PREVALENCE OF MULTI DRUG RESISTANT *Klebsiella pneumoniae* AND GENETIC ANALYSIS OF CARBAPENEM RESISTANT GENES

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By

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Declaration

I hereby declare that the thesis entitled **"PREVALENCE OF MULTI DRUG RESISTANT *Klebsiella pneumoniae* AND GENETIC ANALYSIS OF CARBAPENEM RESISTANT GENES"** is based on work carried out by me and that 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. Dr. Rajani Malla and Mr. Mitesh Shrestha. I will have no objection for availability of the thesis for photocopy and inter – library loan for the purpose of scholarly research.

Nutan Thakur

Roll No. BT 164

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Glossary Acronyms

µg	Microgram
µl	Microliter
µm	Micrometer
AFLP	Amplified Fragment Length Polymorphism
AMC	Amoxycillin – Clavulanic acid
ATCC	American Type Culture collection
BIMPs	Bacterial Integral Membrane proteins
CA	Clavulanic Acid
CAI	Community Acquired infection
CAZ	Ceftazidime
CDC	Center for Disease Control
CLSI	Clinical and Laboratory Standards Institute
CRE	Carbapenem Resistant Enterobacteriacae
CTX	Cefotaxime
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
ESBL	Extended Spectrum Beta- Lactamase
Fig	Figure
HAI	Hospital Acquired Infection
ICU	Intensive Care Unit
IPM	Imipenem
LPS	Lipopolysaccharide

MBL	Metallo Beta lactamase
MDR	Multi Drug Resistance
MEM	Meropenem
MHA	Mueller Hinton Agar
ml	Millimeter
MLST	Multi Locus Sequence Typing
mm	Millimeter
NA	Neutient Agar
NNIS	National Nosocomial Infection Surveillance system
No.	Number
OMPs	Outer Membrane Protein
PBPs	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PCR-ESI-MS	PCR- Electrospray Ionization Mass Spectrometry
PDR	Pan Drug Resistance
PFGE	Pulse Field Gel Electrophoresis
REP-PCR	Repetitive Extragenic Palindromic Polymerase Chain Reaction
RNA	Ribonucleic Acid
WHO	World Health Organization
ZOI	Zone of Inhibition
NHSN	National Healthcare Safety Network
RFLP	Restriction Fragment Length Polymorphism
KPC	*Klebsiella Pneumoniae* Carbapenemase
NDM	New Delhi Metallo Beta - Lactamase

OXA	Oxacillinase
ASM	American Society of Microbiology
bp	Base Pair
NAM	N-acetyl muramic acid
NAG	N-acetyl glucosamine
NNIS	National Nosocomial Infection Surveillance system

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ABSTRACT

One of the most common opportunistic enterobacteriaceae associated with community infection. Antimicrobial resistance is associated with high medical costs and has a significant impact on the effectiveness of antimicrobial agents that could be fatal and may increase mortality rates. In addition, multidrug resistance (MDR) could be menace in disease control by intensifying the possibility of spreading of resistant pathogens, due to, declining efficacy of treatment that may result in prolonged time of infection in patient and could be a potential threat.

According to World Health Organization's critical priority list, it has become essential to control multidrug resistant gram negative bacillus *Klebsiella pneumoniae* that is due to the evolution and rapid spread of plasmid encoded extended spectrum β – lactamases, Metallo – β – lactamase and other genes conferring cross-resistance to different antibiotics. Hence this study was carried out to analyze the frequency of resistance in *K. pneumoniae*. Total 61 *K. pneumoniae* isolates were identified from different clinical samples collected from Institute of Medicine (IOM), Microbiology Department between 1st of March and 16th of May 2016. The organisms were subcultured on nutrient agar and antibiogram assay was done by disk diffusion method. Further more, ESBL and MBL producers were detected by the double disk test and Imipenem – EDTA test respectively. ESBL and MBL producers carbapenem resistant were selected for the further analysis. The DNA was extracted by CTAB method and PCR was performed using primer sets designed using information present at NCBI database. The sequencing of the positive PCR result was done to determine their mobility among the isolates.

The incidence of infection caused by *K. pneumoniae* was high with respiratory tract infection followed by urinary tract infection and the frequency of sputum and urine were 40.9% and 26.22% respectively. More analyzed with antibiotic sensitivity test followed by test for extended spectrum β – lactamase (ESBL) and metallo – β – lactamase (MBL) producing strains according to the standard microbiological methods (CLSI). The study revealed that 73.77% *K.*

pneumoniae strains were MDR with 26.22% non – MDR. Majority of the strains showed resistance to third generations of cephalosporin. Usage of carbapenem drugs i.e. Imipenem, Meropenem and Etrapenem showed 77.6%, 57.4% and 71.5% sensitivity respectively. Among 40 *K. pneumoniae* isolates, 4.91% of *K. pneumoniae* were ESBL producer while 40.9% were MBL producer strains. About 20% of MDR *K. pneumoniae* showed resistance to amoxy-clavulinic acid (20/10 mcg) instead of resistance to Ceftazidime and carbapenem. These resistant strains could be amp'C' type because amp C are not inhibited by Clavulanic acid. The PCR analysis of *K. pneumoniae* isolates detected the presence of two types of carbapenemase enzymes including the most prevalent types including NDM – 1 and OXA – 48 genes. A total of 6 isolates were found to possess blaNDM gene while 3 had blaOXA gene. The sequence of genes showed mutations that could potentially lead to stronger carbapenemase activity.

These enzymes are frequently found on mobile genetic elements and have the potential to spread rapidly. As observed from the preliminary result of the study, preventing both transmission and infections has become significant public health concern and this study also highlights the necessity for important antimicrobial resistance management strategies.

Keywords: Carbapenemase, Extended spectrum β – lactamase, *Klebsiella pneumoniae*, Metallo–β – lactamase, Multi Drug Resistance.

CHAPTER I

INTRODUCTION

1.1 Background

1.1.1 Multidrug Resistance *Klebsiella pneumoniae*

Klebsiella pneumoniae is a gram negative lactose fermenting encapsulated opportunistic bacilli and gamma – proteobacterium of the family *enterobacteriaceae* that are disseminated in hospital surroundings, natural environments and normal flora of mouth, skin and intestines, responsible for most of community and hospital acquired infection that primarily affect immunocompromised patients (Nordmann *et al.,* 2009). Klebsiella has become one of the most significant pathogen in a hospital settings causing nosocomial and community acquired infection with outbreaks of 20% that is raising antibiotic resistance which could be a potential problem because of the carrier prevalence rate of 1 – 6% (Woldu *et al.,* 2016).

Multidrug resistance among gram – negative bacteria is defined as resistance to three or more of the following antimicrobials: Ceftazidime, Ciprofloxacin, Carbapenem, Gentamycin, Ampicillin/Sulbactam, or Piperacillin/Tazobactam (Kumarasamy *et al*., 2010).The growth of MDR is a natural event and due to increase rise in the number of immunocompromised conditions i.e. HIV-infection, diabetic patients, organ transplantation individuals, and severe burn patients whose body could be an easy target for hospital acquired infectious diseases. These avenues are the main contributing factors to promote spread of MDR (Tanwar *et al*., 2014). Especially gram negative bacteria have increased resistant to the current antibiotics and drug development programme needs to be carried out on a large scale to provide therapeutic coverage in the near future. Repeated courses of antimicrobial therapy are common in acutely ill and abuse of antibiotics benefits microbes to undergo mutations which help them to resist antibiotics and give rise to dominant resistant strains (Kumar, 2015).

K. pneumoniae have an elevated intensity of intrinsic resistance to antimicrobial agents and a capacity to develop into an even extensively drug resistant strain. The particularity are caused by selective pressure of mutations in chromosomal genes that causes the production of ESBL and Amp *C* hyper expression, decreased expression of porin protein, and over expression of efflux pumps. In addition, *K. pneumoniae* are able to gain other resistant

genes by horizontal transfer of mobile genetic elements coding for different class of carbapenemases because these genes could be disseminated horizontally in transfer of resistance determinants, ESBL and MBL have become a serious concern in hospitals worldwide. Such acquired ESBLs and MBLs include the KPC, OXA and IMP, VIM types SPM – 1, GIM – 1, SIM – 1, AIM – 1, KHM – 1, NDM – 1, and SID – 1 respectively. These genes are normally encoded in class 1 integrons along with other resistance determinants, such as the aminoglycoside modifying enzymes. The integrons are frequently located in plasmids or transposons that makes easier to transfer these genes in others. The spreading of such transferable elements contributes toward the global spread of resistance mechanism (Bushman, 2002).

1.1.2 Carbapenems

Carbapenems (Imipenem, Meropenem, Doripenem, and Ertapenem) are bactericidal β – lactam antibiotics that have an extremely broad spectrum in the activity. They are used against most of *enterobacteriaceae* including those that produce amp C β– lactamase and extended-spectrum β – lactamase (ESBL) (Halat, 2016). Much multidrug resistant hospital – acquired bacteria are sensitive only to carbapenems. However, expanded and unjustified use of carbapenems has resulted in emergence of carbapenem resistance as well.

The persistent exposure of bacterial strains to a multitude of β – lactams has induced active and constant production as well as evolution of β – lactamases in these bacteria, extending their activity even against the novel β – lactam antibiotics (Kumarasamy *et al.*,2010).The strains producing β – lactamase that hydrolyzes extended spectrum cephalosporins with an oxyimino side chain i.e. cefotaxime, ceftriaxone, cefepime, ceftazidime as well as monobactams and aztreonam, are called extended spectrum β-lactamases (ESBLs) producer and the β – lactamase is characterized by the ability to hydrolyzes all β-lactam antibiotics except resistance to the commercially available β – lactamase inhibitors (clavulanic acid and tazobactum). Among these enzymes that require a zinc cation for the hydrolysis of β – lactam rings and their activity is inhibited by EDTA, are called Metallo – β – lactamase (MBLs) (Pitout and Lauplan, 2008).

1.1.3 Resistance to Carbapenems

Carbapenemase (ESBL or MBL) are mainly transferable enzymes that hydrolyze carbapenems even at low level together with other β – Lactams and can be emerged through horizontal gene transfer. These are major public health danger which can increase the rate of mortality and decline the choice of appropriate antibiotic therapy. The highly mobile genetic elements of carbapenemase genes contribute to their rapid spread and frequent transfer of multiple other antibiotic resistance genes among bacteria. However, some of them are chromosomally encoded (Toleman *et al*., 2009). These mobile genetic elements frequently contain different resistance genes which lead to rapid evolution of MDR bacteria. There are two main molecular families of carbapenemases: serine carbapenemases, which is based on presence of serine in their active site and metallo – carbapenemases, which are class B of metallo – β – lactamases (MBLs) having at least one zinc atom at their active site. Serine carbapenemases are again classified into class A carbapenemase, class D oxacillinase and class C cephalosporinase ESBLs (Brink *et al.,* 2013).

Carbapenem Resistant *Enterobacteriaceae* (CRE) is a family of germs that are difficult to treat because they have high levels of resistance to antibiotics. *Klebsiella* species and *Escherichia coli* are examples of *enterobacteriaceae* that can become carbapenem-resistant. The carbapenemase enzymes found commonly are KPC (*Klebsiella pneumoniae* carbapenemase), IMP (first indication of mobile MBLs), VIM (Verona integron-encoded MBL), NDM (New Delhi Metallo-β-lactamase) and OXA (oxacillinase). These are enzymes that break down carbapenems and make them ineffective (Centers for Disease Control and Prevention, 2016).

New Delhi Metallo – β – lactamase (NDM-1) is one of the recent emerging carbapenemase among gram negative bacteria. It was first identified from *Klebsiella pneumoniae* isolate recovered from a patient at a hospital in New Delhi, India in 2008. It has been transferred to many species of *enterobacteriaceae* in various countries. NDM – 1 is most frequently identified in the Indian subcontinent, followed by the Balkans region and the Middle East, and is mainly associated with community-acquired infections (Nordmann *et al.,* 2012).

However, various mechanisms, other than KPC and NDM, may result in carbapenem resistance because of IMP, VIM, OXA genes are responsible. Carbapenem resistance can be

mediated by production of carbapenemases or by the combination of outer membrane porin expression disruption and production of various β – lactamases (Nordmann *et al*., 2009).

The “golden era” when modern drug saved life by antibiotic treatment is under serious threat. In 2013, the Centers for Disease Control and Prevention (CDC) released a milestone report on “Antibiotic Resistance Threats”. Carbapenem – resistant *enterobacteriaceae* were deemed to be of serious threat. CRE which includes *K. pneumoniae* and *Escherichia coli,* are resistant to almost all currently available antibiotics. Almost 50% of patients who develop blood stream infections with these organisms die from the infection. Carbapenem resistance is typically mediated by the production of β – lactamases, and patients with CRE infections are treated with last – resort antibiotics such as colistin. Carbapenem is generally used as "drug of last resort” i.e. fourth generation of antibiotics for gram negative bacterial infections. CRE also acts as the new "superbug". Tom Frieden (the head of the Center for Disease Control) has referred to CRE as "nightmare bacteria” (Alekshun and Levy, 2007).

The increasing prevalence rate of ESBL producing organisms is responsible for increased use of carbapenems. But extensive and unnecessary use of carbapenems has mediated the emergence of carbapenem resistant bacteria by production of carbapenem hydrolyzing enzyme, metallo – β – lactamase (Ye, 2012).

Carbapenem resistance in *K. pneumoniae* may be due to other causes also, that these include combinations of outer-membrane permeability loss, β – lactamase production and the production of metallo – β – lactamases, such as those of the IMP or VIM groups. With the exception of Greece, most countries have been observing spared with the widespread occurrence of IMP – or VIM – producing *K. pneumoniae* (Aktas, 2012).

Treatment of these multiple drug resistant organisms has become a deep scientific concern. Recently, a significant increase in the incidents of ESBL and MBL related infections has been observed throughout the globe (Oberoi, 2012).

1.2 Current trends

During the last decade, infection due to carbapenem resistant *Klebsiella pneumoniae* (CRKP) has been reported throughout the world, spreading from the United States in 2001. Rapid and global dissemination of CRKP is perceived as a gigantic alarm in hospital settings. It can cause various infections including primary bacteremia, urinary tract infections, pneumonia, intra-abdominal infections, and wound infections (Hackel *et al.,* 2013). The crude mortality rate of CRKP infections ranges from 30% to 44%. It increases remarkably to 71.9%, when in the case of bacteremia. As with most nosocomial pathogens, multiple – drug resistance offers natural selective advantage, which allows such organisms to persist as a normal flora as well as in the hospital environment, in which antibiotic usage is extensive. More specific to *K. pneumoniae* is its capacity to silently colonize patients. These silent carriers act as reservoirs for transmission that spread and cause outbreak. Horizontal transfer of antibiotic resistant genes is one of the most important mechanisms for the dissemination of multidrug resistance among bacteria. The transmission of plasmids which are highly diverse with respect to size, modes of replication and transcription, host ranges and genes also acts as one of the most efficient mode of gene transfer (Gupta *et al.,* 2015).

According to recent work done in Nepal, prevalence of MDR among gram-negative bacteria, 72 % were found to be MDR while only 7.3% were ESBL producers and around 20% were MBL producers. Regular and reliable evaluation of the prevalence, etiologic agents, and predisposing factors of different community and nosocomial infection is essential in developing countries like Nepal in order to reduce its devastation effects in immunocompromised patients. It is very essential to have a regular and routine practice of monitoring of ESBL producing clinical isolates in labor (Thapa *et al.,* 2016).

Patan hospital was challenged with identification of source and control of outbreak caused by *K. pneumoniae* in neonatal units. There was 55% *K. pneumoniae* isolates that were multidrug resistant and extended spectrum β – lactamase producers. These isolates showed 30% resistance to ertapenem and imipenem. Molecular characterization revealed that 22% carbapenem resistant due to NDM – 1 gene encoding β - lactamase enzyme. The infection outbreak was finally controlled by cleaning methods and identifying an intermittently leaking roof. There is very low treatment option for Infections with extended spectrum β – lactamase and metallo– β – lactamase producing *K. pneumoniae* (Amatya *et al.,* 2014).

A recent study done in Tribhuvan University Teaching hospital (TUTH) revealed that gram – negative bacteria were the major cause of MDR infection i.e. around 84 %. Metallo – β – lactamase (MBL) producer was found to be in range of 15 – 30% in different gram negative bacteria. MBL was detected by both double disk synergy test and combined disk methods. This data can be used as base – line information of this novel type of β – lactamase in contest of Nepal but these MBL producers were not confirmed by molecular identification of MBL genes (Pokhrel *et al.,* 2012).

A new metallo – β – lactamase i.e. NDM – 8 was reported in a multidrug-resistant *Escherichia coli* isolate, IOMTU11 (NCGM37), isolated from the respiratory tract of a patient in Nepal. The amino acid sequence of NDM-8 has substitutions at positions 130 (Asp to Gly) and 154 (Met to Leu) in comparison with NDM – 1. NDM – 8 showed enzymatic activities against β-lactams similar to those of NDM – 1 (Tada *et al.,* 2013).

The most recent and upsetting development is the rapid rise in OXA – 48, particularly in *K. pneumoniae.* OXA – 48 – producing *enterobacteriaceae* was first identified in Turkey in 2001, and then it has been reported from several countries in the Middle East, North Africa and Europe (Kilic *et al.*, 2015).

1.3 Hypothesis

The research work aims in understanding the prevalence of carbapenem resistant *Klebsiella pneumoniae* in tertiary hospital settings of Kathmandu.

Null Hypothesis: The samples collected of *K. pneumoniae* are not carbapenem resistant.

Alternative Hypothesis: There is emerging threat for the increased incidence of carbapenem resistant *K. pneumoniae*.

1.4 Objectives

1.4.1 General objective

Study the molecular mechanism prevalent among the carbapenem resistant *Klebsiella pneumoniae* in the Nepalese context.

1.4.2 Specific objective

- Perform the antibiogram assay and determination of the resistance pattern for *K. pneumoniae.*
- Detection of the ESBL and MBL producing isolates by double disk test.
- Identification of the carbapenem resistant strains among the ESBL and MBL producing isolates.
- Extraction of the genomic DNA from the isolates.
- PCR amplification of the gene encoding the carbapenemase using specific primers.
- Perform sequencing and analyze as well as annotate the responsible genes using bioinformatics tools
- Identification of novel mutations and registration into the database.

1.5 Rationale of the study

Antimicrobial resistance is associated with high mortality rates and high medical costs. MDR provokes obstruction in disease control by intensifying the possibility of spread of resistant pathogens, thus, diminishing the efficacy of treatment and resulting in prolonged time of infection for patient.

ESBL and MBL producing bacteria do not respond to even fourth generation cephalosporin *in vivo* although they are susceptible to *in vitro* test as well as they are associated with multidrug resistance. Most of the laboratories lack the facility to detect ESBL and MBL producing bacteria which results in treatment failure. Detection of ESBL and MBL producing strains of *K. pneumoniae* possess great significance to control many outbreaks in hospitals. An increased resistance to antibiotics has been reported in *K. pneumoniae*, due to the evolution and rapid spread of plasmid encoded extended spectrum β – lactamases and other genes conferring cross – resistance to additional antibiotics. This is one of the main

concerns due to the increasing cost of antibiotic treatment and the spread of multidrug resistance to more pathogenic microorganisms. This study was undertaken to analyze the extent of the problem, to identify the most prevalent MDR isolates of *K. pneumoniae*.

Although ESBL and MBL production has been reported in a variety of gram – negative rods, *K. pneumoniae* is one of the organisms most likely to produce ESBLs and MBLs. The frequency of ESBL and MBL production varies substantially from region to region and is increasing significantly due to transferable genes. This rapidly increase in prevalence of ESBL and MBL production among *enterobacteriaceae* is the result of selection pressure due to deregulated prescription and often misuse of broad-spectrum antibiotics, including cephalosporins, both in hospitals as well as in the community settings.

1.6 Scope of the study

Antibiotic resistance is a problem of deep scientific worry both in hospital and community settings. It causes many epidemiological outbreaks in hospitals so the rapid detection in clinical laboratories is essential for the potential recognition of antimicrobial resistant strains. MDR gram – negative rods are becoming an increasingly difficult problem in world wide. CRE is an emerging group of gram – negative bacilli that have become resistant to all or nearly all antibiotics and are causing healthcare – associated infection and significant morbidity and mortality.

Nowadays in medical practice, newer antibiotics have been used extensively that has resulted in emergence and rapid dissemination of resistant bacterial strains. The organism's intrinsic mutation in the gene is the main cause for emergence of resistant organisms. Besides this, the under use or misuse of the antibiotics, inadequate prescription by the physician and patient's self – prescribing habit could have been the contributing factors for the emergence of MDR organisms.

The *enterobacteriaceae* have become resistant to fourth – generation cephalosporin due to the production of ESBL and MBL, leading to difficulty in treatment. The resultant increased use of broad spectrum agents and carbapenems has contributed to resistance due to selection pressure and amplified the emergence of increasingly resistant organisms. Production of extended – spectrum β – lactamases (ESBLs) and metallo – β – lactamases

(MBL) are the significant resistance – mechanisms. These impede the antimicrobial treatment of infections caused by *enterobacteriaceae* and are serious threat to the currently available antibiotic weapons. The resilience of bacteria and their resistance mechanisms are of novel interest in the clinical setting, as antibiotic resistant bacteria are posing a challenge to health care professionals.

Carbapenem resistance has been observed in a number of isolates by routine drug susceptibility test done in microbiology laboratory but the magnitude of these problem and specific genes responsible for this resistance is not clear. This study aims in providing information to clinicians and microbiologists to understand the molecular spectrum of carbapenem resistance in *K. pneumoniae* in hospital samples of IOMTUTH, Kathmandu, Nepal.

It is therefore of crucial importance to detect ESBL and MBL producing strain for optimal treatment of patients and to control the dissemination of resistance. Unfortunately, there are few studies done at the molecular level for ESBL and MBL *K. pneumoniae* in Nepal.

CHAPTERII

LITERATURE REVIEW

2.1 *Klebsiella pneumoniae*

The genus *Klebsiella* refers to the non-motile, non-spore forming and lactose-fermenting, oxidase negative and gram – negative bacteria with a major polysaccharide capsule of extensive width which gives the colony that glistening and mucoid appearance on agar plates (Hawkey *et al.,* 1998). *Klebsiella* is rod shaped, 0.3 – 1 µm in diameter and 0.6 – 6 µmin length arranged singly, in pairs or in short chains. *Klebsiella* is facultative bacteria and the colonies appear large, mucoid, and red with diffusing red pigment on MacConkey agar indicating fermentation of glucose and acid production. *Klebsiella* are normal inhabitant of the intestinal tract of human and animal, soil, water and botanical environment (Grimont *et al.,* 2006).

2.2 Scientific classification (Schroeter, 1886; Trevisan, 1887)

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Klebsiella*

Species: *K.pneumoniae*

(Binomial name– *Klebsiella pneumoniae*)

2.3 Antibiotics

Small molecules those are naturally produced by of the microorganisms like fungi and certain species of bacteria having bactericidal or bacteriostatic properties are commonly referred as antibiotics and they are either semi- or fully synthetic compounds (Courvalin *et al*., 2006). Antibiotic was first discovered by the Scottish scientist Alexander Fleming in

1928. He observed a mold of *Penicillium notatum* produced a diffusible antibacterial agent which was later identified as penicillin, which inhibited the growth of *Staphylococcus aureus* (Lapage, 1946).

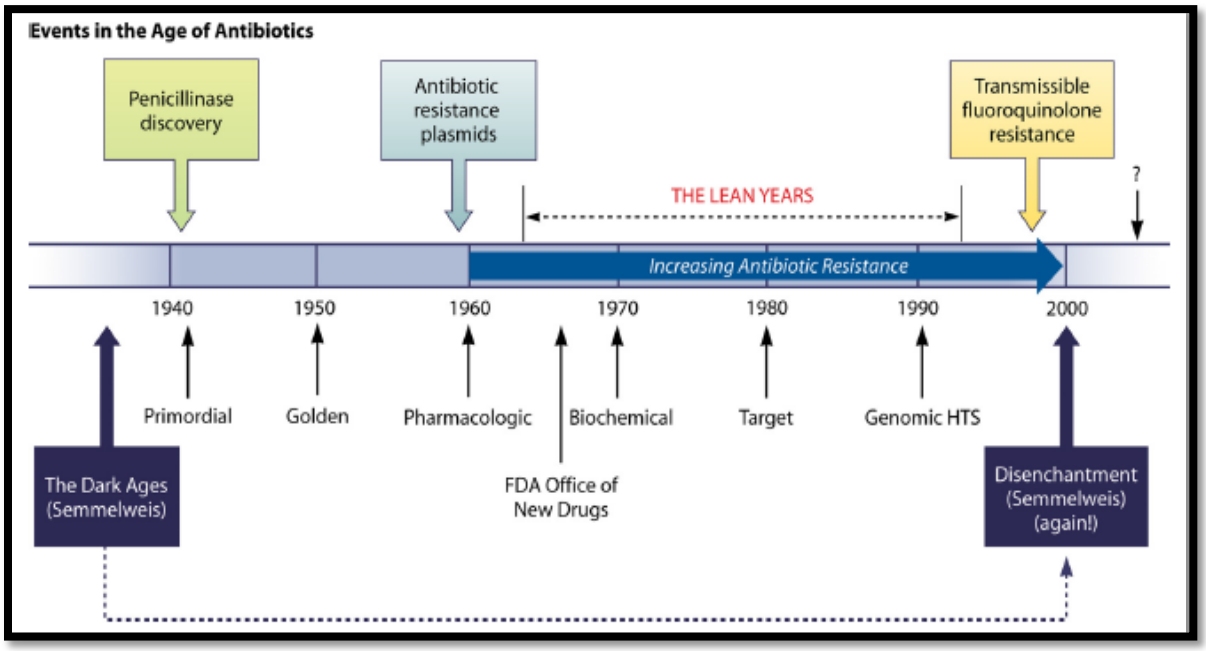

Figure 2.1: History of antibiotic innovation and associated development of antibiotic resistance (Adapted from Davies and Davies, 2010).

All different antibiotics consist of specific molecular structure which acts against the defined targets within the bacterial cells. These targets are usually the key steps of biochemical pathways in bacteria (nucleic acids replication, transcription and translation, protein synthesis, metabolism) or the structural components (cell wall and cytoplasmic membranes) (Shaikh *et al.,* 2015)

2.4 Classification of antimicrobials

The major families of antimicrobials are Beta-Lactams (Penicillin and Cephalosporin), Macrolides, Fluroquinolones, Tetracycline and Aminoglycosides (Moore, 2013).

2.4.1 β – Lactams

β – lactams are one of the most commonly prescribed for gram negative and gram positive bacteria as broad spectrum antimicrobials in human chemotherapy in the worldwide. They work against the cell wall biosynthesis of bacteria (Thompson *et al.*, 1993)**.** The oldest type of β – lactams is penicillin which have a common chemical structure as cephalosporin i.e. β–lactam ring. This antibiotic is generally bactericidal in nature e.g. penicillin, ceftazidime, carapenemetc (Barrons *et al.*, 1992).

2.4.2 Macrolides

Macrolides are bacteriostatic antibiotics that inhibit protein synthesis. These antibiotics are obtained from the Streptomyces bacterium. They all have a macrocyclic lactone chemical structure (Periti *et al.*, 1993)**.** Clarithromycin and azithromycin are used to treat respiratory tract infections because of their substantial ability to permeate the lung region e.g. azithromycin and erythromycin (Tenson *et al.,* 2003).

2.4.3 Fluoroquinolones

Fluoroquinolones belong to the family of quinolones (Briasoulis *et al.*, 2011).These antibiotics are synthetic antibiotics and are not derived from bacteria. They are broad-spectrum bacteriocidal antibiotics that are easily absorbed into the body. Fluoroquinolones act by inhibiting bacteria ability to produce DNA and thus can't reproduce e.g. ciprofloxacin and levofloxacin (Kushner *et al*, 2001).

2.4.4 Tetracycline

They are broad-spectrum bacteriostatic antibiotics. The chemical structure consists of four rings. They are derived from Streptomyces bacteria**.** Tetracyclines may be effective

against a wide variety of microorganisms, including rickettsia and amebic parasites e.g. tetracycline and doxycycline (Falagas *et al.*, 2008).

2.4.5 Aminoglycosides

Aminoglycosides are derived from *Streptomyces griseus*. They are bactericidal and inhibit the production of proteins (Selimoglu, 2007). This antibiotic is used against the gram-negative bacteria and can be used in combination with cephalosporin or penicillin (Lopez-Novoa *et al.*, 2011). Bacteria can easily become resistant to aminoglycosides e.g. amikacin and gentamicin.

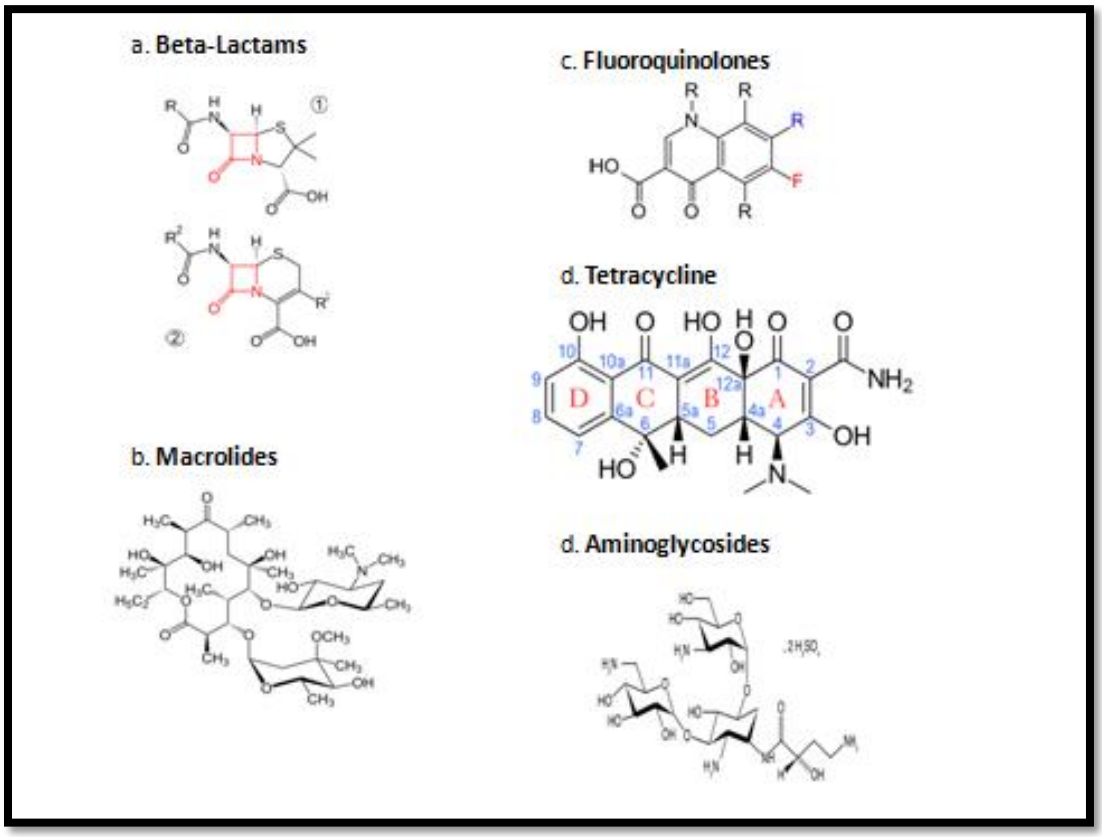

Figure 2.2: Chemical structures of the main groups of antimicrobials. (a) General structure of β – lactams (Penicillins & Cephalosporin). (b) General structure of Macrolides. (c) General structure of Fluoroquinolones. (d) General structure of tetracycline (e) General structure of Aminoglycosides

2.4.1.1 Structure and mechanism of action of β – lactams

The β – lactam antibiotic contains a hetero aromatic ring composed of two carbon atoms in addition to the third carbon in the carbonyl group. The β– lactam antibiotics can be divided into six groups i.e. penicillins, cephalosporins, carbapenems, cephamycins, monobactam and β– lactamase inhibitors (Neuhauser *et al.,* 2008). All of these compounds contain the β –lactam ring in their core structures.

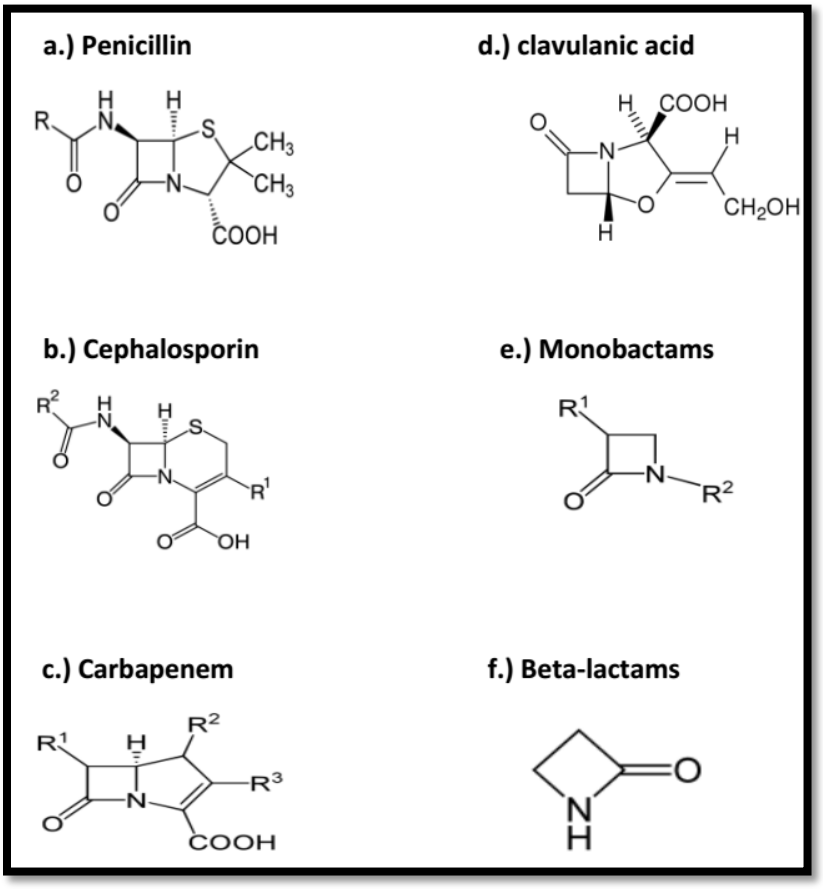

Figure 2.3: Chemical structures of the main groups of β-lactam family of antimicrobials. (a) General structure of penicillins. (b) General structure of cephalosporin. (c) General structure of carbapenem. (d) β – lactamase inhibitor (clavulanic acid) (e) Core structure of monobactams (a) β – lactams

Bacterial cell wall consists of peptidoglycan layer which is made of two types of disaccharides, the N – acetyl glucosamine (NAG) and the N – acetyl muramic acid (NAM). An individual peptidoglycan layer is a set of parallel glycan chains that are cross linked to each

other. The individual chains are made up of NAG and NAM linked through amide bonds in a 'head-to-tail' fashion. These amide bonds are produced in a transglycosylation reaction catalyzed by enzymes called penicillin binding proteins (PBPs) (Scheffers *et al.,* 2005).

β – Lactam antibiotics act on bacteria by inhibiting the bacterial enzymes, transpeptidases and carboxypeptidases, positioned in the cytoplasmic membrane which catalyses synthesis of the cross-linked peptidoglycan (Spratt, 1994). The cell wall of *K. pneumoniae* consists of an inner cytoplasmic membrane and outer layer consisting of lipopolysaccharides (LPS) and lipoproteins. A space between the inner cytoplasmic membrane and outer lipid membrane is called periplasm. This space contains a loose network of peptidoglycan. Gram – negative bacteria have thinner layer of peptidoglycan covered by a lipid bilayer outer membrane. The cross – linking is catalyzed outside the cytoplasmic membrane by a group of membrane – bound bacterial enzymes known as the cell-wall transpeptidases. Peptidoglycan is very important component of the bacterial cell wall. It protects the organism from osmotic rupture, determines cell shape, and is very essential for cell growth.

β – Lactam antibiotics are analogous of the terminal amino acid (D – alanyl – D – alanine) residues on the precursor NAM/NAG-peptide subunits of the peptidoglycan layer. In the presence of the β– lactam antibiotics, the transpeptidases and carboxypeptidases combine with acyl – D – alanyl – D – alanine to produce a lethal serine-ester-linked acyl (penicilloyl, cephalosporoyl) enzyme complex. The β– lactam enzyme complex is very stable, and blocks the normal transpeptidation process. This results in disruption of synthesis of the cell wall and makes the bacteria highly susceptible to cell lysis and death (Wilke, Lovering *et al.,* 2005).

2.4.1.2 Carbapenem

Carbapenems are a class of β – lactam, broad spectrum antibiotics which act by inhibiting the cell wall synthesis and are identified to be the most effective and last choice of drug against gram negative infection (Bradley, 1999). Carbapenem is a mainstay of therapy in patients with serious hospital acquired infection. Carbapenem can be grouped as:

- Group 1: It includes broad-spectrum carbapenem, with limited activity against non-fermentative gram negative bacilli, particularly suitable for community acquired infection (e.g. Ertapenem).

- Group 2: It includes broad spectrum carbapenem, with activity against non-fermentative gram negative bacilli that are particularly suitable for nosocomial infections (e.g. Imipenem and Meropenem).

2.5 Antimicrobial Resistance

Antimicrobial resistance is the acquired ability of the pathogens to withstand an antibiotic that kills off its sensitive counterparts. Usually resistance arises from random mutation in existing genes that already serves a similar purpose. Exposure to antibiotics and others antimicrobial products whether in the human body, in animals, or the environment, applies selective pressure that encourages resistance to emerge, favoring both 'naturally resistant strains' and which have 'acquired resistance'(Davies *et al.,* 2010).

Resistance is neither a new phenomenon nor unexpected in an environment in which potent antimicrobial agents are used. The diversity of the microbial world and the relatively specific activities of antimicrobial agents virtually ensure wide spread resistance among bacteria. Resistance as a clinical entity is essentially a relative phenomenon that exists as a gradient which reflects the phenotypic and genotypic variation in natural microbial population (Forbes *et al.,* 2008).

Multidrug resistance has been defined by various researchers and organization in different ways in different clinical settings. MDR strain is generally defined as the strain that shows resistance to two or more group of antibiotics among the six commonly prescribed drugs (CDC, 2006).

Other bacteria are called extensively drug resistant (XDR) and are susceptible to drugs in only one or two antimicrobial categories. Pandrug resistance (PDR) is defined as resistance to all agents in all currently available antimicrobial categories.

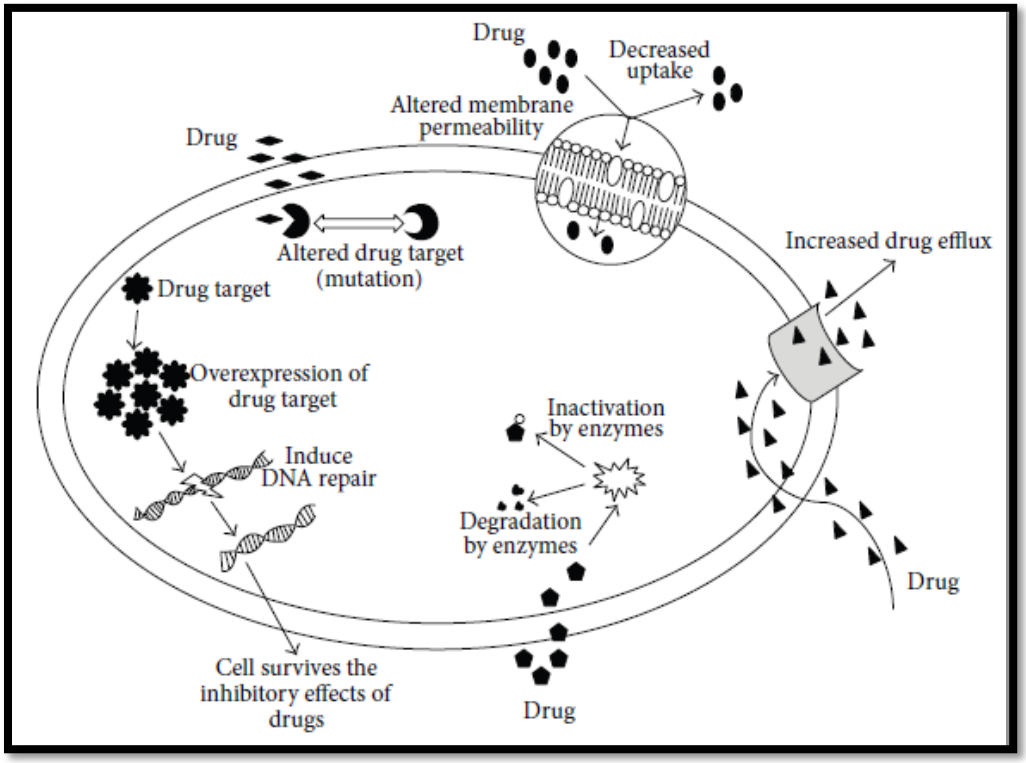

Figure 2.4 Modes of Antibiotic resistance (Tanwar *et al.,* 2014)

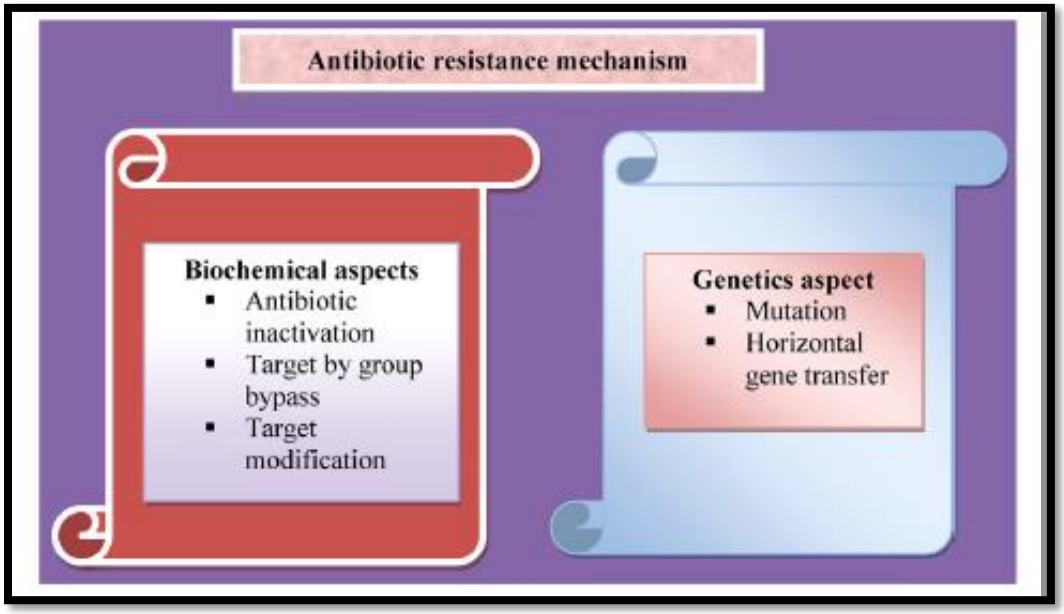

Figure 2.5: Biochemical and genetic aspects of antibiotic resistance mechanisms (Shaikh, 2015)

Resistance in microorganisms can be attributed to various mechanism either acting or in-combination. The various mode of mechanism are as following:

2.5.1 Environmentally mediated antimicrobial resistance

Environmentally mediated resistance is defined as resistance that results from physical or chemical characteristics of the environment that either directly modifies the antimicrobial agent or changes normal physiologic response to the drug of microorganisms. Examples of environmental factors include pH, anaerobic atmosphere, cation concentration and thymine dimer. Antibacterial activities of erythromycin and aminoglycosides diminish with decreasing pH while the activity of tetracycline decreases with increasing pH (Martinez, 2009). Aminoglycoside activity requires intracellular uptake across the cell membrane, much of which is driven by oxidative processes such that in the absence of oxygen, uptake and subsequently the antimicrobial activity are substantially diminished.

2.5.2 Microorganism mediated antimicrobial resistance

Microorganism mediated resistance refers to antimicrobial resistance that is due to genetic traits of the microorganisms and is the type of resistance that *in – vitro* susceptibility testing methods are targeted to detect. Organism based resistance can be categorized into intrinsic and acquired resistance.

2.5.3 Genetic basis of antimicrobial resistance

The genetic changes from antibiotic sensitivity to resistance may come about in bacteria by following modes:

2.5.3.1 Spontaneous mutation

The appearance of resistant microorganisms can be explained by the relatively infrequent occurrence (approximately 1 per 10^7 cells per cell division) of spontaneous gene mutations which confer drug resistance. There is a substantial number of biochemical mechanisms of antibiotic resistance that are based on mutational events, like the mutations in gene sequences encoding the target of certain antibiotics (e.g. resistance to rifampicin and fluoroquinolones is caused by mutations in the genes encoding the targets of these molecules, RpoB and DNA – topoisomerases, respectively) (Ruiz, 2003). The variation in the

expression of antibiotic uptake or efflux systems may also be modified by mutation (e.g. the reduced expression or absence of the Opr D porin of *Pseudomonas aeruginosa* reduces the permeability of the cell wall to carbapenems) (Kolayli, 2004).

2.5.3.2 Acquisition of resistance genes

A principal mechanism for the spread of antibiotic resistance is by horizontal gene transfer of resistance conferring genetic material. Resistance genes for most antibiotics exist in microbial world, either in the species that produces the antibiotic or within species that live in the same ecological niche of the antibiotics producers (Mosig, 2002). Bacteria acquire these resistant genes in various ways:

2.5.3.2.1 Conjugation

Conjugation is the physical contact between two genetically different bacterial cells of the same or closely related species. There is no exchange of genetic material during conjugation; only unilateral transfer (Capone, 1982). Genetic material that mediates resistance is most often transferred as plasmids or transposons. Resistance may, therefore, pass between species, including from commensals to pathogens, and vice – versa (Hawkey, 1998).

2.5.3.2.2 Transduction

Transduction is the transfer of genetic information between bacteria by phages. In the clinical setting, transduction may be more important in spreading resistance among gram-positive bacteria than gram negative bacteria.

2.5.3.2.3 Natural Transformation

In this process a free DNA molecule is transferred from a donor to a recipient bacterium. The DNA released from the donor cell upon cell lyses may be absorbed by competent cells and integrated into their genomes.

2.5.3.2.4 Transposons

Transposons are the non – replicative elements known to code for resistance to antibiotics. They encode their own ability to travel between replicons and sometimes even code for their conjugation allowing them to transfer within bacterial chromosomes.

2.5.3.2.5 Integrons

These are the potentially mobile elements (namely transposons or defective transposon derivative) that constitute a site specific recombination system capable of integrating and expressing the genes cassette structures (Davies *et al.,* 2010). The structure and gene capture mechanism is shown as:

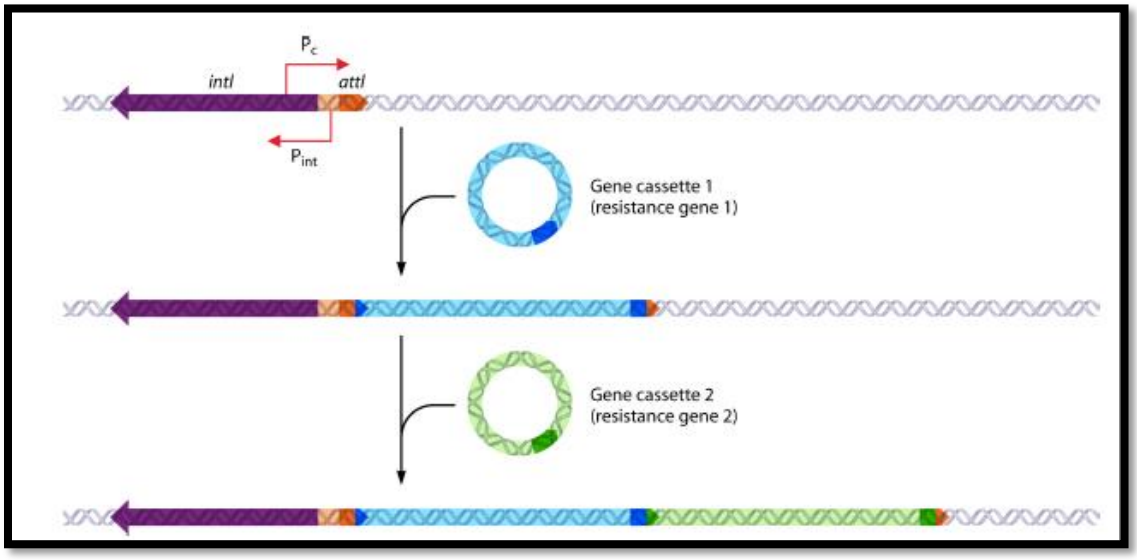

Figure 2.6 Integron structure and gene capture mechanism

2.5.4 Biochemical basis of antimicrobial resistance

The following are the important possible mechanisms by which cells might resist the toxic effects of growth inhibiting drugs:

2.5.4.1 Antibiotic inactivation

2.5.4.1.1 By hydrolysis

Several antibiotics have chemical bonds such as amides and esters which are hydrolytically prone. Numerous enzymes are known to damage antibiotic activity by targeting and cleaving these bonds. These enzymes can often be produced by the organism itself. Extended spectrum beta - lactamases (ESBLs) mediate resistance to all penicillins, third generation cephalosporins (e.g. ceftazidime, cefotaxime, and ceftriaxone) and aztreonam, but not to cephamycins (cefoxitin and cefotetan) and carbapenems (Sleeman *et al.,* 2004).

2.5.4.1.2 By redox process

The pathogenic bacteria infrequently mediate oxidation or reduction of antibiotics. However, there are a few examples of this strategy (Yang *et al.,* 2008). One is the oxidation of tetracycline antibiotics by the TetX enzyme.

2.5.4.1.3 By group transfer

The most diverse family of resistant enzymes is the group of transferases. These enzymes inactivate antibiotics (aminoglycosides, chloramphenicol, streptogramin, macrolides or rifampicin) by chemical substitution (adenylyl, phosphoryl or acetyl groups are added to the periphery of the antibiotic molecule). The modified antibiotics are impaired in their binding to a target. Chemical strategies include *O* – acetylation and *N* – acetylation, *O* – phosphorylation (Matsuoka and Sasaki, 2004), *O* – nucleotidylation (Brisson – Noel *et al.,* 1988), *O* – ribosylation, *O* – glycosylation, and thiol transfer. These covalent modification strategies all require a co – substrate such as ATP, acetyl – CoA, NAD^+, UDP – glucose, or glutathione for their activity and consequently these processes are restricted to the cytoplasm.

2.5.4.2 Antibiotic resistance via target modification

The second major resistance mechanism is the modification of the antibiotic target site so that the antibiotic loses its bind property. However, it is possible for mutational changes to

occur in the target that reduce susceptibility to inhibition while retaining cellular function (Spratt, 1994).

2.6 Spread of Antimicrobial Resistance

Antibiotics resistance arises by chance through mechanisms that may represent the legacy of natural competition among microorganism. The mechanism, genes, and pathways of antibiotics production and resistance help microorganism compete for niches in the nature therefore they are fundamental components of microbial life and represents normal evolutionary phenomena. Selection for antibiotics resistance takes place anywhere an antibiotic is present, may it be on skin, gut, other parts of human bodies or in the external environment. The factors playing role in increase/decrease of the prevalence of the resistant strains include

- Host and clone specificity
- Plasmid and clone specificity
- Virulence
- Interaction with other commensals
- Duration of the selection pressure and
- Virulence gene expression (WHO, 2004)

The emergence of antimicrobial resistant phenotype is inevitably linked to the clinical use of antimicrobial agents against which the resistance is directed. The two major reasons for this association are

- Not testing for resistance to antibiotics that are not in clinical use.
- Nature abhors vacuum, and so when an effective antimicrobial eliminate susceptible members of the flora, resistant varieties soon fills the niche.(Franco – Paredes, 2009)

Soon, new phenotypes with resistant genes spread and favored by the degree of resistance expressed, the ability of the organism to tolerate the resistance mechanism, linkage to the others genes, sites of primary colonization etc. The rapidity and completeness of the resistant gene spread are often unpredictable. Essentially any of the accessory genetic elements found in bacteria are capable of acquiring genes and promoting their

transmission. The type of element involved varies with the genus of the pathogen. These resistant elements are disseminated in the environments as shown in Fig. 2.7.

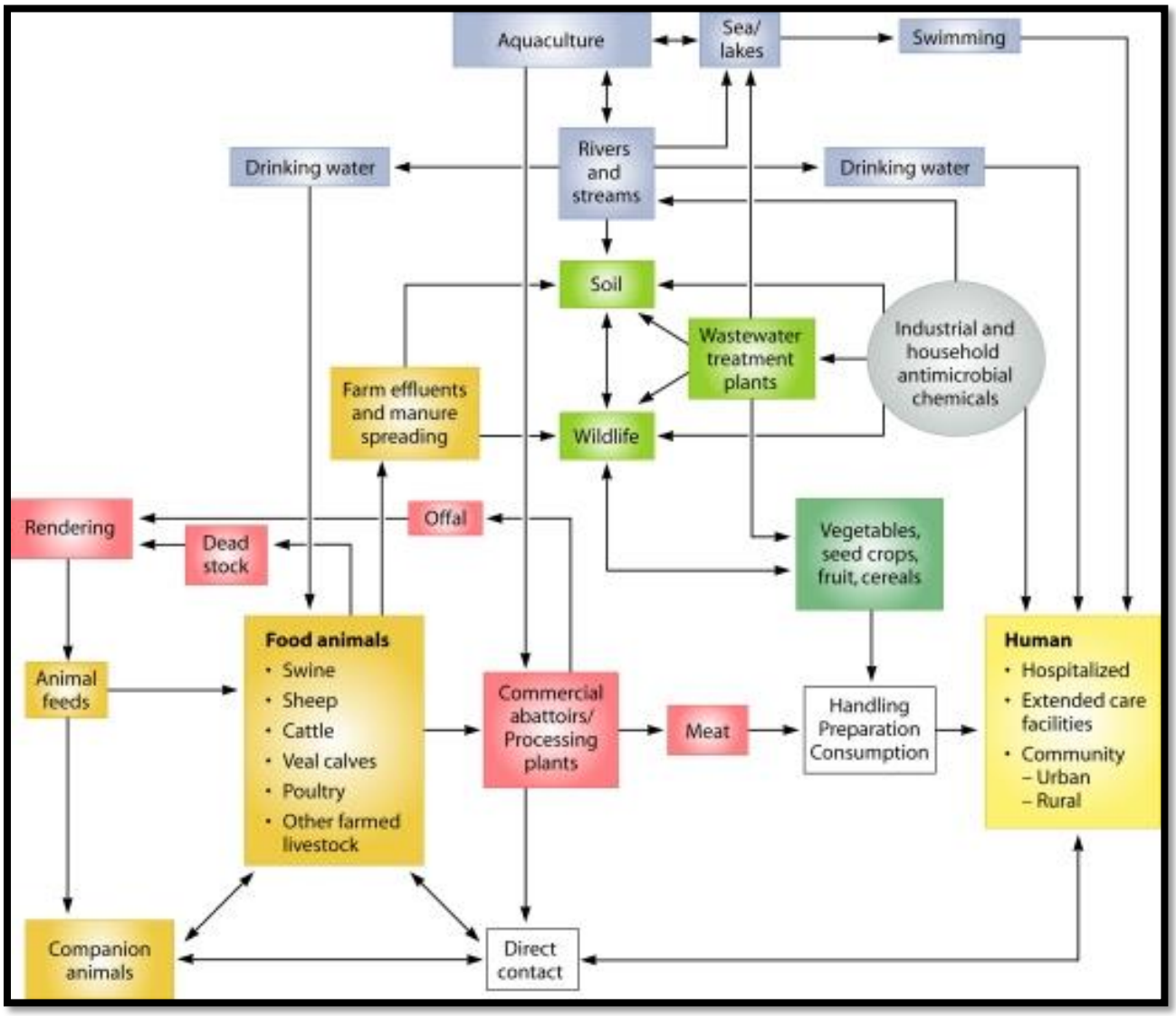

Figure 2.7: Dissemination of antibiotics and antibiotic resistance within agriculture, community, hospital, wastewater treatment and associated environments. (Doyle, 2006)

2.7 Mechanisms of resistance to β- lactam antibiotics

Bacteria avoid the bactericidal effect of β– lactam antibiotics by four major ways:

- Alteration of penicillin binding proteins (PBPs),
- Lack or diminished expression of outer membrane proteins (OMPs),

- Active efflux pumps promoting transport of antibiotic from within cell to the external environment resulting in an intermediate level of resistance, and
- Production of β– lactamases that hydrolyze β– lactam ring and render the antibiotic inactive before it reaches penicillin-binding protein (PBP) target.

2.7.1 PBPs modifications

PBPs are mainly divided into two subgroups: low molecular mass (LMM) and high molecular mass (HMM) enzymes. The HMM enzymes are further subdivided into bi – functional class A enzymes and mono-functional transpeptidase class B enzymes (Wilkem *et al.,* 2005). The penicillin-binding module contains three conserved motifs that form active cavity. They are the Ser – X – X – Lys (SXXK), the Ser – X – Asn (SXN), and the Lys – Thr/Ser – Gly (KT/SG) motifs (Hakenbeck, 1998).

PBPs, as the name suggest, have ability to bind penicillin as well as possess affinity to other β- lactam antimicrobials. There is a range of diverse PBP enzymes in the unrelated microorganisms.

The mode of action for β – lactam antimicrobials goes as follows: PBPs bind the β-lactam antimicrobials instead of their own substrates, D-alanyl D-alanine. However, the bond formed has a covalent nature resulting in inactivation of the PBPs. The cell wall synthesis is stalled and bacterium undergoes destruction during its growth or upon cell division. In other words, β- lactams are bactericidal to growing or dividing cells only. Efficacy of given β – lactam against the bacterium depends on ability of the compound to reach its target PBPs. In gram positive bacteria, cell envelope is composed of only one plasma membrane and thick outer cell wall comprised of many layers of peptidoglycan, hence the PBPs are more directly accessible for β – lactam antimicrobials. In gram negative bacteria (among others *enterobacteriaceae*) the cell envelope consists of the plasma membrane, the cell wall made up of a thinner peptidoglycan matrix and then the outer cellular membrane (Figure 2.8) (David, 1982).

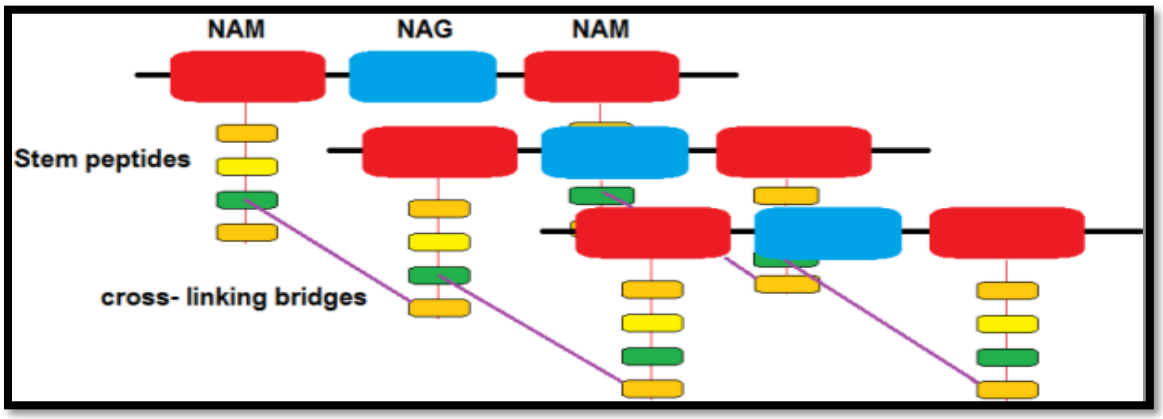

Figure 2.8 Schematic overview of the arrangement of cross – linked peptidoglycan chains within the bacterial cell wall (Foster, 2015).

There are several PBP mediated β– lactam resistance, including:

1. Point mutations altering an amino acid.
2. The acquisition of foreign PBP resistant to β– lactam antibiotics
3. Recombination between susceptible PBPs and those of less susceptible species. Recombination produce hybrid protein, presents slightly less susceptibility to β– lactams (Capone, 1982).
4. Over expression of a PBP is responsible for both natural insensitivity and acquired intrinsic resistance to penicillin in enterococci (Woodford, 2001).

2.7.2 Permeability – based resistance

The outer membrane of Gram negative bacteria plays an important role serving as a diffusion barrier for penetration of hydrophilic compounds. Gram positive bacteria the β– lactam can easily reach the cytoplasmic membrane, whereas in Gram negative bacteria crossing of outer membrane are essentially done through protein channels named as porins (Cohen, 2014).

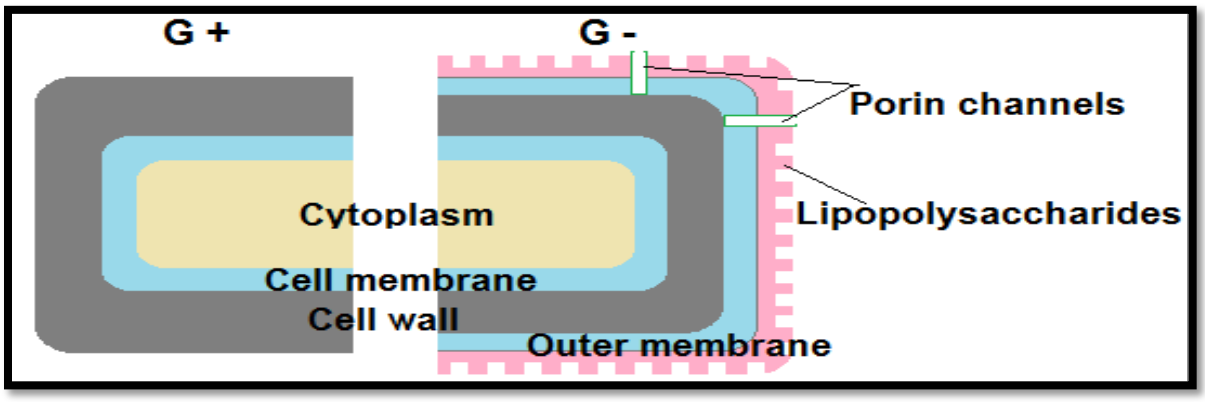

Figure 2.9 Schematic comparison of the cell envelopes in Gram positive and Gram negative bacteria (Hantke, 2005).

In *Klebsiella* spp. OmpK36, OmpK35 and OmpK34 are porins that permit the general diffusion of small polar molecules. A loss of either of these porins has been related to antibiotic resistance. *K. pneumoniae* strains usually express OmpK35 and OmpK36; the ESBL-producing strains commonly express only one of these, normally OmpK36, or no porin at all (Ghafourian, 2011).

2.7.3 Efflux pump

Efflux pump is third mechanism involved in resistance to β– lactam antibiotics. Multi drug resistance mediated by drug efflux pump remains the predominant mechanism of MDR. The over expression of genes encoding ATP – binding cassette (ABC) transporter membrane proteins (e.g., P – glycoprotein (Pgp)), also known as the multidrug efflux pumps that are responsible for the export or expulsion of drugs out of cell, usually generates MDR and continues cellular functions without any interference. Porin proteins transport the antibiotic from inner part of the cell to the external environment. One of the characteristic of efflux pumps is the variety of molecules they may transport, due to poor substrate specificity. Thus, this multidrug efflux system plays an important role in providing resistance to a very wide range of compounds in Gram-negative bacteria (Nikaido *et al.,* 2009).

Bacterial efflux systems generally fall into five classes,

- Major facilitator (MF) super family,
- ATP-binding cassette (ABC) family,

- Resistance-nodulation-division (RND) family,
- Small multidrug resistance (SMR) family
- Multidrug and toxic compound extrusion (MATE) family (Langton, 2005)

The resistance nodulation division (RND) is one of the multidrug efflux pumps. Examples of this super family include the AcrAB system of *E. coli*. The substrates of this family are diverse and include antibiotics, dyes, and detergents (Fernando *et al.,* 2013). However, members of the MFS, MATE and SMR families also show a limited ability to promote resistance to some biocides and antibiotics (Poole, 2004).

2.7.4 Enzyme production

The most common mechanism of resistance to β– lactam antibiotics is the production of β– lactamase enzymes. The β– lactamases confer significant antibiotic resistance to their bacterial hosts by hydrolysis of the amide bond of the β– lactam ring. Classes A, C and D include enzymes that hydrolyze their substrates by forming an acyl enzyme complex through an active site serine, where as class B β– lactamases are metallo - enzymes that utilize at least one active – site zinc ion. These enzymes are especially important in gram – negative bacteria as they constitute the major defence mechanism against β– lactam drugs (Lovering *et al.,* 2005).

There is no consensus on the precise definition of ESBLs. They are a group of enzymes that break down antibiotics belonging to the penicillin and cephalosporin groups that render them ineffective. ESBL has generally been defined as transmissible β – lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam and are encoded by genes that can be exchanged between bacteria. The currently most common genetic variant of ESBL is CTX – M (Carey and Bonomo 2005; Walsh, 2005).

ESBLs are grouped in two groups namely class A and class D ESBLs (Bush and Jacoby, 2010). ESBLs are most prevalent in *Klebsiella spp*. and their epidemiology shows a mixture of mutations, plasmid transfer and clonal spread. About 500 variants of the clavulanic acid – inhibited form (TEM, SHV, CTX-M, KPC, OXA) have been described worldwide. ESBL variants arise from point mutations in the blaTEM*,* blaSHV*,* or blaCTX genes resulting in alterations of

amino acid sequence of the enzyme. ESBLs gene harboring organisms are also resistant to other classes of antibiotics, such as aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and sulfonamide (Shahid *et al.,* 2011).

Table 2.1: β- lactamase classification schemes modified from (Bush and Jacoby, 2010)

Bush-Jacoby Group	Ambler Class	Main substrate	Inhibited by		Representative Enzymes
			CA/TZB	EDTA	
1	C	Cephalosporins	-	-	AmpC, P99,ACT – 1, CMY – 2, FOX – 1,MIR – 1 GC – 1, CMY – 37
1e	C	Cephalosporins	-	-	
2a	A	Penicillins	+	-	PC1
2b	A	Penicillins,earlyCephalosporins	+	-	TEM – 1, TEM – 2, SHV – 1, TEM – 3, SHV – 2, CTXMs, PER, VEB
2be	A	Extended-spectrum cephalosporins, monobactams	-	-	TEM – 30, SHV – 10
2br	A	penicillins	-	-	
2ber	A	Extended-spectrum cephalosporins, monobactams	-	-	TEM – 50 PSE – 1, CARB – 3 RTG – 4
2c	A	Carbencillin	+	-	
2ce	A	Carbencillin, cefepime	+	-	OXA – 1,OXA – 10
2d	D	Cloxacillin	V	-	OXA – 11, OXA – 15
2de	D	Extended-spectrum cephalosporins	V	-	OXA – 23, 0XA – 48

2df 2e 2f	D A A	Carbapenems Extended-spectrum cephalosporins Carbapenems	V + V	- - -	 CEPA KPC – 2, IMI – 1, SME – 1
3a (B1) 3b (B3)	B B	Carbapenems (B2) Carbapenems	- -	+	IMP – 1, VIM – 1,IND – 1, CcrAL1, CAU – 1, GOB – 1,FEZ – 1CphA, Sfh – 1
4	Unknown	-			

(V), variable, (+), Yes, (-), No, CA, Clavulanic acid, TZB, Tazobactam

2.8 Epidemiology of Carbapenem Resistant *K. pneumoniae*

Some features linked to the epidemiology of the carbapenemase producing strain are as follows:

1. The primary reservoir is very likely to be of concern because a specific enzyme will emerge in a certain geographical area where some favorable surroundings exist, such as a high – density population, poor hygiene, and high selective pressure due to overuse and misuse of antibiotics (Woldu, 2016).

2. The genetics of the carbapenemase gene (integron or transposon structures and plasmids) are prone to enhance gene plasticity and mobility this could subsequently favor horizontal gene transfer. The dissemination of carbapenemase gene depends on broad host ranges that will eventually amplified through replication. The emergence of one gene in a so – called thriving clone can favor the initial spread of a carbapenemase through the corresponding bacterial host.

3. The level of human population interactions once a reservoir has been constituted. However, the emergence of a carbapenemase occurs in a certain geographical region where

the population is movable (very commonly worldwide-related diaspora, tourism, or medical tourism), then the likely hood of seeing that resistance determinant emerging worldwide is high (Nordmann *et al.*, 2014).

The mortality rates from MDR *K. pneumoniae* infections range from about 25 to 50% (Gary *et al.,* 2006). Mortality rate rises as high as 50% and approach 100% in hospitalized, immunocompromised patient with diseases such as diabetes mellitus. Community acquired bacteremia usually caused by urinary tract infection, vascular catheter infection, and cholangitis (Gupta *et al.,* 2015). The *Klebsiella* species are second only to *E. coli* causing bacteremia representing 3 – 8% of all nosocomial bacterial infection (Bortlett *et al.*, 1998).

Since 1981, a characteristic syndrome of community – acquired *K. pneumoniae* septicemia with liver abscess has been reported in Taiwan. This syndrome is characterized by high mortality (10 to 40%) and some cases have been complicated by meningitis or endophthalmitis (Esther *et al.,* 2006). The disease has also been reported in North America and Europe. The symptoms of the disease characterized by fatigue, anorexia, nausea, diffuse abdominal discomfort, pleuritic chest pain, jaundice and fever (Kirsten *et al*., 2007).

Carbapenem Resistant *Enterobacteriaceae* (CRE) appears to have been uncommon in the United States before 1992. By analyzing data of the National Nosocomial Infection Surveillance (NNIS) system from 1986 to 1990, Gaynes *et al.* found that only 2.3% of 1825 Enterobacter isolates tested non – susceptible to Imipenem. However, over the last decade CRE have been reported more commonly. In the Meropenem Yearly Susceptibility Test Information Collection Program, Meropenem resistance among clinical isolates of *Klebsiella pneumoniae* was found to increase drastically from 0.6% in 2004 to 5.6% in 2008. Moreover according to the National Healthcare Safety Network (NHSN) in 2006–2007, carbapenem resistance was reported in up to 4.0% of *Escherichia coli* and 10.8% of *K. pneumoniae* isolates in certain device-associated infections (CDC, 2011).

2.9 Geographical distribution and mutational aspects of carbapenemase produced by Gram-negative bacilli worldwide

2.9.1 Class A Carbapenem – hydrolyzing β – lactamases

The main disseminating agent of *Klebsiella Pneumoniae* Carbapenemase is *Klebsiella pneumoniae* in the USA, Israel, Greece, and Italy. Recently KPC enzymes are at the moment the most clinically pivotal enzymes among the class A carbapenemases bacterial isolates.

KPC have been mainly known in *K. pneumonia*e. This pathogen is a common nosocomial pathogen and shows high levels of resistance not only to carbapenems but also to most β – lactams, including broad-spectrum cephalosporins (Poirel *et al.*, 2013).

The first KPC producer strain of *K. pneumoniae* was identified in 1996 on the eastern coast of the USA (Briasoulis *et al.*, 2011), and since then many variants have been identified. There are many KPC variant; all emerged from point – mutation derivatives of a common amino acid sequence. Nowadays, KPC producers are disseminated globally and have been recognized in many gram negative species. In Latin America, KPC producers are endemic in certain region, such as in Colombia and Argentina (Bonomo *et al.*, 2013). The endemic areas in Europe are Greece and Italy. In Israel, endemicity of KPC producers has been verified by many studies, with many cases of nosocomial infection, as well as some distinct cases occurring in the community acquired infection (Naas *et al.*, 2009). The degree of the distribution of KPC in Southeast Asia is not well identified; however, China is measured to be a country where some areas are facing endemic situation (Munoz – Pricea *et al.*, 2013).

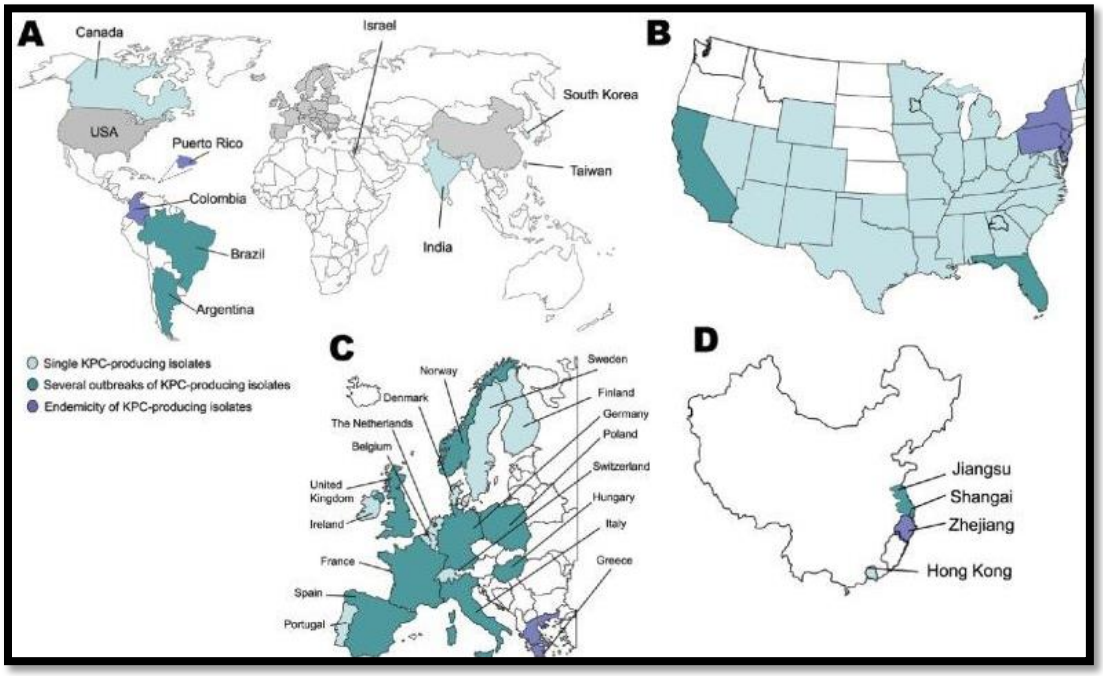

Figure 2.10 Distribution of KPC (CDC, 2011)

2.9.2 Class B Metallo – β – lactamases (MBLs)

MBLs are intrinsic in most of opportunistic bacterial species and have been recognized as acquired enzymes since the early 1990s, both in *Pseudomonas* or *enterobacteriaceae*. VIM and IMP are the most common group of acquired class B MBLs found in *enterobacteriaceae*,

together with the emerging NDM group. Although they have been reported worldwide, the VIM producers among *enterobacteriaceae* are highly prevalent in the Southern part of Europe and around the Mediterranean Sea that was first reported in Italy by Cornaglia *et al.* The IMP producers remain mostly located in Asia (Walsh *et al.*, 2005).

At present time, NDM – 1 (New Delhi metallo – β – lactamase) is clinically significant carbapenemase, which was identified in *K. pneumoniae* and *Escherichia coli* isolates in 2009 from a Swedish patient of Indian origin hospitalized in Orebro, Sweden, after a hospital stay in New Delhi (Poirel *et al.*, 2011). NDM – 1 has very little similar characteristics with other MBLs. The VIM – 1/VIM – 2 shares only 32.4% amino acid similarity. NDM – 1 efficiently hydrolyses a broad range of β – lactams, including penicillins, cephalosporins, and carbapenems, but sparing monobactams such as aztreonam (Giske *et al.*, 2009). Eight variants of NDM – 1 enzyme have been published (NDM – 1 to NDM – 8). Most of these enzymes have originated from Asia (Dahal *et al.*, 2013). When compared with NDM – 1, the NDM – 4, NDM – 5 and NDM – 7 variants have acquired increase hydrolyzing activity towards carbapenems (Prasad *et al.*, 2014). Recently 17 variants have been published i.e. NDM1 – 17. Only one substitution of nucleotide mainly at position 154 was common but in some variants two substitutions of nucleotides are also reported. In NDM – 2 and NDM – 3, Proline is substituted to Alanine at position 28 and Aspartate to Asparagine at position 95 respectively. In NDM – 10, there are maximum numbers of substitution mutation (Khan *et al.*, 2017). The antibiogram pattern analysis showed the orderly correlation with other antibiotic resistance determinants, such as plasmid mediated Amp C cephalosporinases, clavulanic acid – inhibited expanded – spectrum β – lactamases, and other types of carbapenemases (OXA-48, VIM and KPC types) (Woodford *et al.*, 2012). As a result, many of the NDM – 1 producer remain susceptible only to colistin, fosfomycin, and tigecycline (Rossolini *et al.*, 2011). The main well – known reservoir of NDM – producing *enterobacteriaceae* is the Indian subcontinent (Pakistan, India, and Sri Lanka) (Poirel *et al.,* 2011). The NDM producers have been widely found not only among patients from the Indian subcontinent, but also in the soil (Walsh *et al.*, 2011), hence, it is expected that the environment is already heavily contaminated with NDM producers. The prevalence of carriage is anticipated to be 5–15% in that region (Kazi *et al.*, 2013). Consequently, NDM producers among *enterobacteriaceae* have been reported in approximately all parts of world (Shaikh *et al.*, 2014). NDM producers are currently the most prevalent carbapenemase in European countries such as UK and France (Williamson *et al.*, 2012). The main reason behind the spread of NDM producer is intercontinental travel. Thus, the

recognition of NDM producers is thought to be not constantly related with an Indian sub continental origin (Zhang *et al.*, 2014).

K. pneumoniae is one of the major causes of hospital and community – acquired infections. Antibiotic resistance stirring in community settings is very difficult to control, and feces is the source of fast spread of NDM producers into the environment in Southeast Asia. It may therefore be expected that the outbreaks caused mostly by NDM – producing *K. pneumoniae* could be expected to be increasingly reported throughout the globe and, concomitantly, a slow but progressive increase in the prevalence rate of NDM – producing *K. pneumoniae* will be reported (Poirel *et al.*, 2013).

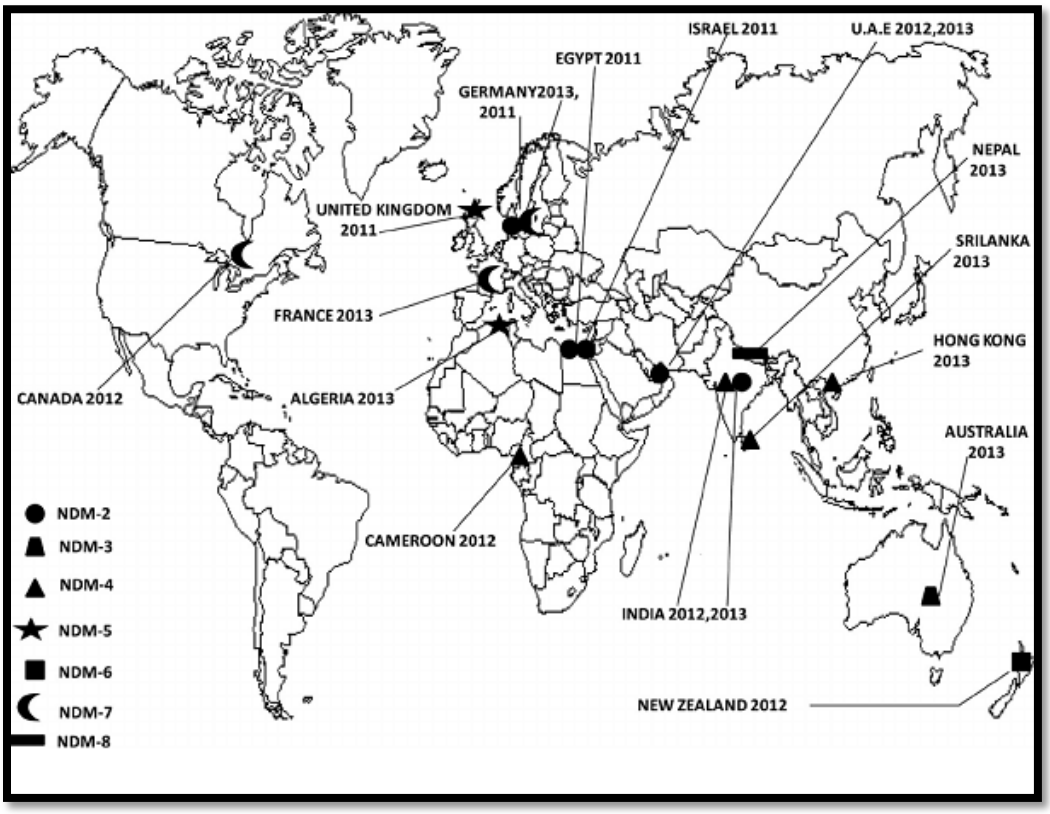

Figure 2.11 Distribution of NDM (Yang *et al.*, 2014)

Table 2.2: Genetic variations among the NDM – 1 and its variants (Mutation at various nucleotide positions leading to the occurrence of NDM variants) (Khan *et al.*, 2017)

NDM-1	Its variants (substitution of nucleotide at different nucleotide position)
NDM-2	Proline 28 to Alanine
NDM-3	Aspartate 95 to Asparagine
NDM-4	Methionine 154 to Leucine
NDM-5	Valine 88 to Leucine Methionine 154 to Leucine
NDM-6	Alanine 233 to Valine
NDM-7	Aspartate 130 to Asparagine Methionine 154 to Leucine
NDM-8	Aspartate 130 to Glycine Methionine 154 to Leucine
NDM-9	Glutamic Acid 152 to Lysine
NDM-10	Arginine 32 to Serine, Glycine 36 toAspartic acid, Glycine 69 to serine, Alanine 74 to threonine, Glycine 200 to Arginine
NDM-11	NA
NDM-12	Glycine 222 to Aspartic acid and Methionine 154 to Leucine
NDM-13	Aspartic acid 95 to Asparagine and Methionine 154 to Leucine
NDM-14	Aspartic acid 130 to Glycine
NDM-15	Alanine 233 to valine Methionine 154 to Leucine
NDM-16	Arginine 264 to Histidine
NDM-17	Valine 88 to Leucine, Methionine 154 to Leucine and Glutamic acid 170 to Lysine

2.9.3 Carbapenem – hydrolysing Class D β – lactamases

Oxacillinase (OXA) is class D β – lactamase. There are >400 variants of these enzymes but only some variants have actually carbapenemase activity (Nordmaan *et al.*, 2011). The OXA do not hydrolyze expanded – spectrum cephalosporins and possibly weak carbapenemase

activity, which does not confer high – level resistance to carbapenems if it is not associated with other factors, such as porin defect (Naas *et al.,* 2010). Even though the OXA variants have been identified in Acinetobacter, OXA – 48 and its derivatives have been also identified in *enterobacteriaceae*. The first OXA – 48 producers to be reported was *K. pneumoniae* isolated in Turkey in 2003. OXA-48 producers have been mainly reported in Turkey, often being the causes of nosocomial outbreaks (Poirel *et al.*, 2004). Nowadays OXA – 48 – producer have broadly spreaded throughout the European countries, and corresponds to North African countries (Aktas *et al.*, 2008). The OXA – 48 producing *K. pneumoniae* hospital outbreaks have been reported in many countries, including France, Germany, Switzerland, Spain, Netherlands, and the UK. The main factors behind the thriving spread of the OXA – 48 within a variety of enterobacterial species is the high transfer efficiency of the plasmid on which OXA – 48 gene is present (Woodford *et al.*, 2012). OXA – 48 producing isolates have been reported in the Middle East, in countries such as Lebanon, the Sultanate of Oman, Saudi Arabia, and Kuwait. The same OXA-48-producing *K. pneumoniae* isolate of sequence type 395 has been identified in Morocco, France, associated with other factors, such as permeability defects (Balkhy *et al.,* 2014).

It is remarkable that the recently identified incidence of OXA – 48 producers in Israel was confirmed to be linked with medical tourism (Briasoulis *et al.*, 2011). The OXA – 48 was considered to be almost completely absent from the Americas, but the works have shown the emergence of OXA – 48 – producing *K. pneumoniae* in the USA (Lascols *et al.*, 2013). There is a point – mutation in OXA – 48, named OXA – 181. It shares the same hydrolytic properties but the genetic structure surrounding blaOXA – 181 was found to be distinct from that associated with blaOXA – 48, indicating that the current disseminations are not related to each other (Potron *et al*, 2014). The OXA – 181 was been identified in enterobacterial isolates from India and from patients with relation with the Indian subcontinent is thought to have spread. The blaOXA – 181 genes has been identified in many different countries, such as France, the UK, Norway, Romania, the Sultanate of Oman, Canada, Australia, New Zealand, Singapore, and Sri Lanka, and a link with India has been systematically observed (Corcoran *et al.*, 2013).

The OXA – 204 has been reported in *K. pneumoniae* isolates recovered from patients having a connection with Algeria or Tunisia. OXA – 204 has two amino acid substitutions as

compared with OXA – 48, and with the substrate profile that is very similar to that of OXA – 48 (Potron *et al.*, 2013).

In addition OXA – 232 has been reported in *K. pneumoniae* isolates in France, from patients who had been traveled from Mauritius or India. Compared to OXA – 48, it has five amino acid substitutions but it is only point – mutant derivative of OXA – 181. However, OXA – 232 has weaker ability to hydrolyze carbapenems than others (Lando *et al.*, 2014).

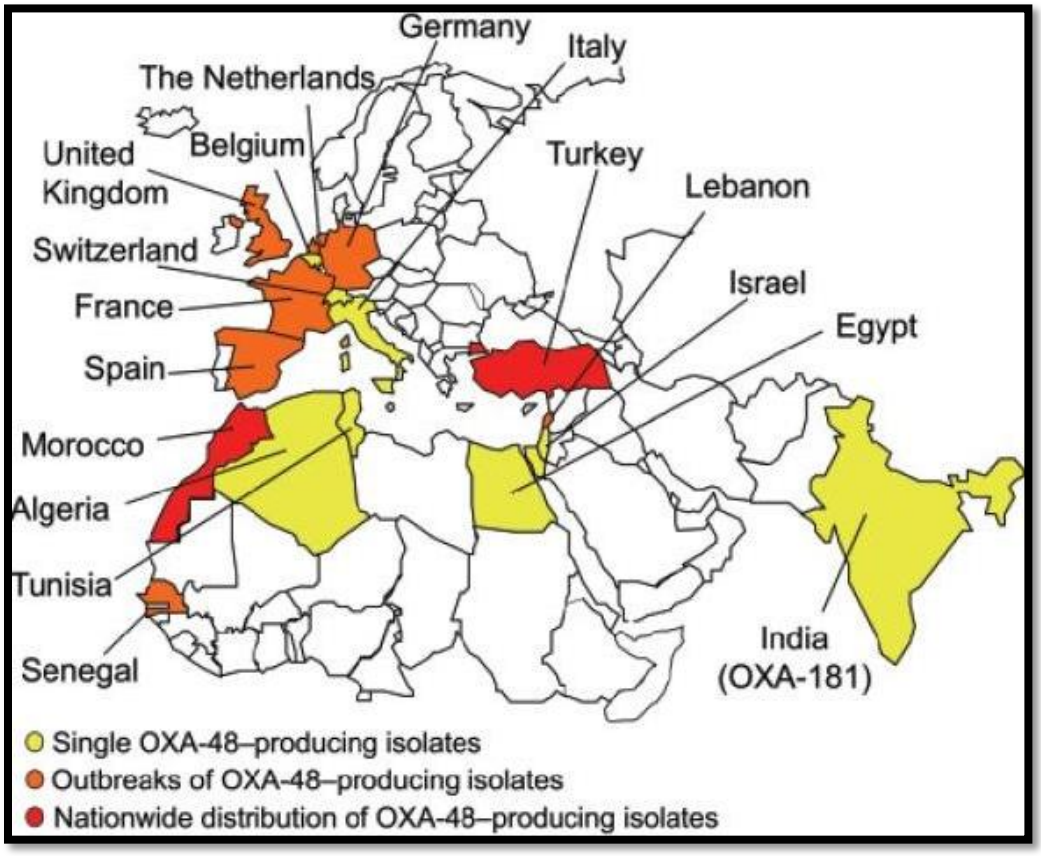

Figure 2.12 Distribution of OXA (CDC, 2011)

CHAPTER III

MATERIALS AND METHODS

3.1 Sampling Location

K. pneumoniae (n=61) were collected from Microbiology Department, Teaching Hospital, Maharajganj and further research activity were carried out at Central Department of Biotechnology, Tribhuvan University, Kirtipur.

3.2 Ethical consideration

Permission was taken from the central department of biotechnology. Oral consent was taken from the Head of Microbiology Department, Teaching Hospital, Maharajganj to obtain bacterial samples.

3.3 Sample under study

K. pneumoniae isolated from Clinical samples (blood, pus, sputum, throat swab, sterile fluids) was received for our study from Microbiology laboratory of Teaching hospital.

3.4 Time Period

The positive samples for *K. pneumoniae* were collected from Teaching hospital from1st March to 16th May 2016.

3.5 Transportation and preservation of isolates

K. pneumoniae was preserved in semi – solid media at temperature of 2 – 8^{o}C at Teaching hospital and the strains were collected in cold chain box and transferred to Central Department of Biotechnology and stored at 2 – 8^{o}C before start of the study. Glycerol stock was prepared for all samples.

3.6 Confirmation of *Klebsiella pneumoniae*

The most important task in bacteriology is to identify the pathogens from the clinical sample so that appropriate treatment can be instituted. Hence, a series of staining and biochemical tests were performed in order to identify the organism.

3.6.1 Staining reaction

Differential and special stains are necessary to bring out characteristics like: gram negative or gram positive bacteria.

Gram staining (Kaplan *et al.*, 1932)

A heat fixed bacterial smear was prepared and flooded with crystal violet for 1 minute, then washed with water. The smear was flooded with iodine for 1 minute, and then washed with water. The smear was decolourized with ethanol-acetone quickly and then washed with water. The smear was flooded with safranin for 1 minute, then wash with water. Finally the smear was blotted in air and observed under microscope.

Interpretation: Gram positive bacteria – violet and Gram negative bacteria – pink

3.6.2 Morphology of bacterial colony

Shape: circular, irregular, radiate or rhizoid.

Size: diameter in mm

Elevation: flat, raised, low convex, dome shaped

Margin: Entire, wavy, lobate, filiform

Surface: smooth, wavy, rough, granular, papillate, glistening etc.

3.6.3 Biochemical tests (Hansen *et al.*, 2004)

3.6.3.1 Indole test

Tryptone broth was inoculated with the test organism and incubated for 18 to 24 hrs at 37^{o}C. Fifteen drops of Kovac's reagent was poured down the inner wall of the tube.

Positive Result: Development of bright red color at the interface of the reagent and the broth within seconds after adding the reagent is indicative of the presence of Indole.

3.6.3.2 Methyl Red/Voges– Proskauer (MR/VP)

MR test

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

Positive Result: Red (indicating pH below 6), and

Negative Result: Yellow (indicating no acid production).

VP test

Pure culture of the test organism was inoculated into MR/VP broth and incubated for 24 hrs at 37^{o}C. One ml of the broth was aliquoted to a sterile test tube and 0.6ml of VP (A) was added followed by 0.2ml of VP (B) Shaked the tube gently to expose the medium to atmospheric oxygen and allowed the tube to remain undisturbed for 10 to 15 min.

Positive Result: Pinkish red color at the surface of the medium, and

Negative Result: Yellow color at the surface of the medium.

3.6.3.3 Citrate Utilization test

Inoculum was streaked over the slant of Simmon's citrate agar in a tube and incubated for 24 – 48 hrs.

Positive Result: Growth on the slant and change in color to blue of the medium.

3.6.3.4 Motility Test

Pure culture was stabbed motility media with inoculating needle.

Positive Result: If a bacterium is motile, there will be growth going out away from the stab line, and

Negative Result: If a bacterium is not motile, there will only be growth along the stab line.

3.6.3.5 Triple Sugar Iron Agar (TSI) test

With a straight inoculating wire, the top of a pure colony was touched. TSI was first stabbed through center of the medium to the bottom of the tube and then streaked the surface of the agar slant. The cap was left on loosely and incubated the tube for 18 – 24 hours at 37^{o}C in an incubator.

Results Interpretation:

1. Alkaline slant/no change in the butt (K/NC) = Glucose, lactose and sucrose non-utilizer (alkaline slant/alkaline butt)

2. Alkaline slant/acid butt (K/A) = Glucose fermentation only.

3. Acid slant/acid butt (A/A), with gas production = Glucose, sucrose, and/or lactose fermenter.

4. Alkaline slant/acid butt (K/A), H2S production = Glucose fermentation only.

3.6.3.6 Catalase test

This test demonstrates the presence of enzyme catalase in the organism. The enzyme catalase mediates the breakdown of hydrogen peroxide (H_2O_2) into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide (30% for slide test), and the rapid effervescence of O_2 bubbles occurs. The lack of catalase is indicated by a lack of bubble production.

$$2H_2O \longrightarrow 2H_2O + O_2 \text{ (gas bubbles)}$$

Positive Result: Rapid effervescence of O_2 bubbles occurs.

Negative Result: Lack of bubble production.

3.6.3.7 Oxidase test

Oxidase test is used to determine the presence of bacterial cytochrome oxidase enzyme using the oxidization of the substrate “tetramethyl – p – phenylene diamine dihydrochloride” to indophenol a dark purple colored end product.

Positive Result: Presence of oxidase indicated by the development of a dark purple colour.
Negative Result: No colour development indicates a negative test and the absence of the enzyme

3.6.3.8 Urea Hydrolysis tests:

Urea broth was inoculated with inoculating loop. The tube was incubated for 24 – 72 hours at 37°C. Urea broth is a yellow-orange color. The enzyme urease will be used to hydrolyze urea to make ammonia.

Positive Result: If ammonia is made, the broth turns a bright pink color, and is positive.

Negative Result: If test is negative, broth has no color change and no ammonia is made.

3.6.3.9 Nitrate reduction test

The test organism was inoculated into nitrate broth and incubated for 18 – 24 hours at 37°C. After incubation, alpha - naphthylamine and sulfanilic acid were added.

Positive Result: These two compounds react with nitrite and turn red in color, indicating presence of nitrite.

Negative Result: If there is no color change at this step, nitrite is absent.

3.7 Kirby - Bauer antibiotic sensitivity testing (CLSI, 2009)

The antibiotic sensitivity tests of all 61 strains of *K. pneumoniae* isolated from clinical specimen were tested for antimicrobial susceptibility by Kirby – Bauer method on Mueller – Hinton Agar (MHA) medium according to criteria recommended by Clinical and Laboratory Standards Institute (CLSI). Following antimicrobial agents (Hi – Media) were used for antibiotic susceptibility testing: Amikacin (30 µg), Ampicillin (10 µg), Cotrimoxazole (1.25/23.75 µg), Ertapenem (10 µg), Aztreonam (30 µg), Gatifloxacin (5 µg), Cefazolin (30 µg), Imipenem (10 µg), Cefotaxime (30 µg), Norfloxacin (10 µg), Ceftazidime (30 µg), Tetracycline (30 µg), and Ciprofloxacin (5 µg). The antibiotic discs were used of Himedia. Mueller Hinton agar was prepared at 4 mm deep, poured into Petri dishes. The MH agar plates were labeled for their respective sample number and also marked using dots, where the antibiotic disks will be kept. Then Inoculation was done with a broth culture diluted to match a 0.5 McFarland turbidity standard, which is roughly equivalent to 150 million cells per ml. Using an aseptic technique, a sterile swab was placed into the broth culture of a specific sample of *K. pneumoniae* and excess liquid was removed by gently pressing or

rotating the swab against the inside of the tube. The following pattern was used for swabbing:

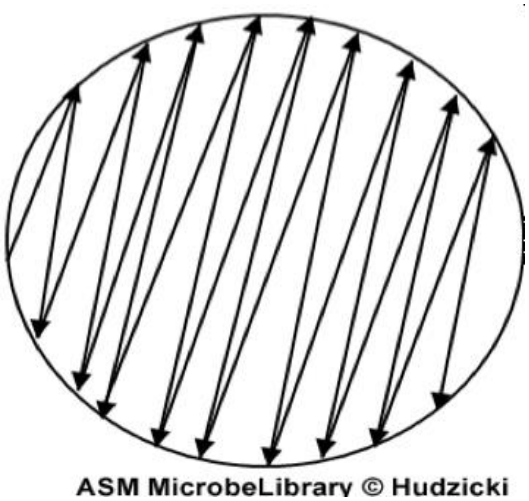

Figure 3.1 Swabbing Pattern (Adapted from Hudzicki, 2009)

The surface of the plate was swabbed thoroughly, making sure to cover the entire surface and the plate was turned approximately 60 degrees and the previous step was repeated (2nd swabbing) followed by further repetition (3rd swabbing).The swab was discarded in a bleach – containing beaker. One antibiotic disk was placed onto the surface of the agar using aseptic technique as follows: the tips of the forceps was heated by placing them just inside the opening of the Bunsen burner for 5 – 10 seconds and forceps was cooled by waving them in the air for about 10 seconds. Then, test disk was picked up carefully with the forceps and it was gently placed in the appropriate spot on the agar surface. The procedure was again repeated with the second antibiotic disk. The surface of the plates had completely dried (it may help to leave the lid slightly open for 3 – 5 minutes) and finally plates were incubated at 37^{o}Cfor 18 hours. After overnight incubation, the diameter of zone of inhibition (ZOI) of each disk was measured (including diameter of the disk) and recorded in millimeter. It was then compared with standard chart developed by Kirby – Bauer to determine bacterial susceptibility towards different antimicrobial agents in terms of 'sensitive', 'resistant' and moderately sensitive (intermediate). The measurement were made with a ruler on the under surface of the plate without opening the lid.

3.8 Detection of ESBL Production:

K. pneumoniae suspected to be producers of ESBLs enzymes were tested with the follow confirmation tests:

3.8.1 Screening test

The initial screening test for the production of ESBL was performed by using ceftazidime (CAZ) 30 µg and cefotaxime (CTX) 30 µg disks (Himedia, India). If the zone of inhibition were $\leq$17 – 22 mm for ceftazidime and $\leq$ 22 – 27 mm for cefotaxime, the isolate was considered as a potential ESBL producer as recommended by CLSI (CLSI, 2007). The organism was swabbed on to a MHA plate as done for screening test in antibiotic sensitivity test.

3.8.2 Double – Disc Test (DDT) (Pfaller *et al.*, 2006)

DDT permits to evaluate the inhibition of ESBL activity by Clavulanic acid. Discs containing cephalosporin (cefotaxime or ceftriaxone, ceftazidime, cefepime) are applied next to a disc with clavulanic acid, amoxicillin + clavulanic acid or ticarcillin + clavulanic acid. Positive result is indicated when the inhibition zones around any of the cephalosporin discs are augmented in the direction of the disc containing clavulanic acid. The distance between the discs is critical and 20 mm center to centre has been found to be optimal for cephalosporin 30 µg discs; however it may be reduced (15 mm) or expanded (30 mm) for strains with very high or low resistance level, respectively.

3.9 Detection of MBL Production (Franklin *et al.*, 2006)

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

I. Imipenem and Imipenem(IMP) + EDTA
II. Meropenem and Meropenem(MRP) + EDTA

Test organisms were inoculated onto Mueller Hinton agar plates as recommended by the CLSI. The test strain were suspended to the turbidity of the McFarland No. 0.5 tube and used to swab and inoculated in a Mueller Hinton agar plate. Two 10 µg Imipenem or Meropenem disks (Becton Dickinson) were placed on the plate, and appropriate amounts of

10 µL of ethylene diamine tetracetic acid (EDTA) (Sigma Chemicals, St. Louis, MO) solution were added to one of them to obtain the desired concentration (750 µg) of EDTA. The inhibition zones of the imipenem and Imipenem – EDTA disks were compared after 16 to 18 hrs of incubation at 37^{o}C. In the combined disc test, if the increase in inhibition zone with the Imipenem and EDTA disc was ≥ 7 mm than the Imipenem disc alone, it was considered as MBL positive.

3.10 Genomic DNA Extraction from Bacteria (Meade *et al.,* 1982)

Bacteria from a saturated liquid culture were lysed and proteins removed by digestion with proteinase K. Cell wall debris, polysaccharides, and remaining proteins were removed by selective precipitation with CTAB, and high molecular weight DNA was recovered from the resulting supernatant by using isopropanol precipitation. Five ml Lauryl broth was inoculated with the bacterial strain of interest and incubated at 37^{o}Cuntil the culture was saturated**.** From the primary culture, 1.5 ml of the culture was centrifuged at 12,000 rpm for 2 min. The supernatant was discarded and resuspended in 567 µl TE buffer by repeated pipetting. 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added. They are mixed thoroughly and incubated for 1 hour at 37^{o}C (Solution should become viscous as the detergent lyses the bacterial cell walls. One hundred µl of 5 M NaCl was added and mixed thoroughly. (This step is very important since a CTAB/Nucleic acid precipitate will form if salt concentration drops below about 0.5 M at room temperature (The aim here is to remove cell wall debris, denatured protein, and polysaccharides complexed to CTAB, while retaining the nucleic acids in solution. 80µl of CTAB/NaCl solution was added and mixed thoroughly and incubated for 10 min at 65^{o}C. An approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol was mixed thoroughly and centrifuged at 12,000 rpm for5 minutes (This extraction removes CTAB protein/polysaccharide complexes, a white interface should be visible after centrifugation). Aqueous supernatant was transferred to a fresh tube, leaving the interface behind and equal volume of Phenol – Chloroform – Isoamyl alcohol was added, mixed thoroughly and centrifuged in a microcentrifuge at 12,000 rpm for 5 minutes. The supernatant was transferred to a fresh tube and 0.6 times volume of isopropanol was mixed to precipitate the nucleic acids. The tube was shaken back and forth until a stringy white DNA precipitate becomes clearly visible and incubated in ice for 10 – 15 minutes. After ice incubation the microcentrifuge was centrifuged at 12,000 rpm for 12 minutes and the DNA was washed with 70% ethanol to remove residual CTAB and re – centrifuged at 12,000 rpm for 5 minutes at room temperature to re – pellet it. The

supernatant was removed carefully and the pellet was dried at 37℃ in incubator. Finally the pellet was re – dissolved in 50 µl TE buffer.

3.11 Gel electrophoresis

Agarose gel electrophoresis is an efficient technique to separate DNA molecules according to their molecular weights. Agarose (0.8 %) was dissolved in 100 ml 1 X TAE buffer and was boiled to dissolve completely. Ethidium bromide was added from stock solution of 10mg/ml at a concentration of 0.5 µg/ml. Gel was cooled down and poured onto a gel casting tray and allowed to set. DNA samples were loaded and electrophoresis was carried at a constant voltage of 100V. After 45 minutes, the gel was observed under BIO RAD Gel doc.

3.12 Polymerase Chain Reaction (Garibyan *et al.*, 2013)

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single copy of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The technique amplifies specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality. One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand.

Table No 3.1: Primer sequence and amplicon size

Gene	Primer	Sequence	Tm (°C)	Amplicon size(bp)
BlaNDM Internal	Forward primer	5’ – GGCCAGCAAATGGAAACTGG – 3’	59.4	460
	Reverse primer	5’ – AATACCTTGAGCGGGCCAAA – 3’	57.3	

BlaNDM Full length	Forward primer	5’ – AATGCTGAATAAAAGGAAAACT – 3’	50.9	869
	Reverse primer	5’ – GGCAGATTGGGGGTGA – 3’	54.3	
BlaOXA	Forward primer	5’ – GAATGCCTGCGGTAGCAAAG – 3’	59.4	192
	Reverse primer	5’ – GGGCGATCAAGCTATTGGGA – 3’	59.4	

Table 3.2 Composition of PCR reaction mixture using Master Mix (2X)

S. No.	Reagents	Volume (µL)
1.	NFW	9.5
2.	NEB Master mix	12.5
3.	Forward Primer	1.0
4.	Reverse Primer	1.0
5.	Template(100 ng)	1.0
Total		25

Table 3.3 Composition of PCR reaction mixture using individual components of Q5’High – Fidelity DNA Polymerase (BioLabs) (25 µL Reaction for sequencing)

S. No.	Reagents	Volume (µL)	Final conc.
1.	5X Q5 Reaction Buffer	5	1X
2.	10 mM dNTPs	0.5	200 µM
3.	10 µM Forward primer	1.25	0.5 µM
4.	10 µM Reverse primer	1.25	0.5 µM
5.	Template DNA (100ng)	1.0	100 ng/25 µl
6.	Q5 High- Fidelity DNA Polymerase	0.25	0.5 U/25 µl

7.	5XQ5 High GC Enhancer(Optional)	5.0	1X
8.	Nuclease Free Water	10.75	

Table 3.4 PCR conditions for different genes

Gene	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Hold
BlaNDM (Internal)	95 °C, 3min	95 °C, 1 min	50°C, 31 sec	73°C, 1 min	72 °C, 5 min	4 °C
		29 cycles				
BlaNDM (Full length)	95 °C, 2 min	95 °C, 30 sec	56°C, 30 sec	72 °C, 90 sec	72 °C, 5 min	4 °C
		Decrease 0.5 °C/cycle , 14 cycles				
		95 °C 30 sec	49 °C, 30 sec	72^0 C, 90 sec		
		19 cycles				
blaOXA (internal)	95 °C, 2 min	95 °C, 30 sec	60°C, 30 sec	72 °C, 80 sec	72 °C, 5 min	4 °C
		29 cycles				

3.13 Sanger Sequencing:

Sanger et al., (1974) used the principles of DNA replication in the development of the process now known as Sanger dideoxy sequencing. This process takes advantage of the ability of DNA polymerase to incorporate 3'- dideoxynucleotides, nucleotide base analogs that lack the 3'-hydroxyl group essential in phosphodiester bond formation. Sanger dideoxy sequencing requires a DNA template, a sequencing primer, DNA polymerase, nucleotides (dNTPs), dideoxynucleotides (ddNTPs), and reaction buffer. The Sanger method chain termination reactions are still used, but pouring, running, & reading polyacrylamide gels has been replaced by automated methods. Instead of labeling the products of all 4 sequencing reactions the same (with a radioactive deoxynucleotide), each dideoxynucleotide is labeled

with a different fluorescent marker. During capillary electrophoresis the fluorescently labeled DNA fragments, separated by size, move across the path of a laser beam. The laser 32 beam causes the dyes on the fragments to fluoresce. An optical detection device on Applied Biosystems genetic analyzers detects the fluorescence. The Data Collection Software converts the fluorescence signal to digital data, then records the data in a *.ab1 file. Because each dye emits light at a different wavelength when excited by the laser, all four colors, and therefore all four bases, can be detected and distinguished in one capillary injection.

3.14 Sequence analysis by using Bioinformatics tools

For sequencing purposes, full length primers for blaNDM and internal primers for blaOXA were used and the products were sent for sequencing to Xcelris Labs Limited, Ahmedabad, India. Preliminary analysis of the sequence obtained was done using Chromas Lite 2.1.1 followed by sequence editing in Codoncode Aligner. Sequence alignment was done using the software MEGA 6.06 by Clustal W. Final sequence was subjected for BLAST to compare with existing sequences in NCBI database.

CHAPTER IV

RESULT

4.1 Frequency of specimen sources of *Klebsiella pneumoniae*

The samples were collected from 1st March to 16th May 2016 from Tribhuvan University Teaching Hospital, Maharajganj, Kathmandu. During this period, a total of 61 *K. pneumoniae* isolates were recovered from different clinical specimens (urine, feces, sputum, swab, pus, tissue and blood). In this study, *K. pneumoniae* isolates were arranged according to clinical source of samples. The most prevalent infection caused by *K. pneumoniae* was respiratory tract infection as depicted by the number of isolates in sputum, which was followed by urinary tract infection and the sample sources of *K. pneumoniae* from sputum; urine and pus were found to be distributed with 40.9%, 26.22% and 14.7% respectively as were characterized as following.

Table 4.1: Frequency of specimen sources of *Klebsiella pneumoniae*

Sample Source	**Frequency**
Sputum	25
Urine	16
Swab	4
Pus	9
Tissue	3
Blood	4

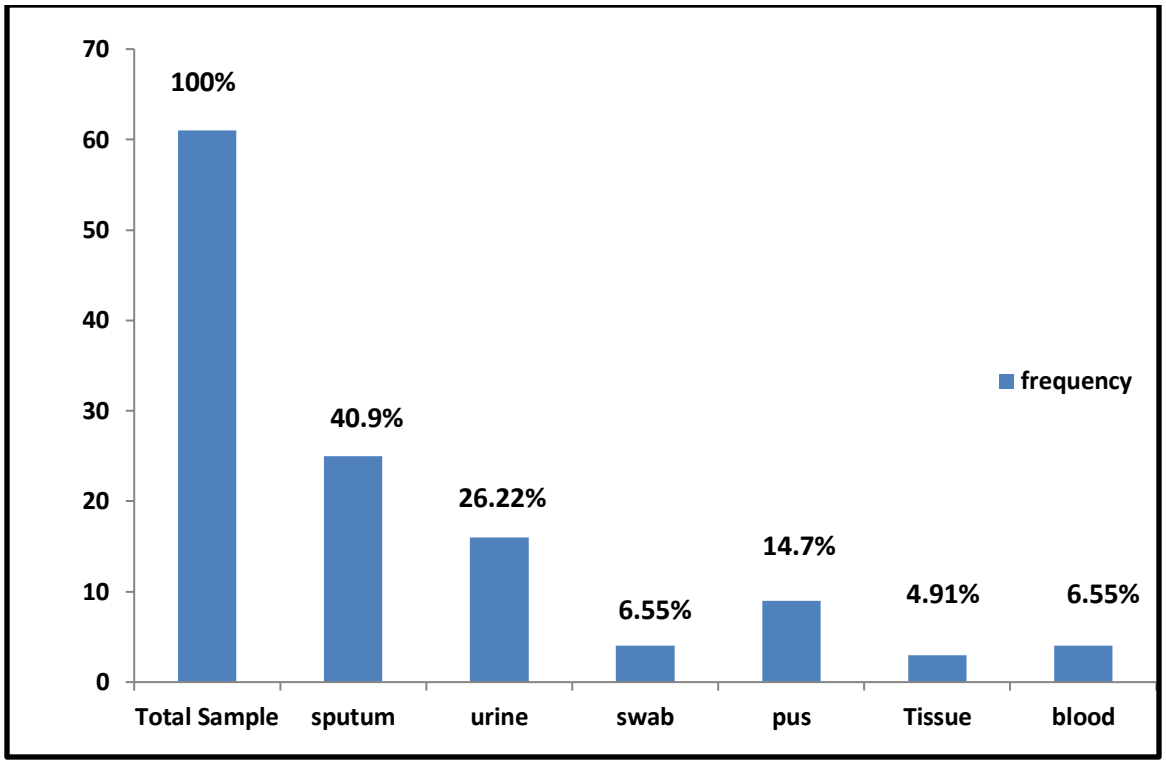

Figure 4.1: Distribution of frequency of specimen sources of *Klebsiella pneumoniae* isolates collected.

4.2 Confirmation of *Klebsiella pneumoniae*

The isolates were observed circular, convex, entire margin with slightly gummy looking as mucoid colonies and pink colored bacilli was seen on grams staining. The figure 4.2 showed the cultural characteristic and Figure 4.3 showed the grams staining of *K. pneumoniae.*

Figure 4.2: Colony morphology of isolate On nutrient agar

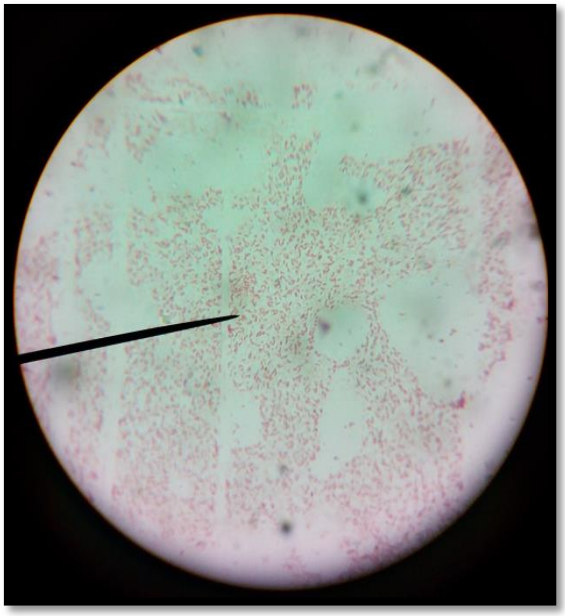

Figure 4.3: Grams staining of isolates

The biochemical tests was performed where TSI showed yellow/yellow with bubble and SIM showed negative without color with Kovac reagents, no growth like string, no black color in tube. Citrate test showed blue coloration of media whereas nitrate test showed red color with nitrate reagent. Urease test was positive with the production of pink color in media. MR/VP test was showed same color of broth as pervious in MR and darker color in VP broth. The biochemical test was shown in Figure 4.2.

Figure 4.4: Biochemical test of isolate; TSI, SIM, Citrate, Nitrate, Urease, MR and VP

4.3 Antibiotic susceptibility test pattern of *K. pneumoniae* strains detected by the double disk diffusion method

Majority of *K. pneumoniae* strains were significantly resistant to Cefotaxime(64.8%), Piperacillin/Tazobactam(84.2%),Ceftazidime(94.7%),Doxycycline(82.3%),Amoxicillin/Clavulanic acid(89.6%), Cefepime(82.3%) and Ceftriaxone(60%) antibiotics. But *K. pneumoniae* towards Gentamicin, Cotrimoxazole, Ciprofloxacin, Amikacin and Polymixin-B showed tremendous level of sensitivity. All of the *K. pneumoniae* were noticed to be resistant to Ampicillin/Sulbactam, piperacillin, Nitrofurantoin i.e. 100% resistant to those drugs.

Among, *K. pneumoniae* isolates around 77.6%, 57.4% and 71.5% exhibited sensitivity towards carbapenem drugs i. e. Imipenem, Meropenem and Etrapenem respectively but there was no resistance for colistin.

Table 4.2: Antibiotic susceptibility test pattern of *K. pneumoniae* isolates

Antibiotic name	**Sensitive%**	**Resistant%**
Cefotaxime(CTX) - 30mcg	35.2	64.8
Ampicillin/Sulbactam(A/S) - 10/10mcg	0	100
Piperacillin/Tazobactam(PIT) - 100/10mcg	15.8	84.2
Ceftazidime(CAZ) - 30mcg	5.3	94.7
Doxycycline (DO) - 30mcg	17.7	82.3
Amoxycillin/Clavulanic acid(AMC) - 20/10mcg	10.4	89.6
Gentamicin(GEN) - 10mcg	60	40
Ceftriaxone(CTR) - 30mcg	40	60
Cefixime(CFM) - 5mcg	43	57
Levofloxacin(LE) - 5mcg	43.5	56.5
Co-Trimoxazole(COT) – 1.25/2.75 mcg	65.3	34.7
Ciprofloxacin(CIP) - 5mcg	58	42
Cefepime(CPM) - 30mcg	17.7	82.3
Chloramphenicol(C) - 30mcg	54.6	45.4
Polymixin-B(PB) - 300 units	88.9	11.1
Amikacin(AK) - 30mcg	55.6	44.4
Colistin(CL) - 10mcg	100	0
Nitrofurantoin(NIT) - 300mcg	0	100
Norfloxacin(NX) - 10mcg	75	25
Piperacillin(PI) - 100mcg	0	100
Imipenem(IPM) - 10mcg	77.6	22.4
Meropenem(MRP) - 10mcg	57.4	42.6
Ertapenem(ETP) - 10mcg	71.5	28.5

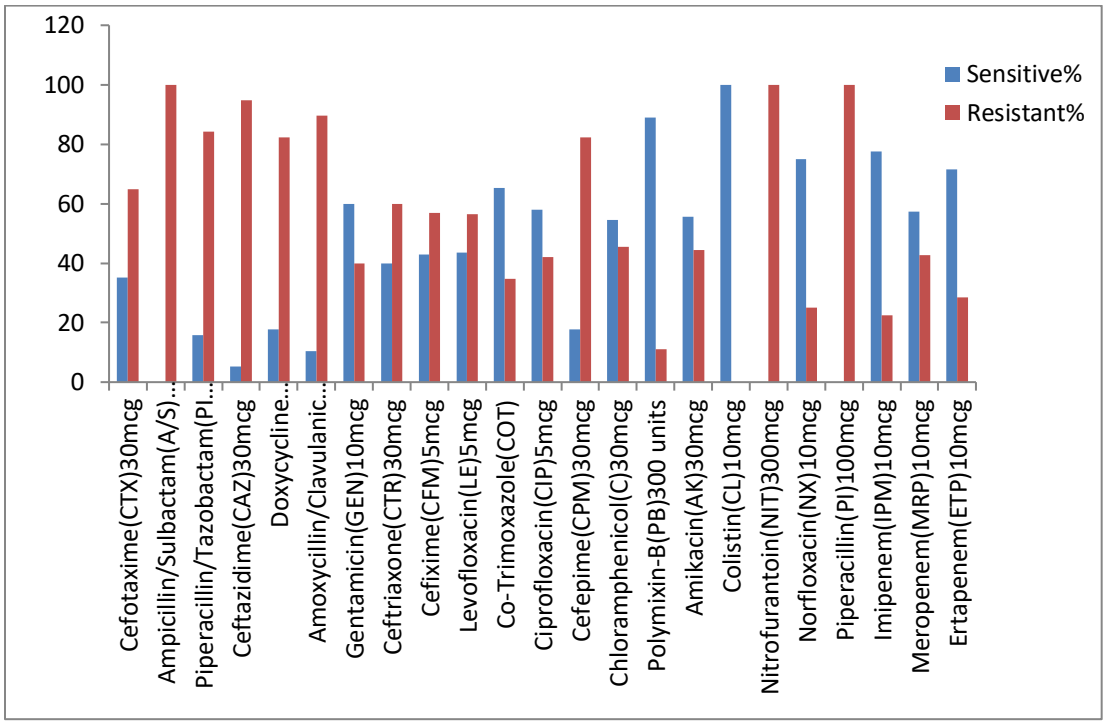

Figure 4.5: Antibiotic sensitivity pattern to differrent antibiotics used for most of gram negative bacteria of *K. pneumoniae* by disk diffusion method

4.4 Prevalence of MDR *K. pneumoniae*

Out of 61 *K. pneumoniae* strains, 45(73.22%) were MDR while only 16(26.22%) were non – MDR isolates.

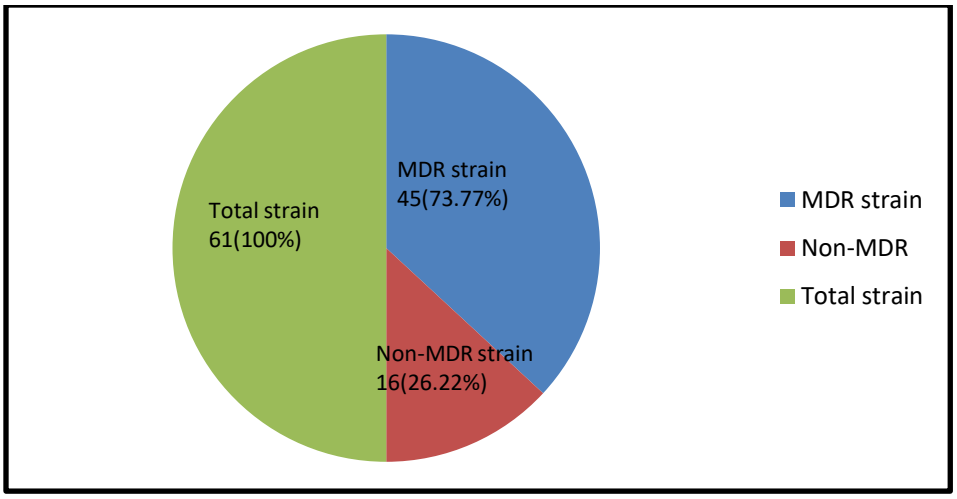

Figure 4.6: Prevalence of MDR and non-MDR *K. pneumoniae*

4.5 ESBL and MBL producer *K. pneumoniae* Strains

The *K. pneumoniae* isolates were further screened for the production of ESBL and MBL activity first by phenotypic methods. Only (n=3) 4.91% *K. pneumoniae* were ESBL producer (Figure 4.7) with MBL producer while all carbapenem resistant strains (n=25) 40.9% were MBL producer. Most of the MDR 20 (32.78%) *K. pneumoniae* showed resistance to amoxy-clavulanic acid (20/10 mcg) instead of, resistant to carbapenem and also third generation of cephalosporin group of drug i.e. cefotaxime. The maximum strains could be amp C type ESBL enzyme producer.

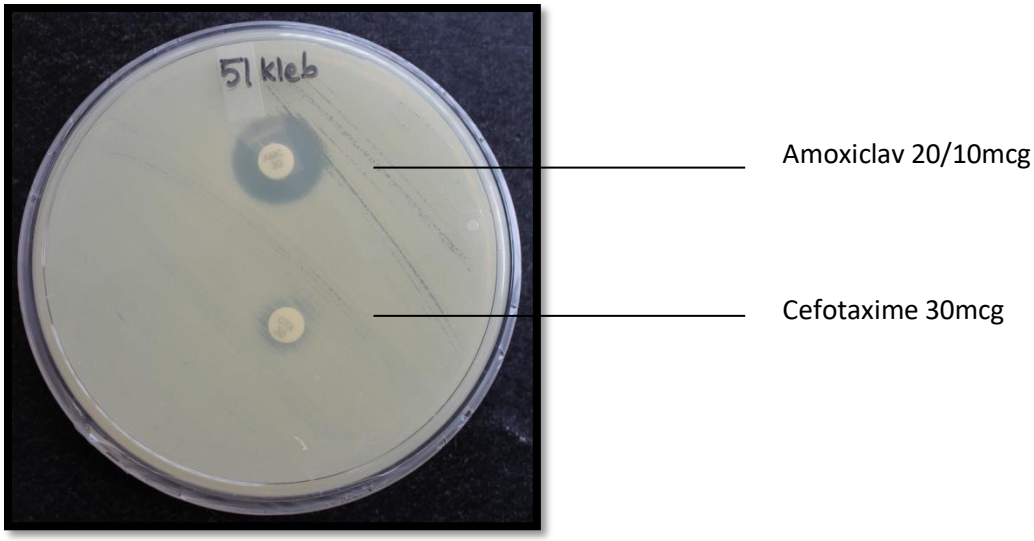

Figure 4.7: Positive combined disc test for detection of ESBL producer by double disc test

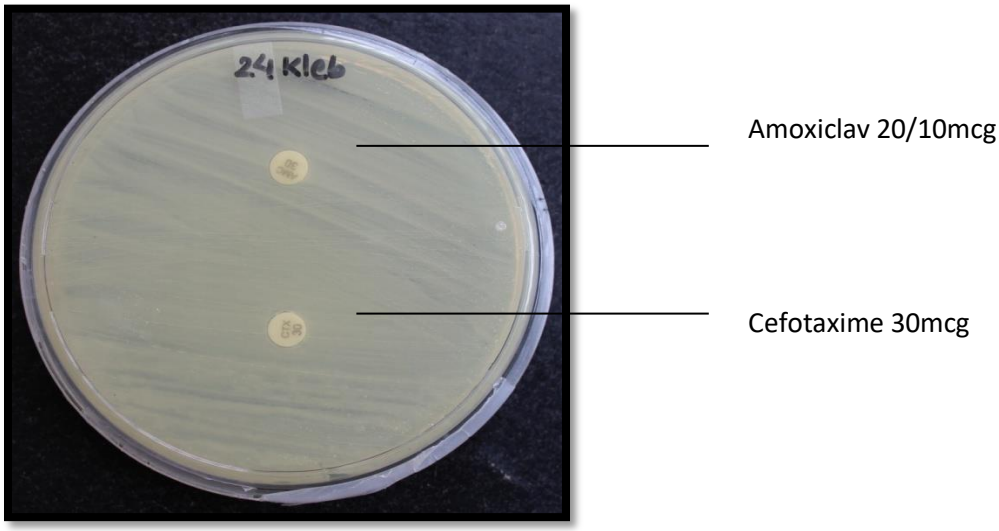

Figure 4.8: Combined disc test for detection of ESBL producer by double disc test (suspected for amp C type)

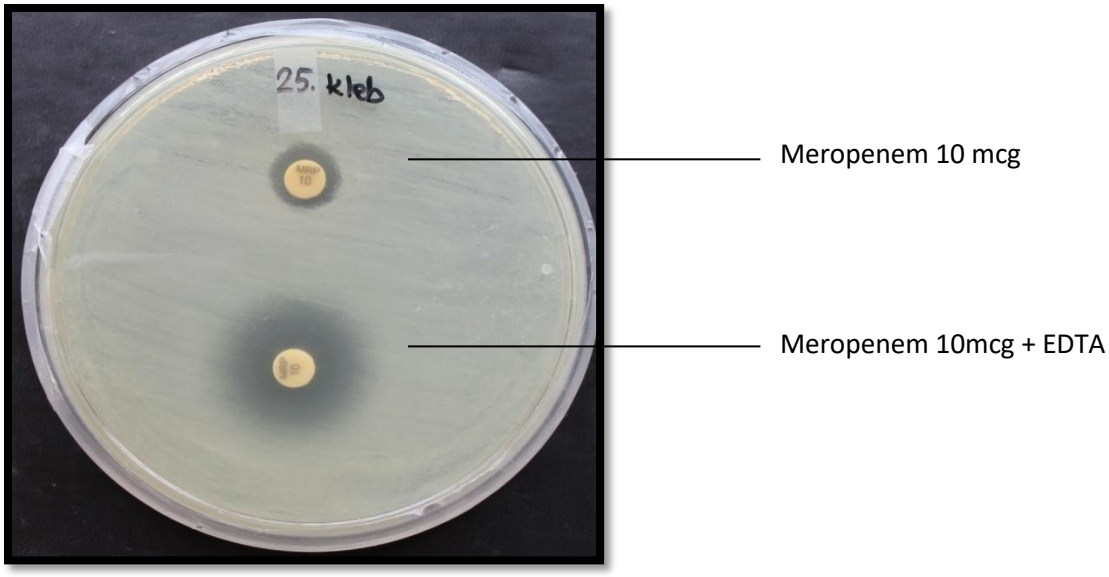

Figure 4.9: Positive combined disc test for detection of MBL Producers by EDTA – Meropenem synergy test

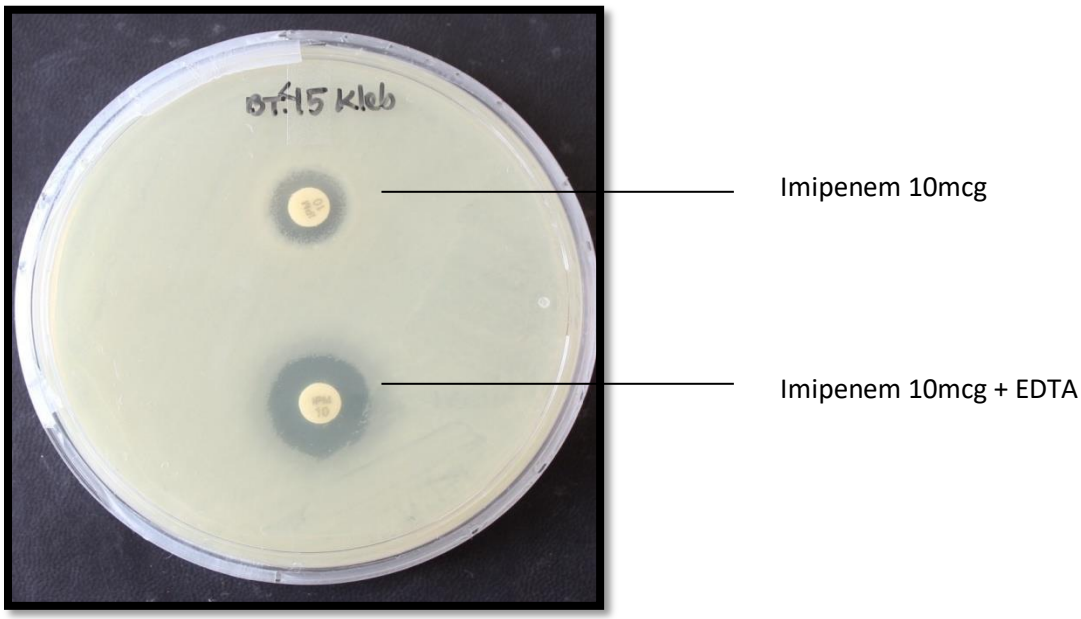

Figure 4.10: Positive combined disc test for detection of MBL Producers by EDTA – Imipenem synergy test.

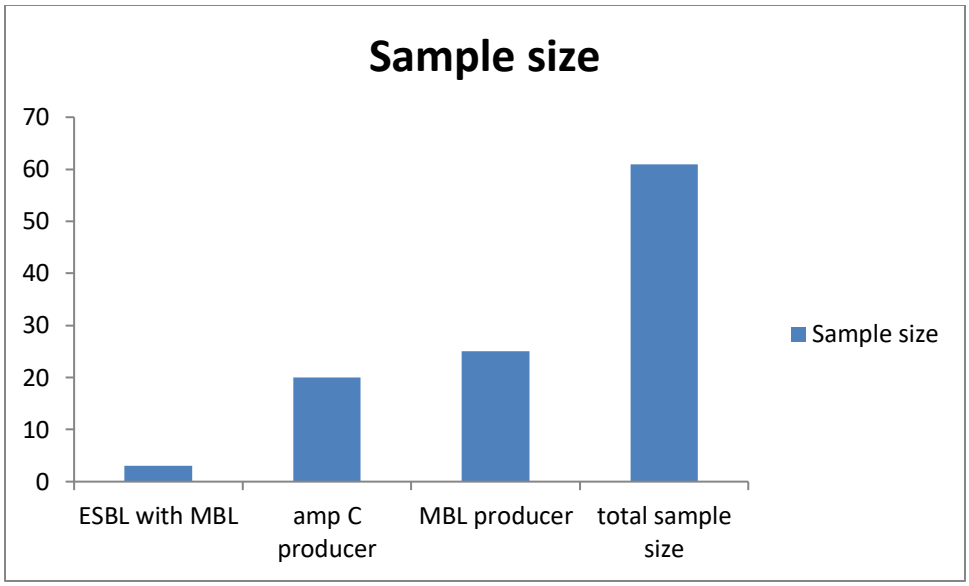

Figure 4.11: Temporal distribution of ESBL and MBL producer strain of MDR *K. pneumoniae.* 25 isolates are MBL.

4.6 DNA extraction of carbapenem resistant strains

Genomic DNA was extracted from bacterial cultures of Carbapenem resistant *K. pneumoniae* strains using CTAB method. DNA samples were electrophoresed and checked on 0.8% agarose gel. These were extracted without any contaminations.

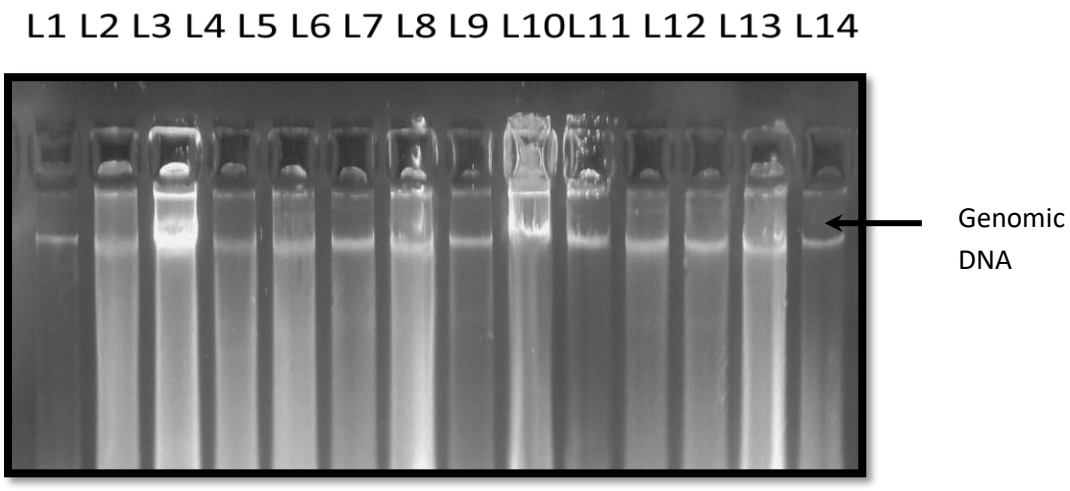

Figure 4.12: Gel image of Genomic DNA of *K. pneumoniae* strains. Lane(L);L1-14-sample no.

4.7 Optimization & detection of carbapenem resistant genes (blaNDM and blaOXA):

PCR amplifications were performed to detect the blaNDM and blaOXA genes in these isolates that were confirmed phenotypically as ESBL and MBL producers by double disk and EDTA test respectively. The resulting amplifications demonstrated that all 3 phenotypically positive ESBL – producing isolates carried a blaOXA gene while 6 out of 25 phenotypically positive MBL producing isolates harbored a blaNDM gene. Amplified products of genes were electrophoresed on 2 % Agarose to check the correct amplification of specific gene size (bp). The internal primer (amplicon size 460bp) and the full length primer (amplicon size 869bP) were used only for the confirmation of occurrence and the mutational analysis of blaNDM genes respectively.

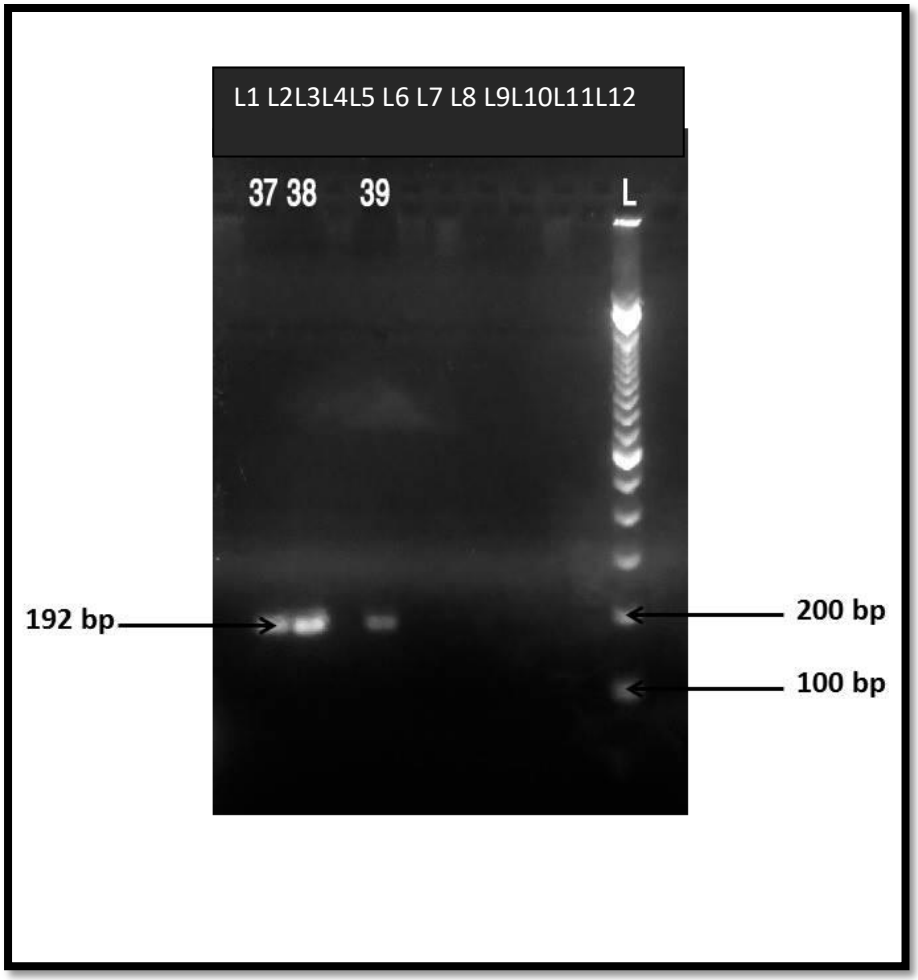

Figure 5.1: 2% Gel electrophoresis of showing results of PCR for the detection of internal bla OXA gene; Product Size: 192 bp. Lane (L); L1-L12. L2: sample no. 37, L3: sample no. 38, L5: sample no. 39, L12: Ladder 100bp (Thermo fisher, 15628019).

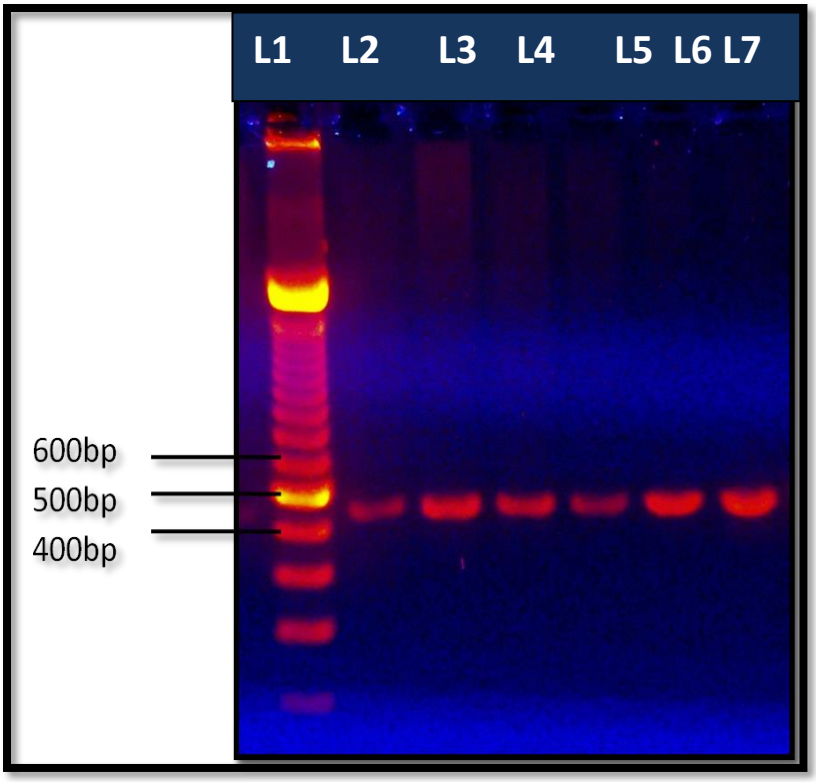

Figure 5.2:2% Gel electrophoresis of showing results of PCR for the detection of internal blaNDM gene; Product Size: 460 bp. Lane (L); L1 – L7; L1: Ladder 100bp (NEB, N3231S), L2: sample no. 16, L3: sample no. 17, L4: sample no. 18, L5: sample no. 19, L6: sample no.20 and L7: sample no. 21

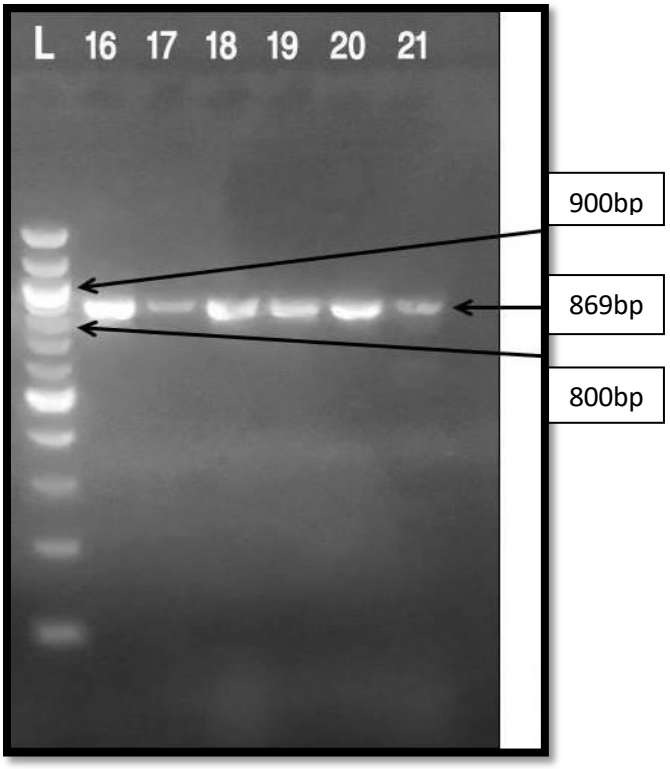

Figure 5.3:2% Gel electrophoresis of showing results of PCR for the detection of full length blaNDM gene; Product Size: 869 bp. Lane (L); L1 – L7; L1: Ladder 100bp (NEB, N3231S), L2: sample no. 16, L3: sample no. 17, L4: sample no. 18, L5: sample no. 19, L6: sample no.20 and L7: sample no. 21

4.7.1 Distribution of the carbapenem resistant genes in *K. pneumoniae*

This study was carried out to assess the distribution of carbapenem resistant gene in *K. pneumoniae* which is second common mechanism than diminished expression or loss of the OmpK35, OmpK36 porins in *Klebsiella pneumoniae* among enterobacteriaceae (Ghafourian, 2011). Another part of this study was to determine the mutational analysis of blaNDM gene by sequencing of positive *K. pneumoniae* strains in contest of Nepal.

In our study, out of 25, 6 MBL producer strains of *K. pneumoniae* were found to be blaNDM-1 gene positive.

Table No.4.3: Frequency of the carbapenem resistant genes in *K. pneumoniae* isolates

Blagenes	No. of positive strain	Percentage
blaNDM	6	24%
blaOXA	3	12%
Other mechanisms (unknown)	16	64%

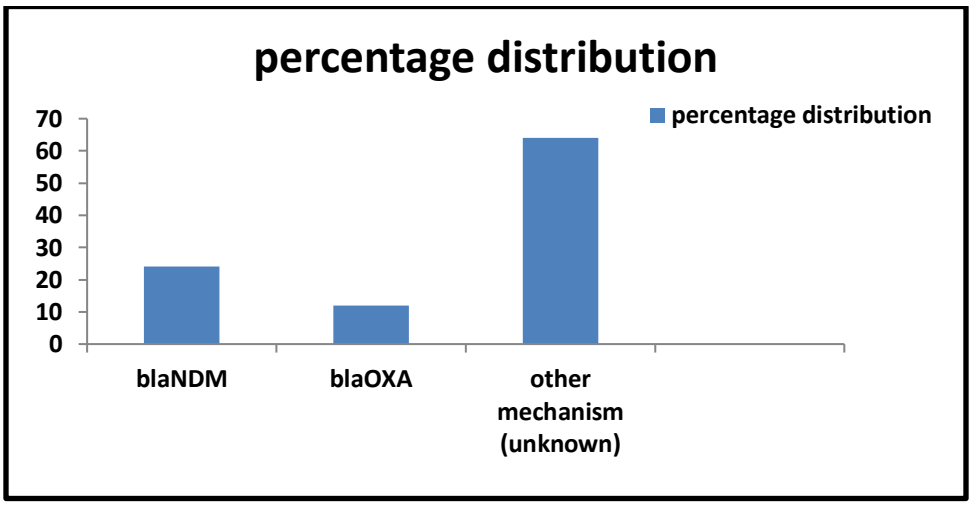

Figure 5.4: percentage distribution of the carbapenem resistant genes among 25 ESBL and MBL producer *K. pneumoniae* isolates.

4.8 Sequencing of ESBL and MBL gene

PCR products of amplified OXA-48 and NDM-1 genes were sent for sequencing to Xcelris Labs Pvt. Ltd., Ahmadabad, Gujarat, India. The chromatogram obtained was subjected to base calling in Chromas lite version 2.6.4 while the sequences were analyzed in Mega Version 6 for blaOXA and Codon Code Aligner for blaNDM gene. Once the sequences were obtained, they were subjected to BLAST and multiple sequence alignment was done in Clustal Omega.

The resulting sequences of blaOXA were compared to gene sequences submitted in NCBI site using BLAST program which showed 100% identity to sequences with gene bank accession no.KU878974.1. OXA-48 showed identity to OXA-517 variant of OXA genes.

The resulting full length sequences of blaNDM were compared to gene sequences submitted in NCBI site using BLAST program which showed similarity to the sequences with gene bank accession no.KY446367.1.

Then the internal sequences of OXA and full length of blaNDM gene were aligned with the reference sequence in Clustal Omega and Phylogenetic tree drawn is illustrated below respectively.

4.8.1 Multiple nucleotide sequence alignment for blaOXA gene

A substitution of nucleotide from G to C at position 56 of blaOXA gene in sample no. 39 only and other two samples showed same sequence as the reference sequence KU878974.1.

```
KU878974.1:51-242     AATGCCTGCGGTAGCAAAGGAATGGCAAGAAAACAAAAGTTGGAATGCTCACTTTACTGA
Sample37 (BT.12)      AATGCCTGCGGTAGCAAAGGAATGGCAAGAAAACAAAAGTTGGAATGCTCACTTTACTGA
Sample38 (BT.31)      AATGCCTGCGGTAGCAAAGGAATGGCAAGAAAACAAAAGTTGGAATGCTCACTTTACTGA
Sample39 (BT.51)      AATGCGTGCGGTAGCAAAGGAATGGCAAGAAAACAAAAGTTGGAATGCTCACTTTACTGA
                      ***** **********************************************************
KU878974.1:51-242     ACATAAATCACAGGGCGTAGTTGTGCTCTGGAATGAGAATAAGCAGCAAGGATTTACCAA
Sample37 (BT.12)      ACATAAATCACAGGGCGTAGTTGTGCTCTGGAATGAGAATAAGCAGCAAGGATTTACCAA
Sample38 (BT.31)      ACATAAATCACAGGGCGTAGTTGTGCTCTGGAATGAGAATAAGCAGCAAGGATTTACCAA
Sample39 (BT.51)      ACATAAATCACAGGGCGTAGTTGTGCTCTGGAATGAGAATAAGCAGCAAGGATTTACCAA
                      ************************************************************
KU878974.1:51-242     TAATCTTAAACGGGCGAACCAAGCATTTTTACCCGCATCTACCTTTAAAATTCCCAATAG
Sample37 (BT.12)      TAATCTTAAACGGGCGAACCAAGCATTTTTACCCGCATCTACCTTTAAAATTCCCAATAG
Sample38 (BT.31)      TAATCTTAAACGGGCGAACCAAGCATTTTTACCCGCATCTACCTTTAAAATTCCCAATAG
Sample39 (BT.51)      TAATCTTAAACGGGCGAACCAAGCATTTTTACCCGCATCTACCTTTAAAATTCCCAATAG
                      ************************************************************
KU878974.1:51-242     CTTGATCGCC
Sample37 (BT.12)      CTTGATCGCC
Sample38 (BT.31)      CTTGATCGCC
Sample39 (BT.51)      CTTGATCGCC
                      **********
```

4.8.2 Multiple nucleotide sequence alignment for blaNDM (Sample no. 16)

There were substitutions of nucleotides A to nucleotide G at position 496 in sample no. 16, T to A at position 618, C to T at position 624 of sample no.16. Similarly, nucleotide substitution occurred at position 632, A to C; at position 639, C to G, at position 640, A to C and at position 644, C to A in sample no. respectively.

But remaining sample sequences showed same sequence to reference sequence of bla NDM-1 (KY446367.1). There was no substitution of nucleotides in sample no. 17, 19, and 20 while mutations were observed in sample no. 16, 18 and 21. While analyzing the sequence of sample 18, a substitution of nucleotide at two different sites i.e. at position 262 G to T and at position 460 A to C, respectively, was identified. In sample no. 21, only one substitution occurred at position 797 of G to C.

```
KY446367.1      ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
Sample16 (BT.1) ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
                ************************************************************
KY446367.1      TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
Sample16        TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
                ************************************************************
KY446367.1      ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
Sample16        ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
                ************************************************************
KY446367.1      CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
Sample16        CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
                ************************************************************
KY446367.1      AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
Sample16        AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
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KY446367.1      ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
Sample16        ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
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KY446367.1      GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
Sample16        GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
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KY446367.1      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
Sample16        GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
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KY446367.1      CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
Sample16        CTGACTTTCGCCGCCGATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
                *************** ******************************************
```

```
KY446367.1     AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600

Sample16       AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600

               ************************************************************
KY446367.1     ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
Sample16       ACCGACATCGCTTTTGGAGGCTGTCTGATCACGGACAGGGCAGGACAAGTCATTCCCCCAT 660
               ***************** ***** ******* ******  **** ****** **  * **
KY446367.1     CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
Sample16       CT------------------------------------------------------------ 662
               **
KY446367.1     CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
Sample16       ------------------------------------------------------------ 662
KY446367.1     CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
Sample16       -------------------------------- 662
```

4.8.3 Multiple nucleotide sequence alignment for blaNDM (Sample no. 17 - 21)

```
KY446367.1       ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
Sample18 (BT.43) ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
Sample17 (BT.40) ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
Sample19 (BT.52) ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
Sample20 (BT.57) ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
Sample21 (BT.61) ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
                 ************************************************************
KY446367.1       TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
Sample18         TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
Sample17         TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
Sample19         TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
Sample20         TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
Sample21         TTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAA 120
                 ************************************************************
KY446367.1       ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
Sample18         ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
Sample17         ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
Sample19         ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
Sample20         ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
Sample21         ACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAG 180
                 ************************************************************
KY446367.1       AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
Sample18         CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
Sample17         CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
Sample19         CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
Sample20         CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
KY446367.1       CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
Sample21         CACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTC 240
                 ************************************************************
Sample18         AGGGATGGCGGCCGCGTGCTGTTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
Sample17         AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
Sample19         AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
Sample20         AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
Sample21         AGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG 300
```

```
                  ********************* ************************************
KY446367.1        ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360

Sample18          ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360

Sample17          ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
Sample19          ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
Sample20          ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
Sample21          ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCAC 360
                  ************************************************************
KY446367.1        GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
Sample18          GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
Sample17          GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
Sample19          GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
Sample20          GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
Sample21          GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT 420
                  ************************************************************
KY446367.1        GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
Sample18          GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGC 480
Sample17          GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
Sample19          GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
Sample20          GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
Sample21          GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC 480
                  ************************************ ********************
KY446367.1        CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
Sample18          CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
Sample17          CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
Sample19          CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
Sample20          CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
Sample21          CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC 540
                  ************************************************************
KY446367.1        AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
Sample18          AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
Sample17          AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
Sample19          AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
Sample20          AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
Sample21          AAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC 600
                  ************************************************************
KY446367.1        ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
Sample18          ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
Sample17          ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
Sample19          ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
Sample20          ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
Sample21          ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAAT 660
                  ************************************************************
KY446367.1        CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
Sample18          CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
Sample17          CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
Sample19          CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
Sample20          CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
Sample21          CTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTC 720
                  ************************************************************
```

```
KY446367.1      CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
Sample18        CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
Sample17        CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
Sample19        CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
Sample20        CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
Sample21        CCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACT 780
                ************************************************************
KY446367.1      CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
Sample18        CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
Sample17        CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
Sample19        CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
Sample20        CATACGGCCCGCATGGCCGACAAGCTGCGCTGA 813
Sample21        CATACGGCCCGCATGGCCCACAAGCTGCGCTGA 813
                ********************* **************
```

4.8.4 Phylogenetic Relationship of the sequences

The Phylogenetic tree of the samples with the reference sequence submitted to NCBI, gene bank showed the evolutionary interrelations of the variants of blaOXA gene derived from a common ancestral gene. The ancestor is in the tree “trunk”; organisms that have arisen from it are placed at the ends of tree “branches.” The distance of one group from the other groups indicates the degree of relationship i.e. closely related groups are located on branches close to one another reference gene. The blaOXA gene amplified showed the similarity to blaOXA-48 variant. The sample no.37 and 38 have similarity to the ancient type of blaOXA gene but sample no. 39 has same identity to reference gene KU878974.1 (blaOXA-48). However, these all blaOXA gene showed the same diversity.

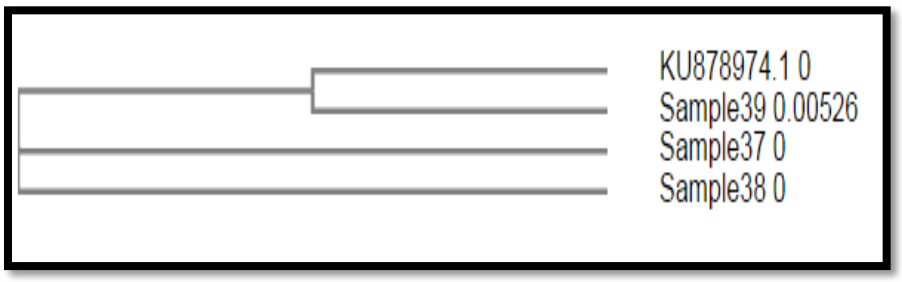

Figure 5.5: Phylogenetic tree of blaOXA gene amplified

The blaNDM gene isolated from our samples showed the similarity to blaNDM-1 sequence. The sample no. 16, 17, 20 and 21 represents same groups of descendent variants and their nodes represent the common ancestors of those descendants i.e. blaNDM-1 gene (Reference KY446367.1) gene whereas sample no. 18 and 19 are sister taxas of blaNDM-1 (Reference KY446367.1) gene.

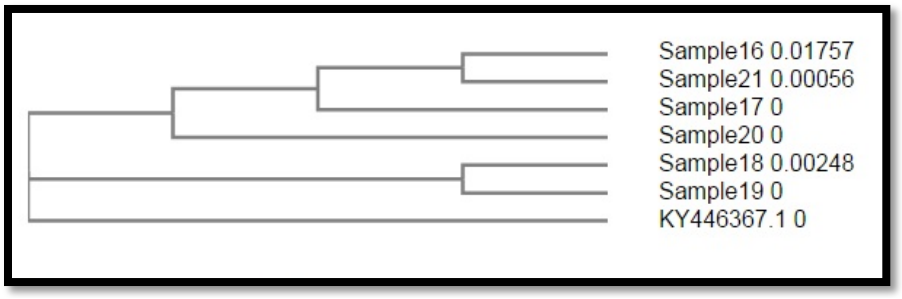

Figure 5.6: Phylogenetic tree of blaNDM gene amplified

4.8.5 Multiple protein sequence alignment for blaOXA

Our sequencing result showed that the amplified blaOXA have the similar identity to blaOXA-48 gene submitted to NCBI, gene bank, reference no. KU878974.1. sample no.39 has a substitution mutation at position 18 proline to arginine and other sample no. 37 and 38 showed 100% similarity.

```
KU878974.1              MPAVAKEWQENKSWNAHFTEHKSQGVVVLWNENKQQGFTNNLKRANQAFLPASTFKIPNS
37(BT.12)               MPAVAKEWQENKSWNAHFTEHKSQGVVVLWNENKQQGFTNNLKRANQAFLPASTFKIPNS
38(BT.31)               MPAVAKEWQENKSWNAHFTEHKSQGVVVLWNENKQQGFTNNLKRANQAFLPASTFKIPNS
39(BT.51)               MRAVAKEWQENKSWNAHFTEHKSQGVVVLWNENKQQGFTNNLKRANQAFLPASTFKIPNS
                        * ************************************************************

KU878974.1              LIA
37(BT.12)               LIA
38(BT.31)               LIA
39(BT.51)               LIA
                        ***
```

4.8.6 Multiple protein sequence alignment for blaNDM (Sample no. 16)

There were substitutions of proteins in sample no. 16, (Glutamine 106 arginine), (Lysine 211 threonine). Similarly, amino acid substitution occurred (Serine 213 arginine), (Serine 213 arginine), (Lysine 214 glutamine), (Alanine 215 asparagine).

But remaining sample sequences showed same sequence to reference sequence of bla NDM-1 (KY446367.1). There was no substitution of nucleotides in sample no. 17, 19, and 20 while mutations were observed in sample no. 16, 18 and 21. While analyzing the sequence

of sample 18, a substitution of nucleotide at two different sites i.e. at (Valine 88 Valine) and (Methionine 154 leucine) respectively could be observed. In sample no. 21, only one substitution occurred at (Asparagine 167 Histidine).

```
16 (BT.1)              MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
KY446367.1             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
                       ************************************************************

16                     HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
KY446367.1             HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
                       ************************************************************

16                     AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAADGWVEPATAPNFGPL
KY446367.1             AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
                       ******************************************:***************

16                     KVFYPGPGHTSDNITVGIDGTDIAFGGCLITDRQDKS
KY446367.1             KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKS
                       *****************************.*:::**
```

4.8.7 Multiple protein sequence alignment for blaNDM (Sample no. 17-21)

```
18(BT.43)              MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
KY446367.1             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
17 (BT.43)             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
19 (BT.52)             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
20 (BT.57)             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
21 (BT.61)             MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQ
                       ************************************************************

18                     HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
KY446367.1             HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
17                     HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
19                     HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
20                     HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
21                     HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTH
                       ************************:***********************************

18                     AHQDKMGGMDALHAAGIATYANALSNQLAPQEGLVAAQHSLTFAANGWVEPATAPNFGPL
KY446367.1             AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
17                     AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
19                     AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
20                     AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
21                     AHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL
                       ************************************************************

18                     KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
KY446367.1             KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
17                     KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
19                     KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
20                     KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
21                     KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAF
                       ************************************************************
```

```
18                  PKASMIVMSHSAPDSRAAITHTARMADKLR*
KY446367.1          PKASMIVMSHSAPDSRAAITHTARMADKLR*
17                  PKASMIVMSHSAPDSRAAITHTARMADKLR*
19                  PKASMIVMSHSAPDSRAAITHTARMADKLR*
20                  PKASMIVMSHSAPDSRAAITHTARMADKLR*
21                  PKASMIVMSHSAPDSRAAITHTARMAHKLR*
                    ************************** ***
```

4.8.8 Phylogenetic Relationship of all variants of blaNDM nucleotide sequences

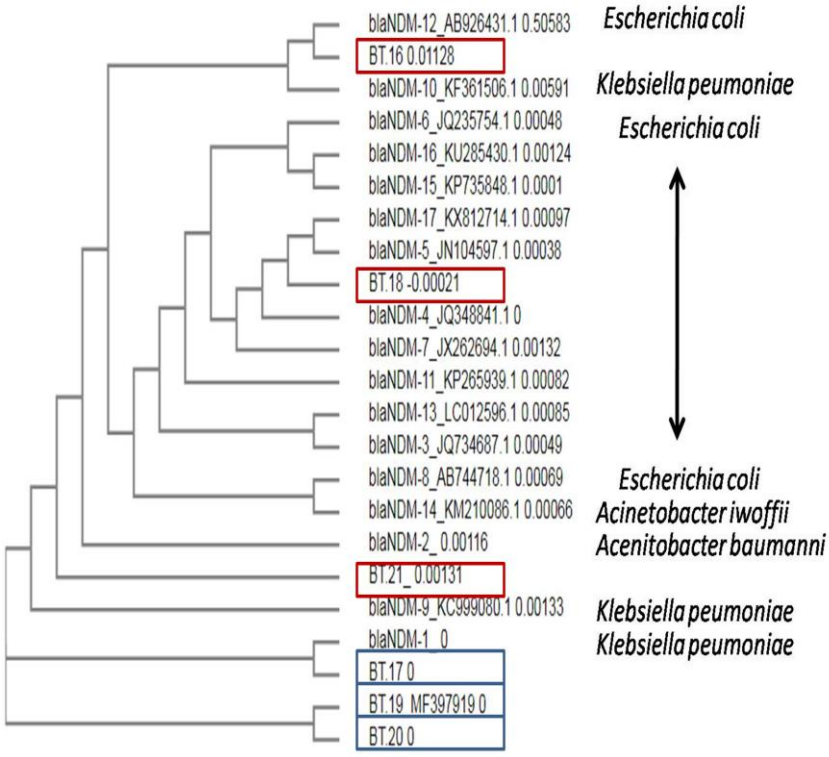

Figure 5.7: Phylogenetic Relationship of all variants of blaNDM nucleotide sequences

4.8.9 Phylogenetic Relationship of all variants of blaNDM protein sequences

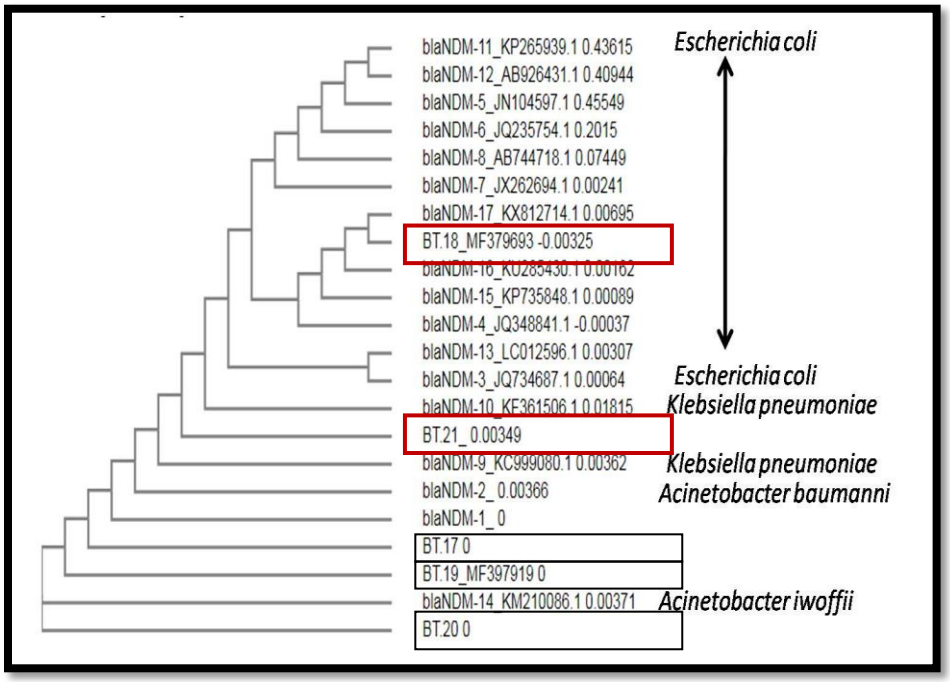

Figure 5.8: Phylogenetic Relationship of all variants of blaNDM peptide sequences

4.8.10 3D Structure of NDM-1 protein

3D Structure of NDM-1 and mutated sample were first predicted by Phyre2 and then analyzed by JSmol. The active site of 3D structure of protein showed two zinc ions as spherical balls linked by Histidine proteins at 120 and 122 positions.

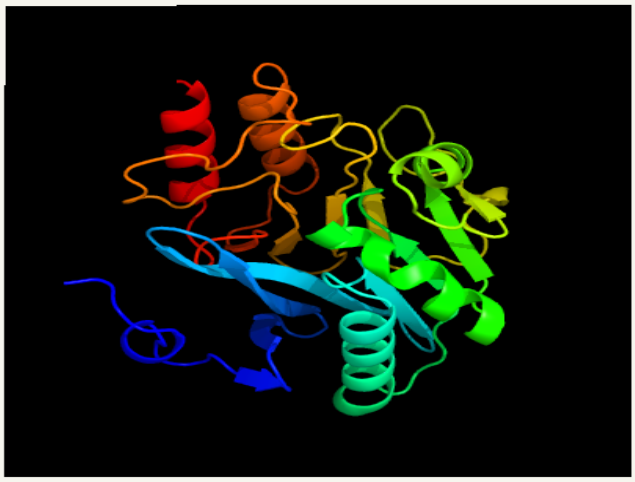

Image coloured by rainbow N → C terminus
Model dimensions (Å): **X**:45.556 **Y**:46.124 **Z**:42.110

Model (left) based on template c3spuB_
Top template information
PDB header:hydrolase
Chain: B: **PDB Molecule:**beta-lactamase ndm-1;
PDBTitle: apo ndm-1 crystal structure
Confidence and coverage
Confidence: **100.0%** Coverage: **88%**
238 residues (88% of your sequence) have been modelled with 100.0% confidence by the single highest scoring template.
3D viewing
Interactive 3D view in JSmol
For other options to view your downloaded structure offline see the FAQ

Figure 5.9: 3D structure of NDM-1 protein by Phyre2

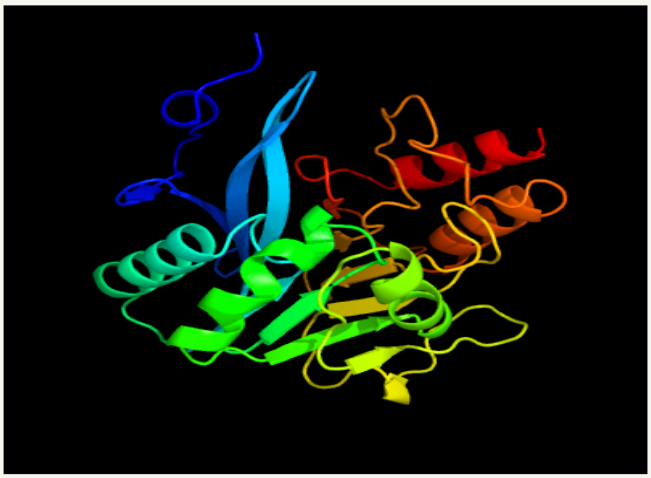

Image coloured by rainbow N → C terminus
Model dimensions (Å): **X**:45.556 **Y**:46.124 **Z**:42.110

Model (left) based on template c3spuB_
Top template information
PDB header:hydrolase
Chain: B: **PDB Molecule:**beta-lactamase ndm-1;
PDBTitle: apo ndm-1 crystal structure
Confidence and coverage
Confidence: **100.0%** Coverage: **88%**
238 residues (88% of your sequence) have been modelled with 100.0% confidence by the single highest scoring template.
3D viewing
Interactive 3D view in JSmol
For other options to view your downloaded structure offline see the FAQ

Figure 5.10: 3D Structure of NDM mutated sample (18) protein by Phyre2

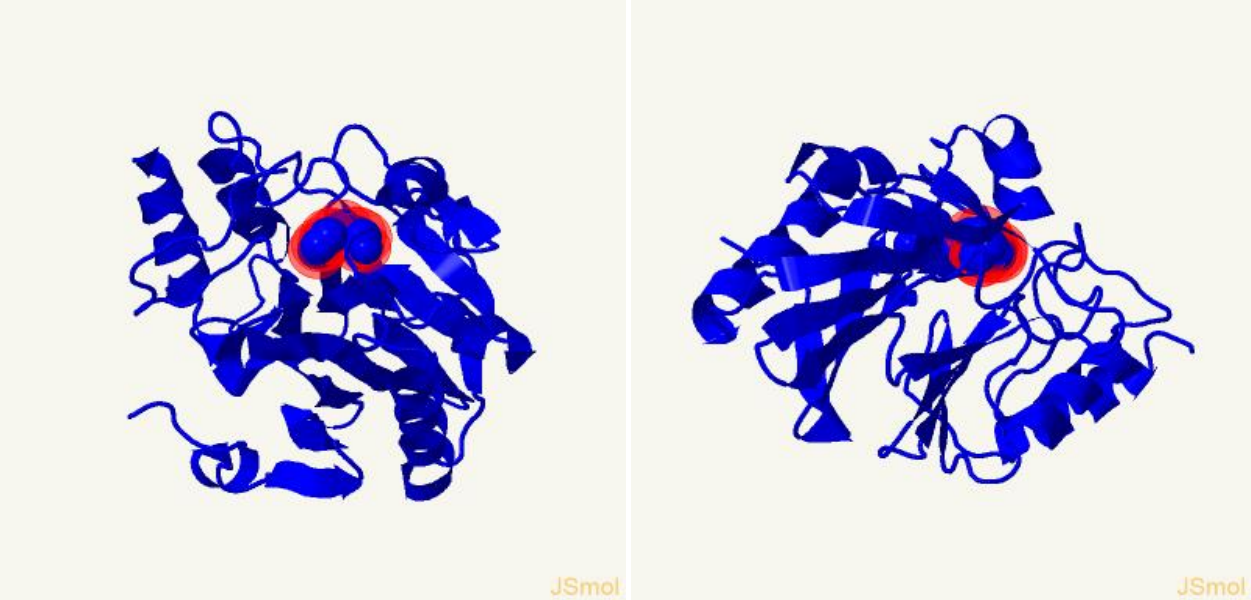

Figure 5.11: 3D Structure of NDM-1 protein by JSmol

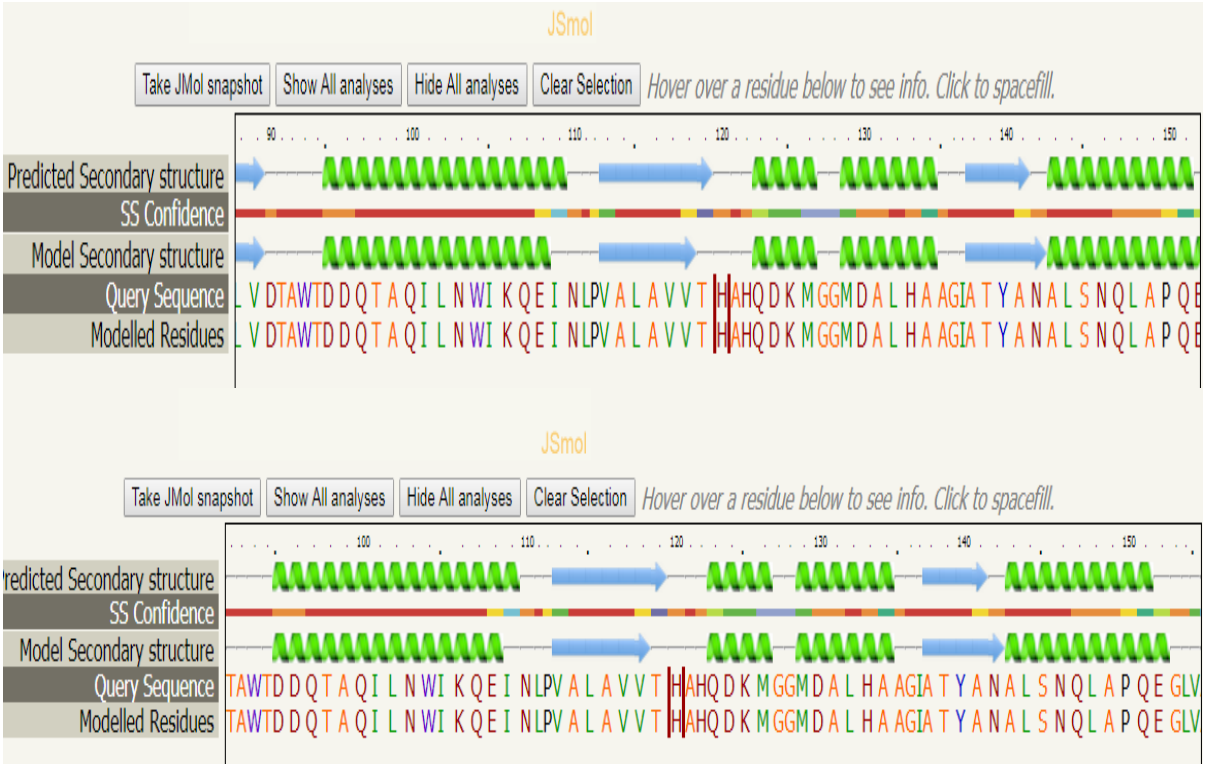

Figure 5.12: Secondary structure of NDM-1 protein and mutated NDM sample

Chapter V
Discussion

K. pneumoniae has become one of the major bacterium of *enterobacteriaceae* family which causes community and hospital acquired infection in our surroundings. It is important to investigate relationship between phenotyping, genotyping and drug resistance from epidemic or outbreak strains in the hospital. Moreover, *K. pneumoniae* could acquire different drug resistance by acquiring of the CTX, KPC, NDM and OXA genes which were considered to be one of the major drug resistances inducing mechanism (Livermore *et al.*, 2012).

In this current study, the distribution of specimen sources for *K. pneumoniae* represented 40.9%, 26.22% and 14.7% from sputum, urine and pus respectively. The most frequent infection caused by *K. pneumoniae* was respiratory tract infection followed by urinary tract infection. Similar results were reported previously in Nepal, where it was observed to be the ensuing common cause of urinary tract infections after *Escherichia coli* (Baral *et al.,* 2013). On the other hand, Yusuf *et al* identified that *K. Pneumoniae* as the main regular bacterium isolated from the culture urine specimen representing 41.8% of all specimens (Pfeifer *et al.*, 2010). Recently, the source distribution of *K. pneumoniae* was found to be higher from blood, pus, sputum and urine respectively in Nepal (Bora *et al.,* 2014). The most important pathogenic consequence of *Klebsiella pneumoniae* in human health is a severe life menacing diseases (Halaby *et al.,* 2012). The death rate may reach up to100% for persons with alcoholism and bacteremia (Hornsey *et al*., 2011).

In addition to this work, the antibiogram assay of MDR bacteria could be extremely significant to prevent the emergence of drug resistant bacteria. The resistance of *K. pneumoniae* against Ceftazidime, Cefotaxime, Cefixime, Imipenem and Meropenem were 94.7%, 64.8%, 57%, 22.4% and 24.5% respectively. Our findings indicate that overall

susceptibility rates to ampicillin and third generation broad spectrum cephalosporin among *K. pneuminae* isolates have highly decreased in the present study along with cephalosporin used in this investigation, i.e. Ceftazidime was relatively less effective on *K. pneumoniae* isolates. In comparison with previous study done in Nepal by Thakur *et al*, (2013) and Amataya *et al,* (2014) as well as in Iran done by Yonesian and Hadadi *et al,* (2008), all of the above study showed similar resistivity pattern except ceftazidime which showed the high result.

The result of our investigation raises more concern because the samples investigated showed increasing resistance to β – lactam antibiotics among Nepalese *K. pneumoniae* isolates. The increase in resistance against ceftazidime could be due to exploitation of cephalosporin in infection that could have built resistance to first and second generation cephalosporin. The main reason could be patient to patient transmission, transfer of patients between wards and the presence of substantial number of asymptomatic carriers in our hospital. In work done in Dhaka University, *K. pneumoniae* was 80 – 100% sensitive to Imipenem regardless of whether they possessed variable resistance to other frequently used antibiotics (Shaikh *et al.,* 2015).

Carbapenem have become the preferred treatment for serious *K. pneumoniae* infections in many centers, and have retained better activity than other antimicrobial agents. However, the number of reports of carbapenem resistance is on growing steadily than previous studies from Nepal (Mishra *et al.,* 2012) raising concern. Carbapenem resistant *K. pneumoniae* isolates are reported worldwide (Chen *et al.*, 2016). Although we only tested susceptibility to Imipenem and Meropenem was tested, it was observed with high resistance to carbapenem in the present study. Emergence of resistance against these antibiotics, although gene mediated resistance investigation data is not enough, it can be presumed that the threat is to be considered. Carbapenem have been used as a drug of choice since few decades, but there is some evidence that increased and uncontrolled use of these antibiotics favor resistance to carbapenems that could also be detected. A

combination of several mechanisms such as loss of outer membrane proteins, altered Penicillin binding proteins (PBPs) and acquired carbapenemases including class B, A and D β-lactamase (Gupta *et al.,* 2015) could be the mechanism. Increased resistance to carbapenem causes a real concern over an approaching threat of untreatable *K. pneumoniae* infections.

In the present study, greater percentages (45; 73.22%) were MDR while only (16; 26.22%) were non – MDR. There is observable increase in MDR in comparison to the prior study done in Nepal and Dhaka (Baral *et al.,* 2013 and Pfeifer *et al.,* 2010) indicates that resistance is building. This high level of resistance could be due to the fact that antibiotics are simply accessible without doctor's prescription in developing countries like Nepal. Other main reason would be self – medication, which is a common practice that probable the input basis of antibiotic resistance. Poor hospital control policy also encourages the emergence of resistivity among isolates. The prevalence of these multidrug resistant *K. pneumoniae* strains leaves limited clinical options for treatment (Tran *et al.,* 2015).,underscoring the need to develop novel antibiotics for gram negative bacterial pathogens in particular. Strict infection control measures remain an important method for controlling the spread of MDR *K. pneumoniae* in hospital and community.

Majority of the strains could be amp C type enzyme producer with porin loss which provides resistivity to third generation cephalosporin and even carbapenem. Out of 61 isolates, 3 isolates (4.91%) were identified as extended spectrum β – lactamase producers by double disk test and most of MDR *K. pneumoniae* could be amp C type of ESBL which were resistant to all first, second and third line of beta- lactam antibiotics. Only 3 strains showed increased sensitivity to clavulanic acids so they are called ESBL producer. But not inhibited in case of amp C type. The most common mechanism of resistance to carbapenem could be decreased expression or loss of the OmpK35, OmpK36 porins in *K. pneumoniae*. Similar results were found in research conducted at a tertiary hospital in Mwanza, Tanzania, where the ESBL prevalence was 64% in *K. pneumoniae* (Mshana *et al.,* 2009). Ashrafian *et al.* found

that 43% of *K. pneumoniae* was ESBL positive in their study conducted at the Imam Reza hospital of Mashhad, IR Iran. This study indicated the high prevalence of ESBL producing *enterobacteriaceae* family especially in patients (Ashrafian *et al.*, 2013). However, increased prevalence rates of ESBLs producing *K. pneumoniae* was reported by other studies i.e. the prevalence was in the range of 7.5% to 22.8% and 8.5% to 29.8% in *K. pneumoniae* in Taiwan and Korea respectively (Shaikh *et al.,* 2014). A multicenter study in mainland of China showed that the prevalence of ESBL production in *K. pneumoniae* isolates increased from 11% in 1994 to 34% in 2001 (Wang *et al.,* 2008). In Pakistan, the prevalence of the ESBLs producing *K. pneumoniae* was 36 % (Ghafourian, 2011).

In this study, 25 (40.9%) isolates were MBL producer with resistance to carbapenem. Previous study from an another tertiary care hospital in Nepal reported comparatively lower incidence of MBL producing gram negative bacteria (1.3%) in lower respiratory tract specimens. In context of other study, 71.79% of *K. pneumoniae* isolates were found to be MBL producer in Nepal (Bora *et al.,* 2014).The study conducted in India showed prevalence of MBL was between 31% and 55% among multidrug resistance *enterobacteriaceae* (Kumarasamy *et al.,* 2010). There is increasing incidence of metallo – β – lactamase than ESBL producing *K. pneumoniae*. The MBL producer *K. pneumoniae* are hard to treat because they hydrolyze strongly even the last line drugs and are inhibited by EDTA only (Khan *et al*, 2017). Class B carbapenemases are also known as metallo – β – lactamase since they contain two zinc ions in their active site which coordinate and present polarized water ions for the oxyanion attacks on the β –lactam ring. Interaction of the β – lactams with zinc ions in the active site of the enzyme is the mechanism of hydrolysis, resulting in the distinctive trait of their inhibition by EDTA, a chelator of Zn^{2+} and other divalent cations (Wright, 2005). Recent studies from different parts of Asia have also demonstrated increasing incidence of MBL production in *enterobacteriaceae* isolates (Datta *et al.,* 2012). In general, production of MBL in *enterobacteriaceae* isolates currently follows an increasing prevalence pattern and the prevalence rate may vary greatly in different geographical areas and from institute to institute (Bora *et al.,* 2014). The reason behind this increasing prevalence could be the

genetic relation between MBL and aminoglycoside resistance genes that magnify the problem (Yong *et al.,* 2009)). The problem of correct treatment regimen is also amplified by lack of new antimicrobials that possess broad – spectrum potency against clinically significant bacteria (Ansari *et al.,* 2016). The spread of MBL genes is likely to escalate and further highlight the importance of international resistance surveillance programs such as SENTRY, MYSTIC, the Alezander project and EARSS in reporting the emergence and epidemic spread of these remarkable but menacing enzymes (Walsh *et al.*, 2005).

Rates of cephalosporin resistance are lower in other countries but the growing prevalence of ESBL producers is sufficient to drive a greater reliance on carbapenems. Consequently, there is selection pressure for carbapenem resistant *K. pneumoniae*, and its resistance emergence is a worldwide public health concern since there are few antibiotics in reserve beyond carbapenem. The major reason for this emergence of resistance is likely due to inappropriate prescribing of antibiotics, incorrect dose or duration of use and antibiotic use without prescription.

All 3 phenotypically positive ESBL and MBL producing isolates carried only blaOXA gene, 6 out of 25 phenotypically positive MBL producing isolates harbored a blaNDM gene. Paton *et al* described the first OXA β –Lactamase with carbapenemase activity in 1993 (Walther-Rasmussen *et al*, 2006). OXA-48-producing *Enterobacteriaceae* (OPE) was first identified in Turkey in 2001, and then it has been reported from several countries (Aktas, 2013). Class D β– lactamases (oxacillinases) have carbapenemase activity and majority of them are encoded by plasmid (Mitchell, 2014). The most recent and worrying threat is the rapid rise of OXA – 48, particularly in *K. pneumoniae.*

In our study, New – Delhi MBL (NDM – 1) is more prevailing and has been reported to spread hastily over Asian continent and international travel has a significant impact on the spread of NDM – 1 (Nordmann *et al.,* 2011). Although the diversity of the NDM-1 is low,

NDM – 1 is the main type among the other variants of NDM. Distribution often involves the transfer of the blaNDM – 1 gene among promiscuous plasmids which can integrate on chromosome. Bacteria which contain NDM – 1 are typically resistant to almost all beta-lactam antibiotics so that reliable detection and surveillance is crucial (Poirel *et al.*, 2011).

Scientists claim that blaNDM – 1 is widely distributed among *enterobacteriacae* and has a rapid distribution around the world (Yamamoto, 2011). It is a very challenging task to try to stop the growing resistance to antibiotic stemming from this part of the world as exemplified by the rapid spread of the carbapenemase NDM – 1 (Jamale *et al.*, 2015).

In this study, there is absence of *Klebsiella pneumoniae* Carbapenemase (KPC) gene in all carbapenem resistant isolates. However, in other country KPC – types are the most clinically common enzymes. The first KPC producer was identified in 1996 in the eastern United States. Over the few years, KPC producers have been spread around the world and described across United States (Nordmann *et al.*, 2011).

Our sequencing result showed that the amplified blaOXA have the similar identity to blaOXA-48 gene submitted to NCBI, gene bank, reference no. KU878974.1. Sample no.39 has a substitution mutation which leads missense mutation because CCU codes for proline where the substituted amino acid CGU codes for arginine at position 18. So there could be additional harmful evolution of new variants of blaOXA – 48 in our context to Nepal.

In case of blaNDM genes amplified have the similar identity to blaNDM-1 gene submitted to NCBI, gene bank, and reference no. KY446367.1. Sample no.16 has much substitution mutation which leads two silent mutation at 618 and 624 position because GGT codes for glycine where the substituted amino acid GGA also codes for glycine. Some substitution occurred which lead to some new variants of blaNDM gene. At amino acid position 106,

211, 213, 214 and 215 lead to new amino acid i.e. glutamine to arginine, lysine to threonine, serine to arginine, lysine to glutamine and alanine to asparagines in sample no.16 by substitution of nucleotides respectively. Similarly analyzing the sequences of sample no. 17, 18, 19 and 20, only sample no.18 and 21 showed substitution mutation at two sites. In sample no. 18, at amino acid position 88 leads to silent mutation because valine substituted by valine but at amino acid position 154 lead to methionine to leucine.

In sample no. 21, asparagine was substituted to histidine at amino acid position 167. By comparing with other study, there have been reported 17 variants of New Delhi metallo- β-lactamase-1 (NDM-1) in worldwide (Khan *et al.*, 2017). These all variants of NDM-1 have one or more than one substitution of nucleotide which results in different amino acid at that position. Of these variants, NDM-2 had a substitution of alanine at position 28 in place of proline, in *Acinetobacter baumannii* (Du *et al.*, 2016). Another variant NDM-3 with an amino acid substitution of Aspartate to Asparagine at position 95 was observed in *E. coli* (Rogers *et al.*, 2013). Among 9 NDM variants, substitutions of amino acids were identified at 7 different positions (28, 88, 95, 130, 152, 154, and 233), but which position(s) plays a critical role in the enzymatic activities, remained unclear (Tada *et al.,* 2014). NDM-4 variant showed substitution of amino acid from 154th Methionine to Leucine in *E. coli*. NDM-4 β-lactamase hydrolyzed all tested β-lactams except for aztreonam, just similar to other MBLs (Nordmann *et al.*, 2012). The substitution of Valine by Leucine at position 88 and Methionine by Leucine at position 154 was found in NDM-5, which was first detected in *E.coli* (Hornsey *et al.*, 2011). NDM-5 shows greater hydrolytic activity than NDM-1 toward carbapenems, cefotaxime, cephalotin and ceftazidime (Du *et al.*, 2016). NDM-6 showed substitution of Alanine to Valine at 233 positions, in E. coli (Kaase *et al.*, 2011).

Carbapenem hydrolyzing activity was gradually reduced from NDM-7 to NDM-5, NDM-6 and NDM-1 (Rahman *et al.*, 2014). NDM variants were found associated with all other groups of antibiotic resistance enzymes encoding genes i.e. ESBL, carbapenemase, Amp C. In blaNDM and its variants, due to genetic co-existence of other antibiotic resistant markers, there are limited options left to treat infections (Sidjabat *et al.*, 2015).

Recently, metabolite aspergillomarasmine A (AMA) which is found in fungi and its natural LLL isomer were identified to be effective inactivators of NDM-1 enzyme both in vivo and in vitro (Albu *et al.*, 2016). Also, the combination of levofloxacin and tigecycline was recently reported to successfully treat nosocomial pneumonia caused by NDM-1 producing Raoultellaplanticola (Pan *et al*, 2016).

Conclusion

In this study amikacin, cotrimoxazole, imipenem, polymyxin B and colistin demonstrated tremendous level of sensitivity against *K. pneumoniae*. Therefore, use of these antibiotics should be restricted to severe infections especially in critically ill patients, in order to stay away from emergence and rapid spread. Carbapenem resistance not only has enormous therapeutic implications, but is also important from the point of view of infection control. Such stains are known for rapid intra institutional spread and therefore, must be notified to infection control teams.

It is important to implement an easy, discriminatory, reproducible and cheap detection of MβL, ESβL and AmpC producers in our hospitals. Double disk synergy method is not only cost effective and highly sensitive but also easy to perform for detection of ESBL and MBLs.

Regular antimicrobial susceptibility surveillance is essential. An effective national and state level area wise monitoring of the resistance patterns antibiotic policy and draft guidelines should be introduced to preserve the effectiveness of antibiotics and for better patient management.

The vast evolution of resistant gene due to the selection stress and their spread among the non-pathogenic bacteria through horizontal gene transfer is one of the disturbing hazards to the health worker in the hospital settings in order to control infections. OXA-48 and NDM-1 and its variants producing pathogens were one of the alarming challenges, which have become even more critical. Now this is a point to consider practically the ways to check infections from hospital settings and to manage internationally for surveillance of such resistant genes producing pathogens. Accurate infection control guidelines need to be implemented worldwide. Surveillance should also be carried out to identify undetected asymptomatic carriers of carbapenem-resistant bacteria.

Recommendations:

- Nationwide survey should be carried out to understand the status of drug resistance in *Klebsiella pneumoniae.*
- Government level policy should be devised for implementation of Antimicrobial stewardship throughout the country.
- Antibiotics prescriptions should be strictly regulated such that instances of emergence of multi drug pathogens could be avoided.

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APPENDIX

50 x 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 volume 1000 ml to be made with Distilled Water

LB medium (Luria Bertani Medium)(pH 7.2)

Tryptone – 10 grams

Yeast Extract – 5 grams

Sodium Chloride – 10 grams

MacFarland Standard Composition

McFarland Standard No.	0.5
1.0% Barium chloride (ml)	0.05
1.0% Sulfuric acid (ml)	9.95
Approx. cell density (1X10^8 CFU/ml)	1.5

Tris– EDTA

10 mMTris (pH 8.0)

1 mM EDTA

Sequences of blaOXA gene from the isolated samples along with the reference sequence

>KU878974.1:51-240 Klebsiella pneumoniae strain 1219 OXA-48 family carbapenem-hydrolyzing class D beta-lactamase OXA-517 (blaOXA) gene, blaOXA-517 allele, complete cds

AATGCCTGCGGTAGCAAAGGAATGGCAAGAAAACAAAAGTTGGAATGCTCACTTTACTGAACATAAATC
ACAGGGCGTAGTTGTGCTCTGGAATGAGAATAAGCAGCAAGGATTTACCAATAATCTTAAACGGGCGA
ACCAAGCATTTTTACCCGCATCTACCTTTAAAATTCCCAATAGCTTGATCGCC

>Sample37

AATGCCTGCGGTAGCAAAGGAATGGCAAGAAAACAAAAGTTGGAATGCTCACTTTACTGAACATAAATC
ACAGGGCGTAGTTGTGCTCTGGAATGAGAATAAGCAGCAAGGATTTACCAATAATCTTAAACGGGCGA
ACCAAGCATTTTTACCCGCATCTACCTTTAAAATTCCCAATAGCTTGATCGCC

>Sample38

AATGCCTGCGGTAGCAAAGGAATGGCAAGAAAACAAAAGTTGGAATGCTCACTTTACTGAACATAAATC
ACAGGGCGTAGTTGTGCTCTGGAATGAGAATAAGCAGCAAGGATTTACCAATAATCTTAAACGGGCGA
ACCAAGCATTTTTACCCGCATCTACCTTTAAAATTCCCAATAGCTTGATCGCC

>Sample39

AATGCGTGCGGTAGCAAAGGAATGGCAAGAAAACAAAAGTTGGAATGCTCACTTTACTGAACATAAATC
ACAGGGCGTAGTTGTGCTCTGGAATGAGAATAAGCAGCAAGGATTTACCAATAATCTTAAACGGGCGA
ACCAAGCATTTTTACCCGCATCTACCTTTAAAATTCCCAATAGCTTGATCGCC

Sequences of blaNDM gene from the isolated samples along with the reference sequence

>KY446367.1 Klebsiella pneumoniae subsp. pneumoniae strain ST11 New Delhi metallo-beta-lactamase (blaNDM-1) gene, complete
cdsATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCATTGATGCTGAGCG

GGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTG
GTTTTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTT
CCAACGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCC
AGATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACA
AGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCC
CGCAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCG
CCCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACG
GCACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCG
ACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGCC
ATTCCGCCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGACAAGCTGCGCTGA

>Sample16

ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCATTGATGCTGAGCGGG
TGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTT
TTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCA
ACGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG
ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAG
ATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCG
CAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCGATGGCTGGGTCGAACCAGCAACCGCGCC
CAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC
ACCGACATCGCTTTTGGAGGCTGTCTGATCACGGACAGGCAGGACAAGTCATTCCCCCATCT

>Sample17

ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCATTGATGCTGAGCGGG
TGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTT
TTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCA
ACGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG
ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAG
ATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCG
CAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCC
CAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC
ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGAC
ACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGCCAT
TCCGCCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGACAAGCTGCGCTGA

>Sample18

ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCATTGATGCTGAGCGGG
TGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTT
TTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCA
ACGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGTTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAGA
TCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAGA
TGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGC
AAGAGGGGCTGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCC
AACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCA
CCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACA
CTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGCCATT
CCGCCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGACAAGCTGCGCTGA

>Sample19

ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCATTGATGCTGAGCGGG
TGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTT
TTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCA
ACGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG
ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAG
ATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCG
CAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCC
CAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC
ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGAC
ACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGCCAT
TCCGCCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGACAAGCTGCGCTGA

>Sample20

ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCATTGATGCTGAGCGGG
TGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTT
TTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCA
ACGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG
ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAG
ATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCG
CAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCC
CAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC
ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGAC

ACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGCCAT
TCCGCCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGACAAGCTGCGCTGA

>Sample21
ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCATTGATGCTGAGCGGG
TGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTT
TTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCA
ACGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAG
ATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAG
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CAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCC
CAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGC
ACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGAC
ACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGCCAT
TCCGCCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCCACAAGCTGCGCTGA