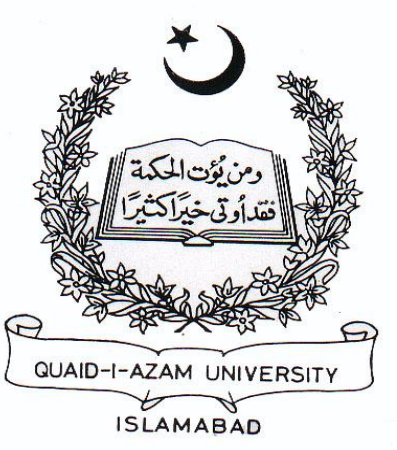
Plasmid Profile Analysis of NDM-1 Positive Isolates from Clinical and Environmental Settings



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2013

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A thesis submitted in the partial fulfillment of the requirement for the degree of Master of Philosophy in

Microbiology

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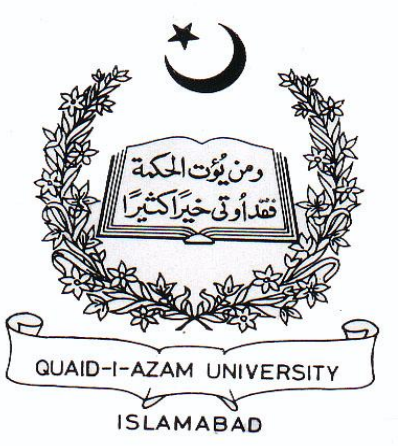

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(We commence) with the name of Allah
The most gracious (To begin with)*
The most Merciful (To the end)**

Dedicated

To
My beloved parents,

My beloved brother Walayat Khan

For their support, prayers and encouragement

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*All praises for **Almighty Allah** the most compassionate, the most beneficent and ever merciful, who gives me the power to do, the sight to observe and mind to think and judge. Peace and blessings of **Almighty Allah** be upon **His Prophet Hazrat Muhammad (P.B.U.H)** who exhorted his followers to seek knowledge from cradle to grave.*

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*May **Almighty Allah** shower **His** blessings and prosperity on all those who assisted me in any way during my research work.*

Amir Afzal Khan

DECLARATION

I hereby declare that the work presented in the following thesis is my own efforts and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Amir Afzal Khan

CERTIFICATE

This thesis by **Amir Afzal Khan** is accepted in its present form by the department of Microbiology, Quaid-i-Azam University, Islamabad, for the requirement of the degree of Master of Philosophy in Microbiology.

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Date: / /

List of Contents

List of Abbreviation

%	Percent
μg	Microgram
μl	Microlitre
ARG	Antibiotic Resistance Genes
ATCC	American type Culture collection
$CaCl_2$	Calcium Chloride
CLSI	Clinical Laboratory Standard Institutes` criteria
CRE	Carbapenem-resistant *Enterobacteriaceae*
E.coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic Acid
ESBL	Extended Spectrum Beta lactamases
GNB	Gram negative bacteria
LB	Luria Bertanai broth
MBL	Metallo-beta-lactamase
MDR	Multi-Drug Resistance
MHA	Mueller Hinton Agar
NaOH	Sodium Hydroxide
NDM-1	New Delhi Metallo-β-lactamase
NDM-1	New Delhi metallo-β-lactamases
PBP	Penicillin binding protein
PCR	Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
UK	United Kingdom
USA	United State of America
XDR	Extensively Drug Resistance

List of Tables

List of Figures

Abstract

Abstract

Multidrug resistance Gram negative bacteria are a great threat to public health. Metallo-β-lactamaese producing bacteria are becoming emerging threat by their increasing resistance to β-lactam antibiotics. The New Delhi metallo-β-lactamases (NDM-1) is a novel type of metallo-β-lactamase named after the city of origion which remains last choice of drug for the treatment of MDR microbial infection. NDM-1 was identified for the first time in clinical isolates of *K. pneumoniae* and *E. coli* from a Swedish patient who had sought medical care in New Delhi, India in 2008.

Total of 36 NDM-1 positive isolates (meropenem resistance) in which 5 were environmental and 31 were clinical. Tested strains were *E. coli, Pseudomonas spp, K. pneumoniae* and *Enterobacteriaceae*. Plasmid profiling of these isolates shows that all the tested isolates carry plasmid of different sizes and numbers. Plasmid stability of 3 isolates (*E. coli, Pseudomonas, Enterobacteriaceae*) in antibiotic free media was tested. All plasmids were stable after 15 passages of these strains in antibiotic free media. *Pseudomonas spp.* and *E. coli* lost plasmid during 8 and 10 passage respectively. To check the plasmid DNA transferability, we transformed plasmid DNA from *Pseudomonas* (meropenem resistance) to *Pseudomonas* (meropenem sensitive) and *Pseudomonas* ATCC (9027) by calcium chloride transformation method, growth on meropenem containing media plates. showed the successful transformation of the plasmid DNA to the meropenem sensitive.

Chapter 01

Introduction

Introduction

1.1 Resistance in Gram Negative Bacteria

Multidrug resistant (MDR) Gram negative bacteria (GNB) are a great threat to public health. MDR is defined as a pathogen showing resistance against three or more antibiotics at the same time (Obritch *et al*., 2005). Pan drug resistance (PDR) is a property of strains that shows resistance to all standardized antimicrobials particularly ceftazidime, cefepime, imipenem, meropenem, pipracillin/tazobactum, ciprofloxacin and levoflaxacin. While extensive drug resistance (XDR) is the ability of pathogens to significantly resist the potentially effective antibiotics (Falagas and Karageorgopoulos, 2008). In Gram negative bacteria, more antibiotic resistance has been observed due to which there are leaving very few antibiotics choices against them (Baiden *et al*., 2010). The most frequent type of resistance among GNB is due to the presence of β-lactamases encoded by gene carried either on plasmid or chromosome (Alhambra *et al*., 2004). Chromosomally mediated β-lactamases are present in many genera of GNR (Bradford, 2001). First plasmid mediated β-lactamase TEM-1 was reported in 1960, which spread quickly throughout the world and frequently identified in *Enterobacteriaceae* Other β-lactamases SHV-1 found to be present in *E.coli* and *K.pneumoniae* (Bradford, 2001). Resistance to broad-spectrum antimicrobials, e.g. Extended-spectrum cephalosporins, is a well-recognized problem to *Enterobacteriaceae* (Jacoby *et al*., 2005). carbapenems have served as an important antimicrobial class for the treatment of these organisms and resistance to carbapenems is uncommon among *Enterobacteriaceae* in the United States till date. However, the emergence of novel β-lactamases with direct carbapenem-hydrolyzing activity has contributed to an increased prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE). CRE are particularly problematic with the frequency similar to that of *Enterobacteriaceae* (Hidron *et al*., 2008). The high mortality rate has been associated with infections caused by CRE (Bratu *et al*., 2005; Schwaber *et al*., 2008) and the widespread transmission of potential carbapenem resistance is due to mobile genetic elements (Watanabe *et al*., 1991; Yigit *et al*., 2010)

1.2 What is NDM-1?

The *bla*$_{NDM-1}$ gene encodes New Delhi metallo-β-lactamase (NDM-1) which is a β-lactamase protein encoded by this gene and has 269 amino acids with a molecular mass of approximately 27.5 KD (Ghafur, 2010). Presence of NDM-1 makes a bacteria resistant to salbactam, clavulanic-acid and a lot of commercially accessible β-lactamase inhibitors. NDM-1 belongs to class B- carbapenemases. The class B carbapanemases have characteristic property of having $Zinc^{+}$ ion at their active site. EDTA is a zinc chelating agent and inhibits enzyme activity (Queenan and Bush, 2010). Metallo-β-lactamases also hydrolyze pencillins and cephalosporin but azteronem may not be hydrolyzed (Queenan and Bush, 2010). The *bla*$_{NDM-1}$ gene is accompanied by other antibiotic resistance genes like chloramphenicol, rifampicin, ciprofloxacillin and erythromycin. It is also accompanied by a genetic element, CMY-4 which is a broad spectrum β-lactamase and encodes for its efflux pump and promoter. A strain carrying *bla*$_{NDM-1}$ gene and other accompanying genes result in highly resistant phenotype (Moellering, 2010). It was reported that *bla*$_{NDM-1}$ also coexisted with aminoglycoside resistance gene like armA and *bla*$_{OXA}$ (Karthikeyan *et al*., 2010). Researchers have also observed that *Citrobacter freundii* carries *bla*$_{NDM-1}$ gene was accompanied by 9 different types of β-lactamases (Poirel *et al*., 2011). Plasmids carrying *bla*$_{NDM-1}$ gene may be transferred to other bacteria by conjugation, which shows its pathogenesis and mobility (Bonomo, 2011). In the absence of any characteristic of disease, positive NDM-1 isolate can get colonized in the gut. Mostly these microorganisms are normal commensals of the gut and it is a difficult task to screen out these microorganisms (Bonomo, 2011).

1 2 Genetics of NDM-1

The original source of *bla*$_{NDM-1}$ gene is still unknown but it is postulated that it has been captured from chromosome location of some unknown environmental microorganism by mobile DNA (Poirel *et al*., 2011). Clinical *K.pneumoniae* and *K.coli* with NDM-1 belong to a range of sequence types (Poirel, L. *et al*. 2011). The *bla*$_{NDM-1}$ gene can be carried by different types of plasmid (Inc/C, Inc/F,Inc/M,or untypeble) and more rarely, becomes chromosomally integrated (Samuelsen *et al*., 2010). Many of its host plasmids have a broad host range and are readily transferred by horizontal gene transfer in the related

Gram negative groups including *Enterobacteriaceae, Pseudomonas*, *Stenotrophomonas*, and *V.cholerae* (Walsh *et al*., 2011). Transfer of this gene is more efficient around 30°C corresponding to the average temperature during several month of the year in many Asian countries (Walsh *et al*., 2011).

1.3 Plasmid carriage of *bla*$_{NDM-1}$ gene

The *bla*$_{NDM-1}$ gene has been detected both on chromosome as well as on plasmid (Lagesen *et al*., 2007; Leung *et al*., 2011). Plasmid of 180 kb was initially reported to carry *bla*$_{NDM-1}$ gene (Yong *et al*., 2009). A successive study identified the gene on plasmids of various sizes ranging from 50-300kb that belonged to unlike Incompatibility group, including FI/FII, A/C, and an un-typed group. Large plasmids of various sizes carry *bla*$_{NDM-1}$ gene (Yong *et al*., 2009). Plasmid carrying *bla*$_{NDM-1}$ gene can easily be transferred to *Enterobacteriaceae* and *Escherichia coli* (Kumarasamy *et al*., 2010). This gene has also been found on plasmids carried by *Providencia spp.*, *Morganella morganii.*, *Citrobacter freundii., Proteus spp., Enterobacter cloacae*, *E. coli*, *K. oxytoca*, and *K. pneumonia* (Yong *et al*., 2009; Rolain *et al*., 2010). P271A, pNDM10505, pNDM-KN, pNDM-1-DoK01, pHK-NDM are the five plasmids which bear *bla*$_{NDM-1}$ gene and have been fully sequenced (Carattoli, 2009, Leung *et al*., 2011). *bla*$_{NDM-1}$ gene carrying plasmid pHK-NDM is an IncL/M 88,803 bp in size. Besides this, it also carries 16S rRNA methylase armA gene, which shows high resistance to aminoglycoside and β-lactams. This gene is also correlated to the plasmids carrying *bla*$_{CTX-M-3}$ gene (Leung *et al*., 2011). *bla*$_{NDM-1}$ gene carried by a plasmid p271A is 35947 bp in size, as a single resistance gene on while a new replicate gene was identified within an IncN2-type plasmid scaffold (Poirel *et al*., 2011). Plasmids pNDM10505, pNDM-KN and pNDM-1_Dok01 belong to IncA/C broad host range plasmid family. Plasmids carrying *bla*$_{NDM-1}$ gene are also able to carry the additional resistance genes, rmtC or armA, 16S RNA methylase gene and *bla*$_{CMY-2}$ gene. Their scaffold are extremely related to those of other Inc/C but in Canada, Europe and USA, the spread of *bla*$_{CMY-2}$ like gene in *Enterobacteriaceae* has been reported due to *bla*$_{NDM-1}$ negative plasmid (Sekizuka *et al*., 2011; Carattoli *et al*., 2012). It is significant that *bla*$_{NDM-1}$ gene was frequently reported to be positioned on plasmid supports. Moreover, it is recently identified that *Acinetobacter baumannii* carry *bla*$_{NDM-1}$ and

bla$_{NDM-2}$ on its chromosome and these have also been observed on the genome of *Pseudomonas aeruginosa* (Jovcic *et al*., 2011; Kaase *et al*., 2011). The region of the plasmid in which *bla*$_{NDM-1}$ gene is present have a high G+C% islands (61.89%; the average G+C% of the strains is 38.85%) flanked by two insertion sequence elements (ISAba11 and ISAba125). This shows that recombination plays a role in the transfer of these islands from waste and seepage sources, *Vibrio cholera*, *Citrobacter freundii*. In *E. coli*, the Inc/C plasmid has been identified in New Delhi isolates (Walsh *et al*., 2011). Overall, Inc/C type plasmid having broad host range and *bla*$_{NDM-1}$ gene has been reported from New Delhi both in environmental and clinical isolates (Walsh *et al*., 2011). Plasmid encoding *bla*$_{NDM-1}$ may be transfered is a temperature dependent process which is higher at 30°C as compared to 25°C or 37°C.

1.4 The Epidemiology of NDM-1

NDM-1 is a braod-spectrum β-lactamase that is able to hydrolyze most of available β-lactams antibiotics (Walsh *et al*., 2005). NDM-1 positive bacterial infection was first reported from UK, India and Pakistan (Kumarasamy *et al*., 2010). The spreading and dissemination of NDM-1 positive strains is occurred worldwide in countries including United States, Canada, Sweden, Japan, China, Australia, Oman, Africa, Belgium, France, Austria, Netherlands and United Kingdom (Rolain *et al*., 2010). Later studies documented the presence of *bla*$_{NDM-1}$ in a variety of isolates coming from different geographical areas (Kumarasamy *et al*., 2010). Resistant *Enterobacteriaceae* from UK and India were screened for the presence of NDM-1 and it was found that among 47 carbapenem resistant isolates, 13% were NDM- 1-positive. Isolates came from patient with a variety of infections such as community acquired, sepsis, wound infection, pneumonia and urinary tract infections (Kumarasamy *et al*., 2010). The same study reported *bla*$_{NDM-1}$ gene in *Enterobacteriaceae* collected from different areas of Indian subcontinent including Port Blair, Hyderabad, Kolkata, Pune, Bangalore, Varanasi, Mumbai, Guwahati and Delhi in India, Sheikhupura, Rahim Yar Khan, Lahore, Karachi, Hafizabad, Gujarat, Faisalabad and Charsadda in Pakistan and Dhaka in Bangladesh (Kumarasamy *et al*., 2010). The investigators also described that *Enterobacteriaceae* carrying carbapanemases increased rapidly in 2008 and 2009 in UK where among 73

NDM-1 positive isolates were 32 (44%) collected from 25 labs in UK. From 29 patients in UK, 17 had travelled to Pakistan or India within the previous year and during travel, 14 had been hospitalized due to renal or bone marrow transplantation, elective cosmetic surgery, dialysis and surgery (Kumarasamy *et al*., 2010). $bla_{\text{NDM-1}}$ carrying bacteria have also been found from the patients in other countries with a history of travelling to Pakistan or India and hospitalization. The spread of pathogenic microorganisms carrying $bla_{\text{NDM-1}}$ gene has now become a major global health threat (Liang *et al*., 2001). Recent findings suggest that Balkan states and Middle East might act as a secondary reservoir for the spread of NDM-1 because NDM-1 is thought to spread from Indian subcontinent to other countries.

1.5 Environmental Sources

Nordmann *et al.*, (2010) and Walsh *et al*., (2010) analyzed seepage and tape water samples in New Delhi and observed that NDM-1 was found in a wider diversity of Gram negative bacteria including *Enterobacteriaceae, Vibrio cholera, Pseudomonas spp, Aeromonas spp* and *Stenotrophomonas.* All *Enterobacteriaceae,* $bla_{\text{NDM-1}}$ positive isolates were MDR involved in the community acquired infection. Transmition occurred through fecal to oral by contaminated food and water (Walsh *et al*., 2011).

1.6 Clinical Sources

NDM-1 carrying *Enterobacteriaceae* has been reported from different clinical setups indicating the broad spectrum of the pathogens causing disease (Kumarasamy *et al*., 2011; Nordmann *et al.*, 2011). Most of the NDM-1 carrying clinical pathogens are both nosocomial and community acquired (Poirel *et al*., 2010; Jovcic *et al*., 2011a). Oral-fecal route is thought to be the main cause of acquiring $bla_{\text{NDM-1}}$ carrying oraganisms (Leverstein *et al*., 2010; Walsh *et al*., 2011). Though it is critical to treat infection with NDM-1 positive bacteria because of enhanced antibiotic resistance, however no enhanced Pathogenicity or virulence has been reported. Other clinical isolates of *Enterobacteriaceae* such as *Enterobacter cloacae, Klebsiella oxytoca, Proteus mirabilis, Citrobacter freundii* and *Providencia spp,* have been also reported for carrying $bla_{\text{NDM-1}}$ besides *K. pneumoniae* and *E. coli*. Different researchers have also identified NDM-1 in *A. baumannii* around the globe (Kumarasamy *et al*., 2011; Nordmann *et al*., 2011; Chen

et al., 2011; Karthikeyan *et al*., 2011). The presence of *bla*$_{NDM-1}$ has also been documented in waste and tape water samples in New Delhi with diversity of GNR including; *Enterobacteriaceae*, *Shigella spp*, *Pseudomonas spp*, *Strenophomonas spp*, *Aeromonas spp*, and *Vibrio cholera* (Walsh *et al*., 2011).

1.7 Aims and objectives

- Plasmid profile analysis of Gram negative bacteria isolated from clinical and environmental settings carrying *bla*$_{NDM-1}$ gene
- To access the stability of plasmids carrying the *bla*$_{NDM-1}$ gene.
- To access the transferability of *bla*$_{NDM-1}$ gene carrying plasmid.

Chapter 02

Literature Review

Literature Review

Resistance to antibiotics is a worldwide problem Studies have revealed that *Enterobacteriaceae* like *Klebsiella* and *E.coli* isolated from Indian origion show resistance to many antibiotics including the most effective antibiotic like carbapenems. Carbapenemases are enzymes; NDM-1 (New Delhi Metallo-β-lactamase-1) is a newly added enzyme responsible for this resistance. Which belong to class B Carbapenemases that require zinc ion at active site. The gene $bla_{\text{NDM-1}}$ is responsible for that enzyme. Few techniques can be used for the screening of $bla_{\text{NDM-1}}$ *Enterobacteriaceae* in laboratory. Disk approximation test or modified Hodge test can be used for the metallo-β-lactamase production. PCR can also be used for the detection of $bla_{\text{NDM-1}}$ gene by using specific primer for the target gene**.** High Mortality and Morbidity rate are associated with such infectious bacteria.$bla_{\text{NDM-1}}$ gene harboring *Enterobacteriaceae* are susceptible to two classes of antibiotics tigycycline and colistin which show in vitro activity. Thus both of these antibiotics are questionable as far as their safety profile is concerned. There is a need of safe antibiotics for the treatment of such kind of infection and vigorous screening of microorganism for $bla_{\text{NDM-1}}$ gene (Charan *et al*., 2012).

2.1 Carbapenemases

Carbapenemases characterize the most flexible family of β-lactamases that have no resemblance with other β-lactam hydrolyzing enzymes. Carbapanemases are almost hydrolysable enzymes while most are resilient to commercially practicable β- lactamase inhibitor (Livemore *et al*., 2006; Ohara *et al*., 1998; Wang and Mi, 2004). Several investigators have referred the term carbapanemases as they hydrolyze the carbapenems (Vourli *et al*., 2004; Queenan and Bush, 2007).

Mechanism of action of carbapenems is similar to other β-lactam antibiotics i.e. cell wall synthesis is inhibited in bacteria by binding and inactivating penicillin-binding proteins (PBPs). Bacteria resist carbapenems by using one of three adaptive machanisms; 1) bring changes in their PBPs, 2) degrade carbapenems by enzyme metallo-β-lactamase or 3) loss of outer membrane specific porin protein. Carbapenems (meropenem, doripenem,

imipenem) have broad spectrum invitro activity against anaerobic, Gram-positive and Gram-negative bacteria (Zhanel *et al*., 2007).

2.2 Beta- Lactamases classification

β- lactamases can be classified in two ways i.e. through amino acid sequence which are molecularly characterized, and they classified into four classes(ABCD). The three classes A, C, D, use serine for β-lactam hydrolysis, while class B uses Zinc ion for β-lactam hydrolysis .The updated functional classification scheme is based on the 1995 proposed by Bush *et al*., (2010) Updated scheme includes; group 2 (classes A and D) broad-spectrum, inhibitor-resistant and extended-spectrum-lactamases and serine carbapenemases; group 3 metallo -lactamases, and group1 contains (class C) cephalosporinases. The major groups also have other subgroups, which are based on specific attributes of individual enzyme (Bush *et al*., 2010).

2.2-1 Class A: Carbapanemases

Class A Carbapanemases have been described in unusual condition; some are chromosomally encoded (Sme, SFC-1, IMI, NmcA,) and other are plasmid encoded (IM1-2, *Klebsiella pneumonia* carbapenemases [KPC]. To some extent they are inhibited by clavulanic acid, but effectively hydrolyze carbapenems (Queenan *et al*., 2007). The most clinically important in this group are KPCs. In 1996 in *K. Pneumoniae* the first KPC was identified in eastern United States (Yigit *et al*., 2001). Now the KPCs producers have spread worldwide in few years near the eastern united state and especially in Greece, Colombia Puerto Rico, Israel, and China (Nordmann *et al*., 2009; Navon-Venezia *et al*., 2009). The KPC producers are identified in European country which causes the nosocomial infections, mostly *K.pnuemoniea* and to a much smaller extent from enterobacterial species and from *E.coli* especially in Israel (Nordmann *et al*., 2009). A single *K.pneumoniae* clone (sequence type [ST]-258) was identified, which are responsible for the spread of bla_{KPC} gene. Several KPC clones are disseminated within a given geographical site that has a different multilocus sequence type, plasmid structure, number and size, but the bla_{KPC} genes are linked with a single genetic element (transposan Tn4401) (Cuzon *et al*., 2010). The KPC producers which are associated with community acquired infection are rarely reported, with the exception of isolates from

Israel which was reported only a few years back. The level of resistance to carbapenems of KPC producers may vary markedly; ertapenem is the carbapenems that has the lowest activity (5–7), KPC producers are typically multidrug resistant (especially to all β-lactams), and also the treatment of KPC related infections is so difficult (Nordmann *et al*., 2009). The multidrug resistance KPC producer is resistant to many antibiotics especially to all β-lactams, and there is limited curative option. Mortility rate with KPC producer are high >50% (Borer *et al*., 2009; Patel *et al*., 2008; Schwaber *et al*., 2008).

2.2-2 Class B: Metallo-β-Lactamases

Class B metallo-β-lactamases (MBLs) generally of contain Verona integron–encoded metallo-β-lactamase IMP types, VIM, and the latest NDM-1 type (Queenan *et al*., 2007). In 1991, the first acquired IMP, MBL was reported in Serratia marcescens in Japan (Ito *et al*., 1995). Endemicity of IMP type and VIM type enzyme reported in Taiwan, Japan and Greece, although the outbreaks and single report of IMP and VIM producer were reported in many countries. Except azteoronem, all β-lactams are hydrolyzed by this enzyme (Walsh *et al*., 2005). Multidrug resistant *K. pneumoniae* are acquired from hospital are mostly responsible for the production of β-lactamases (Queenan *et al*., 2007; Walsh *et al*., 2005). Date rate of MBL producers is in range from 18% to 67% (Daikos *et al*., 2009). New Delhi mettallo-β- lactamase (NDM-1) discovered in 2008 in a Sweden patient in *Enterobacteriaceae,* which carry the $bla_{\text{NDM-1}}$ gene, are now focused worldwide (Yong *et al*., 2009; Nordmann *et al*., 2011). Except in south and Central America, on all continents the NDM-1 producer are known, which show a direct link with the Indian subcontinent in most cases. In Canada and united state few cases are reported. NDM-1 producer finding in Middle East and Balkan state showed that these regions are the secondary reservoir for that gene (Nordmann *et al*., 2011).

In addition to numerous other carbapanemases genes, the $bla_{\text{NDM-1}}$ gene is associated with non-clonally related species and isolates rather than a single clone, $bla_{\text{NDM-1}}$ gene has been identified in *K.pneumonia* and in *E.coli* mostly, and to a smaller level in other *Enterobacterial* species. The carbapenems resistance levels of NDM-1 producers may vary. $bla_{\text{NDM-1}}$ gene carrying plasmid are dissimilar, along with carbapanemases it also carries a number of resistance gene which are, plasmid-mediated such as,

cephalosporinase genes, ESBL genes, pandrug resistance macrolide resistance genes (esterase), carbapenemases genes (oxacillinase-48 [OXA-48] types, VIM types), amino glycoside resistance genes (16S RNA methylases), sulfa-methoxazole resistance genes, rifampin (rifampin-modifying enzymes). In a single isolate there is a rare chance of high number of antibiotic resistance gene, even among other carbapanemase producers. Tigycycline and colistin is most effective drug against NDM-1 producer, and to some extent to fosfomysin (Kumarasamy *et al*., 2010; Nordmann *et al*., 2011). The characteristics of NDM-1 are very disturbing for public health globally; in addition to other carbapanemases there characteristics are;

1) The $bla_{\text{NDM-1}}$ gene spread in environment in the unrelated species, not in a single species.
2) Frequent acquisition by a typical nosocomial pathogen *K.pneumoniae*, and the most common community acquired pathogen such as *E.coli.*
3) Size of reservoir the subcontinent has >1.4 billion person, NDM-1 producer in some area of Pakistan is <20 %, particularly NDM-1 gene have been identified in *E.coli* ST type 131 which is a source of community acquired infection (Coque *et al*., 2008).

In children the most common cause of diarrhea is *E.coli*. These pathogenic strains e release in the environment and increase the risk, and also spread in human. In New Delhi, among many unrelated Gram negative species, in the environmental and tape water the NDM-1 producers have been identified (Walsh et *al*., 2011).

2.3 Types of MBL

Following are the acquired metallo-β-lactamases are the IMP-type, VIM-type and NDM-1 type.

2.3-1 IMP-Type

The IMP type enzyme, first detected in Japan in the late 1980 (Osano *et al*., 1994), and now in Gram negative bacteria non fermenter (mostly in *P.aeruginosa* and *acinetobacter spp*) and in *Enterobacteriaceae* it is prevalent worldwide. The IMP variant are (IMP-1, IMP-4, IMP-7) which distribute worldwide and know their potential for intercontinental spread (Miriagou *et al.*, 2010).

2.3-2 VIM-Type

VIM type enzymes were first detected in *P.aeruginosa* and in other Gram negative bacteria non fermenting species of *Enterobacteriaceae.* The VIM type metello-β-lactamases show even broader substrate specification then do the IMP-types, being able to hydrolyse 6 α methoxy pencillin. Furthermore the VIM-type enzyme is unique in the metallo-β-lactamases because they have a high affinity for carbapenems (Docquire *et al*., 2003).

The NDM-1 is one of the latest additions to acquire metallo-β-lactamases. And its propensity for intercontinental dissemination is of great concern. NDM-1 was first detected in a strain of *K. pneumoniae* isolated in 2008 in a patient returning to Sweden from India, where NDM-1 is widespread in *Enterobacteriaceae* (Kumarasamy *et al*., 2010).

2.3-3 Class D Enzymes of the OXA-48 Type

In a *K. pneumoniae* strain, in Turkey the OXA-48 producer was first identified in 2003 (Poirel *et al*., 2004). Since then from Turkey the OXA-48 producer reported extensively in nosocomial outbreak (Carrer *et al*., 2004; Poirel *et al*., 2011). Now it is distribute worldwide in Africa, Europe, in the southern and eastern part of the Mediterranean Sea (Nordman *et al*., 2004; Poirel *et al*., 2011). In Canada and United State, the OXA-48 has not been identified. OXA-181 which is a point mutant analog of OXA-48 have similar carbapenems activity, recognized in strains of India (Castanheira *et al*., 2006-2007; Kalpoe *et al*., 2011). The OXA-48 producer are now identified in Spain, Germany, France, United Kingdom, Netherland, through hospitalized patients from disease endemic areas that are the source of hospital outbreak. A plasmid of 62.5kb is associated with the resistance and dissemination of OXA-48 producing clones, which was previously identified in approximately 70kb plasmid. The OXA-48/OXA-81 is unusual because they weakly hydrolyze broad spectrum cephalosporin, and carbapenems, such as azteronem, and ceftazidime (Poirel *et al*., 2004; Castanheira *et al*., 2006-2007). They are resistant to amoxicillin/clavulanic acid, and their activity is not inhibited by clavulanic acid or EDTA. Although reported in a variety of *Enterobacterial* species, mostly in *E.coli* and *K.pneumoniae* the OXA-48 producer have been identified, which show higher resistance

level usually to carbapenems when ESBL and permeability defects are associated. The most difficult identified carbapanemases producer is the OXA-48type producer. So, their exact incidence could not be underestimated. The exact mortality rate due to OXA-48 producer remains unknown.

2.4 Global Threat

Multidrug resistance *E.coli* was isolated in Australia which carries the β-lactamases. It is the first coming out of $bla_{\text{NDM-1}}$ gene in Australia, when its molecular characterization was done. And it also carry the other β-lactamases CTX-M-15 together with two 16S rRNA methylases, namely, ArmA and RmtB, which confer a high level resistance to aminoglycosides (Poirel *et al*., 2010).

In one study in India in 2006 and 2007, carbapenems resistant *Enterobacteriaceae* are isolated out of 39, 15 strain carry $bla_{\text{NDM-1}}$ gene and 10 isolate have the carbapenems variant the bla_{OXA} 181, $bla_{\text{VIM-6}}$ are carried by one of *E.cloacae* and the *K.pneumoniae* strain which already have $bla_{\text{OXA-}}$181, also carry the $bla_{\text{VIM-5}}$. Patterns of Multiple pulsed-field gel electrophoresis and Clonal diffusion within and among sites were observed. Isolate producing $bla_{\text{NDM-1}}$ gene was spread in Indian health concern facility as early as 2006 (Castanheira *e*t *al.,* 2010).

NDM-1 is a new type of metallo-β-lactamases. *Enterobacteriaceae* carry the $bla_{\text{NDM-1}}$ gene is known worldwide. Bacteria producing $bla_{\text{NDM-1}}$ gene not only resistance to carbapenems, but it also show high resistance towards many classes of antibiotics. Due to this high resistance, these bacteria may be identified as well as they cause severe infection and it is necessary to avoid their transmition. Researchers have reported in Taiwan that a *klebsiella pneumonia* carry the $bla_{\text{NDM-1}}$ gene were colonized in a patient with a wound (Shin *et al*., 2010).

Dermott *et al*., (2012) reported the *Klebsiella pneumoniae* having NDM-1 in Ireland, which was resistant to many antibiotics classes as well as carbapenems. Plasmid 0f 98 kb size carries the $bla_{\text{NDM-1}}$ gene, and the presence of this gene was confirmed by PCR analysis. This organism was isolated from the child borned in India and moved to the

Ireland at four month age. In Ireland this was the first finding of *Enterobacteriaceae* strain that carry the *bla*$_{\text{NDM-1}}$ gene.

Carbapenems resistant Gram negative bacteria forwarded for the screening of *bla*$_{\text{NDM-1}}$ gene. To date, 321 carbapenems-resistant isolates, encompassing 11 bacterial species, have been tested. In Afghanistan from a military hospital two strains of *Providencia stuartii* were found *bla*$_{\text{NDM-1}}$ positive, both strains were the same when analyzed by pulsed-field gel electrophoresis (PFGE). The large plasmid pMRO211 carry the *bla*$_{\text{NDM-1}}$ gene is 178277bp in size which is sequenced by emulsion PCR and pyrosequencing which belongs to incompatibility group A/C. These plasmids carry many antibiotic resistance genes and have considerable homology to pAR060302 which was isolated from *E.coli.* The plasmid also shares common element with the pNDM-HK plasmid, sul 1, *bla*$_{\text{NDM-1}}$, armA. Still the gene orientation is inverted and from pMR0211 this 3 fragment region is absent. Plasmid pMRo211 also contain extra genes including the bla (OXA-10) gene the quinolone resistance gene qnrA, a gene with highest homology to a U32 family peptidase from *Shewanella amazonensis* the amino glycoside-modifying enzyme loci aadA and aac (6'). It is especially worrisome to find this gene in an intrinsically colistin-resistant species such as *Providencia stuartii*, as it renders the organism resistant to nearly every available antibiotic. Multiple insertion sequences and transposons flanking the region containing the *bla*$_{\text{NDM-1}}$ gene are present (McGaan *et al*., 2012).

NDM1 positive isolates identified in Chennai was 44,37in UK, 73 in Pakistan and India, and 26 in Haryana. Mostly the *E.coli* and *Klebsiella pneumonia* carry the *bla*$_{\text{NDM-1}}$ gene which shows resistance to mostly all antibiotics except colistin and Tigycycline**.** From Haryana the *Klebsiella pneumonia* isolate were Clonal but from the Chennai and UK the NDM-1 producer were clonally assorted**.** Most isolates carried *bla*$_{\text{NDM-1}}$ gene on plasmid, Plasmid from Chennai and UK were readily transferable While from Haryana mostly plasmid are non conjugative. Many patients from UK have NDM-1 positive which were travelled to Pakistan or India in the past year, or association with these country (Kumarasamy *et al*., 2010).

2.5 Environmental aspects

In New Delhi from Sept 26 to October 2010, 50 tap water, 171 seepage samples, and 70 samples of sewage effluent were collected from the Cardiff waste water treatment. They found two $bla_{\text{NDM-1}}$ gene out of 50 from drinking water sample and 51 out of 171 seepage sample, and the gene was absent in Cardiff samples. Out of 171, 12 NDM-1 positive bacterial isolates were grown and from tap water samples 2 out of 50, and 51 out of 171 from the seepage sample, and the Cardiff water sample from which no $bla_{\text{NDM-1}}$ gene is identified. From the seepage samples, 12 samples out of 171 are grown, which carry the $bla_{\text{NDM-1}}$ gene and from the water samples 2 out of 50, the other 11species such as *Vibrio cholera* and *Shigella boydii* in which $bla_{\text{NDM-1}}$ gene was not previously reported. From the samples, 20 isolates carry the bla_{NDM1} gene on plasmid size range from 140 to 400 kb. *Vibrio cholera* and *Aeromonas* isolate carry the same $bla_{\text{NDM-1}}$ gene, but it located on chromosome. Conjugative transfer was more common at 30°C than at 25°C or 37°C (Walsh *et al*., 2011).

The spread of $bla_{\text{NDM-1}}$ gene is gaining global attention. Various bacteria carry the $bla_{\text{NDM-1}}$ gene on large plasmids, while it was first found in *Klebsiella* pneumonia. Hongyan *et al*., (2012) reported the complete sequence of pNDM-BJ01, and its alternative pNDM-BJ02 plasmid, which have the $bla_{\text{NDM-1}}$ gene in clinical isolates of *Acinetobacter iwoffi* strains. A novel plasmid with an unknown protection mechanism which not include in any of the known incompatibility group, reported a size of 47.3 kb which is known as pNDM-BJ01 **(**Hongyan *et al*., 2012**)**.

From municipal waste water treatment plant effluents in the Czech Republic, the multidrug resistance bacteria *Klebsiella* and *E.coli* species carrying extended spectrum β-lactamases have been isolated. The recent spread and emergence of carbapenemases encoding gene universal in *Acinetobacter baumannii*, *Enterobacteriaceae,* and *Pseudomonas aeruginosa*, especially OXA, IMP, VIM, KPC, and NDM, which shows how antibiotic resistance gene can quickly increase in the bacterial population as a model of self-interested gene development and in public drinking water in New Delhi several bacteria were found which carry the $bla_{\text{NDM-1}}$ gene (Sekizuka *et al*., 2011).

2.6 Clinical Review

In India in 2009 a *Klebsiella pneumonia* isolate characterized which were carbapenems resistant carry the $bla_{\text{NDM-1}}$ gene, and from the Swedish patient the *E. coli* who had sought medical care in New Delhi, India. Colistin was the only antibiotic which is more effective against these strains. The $bla_{\text{NDM-1}}$ gene encoding the β- lactamases has become visible with the world wide detection of NDM-1 producers (Poirel *et al*., 2009).

An Indian origin Swedish patient acquired a urinary tract infection caused by *Klebsiella pneumonia* strains which are carbapenems resistance, when traveled to New Delhi. The *Klebsiella pneumoniae* 05-506 isolate acquire the metallo- β- lactamase but was negative for earlier identified MBL genes. In addition to *K. pneumoniae* 05-506, 140 kb plasmid was found to carry $bla_{\text{NDM-1}}$ gene in an *E.coli* strains isolated from the patient feces, inferring the possibility of in vivo conjugation. The extensive resistance passed on this plasmid is an extra distressing progress in India. Though the carbapenemases are more and more being reported, the most common of these would showed to be KPC, which are recently been characterized in the India, Israel, United States , China, Turkey, Nordic countries and the United kingdom (Rolain *et al*., 2012).

The Gram negative bacterium which produces ESBL is multidrug resistance against which the carbapenems are the most practical antibiotics. Resistance to carbapenems is due to the production of β-lactamase and by loss of outer membrane proteins that is capable of hydrolyzing carbapenems. The patient hospitalized in Asia pacific region carry the new type of β-lactamases known as NDM1. This Plasmid carry the broad resistance which is a matter of concern for India, several bacterial infections can be treated by β-lactam drug, carbapenems often becomes the last remedy. Resistance to Carbapenems is due to the carbapenemases, when it emerged and spread from hospital acquired infection since 2000. Carbapenemases fluctuate from one another, including class A and class D (serine carbapenemases) and class B (metallo-β- lactamases). In United States, Israel, United Kingdom, and southern Europe and in Asia the KPC-type class –A Carbapenems from *Klebsiella pneumonia* are most widespread which the most common Carbapenemase in *Enterobacteriaceae*. Antimicrobial resistance in bacteria is mainly due to the population movement and globalization. Due to discovery in New Delhi it is

named as NDM1 because of its novelty. Worldwide dissemination of a new 'superbug' is the issue of concern, since an alarming report was from Asia Pacific region An *E.coli* strains (DVR22) which is carbapenems resistant, isolated from the patient stool specimen which have traveler diarrhea, who had travelled to India. Molecular identification shows the presence of bla_{NDM-1} gene. A plasmid of 300kb which is a conjugative plasmid carry the bla_{NDM-1} gene, and it also contained blaTEM-1, armA, blaDHA-1, blaCTX-M-15 gene; In addition, bla_{NDM-1} was preceded by an ISAba125 insertion element only found in *Acinetobacter spp* (MarSole *et al*., 2011).

The recent increase of the bla_{NDM-1} gene encoding the metallo-β-lactamases in *Enterobacteriaceae* is associated to a variety of neighboring genetic structure and plasmid scaffolds**.** With the help of high density pyrosequencing, the whole sequence of plasmid pGUE-NDM carrying the bla_{NDM-1} gene was determined and a comparative genomic analysis was performed with other bla_{NDM-1} negative IncFII. *E.coli* carrying the plasmid pGUE-NDM confers resistance to many antibiotic molecule including sulfonamides, Trimethoprim, aminoglycosides and β-lactams. The plasmid size is 87,022bp, and along with two β-lacatmase gene bla (OXA-1), bla_{NDM-1} it also carry three aminoglycoside resistance gene aacC2, aacA4, aadA2. A multidrug resistant locus contained a module encompassing bla_{NDM-1} gene as indicated by comparative analysis and it is basically conserved among different structures identified in other enterobacterial isolates. This element was constituted by the bla_{NDM-1} gene, a bleomysin resistance gene and a fragment of insertion sequence ISAba125. The plasmid IncFII type carry the bla_{NDM-1} gene is first characterized. Such alliance between IncFII plasmid and the bla_{NDM-1} gene backbone is awfully irritating considering that this plasmid type is known to spread well, as exemplified with the global dissemination of bla (CTX-M-15) born IncFII plasmids (Bonnin *et al*., 2012).

Genetic features associated with the bla_{NDM-1} gene were investigated in, 1 *Proteus mirabilis*, 1 *Citrobacter freundii*, 6 *Escherichia coli*, 1 *Providencia stuartii*, and 7 *Klebsiella pneumoniae*, are isolates of worldwide origin. For Both *K.pneumoniae* and *E.coli* Clonal diversity was observed. The plasmid encoded bla_{NDM1} gene are carried by different plasmid type (IncL/M, IncF, IncA/C, or untypable and in two isolates it is

chromosomally encoded. The *bla*$_{NDM-1}$ plasmids coharbored a variety of resistance determinants, including quinolone resistance genes and 16S RNA methylase genes and β-lactamase genes (Poirel *et al*., 2011).

Plasmid mediated *bla*$_{NDM-1}$ gene encoding the metallo-β-lactamase is dispersed globally, mostly in members of the *Enterobacteriaceae* which origin is India subcontinent (Jeanette *et al*., 2012).

The multidrug resistance *E.coli* strains 271 carrying the blaNDM1 gene which is plasmid encoded were analyzed by high-throughput genome sequencing. A plasmid(p271A) of 35.9 kb size carry the *bla*$_{NDM-1}$ gene and obsessed an IncN-type backbone that harbored a novel replicase possession of the *bla*$_{NDM-1}$ gene on plasmid p271A had been likely the result of a cointegration incident involving the transposase of Tn5403 (Poirel *et al*., 2011).

In *Klebsiella pneumonia* and *E.coli* the *bla*$_{NDM-1}$ gene was initially identified but recently reported to arise in *Providencia spp*. *Enterobacter cloacae* isolates (24, 32, 40), *Morganella morganii*. Significantly *bla*$_{NDM-1}$ reported mainly plasmid. Studies shows that plasmid (p271A) in *E.coli* harbored the *bla*$_{NDM-1}$ gene that does not carry other résistance determinants and that was not typable by using by using the PCR based replicon typing (PBRT) technique. In *E.coli bla*$_{NDM-1}$ gene carried by plasmid p271A (Yong *et al*., 2009).

A 180 kb plasmid originally carry the *bla*$_{NDM-1}$ gene, but the incompatibility group (Inc) could not be defined. Plasmid of various sizes (50-300kb) carries the *bla*$_{NDM-1}$ gene that belongs to diverse Inc groups, including FI/FII, A/C, and an untyped group. In *vibrio cholera, E.coli* and *Citrobacterbacter freundii* isolates from New Delhi waste seepage the IncA/C plasmid have been identified. *bla*$_{NDM-1}$ gene carrying plasmid IncL/M pNDM-HK first complete sequence has been reported. An *E.coli* NDM-1_Dok) 01 strain carrying the *bla*$_{NDM-1}$ gene on Inc/C pNDm-1_DOKo1 plasmid isolated from the first case in Japan has completely sequenced (Charan *et al*., 2012).

Study revealed that *bla*$_{NDM-1}$ gene carrying *Enterobacteriaceae* was present in New Delhi from as early as 2006. A case report by yong et al, 2009 study show significant addressing the problem that multidrug resistance microorganism carrying the *bla*$_{NDM-1}$ gene. From 59 year old Swedish patient local of Indian origin patient multidrug resistance

Klebsiella pneumonia isolated from urine sample carry the *bla*$_{\text{NDM-1}}$ gene is the first case which is reported. The patient was suffering from type 2 diabetes with a history of multiple strokes. During his stay in India, he was operated for gluteal abscess at Ludhiana (Punjab), and for decubital ulcer at New Delhi (Yong *et al*., 2009).

In an *E.coli* a plasmid of 140 kb was found harbouring the *bla*$_{\text{NDM-1}}$gene, as known in *K. pneumoniae*. Due to this plasmid the broad spectrum resistance is a further perturbing development for India, shows high level of antibiotic resistance (Villa *et al*., 2012).

A plasmid of 250kb in size carry the *bla*$_{\text{NDM-1}}$gene was identified in New Delhi, India, in some strains which are isolated from public tape water and seepage samples, the plasmid is non-typeable by using the current PCR-based replicon typing (PRBT) scheme. Conjugative transfer was more common at 30°C (Islam *et al*., 2012).

Chapter 03

Materials and Methods

Materials and Methods

The experimental design was made to carry out the plasmid profile, plasmid stability and plasmid transformation of Gram negative bacteria carrying the *bla*$_{NDM-1}$ gene in Clinical and Environmental isolates

3.1 Isolation of resistant strains

Each isolate was inoculated on MacConkey media supplemented with meropenem antibiotic 0.5g/1000ml to isolate resistant gram negative bacteria producing metallo-β-lactamase. Isolated strains were further subcultured on meropenem MacConkey media plates for obtaining pure and resistant strains.

3.2 Detection of MBL in isolated strains

The isolated resistant strains were further detected for metallo-β-lactamase (MBL) production. The test organisms were inoculated on Mueller-Hinton Agar (MHA) plates. The combined disc was prepared by pouring 4µl of 5M EDTA (3.72gm/20ml) on meropenem disc and incubate for 5 minutes at 37°C and kept in fridge at 4°C until used. The strain were spread with swab on autoclaved fresh MHA plates and meropenem with EDTA disc and simple meropenem disc were placed on MHA plates and kept in incubator for 24 hours at 37 °C. The inhibition zones of meropenem and meropenem-EDTA disc were compared after 24 hour. All the pure MBL producing strains were preserved on nutrient agar slants and store at 4°C.

3.3 Identification of isolates

All the isolates were first identified by sub culturing on *Pseudomonas* Cetrimide agar and *Samonella Shigella* agar to identify directly *Pseudomonas, Salmonella, and Shigella* spp. the culture was taken and streaked by loop on these media plates. They were incubated for 24 hour at 37°C.

3.4 Biochemical characteristics

Isolates strains of bacteria were biochemically characterized according to Bergay,s manual of determinative Bacteriology(9th edition) and the following tests were performed.

3.4-1 Oxidase test

This test was done to distinguish among groups of bacteria on the basis of cytochrome Oxidase activity. Oxidase enzyme plays a vital role in the operation of electron transport system during aerobic respiration. *Pseudomonas* is Oxidase positive and *Enterobacteriacece* are Oxidase negative.

A piece of filter paper was placed in clean Petri dish and 2-3 drops of freshly prepared reagent Tetramethyle –p-phenyl –diamine- dihydrochloride was added. Using a piece of stick or glass rod, a colony of test organism was streaked on the filter paper. A development of blue colour indicates positive results.

3.5 Plasmid DNA isolation

E. coli was grown in Lauria-Bertani (LB) containing meropenem antibiotic (0.5mg/L). LB medium (5ml) containing *E. coli* cells was centrifuged at 14000 rpm for 1 minute to separate the bacterial cell in form of pellet and added 1ml TE Buffer (1X) to dissolve the pellet completely. The suspension was centrifuged for 3 minute and supernatant was removed. Solution 1 (100µl) was added to the pellet and kept for 5 minutes and then added solution II (150µl) and mixed well by inverting gently 4-5 times followed by addition of Solution III (200µl) and mixed well. Equal volume of chloroform was added and centrifuged for 15 minutes at 14000 rpm. Supernatant was transferred in a fresh eppendorf tube and supernatant was discarded. DNA was precipitated by ice cold isopropanol (600µl) and centrifuged at 14000rpm for 5 minutes. Supernatant was discarded and pellet was washed with 70% chilled ethanol. DNA pellet was air dried followed by dissolution in TE buffer (50µl) and was stored at -20°C.

Lysis solution I (100ml)

50mM glucose 0.9008g

25mM tris-Cl (M.W=121.1) PH=8 0.3025g

10mM EDTA 0.372g

Store at 4°C

Lysis solution II 10ml freshly prepared

0.2N NaOH (0.8g)

1% SDS (100 mg)

Lysis solution III 100ml

5M potassium acetate 29.25g

Glacial acetic acid 11.5 ml

Water 28.5ml

3.6 Plasmid Stability assays

For the plasmid stability, *E.coli, Enterobacteriaceae* and *Pseudomonas spp*. were taken from preserved bacterial culture at -70°C and refreshed on nutrient agar plates containing meropenem. bla_{NDM-1} positive isolates were grown on antibiotic free and meropenem (0.5mg/ml) containing media. To assess the plasmid stability, isolates were grown over night in antibiotic free media and plasmid was isolated while proceed another overnight grown culture for the plasmid extraction. The same process was repeated till 15 passages.

3.7 Plasmid DNA Transformation

Preparation of competent cells,

Pseudomonas strain was grown overnight at 37°C in 5 ml LB broth and 1ml of this overnight culture was diluted in 50ml fresh LB medium followed by keeping in shaking incubator for two hours at 37°C. Culture (1ml) takes in 2ml Eppendorf tube and spin at 4000rpm for 3 minutes. Supernatant was discarded and pellet was resuspended in freshly prepared 500µl $CaCl_2$ (0.1M) solution and kept on ice for 30 minutes followed by spinning for 30 second at 6000rpm at 4°C. Hole in Bacterial pellet indicated that the cell were competent but in our experiment hole was not formed. Supernatant was removed and pellet was resuspended in 100 µl of $CaCl_2$ (0.1M).

Transformation of plasmid DNA

Plasmid DNA of the meropenem resistance strain was added to the competent cell and mixture was kept on ice for 30 minutes followed by heat shock in water bath for 5 minutes at 42°C. LB (400µl) was added to the cell for the expression of plasmid encoded

antibiotic resistance marker beta-lactamases gene and kept for 1 hour at 37°C in shaking incubator. At the end, cells (100µl) were spreaded on selective medium plate with and without meropenem antibiotic to check the transformation.

3.8. PCR Reaction

The strains were screened for the presence of known mobile MBL genes (*bla* $_{NDM-1}$) by PCR with the primer reported in Gene bank (National Center for Biotechnology Information, National institute of Health, MD, USA) Database entry AB571289.1 (http;//www.ncbi.nlm.nih.gov/nuccore/300422615).

The forward primer NDM-1 gf 5`-ACC GCC TGG ACC GAT GAC CA-3` and Reverse primer NDM-1gr 5`-GCC AAA GTT GGG GGC CGC GGT TG-3` were used.

PCR conditions were the following; initial denaturation at 95C° for 5 minute; 95C° for 1 minute, extension at 72 C°for 1 minute and annealing at 52 C° for 1 minute is proceed for 30 cycles. The amplified DNA is stored at -4 C°.

3.9. GEL Electrophoresis

PCR products were loaded on 1% agarose gel. Electrophoresis was carried out for 1 hour at 80 V. The bands were visualized in UV Transilluminator (UVltec, EEC) and digital photograph was taken.

Chapter 04

Results

Results

The present study was conducted to analyze and characterize the plasmid profile of $bla_{\text{NDM-1}}$ gene harbouring Gram negative bacteria isolated from environmental and clinical sources, previously characterized by M.Phil research fellows. The environmental isolates were taken from rivers, lakes, seepage, and drinking water from Islamabad while the clinical isolates were obtained from Pakistan Institute of Medical Sciences, a tertiary care hospital in Islamabad.

4.1 Bacterial Strains

A total of 36 isolates were included in this study where 5 were from environmental source and 31 were clinical. Bacterial strains were first identified by sub culturing on specific and differential media. MacConkey agar medium was for differentiation between lactose fermenter and non-fermenter isolates. Out of 36 strains, 10 were *Enterobacteriaceae spp*., 12 were *Pseudomonas spp.*, 10 were *K. pneumoniae*, and 4 were *E.coli.*

4.2 Biochemical characteristics

Isolated strains of bacteria were biochemically characterized according to Bergay's manual of determinative bacteriology (9^{Th} edition).

4.3 Plasmid Profile analysis

The plasmid profile of *Enterobacteriaceae spp*., *Pseudomonas spp.*, *K.pneumoniae,* and *E.coli* shows that these NDM-1 positive isolates carry different sizes of plasmid.

4.3.1 Plasmid profile of *Enterobacteriaceae spp.*

Plasmid profile analysis of *Enterobacteriaceae spp*. shows different plasmids carried by these isolates. Out of 10 isolates, 2 isolates showed 3 plasmids, 7 isolates displayed 2 plasmids while one plasmid was seen in 1 isolate. All the plasmids were of >10kb in size. The plasmids separated on gel are shown in Figure 1 and the details are given in Table 1.

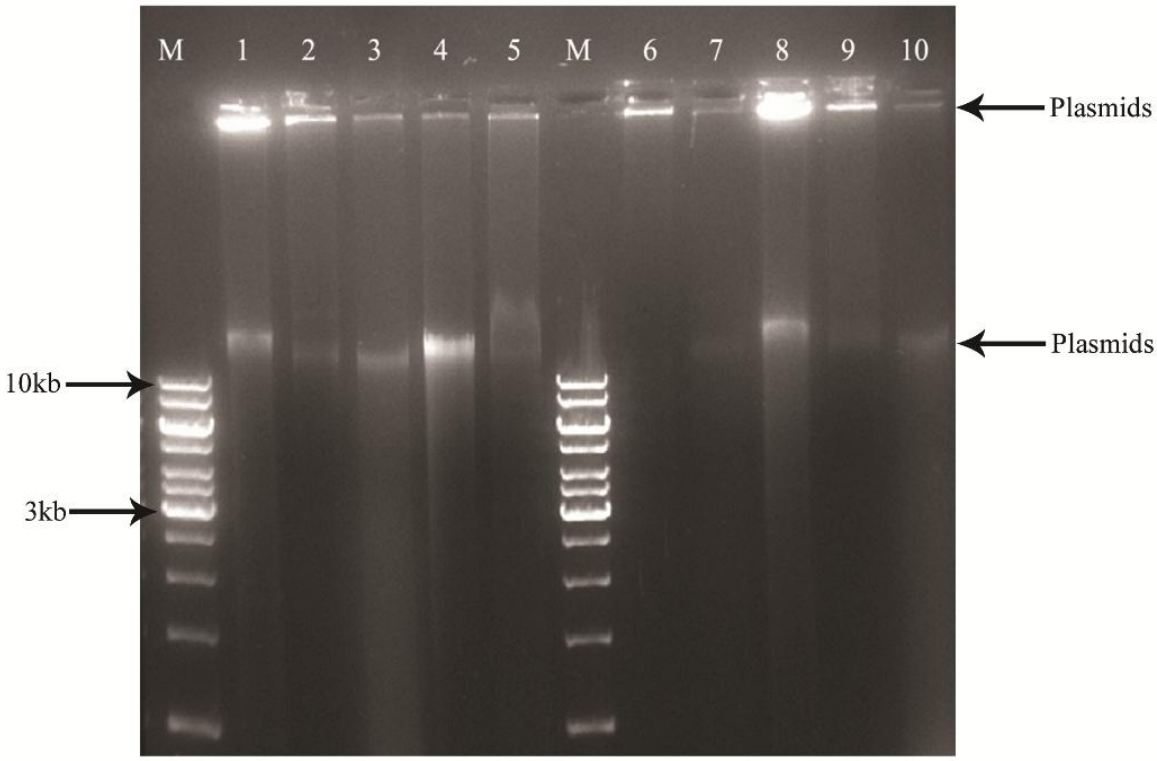

Figure 1: Plasmid profile of *Enterobacteriaceae spp.* Lanes 1-10 show the plasmids (highlighted by arrow) carried by these isolates and lane M shows 1 kb DNA ladder.

Table 1: Plasmids of *Enterobacteriaceae spp.*

S. No	Strains code	Plasmids	Plasmid Size
1	P-8	Two	>10kb
2	P-6	Three	>10kb
3	7	Two	>10kb
4	1	Two	>10kb
5	3	Three	>10kb
6	FE	One	>10kb
7	6	Two	>10kb
8	Alm +	Two	>10kb
9	9	Two	>10kb
10	308	Two	>10kb

4.3.2 Plasmid profile of *Pseudomonas spp*

Plasmid profile of *Pseudomonas spp* shows different plasmids carried by these isolates. Out of 10 isolates, 5 isolates showed 2 plasmids, one isolate showed 6 plasmids, 2 isolates showed 1 plasmid, one isolate showed 4 plasmids, while no plasmid was seen in 1 isolate. All plasmid were of >10kb in size. The plasmids separated on gel are shown in Figure 2 and the details are given in Table 2.

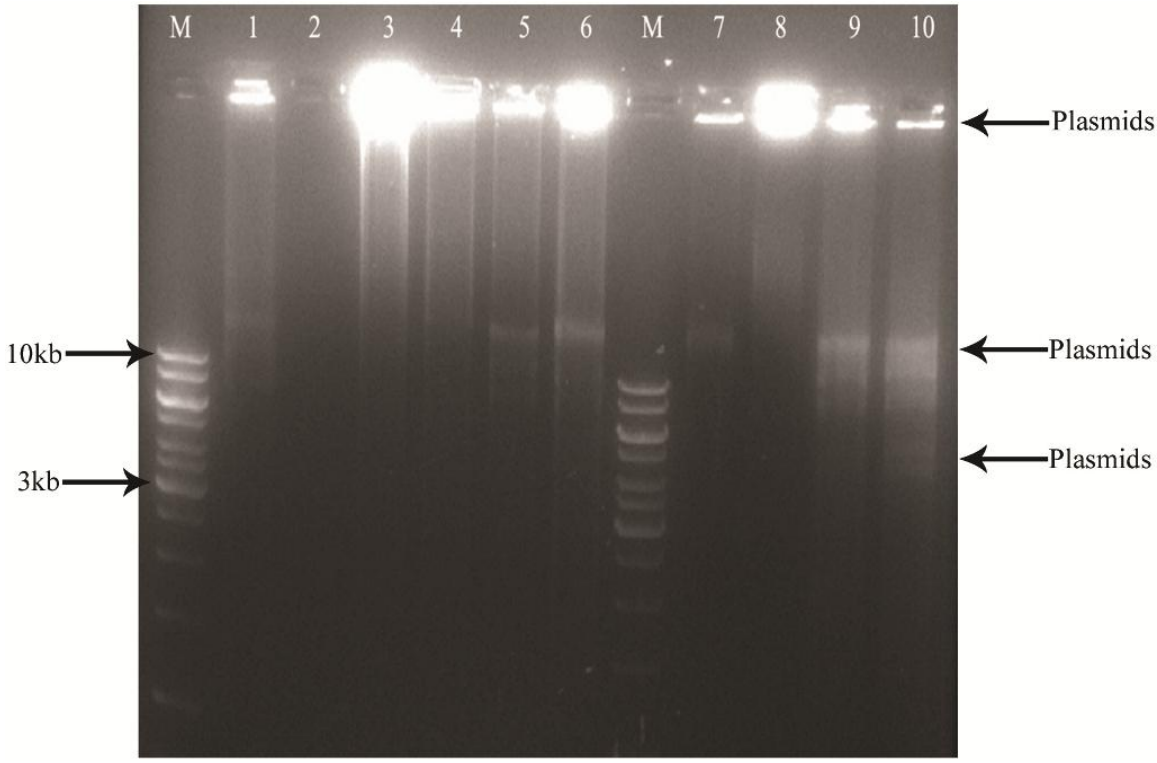

Figure 2: Plasmid profile of *Pseudomonas spp.* Lanes 1-10 show the plasmids (highlighted by arrow) carried by these isolates and lane M shows 1 kb DNA ladder.

Table 2: Plasmid profile of *Pseudomonas spp*

S. No	Strains code	Plasmids	Plasmid Size
1	61-P	Two	>10kb
2	P-12	zero	-
3	P-1	One	>10kb
4	7491	Two	>10kb
5	12-P	Two	>10kb
6	17-P	Two	>10kb
7	P-7	Two	>10kb
8	10-P	One	>10kb
9	22-P	Four	Two were >10kb; one is 10 kb, and one is ~8 kb
10	55-P	Six	Two are >10kb; one is 10kb, one is 8kb, one is 4kb, and one is ~3.2 kb

4.3.3. Plasmid profile of *K. pneumoniae*

Plasmid profile of *K.pneumoniae* shows different plasmids carried by these isolates. Out of 10 isolates, 5 isolates showed 2 plasmids, 3 isolates showed 1 plasmid while 4 plasmids were seen in 1 isolate. All plasmid were of >10kb in size and shown in Figure 3. The plasmid details are given in Table 3.

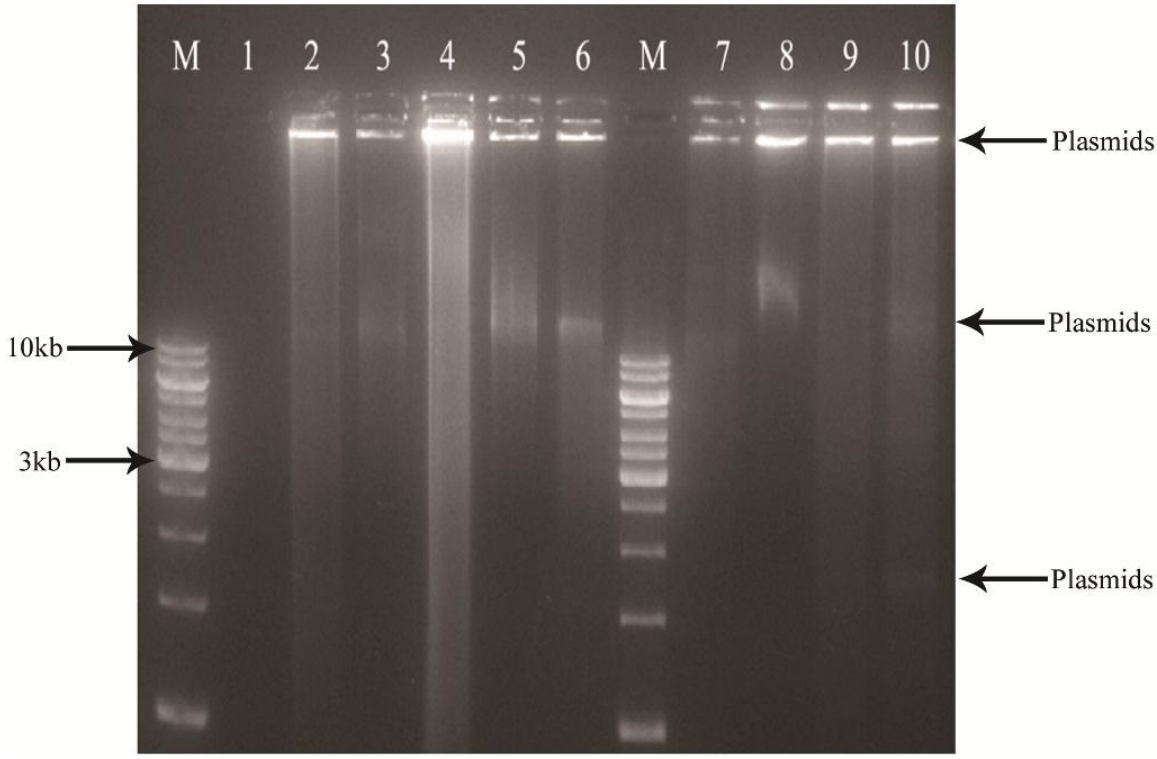

Figure 3: Plasmid profile of *Klebsiella pneumoniae.* Lanes 1-10 show the plasmids (highlighted by arrow) carried by these isolates and lane M shows 1 kb DNA ladder.

Table 3: Plasmids of *K. pneumoniae*

S. No	Strains code	Plasmids	Plasmid Size
2	2 KP	zero	
3	12	Two	>10kb
4	11	Three	>10kb
5	P-9	One	>10kb
6	P-4	Two	>10kb
7	P-15	Two	>10kb
9	5 KP	Two	>10kb
10	P-40	Two	>10kb
11	P-3	One	>10kb
12	3898	Four	Three plasmids >10kb, and one plasmid is ~1.7kb

4.3.4 Plasmid Profile of *E. coli*

Plasmid profile of *E.coli* shows different plasmids carried by the environmental and clinical isolates. Out of four isolates, three isolates showed one plasmid while in one isolate, two plasmids were seen. The plasmids were of >10kb in size and are shown in Figure 4 with details in Table 4.

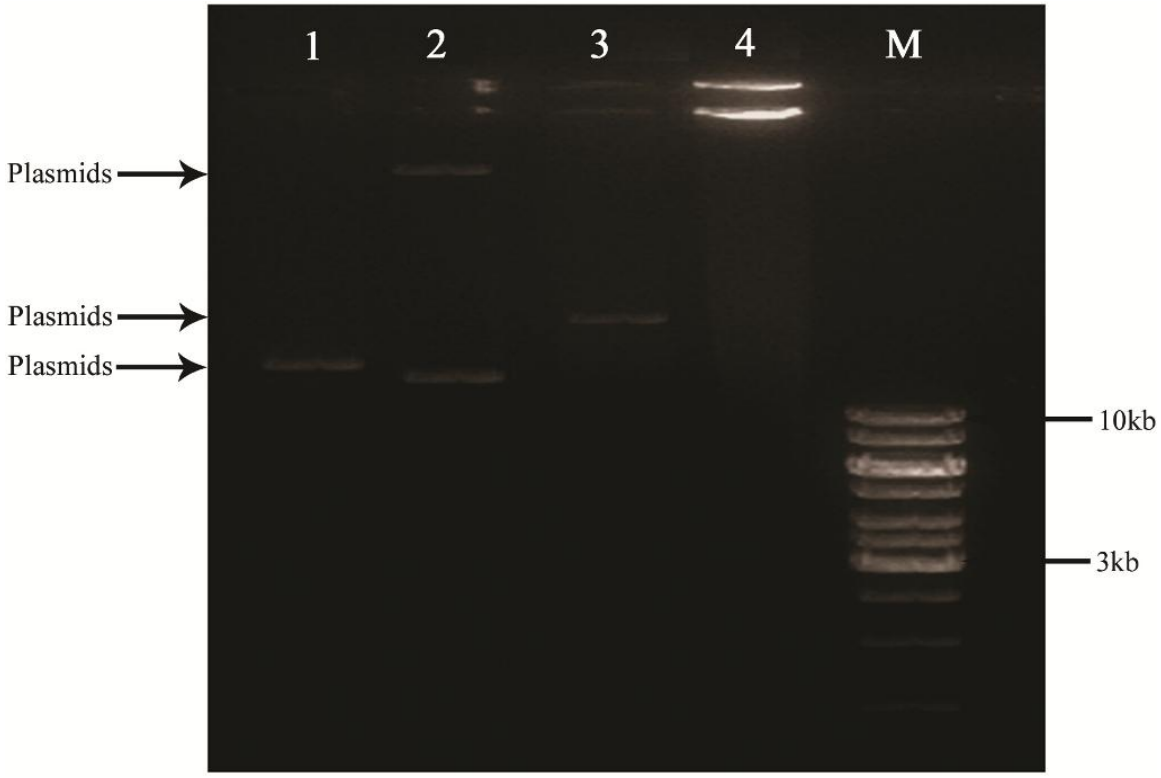

Figure 4: Plasmid profile of *E. coli*

Table 4: Plasmids of *E. coli*

S.No	**Strain code**	**Plasmids**	**Plasmid size**
1	10-E (Environmental isolate)	1	>10kb
2	10-E (Clinical isolate)	2	>10kb
3	3(Clinical isolate)	1	>10kb
4	21(Clinical isolate)	1	>10kb

4.4 Plasmid Stability assay

We assessed plasmid stability by serial passage of NDM-1 positive isolates on antibiotic free and 0.5mg/L meropenem containing media. Three candidate strains; *E.coli, Enterobacteriaceae, Pseudomonas* were selected for this experiment. Passages were done every 24 hours up to 15 passages. All the plasmids displayed by the three strains remained stable up to passage 7 as shown in Figures 5, and 6 with details in Tables 5 and 6. Towards the 8th passage, one plasmid of *Pseudomonas* was lost which is shown in Figure 7 while at 10th passage; one plasmid of *E.coli* was lost. All other plasmids remained stable upto15 passages. The plasmids are shown in Figures 8 and 9 with the details in Tables 8 and 9.

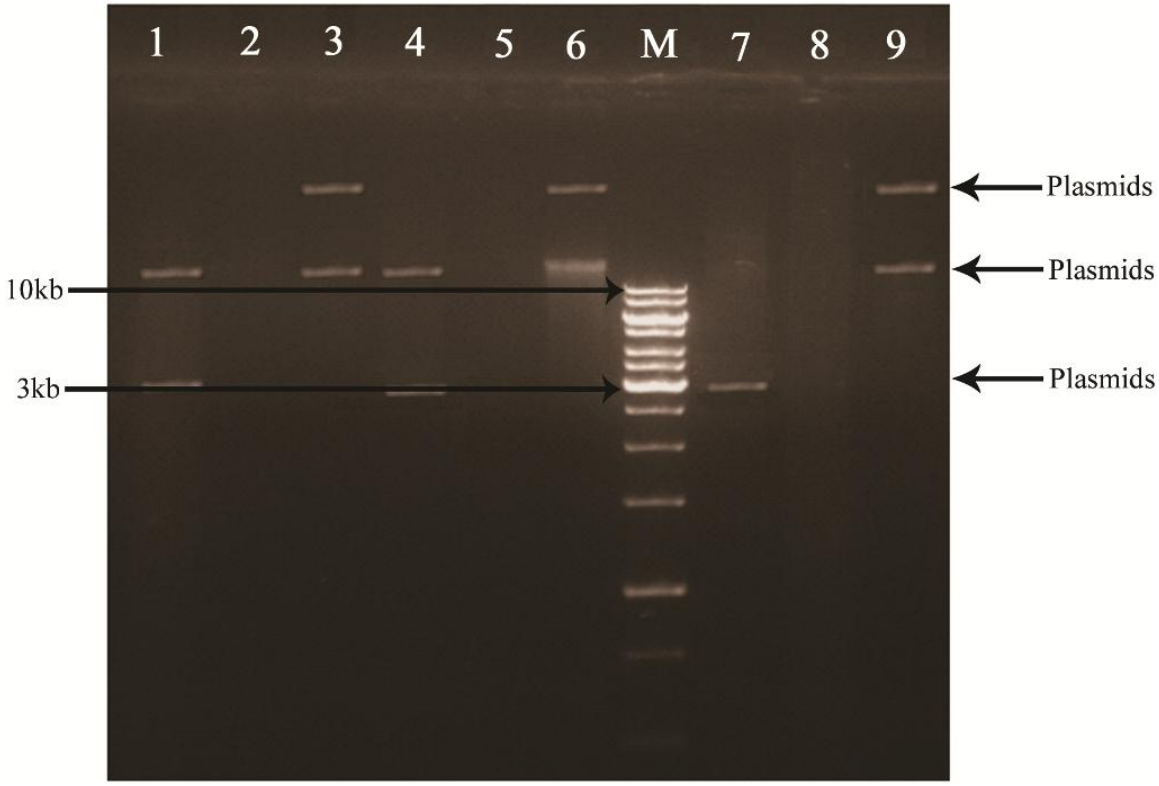

Figure 5: Three passages of plasmid stability: Lane 1, 2, 3 are the result of 1st passage, Lane 4, 5, 6 are the result of 2nd passage, Lane 7, 8, 9 are the result of 3rd passage.

Lane 1, 4, 7, shows *E. coli,* Lane 2, 5, 8, shows *Enterobacteriaceae spp,* Lane 3, 6, 9 shows *Pseudomonas spp*.

Table 5: Passages 1, 2, and 3 of plasmid stability.

Passage No 1 (From lane 1 to lane 3)				
Strain name	**Plasmids.**	**Size**	**Stable**	**Unstable**
1. *E.coli*	Two	One >10kb, and one is 3kb	Yes	Nil
2. *Enterobacteriaceae* spp	One	>10kb	Yes	Nil
3. *Pseudomonas spp.*	Two	>10kb	Yes	Nil
Passage No 2 (From Lane 4 to 6)				
4. *E.coli*	Two	One >10kb and one is 3kb	Yes	Nil
5. *Enterobacteriaceae* spp	One	>10kb	Yes	Nil
6. *Pseudomonas*	Two	>10kb	Yes	Nil
Passage No 3 (From Lane 7 to 9)				
7. *E.coli*	Two	One >10kb and one is 3kb	Yes	Nil
8. *Enterobacteriaceae* spp	One	>10kb	Yes	Nil
9. *Pseudomonas spp.*	Two	>10kb	Yes	Nil

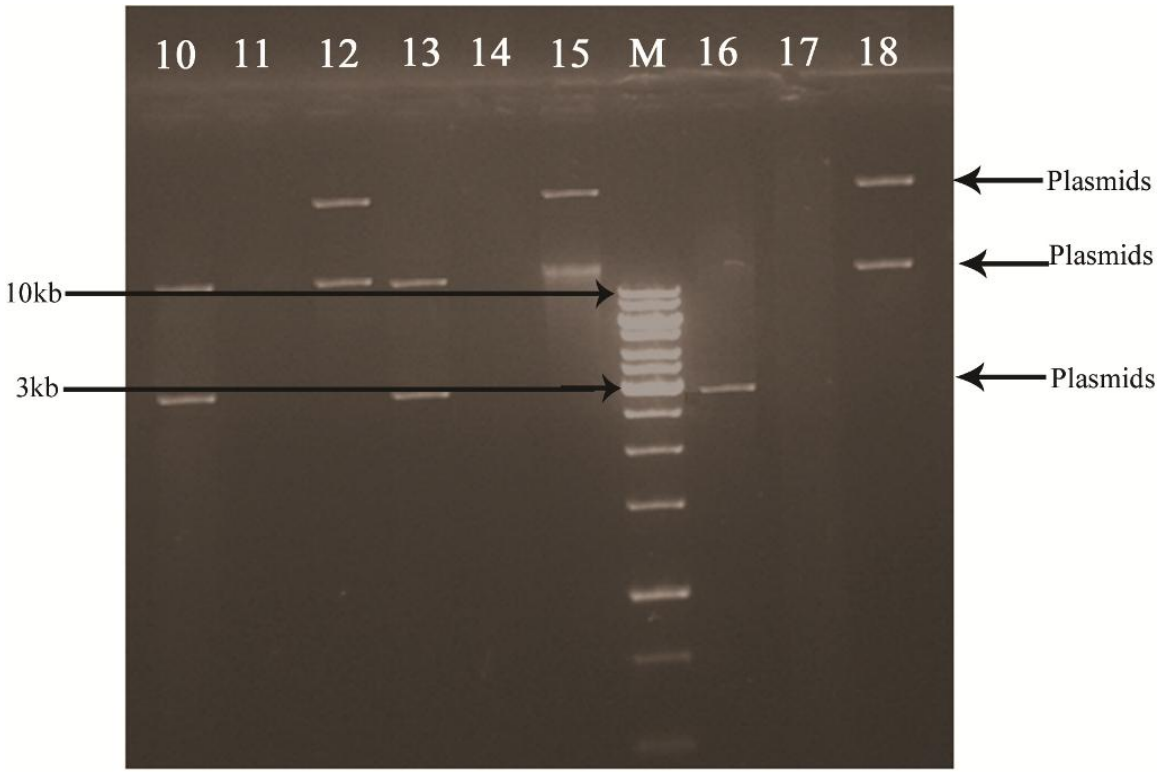

Figure 6: Three passages of plasmid stability: Lane 10, 11, 12 are the result of 4th passage, Lane 13, 14, 15 are the result of 5th passage, Lane 16, 17, 18 are the result of 6th passage.

Lane 10, 13, 16, shows *E. coli*, Lane 11, 14, 17, shows *Enterobacteriaceae spp,* Lane 12, 15, 18 shows *Pseudomonas spp*.

Table 6: Passages 4, 5 and 6 of plasmid stability

Passage No 4 (From lane 10 to lane 12).				
Strain name	**Plasmids**	**Size**	**Stable plasmid**	**Unstable plasmid**
10. *E.coli*	Two	One >10kb, and one is 3kb	Yes	Nil
11. *Enterobacteriaceae* spp	One	>10kb	Yes	Nil
12. *Pseudomonas spp.*	Two	>10kb	Yes	Nil
Passage No 5. (From Lane 13 to 15)				
13. *E.coli*	Two	>10kb, and one is 3kb	Yes	Nil
14. *Enterobacteriaceae spp*	One	>10kb	Yes	Nil
15. *Pseudomonas spp.*	Two	>10kb	Yes	Nil
Passage No 6. (From Lane 16 to 18)				
16. *E.coli*	Two	>10kb, and one is 3kb	Yes	Nil
17. *Enterobacteriaceae* spp	One	>10kb	Yes	Nil
18. *Pseudomonas spp.*	Two	>10kb	Yes	Nil

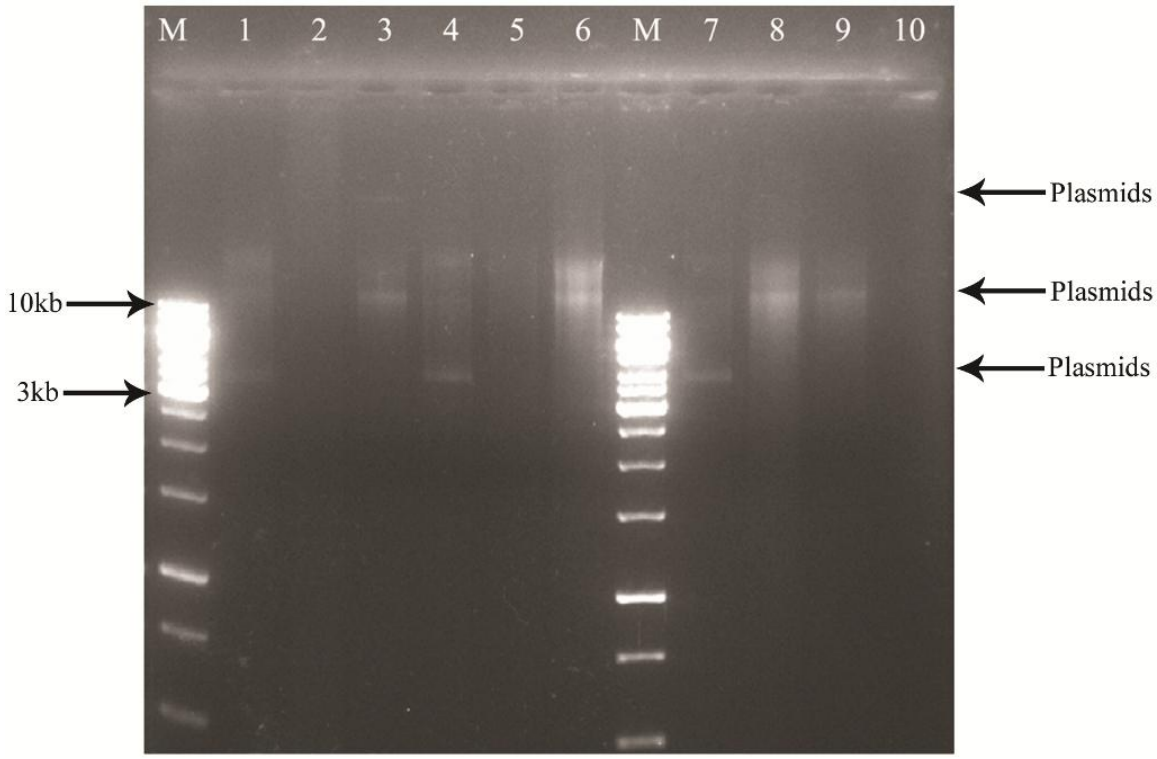

Figure 7: Three passages of plasmid stability Lane 1, 2, 3 are the result of 7th passage, Lane 4, 5, 6 are the result of 8th passage, Lane 7, 8, 9 are the result of 9th passage.

Lane 1, 4, 7, shows *E. coli*, Lane 2, 5, 8, shows *Enterobacteriaceae spp*, Lane 3, 6, 9, shows *Pseudomonas spp*.

Table 7: Passages 7, 8, and 9 of plasmid stability.

Passage No 7 (From lane 19 to 21)				
Strain name	**Plasmids**	**Plasmid size**	**Stable**	**Unstable**
1. E.coli	Two	>10kb, and one is 3kb	Yes	Nil
2. Enterobacteriaceae spp	One	>10kb	Yes	Nil
3. Pseudomonas	Two	>10kb	Yes	Nil
Passage No 8 (From Lane 22 to 24)				
4. E.coli	Two	>10kb, and one is 3kb	Yes	Nil
5. Enterobacteriaceae spp.	One	>10kb	Yes	Nil
6. Pseudomonas	One	>10kb	One stable plasmid	One plasmid >10kb is lost
Passage No 9 (From Lane 25 to 27)				
7. E.coli	Two	>10kb, and one 3kb	Yes	Nil
8. Enterobacteriaceae spp	One	>10kb	Yes	Nil
9. Pseudomonas	One	>10kb	Yes	Nil

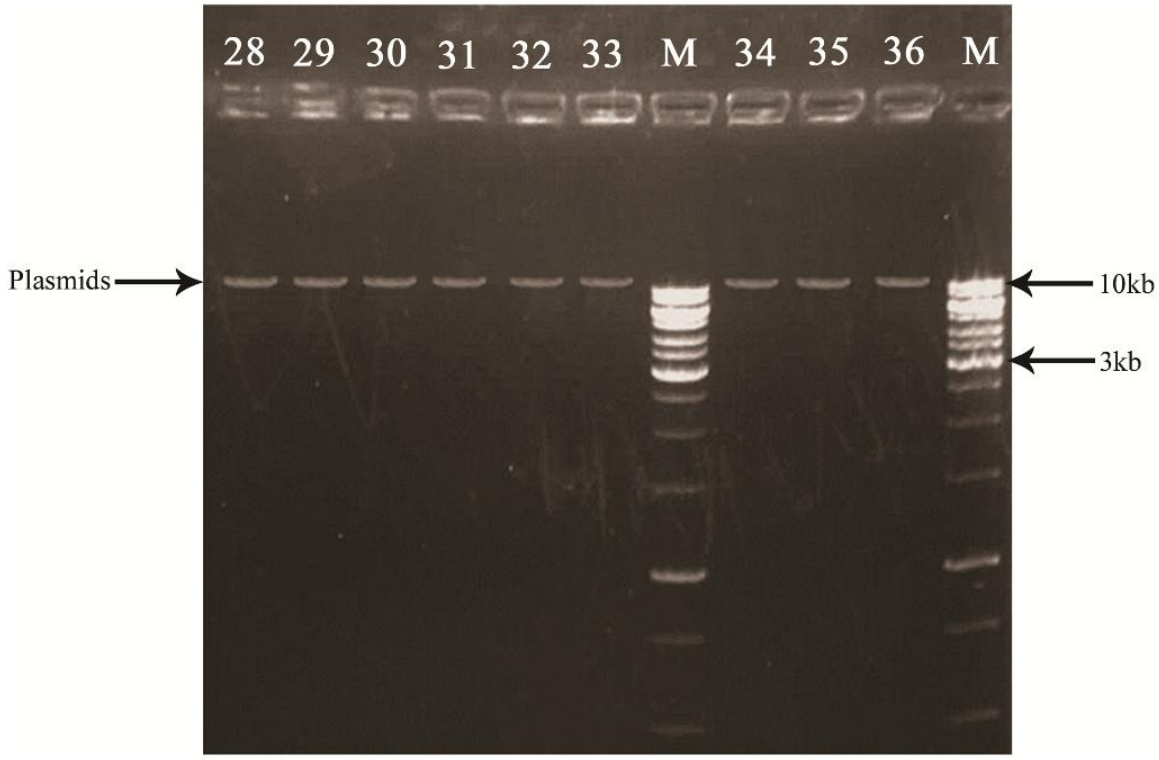

Figure 8: Three passages of plasmid stability Lane 28, 29, 30 are the result of 10th passage, Lane 31, 32, 33 are the result of 11thpassage, Lane 34, 35, 36 are the result of 12th passage.

Lane 28, 31, 34, shows *E. coli*, Lane 29, 32, 35, shows *Enterobacteriaceae spp*, Lane 3o, 33, 36, shows *Pseudomonas spp*.

Table 8 shows Passages 10, 11, and 12 of plasmid stability.

Passage No 10 (From lane 28 to lane 30)				
Strain name	**Plasmids**	**Plasmid Size**	**Stable**	**Unstable**
28. E.coli	One	>10kb	One stable plasmid >10kb	One plasmid of 3kb is lost
29. Enterobacteriaceae spp	One	>10kb	Yes	Nil
30. Pseudomonas	One	>10kb	Yes	Nil
Passage No 11 (From Lane 31 to 33)				
31. E.coli	One	>10kb	Yes	Nil
32. Enterobacteriaceae spp	One	>10kb	Yes	Nil
33. Pseudomonas	One	>10kb		Nil
Passage No 12 (From Lane 34 to 36)				
34. E.coli	One	>10kb	Yes	Nil
35. Enterobacteriaceae spp	One	>10kb	Yes	Nil
36. Pseudomonas	One	>10kb	Yes	Nil

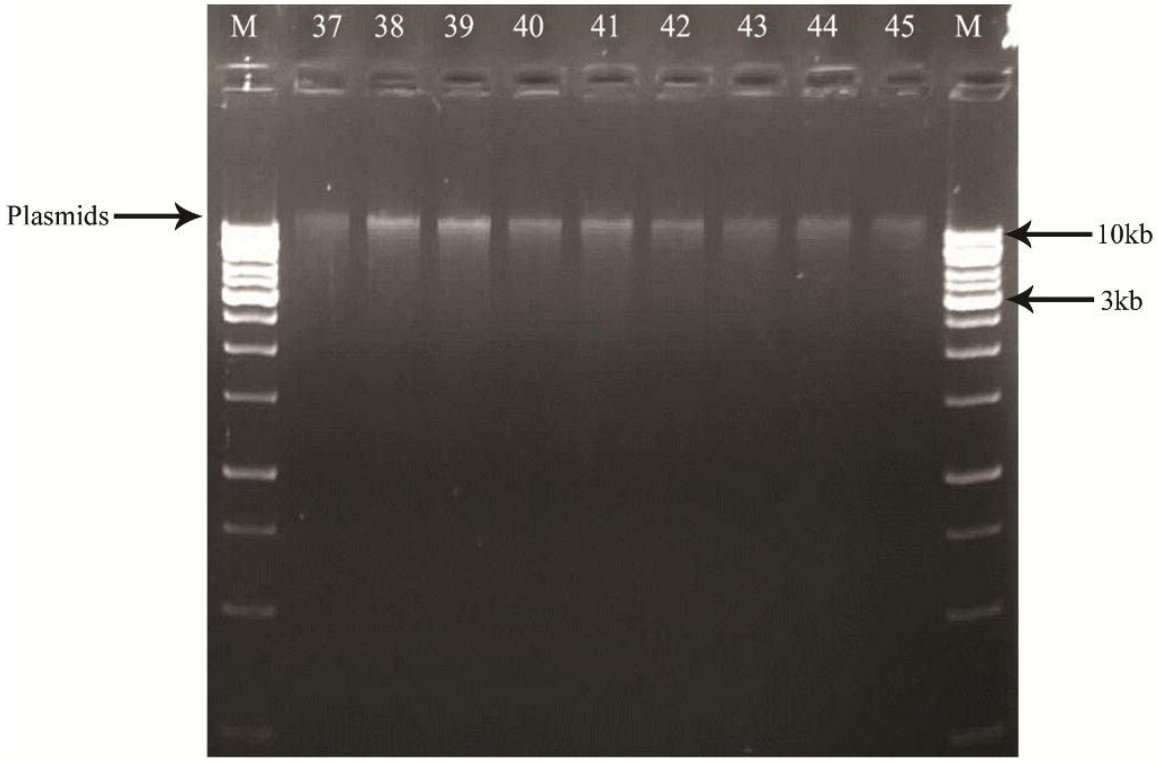

Figure 9: Three passages of plasmid stability: Lane 37 38, 39 are the result of 13th passage, Lane 40, 41, 42 are the result of 14th passage, Lane 34, 35, 36 are the result of 15th passage.

Lane 37, 40, 43, shows *E. coli*, Lane, 38, 41, 44, shows *Enterobacteriaceae spp*, Lane 39, 42, 45, shows *Pseudomonas spp*.

Table 9: shows Passages 13, 14, and 15 of plasmid stability.

Passage No 13 (From lane 28 to lane 30)				
Strain name	**Plasmids**	**Size**	**Stable plasmid**	**Unstable**
37. E.coli	One	>10kb	Yes	Nil
38. Enterobacteriaceae spp.	One	>10kb	Yes	Nil
39. Pseudomonas	One	>10kb	Yes	Nil
Passage No 14 (From Lane 31 to 33)				
40. E.coli	One	>10kb	Yes	Nil
41. Enterobacteriaceae spp.	One	>10kb	Yes	Nil
42. Pseudomonas	One	>10kb	Yes	Nil
Passage No 15 (From Lane 34 to 36)				
43. E.coli	One	>10kb	Yes	Nil
44. Enterobacteriaceae spp.	One	>10kb	Yes	Nil
45. Pseudomonas	One	>10kb	Yes	Nil

Plasmid DNA transformation

We assessed Plasmid DNA transformation from *Pseudomonas* (meropenem resistance) to Pseudomonas (meropenem sensitive) strains and Pseudomonas ATCC (9027), by Calcium Chloride method. Plasmid DNA transformed successfully from *Pseudomonas* (meropenem resistance) to Pseudomonas ATCC (9027), Pseudomonas (meropenem sensitive) the detail showed in figure No 10.

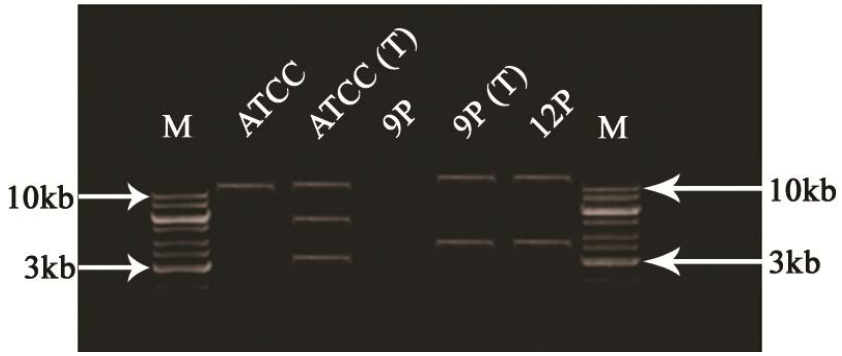

Figure 10 Transformation of plasmid DNA; M shows 1 kb DNA ladder.
ATCC *Pseudomonas*, ATCC (T) *Pseudomonas* (tranformant), 9P *Pseudomonas* (meropenem sensitive), 9P (T) *Pseudomonas*, (transformant), 12P *Pseudomonas* (meropenem resistance).

Molecular identification of *bla*$_{\text{NDM-1}}$

The plasmid DNA of metallo-β-lactamase producing Gram negative isolates was isolated by alkaline lysis method and kept at -4C°.The PCR was performed for detection of *bla*$_{\text{NDM-1}}$ gene in each plasmid DNA sample. The amplified PCR product was run on 1% gel. All the *bla*$_{\text{NDM-1}}$ gene amplified products i.e. *Enterobacteriaceae, Pseudomonas spp,* clinical and environmental isolates of *E. coli* respectively and *Klebsiella pneumonia,* were run at the single 1% agarose gel and digital photograph image are shown in (figure 11).

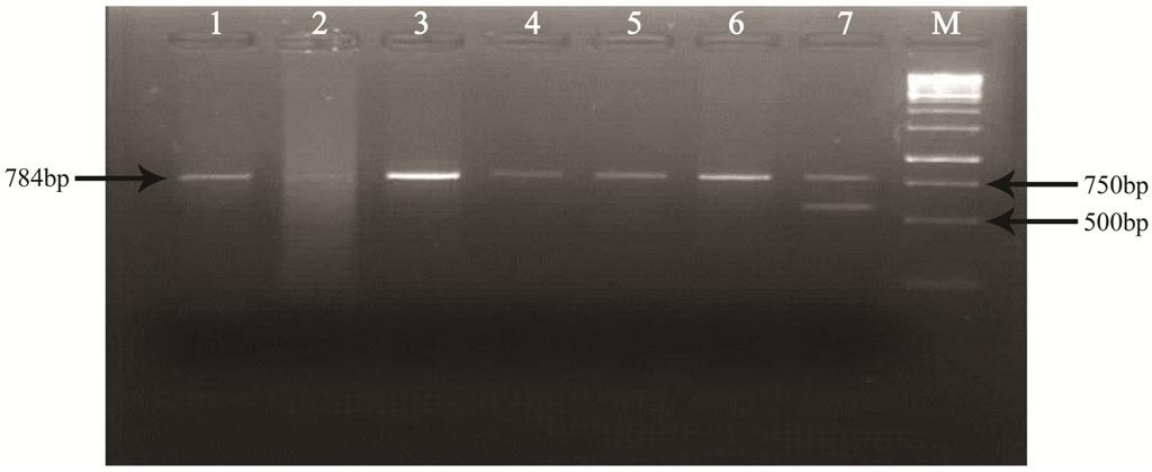

Figure 11: PCR product of 784bp for *bla*$_{\text{NDM-1}}$
Lane 1 shows *Enterobacteriaceae*, Lane 2,3 show *Pseudomonas spp*, Lane 4,5 show clinical and environmental isolates of *E. coli* respectively while Lane 6 & 7 show *Klebsiella pneumonia*. M shows 1kb DNA ladder.

Chapter 05

Discussion

Discussion

In this study we have analyzed the plasmids of Gram negative isolates harboring *bla*$_{\text{NDM-1}}$ gene. We focused on the plasmids profile, stability, and transferability.

The addition of NDM-1 production has the ability to turn these bacteria into true superbugs which are resistant to virtually all commonly used antibiotics The NDM-1 resistance has been observed both on chromosome and mostly on plasmid The NDM-1 encoding gene is located on different large plasmids that are easily transferable to susceptible at a high frequency (Yong *et al*., 2009). Most plasmid detected in bacteria was easily transferable and capable of wide rearrangement suggesting a widespread transmition and plasticity among bacterial population (Jean Mare *et al.,* 2010).

All the microbes showed high resistance against all antimicrobials ampicillins co-amoxiclav, cephalosporin, and amikacin, ciprofloxacin and carbapenems (imepenem, carbapenems).most effective drug was Polymyxin B .similarly an Indian study reported the *K.pneumoniae* which were showed resistance to imepenem and the most effective drug against these isolates were colistin (Roy *et al*., 2011).

Plasmid profiling of these isolates including *Escherichia coli, Klebsiella pneumoniae, and Pseudomonas spp* showed that these isolates carry plasmid of different sizes and numbers, mostly of >10kb in size.

In one study Walsh, T R., *et al*., (2011) reported that all the plasmids from *Enterobacteriaceae* isolates were the same size (140 kb). While in our study plasmid isolate from *Enterobacteriaceae* showed different sizes. Mostly are >10kb. Dermott *et al*., (2012) reported that in *Klebsiella pneumoniae* a Plasmid of 98 kb size carries the *bla*$_{\text{NDM-1}}$ gene. Kumarasamy *et al*., (2010) also reported that in Indian non Clonal isolate of *Klebsiella pneumoniae* carry plasmid of different sizes which range from 50kb to 350kb, while in other Clonal isolates the plasmid size was 118kb or 50kb, and also from the UK isolates which carry a plasmid ranged from 80kb to more than 500kb. While in our study plasmid isolate from *Klebsiella pneumoniae* showed different sizes. Mostly are >10kb.

Walsh *et al*., (2011) reported in the literature that Passaging over 14 days in media with and without meropenem 0·5 mg/L showed that all the *Enterobacteriaceae*, carried stable *bla*$_{\text{NDM-1}}$ plasmids; those have a same size of 140kb In our study after the 15 passage of

the *Enterobacteriaceae* in antibiotic meropenem (0·5 mg/L) free media no plasmid was lost and showed the stable plasmid, which are >10kb in size.

In the literature Walsh *et al*., (2011) reported that *Pseudomonas putida*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas oryzihabitans*, *Pseudomonas aeruginosa*, lost their plasmids within 48 h (two passages) without meropenem (0·5 mg/L)containing media. In one study in China Wang Y *et al*., (2012) reported that a plasmid was unstable if it was lost by the isolate in antibiotic free media after the three consecutive passages a similar results was also observed by Walsh *et al*., (2011) that the plasmid was considered unstable if the plasmid was lost by 2 sequential passages. While in our study the *Pseudomonas spp* lost their plasmid during 8 passages in antibiotic free media.

In our study *Pseudomonas spp* carry the stable plasmid >10kb in size after 15 passages in antibiotic free media. Similar results were observed in one study by Walsh *et al*., (2011) that *Pseudomonas pseudoalcaligenes*, *Pseudomonas putida* carry the stable plasmid after passing over 14 days in antibiotic free media.

In the literature it was reported by Islam *et al*., (2012) that highest no of plasmid reported in *Klebsiella pneumoniae* ranged from 2 to 8.while In our study the highest no of plasmid reported in *Klebsiealle pneumoniae* is 4.

In our study it was observed that *Escherichia coli* carry the stable plasmid after the 15 passaging in antibiotic (meropenem) free media. Similar results were found by Walsh *et al*., (2011) that *Escherichia coli* carry the stable plasmid after the 14 passages in antibiotic (meropenem) free media.

In our study we observed that *Pseudomonas spp* carry different sizes of plasmid mostly are >10kb in size in another study Walsh *et al*., (2011) reported that different sizes of plasmid i.e. range in size from 250kb to 280kb were carried by the *Pseudomonas spp*.

In one study (Subrata *et al.,* 2006) reported that $CaCl_2$ mediated transformation method were not more efficient, because of majority of Plasmid DNA molecule were not taken by the recipient cell. In our study it was confirmed that a CaCl2 method of plasmid DNA transformation was more efficient because we were succeeded in the plasmid DNA transformation.

Plasmid carry the bla_{NDM-1} gene were readily transferable from one bacterial strain to other, of the same or related species and may alter the plasmid DNA in one study in India

when the observer see the plasmid in the transconjugant, plasmid size was alter, in rare cases there is the gaining of DNA but in most cases lost the DNA Kumarasamy *et al*., 2010,while we also observed that in one transconjugant the plasmid size was alter, but in the other transconjugant it remains the same when examined by 0.7% agarose gel electrophoresis.

Raja *et al*, 2009 and Selven, (2009) reported that a plasmid of 23kb in size was transformed by heat shock and transformed the plasmid DNA from *Pseudomonas aeruginoasa* to *E.coli* from the tranconjugant the same plasmid were obtained by the help of gel electrophoresis. In our study we observed the plasmid DNA transformation by heat pulse, by the analysis of transconjugant we found one the same size plasmid in the transforment, while the other plasmid was alter.

Manjusha and Sarita, (2011) reported that in antibiotic resistance, plasmid play a very important role ,which was confirmed by the transformant which become resistance after the uptake of plasmid DNA carry antibiotic resistance gene. In this study they found that the associated antibiotic resistance and plasmid can be transferred to the recipient cell by conjugation and transformation. In our study it was also confirmed by gel electrophoresis the plasmid DNA transformation, and the transformant becomes resistance to selective antibiotics.

Conclusion

Conclusion

- In this study MBL producing strains (*E.coli, K.pneumoniae, and Pseudomonas spp)* harbouring the bla_{NDM-1} gene carry the plasmids of different sizes and numbers.
- The present study reveals that the Gram negative isolates harbouring the bla_{NDM-1} gene (*E.coli, K.pneumoniae, and Pseudomonas spp)* carry the stable plasmid after 15 passaging in antibiotic free media meropenem (0·5 mg/L).
- Bacterial strains harbouring (MBL) gene bla_{NDM-1} gene also carry the unstable plasmid which was lost when passaging in antibiotic free media.
- The present study also reveals that the plasmid DNA from *Pseudomonas* (meropenem resistance) was transformed to *Pseudomonas* (meropenem sensitive) through $CaCl_2$ transformation method.

Future Aspects

Future Aspects

We can proceed the present project in different direction

- Determination of such type of plasmid which carry the *bla*$_{\text{NDM-1}}$ gene in our own isolates.
- Also the determination of other antibiotic resisitence gene on the plasmid carrying the *bla*$_{\text{NDM-1}}$ gene.
- Sequencing of these gene to find out subtypes.
- Conjugation assay of *bla*$_{\text{NDM-1}}$ gene.

Chapter 06

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