Genetic Characterisation of MBL positive Pseudomonas and Enterobacteriaceae

A Thesis submitted for the degree of

Doctor of Philosophy at Cardiff University

Siham Rajab Agouri

2014

Department of Infection, Immunity & Biochemistry,

School of Medicine, Cardiff University

UNITED KINGDOM

Summary

It is now widely regarded that the increase in resistance in Enterobacteriaceae is a serious threat to human health. Furthermore, these same isolates possess heavy metal and toxin-antitoxin genes which play a pivotal role in creating selective pressure for the maintenance of plasmids that also carry the antibiotic resistance mechanisms. *Pseudomonas* spp., *Klebsiella pneumoniae*, *Escherichia coli* and other Gram-negative bacteria, in many countries, express multi-drug resistant (MDR) and extensively drug resistant (XDR) phenotypes. The dissemination of resistance genes among *Pseudomonas* spp. and Enterobacteriaceae strains has escalated due to the increased presence of mobile genetic elements such as plasmids, transposons and integrons.

In this thesis, my work focuses on the phenotypic and molecular characterization of MBLs and ESBLs genes, in Pseudomonas and Enterobacteriaceae; and plasmid-mediated heavy metal and TA systems produced by Enterobacteriaceae.

The *bla*$_{\text{VIM-2}}$ metallo-β-lactamase (MBL) gene has spread worldwide and is commonly present in class 1 integrons. Class 1 integrons, carrying *bla*$_{\text{VIM-2}}$ and other cassette genes, are increasingly associated with certain transposons such as Tn*402*/Tn*5090*. A *bla*$_{\text{VIM-2}}$ was identified from MDR *P. aeruginosa* 301-5473 and *P. fluorescens* 43-14926, and was located on an unusual class 1 integron embedded in a complete Tn*402*-like transposon. Complete sequences of this unusual class 1 integron lack the 3'CS *qac*E/*sul*1 gene fusion and instead possess a transposition module. *P. aeruginosa* 301-5473 revealed it to comprise *bla*$_{\text{OXA-2}}$, *aac*A4, *aad*B, and *qac*G, while in *P. fluorescens* (43-14926) it showed an *aac*A5 gene.

The work in this thesis also set out to determine how stable *bla*$_{\text{NDM-1}}$ plasmids are in Enterobacteriaceae, without selection, for an extended period of time. The *S*1 PFGE results showed that *bla*$_{\text{NDM-1}}$ had a diverse plasmid size range (from <50 kb to >400 kb). Moreover *bla*$_{\text{NDM-1}}$ is stable in most of the isolates. In *K. pneumoniae* strain, K15, the plasmid was not stable with respect to plasmid size. IncX, IncF, and IncA/C were common in all isolates. The *bla*$_{\text{NDM-1}}$ positive plasmids were able to transconjugate to *E. coli* J53 from approx. 80% of the donor isolates. Furthermore, the immediate genetic structure surrounding *bla*$_{\text{NDM-1}}$ plasmid was also studied and showed that the remnant of insertion sequence ISAba*125* was present upstream of the *bla*$_{\text{NDM-1}}$ gene and, similarly, the *ble*MBL gene was identified downstream of the *bla*$_{\text{NDM-1}}$ in most isolates.

Heavy-metal resistance in Enterobacteriaceae isolates was studied. Three groups of the isolates; G1-NDM1 positive isolates [India and UK], G2- ESBLs positive isolates [India] and G3- susceptible/ sensitive isolates [UK] showed different degrees of resistance to copper, silver, mercury, and arsenic. Resistance to heavy metals occurred at a higher rate in NDM-1 positive strains (G1) than ESBL-positive strains (G2). The majority of the sequenced *mer*A, *ars*A, *sil*C and *pco*A genes displayed (99.3%- 100%) nucleotide identity and (100%) protein identity to the same genes/proteins registered in the databases. The *S*1 digestion results showed that heavy metals genes are found both on plasmids and/or chromosomes. Most heavy metal resistance systems were found on plasmids and were transferred by conjugation.

The Enterobacteriaceae genome contains genes encoding toxin–antitoxin systems (TA systems). TA systems were identified in most of isolates. 86% of NDM-1 and CTX-M-15 positive isolates showed the presence of eight T-AT systems (pemKI, ccdAB, relEB, vagCD, pndCA, parDE, hok/sok, and srnBC) in their genome. PemKI, ccdAB, relBE, vagCD and hok/sok were the most frequently represented systems. Indian isolates harbouring $bla_{\text{CTX-M-15}}$ have more T-AT systems than those possessing $bla_{\text{NDM-1}}$. *S*1 results revealed that *pem*KI, *vag*CD, and *hok*/*sok* genes were located on high molecular weight plasmids ranging from 20 to 400kb in the UK isolates. However, *ccd*AB and *srn*BC were detected on plasmids ranging from 50 to 150kb. Moreover, the *rel*BE system was located on the chromosome. Conjugation experiment results showed that transmissible plasmids for TA systems were found in a number of the J53 transconjugants.

The data from my thesis infers that the plasmids carrying $bla_{\text{NDM-1}}$ are diverse and stable in Enterobacteriaceae. The same plasmids can carry both TA systems and heavy metal genes and suggest that the maintenance of plasmids carrying resistant genes may be maintained by factors other than antibiotics.

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful

First and foremost, all praises to Allah for the strength and his blessing in completing this thesis. I would like to thank my supervisor Prof. Timothy Rutland Walsh for all his endless help, support, guidance and supervision. I extend my special thanks to my second supervisor Dr. Mark Alexander Toleman for his very generous help and his insightful thoughts and suggestions.

Special thanks to Mrs Janis Weeks for her constant encouragement and support. My thanks go to Dr Henry Ryley for his help to collect some information. I am beholden to Dr. Mandy Wootton and all staff at her laboratory for their help and collaboration in identification of isolates in this thesis.

Special thanks to Dr. Amands Tonks, Dr. Lim Jone and Dr. Asife Nazam, for her/ his constant support and help. I would like to thank Prof. Salha Ben-Gwirif for her help, support , taught me in undergraduate and in Master degree and until now and introduce my thanks to the spirit of Dr. Fathi Amoshate.

I extend my special thanks to all staff and colleagues in section of Medical Microbiology, University Hospital of Wales for constant support.

Finally, I could not have done this work without the love and support my Family, my father, Rajab Agouri whom I love, who prayed for me, helped me, encouraged me from the beginning study and now, I Send my thanks to his soul in paradise, my beloved husband Abdw Al-Eskandrani – thanks for his constant encouragement from the very beginning. My Sisters, particular, old sister , Salha Rajab, and uncle Abd-Allah Al- Warfali, without their help and support I would not have been able to concentrate on this study, and to my lovely little boy Mohammed.

This thesis is dedicated to the soul of my parents, Rajab and Aisha, my beloved husband and child, Abdw and Mohammed.

Declaration

I declare that the work in this was carried out in the accordance with the Regulation of the University of Cardiff. This thesis represents my own work except where indicated by special references in the text.

SIGNRD

DATE

Publication and presentations

Posters Presentation

- Major genetic plasticity found in NDM-1 plasmid grown under different conditions. Siham R. Agouri, Janis Week, Timothy R. Walsh and Mark A. Toleman, Milan, Italy, 2011

- Determination of Heavy Metals resistance and Addiction Systems in Gram –negative bacteria strains isolated from the United Kingdom and India. S. Agouri, T. Walsh, M. Toleman. Cardiff Institute of Infection and Immunity Annual meeting 2012, Cardiff.

- Identification of addiction modules (T-AT systems) of $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$ encoding genes in Enterobacteriaceae strains. Agouri SR, Toleman MA, Walsh TR, Cardiff University, school of Medicine ,UK. ECMD, Berlin, Germany, 2013

- Stability and transferability of plasmid mediated bla NDM-1 gene in Gram-negative bacteria. Agouri SR, Toleman MA, Walsh TR. Cardiff University, School of Medicine, UK, ECMD, Berlin, Germany, 2013

- Occurrence of Heavy metals resistance genes in metallo-beta-lactamase and extended spectrum –beta- lactamase producing Enterobacteriaceae strains isolated from the UK and India. Agouri SR, Toleman MA, Walsh TR. Cardiff University, School of Medicine, UK, ECMD, Berlin, Germany, 2013

List of Abbreviations

*aac*A	aminoglycoside acetyl transferase
*aac*C	6-N-aminoglycoside acetyltransferases
*aad*B	aminoglycoside-2"-adenyltransferase
ABC	ATP- binding cassette
ACT	the chromosomally encoded, clavulanate- resistant AmpC cephalosporinases of *Enterobacter* spp.
AmpC	Ampicillin
*att*I	attachment site
Bla	β-lactamase
Ccd	coupled cell division
Clp	Kind of cellular proteases
CMY	the chromosomally encoded, clavulanate-resistant AmpC cephalosporinases of *Citrobacter freundii*
CS	conserved segment
CTX-M	Cefotaximase
dfrB	trimethoprim encode dihydrofolate reductases
DHA	the chromosomally encoded, clavulanate- resistant AmpC cephalosporinases of *Morganella morganii*
DIM	Dutch imipenemase
DNA	Deoxyribonucleic acid
Doc	phd–doc system / stable inheritance of the plasmid
EDTA	Ethylenediaminetetraacetate
EPS	Efflux pumps systems
ESBLs	Extended spectrum β-lactamases
G+C	guanine-cytosine content
GIT	Gastrointestinal tract
GNB	Gram-negative bacilli
HGT	Horizontal gene transfer
HicA	T-AT system
HigB	host inhibition of growth system
ICEs	Integration and conjugative element
IMP	Imipenemase
Inc	Incompatibility group
*int*I	Integrase gene
IR	Inverted repeat sequence
IS	Insertion sequence
IS*CR*	Insertion sequence common region
Kb	Kilo base
KHM	Kyorin Health MBL
KPC	*K. pneumoniae* carbapenemase
LGT	Lateral Genetic Transfer
Lon	Kind of cellular proteases
MATE	Multidrug and toxin extrusion
MazF	Toxin-antitoxin system involved in bacterial programmed cell death
MBLs	Metallo- beta- lactamases
MDR	Multidrug resistance

merA	enzyme mercuric reductase
MFS	Major facilitator superfamily
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
MOB	Mobility gene
MPF	Mating pair formation
mRNA	Messenger RNA
MRSA	Methicillin resistant *S. aureus*
NDM	New –Delhi metallo-β-lactamase
OD	Optical density
OM	Outer membrane
ORF	Open reading frame
*ori*T	Origin of transfer
OXAs	Oxacillinases
P	promoter
PAI	pathogenicity islands
par	Partitioning gene
ParE	plasmid maintenance system
PBPs	Penicillin binding proteins
PCD	programmed cell death
PCR	Polymerase Chain Reaction
PFGE	Pulsed field gel electrophoresis
PSK	Post segregation killing system
qacE	Quaternary ammonium compound
qacG	Quaternary ammonium compound
RelE	T-AT system
Rep	replication initiator proteins
Res site	Resolution site
RND	Resistance-nodulation division
*S*1	nuclease enzyme
SDS	Sodium dodeca sulphate
SHV	Sulfhydryl variable
SMR	Small multi- drug resistance
*sul*1	Sulphonamide resistance gene
SXT	Trimethoprim sulphamethoxazole
T4SS	Type 4 secretion system
T-AT	Toxin- Antitoxin system
TEM	Temoneira
Tn	Transposon
TniA	transposase
TnpR	reslovase
UTI	Urinary tract infection
Vap	virulence associated protein
VIM	Verona Imipenemase
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *Staphylococcus aureus*
ω-ε-ξ	omega–epsilon–zeta
XDR	Extensively drug resistance
Zeta toxin	T-AT system

List of Figures page

List of Table page

Contents page

Chapter One

General Introduction

1. Introduction.

1.1 Enterobacteriaceae.

Bacteria are classified as Gram-positive or Gram-negative based on the structure of their cell wall (Figure 1.1). This can be determined by Gram staining. The main difference between them is that Gram-negative bacteria have two double layered membranes with a very thin peptidoglycan layer located between the membranes, whilst Gram-positive bacteria only have a single layered membrane and a thick, 20-80 nm, peptidoglycan layer facing outwards from the cell. The peptidoglycan layer of Gram-negatives is 7-8 nm. The Enterobacteriaceae is a family of Gram-negative bacteria classified within γ-proteobacteria. They include many species, but only a limited number are of clinical relevance. The most studied bacteria of clinical importance in this family are *Escherichia coli, Klebsiella* spp., *Salmonella enterica, Shigella* spp., *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., *Morganella morganii* and *Yersinia* spp.

Many Enterobacteriaceae are rod-shaped, 1-5μm in length, non-sporulating, facultative bacteria. They ferment sugars (e.g. glucose and lactose) to a variety of end products and reduce nitrate to nitrite; they produce catalase and are oxidase negative. All these conditions help identify them in the clinical microbiological laboratory. They are common colonizers of the gastrointestinal tract (GIT) and are important for the health of both humans and most animals as part of natural bacterial flora. Enterobacteriaceae are also widespread in the environment. However, they can also be pathogenic causing infections in the central nervous system, lower respiratory tract, bloodstream, gastrointestinal and urinary tracts. They have many features that aid their survival, the most common being endotoxins, capsules, antigenic phase variation, type III-secretion systems, sequestration of growth factors and resistance to serum killing (Murray *et al*., 2009).

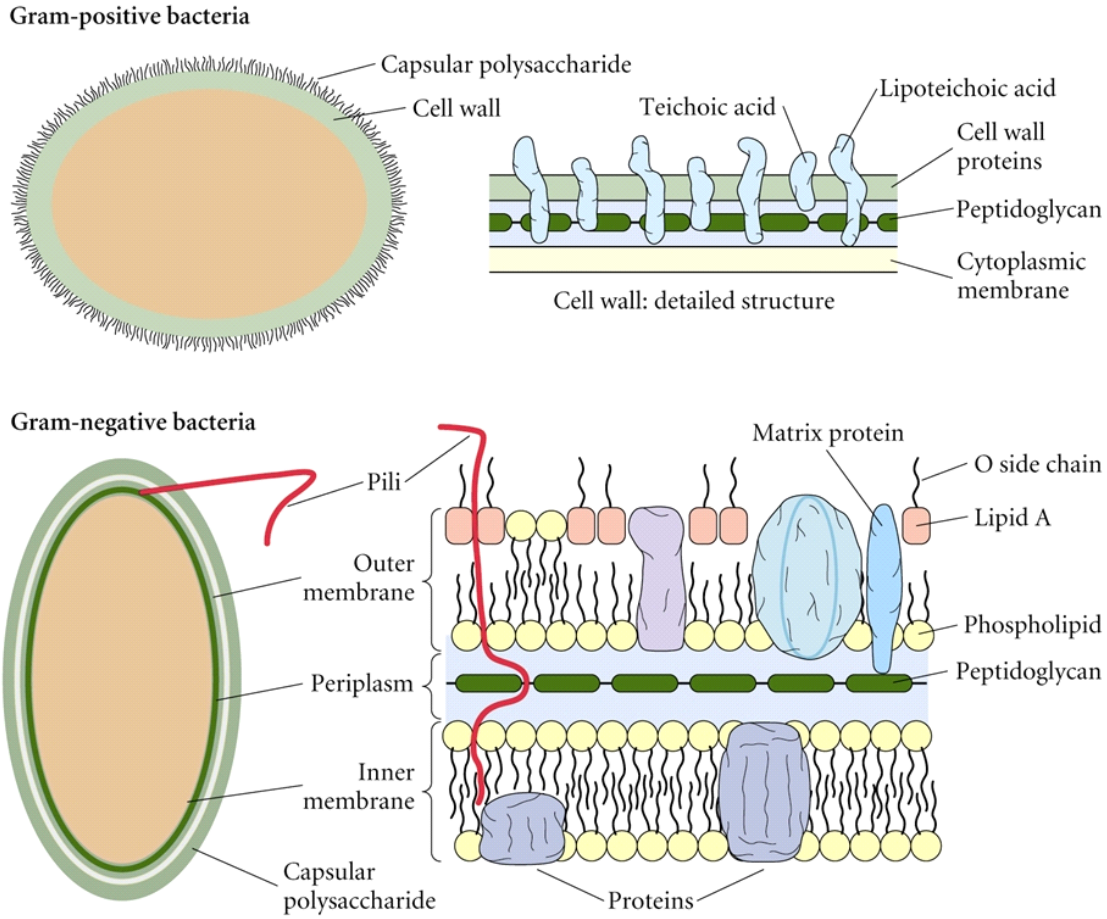


Figure 1.1 Differences in the cell wall configuration between Gram-positive and Gram-negative bacteria. Adapted from http://www.ppdictionary.com/bacteria/gpbac/bacteria_comparison.jpg

1.2 Antibiotics.

1.2.1 Definition of antibiotics.

The term antibiotic refers to a substance produced by microorganisms that inhibits or kills other microorganisms (Harremoës *et al*., 2001). Today, it is widely used and accepted to include synthetic substances that inhibit or kill microorganisms as well as naturally occurring compounds. Antibiotics are classified as bactericidal, exemplified by penicillin, or bacteriostatic, for example, chloramphenicol. Bactericidal antibiotics cause bacterial cell death, whilst bacteriostatic antibiotics prevent the bacteria from growing (Walsh 2010). Antibiotics can be classified according to their various mechanisms of action (Table 1.1) such as (1) interference with cell wall synthesis, (2) inhibition of protein synthesis, (3) interference with nucleic acid synthesis, (4) inhibition of metabolic pathways, and (5) disruption of bacterial membrane structure (Figure 1.2) (Levy and Marshall 2004; Tenover 2006).

Table 1.1 Mechanisms of action of antibiotics.

Mechanism of action	Antimicrobial agent(s)
1. Interference with cell wall synthesis	β- Lactams: penicillins, cephalosporins, carbapenems, monobactams. Glycopeptides: vancomycin, teicoplanin
2. Inhibition of protein synthesis:	Macrolides, chloramphenicol, clindamycin, quinopristin-dalfopristin, linezolid Aminoglycosides, tetracyclines
3. Interference with nucleic acid synthesis **-Inhibition of DNA synthesis** **-Inhibition of RNA synthesis**	Fluoroquinolones, rifampicin
4. Inhibition of a metabolic pathway	Sulfonamides, folic acid analogous
5. Disruption of bacterial membrane structure	Polymyxins, daptomycin

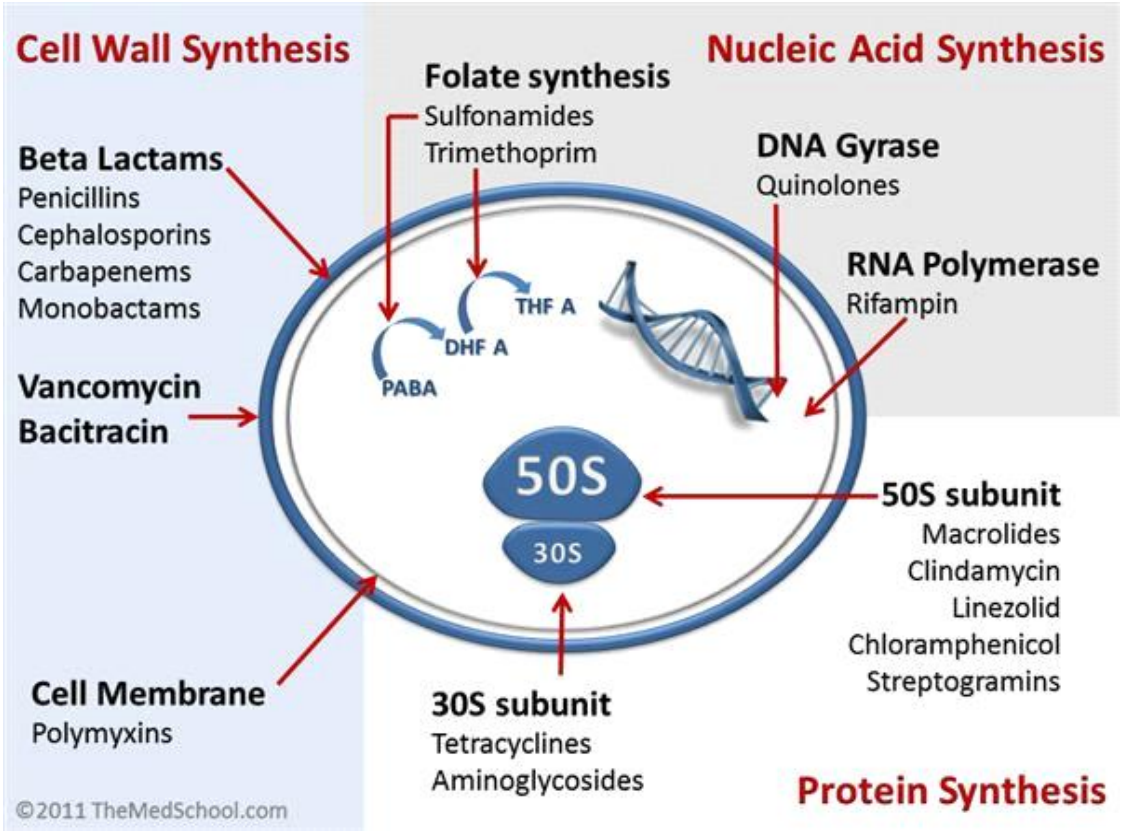


Figure 1.2 Mechanisms of antibiotics action. Adapted from http://commons.wikimedia.org/wiki/file%3Antibiotics_Mechanisms_of_action.png

1.2.2 The emergence of antibiotics.

Antibiotics were introduced into medication well over 70 years ago, considerably changing life expectancy from certain diseases (Lesche, 2007). Antibiotics have enabled the successful treatment of life-threatening contagions and enabled safe surgeries, previously thought to be untreatable due to the high hazards of infection. The first antibiotic, Salvarsan, was used to treat syphilis in 1910 (Harremoës *et al*., 2001). The use of antibiotics expanded in the 1930's with the discovery of sulphonamide, a synthetic antibiotic with broad antibiotic activity against Gram-positive bacteria, but with no effect on Enterobacteriaceae (Lesche 2007). In 1928, Alexander Fleming discovered penicillin and, after purification and mass production in 1941, it was used to treat numerous bacterial infections (Quirke 2001). Despite penicillin's early discovery, it was not commercially available for clinical use until the early 1940s owing to difficulties in its purification and production (Quirke 2001; Harremoës *et al*., 2001). In the following years, with the discovery of numerous new antibiotics, their use rose exponentially and several new classes of both synthetic and naturally-occurring antibiotics were discovered, including aminoglycosides in 1943 and tetracycline in 1955. By the end of the 1960's, companies also began modifying the molecular structure of existing antibiotics to overcome bacterial resistance mechanisms and improve solubility etc. (Projan and Shlaes 2004). It was not until the late 1990s and early 2000s that further new classes of antibiotic, such as daptomycin, tigecycline and linezolid, were discovered and made available for clinical use. With the unregulated availability of antibiotics, resistance was an inevitable outcome. Now, it is estimated that more than 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the antibiotics which are used to treat them. Antibiotic resistance continues to expand for many reasons, including extensive use of broad-spectrum antibiotics in hospitalized patients and in the community, use of antibiotics in animals as growth enhancers, increased international travel, poor hospital sanitation and abuse of antibiotics in both poor and wealthy countries (Kapil 2005; Piéboji *et al*., 2004).

1.2.3. Antibiotic resistance.

Antibiotic resistance is defined as "the ability of a microorganism to resist antibiotic stress and survive" (Walsh 2000). In contrast, the susceptible bacteria would be eliminated. Over the last 75 years, the continued use of antibiotics has led to bacteria procuring and expressing various resistance mechanisms mediating resistance to one or more drugs: i.e. multidrug resistance,(MDR) (Giedraitienė *et al*., 2011) or, where only one or two classes are left, extensive drug resistance (XDR). MDR/XDR has appeared in *P. aeruginosa*, *Acinetobacter baumannii*, *E. coli*, and *K. pneumoniae*, producing extended-spectrum β-lactamases (ESBL), vancomycin-resistant *enterococci*, *Enterococcus faecium* (VRE), MRSA, vancomycin-resistant *Staphylococcus aureus* VRSA, XDR- *Mycobacterium tuberculosis* (Alekshun and Levy 2007) *Salmonella enterica* serovar *typhimurium*, *Shigella dysenteriae*, *Stenotrophomonas*, and *Burkholderia* (Džidic *et al*., 2008).

Antibiotic resistance mechanisms are either intrinsically mediated or acquired. The intrinsic resistance mechanism refers to the existence of chromosomal resistance genes or mutations in other genes/adjacent DNA altering their expression (Davies and Davies 2010); for example, *Mycoplasma* intrinsically shows resistance to β-lactams and other cell-wall targeting antibiotics due to an alternate cell wall (Allen *et al*., 2010). In contrast, acquired resistance mechanisms are gained by bacteria through horizontal gene transfer (HGT) mechanisms (Sykes 2010; Rowe-Magnus *et al*., 1999); for example, many β-lactamase genes are acquired by bacteria through mobile genetic elements (MGEs) such as plasmids (Jeong *et al*., 2003; Toleman *et al*., 2004), transposons (Toleman *et al*., 2003) and insertion sequence common region (ISCR) elements (Toleman *et al*., 2006). A more detailed description of HGT will be discussed later. In principle, a bacterial cell can become resistant to any antibiotic by three mechanisms: a) enzymatic inactivation or modification of the drug; b) reduced drug accumulation at the active site by decreased cell permeability and/or increased efflux from the cell surface; or c) alteration or modification of its targets (Figure 1.3) (Kunz and Brook 2010). A brief overview of the different mechanisms will be given below to focus on resistance to β-lactams. A more detailed characterization of β-lactamases will be discussed later.

1.2.4. Mechanisms of antibiotic resistance.

1.2.4.1. Reduced permeability and active efflux.

In many Gram-negative bacteria, outer membranes (OM) have protein channels, formed by porin proteins, to allow molecules to pass through the OM and enter the cell (Nikaido 2003). These molecules include nutrients as well as antibiotics such as β-lactams or fluoroquinolones. These proteins form channels that traverse the OM and end in the periplasm. In order to prevent the entry of antibiotics, bacteria can reduce the access of antibiotics (also called flux) by changing the OM. Gram-negative bacteria like *K. pneumoniae, P. aeruginosa,* and *A. baumannii* show resistance to antibiotics like β-lactams by altering the porins or by down- regulating expression of the porins (Hancock 1998). As an example, a combination of deletion of OM porins, with the increased expression of plasmid-mediated AmpC β-lactamases, in *K. pneumoniae* can confer resistance to imipenem (Cao *et al*., 2000). Many studies have shown that the combined presence of ESBLs and altered OM can mediate carbapenem resistance in *A. baumannii, P. aeruginosa* and other Gram-negative bacteria (Li and Nikaido 2009).

Efflux pump systems were discovered in the 1970s in *E. coli* and conferred resistance to tetracycline (Ball *et al*., 1980). To date, five major efflux pump families have been reported: ABC (ATP-binding cassette) superfamily, SMR (small multi-drug resistance), RND (resistance-nodulation division), MFS (major facilitator superfamily), and MATE (multidrug and toxin extrusion) superfamilies (Li and Nikaido, 2009). *P. aeruginosa* harbours several efflux pumps, including MexAB-OprM, MexCDOprJ and MexXY-OprM, with different spectra of substrate profiles that include various groups of antibiotics including β-lactams (Masuda *et al*., 2000). Exopolysaccharides (EPS) are responsible for expelling antibiotics from bacterial cells (Kumar and Schweizer 2005). EPS play an important role in the physiology and homeostasis of bacteria (Mirelman *et al*., 1974). Furthermore, EPS are useful tools for the cell to remove other molecules like heavy metals, dyes or detergents (Livermore 1995). While diverse mechanisms account for different resistance forms, the over-expression of efflux pumps extruding antibiotics is a major resistance mechanism for clinical isolates (Majiduddin *et al*., 2002).

1.2.4.2. Enzymatic inactivation or modification of antibiotics.

The enzymatic inactivation or modification of the drug is the most common mechanism of antibiotic resistance in many bacteria. The genes that encode these enzymes are often associated with MGEs such as plasmids, transposons or integrons (Azucena and Mobashery 2001). Most of the antibiotics are characterized by ester or amide bonds, which are hydrolytically susceptible, targeted by certain bacterial enzymes (e.g. β- lactamases) which make them inactive (Wright 2005).

1.2.4.3. Target alteration.

Bacteria can alter the targets of antibiotics which structurally reduce the affinity for antibiotics; for instance, modification of penicillin binding proteins (PBPs,) the important targets for β-lactams, reduces their affinity for β-lactams (Macheboeuf *et al.,* 2006). Resistance to quinolones is caused by chromosomal mutation encoding substitutions in the DNA gyrase or topoisomerase IV (Patzer *et al*., 2004; Sharma and Mohan, 2006).

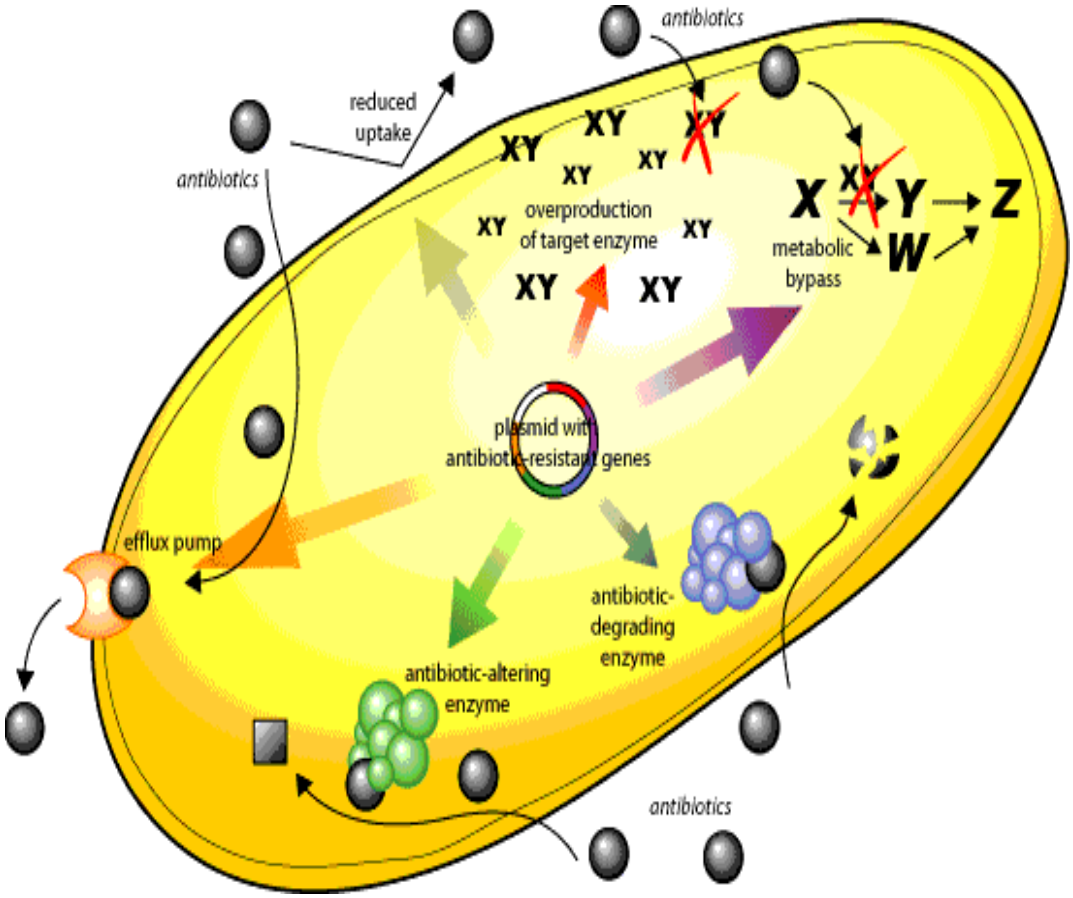


Figure 1.3 Mechanisms of antibiotic resistance. Adapted from

http://www.scq.ubc.ca/wp-content/uploads/2006/08/antibiotresist.gif

1.3. β-Lactam antibiotics.

Since the discovery of penicillin, β-lactams have been the most significant antibiotic group for the past 70 years and are used to treat infections caused by both Gram-negative and Gram-positive bacteria (Siu 2002). β- Lactams can be classified into four main groups based on the structure and discovery; penicillins, cephalosporins, carbapenems, and monobactams. The four different classes of β-lactam antibiotics are described briefly below.

1.3.1 Penicillins.

Penicillins have low toxicity and mostly show high activity against Gram-positive bacteria. Resistance to penicillin, such as that shown by *S. aureus* and Enterobacteriaceae, can occur via several mechanisms, including the production of the enzyme β-lactamase (Waksman and Flynn 1973; Walsh 2000). Overall, the basic structure of penicillins includes a thiazolidine nucleus attached to the β-lactam ring and a side chain at the C6 position (Hodgkin 1949). The side chain represents different groups of penicillins: penicillin G (group 1), penicillin M (group 2), penicillin A (group 3), 6-α-substituted penicillins (group 4), α-carboxy- and α-sulfopenicillins (group 5), amidinipenicillins (group 6), and oxyimino penicillins (group 7) (Saradhi 2012).

1.3.2. Cephalosporins

The first cephalosporin was discovered from a fungal culture of *Cephalosporium acremonium* in 1945, now renamed *Acremonium chysogenum*, and was discovered by a Sardinian public health official, Brotzu. He found that it had an antimicrobial effect on *S. typhi*, the cause of typhoid fever (Campos *et al.* 2007). Cephalosporins are used to treat bacterial infections caused by both Gram-negative and Gram- positive bacteria. The cephalosporin's core structure has the β-lactam ring fused to a six-membered dihydrothiazine ring, with a sulphur atom at position 1 (Bryskier 1997). Cephalosporins are catagorized by their antimicrobial activity and historical development into four generations (Essack 2001). The 1st generation cephalosporins (e.g. cefazolin, cephaloridine, and cephalothin) have better activity against Gram-positive than Gram- negative bacteria. 2nd generation cephalosporins (e.g. cefoxitin, and cefuroxime) have increased Gram-negative activity while retaining some Gram- positive activity and are more resistant to β-lactamases. The 3rd generations (e.g. ceftazidime, cefotaxime, and cefixime), with some exceptions, have a better Gram negative activity to Enterobacteriaceae with

less Gram-positive activity. The 4th generation (e.g. cefepime, cefpirome, and cefozopran) show high activities to both Gram-positive and Gram-negative bacteria and also are more stable against β-lactamases (Bryskier 1997). The cephamycins are structurally similar to cephalosporins, but the cephalosporin nucleus is fused with a 7-alpha-methoxyl group. The other group gives high-level resistance to class A β-lactamases (Essack 2001). The first semi synthetic cephamycin was cefoxitin.

1.3.3. Carbapenems

The first carbapenem was thienamycin, isolated from a culture of *Streptomyces cattleya* (Weaver *et al*., 1979). Carbapenems foster less resistance than other β-lactams because of their stability against hydrolysis by many β-lactamases, including AmpC enzymes and ESBLs (Bassetti *et al*., 2013). Imipenem (N-formimidoyl thienamycin) is a chemically stable compound compared to thienamycin and was the first carbapenem approved for clinical use (Hellinger and Brewer 1999). Meropenem has one of the largest ranges of antimicrobial activity available (Pfaller and Jones 1997) and, due to its stability against most β-lactamases, is often used against many MDR/XDR life threatening infections in infants and adults (Cohen-Wolkowiez *et al*., 2012). Other carbapenems approved for clinical use include ertapenem and doripenem (Nicolau 2008; Paterson and Depestel 2009).

1.3.4. Monobactams.

Monobactams are β-lactam compounds where the β-lactam ring is a single cyclic structure and not fused to another ring. They are active only against Gram-negative bacteria.

SQ-26180 was the first monocyclic β-lactam derived naturally from *Chromobacterium violaceum* (Sykes *et al*., 1981) and was subsequently developed in 1985 after demethoxylation at the C3 position and substitution with a 2-amino-5 thiazolyloxime moiety into aztreonam (Sykes and Bonner 1985). Aztreonam is the only monobactam in clinical use and displays high activity against Enterobacteriaceae and good efficacy against *P. aeruginosa* (Sykes *et al*., 1988).

BAL30072 (SFM) is a new monocyclic β-lactam antibiotic with potent antimicrobial activity against a broad range of Gram-negative bacteria. It is a siderophore-monobactam with potent *in vitro* activity against MDR Gram-negative bacilli, representing a likely choice in treating carbapenem-resistant pathogens (Page *et al*., 2010). An overview of the antibiotics spectrum of β-lactams is presented in Table 1.2.

Table 1.2 Classes of β-lactams and the antibacterial spectrum

Chemical class	Examples	Spectrum of activity	
		Gram- negative	Gram-positive
Beta-lactams (penicillins)	Penicillin-G Penicillin-M	-	+
Semi-synthetic β-Lactams	Amoxicillin, Ampicillin	+	+
Beta-lactams (Cephalosporins)	1st generation Cephalothin, Cefazolin	±	+
	2nd generation Cefoxitin, Cefuroxime	+	±
	3rd generation Ceftazidime Cefotaxim	+	±
	4th generation Cefepime, Cefpirome	+	+
Penems	Carbapenems Imipenem, Meropenem	+	+
Monobactams	Aztreonam	+	+

+: good activity; ±: reduced activity.

1.4. β-lactamases

Hydrolysis of β-lactam antibiotics by β-lactamases is the most common resistance mechanism in clinically important Gram-negative bacteria (Bush and Jacoby 2010). The term β-lactamases refers to an enzyme inactivating the β-lactam by hydrolyzing the amide bond of its β-lactam ring (Siu 2002). They were first identified in *E. coli* before use of penicillin treatment (Spink and Ferris 1947). Now, there are more than 500 β-lactamases documented (www.lahey.org/studies.webt.htm). The substrate and inhibition profiles vary between different types of β-lactamases (Bush 2010). The spread of β-lactamases is often associated with plasmid encoded ESBLs, mainly CTX–M, *K. pneumoniae* carbapenemase (KPC) and MBLs (VIM, IMP and NDM-1) (Pitout 2010). In Gram- negative bacteria, β-lactamases usually accumulate in the periplasm where they hydrolyze the antibiotic. However, in Gram–positive bacteria β-lactamases are released into the surrounding environment as exo-enzymes to hydrolyze the drug ‘‘outside the cell’’ (Ghuysen 1991).

1.4.1 Classification of β-lactamases

β- Lactamases have been classified either on their functional characteristics (Bush *et al.*, 1995) or their primary structure based on amino acid similarities (Ambler 1980). The simplest classification is the Ambler molecular scheme where β-lactamases are divided into four molecular classes (A, B, C, and D) based on amino acid active motifs (Ambler *et al*., 1991; Jaurin and Grundstrom, 1981). β- Lactamases can be grouped structurally into two super families: serine β-lactamases (classes A, C, and D) and metallo-β-lactamases (class B). Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine residue, whereas class B β -lactamases are metallo-enzymes that use at least one zinc ion in their active site to co- ordinate polarized water molecules responsible for the β-Lactam hydrolysis (Bush and Jacoby 2010).

The Bush and Jacoby classification scheme of β-lactamases is based on substrate/inhibitor specificity (functional classification) (Bush *et al*., 1995) and has been further updated to accommodate newly discovered β-lactamases (Bush and Jacoby 2010).

1.4.1.1 Class A β-lactamases (Extended spectrum β-lactamases (ESBLs).

Class A β-lactamases are the largest group of β-lactamases and are generally inhibited by serine β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Paterson 2006). This class includes TEM, SHV and CTX-M-type groups and are mainly associated with ESBLs (Jacoby and Munoz-Price 2005; Walther-Rasmussen and Høiby 2007). The first CTX-M was discovered in 1989 and over 120 CTX-M types have been detected to date. CTX-M-14 and CTXM-15 are the most important genotypes worldwide. Disseminating CTX-M types are often associated with insertion sequence common regions (IS*CR*s). CTX-M-15 has become the most widespread ESBL globally (Coque *et al*., 2008). Class A enzymes can be chromosome and/ or plasmid mediated. KPC is the most clinically important of the Class A types as these enzymes are located on transmissible plasmids and confer resistance to all β-lactams (Yigit *et al*., 2001). KPC can be transmitted from *Klebsiella* to other genera including *E. coli, P. aeruginosa, Citrobacter, Salmonella, Serratia,* and *Enterobacter* spp (Marchaim *et al*., 2008).

1.4.1.2. Class C B-lactamases.

Class C β-lactamases can be encoded on chromosomes of many Gram- negative bacteria (Jacoby 2009), the paradigm being AmpC. AmpC enzyme has been described in many Gram-negative including *C. freundii, Enterobacter* spp., *E. coli, M. morgannii, P. rettgeri, Providencia stuartii, P. aeruginosa,* and *S. marcescens* (Philippon *et al*., 2002). They are more active against cephalosporins than penicillins and are usually resistant to inhibition by clavulanic acid and active against cephamycins such as cefoxitin (Bush and Jacoby 2010). When over- expressed, class C enzymes can, together with decreased OM flux, assist with resistance to carbapenems, especially ertapenem (Quale *et al*., 2006). Plasmid-mediated class C enzymes include CMY, ACT, DHA, FOX, and MIR of which the most common is CMY-2 (Jacoby 2009).

1.4.1.3. Class D β-lactamases.

Class D β–lactamases are termed oxacillinase or OXA- type β-l-lactamases (OXAs) because of their ability to hydrolyze oxacillin (Poirel *et al*., 2010b). These enzymes also hydrolyze cloxacillin, cephalosporins and some carbapenems. OXA enzymes are usually not affected by β-lactamase inhibitors but may be inhibited *in vitro* by sodium chloride (Poirel *et al*., 2010a). OXA genes are embedded into class 1 integrons and recent studies have shown that other MGEs, including insertion sequences (IS) and transposons (Tn), are associated with OXA

genes. The OXA group demonstrating carbapenemase activity is divided to five subfamilies: OXA-23, OXA-24/OXA40, OXA-48/181, OXA-58 and OXA-51. The first four groups are carried on transmissible plasmids, while the last group, OXA-51, is chromosomally located and intrinsic in *A. baumannii* species. OXA48/181 is specific to Enterobacteriaceae and is a growing problem in North Africa, Turkey and Greece (Poirel and Nordmann 2006).

1.4.1.4. Class B β-lactamases.

Class B β-lactamases are known as MBLs, which are named for their dependence upon zinc for efficient hydrolysis of β-lactams. The first MBL enzyme detected was in 1966 in *Bacillus cereus* and then in *Stenotrophomonas* in the 1980s (Queenan and Bush 2007). Initially, MBLs were discovered in environmental and opportunistic Gram- negative bacteria; however, about three decades later, MBLs were identified in clinically important pathogenic bacteria (Cornaglia *et al*., 2011). Chromosomally encoded MBLs include *B. cereus* (BcII) (Lim *et al*., 1988), *Bacteroides fragilis* (CcrA) (Rasmussen *et al.,* 1990), *Chryseobacterium meningosepticum* (BlaBs) (Bellais *et al.*, 2002), *Flavobacterium johnsoniae* (JOHN-1) (Naas *et al*., 2003), *Aeromonas hydrophila* (CphA) (Massidda *et al*., 1991), *A. veronii* (ImiS and AsbM1) (Yang and Bush 1996), *S. maltophilia* (L1) (Ullah *et al*.,1998), *Legionella gormannii* (FEZ-1) (Boschi *et al*., 2000), and *Caulobacter crescentus* (Mbl1b and CAU-1) (Simm *et al*., 2001). MBLs have been subdivided based either on structure (subclasses B1, B2, and B3) or function (subgroups 3a, 3b, and 3c) (Bush and Jacoby 2010). A class B1 and B3 have two zinc ions in their active sites and class B2 has only one zinc ion. IMP, VIM, GIM, SPM-1 and NDM-1 belong to MBL class B1. MBL genes can be located on plasmids and/ or chromosomes and many are associated with integrons and/ or transposons such as Tn*402* (Marchiaro *et al*., 2010; Borgianni *et al*., 2011; Santos *et al*., 2010).

The most common types of acquired MBLs identified in Enterobacteriaceae include the IMP and VIM groups, together with the emerging NDM group, where the group KHM-1 is rare (Walsh *et al*., 2005). Generally, the level of carbapenem resistance observed for MBL-producing strains varies and attributed mortality, associated with MBL production, ranges from 18% to 67% (Daikos *et al.*, 2009).

The first MBL, IMP-1, was described in Japan in 1991 from a *S. marcescens* isolate (Watanabe *et al*., 1991).

Perhaps the most noteworthy is the New Delhi metallo-β-lactamase 1 (NDM-1), first identified from patients hospitalized in India, and now reported worldwide. The Indian subcontinent is clearly the main reservoir of NDM-1 producers (Khan and Nordmann 2012). The $bla_{\text{NDM-1}}$ is not associated with a single species or with a specific plasmid backbone, but has been identified harboured on different plasmid types from unrelated Gram- negative bacteria (Poirel *et a*l. 2011). Plasmids carrying the $bla_{\text{NDM-1}}$ gene are diverse in size, incompatibility groups and associated resistance genes. The NDM-1 gene is located on a mobile genetic element and the pattern of spread appears to be more complex (Yong *et al*., 2009; CDC 2010). Many studies have reported that $bla_{\text{NDM-1}}$ positive isolates are mostly nosocomial *K. pneumoniae* isolates, but the $bla_{\text{NDM-1}}$ gene has also been identified in community-acquired *E. coli* (Nordmann *et al*., 2012). Furthermore, NDM-1- producing strains have been found in the environment, e.g. from tap water from New Delhi, in many diverse Gram- negative species including environmental bacteria such as *Vibrio cholera* (Walsh *et al*., 2011). Currently, eight variants of NDM enzyme have been identified. Most NDM-producing strains described are enterobacterial species and *P. aeruginosa* (Khajuria *et al*., 2013; Jovcic *et al*., 2011). The NDM-2 variant has been identified in *A. baumannii* (Kaase *et al.,* 2011), the NDM-3-producing *E. coli* strain is from Australia, the NDM-4-producing *E. coli* strain from India the NDM-5-producing *E. coli* strain from the United Kingdom, the NDM-6-producing *E. coli* strain from New Zealand, the NDM-7-producing *E. coli* strain from Canada and the NDM-8 producing *E. coli* strain from Nepal (Tada *et al*., 2013).

1.5. Epidemiology of NDM-1

NDM-1 is the most prevalent recently detected transferable class B β- lactamase. It was first described in 2008 in *K. pneumoniae* and *E. coli* isolated in Sweden from an Indian patient c hospitalized in New Delhi, India (Yong *et al*., 2009). He was originally from India but, having lived in Sweden for many years, returned to India. The NDM-1 gene can hydrolyze all penicillins, cephalosporins and carbapenems, but not aztreonam (Kumarasamy *et al*., 2010). Most NDM- positive bacteria are mainly resistant to other antibiotic classes and carry many other resistance mechanisms (e.g. to aminoglycosides and fluoroquinolones) (Muir and Weinbren 2010). NDM-1 was found in many isolates, mostly *K. pneumoniae* and *E. coli,* collected in the UK, India, Pakistan and Bangladesh in 2009, indicating that it had been well established before first discovery (Kumarasamy *et al.,* 2010). Reports from India show significant local prevalence (Castanheira *et al*., 2011; Roy *et al.,* 2011). A hospital in Varanasi (North) identified an NDM-1 prevalence rate of 6.9% among 780 successive isolates taken from

outpatients and hospitalized patients between February 2010 and July 2010 (Seema *et al*., 2011), showing remarkably similar rates to NDM-carrying Enterobacteriaceae isolated from an important hospital in Mumbai (Desphande *et al*., 2010a; Desphande *et al*., 2010b). 18.5% of outpatients in Rawalpindi, Pakistan carried NDM-1 positive bacteria as part of their normal gut flora (Perry *et al.*, 2011). These and other studies, point to widespread occurrence on the Indian subcontinent (Castanheira *et al.*, 2011). Since mid-August 2010, NDM-1-producing bacteria have been reported internationally, except in Central and South America (Nordmann *et al*., 2011a)

Currently, results suggest that the Balkan states and the Middle East might act as secondary reservoirs for the spread of the $bla_{\text{NDM-1}}$ gene (Nordmann *et al*., 2011b). Enterobacteriaceae, harbouring the NDM-1 gene, have been recovered from many clinical settings including UTI, septicaemia, pulmonary infections, peritonitis, and soft tissue infections (Nordmann *et al*., 2011; Kumarasamy *et al*., 2010). NDM-1-producing bacteria have been recovered from the intestinal flora of travellers returning from India and undergoing microbiological examination for unrelated diarrheal symptoms (Leverstein-Van *et al*., 2010). This is in keeping with observed environmental pollution by NDM-1-producing bacteria in New Delhi (Walsh *et al.*, 2011). This distribution suggests the association of the $bla_{\text{NDM-1}}$ gene with promiscuous plasmids. NDM-1 was found in a widespread diversity of Gram-negative species including Enterobacteriaceae, *Pseudomonas* spp., *Stenotrophomonas* spp., *Aeromonas* spp. and *V. cholera*, isolated in New Delhi from sewage and tap water samples (Walsh *et al*., 2011).

1.6. Mobile genetic elements (MGEs).

The first MGEs discovered in bacteria were phages (Duckworth 1976) and plasmids (Lederberg 1952). The diversity of MGEs has been described to include plasmids, bacteriophages, genomic islands (GIs), integrative and conjugative elements (ICEs), insertion sequences (ISs), transposons (Tns) and integrons. The basis for this classification was that these elements share similar characteristics, such as the excision by site-specific recombination, transfer by conjugation and integration by recombination between a specific site of elements and a site in the host's genome (Aminov 2011). MGEs' sizes vary from small IS elements (usually 700 to 2,500 bp) (Mahillon and Chandler 1998) to large mega-plasmids of *Sinorhizobium meliloti* (1.35 and 1.68 Mb) (Barnett *et al*, 2001; Capela *et al*., 2001). The majority of self-transmissible genetic elements have sufficient capacity to carry multiple genes, including those

encoding for antibiotic, heavy metal and biocide resistances, metabolism of various substrates and other auxiliary functions (Wiedenbeck and Cohan, 2011).

1.6.1 Plasmids.

Plasmids are double stranded, circular or linear, extra-chromosomal DNA self-replicating genetic elements. Plasmids were first defined in 1952 by Lederberg, who used the term to describe extra-chromosomal genetic determinants (Lederberg 1952). Plasmids do not carry essential genes for the growth of host cells under non stressed conditions (Carattoli 2011) but, under certain conditions, may confer a selective benefit. This might include enzymes for utilization of unusual carbon sources (Zhang *et al*., 2011), resistance to antimicrobials such as heavy metals (Silver and Misra 1988), antibiotics (Bennett 2008; Schwarz *et al*., 2001) and production of toxins and other proteins that allow the successful infection of the bacterial cell (Sengupta and Austin 2011).

Plasmids scaffolds are scattered with MGEs (i.e. integrons and transposons) that can move genes around within the plasmid as well as between chromosome and plasmid/s. Plasmids contain genes that are beneficial for the survival of bacteria e.g. virulence genes. Plasmids have also developed ways to protect their persistence in bacterial cells, e.g. through toxin-antitoxin systems. Such systems act by removing cells that have lost the plasmids after cell division. Furthermore, to ensure their longevity and stability, many plasmids have toxin-antitoxin (TA) systems (see later) that can kill or destroy bacteria that have not inherited plasmids (Bennett 2008).

Plasmids are usually classified by incompatibility (Inc) groups, defined as the inability of two plasmids to be propagated simultaneously in the same cell (Holt *et al*., 2007). Incompatibility groups have been proposed by genetic relatedness and pilus structure: the IncF group (IncF, IncS, IncC, IncD, IncJ), the IncP group (IncP, IncU, IncM, IncW), the Ti plasmids group (IncX, IncH, IncN, IncT) and the IncI group (IncI, IncB and IncK) (Hawkey and Jones 2009). In 2004 and 2009, a new classification of plasmids based on plasmid mobility (MOB classification) was proposed (Garcill´an-Barcia *et al.,* 2011). Genetic organization of plasmids usually entails a specific structure facilitating stability; transfer and replication (Figure 1.4). Together, these modules constitute the plasmid's backbone (Garcill´an-Barcia *et al*., 2011).

1.6.2. Transposons.

Transposons were discovered by Barbara McClintock in 1948, when she worked with maize in the U.S.A. A transposon (also called a jumping gene) is a DNA sequence that can move from one area of DNA to another or between genetic structures by a process called transposition. Transposition is an extremely important mechanism for the natural transfer of antibiotic resistance genes from one DNA structure to another (Greenwood *et al.,* 2006). Transposons are classified into two classes based on their mechanism of transposition: 1) Class I, Retro transposons (work by copying themselves and pasting copies back into the genome in multiple places) and 2) Class II, DNA transposons (work by cutting and pasting using transposase enzyme such as Tn*7)*. The typical class 1 integron is found embedded in transposons such as Tn*402*/Tn*5090*, Tn*6007*, Tn*21* and Tn*1696*, or defective transposon derivatives (Toleman *et al*., 2005; Siarkou *et al*., 2009; Nagy & Chandler 2004). Non-integron transposons include Tn*3*, that has a short inverted-repeat sequence (IR) (often 38 bp) at each end, transposition genes, tnpA (transposase), and tnpR (resolvase) with a short direct repeat at either end of the transposon (5 bp) (Greenwood *et al*., 2006). The complex transposon structure consists of two copies of an insertion sequence (IS) on either side of a resistance gene(s) such as Tn*10*. The Tn*402*/Tn*5090* family of transposons has four transposition genes (tniA, tniB, tniQ, and tniR) also called the tniC module; a resolution site (*res* site) that is located between tniR and tniQ and two inverted repeats, IRi and IRt, that are found at the ends of the transposon. The Tn*402*/Tn*5090* that lacks the IRt end (at 3`-CS) appears to be a non-mobile remnant of Tn*402*-like transposon (Toleman *et al*., 2004).

1.6.3. Integrons.

Integron was defined by Hall and Collis as a genetic element capable of capturing, integrating and expressing gene cassette coding proteins related to antibiotic resistance (Hall and Collis 1995). Integrons contain the integrase gene (*int*I), attachment site (*att*I) and promoter (P) (Figure 1.5) (Stalder *et al*., 2012).

All classes of integron contain two conserved segments (5'CS) and (3'CS), the integrase gene and the cassette integration site (Mirnejad *et al*., 2013). To date, several classes of integron have been described (Peymani *et al*., 2012). Class 1 integrons are the most common and widely distributed among Gram-negative bacteria and are associated with functional and non-functional transposons derived from Tn*402*, which can be further embedded in larger transposons such as

Tn*21*. Class 1 integron is composed of the 3'conserved sequence area (3'CS) and includes two open reading frames (ORFs), the *qac*EΔ1 gene, which confers resistance to quaternary ammonium compounds, and the *sul*1 gene which confers resistance to sulphonamides. Class 2 integrons are found in transposon Tn*7* derivatives. The integrase gene of class 2 integron is *int*I2. Its 3' CS has five tns genes that are responsible for the mobility of transposons. Class 3 Integrons also have been reported, but the 3' CS is still not well described (Mirnejad *et al*., 2013). Class 3 integrons (Arakawa *et al*., 1995) are also thought to be located in a transposon (Collis *et al*., 2002) and are less prevalent than class 2. The class 4 integron is embedded in a subset of the integrative and conjugative element SXT found in *V. cholerae* (Hochhut *et al*., 2001). Class 5 integrons are located in a compound transposon carried on the pRSV1 plasmid of *Alivibrio salmonicida* (Sorum *et al*., 1992).

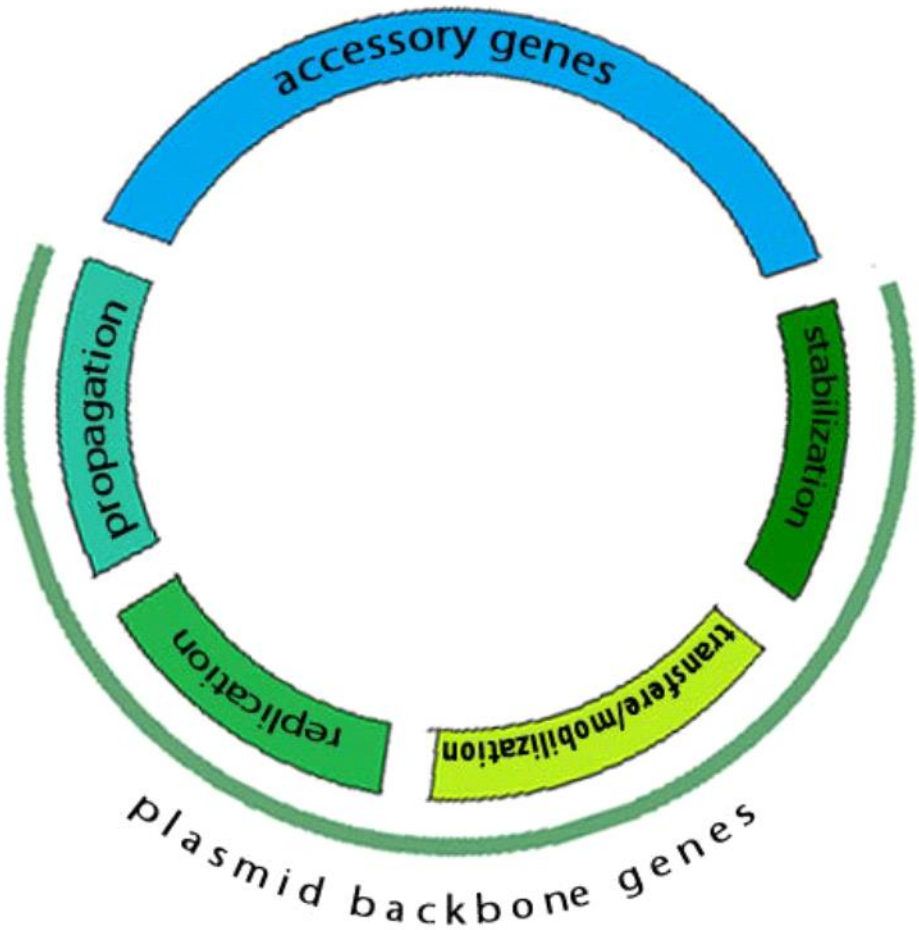


Figure 1.4 Schematic view of plasmid modular.

1.6.4. Insertion sequences (IS).

Insertion sequence is one of the important elements responsible for mobilizing genes. IS elements are short sequences (~700-2500 bp), flanked by inverted repeat sequences, which only encode genes involved in transposition. These structures can be inserted or excised by transposases (Murray *et al*., 2009). Many IS elements are found as part of compound transposons, or associated with class 1 integron, either localized on plasmids and/or chromosomes. Two IS elements surrounding a sequence (e.g. an antibiotic resistance gene) include a transposon. Transposons cannot replicate on their own, but they compose an effective way of transferring genes between plasmids and chromosomes (Bennett 2008; Harbottle *et al*., 2006). IS elements can be responsible for insertion mutations, genome rearrangements and enhanced spread of resistance and virulence determinants within species (Murray *et al*., 2009).

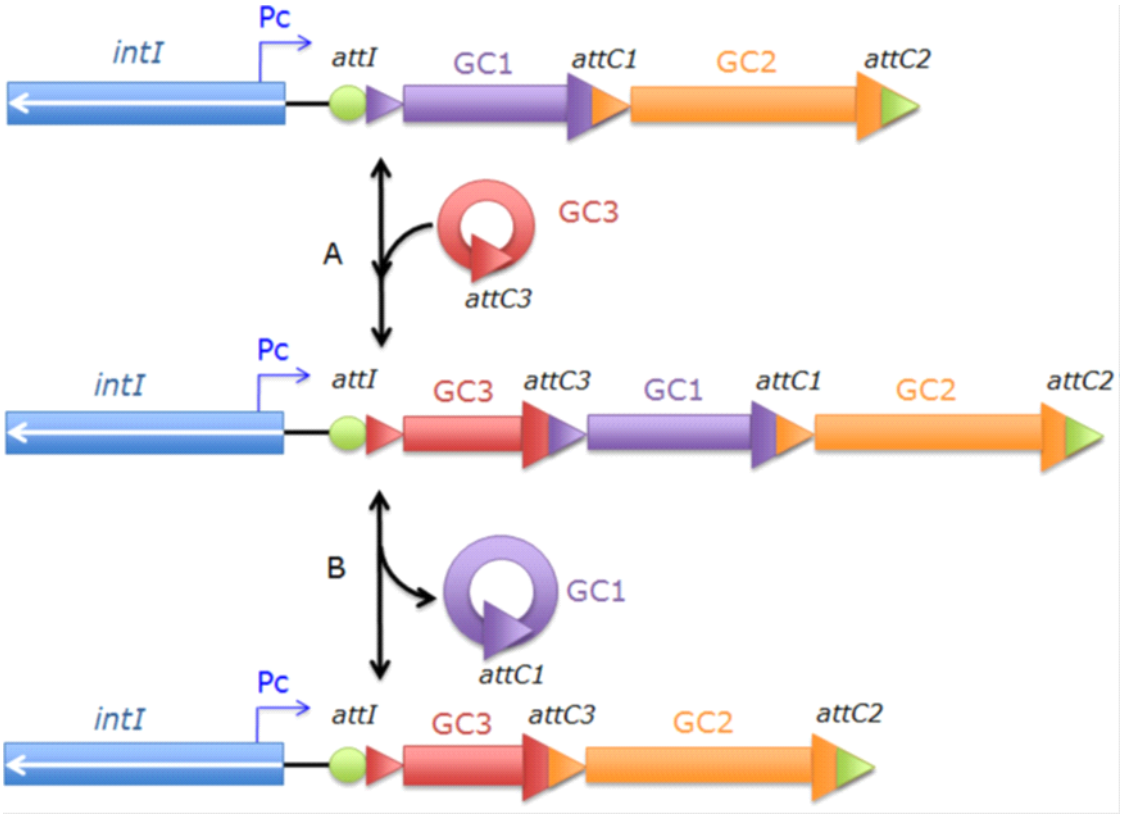


Figure 1.5 General organization of an integron and gene cassette (GC) recombination mechanism. The *Int*I1 protein catalyzes the insertion (A) and excision (B) of the GC in the integron, with GC integration occurring at the attI recombination site. Pc: gene cassette promoter; attI: integron recombination site; *att*C1, *att*C2, andattC3: *att*C GC recombination sites; *int*I: the integrase gene; GC1, GC2, GC3 are the gene cassettes, and arrows indicate the direction of coding sequences. Adapted from (Barraud and Ploy 2011)

1.6.5. Insertion Sequence with Common Regions (ISCRs).

IS*CR* elements are more recently found (1990) mobile genetic structures, also called common regions or *Orf*513 (Stokes *et al*., 1993). Currently, there are 23 different types of IS*CR* elements that differ in the amino acid sequence of their transposases and are divided into three groups based on their G+C% and the amino acid sequence of their transposases (Toleman & Walsh, 2008; Toleman *et al*., 2011). IS*C*R elements can move genes through rolling-circle transposition. All IS*CR* elements discovered have been found in the immediate vicinity of horizontally acquired genes, the majority being antibiotic and antimicrobial resistance genes. IS*CR* elements are often found close to the conserved 3'-end of class 1 integrons and have been found to mobilize various ESBL and MBL genes (Toleman *et al.*, 2006).

1.6.6. Integrative and Conjugative Elements (ICEs).

ICEs are a class of self-transmissible mobile elements that have been found in both Gram-negative and Gram-positive bacteria (Wozniak and Waldor, 2010; Burrus *et al*., 2006). These elements work as mediators of horizontal gene transfer in bacteria (*via* conjugation) (Balado *et al*., 2013). ICEs are, indeed, able to self-transfer from a donor to a recipient cell and integrate into the host chromosome (Hochhut and Waldor, 1999). Initially, they were described as conjugative plasmids (namely; R factors) (Burrus *et al*., 2002). Subsequently, they were found unable to replicate and were classified as conjugative transposons that transfer to recipient cells via a non-replicative circular intermediate (Burrus *et al.*, 2002). The size of ICEs can range from 30 kb to several hundred kb (Wozniak and Waldor 2010). ICEs have been found in *V. cholerae* O1 and O139 isolates from Asia. Furthermore, ICEs of the SXT/R391 family have been described in *V. cholerae* non-O1 and non-O139 (Taviani *et al*., 2009).

1.7. Horizontal Gene Transfer (HGT).

Lateral gene transfer, recently the called Lateral Genetic Transfer (LGT) is also known as Horizontal Gene Transfer (HGT) (Frost *et al.,* 2005). The evolutionary importance of HGT in bacterial cells was seen in the spread of penicillin resistance through plasmid transfer across Enterobacteriaceae (Datta and Kontomichalou 1965). HGT is an essential route in moving and rearranging DNA in prokaryotes and plays a key role in microbial evolution. The spread of resistance genes is a direct result of HGT and this has vast complications for human health (Gogarten *et al*., 2002; Stokes and Gilling 2011).

Stokes and Gilling have defined HGT as the process whereby DNA from one cell is physically transferred from one cell to another cell without an absolute need for cell division and the incorporation of that DNA into the recipient's DNA such that it can be stably inherited (Stokes and Gilling 2011).

HGT requires three mechanisms to gain the bacterial cell foreign DNA (Figure 1): 1) transformation (the uptake of DNA from the environment by bacteria), 2) transduction (the transfer of DNA between cells via a phage vector), or 3) conjugation (transfer of DNA by direct cell-to-cell contact often mediated by conjugative plasmids) (Da Silva and Mendonça 2012).

1.7.1. Conjugation.

Conjugation mediates the exchange of large mobile genetic elements, namely plasmids. Conjugation is believed to be the most important process responsible for short-term bacterial adaptation (Norman *et al*., 2009) as it can transfer genetic material even between phylogenetically unrelated organisms (Dröge *et al.,* 1999). Conjugation is a process whereby a conjugative plasmid is transferred between bacteria via a contact dependent semi conservative replication process. Conjugation requires two sets of genes, the mobility (MOB) genes and the mating pair formation (MPF) genes; furthermore, it needs an origin of transfer (*ori*T) (Smillie *et al.,* 2010). MOB genes code for a relaxase and DNA processing protein, responsible for the relaxosome, and for the coupling protein that links the relaxosome to the mating channel. MPF genes encode for the membrane–associated mating pair formation complex, a form of type 4 secretion system (T4SS) that provides the mating channel (Smillie *et al*., 2010).

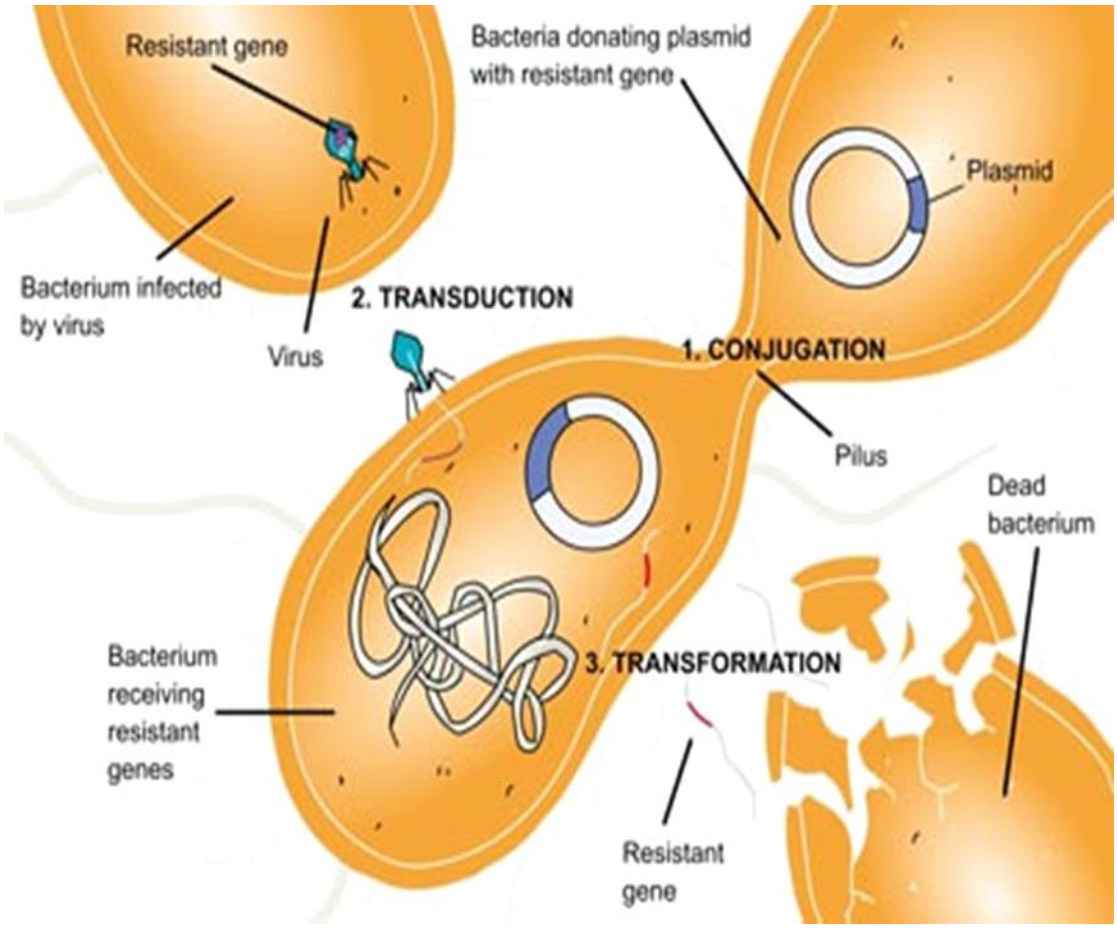


Figure 1.6 Mechanisms of horizontal gene transfer include transformation, conjugation and transduction. Adapted from http://guardianlv.com/wp-content/uploads/2013/10/Mechnisms-of-horizontal-gene-transfer.jpg

The first step in the conjugation process is the mating pair formation, whereby the donor and recipient connect physically. The second step involves relaxase-mediated nicking of the plasmid at *ori*T and formation of the relaxosome (coupling of single-stranded DNA and a protein complex). The relaxosome docks to the coupling protein that helps mediate transport through the T4SS into the recipient cell, followed by the establishment and replication of plasmids in the recipient (Grohmann *et al*., 2003). Mobilizable plasmids lack functions that enable cells to couple (MPF) before DNA transfer, but usually encode the MOB functions needed specifically for the transfer of their own DNA. Transfer can happen if a conjugative element supplies the MPF genes *in trans* (Smillie *et al*., 2010). The simplest mobilizable plasmids contain only the *ori*T and will need both MOB and MPF genes supplied *in trans* to be mobilized (Snyder and Champness 2003).

1.8. Heavy Metals.

1.8.1. Introduction.

Microbial resistance to heavy metals is a global problem in humans. Heavy metal pollutants spread widely and because they don't decompose, they are forever present in the environment. Heavy metals are used in various industries from which effluents are consistently discharged into the environment (Nanda *et al*., 2011) and not only cause health hazards, but disturb the environmental status of the biota (Moore *et al*., 1984).

Many heavy metals like zinc, chromium, cobalt, nickel, manganese, and iron are necessary for living organisms and are known trace elements (Bruins *et al*., 2000) at a particular concentration level. On the other hand, other heavy metals have no biological role and the organisms need them even at low concentrations (cadmium, mercury, lead etc.) (Laila *et al*., 2011). These metals play a vital role in some enzyme activities and cofactors (Amalesh *et al*., 2012); they are stabilizers of protein structures and play a role in protecting the osmotic balance (Bruins *et al*., 2000). However, these metals can become toxic for bacteria at high concentrations (Amalesh *et al*., 2012) by binding to enzymes and DNA (Lopez-Maury *et al*., 2002; Morozzi *et al*., 1982), damaging the DNA (Bruins *et al*., 2000), inhibiting enzyme activity (Nweke *et al*., 2007) and reducing diversity of microbial populations (Anne *et al*., 1999). Heavy metals have been used in water treatment, in clinical hospitals (medication and hygiene), in dental amalgams and in antimicrobial products (Torres-Urquidy and Bright 2012).

Bacteria have adapted to heavy metals by a variety of chromosome-, plasmid- and transposon- mediated resistance mechanisms (Said *et al*., 1991). Essential metal resistance systems are usually chromosome-based and more complicated than plasmid encoded systems. However, plasmid-encoded systems are usually toxic-ion efflux mechanisms. This suggests that ion efflux mechanisms are more likely to be mobilized to other organisms (Bruins *et al*., 2000). Bacterial resistance to heavy metals is common and frequencies range from a few per cent in "clean" environments to as much as 100% in heavily contaminated environments (Silver and Phung 1996).

The MIC is defined as the minimum concentration of a heavy metal at which microbial growth is completely inhibited (Yilmaz 2003). However, a problem remains in defining the concentrations that distinguish metal-resistant from metal-sensitive bacteria. Media ingredients, pH and culture conditions can affect heavy metal ion activity. Metal ions and media components may react antagonistically, which makes it difficult to prove the toxic effect of metals to bacteria (Margesin and Schinner 1996).

Resistance to mercury, silver, copper and arsenic metals were selected in this thesis.

1.8.2. Plasmid-mediated heavy metal resistance in Enterobacteriaceae.

As described, bacterial plasmids have genes that provide extra functions for the cell, among this is resistance to heavy metals. Numerous bacteria have heavy metal resistance mechanisms to metals such as Cu^{2+}, Ag^{+}, AsO_{2}^{-}, Cd^{2+}, Ni^{2+}, Pb^{3+}, Zn^{2+}, Cr^{2+} etc. (Silver and Misra 1988). These resistance systems are often found on plasmids and transposons (Rajini and Mahadevan 1989), with a few exceptions being reported on the chromosome (Wang *et al*., 1987). The same mechanisms of resistance occur in bacteria isolated from soil, water and clinical and industrial waste (Silver and Misra 1984). Plasmids mediating resistance to heavy metals also carry other genes encoding for, for example, antimicrobial resistance (Rajini and Mahadevan 1993; Karbasizaed *et al*., 2003).

In one study, 25 heavy metal resistant isolates were isolated from the Alzahra Hospital of Isfahan, Iran. 15/ 25 were *E. coli* and *K. pneumoniae*. Most of these bacteria demonstrated resistance to four heavy metals (Hg, Cu, Pb and Cd) and, in most of the 25 isolates, plasmids of different molecular size were observed carrying specific mechanisms of heavy metal resistance (Karbasizaed *et al*., 2003, Zolgharnein *et al*., 2007).

1.8.3 Mercury (Hg).

Mercury is the heavy metal most toxic to bacteria because it binds to sulfhydryl groups and inhibits enzyme activity (Foster 1983). Mercury is found in the environment in diverse forms such as Hg^0 (elemental), Hg_2^{2+} and Hg^{2+} (inorganic salt), organic (alkylated) or sulfidic (cinnabar).

Mercury resistance is one of the most sophisticated and well- known bacterial heavy metal resistant mechanisms in Gram-positive (*S. aureus*, *Bacillus* sp.) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *S. marcescens,* and *Thiobacillus ferrooxidans*) (Pan-Hou *et al*., 1981). Bacteria may respond to Hg^{2+}exposure using distinct strategies, e.g., reduced uptake of Hg^{2+} (Pan-Hou *et al*., 1981), conversion to insoluble mercuric sulphide (Pan-Hou *et al*., 1981) or dimethylmercury sulphide (Baldi *et al*., 1993), and reduction to elemental Hg (Barkay *et al*., 2003). The enzymatic reduction of Hg^{2+} to Hg^0 is the most descriptive and widespread mechanism of resistance in many bacteria (Osborn *et al.,* 1997). The enzymatic reduction of Hg^{2+} is catalyzed by products of the *mer* operon (Figure 1.7). The mercury resistance operon system has several genes, *mer*RTPA, with or without accessory genes (*mer*B, C, D, E and G) (Barkay *et al*. 2003). The *mer* system is illustrated in Figure 1.7 and includes uptake and transportation of Hg^{2+} by the periplasmic protein, MerP, and the inner membrane protein MerT. MerE is a membrane protein that probably acts as a broad mercury transporter mediating the transport of methylmercury and Hg^{2+} (Kiyono *et al*., 2009). The cytosolic mercuric reductase MerA reduces Hg^{2+} to less toxic elemental mercury (Barkay *et al*., 2003). *Mer*B encodes an organomercurial lyase that catalyses the protonolytic cleavage of carbon-mercury bonds (Misra 1992). *Mer*G encodes the cellular permeability to organomercurial compounds reduction (Kiyono *et al*., 1999). *Mer*D acts as a distal co-repressor of transcriptional activation (Champier *et al*., 2004). MerR is the activator or repressor of the transcription of *mer* genes in the presence or absence of mercury ions, respectively (Ni'Bhriain *et al*., 1983; Permina *et al*., 2006). Mercury stresses the transcriptional activator MerR and then triggers expression of the structural *mer* genes (Brown *et al*., 2003). The first description of transposon (Tn)-associated resistance was Tn*501*. Tn*501* mer operon was first isolated from the plasmid pVS1 from a *P. aeruginosa* strain isolated in Australia (Barrineau *et al*., 1984). This mer operon has five genes: *mer*R, *mer*D, *mer*T, *mer*P and *mer*A. In addition, Tn*21* has the same genes but with the transport gene, *mer*C, that was originally isolated from plasmid NR1 from *S. flexneri* in Japan (Nakaya *et al*., 1960). *S.*

marcescens has plasmid pDU1358 and carries the lyase gene, *mer*B, which differs from the above two mer operons (Griffin *et al*., 1987).

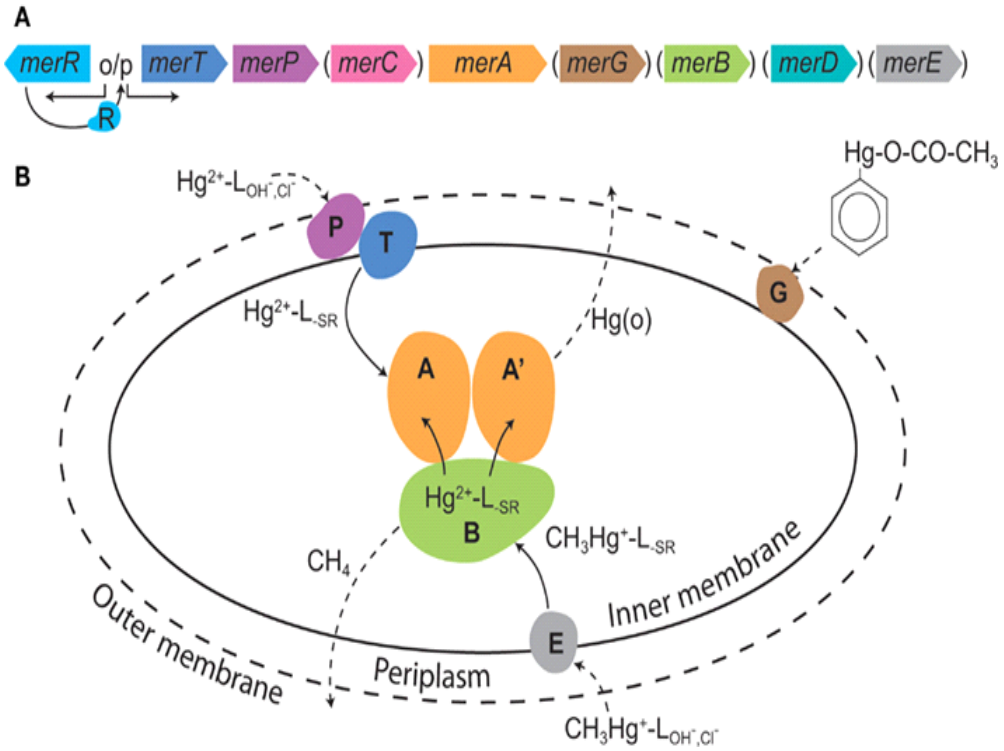


Figure 1.7 The mer system. (A) A basic *mer* operon with genes in parentheses describes those that are present in some, but not the majority of, operons. (B) The *mer*-encoded mercury detoxification mechanisms in bacterial cells. The cell outer layer is illustrated by a broken line showing that not all bacteria have an outer membrane; broken line arrows represent diffusion; solid line arrows indicate transporter transformations; L= ligand with subscripts denoting the ligand type. The colours of various Mer proteins correspond with the colours of the genes that encode for these proteins in (A). Adapted from (Lin *et al*., 2012)

1.8.4. Copper (Cu).

The oldest recorded medical use of copper was to sterilize wounds and drinking water (Dollwet *et al*., 1985), in the treatment of such diseases as headaches, ear infection, burns and intestinal worms, and in hygiene (Grass *et al*., 2011). Using copper in medicine became widespread in France in 1832, to treat cholera and then to treat tubercular infections, syphilis, eczema, and lupus (Dollwet *et al*. 1985). Copper continued to be used as an antimicrobial agent even after the introduction of antibiotics in 1932.

At low concentrations, microorganisms require copper ion as co-factors for metallo proteins, but are highly toxic to living cells when present in high concentrations (Souli *et al*., 2012). Bacterial resistance to copper (Cu) was observed as early as 1983 (Andersen *et al*., 1991). These resistant mechanisms are: 1) Sequestration of copper ions out of cells, 2) Relative impermeability of the outer and inner bacterial membranes to copper ions, 3) Metallothionein-like copper-scavenging proteins in the cytoplasm and periplasm and 4) Active extrusion of copper from the cell (Grass *et al*., 2011).

Bacterial copper resistance has been detected on plasmids (*E. coli* and *P. syringae)* and on the chromosome (*Xantomonas campestris*). The mechanism of resistance in *E. coli* and *Pseudomonas* is the same but for the differences in the nomenclature of the genes. In *E. coli*, a plasmid carries the copper resistance locus consisting of four genes called *pco*A, *pco*B, *pco*C and *pco*D, and in *Pseudomonas*, it consists of *cop*A, *cop*B, *cop*C, and *cop*D (Silver and Ji 1994).

Many studies have confirmed that the ATPase efflux mechanism exists for the copper ion Cu (II) in Gram-positive and in Gram-negative bacteria (Bruins *et al*., 2000; Rademacher and Masepohl 2012). One of the best studied copper resistance and transport systems is found in the Gram-positive enteric bacterium, *Enterococcus hirae (Streptococcus feacalis)* (Solioz *et al*., 2010). In this system *cop* operon consists of four genes: *cop*A, *cop*B, *cop*Z and *cop*Y (Solioz and Odermatt 1995) (Figure 1.8). *Cop*A is responsible for encoding a copper uptake ATPase and *cop*B responsible for detoxification and a P-type efflux ATPase. *Cop*Z and *cop*Y genes both encode a P-type ATPase and both regulate the cop operon (Burins *et al*., 2000; Silver 1996).

The copper resistance system in *E. coli* consists of *pco*ABCDE and *pco*RS genes. This resistance system is based on an efflux mechanism. The *pco* operon in *E. coli* is closely related to the *cop* operon in *Pseudomonas*, (*cop*ABCD and *cop*RS) (Silver 1996). PcoR is the DNA-

binding responder protein and PcoS the sensor protein. PcoD is found in the inner membrane, PcoB is located in the outer membrane and PcoA and PcoC are found in the periplasm. The storage of excess copper in the periplasmic membrane protects cells from copper toxicity. Efflux proteins are often encoded by plasmid-carried *pco* genes, which in turn are dependent on the expression of the chromosomal cut gene (*cut*ABCDEF) (Cooksey 1993). Cooksey (1993) also explains that most bacterial species in the environment have acquired at least one of the management systems of copper, and that the development of copper resistance may have come about by the modification of copper uptake genes found on chromosomes.

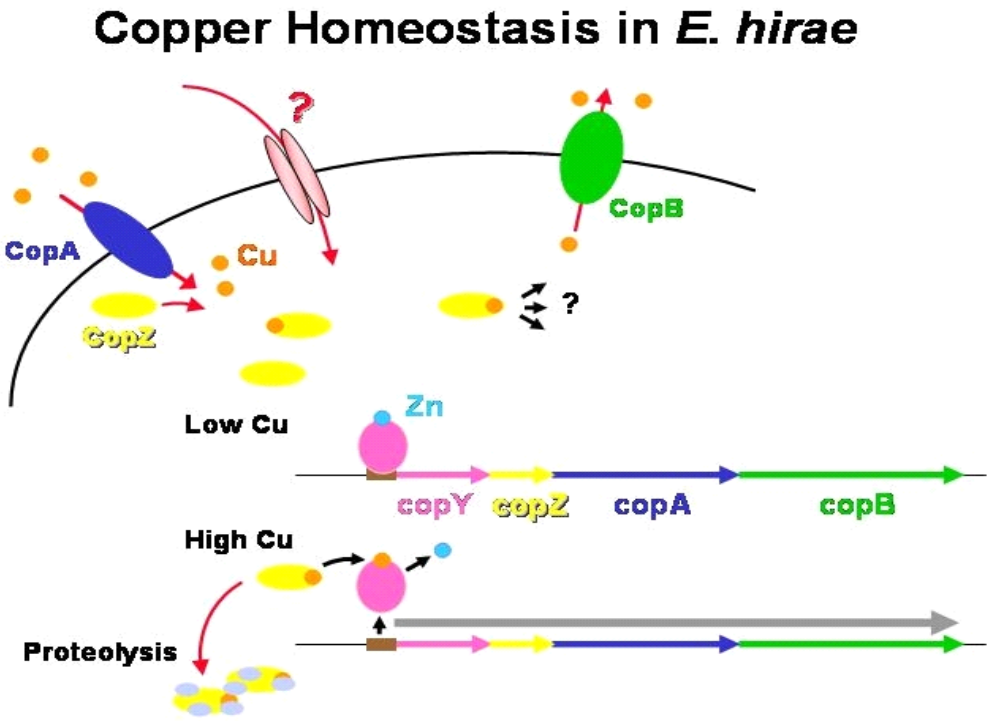


Figure 1.8 Copper homeostasis in *Enterococcus hirae.* Copper enters cells via copA or unknown channels. Excess copper is picked up by the copZ chaperone and delivered to copB for secretion. The copY is repressor for induction of the cop operon. Excess copZ is proteolyzed. At low copper, Zn(II)copY is bound to the promoter and transcription of the cop operon is rejected, while at high copper, if copper is excessive, Cu(I) copZ donates copper to Zn(II) copY repressor, which then loses the bound zinc and dissociates from the DNA, allowing transcription to proceed. To prevent cell damage, the level of Cu (I), copZ, is not allowed to rise beyond a certain level and excess Cu (I) copZ is degraded by a copper- activated protease. Adapted from (Solioz *et al*., 2010)

1.8.5. Arsenic (Ars).

Arsenic is commonly found in the environment and is toxic to bacteria as it inhibits enzymes such as kinases (Foster 1983). Arsenic has two soluble inorganic forms, arsenite^{3+} and arsenate$^{5+.}$ These compounds can enter drinking water from natural sources and have caused poisoning in Taiwan, Chile, Argentina, Bangladesh and West Bengal. Furthermore, arsenic poisoning has been detected in Cambodia, Vietnam, Nepal, China, Bolivia and Mexico (Anyanwu and Ugwu 2010).

Arsenic uptake by bacteria is mediated by phosphate transporters and is pumped back out of cells by an efflux pump (Nies and Silver 1995). Several arsenic resistance mechanisms have been characterized. The plasmid-mediated systems of arsenate and arsenite efflux have been determined in Gram-negative bacteria (Chen *et al*., 1986). Although arsenic is toxic to most microorganisms, some of them have evolved mechanisms to get energy by either oxidizing or reducing arsenic (Anyanwu and Ugwu 2010). The arsenic operon has been found on transmissible plasmids and on the chromosomes in Gram-negative and Gram-positive bacteria (Silver 1998).

The Ars operon consists of either *ars*RDABC or *ars*RBC genes (Ji and Silver 1995). The five genes, *ars*R, *ars*D, *ars*A, *ars*B and *ars*C are found on *E. coli* plasmid R773, whilst the *S. aureus* plasmid can have three genes, *ars*R, *ars*B, and *ars*C (Figure 1.9) (Jareonmit *et al.,* 2010; Silver and Ji 1994). *Ars*R encodes a transacting repressor protein and can be induced *in vivo* by arsenate, arsenite and bismuth (Ji and Silver 1995). *Ars*B encodes ArsB proteins giving partial resistance to arsenate and arsenite, where the high-level of resistance occurs with *ars*A and *ars*B. The last gene is *ars*C, which encodes a reductase that converts As (V) to As (III) (Jareonmit *et al*., 2010). The *ars*R, *ars*B and *ars*C could be present on the chromosomes of *E. coli* and *P. aeruginosa.*

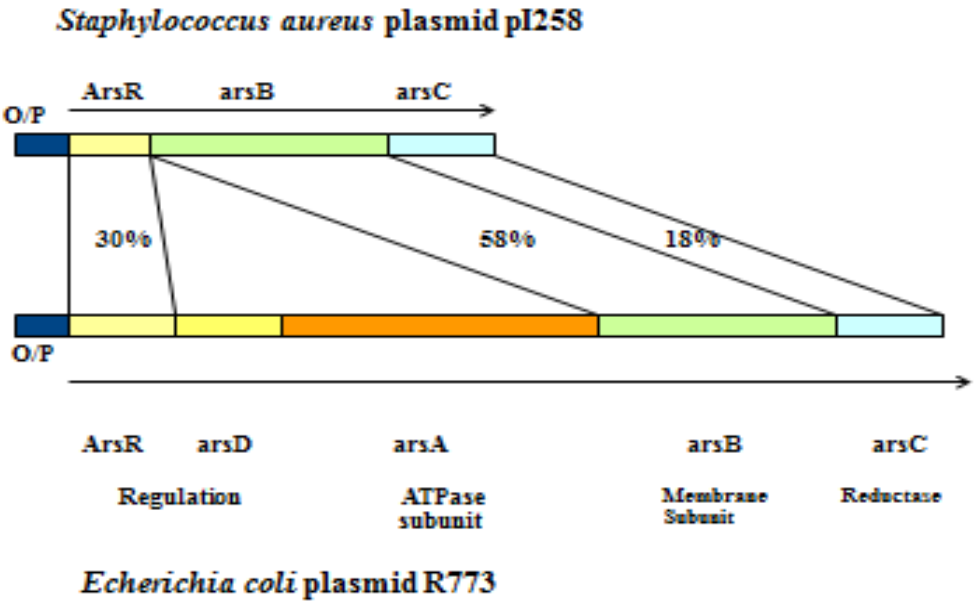


Figure 1.9 A basic arsenic operon in *E. coli* and *Staphylococcus aureus.* Adapted from (Silver and Ji 1994)

1.8.6. Silver (Ag).

Silver, in soluble form, is highly toxic which is reduced when the silver complexes with chloride, sulfide or phosphates. Silver kills bacteria by exerting a toxic effect on cell surface compounds (Belliveau *et al*., 1987), inhibiting respiration (Bragg and Rainnie, 1974) and uncoupling adenosine triphosphate synthesis (Chappell and Greville 1954; Feng *et al*., 2000). Before the beginning of antibiotic treatment, silver was used in antiseptics to treat infections in open wounds and burns (Rai *et al*., 2012). Recently, silver has been used in different hospital practices such as catheters, surgical apparatuses, and surgical masks (Wright *et al*., 2012).

Use of silver compounds has predictably led to bacterial resistance. This has been observed in burn infections treated with silver salts and in hospitals that use silver-coated catheters (Wright *et al*., 2012). There are several types of silver compounds that have been used as antimicrobial for hundreds of years and include silver nitrate (AgNO3), silver sulfadiazine (AgSD), silver zeolite, silver powder, silver oxide, silver chloride and silver cadmium powder (Rai *et al*., 2012).

Resistance to AgNO3 is dependent upon whether the halide ions exist. Susceptible cells can extract Ag^{2+} from an $AgCl_2$ precipitate, whereas resistant cells cannot. Thus, silver resistance appears to be due to a function which is probably located at the cell surface and which prevents cells from extracting silver, which is in an insoluble state (Foster 1983). The first silver resistance encoded by plasmids was reported by McHugh *et al*., in *S. typhimurium* pMG101 which contained seven genes and two open reading frames of unknown function (McHugh *et al*., 1975). The first gene in the silver operon (Figure 1.10) is *sil*E; a periplasmic metal- binding protein. SilS and SilR are a two component membrane sensor kinase and a transcriptional regulatory/responder protein. SilCBA protein complex consists of SilA, an inner- membrane proton/cation antiporter; SilB, a membrane protein and SilC, an outer membrane protein. The last gene, *sil*P, encodes a P-type ATPase (Chopra 2007).

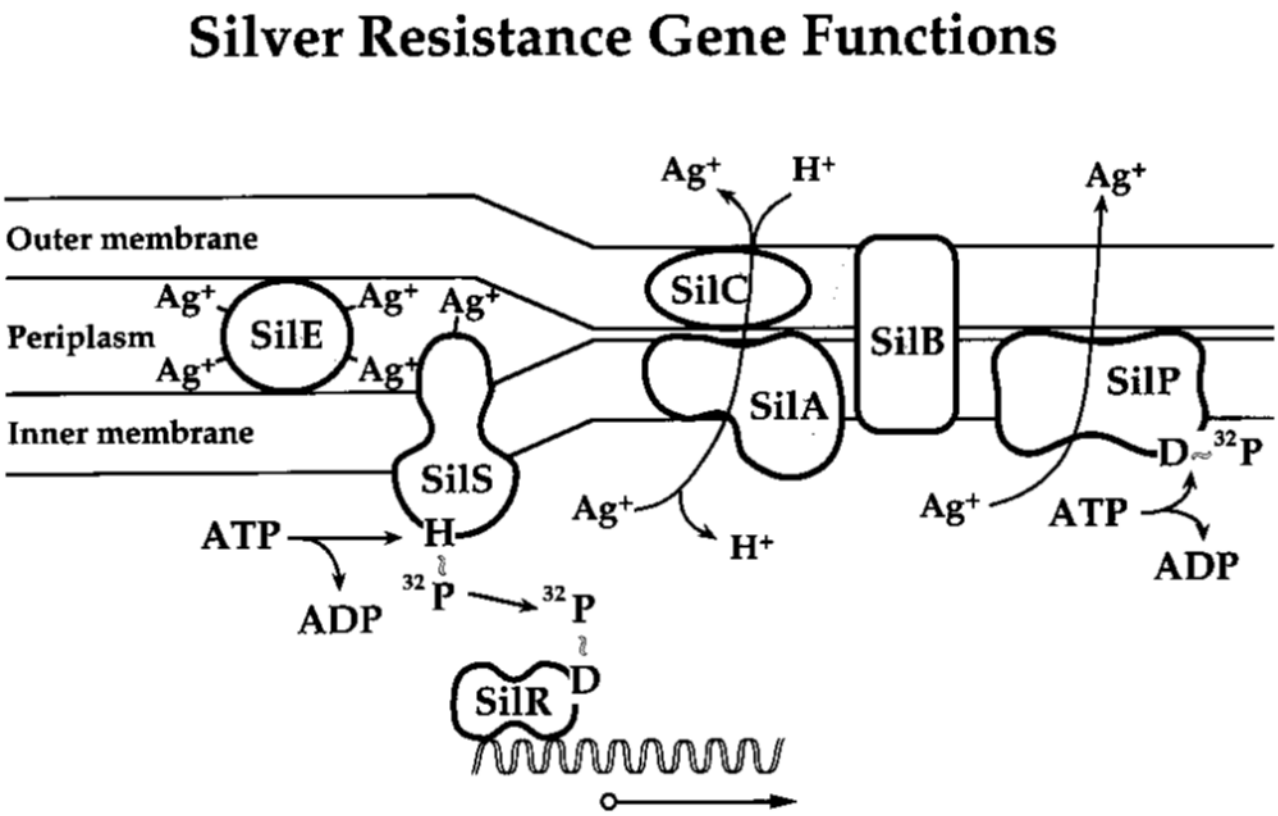


Figure 1.10 A basic silver operon. The first model for proteins and functions of the plasmid silver resistance determinant. Adapted from (Silver 1998)

1.8.7. Antibiotic and heavy metals resistant bacteria.

Co-resistance to both antibiotics and heavy metal are an increasing problem today in clinical and environmental bacterial populations. The increased use of antibiotics and heavy metal in health care and industries create selective pressure for bacteria in polluted environments.

In 1997, a study showed that heavy metals tend to target multiple sites on or within the bacterial cells and have broad-spectrum activity, where antibiotics tend to target specific sites on or within a bacterial cell and have a narrower spectrum of activity (Davies 1997). Nakahara *et al*., (1978), studied resistance to heavy metals and antibiotics in 746 clinical isolates of Enterobacteriaceae isolated from hospitals in Japan. Most of these isolates were multiple-metal-resistant and drug-resistant, and approx. 30% of total isolates were heavy metal-resistant but drug-sensitive, whereas the frequency of metal-sensitive and antibiotic-resistant was 0%.

A growing body of evidence indicates that heavy metal and antibiotic resistance are often found together in many clinical isolates and that metal and antibiotic resistance are closely associated (Timoney *et al*., 1978; Hassen *et al*., 1998). Because heavy metal and antibiotic resistance genes are often found on the same mobile genetic element, heavy metal contamination can promote the emergence of antibiotic resistances in exposed bacteria (Knapp *et al.*, 2011).

Since the 1970s, several studies have reported that antibiotic resistant bacteria may arise through co-resistance (genetically) or cross-resistance (physiologically) to metals (Seiler and Berendonk 2012).

Cross-resistance occurs when the genes specifying resistant phenotypes are located together on the same genetic element such as plasmids, transposons or integrons (Chapman 2003). The methicillin resistant *S. aureus* (MRSA), for example, is considered MDR for the accumulation of resistance mechanisms to, among others, β-lactams, aminoglycosides or fluoroquinolones. In addition MRSA exhibits cross-resistance within specific antibiotic classes, such as cross-resistance to all fluoroquinolones for the mutations in the topoisomerases genes (*par*C) and/or *gyr*A) or macrolides owing to presence different *erm*, *mef* or *mrs* genes (Winston and Chambers 2009).

Several studies demonstrated that metal and antibiotic resistance genes are linked, particularly on plasmids, as shown by transformation, plasmid curing and plasmid sequencing

(Chapman 1998). However, other studies have shown that increasing heavy metal levels lead to a decrease in antibiotic resistance (Holzel *et al*., 2012).

1.9. Toxin – Antitoxin Systems

1.9.1. Discovery and Function of Toxin-Antitoxin Systems.

The Toxin-Antitoxin system is abbreviated as the TA system. TA systems were first identified on prokaryotic plasmids involved in the prevention of post segregation plasmid loss (Gerdes *et al*. 1986). TA systems are found on many bacterial/archael chromosomes (Pandey and Gerdes 2005), and have been linked to the response of bacteria to antibiotics (Agarwal *et al*., 2010; Christensen-Dalsgaard *et al*., 2010). TA systems play a pivotal role in bacteria, including programmed cell death (Hazan *et al*., 2004), stress response (Christensen *et al*., 2001), bacterial persistence (Rotem *et al.*, 2010), regulation of biofilm formation (Wang and Wood, 2011) and protection from bacteriophages (phages) (Fineran *et al*., 2009). Therefore, the main role suggested for TA systems is the control of growth under stress conditions (Gerdes 2005). In all types of TA systems, the cells are "addicted" to the antitoxin, since its production is important for cell survival. Consequently, these systems are also called addiction modules (Syed and Lévesque 2012). Wagner and Unoson have found that chromosomal TA systems play a role in the persister formation (Wagner and Unoson 2012). Type II TA systems seem to be widely spread by horizontal gene transfer (Mruk and Kobayashi 2014).

Bacterial genomes contain small genetic modules encoding two genes; a stable toxin and unstable antitoxin. These modules are called toxin–antitoxin (TA) systems (Syed and Lévesque 2012; Ren *et al.*, 2012). The TA operons are regulated by the binding of the antitoxin to an operator region located nearby or within the promoter of the operon. The antitoxin by itself binds poorly to its DNA target, but the TA complex, generated by antitoxin–toxin interactions, increases the strength of binding leading to efficient self-regulation of its cognate operon (Moreno-Co´rdoba *et al*., 2012).

TA systems are classified into five types or classes. Type I (class I) is where the toxin gene expression is regulated by antisense RNA transcribed from the toxin gene, but in the reverse direction (Fozo *et al*., 2008; Gerdes and Wagner 2007) (Figure 1.11, a). Type I TA systems (e.g., *hok/sok, bsr*G/SR4, *tis*B/ IstR1 and *sho*B/*Ohs*C) have been discovered in Gram-negative and

Gram-positive bacteria. The first and best studied type I system is hok/sok of *E. coli* pR1, which was detected by Kenn Gerdes and Molin in 1986, and then found to be also encoded in the genomes of several Enterobacteriaceae (Gerdes and Molin 1986). Moreover, chromosomally encoded Type I systems are often present in multiple copies (Wagner and Unoson 2012); Type II is where the toxin is neutralized by forming a complex with its cognate antitoxin (Yamaguchi and Inouye 2009). The genetic organization of a type II TA is represented in Figure 1.11. Type III, is where the antitoxin is an RNA molecule that binds to the toxin (Fineran *et al*., 2009) (Figure 1.11, c). The type I and II TA systems are often located on plasmids, prophages and integrons (Mruk and Kobayashi 2014). Furthermore, both type I and II systems were originally recognized through their role in plasmid maintenance by post segregation killing (Fozo *et al*., 2010; Leplae *et al*., 2011; Mruk and Kobayashi, 2013). TA systems of Types IV and V contain a small proteinacious antitoxin that can render the toxin inactive by either protein-protein interaction, by cleavage of toxin mRNA, or by binding to the toxin's target structure (Schuster *et al*., 2013).

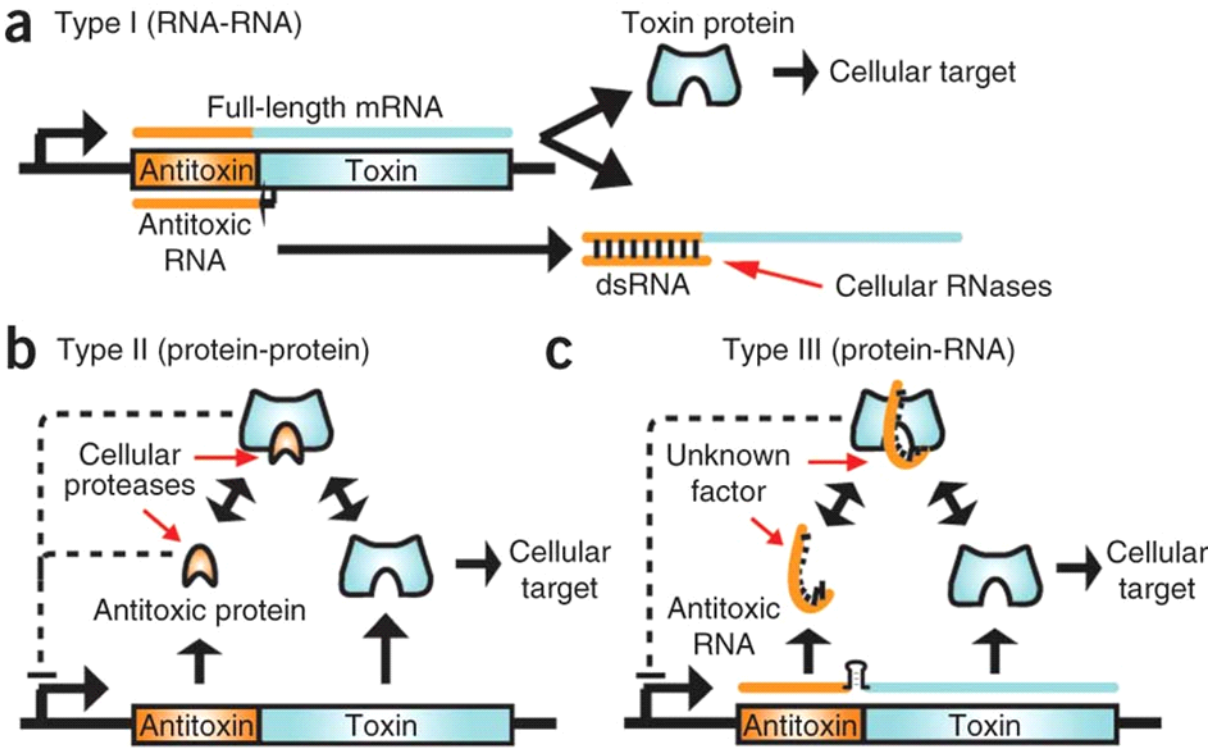


Figure 1.11 Genetic organization and components of a typical type I, II and III TA locus. In general, the antitoxic gene (orange) precedes the toxin gene (cyan), as part of two genes operon. (a) In type I TA systems, the short antisense antitoxic RNA forms a duplex with a short region of the full-length mRNA. This duplex prevents translation of the toxin gene and promotes degradation by a cellular RNase such as RNase III. (b) In type II TA systems, the protein antitoxin forms a complex with the protein toxin. Either as a complex or by itself the antitoxic protein often negatively regulates transcription of the operon. Cellular proteases such as Lon and Clp degrade the antitoxin, releasing the toxin. (c) In Type III TA systems, a proteinaceous toxin interacts with an antitoxic RNA generated from a tandem array of repeated sequence. A transcriptional terminator (black stem-loop) between the antitoxin and toxin genes regulates transcript levels. As yet unidentified cellular factor(s) are predicted to either, degrade the antitoxic RNA, decrease the level of transcription from the locus, or release the toxin, allowing the toxin to take effect in each case. Adapted from (Blower *et al.*, 2011)

1.9.2. CcdB toxin family.

Ccd (coupled cell division) family has been found only in Gram-negative bacteria. Most extensively studied *ccd* toxin is located on the F sex factor plasmids of *E. coli*. F plasmid encompasses a pair of genes, *ccd*A and *ccd*B, whose protein gene products are involved in plasmid maintenance (Bernard and Couturier 1992). Recently, it has been shown that different bacterial strains also contain *ccd* homologues in their chromosomes. The organization of *ccd*A_F/B_F genes in an operon includes the antitoxin *ccd*A_F gene located upstream of the toxin *ccd*B_F gene. $CcdA_F$ antitoxin prevents $CcdB_F$ toxicity by creating a close $CcdA_F$–$CcdB_F$ complex (Afif *et al*. 2001).

$CcdB_F$ toxin inhibits DNA replication by DNA gyrase poisoning (Bernard and Couturier 1992). The discovery of chromosomal *ccd* genes has led some study groups recommending that chromosomal TA systems can selectively advantage their host in post segregational killing conditions. However, in an *E. coli* strain containing the *ccd*ABEch genes inserted into its chromosome, no post segregational killing was observed upon the loss of plasmids carrying $CcdAB_F$ TA system (Wilbaux *et al*., 2007). Subsequently, chromosomal TA systems were hypothesized to act as anti-addiction modules.

1.9.3. ParE toxin family.

ParE toxin family is common in both Gram-positive and Gram-negative bacteria. It has been discovered on plasmid RK2 (Roberts and Helinski 1992). The *par*E gene encodes a toxin and *par*D encodes an antitoxin. The ParE toxins inhibit bacterial cell division and reduce viability, presumably because of their capacity to damage DNA (Jiang *et al*. 2002). The plasmid-borne ParDE TA system has been involved in plasmid maintenance by inhibiting the growth of plasmid-free cells. Correspondingly, it has been suggested that chromosomal par TA systems may be stabilizers of genomic regions to prevent their loss during evolution (Syed and Lévesque 2012).

1.9.4. RelE toxin family.

RelBE systems are extensively distributed in archaea, gram negative bacteria (*E. coli* and *Caulobacter crescentus*) and Gram-positive bacteria (*M. tuberculosis*, *Streptococcus pneumoniae*, and *S. aureus*) (Fiebig *et al*. 2010; Francuski and Saenger 2009), Furthermore, they are commonly chromosomal toxins. The RelEB family contains the *Rel*E toxin, which is stable

and the *Rel*B antitoxin, which is unstable (Overgaard *et al*., 2009). In *E. coli*, RelBEF system has three genes: *rel*E gene encodes a cytotoxin lethal or inhibitory to host cells, while *rel*B gene encodes its cognate antitoxin auto regulated (Gotfredsen and Gerdes 1998). The relF is an operon. RelE is an endoribonuclease that inhibits protein synthesis by cleaving mRNA through its association with the ribosome (Pedersen *et al*., 2003). The RelBE chromosomal TA system is hypothesized to modulate cell growth to cope with stress and survive adverse environmental conditions. When protein synthesis is inhibited, RelE toxin stimulates a dormant state (stasis). On returning to proper conditions, production of RelB antitoxin continues, resulting in a reversal of RelE-induced translation blockade (Syed and Lévesque 2012).

1.9.5. Doc toxin family.

Phd–doc TA system is responsible for stable inheritance of the plasmid prophage of bacteriophage P1 in *E. coli* and represents the archetype of a very small family of TA modules (Gerdes *et al*., 2005). The *doc* operon encodes the Doc toxin (death on curing) and Phd antitoxin (prevent host death). When the plasmid prophage is lost, the unstable phd antitoxin is rapidly degraded by the host-encoded ClpXP protease (Lehnherr and Yarmolinsky 1995). Doc toxin targets the translation process during the inhibition of the elongation step by associating with the 30S ribosomal subunit, causing cell growth arrest on curing (Liu *et al*. 2008). Chromosomal homologues of the phd/doc family are found in Gram-negative and Gram–positive bacteria and a few archaea (Gerdes *et al*., 2005).

1.9.6. HigB toxin family.

The HigB (host inhibition of growth) toxin family has been found in both Gram-positive and Gram-negative bacteria. It has been found on chromosomes in different pathogenic bacteria, including: *V. cholerae*, *S. pneumoniae*, *E. coli* CFT073 and *E. coli* O157:H7 strains (Pandey and Gerdes 2005). Christensen-Dalsgaard and Gerdes (2006) reported that the presence of the HigBA systems in *V. cholerae* could play a role in stabilization of chromosomes (Christensen-Dalsgaard and Gerdes 2006). The HigBA operon has been initially identified in Rts1 plasmid of *P. vulgaris* (Tian *et al*., 1996) and stabilizes plasmids by inhibiting the growth of plasmid-free cells (Gerdes *et al*., 2005).

1.9.7. HipA toxin family.

*Hip*A (high persister) gene is one of the several TA modules present in bacteria. Both *hip*B and *hip*A are organized in an operon with the gene encoding the antitoxin, *hip*B, is located upstream of *hip*A, the toxin gene (Schumacher *et al*. 2009). The *hip*A gene was the first gene related to persistence (Moyed and Bertrand 1983). Persisters are cells that neither grow nor die in the presence of the antimicrobial drugs. Persisters' cells are not mutants, but phenotypic variants of regular cells that form randomly in microbial populations (Lewis 2010). In *E. coli*, HipA is suggested to be responsible for persisted state in which bacteria tentatively stop growing and thus avoid the killing effects of antimicrobial drugs that block cell wall synthesis (Silvaggi *et al*., 2005).

1.9.8. MazF toxin family

MazF toxin is a chromosomal TA system. The *maz*EF operon encodes two proteins MazE and MazF that form a stable complex under normal conditions, but under stress (e.g., amino acid starvation, DNA damage, oxidative stress, and antibiotics), degradation of MazE antitoxin occurs. MazEF TA has been involved in bacterial programmed cell death (PCD). PCD can be defined as any cell death mediated by a genetically regulated intracellular death program (Lewis 2010). Chromosomally encoded T-AT systems are believed to be advantageous to cell survival by being part of global stress response system (Syed and Lévesque 2012). In *E. coli* chromosome, MazF toxins inhibit protein synthesis by inducing cleavage of translated mRNAs during nutrient starvation (Christensen *et al*., 2003).

1.9.9. VapC toxin family.

The TA has been named *vap* for virulence associated protein (Katz *et al*., 1994). The first VapBC TA module has been identified in Gram-negative bacteria, *Dichelobacter nodosus*. It has been found as part of a transmissible genetic element. The *vap* genes are recognized as a part of pathogenicity islands (PAI), a group of laterally-transferred genes in the bacterial genome, which help the organism explore and adapt to new environmental niches (Ren *et al*., 2012). The VapBC toxin family is the largest family of TA systems and has been identified in archaea and Gram-positive bacteria. *M. tuberculosis* has several VapBC homologues, more than any other prokaryote (Makarova *et al*., 2009). VapC protein acts to inhibit translation, presumably by its

ability to cleave mRNA. The chromosomal *vap*BC genes encode the VapC toxin preceded by the VapB antitoxin, which has a transcription factor domain (Robson *et al*., 2009).

1.9.10. HicA toxin family.

HicAB TA family has been found in bacteria and archaea. The *hic*AB locus has been described first as an insertion sequence in several strains of *H. influenzae* (Syed and Gilsdorf 2007).

1.9.11. Zeta toxin family.

Zeta toxin family has been so far found only in Gram-positive bacteria. The first omega–epsilon–zeta (ω-ε-ξ) TA family was initially discovered in the pSM19035 plasmid of *S. pyogenes* and is the only TA family that consists of three components. The omega–epsilon–zeta operon is a novel plasmid addiction system in which the epsilon and zeta genes encode an antitoxin and toxin, respectively, while omega plays an autoregulatory function. The ξ gene was determined to be a cytotoxin by its toxic activity in *B. subtillis, E. coli*, and *Saccharomyces cerevisiae* (Zielenkiewicz and Ceglowski 2005). The ω gene regulates transcription of the entire locus as it encodes for an autorepressor. The ω gene product strictly regulates the ω-ε-ξ locus, and it is not directly involved in the killing-anti-killing mechanism (De la Hoz *et al*., 2000).

1.9.12. Post Segregational Killing system (PSK).

RelE and ParE proteins of *E. coli* were the first model proteins to investigate the role of TA systems. These proteins were among the functionally best characterized toxins of the post-segregational killing system (PSK). This is a widespread mechanism that helps low copy number plasmids to be maintained in their bacterial hosts (Yarmolinsky 1995). In PSK system, when a TA plasmid producing both the toxin and antitoxin is lost from the cell, the unstable antitoxin is quickly degraded, whereas the stable toxin persists long enough to kill the new plasmid-free cell (Pedersen and Gerdes 1999; Mruk and Kobayashi 2014). Plasmid-borne TA system is often responsible for the post segregation. These types of systems, also known as "addiction modules," confirm that only cells that have a plasmid are maintained in the population (Silvaggi *et al*., 2005). This regulated killing is, in all cases, based on a toxin-antitoxin principle.

1.10. Hypothesis and the aim of study.

Enterobacteriaceae harbouring NDM-1 genes have been recovered from many clinical and environmental isolates. Hitherto, it is unknown whether antibiotic resistance is solely driven by the consumption of antibiotics. Particularly in South Asia, there may be other factors that drive the spread of resistance genes on the plasmids they are carried on. Therefore, the hypothesis "Is the acquisition and stability of the NDM-1 gene related to the presence of other resistance genes and other systems such as toxin –antitoxin system?" Is valid and worthy of examination.

Given the emergence of Enterobacteriaceae as a major clinical pathogen with high degrees of genomic plasticity and diversity, I aimed to study Enterobacteriaceae from Indian and UK isolates to determine some of the genetic characteristics present in isolates from these two countries.

The main aims of the thesis were:

- To determine and complete the structure of the nucleotide sequence in an unusual class 1 integron carrying MBLs, embedded in Tn*402*- like transposon in two different *Pseudomonas* species, and to detect whether VIM-2 is located on a plasmid or chromosome.

- To evaluate the stability of $bla_{\text{NDM-1}}$ in several clinical isolates (parents and transconjugants) from India.

- To determine the molecular background of the resistance phenotype for heavy metals, in such as *cop*A, *sil*C, *ars*A, and *mer*A genes, and to evaluate the prevalence of heavy metals among clinical isolates and determine the mechanism of their resistance.

- To ascertain the prevalence of the usual type of toxin –antitoxin systems among clinical isolates of Enterobacteriaceae from India and the UK.

Chapter Two

Materials and methods

2. Materials and methods

2.1. Clinical bacterial strains

2.1.1 Bacterial isolates were collected from Haryana, (*K. pneumonia*, n=5, symbolized K) and Chennai (*K. pneumoniae* n=11; *K. oxytoca* n=1 and *E. coli* n=5; *Enterobacter cloacae* n=4 and *Protuse rettgeri* n=2, all symbolised IR (Appendix A, Table A-1) from the northern and southern Indian subcontinents, respectively. All isolates were NDM-1 positive strains and collected for the study by Kumaarasamy *et al*., 2010.The isolates were derived from the Appollo hospital and the Jagadhri District Hospital, respectively. Clinical information was limited, although these isolates were from human sources, and from blood stream, lower respiratory or urinary tract infections. In our study, I referred to these isolates as Group 1.

2.1.2 The UK NDM-1 positive isolates were recovered from Public Health England Reference Laboratory, Colindale (*K. pneumoniae* n=15; *E. coli* n=6; *Citrobacter* sp. n=2; *Enterobacter* sp. n=2; *Acinetobacter baumanii* n=3; *K. oxytoca* n=1 and *Enterobacter cloacae* n=3) all symbolised N (Appendix A, Table A-1). The isolates from the UK reference Laboratory were from various hospitals and cities. I also referred to this collection as Group 1.

2.1.3 ESBL positive strains were random isolates of burns, blood or urine clinical isolates collected from different Indian cities (Haryana, Delhi, Mumbai, Kerala and Vellore) in1998. These isolates are *K. pneumoniae* n=17*; E. coli* n=14; *Salmonella* sp. n=4; *Enterobacter* sp. n=3; *P. stuanti* n=1 (Appendix A, Table A-1).I referred to this collection as Group 2.

2.1.4 The UK isolates were recovered from Cardiff Central Laboratory, Public Health Wales (2010). These strains, *K. pneumoniae* n=10 and *E. coli* n=10 (symbolized FF) (Appendix A, Table A-1), are susceptible/sensitive to all tested antimicrobials. I referred to this collection as Group 3.

2.1.5 VIM-2 positive *P. aeruginosa* (301-5473) was isolated in France in 2007 and *P. fluoresces* (43-14926) was isolated in Chile in 2002 (Appendix A, Table A-1).

All isolates were kept as stocks at -80ºC and all have antibiotic- resistant plasmids.

2.2. Laboratory bacterial strains used

The laboratory strain *E. coli* J53 Azi^R (Nordman, A.*et al*., 2008) was used as a recipient cell in conjugation experiments. *K. pneumoniae* Kp-05-506 was used as positive control for the NDM-1 gene. *E. coli* ATCC 25922 (Nordman *et al*., 2008), which is commonly used in antibiotic sensitivity testing, was used as a control.

2.3 Ethical Considerations

Given the nature of our study, and the limited amount of patient information required for each clinical isolate, ethical approval was not deemed necessary.

2.4 Safety considerations

Regulation and safety were undertaken according to the Ionizing Radiation Regulations, 1999.

2.5 Chemicals reagents.

All common chemicals were purchased from Sigma Chemical Co. or BDH Chemical Ltd. All media were purchased from either Oxoid Laboratories or Fisher Scientific Laboratories. Radiolabelled Phosphorus ^{32}P was purchased from PerkinElmer, Boston, MA02118, and United States of America (USA). Lambda Ladder PFGE Marker was obtained from New England Bio-labs Inc., PCR Master Mix was purchased from Thermo Fisher Scientific, AB Gene House, Blenheim Road, Epsom, and Surrey, UK.

2.6 Enzymes and commercially prepared kits

All restriction endonucleases were purchased from Ferments Life Sciences. QiaGen Miniprep kit and PCR Gel extraction Kit were supplied from QIAGEN GmbH, D- 40724 Hilden, and North Manchester. Random Primer Labelling kits were purchased from Agilent Stratagene Products, USA.

2.7 Media

All media were prepared and autoclaved by autoclaving at 0.7kg cm^{-2} for 20min at 120^0C before use. The used growth media as following were:

2.7.1. Luria Bertani Broth (L.B. broth) was made up according to the manufacturer's instructions (Fisher Scientific Ltd).

2.7.2. Luria Bertani Agar (L.B. agar) was made up according to the manufacturer's instructions (Fisher Scientific Ltd).

2.7.3 Mueller-Hinton Agar (M.H. agar) was made up according to the manufacturer's instructions (Oxoid Laboratory).

2.7.4 Mueller-Hinton broth (M.H. broth) was made up according to the manufacturer's instructions (Oxoid Laboratory).

2.7.5. UTI media (Brilliance TM UTI Clarity Agar, CM110) was made up according to the manufacturer's instructions (Oxoid).

2.7.6 Nutrient Broth media (N.B.) was made up according to the manufacturer's instructions (Oxoid).

2.7.7 Chromogenic agar was made up according to the manufacturer's instructions (Oxoid).

2.8 Cultivation of bacteria

All cultivation of bacteria was carried out using enriched agar and/or liquid media. All cultures of bacteria were incubated overnight at 37ºC unless otherwise stated.

2.9 Solutions and buffers.

Sterile molecular grade water and buffers were purchased from Sigma Aldrich, Poole, UK.

2.10 Determination of antimicrobial susceptibility.

The MIC is defined as the lowest concentration of an agent that completely inhibits growth of the bacteria. The MICs were determined according to the Clinical Laboratory Standards Institute (CLSI, USA) by the Phoenix 100 (Becton Dickinson) and by Micro-Titer (for heavy metals). The antibiotics used in this experiment are recorded in Appendix A, Table A-2and the heavy metals used in Appendix A, Table A-3.

2.10.1 Determination of the MICs by Phoenix 100

The Phoenix 100 determines which antimicrobials will be most effective in treating an organism. The organism is tested against various concentrations of antimicrobials, determining the organism's resistance (ineffective) or susceptibility (effective) to the antimicrobials. The

antibiotics used include; Amikacin, Amoxicillin-Clavulanate, Ampicillin, Aztreonam, Cefotaxime, Ceftazidime, Cefuroxime, Cephalexin, Ciprofloxacin, Colistin, Ertapenem, Fosfomycin, Gentamicin, Imipenem, Meropenem, Ntrofurantion, Piperacillin-Tazobactam, Tobramycin, Trimethoprim, and Trimethprim-Sulfamethoxazole.

2.10.2 Determination of the MICs by Micro titer

All Enterobacteriaceae strains (recipient, donor and mated strains) were grown in 20 ml of NB with shaking at 150 rpm for 5 hrs. at37ºC. All isolates were tested to determine the MICs of four heavy metals. The MICs of heavy metals were tested by serial dilution in MH broth. Stock solutions (100mg/ml) of heavy metals salts, such as $HgCL_2$ (Sigma-Aldrich), $Cl_2Cu.2H_2O$ Sigma, life science), AgCL (Sigma-Aldrich) and $NaAsO_2.7H_2O$ (Sigma, life science,) were prepared in molecular-grade water and sterilized by filtration. The stock solutions for As and Cu were diluted at 1/10(used concentrations were 5, 10, 20, 40, 80, 160, 320, 625, 1250, 2500 and 5000 µl/ml); Sil and Mer at 1/100 (used concentrations were 0.5, 1, 2, 4, 8, 16, 32, 64, 125, 250 and 500 µl/ml).75µl of MH broth was transferred separately in a 96-well micro titer plate, then 75µl of each heavy metals solution was transferred by using serial dilutions. Finally, to each well of the 96-well plate, 20µl of a bacterial suspension, with a turbidity equivalent to 1/10 of 0.5 McFarland Standard was added. Plates were incubated at 37ºC overnight. MH broth without heavy metals was used as a control. After overnight incubation, the MIC of heavy metals against the isolates was determined.

2.11 Molecular Analysis

2.11.1. Isolation of genomic DNA from bacterial cells

Separate methods were used for the large-scale isolation of genomic DNA from bacterial cells, while a QiaGen Miniprep kit (QIAGEN GmbH, D- 40724 Hilden, Skelton House, Lloyds Street, North Manchester, M15 65K, UK) was used for small-scale isolation of DNA from bacterial cells.

2.11.1.1. Large scale isolation of bacterial genomic DNA

A lawn of bacteria was grown on an agar plate. About half of a confluent plate was scraped and suspended in 9.5ml of TE buffer (pH8) in a 50 ml Falcon tube. 0.5mL SDS (10%) and 50µl of 20mg/ml proteinase K was added, mixed and incubated for one hour at 37ºC. 1.8ml of 5M NaCl was added, mixed thoroughly and left for five to ten mins at 65ºC. An equal volume

of (24:1 v/v) phenol-chloroform-isoamyl alcohol was added and left on a rotary mixer for at least 30 mins. Centrifugation at 5000rpm was carried out for about 30 mins until two distinct layers with a protein interface were seen. The aqueous layer (top layer) was transferred to a clean tube and approximately the same volume of propanol was added to the new tube and mixed gently until the DNA precipitated. The DNA precipitate was transferred to 1 ml of 70% ethanol for washing. The ethanol was removed and discarded and the tube then left open in an incubator for 20 mins to evaporate the remainder. 1ml of water was added to dissolve the dried DNA. Genomic DNA was stored at -80 ºC.

2.11.1.2 Isolation of plasmid DNAs.

The plasmids DNAs were isolated from the Enterobacteriaceae strains by a minipreparation method using the QiaGen Miniprep kit (QIAGEN GmbH, D- 40724 Hilden, Skelton House, Lloyds Street, North Manchester M15 65K, UK.

2.11.2 Polymerase Chain Reaction analysis

The polymerase chain reaction was used to amplify genes from tested strains. For this method it was first necessary to design oligonucleotide primers corresponding to published genes. The following sections outline the design of primers and the setting up of PCR.

2.11.2.1 Criteria for the design of oligonucleotide primers

As outlined by Sambrook *et al*., 1989, the following criteria were considered:

1. The length of each primer was at least 18 bases to allow for specific binding to the template.
2. The primers contained no self-complementary regions and were not complementary to each other.
3. The base composition was chosen such that the primer pairs had similar annealing temperatures.

2.11.2.2 Designing of primers for polymerase chain reaction (PCR)

Oligonucleotide primers were designed using a primer designing program (Primer 3) from Geneious Pro 5.5.6 (Biometric Inc., San Francisco, USA). Primer sequences are given in Appendix A, Table A-4.

All primers were commercially synthesized by Invitrogen (Life Technologies, UK) with cartridge purification. All primers were obtained as a dried primer and were resuspended in sterile dH_2O and stored at 4 ºC.

2.11.2.3 Preparation of DNA template.

Plasmid extraction was carried out using a QiaGen Miniprep kit (QIAGEN GmbH, D-40724 Hilden, North Manchester, UK) from all tested strains.

2.11.3.4. Polymerase Chain Reaction protocol.

Each mixture for PCR analysis contained 5µl of a 2x working concentration of Extensor Hi- Fidelity PCR Master Mix (Thermo Fisher Scientific ABgene House, Blenheim Road, Epsom, Surrey, UK) containing dNTPs, MgCl2 and Taq DNA polymerase, 1µl of strain's plasmid as template, 0.5µl each of the forward and reverse primers, and made up to 10µl final volume with molecular biology grade water. The PCR tube was then placed in a G-Storm PCR machine (Gene Technologies). PCR program reactions were performed under differing conditions depending on the primers used (See Appendix A, Table A-**5)**. A negative control, without a template, was used.

2 .12 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to detect the PCR product size (DNA fragments). Hi-Res Standard Agarose (Gene flow, AGTC Bio products, Paul Fisher House, Elmhurst Business Park, Elmhurst Lichfield, Staffordshire, UK) was used at 1% (w/v) in 1XTBE buffer (900ml autoclaved water and 100ml 10XTBE buffer (Tris108g, Boric acid54g, NaEDTA7.44g, water to 1 litre), autoclaved before use). 1µl/ml Ethidium bromide (Et Br) (Fisher Bio reagents) was added.5µl of loading buffer (10X) was added to 10µl of each sample of DNA and the mixture loaded into the agarose gel. 5µl of Smart ladder MW 1700-02 (0.25g/l Bromophenol blue,0.25 g/l Xylene cyanol, 25g/l Ficoll 400, 1g/l Sodium Azide, 1/1000 Chloroform and TE (Tris

10mM, EDTA 1mM, pH 8, Eurogentec) was loaded alongside the samples on the agarose gel for size comparison. Gels were run at 250 V for 25-30 min in 1X TBE buffer (above).DNA patterns were visualized by UV illumination and recorded photographically by UVP-Gel-Doc-IT^{TM} Imaging system (Bio-Rad House, Maxted Road, Hemel Hempstead, and Hertfordshire) and the images saved for analysis.

2.13 Purification of DNA patterns from agarose gel.

PCR products (DNA patterns) were excised from the gel and purified using a QIAquick Gel Extraction Kit (250) QiaGen (QIAGEN GmbH, D- 40724 Hilden, Skelton House, Lloyds Street, North Manchester, UK) according to the manufacturer's instructions.

2.14 DNA Sequencing

DNA sequencing of purified PCR products was done by DNA Sequencing Core, Cardiff University, UK. 10µl PCR product (200 ng) and 1µl primer were added to 19µl molecular grade water. Sequences were analyzed using NCBI BLAST option on the website http://www.ncbi.nlm.nih.gov/BLAST.cgi.

2.15 PCR-based replicons typing.

PCR- based replicon typing was undertaken to identify the plasmids' origin of replication as described by Carattoli *et al*., 2005. This protocol is used to classify the main known incompatible plasmid groups FIA, FIB, FIC, HI1, HI2, I1, I_{γ}, L/M, N, P, W, T, A/C, K, B/O,X, Y, F and FIIA. A positive control was used to compare plasmid size. Primers sequences are given in Appendix A, Table A-6. The PCR conditions used were as follows; 94ºC for 5min, 94ºC for 1min, 60ºC for 30s (for F simplex PCR the annealing temperature was 50ºC for 30sec), 72ºC for 1min and 72ºC for 5min.The total number of cycles was 35.

2.16 Conjugation experiments

Conjugation assays were performed using *E. coli* J53^{AZR} as a recipient. Fresh colonies of parent and recipient strains were grown individually in LB broth media (Luria Bertani Broth, Fisher Scientific, USA Products) in 50 ml Falcon tubes and incubated overnight at 37^{0}C. Mating assays were undertaken in 2ml volumes.10^{8} colony- forming units of the donor strain and 10^{7} colony- forming units of the recipient *E. coli* J53wereadded to 2ml of fresh LB broth media and incubated overnight at 37ºC in a shaking incubator. After overnight incubation, the mating

suspension was diluted in normal saline (10^{-5}) and plated on UTI agar (Oxoid) to confirm purity. For selection assays, transconjugants were selected by plating 100µl on UTI media (BrillanceTM UTI Clarity Agar, CM1106, Oxoid) containing sodium azide (100µg/ml) and Ceftazidime (30µg/ml). Plates were incubated overnight at 37ºC.Toconfirm that conjugation had taken place in *E coli* $J53^{AZR}$; PCR analysis was performed to test the presence of the MBL gene in transconjugants. The PCR was performed using the forward and reverse primers from Appendix A, Table A-4. The transconjugants were stored at -80ºC for further analysis.

2.17 Plasmid Stability

Ten parents and 8 transconjugants from NDM-1-producing clinical isolates containing plasmids carrying $bla_{\text{NDM-1}}$ of different sizes were used. A single colony from the strains was grown overnight in 1ml of nutrient broth (NB) without antibiotic selection and incubated at 37ºC. Serial passaging daily was carried out in the absence of antibiotic selection by transferring 1ml of overnight culture to 4ml of fresh NB and reincubating. This procedure was performed for fourteen days. For each daily sub culturing, 10µl of overnight culture was taken and plated on chromogenic agar to test for purity and 1ml of each sample was taken and genomic DNA extracted as described in section 2.12.1.2. PCR analysis was performed to verify the presence of the NDM-1 gene and to verify the rep region of the plasmid backbone. The forward and reverse primers were used from Appendix A, Table A-4. In addition, PFGE was performed to establish the location of the NDM-1 gene, whether on plasmids, chromosomes or both.

2.18 Pulsed field gel electrophoresis (PFGE) Analysis

The PFGE technique uses molecular scissors, so-called restriction enzymes. These enzymes cut whole genomic bacterial DNA at certain sites known as restriction sites. These molecular cutters are selected to create a small number of DNA pieces that can be separated based on size and used to discriminate between strains. Typically these DNA fragments are large and require be specially treating and separating to generate a DNA fingerprint.

2.18.1 Determination of chromosomal and plasmid mediated resistance genes.

2.18.1.1 Genomic DNA preparation

PFGE analysis is used to separate large DNA fragments created by digestion of total genomic DNA with restriction endonuclease enzymes that cut DNA infrequently. Bacterial genomic DNA was used to prepare plugs by taking one loop of a fresh overnight growth of colonies on an agar plate and suspended in 3ml of sterile normal saline.1ml of the bacterial suspension was transferred to a cuvette to measure the optical density at 600nm (OD600) of each strain. The OD600 formula was used (1.5/measured OD multiplied by 300) to adjust the volume of cells to equivalent of 300µl in accordance to the OD600 .The suspension was transferred to an Eppendorf tube and centrifuged at 13,000 rpm for 30secs using a mini-centrifuge (Minispin centrifuge, Hamburg, Germany). The supernatant was removed using a pipette and the pellet resuspended in 300µl of sterile normal saline. The cell suspension tube was incubated at 50ºC in the block heater in preparation for the lysis step.

2.18.1.2 Lysis of cells in agarose plugs

The cell suspension was lysed by adding 2-4 drops of 25mg/ml lysozyme. 300µl of Low Melting Agarose 2.5%(w/v) was heated and stored in a 54ºC water bath and was quickly added to the pre-heated tube and gently mixed and dispensed into disposable moulds and then allowed to solidify at 4ºC for 10min. The agarose plugs were then transferred into 2mls of lysis buffer (50 mM NaCl, 0.2% sodium deoxycholate,10 mM Tris-HCl, pH 7.2, 0.5% N-Lauroylsarcosine)supplemented with 80µl of 25mg/ml lysozyme and the plugs incubated at 37ºC for 1.5hrs. After incubation, the plugs were washed with 2ml of 1XTE buffer(10mM Tris-HCl, 50 mM EDTA, pH 8.0, Bio-Rad) for 30min at 37ºC with shaking. The 1X TE buffer was replaced with 2mls of proteolysis buffer (100 mM EDTA, pH 8.0, 0.2% sodium deoxycholate, 1% N-Lauroylsarcosine; Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX) supplemented with 10µl of 20mg/ml of Proteinase K and incubated for 18hrs at 50ºC. After further incubation, the plugs were washed five times for 30min each wash with 1XTE buffer in a shaking incubator at 37ºC.Plugs were stored in 1XTE buffer at 4ºC or digested immediately.

2.18.1.3 Plug Digestion.

Digestion of DNA in plugs was performed using the *S1* nuclease enzyme according to the manufacturer's instructions. One plug of each strain was washed with 1ml of 0.1X TE buffer for 30min at 37ºC. The plugs were then separately washed twice with 300µl of 2XS1 buffer (Ferments, Sheriff Houtton Industrial Park, York, UK) for 15min at 4ºC and at room temperature. The buffers were removed and replaced with 300µl of 1X S1 buffer at 4ºC and at room temperature. Subsequently, the 1X*S*1 buffer was removed and 1µl of 2U of *S1* endonuclease (Promega, USA) was added and incubated for a further 4hr at 4ºC.

2.18.2 Pulsed Field Gel Electrophoresis (PFGE).

Whole genomic DNA of the NDM-1 positive strains (from Group1; G1) was used to prepare plugs, as in section 2.18.1.1, then the plugs were lysed as in section2.18.1.2. Digestion was performed using the *Xba*1 endonuclease enzyme according to the manufacturer's instructions. One plug of each strain was washed with 1ml of 0.1X TE buffer for 30min at 37ºC. The plugs were then separately washed twice with 300µl of 2X*Xba*1 fast digest buffers (Ferments, Sheriff Houtton Industrial Park, and York, UK) for 15min at 4ºC and at room temperature. The buffers were removed and replaced with 300µl of 1X*Xba*1 fast digest buffer for 15min at room temperature. The 1X*Xba*1 buffer was removed and 2.5µl of Xba1 enzyme added in 100µl of 1X*Xba*1 buffer, then incubated overnight at 37ºC.

2.19 Electrophoresis conditions

Once digestion was completed, separation of *Xba*1 and *S*1 digested DNA was performed by using PFGE apparatus (CHEF-DRIII system, Bio-Rad Laboratories, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX).PFGE gels were prepared as follows; 0.88% (w/v) agarose in 0.5X TBE buffer (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA, pH 8.0;Bio-RadHouse, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX) plus 20µl of Ethidium bromide was added to stain the gels. Plugs were loaded into the PFGE gel and the gels run in the PFGE tank (Bio-Rad CHEF-DRIII, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX), using TBE buffer (0.5X Tris borate, 0.5 mM EDTA), for 20hrs at 120º angle, 6.0volt/centime gradient and linear ramp factor. The cooler was set for 14ºC and initial

and final switches were set to 5 and 45 seconds respectively. Lambda marker DNA was used as a marker to establish DNA size.

2.20 Gel Visualization

The resultant PFGE gels were photographed on a gel doc machine and dried overnight at 50ºC on a Whatman filter paper (15 cm X 15 cm) blotting paper; the gels were then re- hydrated using autoclaved water for 30min, denatured using denaturing solution (0.5Msolution NaOH, 1.5M NaCl) for 30min at room temperature (RT), and subsequently neutralized using a neutralizing solution (0.5M Tris-HCl, pH 7.5, 1.5M NaCl) for 30min at RT. The gels were transferred to a hybridization tube containing pre-hybridization solution(20XSSC 6mls,5%Ficoll 400µl, Polyvinylpyrrolidone400(PVP) 400µl, Herring testes DNA 300µl,UHT milk 400 µl,10%SDS1mls, water11.5mls) at 65ºC.

2.21 Southern blot hybridization and ^{32}P labelling

Pulsed-field gels were photographed and dried on blotting paper (Whatman GB004) overnight at 50°C. The gel was then re-hydrated, denatured (0.5 M NaOH, 1.5 M NaCl, 30 min) and neutralized (0.5 M Tris–HCl, pH 7.5, 1.5 M NaCl, 30 min) before incubation overnight at 65°C in pre-hybridization solution and probed with DNA probes. The probes were prepared by PCR with custom-designed primers (for example *Ars*A, *Mer*A, *Pco*A and *Sil*C genes), and labelled with ^{32}P dCTP (Redivue, GE Healthcare, Amersham, UK) using a Prime-it II Random Primer Labelling Kit (Stratagene, Amsterdam, Netherlands). Gels were hybridized overnight at 65°C, washed with 2X SSC/0.1% SDS and then 0.1X SSC/0.1% SDS for 1hr before autoradiography.

Chapter Three

Completion of the nucleotide sequence of an unusual class 1 integron carrying *bla*$_{\text{VIM-2}}$ embedded in complete Tn*402*- like transposon in *Pseudomonas aeruginosa* and *P. fluorescens*

3.1 Introduction

MBL mediated carbapenem resistance has been increasingly described in *Pseudomonas* and other Gram-negative bacilli (GNB) in several countries (Samuelsen *et al*., 2010).

The genus *Pseudomonas* contains non-fermenting Gram-negative bacteria that are unable to ferment sugars to generate energy for cell functioning. Members of this group are opportunistic pathogens and they cause diseases in hospitalized patients; primarily pneumonia, urinary tract infections (UTI) and skin and soft tissue infections (Sacha *et al*., 2008; McGowan *et al*., 2006).

Non-fermenting bacteria colonize in nature (water and soil); also they may be isolated from ventilator machines, mattresses and other equipment, as well as from the skin of healthcare staff (McGowan *et al*., 2006). MBL positive *Pseudomonas* clinical strains, resistant to most β-lactams, have been proposed to act as likely reservoirs of MBL genes (Almuzara *et al*., 2007; Lee *et al*., 2002). The VIM- type MBL family was first described in a *P. aeruginosa* isolate in Verona (Italy) in 1997 and the dominant type of VIM (VIM-2) has been isolated from strains in most countries within Europe (Toleman *et al*., 2005). The first isolates reported to carry the $bla_{\text{VIM-2}}$ gene were from Portugal in 1995 (Cardoso *et al*., 2002) and from France in 1996 (Poirel *et al*., 2000).

P. aeruginosa is amongst the most notorious nosocomial pathogens, often causing severe infections in immunocompromised and critically ill patients, due to its ubiquitous nature, ability to colonize and persist in hospital reservoirs, and noteworthy intrinsic antibiotic resistance (Viedma *et al*., 2013). Furthermore, *P. aeruginosa* can also acquire antibiotic resistance mechanisms through horizontal transfer of genetic elements (integrons, transposons, and plasmids) that carry resistance genes as well as mutational resistance (e.g. mutation in DNA topoisomerases) (Lin *et al*., 2012).

Despite the fact that mutational events contribute to the bacterial adaptation, and hence antibiotic resistance, horizontal gene transfer is the main cause of the rapid proliferation of antibiotic-resistance genes across a wide diversity of bacteria, not least *P. aeruginosa*. In the case of non-fermenting bacteria, many of the horizontal gene transfers have been shown to occur in the environment (Stalder *et al*., 2012).

P. fluorescens is a highly heterogeneous species and saprophytic member. It is found in large numbers in the main natural environments and forms associations with plants. Remarkably, *P. fluorescens* includes some strains suspected to be opportunistic human pathogens. Recently, several studies highlighted the infectious potential of some *P. fluorescens* clinical strains (Sperandio *et al*., 2012).

Hitherto, six types of MBL genes have been reported amongst *P. aeruginosa* (IMP, VIM, SPM, GIM AIM and NDM-1).SPM, GIM and AIM have scarcely been detected beyond their region of first detection, i.e. the propagation of SPM types is restricted to Brazil, while GIM and AIM types are scarce and limited to Germany and Australia respectively. However, VIM and IMP genes display a worldwide dissemination and continue to be detected at increasing rates worldwide and NDM positive *P. aeruginosa* is present all across Southern Asia.VIM-2 producing *P. aeruginosa* has been responsible for clonal spread and nosocomial outbreaks (Van der Bij *et al*., 2011; Livermore *et al.*, 2012).

To date, there are 37 documented diverse VIM- types (www.lahey.org). The *bla*$_{\text{VIM-2}}$ represents the most distributed MBL gene worldwide and is commonly present in class 1 integrons (Hawkey *et al*., 2009; Toleman *et al*., 2007).Class 1 integron plays a key role in the spread of antibiotic resistance genes (Labbate *et al*., 2008).Class 1 integron commonly exists either on plasmids or transposons, or on both, and is transferred along with them (Wei *et al*., 2011). Most class 1 integrons recovered from clinical isolates are linked to a transposon, i.e. Tn*21* or Tn*402*. Tn*402* consists of four adjacent genes comprising a set of transposition genes (tniR, -Q, -B, and -A; also called *tni* or *tni*C module) (Labbate *et al*., 2008, Post *et al*., 2007). Numerous class 1 integrons have the *tni* module of Tn*402* but with a vast variety of gene cassettes (Post *et al*., 2007).

This chapter describes the nucleotide sequence in an unusual class 1 integron embedded in a complete Tn*402*- like transposon. It was identified in *P. aeruginosa* 301-5473 isolated in France, in 2007, and in *P. fluorescens* 43-14926, isolated in Chile in 2002.

3.2 Nucleotide sequence of an unusual class 1 integron carrying *bla*$_{VIM-2}$ embedded in completeTn*402*- like transposon in *P. aeruginosa* strain 301-5473, isolated in France 2009.

3.2.1-Results

3.2.1.1 Antimicrobial susceptibility testing

MICs were carried out by Phoenix 100 assay. Resistance results to carbapenems show that the MICs of imipenem and meropenem were high (Table 3.1). The higher MICs were noted for aztreonam whereas the lowest were for gentamicin, tobramycin and cefazolin (Table 3.1).In the case of aminoglycosides, *P. aeruginosa* 301-5473 showed resistance to amikacin. Moreover, it showed resistance to ertapenem, ceftazidime, cefotaxime, trimethoprim and trimethoprim-sulfamethoxazole.

3.2.2 PCR experiments

PCR amplification of *P. aeruginosa* 301-5473 DNA was carried out by using primers (Appendix A, Table A-5) to fill unknown nucleotide sequence between *bla*$_{VIM-2}$ and *tni*C genes. The *bla*$_{VIM-2}$ and tniC genes had been previously identified. The 2.5kb product was separated by agarose gel electrophoresis, purified and sequenced.

3.2.3 Conjugation assay

A conjugation experiment between *P. aeruginosa* and azide-resistant *E. coli* J53 was unsuccessful. A search for plasmid DNA in this isolate failed. The *bla*$_{VIM-2}$ gene was considered likely to be chromosomally located.

Table 3.1 Antimicrobial susceptibility profiles of the *bla*$_{VIM-2}$ - producing *P. aeruginosa*

Antimicrobial agent	MIC (µg/ml) *P. aeruginosa*
Cefazolin	>4
Aztreonam	16
Cefuroxime	>8
Ceftazidime	>8
Imipenem	>8
Meropenem	>8
Amikacin	>16
Gentamicin	>4
Tobramycin	>4
Nitrofurantoin	>64
Trimethoprim- Sulfamethoxazole	>4/76
Trimethoprim	>4
Piperacillin-tazobactam	>16/4
Amoxicillin-Clavulanate	>8/2
Cefotaxime	>4
Cephalexin	>16
Colistin	< =1
Ertapenem	>1
Fosfomycin	>64
Ampicillin	>8
Ciprofloxacin	>1

3.2.4 DNA Sequencing Analysis

The nucleotide and amino acid sequences were analyzed using Geneious Pro5.6.5 software. Sequence analysis showed that downstream of *bla*$_{VIM-2}$ gene there were four cassette genes (Figure 3.1).The first gene, a *bla*$_{OXA-2,}$ was identified encoding a narrow-spectrum oxacillinase, which had a G+C content of 50.9%. The *bla*$_{OXA-2}$ displayed 100% identity with*bla*$_{OXA-2}$ from *P. aeruginosa* strain 4122(Accession no.AY507153) (see Appendix B.1), *S. enterica* subsp. *enterica* serovar T*yphimurium* (Accession no.AM237806) and *P. aeruginosa* (Accession no.EF184216). The *bla*$_{OXA-2}$ had 828 nucleotides encoding 286 amino acids. The OXA-2 showed 100% amino acid identity with OXA-2 from *P. aeruginosa* (Accession no.AY507153) (see Appendix B.2).

The second gene, an*aac*A4, encoded an AAC (b`)Ib type aminoglycoside acetyl transferase, which had a G+C content of 54.2%. The *aac*A4 gene displayed 100%nucleotide identitywith*aac*A4 gene from *P. aeruginosa* strain 4122 (Accession no.AY507153) (see Appendix B.3& B.4).

The third gene, an *aad*B, encoded an aminoglycoside-modifying enzyme (aminoglycoside-2''-adenyltransferase) conferring resistance to gentamicin and tobramycin, which had a G+C content of 57.7% (see Appendix B.5 & B.6).

The last gene cassette, a *qac*G gene, showed 100% identity nucleotide sequence with *qac*G from *P. aeruginosa* strain 4122 (Accession no.AY507153) (see Appendix B.7). The *qac*G encoded quaternary ammonium compound resistance. Moreover, *qac*G gene had a G+C content of 47.5%.*qac*G gene, specifically, encoded resistance to benzalkonium chloride (Heir *et al*., 1999). The *qac*G gene had 324 nucleotides encoding108amino acids (See Appendix B.8).

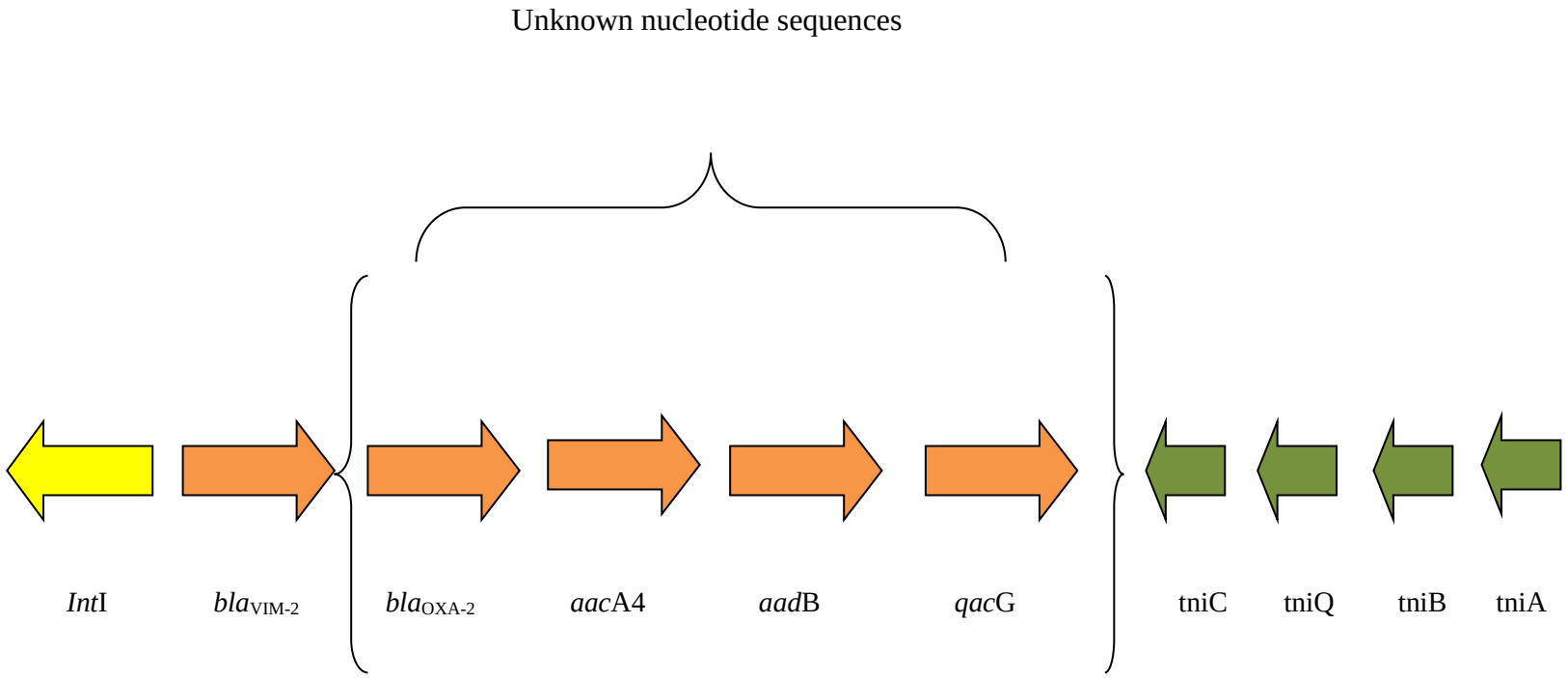


Figure 3.1 Schematic representation of genetic map of unusual class 1 integron carrying *bla*$_{VIM-2}$ embedded in a complete Tn*402*- like transposon in *P. aeruginosa* 301-5473. *Int*I is identified by a yellow arrow, genes cassettes are represented by orange arrows and TniC module genes are designated green arrows.

3.3-Completion of the nucleotide sequence of an unusual class 1 integron carrying *bla*$_{VIM-2}$ embedded in a complete Tn*402*- like transposon in *P. fluorescens* 43-14926

3.3.1 Results

3.3.1.1 Antimicrobial susceptibility testing

P. fluorescens 43-14926 was obtained from Latin American medical centres in Chile. MBL phenotyping was carried out by the disk approximation method (imipenem, meropenem and ceftazidime) and E-test MBLs strips. Antimicrobial susceptibility testing of multidrug resistant *P. fluorescens* 43-14926, an isolate recovered from a blood culture in December 2002, showed that it was resistant to ceftazidime (MICs>16 µg/ml), cefepime (MICs, 16 µg/ml), tobramycin (MICs>16 µg/ml), imipenem (MICs>8 µg/ml), meropenem (MICs>8 µg/ml) and amikacin (MICs, 8 µg/ml) (Table 3.2).

3.3.3 Conjugation assay

Experiments in conjugation between the clinical isolate 43-14926 and *E. coli* J53 did not yield any transconjugants. These data imply that *bla*$_{VIM-2}$ is probably located on the chromosome.

3.3.4 PCR and DNA sequencing analysis

The nucleotide sequences of *P. fluorescens* (43-14926) integrons were determined by PCR with specific primers to amplify the gaps downstream of *bla*$_{VIM-2}$ and upstream of the tniC genes. The use of *bla*VIM-2-FF and tniC-R primers, followed by sequencing, revealed that an *aacA*5 gene, encoding an AAC (6`)-Ib type aminoglycoside acetyltransferase, was located immediately adjacent to the *bla*$_{VIM-2}$ gene (Figure 3.2). The sequence of *aac*A5 gene in *P. fluorescens* showed 99.8% nucleotide identity with*aac*A5 from *P. aeruginosa* (Accession no.AJ515707) (Figure 3.3). The *aac*A5 gene sequence in *P. fluorescens* 43-14926, showed difference in one nucleotide compared to *P. aeruginosa* (Accession no.AJ515707);

A→T (512). Similarly, aacA5 showed 99.4 % identity with aacA5 from *P. aeruginosa* (Accession no.AJ515707), Furthermore, there is difference in one substitution; Asp 171 Val (Figure 3.4).

To our knowledge, this is the first report on the dissemination of *bla*$_{VIM-2}$, *aac*A5 embedded in Tn*402*- like transposon from a *P. fluorescens* isolate.

Table 3.2Antimicrobial susceptibility profiles of the *bla*$_{VIM-2}$- producing *P. fluorescens* 43-14926 isolate.

Antimicrobial agent	MIC(µg/ml) *P. fluorescens* 43-14926
Cefazolin	>16
Cefoxitin	>32
Aztreonam	>16
Cefuroxime	>16
Ceftriaxone	>32
Ceftazidime	>16
Cefepime	16
Imipenem	>8
Meropenem	>8
Amikacin	8
Tobramycin	>16
Gentamicin	>8
Netilmicin	>32

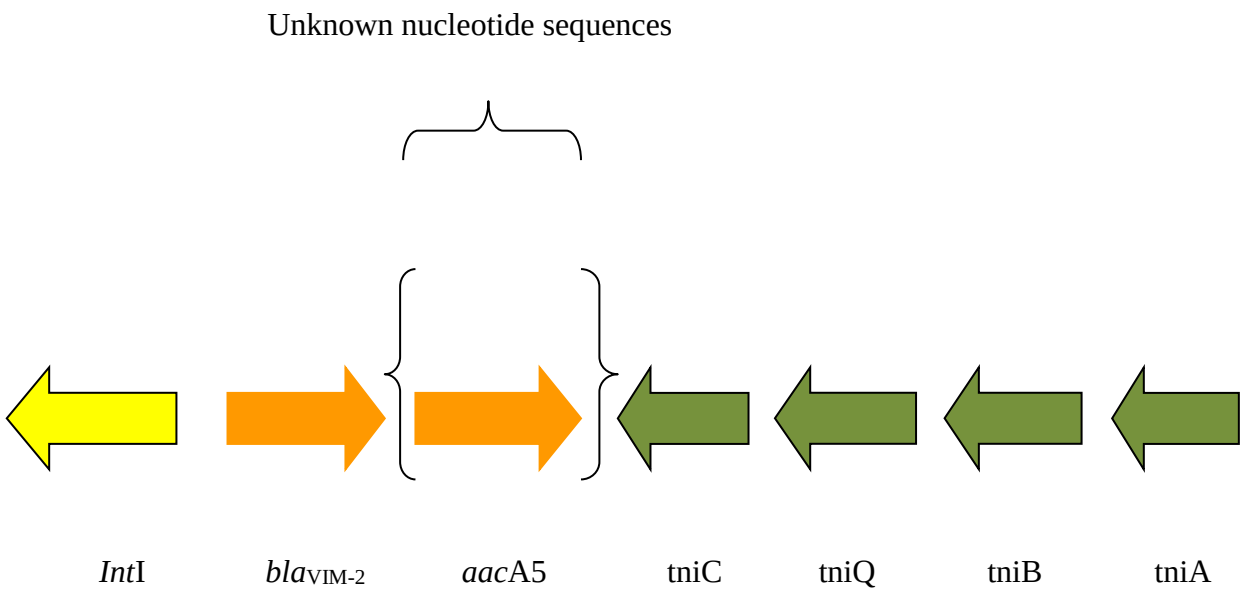


Figure 3.2 Schematic representation of the genetic map for class 1 integron carrying *bla*$_{VIM-2}$ embedded in a complete Tn*402*- like transposon in *P. fluorescens* 43-14926. *Int*I is identified by a yellow arrow, genes cassettes are represented by orange arrows and *tni*C module genes are designated green arrows

Figure 3.3 Nucleotide alignment of *aac*A5 gene of *P. fluorescens* 43-14926 (1) to *aac*A5 gene from *P. aeruginosa* (Accession No. AJ515707) (2), Length: 519 bp, identical sites: 518 bp (99.8% identity).

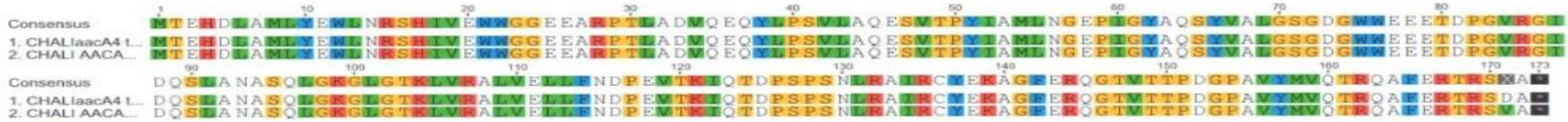


Figure 3.4 Protein alignment of aacA5 gene of *P. fluorescens* 43-14926 (1) to aacA5 gene from *P. aeruginosa* (Accession No. AJ515707) (2), Length: 173 AA, identical sites: 172 AA (99.4% identity).

3.4. Discussion

The variety of mobile genetic elements, described by several studies (Wozniak and Waldor 2010; Bertels and Rainey 2011), displays those, beyond HTG, the loss and acquisition of functional modules play a major part in the processes of rapid bacterial adaptation and development of resistance. Integrons are some of the most common genetic elements involved in this adaptation of bacteria (Stalder *et al*., 2012). The essential components of an integron include three key elements: the *int*I gene, a specific recombination site *att*I, and a promoter, Pc, which promotes the expression of any suitably integrated gene(s) (Hall and Collis 1995; Boucher *et al.,* 2007).

Class 1 integrons are a major medical problem in clinical bacteria. Most clinical-type class 1 integrons are descendants of a class 1 integron that is associated with a specific transposon, Tn*402*/Tn*5090* is characterized by the presence of a transposition module comprising a set of four genes (*tni*R, *tni*Q, *tni*B, and *tni*A) (Labbate *et al*., 2008; Tato *et al.,* 2012). The integron/transposon is structurally related to the functional ancestor, Tn*402*, which was first reported in 1994 (Rådstrom *et al*., 1994).

Presence of resistance genes on transposons provides a platform for moving from one plasmid to another and, once presents on a plasmid, can rapidly spread from one bacterium to another. Additionally, if the plasmid has the ability to be transferred to, and maintained in, more than one bacterial species, and rapid antibiotic resistance could happen (Hall 1997).

Many antibiotic-resistant bacteria and resistance genes' mobile structures have been detected in many environmental samples, such as hospital wastewater, sewage, domestic wastewater and rivers polluted with contaminated water. Wastewater which is being discharged from hospitals can be dangerous to public

health and the ecological balance since it may contain many kinds of deleterious waste such as radioactive, chemical and medication wastes and, not least, MDR pathogenic bacteria (Sharpe 2003).

Several studies have demonstrated that hospital wastewaters are highly selective environments and that they contribute to the high degrees of resistant bacteria, particularly *Pseudomonas* spp, that are being discharged into the natural environment (Fuentefria *et al*., 2011).

In this study, I have analyzed the nucleotide sequence of an unusual class 1 integron,harbouring*bla*$_{\text{VIM-2}}$thatis associated with a complete Tn*402*-like transposon ina carbapenem resistant *P. aeruginosa* clinical isolate (301-5473) from France and in *P. fluorescens, a* clinical isolate (43-14926) from Chile. The horizontal transfer of genetic material within and among different microbial species has been involved in the emergence of antibiotic resistance genes (Juan *et al*., 2010).MBL-producing *P. aeruginosa* strains have been responsible for large occurrences in many hospitals worldwide (Queenan *et al*., 2007). Horizontally acquired MBLs were first described in the early 1990s and presently contain nine groups of enzymes (IMP, VIM, SPM, SIM, GIM, DIM, AIM, KHM and NDM) (Juan *et al*., 2010). Rapid and prevalent emergence of resistance, and more significantly similar patterns of resistance, has been encountered in phylogenetically diverse Gram-negative clinical isolates on an increasing scale (Weldhagen 2004).

Several studies have been reported recently showing that the Tn*402* class 1 cassette array includes a diverse gene cassette. These examples were found in clinical environments. They include the cassettes *aac*C7, *bla*$_{\text{VIM-2}}$, *dfr*B5 and *aac*C6-II from an Indian *P. aeruginosa* isolate, and *aac*A7, *bla*$_{\text{VIM-2,}}$ and *dfr*B5 from *P. aeruginosa* isolates from the United States and Russia (Labbate *et al*., 2008, Samuelsen *et al*., 2010). Thus, the occurrence of the *tni*C-like transposon in *P. aeruginosa* isolates from different geographic locations suggests that the transposon is itself transferable and also responsible for the spread of *bla*$_{\text{VIM-2}}$.

Furthermore, the clinical isolates highlight the fact that MDR class 1 integrons also have transposon functionality that may spread these structures rapidly (Toleman *et al*., 2007). The results show the unusual class 1 integron in isolate 43-14926to have *bla*$_{\text{VIM-2}}$ and *aac*A4 gene cassette accompanied by complete *tni*C gene, to our knowledge the first report of such an arrangement in *P. fluorescens.* However, the gene cassette array of *bla*$_{\text{VIM-2}}$and *aac*A5, as

well as a complete *tni*C module, has been described recently in *P. putida* clinical strains isolated in the Balearic Islands, Spain (Juan *et al*., 2010), and in *P. aeruginosa* strain isolated in Argentina (Marchiaro *et al*., 2010).These results suggest that these elements (integrons and transposons) are used as natural genetic vehicles, enabling the efficient spread between bacterial individuals of similar or diverse species (Cambray *et al*., 2010).The majority of MBL genes are located on the chromosome (Samuelsen *et al*., 2010 and Lauretti *et al*., 1999). Furthermore, acquired MBL genes in *P. aeruginosa* are often carried on plasmids and are not usually transferred by conjugation, at least, to *E. coli* (Jeong *et al*., 2009)

Class 1 integrons are most widespread and clinically important. They are detectable in 22% to 59% of Gram-negative clinical isolates (Labbate *et al*. 2009) and they have been occasionally detected in Gram-positive bacteria (Martin *et al*. 2013, Nandi *et al*., 2004, Nesvera *et al.,* 1998, Shih *et al*., 2006). They are associated with functional and non-functional transposons derived from Tn*402* which can be further inserted in larger transposons, for instance Tn*21*. A recent study of the complete sequence of Tn*402* (Radstrom *et al*., 1994) revealed that this transposon is a class 1 integron. In addition to the integrase module and three integrated cassettes, Tn*402* includes a set of four putative transposition genes that are required for transposition (Kholodii 1995).

Chapter Four

Characteristics and stabilization of *bla*$_{\text{NDM-1}}$ gene in Enterobacteriaceae

4.1 Introduction

Plasmids play an important role in spreading carbapenem resistance, specifically VIM, IMP, and NDM (MBL). Plasmids act as vehicles that acquire mobile genetic elements (insertion sequences, transposons) that subsequently mobilize antimicrobial resistance genes (Carattoli 2013).

NDM-1-mediated resistance to carbapenems is increasingly reported internationally. Although the Indian subcontinent is considered a reservoir of NDM-1 producers, *bla*NDM-1 has been reported in the United Kingdom. Moreover, Balkan states and the Middle East regions could also be potential reservoirs (Poirel *et al*., 2011).The NDM-1 gene was later detected in the United States, Europe and Canada (Nazir *et al*., 2012).

The NDM-1 gene is mostly located on plasmids and on different plasmid types such as IncL/M, IncA/C, IncF, IncHI, IncN and IncHI1 NDM-1. These different plasmids can distribute $bla_{\text{NDM-1}}$ gene in non-clonally related Enterobacteriaceae. Nevertheless, two plasmids types, IncA/C and IncHI1, are more commonly associated with $bla_{\text{NDM-1}}$ (Carattoli, 2013).

Many reports show that the majority of bacteria carry$bla_{\text{NDM-1}}$ on different sizes of plasmids (Yong *et al*., 2009; Kumarasamy *et al*., 2010). Enterobacteriaceae, *K. pneumoniae* and *E. coli* are the most frequent host species to the NDM-1 gene, but it can also be found in *K. oxytoca, C. freundii*, *E. cloacae*, *Morganella morganii*, *Proteus* spp. and *Providencia* spp (Struelens *et al*., 2010).

Several natural plasmids are maintained at their original copy number in the growing bacterial population, controlling their concentration and regulating their replication rate (Nordstrom 2005). Replicons are regions of DNA or RNA that replicate from a single origin of replication; contain the *ori* but also the genes encoding particular replication initiator proteins (Rep) that bind to the *ori* and their regulating factors (Nordstrom2006).

Plasmid stability is determined by genetic and environmental factors. Genetic factors include; 1) Plasmid DNA sequence effects (point mutation, deletion, insertion or rearrangement in the plasmid DNA); 2) Plasmid copy number; 3) replication patterns; and 4)

host background. Environmental factors involve; 1) Culture formulation; 2) Dissolved oxygen tension; 3) Temperature; and 4) Dilution rate (Silva *et al*., 2012).

Plasmid instability is the tendency for plasmids in transformed cells to lose their properties owing to structural changes in the plasmid, or loss of plasmids or co-integration between plasmids. Plasmid instability can take on two forms: structural instability and segregational instability. Structural instability is caused by deletion, insertion, or rearrangement of DNA. Segregational instability is caused by defective partitioning of plasmids between the daughter cells during cell division (Primrose *et al*., 1981; Kumar *et al*., 1991; Silva *et al*., 2012). Genetic instability in combination with environmental factors (i.e. no selective pressure) can lead to loss of plasmids. During culture, cells lacking the plasmid appear which can often coexist and compete with the plasmid-containing population (Zhang *et al*., 2011).

The analysis of structural instabilities is important to understand the spread of AR and some methods are reported. When all daughter cells get at least one plasmid during cell division, the population is segregationally stable (Friehs 2004).

This chapter studies the characteristics and stability of plasmids bearing the $bla_{\text{NDM-1}}$ gene in Enterobacteriaceae.

4.2 Results

4.2.1 Antibiotic susceptibility

All isolates producing NDM-1 were resistant to several antibiotic classes (Table4.1). Ten isolates from Chennai and Haryana were similarly resistant to all β-lactam antibiotics, fluoroquinolones, and aminoglycosides. All isolates were resistant to aztreonam, cefepime, ceftazidime, and amikacin (MIC≥ 64 µg/ml), ciprofloxacin (>8 µg/ml), gentamicin (>32 µg/ml), imipenem (8-64µg/ml) and meropenem (16->32µg/ml), piperacillin (>64 µgm/l) and piperacillinetazobactam (>64 µg/ml). *K .pneumoniae* IR25 was resistant to tigecycline (MIC 8 µg/ml) and colistin (MIC >32 µg/ml) (Table 4.1); however, other NDM-1 positive strains were sensitive (MICs>1µg/ml). Minocycline MICs for the Haryana isolates, *Kpneumoniae*K15 and *Kpneumoniae*K7, were 1 and 2 µg/ml respectively. Tigecycline MICs for eight isolates isolated in Chennai, were <= 0.25- 8µg/ml.

4.2.2 Confirmation of the NDM-1 gene and others in Enterobacteriaceae isolates.

Preliminary screening for*bla*$_{\text{NDM-1}}$ positive *K. pneumoniae* (K15, K7, IR18K, and IR28K isolates),*K. oxytoca* (IR61) and *E. coli* (IR5, IR22, IR26 and IR29) was performed by PCR amplification with primers NDM-1 FR and NDM-1RE specific to the NDM-1(section 2.11.2). In all isolates, NDM-1 gene size was 800 bp. In addition, PCR was used with specific primers to detect ESBL gene such as*bla*$_{\text{CTX-M-15}}$and the genes *rep*A and *rep*B. All *bla*$_{\text{NDM-1}}$ – positive isolates harboured the *bla*$_{\text{CTX-M-15}}$ gene. Moreover, *rep*A and *rep*B genes were screened for in *K pneumoniae* K15 during the first and the sixth days through the passaging experiment.

4.2.3 PCR mapping of the *bla*$_{\text{NDM-1}}$ surrounding sequences

To determine the genetic structure surrounding the *bla*$_{\text{NDM-1}}$ gene, DNA of NDM-1 positive strains was used as a template for PCR with primers designed from the *bla*$_{\text{NDM-1}}$ gene sequence (Appendix A, Table A- 4). *E. coli* IR5, *E. coli* IR22, *E. coli*IR26, *E. coli* IR29, *K. pneumoniae* K15, *K. pneumoniae* K7, *K. pneumoniae* IR28K and *K. oxytoca* IR61 all showed part of IS*Aba125*, an IS element that was present upstream of *bla*$_{\text{NDM-1}}$. We also identified a

gene named *ble*$_{MBL,}$ which encoded resistance to bleomycin, immediately downstream of the *bla*$_{NDM-1}$ gene. However, the IS*26*element identified in *K. pneumoniae* IR18 and *K. pneumoniae* IR25 (Figure 4.2) was present upstream of *bla*$_{NDM-1}$. The diversity of genetic features associated with the *bla*$_{NDM-1}$ gene may explain its current high rates of spread worldwide.

Table 4.1 Susceptibility profile of clinical NDM-1 producers isolated from Indian subcontinent

Character/strains	IR22	IR25	IR26	IR61	IR5	IR18K	IR29	K7	K15	IR28K
Ampicillin	>64	>64	>64	>64	>64	>64	>64	>64	>64	> 64
Aztreonam	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Cefotaxime	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Ceftazidime	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Cefpirome	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Imipenem	16	8	32	16	32	16	16	64	64	16
Meropenem	32	16	>32	16	>32	32	>32	>32	>32	32
Ertapenem	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
Ciprofloxacin	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
Tobramycin	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Amikacin	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Gentamicin	>32	>32	>32	32	>32	>32	>32	>32	>32	>32
Minocycline	4	>32	4	2	32	>32	0.25	16	8	>32
Tigecycline	1	8	0. 5	0.5	1	2	<=0.250	2	1	2
Colistin	<=0.5	>32	<=0. 5	<=0.5	<=0.5	<=0.5	<=0.5	1	<=0.5	<=0.5

***K. pneumoniae* JX988621.1**

K. pneumoniae IR18

K .pneumoniae IR25

Transposase IS*26*

IRR

***E. coli* JF714412.2**

E. coli IR5

E. coli IR22

*E.coli*IR26

E. coli IR29

K. oxytoca IR61

K. pneumoniae K15

K. pneumoniae K7

K. pneumoniae IR28K

Transposase IS*Aba125*

***bla*NDM-1**

***ble*MBL**

Figure 4.1 Schematic drawing showing the *bla*NDM-1-associated genetic structures

Table 4.2 Genetic features associated with the *bla*NDM-1 gene

Strains	City of isolation	Plasmid type carrying bla_{NDM-1}	size Kb	$bla_{CTX-M-15}$	IS	ble_{MBL}	bla_{NDM-1}
K. pneumoniae K15	**Haryana**	**IncX**	**60Kb**	+	+	+	+
		Inc F	**150Kb**		**(ISAb125)**		
K. pneumoniae K7	**Haryana**	**untypeable**	-	+	+	+	+
					(ISAb125)		
K. pneumoniae IR18K	**Chennai**	**Inc AC**	**200Kb**	+	**+ (IS26)**	+	+
		Inc F	**110kb**				
		incF	**90kb**				
K. pneumoniae IR28K	**Chennai**	**untypeable**	-	+	+	+	+
					(ISAb125)		
K. pneumoniae IR25	**Chennai**	**untypeable**	-	+	+	+	+
					(IS26)		
E. coli IR5	**Chennai**	**untypeable**	-	+	+	+	+
					(ISAb125)		
E. coli IR22	**Chennai**	**untypeable**	-	+	+	+	+
					(ISAb125)		
E. coli IR26	**Chennai**	**untypeable**	-	+	**+(ISAb125)**	+	+

4.2.4 Plasmid typing

Plasmid incompatibility groups were assigned by using the PCR–based replicon typing method (Carattoli *et al*., 2005). One plasmid (*Kp* pk7) carrying the *bla*$_{NDM-1}$ gene, from Haryana, could not be typed but two other plasmids, carrying the NDM-1 gene isolated from *K. pneumoniae* K15, were incX and incF. NDM-1 positive *K. pneumoniae* IR18K and *K. oxytoca* IR61 were carrying IncA/C. Moreover, NDM-1 positive *K. pneumoniae* IR18K carried IncF plasmid (Table 4.2).

4.2.5 Stability of plasmid bearing NDM-1 gene in the Enterobacteriaceae

Measurement of plasmid stability is the number of cells subsequently capable of forming colonies on a non-selective medium. The *bla*$_{NDM-1}$ gene stability in *K pneumoniae* K15, *K pneumoniae* K7, *K pneumoniae* IR18K, *K pneumoniae* IR28K, *K pneumoniae* IR25, *K. oxytoca* IR61, *E coli* IR5, *E coli* IR22, *E coli* IR26, and *E coli* IR29 was studied in absence of antibiotic selection. It was found that the genetic structural integrity of *bla*$_{NDM-1}$ was stable in most isolates in the absence of antibiotic selection. This observation was confirmed at least twice in each case.

4.2.7 Plasmid analysis and back probing with *bla*$_{NDM-1}$

NDM-1plasmidstability in Enterobacteriaceae was studied by serial passaging in liquid culture. To determine the genomic location of the NDM-1 gene, *S*1 digests and probing with NDM-1 radio- labelled *bla*$_{NDM-1}$ Probe DNA fragments was carried out using the protocol outlined in section 2.21.The movement and stability of *bla*$_{NDM-1}$ gene between plasmids and chromosome were detected during the time of the experiment, which was for 14 days.

4.2.7.1 Detection of chromosomal and plasmid mediated *bla*$_{NDM-1}$ stability for *K. pneumoniae*

*S*1-PFGE and probing results revealed that *K. pneumoniae* K15and *K. pneumonia* IR25havemore than one plasmid carrying *bla*$_{NDM-1}$.Isolate K15 was found to carry *bla*$_{NDM-1}$ on 60 kb and 100kb plasmids (Figure 4.2) and isolate 1R25 was found to carry *bla*$_{NDM-1}$ on

100 kb and 300 kb plasmids (Figure 4.3). However, isolates IR18K and K7were found to carry*bla*$_{\text{NDM-1}}$ on a100 kb plasmid, (Figure 4.4), and *K. Pneumoniae* IR28K has just one 300 kb NDM-1 plasmid (Figure 4.5). Chromosomal bands in the autoradiograph suggest *bla*$_{\text{NDM-1}}$is located on the chromosome of *K. pneumoniae* IR25 and *K. pneumoniae* IR28K strains (Figure 4.3 and Figure 4.5).

PFGE analysis over 14 days used *Xba*1 restriction enzyme to detect the number of NDM-1genes among strains producing NDM-1. A more complicated picture is observed in *K. pneumoniae* K15, which was grown at 37^0C without antibiotic pressure (Figure 4.6), where it was found to have five copies of the NDM-1 gene on <50 kb, 50 kb, 60kb, 120 kb and 220 kb plasmids. Isolate IR18K, digested with *Xab*1 enzyme, has three copies of the NDM-1 gene at 20 kb, 50 kb, and 100 kb (Figure 4.7).

Overall, *bla*$_{\text{NDM-1}}$ was stable in all *K. pneumoniae* isolates (except isolate K15), and was located on plasmids and chromosomes with differing plasmid sizes and number of copies.

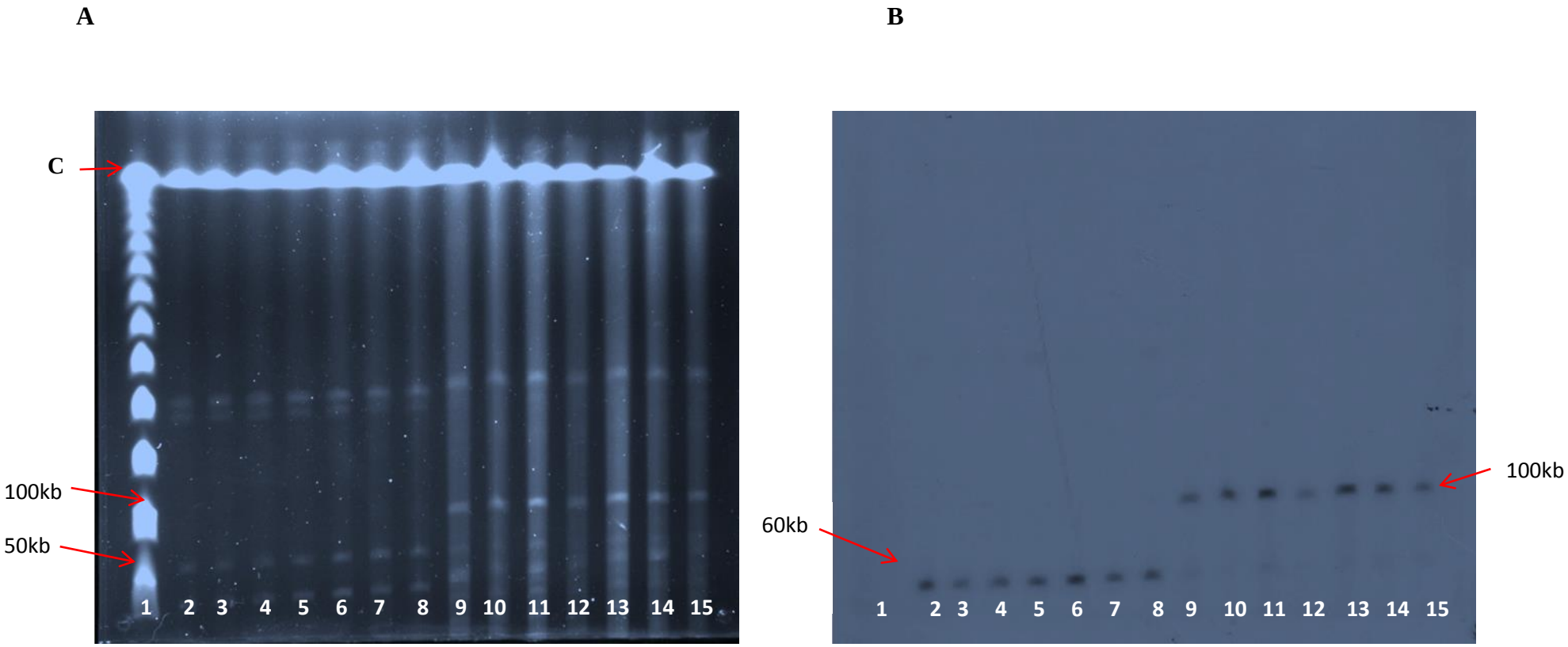


Figure 4.2 Pulsed-field gel of genomic DNA from *K. pneumoniae* K15. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B). Hybridization of gel A with *bla*$_{NDM-1}$gene probe. Lane 1, lambda ladder pulsed-field gel marker; Lane 2=D1 (day one); Lane 3= D2; Lane 4= D3; Lane 5=D4; Lane 6=D5; Lane 7= D6; Lane 8= D7; Lane9= D8; Lane10=D9; Lane11=D10; Lane12=D11; Lane13=D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base pairs.

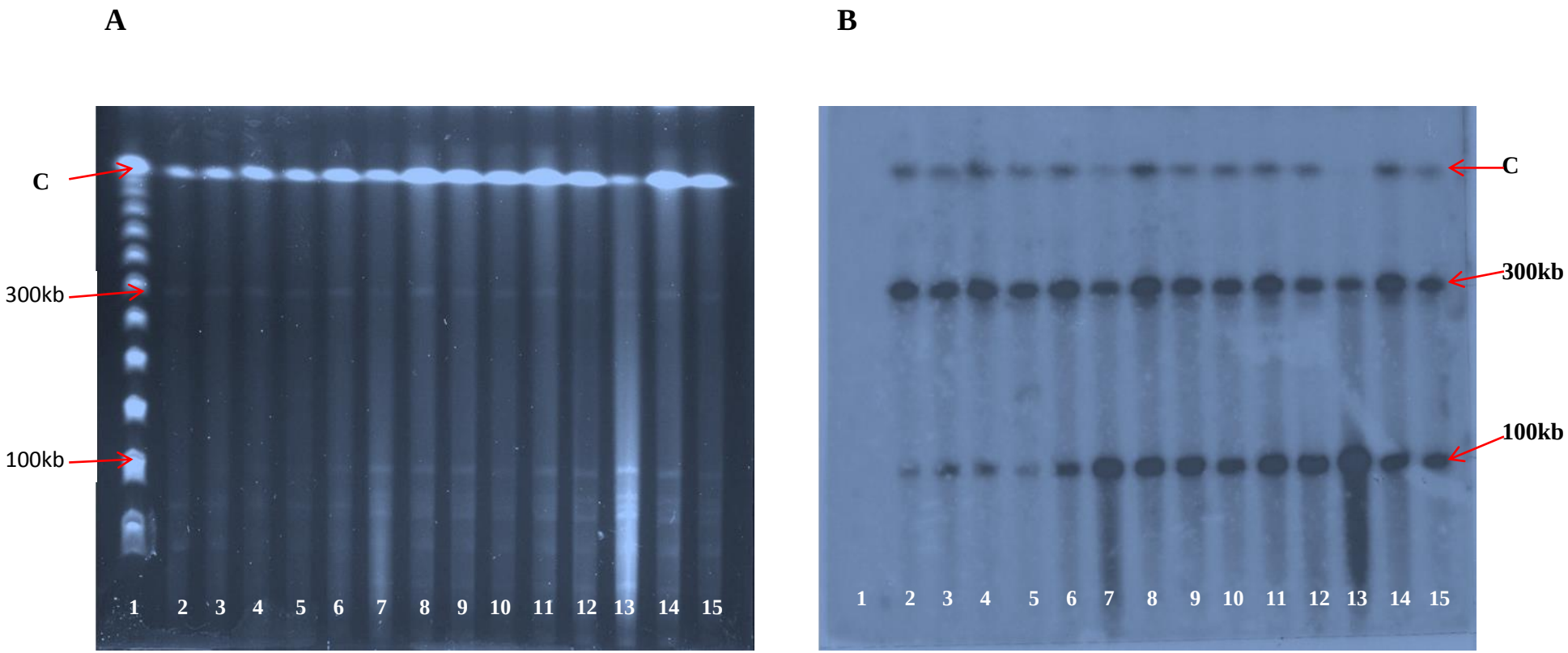


Figure 4.3 Pulsed-field gel of genomic DNA from *K. pneumoniae* IR25 strain (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2=D1 (day one); lane 3=D2; Lane 4= D3; Lane 5= D4; Lane 6=D5; lane 7=D6; Lane 8= D7; Lane9=D8; Lane10=D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14=D13 and Lane 15= D14. C= chromosome. Kb= kilo base pairs.

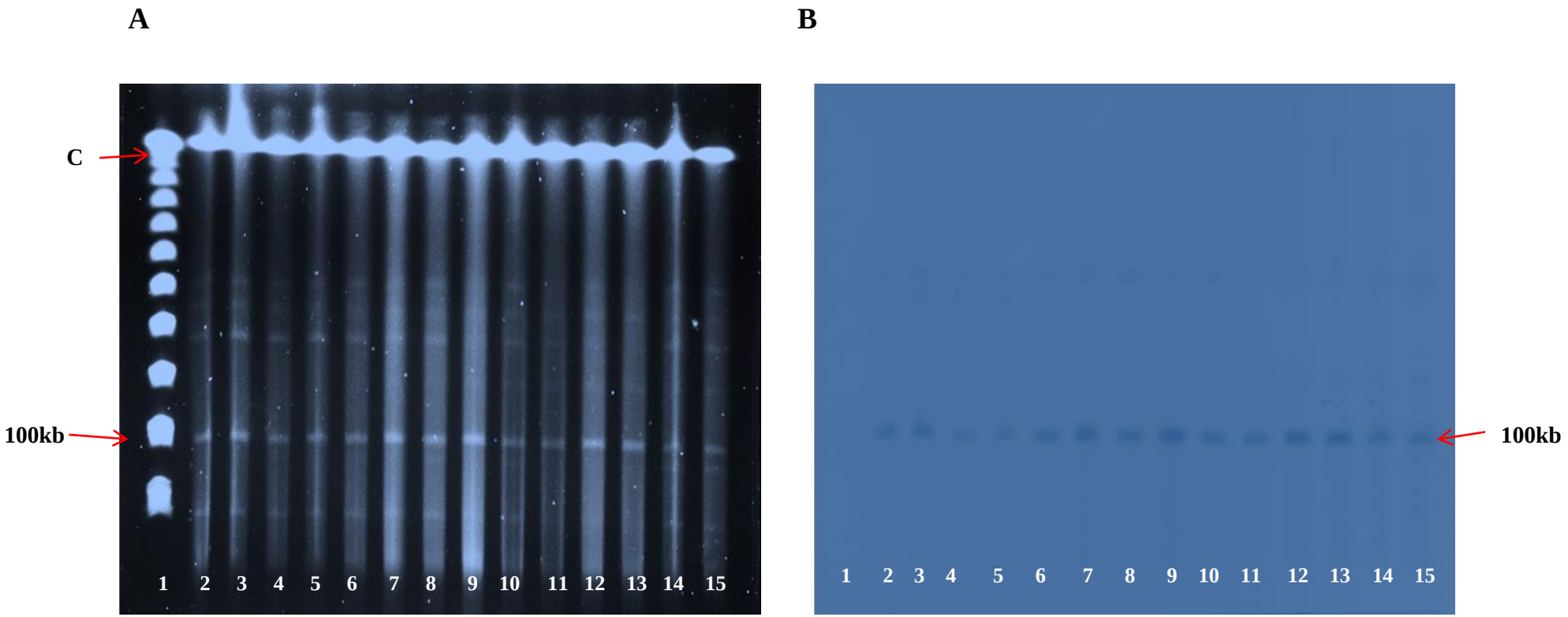


Figure 4.4 Pulsed-field gel of genomic DNA from *K. pneumoniae* IR18K strain. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2=D1 (day one); Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6=D5; Lane 7= D6; Lane 8= D7; Lane9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13=D12; Lane 14= D13 and Lane 15= D14 C= chromosome. Kb= kilo base pairs.

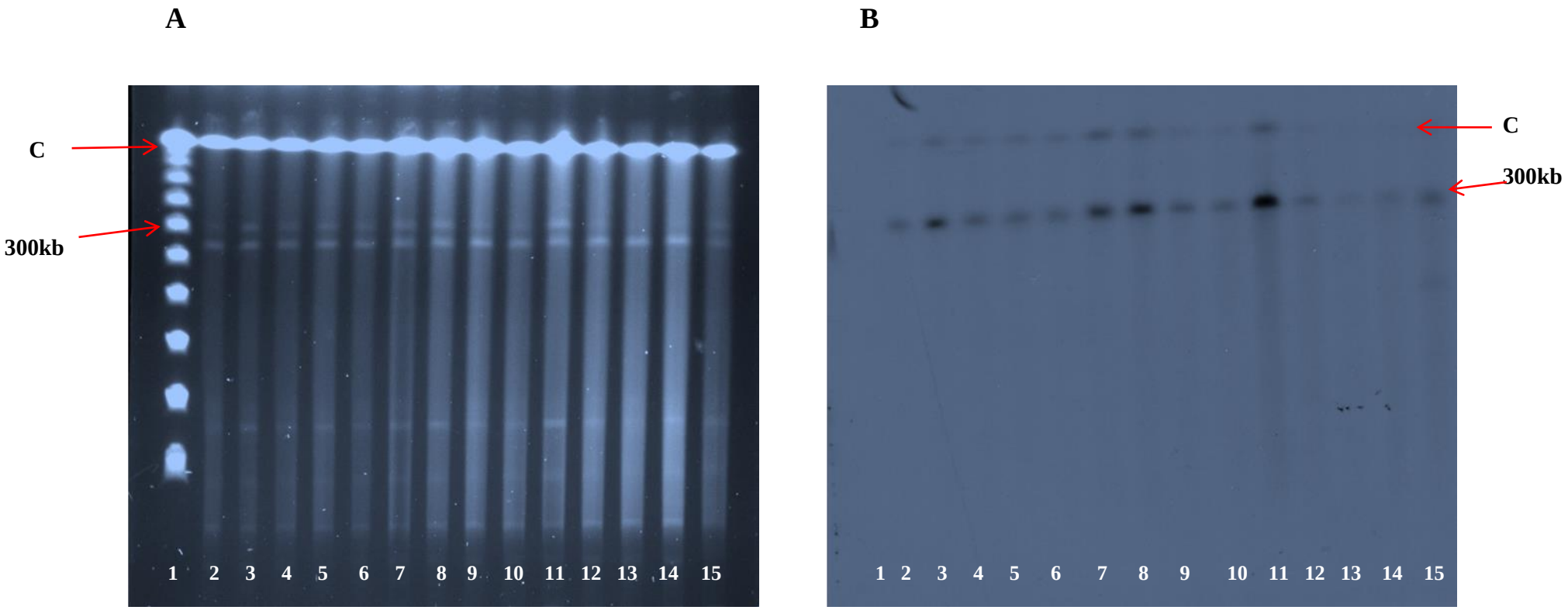


Figure 4.5 Pulsed-field gel of genomic DNA from *K. pneumoniae* IR28K strain. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2=D1(day one); Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6=D5; Lane 7= D6; Lane 8= D7; Lane9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15,=D14. C= Chromosome. Kb= kilo base pairs.

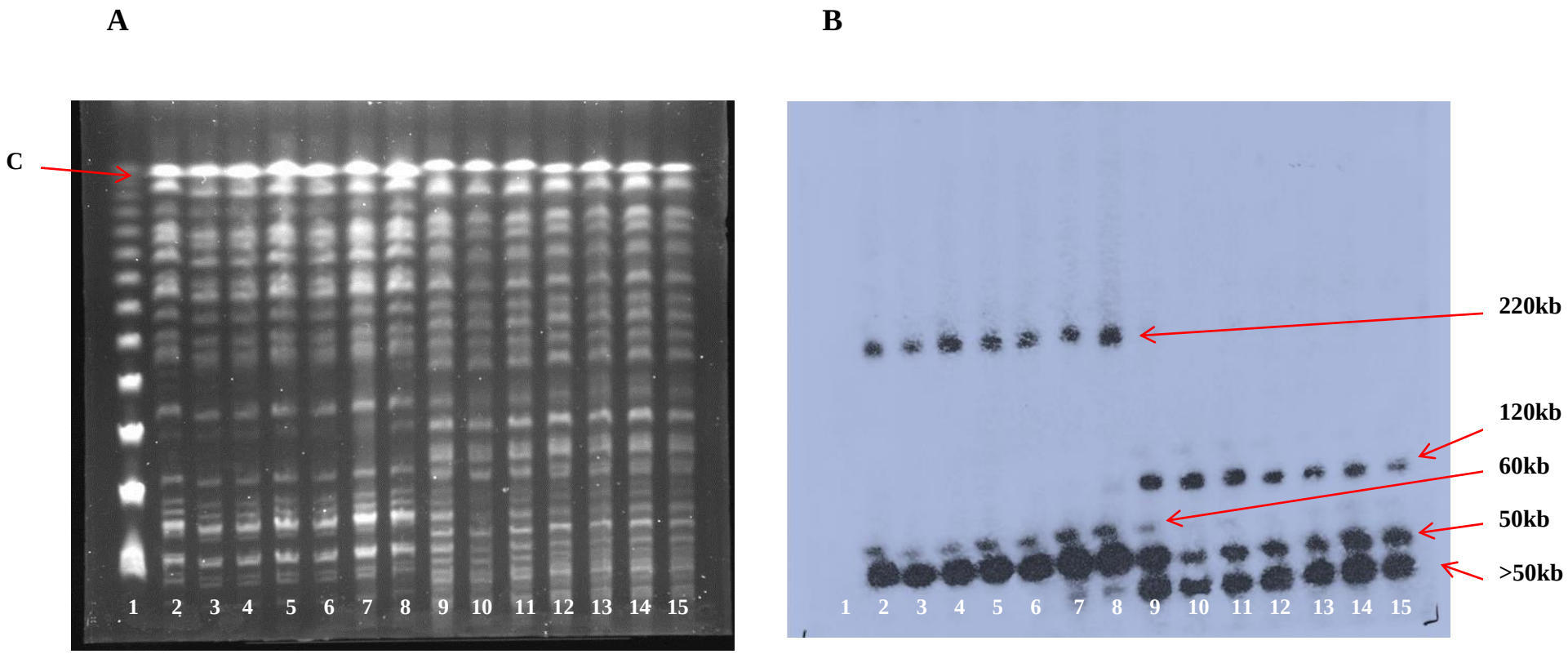


Figure 4.6 Pulsed-field gel of genomic DNA from *K. pneumoniae* K15 strain.(A) Gel digestion with *Xba*l restriction enzyme. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1(day one); Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14.C= chromosome. Kb= kilo base pairs.

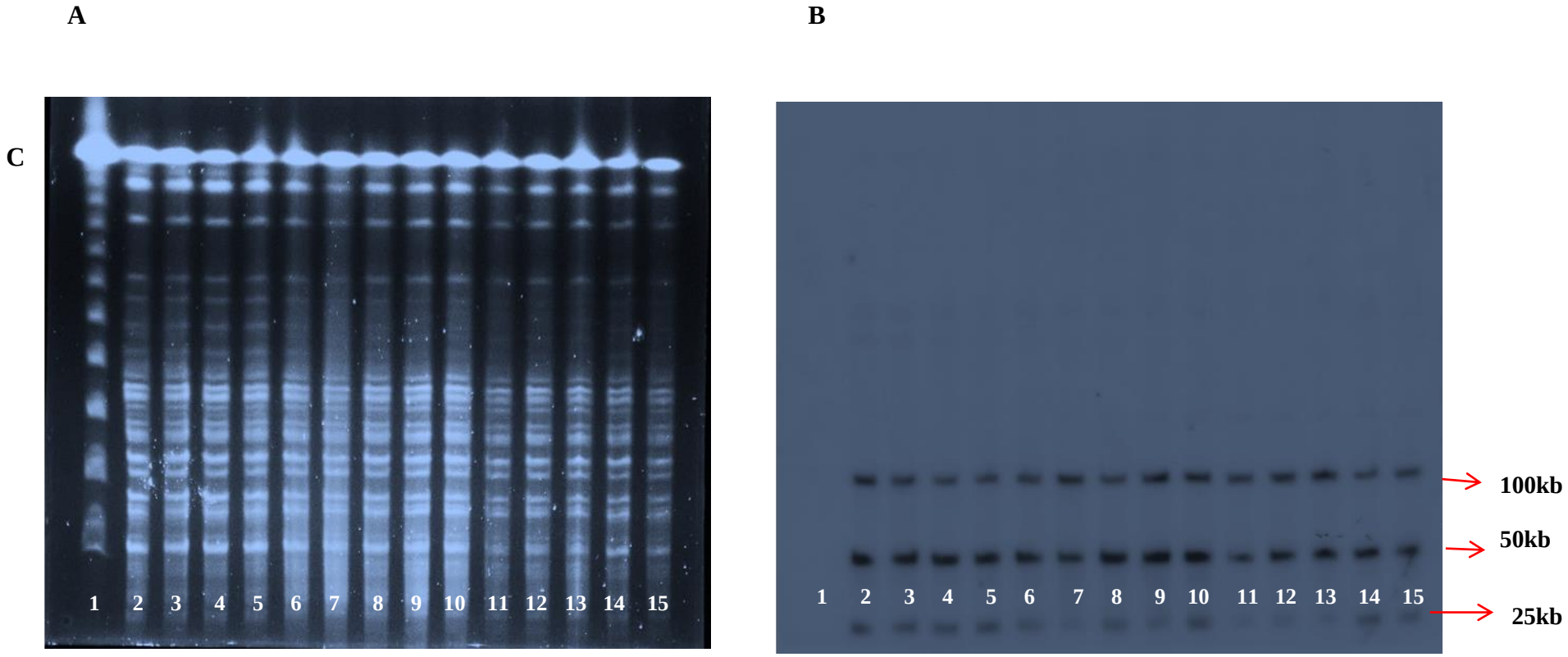


Figure 4.7 Pulsed-field gel of genomic DNA from *K. pneumoniae* IR18K strain.(A) Gel digestion with *Xba*l restriction enzyme. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lane 1, lambda ladder pulsed-field gel marker; Lane 2=D1(day one); Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base pairs.

4.2.7.2 Detection of chromosomal and plasmid carrying *bla*$_{\text{NDM-1}}$ stability for *E. coli*

To determine the genomic location of the *bla*$_{\text{NDM1}}$ genes, *S*1 digests and probing with radio-labelled *bla*$_{\text{NDM-}}$1 DNA fragments were carried out by using the protocol outlined in section. 2.22. The *E. coli* IR5isolatewas found to carry *bla*$_{\text{NDM-}}$1 on 80 kb and 90 kb plasmids. However, isolate IR29 has four NDM-1 plasmids; 150 kb, 250 kb, 300 kb and 350 kb (Figure 4.8). Furthermore, *S*1 results for isolate IR29 suggest that the *bla*$_{\text{NDM-1}}$ gene is present on the chromosome. Isolate IR29 displayed rearrangement of the *bla*$_{\text{NDM-1}}$ plasmid from 250 kb to 300 kb on the fourth day (Figure 4-8). The *bla*$_{\text{NDM-1}}$ gene was identified on 100 kb and 150 kb in isolates IR26 and IR22, respectively.

*Xba*1 digests were carried out on *E. coli* isolates IR26, IR5, and IR22. *Xba*1 results for isolate IR26 show two *bla*$_{\text{NDM-1}}$gene copies (100 and 150kp (Figure 4.9); IR5 has two copies of NDM-1 (140 kb and 150 kb) (Figure 4-10); IR22 carries two copies of NDM-1 at ≥ 150kb. Isolate IR29 has four copies of NDM-1 gene of different sizes.

Generally, NDM-1 gene was stable through *E. coli* isolates and the NDM-1 genes carried were of different sizes. Only in one isolate, IR29, the NDM-1 gene was not stable and, in addition, harboured the NDM-1 gene on the chromosome.

4.2.6.3 Detection of chromosomal and plasmid harbouring *bla*$_{\text{NDM-1}}$ stability for *K. oxytoca.*

K. oxytoca IR61 isolate was found to carry *bla*$_{\text{NDM-1}}$ on 150 kb (Figure 4-11). Genomic DNA of *K. oxytoca* IR61 was digested with *Xba*1 to detect the *bla*$_{\text{NDM-1}}$copy. *Xba*1- PFGE gel result of *K. oxytoca* IR61 is shown in Figure 4.12 .This result shows that isolate IR61 has three copies of *bla*$_{\text{NDM-1}}$ at 25 kb, 40 kb, and 140 kb. *K. oxytoca* IR61's plasmid bearing *bla*$_{\text{NDM-1}}$ gene was stable during the course of passaging.

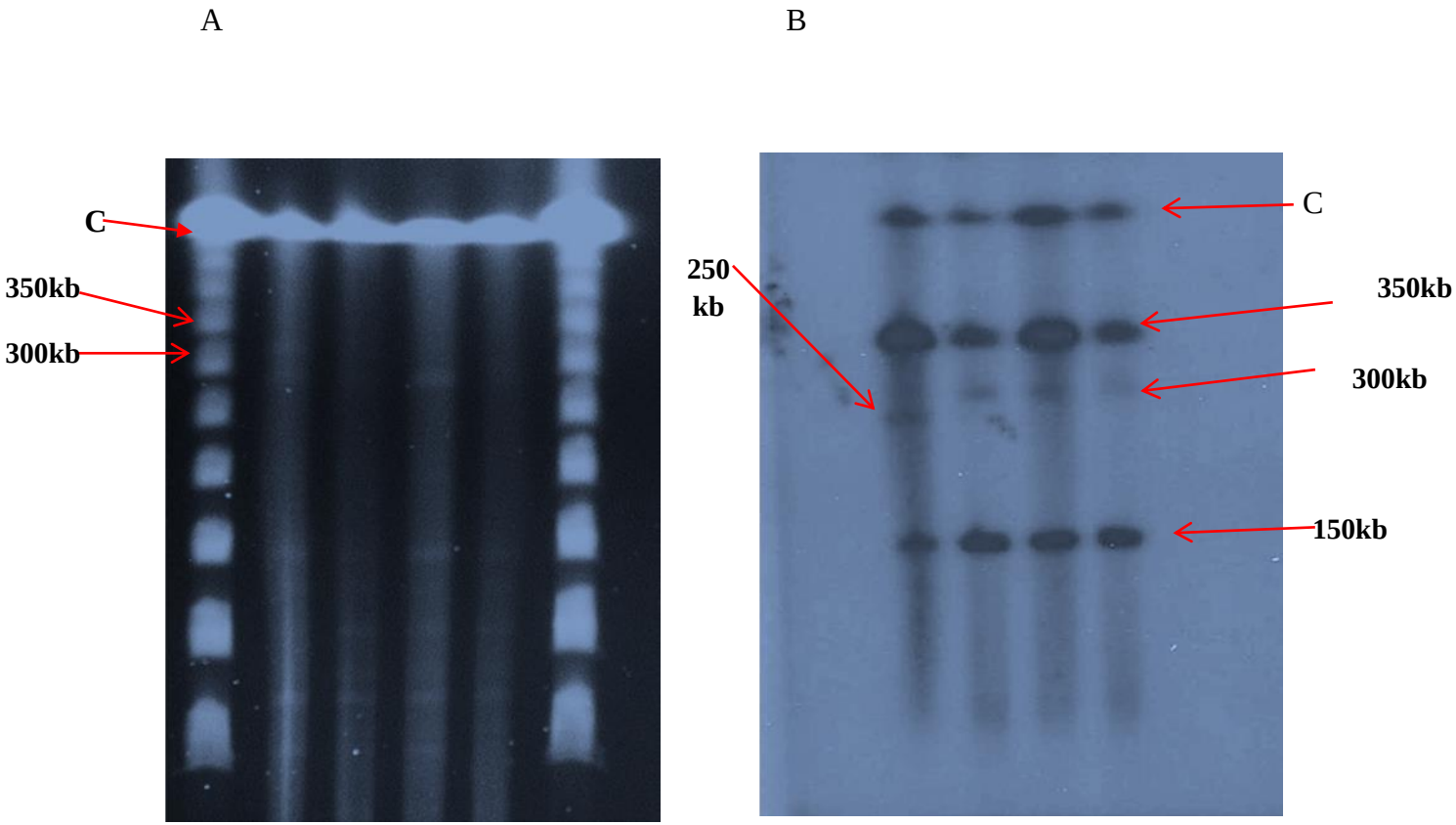


Figure 4.8 Pulsed-field gel of genomic DNA from *E. coli* IR29 strain. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lanes 1&6 =lambda ladder pulsed-field gel marker; Lane 2,=D0 (day zero); Lane 3= D4; Lane 4= D8; Lane 5= D12. C= chromosome. Kb= kilo base pairs.

A

B

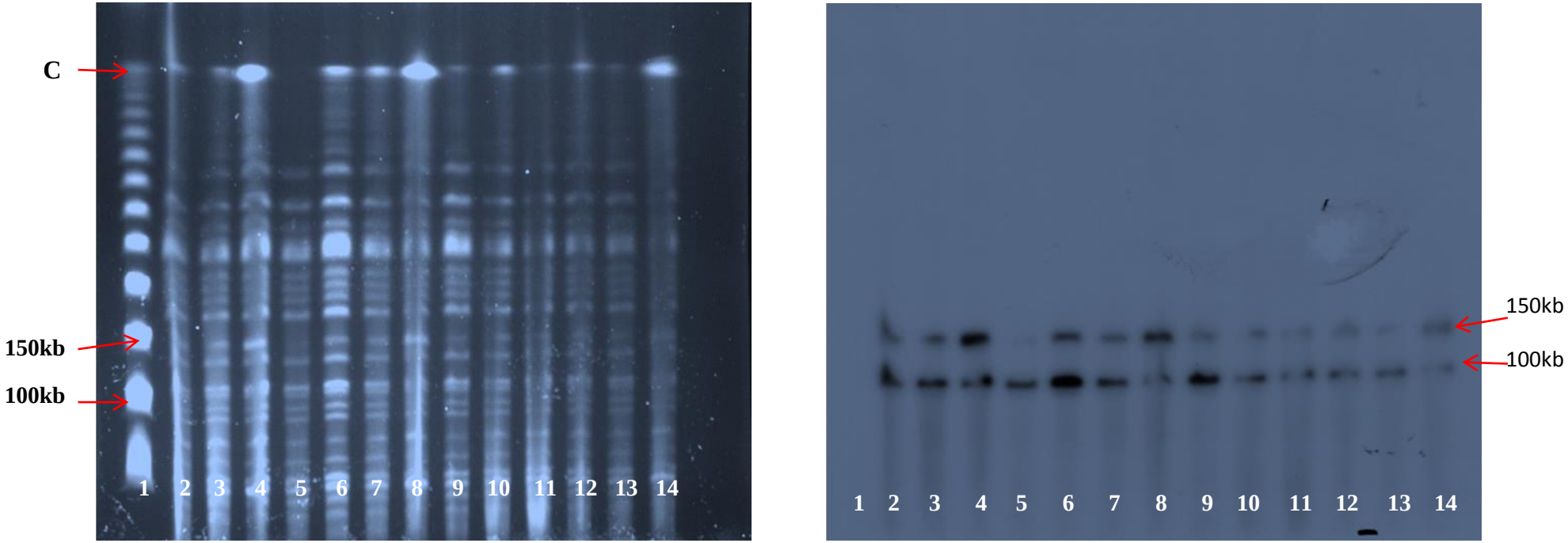


Figure 4.9 Pulsed-field gel of genomic DNA from *E. coli* IR26 strain. (A) Gel digestion with *Xba*l restriction enzyme. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1 (day one); Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane 9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; and Lane 14= D13. (Note, the plug is lost in day 14).C= chromosome. Kb= kilo base pairs.

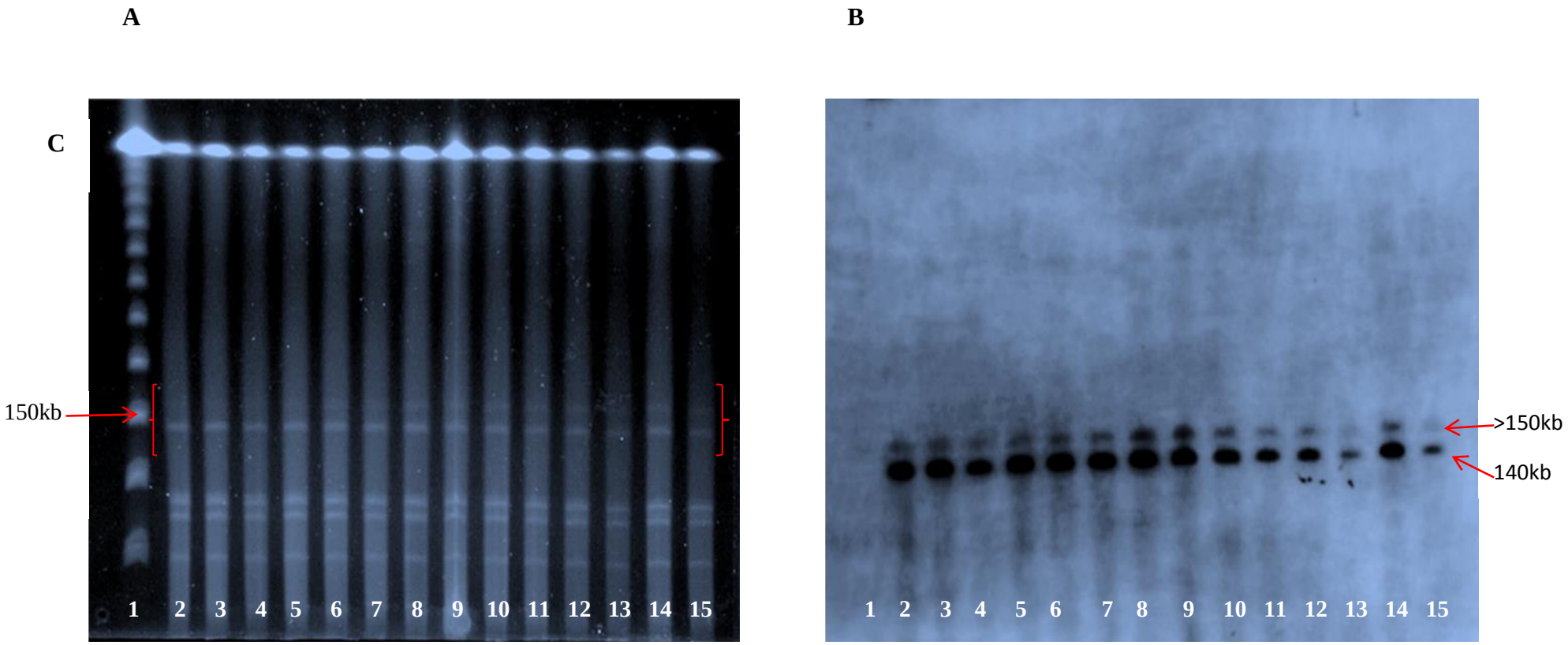


Figure 4.10 Pulsed-field gel of genomic DNA from *E. coli* IR5 strain. (A) Gel digestion with *Xba*l restriction enzyme. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1(day one); Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane 9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base pairs.

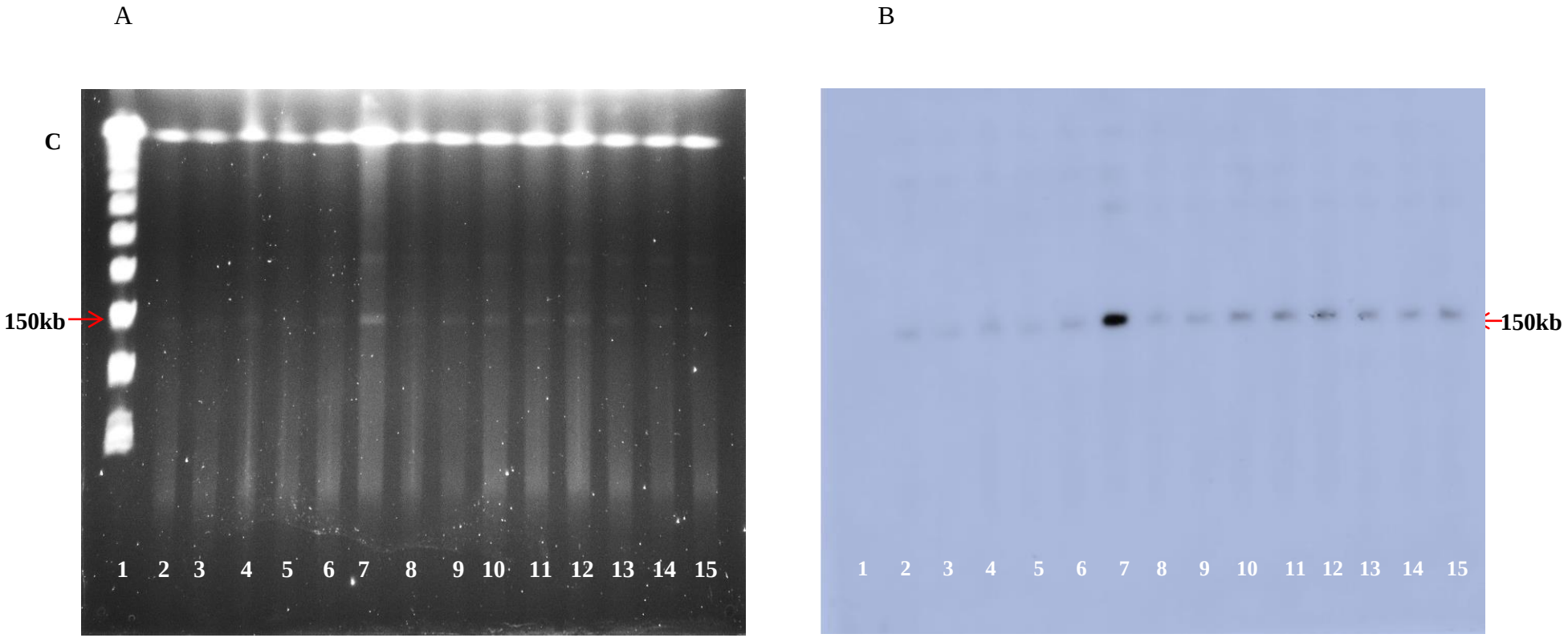


Figure 4.11 Pulsed-field gel of genomic DNA from *K. oxytoca* IR61 strain. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1(day one); Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane 9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base pairs.

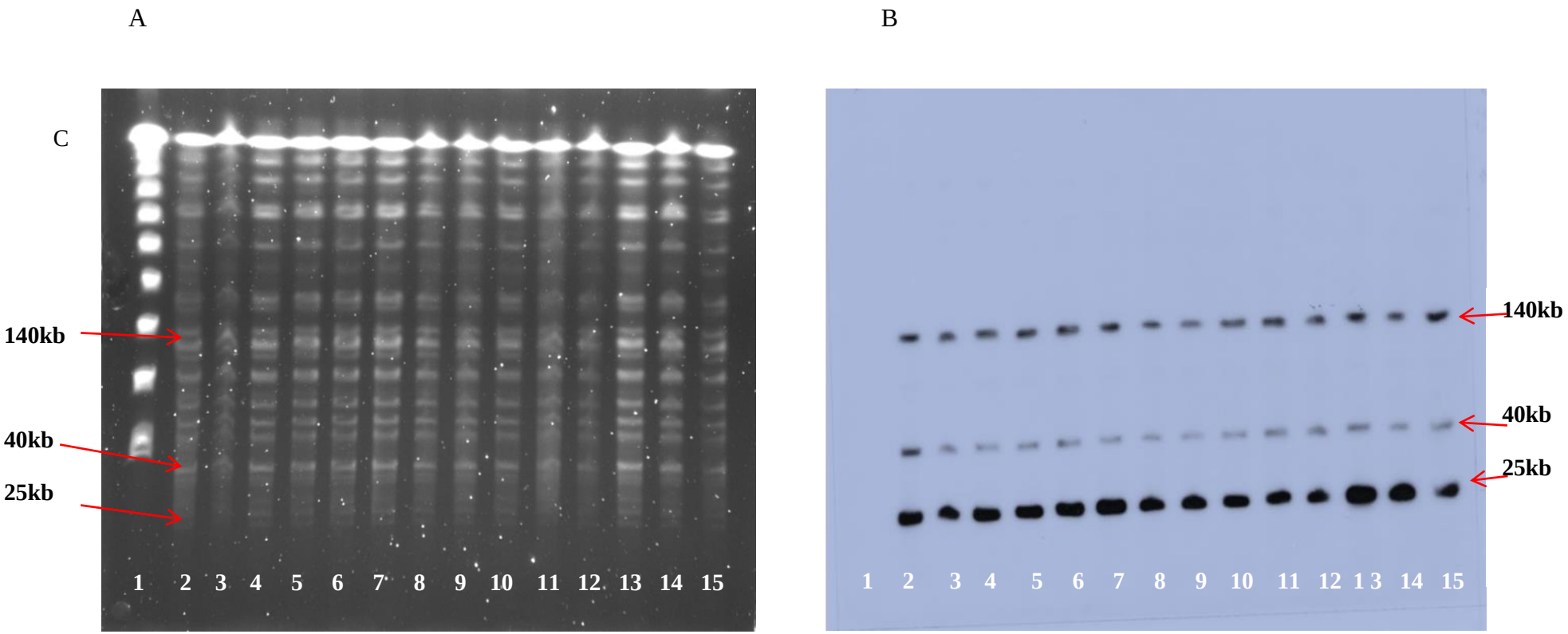


Figure 4.12 Pulsed-field gel of genomic DNA from *K. oxytoca* IR61 strain. (A) Gel digested with *Xba*1 restriction enzyme. (B) Hybridization of gel A with *bla*$_{NDM-1}$ gene probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1(day one); Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6=D5; Lane 7= D6; Lane 8= D7; Lane 9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14.C= chromosome. Kb= kilo base pairs.

4.3 Discussions

Most NDM-1 positive isolates used were susceptible to colistin, minocycline and tigecycline. This result agrees with Kumarasamy`s results (Kumarasamy *et al*., 2010). However, some Indian isolates are described as being colistin and tigecycline resistant, leading to being pan-resistant isolates (Walsh 2010). Our results showed that most isolates had resistance to carbapenems, fluoroquinolones and aminoglycosides.

Plasmid incompatibility groups were determined by using the PCR-based replicon typing method (Carattoli 2005) and classifying different plasmid scaffolds carrying the *bla*$_{\text{NDM-1}}$ gene. These results indicated that, except for three strains, *K pneumoniae* K15, *K pneumoniae* IR18K and *K oxytoca* IR61, the typing method failed to identify plasmid scaffolds in other strains, whereas, IncX, IncF, and IncA/C were common in Indian isolates. The results were consistent with Kumarasamy and his team and Walsh and his colleagues (Walsh *et al*., 2011; Kumarasamy *et al*., 2010). These data show that the current spread of *bla*$_{\text{NDM-1}}$ is not associated with a single plasmid. Numerous plasmids had a narrow host range, such as the IncF plasmids, and thus able to spread among Enterobacteriaceae only; others were of broad host range, such as the IncA/C types, and were able to replicate in *Acinetobacter* and *Pseudomonas* species (Poirel *et al*., 2011).

Genetic structures surrounding *bla*$_{\text{NDM-1}}$ showed that remnants of insertion sequence IS*Aba125* were systematically present upstream of the *bla*$_{\text{NDM-1}}$ gene. IS*Aba125* is an element belonging to the IS*30* family (Poirel *et al*., 2011). IS*Aba125* has only been designated in *Acinetobacter* spp and has not been found to be linked to *bla*$_{\text{NDM-1}}$ before, suggesting that horizontal transfer between *Acinetobacter* and Enterobacteriaceae has occurred (Sole *et al*., 2011). Furthermore, IS*26* was identified in *K. pneumoniae* IR18 and *K. pneumoniae* IR25 (Figure 4.1). The IS*26* element, belonging to the IS*6* family, is common among Enterobacteriaceae plasmids. It has been reported in the immediate region of the *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA}}$ and *bla*$_{\text{SHV}}$ classes of β-lactamases that were part of a transposon-like structure in various plasmids. Among *E. coli* isolates in Germany, integration of the *bla*$_{\text{CTX-M}}$/IS*26* transposon-like structure in plasmids of different replicon types has been described and also chromosomal integration of CTX-M-3 with two IS*26* elements (Cullik *et al*., 2011).

Downstream of *bla*$_{NDM-1}$, the *ble*$_{MBL}$ gene was identified in most of the isolates. This would suggest that an IS*Aba125*-related initial mobilization mechanism had been responsible for the joint acquisition of both the *bla*$_{NDM-1}$ and *ble*$_{MBL}$ genes. It also suggests that both genes originated from the same progenitor (Poirel *et al*., 2011).

Since *bla*$_{NDM-1}$ is flanked by IS insertion elements, we cannot neglect the possibility of integration of the gene into a plasmid and subsequent horizontal spread. The plasticity of the genetic structure surrounding *bla*$_{NDM-1}$, with DNA seemingly originating from both Enterobacteriaceae and *A. Baumannii* explains the observed, rapid spread of this gene within many Gram-negative bacteria species and across genera (Pfeifer *et al*., 2011).

Replication locus *rep* genes of bacteria have been associated with stability during replication of the inherent plasmid (Zhang *et al*., 1996). The replication (*rep*) and partitioning (*par*) systems are often located and regulated separately in plasmid genomes (Pappas and Winans 2003; Pappas 2008). *Rep*A, B and C plasmids, commonly found in α-proteobacteria, are categorized by the existence of a *rep*A, B or C operon, which carries all the elements required for plasmid replication and segregation. Generally, *rep*A, B, and C operons consist of three protein-encoding genes, an antisense RNA gene, and at least one centromere-like region (Cervantes-Rivera 2010).In 1984, Futcher and Cox showed that plasmids containing both *rep* loci are considerably more stable than those lacking one or both loci (Zhang *et al*., 2011).

The*S1* PFGE results showed that *bla*$_{NDM-1}$ itself is unusually mobile and moves between different plasmids. In this study, Enterobacteriaceae carried different sized plasmids bearing *bla*$_{NDM-1}$. However, another recent study showed that the NDM-1 positive Enterobacteriaceae harboured similarly sized plasmids (Walsh *et al*., 2011). The Indian strains had a diverse range of *bla*$_{NDM-1}$ harbouring plasmid sizes (from 25kb to>400kb). Three isolates; *K. pneumonia* IR28K, *K. pneumonia* IR25 and *E. coli I*R29 carried *bla*$_{NDM-1}$ upon the chromosome which has seldom been reported. In most isolates *bla*$_{NDM-1}$ was located on more than one plasmid.

Plasmid stability was studied in our isolates by using serial dilution. The *S*1 PFGE results showed that the $bla_{\text{NDM-1}}$ gene was stable in most isolates.

Stability of the plasmid is mediated by several factors such as; 1) Plasmid load; 2) Plasmid copy number; 3) Replication systems; 4) Substrate type; 5) Medium composition; 6) Host background; 7) Culture conditions; 8) Temperature of culture; 9) Plasmid isoform; and 10) antibiotic concentration (Silva *et al*., 2012). Also, there are other factors affecting plasmid structural stability such as plasmid size (Ertl and Thomsen 2003), direct repeats (Hadj *et al*., 2008), inverted repeats (Bi and Liu, 1996) and insertion sequences (Valesova *et al*., 2005). All these factors can elevate the rate of spontaneous point mutations or promote recombinations between intergenomic or directly repeated sequences (Oliveira *et al*., 2009).

Culture conditions such as dissolved oxygen concentration and pH can affect plasmid stability. A decrease in the dissolved oxygen concentration level in growth medium could be harmful to plasmid maintenance (Goyal *et al*., 2009; Krishna *et al*., 2008) since sufficient dissolved oxygen is essential for maintaining plasmid stability. Furthermore, a low pH medium may cause stress to the bacterial cells resulting in plasmid loss from the high-density cultures (Chen *et al*., 1992). The composition of the media may also impact on plasmid segregational stability (O'Kennedy and Patching 1997). However, it was shown that the use of complex nitrogen sources such as yeast extract or tryptone did not decrease plasmid stability (Goyal *et al*., 2009).Some studies suggest that use of these nitrogen sources resulted in increased plasmid stability (Matsui *et al*., 1990).

Other factors, such as the antibiotic resistance gene and plasmid copy number, can also affect plasmid segregational stability. With regard to plasmid copy number, use of high copy plasmids is believed to induce a higher metabolic burden on the host strain, causing a decrease in plasmid DNA productivity due to reduced cell growth (Jones *et al*., 2000). With respect to antibiotic resistance, in the presence of selective pressure, e.g. kanamycin, resistant plasmids are more stable than ampicillin resistant plasmids (French and Ward, 1995). There is an additional stability system required in the bacteria, such as the toxin/antitoxin (TA) system (Diago-Navarro *et al*., 2010). This system will be shown and discussed in chapter seven.

Plasmid instability is generally originated from either structural instability caused by changes in plasmid itself, for instance point mutation, deletion, insertion or rearrangement in

the plasmid DNA, or segregational instability caused by defective partitioning of plasmids between the daughter cells during cell division (Silva *et al.*, 2012). Structural plasmid instability could arise from the increased expression of mobile and recombinogenic DNA such as transposons, IS elements and phage-related genes (Haddadin and Harcum 2005). Plasmid structural instability can be seen as a result of the mutations and other genetic divergences caused by these mobile DNA sequences in plasmid carrying cells (Haddadin and Harcum 2005).

Chapter Five

The study of transfer of plasmid-harboured *bla*$_{\text{NDM-1}}$ gene in Enterobacteriaceae.

5.1 Introduction

DNA plasmids can confer resistance to major classes of antibiotics such as aminoglycosides, tetracyclines, chloramphenicol, sulphonamides, trimethoprim, macrolides and quinolones (Carattoli 2009) and they acquire mobile genetic elements that mobilize the antibiotic resistance genes. Plasmids play a significant role in horizontal transfer of resistance determinants among bacteria (different species, genera and kingdoms) depending on their narrow or broad host range, conjugative properties and efficiency of conjugation (Thomas and Nielsen 2005; Carattoli 2013).

Conjugation is a very important phenomena and an efficient method for intra- or inter-species genetic transfer. The first discovery of the conjugation process was in 1946 by Lederberg and Tatum in *E. coli* K-12 (Lederberg and Tatum 1946). Conjugal plasmid transfer, in both Gram- negative and Gram –positive bacteria, has been well documented (Possoz *et al.*, 2001).

The rapid spread of NDM-1-producing Gram-negative species has been highlighted in many reports published in the last two years (Nordmann *et al*., 2012). In Enterobacteriaceae, *bla*$_{\text{NDM-1}}$ has been shown to be carried by a range of diverse plasmids, typeable and non-typeable (Nordmann *et al*., 2011b; Poirel *et al*., 2011).

The aim of the study was to determine whether different plasmids harbouring the *bla*$_{\text{NDM-1}}$ gene could be transferred by conjugation to *E. coli* J53 and also to assess the stability of plasmids carrying *bla*$_{\text{NDM-1.}}$

5.2 Results

5.2.1 Plasmid bearing *bla*$_{\text{NDM-1}}$ gene transfer by conjugation

Conjugation experiments were performed, as described in section 2.17, with NDM-1 producers as donors (*K. pneumoniae* K15, K7, IR25, IR18K and IR28K; *K. oxytoca* IR61 and *E. coli* IR5, IR22, IR26 and IR29) and azide- resistant *E. coli* J53 as a recipient using a selection based on ceftazidime (30mg/ml) and azide (100 mg/ml). Except for two strains (*K. pneumoniae* IR18K and IR28K), most of the isolates gave transconjugants. Some donors have more than one plasmid carrying *bla*$_{\text{NDM-1}}$ but only one plasmid has been transferred to the recipient strain by conjugation suggesting that several *bla*$_{\text{NDM-1}}$ plasmids were not self–conjugative and/or required a helper plasmid for their mobilization. Where conjugation failed in some donors it is likely that either 1) the plasmids carrying NDM-1 are located on large size plasmids, including (toxic) DNA, which can lead the cell machinery to degrade it or; 2) the plasmid may be broken during conjugation and may not be able to re-circularize, which once again leads to DNA degradation. Despite the fact the conjugation experiment failed under laboratory conditions, since the NDM-1 gene is located on a plasmid, it can, theoretically, promote its dissemination. In its natural environment, exchange of DNA between bacteria can occur when appropriate conditions in the surroundings are achieved. Under laboratory limitations, those conditions are not always easy to emulate.

5.2.2 Molecular characterization of transconjugant

All 8 transconjugants; *K. pneumonia* pK15, pK7, and pIR25 transconjugants; *K. oxytoca* pIR61 transconjugant; and *E. coli* pIR5, pIR22, pIR26, and pIR29 transconjugants were checked for the presence of *bla*$_{\text{NDM-1}}$ gene by using the PCR method described in section 2.11.2. All were positive for the *bla*$_{\text{NDM-1}}$ gene. The transconjugant product size of the *bla*$_{\text{NDM-1}}$ gene was 800bp.

5.2.3 Stability of the plasmid- borne NDM-1 gene in transconjugants

The plasmid stability determines the proportion of cells maintaining the plasmid. I studied the stability of plasmids carrying *bla*$_{\text{NDM-1}}$ at 37ºC without selective antibiotic pressure. The stability of plasmids in *K. pneumonia* pK15, pK7, and pIR25 transconjugants, *K. oxytoca* pIR61 transconjugant and *E. coli* pIR5, pIR26, pIR22 and pIR29 transconjugants was studied by using serial passaging in liquid culture.

Passaging over two weeks in liquid media without antibiotics showed that most of the transconjugants had one NDM-1 plasmid. This observation was repeated at least twice in each case to verify previous results. However, in the *E. coli* IR22 transconjugant the two plasmids were either integrated or one of them was lost.

5.2.4 Investigation of transconjugant genomic DNA and back probing with *bla*$_{\text{NDM-1}}$

To determine the genomic location of the NDM-1 genes, plasmids from *K. pneumoniae* (pK15, pK7, pIR25) transconjugants, *E. coli* (pIR5, pIR22, pIR26, pIR29) transconjugants and *K. oxytoca* pIR61 transconjugant were isolated and digested with *S*1 nuclease, then probed with NDM-1-radio-labelled *bla*$_{\text{NDM-1.}}$ Probe DNA fragments using the protocols outlined in section 2.22.

In *K. pneumoniae* pK15, pIR25, pK7, *K. oxytoca* IR61 and *E. coli* IR5, IR26 transconjugants, *bla*$_{\text{NDM-1}}$ was seen on the same sized plasmid construct as in the corresponding parent. This is evidence of the conventional conjugation process of a resistance plasmid transferring the resistant determinant between cells.

The data clearly show that a plasmid was demonstrated in most transconjugants, while *E. coli* pIR22 transconjugant showed two plasmids. In *K. pneumoniae* pK15, the transconjugant had a 60 kb plasmid carrying *bla*$_{\text{NDM-1}}$ (Figure 5.1). However, *E. coli* pIR5 and pIR26 transconjugants and *K. oxytoca* pIR61 transconjugant possessed 150 kb plasmids carrying *bla*$_{\text{NDM-1}}$ (Figure 5.2; 5.3 and 5.4).

The *bla*$_{NDM-1}$ in *E. coli* IR29 and *K. pneumoniae* K7 transconjugants were found upon the 100 kb plasmid identified in Figures5.5 and 5.6, respectively.

The chromosomal bands in the autoradiograph gels displayed the *bla*$_{NDM-1}$ gene present upon the chromosomes of *K. pneumoniae* IR25 (Figure 5.7) and *E. coli* IR26 (Figure 5.3). This highlights the genetic plasticity of *bla*$_{NDM-1}$ during conjugation.

Interestingly, the *E. coli* pIR22 transconjugant showed that two plasmids were transferred, with very little difference in size (≥ 150 kb), between day one and day seven, but after the eighth day, the NDM-1 plasmid appeared to be lost and / or included in the appearance of a smaller plasmid at 40 kb (Figure 5.8 highlighted in yellow). This small plasmid does not carry *bla*$_{NDM-1}$ (Figure 5.8). Therefore, one copy of *bla*$_{NDM-1}$ was lost in a deletion event creating the new 40 kb plasmid. This 40 kb plasmid also appears at a higher copy number than the original 150 kb plasmid as observed in the intensely stained band in Figure 5.8 A.

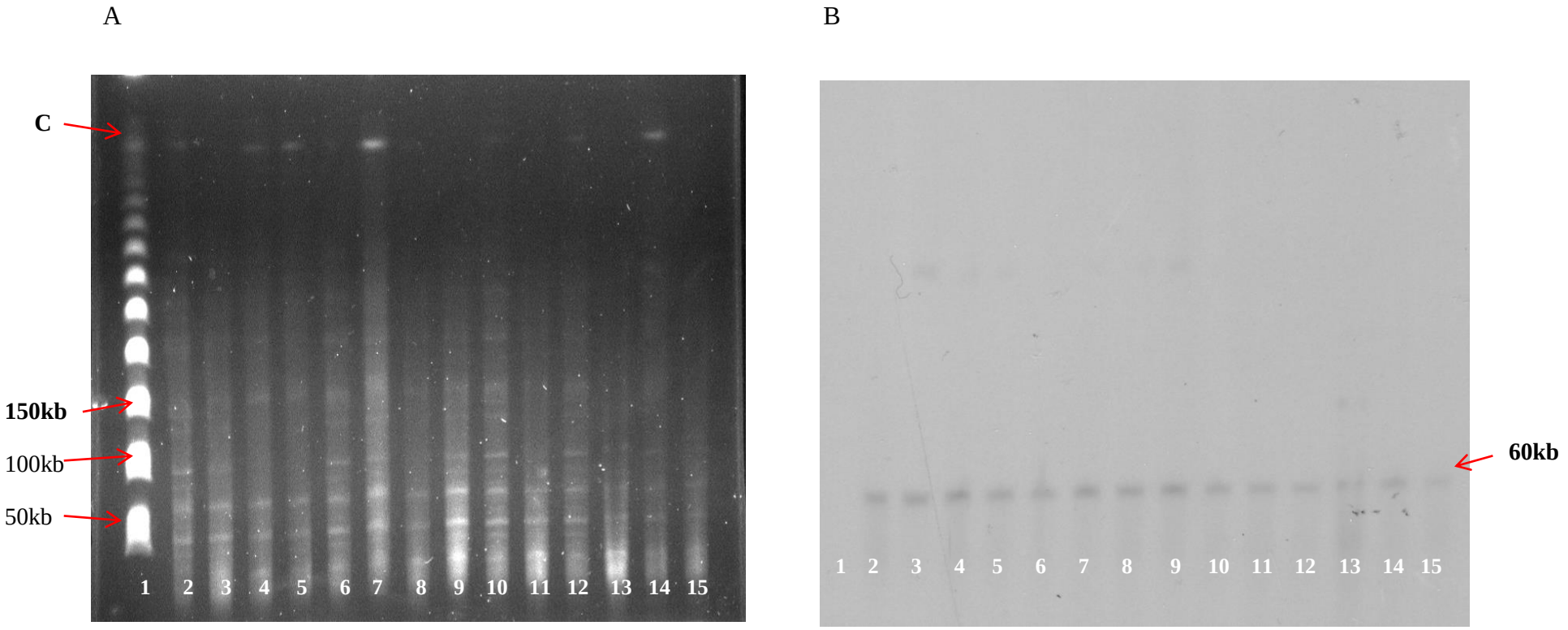


Figure 5.1 Pulsed-field gel of genomic DNA from *K. pneumoniae* transconjugant (pK15) isolate. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1; Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane 9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base.

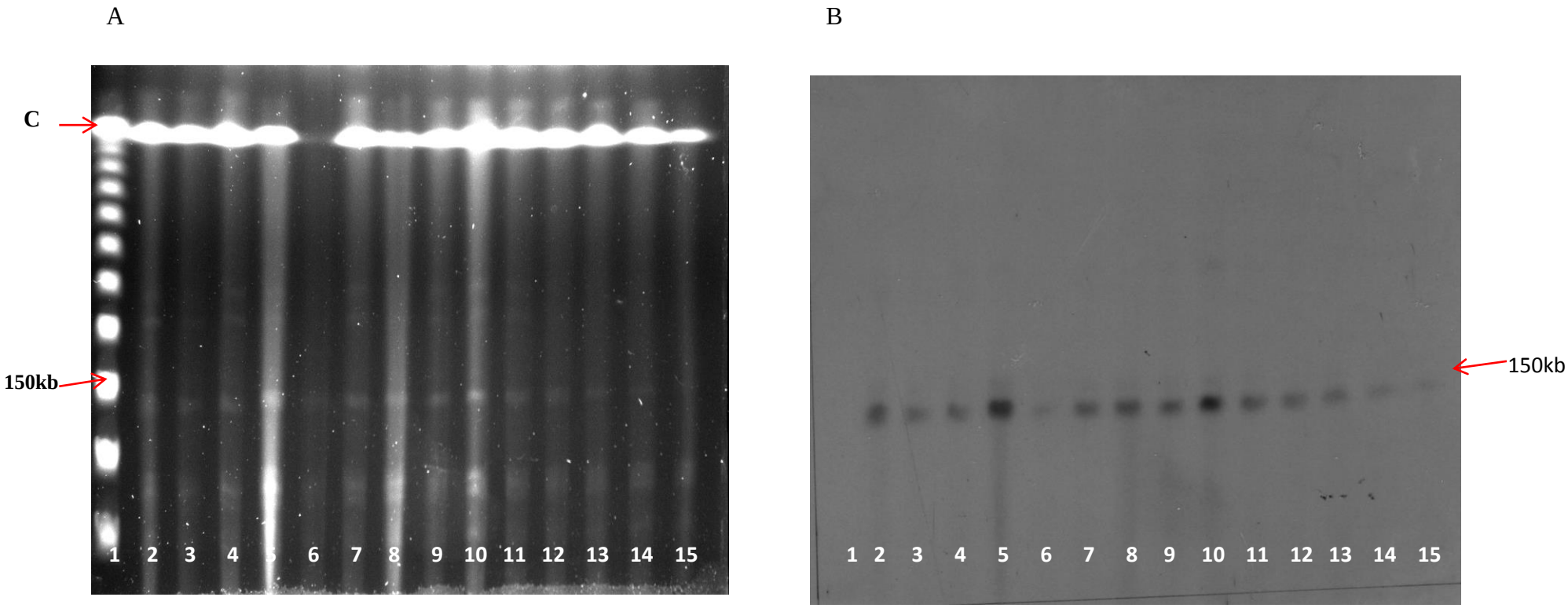


Figure 5.2 Pulsed-field gel of genomic DNA from *E. coli* transconjugant (pIR5) isolate. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2=D1; Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6=D5; Lane 7= D6; Lane 8= D7; Lane9= D8; Lane10= D9; Lane11,=D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base.

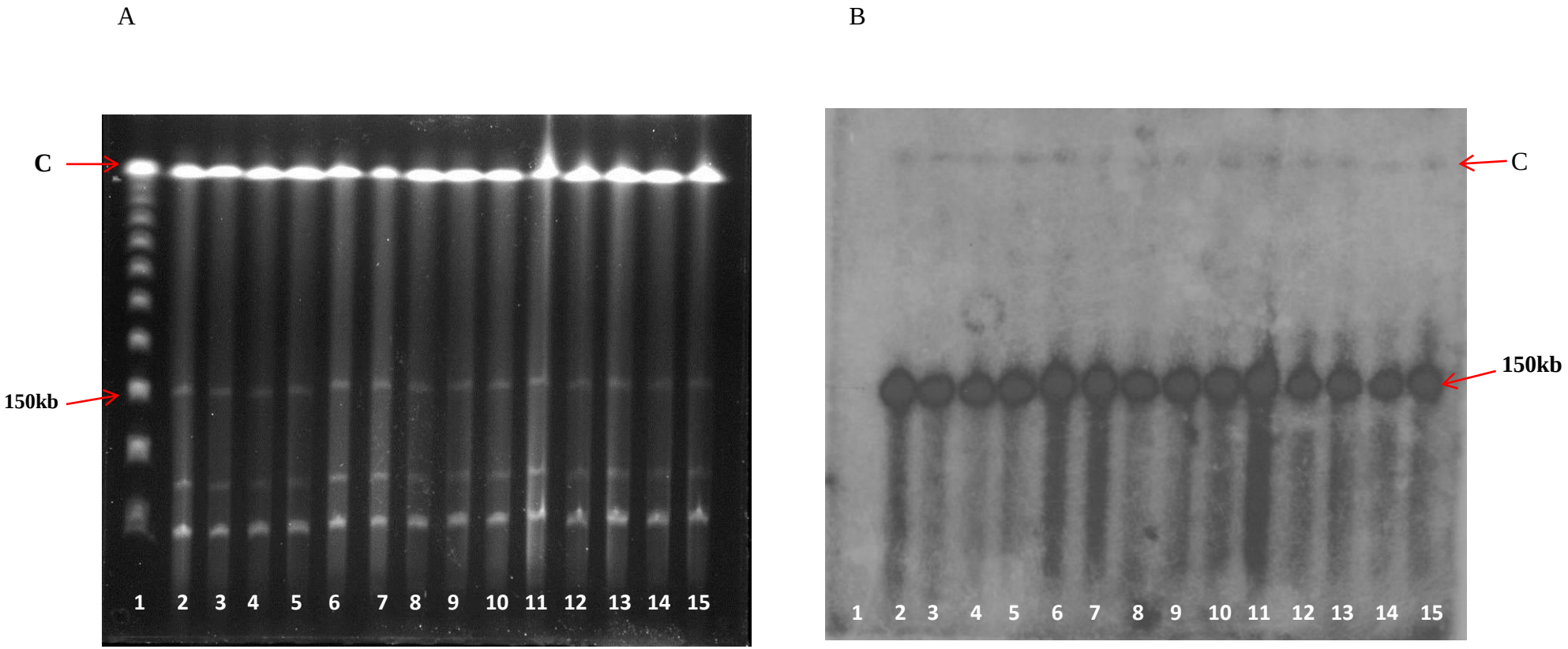


Figure 5.3 Pulsed-field gel of genomic DNA from *E. coli* transconjugant (pIR26) isolate. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1; Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base.

A B

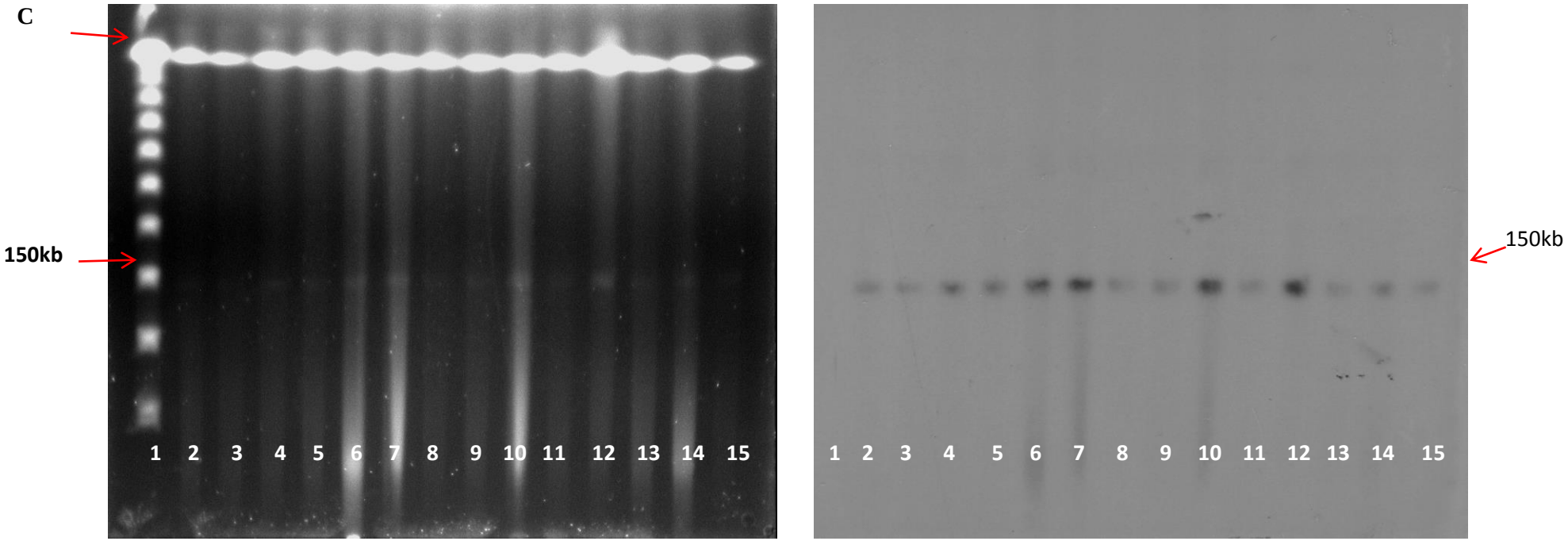


Figure 5.4 Pulsed-field gel of genomic DNA from *K. oxytoca* transconjugant (pIR61) isolate.(A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ probe. Lane 1, lambda ladder pulsed-field gel marker; Lane 2,D1; lane 3, D2; lane 4, D3; lane 5, D4; lane 6,D5; lane 7, D6; lane 8, D7; lane9, D8; lane10, D9; lane11, D10; lane12, D11; lane13, D12; lane 14, D13 and lane 15, D14.C= chromosome. kb= kilo base.

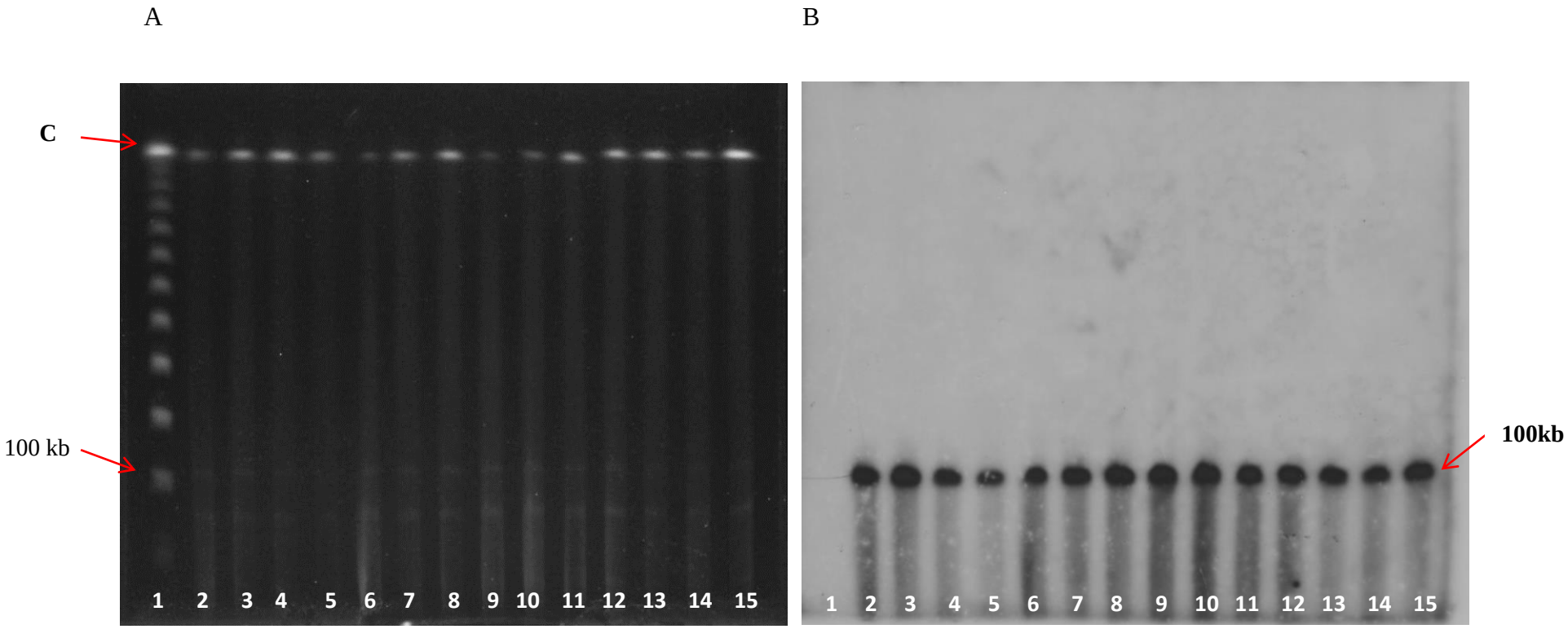


Figure 5.5 Pulsed-field gel of genomic DNA from *E. coli* transconjugant (p IR29) isolate. (A) Plasmid size differentiation by digestion with S1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1; Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base.

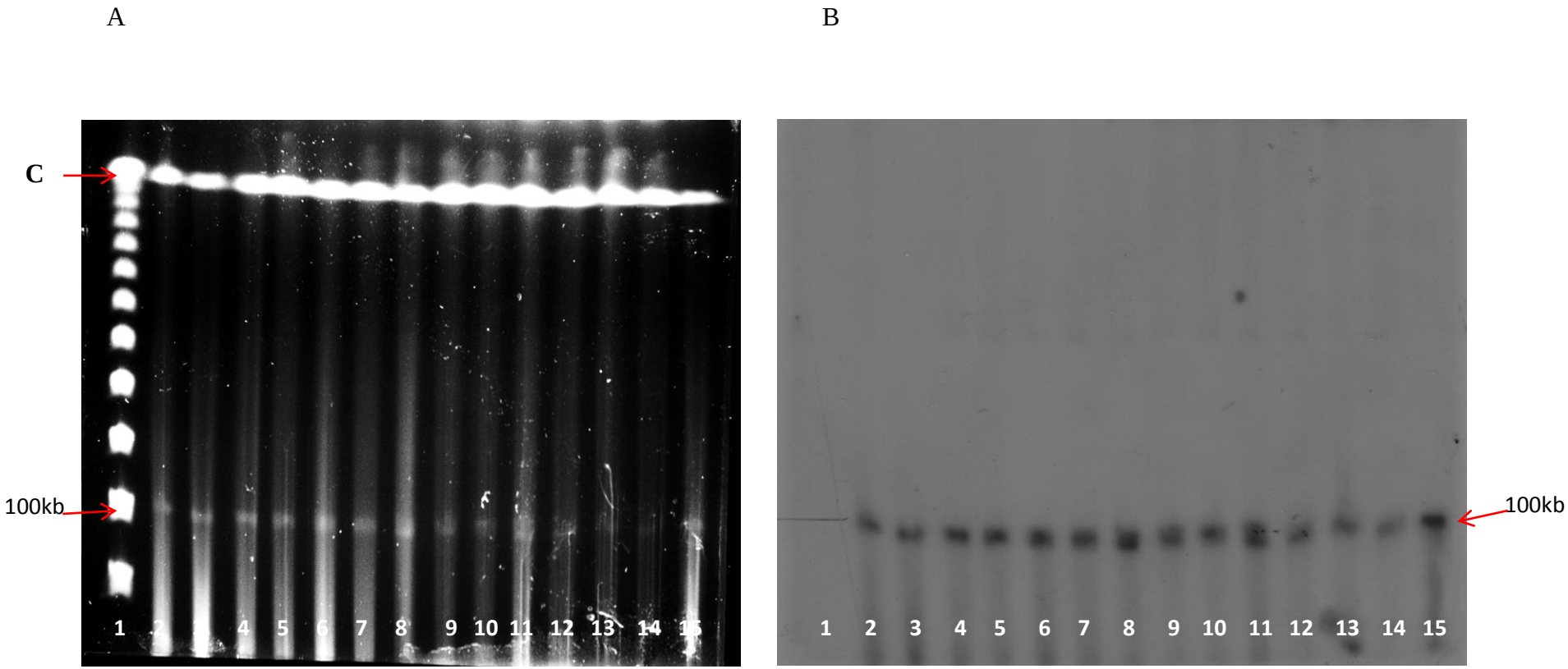


Figure 5.6 Pulsed-field gel of genomic DNA from *K. pneumoniae* transconjugant (pK7) isolate.(A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1; Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane9= D8; Lane10= D9; Lane11= D10; Lane12 D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base.

A

B

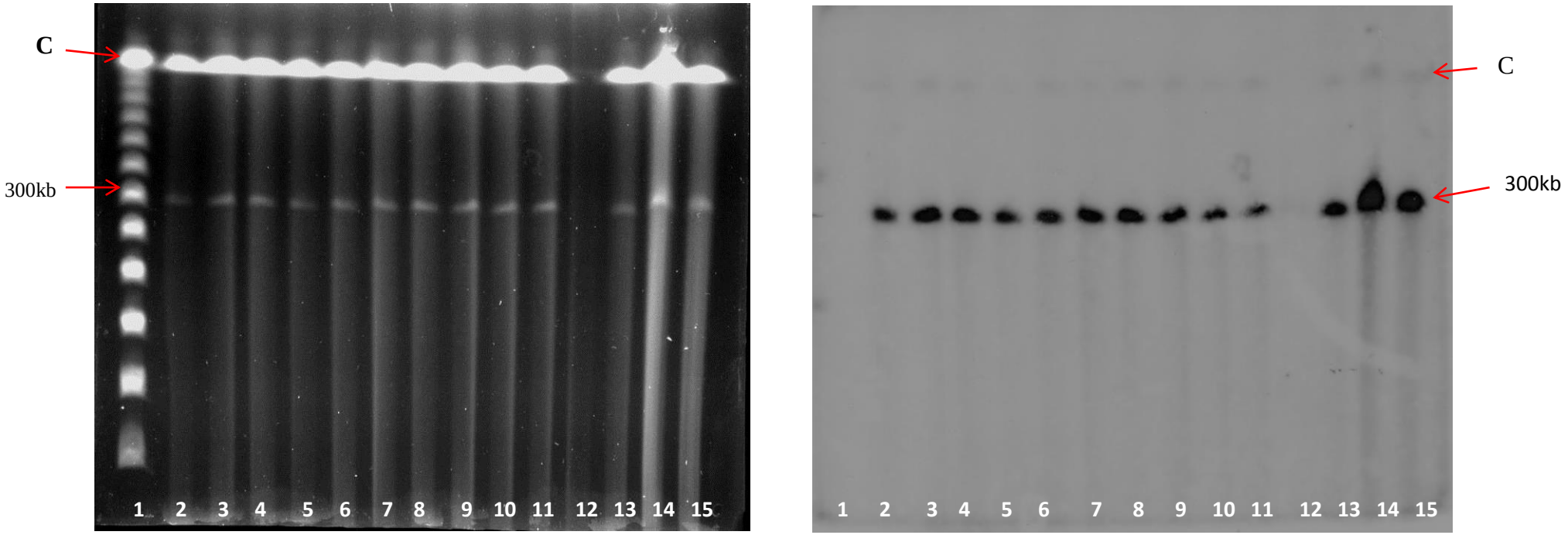


Figure 5.7 Pulsed-field gel of genomic DNA from *K. pneumoniae* transconjugant (pIR25) isolate. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2= D1; Lane 3= D2; Lane 4= D3; Lane 5, D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane 9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base.

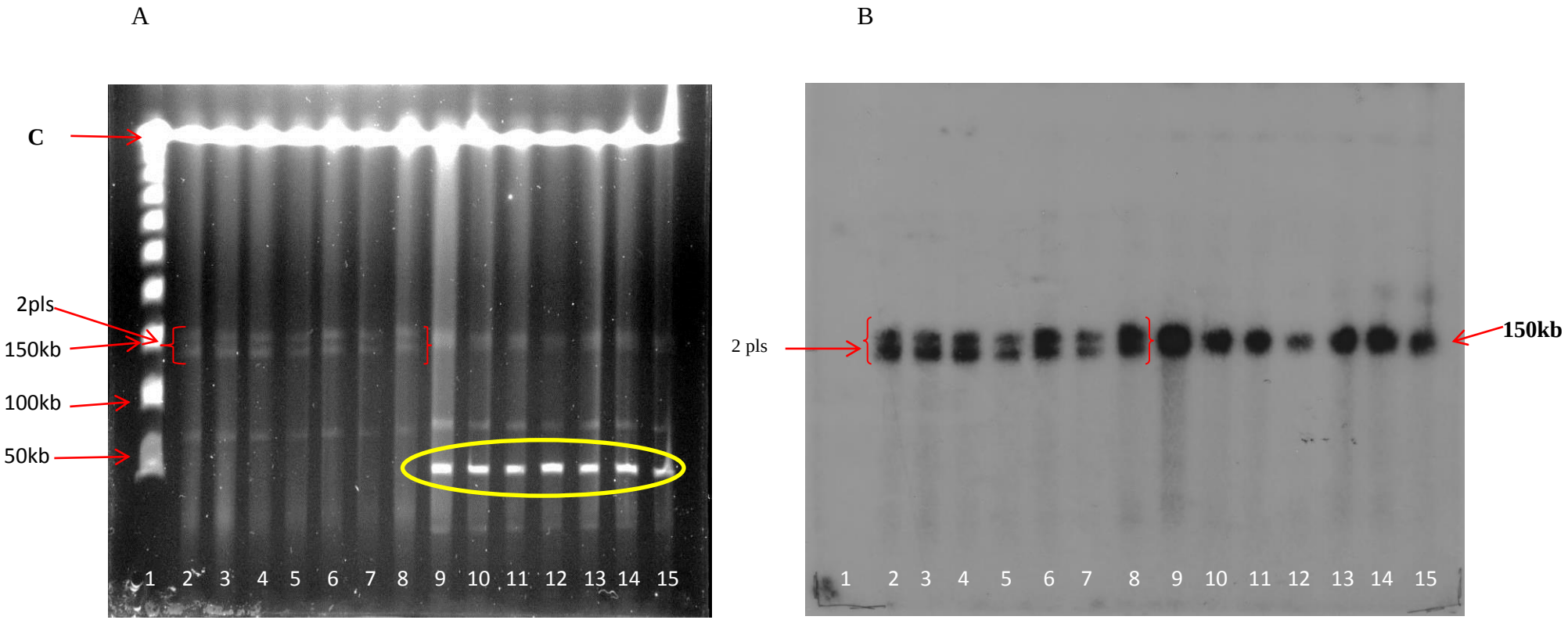


Figure 5.8 Pulsed-field gel of genomic DNA from *E. coli* transconjugant (pIR22) isolate. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *bla*$_{NDM-1}$ probe. Lane 1= lambda ladder pulsed-field gel marker; Lane 2=D1; Lane 3= D2; Lane 4= D3; Lane 5= D4; Lane 6= D5; Lane 7= D6; Lane 8= D7; Lane 9= D8; Lane10= D9; Lane11= D10; Lane12= D11; Lane13= D12; Lane 14= D13 and Lane 15= D14. C= chromosome. Kb= kilo base.

5.3 Discussion

The ease of dissemination of the plasmid-borne*bla*$_{NDM-1}$ gene was apparent by the ability to obtain transconjugants for most clinical donor isolates. Similar findings on*bla*$_{NDM-1}$ plasmid transmissibility has been noted (Poirel *et al*., 2011; Walsh *et al*, 2011). Transconjugants were obtained from eight clinical isolates, indicating that the *bla*$_{NDM-1}$ gene was located on a conjugative plasmid.

In some isolates, the transmission of plasmids harbouring NDM-1 gene failed and could be explained by unsuccessful conjugation in these isolates (*K. pneumoniae* IR18K and IR28K). Either the antibiotic used in the selection was not appropriate to these plasmids, or the NDM-1 gene was located on a non-transferable plasmid, and/or the NDM-1 gene was located on a very large size plasmid. It could also be that these plasmids need a helper/facilitator plasmid to transfer the plasmid carrying *bla*$_{NDM-1}$from donor cells to the recipient. In addition, it might be noted that temperature could lead to failure of plasmid transfer (Stoughton *et al*., 2013). Temperature sensitive conjugative plasmids have been described previously (Sherburne *et al*., 2000). In this study, the NDM-1 plasmid transferred to *E. coli* J53 was of a high frequency at 37ºC. These results are inconsistent with the Walsh *et al*. study, where the transfer of the NDM-1 plasmid was highest at 30ºC (Walsh TR., *et al*., 2011). Potron *et al*., (2011) noted that a temperature of 30°C appeared to enhance conjugation for three of the five studied plasmids as shown with other clinical isolates (Walsh *et al*., 2011). 30°C corresponds to the natural temperature reached in several places in India (Potron *et al.*, 2011). Aside from temperature, other factors such as; organic solvents, pH shift, starvation and ⁄ or restriction in carbon availability have been reported to impact on the ability of donor and recipient cells to receive plasmids in inter and intra species transfers (Mc Mahon *et al*., 2007). Mc Mahon *et al*. reported that low pH, high salt and low temperatures increase the rates of horizontal transmission of selected plasmids between *E. coli* strains, and between *E. coli* and *S. typhimurium* DT104 strains (Mc Mahon *et al*., 2007).

Muela *et al*. (1994) described the finding that environmental stress, such as starvation, affects the ability of donor cells to transfer plasmids, but does not affect the ability of recipient

cells to accept plasmids. However, it is suggested that environmental stresses such as heat, organic solvents, pH changes or detergents, increase the fertility of recipient cells while having little effect on the donor cells (Schafer *et al*., 1994).

Most of the transconjugants proved to be stable, meaning that these isolates showed no plasmid loss over the time of our experiment. However, other studies have revealed that the stability of some plasmids can be changed by different selection pressures such as antibiotics and temperature (Wong *et al*., 2010).

Previous studies revealed that the genes encoding NDM-1 were mostly located on plasmids, usually adjacent to insertion elements, which may facilitate the intra- or interspecific transmission of $bla_{\text{NDM-1}}$ (Kumarasamy *et al.,* 2010; Poirel *et al*., 2010). In this present study, I have confirmed that the $bla_{\text{NDM-1}}$ genes of each transconjugant were located on various plasmids that ranged from >50 to 300 kb in size. Most of the transconjugants carried similarly sized (150 kb) plasmids, corresponding to the size reported for the $bla_{\text{NDM-1}}$ plasmid by other studies (Kumarasamy *et al*., 2010). Several studies revealed that the NDM-1 gene is widely disseminated in environmental (in water for drinking and food preparation and seepage samples) as well as clinical isolates in New Delhi (Walsh *et al*., 2011).

Plasmid stability was assessed by serial passage of NDM-1–positive isolates on antibiotic-free media and showed that the $bla_{\text{NDM-1}}$ gene was stable in most transconjugants. There are several factors that could have a role in the stability of the plasmid, for example, plasmid load, plasmid copy number, replication systems, medium composition, host background, culture conditions and antibiotic concentration (Silva *et al*., 2012). Also, there are other factors affecting plasmid structural stability such as plasmid size (Ertl and Thomsen, 2003), direct repeats (Hadj Kacem *et al*., 2008), inverted repeats (Bi and Liu, 1996) and insertion sequences (Valesova *et al*., 2005).

Plasmid instability is commonly caused by changes in the plasmid itself; for instance, point mutation, deletion, insertion, or segregational instability caused by defective partitioning of plasmids between the daughter cells during cell division (Silva *et al*., 2012).

Plasmid structural instability could also arise due to the increased expression of mobile and recombinogenic DNA such as; transposons, IS elements and phage-related genes (Haddadin and Harcum, 2005). Plasmid structural instability can be seen as a result of the mutations and other genetic divergences caused by these mobile DNA sequences carried on either the chromosome and/ or on other plasmids (Haddadin and Harcum, 2005).

Overall, the NDM-1 plasmids were conjugative transferring their resistance to the *E. coli* J53. Furthermore, in most transconjugants the $bla_{\text{NDM-1}}$ gene remained located upon the same plasmid as its parent's and was stable.

Chapter Six

Characterization of heavy metals genes in Enterobacteriaceae carrying MBLs plasmids

6.1 Introduction

Antibiotic sensitive and resistant bacteria are both ubiquitously present in the environment. The population of bacteria in hospital environments and nature are not only selected in the presence of antibiotics, but also in the presence of other antimicrobial agents; e. g. resistance to heavy metals (Chattopadhyay *et al*., 2011). Many metals, like zinc, chromium, cobalt, nickel, manganese, and iron, are necessary for living organisms and are known trace elements (Bruins 2000) because of their necessary requirement at small concentrations cadmium, mercury, lead etc. (Laila *et al*., 2011). These metals play a vital role in enzyme activities and act as cofactors (Amalesh *et al*., 2012). However, these metals can become toxic at high concentration levels (Amalesh e*t al.*, 2012), due to binding to enzymes and DNA (Lopez-Maury *et al*., 2002), and thus have detrimental effects on bacteria. Such effects might be; increasing lag-phase (Morozzi *et al.*, 1982); damaging the DNA structure (Bruins *et al*., 2000); inhibiting the enzyme activity (Nweke *et al*., 2007) and reducing the diversity of the microbial population (Anne *et al*., 1999).

During the last decade, several researches reported that antibiotic resistant bacteria may arise through co- or cross-resistance to metals (McArthur and Tuckfield 2000; Berg *et al*., 2005; Akinbowale *et al*., 2007). Cross- resistance can occur when different antimicrobial agents (antibiotics and heavy metals) attack the same target, initiate a common pathway to cell death or share a common route of access to their respective targets e.g. efflux and/or outer membrane porins. Also, co-resistance mechanisms can occur when the genes specifying resistant phenotypes are located together on the same genetic element, such as a plasmid, transposon or integron (Chapman 2003). Several studies have demonstrated that metal- and antibiotic resistance genes are linked, particularly on plasmids. The evidence for co-resistance as a mechanism of antibiotic–metal co-selection originated from studies that used transformation, plasmid curing and plasmid sequencing approaches (Chapman 1998). These studies in environmental isolates showed metal concentrations correlate with increased phenotypic or genotypic antibiotic resistance. However, some studies showed that increasing concentrations of heavy metals lead to a decrease of antibiotic resistance (Holzel *et al*., 2012).

Resistance to an antimicrobial agent (antibiotic and heavy metal) can occur either by 'intrinsic' or 'acquired' mechanisms. Acquired resistance can arise by either mutation or acquiring various types of genetic material. Intrinsic resistance is a phenotype shown in bacteria before the use of an antimicrobial agent, i.e. a natural resistance property of bacterium (McDonnell *et al*., 1999). Resistance to antibiotics and heavy metals can be conferred by chromosomal or mobile genetic elements (e.g. plasmids and transposons) (Silver 1996; Krulwich *et al*. 2005).

A 1997 study showed that heavy metals tend to target multiple sites on or within microbial cells and have broad-spectrum activity, whereas antibiotics tend to target specific sites on or within a microbial cell and have a narrower spectrum of activity (Davies 1997). Bacteria having to face and to survive heavy metal pressure, have evolved several mechanisms including: 1. enzymatic detoxification; 2. converting a more toxic substance to a less toxic one, or less available metal ion species; efflux 'pumping' out from the cells of toxic ions; and 3. bioaccumulation (binding of toxic metal ions to bacterial proteins or polypeptides (Ji and Silver 1995). The most important mechanism of such systems is efflux pumps. Resistance mechanisms to antibiotics include; reduction of membrane permeability to antibiotics; drug inactivation; rapid efflux of the antibiotic; and mutation of cellular target(s) (Krulwich *et al*., 2005).

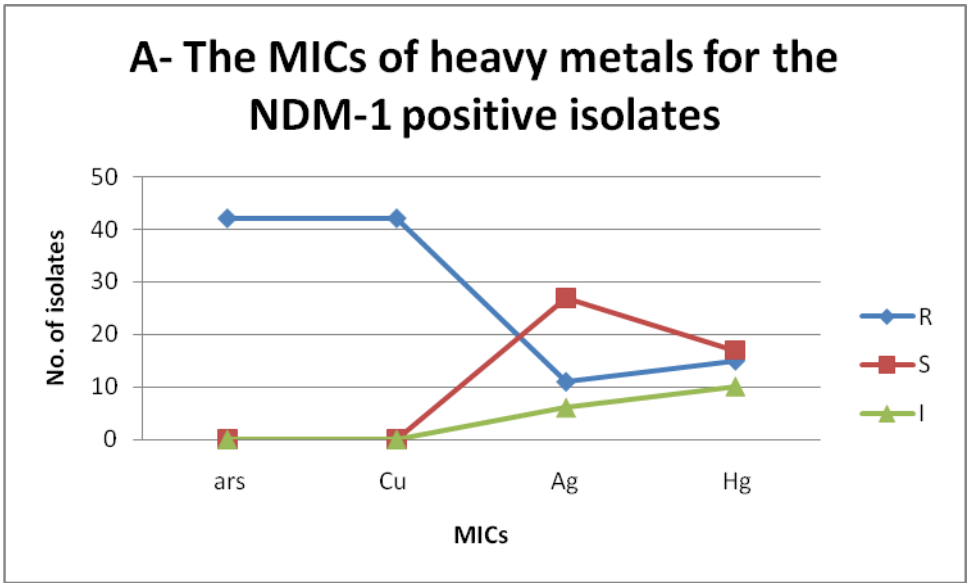
This study was performed to determine heavy metal- resistant bacteria and to detect the genetic location of heavy metal genes in clinical Enterobacteriaceae isolated in the UK and in India.

6.2 Results

6.2.1 Determination of minimum inhibitory concentration (MIC)

The isolates showed different levels of MICs to the heavy metals; MICs of Cu^{2+}, Ag^{2+}, Hg^{2+} and $As^{2+}$for bacterial isolates are shown in Figure 6.1. All isolates of Enterobacteriaceae and *E. coli* 25922(control strain) showed high MICs to arsenic and copper, displaying growth up to >5000µg/ml and 2500µg/ml, respectively. The MICs of mercury and silver fluctuated between <1- 63 µg/ml. ESBL positive *E. coli* isolates (group 2) results showed that the MICs were sensitive to mercury and silver compared with tested isolates in group 1 and group 3. Out of 102 isolates, 100 (98%) showed high MICs to arsenic, and 102 (100%) showed high MICs to copper. The pattern of MICs was in the order: $Cu^{2+} > As^{2+} > Hg^{2+} > Ag^{2+}$.

The MICs were also studied for all transconjugants. A total of 19 transconjugants had high MICs to arsenic and copper. All transconjugants which had high MICs to arsenic and copper exhibited MICs of 5000µg/ml and 1250µg/ml, respectively. For the other metals, Ag and Hg, the MIC values were more variable. With the exception of TCIR25, all transconjugants had low MICs to mercury. 16 out of 19 transconjugants had low MICs to silver. *E. coli* 25922 gave a different MIC value.



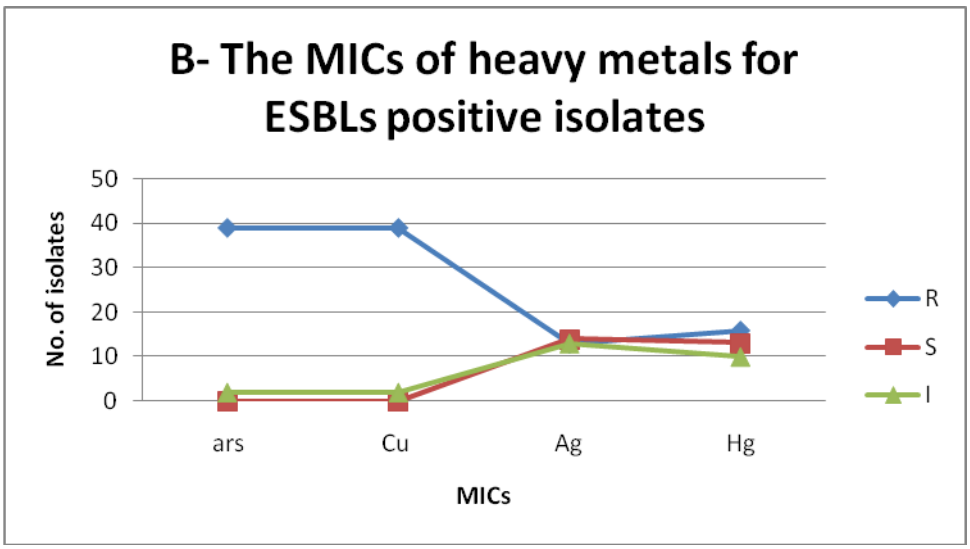


Figure 6.1- The MICs of heavy metals. A- for NDM-1 producing Enterobacteriaceae; B- for ESBL producing Enterobacteriaceae: Ars= 313- >5000 µg/ml; R: Cu=625- 2500 µg/ml; R:Ag= 32- 500µg/ml; R: Hg= 32-63µg/ml; S: ars-0 µg/ml, S: Cu=0µg/ml; S: Ag= 1-8 µg/ml; S: Hg= 2-8 µg/ml; I: Ars=0µg/ml; I: Cu=0 µg/ml; I: Ag= 16 µg/ml; I: Hg= 16µg/ml. R:Resistant; S:Sensitive; I:Intermediate.

6.2.2 Molecular investigation of copper, mercury, silver and arsenic genes

Presence of metal resistance-encoding genes *pcoA, silC, arsA* and *merA* was determined by PCR using specific primers. Positive amplification bands were obtained for *pco*A, *sil*C, *ars*A and *mer*A genes in most isolates. PCR results of *sil*C and *mer*A gene detection showed that in NDM-1 positive isolates (group1), 29 out of 39 have both genes; furthermore, 4 out of 20 of sensitive isolates from Cardiff (group 3) gave PCR positive results for both genes (*sil*C and *mer*A genes). All metals were detected among ESBL- positive clinical isolates (group 2) dating back to 1997 and isolated from India, but less frequently in NDM-1 positive clinical isolates (group 1). The *ars*A gene was less prevalent in ESBL positive strains (group 2); however, *mer*A gene had a low incidence in sensitive strains (group 3). Many strains gave negative results for the prevalent of heavy metal genes, however, were resistant to some ions (such as, ars and Cu) (according to MICs results). Our findings showed that the heavy metal genes were widely disseminated in the NDM-1–producing Enterobacteriaceae (group 1).

To determine and confirm the homology of our amplicons to published sequences *ars*A, *mer*A, *pco*A, and *sil*C genes, gene sequences were selected for the alignment of nucleotide and translated amino acid sequences (Table-6.1).

The *ars*A gene in *E. coli* N14, *K. pneumoniae* IR18K, and *E. coli* IR5 displayed 100% nucleotide identity with the *ars*A gene from *K. pneumoniae* plasmid pKN-LS6 (Accession No.JX442974), *Acidiphilium multivorum p*KW301 (Accession No. AB004659), and *K. pneumoniae* strain ST258 pKPN-IT (Accession No.JN233704), respectively (Table 6.1; see Appendix B.9, B. 11, B.13).

The *mer*A gene sequence from *E. coli* N14 and *K. pneumoniae* E5/14 revealed 100% nucleotide identity with the *mer*A gene from reference strains from the database (Table 6.1) (see Appendix B.15).

The *pco*A genes from *E. cloacae* N4, *Citrobacter freundii* N13, *K .pneumoniae* K15, *K. oxytoca* IR61, and *E. coli* IR22, are closely related (99.7%, 100%, 100%, 100%, 100%) to the *pco*A gene in *E. cloacae* NCTC9394 (Accession No.FP929040), *K.*

pneumoniae plasmid pKN-LS6 (Accession No.JX442974), *K. pneumoniae* KPX plasmid pKPX-1 (Accession No AP012055), *K. pneumoniae* subsp. pneumoniae MGH 78578 (Accession No AP006726), and *K. pneumoniae* subsp. pneumoniae MGH 78578 (Accession No CP000648), respectively. The sequence of the *pco*A gene in *E. cloacae* N4 showed difference in two nucleotide compared with *E. cloacae* NCTC9394 (Accession No.FP929040); A→G (393), and A→G (555) (see Appendix B 17). There was no difference in protein sequences indicating silent mutations (see Appendix B. 18).

The sequence of *sil*C gene from *K. pneumonia* N9 displayed 99.3% nucleotide identity with *sil*C from *E. coli* 55989 (Accession no CU928145) (see Appendix B19). Similarly, SilC protein showed 99.6% identity with SilC protein from *E. coli* 55989. In addition, there is difference in one substitution; Glu154 Gln (see Appendix B .18). However, the *sil*C genes from *E. coli* N15 and *K. pneumoniae* N21 are closely related (100%, 100%) to the *sil*C gene from *E. cloacae* subsp. cloacae ATCC13047 (Accession no CP001918), and *Cronobacter turicensis* z3032 (Accession No.FN543096), respectively (see Appendix B.21, B.22, B.23 and B. 24).

Generally, the high level of the similarity in the nucleotide and amino acid sequences was shown in most of heavy metal genes (Table 6.1).

Table 6.1 Types of heavy metal genes and similarity to sequences reported in the literature.

H. M gene	Isolate	ClustalW & X results			Most closely related H.M
		Identities (%)		Sequence ID	
		DNA	A. A		
***Ars*A**	*E. coliN14*	100	100	JX442974	*Klebsiella pneumoniae* plasmid pKN-LS6
	K. pneumoniae IR18K	100	100	AB004659	*Acidiphilium multivorum* pkw301
	E. coli IR5	100	100	JN233704	*K. pneumoniae* strain ST258 Pkpn-IT
***Mer*A**	*K. pneumoniae N2*	100	100	CP006056.1	*Salmonella enterica* subsp. enterica serovar Cubana str CFSAN002050
	E. coli N14	100	100	CP006056.1	*Salmonella enterica* subsp. *enterica* serovar Cubana strCFSAN002050
	K. pneumoniae E5/14	100	100	KC405622.1	*K. pneumoniae* Pkpc-lk30
***Pco*A**	*Enterobacter cloacae N4*	99.7	100	FP929040	Enterobacter cloacae NCTC9394
	Citrobacter freundii N13	100	100	JX442974	*Klebsiella pneumoniae* plasmid pKN-LS6
	K. pneumoniae K15	100	100	AP012055	*K. pneumoniae* KPX plasmid Pkpx-1
	K. oxytoca IR61	100	100	AP006726	*K. pneumoniae* NTUH-K2044
	E. coli IR22	100	100	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578
***Sil*C**	*K. pneumoniae N9*	99.3	99.6	CU928145	*E. coli 55989*
	E. coli N15	100	100	CP001918	*Enterobacter cloacae* subsp. cloacae ATCC13047
	K. pneumoniae N21	100	100	FN543096	*Cronobacter turicensis Z3032*

6.2.3 Conjugation experiments

Conjugations were performed. The NDM-1 plasmids were selected in the basis of ceftazidium resistance and then transconjugants were examined to see if they harbour heavy metals genes.

The results showed that transfer of NDM-1 plasmids successful in 5 out of 26 strains. To verify that transferred NDM-1 plasmid encodes *mer*A, *ars*A, *pco*A and *sil*C genes, PCR experiments were performed (see section 2.12.2 and Appendix A-2). The PCR results for transconjugants TCIR25, TCIR36, and TCIR21 were positive for *mer*A gene, TCIR3 was positive for *ars*A gene and TCIR44 was positive for *sil*C gene.

6.2.4 Rate of recurrence of heavy metals among Enterobacteriaceae

Presence of the dissimilar numbers of heavy metals in isolates is shown in Table 6.2**.** NDM-1 positive isolates (group 1) have four heavy metals genes (*ars*A, *pco*A, *mer*A, *sil*C) more than the other groups 2 and 3. In the first group, the percentage of isolates which harboured one metal was 12.8% and three metals 15.3%. Out of 41, 10 (24.3%) ESBL positive isolates (group 2) harboured one metal. Furthermore, the numbers of isolates that carry two or three metals are equal.

In group 3, unlike the other groups, the results did not show any isolate having a heavy metal. Presence of metals in the third group is almost non-existent. Overall, the NDM-1 positive isolates possessed more heavy metals.

Table 6.2 Frequency of double or multi-resistance to heavy metals between Enterobacteriaceae

NO. Of HM	No. of resistant strains in microbial groups		
	G1 N=39	G2 N=41	G3 N=20
1	5(12.8%)	10(24.3%)	0
2	9(23%)	6(14.6%)	4(20%)
3	6(15.3%)	6(14.6%)	1(5%)
4	16(41%)	3(7.3%)	1(5%)

G1=NDM-1 positive strains, The UK& India, G2= ESBL positive strains, India, G3= sensitive strains, Cardiff, UK

6.2.5 Detection of chromosomal and plasmid mediated *ars*A, *mer*A, *pco*A and *sil*C genes in Enterobacteriaceae

S1-PFGE results revealed the presence of differences in the molecular weight of plasmids, ranging from 50 kb to >450 kb, in all isolates carrying heavy metal genes.

In addition, there is diversity between the metals' gene locations between chromosomes and plasmids (Table 6.3). *Ars*A and *mer*A genes were found on plasmids in large numbers of isolates; where plasmids sizes fluctuate from 50 kb and 550 kb.

The group 1 isolates, *K. pneumonia* (N1, N11, N18, N27, IR25 and IR28K) and *Enterobacter* sp N26 were found to carry *ars*A gene on the large plasmids, ranging from 200 kb to 300 kb (see Figure 6.2, Figure 6.3, and Figure 6.4 highlighted in green). PFGE and Southern blot analyses indicated that the *ars*A gene was located on different sizes of plasmid per bacterium, *K. pneumoniae* N21 and *E. coli* N14 have three different size plasmids carrying *ars*A gene and *K. pneumoniae* N17 and N16 have two (Figure 6.2 and Figure 6.3 highlighted in blue).Chromosomal bands in the autoradiograph show *ars*A gene is present upon the chromosome of 19 out of 39 NDM-1 positive isolates; *K. pneumoniae* N7, N8, N11, N21, IR25, IR18K, IR28K, *E. coli* N14, IR29, and *C. freundii* N13 (see Figure 6.2, 6.3, 6.4 highlighted in yellow).

In group 2, ESBL resistant *K. pneumonia*, the PFGE and Southern blot analyses indicated that the *ars*A gene was located on one plasmid per bacterium, such as in *K. pneumoniakp*C5/7, *Kp*A5/7, and *Kp*D5/12 (225 kb, 190 kb, 500 kb) (see figure 6.5 highlighted in white). With regard to the chromosomal locations of *ars*A gene in group 2, the *ars*A was detected in *Kp*D5/12 (Figure 6.5 highlighted in yellow).

In group3, *K. pneumoniae* FF160, FF296, and FF101 were found to carry *ars*A on different sized plasmids (Data not shown).

Table 6.3 Summary of position of heavy metals genes on genomic DNA in tested strains

Gene	Location of heavy metals genes in microbial groups (PFGE and hybridizations results)					
	Group 1 n=43		Group 2 n=41		Group 3 n=20	
	P	C	P	C	P	C
***Ars*A**	22	19	6	2	2	0
***Pco*A**	17	11	12	3	5	0
***Sil*C**	17	23	11	15	4	0
***Mer*A**	28	18	13	5	2	0

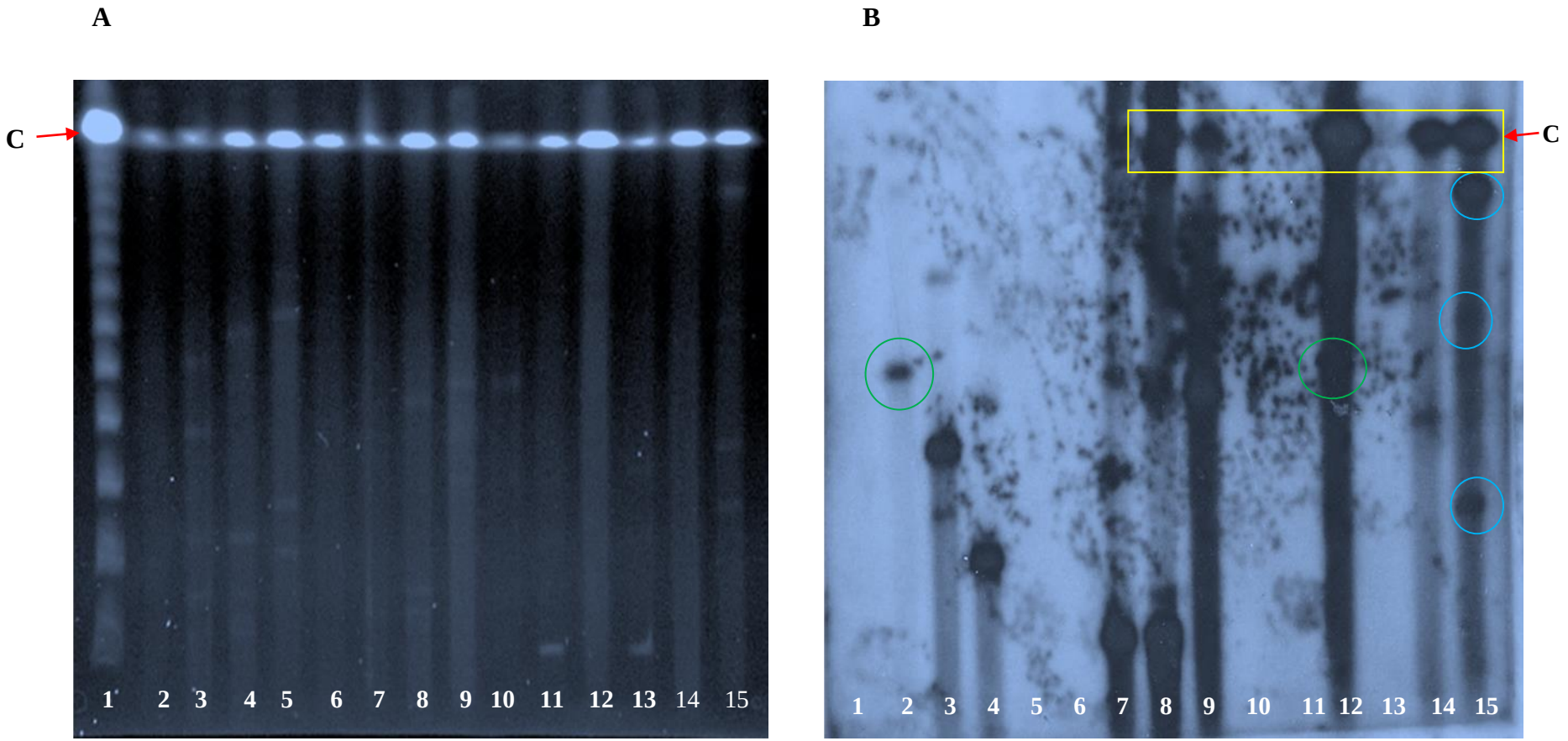


Figure 6.2 Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from the UK. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *ars*A gene probe. Lane 1: Marker. Lane 2= KpN1.Lane 3=KpN2. Lane 4= *C. freundii* N3. Lane 5= *Ent. cloacae* N4. Lane 6= *Enterobacter* sp. N5. Lane 7= *E. coli* N6. Lane 8= Kp N7. Lane 9= KpN8. Lane 10=KpN9. Lane 11= Kp N10. Lane12= KpN11. Lane13=Kp N12. Lane 14= *C. freundii* N13.Lane 15=*E. coli* N14.

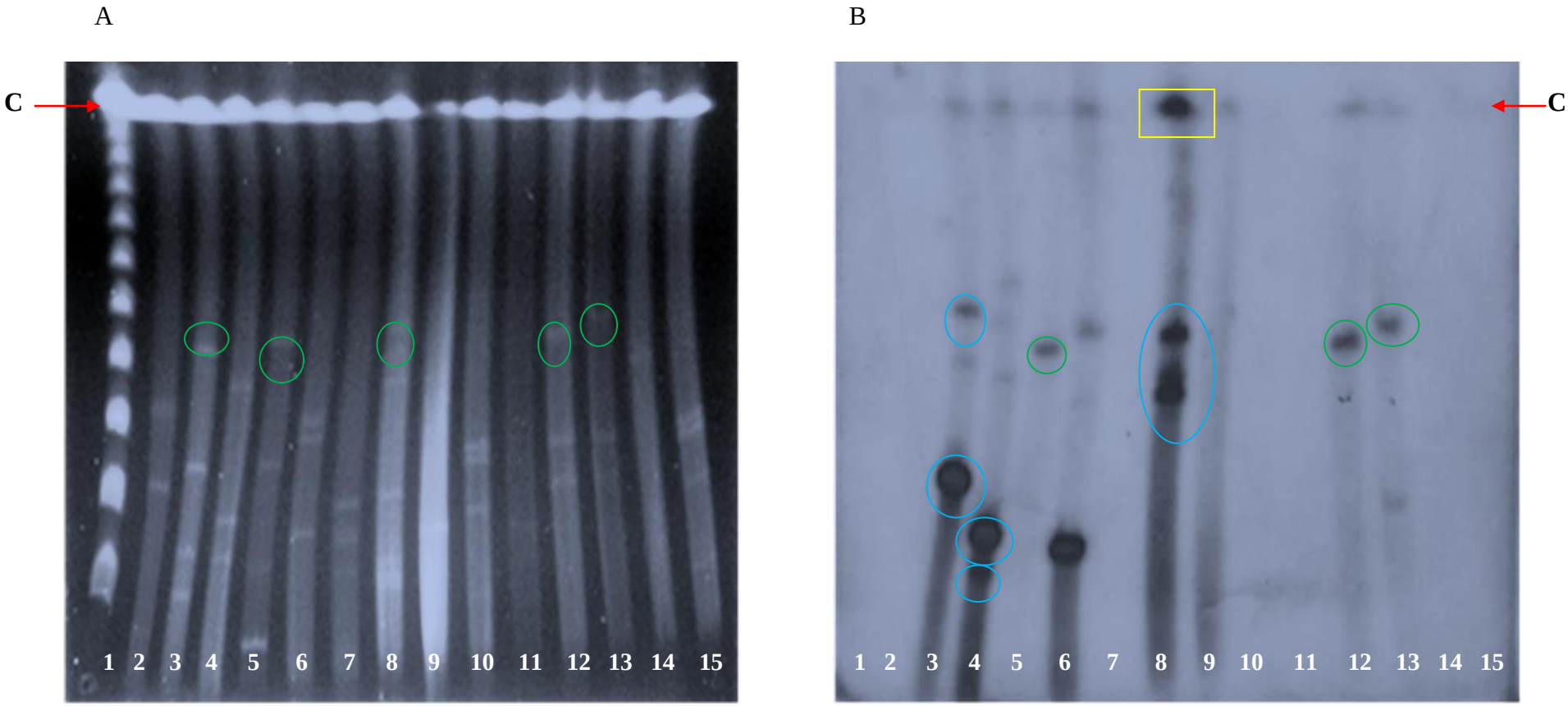


Figure 6.3 Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from the UK.(A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *ars*A gene probe. Lane 1: Marker. Lane 2:*E.coli* N15. Lane 3:*Kp*N16. Lane 4:*Kp*N17. Lane 5:*Kp*N18. Lane 6:*Kp*N19. Lane 7: *E. coli* N20. Lane 8:*Kp*N21. Lane 9:*Kp*N22. Lane 10:*E.coli*N23. Lane 11: *Acinetobacter baumanii* N24. Lane 12: *Enterobacter* sp.N26. Lane 13:*Kp*.N27. Lane 14:*K. oxytoca* N28. Lane 15:*E.coli*N29. *Kp= K. pneumoniae.* C=chromosome. Kb= kilo base

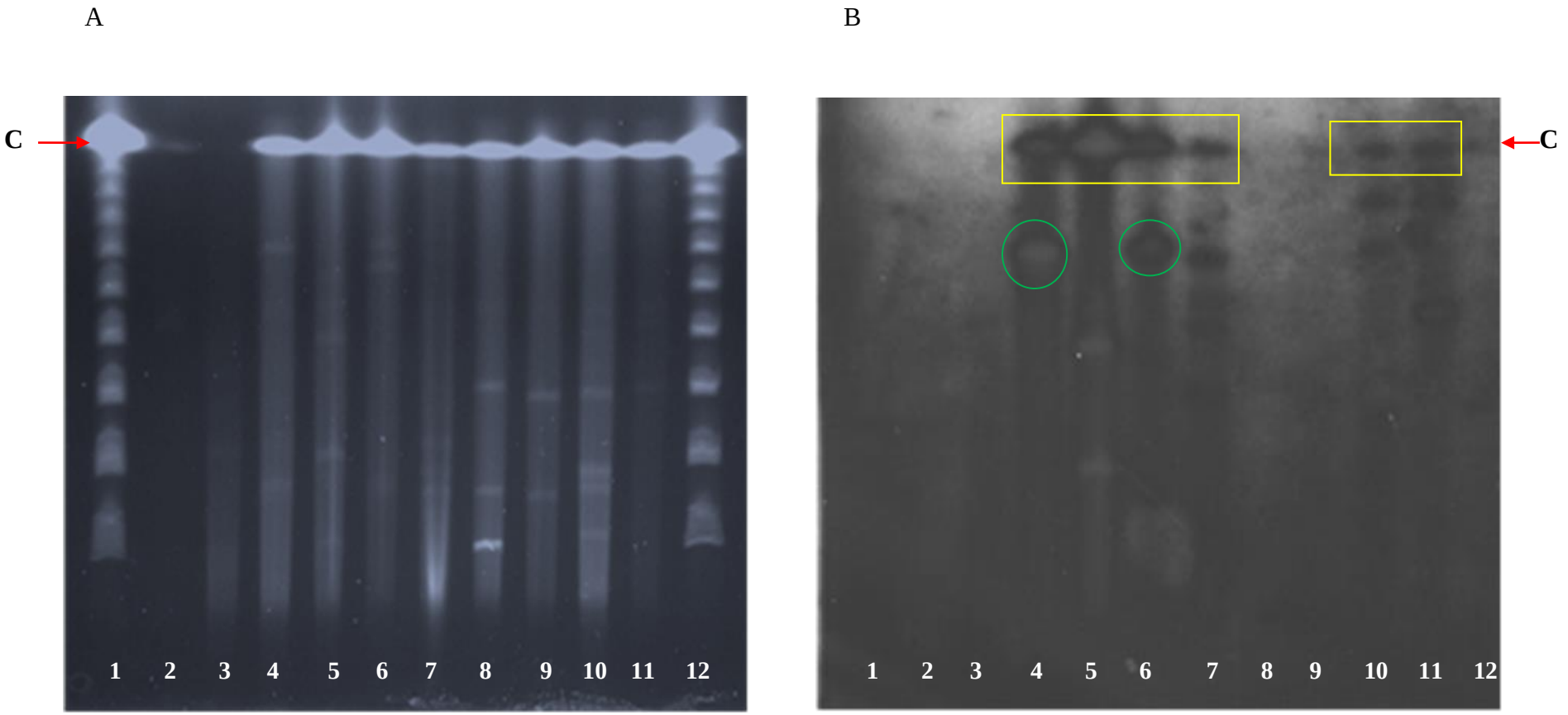
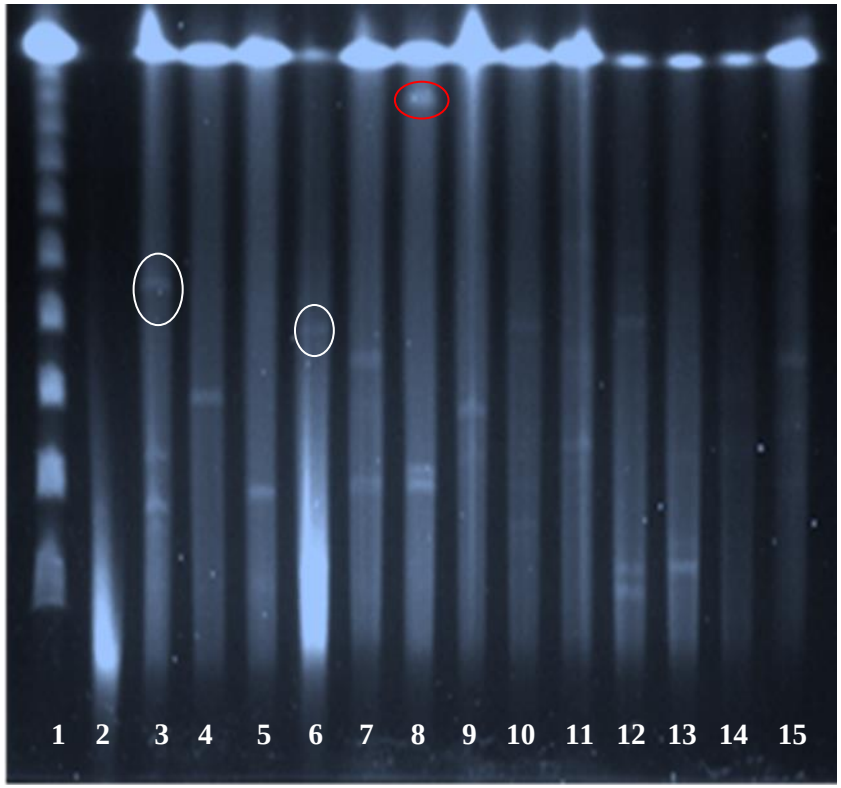
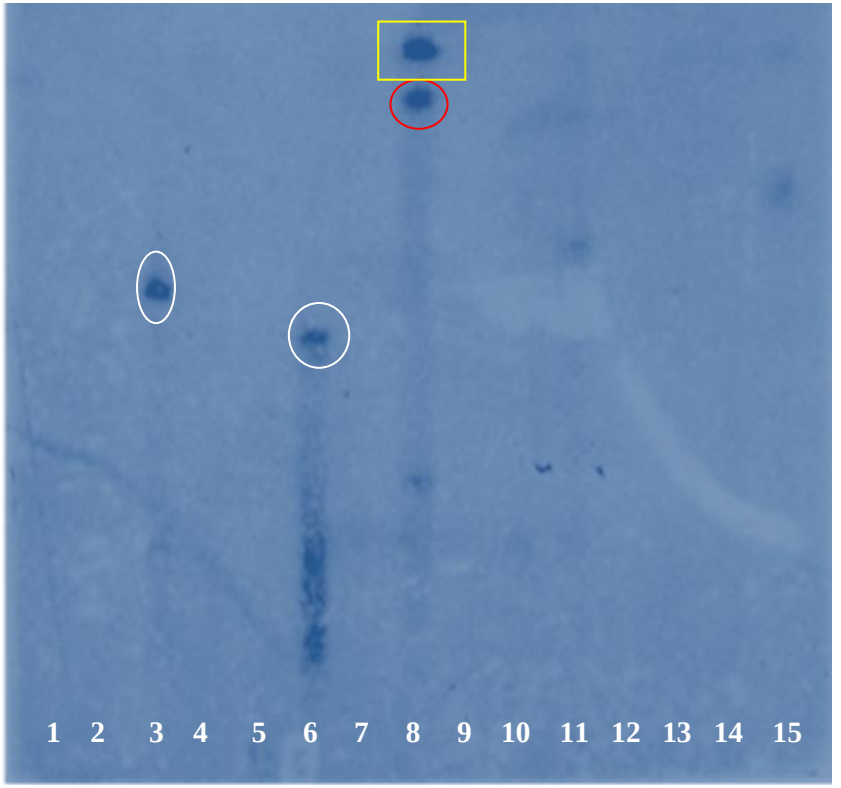


Figure 6.4 Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from India.(A) Plasmid size differentiation by digestion with S1 nuclease. (B) Hybridization of gel A with *ars*A gene probe. Lane 1= Marker. Lane 2= *Kp*K15. Lane 3= *Kp*K7. Lane 4= *Kp*IR25. Lane 5= *Kp*IR18K. Lane 6= *Kp*IR28K. Lane 7= *E. coli* IR29. Lane 8= *E. coli* IR26. Lane 9= *E. coli* IR22. Lane 10= *E. coli* IR5. Lane 11= *K. oxytoca* IR61. Lane 12= Marker. *Kp*= *K. pneumoniae*. C= chromosome. Kb= kilo base

A

B





C

Figure 6.5 Pulsed-field gels of genomic DNA of ESBL- positive strains, isolated from India.(A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *ars*A gene probe. Lane 1= Marker. Lane 2= *Kp*A5/3. Lane 3=*Kp*A5/7. Lane 4= *Kp*A5/4. Lane 5= *Kp*C5/8. Lane 6= *Kp*C5/7. Lane 7= *Kp*C5/5. Lane 8=*Kp*D5/12. Lane 9= *Kp*D5/4. Lane 10= *Kp*E5/14. Lane 11= *Kp* E5/17. Lane 12= *Kp*G5/2. Lane 13=*Kp*G5/6. Lane 14=*Kp*G5/11. Lane 15= *Kp*I5/5. Kp= *K. pneumoniae*. C= chromosome. Kb= kilo.

*S*1 –PFGE showed that the *mer*A gene was found on chromosomes and plasmids, in group 1 and 2; however, in group 3, *mer*A was located on plasmids. The *mer*A gene existed more in genomic DNA in the first group of isolates (63%).The largest plasmid carrying *mer*A gene was found at 450 kb in isolate *K. pneumoniae* N7 and on 300 kb in *K. pneumoniae* N8 and *E. coli* N14 (Figure 6.6 highlighted in red).

In group 1, the *mer*A gene was located on more than one plasmid (Figure 6.6 highlighted in red).*E. coli* N6 was carrying six plasmids with *mer*A, ranging from 50 kb to 250 kb, and a further four isolates (*K. pneumoniae* N2, N7, N8 *E. coli* N14) were carrying three plasmids, ranging from 50 kb to 400 kb, with the *mer*A gene (see figure 6.6 highlighted in red). Moreover, other isolates, *K. pneumoniae* IR18K, *K. oxytoca* IR61, and *E. coli* IR5, IR29 had three plasmids, ranging from 50 kb to 350 kb, with the *mer*A gene (data not shown). The *mer*A gene was identified on the chromosome in *K. pneumoniae* IR25, IR18K, IR28K, N7, N11, *K. oxytoca* IR61, *E. coli* IR29, IR5, N6, N14and *C. freundii* N13 (some data shown in Figure 6.6 highlighted in yellow). Also, the *mer*A gene detected in group 2 was located in 51% of isolates. In this group (G2), the isolates, *E. coli* H4/5, *Salmonella* sp, and *Enterobacter* I2/5, I2/2, F/6 had the *mer*A gene located on one or two plasmids ranging from 100 kb to 300 kb. In addition, *mer*A was seen on the chromosome in *Enterobacter* I2/5 and I2/2 (data not shown). *K. pneumoniae* FF160 and FF11 (group 3) recorded the location of *mer*A only on a plasmid. The group 1 isolates, *K. pneumoniae* N7, N8, N9 were to found to carry *pco*A gene on 200 kb, 175 kb, and 210 kb respectively (Figure 6.7 highlighted in green). Isolate *E. coli* N6 had three plasmids carrying *pco*A gene and isolates; *E. cloacae* N4 and *E. coli* N14 had two plasmids harbouring the *pco*A gene (Figure 6.7 highlighted in white). Chromosomal bands in the probing gel showed the *pco*A gene to be present upon the chromosome of 12 isolates from group one (Figure 6.7 and Figure 6.8 highlighted in yellow). Results obtained from *S*1- PFGE showed that the *sil*C gene was located on the chromosome of most of the isolates in groups one and two (see Figure C.1and C.2, C.3, C.4 in Appendix C, highlighted in yellow). Furthermore, most isolates from the all three groups were found to carry *sil*C gene on one plasmid, ranging from 50 kb to 300 kb (see Figure C.1 and C.2, in Appendix C, highlighted in red). Isolate, *K. pneumoniae* G5/2 was carrying the *sil*C gene on three plasmids (Figure C.3, Appendix C

highlighted in white). In group 3, 5 isolates of *K. pneumoniae* were carrying *sil*C gene, 2/5 isolates on the chromosome (data not shown).

A B

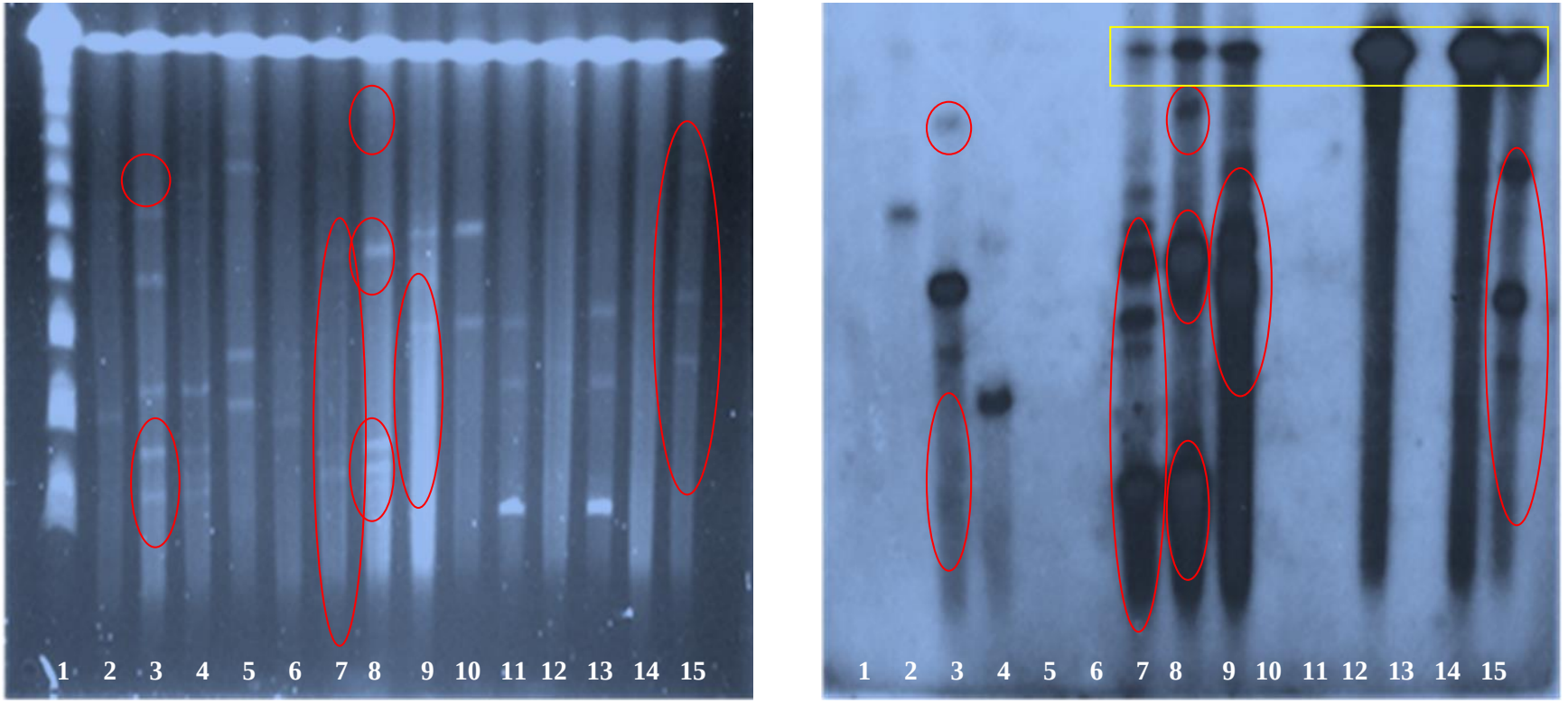


Figure 6.6 Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from the UK.(A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *mer*A gene probe. Lane 1= Marker. Lane 2= *Kp*N1. Lane 3=*Kp*N2. Lane 4= *C. freundii* N3. Lane5= *E. cloacae* N4. Lane6= *Enterobacter* sp. N5. Lane7= *E. coli* N6. Lane8= *Kp*N7. Lane 9=*Kp* N8. Lane10: *Kp*N9. Lane11= *Kp* N10. Lane12= *Kp*N11. Lane13= *Kp*N12. Lane 14= *C. freundii* N13. Lane 15=*E. coli* N14. Kp= *K. pneumoniae*. C= chromosome. Kb= kilo.

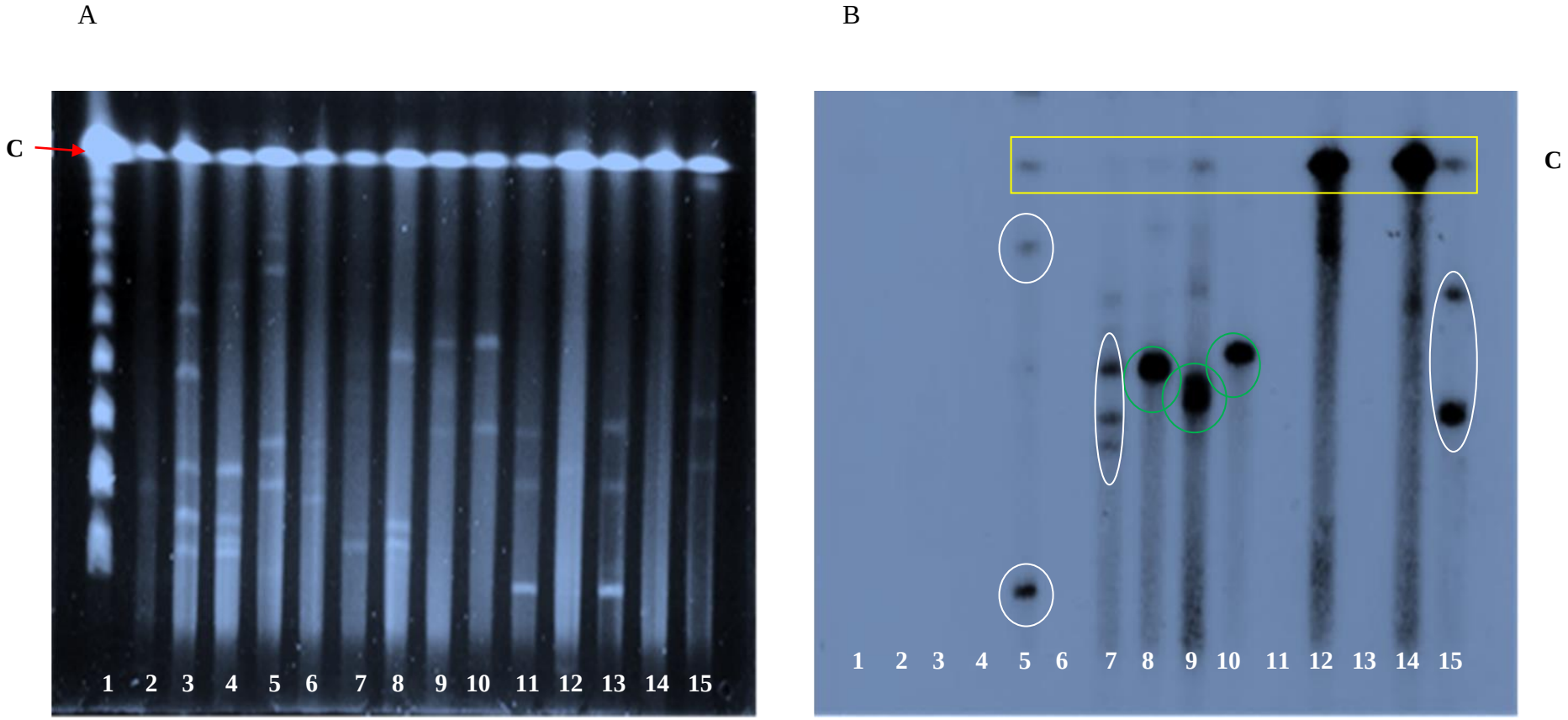
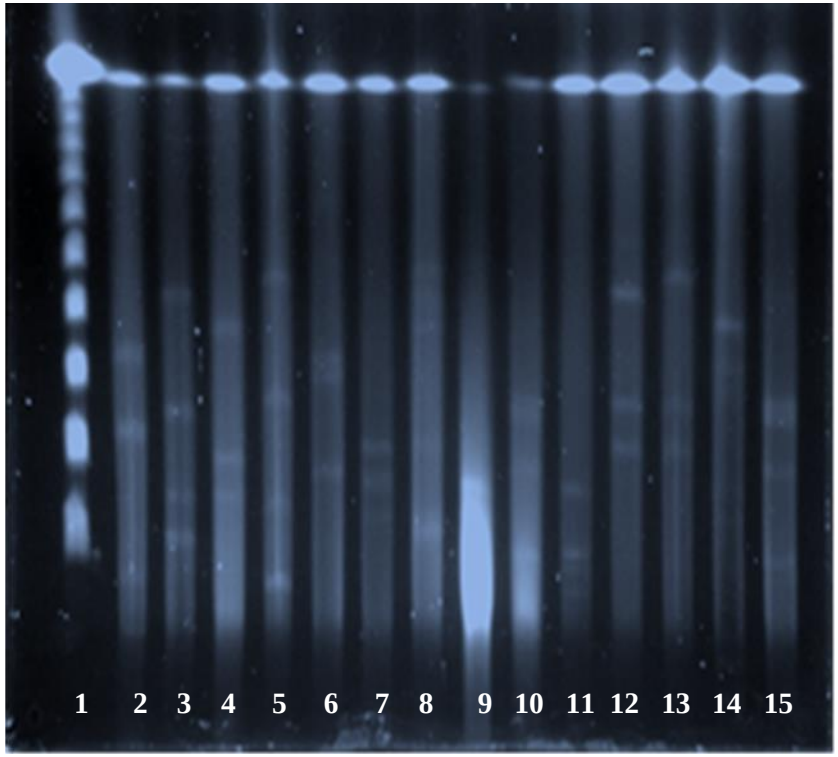


Figure 6.7 Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from the UK. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *pco*A gene probe. Lane 1= Marker. Lane 2= *Kp*N1. Lane 3= *Kp*N2. Lane 4= *C. freundii* N3. Lane 5= *E. cloacae*N4. Lane 6= *Enterobacter* sp.N5. Lane7= *E. coli* N6. Lane 8=*Kp*N7. Lane 9= *Kp*N8. Lane10= *Kp*N9. Lane11= *Kp*N10. Lane12= *Kp*N11. Lane13=*Kp*N12. Lane 14= *C. freundii* N13. Lane 15:*E. coli* N14. *Kp*= *K. pneumoniae*. C= chromosome. Kb= kilo.

A



B

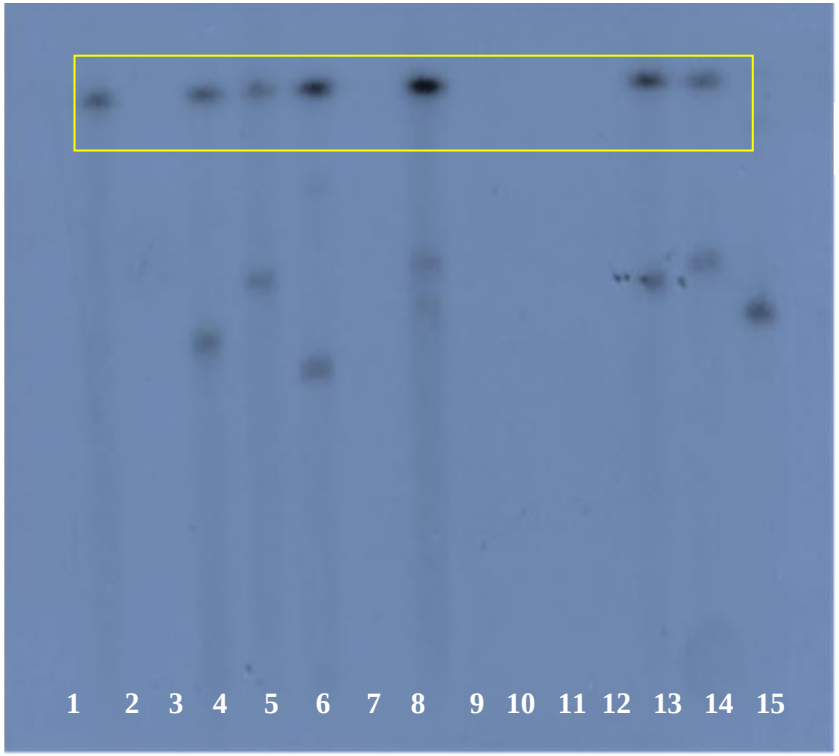


Figure 6.8 Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from the UK. (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *pco*A gene probe. Lane 1= Marker. Lane 2= *E. coli* N15. Lane 3= *Kp*N16. Lane 4=*Kp*N17. Lane 5= *Kp*N18. Lane 6= *Kp*N19. Lane 7= *E. coli* N20. Lane 8= *Kp*N21. Lane 9= *Kp*N22. Lane 10= *E. coli* N23. Lane 11= *Acin. baumanii* N24. Lane 12= *Enterobacter sp*.N26. Lane 13= *Kp*.N27. Lane 14= *K. oxytoca* N28. Lane 15= *E. coli* N29. *Kp* = *K. pneumoniae*. C= chromosome. Kb= kilo

6.3 Discussion

The objectives of this study were to screen the resistance of Enterobacteriaceae to heavy metals, and highlight the presence of plasmid and chromosome-mediated heavy metal resistance. The study of resistance to four different heavy metals was investigated. The MIC value of copper ranged from 625-2500 µg/ml in all isolates. Moreover, the arsenic resistance was clear in all three groups, up to >5000µg/ml. In terms of, silver and mercury, the MICs fluctuated. This suggests that copper and silver resistance occurs possibly through a shared mechanism such as an efflux pump that is able to remove both metals ions from the cell (Torres-Urquidy *et al*., 2012). Not all isolates were resistant to both ions Ag and Mer, some isolates were sensitive. Another study reported high MICs of Cu^{+2} (1000µl/ml) and As^{+2} (800 µl/ml) in soil bacteria isolated from Egypt (Bahig *et.al.* 2008). In India, about one hundred and eighty tons of mercury salts are discharged annually (Rani and Mahadevan 1993). Such pollutants would clearly lead to selection pressure of metal- resistance development in organisms (Hada and Sizemore 1981; Malik and Ahmed 1994). In this thesis, Enterobacteriaceae showed highest MICs for arsenic and copper, and also showed resistance to multiple metals ions. However, it is well known that there are no acceptable concentrations of heavy metals ions which can be used to distinguish metal resistant or sensitive bacteria (Malik and Jaiswal, 2000). Subsequently, the sensitive or resistant ranges of MIC have been determined on the basis of the control strains (standard strains) (Unaldi Coral *et al*., 2005). Therefore, when I used *E. coli* ATCC 25922 as a standard control, all isolates were resistant for arsenic and copper. Multiple resistance and tolerance mechanisms are common phenomena among heavy metals resistant bacteria (Amalesh *et al*., 2012). I have observed that all isolates showed more resistance and sensitivity to four heavy metals ions; Cu, Ars, Hg and Ag. Both *K. pneumoniae* and *E. coli* strains have multiple resistance properties to Ars, Cu, Sil and Mer. In fact, resistance to arsenic and copper were frequently found in bacteria isolated before the antibiotic era (Hughes and Datta 1982). Analysis of heavy metal resistance profiles clearly showed higher occurrence of resistance among NDM-1 positive strains (G1) than ESBL positive strains (G2). Differences in metal resistance phenotype were observed among Enterobacteriaceae.

Sequence analysis of heavy metal genes was performed in some isolates to detect similarity to *mer*A, *pco*A, *sil*C and *ars*A genes in the published sequences in databases.

Nucleotide and protein alignments were performed with Geneious pro 5.6.5 software. The majority of the sequenced *mer*A, *ars*A, *sil*C and *pco*A genes were most similar at both nucleotide (99.3%-100%) and protein (99.6%- 100%) levels to reference strains in the databases.

Intensive use of antibiotics and heavy metals, in hospital and in the environment, is creating a powerful selective pressure favouring the acquisition and dissemination of these antimicrobial agents among bacterial populations. The multiple resistances to heavy metals were described in several studies. Silver (1996), referred to the metal resistance of the bacteria being interrelated to each other. In our results, Arsenic and copper resistant isolates were determined in groups 1, 2, and 3. The explanation for these results is that isolates from India (G2), or from those who came back from India (G1), have originated from contaminated sites, from medical waste or hospital wastewater, or from contaminated hospital equipment. Other studies have investigated the higher number of heavy metal resistant isolates in different sites attributed to selective pressure by heavy metals. Heavy metal resistance in bacteria could reflect the level of ecological contamination with toxic metals and/or is probably directly related to exposure of bacteria to heavy metals (Aiking *et al*., 1984). Furthermore, resistance to metals may be related to the products of capsular polysaccharides often present in some organisms which are able to combine with heavy metals to protect themselves from metal toxicity (Adarsh *et al*., 2007). Also, plasmid–borne heavy metals resistance and transferable plasmids in nature leads to the spread of resistance among the sensitive bacteria including coliforms (Nyamboya *et al*. 2013). However, heavy metals resistant or sensitive bacteria may exist in uncontaminated environments (Unaldi Coral *et al*., 2005). Baath (1989) found that presence of heavy metals resistant bacteria, in environments never exposed to any concentration of heavy metals indicates that heavy metals tolerant species already exist in non-polluted habitats (Baath 1989).

In addition of Enterobacteriaceae were multiply metal resistant were also multiply antibiotic resistant. NDM-1 positive isolates, *K. pneumoniae*, *E. coli*, *Enterobacter*, *Acinetobacter* and *Citrobacter* from India and the UK have exhibited resistance to

antibiotics. In the previous study, NDM-1 positive strains exhibited highest resistance to carbapenems: Imipenem (16- 64mg/l), Ertapenem (>16mg/l) and Meropenem (16->32mg/l). In Group 2, the frequency of resistance to ESBL antibiotics was observed among Enterobacteriaceae (*K. pneumoniae, E. coli*, *Enterobacter* sp. and *Salmonella* sp.) from India. A high degree of heavy metals resistance associated with multiple antibiotic resistances was discovered in different sources of bacteria (Edward *et al*., 2009; Verma *et al*., 2001). Bacteria that are resistant to antibiotics and heavy metals have been isolated from nosocomial and burn wound infection cases (Calomiris *et al*., 1984; Poiata *et al*., 2000). Observations made with respect to metal-antibiotic-double resistance were also reported by Berg *et al.*, 2010. Furthermore, our study revealed that some isolates did not have the genes for metal resistance, but they have the genes for antibiotics resistance; for example, ESBL- positive strains gave negative PCR results to *ars*A gene (85%, 35/41), to *mer*A and *pco*A genes (65.8%, 27/41), and to *sil*C gene (56%, 23/41). In carbapenem resistant isolates (39 isolates from India and the UK) (G1) 16, 41% were negative for the *ars*A gene, 15, 38.7% were negative for *pco*A gene, 12, 30.7% were negative for *mer*A gene and 10, 25.6% were negative for *sil*C gene. These results could prove the lack of relationship between antibiotic and heavy metal resistance and suggest that the two types of resistance are not-or only rarely, co-selected. These results agree with Ame`lie Deredjian and his team (2011). They have revealed that *P. aeruginosa* strains isolated from different sites (hospital and environmental sites) displayed strong resistance to antibiotics and were less resistant to heavy metals. This difference in response of isolates could be due to the selectivity of microbial culture techniques employed especially with regard to the nature and specificity of growth media (Nyamboya *et al.* 2013). In addition, difference in toxicity could be due to several factors; including bioavailability, chemical form, conditions of metabolic activity and other bacterial species related factors (Yue *et al*., 2007).

Bacteria apply various resistance mechanisms in response to heavy metals (Edward *et al*., 2009). The mechanisms of resistance are: i) efflux pump mechanisms (remove toxic ions that entered the cell by transport systems), and ii) detoxification (converts a more toxic ions to less toxic or less available metallic ions species).The efflux pump mechanism uses either ATPase (as the arsenite ATPase of Gram negative bacteria), or chemiosmatic (as the divalent cation efflux system of *Alcaligenes*) (Ji and Silver 1995). This is evidence

that there is no general mechanism for resistance to heavy metals (Ji and Silver 1995). Bacterial resistance of diverse heavy metals is possibly encoded by genes located on transposons, plasmids or chromosomes (Silver and Phung 1996; Silver *et al*., 2000; Bruins *et al*., 2000; Laila 2011). I carried out PFGE and *S*1 digestion experiments to detect the heavy metal genes location. I found high frequency of heavy metal, *ars*A, *pco*A, *mer*A, and *sil*C genes were located on plasmids and chromosomes, in group 1. Previous works reported that the resistance genes to heavy metals are frequently located on plasmids or transposons (Cervantes *et al*., 1991). Some bacteria, isolated from nosocomial infections harboured a conjugative plasmid encoding resistance to antibiotics and toxic heavy metal (Poiata *et al*., 2000). A similar study also revealed that *S. abortus* equi has transferable plasmids encoding resistance to heavy metals and antibiotics (Anjali *et al*., 2000). Most heavy metal resistance systems are found on plasmids and are transferred from cell to cell. Plasmids conferring resistance to antibiotics and heavy metals have been shown in *Salmonella* typhimurium isolated from burn wounds treated with silver nitrate solution (McHugh *et al*., 1975). Timoney *et al*., (1978) have demonstrated linkage between $Hg^{2+,}$ $Cd^{2+,}$ Zn^{2+} and ampicillin resistance.

Chapter Seven

Characterization of Toxin- antitoxin systems of plasmid NDM-1 positive Enterobacteriaceae

7.1 Introduction.

Bacterial genomes can contain small genetic modules encoding two genes: a stable toxin and its unstable antitoxin. These modules are called toxin–antitoxin (TA) systems (Syed and Lévesque 2012; Ren *et al*., 2012). Bacterial TA systems are divided into three different types depending on the antitoxin nature and the mode of action of the antitoxin component (Blower *et al*. 2012). Although toxins are always proteins in type II and type III, antitoxins are either RNAs (types I and III) or proteins (type II) (Magnuson 2007).

In all types of TA systems, the cells are "addicted" to the antitoxin, since its production is critical for cell survival. Consequently, these systems are also called addiction modules (Syed and Lévesque 2012).

In Type I TA loci, the antitoxins are small antisense RNAs that inhibit translation of the toxin genes (Fozo *et al*., 2008; Gerdes and Wagner 2007). Moreover, type I TA systems seem to be less represented in microbial chromosomes than the mainly studied and characterized type II TA systems, where type II TA systems are thought to be part of the mobilome and to move through lateral gene transfer (Fozo *et al*. 2010; Koonin and Wolf 2008). Furthermore, in type II loci, the antitoxins are proteins that combine with and neutralize the toxins (Gerdes *et al*., 2005). Type I and type II TA systems were first designated in the 1980s as addiction modules that contribute to plasmid maintenance, a mechanism called post segregational killing (Gerdes *et al*. 1986).Type III TA loci encode small RNAs that combine with, and neutralize, the toxins (Fineran *et al*., 2009).

TA systems are found on numerous bacterial/archael chromosomes (Pandey and Gerdes 2005). TA systems have been linked to antibiotic response, persistence and dormancy in pathogenic bacteria (Agarwal *et al*., 2010; Christensen-Dalsgaard *et al*., 2010).

TA systems play a pivotal physiological role in bacteria, including programmed cell death (Hazan *et al*., 2004), stress response (Christensen *et al*., 2001), bacterial persistence (Kim and Wood, 2009; Rotem *et al*., 2010), regulation of biofilm formation (Kim and Wood *et al*., 2009; Wang and Wood 2011) and protection from bacteriophages (phages) (Fineran *et al*., 2009). Therefore, the main role suggested for TA systems is the control of growth under stress conditions (Gerdes and Wagner 2007).

This chapter describes the characterization of TA systems (Type I and II) and their location on bacterial genomes, in clinical strains of Enterobacteriaceae from the United Kingdom and India.

7.2 Results

7.2.1 Presence of TA genes on Plasmids isolated from Enterobacteriaceae

In an attempt to define the prevalence of TA systems in clinical Enterobacteriaceae, a total of 109 different clinical isolates were initially tested by PCR amplification (Section 2.12.2). Specialized primers were used, whereby each primer set would specifically amplify the genes of one TA systems (see appendix A-2). These include eight TA systems that represent the main TA systems in Gram negative bacteria (*pem*KI, *ccd*AB, *rel*EB, *vag*CD, *pnd*AC, *par*DE, *hok/sok*, and *srn*BC). The PCR results revealed that TA systems are ubiquitous in most of isolates. Altogether, 8 plasmid TA systems were detected among NDM-1 (Group1) and CTX-M-15 (Group 2) (Table 7-1). The two systems most prevalent in NDM-1 isolates (G1) isolated from the UK were *vag*CD (23/32, 71.5%) and *rel*BE (14/32, 43.75%), however, *vag*CD (7/10, 70%) and *hok/sok* (6/10, 60%) were more prevalent in the Indian isolates. In group 2, the *hok/sok* (24/41, 58.5%) and *pem*KI (18/41, 43.9%) genes were most frequently found. In addition, *par*DE (1/42, 2.43%) occurred less among the CTX-M-15 producing Indian isolates (G2). SrnBC system was not detected in *K. pneumoniae* or *K. oxytoca* group 1.

Sequencing was used to confirm the homology of our amplicons to TA systems, a subgroup of PCR amplicons were selected for DNA sequencing and amino acid variation (ClustalW) (Table 7.2). DNA sequencing of the pemKI operon amplicon revealed that *pem*K toxin and *pem*I antitoxin genes were identified in *K. pneumoniae* N7, K15, *E. coli* IR26 transconjugant, *Enterobacter* I2/5 and *E. coli* G4/12 and the identity was 100% with other genes from reference strains (see Table 7.2) (See an example in Appendix D.1, D.2). *ccdA*B in *E. coli* I4/3 and *Kp* E5/19 display 100% identity with the *ccd*AB genes of *E. coli* 025b:ST131 Str.JIE186p (Accession no.JX077110) (see Appendix D.3, D.4) and uncultured bacterium (Accession no.JX127248), respectively.

The *pnd*C gene of *E. coli* I4/13 was identified and comprises 26 amino acid and displays 94.1% amino acid identity with the PndC protein encoded by plasmid pEC_ Bactec, characterised from *E. coli* strain (Accession no. GU371927). There is variation between amino acid sequences of *E. coli* I4/13 and *E. coli* plasmid pEC_ Bactec reference.

*E. coli*I4/13 has the substitutions Ser, Leu, and Arg at residues 6, 16, 17, respectively (see Appendix D. 5, D.6).

The *vag*C genes in *K. pneumoniae* N1 displayed 100% identity with the *vag*C gene from *K. pneumoniae* pKPC-LK30 (Accession no. KC405622), however, in *K. pneumoniae* N9 showed (98.7%) identity with the *vag*CD genes on the IncI1 plasmid from *S. enterica* subsp. enterica serovar typhimurium (Accession no. EF141186). The encoded *vag*C gene shows 96.1% identity with the VagC protein encoded by the *vag*C gene from IncI1 plasmid isolated from *S. enterica* subsp. enterica serovar typhimurium strain (Accession no. EF141186). There is variation in these substitutions: Thr-34 instead of Met-34 sequence and Ile-39 instead of Ser-39 (see appendix D.7, D8).

The *hok* gene in *K. pneumoniae* A5/3 displays 99.3% nucleotide identity with the *hok* gene in *E. coli* NMI5428/11 plasmid pMC-NDM (Accession no. GU595196). The Hok protein shows 96.1% identity with Hok protein in *E. coli* strain (Table 7.2). The *mok*/*sok* genes in TCECIR29 have 100% identity with the *mok / sok* genes found in *E. coli* ApECO1-CoIBM (Accession no. DQ381420). For Type-I, srnBC system, only *srn*B toxin gene was detected in *E. coli* N20 and TCECIR22 producing NDM-1 by using DNA analysis (Table 7.2)

Table 7.1 PCR results for T-AT genes in Enterobacteriaceae

Code Isolate	Organism	T-AT system
N1	***K. pneumoniae***	***VagC/D***
N2	***K. pneumoniae***	***Pemk,ccdA/b, VagC/D***
N3	***C. freundii***	***Pemk, VagC/D***
N4	***E. cloacae***	***CcdA/B, VagC/D***
N5	***Enterobacter sp***	***RelE***
N6	***E. coli***	***Pemk, relE, VagCD, Hok/Sok***
N7	***K. pneumoniae***	***Pemk, relE, VagCD, Hok/Sok***
N8	***K. pneumoniae***	***VagCD, Hok/Sok***
N9	***K. pneumoniae***	***Neg.***
N10	***K. pneumoniae***	***VagCD***
N11	***K. pneumoniae***	***VagCD***
N12	***K. pneumoniae***	***VagCD***
N13	***C. freundii***	***VagCD, Hok/Sok***
N14	***E. coli***	***Neg.***
N15	***E. coli***	***Pemk, ccdAB, relE, Hok/sok, SrnBC***
N16	***K. pneumoniae***	***Pemk, relE, VagC/D,***
N17	***K. pneumoniae***	***VagCD***
N18	***K. pneumoniae***	***RelE***
N19	***K. pneumoniae***	***RelE, VagCD***
N20	***E. coli***	***Pemk ,ccdAB, relE, VagCD, SrnBC***
N21	***K. pneumoniae***	***Pemk, relE, VagCD, Hok/Sok***
N22	***K. pneumoniae***	***Pemk***
N23	***E. coli***	***Pemk, relE, VagCD, SrnBC***
N24	***A. baumanii***	***ParDE***
N25	***A. baumanii***	***Neg.***

Table 7.1 PCR results for T-AT genes in Enterobacteriaceae

Code Isolate	Organism	TA systems
N26	***Enterobacter sp***	***RelE,ParD/E,VagC/D***
Code isolates	**Organism**	**TA Systems**
N27	***K. pneumoniae***	***RelE, VagCD***
N28	***K. oxytoca***	***Pemk, vagCD***
N29	***E. coli***	***Pemk, vagCD, pndCA, SrnBC***
N30	***A. baumanii***	***Neg.***
N31	***E. cloacae***	***RelE, VagC/D***
N32	***E. cloacae***	***RelE, parDE, VagCD***
K15	***K. pneumoniae***	***RelE, VagCD, pemKI***
K7	***K. pneumoniae***	***RelE, VagCD***
IR25	***K. pneumoniae***	***VagCD, Hok/sok***
IR18k	***K. pneumoniae***	***Pemk, ccdAB, relE, ParDE, VagCD***
IR28k	***K. pneumoniae***	***ParDE, hok/sok***
IR29	***E. coli***	***CcdAB, hok/sok***
IR26	***E. coli***	***Pemk, ccdAB, VagCD, hok/sok, srnBC***
IR22	***E. coli***	***Pemk, ccdAB, VagCD, hok/sok, srnBC***
IR5	***E. coli***	***Pemk, ccdAB, VagCD, hok/sok, pndCA, srnBC***
IR61	***K. oxytoca***	***ParD/E***
TC-IR25	***K. pneumoniae: J53***	***Hok/sok***
TC- K15	***K. pneumoniae: J53***	***Neg.***
TC- IR29	***E. coli:J53***	***hok/sok***
TC- IR26	***E. coli:J53***	***PemKI, ccdA, hok/sok, srnB***
TC- IR22	***E. coli:J53***	***PemKI, ccdA, hok/sok, srnB***
TC- IR5	***E. coli:J53***	***PemKI, ccdA, vagCD, hok/sok, pndCA, srnBC***
TC- IR8	***K. pneumoniae:J53***	***Neg.***
TC-IR34 SC1	***K. pneumoniae:J53***	***Neg.***
TC- IR57	***Pr. rettgeri:J53***	***CcdA, hok, srnB***

Table 7.1 PCR results for T-AT genes in Enterobacteriaceae

Code isolate	Organism	TA systems
TC-IR57 SC1	***Pr. rettgeri:J53***	***CcdA, hok***
TC- K3	***K. pneumoniae:J53***	***CcdA, hok***
TC- 20K	***K. pneumoniae:J53***	***Pemk,ccdA, hok***
TC- IR44	***E. cloacae:J53***	***CcdA, hok, pndAC, srnB***
TC- IR8	***K. pneumoniae:J53***	***Hok***
TC- IR19K	***K. pneumoniae:J53***	***PemkI, CcdA, hok, pndAC, srnB***
TC- K6	***K. pneumoniae:J53***	***Neg.***
TC- IR21	***K. pneumoniae:J53***	***PemK, hok, pndC***
TC- HR5	***E. coli:J53***	***CcdA, pndC***
TC- IR21	***K. pneumoniae:J53***	***PemKI, hok***
TC- IR3	***K. pneumoniae:J53***	***PemK, hok, pndC***
TC- IR12	***E. coli:J53***	***PemKI, ccdA, parD, pndC, srnB***
TC- IR91	***E. coli:J53***	***PemKI, ccdA, pndC***
TC- IR3	***K. pneumoniae:J53***	***pndC***
TC- IR36	***E. cloacae:J53***	***pndC***
TC- IR38	***E. cloacae:J53***	***PndC, srnB***
TC-IR44	***E.cloacae:J53***	**Neg.**
***Kp* A5/3**	***K. pneumoniae***	***Pemk, Hok/sok***
***Kp* A5/7**	***K. pneumoniae***	***Pemk, Hok/sok***
***Kp* A5/4**	***K. pneumoniae***	***VagC/D***
***Kp* C5/8**	***K. pneumoniae***	***Pemk ,ccdA/B, hok/sok,***
***Kp* C5/7**	***K. pneumoniae***	***VagC/D***
***Kp* C5/5**	***K. pneumoniae***	***Pemk, ccdA/B, hok/sok,***
***Kp* D5/12**	***K. pneumoniae***	***Pemk, VagC/D, Hok/Sok***
***Kp* D5/4**	***K. pneumoniae***	***VagC/D***
***Kp* E5/14**	***K. pneumoniae***	***VagC/D, Hok/Sok***
***Kp* E5/17**	***K. pneumoniae***	***VagC/D***
***Kp* G5/2**	***K. pneumoniae***	***Pemk ,hok/sok***

Table 7.1 PCR results for ESBLs and T-AT genes in Enterobacteriaceae

Code a isolate	Organism	TA systems
***Kp* G5/6**	***K. pneumoniae***	**Neg.**
***Kp* G5/11**	***K. pneumoniae***	***VagCD***
***Kp*. I5/5**	***K. pneumoniae***	***Pemk, relE, vagCD***
***Kp*. F5/6**	***K. pneumoniae***	***Pemk, hok/sok***
***Kp* B6/1**	***K. pneumoniae***	***PndCA, ccdA***
***Kb.* E5/19**	***K. pneumoniae***	***VagCD, ccdA***
***Kp*. B5/11**	***K. pneumoniae***	***CcdA, Hok/sok ,pndCA, SrnBC***
***E coli* A4/8**	***E. coli***	***Pemk, hok/sok***
***E coli* F4/3**	***E. coli***	***ccdAB, hok/sok***
***E coli* B4/6**	***E. coli***	***ccdAB, SrnBC***
***E coli* A4/11**	***E. coli***	***Pemk ,ccdAB VagCD, Hok/Sok, SrnBC***
***E coli* C4/3**	***E. coli***	***Hok/sok ,pndCA***
***E coli* E4/4**	***E. coli***	***Hok/sok, pndCA***
***E coli* D4/12**	***E. coli***	***Pemk, hok/sok, srnBC***
***E coli* C4/12**	***E. coli***	***CcdA, pndCA***
***E coli* G4/12**	***E. coli***	***Pemk, ccdAB, , hok/sok ,pndCA, srnBC***
***E coli* I4/9**	***E. coli***	***Pemk, ccdAB, hok/sok, srnBC***
***E coli* I4/3**	***E. coli***	***Pemk, ccdAB, Hok/Sok, SrnBC***
***E coli* I4/13**	***E. coli***	***Pemk ,ccdAB, VagCD***
***E. coli* H4/5**	***E. coli***	***Pemk, VagC/D ,Hok/Sok***
***Salmo*.H6/20**	***Salmonella sp.***	***RelE, VagC/D ,Hok/sok***
***Salmo*. G6/9**	***Salmonella sp.***	***CcdA/B, VagC/D, Hok/sok***
***Salmo*.G6/13**	***Salmonella sp.***	***Neg.***
***Enterobacter* I 2/5**	***Enterobacter***	***Pemk, vagC/D, Hok/sok***
***Enterobacter* I 2/2**	***Enterobacter***	***VagC/D, Hok/sok***
***Enterobacter* F2/6**	***Enterobacter***	***Neg.***
***Salmonella* sp**	***Salmonella sp***	***Neg.***
***P. stuarti* B1/10**	***P. stuarti***	***Neg.***
***Salmonella* sp**	***Salmonella sp***	***Neg.***
E. coli	***E. coli***	***PemK, ccdA , parD, VagC/D,HOK/SOK***

Table 7- 2 Types of Toxin-Antitoxin systems and similarity to sequences reported in the literature.

Family/ type	TA system	Toxin	Antitoxin	Isolates	ClustalW & X Results Identities %		Sequences ID	Most closely related TA systems
					DNA	AA		
MazEF/ T-II	**PemK/I**	***Pem*K**	***Pem*I**	TC *E.coli*IR26	**100**	**100**	CU928148	*E. coli Strain UMNO26plasmidp1ESCUM*
		***Pem*K**	***Pem*I**	*KpK15*	**100**	**100**	CP001856	*E. coli* O83:H1 str. NRG857C
		***Pem*K**	***Pem*I**	*Enterobacter*I2/5	**100**	**100**	FJ876827	*K. pneumoniae*
		***Pem*K**	***Pem*I**	KpN7	**100**	**100**	JF274991	*Salmonella enterica* subsp. *enterica* serovair *typhimurium*
		***Pem*K**	***Pem*I**	*Ec*G4/12	**100**	**100**	KC788405	*E. coli*
CcdAB/ T	**CcdAB**	***ccd*B**	***ccd*A**	*KpC5/8*	**100**	**100**	AB364141	*S. pombe* expression vector pDUAL-HFG21C
		***ccd*B**	***ccd*A**	*Ec*I4/3	**100**	**100**	JX077110	*E. coli* 025b:ST131 Str.JIE186p
		***ccd*B**	***ccd*A**	*KpE5/19*	**100**	**100**	JX127248	Uncultured Bactirum
VagCD/T -II	***vagCD***	**-**	***vag*C**	*Kp*N1	**100**	**100**	KC405622	*K. pneumoniae* p kPC-LK30

TA=Toxin-Antitoxin gene; *pem*kI= plasmid emergency maintenance; *ccd*AB= coupled cell division; *rel*BE= relaxed control of stable RNA synthesis; *par*DE= plasmid maintenance; *vag*CD= virulence-associated protein; *hok/sok*= host-killing; *pnd*CA= promotion of nucleic acid degradation; *srn*BC= stable RNA. AA= amino acid. *Kp= K. pneumoniae*. *Ec= E. coli*.

Table 7-2 Types of Toxin-Antitoxin systems and similarity to sequences reported in the literature.

Family/ Type	TA system	Toxin	Antitoxin	Isolates	ClustalW & X Results Identities %		Sequences ID	Most closely related TA systems
					DNA	AA		
VagCD/ T-II	**vagCD**	**vagD**	**vagC**	*Kp*N9	**98.7**	**96.1**	EF141186	*IncI1 plasmid source Salmonella enterica subsp. enterica serovar typhimurium*
		-	**vagC**	TC*EC*IR5	**100**	**100**	GU595196	*K. pneumoniae* strain pKpQIL
Hok/sok or Hok/mok T-I	***Hok/sok or Hok/mok***	***hok***	**-**	*Kp*A5/3	**99.3**	**96.1**	GU371929	*E. coli* p EC_ L46
		hok	**-**	*Kp*N7	**100**	**100**	HG003695	*E. coli* strain NMI5428/11 plasmid p MC-NDM
		hok	**-**	*Enterobacter*I2/5	**99.3**	**89.4**	KF719970	*K. pneumoniae* strain ST48 plasmid pKp09085
		-	**mok, sok**	TC*EC*IR29	**100**	**100**	GU371928	*E. coli*plasmid p EC_L8
		-	**mok, sok**	*Kp*A5/3	**100**	**100**	DQ381420	*E. coli* ApECO1-CoIBM
PndAC/ T-I	***PndAC***	***pnd*A**	**pndC**	EcI4/13	**98.1**	**94.1**	GU371927	*E. coli* plasmid pEC_Bactec
		pndA	**pndC**	*Kp*N8	**100**	**100**	AB027308	IncI1 plasmid source plasmid R64
SrnBC/ T-I	***SrnBC***	**srnB**	**-**	*Ec*N20	**98.7**	**96.2**	JX077110	*E. coli* 025b:ST131 Str.JIE186p
		srnB	**-**	EcTCIR22	**100**	**100**	JN232517	E. coli strain7A8

TA=Toxin-Antitoxin gene; *pem*kI= plasmid emergency maintenance; *ccd*AB= coupled cell division; *rel*BE= relaxed control of stable RNA synthesis; *par*DE=plasmid maintenance; *va*gCD= virulence-associated protein; *hok/sok*= host-killing; *pnd*CA= promotion of nucleic acid degradation; *srn*BC= stable RNA. AA= amino acid. *Kp*= *K. pneumoniae*. *Ec*= *E. coli*.

7.2.2 Transmission and Presence TA system genes isolated from transconjugant plasmids

Conjugations were performed. The NDM-1 plasmids were selected in the basis of ceftazidium resistance and then transconjugants were examined to see if they harbour TA genes. The presence of the TA genes was confirmed by PCR in all transconjugant isolates with specific primers for TA systems (see section 2.12.2and Appendix A-2). The transfer of TA plasmids was not successful in five strains. These strains (TCK15, TCIR8, TCIR34, TCK6 and TCIR44) gave negative PCR results for all TA systems (*pem*KI, *ccd*AB *rel*EB, *vag*CD, *pnd*AC, *par*DE, *hok/sok*, and *srn*BC). Overall, none of the transconjugants harboured the *re*lEB system. However, the *ccd*AB, *hok/sok* and *srn*BC were detected in most of the transconjugants. The TA genes most prevalent among the transconjugants were: *hok/sok* (15/26, 57.69%) and *ccd*AB (12/26, 46.1%). Most of the parent's isolates have the *vag*CD system. However, only one transconjugate, TCIR5, carried *vag*CD.

7.2.3 Detection and location of TA systems among NDM-1 and CTX-M-15 producing Enterobacteriaceae

To determine the genomic location (plasmid or chromosome) of TA genes, *S*1 nuclease digests and probing with a radio labelled TA DNA fragments was carried out using the protocol in section 2.19 and 2.22. The *S*1 digests and repeated probing revealed that the TA genes were not detected in 10 isolates. Data from all isolates showed that there are diverse TA genes locations (Table 7.3), while different numbers of isolates were shown to contain more than one TA genes per parent/transconjugant (Table 7.4). *Hok/sok* genes are located on a plasmid in a large number of isolates from group 2 and group 1, where the plasmids sizes are diverse and, fluctuated from 50 and 330 kb (Figures 7.1, 7. 2, 7. 3, and7.4). The group 2 isolates, *K. pneumoniae* A5/7, C5/8, Kp C5/5, D5/12, and G5/2 carry two plasmids carrying *hok/sok* genes (Figure 7.1 highlighted in red). However, *Enterobacter* I2/5 isolate has three plasmids carrying *hok/ sok* genes (Figure 7.3 highlighted in red).

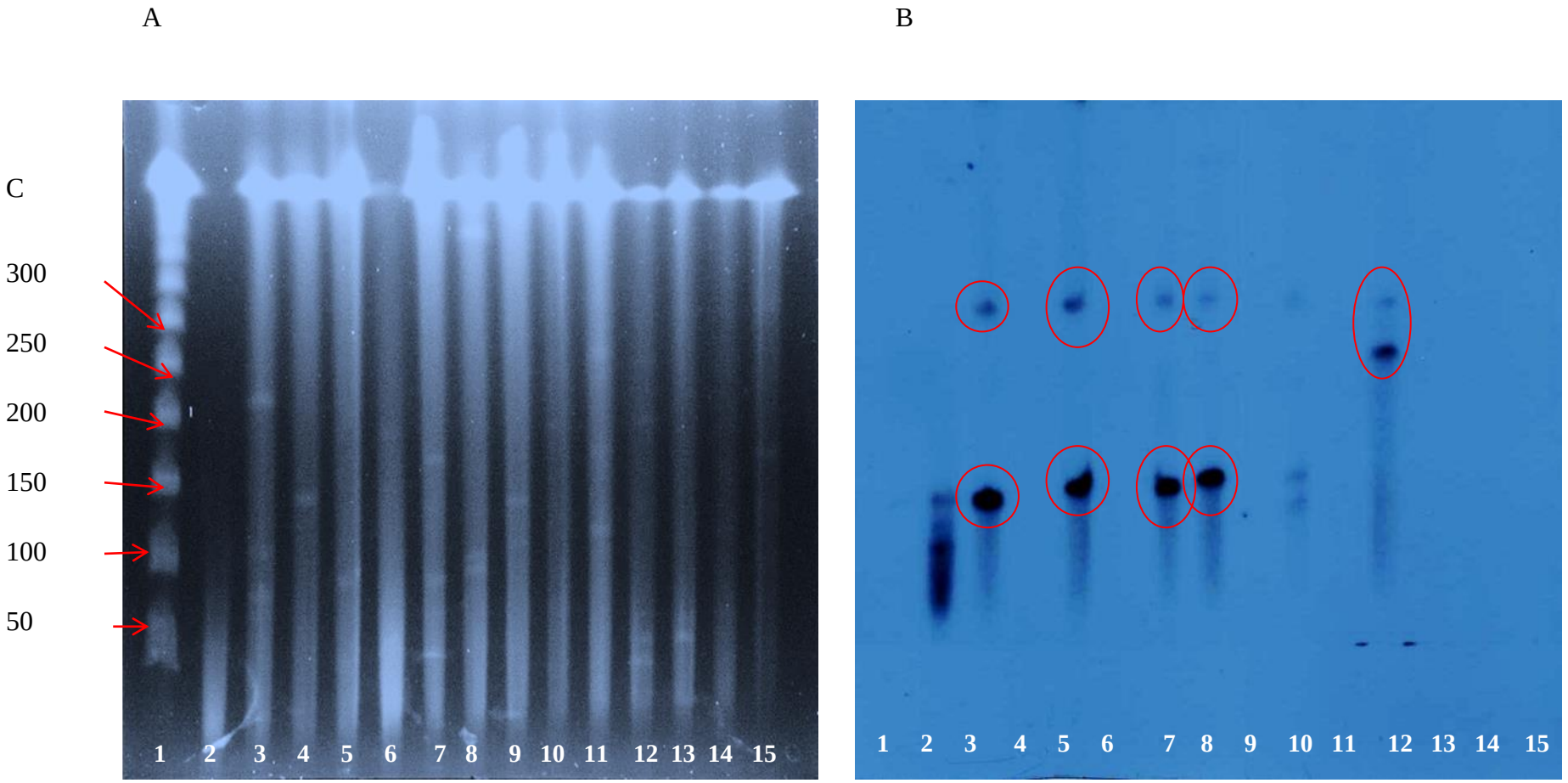


Figure 7.1 Pulsed-field gels of genomic DNA of CTX-M-15 positive strains, isolated from India (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A *with hok/sok* gene probe. Lane 1= Marker. Lane 2= *Kp*A5/3. Lane 3=*Kp*A5/7. Lane 4= *Kp* A5/4. Lane 5=*Kp*C5/8. Lane 6=*Kp*C5/7. Lane 7= *Kp*C5/5. Lane 8= *Kp*D5/12. Lane 9= *Kp*D5/4. Lane10= *Kp*E5/14. Lane11= *Kp*E5/17. Lane12=*Kp*G5/2. Lane13=*Kp*G5/6. Lane 14= *Kp* G5/11. Lane 15= *Kp* I5/5. C= chromosome. Kb= kilo base pairs.

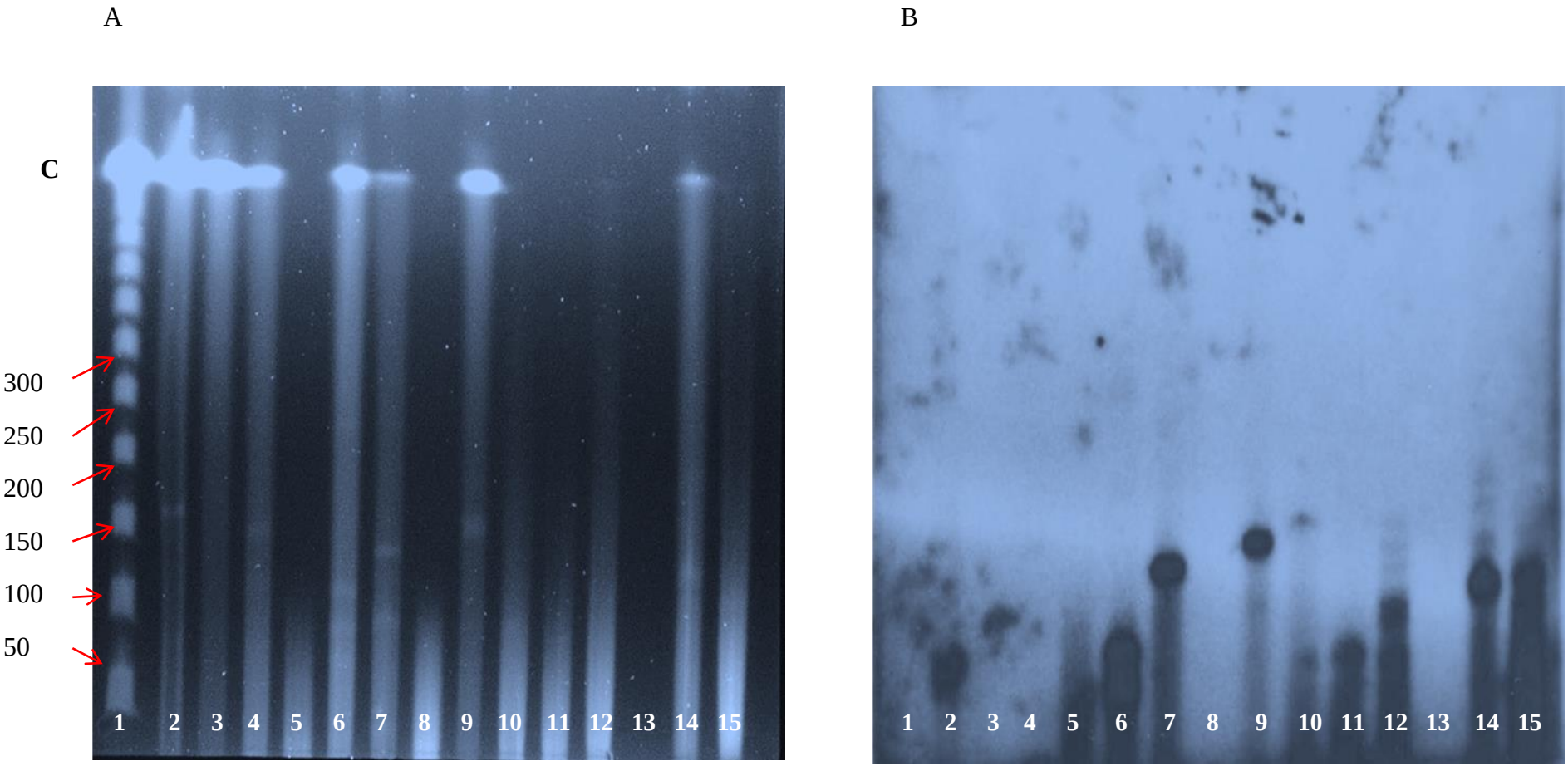


Figure 7.2Pulsed-field gels of genomic DNA of CTX-M-15 positive strains, isolated from India (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *hok/sok* gene probe. Lane 1= Marker. Lane 2= *Kp*F5/6. Lane 3= *Kp*B6/1. Lane 4= *Kp*E5/19. Lane 5= *Kp*B5/11. Lane 6= *E. coli* A/8. Lane 7= *E. coli* F4/3. Lane 8= *E. coli* B4/6. Lane 9= *E. coli* A4/11. Lane10= *E. coli* C4/3. Lane11= *E. coli* E4/4. Lane12= *E. coli* D4/12. Lane13= *E. coli* C4/12. Lane 14= *E. coli* G4/12. Lane15: *E. coli* I4/9

A

B

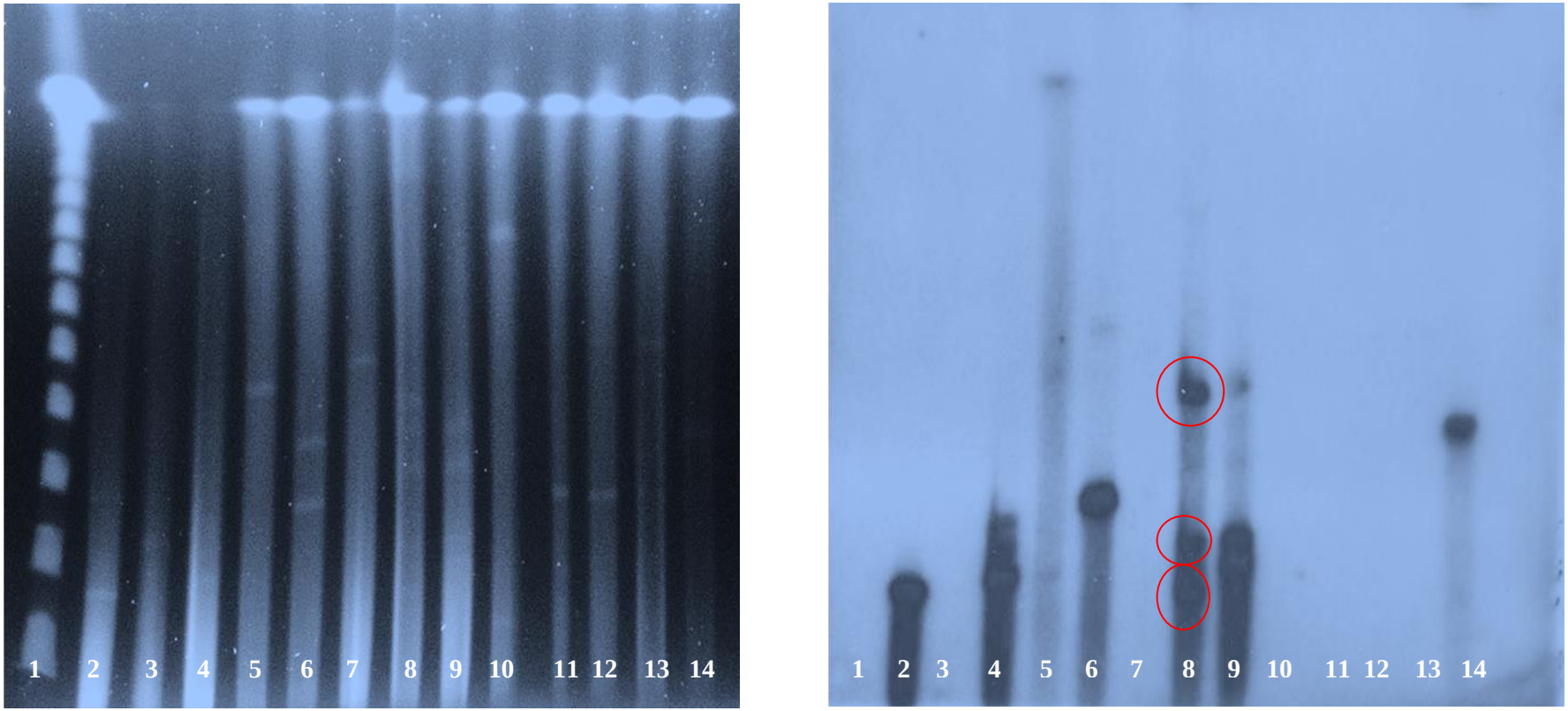


Figure 7.3 Pulsed-field gels of genomic DNA of CTX-M-15 positive strains, isolated from India (A) Plasmid size differentiation by digestion with S1 nuclease. (B) Hybridization of gel A with *hok/sok* gene probe. Lane 1= Marker. Lane 2= *E. coli* I4/3. Lane 3= *E. coli* I4/13. Lane 4= *E. co*li H4/5. Lane 5= *Salmo*.H6/20. Lane 6= *Salmo*.G6/9. Lane7= *Salmo*.G6/13. Lane 8= *Enterobacter*I2/5. Lane 9= *Enterobacter*I2/2. Lane10= *Enterobacter*F2/6. Lane11= *Salmonella*. Lane12=*P.stuanti*B1/10. Lane13= *Salmonella* sp. Lane14= *E. coli*. C= chromosome. Kp =kilo base

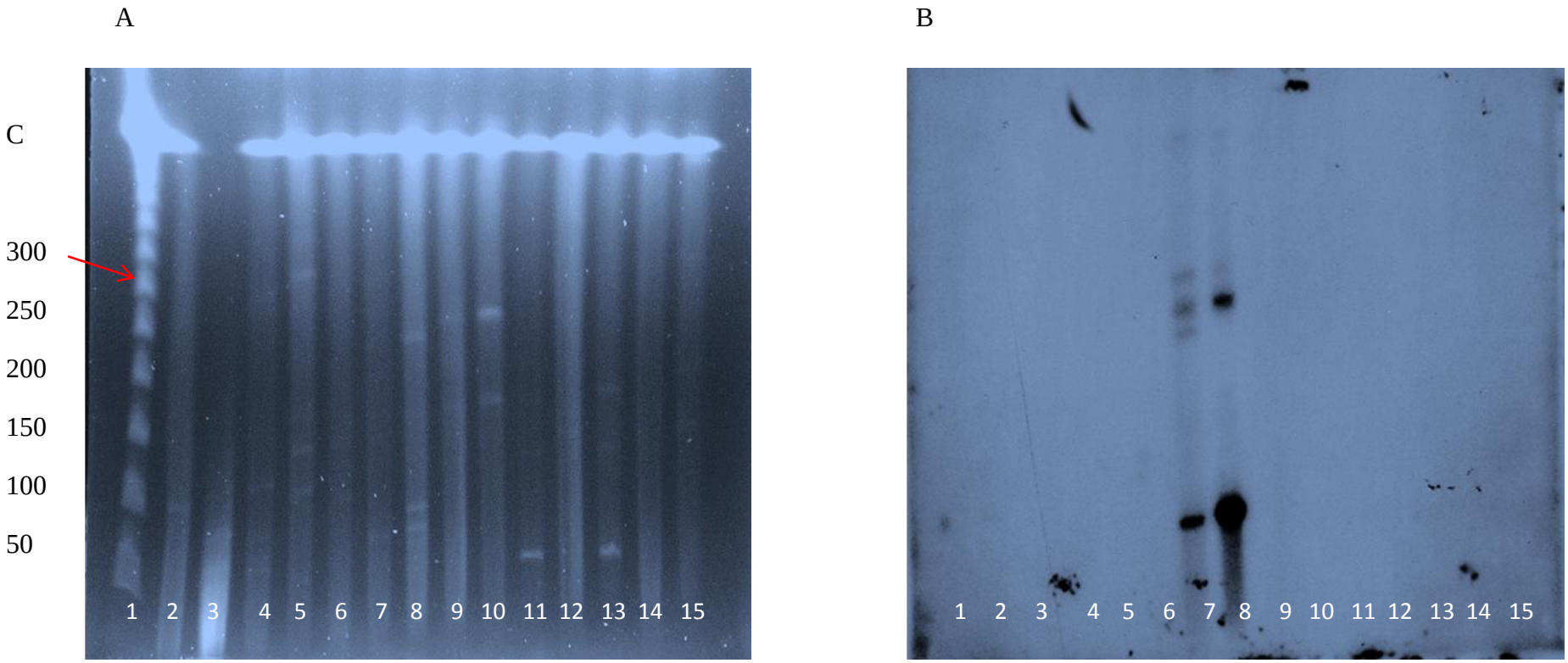


Figure 7.4 Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from the UK (A)Plasmid size differentiation by digestion with *S*1 nuclease.(B)Hybridization of gel A with *hok/sok* gene probe. Lane 1= Marker. Lane2=*Kp*N1. Lane 3= *Kp*N2. Lane 4= *C. freundii* N3. Lane 5= *E. cloacae* N4. Lane 6=*Enterobacter sp*N5. Lane7=*E. coli* N6. Lane 8= *Kp*N7. Lane 9= *Kp*N8. Lane10= *Kp*N9. Lane11=*Kp*N10. Lane12=*Kp*N11. Lane13=*Kp*N12. Lane14=*C. freundii* N13. Lane15=*E. coli* N14. C= chromosome. Kp =kilo base

14 out of 42 of NDM-1positive isolates (G1) carried *rel*EB on their chromosome (Figure 7.5 & Figure 7. 6 highlighted in yellow).

The isolates, *Enterobacter* N26 and N27 were found to carry *par*DE on 260 kb and 270 kb plasmids respectively. Moreover, they carried the *par*DE on their chromosome (Figure 7.7 highlighted in yellow).

*S*1-PFGE results indicated that *pem*KI genes were located on different size plasmids per isolate, such as *K. pneumoniae*A5/3 (Figure 7.8 highlighted in yellow), *K. oxytoca* IR61 has three plasmids carrying *par*DE genes, and *K. pneumoniae* E5/17 has three plasmids carrying *vag*CD genes (Figure7.9-B1 highlighted in yellow).

The majority of CTX-M-15 producing strains harboured two TA genes on their plasmids compared with other strains. In G2, p*arDE* and *vag*CD genes were more common on the chromosome than on plasmids.

In the isolates from group 1, the *srn*BC was more common among *E. coli* harbouring $bla_{\text{CTX-M-15}}$ gene. However, the *srn*BC gene was completely absent in other different bacteria such as, *K. pneumoniae*, *E. cloacae*, *Salmonella* sp., *A.baumanii*, *K. oxytoca* and *C. freundii.*

Isolates were positive for *srn*BC, these were found on plasmids. The size of *srn*BC plasmid was 80 to 100 kb in NDM-1 positive isolates, for example, *E. coli* N15, *E. coli* N20, *E. coli* N23 and *E. coli* N29. However, in the CTX-M-15 producing *E. coli*, the sizes of *srn*BC plasmids range from 70 kb to 150 kb. The *srn*BC plasmid size in the *E. coli* IR22 and *E. coli* IR26 harbouring $bla_{\text{NDM-1}}$ was 150 kb (data not shown). *Srn*BC was not detected in *K. pneumoniae* or *K. oxytoca* in group 1.

A

B

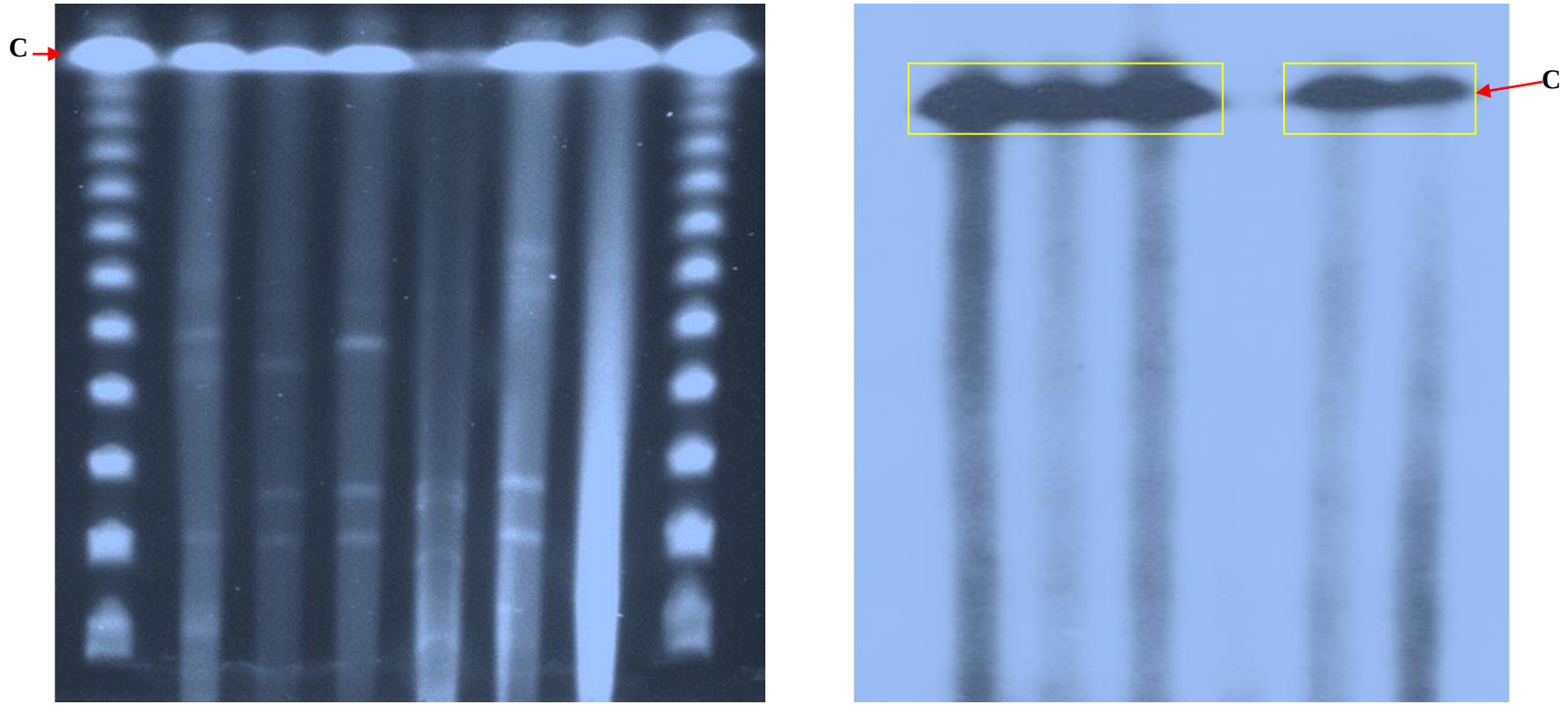


Figure7.5Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from the UK (A) Plasmid size differentiation by digestion with *S*1 nuclease (B) Hybridization of gel A with *rel*EB genes probe. Lane 1= Marker. Lane 2= *Kp*N21. Lane 3= *Enterobacter* sp N26. Lane 4= *Kp*N27. Lane 5= *E. coli* N29. Lane6= *E. cloacae* N31. Lane7= *E. cloacae*N32. Lane 8= M. C= chromosome. Kp=kilo base.

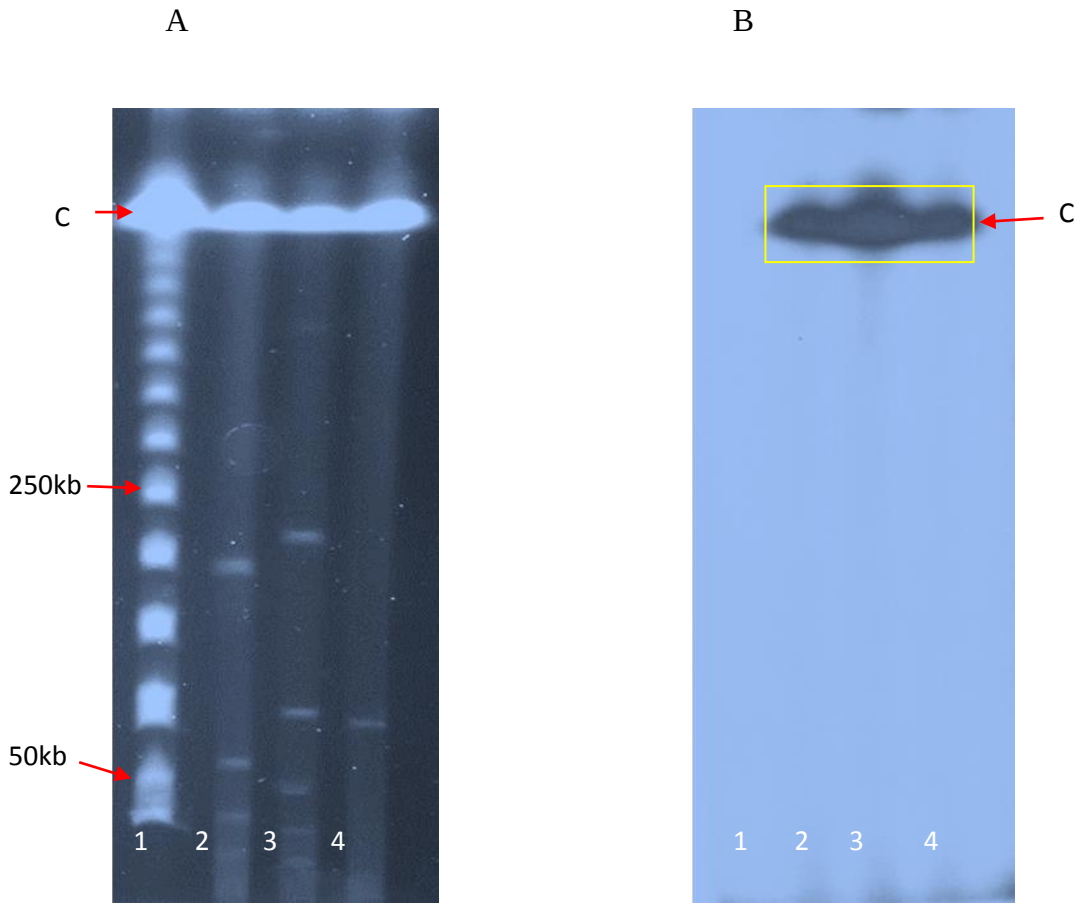


Figure 7.6 Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from India (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *rel*EB gene probe. Lane 1= Marker. Lane 2= *Kp*K15. Lane 3= *Kp*K7. Lane 4= *Kp*IR18K. C= chromosome. Kp =kilo base.

Table 7.3 Location of TA systems on genomic DNA in Enterobacteriaceae

	location of TA system on	Toxin- anti-Toxin systems							
		***pem*KI**	***ccd*AB**	***rel*EB**	***par*DE**	***vag*CD**	***hok/sok***	***pnd*CA**	***srn*BC**
UK and India **NDM-1-producing strains** **N=42**	**TA plasmid**	**11**	**3**	**0**	**0**	**4**	**6**	**1**	**4**
	TA chromosome	**0**	**1**	**14**	**1**	**11**	**0**	**0**	**0**
	TA plas. & chromo.	**0**	**0**	**0**	**2**	**8**	**0**	**0**	**0**
India **CTX-M-15- producing strains N=40**	**TA plasmid**	**17**	**14**	**0**	**0**	**13**	**23**	**6**	**7**
	TA chromosome	**0**	**0**	**2**	**1**	**3**	**0**	**0**	**0**
	TA plas. & chromo.	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**

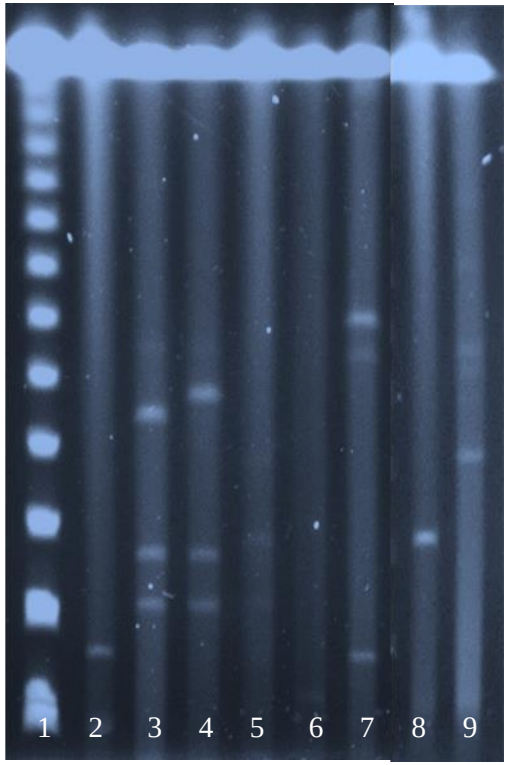
TA=Toxin-Antitoxin gene; *pem*KI= plasmid emergency maintenance; *ccd*AB=coupled cell division; *rel*BE=relaxed control of stable RNA synthesis; *par*DE=plasmid maintenance; *vag*CD=virulence-associated protein; *hok/sok*= host-killing; *pnd*CA=promotion of nucleic acid degradation; *srn*BC=stable RNA.

Table 7.4 Frequency of appearance of TA systems among Enterobacteriaceae

Place of isolates / No. Of TA	UK NDM-1 N=32	India NDM-1 N=10	Transconjugants NDM-1 (India) N=26	India CTX-M-15 N=41	Total N=109 %
0	4	0	5	6	(15)13.76%
1	8	1	5	5	(19)17.4%
2	8	5	5	14	(32) 29.3%
3	4	0	5	11	(20)18.3%
4	5	0	3	2	(10) 9.17%
5	2	3	2	3	(10) 9.17%
6	0	1	1	0	(2) 1.83%

TA= Toxin-Antitoxin system.

A



B

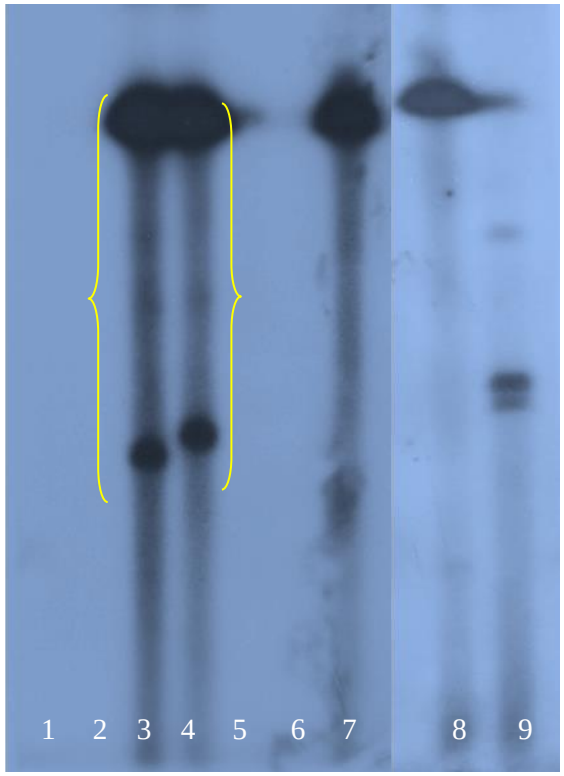
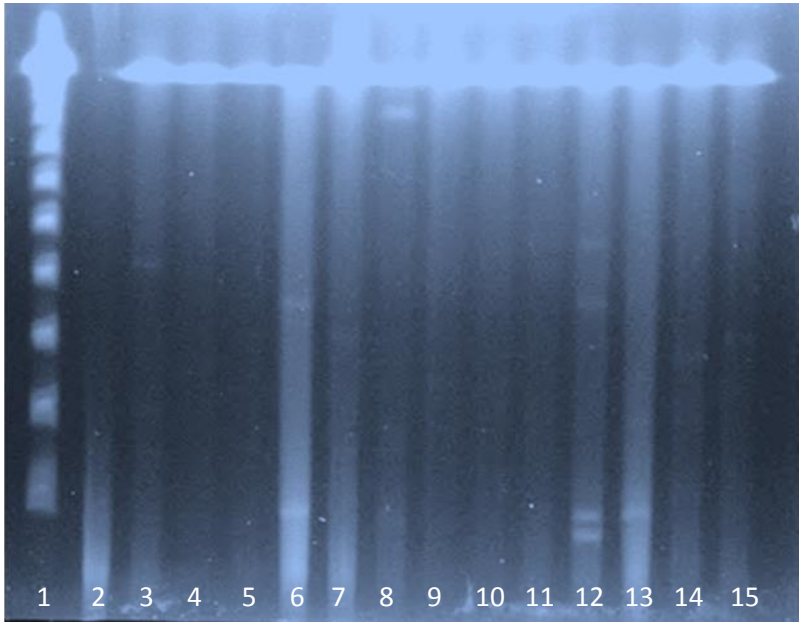


Figure 7.7 Pulsed-field gels of genomic DNA of NDM-1 positive strains, isolated from the UK (A) Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *par*DE gene probe. Lane 1= Marker. Lane 2= *A. baumanii* N24. Lane 3= *Enterobacter* sp N26. Lane 4= *Kp*N27. Lane 5= *K. oxytoca* N*28*. Lane 6= *E. cloacae* N32. Lane7= *Kp*IR28K. Lane 8= *Kp*IR28K. Lane 9=*K. oxytoca* IR61. C= chromosome. Kp =kilo base

A



B

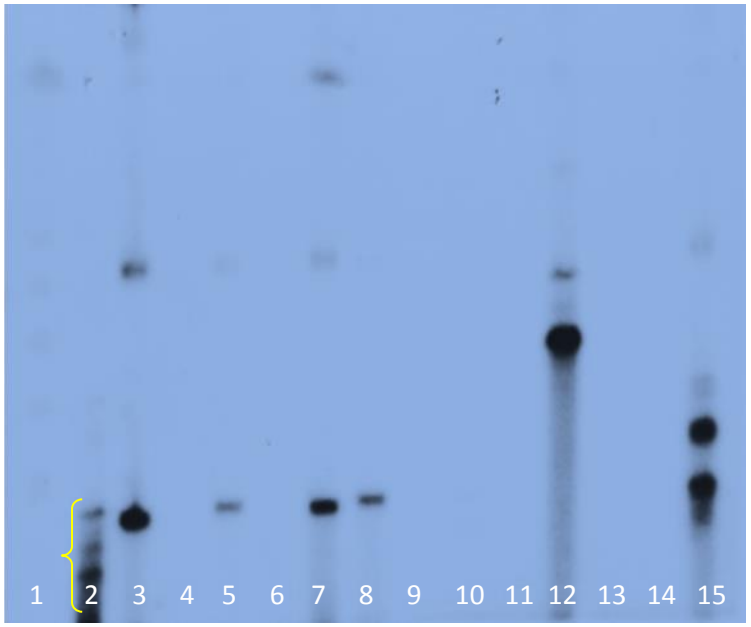


Figure 7.8 Pulsed-field gels of genomic DNA of CTX-M-15 positive strains. (A). Plasmid size differentiation by digestion with *S*1 nuclease. (B) Hybridization of gel A with *pem*KI gene probe. Lane 1= Marker. Lane 2=*K. pneumoniae* A5/3. Lane 3= *Kp*A5/7. Lane 4= *Kp*A5/4, Lane 5= *Kp*C5/8. Lane 6= *Kp* C5/7. Lane7= *Kp* C5/5. Lane 8= *Kp* D5/12. Lane 9= *Kp* D5/4. Lane 10= *Kp*E5/14. Lane 11= *Kp*E5/A. Lane 12= *Kp* G5/2. Lane 13= *Kp*G5/6. Lane 14= *Kp* G5/11. Lane 15=*Kp* I5/5. C= chromosome. KB =kilo base pair.

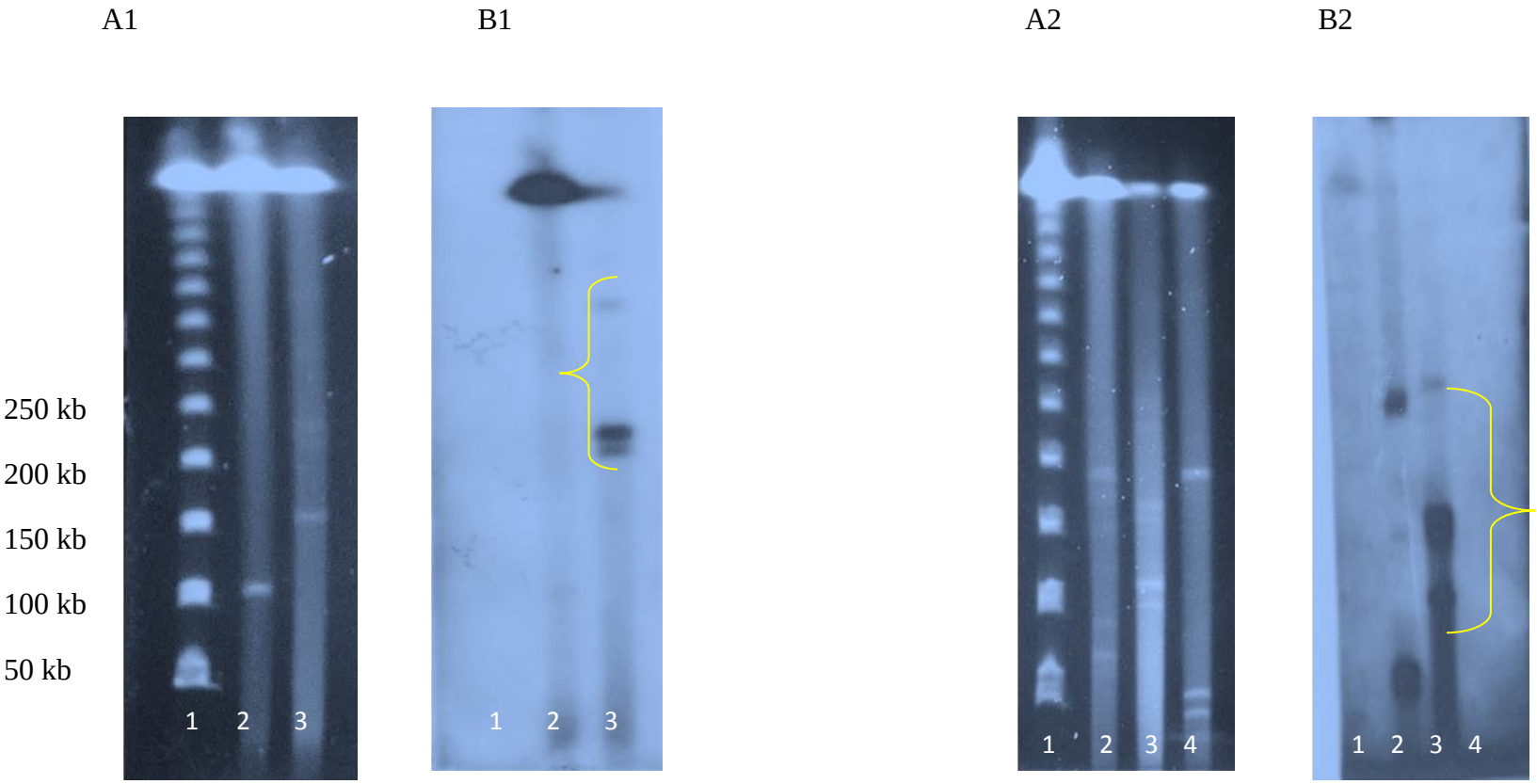


Figure 7.9 Pulsed-field gels of genomic DNA of CTX-M-15 positive strains and NDM-1 positive strains, isolated from India (A1 and A2) Plasmid size differentiation by digestion with *S*1 nuclease. (B1) Hybridization of gel A1 with *par*DE gene probe; Lane1= Marker. Lane 2= *K. pneumoniae* IR18K. Lane 3= *K. oxytoca* IR61. (B2) Hybridization of gel A2 with *vag*CD gene probe; Lane 1= Marker. Lane 2= *K. pneumoniae* E5/14. Lane 3= *K. pneumoniae* E5/17. Lane 4= *K. pneumoniae* G5/2 C=chromosome. Kb= kilo base pair.

7.2 Discussion

TA systems contribute to the stability and maintenance of plasmids in hosts and facilitate additional spreading even in the absence of antibiotic selection (Tamang *et al*., 2013). The molecular characteristics of TA system harbouring Enterobacteriaceae from clinical samples in the UK and India were investigated in this study. Overall, 94 out of 109 of NDM-1 and CTX-M-15 producing isolates revealed the presence of eight TA operons (*pem*KI, *ccd*AB, *rel*EB, *vag*CD, *pnd*CA, *par*DE, *hok/sok*, and *srn*BC) in their genome. Although all eight TA systems were detected among these strains; *pem*KI, *ccd*AB, *rel*BE, *vag*CD and *hok/sok* were the most frequently represented systems. Indian isolates harbouring $bla_{\text{CTX-M-15}}$ have more TA systems than $bla_{\text{NDM-1}}$producing isolates. These results were interesting when compared with previous molecular studies of ESBL- producing *E. coli* strains which reported, the dissemination of various TA systems on plasmids bearing the $bla_{\text{CTX-M}}$ gene in French isolates of *E. coli* producing CTX-M-15 (Mnif *et al*., 2010). There is complete or partial absence of certain TA systems in the transconjugants compared to parent isolates. This might be explained by the failure of transfer of NDM-1 positive plasmids bearing certain of these TA systems. In addition, it suggests that certain types of TA systems are located on the chromosome or may be located on non-conjugative plasmids. Multiple TA systems were identified on single plasmids harbouring $bla_{\text{CTX-M-15}}$ and $bla_{\text{NDM-1}}$ genes among clinical Enterobacteriaceae isolated from India and the UK. Our results were consistent with Doumith *et al*, who reported that several TA systems were detected in multi-resistant plasmids bearing $bla_{\text{CTX-M}}$ genes among clinical *E. coli* isolated in the UK (Doumith *et al*., 2012).

In addition, these strains having multiple TA systems were also resistant to multiple antibiotics. The NDM-1 positive *K. pneumoniae*, *E. coli*, *Enterobacter*, *Acinetobacter* and *Citrobacter* spp. isolated in India and the UK have exhibited resistance to antibiotics.

Type II TA systems were originally found in low copy number plasmids of *E. coli* where they were believed to be addiction modules to stabilize these systems (Tripathi *et al*., 2012). Recently, the detection of their high prevalence on the chromosomes of free-living bacteria (Pandey and Gerdes, 2005; Leplae *et al*., 2011) led to the suggestion that chromosomal type II TA systems are stress-response elements contributing to prokaryotes' adaptation to stressful environments (Ning *et al*., 3013).

TA systems have been identified on the chromosomes of many different bacteria (Sberro *et al*., 2013; Shao *et al*., 2011). In addition, they have been found on plasmids within certain (commonly in Gram-negative) bacteria (Hayes 2003). On plasmids, the function of TA systems seems to ensure dissemination of bacteria harbouring the plasmid through the post segregational killing of plasmid-free daughter cells (Moritz and Hergenrother 2007)

The diversity in location (TA-plasmid and TA-chromosome) and size of the TA system were detected in this study. In NDM-1 producing isolates in the UK, the southern blot analysis results revealed that *pem*KI, *vag*CD, and *hok/sok* genes were located on high molecular weight plasmids ranging from 20 to 400kb. However, *ccd*AB and *srn*BC were detected on plasmids ranging from 50 to 150kb.

The roles of chromosomal (TA) systems in the regulation of bacterial growth have been known in many bacteria, mainly *E. coli*, to cope with several stresses by reducing growth, inhibiting growth or killing a subpopulation of cells (Yamaguchi *et al*., 2011 ; Erental *et al*., 2012).

*Rel*BE encoded by *rel*E, an RNA endonuclease, is a stable, toxin and *rel*B, as a counterpart is an unstable, antitoxin. This system is believed to have originated from the *E. coli* chromosome (*rel*BE-chromosome) (Syed and Lévesque, 2012; Moreno-Cordoba *et al*., 2012; Christensen *et al*., 2001). Our results agreed with this finding and revealed that the *rel*BE system was located on chromosome. However, the *rel*BE genes have been found in vancomycin-resistant *Enterococci* on their plasmids (Moritz and Hergenrother 2007).

*Vap*BC genes, also called *vag*CD were first identified on the chromosome of strains of *Dichelobacter nodosus* and *Leptospira interrogans* (Gerdes *et al*., 2005; Ren *et al*., 2012). It was also identified on a *S. dublin* virulence plasmid (Gerdes *et al*., 2005). *Vap*BC are found as transmissible genetic elements for the transfer of virulence determinants (Ren *et al*., 2012). The *vap* genes are recognized as a part of pathogenicity islands (PAI); they are transferred using horizontal gene transfer by a plasmid, phage, or conjugative transposon. Therefore, PAIs contribute to the bacteria`s ability to evolve and adapt to new ecological niches (Hacker *et al*., 1997). The pathogenesis of some bacteria is dependent on the existence of virulence-associated plasmids and the expression of the virulence-associated factors such as protein C/D (VapC/VagD). Consequently, it seems that any isolate carrying the virulence-associated plasmid

appears more capable of causing disease (Stoughton *et al*., 2013). Our results revealed that the existence of *vag*D (toxin protein) appears relatively frequently in isolates harbouring $bla_{\text{NDM-1}}$.

The *ccd* systems (coupled cell division) of plasmid F was the first proteic bacterial toxin-antitoxin (TA) system identified (Smith *et al*. 2012). *Ccd*B encodes a toxin; it is a DNA gyrase poison that entraps a cleavage complex between gyrase and DNA (Mun˜oz-Gomez *et al*., 2005). In the presence of its antitoxin, *ccd*A, *ccd*B is sequestered in the form of a *ccd*AB complex. *Ccd*A can neutralize the toxic activity of *ccd*B by forming a non-covalent complex (De Jonge *et al*., 2012). However, if the cell loses the F-plasmid, the labile *ccd*A is degraded by the ATP dependent Lon protease (Van *et al*., 1994), releasing *ccd*B from the complex to act on its target DNA gyrase, which finally leads to cell death. The F-plasmid originates from *ccd* operon, whether located on a multi copy plasmid or in a single copy on the *E. coli* chromosome, and plays a significant role in the generation of persistence, in addition to its role in plasmid maintenance (Tripathi *et al*., 2012).

In 2005 Pandey & Gerdes assumed an extensive bioinformatics search of seven TA systems across the completely sequenced genomes of 126 bacteria. The two largest TA families were *Vap*BC and *Rel*BE. These families were represented across bacteria and archaea, whereas *maz*EF, *par*DE, *hig*BA (a member of the *rel*BE family) and *ccd* were all constrained to bacteria (Pandey and Gerdes, 2005). *Vap*BC TA systems were found to be the most common TA systems across archaea. All TA systems were present in both Gram negative and Gram positive bacteria with the exception of the *Ccd* TA system which is only found in Gram negative bacteria.

Members of a given TA family can be found at multiple locations in the genome of multiple bacterial species, suggesting that they arose by horizontal gene transfer (HGT) (Tsilibaris *et al*., 2007). Further evidence is found in that many TA systems are often associated with either mobile genetic elements or, on occasion, pathogenicity islands (Pandey and Gerdes 2005). The phylogeny of TA systems is not consistent with bacterial phylogeny (Magnuson 2007; Pandey and Gerdes, 2005) and their distribution differs greatly between isolates of the same bacterial species, implying that they are mobile (Magnuson 2007; Mine *et al*., 2009)

Conjugation is a highly specific process whereby DNA is transferred from donor to recipient bacteria by a specialized multi-protein complex, termed the conjugation system (Grohmann *et al*., 2003). Our results show that those transmissible plasmids for TA systems were found only in a low number of the transconjugant strains. These results could be explained

by the failure in the conjugation process in other strains; either that the antibiotic used in the selection was not appropriate to other plasmids, or that the TA genes are located on the chromosome, or that these TA genes indicates that they are located on non-transferable plasmids. It could also be that these plasmids need a helper/facilitator plasmid to transfer plasmid from donor cells to recipient cell (Mergeay *et al*., 1985).

Chapter Eight

General Discussion and Conclusion

8.1 Introduction

This thesis describes the genetic context of antimicrobial resistance (antibiotic and heavy metal resistance) genes and their mechanisms of mobility among collected clinical isolates procured from different countries. Data for this thesis can be divided into different sections: 1. Completion of the integrons- transposons sequence in the Pseudomonas spp. 2.Examination of bla_{NDM-1} stability in parent Enterobacteriaceae and transconjugants. 3. Determination of the presence of heavy metal genes in CTX-M-15 -and NDM-1- producing Enterobacteriaceae. 4. Analysis of the toxin–antitoxin systems and their location in Enterobacteriaceae. Data of antibiotic, heavy metal resistance genes and Toxin-antitoxin genes (Table 8.1)

8.2 Are ancestral class 1 integron more clinically relevant than classic 1 integron?

The work in this thesis was described the sequence of Tn*402*- like class 1 integrons in *Pseudomonas* spp from Chile and France. In strains, (*P. aeruginosa* and *P. fluorescence*), the VIM-2 integron was located in the ancestral transposon (Tn*402*- like) suggesting its role in the dissemination of bla_{VIM-2} (Juan *et al*., 2010). The integron lacked the 3` CS region (termed *qac*EΔ1/*sul*1) and associated with the *tni*ABQR genes (Tn*402*-like transposon). In *P. aeruginosa* 301-5473 isolate, which was isolated from France, the completed nucleotide sequence in the truncated class 1 integron consists of: the gene bla_{OXA-2} gene cassette, encoding a narrow-spectrum oxacillinase ; *aac*A4 gene encoding an *aac* (b`) Ib type aminoglycoside acetyl transferase; *aad*B gene encoding an aminoglycoside-modifying enzyme, aminoglycoside-2"-adenyltransferase (conferring resistance to gentamicin and tobaramycin); and *qac*G gene, producing a quaternary ammonium compound resistance protein. This is followed by the Tn*402*-like transposon.

In the other isolate, *P. fluorescence* 43-14926, isolated from Chile, the truncated class 1 integron only possesses *aac*A5 gene cassette, in addition to the bla_{VIM-2} gene cassette. The variable region in this integron is followed by transposition genes (Tn*402*- like transposon). Tn*402,* possessing a variable region carrying aminoglycoside and MBL genes, can easily move this resistance from one genetic structure to another, but the genes themselves cannot transfer from one organism to another without the assistance of other genetic elements such as plasmids (Walsh *et al.,* 2005).

Table 8.1 Summary of antibiotic (AB), heavy metal (HM) resistance genes and toxin-antitoxin (TA) genes in Enterobacteriaceae.

Isolate Code	Organism	AB	HM	TA
N1	*K. pneumoniae*	NDM-1	ArsA/ merA/ silC	VagCD
N2	*K. pneumoniae*	NDM-1	ArsA/ merA	Pemk, ccdAB, vagCD
N3	*C. freundii*	NDM-1	ArsA/ merA	PemK, VagCD
N4	*E. cloacae*	NDM-1	PcoA/silC	CcdAB, vagCD
N5	*Enterobacter sp.*	NDM-1	NA	RelE
N6	*E. coli*	NDM-1	ArsA/merA/pcoA/ silC	Pemk, relE, vagCD, Hok/sok
N7	*K. pneumoniae*	NDM-1	ArsA/merA/pcoA/ silC	Pemk, relE, vagCD, Hok/sok
N8	*K. pneumoniae*	NDM-1	ArsA/merA/pcoA/ silC	vagCD, Hok/sok
N9	*K. pneumoniae*	NDM-1	PcoA/silC	Neg.
N10	*K. pneumoniae*	NDM-1	SilC	VagCD
N11	*K. pneumoniae*	NDM-1	ArsA/merA/pcoA/ silC	VagCD
N12	*K. pneumoniae*	NDM-1	silC	VagCD
N13	*C. freundii*	NDM-1	ArsA/merA/pcoA/ silC	VagCD, Hok/sok
N14	*E. coli*	NDM-1	ArsA/merA/pcoA/ silC	Neg.
N15	*E. coli*	NDM-1	PcoA/silC	PemkI, ccdAB, relE, Hok/sok, SrnBC
N16	*K. pneumoniae*	NDM-1	ArsA/merA	Pemk, RelE, VagC/D
N17	*K. pneumoniae*	NDM-1	ArsA/merA/pcoA/ silC	VagCD
N18	*K. pneumoniae*	NDM-1	ArsA/merA/pcoA/ silC	RelE
N19	*K. pneumoniae*	NDM-1	ArsA/merA/pcoA/ silC	RelE, VagCD
N20	*E. coli*	NDM-1	merA	PemkI, ccdAB, relE, VagCD, SrnBC
N21	*K. pneumoniae*	NDM-1	ArsA/merA/pcoA/ silC	PemkI, RelE, VagCD, Hok/sok
N22	*K. pneumoniae*	NDM-1	ArsA	PemK
N23	*E. coli*	NDM-1	Neg.	pemkI, relE, VagCD, SrnBC
N24	*A. baumanii*	NDM-1	Neg.	ParDE
N25	*A. baumanii*	NDM-1	NA	Neg.

Table 8.1 Summary of antibiotic, heavy metal resistance genes and toxin-antitoxin genes in Enterobacteriaceae.

Isolate code	Organism	AB	HM	TA
N26	*Enterobacter sp.*	NDM-1	*ArsA, merA*	*RelE, ParDE, vagCD*
N27	*K. pneumoniae*	NDM-1	*ArsA, merA, pcoA, silC*	*RelE, VagCD*
N28	*K. pneumoniae*	NDM-1	*merA, pcoA, silC*	*Pemk, VagCD*
N29	*E. coli*	NDM-1	*merA, pcoA, silC*	*PemK, VagCD, PndCA, SrnBC*
N30	*A.. baumanii*	NDM-1	*NA*	*Neg.*
N31	*E. cloacae*	NDM-1	*Neg.*	*RelE, VagCD*
N32	*E. cloacae*	NDM-1	*PcoA, silC*	*RelE, ParDE, VagCD,*
K15	*K. pneumoniae*	NDM-1	*merA, pcoA, silC*	*RelE, vagCD, pemKI*
K7	*K. pneumoniae*	NDM-1	*MerA, pcoA, silC*	*RelE, vagCD*
IR25	*K. pneumoniae*	NDM-1	*ArsA, merA, silC*	*VagCD, hok/sok*
IR18K	*K. pneumoniae*	NDM-1	*ArsA, merA, pcoA, silC*	*PemK, ccdAB, relE, parDE, vagCD*
IR28K	*K. pneumoniae*	NDM-1	*ArsA, merA, pcoA, silC*	*ParDE, hok/sok*
IR29	*E. coli*	NDM-1	*ArsA, merA, pcoA, silC*	*CcdAC, hok/sok*
IR26	*E. coli*	NDM-1	*NG*	*pemK, ccdAB, vagCD, hok/sok, srnBC*
IR22	*E. coli*	NDM-1	*merA, silC*	*pemK, ccdAB, vagCD, hok/sok, srnBC*
IR5	*E. coli*	NDM-1	*ArsA, merA, pcoA, silC*	*pemK, ccdAB, vagCD, hok/sok, pndCA, srnBC*
IR61	*K. oxytoca*	NDM-1	*ArsA, merA, pcoA, silC*	*ParDE*
TC-IR25	*K. pneumonae:EcJ53*	NDM-1	*merA*	*Hok/sok*
TC-K15	*K. pneumonae:EcJ53*	NDM-1	NG	Neg.
TC-IR29	*E. coli:EcJ53*	NDM-1	NG	*Hok/sok*
TC-IR26	*E. coli:EcJ53*	NDM-1	NG	*PemKI, ccdA, hok/sok, srnB*

Table 8.1 Summary of antibiotic, heavy metal resistance genes and toxin-antitoxin genes in Enterobacteriaceae

Isolate code	Organism	AB	H. M	TA
TC-IR61	*K. oxytoca:EcJ53*	NDM-1	NG	Neg.
TC-IR22	*E. coli:EcJ53*	NDM-1	NG	*PemKI, ccdA, hok/sok, srnB*
TC-IR5	*E. coli:EcJ53*	NDM-1	NG	*PemKI, ccdA, vagCD, hok/sok, pndCA, srnBC*
TC-IR8	*K. pneumonae:EcJ53*	NDM-1	NG	Neg.
TC-IR34	*K. pneumonae:EcJ53*	NDM-1	NG	Neg.
TC-IR57	*Pr. rettgeri: Ec J53*	NDM-1	NG	*CcdA, hok, srnB*
TC-K3	*K. pneumonae:EcJ53*	NDM-1	NG	*CcdA, hok*
TC-20K	*K. pneumonae:EcJ53*	NDM-1	NG	*PemK, ccdA, hok*
TC-IR44	*E. cloacae: EcJ53*	NDM-1	SilC	*CcdA, hok, pndAC, srnB*
TC-IR19K	*K. pneumonae:EcJ53*	NDM-1	NG	*PemKI, ccdA, hok, pndAC, srnB*
TC-K6	*K. pneumonae:EcJ53*	NDM-1	NG	Neg.
TC-IR21	*K. pneumonae:EcJ53*	NDM-1	NG	*PemK, hok, pndC*
TC-HR5	*E. coli: EcJ53*	NDM-1	NG	*CcdA, pndC*
TC-IR21	*K. pneumonae:EcJ53*	NDM-1	merA	*PemKI, hok*
TC-IR3	*K. pneumonae:EcJ53*	NDM-1	arsA	*PemK, hok, pndC*

Table 8.1 Summary of antibiotic, heavy metal resistance genes and toxin-antitoxin genes in Enterobacteriaceae

Isolate code	Organism	AB	HM	TA
TC-IR12	*E. coli:EcJ53*	NDM-1	NG	*Pem*KI, *ccd*AB, *parD, pndC, srnB*
TC-IR91	*E. coli:EcJ53*	NDM-1	NG	*Pem*KI, *ccd*AB, *pndC*
TC-IR36	*E. cloacae:EcJ53*	NDM-1	*merA*	*Pnd*C
TC-IR38	*E. cloacae: EcJ53*	NDM-1	NG	*Pnd*C, *srn*BC
KpA5/3	*K. pneumoniae*	CTX-M-15	*Neg.*	*Pem*KI, *hok/sok*
Kp A5/7	*K. pneumoniae*	CTX-M-15	*ars*A, *mer*A, *pco*A, *sil*C	*Pem*KI, *hok/sok*
Kp A5/4	*K. pneumoniae*	CTX-M-15	*pco*A, *sil*C	*Vag*CD
Kp C5/8	*K. pneumoniae*	CTX-M-15	*silC*	*Pem*KI, *ccd*AB, *hok/sok*
Kp C5/7	*K. pneumoniae*	CTX-M-15	*ars*A, *mer*A, *pco*A, *sil*C	*Vag*CD
Kp C5/5	*K. pneumoniae*	CTX-M-15	*silC*	*Pem*KI, *ccd*AB, *hok/sok*
Kp D5/12	*K. pneumoniae*	CTX-M-15	*arsA, silC*	*PemK, vagCD, hok/sok*
Kp D5/4	*K. pneumoniae*	CTX-M-15	*silC*	*VagCD*
Kp E5/14	*K. pneumoniae*	CTX-M-15	*MerA, pco*A, *sil*C	*Vag*CD, *hok/sok*
Kp E5/17	*K. pneumoniae*	CTX-M-15	*ars*A, *mer*A, *pcoA, silC*	*Vag*CD
Kp G5/2	*K. pneumoniae*	CTX-M-15	*mer*A, *pco*A, *sil*C	*Pem*KI, *hok/sok*
Kp G5/6	*K. pneumoniae*	CTX-M-15	*pco*A, *sil*C	Neg.
Kp G5/11	*K. pneumoniae*	CTX-M-15	*sil*C	*Vag*CD
Kp I5/5	*K. pneumoniae*	CTX-M-15	*ars*A, *pco*A, *sil*C	*Pem*KI, *relE*, *vag*CD

Table 8.1 Summary of antibiotic, heavy metal resistance genes and toxin-antitoxin genes in Enterobacteriaceae

Isolate code	Organism	AB	HM	TA
Kp F5/6	*K. pneumoniae*	CTX-M-15	*Neg.*	*Pem*KI, *hok/sok*
Kp B6/1	*K. pneumoniae*	CTX-M-15	*Neg.*	*Ccd*AB, *pnd*CA
Kp E5/19	*K. pneumoniae*	CTX-M-15	*Ars*A, *pco*A, *sil*C	*Ccd*AB, *VagCD*,
Kp B5/11	*K. pneumoniae*	CTX-M-15	*Neg.*	*Ccd*AB, *hok/sok*, *pnd*CA, *srn*BC
Ec A4/8	*E. coli*	CTX-M-15	*Neg.*	*Pem*KI, *hok/sok*
EcF4/3	*E. coli*	CTX-M-15	*Neg.*	*Ccd*AB, *hok/sok*
Ec B4/6	*E. coli*	CTX-M-15	*Neg.*	*Ccd*AB, *srn*BC
Ec A4/11	*E. coli*	CTX-M-15	*Neg.*	*Pem*KI, *ccd*A, *vag*CD, *hok/sok*, *srn*BC
Ec C4/3	*E. coli*	CTX-M-15	*Neg.*	*hok/sok*, *pnd*CA
EcE4/4	*E. coli*	CTX-M-15	*Neg.*	*hok/sok*, *pnd*CA
Ec D4/12	*E. coli*	CTX-M-15	*Neg.*	*Pem*KI, *hok/sok*, *srn*BC
Ec C4/12	*E. coli*	CTX-M-15	*Neg.*	*ccd*AB, *pnd*CA
Ec G4/12	*E. coli*	CTX-M-15	*Neg.*	*Pem*KI, *ccd*AB, *hok/sok*, *pnd*CA, *srn*BC
Ec I4/9	*E. coli*	CTX-M-15	*Neg.*	*PemKI*, *ccdAB*, *hok/sok*, *srnBC*
Ec I4/3	*E. coli*	CTX-M-15	*Neg.*	*Pem*KI, *ccd*AB, *hok/sok*, *srn*BC
Ec I4/13	*E. coli*	CTX-M-15	*mer*A, *pco*A	*Pem*KI, *ccd*AB, *vag*CD
Ec H4/5	*E. coli*	CTX-M-15	*pco*A, *sil*C	*Pem*KI, *vag*CD, *hok/sok*
Salmo. H6/20	*Salmonella* sp	CTX-M-15	*Pco*A, *sil*C	*relE*, *vag*CD, *hok/sok*
Salmo. G6/9	*Salmonella* sp	CTX-M-15	*mer*A	*ccd*AB, *vag*CD, *hok/sok*
Salmo. G6/13	*Salmonella* sp	CTX-M-15	*mer*A, *pco*A, *sil*C	*Neg.*
Enter. I2/5	*Enterobacter*	CTX-M-15	*mer*A, *pco*A, *sil*C	*Pem*KI, *Vag*CD, *hok/sok*

Table 8.1 Summary of antibiotic, heavy metal resistance genes and toxin-antitoxin genes in Enterobacteriaceae

Isolate code	Organism	AB	HM	TA
Enter. I2/2	*Enterobacter*	CTX-M-15	*mer*A	*Vag*CD, *hok/sok*
Enter. F2/6	*Enterobacter*	CTX-M-15	*mer*A	*Neg.*
Salmonella sp	*Salmonella sp*	CTX-M-15	*mer*A	*Neg.*
P. stuarti B1/10	*P. stuarti*	CTX-M-15	*mer*A	*Neg.*
Salmonella sp	*Salmonella sp*	CTX-M-15	*mer*A	*Neg.*
E. coli	*E. coli*	CTX-M-15	*Neg.*	*Pem*KI, *ccd*AB, *par*DE, *vag*CD, *hok/sok*

VIM-2 type has been identified in several Enterobacteriaceae and *Acinetobacter* species, but *P. aeruginosa* remains the most significant known reservoir of these enzymes (Walsh *et al.*, 2005). Interestingly, it would appear that MBL genes, such as VIM-2, are first propagated in *P. aeruginosa* before appearing in *Acinetobacter* and Enterobacteriaceae which may be explained by the transfer of their plasmids into Enterobacteriaceae by HGT (Alvarez-Ortega *et al*., 2011). However, it is noteworthy that $bla_{\text{VIM-2}}$ gene in *Pseudomonas* spp. are mainly carried on the chromosome and not on broad host plasmids (Samuelsen *et al*., 2010). This is likely to be an example of the association between an antibiotic resistance gene and its adjacent mobile genetic elements ensuring the spread of antibiotic resistance genes to a great number of bacteria by the cooperative action of site-specific recombination, transposition, conjugation and homologous recombination (Toleman and Walsh 2010). The capture of the class 1 integron by a Tn*402*-like transposition module has triggered its rapid dissemination by LGT (Gillings *et al*., 2008). Class 1 integrons have been found to be associated with a variety of replicative transposons due to the *res* hunting capacity of the Tn*402*-like transposition system. This "*res* hunting" capacity of the transposon Tn*402* has allowed for further linkage to *res* sites of other transposons and plasmids. Particularly, they have been successful in associating themselves with mercury-resistance (*mer*) transposons, such as some transposons from the Tn*3* family. The acquisition of class 1 integrons carrying antibiotic and quaternary ammonium compound (*qac*) resistance genes may have facilitated the co-selection of *mer* transposons in clinical and farming settings within the last few decades, and *vice versa* (Stokes and Gillings 2011). Transposons belonging to the Tn*21*-subfamily (Liebert *et al*., 1999) most frequently harbour class 1 integrons (Petrova *et al*., 2011). Specific examples of transposons belonging to the Tn*21*-subgroup include Tn*21* itself and Tn*1696* (Partridge *et al*., 2001).

Tn*402* family is especially important in the dissemination of class 1 integrons in pathogens (Minakhina *et al*., 1999). The class 1 integron-transposon association has resulted in an extraordinarily efficient dispersal of this integron and its accompanying gene cassettes. One of the key steps in this spread is the capture of the integron to a Tn*402*-like transposon platform. The Tn*402* transposon (also known as Tn*5090*) (Radstrom *et al*., 1994) comprises a complete transposition module (*tni* module: *tni*R, Q, B, A) bounded by a 25 base pair (bp) of inverted repeats identical to IRi and IRt (Gillings *et al*., 2008; Stokes and Hall 1989; Stokes *et al.*, 2006)

8.3 Why are MBLs genes in *P. aeruginosa* usually found on the chromosome?

The growing threat of antimicrobial resistance in *P. aeruginosa* is displayed by its intrinsic resistance to many antimicrobial drugs and an additional ability to become MDR and XDR. These features are caused by a series of mutations in chromosomal genes that can lead to *amp*C hyper expression, repression or inactivation of *opr*D and overexpression of efflux pumps (Pfeifer *et al*., 2010; Viedma *et al*., 2012; Juan *et al*., 2010). In addition, *P. aeruginosa* is capable of capturing and incorporating clusters of genes, conferring antibiotic resistance and enhancing virulence in conjunction with the emergence and dissemination during the last decade (Queenan and Bush 2007; Mesaros *et al*., 2007; Moya *et al*., 2009) of horizontally acquired resistance genes encoding for class B carbapenemases (MBLs).

A recent study found that *P. aeruginosa* has thirty seven gene positions in the chromosome that have contributed to its intrinsic resistance, and a further forty one loci could potentially be involved in the acquisition of resistance, because mutants in these loci were less susceptible than their parent's strains (Alvarez-Ortega *et al*., 2011). These results indicate that the intrinsic resistant genes of *P. aeruginosa* include numerous elements belonging to diverse functional families and cannot be considered as a specific mechanism of adaptation to the new usage of antibiotics as therapeutic agents (Alvarez-Ortega *et al*., 2011). With respect to this resistance, MDR and XDR *P. aeruginosa* isolates have increased as a consequence of the acquisition of mobile elements such as class 1 integrons and the antibiotic resistance gene cassettes associated with them (Viedma *et al*., 2012).

MBLs can be disseminated horizontally through transfer of DNA segments/ structures and have become a serious concern in hospitals worldwide. Some MBL genes are normally carried in class 1 integrons along with other resistance determinants, for instance, the aminoglycoside-modifying enzymes. The integrons are often located on plasmids and/ or transposons, the dissemination of which contributes to the global spread of MBLs (Poirel *et al*., 2000; Riccio *et al*., 2001). The ability of *P. aeruginosa* to co- regulate different resistance mechanisms makes this pathogen a constantly moving target that continues to challenge therapeutic strategies (Obritsch *et al*., 2004; Leibovici *et al*., 1998; Lister *et al*., 2009). There are a variety of resistance mechanisms encoded on the *P. aeruginosa* chromosome: (i) the AmpC cephalosporinase; (ii) the *Opr*D outer membrane porin, (iii) the multidrug efflux pumps (Lister et al., 2009).

The logical question that arises is why the chromosome, rather than plasmids, seems to be the preferred location for class 1 integrons in isolates of *P. aeruginosa*? One reason for this trend towards integration into the chromosome is that there may be linkage of other resistance genes and/ or other pathogenicity determinants to create strains that are, overall, more resistant and potentially more virulent (Janvier *et al*., 2013). In the case of *P. aeruginosa* this is supported by the recently reported isolation of the *bla*$_{\text{NDM-1}}$ gene located in the chromosome (Janvier *et al*., 2013). In this case, the *P. aeruginosa* isolate HIABP11, had *bla*$_{\text{NDM-1}}$ in association with a class 1 integron closely related to the one described in Tn*6162* (Martinez *et al*., 2013). Analyzing the genomic context of *bla*$_{\text{NDM-1}}$ in *P. aeruginosa* isolate HIABP11 revealed the presence of IS*Pa7* and it is likely that *bla*$_{\text{NDM-1}}$ was mobilized into a new complex class 1 integron by IS*Pa7*, which is a part of a chromosomally located Tn*402*-like structure (Janvier *et al*., 2013). β- Lactam resistance is a particular problem in *P. aeruginosa* clinical isolates (Poole 2011), and this can arise through multiple mechanisms. These mechanisms include both changes to chromosomally located genes as well as the acquisition of mobilized genes by LGT (Lister *et al*., 2009). Therefore, an understanding of the causes of resistance in clinical populations of *P. aeruginosa* requires an assessment of how multiple pathways interact.

8.4 Why did NDM-1 producing strains appear in the Indian subcontinent and now are spread globally?

The progressive dissemination of antimicrobial-resistant bacteria, resulting from use and abuse of antimicrobials (such as antibiotics and heavy metals), has today become a major global health concern. The emergence and global spread of NDM-1 positive Enterobacteriaceae and Gram-negative non-fermenters, which inactivate all β-lactams (apart from aztreonam) including carbapenems, is a major clinical and public health threat. The *bla*$_{\text{NDM-1}}$ appears to have originated in the Indian subcontinent and, subsequently, could be found in several countries worldwide immediately after it was first reported; indeed it has now spread to over 50 countries (Arya and Agarwal 2011; Walsh *et al*., 2011; Kumarasamy *et al*., 2010). The authors of the original epidemiological study were able to determine that the main reservoirs of these resistant strains were located in the Indian sub-continent: India, Pakistan, Bangladesh, and Sri Lanka. The *bla*$_{\text{NDM-1}}$ gene was identified in various species of Enterobacteriaceae; not only *Klebsiella* and *E. coli* but many other non-prevalent bacteria. In other studies, the *bla*$_{\text{NDM-1}}$ was detected most frequently in community-acquired Enterobacteriaceae, in *A. baumannii*, and then reported in *P. aeruginosa*. A significant spread was observed in the UK, which has close links with India and

Pakistan, the former having a strong link with medical tourism (Poirel *et al*., 2010b; Kumarasamy *et al*., 2010). There are several diverse factors promoting $bla_{\text{NDM-1}}$ dissemination.

1) Strong selection pressure: India is considered the main country for the production of drugs (including antimicrobial drugs) in the world. Therefore, all antibiotics are sold freely and used without any control (it is of significant interest whether the Chennai Declaration will help enforce the Indian government ban starting March 1st 2014)

2) The environment is characterized by the non-compliance of fundamental hygiene rules, especially sewerage treatment and potable water. A great proportion of people present with chronic diarrhea and the mortality rate, due to intestinal infections is one of the highest in the world. Walsh and colleagues collected samples in New Delhi, at open sewerage and public water delivery points, and showed that tap water and waste water contained significant quantities of bacteria carrying NDM-1; not only Enterobacteriaceae, *S. maltophilia*, and *Pseudomonas* strains, but also *V. cholerae* (Walsh *et al*., 2011). This study further assessed the transmissibility of $bla_{\text{NDM-1}}$ by mobile genetic elements at different temperatures and found a higher frequency at 30ºC rather than 37ºC indicating rapid spread in the environment/ community (Walsh *et al*., 2011).

3) The transmission and spread of strains is promoted by over-population; 1.3 billion people live in India in slightly more than 3 million km^2. This proximity enhances the risks of person-to-person transmission of intestinal bacteria particularly in urban areas. The high temperatures and high humidity are especially favourable for bacterial growth, especially in India and Bangladesh. The spread of strains in the community is important, meaning that colonization of travellers is possible even if they are not admitted to hospital (Perry *et al*., 2011).

4) The increase of cross-border and cross-continental movements of people has a main impact on the spread of antimicrobial-resistant bacteria (Arya and Agarwal, 2011; Cheng *et al*, 2012). A recent study showed that seven out of eight tourists returning to Sweden from India had been colonized by ESBL- producing gut bacteria that they did not carry before departure, and is further supported by the high concentration of ESBLs in community-onset infections as well as hospital-onset and intra-abdominal infections in India (Walsh *et al*., 2011). Laupland and colleagues found that overseas travel significantly increased the risk of isolating e. g. NDM-1–producing bacteria and that the risk was highest among travellers to India, the Middle East and Africa (Laupland *et al*., 2008). In a recent report from America it was estimated that about 750

000 Americans travelled abroad for medical care in 2007, and as many as 1.6 million will have received healthcare outside of the United States by 2012 (Pillai *et al*., 2011).

8.5 Bacterial cells Persistence and Toxin-Antitoxin systems

Persister cells can be described as a small drug tolerant sub-population of dormant cells amongst a population that is growing normally. They favour the insubordination of chronic infections to therapy, and they are more common in the biofilm; therefore, it is difficult to treat infections caused by pathogenic microbes in biofilms (Beceiro *et al*., 2013). Persisters are frequently forming in *P. aeruginosa* infections in patients with cystic fibrosis (Mulcahy *et al*., 2010) and in other genera such as *S. aureus* (Lechner *et al*., 2012) and *E. coli* (Hansen *et al*., 2008). Persistence is not restricted to bacteria but it has also been detected in *Candida albicans* (Wagner and Unoson 2012). In 1944, Joseph Bigger reported that the bactericidal antibiotics do not completely destroy bacteria within a culture and further demonstrated that, despite lysis of *S. pyogenes* by penicillin, the surviving bacterial sub-population was not penicillin-resistant. Interestingly, these survivors were revealed to be penicillin-tolerant rather than penicillin-resistant (Bigger 1944). This phenomenon occurs because, over time, the rate of killing by antibiotics declines thereby allowing a population of bacteria to survive; this is referred to as persistence (Levin and Rozen 2006). Persisters exemplify the heterogeneity phenotype and cause major health problems since they are recalcitrant to antibiotics (Lewis 2010; Lafleur *et al*., 2010). Persister cells are not genetically different since they are resuscitated as normally growing, antibiotics-sensitive cells that can subsequently generate persisters at a low frequency (Wagner and Unoson 2012). The TA system in bacteria plays a vital role in cell survival under stress conditions (amino-acid starvation, antibiotics treatment, temperature change and oxidative stress) even though TA systems are not necessary for normal cell growth (Yamaguchi *et al*., 2011). Bacterial chromosomes and plasmids have genetic elements called TA systems (Gerdes *et al*., 2005). They comprise two genes expressed as an operon. One of the genes encodes a stable toxin and the other an unstable antitoxin. The data presented in chapter seven revealed that the genes encoding TA were distributed among genomic DNA (plasmids and chromosomes). TA systems constitute a maintenance function in plasmids (Jayaraman 2008). They have been implicated in triggering cell death in response to stress such as starvation, antibiotic pressure, DNA damage and heat (Hazen *et al.*, 2004; Jayaraman 2008). Interestingly, some researchers believe that they do not cause cell death but act as inhibitors of macromolecular synthesis leading to reversible inhibition of growth (bacteriostasis) (Jayaraman 2008). The study of the

persistent cells in *E. coli* K-12, by Keren *et al*. revealed that some well characterized TA modules (*rel*BE and *maz*EF) were among the over-expressed genes in persistent cells. In addition, they found that over-expression of plasmid-borne *rel*E, the toxin gene component of the *re*lBE operon, encoding for a translation inhibitor (Christensen *et al*., 2001), resulted in a 10–10,000-fold increase in persisters in response to fluoroquinolone, cephalosporin and an aminoglycoside. The first gene linked to persisters, *hip*A (Moyed and Bertrand 1983), is also a toxin and its ectopic expression causes multi-drug tolerance (Korch and Hill 2006; Jayaraman 2008). Falla and Chopra (1998) have reported that over-expression of *hip*A increased the production of persisters tolerant to β-lactam and fluoroquinolone antibiotics. These observations imply that *hip*BA has a general role for TA modules in generating persisters in response to antibiotic and other stresses. Interestingly, *ygi*U along with *ygi*T, is the most over-expressed gene in slow-growing cells and causes a dramatic increase in tolerance to fluoroquinolone; indeed more than *hip*A or *rel*E (Shah *et al*., 2006). TA systems *sym*ER, *hok*E, *yaf*N/*yaf*O, and *tis*AB/istr1 are induced by the SOS response (Courcelle *et al*., 2001; Motiejūnaite *et al*., 2007; Singletary *et al*., 2009). Fluoroquinolones induce the SOS response and the ability of ciprofloxacin to induce persister formation has been well noted (Dörr *et al*., 2009). Examination of deletion strains showed that the level of persisters dropped dramatically, from 10- to 100-fold, in a Δ*tis*AB mutant (Dörr *et al*., 2010). This suggests that *Tis*B was responsible for the formation of the majority of persisters under conditions of SOS induction. Pathogens are exposed to many stress factors in the host environment apart from DNA-damaging agents, such as oxidants, high temperature, low pH, etc. It is possible that all stress responses induce the formation of surviving persisters.

8.6 Persistence of antibiotic resistance in bacterial cells

In general, antibiotic resistance can occur either as the intrinsic resistance, i.e. the outcome of genetic change (e.g. mutations), or by the acquisition of antibiotic resistance gene via HGT. Intrinsic resistance is defined as the inherent resistance against an antimicrobial drug which is a characteristic of all, or almost all, representatives of the species (http://www.eucast.org/expert_rules/). There are three causes which are considered the most important of this intrinsic resistance: lack of the target, activity of chromosomally encoded antibiotic-inactivating enzymes and reduced uptake of the antibiotic (reduced permeability of the

cellular envelopes and activity of efflux pumps) (Olivares *et al*., 2013). The acquisition of resistance by the bacterial cells can be divided into two main steps:

1) Fast response, which includes the reorganization of the membrane and its permeability (change in lipopolysaccharide composition (Martins *et al.,* 2013); decrease of porin content (Hajjar *et al*., 2010); and/or over expression of efflux pumps (Li and Nikaido 2009; Nikaido and Pagès 2012).

2) Slow response, which involves genetic changes (Martins *et al*., 2013).

Resistance to antibiotics used to treat serious bacterial infections is increasing every day in both the community and hospital settings. Concentrations of antibiotic in the various environments are considered the main driving force for the selection of resistant bacteria populations (Andersson and Hughes 2011). The upsurge in MDR/XDR Enterobacteriaceae (e.g. *K. pneumoniae, E. coli*, etc.) and non-fermenting Gram- negative bacteria (*P. aeruginosa*) over the past decade is threating the successful treatment of infection (Hawkey and Jones 2009). Under environment conditions, the resistance might be stabilized in bacteria, where antibiotic selective pressure may be very low or lacking altogether. Many factors could lead to the stabilization of the resistance in bacteria, for instance; (1) compensatory mutations that restore fitness without loss of resistance; (2) occurrence of rare cost-free resistance mutations or; (3) genetic linkage and co-selection between resistance mutations and other selected genetic markers (e.g. virulence factors or resistances). In addition to these factors, which could help the resistance stabilization in bacteria, there are many factors that can cause the persistence of antibiotic resistance in a bacterial population; for example; 1) the presence of different drug concentration; 2) co-selection between genetic elements and clones; 3) cost of acquired resistances; and 4) fitness- increasing resistances (Andersson and Hughes 2011). There is historical link between the use of antibiotics and the selection of antibiotic resistance. Tenover and McGowan 1996, explained the term of selective pressure (Baquero *et al*., 1998) as follow; the selective pressure refers to environmental conditions, including not only the use of antibiotics but also any other environmental factors; for instance how the patients are linked by epidemiological features, other drugs (antibiotic or heavy metals), or environmental pollutants. Generally, the selective pressure refers to many factors that allow bacteria with unique mutations or newly acquired characteristics, to survive and proliferate (Baquero *et al*., 1998). By increasing antimicrobial use, the frequency of resistance escalated in many diverse bacteria, mainly in developing countries

where antimicrobials were freely available without prescription. Poor sanitation conditions can further assist spread of these resistant bacteria. Additionally, inadequate healthcare budgets prevent access to effective new antibiotics (Levy and Marshall 2004).

8.7 Plasmid stability

Plasmids can be present in almost all bacteria (Amábile-Cuevas and Chicurel 1992) ranging in size from a few to more than one thousand kilobases (kb) and they can represent a significant ratio of the whole genome. There are several factors affecting plasmid DNA stability. These factors include, culture conditions, antibiotic resistance genes, and plasmid copy number.

A) Culture conditions:

1) Dissolved oxygen concentration is required for maintaining plasmid stability; therefore, a decrease in dissolved oxygen concentration level in growth medium could adversely affect plasmid maintenance (Goyal *et al*., 2009; Krishna *et al*., 2008);

2) Low pH medium may cause stress to the bacterial cells, resulting in plasmid loss from the high-density cultures (Chen *et al*., 1992);

3) The composition of culture media may also impact on plasmid stability (O'Kennedy and Patching 1997). Nevertheless, it has been shown that the use of complex nitrogen sources (yeast extract or tryptone) did not decrease plasmid stability (Goyal *et al*., 2009). In fact, some studies revealed that the use of nitrogen sources resulted in increased plasmid stability (Matsui *et al*., 1990).

B) Antibiotic resistance genes can also influence plasmid stability. It is known that kanamycin resistant plasmids are more stable than ampicillin resistant plasmids (French and Ward 1995). The presence, and type, of selective pressure is also known to play a role in maintaining or improving plasmid segregational stability (Goyal *et al*., 2009; Johansson and Lidén 2006).

C) The copy number influences the plasmid stability (namely, the maintenance of the plasmid within the cells during cell division). A positive effect of a high copy number is the greater stability of the plasmid when the random partitioning occurs at cell division. However, a high copy number of plasmids usually decrease the growth rate, potentially compensating for cells with few plasmids to dominate the culture (Silva *et al*., 2012). Plasmid structural stability also could be affected by several factors such as plasmid size (Ertl and Thomsen, 2003), direct

repeats (Hadj *et al*., 2008), inverted repeats (Bi and Liu 1996) and insertion sequences (Valesova *et al*., 2005). Other environmental stress factors (antibiotic concentration, medium composition, temperature changes and oxygen fluctuations) could potentially elevate the rate of spontaneous point mutations or promote recombination between intergenomic or directly repeated sequences (Oliveira *et al*., 2009). There are several different mechanisms linked with plasmid stability such as, multimer resolution system (*mrs*) (Hayes and Barilla 2006; Shih and Rothfield, 2006), Post - Segregational Killing (PSK) systems or addiction systems (Thisted *et al*., 1994) and partitioning systems (Ebersbach and Gerdes, 2005). All of these mechanisms are important for plasmid stability, and are likely to be found on each plasmid naturally (Sengupta and Austin, 2011). In my research (chapters Four and Five), the findings revealed that the NDM-1 plasmid was stable in most isolates (in India and the UK). Moreover, NDM-1- carrying plasmids are not negatively affected by any factors that affect stability, but the size of plasmids was affected. In order to investigate all plasmid stability systems, further studies need to be done to all NDM-1- carrying plasmids.

8.8 Correlation of heavy metal and antibiotic resistance

Heavy metal contamination, due to natural and anthropogenic sources, is a global environmental concern. Release of heavy metals into human systems, mainly through contaminated water, food, and air, without proper treatment, poses a significant threat to public health. In developing countries, communities need to be aware of these problems and find preventive and remedial solutions for management. Sometimes expensive high-tech remedial measures are not easy for a country like, for example India, and hence emphasis has to be on prevention. Several studies towards remediation have to be encouraged, bearing in mind India's unique difficulties of poverty, crowding and malnutrition (Sahni 2011).

In nature there are about 50 heavy metals of special concern. Consequently, any metal element may be considered a "contaminant" if it occurs where it is unwanted, or in a form or concentration that causes a detrimental effect on humans, or microorganisms, or the environmental (Singh *et al*., 2010). There is a need to be more aware of the impact of the products that we release into the environment such as disinfectants, heavy metals and other antimicrobials that create a selective pressure in the environment (Christopher *et al*., 2014). In India, about one hundred and eighty tons or more of mercury salts are discharged annually (Rani

and Mahadevan 1993). These types of pollutants would clearly provide selection pressure for the development of metal- resistant organisms (Hada and Sizemore 1981; Malik and Ahmed 1994).

In the present study, it was evident that different bacterial strains show differing degrees of resistance and sensitivity to four heavy metals, viz., Ars, Mer, Hg and Ag. The MIC value of copper ranged between 625-2500μg/ml in all tested isolates. In addition, arsenic ion resistance was detected in all three groups. In terms of silver and mercury, the MICs were more varied. This suggests that copper (Cu) resistance in tested isolates also confers resistance to silver (Ag) perhaps through a shared mechanism, for instance an efflux pump (Torres-Urquidy *et al*., 2012). The results obtained in this study are consistent with the data collected by other workers.

Bacterial resistance to heavy metals and antibiotics is an increasing problem worldwide. In fact, antibiotics are becoming less useful to treat, but used more widely against resistant pathogenic bacteria, hence infectious diseases are becoming more difficult and more costly to treat. This is due to the creation of selective pressure in the environment that leads to the mutation of microorganisms, thus allowing them to survive by creating "superbugs" that multiply posing a potential public health hazard (Baquero *et al*., 1998; Christopher *et al*., 2014).

Heavy metals are widespread in industry, hospital effluent and sewage and there has been considerable speculation about the likely genetic association between bacterial tolerance for these metals and multiple antibiotic resistances (Christopher *et al*., 2014). It has been suggested that genes encoding resistance to heavy metals can be located, with antibiotic resistance genes on either the same genetic structure (e.g. plasmid or other MGE), or different genetic structures within the same bacterial strain (Guardabassi and Dalsgaard 2002). Antibiotic resistance in bacteria is more frequently associated with and strongly correlated with, metal resistance and expression of antibiotic resistance may be dependent on exposure to metals (McArthur and Tuckfield 2000; Selvi *et al*., 2012).

In bacterial cells, plasmids carry genes that govern resistances to numerous heavy metals and also genes for antibiotics resistance. Certainly, there are plasmids that affect only antibiotic or heavy metal resistances as well as those that affect both. For both heavy metals and antibiotics, there seems to be a great specificity of the mechanism for each resistance (Silver and Keach 1982).

Genes may be clustered on a plasmid. If all the genes clustered are useful to the bacterium, in terms of survival and persistence of its species, those genes are more likely to be transferred together by using the conjugation process. In an environment with multiple stresses, for instance antibiotics and heavy metals, it would be more ecologically favourable for a bacterium to acquire resistance to both stresses. If the resistance is located on a plasmid, those bacteria with clustered resistance genes are more likely to simultaneously pass on those genes to other bacteria, and those bacteria would then have a better chance of survival. In such a situation, one may suggest an association of antibiotic resistance with metal tolerance (Lawrence 2000).

In chapter Six, I showed that the high frequency of heavy metals genes, arsA, pcoA, merA and silC, were located on both plasmids and chromosomes in Group 1 (NDM-1 positive strains). These results were consistent with previous works (Cervantes *et al*., 1991). Most heavy metal resistance systems are found on plasmids that are capable of HGT.

Association between resistance to antibiotics and heavy metals has been described by several workers where genes encoding resistance to metals were located on transmissible plasmids (Dhakephalker and Chopade, 1994; Máthé *et al*., 2012). The prevalence of such metal tolerant microorganisms is ecologically important, particularly if they are also antibiotic resistant. Under environmental conditions such metal and antibiotic resistant, bacterial populations will adapt faster by the spread of R-factors than by mutation and natural selection, thus leading to a very rapid increase in their numbers (Bhattacherjee 1986).

The results in chapter six show that transferable plasmids, encoding resistance to various heavy metals and antibiotics in Enterobacteriaceae isolates, were clearly observed. Ghosh *et al.*, 2000 have shown transferable plasmids carrying heavy metal and antibiotics genes in *Salmonella abortus* equi (Ghosh *et al*., 2000). Moreover, my results revealed that some isolates did not have genes of metal resistance but they carry genes of antibiotic resistance. These finding suggest a lack of relationship between antibiotic and heavy metal resistance, further suggesting that the two types of resistance are not, or only rarely, co-selected. These results agree with Deredjian and his team (2011).

The spread of multiple antibiotic resistant bacteria is one of the most serious threats to the successful treatment of disease. Although resistance to metal ions is of less clinical concern than resistance to antibiotics, such an association is significant as knowledge of resistance to

metal ions may provide useful information on the mechanisms of antibiotic resistance, plasmid genetics, physiology and ecology of the microbes in polluted environments (Verma *et al.*, 2001).

Microorganisms, tolerant to metals and resistant to antibiotics, have been isolated from clinical and metal contaminated environments (Christopher *et al*., 2014). Henriette *et al*., (1991) showed that, in the bacterial population, there was an association of resistance between zinc and ampicillin, copper and penicillin, cobalt and ampicillin, cadmium and erythromycin, nickel and ampicillin as well as mercury and bacitracin. Calomiris *et al*. (1984) observed a positive correlation between tolerance to Cu^{2+}, Pb^{2+} and Zn^{2+} and multiple antibiotic resistances. They also reported that water isolates resistant to kanamycin were also tolerant to Cu^{2+}, Pb^{2+} and Zn*2*+. These finding propose that combined expression of antibiotic resistance and metal tolerance may not be a chance phenomenon, but rather the effect of selection by metals present in the environment. Also, it has been described that heavy metals, disinfectants, and antimicrobials- all can select for different kinds of bacteria, including those resistant to lifesaving antibiotics (Levy 1998; Moken *et al*., 1997). Patterns of resistance genes, originating in commensal or environmental bacteria and transferring to pathogenic bacteria, have previously been described (Hart 1998).

8.9 CONCLUSIONS

In summary, we can conclude the following:

* The work described in this thesis has given a preliminary understanding of MBLs gene (*bla*$_{\text{NDM-1}}$) in most Enterobacteriaceae isolates and they have been stable.

* The NDM-1 plasmids show diversity in size (>50kb- >450kb).

* *K. pneumoniae* K15 plasmid was not stable.

* The *bla*$_{\text{NDM-1}}$ gene transfers in approx. 80% of cases from enteric bacterial isolates to *E. coli* J53.

* *Pseudomonas* spps possess an unusual class 1 integron- carrying *bla*$_{\text{VIM-2}}$ embedded in complete Tn*402*- like transposon with different cassette genes in the variable regions.

* First report of class 1 integron harbouring *bla*$_{VIM-2}$ and *aac*A5 genes in *P. fluorescens*, , both are embedded in Tn*402*-like transposon.

* Different levels of the minimum inhibitory concentration in various metals; copper, silver, arsenic and mercury in different isolates in India and the UK.

* The incidence of these heavy metals is higher in G1 (NDM-1 positive isolates) than G2 (ESBL- positive isolates).

* Toxin –antitoxin genes were dispersed in most of isolates.

* The transfer of T-AT plasmids was successful in most of isolates.

* Diversity in location of T-TA genes among plasmids and chromosomes.

* There are differences between G1 (NDM-1- producing isolates) and G2 (CTX-M-15 producing isolates) in terms of the location of T- AT genes on genomic DNA.

8.10 Future work

* Transformation experiments which were not done in this study should be conducted to account for the frequent incidence of metal and antibiotic resistance.

* Study effects of the TA system on the stability of NDM-1 plasmids and CTX-M15 plasmids in natural and clinical bacteria.

* Investigate whether the TA systems are essential for the maintenance of the NDM -1 plasmid.

* Determine if additional TA systems are present in NDM-1- and CTX-M15- producing Enterobacteriaceae, and how the expressions of these TA systems are regulated.

* The study should be extended to cover areas around patients in hospital to establish the impact of detergents and other toxicants implicated in the co-selection of antibiotic resistance.

* Additional flora and fauna, including crops and fish which are known to accumulate plenty of heavy metals, should be tested for heavy metal analysis to shed more light on the extent of metal pollution within the aquatic environments in India.

Chapter Nine

References

Adarsh, V.K, *et al*., 2007.Studies on Metal Microbe Interaction of Three Bacterial Isolates from East Calcutta Wetland. *Online Journal of Biological Sciences* **7** (2), pp 80-88, 2007

Afif, H., Allali, N., Couturier, M., and Van Melderen, L. (2001).The ratio between CcdA and CcdB modulates the transcriptional repression of the *ccd* poison–antidote system. *Mol. Microbiol*. **41**(1), 73–82.

Agarwal, S., Mishra, NK, Bhatnagar, S., Bhatnaga, R. (2010).PemK Toxin of *Bacillus anthracis* is a Ribonuclease: an insight into its active site, structure, and function. *Journal of Biological Chemistry* **285**(10), 7254-7270.

Aiking H, Stijnman A, van Garderen C, Van Heerikhuizen H, *et al*.(1984).Inorganic phosphate accumulation and cadmium detoxification in *Klebsiella aerogenes* NCTC 418 growing in continuous culture. *Appl Environ Microbiol.* **47**(2), 374-7

Akinbowale O., Peng H, Grant P and B. MD. (2007).Antibiotic and heavy metal resistance in motile aeromonads and pseudomonads from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. *Int J Antimicrob Aga* **30,** 177-182.

Alekshun MN, Levy SB. (2007).Molecular mechanisms of antibacterial multidrug resistance. *Cell* **128**, 1037-1050

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nature reviews. Microbiology*, **8**(4), 251-259.

Almuzara M, Radice M, de Gárate N, Kossman A, Cuirolo A, *et al*. (2007). VIM-2-producing *Pseudomonas putida*, Buenos Aires. *Emerg Infect Dis* **13** (4), 668-9.

Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martínez JL. (2011). The intrinsic resistome of *Pseudomonas aeruginosa* to β-lactams. *Virulence* 2 (2), 144-6

Amábile-Cuevas CF, Chicurel ME. (1992).Bacterial plasmids and gene flux. *Cell***70** (2), 189-99

Amalesh Samanta, Paramita Bera, Mahamuda Khatun, Chandrima Sinha, Pinaki Pal, Asif Lalee, Anurup Mandal. (2012). An investigation on heavy metal tolerance and antibiotic resistance properties of bacterial strain *Bacillus* sp. isolated from municipal waste. *J. Microbiol. Biotech Res* **2** (1), 178-189.

Ambler RP, Coulson AF, Frère JM, Ghuysen JM, Joris B, *et al.* (1991).A standard numbering scheme for the class A B -lactamases. *Biochem J*.**276**, 269–272.

Ambler R. (1980).The structure of β-Lactamases. *Philos Trans R Soc Lond B Biol Sci*. **289**(1036), 321–331.

Aminov RI (2011). Horizontal gene ex-change in environmental microbiota. *Front Microbiol*. **2**,1-19.

Andersen, G. L., Menkissoglou, O., Lindow, S.E. (1991).Occurrence and properties of copper tolerant strains of *Pseudomonas syringae* isolated from fruit trees in California. *Phytopathology* **81**, 648–656.

Andersson DI, Hughes D. (2011).Persistence of antibiotic resistance in bacterial populations. *FEMS Microbiol Rev***35** (5), 901-911.

Anjali Ghosh, A. Singh, P. W. Ramteke and V. P. Singh.(2000).Characterization of Large Plasmids Encoding Resistance to Toxic Heavy Metals in *Salmonella abortus* equi. *Biochemical and Biophysical Research Communications* **272**, 6-11.

Anne SR., Enger O and Torsvik V. (1999).Abundance and diversity of archaea in heavy metal contaminated soils. *J. Applied Environ. Microbio*l **65**, 3293-3297

Anyanwu CU., Ugwu CE. (2010).Incidence of arsenic resistant bacteria isolated from a sewage treatment plant. *International Journal of Basic & Applied Sciences***10** (06), 64- 78

Arakawa Y, Murakami M, Suzuki K, Ito H, Wacharotayankun R, *et al.* (1995).A novel integron-like element carrying the metallo-beta-lactamase gene *bla*$_{\text{IMP}}$. *Antimicrob Agents Chemother* **39**(7), 1612– 1615

Arya S. C., Agarwal N. (2011). International travel with acquisition of multi-drug resistant Gram -negative bacteria containing the New Delhi metallo-beta lactamase gene, *bla*$_{\text{(NDM-1)}}$.*Travel Med. Infect. Dis*. **9**, 47–48.

Azucena E, Mobashery S. (2001).Aminoglycoside- modifying enzymes: mechanisms of catalytic processes and inhibition. *Drug Resist Update*.**4** (2), 106-117.

Azzoni AR, R. S., Monteiro GA, Prazeres DM. (2007).The impact of polyadenylation signals on plasmid nuclease-resistance and transgene expression. *J Gene Med***9**, 392-402

Baath E. (1989).Effects of heavy metals in soil on microbial processes and populations. *Water, Air, and Soil Pollution***47**, 335-379

Bahig A. E., Aly E. A., Khaled A. A. and Amel K. A.(2008).Isolation, characterization and application of bacterial population from agricultural soil at Sohag Province, Egypt. *Malays. J. Microbiol*., **4**, 42-50.

Bahl MI., Hansen LH, Sorensen SJ. (2009).Persistence mechanisms of conjugative plasmids. *Methods Mol. Biol*. **532**, 73–102.

Balado M, Lemos ML, Osorio CR. (2013).Integrating conjugative elements of the SXT/R391 family from fish-isolated Vibrios encode restriction-modification systems that confer resistance to bacteriophages. *FEMS Microbiol Ecol*. 83(2):457-67

Baldi, F., Fillipelli M. (1993).Methylmercury resistance in *Desulfovibrio desulfuricans* strains in relation to methylmercury degradation. *Appl Environ Microbiol***59** (8), 2479-2485

Ball PR, Shales SW, Chopra I. (1980). Plasmids-mediated tetracycline resistance in *Escherichia coli* involves increased efflux of the antibiotic. *Biochem Biophys Res Commun* **93** (1), 74-81

Baquero F, Negri MC, Morosini MI, Blázquez J. (1998).Antibiotic–selective environments. *Clinical Infectious Disease***27**, 55-61

Barkay T, Miller SM, Summers AO. (2003).Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiol Rev***27** (2-3), 355-384

Barnett MJ, Fisher RF, Jones T, Komp C, Abola AP, Barloy-Hubler F, *et al.*(2001).Nucleotide sequence and predicted functions of the entire *Sinorhizobium meliloti* pSymA mega plasmid. *Proc. Natl. Acad. Sci.U.S.A.* **98** (17), 9883–9888.

Barraud O., Ploy MC. (2011). Actualités sur les intégrons de résistance aux antibiotiques: mise au point. *J. Anti-infectieux***13**, 133–144.

Barrineau P, Gilbert P, Jackson WJ, Jones CS, Summers AO, Wisdom S.(1984).The DNA sequence of the mercury resistance operon of the IncFII plasmid NR1. *J Mol Appl Genet* **2** (6), 601-19

Bassetti M, Merelli M, Temperoni C, Astilean A. (2013). New antibiotics for bad bugs: where are we? *Ann Clin Microbiol Antimicrob* **12(**22), 1-15

Beceiro A, Tomás M, Bou G. (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clin Microbiol Rev* **26** (2), 185-230.

Bellais S, Naas T, Nordmann P. (2002).Genetic and biochemical characterization of CGB-1, an Ambler class B carbapenem-hydrolyzing beta-lactamase from *Chryseobacterium gleum. Antimicrob Agents Chemother***46** (9), 2791-6

Belliveau BH, Starodub ME, Cotter C, Trevors JT. (1987).Metal resistance and accumulation in bacteria. *Biotech Adv.***5** (1), 101-127.

Bennett PM. (2008).Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol* **153**, 347-357.

Berg J, T.-P. A., Nybroe O. 2005.Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field. *Lett Appl Microbiol* **40**, 146-151

Berg J, Thorsen MK, Holm PE, Jensen J, Nybroe O, Brandt KK. (2010).Cu exposure under field conditions co-selects for antibiotic resistance as determined by a novel cultivation independent bacterial community tolerance assay. *Environmental Science of Technology* **44**, 8724–8728

Bernard P, Couturier M. (1992).Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *J. Mol. Biol.***226** (3), 735–745.

Bertels F, Rainey PB. (2011). Within-genome evolution of REPINs: a new family of miniature mobile DNA in bacteria. *PLoS Genet***7** (6), e1002132

Bhattacherjee JW. (1986).Effect of cadmium and zinc on microbial adhesion, growth, and metal uptake. *Bull Environ Contam Toxicol.*36(3):396-400.

Bi X, Liu LF. (1996).DNA rearrangement mediated by inverted repeats. *Proc Natl Acad Sci USA* **93** (2), 819-823.

Bigger JW. (1944).Treatment of staphylococcal infections with penicillin. *Lancet* **244**, 497-500.

Blower TR, Short FL, Rao F, Mizuguchi K, Pei XY, Fineran PC, Luisi BF, Salmond GP. (2012).Identification and classification of bacterial Type III toxin–antitoxin systems encoded in chromosomal and plasmid genomes. *Nucleic Acids Research,* **40** (13), 6158-6173.

Borgianni L, Prandi S, Salden L, Santella G, Hanson ND, *et al.* (2011).Genetic context and biochemical characterization of the IMP-18 metallo- beta- lactamase identified in a *Pseudomonas aeruginosa* isolate from the United States. *Antimicrobial Agents Chemother* **55** (1), 40-145.

Boschi L, Mercuri PS, Riccio ML, Amicosante G, Galleni M, *et al*. (2000).The Legionella (Fluoribacter) gormanii metallo-beta-lactamase: a new member of the highly divergent lineage of molecular-subclass B3 beta-lactamases. *Antimicrob Agents Chemother* **44**(6), 1538-43.

Boucher Y, Labbate M, Koenig JE, Stokes HW. (2007). Integrons: mobilizable platforms that promote genetic diversity in bacteria. *Trends Microbiol* **15**(7), 301-9

Bragg PD, Rainnie DJ. (1974). The effect of silver ions on the respiratory chain of *Escherichia coli*. *Can. J. Microbiol* **20**(6), 883 - 889.

Brown NL, Stoyanov JV, Kidd SP, Hobman JL. (2003). The MerR family of transcriptional regulators.*FEMS Microbiol Rev* **27**(2-3), 145–163

Bruins, MR., Kapil, S and Oehme, F W. (2000).Microbial resistance to metals in the environment. *Ecotoxicol Environ Saf* 45(3), 198-207.

Bryskier A. (1997).Novelties in the field of anti-infective in 1997.*Clin Infect Dis* **27**(4), 865-83

Burrus V, Pavlovic G, Decaris B, Guédon G. (2002).Conjugative transposons: the tip of the iceberg. *Mol. Microbiol*. **46**(3), 601–610.

Burrus V, Marrero J, Waldor MK. (2006). The current ICE age: biology and evolution of SXT-related integrating conjugative elements. *Plasmid* **55**(3), 173–183.

Bush K, Jacoby GA, Medeiros AA. (1995).A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother* **39**(6), 1211-1233.

Bush K. (2010).Alarming β-lactamase-mediated resistance in multidrug resistant Enterobacteriaceae. *Curr Opin Microbiol* **13**(5), 558-564

Bush K, Jacoby GA. (2010).Updated functional classification of β- lactamases. *Antimicrob Agents Chemother* **54** (3), 969-976.

Calomiris, J. J., Armstrong, J.L. and Seidler, R J. (1984).Association of metal tolerance with multiple antibiotic resistances of bacteria isolated from drinking water. *Applied Environmental Microbiology* 47, 1238-1242

Cambray G., Guerout A., Mazel D. (2010).Integrons. *Annu Rev Genet***44**, 141–166.

Campos Muñiz, C., Zelaya TE., Esquivel GR., Fernández FJ. (2007). Penicillin and cephalosporin production: A historical perspective. *Rev Latinoam Microbiol***49** (3-4), 88-98.

Cao VT, Arlet G, Ericsson BM, Tammelin A, *et al*. (2000).Emergence of imipenem resistance in Klebsiella pneumoniae owing to combination of plasmid-mediated CMY-4 and permeability alteration. *J Antimicrob Chemother* **46**(6), 895-900.

Capela D, Barloy-Hubler F, Gouzy J, Bothe G, Ampe F, Batut J, *et al*.(2001). Analysis of the chromosome sequence of the legume symbiont *Sinorhizobium meliloti* strain 1021. *Proc Ntl. Acad. Sci.U.S.A.***98** (17), 9877–9882.

Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ.(2005).Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* **63**(3), 219-28

Carattoli A. (2009). Resistance plasmid families in Enterobacteriaceae. *Antimicrob Agents Chemother* .53(6):2227-38

Carattoli A. (2011). Plasmids in Gram negatives: molecular typing of resistance plasmids. *Int J Med Microbiol* **301**(8), 654-658.

Carattoli, A.(2013).Plasmids and the spread of resistance. *International Journal of Medical Microbiology***303**, 298– 304

Cardoso O, Leitão R, Figueiredo A, Sousa JC, Duarte A, Peixe LV.(2002).Metallo-beta-lactamase VIM-2 in clinical isolates of *Pseudomonas aeruginosa* from Portugal. *Microb Drug Resist.* 8(2):93-7

Castanheira M., Deshpande LM, Mathai D, Bell JM, Jones RN, Mendes RE.(2011).Early dissemination of NDM-1 and OXA- 181-producing Enterobacteriaceae in Indian hospitals: report from the SENTRY Antimicrobial Surveillance Program, 2006-2007.*Antimicrob. Agents Chemother* **55**(3), 1274–1278.

Centers for Disease Control and Prevention (CDC). (2010).Update: detection of a Verona integron-encoded metallo-beta-lactamase in *Klebsiella pneumoniae*, United States, *MMWR* **59** (37), 1212.

Cervantes C, Chávez K, Vaca S. (1991). AB: Mechanisms of bacterial resistance to heavy metals. *Rev. Latinoam Microbiol.* **33**(1) ,61-70.

Cervantes-Rivera R, Romero-López C, Berzal-Herranz A, Cevallos MA. (2010). Analysis of the mechanism of action of the antisense RNA that controls the replication of the *rep*ABC plasmid p42d.*J Bacteriol* **192**(13), 3268-78

Champier L, Duarte V, Michaud-Soret I, Covès J. (2004). Characterization of the MerD protein from *Ralstonia metallidurans* CH34: a possible role in bacterial mercury resistance by switching off the induction of the mer operon. *Mol Microbiol* **52** (5), 1475–1485.

Chapman JS. (1998).Characterizing bacterial resistance to preservatives and disinfectants. *International Biodeterioration & Biodegradation* **41**, 241-245.

Chapman, JS. (2003).Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int. Biodeterior. Biodegradation***51**, 271-276.

Chappell JB, Greville GD. (1954). Effect of silver ions on mitochondrial adenosine triphosphatase. *Nature.*13; **174**(4437):930-1

Chattopadhyay M., Grossart HP. (2011). Antibiotic and heavy metal resistance of bacterial isolates obtained from some lakes in northern Germany. *NSHM Journal of Pharmacy and Healthcare Management* **02**, 74-75.

Chen CM, Misra TK, Silver S, Rosen BP. (1986). Nucleotide sequence of the structural genes for an anion pump: the plasmid-encoded arsenical resistance operon. *J. Biol. Chem.* **261**:15030–38

Chen HC., ***et al*****.** (1992).High-density *Escherichia coli* cultivation process for hyper expression of recombinant porcine growth hormone. *Enzyme Microb Technol* **14**, 321-326.

Chopra I. (2007). The increasing use of silver-based products as antimicrobial agents: a useful development or a cause for concern? *Journal of Antimicrobial Chemotherapy* **59** (4), 587–590.

Christensen SK., Mikkelsen M., Pedersen K., Gerdes K. (2001).RelE, a global inhibitor of translation, is activated during nutritional stress. *Proc. Natl. Acad. Sci. USA* **98**, 14328–14333.

Christensen SK, Pedersen K, and Hansen FG., Gerdes K. (2003) .Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. *J. Mol. Biol* **332** (4), 809–819.

Christensen-Dalsgaard M, Gerdes K. (2006). Two higBA loci in the Vibrio cholerae super integron encode mRNA cleaving enzymes and can stabilize plasmids. *Mol. Microbiol*. **62**(2), 397–411.

Christensen-Dalsgaard, M., Jorgensen, M. G., Gerdes, K.(2010).Three New RelE-Homologous mRNA interferases of *Escherichia coli* Differentially Induced by Environmental Stresses. *Molecular Microbiology***75** (2), 333-348.

Christopher M., Paul O., Hamadi B. (2014).Association of Metal Tolerance with Multidrug Resistance among Environmental Bacteria from wetlands of Lake Victoria Basin. *Agric. Biol. J. N. Am*., **5**(1), 24-32.

Cohen-Wolkowiez M, Poindexter B, Bidegain M, Weitkamp JH, *et al.*(2012).Safety and Effectiveness of Meropenem in Infants with Suspected or Complicated Intra-abdominal Infections. *Clin Infect Dis***55** (11), 1495–1502.

Collis CM, Kim MJ, Partridge SR, Stokes HW, Hall RM. (2002).Characterization of the class 3 integron and the site-specific recombination system it determines. *J. Bacteriol*. **184**(11), 3017–3026.

Cooksey DA. (1993).Copper uptake and resistance in bacteria. *Mol Microbiol* **7**, 1-5.

Coque TM, Novais A, Carattoli A, Poirel L, Pitout J, Peixe L, Baquero F, Cantón R, Nordmann P. (2008).Dissemination of Clonally Related *Escherichia coli* Strains Expressing Extended-Spectrum β-Lactamase CTX-M-15.*Emerg Infect Dis* **14** (2), 195-200.

Cornaglia G, Giamarellou H, Rossolini GM. (2011). Metallo-β-lactamases: a last frontier for β-lactams? *Lancet Infect Dis*. **11**(5), 381-93.

Courcelle J, Khodursky A, Peter B, Brown PO, Hanawalt PC.(2001).Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*. *Genetics* **158** (1), 41–64.

Cullik A, Pfeifer Y, Prager R, von Baum H, Witte W. (2010). A novel IS*26* structure surrounds blaCTX-M genes in different plasmids from German clinical *Escherichia coli* isolates. *J Med Microbiol*.**59**(Pt 5):580-7.

Daikos GL, Petrikkos P, Psichogiou M, Kosmidis C, Vryonis E, *et al.* (2009).Prospective observational study of the impact of VIM-1 metallo-beta-lactamase on the outcome of patients with *Klebsiella pneumoniae* bloodstream infections. *Antimicrob Agents Chemother* **53**(5), 1868-73.

Da Silva GJ, Mendonça N. (2012).Association between antimicrobial resistance and virulence in *Escherichia coli*. *Virulence* **3** (1), 18–28.

Datta N, Kontomichalou P. (1965). Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. *Nature* **208** (5007), 239–241.

Davies J.(1997) Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Found Symp* **207**, 15-27.

Davies J, Davies D. (2010).Origins and evolution of antibiotic resistance. *Microbiology and molecular biology reviews* **74** (3), 417-433

De Jonge N, Simic M, Buts L, Haesaerts S, Roelants K, Garcia-Pino A, *et al*.(2012). Alternative interactions define gyrase specificity in the CcdB family. *Mol Microbiol***84** (5), 965-78

De la Hoz AB., S. Ayora, I. Sitkiewicz, S. Fernandez, R. Pankiewicz, J. C. Alonso, and P. Cegłowski.(2000). Plasmid copy-number control and better-than-random segregation genes of pSM19035 share a common regulator. *Proc. Natl. Acad. Sci.* USA97:728-733.

Deredjian A., Colinon C., Brothier E., Favre-Bonte S., Cournoyer B., Nazaret S. (2011).Antibiotic and metal resistance among hospital and outdoor strains of *Pseudomonas aeruginosa*. *Res Microbiol* **162**, 689–700.

Deshpande P, Rodrigues C, Shetty A, Kapadia F, Hedge A., Soman R. (2010a).New Delhi metallo- beta-lactamase (NDM-1) in Enterobacteriaceae; treatment options with carbapenems compromised. *J. Assoc. Physicians India* **58**, 147-9

Deshpande P, Shetty A, Kapadia F, Hedge A, Soman R, Rodrigues C. (2010b). New Delhi metallo 1: have carbapenems met their doom? *Clin Infect. Dis* **51** (10), 1222.

Dhakephalkar PK, Chopade BA. (1994).High levels of multiple metal resistances and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*. *Biometals* **7** (1), 67-74.

Diago-Navarro E, Hernandez-Arriaga AM, López-Villarejo J, *et al*.(2010).ParD toxin-antitoxin system of plasmid R1--basic contributions, biotechnological applications and relationships with closely-related toxin-antitoxin systems. *FEBS J* **277** (15), 3097-117

Dollwet HA., Sorenson JR. (1985).Historic uses of copper compounds in medicine. *Trace Elem. Med*. **2**, 80–87.

Dörr T, Lewis K, Vulić M. (2009). SOS response induces persistence to fluoroquinolones in *Escherichia coli .PLoS Genet*. **5**(12), e1000760.

Dörr T, Vulić M, Lewis K. (2010). Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli. Plos Biol***8** (2), e1000317

Doumith M, Dhanji H, Ellington MJ, Hawkey P, Woodford N. (2012). Characterization of plasmids encoding extended-spectrum β-lactamases and their addiction systems circulating among *Escherichia coli* clinical isolates in the UK. *J Antimicrob Chemother***67** (4), 878-85

Dröge M., Puhler A., Selbitschka W. (1999).Horizontal gene transfer among bacteria in terrestrial and aquatic habitats as assessed by microcosm and field studies. *Biol. fertile soils***29**, 221-245

Duckworth DH. (1976).Who discovered bacteriophage? *Bacteriol Rev.***40**, 793–802.

Džidic SS., Kos B. (2008).Antibiotic resistance mechanisms in bacteria: biochemical and genetic aspects. *Food Technol Biotechnol***46** (1), 11-21.

Ebersbach G, Gerdes K. (2005).Plasmid segregation mechanisms. *Annual Review Genetics***39**, 453-479.

Erental A, Sharon I, Engelberg-Kulka H. (2012).Two programmed cell death systems in *Escherichia coli:* an apoptotic-like death is inhibited by the mazEF-mediated death pathway. *PLoS Biol.***10** (3), e1001281

Ertl PF., Thomsen LL. (2003).Technical issues in construction of nucleic acid vaccines. *Methods* **31**(3), 199-206

Essack SY. (2001). The development of β-lactam antibiotics in response to the evolution of β-lactamases. *Pharm Res***18** (10), 1391-1399.

Falla TJ., Chopra I. (1998).Joint tolerance to β-lactam and fluoroquinolone antibiotics in *Escherichia coli* results from over expression of *hip* A. *Antimicrob. Agents Chemother* **42**, 3282–3284.

Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO. (2000).A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *J. Biomed. Mater. Res.***52** (4), 662–668

Fiebig A, Castro Rojas CM, Siegal-Gaskins D, Crosson S. (2010). Interaction specificity, toxicity and regulation of a paralogous set of ParE/RelE-family toxin–antitoxin systems. *Mol. Microbiol*. **77**(1), 236–251.

Fineran PC, Blower TR, Foulds IJ, Humphreys DP, Lilley KS, Salmond GP. (2009). The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. *Proc. Natl. Acad. Sci. USA***106** (3), 894–899.

Foster TJ. (1983).Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol Rev.* 47(3):361-409.

Fozo EM, Makarova KS, Shabalina SA, Yutin N, Koonin EV, Storz G. (2010). Abundance of type I toxin-antitoxin systems in bacteria: searches for new candidates and discovery of novel families. *Nucleic Acids Res.*, **38**(11), 3743–3759.

Fozo EM, Kawano M, Fontaine F, Kaya Y, Mendieta KS, *et al.*(2008).Repression of small toxic protein synthesis by the Sib and OhsC small RNAs. *Mol. Microbiol* **70** (5), 1076–1093.

Francuski D, Saenger W. (2009).Crystal structure of the antitoxin–toxin protein complex RelB–RelE from *Methanococcus jannaschii*. *J. Mol. Biol*. **393**(4), 898–908

Friehs K. (2004).Plasmid copy number and plasmid stability. *Adv Biochem Eng Biotechnol* **86**, 47-82.

Frost LS, Leplae R, Summers AO, Toussaint A. (2005).Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol* **3**(9), 722–732.

Fuentefria DB, Ferreira AE, Corção G. (2011). Antibiotic-resistant Pseudomonas aeruginosa from hospital wastewater and superficial water: are they genetically related? *J Environ Manage* **92**(1), 250-5.

Garcillán-Barcia MP, Alvarado A, de la Cruz F. (2011). Identification of bacterial plasmids based on mobility and plasmid population biology. *FEMS Microbiology Reviews***35** (5), 936-56

Gerdes K, Rasmussen PB, Molin S. (1986). Unique Type of Plasmid Maintenance Function: Postsegregational Killing of Plasmid Free Cells. *Proceedings of the National Academy of Sciences*, **83**(10), 3116-3120.

Gerdes K, Christensen SK, Løbner-Olesen A. (2005).Prokaryotic toxin–antitoxin stress response loci. *Nature Rev. Microbiol*. **3**, 371–382.

Gerdes K, Molin S. (1986).Partitioning of plasmid R1.Structural and functional analysis of the parA locus. *J Mol Biol* **190**(3), 269-79

Gerdes K, Wagner EG. (2007). RNA antitoxins. *Curr Opin Microbiol***10** (2), 117–124

Ghosh A, Singh A, Ramteke PW, Singh VP. (2000). Characterization of large plasmids encoding resistance to toxic heavy metals in *Salmonella abortus* equi. *Biochem Biophys Res Commun* **272**(1), 6-11

Ghuysen JM. (1991). Basic research and medicine. *Rev Med Liege* **46** (3), 115-122.

Giedraitienė A, Vitkauskienė A, Naginienė R, Pavilonis A. (2011).Antibiotic resistance mechanisms of clinically important bacteria. *Medicina* (*Kaunas*) 47(3), 137-146.

Gillings M, Boucher Y, Labbate M, Holmes A, Krishnan S, Holley M, Stokes HW.(2008).The Evolution of Class 1 Integrons and the Rise of Antibiotic Resistance. *J Bacteriol* **190** (14), 5095-5100.

Gogarten JP, Doolittle WF, Lawrence JG. (2002). Prokaryotic evolution in light of gene transfer. *Mol Biol Evol***19** (12), 2226–2238.

Gotfredsen M, Gerdes K. (1998). The *Escherichia coli* relBE genes belong to a new toxin–antitoxin gene family. *Mol. Microbiol*. **29**(4), 1065–1076.

Goyal D, Sahni G, Sahoo DK. (2009). Enhanced production of recombinant streptokinase in *Escherichia coli* using fed-batch culture. *Bioresour Technol* **100** (19), 4468-4474.

Grass G, Rensing C, Solioz M. (2011).Metallic Copper as an Antimicrobial Surface. *Applied and Environmental Microbiology* **77** (5), 1541-1547.

Greenwood D., Finch R., Davey P., Wilcox M. (2006).Antimicrobial Chemotherapy.5th edition. Oxford University Press

Griffin HG, Foster TJ, Silver S, Misra TK. (1987). Cloning and DNA sequence of the mercuric- and organomercurial-resistance determinants of plasmid pDU1358. *Proc Natl Acad Sci U S A.* **84** (10), 3112-6

Grohmann E, Muth G, Espinosa M. (2003).Conjugative plasmid transfer in gram-positive bacteria. *Microbiol Mol Biol Rev***67** (2), 277-301.

Guardabassi L and Dalsgaared A. (2002).Occurrence and fate of antibiotic resistant bacteria n sewage. .*Paper presented to Danish Environmental Protection Agency* **722**, 1-59.

Hacker J, Blum-Oehler G, Mühldorfer I, Tschäpe H. (1997). Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol Microbiol* **23** (6), 1089-97.

Hada HS, Sizemore RK. (1981).Incidence of Plasmids in Marine Vibrio spp. Isolated from an Oil Field in the Northwestern Gulf of Mexico. *Applied and Environmental Microbiology* **41**, 199-202.

Haddadin FT, Harcum SW. (2005).Transcriptome profiles for high-cell-density recombinant and wild-type *Escherichia coli. Biotechnol Bioeng* **90** (2), 127-53

Hadj Kacem B, G. J., Gargouri A. (2008).*In vitro* direct repeats-mediated deletion during PCR amplification. *Mol Biotechnol* **40**, 39-45.

Hajjar E, Bessonov A, Molitor A, Kumar A, Mahendran KR, *et al*.(2010).Toward screening for antibiotics with enhanced permeation properties through bacterial porins. *Biochemistry* **49**(32), 6928-6935

Hall RM., Collis CM. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Molecular Microbiology* **15** (4), 593-600.

Hall RM. (1997). Mobile gene cassettes and integrons: moving antibiotic resistance genes in gram-negative bacteria. *Ciba Found Symp.* **207**, 192-202

Hancock RE. (1998).Resistance mechanisms in *Pseudomonas aeruginosa* and other non-fermentative Gram-negative bacteria. *Clinical infectious diseases* **27** (1), S93-99

Hansen S, Lewis K, Vulić M. (2008).Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli. Antimicrob Agents Chemother* **52**, 2718 –2726

Harbottle H, Thakur S, Zhao S, White DG.(2006).Genetics of antimicrobial resistance. *Anim Biotechnol***17** (2), 111-124.

Harremoës, P., D. Gee, M. MacGarvin, A. Stirling, J. Keys, *et al*. (2001). Late lessons from early warnings: the precautionary principle 1896–2000. Environmental issue report 22 European Environment Agency, Copenhagen K, and Denmark. ISBN 92-9167-323-4, Environmental issue report No 22

Hart CA. (1998). Antibiotic resistance: an increasing problem? *British Medical journal* 316 (7140), 612-613

Hassen A., Saidi N., Cherif M., Boudabous A. (1998).Resistance of Environmental Bacteria to Heavy Metals. *Bioresource Technology* **64**, 7-15.

Hawkey PM, Jones AM. (2009). The changing epidemiology of resistance. *J Antimicrob Chemother* **64**: Suppl 1, i3-i10

Hayes F. (2003). Toxins-Antitoxins: Plasmid Maintenance, Programmed Cell Death, and Cell Cycle Arrest. *Science*.**301** (5639), 1496-1499.

Hayes F, Barillà D. (2006).The bacterial segrosome: a dynamic nucleoprotein machine for DNA trafficking and segregation. *Nature Reviews Microbiology* **4**(2), 133-143.

Hazan R, Sat B, Engelberg-Kulka H. (2004). *Escherichia coli* mazEF–mediated cell death is triggered by various stressful conditions; *J. Bacteriol*. **186**(11), 3663–3669

Hellinger WC, Brewer NS. (1999). Carbapenems and monobactams: imipenem, meropenem, and aztreonam. *Mayo Clinic Proc*. **74**(4), 420-434.

Henriette *et al*.(1991).Mercury reductase activity in the adaptation to cationic mercury, phenyly mercury acetate and multiple antibiotics of gram negative population isolated from aerobic fixed bed reactor. *Journal of Applied Bacteriology* **71**, 439-444.

Hochhut B, Lotfi Y, Mazel D, Faruque SM, Woodgate R, Waldor MK. (2001).Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins *Antimicrob Agents Chemother*. **45**(11), 2991–3000.

Hochhut B, Waldor MK. (1999).Site-specific integration of the conjugal Vibrio cholerae SXT element into prfC. *Mol. Microbiol*. **32**(1), 99–110.

Hodgkin DC. (1949).The X-ray analysis of the structure of penicillin. *Adv Sci*.**6** (22), 85-89.

Holt KE, Thomson NR, Wain J, Phan MD, Nair S, Hasan R, *et al*.(2007). Multidrug-resistant *Salmonella enterica* serovar paratyphi A harbours IncHI1 plasmids similar to those found in serovar typhi. *J Bacteriol***189** (11), 4257-4264.

Hölzel CS, Müller C, Harms KS, Mikolajewski S, Schäfer S, Schwaiger K, Bauer J. (2012). Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environ Res*. **113**, 21-7.

Hughes VM and Datta N. (1983).Conjugative plasmids in bacteria of the 'pre-antibiotic' era. Nature 302, 725-726

Jacoby GA. (2009). AmpC beta-lactamases. *Clin Microbiol Rev*. **22**(1), 161-82.

Jacoby GA., Munoz-Price LS.(2005).The new beta-lactamases. *N Engl J Med.* **352** (4), 380-91.

Janvier F, Jeannot K, Tessé S, Robert-Nicoud M, *et al.*(2013).Molecular characterization of *bla*$_{\text{NDM-1}}$ in a sequence type 235 *Pseudomonas aeruginosa* isolate from France. *Antimicrob Agents Chemother*. **57**(7), 3408-11

Jareonmit P, Sajjaphan K, Sadowsky MJ.(2010).Structure and diversity of arsenic resistant bacteria in an old tin mine area of Thailand. *J Microbiol Biotechnol.* 20(1):169-78

Jayaramanj R. (2008). Bacterial persistence: some new insights into an old phenomenon *Biosci*. **33**(5), 795–805

Jeong SH, Lee K, Chong Y, Yum JH, Lee SH, Choi HJ, Kim JM, *et al*.(2003).Characterization of a new integron containing VIM-2, a metallo-β-lactamase gene cassette, in a clinical isolate of *Enterobacter cloacae*. *The Journal of antimicrobial chemotherapy* **51** (2), 397-400

Jeong JH, Shin KS, Lee JW, Park EJ, Son SY.(2009).Analysis of a novel class 1 integron containing metallo-beta-lactamase gene VIM-2 in *Pseudomonas aeruginosa*. *J Microbiol* **47**(6), 753-9

Ji G, and Silver S. (1995).Bacterial resistance mechanisms for heavy metals of environmental concern. *J. Ind. Microbiol.***14**, 61-75.

Jiang, Y., Pogliano, J., Helinski, D.R., Konieczny, I. (2002). ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of Escherichia coli gyrase. *Mol. Microbiol*. **44**(4), 971–979.

Johansson L, Lidén G. (2006).A study of long-term effects on plasmid-containing *Escherichia coli* in carbon-limited chemostat using 2D-fluorescence spectrofluorimetry. *Biotechnol Prog* **22** (4), 1132-1139.

Jones KL, Kim Sw., Keasling JD. (2000). Low-copy plasmids can perform as well as or better than high-copy plasmids for metabolic engineering of bacteria. *Metab Eng* **2**, 328-338.

Jovcic B, Lepsanovic Z, Suljagic V, Rackov G, Begovic J, *et al*. (2011).Emergence of NDM-1 metallo b-lactamase in Pseudomonas aeruginosa clinical isolates. *Antimicrob Agents Chemother* **55** (8), 3929–3931.

Juan C, Zamorano L, Mena A, Albertí S, Pérez JL, Oliver A. (2010). Metallo-b-lactamase-producing *Pseudomonas putida* as a reservoir of multidrug resistance elements that can be transferred to successful *Pseudomonas aeruginosa* clones .*J Antimicrob Chemother***65** (3), 474–478.

Kaase M, Nordmann P, Wichelhaus TA, Gatermann SG, Bonnin RA, Poirel L.(2011).NDM-2 carbapenemase in *Acinetobacter baumannii* from Egypt. *J Antimicrob Chemother* **66**(6), 1260-2

Kapil A. (2005). The challenge of antibiotic resistance: need to contemplate. *Indian J Med Res* **121**(2), 83-91

Karbasizaed V., Badami N., Emtiazi G. (2003). Antimicrobial, heavy metal resistance and plasmid profile of coliforms isolated from nosocomial infections in a hospital in Isfahan, Iran. *Afr. J. Biotechnol***. 2** (10), 379-383

Katz ME, Wright CL, Gartside TS, Cheetham BF, Doidge CV, Moses EK, Rood JI.(1994).Genetic organization of the duplicated vap region of the Dichelobacter nodosus genome. *J Bacteriol*.**176**(9):2663-9.

Khan AU, Nordmann P. (2012). Spread of carbapenemase NDM-1 producers: the situation in India and what may be proposed. *Scand J Infect Dis* **44**(7), 531-5.

Khajuria A, Praharaj AK, Kumar M, Grover N. (2013). Emergence of NDM - 1 in the Clinical Isolates of Pseudomonas aeruginosa in India. *J Clin Diagn Res* **7**(7), 1328-31

Kholodiĭ GIa. (1995). Inversion activity in the resolution of transposons Tn*5053* and Tn402 possessing a non-standardly organized res-region. *Genetika***31** (12), 1698-703.

Kim Y, Wood TK. (2009). Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*. *Biochemical and Biophysical Research Communications* **391** (1), 209-213.

Kiyono M, Pan-Hou H. (1999).The merG gene product is involved in phenylmercury resistance in *Pseudomonas* strain K-62.*J Bacteriol* **181** (3), 762–730.

Kiyono M, Sone Y, Nakamura R, Pan-Hou H, Sakabe K. (2009). The MerE protein encoded by transposon Tn*21* is a broad mercury transporter in *Escherichia coli*. *FEBS Lett* **583** (7), 1127–1131.

Knapp CW, McCluskey SM, Singh BK, Campbell CD, Hudson G, Graham DW.(2011).Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. *PLoS One.* 6(11):e27300

Kollek R, Oertel W, Goebel W. (1978).Isolation and characterization of the minimal fragment required for autonomous replication ("basic replicon") of a copy mutant (pKN102) of the antibiotic resistance factor R1.*Mol Gen Genet*. **162**(1):51-7.

Korch SB, Hill TM. (2006). Ectopic overexpression of wild-type and mutant *hip*A genes in *Escherichia coli*: effects on macromolecular synthesis and persister formation. *J. Bacteriol* **188** (11), 3826–3836.

Krishna Rao DV, Ramu CT, Rao JV, Narasu ML, Bhujanga Rao AK. (2008).Impact of dissolved oxygen concentration on some key parameters and production of rhG-CSF in batch fermentation. *J Ind Microbiol Biotechnol* **35** (9), 991-1000.

Krulwich TA, Lewinson O, Padan E, Bibi E. (2005).Do physiological roles foster persistence of drug/multidrug- efflux transporters? A case study. *Nat. Rev. Microbiol* **3** (7), 566-572.

Kumar A, Schweizer HP. (2005). Bacterial resistance to antibiotics: active efflux and reduced uptake. *Adv Drug Deliv Rev*, **57**(10), 1486-1513.

Kumar PK, Maschke HE, Friehs K, Schügerl K. (1991).Strategies for improving plasmid stability in genetically modified bacteria in bioreactors. *Trends Biotechnol* **9**(8), 279-84

Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, *et al.* (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis* **10** (9), 597–602.

Kunz AN, Brook I. (2010) Emerging Resistant Gram-Negative Aerobic Bacilli in Hospital-Acquired Infections. *Chemotherapy* **56** (6), 492-500.

Labbate M, Roy Chowdhury P, Stokes HW. (2008). A Class 1 Integron Present in a Human Commensal Has a Hybrid Transposition Module Compared to Tn402: Evidence of Interaction with Mobile DNA from Natural Environments. *J Bacteriol* **190** (15), 5318-5327.

Labbate M, Case RJ, Stokes HW. (2009).The integron/gene cassette system: an active player in bacterial adaptation. *Methods Mol Biol* **532**, 103-125.

Lafleur MD, Qi Q, Lewis K. (2010). Patients with long-term oral carriage harbour high-persister mutants of *Candida albicans. Antimicrob Agents Chemother***54** (1), 39-44.

Laila M. Abdelatey, Wagdy K. B. Khalil, Thanaa H. Ali, Karima F. Mahrous. (2011). Heavy metals resistance and gene expression analysis of metal resistance genes in gram – positive and gram-negative bacteria present in Egyptian soils. *Journal of applied sciences in environmental sanitation* **6** (2), 201-211

Laupland KB, Church DL, Vidakovich J, Mucenski M, Pitout JD.(2008).Community onset extended-spectrum beta lactamase (ESBL) producing *Escherichia coli:* importance of international travel. *J Infect* **57** (6), 441-448.

Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, *et al*.(1999).Cloning and characterization of *bla*$_{VIM}$, a new integron-borne metallo-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. *Antimicrob Agents Chemother* **43**(7), 1584–1590

Lawrence JG. (2000).Clustering of antibiotic resistance genes: beyond the selfish operon. *ASM News***66** (5), 281-286.

Lechner S, Lewis K, Bertram R. (2012).*Staphylococcus aureus* persisters tolerant to bactericidal antibiotics. *J. Mol. Microbiol. Biotechnol* **22** (4), 235–244.

Lederberg J. (1952). Cell genetics and hereditary symbiosis. *Physiol. Rev.***32** (4), 403–430.

Lederberg J, Tatum EL. (1946). Gene recombination in *Escherichia coli*.*Nature*.19; 158(4016):558.

Lehnherr H, Yarmolinsky M. (1995). Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.* **92**(8), 3274–3277

Leibovici L, Shraga I, Drucker M, Konigsberger H, *et al*. (1998).The benefit of appropriate empirical antibiotic treatment in patients with bloodstream infection. *J Intern Med.* **244** (5), 379–386.

Leplae R, Geeraerts D, Hallez R, Guglielmini J, Drèze P, Van Melderen L. (2011). Diversity of bacterial type II toxin-antitoxin systems: a comprehensive search and functional analysis of novel families. *Nucleic Acids Res*., **39**(13), 5513–5525.

Lesche J. (2007). The first miracle drugs: how the sulfa drugs transformed medicine. Chapter 3: Prontosil. Oxford University Press: 51.

Levin BR, Rozen DE. (2006).Non-inherited antibiotic resistance. *Nat Rev Microbiol* **4** (7), 556-562.

Levy SB. (1998).The challenge of antibiotic resistance. *Scientific American* **278**, 32-39.

Levy SB, Marshall B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature medicine* **10** (12 Suppl), S122-129

Lewis K.(2010). Persister cells. *Annu Rev Microbiol* **64**, 357-372.

Li XZ, Nikaido H. (2009). Efflux-mediated drug resistance in bacteria: An update. *Drugs* **69**(12), 1555-1623.

Liebert CA, Hall RM, Summers AO. (1999).Transposon Tn*21*, flagship of the floating genome. *Microbiol* 63(3):507-22.

Lim HM, Pène JJ, Shaw RW. (1988).Cloning, nucleotide sequence, and expression of the Bacillus cereus 5/B/6 β-lactamase II structural gene. *J.Bacteriol* **170**(6), 2873–2878

Lin CC., Yee N., Barkay T. (2012). Microbial transformations in the mercury cycle, in Environ-mental Chemistry and Toxicology of Mercury, edsC.Liu, C.Yongand N. O'driscoll (Hoboken, NJ: JohnWiley & Sons, Inc.), 155–191.

Lin CJ, Siu LK, Ma L, Chang YT, Lu PL.(2012).Molecular epidemiology of ciprofloxacin-resistant extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in Taiwan. *Microb Drug Resist* **18**(1), 52-58.

Lister PD, Wolter DJ, Hanson ND. (2009). Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev***. 22**(4), 582-610

Liu M, Zhang Y, Inouye M, Woychik NA. (2008). Bacterial addiction module toxin Doc inhibits translation elongation through its association with the 30S ribosomal subunit. *Proc. Natl. Acad. Sci. U.S.A.* **105**(15), 5885–5890.

Livermore DM. (1995).β-Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev* **8**(4), 557-584.

Livermore DM, Andrews JM, Hawkey PM, Ho PL, Keness Y, *et al.* (2012).Are susceptibility tests enough, or should laboratories still seek ESBLs and carbapenemases directly? *J Antimicrob Chemother* **67**(7), 1569-77.

López-Maury L, Florencio FJ, Reyes JC. (2003). Arsenic sensing and resistance system in the *Cyanobacterium synechocystis* sp. strain PCC 6803. *J.Bacteriol* **185**(18), 5363-5371.

López-Maury L, García-Domínguez M, Florencio FJ, Reyes JC. (2003). A two-component signal transduction system involved in nickel sensing in the Cyanobacterium *synechocystis* sp. PCC 6803.*Mol Microbiol* **43**(1),247-56.

Lupo A, Coyne S, Berendonk TU. (2012). Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Front Microbiol*. 26, 3:18.

Macheboeuf P, Contreras-Martel C, Job V, Dideberg O, Dessen A. (2006).Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. *FEMS Microbiol Rev* **30**(5), 673-691.

Magnuson RD. (2007).Hypothetical functions of toxin-antitoxin systems. *J Bacteriol* **189**(17), 6089-92

Mahillon J., Chandler M. (1998).Insertion sequences. *Microbiol. Mol. Biol. Rev* **62**, 725–774.

Majiduddin FK, Materon IC, Palzkill TG. (2002).Molecular analysis of β-lactamase structure and function. *Int J Med Microbiol* **292**(2), 127-137.

Makarova KS, Wolf YI, Koonin EV. (2009).Comprehensive Analysis of Type 2 Toxin-Antitoxin Systems and Related Mobile Stress Response Systems in Prokaryotes. *Biol Direct* **4**(19), 1-38.

Malik A., M. Ahmad. (1994).Incidence of drug and metal resistance in *E. coli* strains from sewage water and soil. *Chemical and Environmental Research* 3, 3-11.

Malik A., Jaiswal R. (2000).Metal Resistance in *Pseudomonas* strains isolated from soil treated with industrial wastewater. *World Journal and Microbiology and Biotechnology*, **16**, 177-182.

Marchaim D, Navon-Venezia S, Schwaber MJ, Carmeli Y. (2008). Isolation of imipenem-resistant Enterobacter species: emergence of KPC-2 carbapenemase, molecular characterization, epidemiology, and outcomes. *Antimicrob Agents Chemother* **52**(4), 1413-1418.

Marchiaro, P., Viale, AM., Ballerini, V., Rossignol, G., *et al*. (2010).First report of a Tn*402*-like class 1 integron carrying *bla*$_{\text{VIM-2}}$ in *Pseudomonas putida* from Argentina. *J Infect Dev Ctries* **4**(6) 412-416.

Margesin R., *et al*. (1996).Bacterial heavy metal-tolerance - extreme resistance to nickel in *Arthrobacter* spp. strains. *J. Basic Microbiol*. **36,** 269-282

Martins A, Hunyadi A, Amaral L. (2013).Mechanisms of Resistance in Bacteria: An Evolutionary Approach. *The Open Microbiology Journal* **7**, 53-58.

Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. (2000).Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **44**(12), 3322-3327.

Máthé I., Benedek T., Táncsics A., Palatinszky M., *et al*. (2012). Diversity, activity, antibiotic and heavy metal resistance of bacteria from petroleum hydrocarbon contaminated soils located in Harghita County (Romania*) International Biodeterioration & Biodegradation* **73**, 41-49

Matsui T, Sato H, Sato S, Mukataka S, Takahashi J. (1990).Effects of nutritional conditions on plasmid stability and production of tryptophan synthase by a recombinant *Escherichia coli*. *Agric Biol Chem* **54**, 619–624.

McArthur JV, Tuckfield RC. (2000).Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. *Appl Environ Microbiol* **66**, 3722-3726.

McDonnell G, R. AD. (1999).Antiseptics and disinfectants: activity, action and resistance. *Clin Microbiol Rev* **12**, 147-179.

McHugh GL, Moellering RC, Hopkins CC, Swartz MN.(1975).*Salmonella tphimuriumn* resistant to silver nitrate chloramphenicol and ampicillin. *Lancet* **1**(7901), 235-40

McGowan JE Jr. (2006). Resistance in non-fermenting gram-negative bacteria: multidrug resistance to the maximum. *Am J Med* **119**(6 Suppl 1):S29-36

McMahon MA, Blair IS, Moore JE, McDowell DA. (2007).The rate of horizontal transmission of antibiotic resistance plasmids is increased in food preservation-stressed bacteria. *J. Appl Microbiol.*103(5):1883-8.

Mergeay M., Nies D., Schlegel HG, Gerits J., P. Charles P., Gijsegem FV. (1985). *J. of Bacteriology*.162, 328-334.

Mesaros N, Nordmann P, Plésiat P, Roussel-Delvallez M, Van Eldere J, *et al*. (2007). *Pseudomonas aeruginosa*: resistance and therapeutic options in the turn of the new millennium. *Clin Microbiol Infect* **13**(6), 560–578.

Minakhina S, Kholodii G, Mindlin S, Yurieva O, Nikiforov V. (1999). Tn*5053* family transposons are res site hunters sensing plasmidal res sites occupied by cognate resolvases. *Mol Microbiol* **33**(5), 1059-1068.

Mine N, Guglielmini J, Wilbaux M, Van Melderen L.(2009).The decay of the chromosomally encoded ccdO157 toxin-antitoxin system in the *Escherichia coli* species.*Genetics*.181(4), 1557-66.

Mirelman D, Bracha R, Sharon N. (1974).Penicillin-induced secretion of soluble, uncross-linked peptidoglycan by *Micrococcus luteus* cells. *Biochemistry* **13**(24), 5045-5053.

Mirnejad R, Mostofi S, Masjedian F, Fooladi AA. (2013). Antibiotic resistance and carriage class 1 and 2 integrons in clinical isolates of *Acinetobacter baumannii* from Tehran, Iran. *Asian Pac J Trop Biomed* **3**(2), 140-145.

Misra TK. (1992).Bacterial resistance to inorganic mercury salts and organomercurials. *Plasmid* **27** (1), 4–16.

Mnif B, Vimont S, Boyd A, Bourit E, Picard B, *et al*. (2010).Molecular characterization of addiction systems of plasmids encoding extended-spectrum b-lactamases in *Escherichia coli. J Antimicrob Chemother* **65**, 1599–1603.

Moken MC, McMurry LM, Levy SB. (1997).Selection of multiple –antibiotic resistant (mar) mutants of Escherichia coli by using the disinfectant pine oil: roles of the mar and acrAB loci. *Antimicrobial Agents and Chemotherapy* **41**(12), 2770- 2772

Moore JW., *et al*. (1984).Heavy Metals in Natural Waters. Applied Monitoring and Impact Assessment, Springer Verlag, New York. ISBN 0-38790885-4.

Moreno-Córdoba I, Diago-Navarro E, Barendregt A, Heck AJ, *et al*. (2012).The toxin-antitoxin proteins relBE2Spn of *Streptococcus pneumoniae*: characterization and association to their DNA target. *Proteins* **80** (7), 1834-46.

Moritz EM, Hergenrother PJ. (2007). Toxin–antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci. *Proc Natl Acad Sci USA***104** (1), 311–316

Morozzi G, Cenci G, Caldini G.(1982).The tolerance of an environmental strain of *Escherichia coli* to some heavy metals. *Zentralbl Bakteriol Mikrobiol Hyg B* **176**(1), 55-62.

Motiejūnaite R, Armalyte J, Markuckas A, Suziedeliene E. (2007. *Escherichia coli* dinJ-yafQ genes act as a toxin-antitoxin module. *FEMS Microbiol. Lett* **268 (**1), 112–119

Moya B, Dötsch A, Juan C, Blázquez J, Zamorano L, Haussler S, Oliver A. (2009).β-Lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. *PLoS Pathog* **5**(3), e1000353

Moyed HS, Bertrand KP. (1983).HipA, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J. Bacteriol*. **155**(2), 768–775.

Mruk I, Kobayashi I. (2014).To be or not to be: regulation of restriction– modification systems and other toxin–antitoxin systems. *Nucleic Acids Res*. **42**(1), 70-86.

Muela, A., Pocino, M., Arana, I., Justo, J.I., Iriberri, J. and Barcina, I.(1994).Effect of growth phase and parental cell survival in river water on plasmid transfer between *Escherichia coli* strains. *Appl Environ Microbiol* (60) 4273–4278.

Muir A, Weinbren MJ. (2010). New Delhi metallo-b-lactamase: a cautionary tale. *J. Hosp. Infect*. **75**(3), 236–246.

Mulcahy LR, B. J., Lory S, Lewis K. (2010).Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. *J. Bacteriol.*192, 6191–6199.

Muñoz-Gómez AJ, Lemonnier M, Santos-Sierra S, *et al*.(2005).RNase/anti-RNase activities of the bacterial parD toxin-antitoxin system. *J Bacteriol* **187** (9) 3151-3157.

Murray PR, Rosenthal KS, Pfaller MA. (2009).Medical Microbiology, Mosby Elsevier, 7th Edition.

Naas T, Bellais S, Nordmann P. (2003).Molecular and biochemical characterization of a carbapenem-hydrolysing beta-lactamase from *Flavobacterium johnsoniae. J Antimicrob Chemother* **51**(2), 267-273.

Nagy Z, Chandler M. (2004).Regulation of transposition in bacteria. *Res Microbiol* **155** (5), 387-398.

Nakaya R, Nakamura A, Murata Y. (1960). Resistance transfer agents in *Shigella*. *Biochem Biophys Res Commun* **3**, 654-659.

Nanda M., Sharma D., Kumar A. (2011).Removal of Heavy Metals from Industrial Effluent Using Bacteria. *International Journal of Environmental Sciences* **2**(2), 781-787

Nandi S, Maurer JJ, Hofacre C, Summers AO. (2004). Gram-positive bacteria are a major reservoir of Class 1 antibiotic resistance integrons in poultry litter. *Proc Natl Acad Sci U S A.***101** (18), 7118-22

Nazir T, Abraham S, Islam A. (2012). Emergence of potential superbug mycobacterium tuberculosis, lessons from New Delhi mutant-1 bacterial strains. *Int J Health Sci (Qassim)* **6**(1), 87-94.

Nesvera J, Hochmannová J, Pátek M. (1998). An integron of class 1 is present on the plasmid pCG4 from gram-positive bacterium *Corynebacterium glutamicum*. *FEMS Microbiol Lett* **169**(2), 391-395.

Ni'Bhriain NN, Silver S, Foster TJ. (1983).Tn5 insertion mutations in the mercuric ion resistance genes derived from plasmid R100. *J Bacteriol* **155**(2), 690–703.

Nicolau DP. (2008).Carbapenems: a potent class of antibiotics. *Expert Opin Pharmacother* **9**(1), 23-37

Nies DH. (1999).Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* **51**(6), 730-50

Nies DH, Silver S. (1995).Ion efflux systems involved in bacterial metal resistances. *J Ind. Microbiol.***14**(2):186-99.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and molecular biology reviews* 67(4), 593-656

Nikaido H, Pagès JM. (2012).Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol Rev* **36**(2), 340-363.

Ning D, Jiang Y, Liu Z, Xu Q. (2013).Characterization of a chromosomal type II toxin-antitoxin system mazEaFa in the Cyanobacterium Anabaena sp. PCC 7120.*PLoS One***8** (2), e56035.

Nordmann P, Boulanger AE, Poirel L. (2012).NDM-4 Metallo-B-Lactamase with Increased Carbapenemase Activity from *Escherichia coli. Antimicrob Agents Chemother* **56**(4), 2184-6

Nordmann P, Naas T, Poirel L. (2011a).Global spread of carbapenemase producing Enterobacteriaceae. *Emerg Infect Dis* **17**(10), 1791–1798.

Nordmann P, Poirel L, Toleman MA, Walsh TR. (2011b). Does broad-spectrum resistance due to NDM-1 herald the end of the antibiotic era for treatment caused by Gram negative bacteria? *J. Antimicrob. Chemother* **66**(4), 689–692.

Nordström K. (2006). Plasmid R1--replication and its control. *Plasmid* **55**(1), 1-26.

Norman A, Hansen LH, She Q, Sørensen SJ. (2008). Nucleotide sequence of pOLA52: a conjugative IncX1 plasmid from *Escherichia coli* which enables biofilm formation and multidrug efflux. *Plasmid* **60**(1), 59–74.

Norman, A., Hansen, LH, Sørensen, SJ. (2009).Conjugative plasmids: vessels of the communal gene pool. *Phil Trans R Soc* B 364, 2275–2289

Nweke CO., Alisi CS., Okolo JC, Nwanyanwu CE.(2007).Toxicity of zinc to heterotrophic bacteria from tropical river sediment. *Applied Environ. Res*. **5**(1), 123-132.

Nyamboya RA., Okemo PO., Ombori O. (2013). Heavy metal and associated antibiotic resistance of fecal coliforms, fecal streptococci and pathogens isolated from wastewaters of abattoirs in Nairobi, Kenya. *J. Appl. Biosci*. **64**, 4858 – 4866.

Obritsch MD, Fish DN, MacLaren R, Jung R. (2004).National surveillance of antimicrobial resistance in *Pseudomonas aeruginosa* isolates obtained from intensive care unit patients from 1993 to 2002*Antimicrob Agents Chemother.* **48**(12), 4606–4610.

O'Kennedy RD, Patching JW. (1997).Effects of medium composition and nutrient limitation on loss of the recombinant plasmid pLG669-z and beta-galactosidase expression by Saccharomyces cerevisiae. *J Ind Microbiol Biotechnol* **18**(5), 319-325.

Olivares J, Bernardini A, Garcia-Leon G, Corona F, *et al*.(2013).The intrinsic resistome of bacterial pathogens. *Frontiers in Microbiology* **4**(103), pp 1-45

Oliveira PH, Prather KJ, Prazeres DM, Monteiro GA. (2009).Structural instability of plasmid biopharmaceuticals: challenges and implications. *Trends Biotechnol* **27**(9), 503–511.

Osborn AM, Bruce KD, Strike P, Ritchie DA. (1997).Distribution, diversity and evolution of bacterial mercury resistance (mer) operon. *FEMS Microbiol Rev*. **19**(4), 239–262.

Overgaard M, Borch J, Gerdes K. (2009). RelB and RelE of Escherichia coli form a tight complex that represses transcription via the ribbon–helix–helix motif in RelB. *J. Mol. Biol*. **394**(2), 183–196.

Page MG, Dantier C, Desarbre E. (2010).*In vitro* properties of BAL30072, a novel siderophore sulfactam with activity against multiresistant gram-negative bacilli. *Antimicrob Agents Chemother* **54**(6), 2291-302

Pandey DP., Gerdes K. (2005).Toxin–antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Res.* **33**(3), 966–976.

Pan-Hou HS, Nishimoto M, Imura N.(1981).Possible role of membrane proteins in mercury resistance of Enterobacter aerogenes. *Arch Microbiol* **130**(2), 93-95.

Pappas KM. (2008). Cell-cell signalling and the *Agrobacterium tumefaciens* Ti plasmid copy number fluctuations. *Plasmid*.**60** (2), 89-107.

Pappas KM, Winans SC. (2003). The RepA and RepB autorepressors and TraR play opposing roles in the regulation of a Ti plasmid repABC operon. *Mol Microbiol* **49**(2), 441-55

Partridge SR, Brown HJ, Stokes HW, Hall RM. (2001).Transposons Tn*1696* and Tn*21* and Their Integrons In*4* and In*2* Have Independent Origins. *Antimicrob Agents Chemother* **45**(4), 1263-1270

Paterson DL. (2006).Clinical experience with recently approved antibiotics. *Curr Opin Pharmacol* **6**(5), 486–490

Paterson DL, Depestel DD. (2009).Doripenem. *Clin Infect Dis*. 15; 49(2):291-8.

Patzer J, Toleman MA, Deshpande LM, Kamińska W, Dzierzanowska D, *et al*. (2004).*Pseudomonas aeruginosa* strains harbouring an unusual *bla*$_{\text{VIM-4}}$ gene cassette isolated from hospitalized children in Poland (1998–2001). *J Antimicrob Chemother* **53**, 451–456.

Pedersen K, Gerdes K. (1999).Multiple hok genes on the chromosome of Escherichia coli. *Mol. Microbiol*. **32**(5), 1090–1102.

Pedersen K, Zavialov AV, Pavlov MY, Elf J, Gerdes K, Ehrenberg M. (2003).The bacterial toxin RelE displays codonspecific cleavage of mRNAs in the ribosomal A site. *Cell* **112**(1), 131–140.

Permina EA, Kazakov AE, Kalinina OV, Gelfand MS. (2006).Comparative genomics of regulation of heavy metal resistance in Eubacteria. *BMC Microbiol***6**, 49–60.

Perry JD, Naqvi SH, Mirza IA, Alizai SA, Hussain A, Ghirardi S, *et al*. (2011).Prevalence of faecal carriage of Enterobacteriaceae with NDM-1 carbapenemase at military hospitals in Pakistan, and evaluation of two chromogenic media. *J Antimicrob Chemother* **66**(10), 288–294.

Petrova M, Gorlenko Z, Mindlin S. (2011). Tn*5045*, a novel integron-containing antibiotic and chromate resistance transposon isolated from a permafrost bacterium. *Res Microbiol***162** (3), 337-345

Peymani A, Farajnia S, Nahaei MR, Sohrabi N, *et al.* (2012).Prevalence of Class 1 Integron among Multidrug-Resistant *Acinetobacter baumannii* in Tabriz, Northwest of Iran. *Polish Journal of Microbiology* **61** (1), 57–60.

Pfaller MA, Jones RN. (1997).A review of the in vitro activity of meropenem and comparative antimicrobial agents tested against 30,254 aerobic and anaerobic pathogens isolated worldwide. *Diagn Microbiol Infect Dis* **28** (4), 157–163.

Pfeifer Y, Cullik A, Witte W. (2010). Resistance to cephalosporins and carbapenems in Gram-negative bacteria pathogens. *Int J Med Microbiol* **300**(6), 371–379.

Philippon A, Arlet G, Jacoby GA. (2002).Plasmid-determined AmpC-type beta-lactamases. *Antimicrob Agents Chemother* **46**(1):1-11.

Piéboji JG, Koulla-Shiro S, Ngassam P, Adiogo D, Njine T, Ndumbe P. (2004). Antimicrobial resistance of Gram-negative bacilli isolates from inpatients and outpatients at Yaounde Central Hospital, Cameroon. *Int J Infect Dis*. **8**(3), 147-54.

Pillai DR., McGeer A., Low DE. (2011).New Delhi metallo-β-lactamase-1 in Enterobacteriaceae: emerging resistance. *CMAJ* **183**(1), 59-64.

Pitout JD.(2010) Infections with Extended-spectrum-β-lactamase-producing Enterobacteriaceae changing epidemiology and drug treatment Choices. *Drugs* **70**(3), 313-333.

Poiată A, Bădicuţ I, Indreş M, Biro M, Buiuc D.(2000).Mercury resistance among clinical isolate of *Escherichia coli*. *Roum Arch. Microbiol Immunol* **59**(1-2), 71-79.

Poirel L, Collet L, Nordmann P. (2000).Carbapenem-hydrolyzing metallo- lactamase from a nosocomial isolate of Pseudomonas aeruginosa in France. *Emerg Infect. Dis*.**6** (1), 84–85.

Poirel L, H. A. C., Freneaux C, Bernabeu S, Nordmann P. (2010a). Global spread of New Delhi metallo-lactamase 1.*Lancet Infect Dis***10**, 832.

Poirel L, Nordmann P. (2006).Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clin Microbiol Infect*. **12**(9), 826-36

Poirel L, Naas T, Nordmann P. (2010b).Diversity, epidemiology, and genetics of class D beta-lactamases. *Antimicrob Agents Chemother* **54**(1), 24-38.

Poirel L, Dortet L, Bernabeu S, Nordmann P. (2011).Genetic features of bla_{NDM-1}–positive Enterobacteriaceae. *Antimicrob Agents Chemother* **55**(11), 5403–5407.

Poole K. (2011).*Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol*. **2**(65), 1-13.

Possoz C, Ribard C, Gagnat J, Pernodet JL, Guérineau M.(2001). The integrative element pSAM2 from Streptomyces: kinetics and mode of conjugal transfer. *Mol Microbiol*.**42**(1):159-66.

Post V, Recchia GD, and Hall RM. (2007) .Detection of gene cassettes in Tn*402*-like class 1 integrons. *Antimicrob Agents Chemother* **51**(9), 3467-3468

Potron A, Poirel L, Nordmann P. (2011).Plasmid-mediated transfer of the *bla* ($_{NDM-1}$) gene in Gram-negative rods. *FEMS Microbiol Lett.* 324(2):111-6.

Primrose SB, Ehrlich SD. (1981).Isolation of plasmid deletion Mutants and study of their instability. *Plasmid* **6**(2), 193-201.

Projan SJ, Shlaes DM. (2004). Antibacterial drug discovery: is it all downhill from here? *Clin Microbiol Infect*. **10**, 18-22

Queenan AM, Bush K. (2007).Carbapenemases: the versatile β-lactamases. *Clin Microbiol Rev*. **20** (3), 440–458.

Quirke VM. (2001). History of Penicillin. Encyclopedia of life sciences. Oxford Brookes Business School, Wheatley, UK

Rademacher C, Masepohl B. (2012).Copper-responsive gene regulation in bacteria. *Microbiology* **158**(Pt 10), 2451-2464.

Rådström P, Sköld O, Swedberg G, Flensburg J, Roy PH, Sundström L. (1994).Tn*5090* of plasmid R751, which carries an integron, is related to Tn*7*, Mu, and the retroelements. *J Bacteriol* **176**(11), 3257-3268.

Rai MK, Deshmukh SD, Ingle AP, Gade AK. (2012).Silver nanoparticles: the powerful nanoweapon against multidrug resistant bacteria. *J Appl Microbiol* **112**(5), 841–852

Rajini Rani, DB, Mahadevan A (1989). Plasmid encoded metal resistance in bacteria. *J Sci Ind Res* **48**, 338–345.

Rani DB, Mahadevan A. (1993).Patterns of heavy metal resistance in marine *Pseudomonas* MR1.*Indian J Exp Biol*. **31**(8):682-6.

Rasmussen BA, Gluzman Y, Tally FP. (1990). Cloning and sequencing of the class B beta-lactamase gene (ccrA) from Bacteroides fragilis TAL3636. *Antimicrob Agents Chemother* **34**(8):1590-1592.

Ren D, Walker AN, Daines DA. (2012). Toxin-antitoxin loci vapBC-1 and vapXD contribute to survival and virulence in non-typeable *Haemophilus influenza*. *BMC Microbiology* **12**(263), 1-12.

Riccio ML, P. L., Fontana R, Rossolini GM. (2001).In70 of plasmid pAX22, a *bla*$_{\text{VIM-1}}$-containing integron carrying a new aminoglycoside phosphotransferase gene cassette. *Antimicrob Agents Chemother***45**, 1249–1253.

Robson J, McKenzie JL, Cursons R, Cook GM, Arcus VL. (2009).The vapBC Operon from Mycobacterium smegmatis is an Autoregulated Toxin-Antitoxin Module that Controls Growth via Inhibition of Translation. *J Mol Biol*, **390**(3), 353-367.

Rotem E, L. A., Ronin I, Levin-Reisman I, Gabay C, *et al.* (2010). Regulation of Phenotypic Variability by a Threshold-Based Mechanism Underlies Bacterial Persistence. *Proceedings of the National Academy of Sciences* **107**(28), 12541-12546.

Rowe-Magnus DA, Mazel D. (1999).Resistance gene capture. *Current opinion in microbiology* **2**(5), 483-488

Roy S, Singh AK, Viswanathan R, Nandy RK, Basu S. (2011).Transmission of imipenem resistance determinants during the course of an outbreak of NDM-1 Escherichia coli in a sick newborn care unit. *J Antimicrob Chemother* **66**(12), 2773-80.

Sacha P, Wieczorek P, Hauschild T, Zórawski M, Olszańska D, Tryniszewska E. (2008).Metallo-β-lactamases of *Pseudomonas aeruginosa*-a novel mechanism resistance to β-lactam antibiotics. *Folia Histochem Cytobiol* **46**(2), 137-142.

Sahni SK. (2011).Hazardous Metals and Minerals Pollution in India: sources, toxicity and management. A Position Paper, Indian National Science Academy E-mail: angkor@rediffmail.com

Said WA, Lewis DL. (1991).Quantitative assessment of the effects of metals on microbial degradation of organic chemicals. *Appl Environ Microbiol*.**57**(5):1498-503.

Sambrook J., Fritsch EF., Maniatis T. (1989) Molecular Cloning: A Laboratory Manual, (second Eds.) Cold Spring Harbor, New York. Cold Spring Harbor Laboratory Press.545

Samuelsen Q., Toleman MA, Sundsfjord A., Rydberg J., Leegaard TM, *et al*. (2010). Molecular Epidemiology of Metallo-B-Lactamase-Producing *Pseudomonas aeruginosa* Isolates from Norway and Sweden Shows Import of International Clones and Local Clonal Expansion. *Antimicrob Agents Chemother* **54**(1), 346-352.

Santos C, Caetano T, Ferreira S, Mendo S.(2010).Tn5090-like class 1 integron carrying bla_{VIM-2} in a *Pseudomonas putida* strain from Portugal. *Clinical Microbiology and Infectious Diseases*, **16**(10), 1558–1561.

Saradhi BN. (2012).Structural and biochemical investigation of Metallo-β-lactamases; Insights into the antibiotic binding sites. PhD, thesis, University Hospital of North Norway

Sberro H, Leavitt A, Kiro R, Koh E, Peleg Y, Qimron U, Sorek R. (2013).Discovery of functional toxin/antitoxin systems in bacteria by shotgun cloning. *Mol Cell* **50**(1), 136-48

Schafer A., Kalinowski J., Puhler A. (1994) Increased fertility of *Corynebacterium glutamicum* recipients in intergeneric matings with *Escherichia coli* after stress exposure. *Appl Environ Microbiol*. 60, 756–759

Schumacher MA., Piro KM, Xu W., Hansen S, Lewis K, Brennan RG. (2009).Molecular mechanisms of HipA-mediated multidrug tolerance and its neutralization by HipB. *Science.*323**:**396-401.

Schuster CF, P. J., Prax M, Herbig A, Nieselt K, *et al*.(2013).Characterization of a mazEF toxin-antitoxin homologue from *Staphylococcus equorum*. *J Bacteriol* **195**(1), 115-125.

Seema K, Ranjan Sen M, Upadhyay S, Bhattacharjee A. (2011).Dissemination of the New Delhi metallo-beta-lactamase-1(NDM-1) among Enterobacteriaceae in a tertiary referral hospital in north India. *J. Antimicrob. Chemother* **66**(7), 1646–1647.

Seiler C, Berendonk TU (2012).Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Front Microbiol*. 2012 Dec 14; 3:399

Selvi, AT., Anjugam, E., Devi, RA., Madhan, B., *et al*.(2012).Isolation and Characterization of Bacteria from Tannery Effluent Treatment Plant and Their Tolerance to Heavy Metals and Antibiotics. *Asian J. Exp. Biol. Sci*. **3**(1), 34 – 41.

Sengupta M, Austin S. (2011).Prevalence and significance of plasmid maintenance functions in the virulence plasmids of pathogenic bacteria. *Infect Immun* **79**(7), 2502-2509.

Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, Lewis K. (2006). Persisters: a distinct physiological state of *E. coli*. *BMC Microbiol* **6**(53), 1-9

Shao Y, Harrison EM, Bi D, Tai C, He X, Ou HY, Rajakumar K, Deng Z. (2011). TADB: a web-based resource for Type 2 toxin-antitoxin loci in bacteria and archaea. *Nucleic Acids Res*. **39**, D606–D611.

Sharma SK, Mohan A. (2006). Multidrug –resistant tuberculosis: a menace that threatens to destabilize tuberculosis control. *Chest* 1**30**(1), 261-272.

Sharpe M. (2003).High on pollution: drugs as environmental contaminants. *J Environ Monit* **5**(3), 42N-46N.

Sherburne CK, Lawley TD, Gilmour MW, Blattner FR, Burland V, *et al.*(2000).The complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. *Nucleic Acids Res*. **28**(10), 2177-86.

Shih YL, Rothfield L. (2006).The bacterial cytoskeleton. *Microbiol Mol Biol Rev***70** (3), 729-754.

Siarkou VI, Vitti D, Protonotariou E, Ikonomidis A, Sofianou D.(2009). Molecular epidemiology of outbreak-related *Pseudomonas aeruginosa* strains carrying the novel variant $bla_{\text{VIM-17}}$ metallo-beta-lactamase gene. *Antimicrob Agents Chemother* **53**(4), 1325-1330.

Silva F., Queiroz JA, Domingues FC. (2012a).Evaluating metabolic stress and plasmid stability in plasmid DNA production by *Escherichia coli*. *Biotechnology Advances*. 30(3), 691–708

Silva F, Queiroz JA, Domingues FC. (2012b).Plasmid DNA fermentation strategies: influence on plasmid stability and cell physiology. *Appl Microbiol Biotechnol* **93**(6), pp 2571-80

Silvaggi JM, Perkins JB, Losick R. (2005). Small Untranslated RNA Antitoxin in *Bacillus subtilis*. *J Bacteriol*. **187**(19), 6641-6650.

Silver S, Ji G. (1994).Newer systems for bacterial resistances to toxic heavy metals. *Environ Health Perspect*. **102** Suppl 3,107-13

Silver S, Keach D. (1982).Energy-dependent arsenate efflux: The mechanism of plasmid mediated resistance. *Biochemistry* **79**(20), 6114-6118.

Silver S. (1996).Bacterial resistances to toxic metal ions. *Gene* **179**(1), 9-19.

Silver S., Novick A. Gupta A. (2000).Mechanism of resistance to heavy metals and quaternary amines. In Gram-Positive Pathogens, *DC: American Society for Microbiology*, 647-659.

Silver S, Phung LT. (1996). Bacterial heavy metal resistance: new surprises. *Annu Rev Microbiol***50**, 753-789.

Silver S, Misra TK. (1988).Plasmid-mediated Heavy Metal resistances. *Ann. Rev. Microbial***42**, 717-743.

Simm AM., Higgins CS, Pullan ST, Avison MB, Niumsup P, Erdozain O, *et al*. (2001). A novel metallo-beta-lactamase, Mbl1b, produced by the environmental bacterium Caulobacter crescentus. *FEBS Lett* 509(3), 350-354.

Singh SK, Tripathi VR, Jain RK, Vikram S, Garg SK. (2010).An antibiotic, heavy metal resistant and halotolerant *Bacillus cereus* SIU1 and its thermoalkaline protease. *Microbial Cell Factories* **9**(59), 1-7

Singletary LA, Gibson JL, Tanner EJ, McKenzie GJ, *et al*. (2009) .An SOS-regulated type 2 toxin-antitoxin system. *J. Bacteriol*. **191**(24), 7456–7465.

Siu LK. (2002). Antibiotics: action and resistance in Gram-negative bacteria. *J Microbiol Immunol Infect.* **35**(1), 1-11.

Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EP, de la Cruz F. (2010).Mobility of plasmids. *Microbiol Mol Biol Rev*. **74**(3), 434-52.

Smith AB, López-Villarejo J, Diago-Navarro E, Mitchenall LA, Barendregt A, *et al*.(2012). A common origin for the bacterial toxin-antitoxin systems parD and ccd, suggested by analyses of toxin/target and toxin/antitoxin interactions. *PLoS One* **7**(9), e46499.

Snyder L., Champness W. (2003).Molecular Genetics of Bacteria. Washington, DC: American Society for Microbiology

Solé M, Pitart C, Roca I, Fàbrega A, Salvador P, Muñoz L, Oliveira I, Gascón J, *et al*. (2011).First description of an *Escherichia coli* strain producing NDM-1- carbapenemase in Spain. *Antimicrob Agents Chemother* **55**(9), 4402-4

Solioz M, Abicht HK, Mermod M, Mancini S. (2010).Response of gram-positive bacteria to copper stress. *J Biol Inorg Chem*. **15**(1), 3-14.

Sørum H, Roberts MC, Crosa JH. (1992). Identification and cloning of a tetracycline resistance gene from the fish pathogen *Vibrio salmonicida*. *Antimicrob Agents Chemother* **36**(3), 611–615

Souli M, Galani I, Plachouras D, Panagea T, Armaganidis A *et al.*(2012).Antimicrobial activity of copper surfaces against carbapenemase-producing contemporary Gram-negative clinical isolates. *J Antimicrob Chemother* **68**(4), 852-857.

Sperandio D, Decoin V, Latour X, Mijouin L, Hillion M, *et al.*(2012).Virulence of the *Pseudomonas fluorescens* clinical strain MFN1032 towards *Dictyostelium discoideum* and macrophages in relation with type III secretion system. *BMC Microbiol* **12(**223), 1-10

Spink WW, Ferris V. (1947). Penicillin-resistant *Staphylococci:* mechanisms involved in the development of resistance. *J Clin Invest* **26** (3), 379-393.

Stalder T, Barraud O, Casellas M, Dagot C., Ploy MC. (2012).Integron involvement in environmental spread of antibiotic resistance. *Front Microbiol* **3** (119), 1-14

Stokes HW, Hall RM. (1989).A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol* **3** (12), 1669-1683.

Stokes HW and Gillings MR. (2011).Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiol Rev* **35**(5), pp 790-819.

Stokes HW, Nesbø CL, Holley M, Bahl MI, Gillings MR, Boucher Y. (2006).Class 1 integrons potentially predating the association with Tn*402*-like transposition genes are present in a sediment microbial community. *J Bacteriol* **188**(16), 5722-5730.

Stokes HW, Tomaras C, Parsons Y., and Hall RM. (1993).The partial 30-conserved segment duplications in the integrons In6 from pSa and In7 from pDGO100 have a common origin. *Plasmid* **30**, 39–50.

Stoughton W, Poole T, Kuskie K, Liu M, Bishop K, *et al*. (2013). AB: Transfer of the virulence-associated protein a-bearing plasmid between field strains of virulent and a virulent *Rhodococcus equi. J Vet Intern Med*. **27**(6), 1555-1562.

Struelens MJ, Monnet DL, Magiorakos AP, Santos O'Connor F, *et al.*(2010).New Delhi metallo-beta-lactamase-1 producing Enterobacteriaceae: emergence and response in Europe. *Euro Surveill* **15**(46), ii: 19716.

Syed MA, Lévesque CM. (2012).Chromosomal bacterial type II toxin–antitoxin systems. *Can. J. Microbiol* **58**(5), 553–562.

Syed SS., Gilsdorf JR. (2007).Prevalence of hicAB, lav, traA, and hifBC among *Haemophilus influenzae* middle ear and throat strains. *FEMS Microbiol Lett.* 274(2):180-3.

Sykes R. (2010). The 2009 Garrod lecture: the evolution of antimicrobial resistance: a Darwinian perspective. *The Journal of antimicrobial chemotherapy* **65**(9), 1842-1852

Sykes RB, Cimarusti CM, Bonner DP, Bush K, Floyd DM, *et al*.(1981).Monocyclic beta-lactam antibiotics produced by bacteria. *Nature* **291**(5815), 489-491.

Sykes RB, Koster WH, Bonner DP. (1988).The new monobactams: chemistry and biology. *J Clin Pharmacol* **28**(2), 113-119.

Sykes RB, Bonner DP. (1985).Aztreonam: the first monobactam. *Am J Med* **78**(2A), 2-10.

Tada T, Miyoshi-Akiyama T, Dahal RK, Sah MK, Ohara H, Kirikae T, Pokhrel BM. (2013).NDM-8 metallo-β-lactamase in a multidrug-resistant *Escherichia coli* strain isolated in Nepal. *Antimicrob Agents Chemother* **57**(5), 2394-2396

Tamang MD, Nam HM, Gurung M, Jang GC, Kim SR, Jung SC, Park YH, Lim SK. (2013).Molecular characterization of CTX-M β-lactamase and associated addiction systems in *Escherichia coli* circulating among cattle, farm workers and the farm environment. *Appl Environ Microbiol*.**79**(13)3898-905

Tängdén T, Cars O, Melhus A, Löwdin E. (2010). Foreign travel is a major risk factor for colonization with *Escherichia coli* producing CTX-M-type extended-spectrum beta-lactamases: a prospective study with Swedish volunteers. *Antimicrob Agents Chemother* **54** (9), 3564-3568

Tato M, Coque TM, Baquero F, Cantón R. (2010). Dispersal of carbapenemase *bla*$_{\text{VIM-1}}$ gene associated with different Tn*402* variants, mercury transposons, and conjugative plasmids in Enterobacteriaceae and *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **54**(1), 320-327.

Taviani E, Grim C, Chun J, Huq A, Colwell R. (2009).Genomic analysis of a novel integrative conjugative element in *Vibrio cholerae*. *FEBS Letters* **583**(22), 3630–3636.

Tenover FC. (2006).Mechanisms of antimicrobial resistance in bacteria. *Am. J. Infect. Control* **34**(5), S3- S10

Tenover FC, McGowan JE Jr. (1996).Reasons for the emergence of antibiotic resistance. *Am J Med Sci* **311** (1), 9–16

Thomas CM, Nielsen KM. (2005).Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol*.3(9):711-21.

Tian QB,Ohnishi M, Tabuchi A, Terawaki Y. (1996). A new plasmid-encoded proteic killer gene system: cloning, sequencing, and analyzing hig locus of plasmid Rts1. *Biochem Biophys Res Commun.* **220**(2):280-4.

Timoney JF, Port J, Giles J, Spanier J.(1978).Heavy metal and antibiotic resistance in the bacterial flora of sediments of New York Bight. *Appl. Environ. Microbiol* **36**(3), 465-472.

Toleman MA, Biedenbach D, Bennett D, Jones RN, Walsh TR. (2003).Genetic characterization of a novel metallo-β- lactamase gene, *bla*$_{\text{IMP-13}}$, harboured by a novel Tn*5051*-type transposon disseminating carbapenemase genes in Europe: report from the SENTRY worldwide antimicrobial surveillance programme. *The Journal of antimicrobial chemotherapy* **52**(4), 583-590

Toleman MA, Rolston K, Jones RN, Walsh TR. (2004).*bla*$_{VIM-7}$, an evolutionarily distinct metallo-beta-lactamase gene in a *Pseudomonas aeruginosa* isolate from the United States. *Antimicrob Agents Chemother* **48**(1), 329-332.

Toleman MA, Biedenbach D, Bennett DM, Jones RN, Walsh TR. (2005). Italian metallo-beta-lactamases: a national problem? Report from the SENTRY Antimicrobial Surveillance Programme. *J Antimicrob Chemother* **55**(1), 61-70.

Toleman MA, Bennett PM, Walsh TR. (2006).ISCR elements: novel gene-capturing systems of the 21st century? *Microbiology and molecular biology reviews* **70**(2), 296-316

Toleman MA, Vinodh H, Sekar U, Kamat V, Walsh TR. (2007).*bla*$_{VIM-2}$-harboring integrons isolated in India, Russia, and the United States arise from an ancestral class 1 integron predating the formation of the 3' conserved sequence. *Antimicrob Agents Chemother* **51**(7), 2636-2638

Toleman MA, Walsh TR. (2008).Evolution of the ISCR3 group of ISCR elements. *Antimicrob Agents Chemother* **52**(10), 3789-3791.

Toleman MA, Walsh TR. (2010). ISCR elements are key players in IncA/C plasmid evolution. *Antimicrob Agents Chemother* **54**(8), 3534

Torres-Urquidy O, Bright K. (2012).Efficacy of multiple metals against copper- resistant bacterial strains. *J Appl Microbiol* **112**(4), 695–704

Tripathi A, Dewan PC, Barua B, Varadarajan R. (2012). Additional role for the ccd operon of F-plasmid as a transmissible persistence factor. *Proc Natl Acad Sci U S A.* **109**(31), 12497-12502.

Tsilibaris V, Maenhaut-Michel G, Mine N, Van Melderen L. (2007). What is the benefit to *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? *J Bacteriol* **189**(17), 6101-6108.

Ullah JH, Walsh TR, Taylor IA, Emery DC, Verma CS, *et al.*(1998). The crystal structure of the L1 metallo-beta-lactamase from *Stenotrophomonas maltophilia* at 1.7 A resolution. *J Mol Biol* **284**(1) ,125-136.

Unaldi Coral MN., Kormaz H., Arikan B., Coral G. (2005). Plasmid mediated heavy metal resistances in *Enterobacter* sp. isolated from Sofulu Landfill, in Adana, Turkey. *Ann. Microbiol.*, **55**(3), 175-179.

Valesová R, Stepánek V, Vecerek B, Kyslík P. (2005).IS*2*-mediated re-arrangement of the promoter sequence suppresses metabolic burden of the recombinant plasmid. *Folia Microbiol* (*Praha*) **50**(4), 275–282.

Van der Bij AK, Van Mansfeld R, Peirano G, Goessens WH, *et al.* (2011). First outbreak of VIM-2 metallo-β-lactamase-producing Pseudomonas aeruginosa in The Netherlands: microbiology, epidemiology and clinical outcomes. *Int J Antimicrob Agents* **37**(6), 513-518

Van Melderen L, Bernard P, Couturier M. (1994). Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. *Mol Microbiol* **11**(6), 1151-1157.

Van Melderen L, De Bast MS. (2009). Bacterial toxin-antitoxin systems: more than selfish entities? *PLoS Genet*. **5**(3), e1000437

Verma T, Srinath T, Gadpayle RU, Ramteke PW, Hans RK, Garg SK.(2001).Chromate tolerant bacteria isolated from tannery effluent. *Bioresour Technol* **78**(1), 31-35

Viedma E, Juan C, Villa J, Barrado L, Orellana MA, Sanz F, *et al*.(2012).VIM-2–producing Multidrug-Resistant *Pseudomonas aeruginosa* ST175 Clone, Spain .*Emerging Infectious Diseases* **18**(8), 1235-1241.

Waksman SA, Flynn JE. (1973).History of the word 'antibiotic'. *J Hist Med Allied Sci* **28**(3), 284-286.

Walsh C. (2000).Molecular mechanisms that confer antibacterial drug resistance. *Nature* **406**(6797), 775-781.

Walsh TR. (2010).Emerging carbapenemases: a global perspective. *Int J Antimicrob Agents* **36** (SUPPL3) S8–14

Walsh TR, Toleman MA, Poirel L, Nordmann P. (2005). Metallo-b-lactamases: the quiet before the storm? *Clin Microbiol Rev* **18**(2), 306–325.

Walsh TR, Weeks J, Livermore DM, Toleman MA. (2011). Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect. Dis.* **11**(5), 355– 362

Walther-Rasmussen J, Høiby N. (2007).Class A carbapenemases. *J Antimicrob Chemother* **60**(3), 470-82

Wang X, Wood TK. (2011).Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. *Appl. Environ. Microbiol* **77**(16), 5577–5583.

Wagner E, Unoson C. (2012).The toxin-antitoxin system tisB-istR1expression, regulation and biological role in persister phenotypes, RNA Biology. *Landes Bioscience* **9**(12), 1513–1519.

Watanabe M, Iyobe S, Inoue M, Mitsuhashi S. (1991).Transferable imipenem resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **35**(1), 147-151.

Weaver SS, Bodey GP, LeBlanc BM. (1979). Thienamycin: new β-lactam antibiotic with potent broad-spectrum activity. Antimicrob Agents Chemother **15**(4), 518-521.

Wei Q, Jiang X, Li M, Chen X, Li G, Li R, Lu Y. (2011). Transcription of integron-harboured gene cassette impacts integration efficiency in class 1 integron. *Mol Microbiol* **80** (5), 1326-36.

Weldhagen GF. (2004).Integrons and beta-lactamases a novel perspective on resistance. *Int J Antimicrob Agents***23** (6), 556-562.

Wiedenbeck J, Cohan FM.(2011).Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS Microbiol Rev* **35** (5), 957–976.

Wilbaux M, Mine N, Guérout AM, Mazel D, Van Melderen L. (2007). Functional interactions between coexisting toxin–antitoxin systems of the ccd family in *Escherichia coli* O157:H7.*J. Bacteriol* **189** (7), 2712–2719.

Winston L, Chambers H. (2009). Coming home with MRSA. *Arch Intern Med.*10;**169**(15)

Wozniak RA, Waldor MK. (2010). Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* **8**(8), 552-563.

Wright BD, Shah PN, McDonald LJ, Shaeffer ML, Wagers PO, *et al*. (2012). Synthesis, characterization, and antimicrobial activity of silver carbene complexes derived from 4, 5, 6, 7-tetrachlorobenzimidazole against antibiotic resistant bacteria. *Dalton Trans***41** (21), 6500-6506

Wright GD. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. Advanced drug delivery reviews, **57**(10), 1451-1470.

Yamaguchi Y, Park JH, Inouye M. (2011).Toxin–antitoxin systems in bacteria and archaea, *Annu. Rev. Genet.***45**, 61–79

Yamaguchi Y, Inouye M. (2009). mRNA interferases, sequence-specific endoribonucleases from the toxin antitoxin systems. *Prog Mol. Biol. Transl. Sci*. **85**, 467–500.

Yang Y, Bush K. (1996).Biochemical characterization of the carbapenem-hydrolyzing beta-lactamase AsbM1 from *Aeromonas sobria* AER 14M: a member of a novel subgroup of metallo-beta-lactamases. *FEMS Microbiol Lett* **137** (2-3), 193-200.

Yarmolinsky MB. (1995). Programmed cell death in bacterial populations. *Science.*10; **267**(5199):836-7.

Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A., *et al*.(2001).Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **45** (4), 1151-1161.

Yilmaz EI. (2003). Metal tolerance and biosorption capacity of *Bacillus circulans* strain EB1.*Res Microbiol* **154** (4), 409-415

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR. (2009). Characterization of a New Metallo-B-Lactamase Gene, *bla*$_{\text{NDM-1}}$, and a novel Erythromycin Esterase Gene Carried on a Unique Genetic Structure in Klebsiella pneumoniae Sequence Type 14 from India. *Antimicrob Agents Chemother* **53**(12), 5046–5054.

Yue ZB, Yu HQ, Wang ZL. (2007).Anaerobic digestion of cattail with rumen culture in the presence of heavy metals. *Bioresource Technology* **98**(4), 781–786.

Zhang H, Hao Y, Zhang D, Luo Y. (2011).Characterization of the cryptic plasmid pTXW from *Lactobacillus paracasei* TXW. *Plasmid* 65(1), 1-7.

Zielenkiewicz U, Ceglowski P. (2005). The toxin-antitoxin system of the streptococcal plasmid pSM19035. *J Bacteriol* **187**(17)6094-105

http://commons.wikimedia.org/wiki/file%3Antibiotics_Mechanisms_of_action.png

http://www.lahey.org/studies.webt.htm

http:// www.lahey.org

http://www.ncbi.nlm.nih.gov/BLAST.cgi.

http://www.ppdictionary.com/bacteria/gpbac/bacteria_comparison.jpg

http://www.scq.ubc.ca/wp-content/uploads/2006/08/antibiotresist.gif