCTGGCG AGAAATCCTG GCAGGCTCG-
project so      TGCAGTACTC CGACTTCAAC GGCCCCGGCG AGAAATCCTG GCAGGCCCG-
 OprD 18 co      TCCAGTACTC CGACTTCAAC GGTCCTGGCG AGAAATCCTG GCAGGCCCG-
OprD9 reco      TGCAGTACTC CGACTTCAAC GGCCCCGGCG AGAAATCCTG GCAGGCCCG-
OprD36 rec      TGCAGTACTC CGACTTCAAC GGCCCTGGCT AGAAATCCTG GCAGGCCCGA
OPRD37          TGCAGTACTC CGACTTCAAC GGCCCCGGCG AGAAATCCTG GCAGGCCCG-
OprD85 85       TCCAGTACTC CGACTTCAAC GGCCCTGGCG AGAAATCCTG GCAGGCTCG-
OprD92 rec      TGCAGTACTC CGACTTCAAC GGCCCCGGCG AGAAATCCTG GCAGGCCCG-

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1060       1070       1080       1090       1100
NC_002516.      CTACGACCTG AACCTAGCCT CCTATGGCGT TCCCGGCCTG ACTTTCATGG
project so      CTACGA---- ---------- ---------- ---------- ----------
OprD 18 co      CTACGACCTG AACCTGGCCT CCTACGGCGT TCCTGGCCTG ACCTTCATGC
OprD9 reco      CTACGACCTG AACATGGCCT CCTACGGCGT TCCCGGCCTG ACTTTCATGG
OprD36 rec      CTACGACCTG AACCTCGCTT CGTATGGCGT TCCCGGCCTG ACTTTCATGG
OPRD37          CTACGACCTG AACCTCGCCT C-TATGGCGT TCCCGGCCTG ACTTTCATGG
OprD85 85       CTACGACCTG AACCTAGCCT CCTATGGCGT TCCCGGCCTG ACTTTCATGG
OprD92 rec      CTACGACCTG AACCTCGCCT CCTATGGCGT TCCCGGCCTG ACTTTCATGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
```

```
                1110       1120       1130       1140       1150
 NC_002516   TCCGCTATAT CAATGGCAAG GACATCGATG GCACCAAGAT GTCTGACAAC
project so   ---------- ---------- ---------- ---------T GTCTGACAAC
 OprD 18 co  TGCGTTACAT CAATGGTAAG GACATCGACG GTACCAA--- GGTCGATTCC
OprD9 rec    TTCGCTACAT CAACGGTAAG GACATCGACG GCACCAA--- GGTCGACTAC
OprD36 rec   TCCGCTATAT CAATGGCAAG GACATCGATG GCACCAAGAT GTCTGACAAC
OPRD37       TCCGCTATAT CAATGGCAAG GACATCGATG GCACCAAGAT GTCTGACAAC
 oprd85      TCCGCTATAT CAATGGCAAG GACATCGATG GCACCAAGAT GTCTGACAAC
OprD92 rec   TCCGCTATAT CAATGGCAAG GACATCGATG GCACCAAGAT GTCTGACAAC
             ....|....| ....|....| ....|....| ....|....| ....|....|
                1160       1170       1180       1190       1200
NC_002516.   AACGTCGGCT ATAAGAACTA CGGCTACGGC GAGGATGGCA AGCACCACGA
project so   AACGTCGGCT ATAAGAACTA CGGCTACGGC GAGGACGGCA AGCACCACGA
OprD 18 co   AGCTCCTCCT ATGCAGGCC- --TGTACGGC GAGGATGGCA AGCACCACGA
OprD9 reco   AACGTCGGCT ATAAGGGCC- --TGTACGGC GAGGATGGCA AGCACCACGA
OprD36 rec   AACGTCGGCT ATAAGAACTA CGGCTACGGC GAGGACGGCA AGCACCACGA
OPRD37       AACGTCGGCT ATAAGAACTA CGGCTACGGC GAGGACGGCA AGCACCACGA
OprD85 85    AACGTCGGCT ATAAGAACTA CGGCTACGGC GAGGATGGCA AGCACCACGA
OprD92 rec   AACGTCGGCT ATAAGAACTA CGGCTACGGC GAGGACGGCA AGCACCACGA

              ....|....| ....|....| ....|....| ....|....| ....|....|
              1210       1220       1230       1240       1250
NC_002516.   AACCAACCTC GAAGCCAAGT AC-GTGGTCC -AGTCC-GGT CCGGCCAAG-

project so   GACCAACCTC GAAGCCAAGT AC-GTGGTCC -AGTCC-GGT CCGGCCAAG-
 OprD 18     AACCAACCTC GAAGCCAAGT AC-GTGGTCC -AGTCC-GGT CCGGCCAAG-
```

```
OprD9 reco   GACCAACCTC GAAGCCAAGT ACAGTGGTCC -AGTCC-GGT CCGGCCAAG-
OprD36       GACCAACCTC GAAGCCAAGT AC-GTGGTCC -AGTCC-GGT CCGGCCAAA-
 OPRD37      GACCAACCTC GAAGCCAAGT AC-GTGGTCC TAGTCC-GGT CCGGCACAA-
OprD85 85    AACCAACCTC GAAGCCAAGT AC-GTGGTCC TAGTCCAGGT CCGGCCCAAA
OprD92 rec   GACCAACCTG GAAGCCAAGT AC-GTGGTCC -AGTCC-GGT CCGGCCAAG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                 1260       1270       1280       1290       1300
 NC_002516.   -GACC-TGTC GTTCCG-CAT CCGCCAGGCC ---TGGCACC GTGCCAACGC
 project so   -GACC-TGTC GTTCCG-CAT CCGCCAGGCC ---TGGCACC GCGCCAACGC
 OprD 18 co   -GACC-TGTC GTTCCG-CAT CCGCCAGGCC ---TGGCACC GTGCCAACGC
OprD9 reco    -GACC-TGTC GTTCCGACAT CCGCCAGGCC -------GCT ATACCAACGC
OprD36 rec    GGACC-TGTC GTTCCG-C-A T--------- ---------- ----------
OPRD37        GGACC-TGTC GTTCCG-CTA TCACAGTCGC ---C------ ----------
OprD85 85     GGACCGTGTC GTTCCG-CAT CCGCCAAGCC ---TGGCACC GCGCT-----
OprD92 rec    -GACC-TGTC GTTCCG-CAT CCGCCAGGCC ACTTAGCACC GCG--AACAA

            ....|....| ....|....| ....|....| ....|....| ....|....|
                1310       1320       1330       1340       1350
NC_002516.   CGACCAGGGC GAAGGCGACC AGAACGAGTT CCGCCTGATC GTCGACTATC
project so   CGACCAGGCC GAAGGCGACC AGAATCGAGT CCCGCCCAAA TTCCG-----
OprD 18 co   CGACCAGGGC GAAGGCGACC AGAACGAGTT CGGCCTGATC GTC-------

OprD9 reco   CGACGAGGGC GAAGGTGACC AGA------- ---------- ----------
OprD36 rec   ---------- ---------- ---------- ---------- ----------
```

```
OPRD37        ---------- ---------- ---------- ---------- ----------

OprD85 85     ---------- ---------- ---------- ---------- ----------

OprD92 rec    CGACCGACCC GAAGGCGA-- ---------- ---------- ----------
```

Phylogenetic relationship of the sequence:

The phylogenetic tree of the sample with the reference sequence showed the evolutionary interrelation of the oprd gene derived from the common ancestral gene. The amplified OprD gene is a variant from reference gene of OprD extracted from the NCBI. All the Samples were originated from common ancesters.

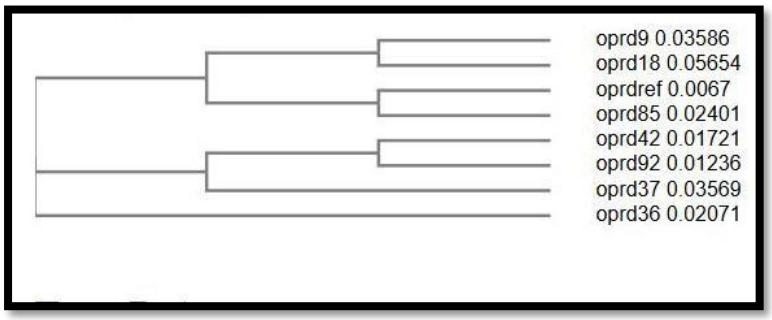

Figure 16 Tree diagram showing alignment between different samples with the reference sequence

4.13.2 Multiple sequence aligment of AmpC gene

Multiple sequence alignment of AmpC gene of 5 sample was performed with the sequence extracted from NCBI AmpC gene (accession number: NC_002516), where the sequence shared 97-99% homology and therefore used as the reference strain for this study. Few variables were obtained in ampC coding region of the clinical samples compared to the refrence such in AmpC 1, AmpC92 and AmpC 96 there is substitution of GC/AT, substitution of G/T in AmpC22, AmpC92, AmpC96, C/T at position 229 of AmpC18,AmpC96 and AmpC92.A/G at postion 252 of AmpC1, AMPc18, AmpC92, AmpC96,insertion of G at position 301 of AmpC92.substitution of A/T in AmpC96 at position 430, C/T in AmpC18 458 position.At position 552 G/C in AmpC1, AmpC92, AmpC96 and G/T in AmpC 18.T/C in at postion 621 of all samples. Insertion of T nucleotide in AmpC 18 at postion 722. Nucleotide substitution at position 785 of ampC22 C/T deletion of nucleotide at postion of 941 of AmpC22 and ampC96.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|

                       10         20         30         40         50

NC_002516.      CCACCCCGGC CATTGCCGGC GAGGCCCCGG CGGATCGCCT GAAGGCACTG

AmpC1           CCACCCCGGC CATTGCCGAT GAGGCCCCGG CGGATCGCCT GAAGGCACTG


AmpC18          CCACCCCGGC CATTGCCGGC GAGGCCCCGG CGGATCGCCT GAAGGCACTG

  AmpC22         CCACCCCGGC CATTGCCGGC GAGGCCCCGG CGGATCGCCT GAAGGCACTG

  AmpC92         CCACCCCGGC CATTGCCGAT GAGGCCCCGG CGGATCGCCT GAAGGCACTG

  AmpC96         CCACCCCGGC CATTGCCGAT GAGGCCCCGG CGGATCGCCT GAAGGCACTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       60         70         80         90        100

 NC_002516.     GTCGACGCCG CCGTACAACC GGTGATGAAG GCCAATGACA TTCCGGGCCT

 AmpC1          GTCGACGCCG CCGTACAACC GGTGATGAAG GCCAATGACA TTCCGGGCCT

  AmpC18         GTCGACGCCG CCGTACAACC GGTGATGAAG GCCAATGACA TTCCGGGCCT

 AmpC22         GTCGACGCCG CCTTACAACC GGTGATGAAG GCCAATGACA TTCCGGGCCT

 AmpC92         GTCGACGCCG CCTTACAACC GGTGATGAAG GCCAATGACA TTCCGGGCCT


 AmpC96         GTCGACGCCG CCTTACAACC GGTGATGAAG GCCAATGACA TTCCGGGCCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
```

```
                    110        120        130        140        150
NC_002516.  GGCCGTAGCC ATCAGCCTGA AAGGAGAACC GCATTACTTC AGCTATGGGC
 AmpC1      GGCCGTAGCC ATCAGCCTGA AAGGAGAACC GCATTACTTC AGCTATGGGC
AmpC18      GGCCGTAGCC ATCAGCCTGA AAGGAGAACC GCATTACTTC AGCTATGGGC
AmpC22      GGCCGTAGCC ATCAGCCTGA AAGGAGAACC GCATTACTTC AGCTATGGGC
AmpC92      GGCCGTAGCC ATCAGCCTGA AAGGAGAACC GCATTACTTC AGCTATGGGC
 AmpC96     GGCCGTAGCC ATCAGCCTGA AAGGAGAACC GCATTACTTC AGCTATGGGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    160        170        180        190        200
NC_002516.    TGGCCTCGAA AGAGGACGGC CGCCGGGTGA CGCCGGAGAC CCTGTTCGAG
AmpC1         TGGCCTCGAA AGAGGACGGC CGCCGGGTGA CGCCGGAGAC CCTGTTCGAG
AmpC18        TGGCCTCGAA AGAGGACGGC CGCCGGGTGA CGCCGGAGAC CCTGTTCGAG
 AmpC22       TGGCCTCGAA AGAGGACGGC CGCCGGGTGA CGCCGGAGAC CCTGTTCGAG
  AmpC92      TGGCCTCGAA AGAGGACGGC CGCCGGGTGA CGCCGGAGAC CCTGTTCGAG
  AmpC96      TGGCCTCGAA AGAGGACGGC CGCCGGGTGA CGCCGGAGAC CCTGTTCGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    210        220        230        240        250
NC_002516.    ATCGGCTCGG TGAGCAAGAC CTTCACCGCC ACCCTCGCCG GCTATGCCCT
 AmpC1        ATCGGCTCGG TGAGCAAGAC CTTCACCGTC ACCCTCGCCG GCTATGCCCT
AmpC18        ATCGGCTCGG TGAGCAAGAC CTTCACCGCC ACCCTCGCCG GCTATGCCCT
AmpC22        ATCGGCTCGG TGAGCAAGAC CTTCACCGCC ACCCTCGCCG GCTATGCCCT
 AmpC92       ATCGGCTCGG TGAGCAAGAC CTTCACCGTC ACCCTCGCCG GCTATGCCCT
 AmpC96       ATCGGCTCGG TGAGCAAGAC CTTCACCGTC ACCCTCGCCG GCTATGCCCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   260        270        280        290        300
NC_002516.  GACCCAGGAC AAGATGCGCC TCGACGACCG CGCCAGCCAG CACTGGCCGG
AmpC1       GGCCCAGGAC AAGATGCGCC TCGACGACCG CGCCAGCCAG CACTGGCCGG
AmpC18      GGCCCAGGAC AAGATGCGCC TCGACGACCG CGCCAGCCAG CACTGGCCGG
```

```
AmpC22        GACCCAGGAC AAGATGCGCC TCGACGACCG CGCCAGCCAG CACTGGCCGG

 AmpC92       GGCCCAGGAC AAGATGCGCC TCGACGACCG CGCCAGCCAG CACTGGCCGG

AmpC96        GGCCCAGGAC AAGATGCGCC TCGACGACCG CGCCAGCCAG CACTGGCCGG

                ....|....| ....|....| ....|....| ....|....| ....|....|

                       310        320        330        340        350

 NC_002516.  -CACTGCAGG GCAGCCGCTT CGACGGCATC AGCCTGCTCG ACCTCGCGAC

AmpC1         -CACTGCAGG GCAGCCGCTT CGACGGCATC AGCCTGCTCG ACCTCGCGAC

AmpC18       -CGCTGCAGG GCAGCCGCTT CGACGGCATC AGCCTGCTCG ACCTCGCGAC

AmpC22       -CACTGCAGG GCAGCCGCTT CGACGGCATC AGCCTGCTCG ACCTCGCGAC

AmpC92       GCACTGCAGG GCAGCCGCTT CGACGGCATC AGCCTGCTCG ACCTCGCGAC

AmpC96       -CACTGCAGG GCAGCCGCTT CGACGGCATC AGCCTGCTCG ACCTCGCGAC

                ....|....| ....|....| ....|....| ....|....| ....|....|

                       360        370        380        390        400
NC_002516.    CTATACCGCC GGCGGCTTGC CGCTGCAGTT CCCCGACTCG GTGCAGAAGG

 AmpC1        CTATACCGCC GGCGGCTTGC CGCTGCAGTT CCCCGACTCG GTGCAGAAGG

 AmpC18       CTATACCGCC GGCGGCTTGC CGCTGCAGTT CCCCGACTCG GTGCAGAAGG

 AmpC22       CTATACCGCC GGCGGCTTGC CGCTGCAGTT CCCCGACTCG GTGCAGAAGG

 AmpC92       CTATACCGCC GGCGGCTTGC CGCTGCAGTT CCCCGACTCG GTGCAGAAGG

 AmpC96       CTATACCGCC GGCGGCTTGC CGCTGCAGTT CCCCGACTCG GTGCAGAAGG

                ....|....| ....|....| ....|....| ....|....| ....|....|

                       410        420        430        440        450

 NC_002516.  ACCAGGCACA GATCCGCGAC TACTACCGCC AGTGGCAGCC GACCTACGCG

 AmpC1       ACCAGGCACA GATCCGCGAC TACTACCGCC AGTGGCAGCC GACCTACGCG

 AmpC18      ACCAGGCACA GATCCGCGAC TACTACCGCC AGTGGCAGCC GACCTACGCG

 AmpC22      ACCAGGCACA GATCCGCGAC TACTACCGCC AGTGGCAGCC GACCTACGCG

 AmpC92      ACCAGGCACA GATCCGCGAC TACTACCGCC AGTGGCAGCC GACCTACGCG

 AmpC96      ACCAGGCACA GATCCGCGAC TACTACCGCC TGTGGCAGCC GACCTACGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
```

```
NC_002516.   CCGGGCAGCC AGCGCCTCTA TTCCAACCCG AGCATCGGCC TGTTCGGCTA
  AmpC1      CCGGGCAGCC AGCGCCTCTA TTCCAACCCG AGCATCGGCC TGTTCGGCTA
  AmpC18     CCGGGCAGTC AGCGCCTCTA TTCCAACCCG AGCATCGGCC TGTTCGGCTA
  AmpC22     CCGGGCAGCC AGCGCCTCTA TTCCAACCCG AGCATCGGCC TGTTCGGCTA
  AmpC92     CCGGGCAGCC AGCGCCTCTA TTCCAACCCG AGCATCGGCC TGTTCGGCTA
  AmpC96     CCGGGCAGCC AGCGCCTCTA TTCCAACCCG AGCATCGGCC TGTTCGGCTA

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   510        520        530        540        550
NC_002516.   TCTCGCCGCG CGCAGCCTGG GCCAGCCGTT CGAACGGCTC ATGGAGCAGC
AmpC1        TCTCGCCGCG CGCAGCCTGG GCCAGCCGTT CGAACGGCTC ATGGAGCAGC
AmpC18       TCTCGCCGCG CGCAGCCTGG GCCAGCCGTT CGAACGGCTC ATGGAGCAGC
AmpC22       TCTCGCCGCG CGCAGCCTGG GCCAGCCGTT CGAACGGCTC ATGGAGCAGC
AmpC92       TCTCGCCGCG CGCAGCCTGG GCCAGCCGTT CGAACGGCTC ATGGAGCAGC
AmpC96       TCTCGCCGCG CGCAGCCTGG GCCAGCCGTT CGAACGGCTC ATGGAGCAGC

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   560        570        580        590        600
 NC_002516.   AAGTGTTCCC GGCACTGGGC CTCGAACAGA CCCACCTCGA CGTGCCCGAG
 AmpC1        AACTGTTCCC GGCACTGGGC CTCGAACAGA CCCACCTCGA CGTGCCCGAG
 AmpC18       AATTGTTCCC GGCACTGGGC CTCGAACAGA CCCACCTCGA CGTGCCCGAG
 AmpC22       AAGTGTTCCC GGCACTGGGC CTCGAACAGA CCCACCTCGA CGTGCCCGAG
 AmpC92       AACTGTTCCC GGCACTGGGC CTCGAACAGA CCCACCTCGA CGTGCCCGAG
 AmpC96       AACTGTTCCC GGCACTGGGC CTCGAACAGA CCCACCTCGA CGTGCCCGAG

              ....|....| ....|....| ....|....| ....|....| ....|....|
                    610        620        630        640        650
 NC_002516.   GCGGCGCTGG CGCAGTACGC CCAGGGCTAT GGCAAGGACG ACCGCCCGCT
 AmpC1        GCGGCGCTGG CGCAGTACGC CCAGGGCTAC GGCAAGGACG ACCGCCCGCT
 AmpC18       GCGGCGCTGG CGCAGTACGC CCAGGGCTAC GGCAAGGACG ACCGCCCGCT
  AmpC22       GCGGCGCTGG CGCAGTACGC CCAGGGCTAC GGCAAGGACG ACCGCCCGCT
  AmpC92       GCGGCGCTGG CGCAGTACGC CCAGGGCTAC GGCAAGGACG ACCGCCCGCT
```

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AmpC96        GCGGCGCTGG CGCAGTACGC CCAGGGCTAC GGCAAGGACG ACCGCCCGCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
NC_002516.    ACGGGTCGGT CCCGGCCCGC TGGATGCCGA AGGCTACGGG GTGAAGACCA
AmpC1         ACGGGTCGGT CCCGGCCCGC TGGATGCCGA AGGCTACGGG GTGAAGACCA
AmpC18        ACGGGTCGGT CCCGGCCCGC TGGATGCCGA AGGCTACGGG GTGAAGACCA
AmpC22        ACGGGTCGGT CCCGGCCCGC TGGATGCCGA AGGCTACGGG GTGAAGACCA
AmpC92        ACGGGTCGGT CCCGGCCCGC TGGATGCCGA AGGCTACGGG GTGAAGACCA
AmpC96        ACGGGTCGGT CCCGGCCCGC TGGATGCCGA AGGCTACGGG GTGAAGACCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
NC_002516.    GCGCGGCCGA CCTGCTGCGC TT-CGTCGAT GCCAACCTGC ATCCGGAGCG
AmpC1         GCGCGGCCGA CCTGCTGCGC TT-CGTCGAT GCCAACCTGC ATCCGGAGCG
AmpC18        GCGCGGCCGA CCTGCTGCGC TTTCGTCGAT GCCAACCTGC ATCCGGAGCG
AmpC22        GCGCGGCCGA CCTGCTGCGC TT-CGTCGAT GCCAACCTGC ATCCGGAGCG
AmpC92        GCGCGGCCGA CCTGCTGCGC TT-CGTCGAT GCCAACCTGC ATCCGGAGCG
AmpC96        GCGCGGCCGA CCTGCTGCGC TT-CGTCGAT GCCAACCTGC ATCCGGAGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                     760        770        780        790        800
NC_002516.    CCTGGACAGG CCCTGGGCGC AGGCGCTCGA TGCCACCCAT CGCGGTTACT
AmpC1         CCTGGACAGG CCCTGGGCGC AGGCGCTCGA TGCCACCCAT CGCGGTTACT
AmpC18        CCTGGACAGG CCCTGGGCGC AGGCGCTCGA TGCCACCCAT CGCGGTTACT
AmpC22        CCTGGACAGG CCCTGGGCGC AGGCGCTCGA TGCCACTCAT CGCGGTTACT
AmpC92        CCTGGACAGG CCCTGGGCGC AGGCGCTCGA TGCCACCCAT CGCGGTTACT
AmpC96        CCTGGACAGG CCCTGGGCGC AGGCGCTCGA TGCCACCCAT CGCGGTTACT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
```

```
NC_002516.    ACAAGGTCGG CGACATGACC CAGGGCCTGG GCTGGGAAGC CTACGACTGG
 AmpC1        ACAAGGTCGG CGACATGACC CAGGGCCTGG GCTGGGAAGC CTACGACTGG
 AmpC18       ACAAGGTCGG CGACATGACC CAGGGCCTGG GCTGGGAAGC CTACGACTGG
 AmpC22       ACAAGGTCGG CGACATGACC CAGGGCCTGG GCTGGGAAGC CTACGACTGG
 AmpC92       ACAAGGTCGG CGACATGACC CAGGGCCTGG GCTGGGAAGC CTACGACTGG
 AmpC96       ACAAGGTCGG CGACATGACC CAGGGCCTGG GCTGGGAAGC CTACGACTGG

              ....|....| ....|....| ....|....| ....|....| ....|....|
                     860        870        880        890        900

NC_002516.   CCGATCTCCC TGAAGCGCCT GCAGGCCGGC AACTCGACGC CGATGGCGCT
 AmpC1       CCGATCTCCC TGAAGCGCCT GCAGGCCGGC AACTCGACGC CGATGGCGCT
 AmpC18      CCGATCTCCC TGAAGCGCCT GCAGGCCGGC AACTCGACGC CGATGGCGCT
 AmpC22      CCGATCTCCC TGAAGCGCCT GCAGGCCGGC AACTCGACGC CGATGGCGCT
 AmpC92      CCGATCTCCC TGAAGCGCCT GCAGGCCGGC AACTCGACGC CGATGGCGCT
 AmpC96      CCGATCTCCC TGAAGCGCCT GCAGGCCGGC AACTCGACGC CGATGGCGCT

            ....|....| ....|....| ....|....| ....|....| ....|....|
                  910        920        930        940        950
NC_002516.  GCAACCGCAC AGGATCGCCA GGCTGCCCGC GCCACAGGCG CTGGAGGGCC
AmpC1       GCAACCACAC AGGATCGCCA GGCTGCCCGC GCCACAGGCG CTGGAGGGCC
 AmpC18     GCAACCACAC AGGATCGCCA GGCTGCCCGC GCCACAGGCG CTGGAGGGCC
 AmpC22     GCAACCACAC AGGATCGCCA GGCTGCCCGC GCCACAGGCG C-GGAGGGCC
 AmpC92     GCAACCACAC AGGATCGCCA GGCTGCCCGC GCCACAGGCG CTGGAGGGCC
 AmpC96     GCAACCACAC AGGATCGCCA GGCTGCCCGC GCCACAGGCG C-GGAGGGCC

              ....|....| ....|....| ....|....| ....|....| ....|....|
                    960        970        980        990        1000

NC_002516.   AGCGCCTGCT GAACAAGACC GGTTCCACCA ACGGCTTCGG CGCCTACGTG
 AmpC1       AGCGCCTGCT GAACAAGACC GGCTCCACCA ACGGCTTCGG CGCCTACGTG
 AmpC18      AGCGCCTGCT GAACAAGACC GGCTCCACCA ACGGCTTCGG CGCCTACGTG
 AmpC22      AGCGCCTGCT GAACAAGACC GGCTCCACCA ACGGCTTCGG CGCCTACGTG
```

```
AmpC92          AGCGCCTGCT GAACAAGACC GGCTCCACCA ACGGCTTCGG CGCCTACGTG

AmpC96          AGCGCCTGCT GAACAAGACC GGCTCCACCA ACGGCTTCGG CGCCTACGTG

                ....|....| ....|....| ....|....| ....|....| ....
                     1010       1020       1030       1040

NC_002516.      GCGTTCGTCC CGGGCCGCGA CCTGGGCCTG GTGATCCTGG CCAA

AmpC1           GCGTTCGTCC CGGGCCGCGA CCTGGGCCTG GTGATCCTGG CCAA

AmpC18          GCGTTCGTCC CGGGCCGCGA CCTGGGCCTG GTGATCCTGG CCAA

AmpC22          GCGTTCGTCC CGGGCCGCGA CCTGGGCCTG GTGATCCTGG CCAA

AmpC92          GCGTTCGTCC CGGGCCGCGA CCTGGGCCTG GTGATCCTGG CCAA

AmpC96          GCGTTCGTCC CGGGCCGCGA CCTGGGCCTG GTGATCCTGG CCAA
```

Phylogenetic relationship of AmpC gene sequence

Different samples were originated from common ancestors and the most similar was found to be AmpC22 with the reference sample sequence extracted from NCBI(NC 002516.2)

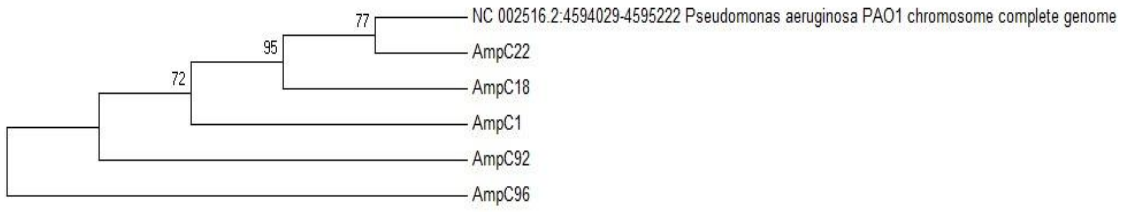

Figure 17 Tree diagram showing alignment between different samples of AmpC gene with the reference sequence

4.13.3 Multiple sequence alignment of blaNdm gene.

Out of 7, only one BlaNDM positive sample were sent for the sequencing and from the result obtained after sequencing were using for sequence analysis and for constructing phylogenetic tree. Refernence used was extracted from the NCBI (accession number: MF379684) was 99% identical. There was one substitution at postion 18(T/G) and insertion of G at postion 7, 794 and deletion of one nucleotide at 407. Phylogenetic tree was contructed with other reference sequence.

```
Query  1   ATGGAATTG-CCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGC  59
           ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct806   ATGGAATTGCCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGC 747

Query 60   ATTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGA 119
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 746  ATTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGA 687

Query 120  AACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCA 179
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 686  AACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCA  627
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Query 180 GCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGT 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 626 GCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGT 567

Query 240 CAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 566 CAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCA 507

Query 300 GATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCA 359
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 506 GATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCA 447

Query 360 CGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTA 419
          |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct 446 CGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGC-TGCGGCGGGGATTGCGACTTA 388

Query 420 TGCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAG 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
          TGCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAG 328

Query 480 CCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGGGCCCAACTTTGGCCCGCT 539
          |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct 327 CCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCT 268

Query 540 CAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGG5 99
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 267 CAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGG 208

Query 600 CACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGGAAGGACAAGTCGCTCGGCAA 659
          |||||||||||||||||||||||||||||||||||| |||| |||||||||||||||||
Sbjct 207 CACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAA 148

Query  660  ACTCGGAGATGCAGACACTGAG  681
             ||||| ||||| |||||||||
Sbjct  147  TCTCGGTGATGCCGACACTGAG  126
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Phylogenetic relationship of the NDM gene sequence

The NDM gene amplified showed the similarity to NDM varients extracted from NCBI, where MF379683 and MF379682 have similarity to the ancient type of NDM gene, while sample Ndm40 has same identity to the MF379684 (reference gene). However, these all NDM gene showed the same diversity.

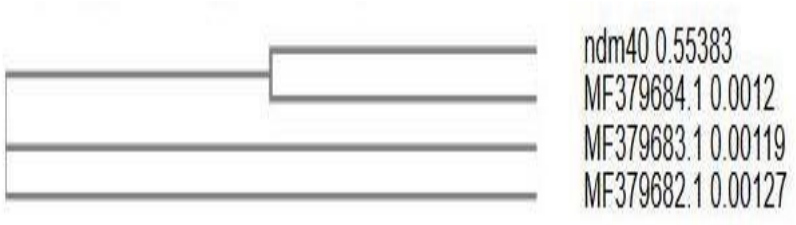

Figure 18 : tree diagram showing alignment between NDM gene samples with the reference sequence.

CHAPTER 5
DISCUSSION

P. aeruginosa is one of the opportunistic pathogens responsible for several infections like, urinary tract infections, respiratory infections, soft and wound tissue infections, and in immunocompromised patients with bacterimia as well as in patient with thermal injuries. *P. aeruginosa* shows resistance to many antibiotics due to various mechanisms such as presence of efflux pumps, outer membrane barriers and endogenous antimicrobial inactivation (Morita et al., 2014).

The choice of suitable antibiotics is hindered by their ability to develop resistance to multiple classes of antimicrobial drugs (Lister et al., 2009). Carbapenems –one of the potent beta lactam antibiotics which are used to treat serious *P. aeruginosa* infections. They are the last resort of drugs. Nevertheless, resistance to them have emerged more frequently and notably in past few years (Lautenbach et al., 2010).

5.1 Distribution of *P. aeruginosa* in various specimens

Our study included *P. aeruginosa* collected from Tribhuvan University Teaching Hospital (TUTH). The specimens from which the *P. aeruginosa* isolates were collected include sputum, pus, blood, urine and swab. Sputum was the major source from where 36 isolates, out of total – 95, were isolated. 31 isolates were obtained from the pus, 16 from swab, 8 from urine and 4 from the blood.

Similar study carried out at Department of Microbiology, Nepal Medical College and Teaching Hospital obtained highest number of *P. aeruginosa* isolates from urine and sputum – 37 each out of 102 specimens, followed by the 10 isolates each from pus and devices. Other sources taken were blood, renal stone and body fluids (Shrestha et al., 2016).This result might vary among hospitals , depending upon the flow ofinfected patients and the facilities at the hospital.

5.2 Antibiotic susceptibility test

In our study we used different class of antibiotics to study the resistance profile of *P. aeruginosa.* Antibiotic susceptibility was confirmed by disc diffusion technique, on Muller- Hinton Medium, the inhibition zone diameter breakpoints used for *P. aeruginosa* were those recommended in the Clinical and Laboratory Standards Institute.

Table 4 summarizes the contribution of different class of antibiotics. The isolates of *P. aeruginosa* found resistant to cefotaxime, ciprofloxacin, ceftazidime, gentamicin, meropenem and imipenem were 84.1%, 76.84%, 73.68%, 61.05%, 60% and 36.84% respectively. Similar results were obtained in the study carried out at Microbiology department, LSBK Memorial Government Medical College, Chattisgarh in 2016. Piperacillin tazobactam was found to be more sensitive - 93%, imipenem 91.41 %, ticarcillin + tazobactum 87%, meropenem 83.5%, levofloxacin 83%, amikacin 78%, piperacillin 75%, aztreonam 64%, gentamicin 53%, ceftazidime 52%, ciprofloxacin 51% and cefepime 48**%** (Yadav, 2017).

Thus, we can infer from this data that the meropenem and imipenem are more effective antibiotics for routine use against *P. aeruginosa* infections as compared to other antibiotics, and cefotaxime was still noted to have the highest resistance. Resistance among bacteria is a result of either overuse and misuse of antibiotics including prescription of antibiotics without establishing bacterial infection, and insufficient dosage. Moreover, antibiotic resistance can also be transferred horizontally between bacteria.

5.2.1 Prevalence of MDR *P. aeruginosa*

In our study, Multi-drug resistant isolates were defined as those resistant against at least three of the four following groups: (1) imipenem and meropenem; (2) ceftazidime and cefotaxime; (3) aminoglycosides and (4) ofloxacine and Among 95 isolates, 73 of them were found to be multidrug resistance *P. aeruginosa*. Thus, our study suggest an increase in MDR *P.aeruginosa* as compared to previous research conducted in 2013 by (Khanal et al., 2013), which In another case, central department of microbiology, Tribhuvan university in 2012 revealed that out of 1060 total samples from Sahid Gangalal Heart Center, Kathmandu, 700 of them were directly taken from patients of which 66 (9.43%)were *P. aeruginosa* and rest 360 samples were taken from the surface swab of which 60 (16.67%)were *P. aeruginosa* isolates. They also found the prevalence of MRDPA - 59(89.4%) in clinical samples while 7(11.7%) in swab samples (Bhandari et al, 2013).

The study of the prevalence of multidrug resistance bacteria has become current issue in Nepal. Various government and non government hospitals, research institutes and laboratories are conducting research on screening of multidrug resistance bacteria in clinical settings but most of them are limited to phenotypic test and only few works are carried out at the molecular level.

5.3 Detection of carbapenem resistance *P. aeruginosa*

Carbapenem is commonly used antibiotics which highly active against gram-positive and gram-negative bacteria including *P. aeruginosa*. However, *P. aeruginosa* possess various mechanisms to overcome the activity of the carbapenem - imipenem and meropenem. This study, out of 73 Multidrug resistance *P. aeruginosa* 61(83.56%) of them were screened out as carbapenem resistance isolates. Further they are grouped into three different types based on the carbapenem susceptibility patterns, they are (30) IMP^{R} MRP^{R}, (4) $IMP^{R}MRP^{S}$, (27) $IMP^{S}MRP^{R}$. This suggests that several mechanisms are concerned for providing resistant against carbapenem. In this study notable difference was observed where most isolates were resistance to meropenem and less to imipenem, this notable difference as compared to others studies may be due to less use of imipenem antibiotics in our antibiotic therapeutics.

Similar case was reported by retrospective study conducted in Departments of Nepalgunj Medical College and Teaching Hospital, Banke, Nepal, 2014 which showed that in 917 samples occurance of multidrug resistance was found to be 24.74%. 15 of

them were resistance to meropenem while none of them were found to be resistant to imipenem (Salman Khan et al, 2014).

5.4 Detection of MBL producer

Metallo-β-lactamases (MBL) are enzymes that belong to class B of Ambler's molecular classification and have their ability to hydrolyze different classes of antibiotics, including the carbapenems. Metallo-Beta-Lactamase (MBL), requires bivalent metal ions, usually zinc for their activity and thus, the activity of MBLs can be inhibited by chelating agents such as EDTA (ethylenediaminetetraacetic acid), which binds to zinc cations.

In this study, Combined Disc Test (CDT) was performed which was simple and accurate for the detection o MBL. Carbapenem disk alone and carbapenem in combination with EDTA were used. Table above showed that among 61 carbapenem resistant isolates around (90.1%)55 of them were MBL producer which prevalence was much higher than those reported in the previous study from Nepal where (30) 75% of MBL producing *P.aeruginosa* was obtained from 40 carbapenem resistance *P. aeruginosa* (Sthapit, 2017).

Similar study, Using combined disc test (meropenem combined with EDTA) has shows 88.46% of the MBL producer (Esther et al, 2017) in 2017.

In Nepal's scenario, prevalence of MBL producer has played major role in carbapenenem resistance. Prevalence of MBL producers from various geographical areas indicate their high frequency in worldwide. In Nepal, the first case of MBL producing *P. aeruginosa* was reported in 2012 (Tada et al., 2017)**.**

5.5 Detection of ESBL producer

Enterobacteriacae families are the most frequently isolated, ESBL – producing strain in the world as compared to ESBL producing *P.aeruginosa* strains. However in recent year, ESBL producing *P.aeruginosa* has been detected and increasingly reported in the most part of the world (Potron et al., 2015). Most of the laboratories fail to detect ESBL producing *P. aeruginosa* as a result, prevalence of ESBL producing strains is higher than currently reported. Also, ESBL enzyme, produced by the *P. aeruginosa* are different from the types of ESBL enzyme produced by the Enterobacteriaceae. PER-1, VEB-1 is reported in *P. aeruginosa* while other SHV, TEM, CTX-M etc are least prevalent. Moreover, the level of AmpC production by *P. aeruginosa* isolates may interfere with or even hide the detection of ESBLs by phenotypic tests (Jiang et al., 2006; Weldhagen et al, 2003).

This results in false susceptibility in routine laboratory testing in spite of enzyme production, prolonged illness and prolonged hospitalization might ensue. CLSI donot identify the ESBL strain as resistant by disc diffusion method rather they are screened and subequently confirmed for ESBL production. There is no recommendation made by CLSI for the detection of ESBL producing *P. aeruginosa* till date. So, in our study we employed the same criteria that were used for Enterobacteriaceae (CLSI, 2015) to detect

Prevalence of ESBL producing *P. aeruginosa*, as the principle remains the same. CLSI 2015 has recommended the use of any of the following antibiotics for screening for ESBL producers. It includes antibiotic discs of ceftazidime, aztreonam, cefotaxime and ceftriaxone (Nithyalakshmi et al, 2016)**.** In our study all MDR isolates of *P. aerugisona* were screened as potential ESBL producer using Ceftazidime 30 mcg and (61.45%) 45 of them were confirmed as ESBL producer by using Ceftazidime alone and ceftazidime – clavulanic acid. Compared to some earlier studies done in Nepal 2016, which was found to be (33.1%) out of 178 isolates (Ansari et al., 2016), our study showed higher prevalence of ESBL-producing *P. aeruginosa.* Similar case of high prevalence of ESBL producer was observed in study carried by (Khanal et al., 2013).

5. 6 Detection of AmpC producer

None of the isolates were found to be cefoxitin resistant AmpC Producer. Similar result in case of Beta-lactamases were found in Nepalese hospital, where only one isolate of *E.coli* was found to be AmpC producer out of 75 gram negative isolates (Mishra et al, 2016). Only few studies have been carried out in AmpC beta lactamase in *P. aeruginosa* in the context of Nepal.

5.7 Preparation of genomic DNA

Genomic DNA extraction of all the 73 MDR positives isolates of *P. aeruginosa* was done by following the standard protocol of Nishiguchi et al. After genomic extraction, the genomic DNA were run in 0.8% agarose gel with Ethidium bromide(EtBr) at 60 Volt for 45 minutes and visualized under UV transilluminator (Nishiguchi et al., 2002)

5.8 Pcr amplification of OprD gene.

Carbapenems are members of the β-lactam antibiotic class. Although carbapenems are used commonly to treat infections caused by *P. aeruginosa*, increased emergence of highcarbapenem resistant isolates have been observed worldwide. Several mechanisms are involved such as loss of OprD, outer membrane protein (Mishra et al., 2016)**.** In our study, 61 isolates were selected for PCR amplification of OprD gene which were resistant to carbapenem antibiotics – imipenem and meropenem. From these, 19(31.14%) showed negative results in pcr amplification which indicates that there was loss of OprD gene. 42 (68.86%) of them showed positive result in pcr amplification with the product size of 1332 bp indicating the presence OprD gene. Several studies have reported the loss of specific outer membrane porin protein in imipenem resistance but it w as also obsereved in low degree of resistance to meropenem. Whereas, in our study loss of OprD gene was observed both in imipenem and meropenem resistant isolates, and imipemem resistant isolates. OprD inactivation confers resistance to imipenem whereas, the mechanisms leading to meropenem resistance seem to be more complex and are very likely to be multifactorial, which involves overproduction of AmpC or overexpression of the efflux pumps such as MexAB-OprM, MexXY-OprM, and MexCD-OprJ (El Amin et al., 2005)**.**

A study on genetic analysis of laboratory derived mutants and clinical isolates has shown that the loss of OprD expression occur at the levels of transcription and translations**.** Mutations (base transitions or deletions) in OprD structural gene generate a premature stop codon and early termination of translation. Deletion also interfere with expression of OprD at the transcriptional level such as large deletion encompassing the promoter, initiation codon and putative Shine-Dalgarno sequence of OprD preventing transcription initiation was observed in study done by Yoneyama and Nakae(Yoneyama et al, 1993). Wolter et al have shown the first report of carbapenem resistance due to OprD gene occurring through insertional inactivation of the OprD gene by insertion sequence (IS) elements (Wolter et al, 2004).

5.9 PCR amplification of blaAmpC gene

Interplay between increased production of AmpC, reduced outer membrane porin OprD expression, and carbapenemase enzyme (MBL and ESBL) which are known to contribute to carbapenem resistance in *P. aeruginosa* (Qual et al, 2006; Tenover et al., 1995) **.** None of the isolates were phenotypically AmpC producer in our study, Therefore, genotypic detection of Ampc gene was essential for all 61 carbapenem resistant isolates, out of which 26 (42.62%) were found to be positive upon amplification. It seems that this is the first report of AmpC gene detection in *P. aeruginosa*, with high frequency in Nepal.

5.10 PCR amplification of blaNDM gene

Pcr amplification of 61 carbapenem resistant *P. aeruginosa* was done, out of which 7(11.4%) of them confirmed positive for blaNDM gene, which was quite comparable to the prevalence of (13.6%) bla NDM gene in *Acinetobacter baumannii* (Wolter et al., 2004)**.** In our study we observed that blaNDM encoding metallo betalactamase is not solely responsible in providing resistance, there might be the presence of other genes such as VIM, IMP, sometimes OXA for producing MBL in *P. aeruginosa*. Several studies demonstrate that, particularly VIM and IMP types are among the most widespread and globally reported carbapenemases. Another reason, for increasing number MBL producing isolates,the presence of bla NDM gene located in their plasmid and which can contribute to the acquisition of new resistance mechanisms (Cornaglia et al, 2011).

5.11 Sequencing of OprD gene, BlaAmpC gene, and BlaNDM gene

After the sequencing of the PCR positive product of OPRD, BlaAmpC, and blaNDM genes, the sequence chromatograms were analyzed using sequencher software and bioinformatics tools such as bioedit tools, NCBI BLAST, T-Coffee multiple sequence alignment tools and phylogenetic tree was constructed. Sequenced pcr products of OprD were found 97-99% homology to OprD gene of PAO1, and therefore it was used as reference sequence as for the futher studies. Various numbers of unusual mutations were observed as compared to the several other literatures related to the OprD gene in *P. aeruginosa*. Mutations include many insertions and deletions of single nucleotides. Large number of deletions was also observed in one of the sample (OprD sop42). This mutation can lead to mutational inactivation, premature termination and translations.

Such mutational changes in the OprD gene causes the conformational change which could lead to carbapenem resistance (H. Li et al., 2012).

Five of the sequenced samples showed the presence of BlaAmpC gene that codes for the AmpC beta lactamase. AmpC gene were also found in the Cefoxitin resistant non AmpC producer phenotypes. Sequence of blaAmpC gene was analyzed, which matched with AmpC gene of *P. aeruginosa* (Gene accession number: NC_002516.2) and hence used as reference sequence. We observed variation in the AmpC gene. PDC-1 variations are seen in the reference sequence. Most of the AmpC gene of the clinical isolates were identical to PA01 AmpC gene. Some of the chances in the nucleotide sequence were observed such as deletion and insertion and substitution. Mutations in β-Lactamase AmpC Increase (Berrazeg et al., 2015).

Multiple sequence aligment was done for blaNDM positive PCR product, which matched with the NDM-1 variants. While alignment was done using the sequence obtained from the NCBI (gene accession: MF37968). Similar study was performed in 2016, Central department of Biotechnology, Nepal. They obtained 3 nobel varients from the NDM-1 such as NDM-8, NDM12, NDM-13. Depending upon the type of mutation in the coding regions upon of the gene sequence have variable effect upon the resistivity pattern. The occurance of varients in, BlaAmpC and BlaNDM gene with the each passing years suggests that the genes are being rapidly spread among the clinical isolates within or even different species (Sthapit, 2017).

CHAPTER 6
SUMMARY

The Prevalence of multidrug resistance *P. aeruginosa* is drastically increasing and has become a global threat. In our study, many of the isolates were found resistant against multiple classes of drugs. Although this study includes carbapenem resistant and MDR isolates, which were also found susceptible to different class of antibiotics such as ceftazidime, indicating the importance of antimicrobial susceptibility testing in choosing of antibiotics for treatment of infections. These MDR–carbapenem resistance isolates were found to be not only the producer of MBL, but also the producer of other enzymes such as ESBL that mediate resistance to wide category of antibiotics including cephalosporin and carbapenem. However, none of the isolates were confirmed as Ampc producer in our study by phenotypic method. Among all the possible carbapenem resistant mechanism - loss of OprD porin, over expression of efflux pump system, production of betalactamase including ESBL, MBL and AmpC are the major contributors. Our study evaluates such resistance coding gene in clinical *P. aeruginosa* isolates by using PCR. Pcr amplification for the OprD gene in carbapenem resistance isolates showed lack of OprD gene in some cases indicating OprD is responsible for outer membrane permeability mostly for antibiotics like carbapenem. While Sequence analysis of the OprD gene revealed that there are small and large fragment deletions, insertions and duplications. This is responsible for, frame shifts in the OprD coding sequence as well as substitutions, resulting in mutational inactivation in OprD gene leading to carbapenem resistance. Through this study we also found the presence of AmpC producer *P. aeruginosa* which was not observed in phenotypic method but were detected (in Carbapenem resistance isolates) by genetotypic method. This false positive result suggests that there are high chances of spreading of AmpC producer in the health care sectors and remain unnoticed. Sequence analysis of AmpC gene verifies the presence of ampc beta lactamase gene in carbapenem resistant isolates and is not solely responsible for carbapenem resistance. They have coupled with other mechanism specially loss of OprD porin leading to carbapenem resistance.

Another interesting finding in our study was the detection blaNDM-1 gene were found in MBL producing carbapenem resistance isolates of *P. aeruginosa*. From this study we came to a conclusion that the multidrug resistance genes coding for OPRD porin, AmpC enzyme, β lactamase (NDM) were found to coexist and interact in the same strain. They are the disturbing factors in the hospital settings in order to control infections. Their rapid spread and evolution of resistance gene has jepordized treatment option which has become even more critical. Therefore, it is necessary to know the prevalence of such pathogens and their mechanism involved in resistance before undergoing antibiotic therapy.

CHAPTER 7
CONCLUSION

According to WHO, P. aeruginosa is categorized as most critical group of multidrug resistance bacteria. They have emerged as serious threat to global health, causing severe infections and causing deaths. Eradication of such 'superbugs' is a worldwide concern since they are capable to resist multiple numbers of antibiotics including carbapenem- powerful drugs of all time through various mechanisms. Multidrug resistant-CRPA shows both intrinsic and acquired resistance mechanisms. Several studies on MDR-CRPA has reported that the broad spectrum resistance typically involves Intrinsic resistance mechanisms such as change in outer membrane permeability due to OprD porin loss, carbapenemase encoding gene, over expression of efflux pump and overproduction of AmpC enzyme. Detection of such carbapenem resistance gene is important for accurate and effective antibiotic therapies. In our study we found that most of the isolates were MDR-CRPA, which were highly sensitive to different classes of antibiotics including carbapenem, third generation of Cephalosporins, second generation Fluoroquinolones and first generation Aminoglycosides. In our samples MBL were the most frequent types of beta lactamase enzymes followed by the ESBL while none of the isolates produced AmpC enzymes. Resistance due to lack of OprD porins in carbapenem resistance isolates of *P. aeruginosa* were predominant. Genes coding for AmpC beta lactamase were found in many isolates which were phenotypically AmpC non producer. this Similarly, the blaNDM was also found in our study which was responsible for enzymetic mechanism of resistance. Such mechanism calls for the rapid detection of responsible gene for antibiotic resistance which has ended the hopes to control infection. Thus, findings of this work provide essential insight into development of effective strategies for national wide control and reducing the rate of untreatable infections in clinical settings. This study also urge for strict consideration method of prescriptions of antibiotics in order to implement antimicrobial stewardship.

APPENDIX

50X Tris Acetate- EDTA (TAE) buffer

242 g Tris base

57.1 ml Glacial Acetic acid

100 ml 0.5 M EDTA (pH 8.0)

Final volume1000 ml to be made with distilled water

LB medium (Luria Bertani Medium)- pH 7.2

Tryptone- 10 grams

Yeast Extract- 5grams

Sodium Chloride- 10grams

McFarland Standard Composition

McFarland standard No.	0.5
1.0% Barium chloride(ml)	0.05
1.0% sulfuric acid (ml)	9.95
Approx. cell density	1.5

Tris-EDTA

10mM Tris(pH 8.0)

1mM EDTA

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