Search for molecular diversity of metallo-β-lactamase genes in eubacterial isolates of Karala and Mahananda rivers of West Bengal

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By

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Under the supervision of

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DEPARTMENT OF BIOTECHNOLOGY
UNIVERSITY OF NORTH BENGAL

February, 2021

Dedicated to….

Maternal Grandmother

Late Smt. Ram Pari

(Whome I Love the most)

And

Revered Grand Father

Late Devendra Rajak

DECLARATION

I hereby declare that the thesis entitled **"Search for molecular diversity of metallo-β-lactamase genes in eubacterial isolates of Karala and Mahananda rivers of West Bengal"** is a genuine research work carried out by me in the Department of Biotechnology, University of North Bengal, Darjeeling-734013, West Bengal, India, under the supervision of **Prof. (Dr.) Ranadhir Chakraborty**, Department of Biotechnology, University of North Bengal, Darjeeling-734013, West Bengal, India. I also affirm that the thesis is the original work and has not been submitted before in part or full for any degree, diploma or any other academic award to this or any other University or Institution.

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CERTIFICATE

The research work presented in this thesis entitled **"Search for molecular diversity of metallo-β-lactamase genes in eubacterial isolates of Karala and Mahananda rivers of West Bengal"** has been carried out under my direct supervision by **Mr Vivek Kumar Ranjan** This work is original and has not been submitted for any degree or diploma to this or any other University or Institution.

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https://en.wikipedia.org/wiki/New_Delhi_metallo-beta-lactamase_1
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3118321/
https://pubmed.ncbi.nlm.nih.gov/21595893/
https://www.researchgate.net/publication/242763314_blaIMP-9_and_Its_Association_with_Large_Plasmids_Carried_by_Pseudomonas_aeruginosa_Isolates_from_the_People's_Republic_of_China
https://www.researchgate.net/publication/11259402_Metallo-_b_-Lactamase_VIM-2_in_Clinical_Isolates_of_Pseudomonas_aeruginosa_from_Portugal
https://www.researchgate.net/publication/7433704_Identification_of_an_OprD_Homologue_in_Acinetobacter_b_aumannii
https://worldwidescience.org/topicpages/m/metallo+beta+lactamase.html
https://www.researchgate.net/publication/286476291_Carbapenem_resistance_Overview_of_the_problem_and_future_perspectives
https://www.researchgate.net/publication/333508615_Distribution_of_carbapenem_resistance_mechanisms_in_clinical_isolatesof_XDRPseudomonas_aeruginosa

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Abbreviations

Abbreviation	Description
AR	Antibiotics resistance
AMR	Antimicrobial drug resistance
ARB	Antibiotic-resistant bacteria
ARG	Antibiotics-resistance genes
WWTP	Waste water treatment plant
ATM	Aztreonam
BLAST	Basic Local Alignment Search Tool
CAZ	Ceftazidime
CDST	Combined disc synergy test
CFU	Colony-forming unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CPE	Carbapenemase-producing enterobacteria
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
gDNA	Genomic DNA
GI	Genomic Island
HGT	Horizontal gene transfer
IMP	Imipenem
ImR	Imipenem-resistant
Kb	Kilobase pair
LEV	Levofloxacin
MBL	Metallo-β-lactamase
MDR	Multi-drug resistant

Abbreviation	Description
Mg	Milligrams
mL	Milliliters
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
mM	Millimolar
MH	Muller-Hinton
NCBI	National Center for Biotechnology Information
NDM	New Delhi metallo-β-lactamase
NGS	Next-generation sequencing
OD	Optical density
OMP	Outer membrane protein
ORF	Open reading frame
PAβN	Phenylalanine-arginine β-naphthylamide
PBP	Pencillin-binding protein
PCR	Polymerase chain reaction
PDR	Pandrug-resistance
Rpm	Revolutions Per Minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RND	Resistance nodulation-cell division
TAE	Horizontal gene transfer
TE	Tetracycline
VIM	Verona integron-encoded metallo-β-lactamase
WHO	World health organization
WGS	Whole-genome sequencing
WWTP	Waste water treatment plant
XDR	Extensively drug-resistant

Abstract

The development and spread of antibiotic-resistant bacteria are now recognised as a key threat to our community, public health and domestic animal. A small number of antibiotics imipenem and meropenem are considered to be the drugs of 'last resort' in the treatment of bacterial infection. Now a day's MBLs producing bacteria easily hydrolyse carbapenem drug-like imipenem and meropenem and bacterial cells become resistant against of these antibiotic groups. In this study, we have investigated the incidence of metallo-β-lactamase (MBL) producing bacteria (specially *bla*$_{NDM}$ producing strains) within abundantly occurring carbapenem-resistant bacterial population in two river water samples, collected in the pre-monsoon (March and April) and post-monsoon (November and December) months of two significant rivers, Mahananda and Karala, bisecting two most populous town, Siliguri and Jalpaiguri respectively, in northern West Bengal, India. Before this study, the presence and significance of MBL producing eubacterial isolates in Mahananda and Karala River remained elusive. MBL-producing bacteria were screened out from imipenem resistant bacteria isolates by the phenotypic (Carba NP test and EDTA inhibition test) and genotypic (multiplex PCR) methods. Overall, 7/1,237 (0.56%) and 8/1,593 (0.50%) MBL -producing bacteria were detected in Mahananda and Karla river water samples respectively. Remarkably, all MBL producing isolates were obtained only from mid-stream (city-based sampling point) sampling point of both the river water samples and conspicuously absent in isolates of up and downstream of both the river. In antimicrobial susceptibility testing, all 15 MBL producing isolates have shown resistance against the 10 different groups of antibiotics and the highest MIC values were observed in all MBL positive isolates against ampicillin molecules. The MDR pattern was detected in all 15 MBL-producing bacterial isolates of both rivers. The metallo-β-lactamase activity shown that the MBLs producing gene appears to be the main reason for carbapenem-resistance in all bacterial isolates. Based on 16S rRNA gene sequences, three different genera among MBL-producers, *Pseudomonas*, *Myroides* (pathogenic organism) and *Acinetobacter* were identified in Mahananda River water samples, and in Karala River water samples, four different genera among MBL-producers, *Pseudomonas*, *Proteus*, *Escherichia* and *Acinetobacter* were detected. Overall, the *Acinetobacter* is the most abundant and common MBL producing genus in both river water samples. PCR products of the MBL-producing

gene were generated and sequenced using primer targeting New Delhi Metallo-β-lactamase (*bla*$_{NDM}$) genes. The complete ORF sequence analysis of *bla*$_{NDM}$ genes showed that the most common MBLs are *bla*$_{NDM-1}$ 10/15 (73.34%) observed in both river water samples and *bla*$_{NDM-7}$ was observed only in the genus, *Escherichia* , of Karala River isolates 3/15 (20%). The result suggests that *bla*$_{NDM-1}$ types of MBLs are common for carbapenem resistance within MBL-producing bacteria present in both the rivers. Class 1 integrons with the frequent presence of *aad*A and *aac*(6´)-Ib gene cassettes in 50 % of NDM-1 bearing isolate is indicative of selective pressures generated out of unregulated use of streptomycin, in agriculture field, owned by the tea cultivators living in locales, drained by these two rivers, in their up and downstream; and amikacin remains as one of the most often prescribed drugs in the highly-crowded government-sponsored 'Sadar' and district hospitals of Siliguri and Jalpaiguri. The isolate, *Pseudomonas* sp. MR 02 was used as a model strain. Whole genome sequence of MR 02 was explored to understand why it was resistant to most of the antibiotic groups. Genome sequence analyses revealed the carriage of 100 distinct genes contributing to antibiotic resistance that made MR 02 a pandrug resistant organism (PDR). Among these 100 genes, 64 genes were identified as an antibiotic-related efflux pump, 14 genes related to OprD porin, 18 genes related to antibiotics hydrolysis and modification, and 4 genes were related to target site modification. Three chromosomally located genes coding for β-lactamases belonging to class A extended-spectrum β-lactamase *bla*$_{PME-1}$, subclass B1 Metallo-β-lactamase *bla*$_{NDM-1}$ and class C β-lactamase *bla*$_{PDC}$ respectively were identified in MR 02 strain. Hence, MR 02 was established as a PDR strain. The antibiotic susceptibility profile of MR 02 correlated with the analyses of antibiotic-resistance genes present in its genome. Another significant attribute of the NDM-producing strain, *Pseudomonas* sp. MR 02, not reported earlier for any PDR or MDR bacterial strain, is its ability to utilize ampicillin molecule as a sole source of carbon, nitrogen and energy source. It was hypothesized that the elevated catalytic efficiency of NDM-1 towards ampicillin leading to very high minimum inhibitory concentration (MIC) might render the host bacterium an opportunity to utilize the drug as a sole source of carbon, nitrogen, and energy. Despite having a 6-aminopenicillanic acid core containing nitrogen in all β-lactam antibiotics, ampicillin possesses a side chain with an additional amino group. From the results derived out of this study, it was revealed that

ampicillin is also being used by MR 02 as a nitrogen source. When MR 02 confronts a new environment where no easily metabolizable carbon and energy source like glucose is available, it confronts a survival challenge and takes an extended lag phase to focus and synthesize enzymes for the utilization of inactivated-ampicillin resource disposed of with the aid of NDM-1. This study has demonstrated that only by the expression of $bla_{\text{NDM-1}}$ in *E. coli* DH5α strain, it was capable of utilizing ampicillin as a carbon source to grow in a minimal medium for β-lactam catabolism. RNA-Seq was used to generate a differential gene expression profile in ampicillin and glucose, grown MR 02 cells and it revealed that the expression of $bla_{\text{NDM-1}}$ was significantly higher [upregulated (log2 fold change 2.00088)]. For ampicillin catabolism to take place, three genetic components are required: (i) New Delhi Metallo-β-lactamase genes (bla_{NDM}) (ii) amidase gene and (iii) PAA pathway-related genes; and absence of any one of the three components would not support bacteria to utilize β-lactam antibiotics as carbon and energy source.

Preface

Each year, we produce, protect and use more antibiotics than the previous year, because these drugs are saving millions of lives from infections. Although these drugs have protective roles against infectious bacteria, due to overuse of it pollution has been speeded in the environment. One of the most noted consequences of overuse or misuse of antibiotic pollution is the increased frequency of antibiotic-resistant bacteria in a different environment.

The river is the main places where bacteria can mingle in a diluted bisque of antibiotics and resistance genes are the recipe for transmitting resistance between different species and potentially converted into new multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria. Bacteria in the river water come from the city "wastewater treatment plants" (WWTPs) because WWTPs cannot remove all bacteria and from villages; and active antibiotic molecules, came from hospital waste, fish farms and pharmaceutical factories. That pollution has a huge potential for detrimental effects on the ecology of our rivers as well as on human and animal health. Therefore, we should monitor antibiotic and antibiotic resistance genes in our river water environment at a regular time interval and these steps will help us for dealing with the problem.

Rivers Mahananda and Karala flow through the middle of Siliguri and Jalpaiguri city respectively. Siliguri and Jalpaiguri are a city in the northeast Indian state of West Bengal and both cities sit in the foothills of the Himalayas. These both river basins sustain life and livelihoods of tea gardeners, fishermen and slum-dwellers. Tea gardeners consume the water resource for tea plantation and drain off the utilized excess water, which carries a variety of pesticides and fertilizers to those rivers. Slum-dwellers exploit the water resource for bathing, washing of clothes etc. Untreated waste water from city, garbage from the market and ash of cremation directly mix up with this river. As a result, the physicochemical and biological characteristics of the river water are gradually changing and producing the harmful effects on aquatic biota and thereby on human beings. Till date, no study has been done on the distribution of metallo-β-lactamase amongst the eubacterial population, especially the members of *Enterobacteriaceae* of this river water system. Therefore it demands a thorough investigation of a riverine bacterial population.

The present study will therefore focus on two city river water environment for studying the prevalence and diversity of the metallo-β-lactamase producers and will also try to forecast the risk associated with the dissemination of the metallo-β-lactamase genes. This study has been undertaken to provide comprehensive descriptive information about the metallo-β-lactamase producing eubacterial population and also to explore the phenotypic resistance pattern. Whole-genome sequencing of NDM-1 producing *Pseudomonas* sp. MR 02 bacteria correlate the genotypic and phenotypic characters and another more important objective of this work is to reveal the role of $bla_{\text{NDM-1}}$ genes in ampicillin catabolism.

General introduction

I. History of antimicrobial drugs (Antibiotics)

The word antibiotic was used for the first time by Selman A. Waksman (1888-1973) in the medical sense in 1943 and they described "antibiotics" as a substance produced by a microorganism that is antagonistic to the growth of other microorganisms (Waksman, 1947). This definition has excludes synthetic antibacterial compounds (compounds that may kill bacteria or inhibits bacterial growth, but not produced by a microorganism) such as the sulfonamides (Scholar & Pratt, 2000; Davies & Davies, 2010). However, in the present time, the word "antibiotic" is used for any drug or compounds that kill bacteria or inhibit bacterial growth.

In 1928, Alexander Fleming (1881-1955) discovered penicillin and after 12 years of discovery, in 1942 three scientists, Ernst Chain, Howard Florey and Edward Abraham successfully purified penicillin which began the era of antibiotics (Tan & Tatsumura, 2015; Jones & Jones, 2014). However, the discovery of penicillin was not a former contribution to the use of antibiotics. There are some good evidences which suggest that antibiotics were used since the ancient period of times. In ancient civilizations, a variety of natural compounds were used as a treatment for infection, for example, herbs, honey, mouldy bread and animal faeces (Keyes *et. al.,* 2003). There are some references, which indicate that in ancient Egypt, China, Serbia, Greece and Rome, where the mouldy bread was used as a treatment for infection. John Parkinson (1567-1640) had also mentioned the benefits of mouldy bread in his book *Theatrum Botanicum,* which was published in 1640 (Gould, 2016). Even some modern antibiotics (devolved in the 20th century) may have been available in the ancient period. A notch of tetracyclines antibiotic has been detected in human skeletons dig out in Nubia and in ancient time Nubia was the part of roman occupied Egypt (Bassett *et. al*., 1980; Nelson *et. al*., 2010).

Most people have heard the story of how Alexander Fleming by coincidence contaminated his agar plates with mould and discovered penicillin back in 1929, but before the invention of penicillin, in 1909 Paul Ehrlich and Sahachiro Hata discovered a novel drug salvarsan, which is employed for treating the sexually transmitted disease *syphilis* that's is caused by the spirochete *Treponema palladium* (Ehrlich & Hata, 1910). Salvarsan was the foremost prescribed drugs until it was replaced by penicillin in the 1940s (Aminov,

2010). The systematic screening method used by Ehrlich and Hata in the invention of salvarsan became the path for identifying novel drugs and led to the discovery of the first sulfa drug (first systematically active antibacterial drug) in 1934, sulfonamidochrysoidine (KI-730, Prontosil), a precursor of the active compound sulfanilamide, which inhibits bacterial folic acid (vitamin B9) synthesis and *de novo* synthesis of nitrogen bases like purines and pyrimidines (Aminov, 2010; Achari *et. al*., 1997).

II. The golden era of antimicrobial drugs

We usually associate the beginning of the modern "antibiotic era" with the discovery of sulfa drug and release of penicillin. After the discovery of both drugs a period of 30 years known as the golden age of antibiotics (1940-1970), in which almost the entire antibiotic drug classes used in the clinic today was discovered (Figure 1) (Gould, 2016 and Clatworthy *et. al*., 2007).

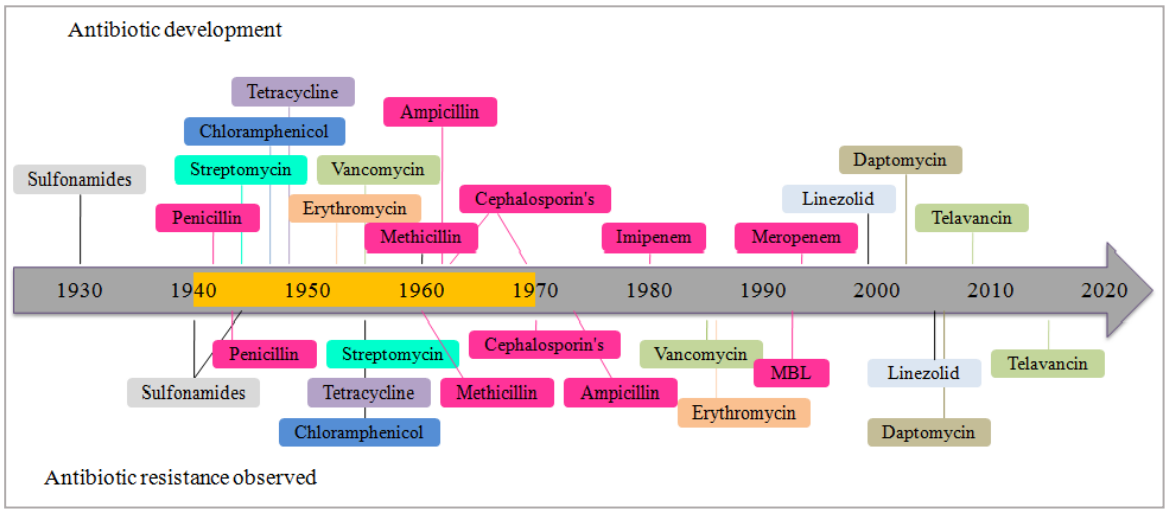


Figure 1: Timeline of new antibiotics developments and antibiotic resistance developments

The upper panel indicates the time at which antibiotics were discovered. The down panel indicates when resistance was observed. Modified from Clatworthy *et. al.*, 2007.

Most of the antimicrobial compounds discovered in the early days of the golden era of antibiotics were isolated from different naturally occurring microorganisms, and this type of research work began worldwide after the isolation of an antimicrobial compound "streptomycin". In 1944 streptomycin was isolated from soil growing bacteria *Streptomyces griseus* (Saga, 2009). Eli Lilly had given a good idea and requested Christian

missionaries to send him a sample from every exotic place that they had visited and through this way they collected the soil samples from around the world. Among them, one soil sample was from Borneo and vancomycin producing bacteria was isolated from these soil samples. In 1952 vancomycin producing *Streptomyces orientalis* was isolated from Borneo soil samples and this vancomycin antibacterial compound was released in 1958 for clinical use (Levine, 2006). Amidst all of this, Scientists were engaged in improving these existing agents so that they could overcome this obstacle of antibiotic resistance. As a result in 1959 first penicillinase-resistant β-lactam antibiotic "methicillin" was found. The penicillin's spectrums of activity pharmacodynamics/pharmacokinetics were improved by the introduction of ampicillin in 1961.

Cephalosporin was first introduced in 1960 and their evolution classified into three generations, according to their spectrum of activity. Third-generation cephalosporin ceftazidime appeared in the late 1970s (Russell, 1957).

The first β-lactamase inhibitors clavulanic acid was identified in 1976, which was isolated from gram-positive bacteria *Streptomyces clavuligerus* (Drawz & Bonomo, 2010). It was introduced for clinical use in combination with amoxicillin and thienamycin. First carbapenem antibiotic imipenem was evolved from Thienamycin in 1983 which was very effective *in vitro* condition; however, it had very short half-life in the human body. After addition of cilastatin in imipenem, the half-life ($t_{1/2}$) of imipenem had increased and the combination of imipenem + cilastatin was available for clinical use around the world. Second, most prescribed carbapenem antibiotics "meropenem" was introduced in 1995 and it had similar activity. It was associated with fewer adverse effects (Papp-Wallace *et. al.*, 2011).

There were plenty of antibiotic compounds discovered during the golden era, along with the start of antibiotics resistant bacteria. However, this was the beginning of the race between the evolution of antibiotic resistance and development and discovery of antibiotics "a race that currently seems to be led by the microorganism".

III. Classification of antibiotics

There are different classifications of antibiotics according to the mode of action, spectrum efficacy, the killing (bactericidal) or inhibitory (bacteriostatic) effects and their

Table 1: Classification of antibiotics, based on to their chemical structure and mechanism of action

Class of antibiotics	Examples	Mechanism of action
Aminoglycosides	Neomycin	Inhibit the bacterial protein synthesis by binding to 50S ribosomal subunit.
	Streptomycin	
Beta-lactam antibiotics		
Carbapenems	Imipenem	Inhibit the bacterial cell wall formation by inhibiting the cross-linking of stem peptides on peptidoglycan chain.
Cephalosporins	Cefepime	
Monobactam	Aztreonam	
Penicillin	Ampicillin	
Fluoroquinolones	Levofloxacin	Inhibit bacterial DNA synthesis by binding to DNA gyrase enzymes.
	Ciprofloxacin	
Folate pathway inhibitors	Trimethoprim Sulphamethaxazole	Folate pathway inhibitors.
Furanes	Nitrofurantoin	Damage bacterial DNA.
Imidazoles	Metronidazole	Inhibit bacterial DNA synthesis.
Lipopeptides	Polymyxins	Alters the bacterial membrane functions.
Lincosamides	Clindamycin	Interfering bacterial protein synthesis.
Macrolides	Erythromycin	Inhibition of bacterial protein synthesis.
	Azithromycin	
Peptides	Bacitracin	Prevent the bacterial cell wall synthesis.
Phenicols	Chloramphenicol	Inhibit the bacterial protein synthesis by binding to 50S subunits.
Phosphonic acids	Fosfomycin	Prevent the bacterial protein synthesis.
Polymyxins	Colistin	Destroying bacterial outer membrane integrity.
	Polymyxin B	
Tetracyclines	Tetracycline	Inhibit the bacterial protein synthesis by binding to 30S subunits.
	Doxycycline	

structural similarities. The classification of antibiotics according to their mode of action depends on the inhibition of a cellular structure or a metabolic channel that's present within the bacterial cell but not in the host cell. Several mechanisms have been reported

like the inhibition of bacterial cell wall synthesis, obstruction with cell membrane functions, inhibition of protein synthesis, and inhibition of nucleic acid synthesis (Levinson, 2008). However, the foremost common way of antibiotics classification is based on their chemical structure (Table 1), as antibiotics having closely related chemical structure tend to have the same antibacterial activity and same spectrum of activity against the host.

IV. Beta-lactam antibiotics

Beta-lactams are antibiotics that contain a β-lactam ring (2-Azeidinone) in their chemical structure (penicillins, cephalosporins, carbapenems and monobactams) (Figure 2). β-lactam antibiotics have a broad spectrum of antimicrobial activity, including gram-positive and gram-negative pathogens. Because of their favourable characteristics, β-lactams are the most broadly used antibiotics worldwide (Livermore *et. al.*, 2006). The β-lactam ring is essential for the antimicrobial activity and which mimics the D, D-alanyl-alanine structure of the pentapeptide chain of the bacterial cell wall. The D, D-alanyl-alanine structure is the site of catalysis for PBPs catalytic domain thus; the β-lactam rings of beta-lactam antibiotic binds irreversibly to the PBPs and inhibit the synthesis of the peptidoglycan layer in the bacterial cell wall (Konaklieva *et. al.*, 2014). All the β-lactam antibiotics are classified into four major groups based on their molecular structure.

(a) Penicillin: These antibiotics were among the first antibiotics prevalent in the treatment of infection caused by gram-positive bacteria. Drugs in the penicillin class work by indirectly bursting the bacterial cell walls. The basic structure of the penicillin group of antibiotics must contain a heterocyclic thiazolidine nucleus which is attached with a β-lactam ring and a side chain at the C6 position (Dalhoff *et. al.*, 2006). Based on the molecular composition of penicillin's side chain represent a different group of penicillin's: benzylpenicillin (Penicillin G), phenoxymethylpenicillin (Penicillin V), Penicillin O etc. However, due to the extensive use of natural penicillin, frequent resistance was observed. Also, the natural penicillins have a limited scope of use due to stability and extraction process.

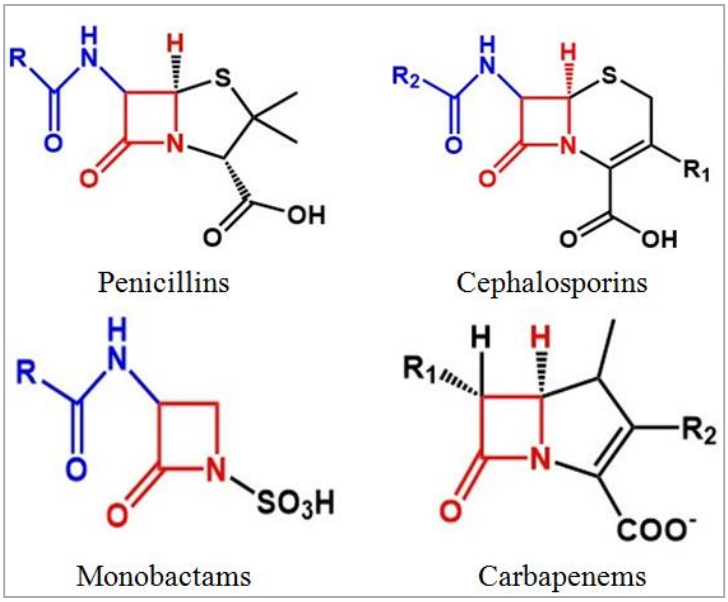


Figure 2: Beta-lactam antibiotics core structure. All β-lactam antibiotics contain the same core structure (highlighted in red)

(b) Cephalosporin: Groups of cephalosporin antibiotics are considered as the largest group of broad-spectrum antibiotics and cephalosporin antibiotic was discovered by the Italian pharmacologist Giusseppe Brotzu from the mould *Cephalosprium* (Torok *et. al*., 2016). They contain a nucleus (7- Aminocephalosporanic acid), which is fused with the β-lactam ring. Based on the antimicrobial activity, development period and modification of cephalosporin, the group has been subdivided into four generations (Table 2).

(c) Carbapenem: These antibiotics are the broad-spectrum β-lactam antibiotics used against all types of multidrug-resistant bacteria. All the carbapenem antibiotics are the most effective against both types of drug resistance microorganism (gram-positive and gram-negative bacteria) and therefore, often it is called the last resort of antibiotics. The widely used carbapenems are imipenem (developed in 1980 by Merck & Co.), meropenem (discovered by Dainippon Sumitomo Pharma).

(d) Monobactam: These antibiotics are the compounds which have a single β-lactam ring without any fused ring (Figure 2). It is narrow-spectrum β-lactam and they are active against only gram-negative bacteria. MBL (bla_{NDM}) positive strains are only sensitive against (Aztreonam) monobactam antibiotics (Aztreonam is the only monobactam antibiotics which is used presently in clinical practices).

Table 2: Generations of cephalosporin antibiotics

1st generation	2nd generation	3rd generation	4th generation	5th generation
Cefadroxil Cephalothin Cephalexin Cefapirin	Cefaclor Cefotetan Cefoxitin Cefprozil	Cefotaxime Cefixime Cefoperazone Ceftazidime	Cefepime Cefpirome Cefquinome	Ceftaroline Ceftobiprole Ceftolozane
Effective against gram positive bacteria and less effective against gram negative	Effective against gram negative bacteria and moderate against gram positive	Effective against gram negative bacteria and less effective against gram positive	Effective against gram negative bacteria and less effective against gram positive	Less susceptible to the development of resistance when combined with β-lactamase inhibitors.

V. Mechanism of resistance to β-lactams

Bacteria employ multiple mechanisms to resist β-lactam antibiotics. These resistance mechanisms have developed in bacteria either via mutation in the bacterial gene or by the acquisition of resistance genes from other bacteria. Bacteria can avoid the bactericidal effect of β-lactam antibiotics by several mechanisms; active efflux pumps promoting the efflux of the antibiotic out of the cell, a mutation in the penicillin-binding protein, lack/reduced the expression of porin channels, inactivation of β-lactam antibiotics by chromosomally encoded β-lactamases or through acquired specific β-lactamases gene (Figure 3) (Wanda, 2018). Often, multiple β-lactam resistance mechanisms could be also present in a single bacterial organism and some cases, they will work synergistically.

i. Reduced permeability: Porin is a channel, present in the outer membrane of a bacterial cell and it facilitates the transport of β-lactam antibiotic molecules across the cell membrane. Mutated porin channel can be reduced or lost to inhibit β-lactam invasion into the cell. *Pseudomonas aeruginosa* OprD porin is the substrate-specific porin and it facilitates the diffusion of imipenem. Modified/mutated OmpC and OmpF outer membrane Porins are a common in *E. coli* and *Enterobacter* spp. and increase the MIC value of antibiotics (Ochs *et. al*., 1999; Wozniak *et. al*., 2012; Novais *et. al*., 2012).

ii. **Efflux pumps:** Efflux pumps actively transport antibiotics and toxic substrates across the cell membrane to outside of the cell. Thus increase the expression of efflux pump genes

in bacterial cell increase the MIC values of antibiotics and decrease the level of antibiotics inside the cell. Total six different types of antibiotic resistance efflux pump have been reported and among them, RND efflux pumps are responsible for β-lactam antibiotic resistance in gram-negative bacteria. MexAB-OprM RND type efflux pump played an important role in carbapenem resistance in *Pseudomonas* spp. (Fernandez and Hancock, 2012).

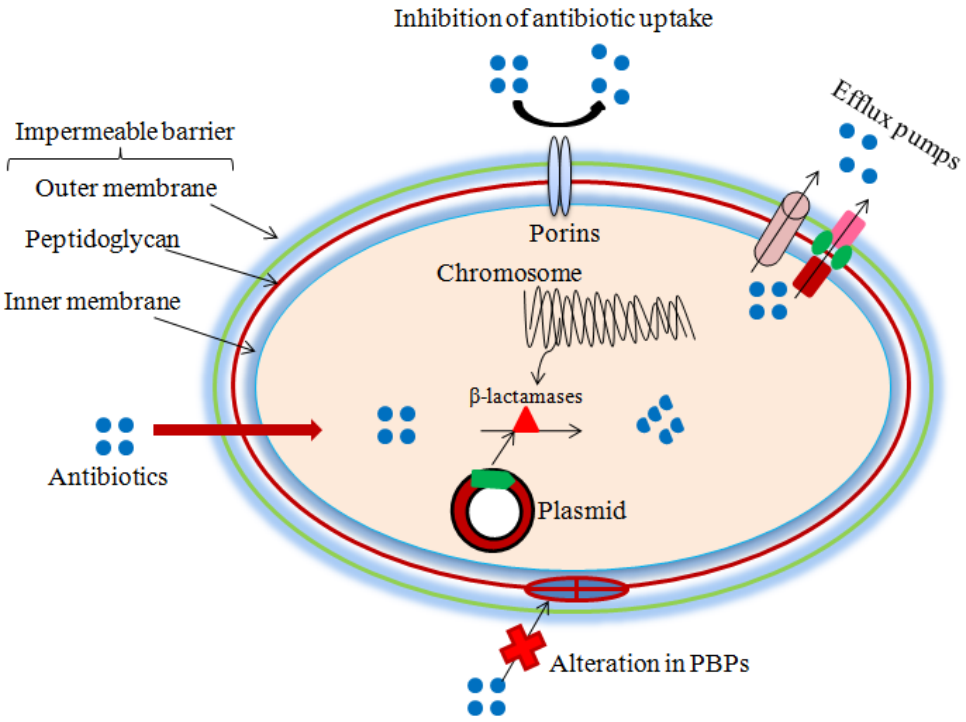


Figure 3: Mechanisms of resistance of β-lactam antibiotics in gram-negative bacteria

This diagram shows the mechanism used by gram-negative bacteria confer resistance to β-lactam antibiotics, which include enzymatic inactivation, activation of different types of efflux pumps, porins channel and alteration of β-lactam binding sites in the cell (Modified from Wanda, 2018).

iii. Penicillin-binding protein: Penicillin-binding protein is the first target of β-lactam antibiotics and due to mutation in PBP site the antibiotic molecule is unable to bind to them and this type of resistance mechanism observed in gram-negative and gram-positive bacteria (Zapun *et. al*., 2018). Due to this alteration or mutation *Staphylococcus aureus* show, methicillin resistance and *Pneumococci* show penicillin resistance (Grebe and Hakenbeck, 1996).

VI. Beta-lactamases

Enzymes are able to reduce the activation barrier of a reaction, thus accelerating reaction rates by many orders of magnitude. For this elemental ability, enzymes are both essential and ubiquitous in all the kingdoms of life (Wilcox, 1996 and Schenk, *et. al*., 2012). Enzymes can catalyze diverse reactions, being involved in a wide range of chemical pathways. For example, hydrolases are enzymes that can hydrolyze ester or amide bonds in specific substrates. Hydrolytic enzymes are involved in many physiological pathways and are necessary to recycle metabolites needed by the cells (Wilcox, 1996; Schenk, *et. al*., 2012 and Meyers, *et. al*., 1996). Among them, the enzyme β-lactamases are produced by both gram-positive and gram-negative bacteria and they hydrolyzed amide bond of the four-membered β-lactam ring of penicillin, cephalosporin, monobactam and cephalosporin group of antibiotics, destroying their antimicrobial activity (Wilcox, 1996; Schenk, *et. al*., 2012; Mitic *et. al*., 2006). In the case of gram-negative bacteria, they accumulate in the periplasmic space of cell wall, whereas in the case of gram-positive bacteria, they get excreted from the cell (Ghuysen, 1991).

Presently, more than 850 types of β-lactamases have been recognized, we speculate that the fast replication rate, recombination rates, and high mutation frequency allow bacteria to adapt to novel β-lactams by the evolution of these β-lactamases (Perez *et. al*., 2007).

VII. Classification of beta-lactamases

Beta-lactamases have been classified based on various criteria such as relative rates of hydrolysis and inhibition of β-lactamases by various compounds and their relation to molecular structure and functional properties. The molecular structure (sequenced based) classification of β-lactamases enzyme was proposed by Ambler (Ambler, 1980) and also called the ambler classification. It is based on amino acid sequence and the nature of the catalytic site. It distinguishes four different classes of the β-lactamases, where class A, C and D contain evolutionary distinct serine β-lactamases and class B contains Zinc metal ions (Table 3) (Ambler *et. al*., 1991). The second classification of β-lactamases was functional classification and it proposed by Bush, Jacoby-Medeiros (Bush and Jacoby 2010) and known as Bush Jacoby classification. It is more complex and based on substrate

affinity and inhibition properties. According to functional classification, the β-lactamases enzyme is divided into four major groups (group 1 to 4), with multiple subgroups. On the basis of functional classification metallo-β-lactamases placed in group 2f and 3 (Table 3).

Table 3: Classification of β-lactamase enzymes (Bush *et. al*., 2010)

Ambler class	Bush Jacoby-Medeiros group	Active site	Enzyme Type	Substrate
A	2b, 2be, 2br, 2c, 2e, 2f	Serine	ESBL (TEM, SHV, CTX-M)	Penicillins, 3 generation Cephalosporins
			Carbapenemase (KPC, GES, SME)	All beta-lactams
B	3	Zn binding thiol group	Carbapenemases (NDM, IMP, VIM)	All beta-lactams
C	1	Serine	AmpC, CMY, FOX	Cephamycins, 3rd Generation cephalosporins
	1e		GC1, CMY-37	Cephamycins, 3rd Generation cephalosporins
D	2d	Serine	ESBL (OXA)	Penicillins, 3rd generation cephalosporins
			Broad spectrum beta-lactamases (OXA)	Oxacillin, ampicillin, cephalothin
			Carbapenemases (OXA)	All beta-lactams

VII. Metallo-β-lactamases

Metallo-β-lactamases are a class of binuclear metallohydrolases and the first metallo-β lactamases (B1 types) was reported in gram-positive bacteria *Bacillus cereus* (Sabath *et. al*., 1966; Hussain *et. al*., 1985). Metallo-β-lactamases have a broad substrate spectrum β-lactamases and it can hydrolyze virtually all β-lactams antibiotics including imipenem. The metallo-β-lactamases enzymes reveal a various range of sequences homology with minimum 25% similarity between some enzymes (Garau *et. al*., 2004). X-ray crystallographic study of metallo-β-lactamases revealed that the group is structurally similar and it has a characteristic αβ/βα sandwich fold with the active site (Ullah *et. al*.,

1998; Concha *et. al*., 1996). The structural fold, a characteristic of MBLs was first identified in 1997 (Bebrone, 2007; Neuwald *et. al*., 1997). This three-dimensional fold structure supports up to six amino acids residues at the active site which manages either one or two zinc metal ions that are responsible for the catalytic mechanism. Enzymes belonging to the MBLs family of β-lactamases share a common three-dimensional structure as well as five conserved motifs in their sequences, i.e. Asp84, His116-X-His118-X-Asp120-His121, His196, Asp221 and His263 (Bebrone, 2007; Crowder *et. al*., 2006; Daiyasu *et. al*., 2001).

Based on the structural homology between two halves of the MBL protein, it has assumed that MBLs may have developed due to a gene-duplication. But no solid evidence has been established yet to substantiate such hypothesis. Rather, a solid authentication has been made that such beta-lactamases evolved from an ancient superfamily of metallohydrolases with distinct activities, called the MBL superfamily (Daiyasu *et. al.*, 2001). Moreover, the αβ/βα fold and a metal-binding motif in the active site are more conserved across the MBL superfamily, which is assumed to have evolved billions of years ago (Daiyasu *et. al.,* 2001). The metal ligands present in MBLs are distinctive to enzymes activity, substantiate that this metal-binding motif has been specific for β-lactam hydrolysis (Gonzalez *et. al.,* 2012).

Based on the sequence homology, substrate binding ability and Zinc ion requirements, the MBLs are divided into three subgroups (B1, B2 and B3) based on amino acid similarity (Bebrone, 2007; Garau *et. al*., 2004; Heinz *et. al*., 2004; Page *et. al*., 2008). Among all the three subclasses, B1 subclass is the most prevalent and structurally most extensively studied class of MBLs (Bebrone, 2007; Bellais, *et. al*., 2002; Carfi A., *et. al*., 1995). Among the class B β-lactamases the most common and biggest MBLs groups are B1 subgroups MBLs, which harbours some good studies MBLs such as Verona integron-encoded metallo-β-lactamases (VIM-1) from *P. aeruginosa*, New Delhi metallo-β-lactamases (NDM-1) *K. pneumoniae*, CcrA metallo-β-lactamases from *Bacteroides fragilis*, BcII metallo-β-lactamases from *Bacillus cereus*, and IMP-1 from *P. aeruginosa*. and Sao Paulo metallo-β-lactamases (SPM-1) from *P. aeruginosa* (Yong *et. al*., 2009; Poirel *et. al*., 2010; Rolain *et. al*., 2010; Zhang *et. al*., 2011; Walsh *et. al*., 2011; Li *et. al*., 2014). Such β-lactamases efficiently hydrolyse various β-lactam antibiotics groups, which

included penicillin's, cephalosporins, and carbapenems. Examples of subclass B2 is ImiS metallo-β-lactamases from *A. veronii* and CphA from *A hydrophila* (Segatore *et. al*., 1993; Crawford *et. al*., 2004) which have poor activity towards penicillins and cephalosporins groups of β-lactam antibiotics but easily hydrolyses the antibiotics of the carbapenems. Finally, subclass B3 is represented by monomeric FEZ-1 metallo-β-lactamases isolated from *F. gormanii*, and L1 metallo-β-lactamases isolated from *S. maltophilia*, both hydrolyse penicillins and cephalosporins groups of β-lactam antibiotics effectively (Costello *et. al*., 2006; Ullah *et. al*., 1998; Hu *et. al*., 2008; Garcia-Saez *et. al*., 2003).

The first NDM-1 was reported in 2009, a Swedish patient, who travelled to New Delhi and got infected with *Klebsiella pneumonia* (Yong *et. al*., 2009). NDM-1 belongs to the B1 subclass of Metallo-β-lactamase family containing two zinc ions and other divalent cations as cofactors. It catalyses almost all classes of β-lactam antibiotics including carbapenems. NDM-1 is more effective and broad in inactivating β-lactam antibiotics than known MBLs. The most common bacteria that make this enzyme are gram-negative bacteria such *Enterobacteriaceae* species, *Acinetobacter* sp., and *Pseudomonas aeruginosa* but the gene of NDM-1 can spread from one species/strain of bacteria to another by horizontal gene transfer (Zheng *et. al*., 2011).

VIII. Environmental perspective of abundance and spread of metallo-β-lactamases

The soil and water environment is a repository of antibiotics resistant bacteria and antibiotic-resistant genes, where these bacteria transmit antimicrobial resistance genes within the same or different genus and species. It is not yet clear whether an antibiotic-resistant bacterium originated from the clinical setting or environment, but it has been confirmed that clinical setting antibiotic-resistant bacteria are also found in the environment setting like soil and water. The antibiotic resistance gene of the clinical setting and soil share almost 100% similarity and these bacteria are a source of these antibiotics resistance genes for human infection (Forsberg *et. al*., 2012). However, now it was confirmed that antibiotic resistance genes are an essential element of bacteria and these types of bacteria come from different sources into the soil and water environment and where they are exchanged or transfer antibiotic resistance genes within the same species or other species. Due to this horizontal gene transformation method, different types of

antibiotic-resistant bacteria are evolving and spread in human and between the various environments (Figure 4).

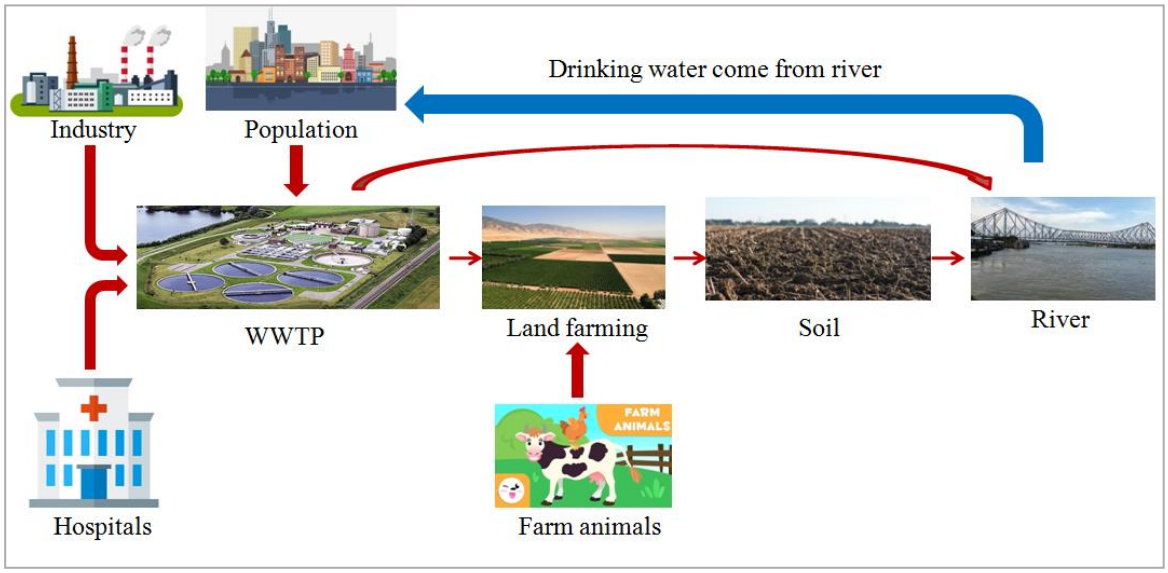


Figure 4: Various routes for antibiotic resistant bacteria and gene spread from human activity origins to the environment

The above figure shows the spread and dissemination routes of antibiotic resistant bacteria and gene between human and different environment, industry, farm animal and agriculture farm (WWTP= waste water treatment plants) (adapted and modified from Berendonk *et . al*., 2015).

Antibiotic-resistant bacteria are present in soil and represent the evolutionary reservoir of resistance for most bacteria. The soil environment is the largest and most divergent among other types of environments. Several studies have provided evidence for the transmission of ARGs between soil bacteria and clinical pathogens (D'Costa *et. al*., 2006).

Some of the most dangerous clinical infections are caused by the metallo-β-lactamase (MBL) producing bacteria (Van Duin & Doi, 2017). Some MBLs genes are plasmid-mediated and some are carried on smaller, independent chromosomes (Queenan & Bush, 2008). Plasmid-mediated MBLs producing genes include variants of New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM) and imipenem resistance metallo-β-lactamase (IMP). These MBLs producing genes are found in environmental samples like soil and water but they are typically found in conjunction with at least one of the other β-lactamase genes (Diene & Rolain, 2014; Meletis, 2016).

The study about MBLs type antibiotic resistance is often focused around clinical setting and it is an important source of MBLs and other types of ARGs or ARB and these MBLs producing bacteria release to the environment through the hospital sewage system to water and soil environment (Ray *et. al*., 2016; Satlin *et. al*., 2017). Two independent studies, one from Bangladesh and another one from Switzerland have reported increased MBLs-producing isolates downstream of hospitals (Islam *et. al*., 2017; Zurfluh *et. al*., 2017). Untreated hospital wastewater has the main source of MBLs producing bacteria in a developing country, which has been reported in several studies. NDM types MBLs have been isolated from hospital wastewater in Seoul, South Korea (Hwang and Kim, 2018). In Saudi Arabia, NDM was detected in municipal wastewater (Mantilla-Calderon *et. al*., 2016). In North China, NDM was detected in the final effluent of WWTP and dewatered sludge, which is often used in agriculture field. VIM was also identified in freshwater and sediment sludge samples of WWTP in Tianjin (Yang *et. al*., 2017). In India, NDM was not detected in WWTP samples, but it was identified in two treated tap water samples from around the Delhi city (Walsh *et. al*., 2011). Water samples from a first Nations Community in Canada also detected VIM types of MBLs in their drinking water, (Fernando *et. al*., 2016). NDM types of MBLs have been isolated from surface water in the Danube River, Brazil (Kittinger *et. al*., 2016). VIM types MBLs have been isolated from water in Canada, Spain and Austria. Sediment sludge is also a reservoir of AR bacteria and a higher concentration of AR bacteria are reported than waterways (Yang *et. al*., 2017). A study of sediment soil in Mula Mutha River, India detected MBLs producing genes prevalence in the upstream region of a city and within the city. Whereas only one type of MBLs VIM was detected in the upstream region of the city and two genes of interest NDM and VIM were detected within the city (Marathe *et. al*., 2017). NDM has been also detected in sediment soil samples from the Scioto River watershed in Ohio, USA (Lee *et. al*., 2019).

IX. Epidemiology

Metallo-β-lactamases (MBL) producing bacterial isolates mainly *Pseudomonas aeruginosa*, *Enterobacteriaceae*, *Acinetobacter* spp. and other genera have a strong impact on clinical and therapeutic decisions. MBL-producing gram-negative bacteria have been reported continuously worldwide. Continues emergence and worldwide spreading of MBLs producing bacteria are becoming major threats to public health. The current

epidemiology of MBLs identification usually follows a pattern of increasing occurrences that are country-specific. Apparently, this depends upon multiple factors, including excessive use of antibiotics, dosing procedure, and clinical practices concerning the isolation of patients with multidrug-resistant pathogens.

IMP-4 was the first MBL found in China, which was detected in *Citrobacter youngae* (Hawkey *et. al*., 2001) and it was subsequently reported in four different unrelated strain of *Acinetobacter* spp. and *Klebsiella pneumoniae* (Chu *et. al*., 2001 and Mendes *et. al*., 2008). IMP-1 has been detected in *P. aeruginosa* and *Enterobacter cloacae* (Cheng *et. al*., 2008; Chen *et. al*., 2009). In 2006 in China the first VIM types MBL VIM-2 was detected in *P. aeruginosa* (Wang *et. al*., 2006). Recently NDM-1, NDM-3 and NDM-5 producing bacterial strain were detected and these MBLs producing strains was isolated from eight city hospital in China from January 2013 to December 2015. (Xiaofeng Hu *et. al*., 2017).

IMP-1 positive *P. aeruginosa* and *Serratia marcescens* which were isolated from Japanese patients were first MBL positive strain detected in Japan (Watanabe *et. al*., 1991; Osano *et. al*., 1994). However, now in Japan, IMP-1 has been found in *Enterobacteriaceae* and other non-fermenting bacterial strain. The VIM type MBLs were later identified in Japan with low prevalence than that of the IMP type MBLs.

A VIM-2 type MBL was common MBL in South Korea and it was detected in various gram-negative bacteria such as; *P. aeruginosa*, *Enterobacteriaceae* and *Acinetobacter* spp. (Walsh *et. al*., 2005; Yum *et. al*., 2002). Various type MBLs like SIM-1, IMP-1 and NDM-1 were reported from South Korea and first NDM-1 producing *Klebsiella pneumoniae* was detected in 2011.

In the Middle East region of Asia, the alleles of VIM and NDM-1 MBLs were identified. VIM-2 MBLs were detected in *P. aeruginosa*, which was isolated from Saudi Arabia and Iran and first NDM-1 was reported from Oman in 2011 (Al-Agamy *et. al*., 2009; Khosravi *et. al*., 2008).

NDM-1 and their alleles are widely spread in Indian subcontinents (Pakistan, Bangladesh, and Nepal). The NDM-12 and NDM-14 first appeared in Nepal. It has now widely identified in *P. aeruginosa*, *Enterobacteriaceae*, *Acinetobacter* spp. and *Klebsiella pneumoniae* (Rolain *et. al*., 2010; Kumarasamy (a) *et. al*., 2010 and Kumarasamy (b) *et.*

al., 2010). Several VIM types MBLs like VIM-2, VIM-5, and VIM-11 are highly widespread in *Pseudomonas* spp. (Castanheira *et. al*., 2009). IMP types MBLs identified in *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Azim *et. al*., 2010). In Asian countries like China, Japan, Korea, India, Pakistan and Gulf countries the most common MBLs are IMP, VIM and NDM-1 among them VIM and NDM most predominant.

Among the European country, Italy was the first country where MBLs were reported, namely the IMP-2 and VIM-1 (Chu *et. al*., 2001 and Cornaglia *et. al*., 1999). Various type of IMP and VIM were reported from Italy, and among them VIM-1, VIM-2 and IMP-13 are the most common (Cornaglia *et. al*., 2007). The novel IMP-12, IMP-13 and VIM-14 was also reported from Italy (Docquier *et. al*., 2003; Pagani *et. al*., 2003 and Mazzariol *et. al*., 2010). NDM-1 positive *E. coli* have also been reported (Poirel *et. al*., 2010).

The first MBL found in France was VIM-2 and it was detected in *P. aeruginosa* (Poirel *et. al*., 2000). The novel MBL IMP-19 was also reported from France, which was detected in *Aeromonas cavial*. NDM-1 and VIM-4 have been detected in a *C. freundii* bacterial isolate in a hospital patient (Poirel *et. al*., 2010).

A. baumannii derived IMP MBL was detected first time in U.K. (Walsh *et. al*., 2005). VIM-2 producing *P. aeruginosa* was identified during a British Society for Antimicrobial Chemotherapy Surveillance Programme (Miriagou *et. al*., 2010). Two novels VIM types MBL VIM-9 and VIM-10 was also reported from UK (49). NDM-1 producing *A. baumannii* also reported (Kumarasamy *et. al*., 2010).

A novel MBL IMP-5 found in *A. baumannii* was the first MBLs which were first time reported in Portugal (Da Silva *et. al*., 2002). The multiple MBLs containing strain was also reported. VIM-2 MBL was also identified in several isolates of *P. aeruginosa* in 1995 (Walsh *et. al*., 2005).

In Germany VIM-1 producing *P. aeruginosa* and *Enterobacteriaceae* was reported (Valenza *et. al*., 2010; Weile *et. al*., 2007). GIM-1 was detected in five isolates of *P. aeruginosa* from different patients in Dusseldorf (Castanheira *et. al*., 2004) and it's highly widespread in *Pseudomonas* spp. and *Enterobacteriaceae* bacterial isolates.

In European countries, the most common MBLs are IMP, VIM, DIM, SIM and NDM and among them, VIM-2 and NDM are most common MBLs widely distributed in European subcontinents.

X. Aims and Objectives

1. To investigate the existence of imipenem resistance bacteria in river water of Karala and Mahananda of Jalpaiguri and Siliguri respectively, in the northern West Bengal, India.
2. To enumerate the incidence of metallo-β-lactamase resistant bacteria in both river water of Karala and Mahananda.
3. To ascertain molecular phylogeny of the metallo-β-lactamase genes carrying isolates.
4. To characterize metallo-β-lactamase genes using molecular techniques of PCR, cloning and sequencing.
5. To ascertain of metallo-β-lactamase genes with mobile DNA elements like integrons.
6. To sequence the entire genome of a selected metallo-β-lactamase-producing bacterial strain *Pseudomonas* sp. MR 02.
7. To elucidate additional role of $bla_{\text{NDM-1}}$ genes, if any, in ampicillin catabolism.

Chapter 1

Isolation, screening and phenotypic characterization of metallo-β-lactamase (MBL) producing eubacterial isolates from Mahananda and Karala River of Siliguri and Jalpaiguri towns of West Bengal

1.1 Introduction

Rivers are the lifeline for all living organisms on our planet earth and they are the veins of our planet, through which life flows. Rivers are not only making our planet livable, but they also make it grossly beautiful, because they are the main important source of freshwater on our planet and the water of the river are invaluable useful for all living things such as man, animals, and plants. It is the source of drinking water, agriculture irrigation, power generation, transport, etc. Since human's life depends so much upon rivers, it is his bounden duty to keep them clean and pollution-free. However, due to rapid industrialization and poor maintenance, rivers become highly polluted. Now they are transformed into a huge dumping area of the city and polluted rivers water are highly dangerous for the human race because it has become a reservoir of pathogenic microbes which can directly affect the human and animals.

The river water environment is one of the most important natural bacterial habitats, which harbour richest bacterial diversity and it is also a major way of spreading of microorganisms in nature (Tamames *et. al.,* 2010). The river is a dumping area for all types of wastewater and human excreta. A man-made environment like, wastewater treatment plant (WWTP) are the most important elements which are responsible for increasing pathogenic bacterial population in river water. Human waste and wastewater are recognized as a major source of antibiotic-resistant bacteria (ARBs) and antibiotics-resistance genes (ARGs). In urban areas, wastewater treatment plant (WWTP) used for the treatment of sewage water, community and household wastewater. Wastewater treatment plants are removed chemical, organic and biological wastes from wastewater and sewage water. WWTPs are the most important collector and distributor of antibiotics resistant bacteria and antimicrobial resistance (AMR) genes because WWTP can only reduce the concentration of antibiotic resistant bacteria. WWTP is the hotspot for pathogenic microbes and spreader of those types of microbes because they collect microbes from hospital and health care sectors and dump into the river after treatment (Jury *et. al.,* 2011; Bouki *et. al.,* 2013).

As a result of decades of use and misuse of antibiotics and other antimicrobial compounds, their functional metabolites, antibiotics and AMRs gene are common and easily distributed in the river water environments. Where they can persist for long periods

many enteric pathogenic bacteria and faecal bacteria, such as *E. coli* and *Enterococci*, are now become multiple drug-resistant, with some high drug-resistance such that infections cannot be easily treated. Several reports have linked hospital isolates of carbapenemase-producing enteric bacteria to the same bacteria found in the river water that was implicated in a possible waterborne community salmonellosis outbreak (Akinyemi *et. al*., 2011) and recently a study of Suzuki has been found that a carbapenem resistant isolates *E. coli* belonging to CC10 and *K. pneumoniae* belonging to ST147 and ST11 in hospital sewage as well as in river water (Suzuki *et. al*., 2020). Water environment is not only reservoirs of both ARBs and ARGs rather it is also a hot spot for horizontal gene transfer enabling the spread of ARGs between bacterial strain and develop new antibiotics-resistant bacteria of human health concern and they are spreading globally, thus posing increased human health risks (Karkman *et. al*., 2018).

Some of the most dangerous antibiotic-resistant infections are caused by MBLs producing *Enterobacteriaceae* (lactose fermenting and non-lactose fermenting), *Pseudomonas* and *Acinetobacter* spp., and is found worldwide (Van Duin & Doi, 2017; Iovleva & Doi, 2017). These MBL positive bacterial strains showed resistance against the antibiotics of the carbapenem group. As carbapenems (including imipenem, ertapenem, meropenem and doripenem) belongs to the group of β-lactam antibiotics, so the development of resistance to carbapenems by enzyme hydrolysis mechanism is associated with resistance to other β-lactam groups of antibiotics (including penicillin and all types of cephalosporins) (Queenan & Bush, 2007). The research work of carbapenem resistance in aquatic environment and hospital has shown some relationship levels of resistance. Harmon and his co-workers (2019) found widespread carbapenem resistant bacterial isolates in water obtained from the different aquatic environment including ponds, lakes and dam in West Coast of the United State. They identified MBLs producing isolates of *Enterobacter asburiae* and *Stenotrophomonas maltophilia*.

MBLs producing bacteria have also been reported from environments other than the clinical (Isozumi *et. al*., 2012). The incidence of NDM-1 metallo-β-lactamase producing bacteria in environmental samples (drinking water), as found in New Delhi has important implications for people living in the city who are dependent on public water and sanitation facilities (Walsh *et. al.*, 2011). The reality is that a substantial proportion of

urban populations dwelling in several old and sprawling new cities of India does not have proper sanitation system and access to clean potable water (highlighted by a recent UN report, accessed March 25, 2011). The existing sewerage system in urban centers in a metropolis like New Delhi can cater to only 60% of the population (Cairncross, 2003; Ensink *et. al*., 2008; Jamwal *et. al*., 2009; Baruah, 2009; Jamwal; Mittal, 2010). The rate of potential risks associated with the 'oral-faecal' transmission of bacteria accelerates with the standards of sanitation and facilitates the continuous spread of genetic pools carrying antibiotic resistance genes from one human sector to another (Mukherjee *et. al*., 2005; Mukherjee & Chakraborty 2006; Mukherjee & Chakraborty 2007). Identification of a significant number of NDM-1 in *E. coli* is an additional source of concern as it suggests that the resistance is being disseminated in the environment as well as in hospitals (Nordmann *et. al*., 2011). NDM-1 producing bacteria are mainly spread via the 'fecal-oral' route, the distribution of NDM-1 within the Indian subcontinent is extremely high may be due to poor sewage systems and pollution of rivers by city wastes (especially hospital wastes).

India has become the focal point of global interest on antibiotic resistance as the detection of class B metallo-β-lactamases (MBLs) NDM-1 containing bacteria elsewhere (Walsh & Toleman, 2011). The study of Deshpande *et. al*., (2010) showed that NDM-1 is widely disseminated in the Indian subcontinent. Several studies have reported the presence of NDM-1 producers from different parts of India (Roy *et. al*., 2011; Castanheira *et. al*., 2011; Sarma *et. al*., 2011). Several reports from many parts of India have shown the increasing co-production of NDM-1 with other carbapenemases among the isolates of *Enterobacteriaceae* and *Acinetobacter* sp. (Karthikeyan *et. al*., 2010; Karthikeyan *et. al*., 2010; Poirel *et. al*., 2011, Lascols *et. al*., 2011).

Pressing demand of the present time is to evaluate the spread of Metallo-β-lactamase among both the hospital-acquired and community-acquired pathogens in the Indian subcontinent. It is also very much essential to extend the search in different environmental settings that facilitates both the evolution and trans-boundary dissemination of drug-resistant microbes.

The first aim of this chapter was to isolate the imipenem resistance bacteria and screen MBL-producing strains from imipenem resistance isolates from Mahananda and

Karala River flowing through Siliguri and Jalpaiguri city respectively of West Bengal, India. The second aim was to phenotypically characterizing the MBLs producing bacterial strains.

1.2. Materials and Methods

1.2.1 Work plan

A flow diagram depicting the workflow for the sequence of the microbiological analysis of both river water samples has been shown in the Figure 1.1

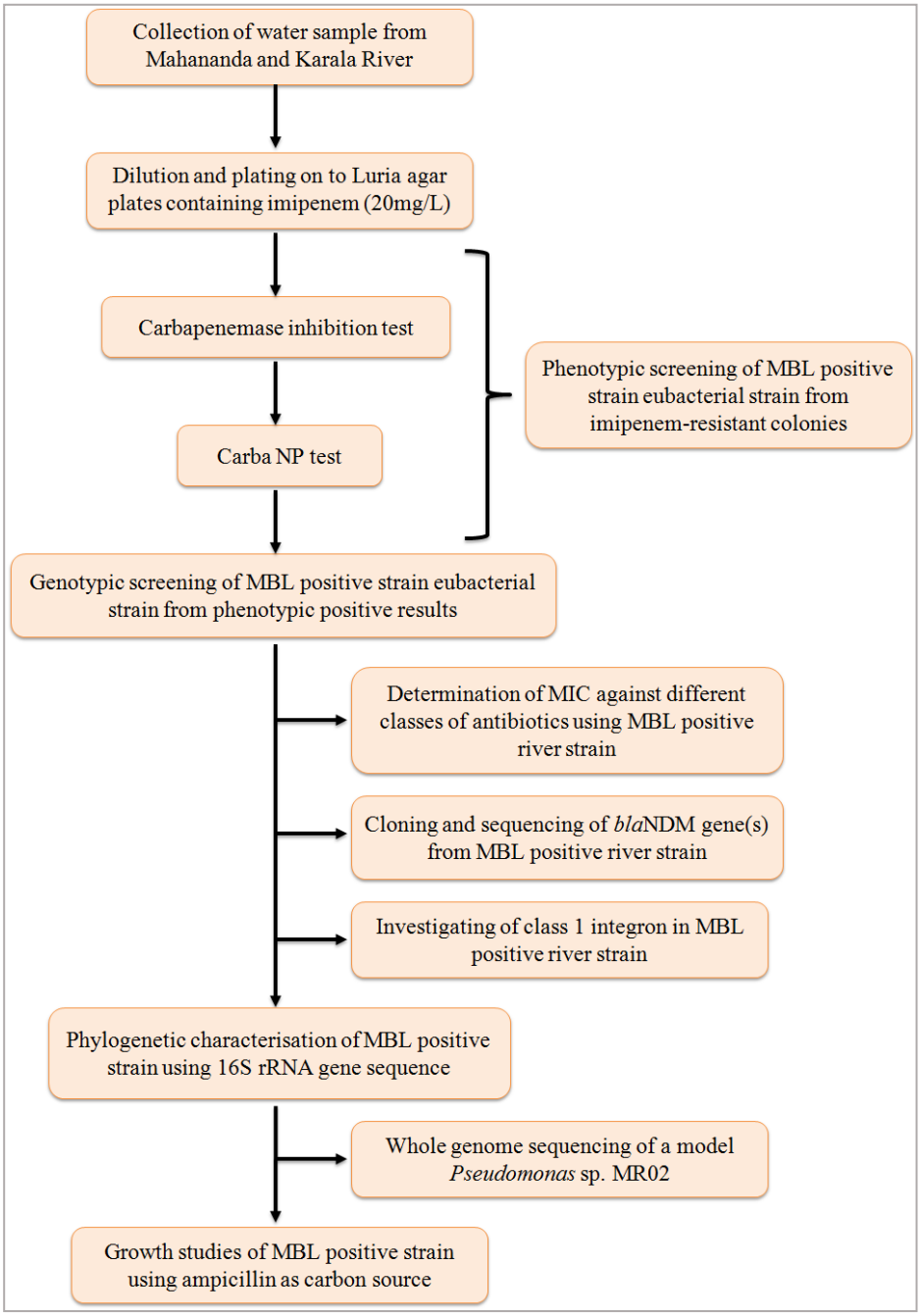


Figure 1.1: Flow diagram depicting the work flow for the methodology used in this study

1.2.2 Sampling station and sample collection from river water

The samples were collected from different locations (n= 6) in both cities Siliguri and Jalpaiguri, West Bengal India. Three locations were selected from Mahananda River representing upstream (SSM I), midstream (SSM II) and downstream (SSM III) for Karala River representing upstream (SSK I), midstream (SSK II) and downstream (SSK III). Four river water samples from each the sampling site were collected [two samples were collected before the monsoon season (March and April) and two samples were collected after the monsoon season (November and December)]. Three grab samples, from each sampling site, were collected from the left, right and middle of the river. All three samples were mixed in equal proportion and treated as a single sample. Altogether twenty-four composite water samples [6 sampling sites (3 sites on individual river × 2 rivers) × 1 composite sample from each site × 4 times sampling] were collected.

1.2.2.1 Selection of sampling station on Mahananda River

After examination of the topographic map of Mahananda River of Northern West Bengal, three sampling stations were selected individually on the river (upstream, midstream, and downstream) for the isolation of MBLs producing strains (Figure 1.2).

The sampling sites were the following:

SSM I (upstream, the entry point of the river into Siliguri city and less populated urban region) – near Champasari (26°79′22.72″ N, 88°42′85.31″ E); the sampling site is weakly affected anthropogenically and less polluted.

SSM II (midstream, under the Mahananda bridge in Siliguri city) – under the Mahananda bridge (26°71'97.20" N, 88°41'96.07" E) situated in the middle of the city; the influx of household, hospital effluents, other forms of city wastes get mixed with the midstream through the several city drainage outlets.

SSM III (downstream, a point where the river leaves the main township) – under the 3rd Mahananda bridge Babupara (26°70'22.62″ N, 88°40'88.64″ E); at Siliguri city.

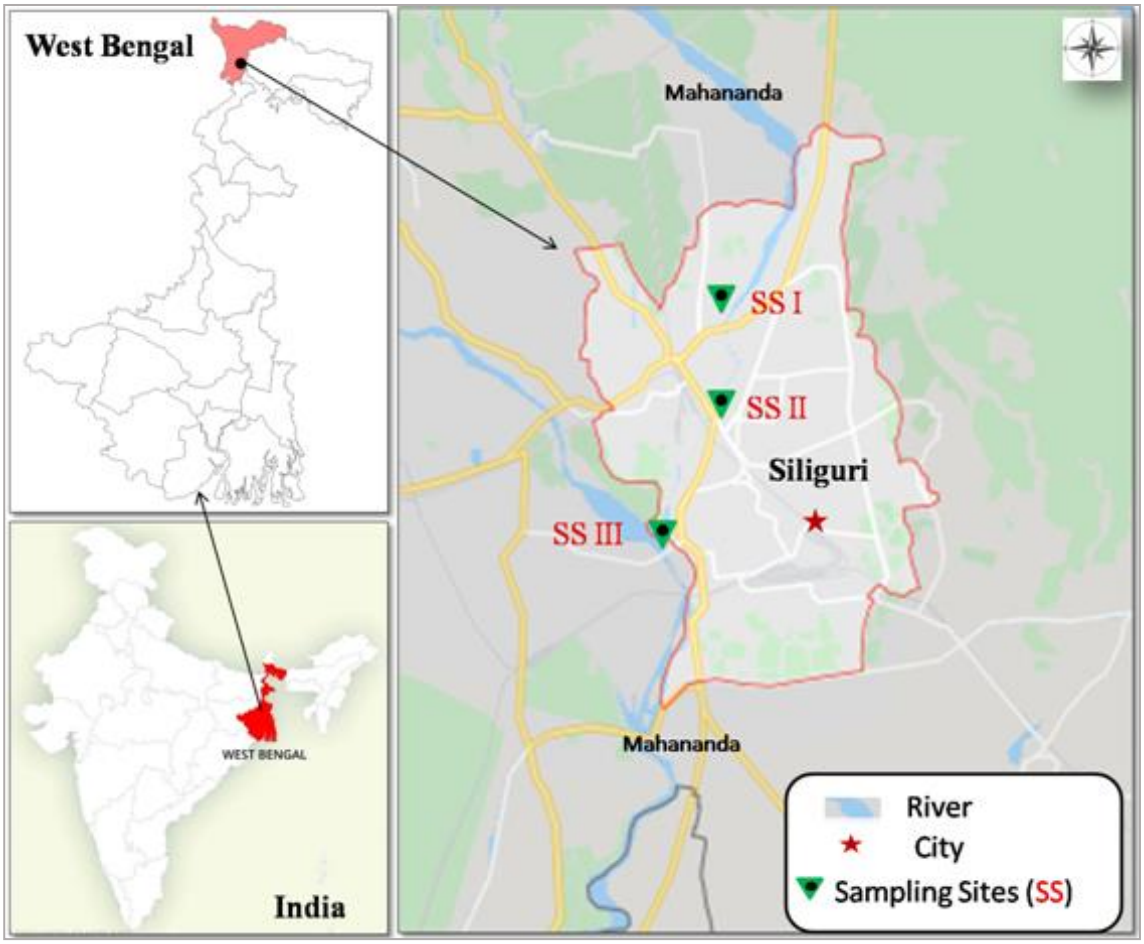


Figure 1.2: Geographical map showing sampling location in Siliguri West Bengal, India

(SSM I) upstream, (SSM II) midstream and (SSM III) downstream of Mahananda River

1.2.2.2 Selection of sampling station on Karala River

After examination of the topographic map of Karala river of Northern West Bengal, three sampling stations were selected individually on the river (upstream, midstream, and downstream) for the isolation of MBLs producing strains (Figure 1.3).

The sampling sites were the following:

SSK I (upstream; entry point of the river into Jalpaiguri city) – near Mohitnagar (26°53'10.29″ N, 88°67'02.04″ E); the sampling site is a less populated rural region.

SSK II (midstream; the midpoint of the river in Jalpaiguri city) – near the Karala river bridge (26°52'80.11″ N, 88°72'60.15″ E) situated very near to the district hospital, the

influx of household, hospital effluents, other forms of city wastes get mixed with the midstream through the several city drainage outlets.

SSK III (downstream; a point where the river leaves the main town) – near Moulayi para (26°48'92.01″ N, 88°73'66.68″ E) of Jalpaiguri city.

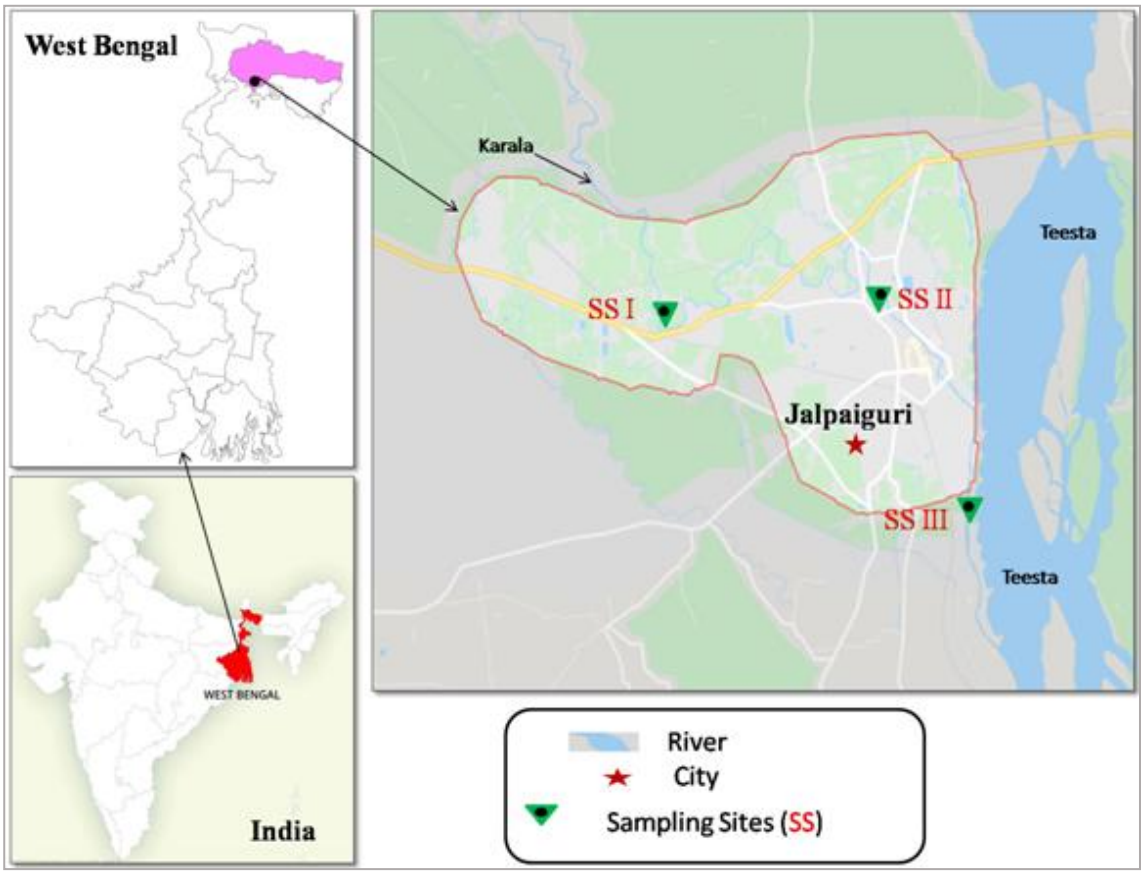


Figure 1.3: Geographical map showing sampling location in Jalpaiguri West Bengal, India

(SSK I) upstream, (SSK II) midstream and (SSK III) downstream of Karala River

1.2.2.3 Collection and transportation of water samples to the laboratory

For sampling, sterilized screw-capped water bottles were used. The bottles were opened underwater, rinsed thoroughly with the river water even it was pre-cleaned and were half-filled by opening and closing the bottles underneath the water. Total 0.5 liter water sample was collected from each sampling point. After collection, the collected samples were kept in the icebox during transportation to the laboratories. The

minimum elapsed time between collection and analysis was not exceeding more than 24 hrs and samples were kept in the refrigerator at 4°C, after reaching the laboratory.

1.2.3 Preparation of antibiotic stock solutions and antibiotic plates

The stock solutions of antibiotics were prepared by dissolving the measured amount of the antibiotic powder in either in sterile water or ethanol or DMSO as per the manufacturer's instructions. The solutions were stored at least once a month at 20°C (Table 1.1).

Table 1.1: Antibiotics solution preparation

Antibiotics	Solubility	Stock concentration (mg/ml)	Working concentration (µg/ml)
Imipenem	Water	10	50
Meropenem	Water	20	50
Cefepime	Water	50	100
Ceftazidime	Water	50	100
Aztreonam	Water	50	50
Ampicillin	Water	100	100
Tetracycline	Ethanol	50	50
Chloramphenicol	Ethanol	50	50
Piperacillin/Tazobactam	Water	50	50
Trimethoprim	DMSO	50	50
Levofloxacin	Water	50	50
Ciprofloxacin	Water	25	50

1.2.4 Isolation of imipenem resistance bacteria from river water samples

The samples were manipulated under aseptic conditions inside the laminar airflow. Before inoculation of the samples on to culture agar plates, water samples were 10-fold serially diluted using phosphate-buffered saline starting from the undiluted specimen 10^0 down to 10^{-3}. Then 100 µl from each dilution was plated onto Luria agar plates supplemented with imipenem (20 mg/L). This isolation technique was performed to enable

the isolation of the imipenem resistance. Cultured plates were incubated at 37°C for 20-24 hrs. The resulting isolated colonies were then counted for each dilution.

1.2.5 Screening of metallo-β-lactamase-producing eubacterial strains from imipenem-resistant colonies

Phenotypic and genotypic methods are used for the screening of metallo-β-lactamases-producing eubacterial strain from imipenem resistance eubacterial isolates. The genotypic method remains the gold standard for the detection of MBLs. Although genotypic method gives more reliable and satisfactory results phenotypic methods are a simple and inexpensive method for the detection of MBLs producers. Whatever but I have used both methods for the MBLs screening in this study. Several different and modified phenotypic and genotypic methods have been reported but in this study, I have used two different phenotypic and one multiplex PCR based genotypic methods for the detection of MBLs positive strain.

1.2.5.1 Phenotypic Screening

1.2.5.1.1 Carbapenemase inhibition test

Replica plating method was employed for phenotypic screening of MBL positive isolates. Discrete colonies evident on imipenem (20 µg/ml) containing MHA plates were numbered serially (1, 2, 3.. n). Approximately, 50 ±10 random numbers (out of >150 discrete colonies) were generated using a research Randomizer tool (https://www.randomizer.org). Colonies corresponding to the random numbers were then picked up with the help of sterile loop and transferred to the respective grids of the first generation master plates of MHA plates containing imipenem (20 µg/ml). The first generation master plates were then replicated on the EDTA impregnated (200µl of 50mM EDTA solution was used) imipenem (20 µg/ml) plates and incubated at 37ºC for 24 hrs. Isolates that did not show any growth on EDTA impregnated plates after proper incubation were considered as potential MBL producers. A schematic representation of the carbapenemase inhibition test method shown in Figure 1.4A.

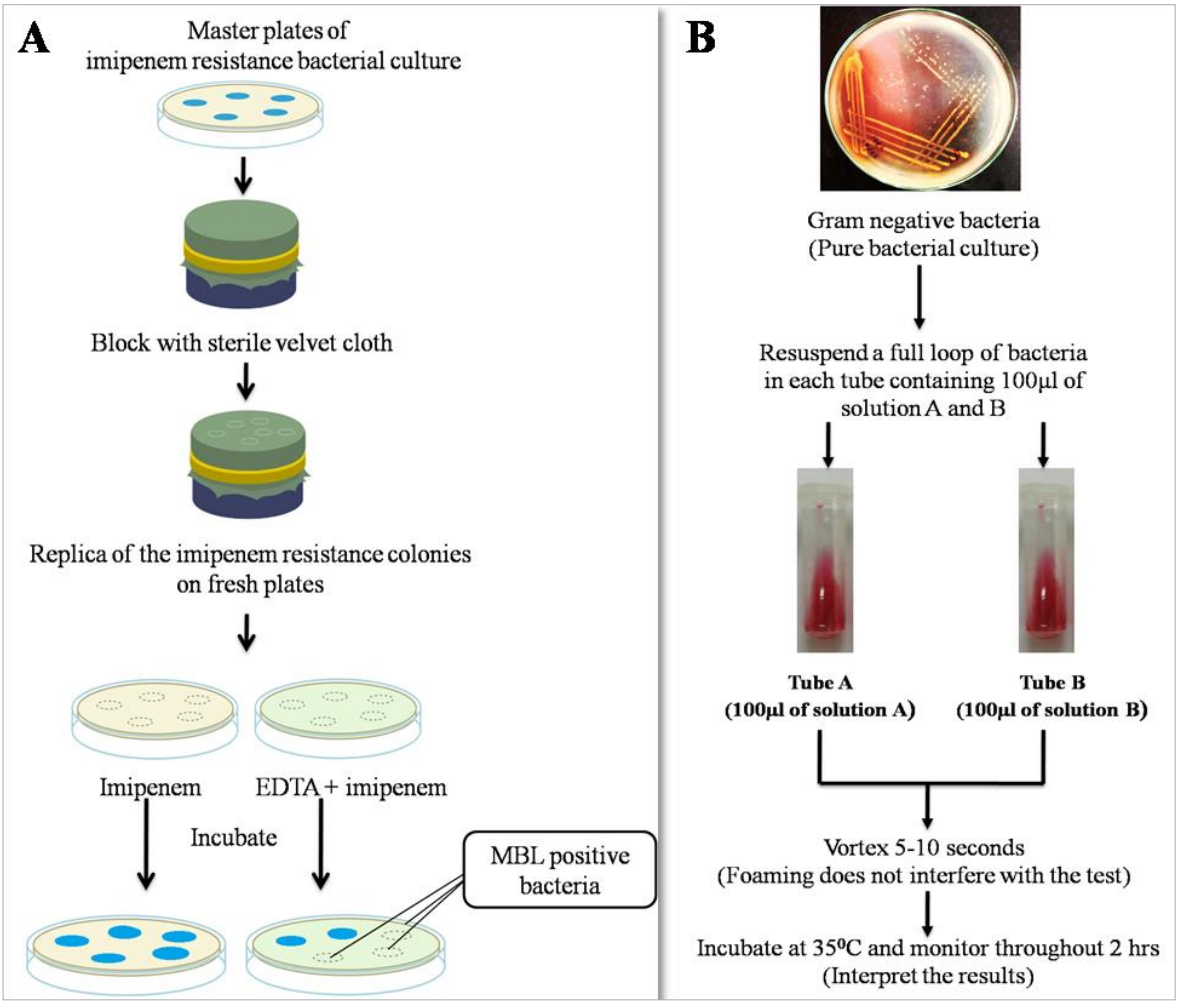


Figure 1.4: Schematic representation of the phenotypic screening protocol for metallo-β-lactamase positive bacteria

(a) Carbapenemase inhibition test (b) Carba NP-test

1.2.5.1.2 Carba NP test

Carba NP test was used for phenotypic screening of MBL positive isolates, CLSI recommends Carba NP test as a confirmatory test for detection of metallo-β-lactamases enzyme production in gram-negative bacteria. Carba NP test based on the principle of acidimetry has been previously described by Nordmann *et. al.*, 2013. This test is based on *in-vitro* hydrolysis of imipenem antibiotics by bacterial carbapenemase enzyme which is detected by a change in pH value using the indicator phenol red and the colour of the tube turned red to orange or yellow.

For Carba NP test two solutions are required one for control (solution A) and another one for the experiment (solution B) condition. The solution A was prepared by adding phenol red (0.05%) and $ZnSO_4.7H_2O$ (0.1 mmol/L) to sterile double distilled water; pH was adjusted to 7.8 ± 0.1, and the solution was stored at 4°C. The solution B was freshly prepared by adding 6 mg/ml imipenem powder (in case of imipenem + cilastatin, the amount of imipenem standard grade powder will be double 12 mg/ml) to solution A and stored at 4°C till it is used.

For Carba NP test purified one loop imipenem resistant bacteria culture was taken from 18 to 24 hrs grown Luria agar plates and were resuspended in 100 µl solution A (tube labelled as A) and one loop bacterial culture were resuspended in 100 µl solution B (tube labelled as B) and vortex for 30 sec. after vortexing, both the sample tube were incubated at 37°C for 2 hrs. The test was considered positive when solution of tube A was become red and solution of tube B was orange/yellow. *E. coli* DH5α was used as a negative control and schematic representation of the Carba NP test method was shown in Figure 1.4B.

1.2.5.2 Genotypic Screening

Multiplex PCR was used for further confirmation of MBL positive phenotype of the isolates that did not grow on EDTA impregnated imipenem plates and gave positive results in Carba NP test. This reaction refers to the use of polymerase chain reaction to amplify different DNA targets (genes) simultaneously (as if performing many separate PCR reactions all together in one reaction). This process amplifies conserved DNA segments of different MBL genes using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler. All carbapenems primers pairs have to be optimized so that all primer pairs can work at the same annealing temperature during multiplex PCR.

The DNA templates for multiplex PCR amplification were prepared as described by (Chakraborty *et. al*., 2013). Phonotypic MBLs positive bacterial isolates were grown in 5 ml LB in presence of ampicillin (50 µg/ml) at 37°C for overnight, then 1 ml of overnight bacterial culture was centrifuged at 6000 rpm for 10 minutes, and the supernatant was removed and wash the bacterial pellets. The equal volume of sterile double distilled water was added, and mixed with the vortex. The bacterial suspension was added to boiling water

bath for 10 minutes, then centrifuge at 6000 rpm for 5 minutes. Finally, the supernatant was used as a template for PCR amplification.

Table 1.2: Primer used in multiplex PCR for different MBLs genes screening

Target gene	Primer Name	Sequence (5' to 3')	Amplicon size (bp)
***bla*IMP**	IMP-F	GGAATAGAGTGGCTTAAYTCTC	232
	IMP-R	GGTTTAAYAAAACAACCACC	
***bla*SPM**	SPM-F	AAAATCTGGGTACGCAAACG	271
	SPM-R	ACATTATCCGCTGGAACAGG	
***bla*AIM**	AIM-F	CTGAAGGTGTACGGAAACAC	322
	AIM-R	GTTCGGCCACCTCGAATTG	
***bla*VIM**	VIM-F	GATGGTGTTTGGTCGCATA	390
	VIM-R	CGAATGCGCAGCACCAG	
***bla*OXA**	OXA-F	GCGTGGTTAAGGATGAACAC	438
	OXA-R	CATCAAGTTCAACCCAACCG	
***bla*GIM**	GIM-F	TCGACACACCTTGGTCTGAA	477
	GIM-R	AACTTCCAACTTTGCCATGC	
***bla*BIC**	BIC-F	TATGCAGCTCCTTTAAGGC	537
	BIC-R	TCATTGGCGGTGCCGTACAC	
***bla*SIM**	SIM-F	TACAAGGGATTCGGCATCG	570
	SIM-R	TAATGGCCTGTTCCCATGTG	
***bla*NDM**	NDM-F	GGTTTGGCGGATCTGGTTTTC	621
	NDM-R	CGGAATGGCTCATCACGATC	
***bla*DIM**	DIM-F	GCTTGTCTTCGCTTGCTAACG	699
	DIM-R	CGTTCGGCTGGATTGATTTG	
***bla*KPC**	KPC-Fm	CGTCTAGTTCTGCTGTCTTG	798
	KPC-Fm	CTTGTCATCCTTGTTAGGCG	

Eleven pairs of primers were designed to amplify conserved fragments of carbapenem resistance genes with sizes from 232 to 798 bp (Table 1.2). For the *bla*IMP, *bla*SPM, *bla*VIM, *bla*GIM and *bla*SIM genes, the primers used were those as previously

Table 1.3: Multiplex PCR reaction mixture

Reaction mixture set-A	Amount(µl)
1X PCR buffer (1.5mM $MgCl_2$)	4.5
dNTPs mix	1.5
IMP-F (238 pmol)	2
IMP-R (95 pmol)	5
VIM-F (276 pmol)	1.8
VIM-R (66.4 pmol)	7
SPM-F (144.5 pmol)	3.5
SPM-R (195.7 pmol)	2.5
Taq polymerase (2U)	2
DNA Template	10
ddH_2O	7.2
Total reaction mixture	50

Reaction mixture set-B	Amount(µl)
1X PCR buffer (1.5mM $MgCl_2$)	4.5
dNTPs mix	1.5
NDM-F (303 pmol)	1.5
NDM-R (131 pmol)	3.5
KPC-F (268 pmol)	2
KPC-R (169 pmol)	3
BIC-F (223 pmol)	2
BIC-R (195 pmol)	2.5
OXA-F (183 pmol)	2.5
OXA-R (94 pmol)	5
Taq polymerase (2U)	2
DNA Template	10
ddH_2O	7.2
Total reaction mixture	50

Reaction mixture set-C	Amount (µl)
1X PCR buffer (1.5mM $MgCl_2$)	4.5
dNTPs mix	1.5
AIM-F (137 pmol)	3.5
AIM-R (259 pmol)	2.0
GIM-F (202 pmol)	2.5
GIM-R (292 pmol)	1.6
SIM-F (227 pmol)	2
SIM-R (109 pmol)	4.5
DIM-F (142 pmol)	3.5
DIM-R (103 pmol)	4.5
DMSO	3
Taq polymerase (2U)	2
DNA Template	10
ddH_2O	7.2
Total reaction mixture	50

described by Ellington *et. al*., 2007. Another set of primers *bla*$_{KPC,}$ *bla*$_{NDM}$, *bla*$_{AIM}$, *bla*$_{DIM}$, *bla*$_{BIC}$, and *bla*$_{OXA-48}$ genes are designed by Poirel *et. al*., 2011 (Table 1.2). 3 multiplex reactions were defined, with no. 1 including detection of *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{SPM,}$ no. 2 including detection of *bla*$_{NDM}$, *bla*$_{KPC}$, and *bla*$_{BIC}$, no. 3 including the detection of *bla*$_{AIM}$, *bla*$_{GIM,}$ *bla*$_{SIM,}$ and *bla*$_{DIM.}$ The reaction mixture is shown in (Table 1.3 A, B, & C). The PCR was done in a Veriti 96 well Thermal cycler (ThermoFisher Scientific). The PCR was done by following the thermal-cycle profile as shown in Figure 1.5. The chemical used in PCR reaction were purchased from Genei labs and ThermoFisher Scientific.

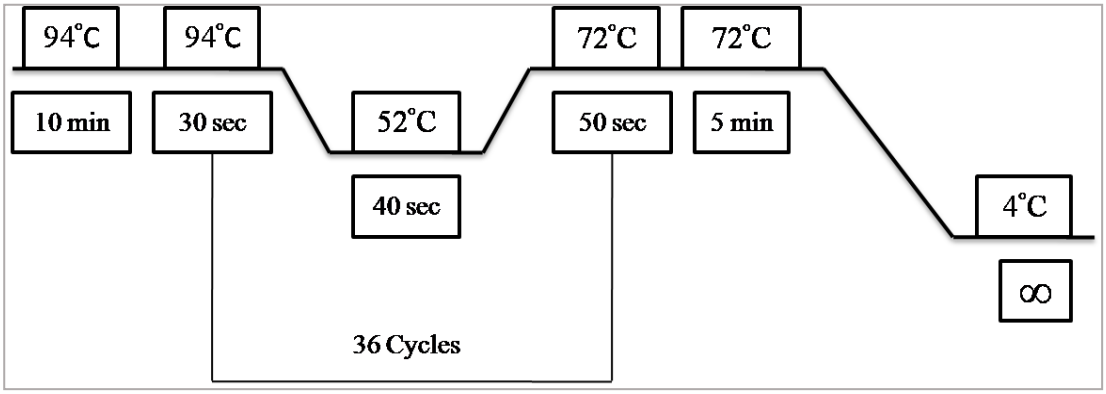


Figure 1.5: polymerase chain reaction setup for multiplex PCR

After PCR amplification, 8 µl of the amplified DNA product was electrophoresis on a 2.0% (w/v) agarose (Seakem LE Agarose, Loanza) gel at 100V for one hour in 1X TAE buffer, and a 500 bp ladder (Promega, Medison, WI), was used as a molecular size marker. Gels were stained with ethidium bromide for visualization.

1.2.6 Subculturing of MBLs positive strain using Luria broth

After the genotypic screening, all the MBL positive colonies, every single colony obtained from the imipenem containing plates were subcultured onto the same type of plates. The subculture plates of MBL positive isolates were then stored at 4°C and used within a month of sub culturing.

1.2.7 Determination of oligotrophic nature of metallo-β-lactamases positive isolates

For determination of oligotrophic nature of MBL positive strain, the inoculums were prepared by transferring a single colony of 24 hrs old cultures of MBL positive isolates into 10 ml sterile LB in 100 ml Erlenmeyer flask. The inoculated flask was incubated at 37°C for overnight at shaking incubator. The culture was harvested by centrifuging at 3000 rpm for 5 min at 4°C and washed twice with sterile distilled water to remove traces of media. The washed pellet was finally suspended in 1 ml sterile distilled water. Aliquots of 100 µl of concentrated (1 × 108 cells/ml) cell suspension (s) were added separately to 10 ml of 0.1X and 0.01X Luria Broth in 10 ml Erlenmeyer flasks. The flasks were incubated at 37°C (without shaking). Growth was observed continuously up to 48 hrs.

1.2.8 Antibiotics susceptibility profile of metallo-β-lactamase producing strains from Mahananda and Karala Rivers

1.2.8.1 Disk diffusion assay

Disk diffusion assay of 15 metallo-β-lactamase positive isolates (7 isolates from Mahananda River and 8 isolates from Karala River) was done by Kirby-Bauer method (Bauer *et. al*., 1966). For disc diffusion assay, were using 22 different antibiotics disc (showing in table 2.1) and each antibiotic disc were dispensed on MH (Muller-Hinton) agar plates inoculated with different MBL positive strains. The impregnated disc containing a standard amount (concentration shown in table 2.1) of each of the antibiotics were placed on MHA plates seeded with the MBLs positive isolates. As the bacteria grow during overnight incubation, the antibiotics molecules diffuse into the agar medium. The resistance pattern of the test organism is proportional to the zone of inhibition produced by the antibiotics used. According to EUCAST breakpoint (EUCAST, 2013), an isolate is considered resistance or sensitive to the all 22 antibiotics used and zone size ranged of particular antibiotics shown in table 1.4. A reference strain of *E. coli* DH5α MTCC 1652 was used as a negative control.

For disc diffusion assay commercially available antibiotic discs (HiMedia Co., India) were used, which was shown in table 1.4.

Table 1.4: List of antibiotic discs and its concentration used in this study

Antimicrobial category	Antimicrobial agents	Antibiotics abbreviation / Disc concentration	Interpretative Criteria					
			Enterobacteriaceae		***Acientobacter* spp.**		***Pseudomonas* spp.**	
			Sen-sitive	**Resi-stant**	**Sen-sitive**	**Res-istant**	**Sen-sitive**	**Res-istant**
			mm or more	**mm or less**	**mm or more**	**mm or less**	**mm or more**	**mm or less**
Aminoglycoside	Amikacin	AK[30]	17	14	18	15	18	15
	Tobramycin	TOB[10]	15	12	17	17	16	16
Carbapenems	Doripenem	DOR[10]	NA*	NA	NA	NA	25	22
	Ertapenem	ETP[10]	25	22	NA	NA	NA	NA
	Imipenem	IPM[10]	22	16	23	17	20	17
	Meropenem	MRP[10]	22	16	21	15	24	18
Cephalosporins	Cefepime	CPM[30]	24	21	19	19	19	19
	Cefotaxime	CTX[30]	26	22	23	14	23	14
	Cefoxitin	CX[30]	19	19	NA	NA	NA	NA
	Ceftazidime	CAZ[30]	21	17	21	17	21	17
	Ceftriaxone	CTR[30]	23	20	21	13	21	13
Fluroquinolones	Ciprofloxacin	CIP[5]	22	19	21	21	25	22
	Lomefloxacin	LOM[10]	22	18	NA	NA	22	18
	Levofloxacin	LE[5]	22	19	18	15	20	17
	Ofloxacin	OF[5]	16	12	16	12	16	12
Glycylcyclines	Tigecycline	TGC[15]	18	15	18	15	18	15
penicillins	Ampicillin	AMP[10]	14	14	NA	NA	NA	NA
	Ticarcillin	TI[75]	23	23	20	14	17	17
	Amoxyclav	AMC[30]	19	19	NA	NA	NA	NA
penicillins + β-lactamase inhibitors	Piperacillin-tazobactam	PIT[100/10]	21	17	21	17	21	14
Monobactam	Aztreonam	AT[30]	24	21	NA	NA	50	16
Polymyxins	Colistin	CL[10]	NA	NA	11	10	11	10

(*NA = Not Applicable)

1.2.8.2 Determination of minimal inhibitory concentration (MIC)

As the isolation concentration for imipenem resistance in this study was 20 μg/ml, it was important to determine the resistance levels of isolated bacteria from both river water samples. The antibiotic susceptibility of the metallo-β-lactamase producing strains was quantified through minimum inhibitory concentration (MIC) determination by broth dilution method, as per EUCAST 2013 and CLSI guidelines and breakpoints (EUCAST, 2013; Wayne, 2017). In addition, positive reference strains *Klebsiella pneumonia*e producing NDM-1 and negative reference strain of *E. coli* DH5α MTCC 1652 were included. MICs of a different group of antibiotics (β-lactams, aminoglycosides, cephalosporins, carbapenems, chloramphenicol, fluoroquinolone, sulfonamides, and tetracycline) were determined by a standard broth macro dilution method, in Mueller-Hinton broth, following CLSI guidelines and breakpoints (Wayne, 2017). The test tube was prepared and incubated for 24 hrs at 37˚C. The inoculums used per experiment was 10^4 to 10^5 cells /ml. MIC was determined as the lowest concentration of antibiotics that completely inhibited growths of the MBL producing bacterial strain in the test tube, and bacterial growth detected by turbidity measurements.

1.2.9 Study of metallo-β-lactamase activity

The metallo-β-lactamse ($bla_{\text{NDM-1}}$) activity in bacterial isolates was confirmed by the micro-broth-dilution method, in Mueller Hinton broth. It is based upon the comparative MIC values of carbapenem (imipenem and meropenem) and cephalosporin (cefepime and ceftazidime) were also determined in the presence of 0.4 mM EDTA and absence of EDTA. The test tube was prepared with Mueller Hinton broth, antibiotics, EDTA and MBL producing bacterial cells and test tube incubated for 24 hrs at 37˚C. The inoculum used per experiment was 10^4-10^5 cells/ml. The metallo-β-lactamase activity was determined as the lowest concentration of antibiotics with fixed concentration of metal chelating agent that completely inhibited growths of the MBLs producing bacterial strain in the test tube, and bacterial growth detected by turbidity measurements. To validate the effect of EDTA or to serve as a control experiment, MBL positive strain were grown in antibiotic-free Luria broth with or without 0.4 mM EDTA (Figure A1.5 and A1.6).

1.2.10 Statistical analysis

Statistical analysis (significance tests), including the t-test and standard deviation were done using GraphPad Quick Calcs (https://www.graphpad.com) and Excel 2016 respectively. The t-test is used to compare whether there are significant differences between two independent variables (imipenem resistance bacterial population). Differences between variables are considered statistically significant when the *P* value is ≥ 0.05 (Mathew & Farewll, 2007)

1.3 Results and discussion

1.3.1 Isolation and enumeration of imipenem resistant bacteria from Mahananda and Karala Rivers

The abundance of imipenem resistant bacteria in the both river water was investigated using culture-based approaches. Water samples were taken from three different sites of each river. Four river water samples from each sampling site were collected [two samples were collected before the monsoon season (March 2015 and April 2015) and two samples were collected after the monsoon season (November 2015 and December 2015)]. Water samples from three different sites of Mahananda River (SSM I, SSM II and SSM III) and Karala River (SSK I, SSK II and SSK III) were taken from 2-3 meters from both riverbank, at a depth 6 inch below the surface of the water. The samples were serially diluted and 100 µl aliquots were plated onto Luria agar plates supplemented with imipenem (20 µg/ml). We have used 20 µg/ml imipenem concentration for isolation of imipenem resistant bacteria from river water and it has a higher concentration than the prescribed concentration (8 µg/ml) as per German DIN 58940 standard (Farzana *et. al*., 2013) because the earlier study was showing that only 6.5 % of the total MBL-producing isolates exhibited ≤ 8 µg/ml MIC towards imipenem; whereas 87% exhibited MIC values ≥ 32 µg/ml so we used a compromise concentration in between 16 and 32 µg/ml imipenem in Luria agar plates for obtaining more number of MBL positive isolates from the pool of imipenem-resistant bacteria isolated from the river water samples.

Total twenty-four water samples [2 rivers × 3 sites on individual river × 4 times sampling (1 composite sampling from each site)] were collected. Numbers of imipenem resistant cultivable bacteria were determined after 24 hrs. The data on the total number of

culturable imipenem resistant bacteria of both rivers (Mahananda and Karala) in different sampling times for three sampling sites are shown in Figure 1.6 and Table A1.1.

Maximum numbers of culturable imipenem resistant bacteria in both rivers (Mahananda and Karala) were detected in water samples of April 2015 (before the monsoon session) and minimum numbers were detected in fourth and last sampling times December 2015 (during winter session).

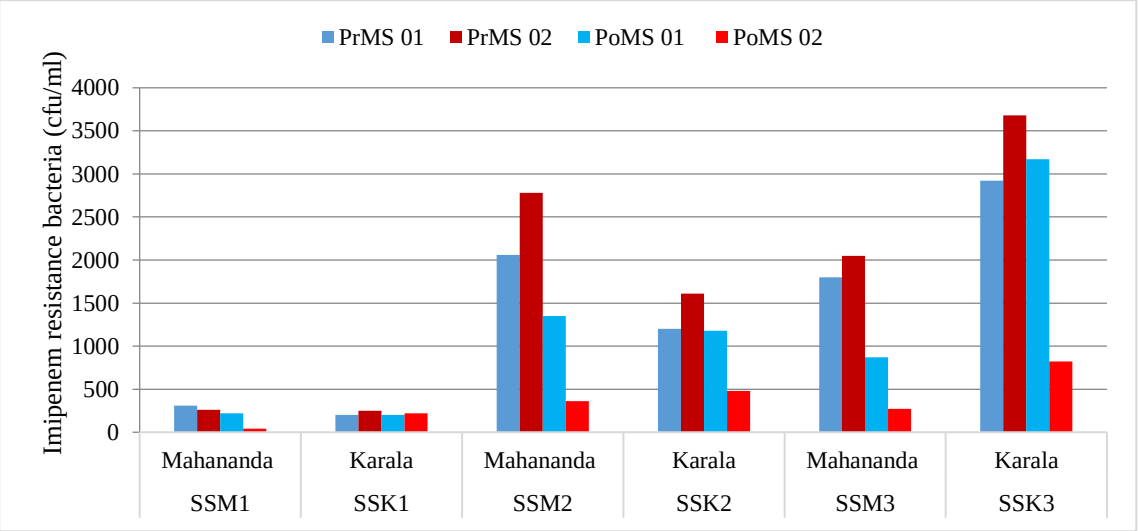


Figure 1.6: Total culturable imipenem resistant bacteria isolated from Mahananda and Karala River water from Siliguri and Jalpaiguri, West Bengal, India

Total No. of imipenem resistance bacterial isolates from three different sampling sites: SSM I, SSM II, SSM III and SSK I, SSK II, SSK III of Mahananda and Karala River respectively. Two sampling was performed before the monsoon and two sampling was performed after the monsoon.

1.3.1.1 Variation in the number of culturable imipenem resistant bacteria isolated from three sampling sites on Mahananda River

In all three sampling sites, the highest no. of imipenem resistant bacteria in Mahananda River water (mean 6.5×10^3 ±SD 1032.55 CFU/ml) (Table A1.2) was detected in SSM II (Midstream) (Figure 1.3). While the lowest numbers were detected in SSM I (upstream) (mean 8.3×10^2 ±SD 117.57 CFU/ml) (Table A1.2) which had significantly lower numbers of imipenem resistant bacteria than in sampling point SSM II and SSM III (Table 1.5). Significant variation in the number of culturable imipenem resistant bacteria (Mahananda river isolates) between individual sampling points was identified using the t-test.

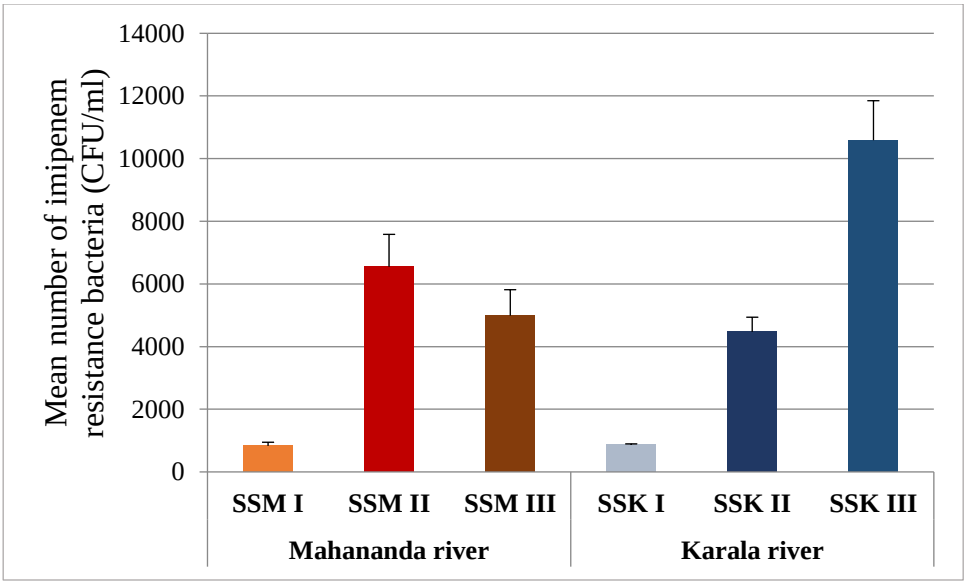


Figure 1.7: Mean total imipenem resistant bacteria isolated from each sampling site on Mahananda and Karala River of Siliguri and Jalpaiguri, West Bengal, India

Mean values ±SD are shown (n=4)

Table 1.5: Pairwise comparison using the t-test showing variation between numbers of culturable imipenem-resistant bacteria between individual sampling sites of each river

Pairwise comparison (Sampling site vs. Sampling site)	***P* value**
Mahananda River	
SSM I vs. SSM II	**0.0332**
SSM I vs. SSM III	**0.0470**
SSM II vs. SSM III	0.5768
Karala River	
SSK I vs. SSK II	**0.0086**
SSK I vs. SSK III	**0.0084**
SSK II vs. SSK III	0.0630

Significant *P* value ≤ 0.05 are marked in bold

1.3.1.2 Variation in the number of culturable imipenem-resistant bacteria isolated from three sampling sites on Karala River

The number of culturable imipenem-resistant bacteria in Karala River water was highest in SSK III (downstream) (mean 1.05×10^4 ±SD 1090.07 CFU/ml) (Table A1.2) While the lowest number was detected in SSK I (upstream) (mean 8.7×10^2 ±SD 23.63 CFU/ml) (Table A1.2) (Figure 1.7) which had significantly lower numbers of imipenem-resistant bacteria than in sampling point SSK II and SSK III (Table 1.5). Significant variation in the number of culturable imipenem-resistant bacteria (Karala River isolates) between individual sampling points was identified using the t-test.

1.3.1.3 Variation in the number of culturable imipenem resistance bacteria isolated from both Mahananda and Karala Rivers

Comparing the both river overall, the mean numbers of imipenem-resistant bacterial counts were slightly higher in the Karala River water than in Mahananda River water and no significant variation were detected in bacterial counts between both river water samples (P=0.5198) using the t-test (Figure 1.8).

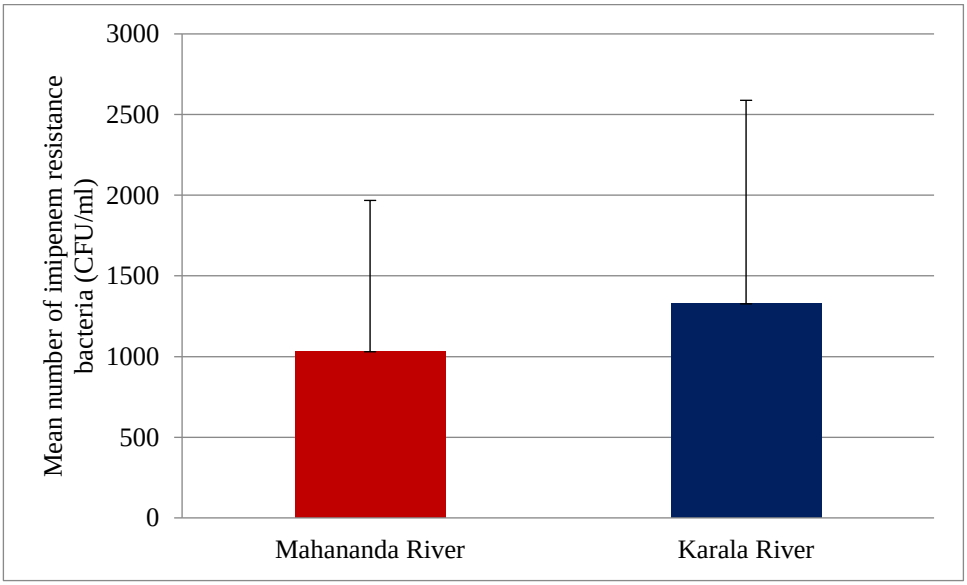


Figure 1.8: Mean total imipenem resistant bacteria isolated from Mahananda and Karala Rivers in West Bengal, India

Mean values ±SD are shown (n=12)

1.3.2 Screening of metallo-β-lactamase-producing eubacterial strains from isolates demonstrating imipenem resistance

A total 665 and 832 imipenem-resistant bacterial isolates were randomly selected from total 1,593 and 1,237 imipenem resistant bacteria isolates from Mahananda and Karala River water samples for phenotypic screening of MBL positive isolates (Table A1.3 and A1.4).

1.3.2.1 Phenotypic screening

1.3.2.1.1 Carbapenemase inhibition test

A total of 665 and 832 imipenem-resistant bacterial isolates were randomly selected from Mahananda and Karala River water samples for phenotypic screening of MBL positive isolates using replica plate method. Primarily, 5.26% (35) of imipenem-resistant bacterial isolates from Mahananda River and 4.68% (39) from Karala River were unambiguously selected as MBL positive strains as they did not show any growth on EDTA impregnated imipenem plates (Figure A1.1 and A1.2).

1.3.2.1.2 Carba NP test

The Carba NP test is a biochemical test for the rapid detection of metallo-β-lactamse production in gram-negative bacteria. We used 35 and 39 isolates for the Carba NP test from Mahananda and Karala River respectively, which did not show any growth on EDTA impregnated imipenem plates. Out of 35 from Mahananda River, seven isolates were detected as positive in Carba NP test and out of 39 isolates from Karala River, eight was detected as positive in Carba NP test (Figure 1.9).

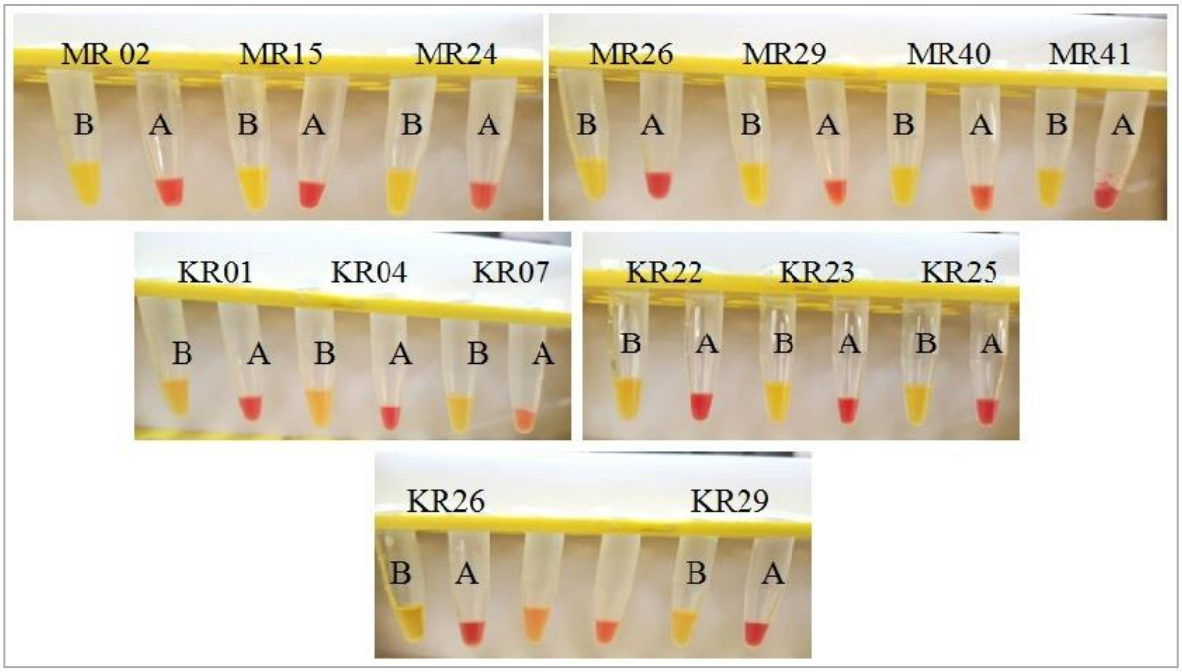


Figure 1.9: Carba NP test results of Mahananda and Karala River MBLs Positive isolates.

(Only positive result are shown in this figure)

1.3.2.2 Genotypic screening of phenotypically-ascertained MBL-positive eubacterial strains using multiplex PCR

Multiplex PCR was used to detect MBL genes within phenotypically ascertained MBL positive isolates. Out of 35 phenotypically ascertained MBL-positive bacteria isolated from Mahananda River, bla_{NDM} gene was detected in 7 (20 %) isolates only (Figure 1.10a). 20.51 % (8 out of 39 isolates) phenotypically ascertained MBL positive isolates from Karala River had bla_{NDM} and 2.56 % (1 out of 39 isolates) had bla_{VIM} genes (Figure 1.10b). Remarkably, MBL positive strains were obtained only from imipenem-resistant bacteria isolated from midstream waters of both the rivers, and conspicuously absent in isolates of up-and downstream of both the rivers.

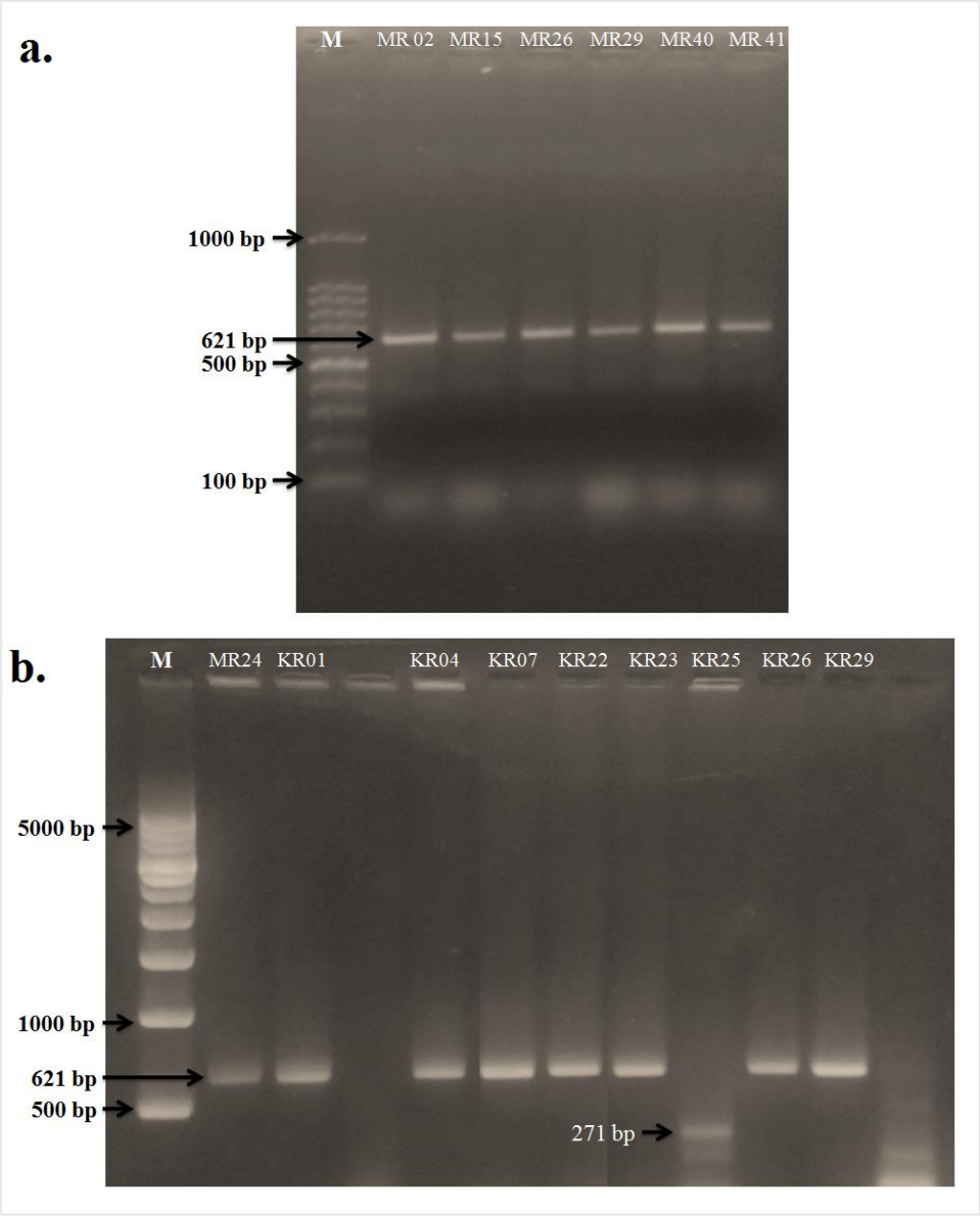


Figure 1.10: Agarose gel electrophoresis of multiplex PCR amplified MBLs genes and loaded on 2% agarose gel

(a) lane M: molecular marker (100 bp), Lane MR 02, MR15, MR26, MR29, MR40 and MR41: PCR amplification of bla_{NDM} (621 bp) from Mahananda River isolates. **(b)** Lane M: molecular marker, Lane MR24, KR01, KR04, KR07, KR22, KR23, KR26 and KR29: PCR amplification of bla_{NDM} (621 bp) from Mahananda and Karala Rivers isolates. Lane KR25: PCR amplification of bla_{VIM} (271 bp) from Karala River isolates.

1.3.3 Determination of the oligotropic nature of metallo-β-lactamase-positive isolates

Facultative oligotrophic bacteria have the flexibility to grow in less as well as the nutrient-rich media. For the detection of the facultative oligotrophic nature of MBLs producing strains, all MBL positive isolates grown in 0.1X and 0.01X Luria broth and growth was observed after 24 hrs of incubation. Except KR29, all the MBL-positive isolates have shown good growth in 0.1X LB. The growth-response was different in 0.01X LB where five isolates (KR07, KR22, KR23, MR15, and MR20) grew well, four (KR01, KR04, MR40, and MR41) grew fairly and the rest have shown poor growth; (Table 1.6). We have classified the growth results into 'good', 'fair' and 'poor' growth in terms of the range of $O.D_{600}$ values obtained for bacterial cultures after 24 h of incubation; 0.5 – 1.0, good; 0.2 – 0.45, fair; and 0.05 – 0.15, poor.

Table 1.6: Determination of the oligotrophic nature of all MBL-positive bacterial isolates in 0.1X and 0.01X Luria Broth

Strain name	0.1X LB	0.01X LB
KR01	+++	++
KR04	+++	++
KR07	+++	+++
KR22	+++	+++
KR23	+++	+++
KR25	+++	+
KR26	+	+
KR29	+	+
MR02	+++	+++
MR15	+++	+++
MR24	+++	+
MR26	+++	+
MR29	+++	++
MR40	+++	++
MR41	+++	++

(+++: Good growth, ++: Fair growth, +: Poor growth).

1.3.4 Antibiotics susceptibility profile of metallo-β-lactamase producing strains from Mahananda and Karala Rivers

Metallo-β-lactamse producing isolates were chosen for further study. A total 15 isolates (6 from Mahananda and 8 from Karala River) were characterized by phenotypic method (this chapter) and molecular methods (chapter 2). The identification of metallo-β-lactamase producing isolates was done by 16S rRNA sequencing, which is reported in chapter 2.

1.3.4.1 Disk diffusion assay

Among these 15 MBL producing isolates two isolates MR 02 and MR15 from Mahananda River and two isolates KR22 and KR23 from Karala River were demonstrated prominent resistance against 22 different antibiotics belonging to different antibiotic groups in disk diffusion test. The zone of inhibition of 22 different antibiotics is demonstrated in Figure A1.3 and A1.4 and zone diameter was represented in table 1.7 and table 1.8 for Mahananda and Karala River MBL isolates respectively. Zone diameter interpretive standard for *Enterobacteriaceae*, *Acinetobacter* and *Pseudomonas* spp. were followed according to the CLSI and EUCAST guidelines (Table 1.4). Three isolates KR04, KR26 and KR29 are showing sensitive against fluroquinolones antibiotics group; Levofloxacin and ofloxacin, sensitive pattern represented by light green color. Four isolates MR26, MR29, MR40 and MR41 showing intermediate resistance pattern against two antibiotics levofloxacin, ofloxacin and intermediate resistance pattern represented in table light pink colour and resistance pattern were represented by light brown color. Isolates KR25, KR26 and KR29 are showing a sensitive pattern against colistin antibiotics.

Table 1.7: Antibiotic susceptibility test results using disk diffusion methods for MBL producing isolates from Mahananda River

Antimicrobial category	Antimicrobial agents	Average zone diameter in mm						
		MR02	MR15	MR24	MR26	MR29	MR40	MR41
		Pseudomonas spp.		*Myroids* *sp.	*Acinetobacter* spp.			
Aminoglycoside	Amikacin	10	10	10	6	0	7	12
	Tobramycin	7	7	0	0	0	13	0
Carbapenems	Doripenem	0	0	15	NA	NA	NA	NA
	Ertapenem	NA	NA	13	NA	NA	NA	NA
	Imipenem	10	10	18	17	17	13	13
	Meropenem	0	0	16	14	14	14	14
Cephalosporins	Cefepime	0	0	13	16	13	10	14
	Cefotaxime	0	0	13	12	11	10	18
	Cefoxitin	NA	NA	12	NA	NA	NA	NA
	Ceftazidime	0	0	8	13	0	0	10
	Ceftriaxone	0	0	8	14	10	9	15
Fluroquinolones	Ciprofloxacin	0	0	16	19	17	11	20
	Lomefloxacin	0	0	14	NA	NA	NA	NA
	Levofloxacin	0	0	18	20 (S)	17(S)	17(S)	19(S)
	Ofloxacin	0	0	16	NA	NA	NA	NA
Tetracycline	Tigecycline	NA	NA	14	13	15	14	15
Penicillins	Ampicillin	NA	NA	12	NA	NA	NA	NA
	Ticarcillin	0	0	14	18	9	14	16
	Amoxyclav	NA	NA	13	NA	NA	NA	NA
Penicillins + β-lactamase inhibitors	Piperacillin-tazobactam	13	13	20	20	18	11	21
Monobactam	Aztreonam	0	0	12	NA	NA	NA	NA

(No guide line for *Myroids* sp. 'Red brown' Resistance, 'light pink' Intermediate, and light green' Sensitive, NA= Not Applicable)

Table 1.8: Antibiotic susceptibility test results using disk diffusion methods for MBL producing isolates from Karala River.

Antimicrobial category	Antimicrobial agents	Average zone diameter in mm							
		KR01	KR04	KR07	KR22	KR23	KR25	KR26	KR29
		Proteus spp.		*Escherichia* spp.			*Pseudomonas* sp.	*Acinetobacter* spp.	
Aminoglycoside	Amikacin	0	0	0	14	0	13	18	18
	Tobramycin	0	8	0	9	0	12	18	22
Carbapenems	Doripenem	NA	NA	NA	NA	NA	0	NA	NA
	Ertapenem	11	13	10	0	0	NA	NA	NA
	Imipenem	13	13	17	0	12	9	17	17
	Meropenem	11	13	17	7	8	0	13	16
Cephalosporins	Cefepime	0	8	0	0	0	0	11	11
	Cefotaxime	20	20	18	0	0	0	14	0
	Cefoxitin	14	12	14	0	0	NA	NA	NA
	Ceftazidime	0	0	0	0	0	0	0	0
	Ceftriaxone	14	8	8	0	0	0	10	10
Fluroquinolones	Ciprofloxacin	17	17	18	0	0	12	17	17
	Lomefloxacin	11	10	11	0	0	0	NA	NA
	Levofloxacin	0	20	0	8	0	0	20	31
	Ofloxacin	0	16	12	0	0	0	19	18
Tetracycline	Tigecycline	0	9	14	0	0	NA	26	25
Penicillins	Ampicillin	10	0	17	0	0	NA	NA	NA
	Ticarcillin	0	9	14	0	0	0	14	14
	Amoxyclav	13	20	11	0	0	NA	NA	NA
Penicillins + β-lactamase inhibitors	Piperacillin-tazobactam	13	21	25	0	0	0	22	36
Monobactam	Aztreonam	0	12	18	0	0	0	NA	NA

1.3.4.2 The MIC of MBL-positive isolates

Antimicrobial susceptibility testing revealed that all the 15 MBL producing isolates from both river exhibited resistance to ten different groups of antibiotics when compared with the CLSI guideline. MICs results for individual Mahananda and Karala River isolates are shown in Table 1.9 and Table 1.10

After identifying the MICs of seven MBL producing Mahananda River isolates, it was found that the MIC to imipenem among these isolates ranged from between 64 to 160 µg/ml and the MIC to meropenem are ranging between 100 to 650 µg/ml. MR26 and MR29 showing less MICs value against all the antibiotics in respect to other isolates. MR 02 bacterial strain showing unexpected high MICs value against 11 different classes of antibiotic group which is used in this study. The highest MICs value was observed in all the 7 MBL-positive isolates of Mahananda River against the ampicillin antibiotic.

The MICs of eight MBL producing Karala River isolates were determined. The MICs to imipenem among these isolates ranged from between 8 to 2700 µg/ml. the highest MIC value against the imipenem were recorded in KR22 strain and lowest value recorded in KR29 strain. KR22 bacterial strain was showing high MIC value against all the antibiotics which used in this study and KR29 showing low MICs value among these isolates. Two Karala River MBL-positives isolates KR22 and KR23 showing unexpected high MICs value against 11 different classes of antibiotic group which is used in this study. The highest MICs value was also observed in all Karala River MBL-positive isolates against the ampicillin antibiotic.

In MICs test, all the 15 MBL-positive isolates showing MIC value above the resistance cut value.

Table 1.9: Minimal inhibitory concentration of MBL producing isolates from Mahananda River

Antimicrobial category	Antimicrobial agents	MIC (µg/ml)						
		MR 02	**MR15**	**MR24**	**MR 26**	**MR29**	**MR40**	**MR41**
Aminoglycosides	Neomycin	600	600	16	32	32	64	8
Carbapenems	Imipenem	160	160	80	80	80	64	100
	Meropenem	650	650	100	400	400	200	300
Extended-spectrum cephalosporins	Cefepime	1600	1600	800	600	600	600	1200
	Ceftazidime	1600	1600	800	800	800	800	1600
Fluoroquinolones	Levofloxacin	600	600	8	8	8	128	64
	Ciprofloxacin	750	750	8	8	8	512	512
Folate pathway inhibitors	Trimethoprim	256	256	256	1500	1500	512	512
Macrolides	Azithromycin	512	512	512	512	512	512	512
Penicillins	Ampicillin	>10000	>10000	2900	1000	1000	2100	5000
Penicillins + β-lactamase inhibitors	Piperacillin/ Tazobactam	≥5000	≥5000	800	900	900	800	2600
Phenicols	Chloramphenicol	750	750	32	4	4	16	32
Tetracyclines	Tetracycline	512	512	32	32	32	16	32
Polymyxins	Colistin	64	64	32	16	16	32	32

Table 1.10: Minimal inhibitory concentration of MBL producing isolates from Karala River

Antimicrobial category	Antimicrobial agents	MIC (µg/ml)							
		KR01	KR04	KR07	KR22	KR23	KR25	KR26	KR29
Aminoglycosides	Neomycin	256	256	256	512	256	512	16	16
Carbapenems	Imipenem	800	200	315	2700	1750	800	150	8
	Meropenem	900	512	512	3400	2200	1200	100	16
Extended-spectrum cephalosporins	Cefepime	6500	7000	5500	7000	4000	3000	2000	200
	Ceftazidime	8000	9000	7000	9000	8000	5500	3000	300
Fluoroquinolones	Levofloxacin	32	16	128	128	128	128	8	8
	Ciprofloxacin	128	128	64	64	64	64	8	8
Folate pathway inhibitors	Trimethoprim	5000	>5000	>5000	>5000	>5000	3000	5000	4000
Monobactams	Azithromycin	3000	2000	6000	3000	2000	2000	32	64
Penicillins	Ampicillin	>10000	>10000	>10000	>10000	>10000	7000	5000	3000
Penicillins + β-lactamase inhibitors	Piperacillin/ Tazobactam	6000	6000	3000	9000	4000	2800	800	256
Phenicols	Chloramphenicol	128	128	128	256	256	16	16	64
Tetracyclines	Tetracycline	128	128	128	128	128	8	16	64
Polymyxins	Colistin	1024	1024	256	256	256	64	64	64

1.3.5 Study of metallo-β-lactamase activity

The MBLs activities were determined by the micro dilution method in the presence and absence of EDTA. The MIC values of carbapenem (imipenem and meropenem) and cephalosporin (cefepime and ceftazidime) were determined in the presence of 0.4mM

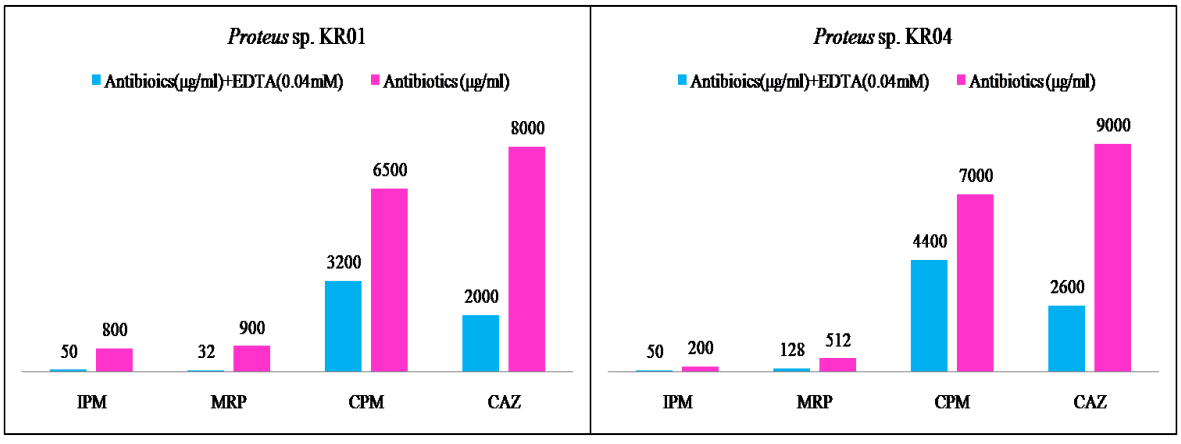
EDTA to demonstrate the reduction in the bla_{NDM} and bla_{VIM} activity due to metal chelating.

The sub-inhibitory concentration of EDTA further reduced the MIC values of Mahananda River MBL producing isolates; MR 02/MR15, MR24, MR26, MR29, MR40 and MR41 by 20, 160, 320, 320, 8 and 12 folds for imipenem, 5, 100, 100, 100, 3 and 18 folds for meropenem 2, 75, 800, 800, 36 and 75 folds for cefepime and 2.5, 75, 200, 200, 50 and 100 folds for ceftazidime antibiotic respectively. Whereas in the case of MR 02/MR15, MR40 and MR41 the MIC values were reduced but the resistant pattern remained unshakable for carbapenem (imipenem and meropenem) and cephalosporin (cefepime and ceftazidime). The MIC value of Mahananda River MBL producing isolates in the presence and absence of EDTA represented through bar graph in Figure 1.11.

Figure 1.11: MBL activity detection in Mahananda river MBL producing isolates

The sub-inhibitory concentration of EDTA further reduced the MIC values of Karala River MBL producing isolates; KR01, KR04, KR07, KR22, KR23, KR25, KR26 and KR29 by 16, 4, 2, 1.7, 1.7, 400, 75 and 8 folds for imipenem, 28, 3.9, 9, 2.1, 2.1, 150, 100 and 16 folds for meropenem 2, 1.6, 1.7, 3.2, 2, 750, 500 and 50 folds for cefepime and 4, 3.4, 1.3, 1.5, 1.3, 750, 375 and 37.5 folds for ceftazidime antibiotic respectively. Whereas in the case of KR01, KR04, KR07, KR22 and KR23 the MIC values were reduced but the resistant pattern remained unshakable for carbapenem (imipenem and meropenem) and cephalosporin (cefepime and ceftazidime) but in case of KR25, KR26 and KR29 the MIC values were reduced to equal of resistance cutoff level or below cutoff level and resistance pattern were converted into sensitive type. The MIC value of Karala River MBL producing isolates in the presence and absence of EDTA represented also through a bar graph in Figure 1.12.

Therefore, in summary, the metallo-β-lactamase activity indicate that four MBL producing isolates MR 02, MR15, MR40 and MR41 from Mahananda river and five MBL producing isolates KR01, KR04, KR07, KR22 and KR23 from Karala river have extra antibiotics resistance gene than NDM which is helping in to increasemeant of the MIC values of carbapenem and cephalosporin group of antibiotic.



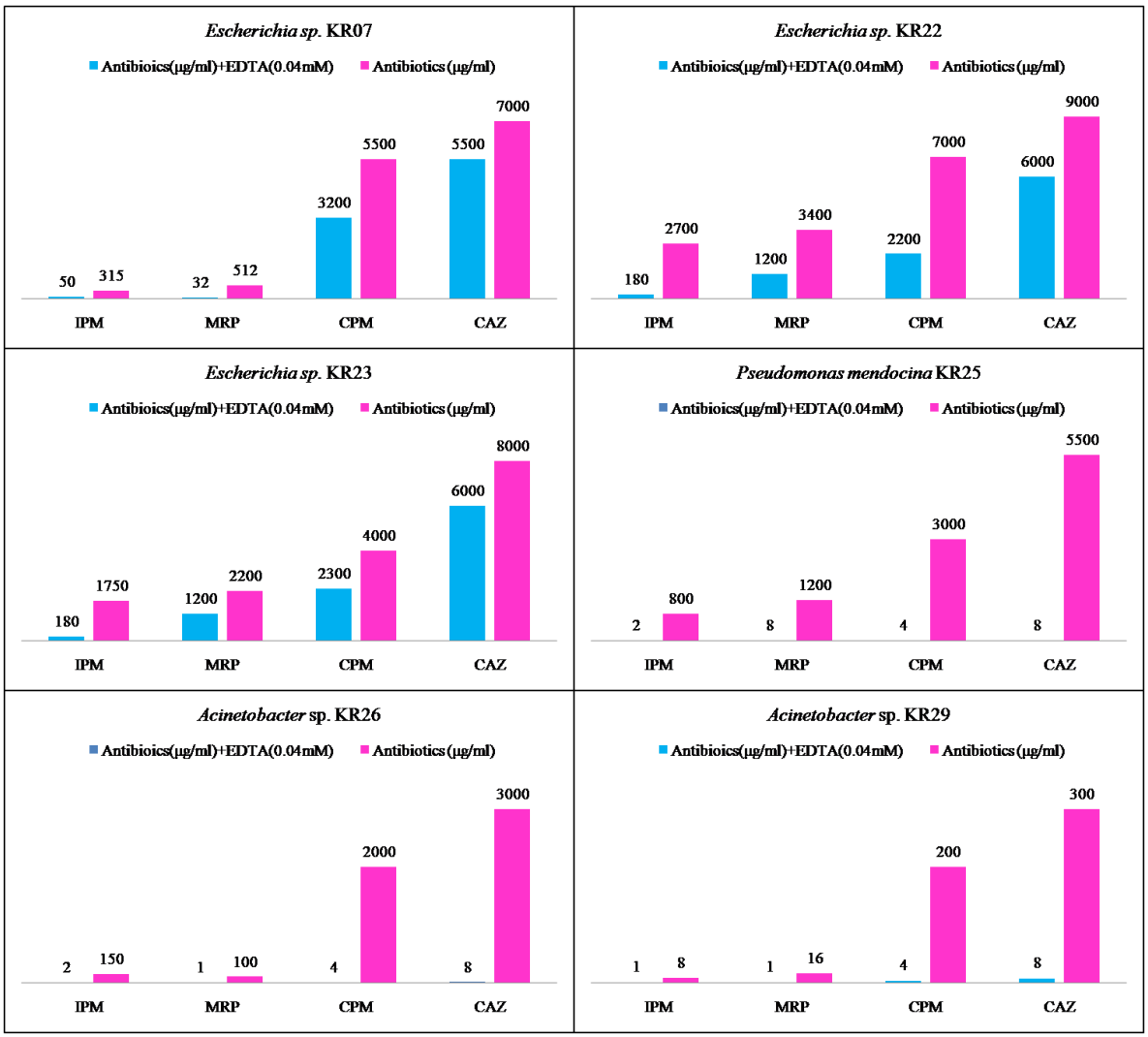


Figure 1.12: MBL activity detection in Karala River MBL producing isolates

MIC value of two cephalosporin cefepime (CPM), Ceftazidime (CAZ) and two carbapenem Imipenem (IMP), Meropenem (MRP) antibiotics in the presence and absence of metal chelator EDTA (0.4mM) and the MIC value expressed in µg/ml

1.4 Conclusion

Several conclusions can be drawn from this study and our study showed that carbapenemase-producing bacteria are present in both rivers water. The *bla*$_{NDM}$ producing strain was widely distributed in both river water samples. The lowest numbers of culturable imipenem-resistant bacteria were detected in a less populated area (SSM I and SSK I) in both rivers. All the MBLs producing strain were detected in the most populated area (SSM II and SSK II) where dumping mostly treated and untreated city wastewater and clinical

wastes. Overall the current results support the hypothesis that city-waste polluted rivers might represent an important reservoir.

We assessed various methods for the screening of MBLs producing strain from imipenem-resistant bacteria and showed that the multiplex PCR method was the most sensitive for the detection of MBL genes in culturable imipenem-resistant river water isolates. Conversely, all three methods had similar sensitivities for the screening of MBLs producing strain in river water isolates.

The overall comparative analysis of both rivers, no significant variation was observed in the mean number of culturable imipenem resistant bacterial counts. We can detect some factors which affect the occurrences of culturable imipenem-resistant bacteria in water environments. However, as there were raining events and winter temperature drop down takes place, our power to detect an effect was limited. These findings can be utilized in the future to improve efficiency in detection of imipenem-resistance bacterial detection in water environments.

On the basis of this study, we can conclude that hospital waste and municipal effluents are the main players to increase pollution and antibiotics resistance in bacteria present in river water environments.

Chapter 2

Molecular characterization and phylogenetic analysis of MBL producing bacteria, and identification of New Delhi metallo-β-lactamase (NDM) variants

2.1 Introduction

Carbapenems (Imipenem, Ertapenem, Doripenem and Meropenem) are members of a group of β-lactam antibiotics are the last choice to treat multidrug-resistant (MDR) bacteria such as *Acinetobacter*, *Pseudomonas*, *Escherichia* and *Klebsiella* species (Nicolau, 2008). However, some strains of these bacterial genera have now developed resistance mechanism against the carbapenem antibiotics. The mechanisms of resistance are variable in gram-negative bacteria and include the production of metallo-β-lactamases enzymes (the most important mechanism), PBPs (penicillin-binding proteins) and the higher expression of efflux pumps (Poirel & Nordmann, 2006). In *Pseudomonas aeruginosa* carbapenems resistance is also developed by a mutation in OprD porin genes leading to a decrease in outer membrane-activeties and specially developing a barrier for imipenem antibiotics (Eichenberger *et. al*., 2019).

The development of metallo-β-lactamase enzymes and their spread among environmental and clinical gram-negative bacteria have detrimental consequences in the treatment of infections. MBL enzymes provide resistance not only to all β-lactams antibiotics group but it also to other groups of antibiotics, especially in the case of NDM-1 in *Enterobacteriaceae and Pseudomonas* spp. (Livermore, 2009). This pattern of resistance has led to the development of pandrug resistant bacteria species which are untreatable and associated with high mortality rates (Zhi-Wen *et. al*., 2015). *Enterobacteriaceae* and gram-negative bacteria developed resistance mechanism to carbapenems by acquiring carbapenemase and MBL genes like KPC, OXA, VIM and NDM (Codjoe & Donkor, 2017).

The emergence of strains producing different types of MBLs has been observed in several countries. In the United Kingdom <1% *Enterobacteriaceae* developed resistance mechanism against the carbapenem by different MBL enzymes (that is 10 fold less prevalent than ESBLs) but in India, 5-18.5% *Enterobacteriaceae* developed resistance mechanism against the antibiotics of the carbapenem by New Delhi metallo-β-lactamase-1 (NDM-1)enzymes (Perry *et. al*., 2011; Bharadwaj *et. al*., 2012; Lascols *et. al*., 2014). In the USA 1.6% and <1% *Enterobacteriaceae* have VIM and NDM MBLs enzymes respectively, which make resistance to bacterial cells against the antibiotics of the carbapenem (CDC, 2013; Chae *et. al*., 2018). Despite their overall infrequency in India

local clusters of MBLs producers are *Enterobacteriaceae, Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter* spp. with VIM and NDM enzymes (Van Duin & Doi, 2017).

Since the discovery of NDM-1 to date, till now (2019) 24 variants of New Delhi metallo-β-lactamse have been identified (http://www.lahey.org/Studies/other.table1). These MBLs variants have been mainly identified in *Acinetobacter*, *Klebsiella* and *Escherichia* species and were found to have variation either by more than one residue at different positions or by replacing single amino acid. The most widespread variants were identified in India and their sub-continent, are NDM-1, NDM-4, NDM-5, NDM-6, and NDM-7 (Khan *et. al*., 2017)

The first aim of this study was phylogenetic characterization of metallo-β-lactamase (MBLs) producing eubacterial strain from both rivers water (Mahananda and Karala River) and the second aim was to amplify full-length amplicons of NDM to confirm the prevalence of NDM variants.

2.2 Matterials and methods

2.2.1 Extraction of crude cell lysate containing DNA

DNA extraction was performed by a method, earlier described method by Chakraborty *et. al*., (2013), taking one loopful colony from a plate and transfer into 1.5 ml microcentrifuge tube containing 200µl sterile distilled water. The cells were mixed by tapping or small vortexing for 10-15 seconds and heated using a water bath at 100^0C for 5 minutes followed by centrifugation at 6000 rpm for 5 minutes. The supernatant containing DNA was used as DNA template in PCR reaction to amplify 16S rRNA gene sequences and full length bla_{NDM} genes.

2.2.2 Amplification of 16S rRNA gene sequences of MBL producing strains

Amplification of 16S rRNA was done using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') universal primers following previously described method by Kumar *et. al*., (2010). PCR primer was synthesized from Eurofins genomics Bangalore India.

PCR amplification was formed in 25 µl reaction volume. Each 25 µl PCR mix contains; 2µL of 10X buffer containing 15mM $MgCl_2$, 1.5µL of 10mM dNTP mix, 6 pmol of each primer, 8µL of template DNA and 1.25 U Taq DNA polymerase. The PCR was done in a Veriti 96 well Thermal cycler (Thermo Fisher Scientific). The PCR was done by following the thermal-cycle profile as shown in Figure 2.1. The chemical used in PCR reaction were purchased from Genei, ThermoFisher Scientific.

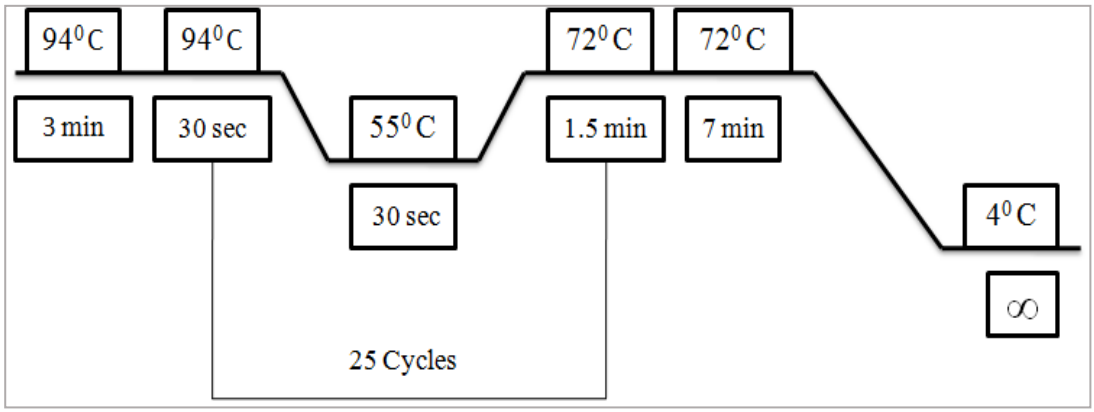


Figure 2.1 Polymerase chain reaction setup for 16S rRNA.

2.2.3 PCR amplification of complete ORF of *bla*$_{NDM}$ genes from MBLs producing bacterial isolates

The complete coding DNA sequence of *bla*$_{NDM}$ was amplified using *Eco*RI-NDM-F (5′-GGGAATTCATGGAATTGCCCAATATTATG-3′) and *Pst*I-NDM-R (5′-AACTGCAGTCAGCGCAGCTTGTCGGCCAT-3′) as the forward and reverse primer following the method described earlier (Tada *et. al*., 2014).

PCR amplification was formed in 25 µl reaction volume. Each 25 µl PCR mix contains; 2µL of 10X buffer containing 15mM $MgCl_2$, 1.5µL of 10mM dNTP mix, 6 pmol of each primers, 5µL of template DNA and 1.25 U Taq DNA polymerase (Genei India), and made up total volume of 25 µl with sterile double distilled water.. The PCR was done in a Veriti 96 well Thermal cycler (Thermo Fisher Scientific). The PCR was done by following the thermal-cycle profile as shown in Figure 2.2.

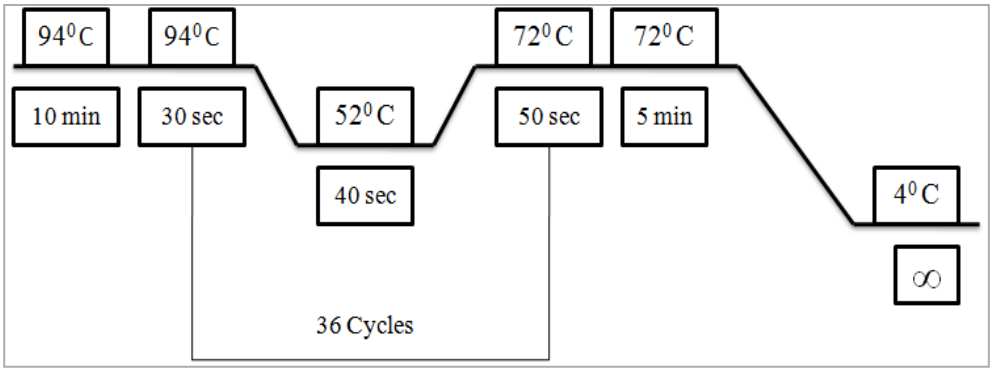


Figure 2.2 Polymerase chain reaction setup for full length ORF of *bla*$_{NDM}$ genes

2.2.4 Detection of amplified 16S rRNA and *bla*$_{NDM}$ PCR products using agarose gel electrophoresis

After PCR amplification, 5 µl of the amplified DNA product to be electrophoresed was mixed with the loading dye (1µl) (Genei India) and pipette into wells within the 1.0% (w/v) agarose (Seakem LE Agarose, Loanza) gel, and a 500 bp ladder (Promega, Medison, WI), was used as a molecular size marker. Gels were stained with ethidium bromide for visualization. DNA was electrophoresed at 80-100V. The resulting amplified DNA fragments were visualized using a UV transilluminator at 302 nm.

2.2.5 Cloning of PCR amplified 16S rRNA and *bla*$_{NDM}$ PCR products for sequencing

The pGEM–T Easy Vector System (Promega Corporation, Madison, USA) was used for the cloning purpose of 16S rRNA and NDM amplified PCR products. For cloning, ligation mixture was prepared according to the manufacturer's instructions and then transformed into competent *E. coli* DH5α cells following heat shock method

2.2.5.1 Preparation of competent *E. coli* DH5α by $CaCl_2$ method

The competent cells prepared by Madel and Higa classic experimental protocol (Mandel and Higa, 1970). Overnight grown *E. coli* DH5α cells (0.01%) were inoculated in 10 ml LB fresh flask and incubated at 37°C until the optical density at 600 nm reached 0.5-0.6. Thereafter, the cells were chilled on ice for 10 min and transfer to pre-chilled 1.5 ml microcentrifuge tube. The cells were harvested via centrifugation at 4200 rpm for 10 min at 4°C. The supernatant was discarded gently. The cell pellet was resuspended in 1.5 ml ice- cold $CaCl_2$ solution (100mM) and incubated for 45 min on ice. Finally, the tubes were

centrifuged as above, and the pellets were resuspended in fresh 300 µl ice cold $CaCl_2$ (100mM) solution.

2.2.5.2 Transformation

The chemically prepared competent cells were transformed with the ligated vector via heat shock method. 10 µl ligated DNA mixture was added aseptically to the 100 µl competent cells and mixed by gentle tapping. Thereafter, the tubes were incubated on ice for 45 min, followed exposure to heat shock at 42°C for 90 seconds. After the heat shock, the tubes were rapidly transferred on ice for 1-2min and to it, 300 µl sterile LB was added. The cells were incubated at 37°C for 45 min. Post incubation 100 µl transformed cells were spread onto the LB plates under appropriate selection pressure. The plates were incubated at 37°C for overnight.

2.2.5.3 Selection of recombinant strain

The recombinants were selected by the complementation method (by blue-white screening). Blue-white screening enabled to discriminate recombinant plasmids (white-colored colonies) from non-recombinant (blue colored colonies). Recombinants were confirmed for the presence of the required insert by a PCR reaction using T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3') primers. PCR amplification was formed in 25 µl reaction volume. Each 10 µl PCR mix contains; 1µL of 10X buffer containing 15mM $MgCl_2$, 0.75 µL of 10mM dNTP mix, 6 pmol of each primers, 1 µL of template DNA and 1 U Taq DNA polymerase (Genei India), and made up a total volume of 10 µl with sterile double distilled water. The PCR was done in a Veriti 96 well Thermal cycler (ThermoFisher Scientific). The PCR was done by following the thermal-cycle profile as shown in Figure 1.3. The chemicals used in PCR reaction were purchased from (Genei India) and ThermoFisher Scientific.

2.2.6 DNA sequencing, alignment and analysis of 16S rRNA gene and *bla*$_{NDM}$ genes

PCR products were sequenced by Eurofins Genomics Pvt. Ltd. (India) and SciGenom Labs (India), using Sanger sequencing. The sequence data were assembled and aligned after removing the vector sequences. Vector sequences were removed from both the ends via Vecscreen online tool (www.ncbi.nih.gov) and for sequence alignment offline tool MEGA X (Molecular Evolutionary Genetics Analysis) was used. The 16S rRNA gene sequence of each of the isolate was used as a query to search for the homologous sequence

in the nucleotide sequence databases by using BlastN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program (Altschul *et. al.*, 1997) and BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for *bla*$_{NDM}$ gene homology study.

2.2.7 16S rRNA gene sequences based phylogenetic analysis

The 16S rRNA sequences showing high similarities were retrieved from the GenBank database and were aligned with 16S rRNA gene sequences of the isolate by using CLUSTAL W in MEGA X software. MEGA X was used for the generation of phylogenetic trees. The evolutionary distances were calculated according to the Kimura 2-parameter method. All phylogenetic trees were constructed by the NJ (neighbor-joining) method. Tree topology was evaluated by the bootstrap analysis (based on 1000 re-samplings).

2.3 Results and discussion

2.3.1 PCR amplification, sequencing and gene sequence identification of DNA for 16S rRNA genes from MBL producing isolates from Mahananda and Karala River

DNA was extracted and PCR amplifications were performed using primer 27F and 1492R targeting the 16S rRNA gene. 16S rRNA genes were amplified from the fifteen MBL producing isolates from both river Mahananda and Karala. The PCR products were cloned in pGEM-T Easy vector prior to sequencing. Sequence analysis of these products showed that these PCR products were specific and related to the 16S rRNA gene by BLASTN program of the NCBI database. Fully constructed 16S rRNA gene sequence of the all MBLs positive isolates were deposited in NCBI database (Table 3.1). These sequences were used as query to search for homologues in the NCBI database by running BLASTN program. After Blast analysis of 16S rRNA sequences indicated that the seven Mahananda river MBL producing isolates under study fell in three bacterial genera, namely, *Acinetobacter*, *Myroides* and *Pseudomonas*. Two isolates MR 02 and MR15 belonged to *Pseudomonas*, one isolates KR24 belonged to *Myroides* and four isolates KR26, KR29, KR40 and KR41 belonged to *Acinetobacter* genera.

Table 2.1: 16S rRNA gene identification of MBL producing bacteria isolated from Mahananda and Karala River

Genus and species identification	Phylum (class)	Number of isolates	Identity % with closest related species sequence	Strain name	NCBI Accession number
Acinetobacter sp.	Proteobacteria (Gammaprotteobacteria)	**6**	97%	MR26	MN252110.1
			97%	MR29	MK816859.1
			99.20%	MR40	MK816860.1
			99.20%	MR41	MK816861.1
			98.91%	KR26	MK490975.1
			99.53%	KR29	MK490976.1
Escherichia sp.	Proteobacteria (Gammaprotteobacteria)	**3**	99.54%	KR07	MN252107.1
			99.54%	KR22	MN252108.1
			99.54%	KR23	MN252109.1
Myroides profundi	Bacteroidetes (Flavobacteria)	**1**	99.51%	MR24	MK816858.1
Proteus sp.	Proteobacteria (Gammaprotteobacteria	**2**	99.40%	KR01	MK490969.1
			99.74%	KR04	MK490970.1
Pseudomonas sp.	Proteobacteria (Gammaprotteobacteria)	**3**	99.70%	MR02	MF401548.1
			99.70%	MR15	MN252105.1
			99.27%	KR25	MK490974.1

Sequencing of the 16S rRNA gene from MBL producing strain isolated from Karala River identified 8 MBL producing bacteria. These isolates represented four genera namely, *Acinetobacter, Escherichia, Proteus* and *Pseudomonas.* Among these eight isolates two isolates KR01 and KR02 belonged to *Proteus,* Three isolates KR07, KR22 and KR23 belonged to *Escherichia,* One isolate belonged to *Pseudomonas* and two isolates KR26 and KR29 belonged to *Acinetobacter*. The two MBL producing genera, *Acinetobacter* and *Pseudomonas* are identified as a common in the both river water.

2.3.2 Phylogenetic analysis

Phylogentic analysis of six strains of *Acinetobacter* sp. together with 35 other similar sequences of same genus, retrieved from NCBI Genbank database were used to construct the phylogenetic tree to understand the nearest neighbour of the study sequences. The genetic divergence and homogeneity of the sequences are apparent in the phylogenetic tree (Figure 2.3). Trees were rooted using the genus *Cellvibrio japonicus* as an outgroup. In evolutionary comparisions with the 16S rRNA gene sequences indicated of these strain BlastN analysis revealed 96.94% and 98.91% similarity with KR26 and MR26 with *A. equi* strain 114 and *A. puyangensis* BQ-1 respectively while MR29 shared 94.93% and 95.35% similarity. One MBL-producing strain KR29 has shown 99.5% similarity with *A. seohaensis* SW-100. Two MBL-producing strain MR40 and MR41 strain showed 99.2% similarity with *A.Variabilis* NIPH2171. In the phylogenetic tree, isolates MR29 and MR26 formed a complete separate cluster with KR26 supported by 100% bootstrap value. Nearest to them was *A. equii* and *A. pragnesis* (Figure 2.3). KR29 branched in a cluster with *A. seohaensis* while MR40 and MR41 formed separate clustered with *A. variabilis* (Figure 2.3). On the basis of 16S rRNA sequence analysis and phylogentic analysis four strains MR26 and MR29 are same species, in the same way MR40 and MR41 are also the same species. All six MBL (bla_{NDM}) producing *Acinetobacter* sp. from both river water revealed novel species.

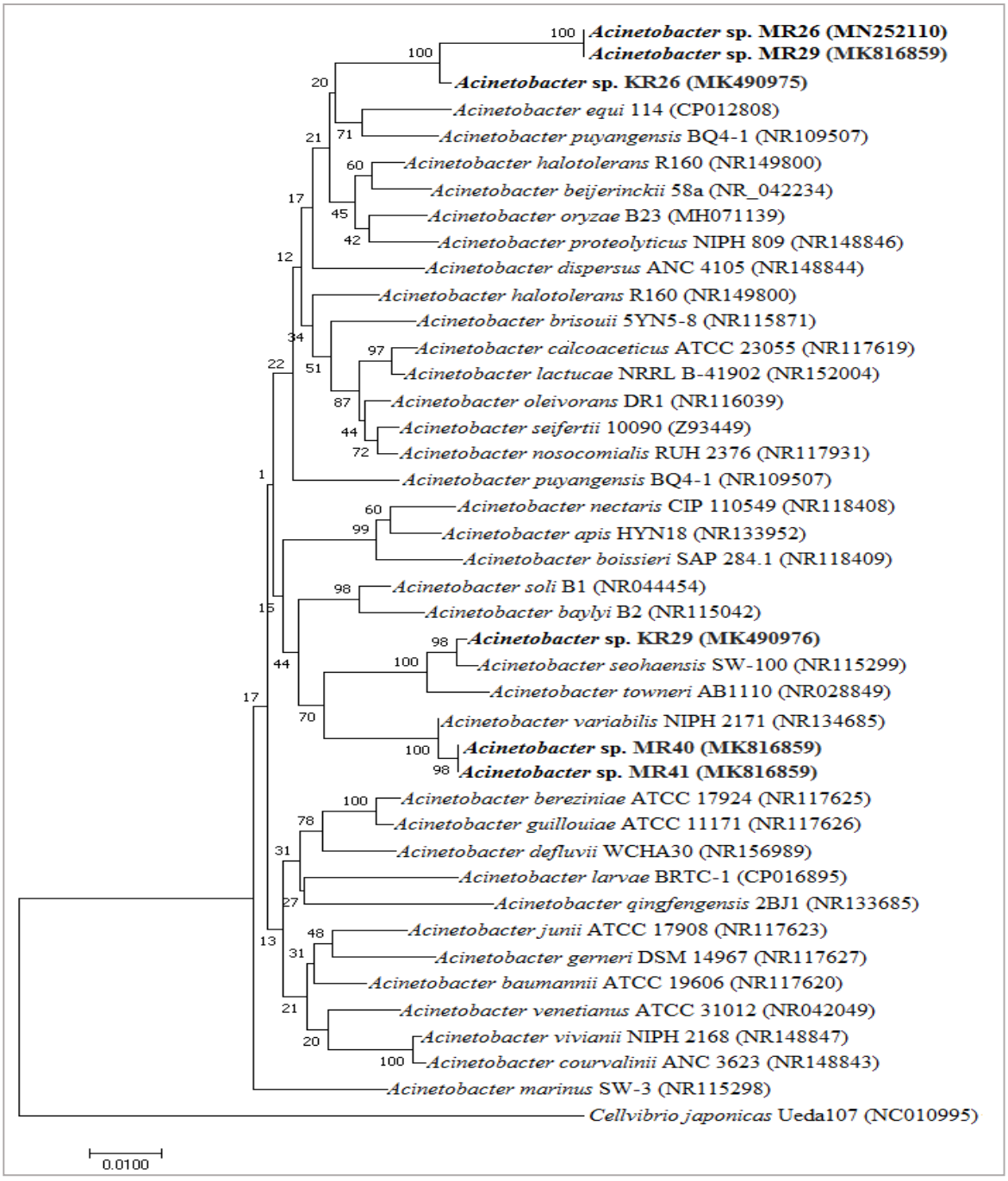


Figure 2.3: Phylogenetic tree of MBL-producing strains MR26, MR29, MR40, MR41, KR26 and KR29

16S rRNA sequence-based phylogenetic tree showing the position of strain MR26, MR29, MR40, MR41, KR26 and KR29 (bold letters) with the other species of genus *Acinetobacter*. *Cellvibrio japonicus* is used as outgroup. The tree was constructed using the Neighbor-Joining method. The numbers at the branching points are the proportion of 1,000 bootstrap resamplings, supporting the tree topology. GeneBank accession numbers are given in parentheses.

Three (20%) MBL positive isolates KR07, KR22 and KR23 were identified as the members of the genus *Escherichia*. They exhibited 99.54% similarity with *Escherichia fergusonii* ATCC35469 and *Escherichia albertii* TW07627 during BlastN analysis. 16S rRNA phylogeny showed that they were branching with *Escherichia albertii* with 95% bootstrap confidence value (Figure 2.4). Trees were rooted using the genus *Morganella morganii* subsp. *Sibonii* DSM 14850 as an outgroup.

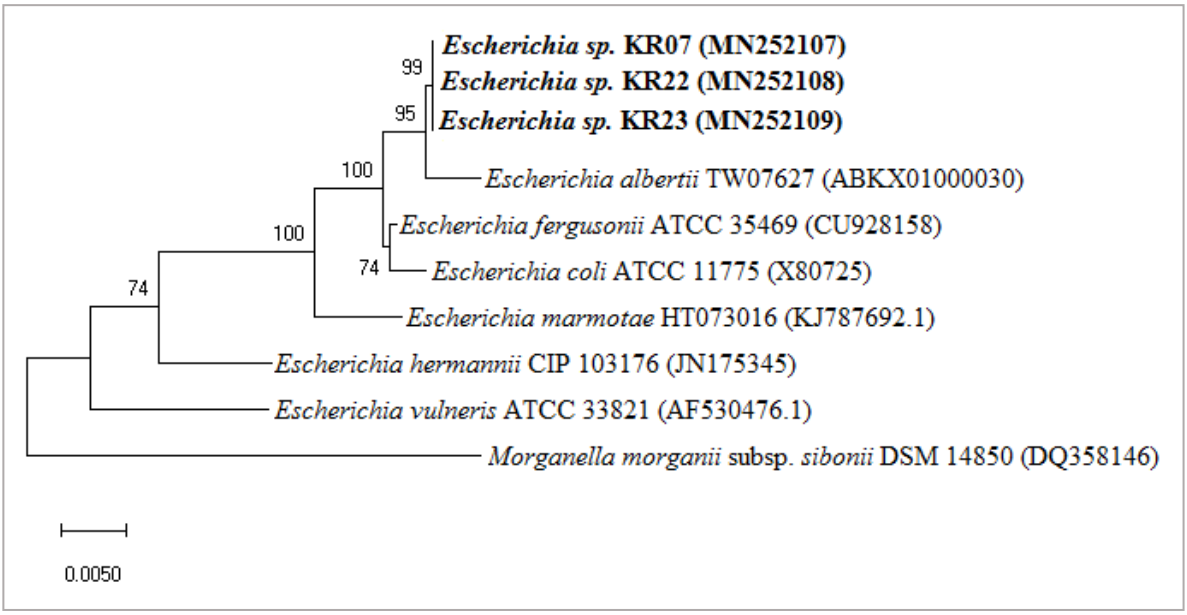


Figure 2.4: Phylogenetic tree of MBL-producing strains KR07, KR22 and KR23

16S rRNA sequence-based phylogenetic tree showing the position of strain KR07, KR22 and KR23 (bold letters) with the other species of genus *Escherichia. Morganella morganii* subsp. *Sibonii*is is used as outgroup. The tree was constructed using the Neighbor-Joining method (N-J). The numbers at the branching points are the proportion of 1,000 bootstrap resamplings, supporting the tree topology. GeneBank accession numbers are given in parentheses.

Only 13.33% (2 of 15) of the MBL positive isolates belonged to the genus *Proteus*. Isolate KR01 exhibited 99.4% similarity with *Proteus vulgaris* NCTC13145^{T} and 99.2% similarity with *Proteus mirabilis* ATCC29906^{T} at nucleotide level while KR04 revealed 99.73% similarity with *Proteus vulgaris* NCTC13145^{T}. In the phylogenetic tree KR04 clustered with *Proteus vulgaris* but the nearest members of KR01 were *P. mirabilis* and *P. columbae* (Figure 2.5). Trees were rooted using the genus *Morganella morganii* subsp. *Sibonii* DSM 14850 as an outgroup.

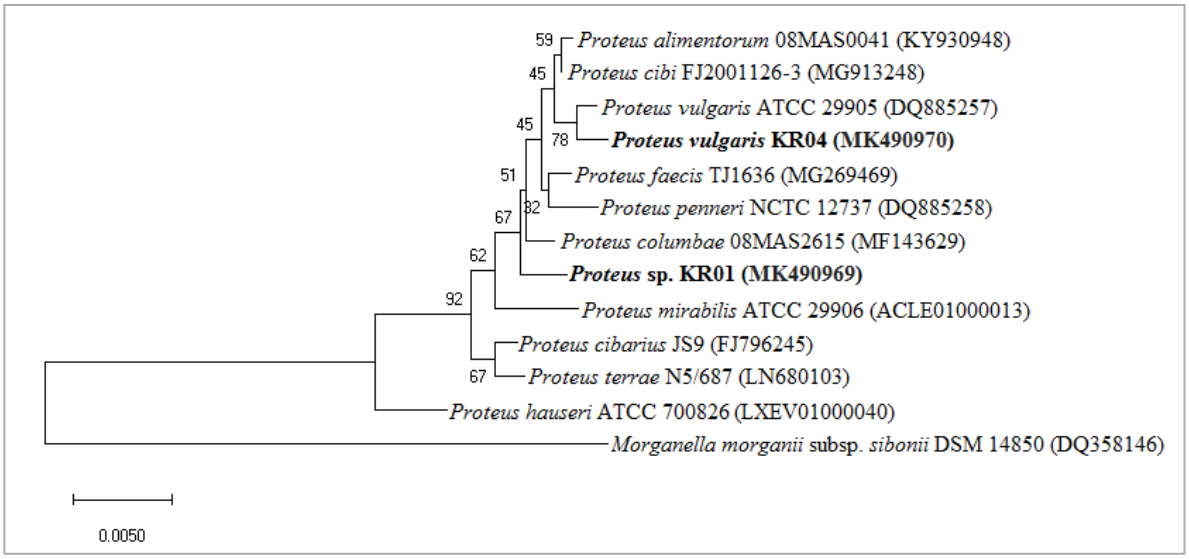


Figure 2.5: Phylogenetic tree of MBL-producing strains KR01 and KR04

Rooted 16S rRNA sequence-based phylogenetic tree showing the position of strain KR01and KR04 (bold letters) with the other species of genus *Proteus. Morganella morganii* subsp. *Sibonii*is used as outgroup. The tree was constructed using the Neighbor-Joining method. The numbers at the branching points are the proportion of 1,000 bootstrap resamplings, supporting the tree topology

The genus *Pseudomonas* was represented by 20% (3out of 15) MBL positive isolates (MR15, MR20 and KR25). Highest similarities at nucleotide level were recorded with *P. mendocina* NCTC10897^{T} (99.71% similarity with KR25), *P. taiwanensis* DSM 21245^{T} (99.6% similarity with MR 02 and MR15) and with *P. plecoglossicida* DSM 15088^{T} (99.4% similarity with MR 02 and MR15). In the phylogenetic tree isolate KR25 branched in a cluster with *P. mendocina* but MR 02 and MR15 clusterted together separately from other members of *Pseudomonas* (Figure 2.6).

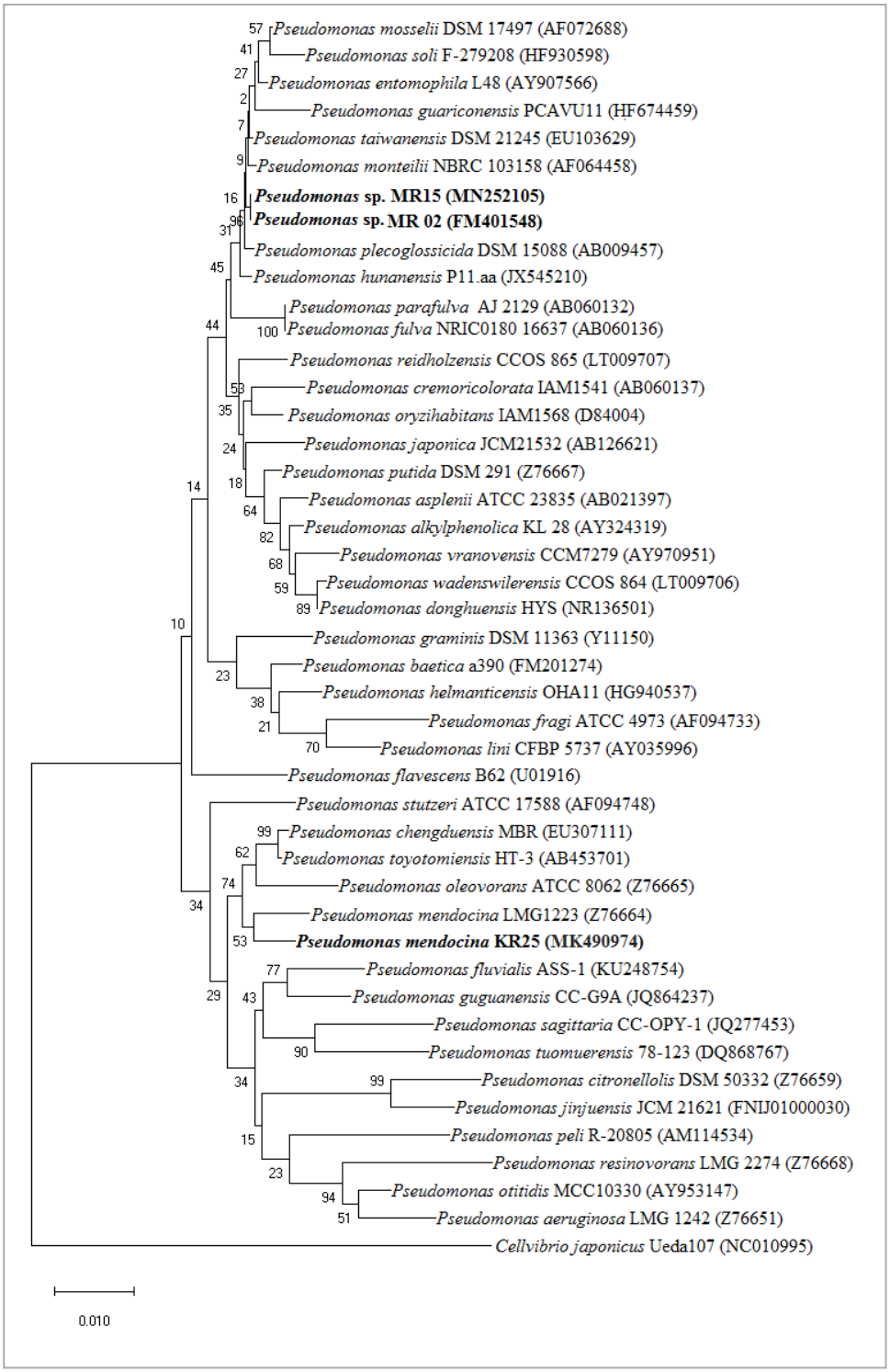


Figure 2.6: Phylogenetic tree of MBL-producing strains MR 02, MR15 and KR25

Rooted 16S rRNA sequence-based phylogenetic tree showing the position of strain MR15, MR20 and KR25 (bold letters) with the other species of genus *Pseudomonas. Cellvibrio japonicus* is used as outgroup. The tree was constructed using the Neighbor-Joining method. The numbers at the branching points are the proportion of 1,000 bootstrap resamplings, supporting the tree topology.

One MBL positive isolates belonged to the genus *Myroides*. Mahananda River MBL positive MR24 isolate exhibited 99.51% similarity with *Myroides profundi* and 99.44% similarity with *Myroides odoratimimus*, at the nucleotide level while MR24 revealed 99.17% similarity with Myroides gitamensis (Figure 2.7). The tree was rooted using the genus *Cellvibrio japonicus* as outgroup.

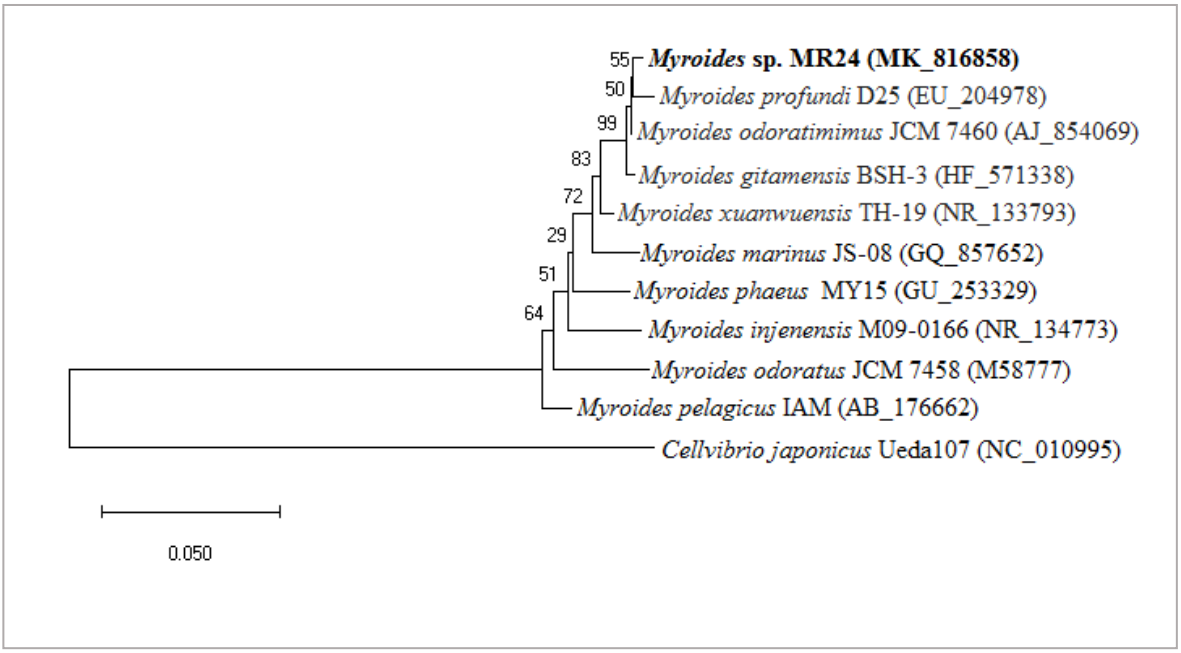


Figure 2.7: Phylogenetic tree of MBL-producing strain MR24

Rooted 16S rRNA sequence-based phylogenetic tree showing the position of MBL positive strain MR24 (bold letters) with the other species of genus *Myroides. Cellvibrio japonicus* is used as outgroup. The tree was constructed using the Neighbor-Joining method. The numbers at the branching points are the proportion of 1,000 bootstrap resamplings, supporting the tree topology.

A comparison of the both river water MBLs isolates (Table 2.1) shows that *Acinetobacter* genera were common to both water environments. The other isolates *Pseudomonas* genera were only common in Mahananda river water and *Escherichia* and *Proteus* were only common in Karala river water samples.

2.3.2 PCR amplification, sequencing and identification of NDM variants in MBL producing river water isolates

Genomic DNA was extracted from the MBL producing isolates and using NDM targeting primer for the complete ORF genes of NDM and to the identified NDM variants

in MBL producing strains. 813 bp PCR products of NDM genes were generated from the Mahananda and Karala River isolates.

Sequence analysis of these products from both river water isolates showed that these PCR products were specific and related to NDM genes. Two different types of NDM alleles were identified, NDM-1 and NDM-7 from both river MBL positive isolates (Table 2.2). NDM-1 was detected in seven MBL producing isolates MR 02, MR15, MR24, MR26 and MR29 from Mahananda River and four isolates KR01, KR04, KR26 and KR29 from Karala river while NDM-7 was found in MBL producing isolates KR07, KR22 and KR23 from Karala river. 11 $bla_{\text{NDM-1}}$ bearing multidrug resistant isolates belonging to four genera *Acinetobacter*, *Myroides*, *Proteus* and *Pseudomonas* and three $bla_{\text{NDM-7}}$ bearing multidrug resistant isolates belonging to one genera *Escherichia*. The only one isolates among these MBL producing isolates, which bearing $bla_{\text{VIM-2}}$ belonging to the genus *Pseudomonas* (gene characterized in chapter 3).

Overall, NDM-1 bearing *Acinetobacter* are most prominent isolates in both river water samples. In India first NDM-1 producing *Acinetobacter* was reported in year 2010 (Karthikeyan *et. al*., 2010) and by looking at the previous study, it showed that the NDM producing *A. baumannii* strain was detected in the sewage water of the hospitals but till this study NDM producing *Acinetobacter* strain report was not done in river water or fish pond water.

Table 2.2: Identification of PCR products of *bla*$_{NDM}$ genes from Mahananda and Karala river MBL-producing bacterial isolates

Strain name	Organism name	Types of NDM	Identity %	NCBI Accession No.
MR 02	*Pseudomonas* sp.	*bla*$_{NDM-1}$	100	PJI70472
MR15	*Pseudomonas* sp.	*bla*$_{NDM-1}$	100	MN251668
MR24	*Myroides profundi*	*bla*$_{NDM-1}$	100	MK834316
MR26	*Acinetobacter* sp.	*bla*$_{NDM-1}$	100	MN251666
MR29	*Acinetobacter* sp.	*bla*$_{NDM-1}$	100	MN251667
MR40	*Acinetobacter* sp.	*bla*$_{NDM-1}$	100	MN251664
MR41	*Acinetobacter* sp.	*bla*$_{NDM-1}$	100	MN251665
KR01	*Proteus* sp.	*bla*$_{NDM-1}$	100	MK834310
KR04	*Proteus* sp.	*bla*$_{NDM-1}$	100	MK834311
KR07	*Escherichia* sp.	*bla*$_{NDM-7}$	100	MK834312
KR22	*Escherichia* sp.	*bla*$_{NDM-7}$	100	MK834313
KR23	*Escherichia* sp.	*bla*$_{NDM-7}$	100	MK834314
KR26	*Acinetobacter* sp.	*bla*$_{NDM-1}$	100	MK834315
KR29	*Acinetobacter* sp.	*bla*$_{NDM-1}$	100	MN251671

2.4 Conclusion

The most abundant MBL producing genera identified were *Acinetobacter* (40%) from both river water samples and two genera *Acinetobacter* and *Pseudomonas* are common in both river's water. Infection from NDM producing bacteria are now increasing at an unprecedented rate in many parts of India and Indian subcontinents like Pakistan, Bangladesh and Nepal. The NDM genes detected in the river water isolates (in the present study) were closely related to clinically important species. This indicates that a potentially

MBLs (Mainly NDM) producing strains are now spread in river water of North Bengal India. The very low number (1 out of 15) of VIM MBL producing bacteria was detected in river water samples (Chapter 3) but it is the most dangerous sign for the health sector. Although the percentage of NDM producing bacteria is very low, but it is dangerous which is forcing us to search for a solution. Otherwise in the coming days the amount of NDM producing bacteria in the rivers of North Bengal will be much higher and that could be a challenge for treatment of infected persons from North Bengal. Three strains KR07, KR22 and KR23 are identified as the same species (Molecular level) and they grouped under *Escherichia* genera but their phenotypic characters are different. Two isolates MR 02 and MR15 are identified as the same species and there phenotypic character is also same but they are highly resistance against all the antibiotics used in this study and may support its role as an opportunistic pathogen. MR 02 used as a model strain for further analysis via whole genome sequencing (Chapter 4) and to understand the mechanism of resistance against all antibiotic classes.

Chapter 3

Detection of class 1 integrons and molecular characterization of antibiotic resistance gene cassettes in MBL-producing bacterial isolates

3.1 Introduction

Microbes are transformed into superbug by molecular evolution. In the molecular evolution, microbes are adopted to several mechanisms for its survival and to maintain genomic plasticity. Horizontal gene transfer (HGT) mechanism is common for molecular evolution in microbes. Antibiotics resistance genes are located on plasmids, transposons, integrons, and chromosome. Plasmid, transposons and integron have an element which plays major roles in horizontal transfer of antibiotic resistance genes (Schwarz & Chaslus-Dancla, 2001). However, numerous studies have shown that many antibiotic resistance genes found on plasmids and transposons in Gram-negative bacteria are located at a unique site within the conserved DNA sequence, leading to the discovery of a new genetic-element called an integron. The term integron was coined by Stokes and Hall in 1989 (Stokes & Hall, 1989; Hall & Collis, 1995). Integrons are generally located on mobile genetic-elements MGEs like transposons and plasmids that could serve as vehicles for the inter- and intra-species transmission of genes. On the basis of recent year report integron have played an important role in horizontal gene transfer of antibiotic resistance gene in bacteria.

Integrons are genetic elements that contain a site-specific recombination system which is able to insert and express a specific DNA element. All integrons contain three key elements: an *intI* gene encoding an integrase, proximal primary recombination site (*attI*) and an integron-associated promoter (Pc) for driving the expression of the newly integrated genes (Messier & Roy, 2001; Partridge *et. al.,* 2000; Collis & Hall, 1995). It has generally defined by the presence of an integrase gene (*intI*) and a proximal primary recombination site (*attI*) (Messier & Roy, 2001) (Figure 3.1). The integrase protein (intI) which belongs to a member tyrosine recombinase family is catalyzes recombination between incoming gene cassettes and the second core components, an integron-associated recombination site, *attI.* On the basis of location of the recombination site in integron they are of two types*:* first *attI*, primary site of attachment of the gene cassettes and second *attC* which is located on the gene cassettes (Poirel *et. al.,* 2009). These sites are recognized by the integrase protein, and once a gene cassette is incorporated in inetgron, then it is expressed by the third core feature, an integron-associated promoter, Pc. Generally most of the gene cassettes have no promoters preceding the genes harbored in them, making the regulation

of the genes within gene cassettes dependent on the upstream Pc. In class 1 integrons, several Pc variants have been reported on the basis of their -35 and -10 hexamer sequences as well as their spacer sequence, and the relative strengths of these Pc variants have been calculated (Papagiannitsis *et. al*., 2009). Apart from the Pc promoter in class 1 integron a second promoter P2 is located about 90bp downstream of the Pc promoter (Figure 3.1). Integrons capture foreign antibiotic resistance genes with the help of integrase, which are expressed under the control of upstream promoters. The amino acid sequence of integrase gene have been used as a basis for dividing integrons into classes those contain *intI1* can be defined as class 1 integron, *intI2* as class 2 integron, and int3 as class 3 integron. Total nine different classes of integrons have been reported but three major integron classes, classes 1, 2, and 3, are the most commonly known to be associated with horizontally transferred resistance genes.

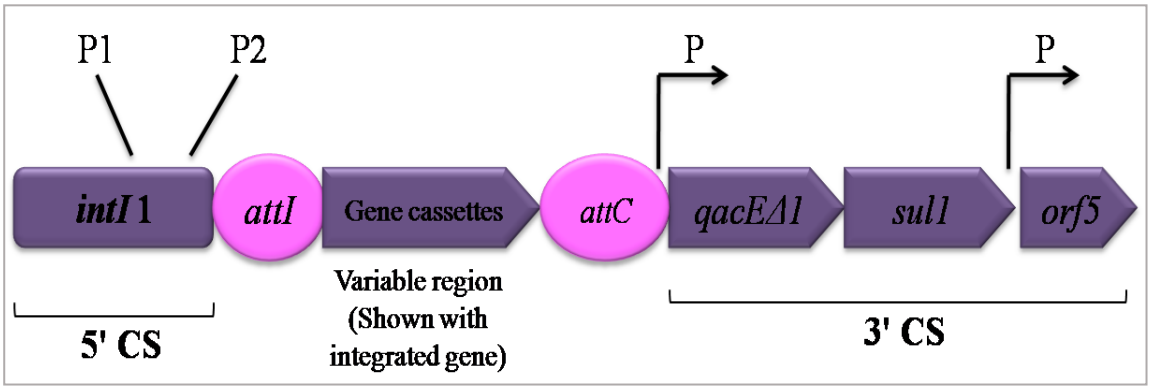


Figure 3.1: General structure of class 1 integron

The basic integron consists of the following: *intI*, a gene for the integron integrase; P1 and P2, an integron-carried promoter; *attI*, the integron associated recombination site; *attC*, the cassettes associated recombination site; *qacEΔ1*, quaternary ammonium sulphate resistance gene; *sul1*, sulfonamide resistance gene; *orf5*, unknown function; CS, conserved segments.

Class 1 integron has been most common and widely distributed integrons in multidrug resistance gram negative clinical isolates (Gillings *et. al*., 2014). It has been consisting of two conserved segments, 5' and 3' conserved sequences, separated by a variable region, which includes one or more genes (Figure 3.1). The 5' conserved sequence (5' CS) of integrons consists of three important elements for incorporation of gene cassettes; a gene encoding a site specific recombinase integrase enzyme (*intI*1), the integrase enzyme belongs to the tyrosine-recombinase family that has an attachment site

where horizontally acquired sequences are integrated (*attI*); and a promoter that drives expression of the incorporated sequence (P), while the 3' conserved sequence (3' CS) is conserved and usually contains a truncated antiseptic resistance gene (*qacE*Δ*1),* conferring resistance to quaternary ammonium compounds (QACs), a sulfonamide resistance gene (*sul1*), and an open reading frame (ORF5), encoding a protein of unknown function (Xu Z *et. al*., 2011; Mazel, 2006) (Figure 3.1). Class 1 integrons carry a pool of gene cassettes, most of which make resistance against a wide range of antibiotics. The most common genes inserted into variable regions as cassettes are antibiotic resistance genes. (Collis *et. al.,* 1992; Levesque *et. al*., 1995). More than 130 antibiotics resistance gene cassettes have been illustrated for class 1 integrons and these antibiotics resistance gene cassettes probably have been accumulating incrementally from diverse phylogenetic backgrounds (Gillings, 2014; Mazel, 2006)

Gene cassettes are small and circular mobile genetic elements, consisting of a single open reading frame and a recombination site. Via specific excision and integration, gene cassettes are integrated between two recombination sites (*attI* and *attC*) and thus become part of the integron (Figure 3.2). Gene cassettes don't have necessarily part of integrons, but once incorporated, they become part of that integron (fluit & Schmitz, 1999). Before the integration gene cassettes are exist as free and circular form; but after the integration they converted into linear sequences that constitute part of a such as a plasmid or bacterial chromosome. Integron gene cassettes do not have replication systems or transposition systems, but it moves with the help of site-specific recombination. The genes (most commonly an antibiotics resistance gene) on the cassette are then bound by the *attI*1 site on the 5' CS side and by *attC* on the 3 CS side. The end 3' CS (59 DNA base) is called *attC* site. The *attC* sites vary in length from 57 bp to 141 bp; however, they share a non coding region of about 25 bp at each end which conforms to consensus sequences (Collis & Hall, 1992; Hall *et al.,* 1994; Stokes *et al.,* 1997). The consensus sequences are consisting of a series of inverted repeats (R″, L″, L′, and R′) which are the integrase binding domains. Among these four binding domains, only two R″ and R′ have conserved sequences, these begin with 5'-RYYYAAC and 5'- GTTRRRY respectively. The *attC* sites begin with an inverted core site 5'-RYYYAAC separated by spacer of 7 or 8 bp and end with a core site 5'-GTTRRRY (Stokes *et. al*., 1997). The *attC* sites in every gene cassettes

are own version and these varations in gene cassettes comes from the orientation of the embedded open reading frame (ORFs).

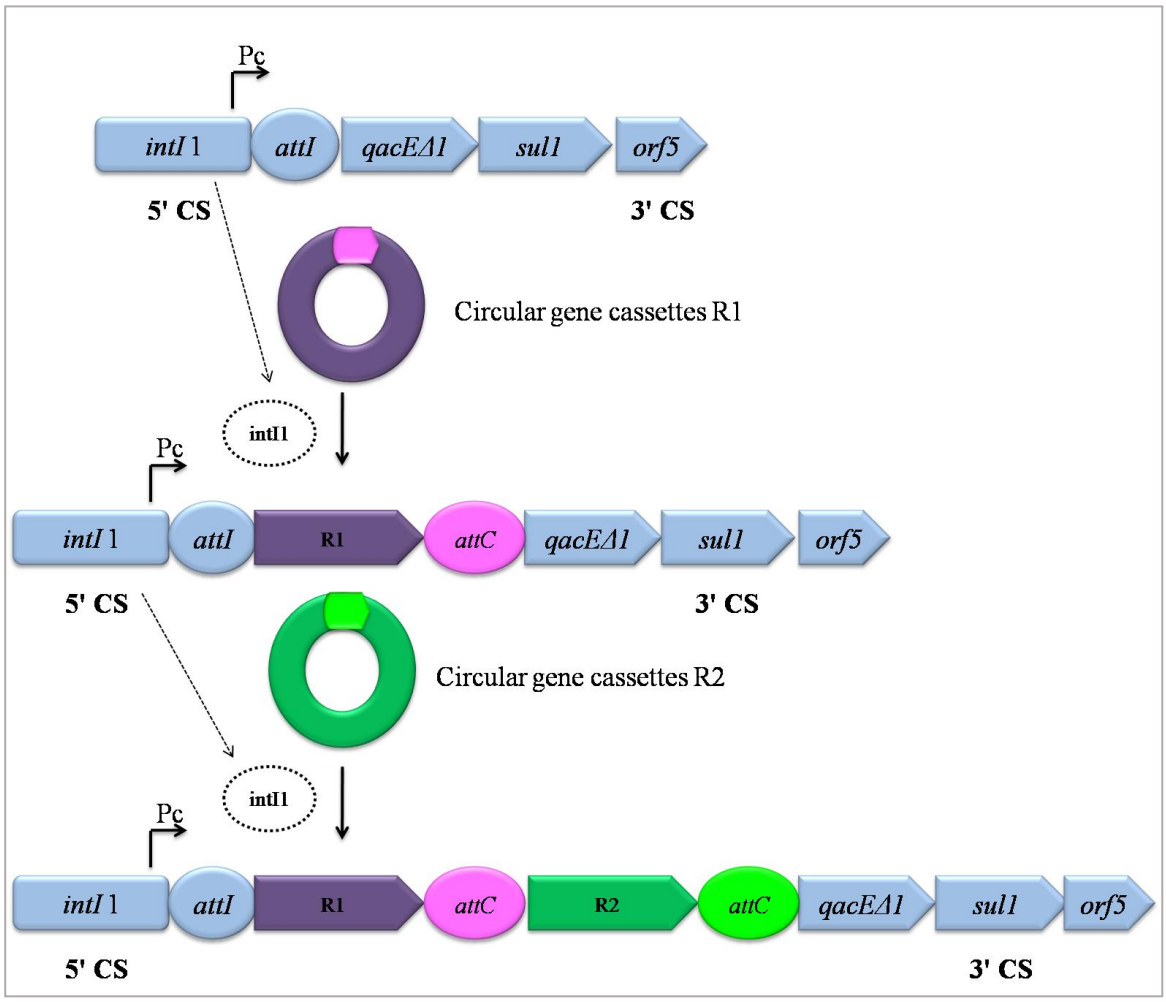


Figure 3.2: Integration of gene cassettes

Integron platform acquires new gene cassettes by recombination between the *attC* site of circular gene cassette and the *attI* site on the integron. The inserted incoming gene cassettes are at a position proximal to the integrase gene *intI* and embedded promoter Pc. Gene cassettes arrays can expand by repeated cassettes acquisition, but gene cassettes can also be excised as closed circles by *attI* × *attC* or *attC* × *attC* recombination.

The *attI* sites two integrase binding domains are present and represented as Land R. The R binding domains contains the canonical sequence 5′-GTTRRRY, with incoming gene cassettes being inserted between the G and T residues. In the *attI* sites of class 1 integrons *attI* sites consisting of two integrage binding sites and it termed as DR1 and DR2. The integron cassette carries one coding sequence but may also carry a variable

number of non-translated DNA bases (Fluit & Schmitz, 1 999). Generally cassettes may carry one gene but in some cases, they carry two or more than two genes. (Holmes *et. al*., 2003). The mobility of gene cassettes is depending on *intI* gene, encoded intI protein and they belongs to the family of integrases. The *intI* gene cassettes can be inserted into bacterial chromosomes or plasmids and their mobility allows genes to transfer into new organisms. Integrons can carry several different gene cassettes and therefore play an important role in the dissemination of multiple antibiotic resistance genes.

Antibiotic resistance gene cassettes have a number of features in common. They are usually mobile, and their cassettes arrays are short and normally encode enzymes which make bacteria resistant against the antibiotics. However, these features don't seem to be inherent properties of their ancestor integrons but have arisen as a result of convergent evolution, driven by the strong selection pressures imposed during antibiotics concentration. Integrons can carry several different gene cassettes and therefore play an important role in the dissemination of multiple antibiotic resistance genes. To date, several different types of antibiotics resistance gene cassettes with nucleotide sequences are reported, which is associated with class 1 integrons. Among them they confer resistance to all known β-lactams (including carbapenem and cephalosporin), all types of aminoglycosides, chloramphenicol, trimethoprim, streptothricin, rifampin, erythromycin, fosfomycin, and tetracycline. (Fluit & Schmitz, 2004; Gillings, 2014). Many studies have been done, characterizing β-lactamase encoding gene cassettes which is integrated into class 1 integron. Class 1 integron is an important source for spreading of β-lactamases genes. The transferable MBLs gene cassettes are commonly integrated into type 1 and type 3 integrons. Till now only two types of MBLs (VIM and IMP) gene cassettes have been reported. Incidentally, NDM gene cassettes are still rare (Arakawa *et. al*., 1995; Lauretti *et. al*., 1999; Poirel *et. al*., 2000). VIM-2 MBL gene cassettes carrying *Pseudomonas* was reported in 2001 in hospital in Zabrze together with the aminoglycoside resistance gene *aacA4* (Walsh *et. al*., 2003). Another report described a group of Pseudomonas isolates from hospital of Warsaw identified in 1998 to 2001. They all carried class 1 integron congaing VIM-4 gene cassettes (Patzer *et. al*., 2004).

The aims of this study were to search, identify, and characterize antibiotics resistance gene cassettes in MBL-positive isolates from Mahananda and Karala rivers water of West Bengal India.

3.2 Materials and methods

3.2.1 PCR based screening of class 1 integrons in MBL positive isolates

A total 15 MBL positive isolates (7 from Mahananda River and 9 from Karala River) was examined for the presence of class 1 integron using the CS-PCR (conserved segment polymerase chain reaction) method described earlier (Chakraborty *et. al.*, 2013). Since upstream primer 5' CS (5'-GGCATCCAAGCAGCAAG-3') and downstream primer 3' CS (5'-AAGCAGACTTGACCTGA-3') used in this PCR anneal specifically in the 5' and 3' CS regions of class 1 integron, the amplicons contained the inserted variable region of antibiotics gene cassettes flanked on both sides by small parts of the CSs. PCR primers were synthesized from BioServe Biotechnologies India Pvt. Ltd. (Hyderabad India).

The DNA templates for PCR amplification were prepared as described by (Chakraborty *et. al.*, 2013). MBLs positive bacterial isolates were grown in 10ml LB in presence of ampicillin (50µg/ml) at 37°C overnight, then 1ml of overnight bacterial culture was centrifuge at 6000 rpm for 5 minute, and the supernatant was discarded. The equal volume of sterile double distilled water was added, and mixed with vortex. The bacterial suspension was added to boiling water bath for 10 minute, then centrifuge at 6000 rpm for 5 minutes. Finally, the supernatant was used as template for PCR amplification.

PCR amplification was performed in 50µL reaction volume. Each 50µL PCR mix contains; 5µL of 10X buffer containing 15mM $MgCl_2$, 3µL of 10mM dNTP mix, 12 pmol of each primers, 8µL of template DNA and 1.25 U Taq DNA polymerase (Genei India), and made up total volume of 50 µl with sterile double distilled water. The PCR was done in a Veriti 96 well Thermal cycler (ThermoFisher Scientific). The PCR was done by following the thermal-cycle profile as shown in Figure 3.3. The chemical used in PCR reaction were purchased from Genei, ThermoFisher Scientific.

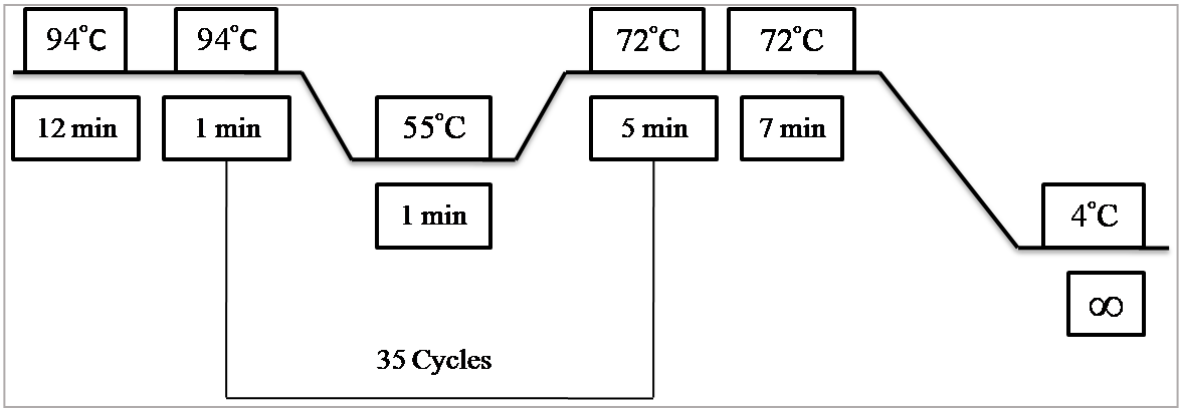


Figure 3.3: Polymerase chain reaction setup for variable region of class 1 integron

3.2.2 Gel electrophoresis and size determination of the CS-PCR products

After PCR amplification, 8 µl of the amplified DNA product were electrophoresed on a 1.0% (w/v) agarose (Seakem LE Agarose, Loanza) gel, and a 500 bp ladder (Promega, Medison, WI), was used as a molecular size marker. Gels were stained with ethidium bromide for visualization.

3.2.3 Cloning and sequencing of class 1 integron amplicons generated with 5 CS and 3 CS primers

3.2.3.1 Gel extraction and purification of the CS-PCR products

The CS PCR product which shows two or more bands, first precipitated by ethanol and then suspended in 20µl sterile double distilled water. 20µl amplified PCR products were loaded in a low melting point agarose gel and were subjected to electrophoresis at 30-35 mV for at least 8-10 hrs, and temperature of running buffer was maintained nearly 4°C. The required bands were cut out from the agarose gel with a sterile scalpel. The DNA samples from the gel was extracted and purified via GSure Gel Extraction Kit (GCC Biotech India) by following the manufacturer's instructions. Finally the purified DNA samples were suspended in the sterile double distilled water/nuclease free water. The purified DNA samples were stored at -20°C for further cloning purpose.

3.2.3.2 Preparation of competent *E. coli* DH5α by $CaCl_2$ method

Competent cells were prepared by $CaCl_2$ method and preparation method was described in chapter 2.

3.2.3.3 Cloning of the purified PCR products

The pGEM–T Easy Vector System (Promega Corporation, Madison, USA) was used for the cloning purpose of CS amplified PCR products. For cloning ligation mixture were prepared according to the manufacturer's instructions and then transformed in competent *E. coli* DH5α cells following heat shock method (method described in chapter 2)

3.2.4 DNA sequencing and *in silico* analyses of the sequences

The recombinant plasmids were used for sequencing of the inserted gene cassettes using primers for T7 and SP6 promoters. The complete sequence for large gene cassettes was obtained by using a primer walking method. Potential open reading frames of gene cassettes were predicated by using the freely available tool NCBI ORF Finder Tool (https://www.ncbi.nlm.nih.gov/orffinder/). BLAST N and BLAST P (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against GenBank database and the Integron database INTEGRALL (http://integrall.bio.ua.pt/) were performed repeatedly for sequence analysis, comparison, annotation and functional analysis (McGinnis & Madden 2004). The nucleotide sequences of class 1 integron variants were deposited in the GeneBank (NCBI), and the accession numbers were obtained for each integron gene cassette (Table 3.1).

3.3 Results and discussion

3.3.1 Detection of class 1 integrons in MBL-positive isolates

Total Seven (2 from Mahananda river and 6 from Karala River) 46.67% out of the 15 MBL positive isolates yielded a PCR product of the class 1 integron with variable length. Except one of the MBL positive isolates (KR23) which yielded a 426 bp product containing only 5'-CS and 3'-CS sequences of the class 1 integron, all contained class 1 integron with variable gene cassettes. Three strains KR01, KR04 and KR07 also carried empty class 1 integron and with gene cassettes congaing class 1 integron. Four MBL positive and class 1 integron positive isolates KR22, KR25, MR40 and MR41 were found to carry two amplicons of different sizes and three KR01, KR04 and KR07 isolates contained only one class 1 integron each (Figure 3.4)

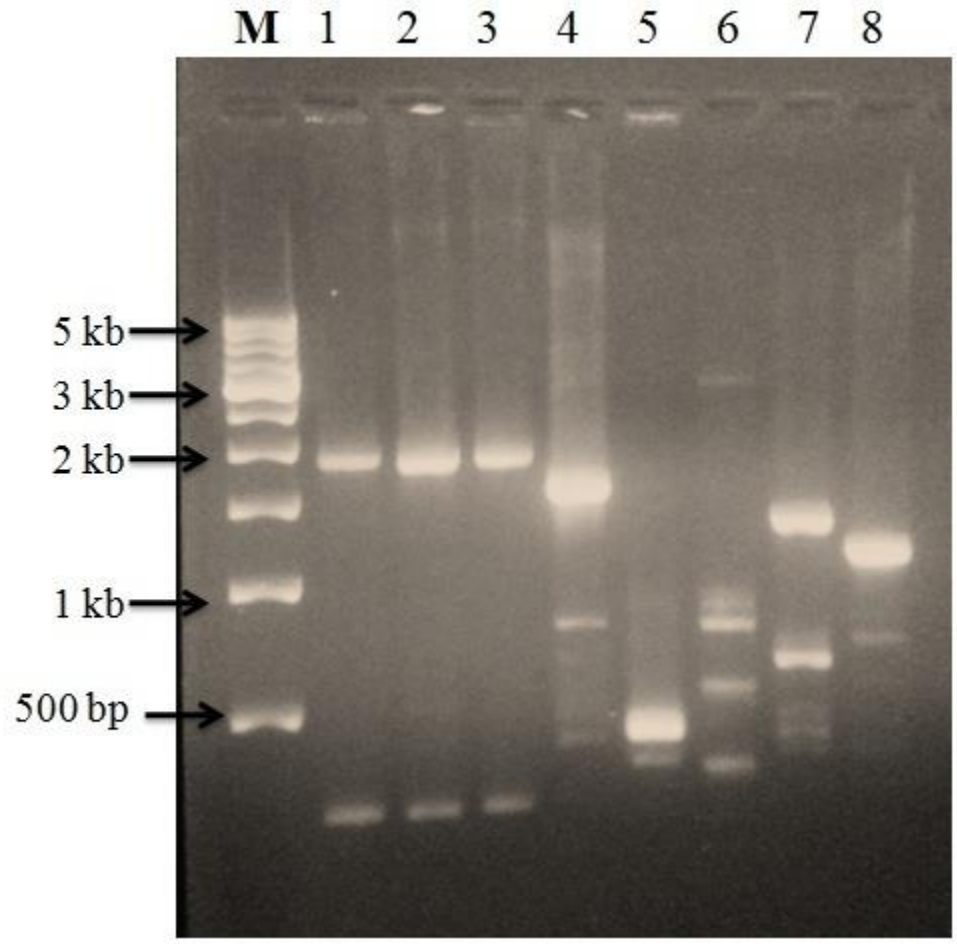


Figure 3.4: Agarose gel electrophoresis of PCR amplification products of different the 5' CS and 3' CS of variable regions of class 1 integrons from MBLs isolates from Mahananda and Karala Rivers

The PCR products were separated by electrophoresis in 1.0% agarose. Lane M, 500 bp DNA ladder; Lane 1, KR01; Lane 2, KR04; Lane 3, KR07; Lane 4, KR22; Lane 5, KR22; Lane 6, KR25; Lane 7, MR40; Lane 8, MR41.

3.3.2 Characterization of antibiotic-resistance gene cassettes in class 1 integron

Total nine different gene cassettes of Class 1 integrons which we classified as type I-IX were identified in 7 (46.67%) out of the 15 MBL positive isolates (Figure 3.4 and Table 3.1). The amplicon lengths, corresponding to the approximate sizes of the inserted gene cassette, varied from 0.7 to 3.5 kb, Sequences derived from the amplicons exhibited significant homology with already existing antibiotic resistance genes. Largest amplicon of nearly 3.5 kb was identified in KR25. Purified CS-PCR products of KR01, KR04, KR07,

Table 3.1: Characteristics of class 1 integron and their associated antibiotic resistance gene cassettes from MBLs positive isolate

Integron type and No.	Isolates	Gene cassettes arrays	Amplicon Size (bp)	Cassette encoded resistance phenotype	Accession No.
I	MR41	*dfrA15*	739	Trimethoprim	MN256775
II	MR40	*dfrA5*	730	Trimethoprim	MN256771
III	MR40	*bla* $_{PSE-1}$	1197	β-lactam	MN256776
IV (2)	KR22,KR25	*aac(6')-Ib*	862	Fluoroquinolone	MN256778
V	MR40	*aadB-aac(6')-Ib*	1400	Streptomycin, Fluoroquinolone	MN256772
VI	KR01	*aac(6')-Ib-aadA2*	1968	Fluoroquinolone, Streptomycin	MN256774
VII (2)	KR04,KR07	*aadA2-aadA1*	1928	Streptomycin	MN256779, MN256780
VIII	KR22	*dfrA17-aadA5*	1661	Streptomycin	MN256773
IX	KR25	*bla*$_{VIM-2}$*-aacA4-aadA1-aadA2*	3449	β-lactam, Fluoroquinolone, Streptomycin	MN256777

KR22, KR25, MR40 and MR41, cloned in pGEM-T Easy Vector, were subjected to DNA sequencing and gene cassettes thereby identified by sequence analysis (Table 3.1). Altogether nine different gene cassettes were identified (Figure 3.5). The most common carriage by integron positive isolates involved aminoglycoside adenyl transferase (*aad*A1, *aad*A2, *aad*A5, *aad*B). The type VII class 1 integron (*aadA*2-*aadA*1) was detected in KR04 and KR07. Aminoglycoside acetyltransferase (*aac(6')-Ib*) was detected in MR40, KR22 and KR25. Dihydrofolate reductase cassettes were next in abundance and found in MR40 (*dfr*A5), MR 41(*dfr*A15) and KR22 (*dfr*A17) (Table 3.1 and Figure 3.5). The gene cassette(s) *aac(6')*-Ib and *aadA*2 of *Proteus sp.* KR01 showed 99.81 and 99.62% similarity with the respective genes from *Morganella morganii* class 1 integron (MF370653); *aad*A1 and *aad*A2 gene cassettes from *Proteus sp.* KR04 and *Escherichia* sp. KR07 revealed

100% similarity with same gene cassettes from *Morganella morganii* (MF370653) and *Escherichia coli* class 1 integron (KF914287). The sequence from 1661 bp amplicon of *Escherichia* sp. KR22 shared 100% similarity with *dfr*A17 and *aad*A5 genes of *Salmonella enterica* (KY399741) class 1 integron; 862 bp amplicon of *Escherichia* sp. KR22 and *Pseudomonas* sp. KR25 revealed 100% similarity with *aac(6')*-Ib from class 1 integron of bacterium AK-MB42 (HE653230). The sequence analysis of 3.5kb amplicon of *Pseudomonas* sp. KR25 exhibited 100% and 99.74% similarity with $bla_{\text{VIM-2}}$ and *aad*A2 gene cassettes of *Pseudomonas aeruginosa* (EF207717) class 1 integron. The analysis of the 1.4 kb amplicon of *Acinetobacter* sp. MR40 showed 99.81% and 100% similarity with *aad*B and *aac(6')*-Ib gene cassettes of *Aeromonas caviae* class 1 integron (KU886278), while the sequence from 730 bp amplicon of *Acinetobacter* sp. MR40 shared 99% identity with the bacterium AK-MB72 class 1 integron *dfr*A5 gene (HE650983). 1197 bp amplicon of MR41 shared 100% similarity with the $bla_{\text{PME-1}}$ *gene* of *Proteus mirabilis* class 1 integron (MF576130) while 730 bp amplicon exhibited 100% similarity with *the dfrA15 gene* of *Acinetobacter baumannii* class 1 integron (HQ880256).

In an earlier study from China, a considerable prevalence of class 1 integrons in multidrug resistant clinical *P. aeruginosa* isolates was observed (Chen *et. al.,* 2009). Out of a total of 19 ORFs of class 1 integron borne gene cassettes, the most abundant ORFs (09/1) was *aad*A family of genes which encode aminoglycoside-3-adenylyltransferases (AAD) which confer resistance to streptomycin and spectinomycin by adenylation, and the second most frequent (04/19) was *aac* (6′) Ib gene which code for aminoglycoside 6′-N-acetyltransferase type I b, an enzyme of clinical importance that catalyzes the transfer of an acetyl group from acetyl CoA to the 6-amino group of aminoglycoside molecules conferring resistance to antibiotics containing the purpuroamine ring including amikacin and kanamycin, found in a wide variety of gram-negative pathogens. Two *Proteus* spp., isolated from Karala river, each bearing class 1 integron having two gene cassettes, *aac* (6/)-I-*aad*A2, and *aad*A2-*aad*A1respectively, were found also to bear $bla_{\text{NDM-1}}$.

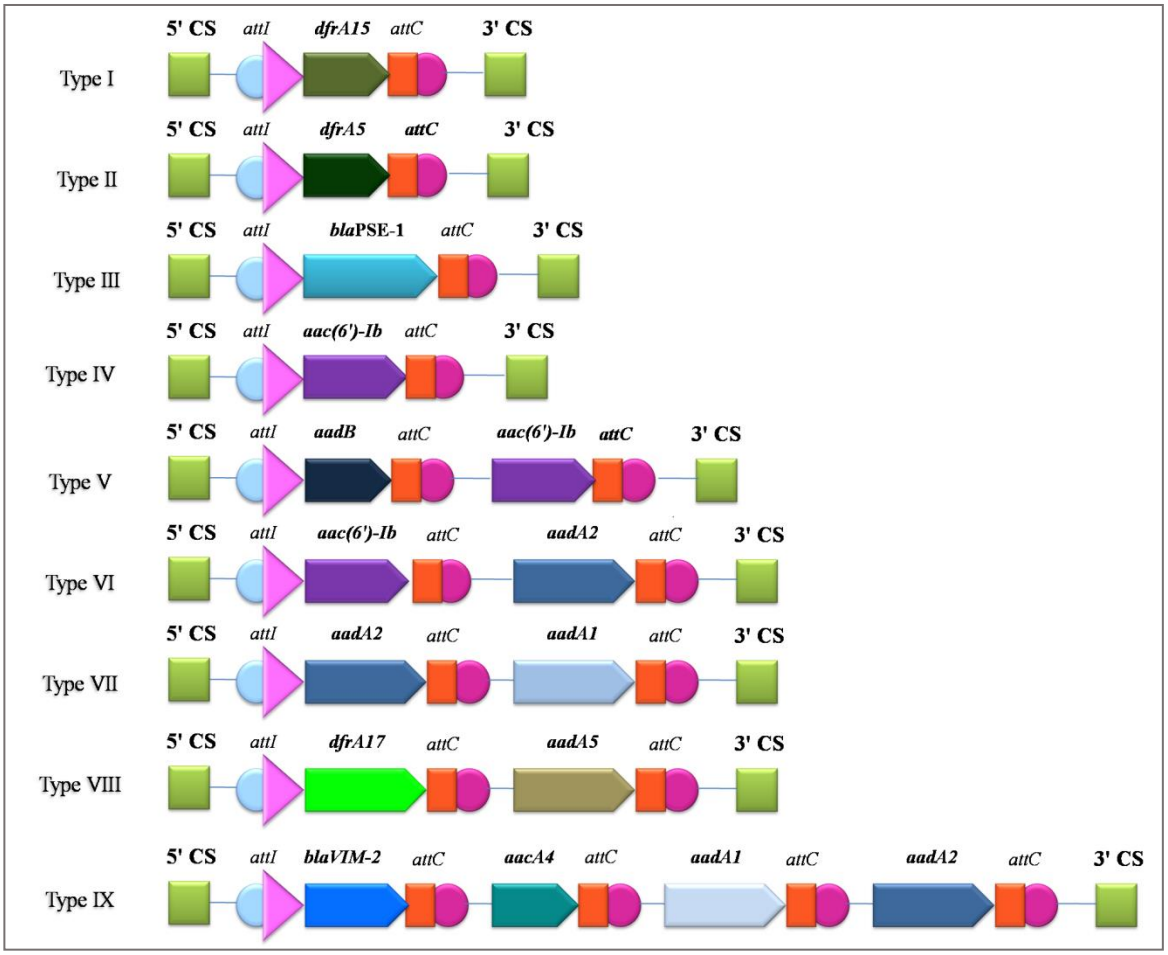


Figure 3.5: Schematic representation of the variable region of class 1 integron identified in MBLs positive isolates from Mahananda and Karala Rivers

Integron type with different cassettes arrays (Type I to IX) are arranged as identified in Table 3.1. Gene cassettes are shows as boxes, with arrows indicating the origination of transcription. The 5' and 3' conserved segments (5' CS and 3' CS) are annotated.

3.4 Conclusion

The results of this chapter revealed that integron structures were prevalent in the MBL positive isolates of the Mahananda and Karala rivers of West Bengal India. The predominance of the selected aminoglycosides (*aac(6')-Ib, aadA*) and trimethoprim (*dfrA*) resistance gene cassettes array, however, indicate that these gene cassettes are more stably incorporated within the integron structures than other. Two class 1 integron gene cassettes type III and IX that confer the extended spectrum β-lactamase and metallo-β-lactamase resistance genes respectively. These types of integrons are more dangerous to infected

patients. A gene cassettes which can carried *bla*$_{\text{NDM-1}}$ gene have been absent in both the rivers water, luckily it is still rare.

Chapter 4

Whole-genome sequencing of a model *Pseudomonas* sp. MR 02 bearing *bla*$_{\text{NDM-1}}$ gene

4.1 Introduction

The infection which is caused by antibiotic-resistant bacteria constitutes one of the most serious threats to human health. If we leave it uncontrolled, even small surgery can become a major risk procedure (Davies, 2011). Controlling of antibiotic resistance bacterial infection is becoming more complex than previous to, because there is a huge difference between antibiotic development and antibiotic resistance character developed by microbes and this difference can be controlled by using rapid and improved diagnostics techniques (WHO, 2012; Livermore & Wain, 2013; Spellberg, 2013). As a result of technological development in recent times, bacterial whole genome sequencing has emerged as a tool in clinical microbiology and research fields that can reduce this gap. Whole genome sequencing of bacteria has emerged as a cost-effective and convenient technique for identification of bacterial behaviours in single assays. Although in traditional method separate technique is used for species identification and virulence and drug resistance character identification. A major advantage of whole genome sequencing is to yield all of the available genetic information in single steps of bacterial culture (Loman, 2012; Loman, 2013).

After the introduction of next-generation sequencing (NGS) in 2005 was enabled to reduce the sequencing cost and facilitated the performance of sequencing in all types of laboratories (Barbosa *et al*., 2014). Several NGS sequencing technology available at the current time, but the general steps remain the same, and it includes sample preparation, DNA sequencing, sequence assembly and bioinformatics analysis (Dark, 2013). These NGS techniques include the Illumina Miseq and Hiseq (both are accurate with low error rates), SOLiD (Sequencing by Oligo Ligation Detection) developed by Life technologies and in several countries, such as USA, United Kingdom and Germany Whole-genome sequencing technique is also being used or in a trial phase in the medical microbiology field, because this technique allows rapid detection of pathogenic character from infected human and to direct appropriate treatment (Pareek *et. al*., 2014; Pecora *et. al*., 2015; Zankari *et. al*., 2013; Koser *et. al*., 2014; Mellmann *et. al*., 2017). A major advantage of whole-genome sequencing is to yield all of the available genetic information in single steps of bacterial culture and directly to identify a microorganism and also establish genotypic to phenotypic relationships (Torok *et. al*., 2012).

The first complete bacterial genome to be sequenced was that of *Haemophilus influenzae* in 1995 and the *Pseudomonas aeruginosa* PA01 was the fifth bacterial strain whose complete whole-genome reported in 2000 (Fleischmann *et. al*., 1995; Stover *et. al*., 2000). *Pseudomonas aeruginosa* ubiquitous in nature, and is present in soil and water as well as on fruit, vegetables and flowers (Kiska & Gilligan, 2003) and it can also be isolated from human, animal and clinical settings (Lederberg *et. al*., 2000). *Pseudomonas aeruginosa* is an opportunistic human pathogen and it is responsible for 18% nosocomial infection in human. They were first isolated and identified in 1850 by Migula (Lederberg *et. al*., 2000). Genus *Pseudomonas* is represented by species that occupy a wide range of niches owing to metabolic and physiological diversity (Kiska & Gilligan, 2003). *Pseudomonas aeruginosa* strain contains approximately 5 to 7 Mbp genome size, and it contains significant numbers of regulatory protein-encoding genes. This indicates that *P. aeruginosa* is capable of responding to various environmental stresses (Moradali *et. al*., 2017). Additionally *P. aeruginosa* has intrinsic multidrug resistance properties due to the presence of three or four component like RND efflux pumps, porin channel and AMPC β-lactamase that physically sequester incoming antibiotics and hydrolyzed incoming β-lactam antibiotics (Aeschlimann, 2012; Jacoby, 2009). Moreover, biofilms act as a protective layer for drug penetration (Stewart, 2000). Genetically it can also receive the antibiotic resistance gene from other species by a horizontal transfer mechanism (Davies and Davies, 2010).

In the current study, NDM-1 producing *Pseudomonas* sp. MR 02 was the only strain found in Mahananda River, which is isolated in two different sampling dates and it has also high MIC value against different antibiotics. However, when applying genotypic screening to detect carbapenem resistance gene, PCR assays to detect only one MBL which was NDM-1, but in the metallo-β-lactamases activity test of MR 02 strain, the MIC value of cephalosporin antibiotics in the presence of EDTA (0.4 mM) was recorded higher than the resistance MIC value (CLSI guideline) and these results indicate that other genes are also playing a role in cephalosporin resistance. NDM-1 metallo-β-lactamases are responsible for hydrolyzing β-lactam antibiotic groups but *Pseudomonas* sp. MR 02 Mahananda river water isolates showing resistance in lactams group antibiotic as well as in other antibiotic groups like aminoglycosides, chloramphenicol, tetracycline, trimethoprim

and fusidic acid. This result highlights the limitations of genotypic and phenotypic test for detecting and allowing characterization of divergent functional genes. Hence in this chapter, NGS to the analysis of multiple drug resistance *Pseudomonas* sp. MR 02 isolates especially. The objective of this chapter was to describe the diversity and distribution of resistance mechanism and also corroborate genotypic-to-phenotypic relationships in MR 02 isolates.

4.2 Materials and Methods

4.2.1 Isolation of genomic DNA from *Pseudomonas* sp. MR 02

Pseudomonas sp. MR 02 genomic DNA was isolated by using phenol: chloroform isolation method described by Furlong *et. al*., (2002) with modifications. The quantity of genomic DNA was measured using a NanoDrop spectrophotometer, while its quality was assessed via agarose gel (0.8%) electrophoresis.

4.2.2 Sequencing of *Pseudomonas* sp. MR 02 genome

The genome of strain MR02 was sequenced by the NGS Laboratory (Eurofins Genomics, India) using de novo sequencing by Illumina NextSeq 500 platform and methods described below.

4.2.2.1 Preparation of 2×150 NextSeq 500 shotgun libraries

The paired-end sequencing libraries were geared up using illumina TruSeq Nano DNA Library Prep Kit. Briefly, approximately 200 ng genomic DNA of MR 02 was fragmented by covaris M220 to generate fragment distribution of 400bp. Covaris shearing makes dsDNA fragments with 3 or 5 overhangs. The fragments were then subjected to end-repair. The 3 to 5 exonuclease activity of this mix removes the 3 overhangs and the 5 to 3 polymerase activity fills in the 5 overhangs followed by adapter ligation to the fragments. Ligated and end-repaired products were size selected by AMPure XP beads. The size selected products were PCR amplified with index primer and indexing adapters were ligated to the ends of the DNA fragments, preparing them for hybridization onto a flow cell. The library was analyzed in a 4200 Tape Station system (Agilent Technologies).

4.2.2.2 Cluster generation and sequencing

After quantity and quality check of library Paired-end Illumina libraries were loaded onto NextSeq 500 for cluster generation and sequencing. Paired-end sequencing

allows the DNA template fragments to be sequenced in both directions on NextSeq500. After both template fragments sequencing, 1.1 GB adapter trimming data of MR 02 was generated, which was required for the genome to be used in a taxonomic purpose (Chun *et. al.,* 2018).

4.2.2.3 Genome annotation and analysis

The high- quality filtered reads were assembled into scaffold using SPAdes genome assembler (Bankevich, 2012). Prokka v-1.12 (Seemann, 2014) was used to predict the genes from final complete assembled scaffolds. BLASTx (NCBI) programme were used for functional annotation of the MR 02 gene, and Blast2GO platform were used for MR 02 gene ontology annotations (Jones *et. al*., 2008).

4.2.3 Identification of antibiotic resistance genes in *Pseudomonas* sp. MR 02

AMR (antimicrobial resistance) genes were identified in draft genome of MR 02 using publicly available comprehensive antibiotic resistance databases (CARD) (Jia *et. al*., 2017). Resistance determinants were identified proviso they conform the criteria of = 85% amino acid identity and =50% sequence length identity to known resistance proteins. Sequences showing = 100% identity and/or sequence length were analyzed by additional BLAST analysis to identify the appropriate resistance genes. The results obtained were then again validated by BLAST analysis against information in the COG, NCBI, CDD and Swiss-Prot databases; manual curation was done for all blast results as per consistency of annotations between different databases. For analysis of chromosomal structural gene mutations, *gyrA* and *parC*, genes, were analyzed for quinolone resistance-determining region (QRDR) mutations, with alignment by ClustalW in Mega version 7 (Park *et. al*., 2011).

4.2.4 Phylogenetic and comparative genomic analysis of *Pseudomonas* sp. MR 02 with reference genome of *Pseudomonas* spp.

Pseudomonas sp. MR 02 were subjected to phylogenetic analysis using four different method viz. 16S rRNA based phylogenetic analysis (chapter 2), bcg tree based phylogenetic analysis, Average Nucleotide Identity (ANI) and *In silico* DNA-DNA hybridization (*i*DDH).

For comparative genome analysis, the complete genome sequence of strain MR02 along with that of twenty *Pseudomonas* type strains were obtained and used as references

for building of phylogenetic tree based on core genes. A phylogenetic tree based on bacterial core genome was constructed by the bcgTree pipeline (Vesth *et. al*., 2013; Ankenbrand & Keller, 2016). The pipeline automatically extracts 107 essential single-copy core genes, found in a majority of bacteria, using hidden Markov models and uses them to reconstruct a phylogenetic tree based on partitioned maximum-likelihood analysis utilizing RAxML program (Stamatakis, 2014).

Average Nucleotide Identity (ANI) was calculated by the recently released OrthoANI program (Lee *et. al*., 2016). The OrthoANI provides a measure of genomic relatedness correlated with the original ANI and both of these algorithms share the same species demarcation cut-off at ≤ 96%. In addition, OrthoANI revolves around the problem of reciprocal inconsistency of the original ANI algorithm. ANI value to define a new species is ≤ 96.

In silico DNA-DNA hybridization (*i*DDH) was estimated by using the Genome-to Genome Distance Calculator (GGDC) version 2.1 Web server (http://ggdc.dsmz.de/ggdc.php) (Auch *et. al*., 2010). Since many of the genomes are incomplete draft genomes, therefore Formula 2 was used for the analysis. Formula 2 calculates *i*DDH estimates independent of genome lengths and is recommended by the authors of GGDC for use with any incomplete/draft genomes. The point estimate plus the 95% model-based confidence intervals were used for analysis. DDH value to define a new species is ≤ 70%.

The contigs sequence of *Pseudomonas* sp. MR 02 was aligned to the complete sequence of *P. putida* NDRC14164 genome using Muscle (Kurtz *et. al*., 2004) for making genome orientation. Four available whole genome sequences of the phylogenetically nearest species *P. plecoglossicida* DSM15088, *P. monteilii* NBRC103158, *P. putida* NBRC14164 and *P. aeruginosa* PA01 were used for the comparative genomic analysis with MR 02. For visualization of circular genome comparison, the BLASTN-based graphical map was generated by BLAST Ring Image Generator (BRIG) 0.95 (Alikhan *et. al*., 2011).

4.2.5 Correlation of phenotypic and genotypic characters of MR 02

Two different types of phenotypic experiments used for antimicrobial susceptibility testing one for efflux pump activity detection and another one for *bla*$_{NDM-1}$ activity (Chapter 1 in section 1.2.9). Each interpretation of antibiotics resistant was compared with the presence of known corresponding resistance genes and/or structural gene mutations.

4.2.5.1 Determination of efflux pump activity in contributing antibiotic-resistance in MR 02

The efflux pumps activity in MR 02 isolates was confirmed by the micro-broth-dilution method, in Mueller Hinton broth. It is based upon the comparative MIC values of antibiotics were also determined in the presence of Phe-Arg-β-naphthylamide (30 μg/ml) and absence of Phe-Arg-β-naphthylamide. The antibiotics were used as phenotypic markers of the RND efflux pumps of interest shown in Table 4.1.

Table 4.1: Examples of RND efflux systems involved in the antibiotic resistance in *Pseudomonas aeruginosa* and *Pseudomonas* species

Efflux pump types	Antibiotic resistance to	References
MexAB-OprM	β-lactams, Azithromycin, Chloramphenicol, Macrolides, Tetracycline, Trimethoprim, Imipenem , Meropenem	Li *et. al*., 1995; Poole *et. al*., 1993; Poole *et. al*., 1996 (a)
MexCD-OprJ	Cephalosporins, Chloramphenicol, Fluoroquinolones, Tetracycline, Imipenem	Poole *et. al*., 1996 (b)
MexEF- OprM	Chloramphenicol, Fluoroquinolones, Imipenem, Meropenem	Kohler *et. al*., 1997
MexJK-OprJ	Aminoglycosides, Ciprofloxacin, Erythromycin, Tetracycline	Chuanchuen *et. al*., 2002
MexVW- OprM	Chloramphenicol, Fluoroquinolones, Tetracycline	Li *et. al*., 2003
MuxABC-OprM	Novobiocin	Mima *et. al*., 2009
TriABC-OprH	Triclosan	Mima *et. al*., 2007

MICs were determined by broth microdilution in the absence or presence of a broad-spectrum inhibitor of RND pumps, namely Phe-Arg-β-naphthylamide (PAβN), also

known as MC-207,110 at a concentration of 50 μg/ml, which fully restored the susceptibility of reference strains to the reporter antibiotics but did not impair the bacterial growth **(**Lomovskaya *et. al*., 2001**)**. The test tube was prepared with Mueller Hinton broth, antibiotics (different concentration), Phe-Arg-β-naphthylamide (fixed concentration) and MBL producing bacterial cells and test tube incubated for 24 hours at 37°C. The inoculums used per experiment was 10^4 to 10^5 cells/ml. Efflux pump activity was determined as the lowest concentration of antibiotics with fixed concentration of efflux pump inhibitor that completely inhibited growths of the MBLs producing bacterial strain in the test tube, and bacterial growth was detected by turbidity measurements.

To detect minimal changes, however, arithmetic dilutions were occasionally used for all the antibiotics used in the study (10 μg/ml increments in the 10–2000 μg/ml range).

4.2.5 Nucleotide sequence accession numbers

The whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number PESJ00000000.

4.3 Results and Discussion

4.3.1 Isolation, qualitative and quantitative analysis of *Pseudomonas* sp. MR 02 genomic DNA

Qualitative analysis of the strain MR 02 genomic DNA was resolved on 0.7% agarose gel at 100 V for approximately 60 min or till sample reached 3/4th of the gel (Appendix A 4.1) and 1 μl of the sample was loaded in nanodrop for quantitative analysis. The quantification of genomic DNA is an important step before starting the genome sequencing. Concentration and A260/280 ratio of genomic DNA of strain MR 02 was 425 and 1.76 respectively. For whole genomic sequencing, the DNA sample preparation is very important as the optimal quantity and quality of genomic DNA is crucial. The high and low concentrations of genomic DNA will affect the sequencing quality; if the DNA concentration is high these will produce overlapping sequencing clusters. On the other hand, if the genomic DNA is low than the optimal amount needed, then it will give the clusters with low density (Buehler *et. al*., 2010)

4.3.2 Whole genome sequencing, genome annotation and analysis of *Pseudomonas* sp. MR 02

The QC passed sample was used to prepare paired end library using Illumina TrueSeq Nano DNA library preparation kit generating a fragment with mean length of 434bp of strain MR 02. The libraries were sequenced on NextSeq 500 using 2×150bp. After sequencing adapter was removed by trimming (trimmomatic v0.35) and obtain high quality data (1.1 GB with 1,074,251,227 bases).

SPAdes genome assembler tool used for de novo assembly of MR 02 high quality read. Arranged genome in scaffolds and detailed assembly statistics were mentioned in the Table 4.2.

Table 4.2: Draft genome assembly statistics of *Pseudomonas* sp. MR 02

Scaffolds	Total size of assembly (bp)	Average size of scaffolds (bp)	N50 (bp)	Max size of scaffolds (bp)	Min size of scaffolds (bp)
90	5,946,931	66,077	144,752	431,092	520

Protein coding sequencing were predicted from the assembled scaffolds using Prokka v-1.12 with default parameters. Statistics of total number of genes, average gene length, maximum and minimum length of the strain MR 02 was 5,661, 923, 12,954, and 59 respectively.

Functional annotation of the MR 02 genes was performed using BLASTx program and it has a part of ncbi blast 2.3.0+ search application. BLASTx find the homologous sequences for the genes against NR (non redundant protein database) from NCBI, Swiss-prot database, KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Clusters of Orthologous Groups) and InterPro database with an E-value $<10^{-5}$. After blast analysis MR 02 draft genome consist of 5,530 genes, out of which 5,493 protein coding genes showing blast hits and 37 protein coding genes showing without blast hit. Top blast hit species distribution of MR 02 showing in Figure 4.1.

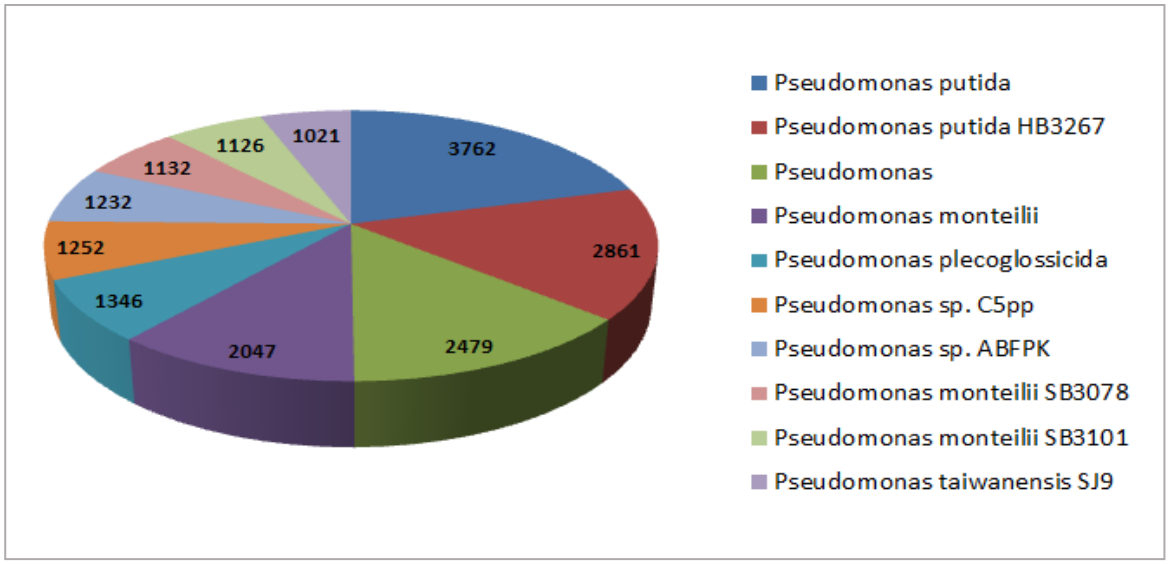


Figure 4.1: Top blast hit species distribution of *Pseudomonas* sp. MR 02

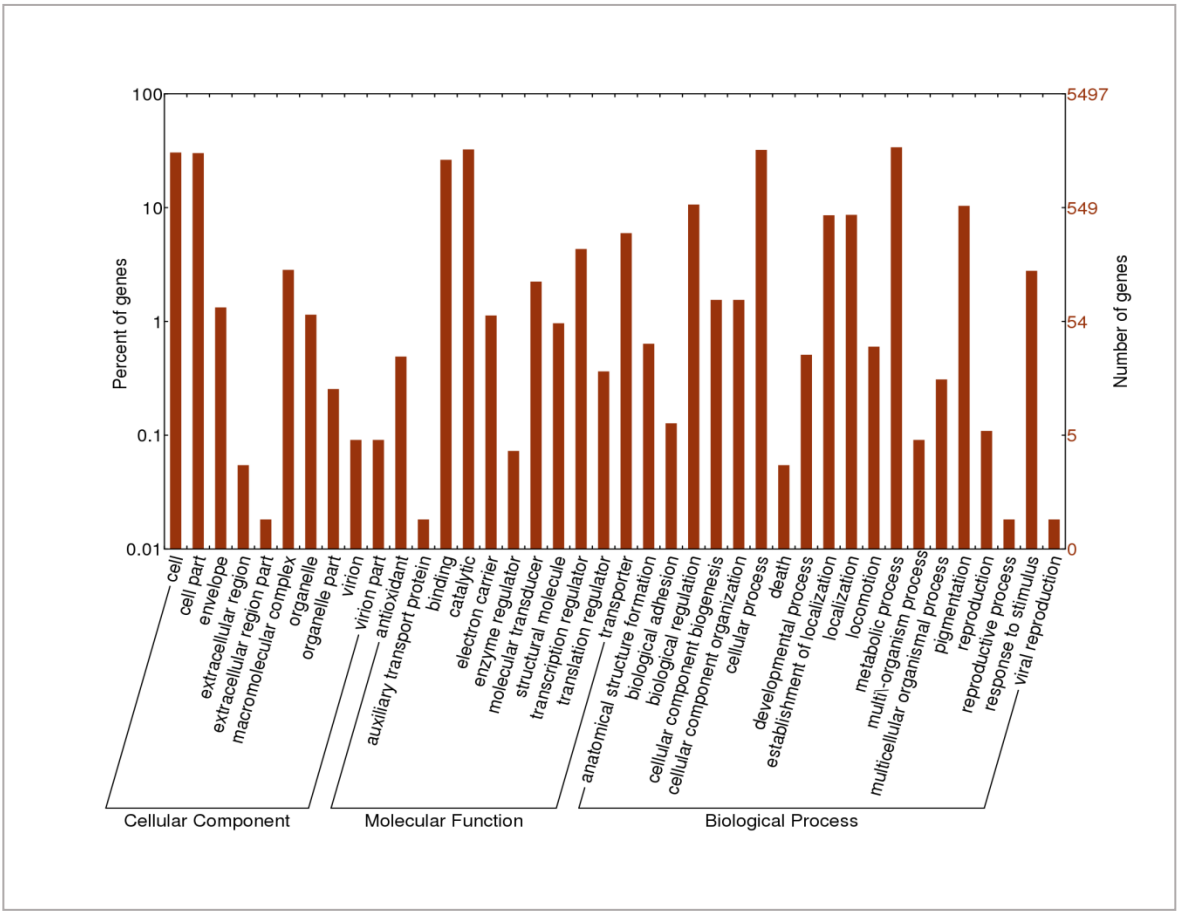


Figure 4.2: WEGO plots of the *Pseudomonas* sp. MR 02

BLASTx results accession IDs (functionally annotated genes) were directly searched in the gene products table of gene ontology database. Gene Ontology mapping provided function of the genes and their product properties. On the basis of genes product properties Blast2Go platform MR 02 genes are grouped into three main domains: Biological processes (2,593), Cellular component (1,687), and molecular function (2,695). Graphical-representation of MR 02 draft genome ontology in figure 4.2. Basic feature of MR 02 draft genome are summarized in table 4.3. Most of the predicted ORFs have the high G+C contents (67.6%).

Table 4.3: Genome features of *Pseudomonas* sp. MR 02

General feature			
Genome size	5,946,931		
G+C content	62.6%		
Coding regions			
Genes (Total)	5,799		
CDS (Total)	5,732 (98.84%)		
Genes (coding)	5,503 (94.89%)		
CDS (coding)	5,503 (94.89%)		
Genes (RNA)	67		
Types of rRNA	16S rRNA	23S rRNA	5S rRNA
Number of rRNA	1	1	2
tRNA	59		
Non-classical RNA	4		
Pseudogenes (total)	229		

4.3.3 Identification of antibiotic resistance in *Pseudomonas* sp. MR 02

Protein sequences of MR 02 draft genome were used for searches against CARD, and NCBI protein smart blast. After comparative analyses of the draft genome of *Pseudomonas* sp. MR 02, we identified a total of 98 genes associated with antibiotic resistance. Total 98 (1.78%) antibiotics resistance genes were compared with *Pseudomonas aeruginosa* PA01 (Table 4.4). 34 (0.67% of total ORFs) intrinsic antibiotics resistance genes are present in *P. aeruginosa* PA01 genome. On the basis of these chromosomally encoded antibiotics resistance gene *P. aeruginosa* is naturally resistance

against β-lactams, some aminoglycosides, nalidixic acid and trimethoprim antibiotics (Wroblewska, 2006; Stover *et. al.*, 2000). Total 8 genes including metallo-β-lactamases *bla*$_{NDM-1}$ gene, that is absent in PA01.

Table 4.4: Total antibiotics predicted genes in *Pseudomonas* sp. MR 02

MR 02				Query Cover with highest homology one	PA01		
Annotated Protein of MR 02	**Name of the Genes**	**Accession No. of annotated protein**	**Number of amino acid Predicted**		**% identity with PA01**	**Accession No.**	**Number of amino acid**
RND	*oprN*	PJI75874·1	509	93%	64%	NP252583	496
MFS	*emrA*	PJI75872·1	286	98%	73%	NP252581	302
MFS	*emrB*	PJI75869·1	513	100%	60%	NP251525	530
MFS	*emrA*	PJI75868·1	353	86%	66%	NP251526	354
RND	*oprM/opmB*	PJI75867·1	483	96%	56%	NP251527	479
PBP2		PJI75810·1	581	99%	79%	NP253108	579
MFS	*mdfA*	PJI75788·1	414	68%	31%	NP252825	402
RND	*mexW/mexI*	PJI75774·1	1014	99%	82%	NP253065	1018
RND	*mexH*	PJI75773·1	382	97%	60%	NP253064	376
Transcriptional regulator	*mexT*	PJI7571·1	307	99%	66%	NP248881	305
Porin	*oprD*	PJI75717·1	418	96%	72%	NP248931	421
RND	*oprM*	PJI75716·1	485	96%	70%	NP249118	485
RND	*mexB*	PJI75715·1	1050	99%	78%	NP249117	1045
RND	*mexA*	PJI75714·1	384	99%	67%	NP249116	383
MFS	*emrB*	PJI75712·1	483	98%	66%	NP248937	501
Porin	*oprD*	PJI75663·1	429	93%	65%	NP249446	427
MATE	*norm*	PJI75496·1	462	98%	61%	NP253981	488
Porin	*oprD*	PJI75483·1	420	93%	66%	NP251390	435
RND	*triA*	PJI75410·1	362	95%	57%	NP248846	383
RND	*triB*	PJI75409·1	455	98%	60%	NP248847	356
RND	*triC*	PJI75408·1	1018	99%	74%	NP248848	1015

Dihydrofolatereductase	*dfrA*	PJI75361·1	171	96%	66%	NP249041	168
Porin	*oprD*	PJI75048·1	429	100%	63%	NP252868	431
RND	*opmH*	PJI74863·1	452	89%	24%	NP250566	425
RND	*mexK*	PJI74743·1	1021	99%	83%	NP252366	1025
RND	*mexJ*	PJI74742·1	366	100%	70%	NP252367	367
DNA topoisomerase IV subunit A	*parC*	PJI74590·1	752	99%	87%	NP253651	754
RND	*opmH*	PJI74580·1	478	100%	73%	NP253661	482
SMR	*emrE*	PJI74577·1	110	100	64%	NP253677	110
RND	*oprN*	PJI74343·1	506	91%	34%	NP251527	479
RND	*oprM*	PJI74333·1	470	98%	60%	NP249118	485
RND	*mexB*	PJI74332·1	1049	99%	64%	NP249117	1046
16S rRNA methyltransferase	*rmtA*	PJI74285·1	332	99%	71%	NP253317	332
RND	*oprJ*	PJI74223·1	417	91%	26%	NP251212	428
RND	*mexY/H*	PJI74224·1	488	37%	31%	NP253064	376
RND	*mexW*	PJI74225·1	1052	98%	36%	NP251210	1051
RND	*mexW*	PJI74256·1	1053	99%	78%	NP251210	1051
RND	*mexV*	PJI74257·1	416	85%	58%	NP251211	484
RND	*opmH*	PJI74258·1	394	95%	51%	NP251212	428
Porin	*oprD*	PJI74259·1	447	99%	58%	NP251195	448
16S rRNA methyltransferase	*rsmG*	PJI74199·1	419	99%	73%	NP255451	214
MFS	*mdtN*	PJI74088·1	291	92%	43%	NP252581	302
RND	*oprM*	PJI74087·1	472	97%	32%	NP251185	472
Porin	*oprD*	PJI74021·1	444	99%	48%	NP248982	460
Porin	*oprD*	PJI73986·1	439	100%	66%	NP251450	425
Porin	*oprD*	PJI73790·1	446	99%	43%	NP249649	443
RND	*cusA*	PJI73799·1	417	96%	49%	NP251212	428
RND	*mexV*	PJI73798·1	399	85%	68%	NP251211	484
RND	*opmH*	PJI73797·1	1048	99%	26%	NP251210	1051
Fluoroquinolone	*gyrA*	PJI73676·1	921	99%	86%	NP251858	923

ABC	*macA*	PJI73394·1	961	98%	98%	NP252924	945
23S rRNA methyltransferase	*rlmN*	PJI73519·1	381	95%	88%	NP252495	379
RND	*mexN*	PJI73468·1	1030	98%	72%	NP250127	1036
Porin	*oprD*	PJI73464·1	444	100%	60%	NP248852	444
RND	*mexM*	PJI73602·1	387	99%	48%	NP250126	385
Porin	*oprD*	PJI73553·1	459	100%	37%	NP249649	476
MFS	*mdtG*	PJI73374·1	401	97%	49%	NP250043	403
RND	*mexY*	PJI73340·1	383	73%	25%	NP253064	376
Porin	*oprD*	PJI73206·1	422	100%	52%	NP249649	443
Aminoglycoside	*Aph*	PJI72935·1	339	--	--	--	--
RND	*mexY/I/B*	PJI72905·1	791	100%	84%	NP251769	793
Porin	*ompA*	PJI72878·1	345	100%	66%	NP250468	350
23S rRNA methyltransferase	*rlmL*	PJI72886·1	730	100%	81%	NP251738	725
RND	*mexI*	PJI72862·1	1026	99%	78%	NP252896	1029
RND	*mexH*	PJI72861·1	365	98%	54%	NP252895	370
Porin	*oprD*	PJI72771·1	417	96%	46%	NP249446	427
RND	*muxC*	PJI72659·1	1035	100%	59%	NP251216	1036
RND	*muxB*	PJI72658·1	1032	98%	72%	NP251217	1043
RND	*muxA*	PJI72657·1	440	95%	56%	NP251218	426
RND	*opmB*	PJI72660·1	495	91%	63%	NP251215	498
β-lactamase	*bla*PDC-1	PJI72364·1	380	93%	52%	NP252799	397
Porin	*oprD*	PJI72044·1	410	94%	54%	NP252826	418
Porin	*oprD*	PJI72100·1	416	99%	48%	NP250903	416
23S rRNA methyltransferase	*rlmL*	PJI71919·1	308	98%	71%	NP250098	313
RND	*opmH/opmB*	PJI71812·1	442	93%	38%	NP252094	451
RND	*oprM/oprJ/opmB*	PJI71694·1	423	98%	92%	NP250566	425
RND	*mexI*	PJI71718·1	821	96%	49%	NP251769	793
ABC	*macA*	PJI71650·1	390	--	--	--	--
ABC	*macB*	PJI71651·1	654	--	--	--	--

RND	*oprM*	PJI71652·1	472	97%	61%	NP251081	474
Sulfonamide resistance	*sul1*	PJI71575·1	279	--	--	--	--
RND	*mexE*	PJI71193·1	413	100%	71%	NP251183	414
RND	*mexF*	PJI71194·1	1059	100%	92%	NP251184	1062
RND	*oprN*	PJI71195·1	471	95%	79%	NP251185	4772
Regulator	*ompR*	PJI71217·1	233	99%	51%	NP250490	235
RND	*mexC*	PJI71218·1	391	91%	51%	NP253289	387
RND	*mexD*	PJI71219·1	1042	98%	61%	NP253288	1043
MFS	*emrA*	PJI71084·1	351	97%	73%	NP251826	355
MFS	*emrB*	PJI71085·1	516	95%	85%	NP251827	499
PBP2	*mrdA*	PJI70786·1	631	98%	70%	NP252692	646
Aminoglycoside	*aac(3)-iiia*	PJI70455·1	264	--	--	--	--
MFS	*floR*	PJI70475·1	404	91%	30%	NP252263	392
Transcriptional Regulator	*tetR*	PJI70477·1	208	--	--	--	--
MFS	*tetG*	PJI70478·1	391	35%	48%	NP249822	422
Bleomycin binding protein	*brp*MBL	PJI70471·1	121	98%	36%	NP251817	267
β-Lactamase	*bla*NDM-1	PJI70472·1	270	--	--	--	--
Aminoglycoside	*ant4-iib*	PJI70457·1	254	--	--	--	--
β-Lactamase	*bla*PME-1	PJI70444·1	309	--	--	--	--

These 98 antibiotics resistance genes are divided into two major groups: (i) the antibiotic efflux pumps (Table 4.5) and (ii) the enzymes that conferred antibiotic resistance (Table 4.6). Genes were also idetified in MR 02 that confer resistance to additional compounds (kanamycin, bleomycin). After annotations of these antibiotics resistance genes in different databases are nearly the same, we can suggest that the prediction results are more authentic.

Efflux pumps may be consist of single or multiple component, but in *Pseudomonas* many pumps have a three component systems and consist of inner membrane and outer membrane components as well as a membrane fusion protein (MFP) situated in the

periplasm. 64 genes are assigned to 21 efflux pump systems belonging to five different transporter families. 48 genes belong to resistance nodulation cell division (RND) family, 11 genes to major facilitator super family (MFS), single gene to multidrug and toxic compound extrusion (MATE) family, single gene to small multidrug resistance (SMR) family, and three genes to ATP-binding cassette (ABC) family (Table 4.5).

Table 4.5: Predicted drug resistance-related porin channels and efflux pumps in *Pseudomonas* sp. MR 02

Porin channels and Multidrug transporter type	Gene locus
Porin (OprD)	PJI75663, PJI75717, PJI75483, PJI75048, PJI74259, PJI73986, PJI74021, PJI73790, PJI7334, PJI73553, PJI73206, PJI72771, PJI72100, PJI72044.
RND efflux pumps	PJI75714_75715_75716,PJI75773_75774,PJI75874,PJI75867_75868_75869,PJI75408_75409_75410,PJI74863,PJI74742_74743,PJI74580,PJI74343,PJI74333_PJI74332,PJI74223_74224_74225,PJI74256_74257_74258,PJI74087,PJI73797_73798_73799PJI73468,PJI73602,PJI73340,PJI72905,PJI72861_72862,PJI72657_72658_72659_72660,PJI71812,PJI71694,PJI71718,PJI71652,PJI71193_71194_71195,PJI71218_71219.
MFS efflux pumps	PJI75872, PJI75868_75869,PJI75712, PJI75788, PJI74088,PJI73374, PJI71084_71085, PJI70475, PJI70478.
SMR efflux pumps	PJI74577
MATE efflux pumps	PJI75496
ABC efflux pump	PJI71650_71651, PJI73394.

14 homologues to the porin protein OprD and only one OmpA porin channel have been found Permeability barrier of outer membrane also play an important antibiotics resistance mechanism in *P. aeruginosa*. Low expression of *opr*D genes is responsible for making resistance against imipenem (carbapenem) antibiotics. Lack of OprD protein leads to reduction of active antibiotics molecules (Imipenem) capable of reaching the target penicillin-binding-protein (PBP) (Kadry, 2003). 14 *opr*D genes are identified in MR 02 (Table 4.5). All 14 *opr*D genes are mutated and unable to producing OprD protein.

Three different β-lactamase enzyme producing genes are present in MR 02. They belong to class A (PME-1 *P. aeruginosa* ESBL), class B (NDM-1), and class C (AMPC)

β-lactamase family (Table 4.6). First report of PME-1 came in 2011. It is grouped in Ambler class A extended-spectrum β-lactamase (ESBL), which is the most common cause of cephalosporin resistance in other Gram negative pathogens, such as *Escherichia coli* and *Klebsiella pneumonia*.

Table 4.6: Predicted drug resistance-related enzymes in *Pseudomonas* sp. MR 02

Drug class	Putative product	Gene locus
Beta-lactams	Class A beta-lactamase	PJI70444
	Class B beta-lactamase	PJI70472
	Class C beta-lactamase	PJI72364
Aminoglycosides	Aminoglycoside N-acetyltransferase	PJI70455
	Aminoglycoside O-phosphotrnsferase	PJI72935
	ANT(4) II family Aminoglycoside nucleotidyltransferase	PJI70457
	16S rRNA methyltransferase	PJI74285
Other	Dihydrofolate reductase	PJI75361
	16S rRNA methyltransferase	PJI74199
	23S rRNA methyltransferase	PJI73519
	23S rRNA methyltransferase	PJI72886
	23S rRNA methyltransferase	PJI71919
	DNA topoisomerase IV subunit A	PJI74590
	Fluoroquinolone	PJI73676
	Sulfonamied resistance	PJI71575
	Fosfomycin thiol transferase	PJI72090

Eight genes responsible for enzymatic degradation of aminoglycosides antibiotics, three genes for aminoglycoside-modifying enzymes (AMEs) and five rRNA methylase genes (two 16S rRNA and three 23S rRNA) have been identified. AMEs attach a phosphate, adenyl or acetyl radical to the aminoglycosides antibiotic molecule, due to this attachments molecular structure of antibiotics are modified, and thus they decrease the

binding affinity of the antibiotics to the target in the bacterial cell (30S ribosomal subunit) (Llano-sotelo *et. al*., 2002). AMEs are divided into three classes: aminoglycoside phosphoryltransferases (APHs), aminoglycoside adenylyltransferases or nucleotidyltransferases (AADs or ANTs) and aminoglycoside acetyltransferases (AACs) (Vakulenko & Mobashery, 2003). On the basis of AMEs classification one AMEs from each group is presents in MR 02 (Table 4.6) and they make resistances against all types of aminoglycosides.

Microbes are resistance *Sul* 1 and *dfr* A genes responsible for mediating resistance against sulfamethoxazole and trimethoprim were also found in MR 02 genome. Dihydrofolate reductase resistance genes show 66% homology with *P. aeruginosa* PA01 whereas *sul* 1 gene is absent in PA01 genome.

Previous studies have shown the involvement of efflux channels and mutations of certain genes (*gyrA*, *gyrB*, *parC*, and *parE*) in the development of resistance to quinolone group of antibiotics in *Pseudomonas aeruginosa* (Nakajima *et. al*., 2002; Wang *et. al*., 2007). Mutated genes like *gyrA* and *parC are* also identified in MR 02 (Table 4.4 & 4.6). Presence of mutated genes like *gyrA* and *parC* along with the abovementioned efflux channels might have played a role in development of resistance to fluoroquinolone in MR 02.

We further analyzed the genomic islands by island viewer (Figure 4.1) and identified 14 antibiotic related genomic islands and 53 open reading frames. Three genes tetR PJI70477.1 (*tet*G regulatory protein) and two ABC efflux pump genes PJI71650_71651 precisely located in a putative genome island in the MR 02 strain, indicating that this gene was probably acquired through horizontal gene transfer; all the remnant genes were not present within any genomic island. One new RND efflux pumps PJI73799_73798_73797 are predicted, which is also located in putative genomic island. On the basis of these results suggest that the antibiotics resistance genes are conserved in *Pseudomonas* sp. MR 02 and are probably responsible for the intrinsic resistance in *Pseudomonas* sp. MR 02.

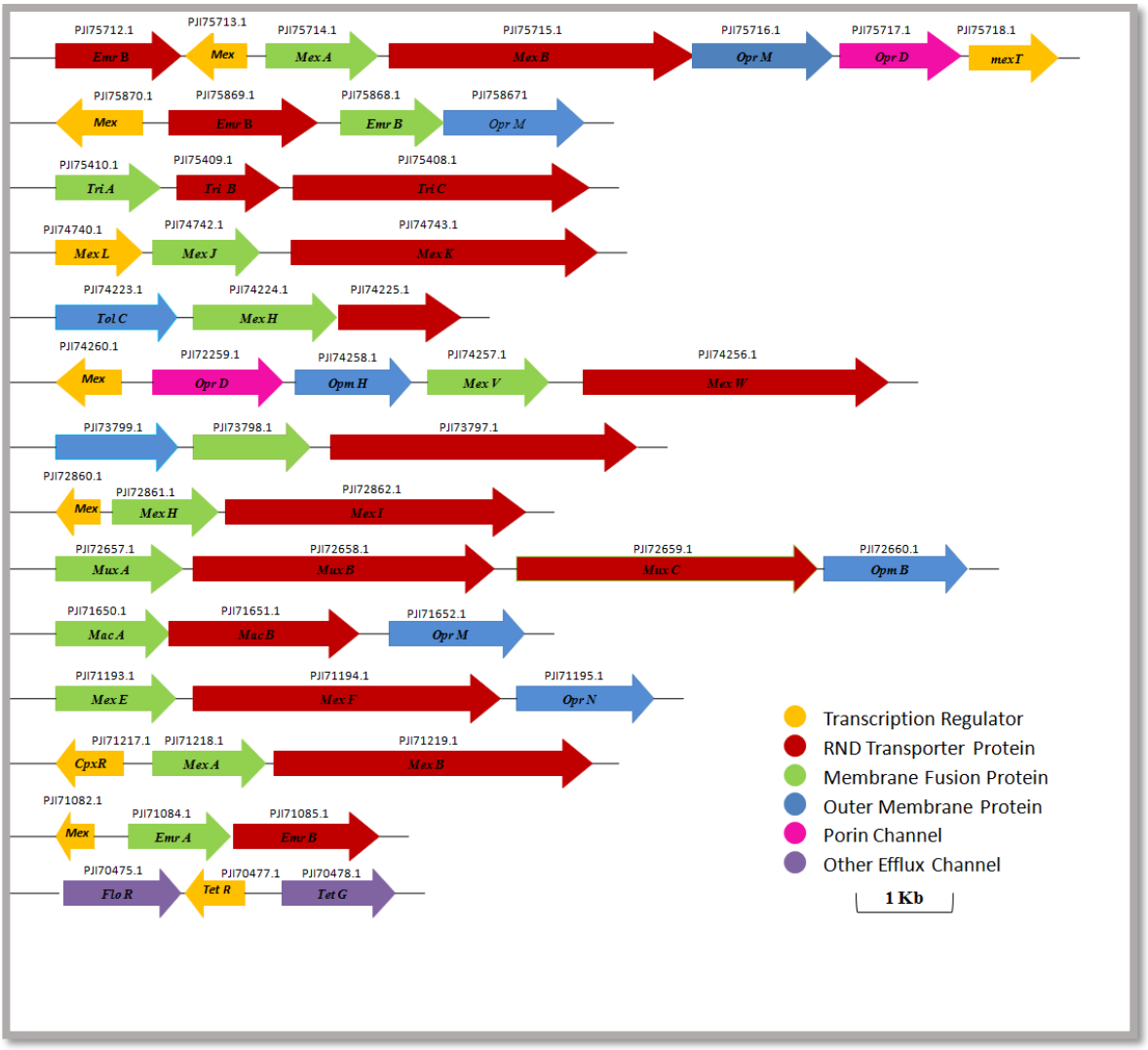


Figure 4.3: A schematic representation of the cluster of antibiotic resistance gene in the draft genome of *Pseudomonas* sp. MR 02

4.3.4 Taxonomic identification of *Pseudomonas* sp. MR 02

16S phylogeny revealed that strain MR 02 is a member of the genus *Pseudomonas*. Strain MR 02 revealed highest levels of sequence similarities with *Pseudomonas taiwanensis* DSM 21245 (99.7 %) followed by *Pseudomonas plecoglossicida* DSM 15088 (99.8%), *Pseudomonas monteilii* NBRC 103158 (99.7%) respectively. *Pseudomonas* sp. MR 02 was clustered in a separate branch with *Pseudomonas taiwanensis* DSM 21245 as its closest relative (Figure 2.6).

Though 16S rRNA is the unrivaled and universally applied marker of choice for most phylogenetic studies, but reliance on this single marker can result in poor resolution as there are several shortcomings in this approach. Genome based phylogeny plays a crucial role in phylogenetics of bacteria by replacing 16S phylogeny. Therefore the results of 16S phylogeny should be considered together with the results of whole genome based data analysis in a systematic process during species delineation. The bcgTree, (Figure 4.4), has revealed that the nearest phylogenomic neighbours of the strain MR 02 were *P. monteilii* CIP 104883, *Pseudomonas putida* NBRC 14164, *Pseudomonas hunanensis* P11.aa, *P. plecoglossicida* DSM 15088, *P. taiwanensis* DSM 21245, and *P. guariconensis* LMG 27394.

Two different *in silico* approaches were used in this study, *in silico* DDH and ANI, to determine the genomic similarities in order to confirm the species status. Both of these have been proposed as robust parameters (ANI value to define a new species is ≤ 96; and DDH value to define a new species is ≤ 70%) that can replace 16S rRNA gene sequence comparison for species delineation in the genomic era. The *i*DDH and ANI values obtained for MR 02 with *P. monteilii* CIP 104883 or *P. putida* NBRC 14164 or *P. hunanensis* P11.aa or *P. plecoglossicida* DSM 15088 or *P. taiwanensis* DSM 21245 or *P. guariconensis* LMG 27394 were 40.5 & 89.9% or 33.1 & 86.9% or 30.6 & 85.6% or 30.4 & 85.5% or 28.7 & 84.5% (Table A4.1 and A4.2). The analyses revealed that strain MR 02 showed maximum relatedness to the genome of the type strain of *P. monteilii*; values with other closely related type strains were even lower. These values qualify MR 02 strains as a distinct species of the genus *Pseudomonas* and different from any of their closest relatives.

The circular maps was used for gene characterization, functional assignment and function-based browsing of the MR 02 genome with 4 phylogenetically nearest species *P. plecoglossicida* DSM15088, *P. monteilii* NBRC103158, *P. putida* NBRC14164 and *P. aeruginosa* PA01. Non homologous regions present in the genome of four different nearest species of *Pseudomonas*, compared with MR 02 genome have been presented where a sub-set of it was related to antibiotic resistance genes. Total 8 genes were found unique to MR 02 (Figure 4.5).

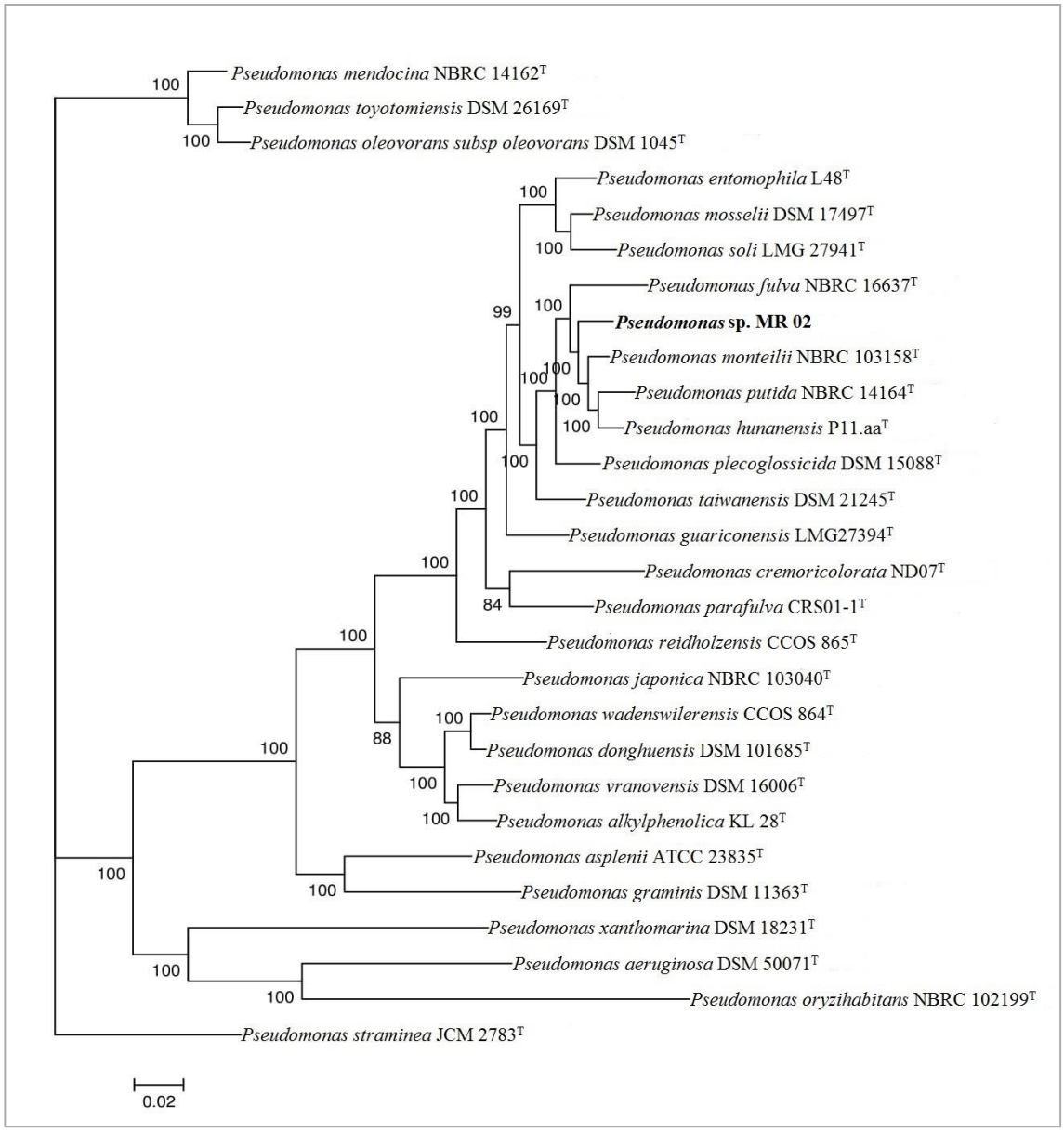


Figure 4.4: A phylogenetic tree based on bacterial core genome

The tree was built by the bcgTree pipeline for *Pseudomonas* sp. MR 02 along with 27 other *Pseudomonas* type strain genomes. Numbers at nodes designate bootstrap support values resulting from 100 bootstrap replicas.

4.3. Comparative genomic analysis of *Pseudomonas* sp. MR 02

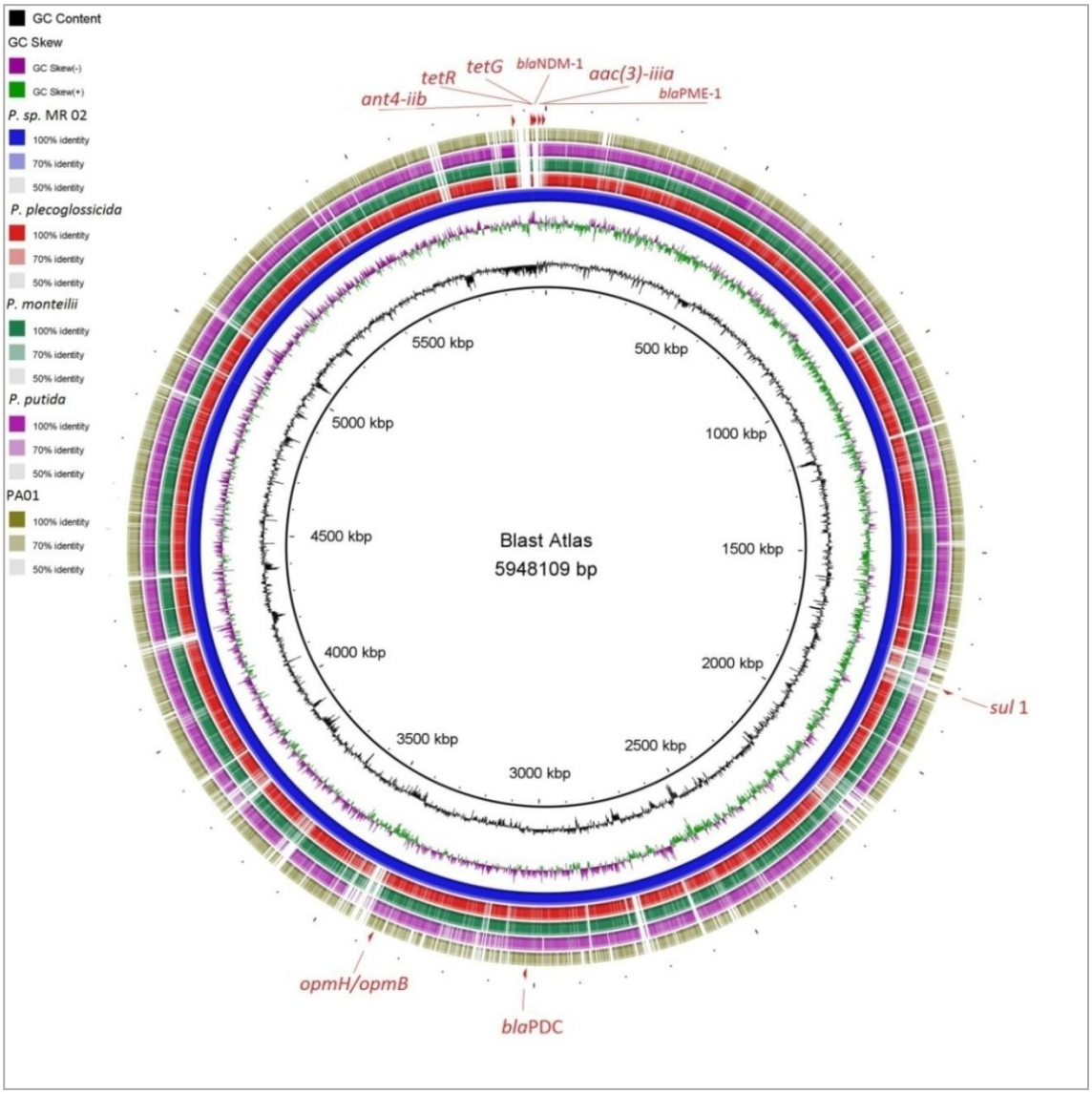


Figure 4.5: Circular representation of the *Pseudomonas* sp. MR 02 draft genome in comparison with four reference genome of *Pseudomonas* genus

Ring from inside to outside (1) GC contents (black) (2) GC skew (purple and green) (3) Coding sequences of *Pseudomonas* sp. MR 02 draft genome (dark blue) (4) BLAST comparison with *P. plecoglossicida* DSM 15088 (red) (5) BLAST comparison with *P. monteilii* NBRC 103158 (green) (6) BLAST comparison with *P. putida* NBRC14164 (violet) (7) BLAST comparison with *P. aeruginosa* PA01 (lime green) (8) Position of some important antibiotic resistance genes (red). Figure generated by BLAST Ring Image Generator v 0.95.

4.3.5 Correlation of phenotypic and genotypic characters of *Pseudomonas* sp. MR 02

Before the correlation of phenotypic characters of MR 02 with annotated 98 antibiotics resistance genes, we design two extra experiments: (i) determination of MIC value of MR 02 in presence or absence of PAβN (efflux pump activities determination), (ii) determination of MIC value of MR 02 in presence of EDTA (0.4 mM) (*bla*$_{\text{NDM-1}}$ activities determination) (Figure 1.10).

4.3.5.1 Determination of efflux pump activity in contributing antibiotic-resistance in *Pseudomonas* sp. MR 02

In the presence of the efflux-inhibitor, PAβN, MICs of chloramphenicol, tetracycline, ciprofloxacin, and levofloxacin were reduced to 2.0, 2.0, 1.3, and 16.6 % respectively of the values shown in absence of PAβN (MIC values without PAβN was taken as 100%) , thus temporarily causing complete reversion of the resistance to susceptible phenotype. Similar reduction in MIC values, but of varying influence, has been observed for erythromycin (reduced to 20 %) and streptomycin (reduced to 33.3%) in presence of PAβN. The reductions in MICs, in presence of the efflux inhibitor, to lesser degrees were obtained with meropenem (reduced to 61.5%) imipenem (reduced to 75%), neomycin (reduced to 66.6%), gentamycin (reduced to 70%), cefepime (reduced to 75%) and ceftazidime (reduced to 75%). Least reduction in MIC in presence of PAβN was observed in case of trimethoprim or azithromycin (reduced to 83.3%) (Figure 4.6). Hence, on analyses, resistance shown by MR 02 against chloramphenicol, tetracycline, ciprofloxacin, and levofloxacin may be solely governed by the efflux pump(s) while resistance to other antibiotics except trimethoprim and azithromycin, were partially dependent on efflux system.

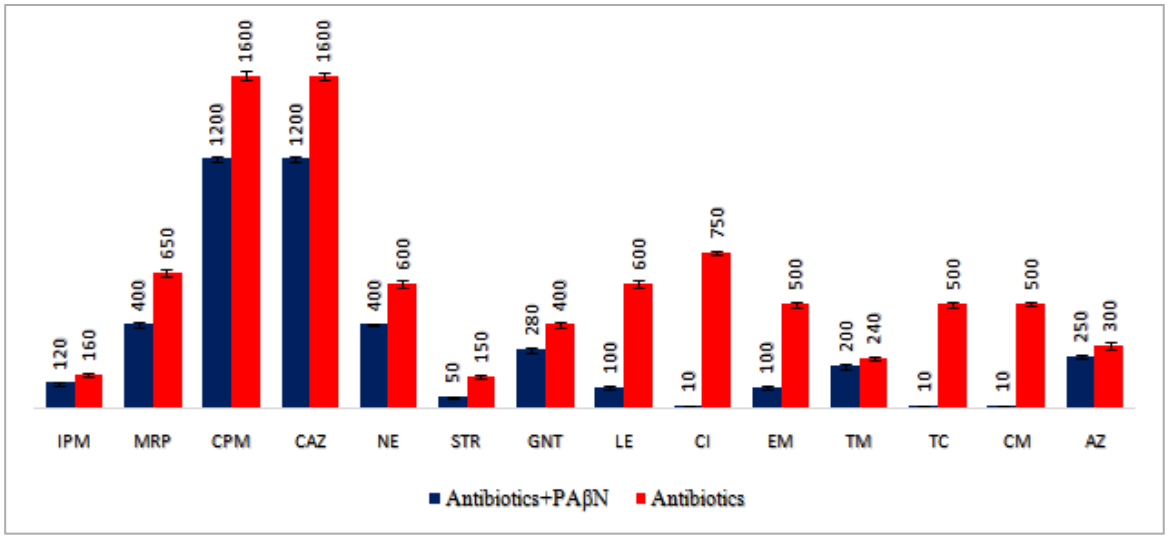


Figure 4.6: Reduction in MIC values of *Pseudomonas* sp. MR 02 in presence and presence of PAβN with antibiotics

MIC values of 14 antibioticsin the presence or absence of efflux pump inhibitor,PAβN (30µg/ml) ; MIC value expressed in µg/ml is shown at the tip of each bar graph. Neomycin(NE), Streptomycin (STR), Gentamycin(GNT), Trimethoprim(TM), Tetracycline(TC), Chloramphenicol(CM), Levofloxacin(LE), Ciprofloxacin(CI), Erythromycin(EM), Azithromycin (AZ).

Overall, phenotypic resistance pattern of MR 02 correlated highly with the predicted 98 resistance genes. Genotypic and phenotypic analysis showing only efflux pumps are responsible for resistance against chloramphenicol and tetracycline in MR 02 (Figure 4.6 and Figure 4.7). A comprehensive genomic distribution of antibiotics resistance genes in MR 02 annotated as per function to confer resistance to every single antibiotics of a total of 14 antibiotics (Figure 4.7). This resulted in an overall sensitivity of 100%. In this study, 0·4 mM EDTA was used to completely inhibit NDM-1 activity; which in turn reduced the MIC value of imipenem up to 95%. One metallo-β-lactamas genes encoding carbapenem resistance enzyme, and this results sensitivity was also 100%.

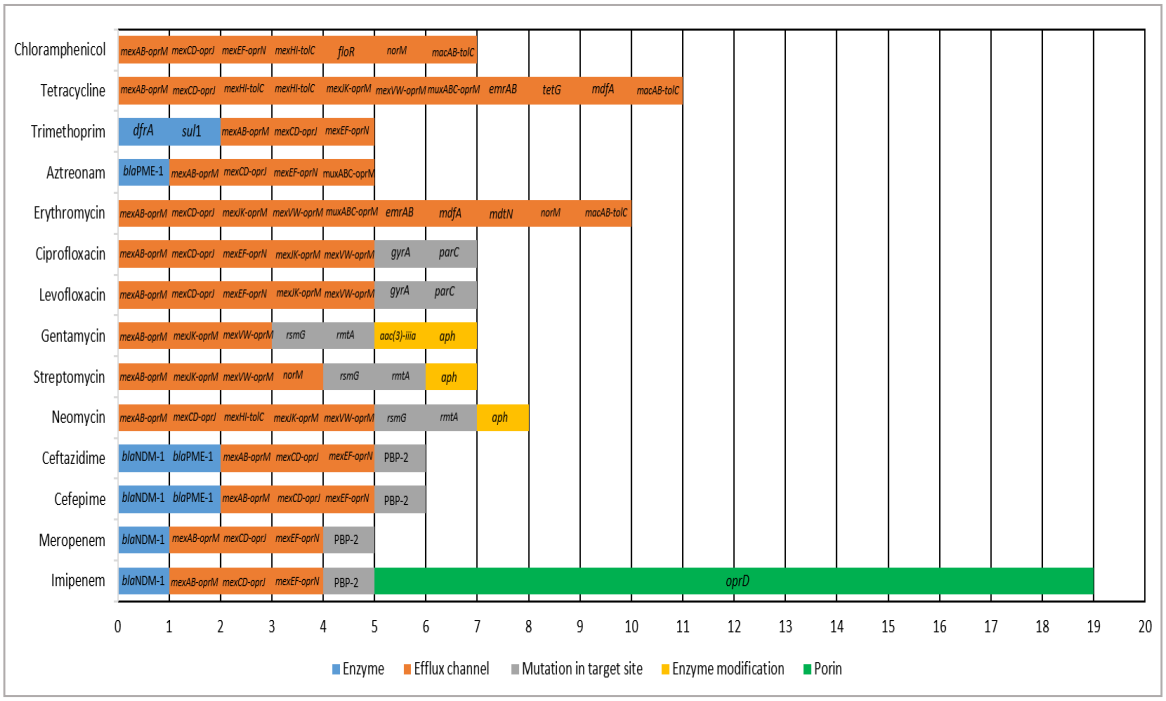


Figure 4.7: A comprehensive genomic distribution of genes in MR 02 annotated as per function to confer resistance to every single antibiotic of a total of 14 antibiotics

4.4 Conclusion

Whole genome sequencing in the current study was done to detect different types of resistance genes in *Pseudomonas* sp. MR 02 which were not detected in the previous chapters by phenotypic and PCR analysis. Whole genome sequencing helps in correlation between genotypic characters and phenotypic characters of *Pseudomonas* sp. MR 02. However, the antibiotic-resistance analysis tool (Resfinder, ARG-ANNOT, and CARD) was not efficient enough to detect the type of antibiotics resistance genes, while the BLAST tool (Smart BLAST) provided more detailed characterization of the ARGs. Although bacterial whole genomic studies are time consuming and expensive, they are more accurate and informative about the genetics of bacteria. Efforts are needed to make genomic study, and especially bioinformatics analysis, easier and more convenient for both laboratory and clinical and diagnostic work.

Pseudomonas sp. MR 02 strain carries chromosomally encoded complete package of resistance genes (efflux pumps, targets site modification, enzyme mortification, mutation in antibiotics binding sites, antibiotics degrading enzymes and non enzymic mechanism)

for aminoglycosides and β-lactams antibiotics. It has genotypically and phenotypically resistance to nearly all clinical trial antibiotics and they have lost susceptibility toward fluoroquinolone, aminoglycosides, monobactam, tetracycline, chloramphenicol, trimethoprim, colistin, cephalosporins, and carbapenem (including last resort antibiotic).

Detailed biochemical, physiological and phylogenetic analyses identified the strain MR 02 as a novel species of the genus *Pseudomonas* under *P. putida* group. In summary, MR 02 is a Pandrug-resistant bacteria and it has all possible arsenals arranged in its genome, ready for encountering representative molecules from any class of antibiotic.

Chapter 5:

Revealing the role of New Delhi Metallo-β-lactamase in metabolism of β-lactam antibiotics as growth substrate

5.1 Introduction

Antibiotics have always been reckoned as one of the wonder drug discoveries of the last century. This is true but increasing antibiotics-resistance in pathogenic bacteria with the associated decrease in the development of new antibiotics drugs threatens us to the return to the dark ages of the pre-antibiotics era. The protective mechanisms in bacteria that have developed include preventing entry of the antibiotics, producing hydrolytic enzymes that destroy or modify the antibiotic drugs, or making changes to the antimicrobial target sites. Therefore, the antibiotic-resistance mechanism of bacteria also could be considered to simply symbolize the Darwinian evolutionary theory. Bacteria naturally developed a resistance mechanism against the microorganism-derived antimicrobial molecules for survival and make many copies of itself in a successive generation. A wide range of biochemical and physiological mechanisms has developed in bacteria to make resistance against antibacterial compounds. In the specific case of antibacterial agent, the complexity of the processes that help to emergence and distribution of resistance can't be exaggerated, and the lack of basic knowledge on resistance mechanism is one of the primary reasons that there has been so little significant success in the effective prevention and control of resistance development.

Now microorganisms are not only developing resistance mechanisms against the antibiotics molecules but it also developed a new way they eat antibiotics as food. Despite the lack of appropriate information on antibiotics-use, 100-200 thousand tons of antibiotics used worldwide every year (Wise, 2002; Kummerer, 2003) and now also recognized have an additional role of antimicrobials. These new additional roles have been developed by bacteria and through these new roles, antibiotics offer carbon and nitrogen or only carbon sources of bacteria. However, remarkable only few works has been reported on the biochemistry of antibiotic catabolism. Recently a research work on penicillin catabolism was published and they find the conserved pathway for penicillin catabolism involving initial hydrolysis catalyzed by β-lactamases the same enzymes that are widely responsible for resistance to the penicillins and β-lactam group of antibiotics (Crofts *et*. *al*., 2018). Based on antibiotics origin antibacterial compounds are classified into three types, natural, semi-synthetic and synthetic. The present period semi-synthetic and synthetic antibiotics are more prescribed drugs. Semi synthetics antibiotics drugs like "Ampicillin" was

introduced for mankind more than 60 years ago. When the amino group added into the benzylpenicillin molecule a new molecule was developed called ampicillin (Witting and Smith 2011). Ampicillin was placed in β-lactams group, which include successive generations of β-lactams (including cephalosporin, and carbapenems) remain the most important Class of antimicrobial agents. Penicillin is a natural compound and these produced by microorganisms long before human use, and hence antibiotic-catabolism may well have ancient origins. Based on the previous study, penicillin catabolism in bacteria proceeds via initial β-lactamase-mediated hydrolysis to originate benzylpenicilloic acid, which acts as a substrate for amidase and benzylpenicilloic acid converted into phenylacetic acid. At the end of the process phenylacetic acid can then undergo conversion into acetyl coenzyme A (CoA) and succinyl CoA via an established process of phenylacetic acid catabolism (Crofts *et. al*., 2018; Teufel *et. al*., 2010). On the other hand, ampicillin is semi-synthetic compounds and it does not easily catabolized due to the amino group, but when this amino group shall be removed then it will easily be catabolized by bacteria. Serine β-lactamase enzyme can only hydrolyze ampicillin molecules and then they only converted into an inactive form, these hydrolyzed product can act as substrate molecules for amidase enzymes, but not make a product for PAA pathway. Because after amidase hydrolysis originates a benzylpeniclloic acids with an amino group do not convert into phenylacetic acid (Figure 5.1). In brief, we can say that for β-lactam antibiotic catabolism required three components and these components are (i) β-lactams resistance genes (ii) amidase genes and (iii) phenylacetic acid catabolism enzymes encoding genes. When these three components shall be present in any microorganism, then antibiotics molecules will be catabolized by those microorganisms. Previous reports have explained only natural antibiotics (penicillin) catabolism, but in this study we explain ampicillin catabolism in MBL positive strain which was isolated from Mahananda and Karala River of Siliguri and Jalpaiguri of West Bengal. All MBL positive isolates showing unexpected high MIC value against penicillin as well as ampicillin molecules. Simply this result indicates MBL positive strain used β-lactam antibiotics as a food (carbon and nitrogen molecules) for growth. Based on NDM-1 and ampicillin binding study, hypothetically we recognized an additional role of NDM-1 in ampicillin catabolism; they could be provided with a carbon and nitrogen source to bacterial cells.

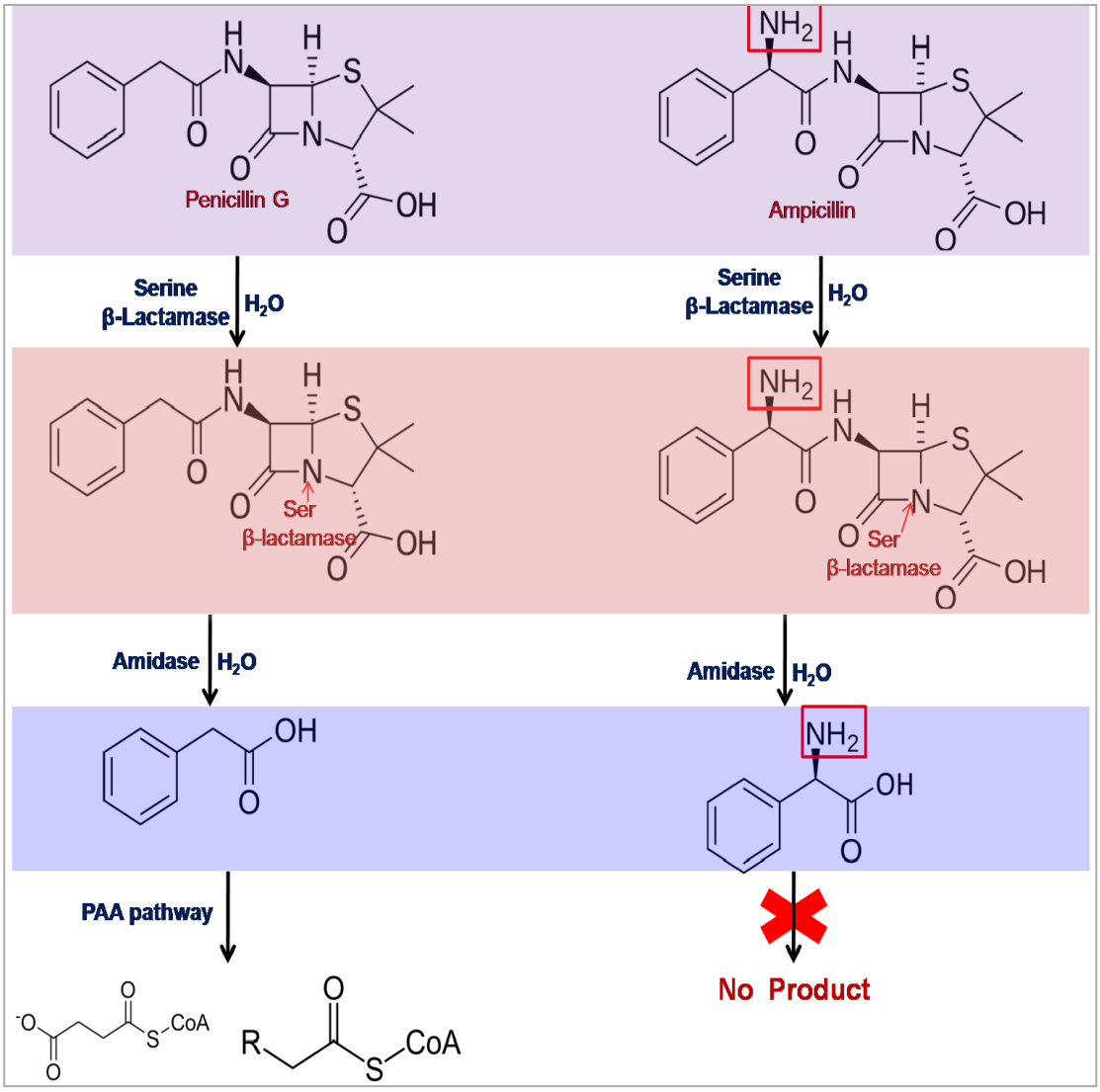


Figure 5.1: Difference between penicillin and ampicillin molecule and their catabolic pathways

This chapter is the soul of the whole study and the first and most important aim of this chapter was to establish a new role of New Delhi Metallo-β-lactamase in the catabolism of ampicillin antibiotics as a growth substrate. We demonstrate that strategy by recombinant *E. coli* DH5α strain, enabling it to grow using ampicillin as its sole carbon source. The second aim of this chapter was to demonstrate the utilization of ampicillin as a sole carbon source in MBL positive strain. All the MBL positive strains were used because all the bacterial strain carries New Delhi Metallo-β-lactamase genes. The third aim of this study was to revelation of ampicillin catabolic pathway in the draft genome of MR 02

strain, although these MR 02 strain was also used ampicillin as a sole carbon source for growth and the whole genome is available, so, this strain can be used as a model strain for analysis of β-lactams catabolic responsible genes and their expression (RNA-seq analysis).

5.2 Materials and Methods

5.2.1 Growth studies of MR 02 demonstrate utilization of ampicillin as the sole source of carbon, nitrogen, and energy

To study the utilization of ampicillin as carbon and nitrogen sources, the inoculum was prepared as follows. MR 02 was inoculated in 10ml Luria Broth containing 50µg/ml Imipenem and was incubated at 30°C in shaking incubator for 18 hrs. 18 hrs grown MR 02 culture was centrifuged in 1.5ml tube at 3000 rpm for 5 minutes and the supernatant was discarded. Then 1.5 ml fresh autoclaved distilled water was added, the cell pellet was dissolved in it following centrifugation at 3000 rpm for 5 minutes and again the supernatant was discarded. The same process was repeated for the second time. The culture was washed a total of two times in minimal media lacking a carbon and nitrogen sources. After that, the fully washed cell pellet was dissolved in 1.5 ml fresh minimal media. From this stock, 1% (200µl) inoculum was taken to inoculate each 100 ml Erlenmeyer flasks containing 20 ml minimal media with different experimental compositions.

The composition of the minimal media used in this study was as follows:

(I) Ampicillin as a sole source of carbon and nitrogen

(II) Ampicillin was used only as a sole source of carbon

For growth on ampicillin as both carbon and nitrogen sources, no dextrose and no NH_4Cl were added in minimal media, but the only different concentration of ampicillin (Himedia) 2 g/L, 4 g/L, 6 g/L with EDTA and without EDTA was added. For growth on ampicillin as the only carbon source, no dextrose but only different concentration ampicillin 2g/L, 4g/L, 6g/L, with EDTA and without EDTA was added. Minimal media were prepared consisting of (per litre) 3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl, 5 g/L NH_4Cl, and 100 mg/l $MgSO_4$. The pH value was adjusted to 7.0 ± 0.2 and sterilized by autoclaving. After inoculation flasks were incubated at 30°C for 216 hrs in a shaking incubator at 120 rpm. Growth was monitored after every 24 hrs at 600 nm in a spectrophotometer (BMG SPECTROstar nano).

The growth of MR 02 in the minimal media containing NH_4Cl with and without EDTA was studied to test the effect of EDTA. All growth experiments included triplicate independent cultures, and OD_{600} values were evaluated as averages with standard error. Growth data were plotted using Microsoft Excel.

5.2.2 Identification of β-lactam catabolism-responsive genes from the draft genome of *Pseudomonas* sp. MR 02

Open reading frames (ORFs) of β-lactam catabolic genes (resistance-associated, catabolism associated and phenylacetic acid catabolism) were identified from the draft genome of MR 02. Sequence lengths were again analyzed by additional BLASTP via the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST).

5.2.3 Establish a new role of *bla*$_{NDM-1}$ in the β-lactam catabolism pathway

Our hypothesis is that if a bacterium will have NDM-1 gene with amidase(s) and the pathway to catabolize phenylacetic acid (PAA), then it will use ampicillin as a carbon and energy source. The amidase(s) and pathway to catabolize phenylacetic acid (PAA) are naturally present in the *E. coli* cell (Teufel *et. al*., 2010; Priyadarshini *et. al*., 2007) and when we will clone and express the NDM-1 gene in the *E. coli* cell, then recombinant cell will also use ampicillin as the carbon source. Thus, the speculation was validated with experiment.

5.2.3.1 Amplification of *bla*$_{NDM-1}$ gene *Pseudomonas* sp. MR 02

The *Pseudomonas* sp. MR 02 *bla*$_{NDM-1}$ full length (813bp) gene was amplified by Polymerase Chain Reaction using primers *Eco*RI (5′-GGGAATTCATGGAATTGCCCAATATTATG-3′) and *Pst*I (5′-AACTGCAGTCAGCGCAGCTTGTCGGCCAT-3′) (Tada *et. al*., 2014). The forward and reverse primers were flanked by the specific restriction endonuclease sites used for cloning the amplified fragments. The reaction mixture and thermal cycle profile for PCR amplification described in previous chapter 2. The Amplified product was checked in 1.5% agarose gel by gel electrophoresis.

5.2.3.2 Cloning vector pHSG398

The plasmid vector pHSG398 (Takara, Japan) of 2227bp length, was used for the cloning of full-length PCR amplified *bla*NDM-1 gene product. pHSG398 is a pUC-type

bacterial cloning vector with a chloramphenicol resistance marker gene. This vector also contains multiple cloning sites (MCS) with the *lacZ* genes (Figure. 5.2).

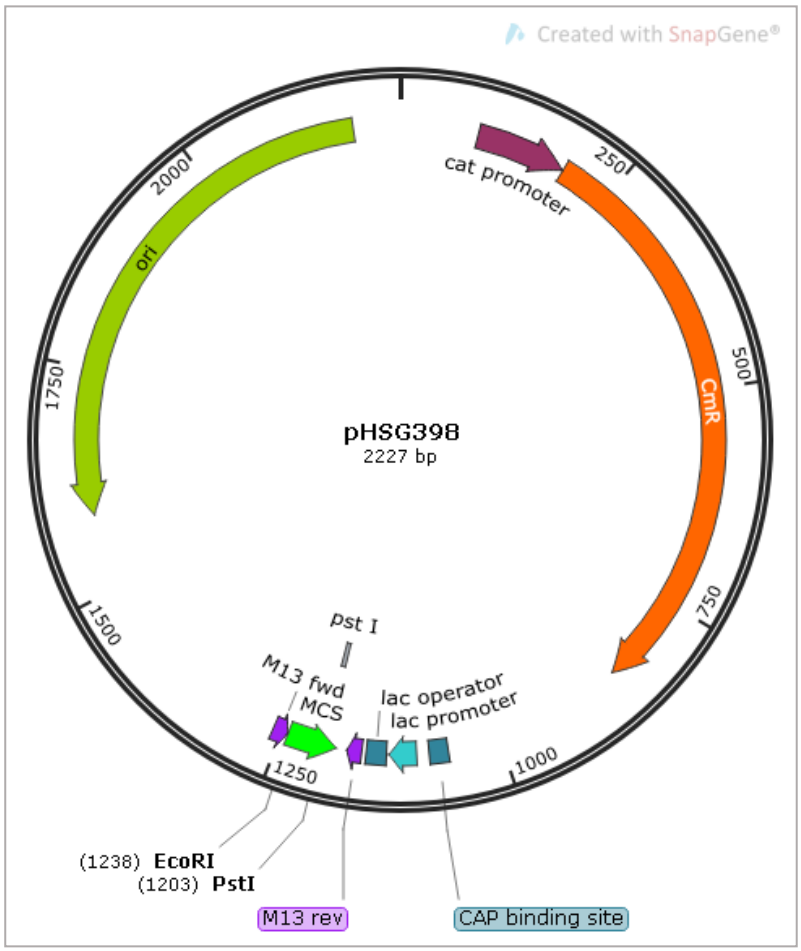


Figure 5.2: A schematic representation of plasmid vector pHSG398 (2227bp)

(Vector map were created in SnapGene tool)

5.2.3.3 Restriction endonuclease (RE) digestion of PCR amplified *bla*$_{NDM-1}$ genes and pHSG398 Plasmid

Restriction double digestion of the amplified PCR product and plasmid vector was carried out using the two endonuclease restriction enzymes *Eco*RI and *Pst*I. The reaction mixture for double digestion was prepared separately for both DNA samples as per the manufacturer protocol (Thermo Fisher Scientific). For this 2.5µl (15 units) *Eco*RI and 5µl (30 units) *Pst*I of required restriction enzymes was added to 1µg DNA samples with tango buffer supplied by the manufacturer and the mixture was incubated overnight at 37°C. The

digested DNA samples were run on the 1% agarose gel to confirm the success of the restriction digestion reaction.

5.2.3.4 Gel extraction of RE digested DNA products

The double digested PCR DNA and vector DNA bands were cut out from the agarose gel (low melting agarose) with a sterile scalpel. The DNA bands from the gel were extracted and purified by the G Sure DNA purification kit (GCC Biotech) follow manufacture instruction. Finally, the purified DNA products were eluted in the nuclease-free water separately and thereafter, the purified DNA products were stored at -20°C for further cloning work.

5.2.3.5 Preparation of competent cell

The competent cells were prepared for the transformation of the recombinant vector (pHSG398+NDM-1) is followed Mandel and Higa classic experiment method (Mandel & Higa, 1970) Chemical use in this method were described in previous chapter 2.

5.2.3.6 DNA ligation

The gel-purified double digested PCR products were ligated with double digested gel-purified pHSG398 vector in molar ratio 1:3 in an appropriate buffer and T4 ligase enzyme (GENEI). The mixture was incubated at 4°C overnight. Thereafter, the ligated DNA products were transformed into *E. coli* DH5α competent cells.

5.2.3.7 Transformation

The chemically prepared competent cells were transformed with the ligated recombinant vector (pHSG398+ bla_{NDM-1}) product via the heat shock method. 20µl (100ng DNA) ligation mixture was added aseptically to the 100 µl competent cells and mixed by gentle tapping and following the transformation method described in previous chapter 2. The transformants were spread onto the LB agar plate containing imipenem (10 mg/l) and chloramphenicol (50 mg/l). The plate was incubated at 37°C for overnight.

5.2.3.8 Screening of the recombinant by PCR methods

Confirmation of the cloned bacterial isolates was done by amplification of the conserved region of bla_{NDM-1} genes by PCR methods followed by PCR method described in previous chapter 1.

5.2.3.9 Determination of minimum inhibitory concentration (MIC) of recombinant *E. coli* DH5α strain

MIC values of the recombinant *E. coli* DH5α (pHSG398+bla_{NDM-1}) were determined by the broth macrodilution method as per CLSI guidelines (Wayne 2017). The experiment was set up in triplicates in the test tube with 3ml MH broth media containing different dilution of antibiotics and 10^6 recombinant *E. coli* DH5α (pHSG398+ bla_{NDM-1}), and wild type *E. coli* DH5α cells were used as a control. The tubes were incubated at 37°C for 16-18 hrs. Thereafter bacterial growth was determined by measuring at 600 nm via spectrophotometer (BMG SPECTROstar nano).

5.2.2.10 Catabolism of ampicillin by recombinant *E. coli* DH5α cell

To study the utilization of ampicillin as carbon sources by recombinant strain of *E. coli* DH5α (pHSG398+bla_{NDM-1}) inoculums and minimal media were prepared as the following method described above section 5.2.1. Ampicillin was used as the carbon source and the concentrations of ampicillin with minimal media used were 0.1 g/L. *E. coli* DH5α+ pHSG398 and *E. coli* DH5α+pGEM-T strain used as negative and positive control respectively. After inoculation flasks were incubated at 37°C for 48 hrs in a shaking incubator at 120 rpm. Bacterial growth was measured by CFU counting method. Samples were taken for plating on LB plates at 12 hrs interval to 48 hrs after starting the experiment. All growth experiments included triplicate independent cultures, and CFU values were evaluated as averages with standard error. Growth data were plotted using Microsoft Excel.

5.2.4 Transcriptome sequencing and bioinformatics analyses of *Pseudomonas* sp. MR 02 to determine expression of gene responsible for ampicillin-catabolism

5.2.4.1 Preparation of sample for transcriptome sequencing

For transcriptome RNA sequencing (RNA-Seq), the sample *Pseudomonas* sp. MR 02 was grown under two different experimental conditions, one with glucose (6 g/L) (control) and another one with ampicillin (6 g/L) (test/experimental) as carbon source containing minimal media (described in section 5.2.1). Culture flasks were incubated at 30°C with aeration until cultures attained log phase and turned turbid (360 hrs).

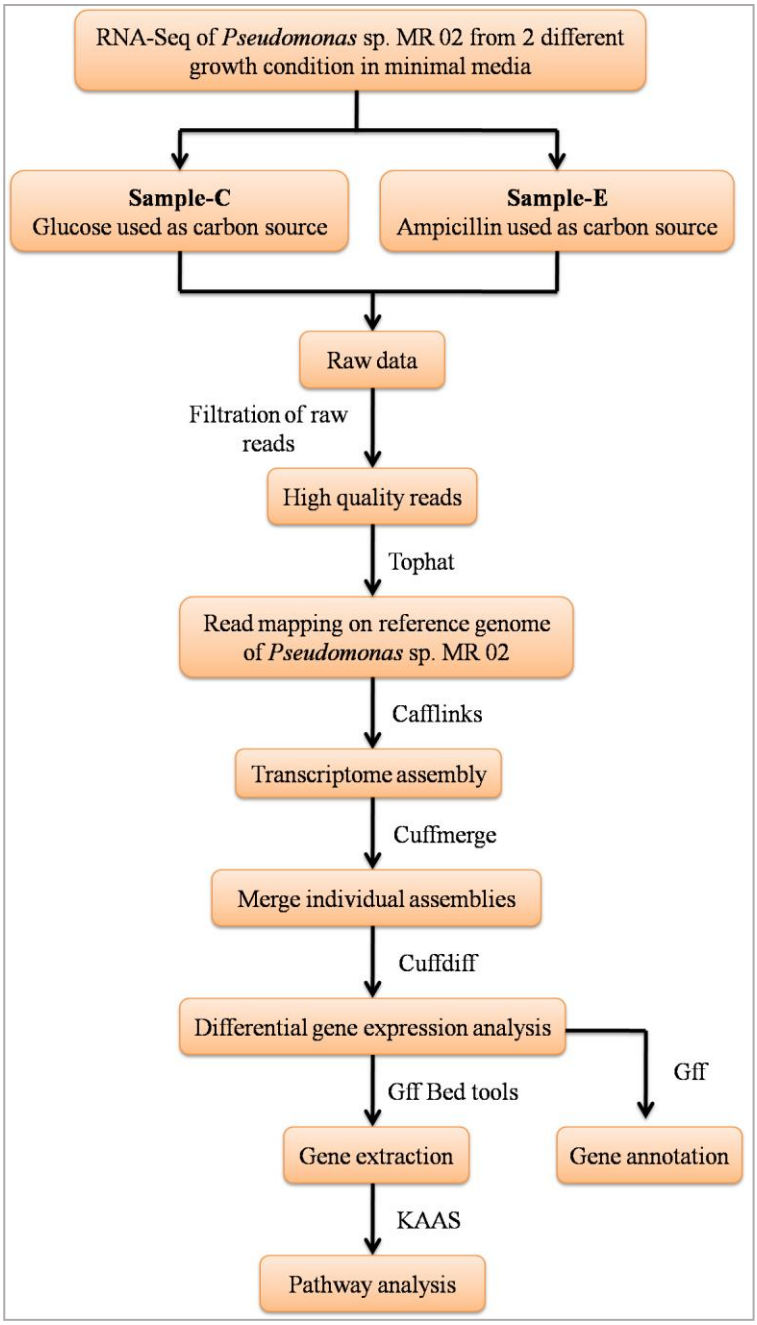


Figure 5.3: Overview of the RNA-Seq analysis steps of *Pseudomonas* sp. MR 02

5.2.4.2 Total RNA isolation, qualitative and quantitative analysis

Total RNA was isolated from both the samples (control 'C' and experiment 'E') using Quick-RNA Miniprep Plus total RNA isolation kit (Zymo Research) by following the standard protocol as described by the manufacturer. Total RNA was dissolved in nuclease-free water and the qualities/purity of RNA was checked by measuring Optical

Density Absorption ratio ($A_{260/280}$) using nanodrop and quantities of the isolated RNA were checked using 1% RNA agarose gel and visualization under U.V. light.

5.2.4.3 cDNA library preparation and sequencing

The paired-end cDNA sequencing libraries were prepared using total RNA per sample using a TrueSeq RNA sample preparation V2 kit (Illumina, San Diego California, USA) as per manufacturer's protocol. Then the cDNA fragments were purified with AMPure XP system and to select a fragment of 150-200bp in length for sequencing. The library quality was checked on Agilent 4200 bioanalyzer using High sensitivity DNA chip. For next-generation sequencing of both samples was performed using paired-end 2×150 bp library of the Next Seq 500 platform.

5.2.4.4 Preprocessing RNA-Seq data and alignment to the reference genome

The raw data of both samples were filtered using Trimmonatic v0.38 and high quality reads were obtained by removing the adapter and low-quality sequences from the raw data. The sequence reads were aligned to reference genome of *Pseudomonas* MR 02 using Top-hat V2.1.1 with default parameters (Trapnell *et. al*., 2009).

The reference genome of *Pseudomonas* sp. MR 02, with a genome size of ~5.9 Mb and the associated annotations were downloaded from NCBI Genome DB (https://www.ncbi.nlm.nih.gov/genome/13508?genome_assembly_id=354794).

5.2.4.5 Differential Gene Expression (DGEs) analysis

Assembled all transcriptome data from RNA-Seq data with help of cufflinks V2.2.1 program and quantifies their expression. The individual gtf files of the assembled transcriptomes were used for differential gene expression analysis using cuffdiff program. There are a total of 5,436 coding genes present in the annotation file of *Pseudomonas* sp. MR 02. Differential gene expression analysis was performed using cuffdiff v2.2.1 to obtain significantly differently expressed genes between control and experiment samples (Trapnell *et. al*., 2013). The analysis was carried out for commonly expressed genes reported between control and experiment samples respectively. FPKM values were used to calculate the log fold change as log2 (FPKM-experimental/ FPKM-control). Log2 fold change values greater than zero were considered up-regulated whereas less than zero were down-relegated along with *P*-value (≥0.05) for statistically significant results.

An average linkage hierarchical cluster analysis was performed on top 50 differentially expressed genes, of the differential genes, expressed combination control vs. experiment, using multiple experiments viewer (MeV v4.9.0) (Eheanor *et. al*., 2011). The heatmap shows the level of gene abundance. Levels of expression are represented as the log2 ratio of gene abundance between control and experiment samples. Differentially expressed genes were analyzed by hierarchical clustering. Heatmaps were created using the log-transformed and normalized value of genes based on Pearson uncentered distance and average linkage method.

5.2.4.6 Pathway analysis of coding DNA sequences by KEGG

The functional annotations of genes were carried out against the curated KEGG GENES database using KAAS ver. 1.6 (KEGG Automatic Annotation Server-. (http://www.genome.jp/kegg/ko.html). The KEGG Orthology database of Prokaryotes family was used as the reference for pathway mapping. The result contains KO (KEGG Orthology) assignments and automatically generated KEGG pathways using KAAS BBH (bidirectional best hit) method against the available database with default parameters (Moriya *et. al*., 2007).

5.2.5 Growth studies of MBL-positive strains to demonstrate utilization of ampicillin as sole source of carbon and energy

To examine the growth of four MBL-positive isolates of the *Proteus*, *Escherichia*, *Pseudomonas*, and *Acinetobacter* genera termed KR01, KR23, KR25 and MR40 (one isolate from each genus) in mineral medium supplemented with ampicillin as a sole source of carbon and energy, the inoculums and minimal media were prepared as following method described above section 5.2.1. Each culture was washed two times in distilled water lacking a carbon source by cell pellet centrifugation (3000 rpm, 5 min). After the final wash, the 10^6 cell suspension was resuspended in 100 ml Erlenmeyer flasks containing 20 ml minimal media with different supplementations. The basal minimal medium, used in this study, contained (g/L): KH2PO4, 3.0; Na2HPO4, 6.0; NaCl, 5.0; and MgSO4, 0.1(pH 7.0 ± 0.2). Ampicillin was used as the carbon source and the concentrations of ampicillin used were 0.5 g/L, 1g/L, 2g/L, and 4g/L. Flasks were incubated at 30ºC for 216 hrs in a shaking incubator at 120 rpm. All growth experiments

included triplicate independent cultures, and OD600 values were evaluated as averages with standard error. Growth data were plotted using Microsoft Excel.

5.3 Results and Discussion

5.3.1 Growth of MR 02 in minimal medium supplemented with ampicillin as a sole source of carbon, nitrogen, and energy in the presence or absence of EDTA

The strain *Pseudomonas* sp. MR 02 isolated from Mahananda River water, before growth study in minimal media with ampicillin as a carbon and nitrogen source and was found to be extensively antibiotic-resistant and it has been established as a pandrug resistance bacterial strain in previous chapter 4. We cultured strain MR 02 with aeration in minimal media containing 2 g/L, 4 g/L and 6 g/L ampicillin as the sole carbon and nitrogen source at 30°C and measured culture density over the 216 hrs (Figure 5.4). The strain *Pseudomonas* sp. MR 02 grew robustly in minimal media supplemented with 6 g/L ampicillin as the sole source of carbon (Figure 5.4 b). MR 02 growth was also observed when we used ampicillin used as both carbon and nitrogen source (Figure 5.4a). The strain MR 02 grow in a specific concentration (nearly half of the MIC value), they do not grow in a dose-dependent manner in both conditions (ampicillin used as an only carbon source or both carbon or nitrogen source), and the growth was observed only in 6 g/L ampicillin. MR 02 cells failed to survive when we added 0.4 mM EDTA (metal chelating agents) in minimal media and the same concentration of ampicillin used as nitrogen (Figure 5.4c) and carbon source (Figure 5.4d). It was found that in minimal medium containing glucose (8.0 g/L) and NH4Cl (2.0 g/L), growth was almost unaffected in the presence of 0.4 mM EDTA (Figure 5.5).

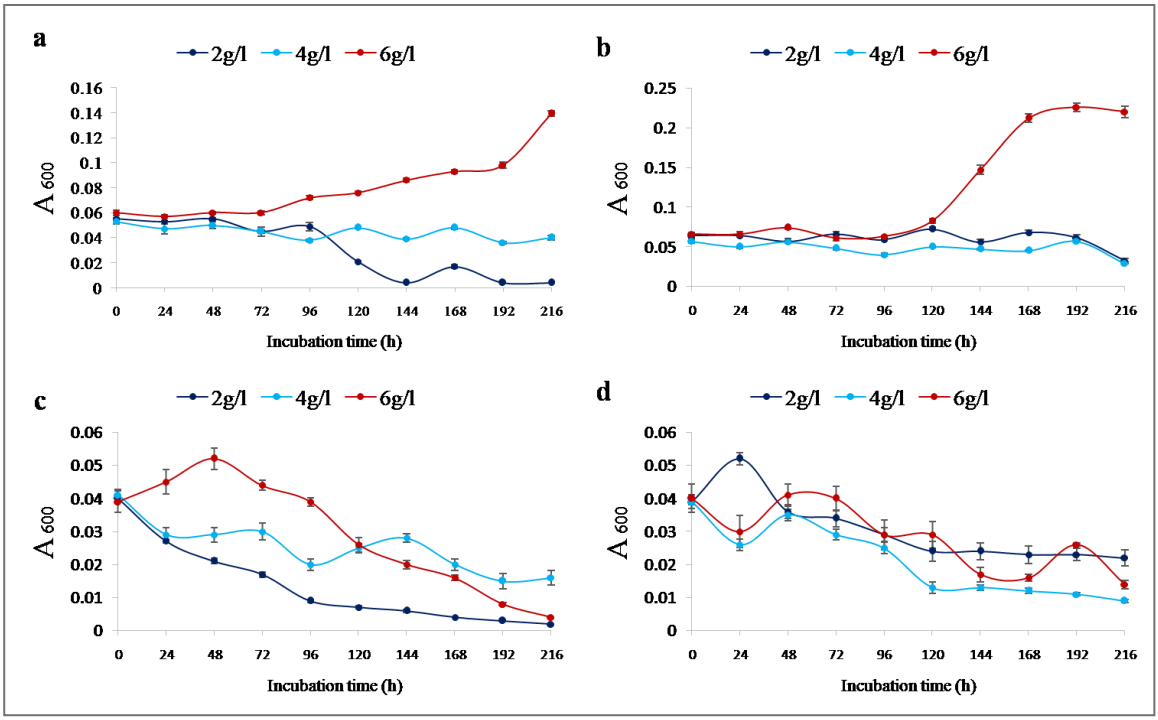


Figure 5.4(a-d): Consumption of ampicillin as a growth substrate by *Pseudomonas* sp. MR 02

Pseudomonas sp. MR 02 used β-lactam as a sole carbon and nitrogen source for growth (a) Growth of MR 02 in the presences of ampicillin as a both sole carbon and nitrogen source (b) Growth of MR 02 in the presences of ampicillin as a only carbon source (c) Growth of MR 02 in the presences of ampicillin as a both sole carbon and nitrogen source with Metallo-β-lactamase Sub-inhibitory concentrations of EDTA (0.4 mM) (d) Growth of MR 02 in the presences of ampicillin as a only carbon source with Metallo-β-lactamase Sub-inhibitory concentrations of EDTA (0.4 mM).

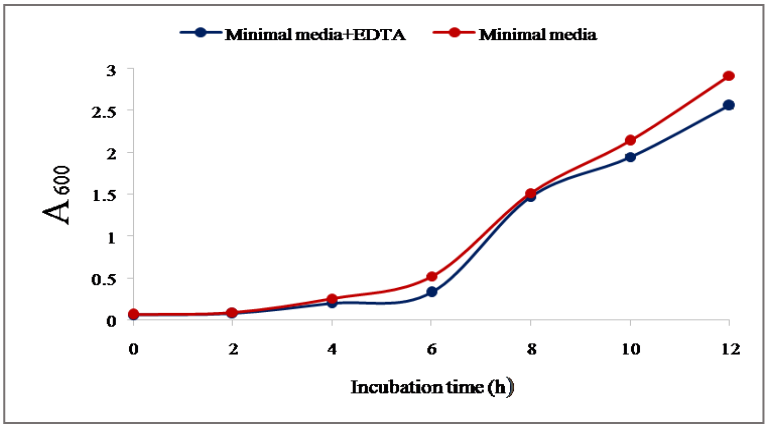


Figure 5.5: Growth of *Pseudomonas* sp. MR 02 in minimal media in the presences or absence of EDTA (0.4 mM EDTA) as a control

5.3.2 Identification of β-lactam catabolism responsible gene

We have an annotated draft genome of MR 02 to explicate the enzymes and pathways utilized during β-lactam catabolism. We annotated three chromosomal encoded β-lactamase genes belonging to class A extended-spectrum β-lactamase *bla*$_{PME-1}$, subclass B1 Metallo-β-lactamase *bla*$_{NDM-1}$, and class C β-lactamase AMPC, genes belonging to structural genes for amidase operon and also genes for the complete phenylacetic acid catabolic pathway. A figure has been constructed with the annotated genes and operons for catabolism of β-lactam antibiotic, ampicillin (Figure 5.6).

Hence, we hypothesize that *Pseudomonas* sp. MR 02 catabolizes ampicillin in following steps: (i) Cleavage of the β-lactam ring and remove an amino group of ampicillin by NDM-1 (ii) amidase enzyme cleaves amide bond of inactivated ampicillin and releasing phenylacetic acid and ammonia molecules (NH_4^+) (iii) phenylacetic acid is routed to central metabolism as a carbon source by the phenylacetic acid catabolic pathway and (iv) two ammonia molecules (NH_4^+) which is routed to central metabolism use as a nitrogen source for amino acid synthesis and other reduced nitrogen-carbon compounds synthesis (Figure 5.7).

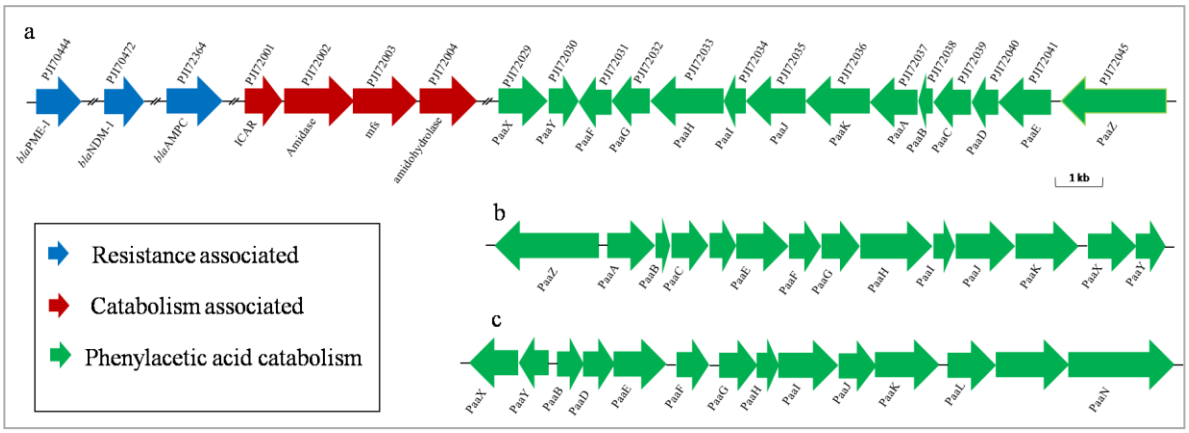


Figure 5.6: Annotations of genes responsible for ampicillin catabolism

(a) Genes and ORF responsible for β-lactam catabolism in *Pseudomonas* sp. MR 02; blue colour indicating genes coding for β-lactamases; red colour indicating genes for amidase operon; and green colour indicating genes for phenylacetate degradation; (b) Complete ORF of phenylacetate degradation in *E. coli* K12; (c) Complete ORF of phenylacetate degradation in *Pseudomonas* sp. PE-S1G-1.

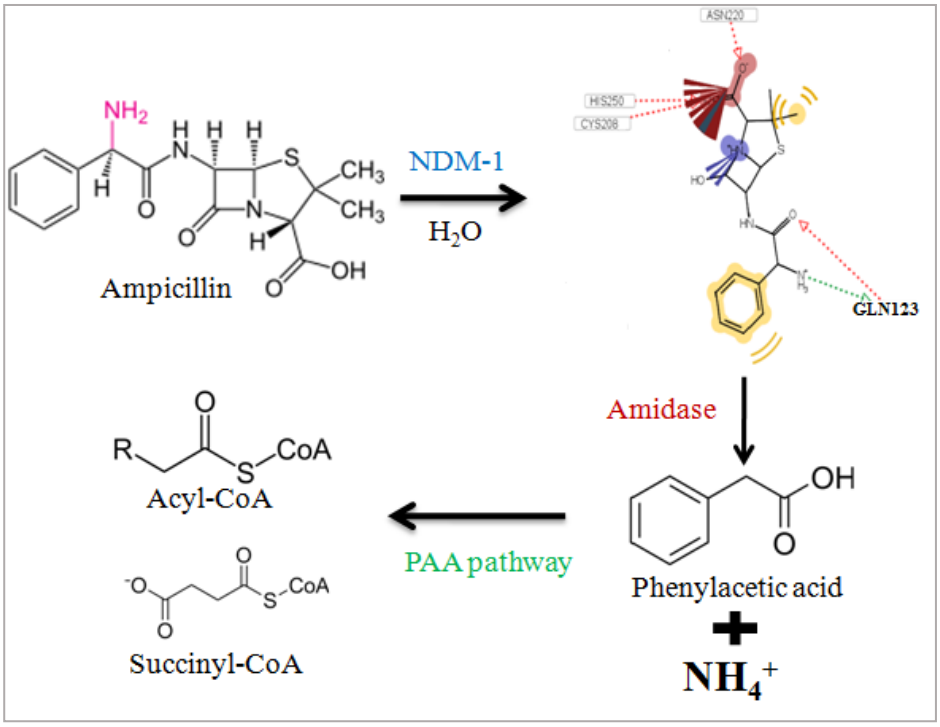


Figure 5.7: Hypothesized pathway for ampicillin catabolism

(Docking image was adopted from Wang *et. al*., 2015)

5.3.3 *E.coli* expressing MR 02 *bla*$_{NDM-1}$ catabolizes ampicillin

Based on our growth studies of MR 02 in minimal medium supplemented with ampicillin as carbon and nitrogen source in presence and absence of EDTA (0.4 mM), we hypothesized an additional attribute of NDM-1 to support the growth of any bacterium to utilize ampicillin as carbon and energy source, provided it has dedicated amidase(s) and the pathway to catabolise phenyl acetic acid (PAA). To determine the necessity of NDM-1 in ampicillin catabolism, the recombinant *E. coli* DH5α cells were used because it having a similar metabolic pathway. Hence the *bla*$_{NDM-1}$ gene of *Pseudomonas* sp. MR 02 was cloned and expressed in *E. coli* DH5α cell. Before the growth study first, we determine the MIC value of recombinant *E. coli* DH5α + NDM-1 strain (Table 5.1). Recombinant *E. coli* DH5α cells showed ampicillin resistance more than or equivalent 256 µg/ml. The recombinant *E. coli* DH5α+ NDM-1 strain was able to grow in ampicillin (100 mg/L) as sole carbon and energy source (Figure 5.6). There by supporting the hypothesis to prove

the associated role of *bla*$_{NDM-1}$ in ampicillin catabolism. Two different recombinant strains E. coli DH5α_pHSG398 and E. coli DH5α_pGEM-T were used as controls (Figure 5.6).

Table 5.1: Antimicrobial susceptibility pattern of *E.coli* DH5α:pHSG398 and *E.coli* DH5α:pHSG398+NDM-1

	MIC (µg/ml)					
	Ampicillin	**Penicillin**	**Imipenem**	**Meropenem**	**Cefepime**	**Ceftazidime**
DH5α+ pHSG398	6	6	0.45	0.9	0.02	0.09
DH5α+ pHSG398/ *bla*$_{NDM-1}$	≥256	≥256	4	16	32	32

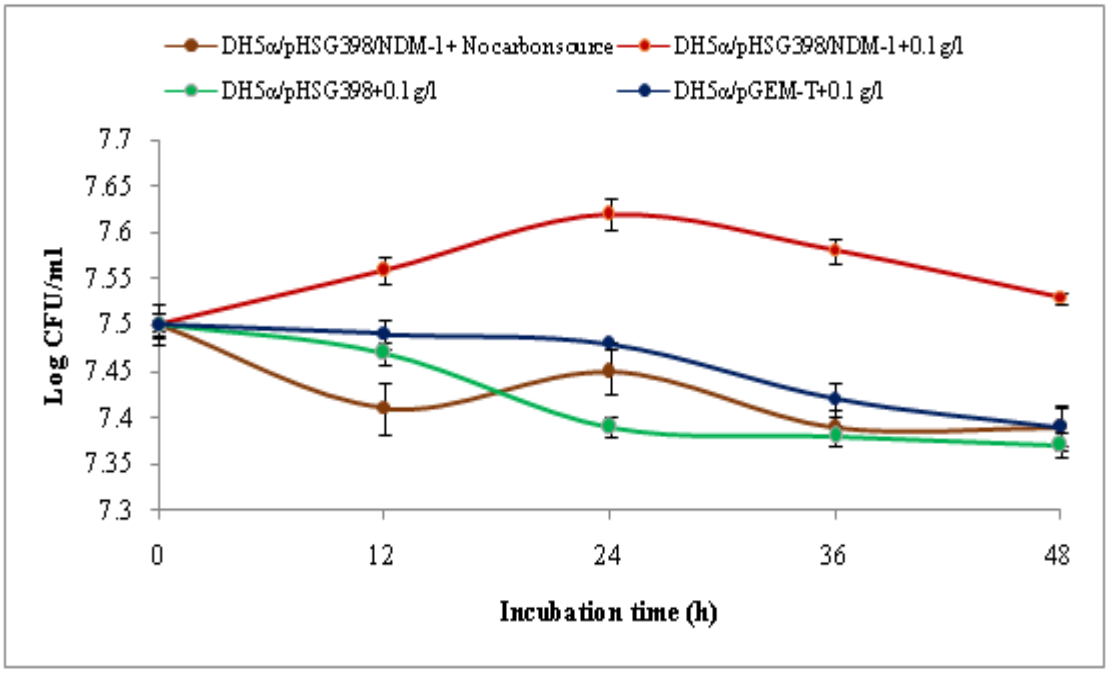


Figure 5.8: Growth of recombinant strain, *E. coli* DH5α: pHSG398+NDM-1 in minimal media with ampicillin as sole carbon

Recombinant strain DH5α/pHSG398 and DH5α/pGEM-T used as negative control and positive control respectively. Data points are average of three experiments with standard error bars. OD_{600} Optical density at 600 nm.

5.3.4 Whole transcriptome sequencing of *Pseudomonas* sp. MR 02

5.3.4.1 Qualitative and quantitative analysis of RNA samples

Qualitative analysis of the total RNA of both control and experiment cells of *Pseudomonas* sp. MR 02 was resolved on 1% agarose gel at 80V for approximately 60 min till sample reached 3/4th of the gel (Appendix A5.1) and 1 µl of RNA of both sample was loaded in nanodrop for quantitative analysis. The quality control (QC) passed samples were used to prepare cDNA library.

5.3.4.2 RNA sequencing, transcriptome assembly and aligning to the reference genome

Two cDNA libraries of *Pseudomonas* sp. MR 02 cultured in minimal media with glucose as carbon source and minimal media with ampicillin as carbon source were sequenced on Illumina Next Seq 500 platform and name as *Pseudomonas* sp. MR 02 control 'C' and *Pseudomonas* sp. MR 02 experiment 'E' respectively. There were a total of 9,211,961 and 8,035,277 raw reads were generated comprising of 2,752,874,653 and 2,400,906,916 nucleotide bases in control and experiment libraries, respectively (Table 5.2). After the quality filtration (mean quality score < 20 Phred score) and adaptor trimming using Trimmomatic v0.38, the high-quality reads were used for reference-based read mapping. The raw reads were submitted to the NCBI database under BioProject number PRJNA612657 with BioSample numbers SAMN14379771 and SAMN14379772. As the draft genome sequence of *Pseudomonas* sp. MR 02 strain has been published, the sequence reads were then aligned to the database for further analysis of gene expression profile. More than 94% of the total reads of both samples could be aligned to the *Pseudomonas* sp. MR 02 draft genome, of which approximately 99% could be identified read mapped and used for gene expression analysis. In taxonomy analysis 63% and 37% reads were aligned, control sample read and experiment sample read with MR 02 draft genome respectively. After genome mapping 3721 and 3710 genes were detected to be expressed in control and experiment respectively with *Pseudomonas* sp. MR 02 respectively. Among these genes, 3707 were commonly expressed between both samples.

5.3.4.3 Differential genes expression analysis

Differential gene expression profile between carbon source as glucose and carbon source as ampicillin transcripts was developed using cuffdiff v2.2.1 to identify genes with differential expression level in the control compared to experiment, initially we used the FPKM method to calculate the expression level of the CDS. Differentially expressed genes were analyzed by hierarchical clustering. A heat map was constructed using the log transformed and normalized value of genes based on person uncentered distance as well as based on average linkage method.

Table 5.2: Differential genes expression summary

Description	Gene counts				
	Upregulated	**Downregulated**	**Exclusive only in control sample**	**Exclusive only in experiment sample**	**Expressed in both**
***Pseudomonas* sp. MR 02, Control vs. Experiment**	93	45	14	3	3707

Based on the common hit accession of functionally annotated CDS in control and expressed strain CDS, a total of 3306 CDS expressing in both sample transcripts of which a total 45 (1.21%) CDS were down-regulated in control strain transcript compare to experiment strain transcript whereas 93 (2.50%) CDS were up-regulated in control compared to experiment with the log 2 fold change value of greater than zero (Table 5.2).

Heatmap was constructed on the average linkage hierarchical cluster analysis, shows the levels of transcript abundance of the top 50 differentially expressed transcripts recognized in control and experiment samples. The heat map represents that out of 50 genes, nearly 50% of the genes were shown to be upregulated and the rest 50% nearly to be down regulated. Among these 50 genes, metallo-β-lactamase NDM-1 gene is present (Figure 5.8). The red and green color in the heat map represent (logarithmic intensity of the expression genes) upregulated and downregulated genes respectively, among the top 50 differentially expressed genes in both control and experiment samples.

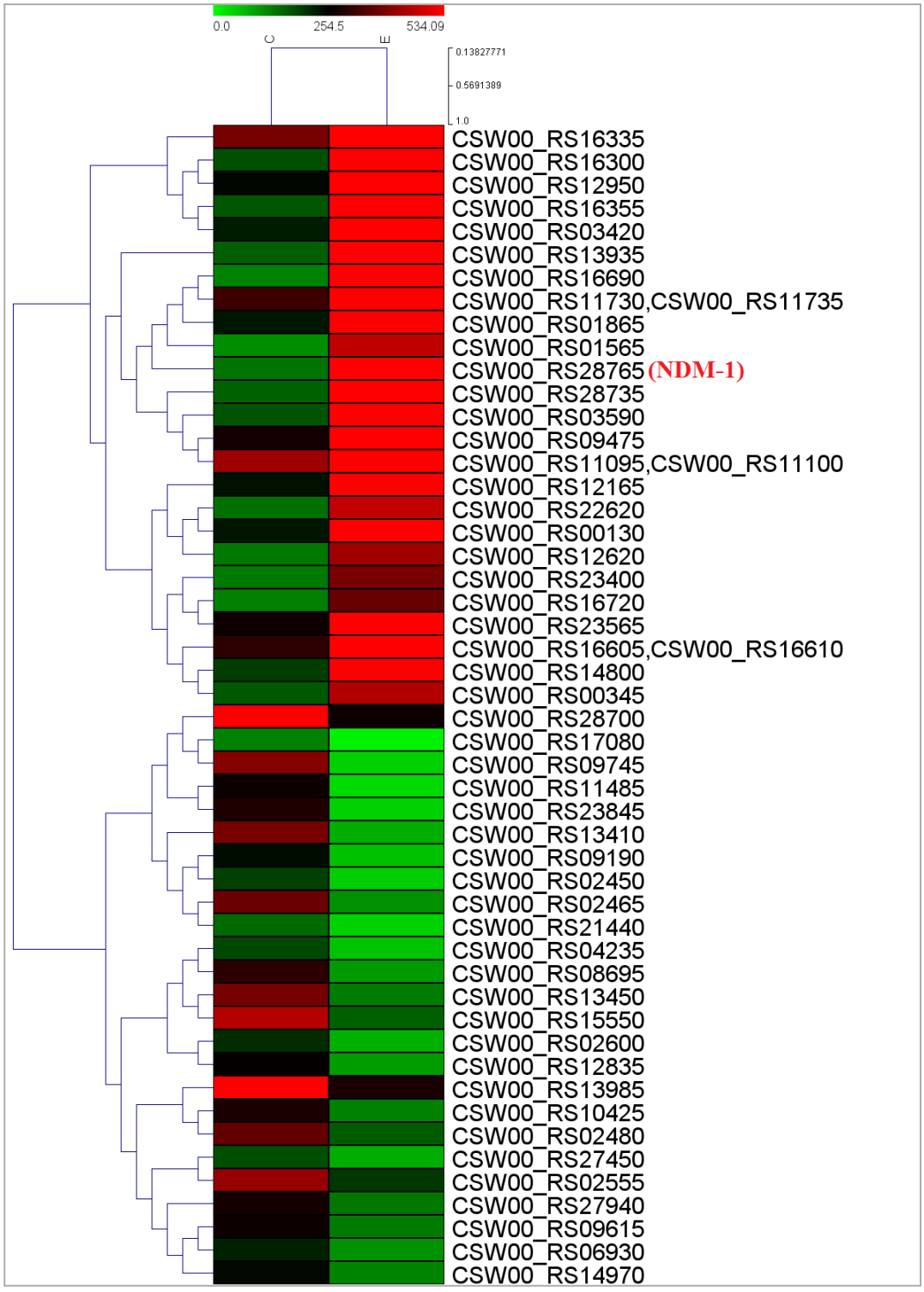


Figure 5.9: Heatmap of top 50 differentially expressed genes of *Pseudomonas* sp. MR 02 grown in glucose and ampicillin as carbon source containing minimal media

5.3.4.4 KEGG pathway analysis of DEGs

Gene ontology enrichment analysis was performed with the 93 CDS up-regulated in control compared to experiment (Appendix A5.1). This study was focused on ampicillin catabolism, how bacteria use ampicillin as carbon source and this is also shown in transcriptome study. The genes identified were found to be classified into 21 functional pathway categories in KEGG. Most of the annotated genes were found to associate with carbohydrate metabolism (138) energy metabolism, lipid metabolism, signal transduction and environmental adaptation (Table 5.3).

Table 5.3: KEGG pathway classification

Pathways	**Gene counts in C vs. E**
Metabolism	
Carbohydrate metabolism	136
Energy metabolism	90
Lipid metabolism	57
Nucleotide metabolism	42
Amino acid metabolism	181
Metabolism of other amino acids	57
Glycan biosynthesis and metabolism	27
Metabolism of cofactors and vitamins	85
Metabolism of terpenoids and polyketides	22
Biosynthesis of other secondary metabolites	28
Xenobiotics biodegradation and metabolism	49
Genetic Information and Processing	
Translation	20
Folding, sorting and degradation	15
Replication and repair	22
Environmental Information and Processing	

Membrane transport	136
Signal transduction	159
Cellular Process	
Transport and catabolism	9
Cell growth and death	17
Cellular community- prokaryotes	92
Cell motility	37
Organism System	
Environmental adaptation	11

5.3.4.5 Study of functional genes in ampicillin catabolism

According to our hypothesis the ampicillin catabolism required three different components (i) metallo-β-lactamase *bla*$_{NDM}$ genes (ii) amidase genes and (iii) PAA pathway genes. Based on the comparative RNA-Seq it was demonstrated that the expression of ampicillin catabolism responsible genes coding for NDM-1, amidase and PAA pathway, there has been a change in the expression of metabolic enzymes to presence of growth requirements in minimal media containing ampicillin as sole carbon source. We identified that of the 138 differentially expressed genes, 93 and 45 genes were significantly upregulated (Table A5.1) and downregulated (Table A5.2) respectively (Figure 5.8), in MR 02 cells when grown in minimal medium containing ampicillin as sole carbon and energy source compared to the cells grown in minimal medium containing glucose as sole carbon and energy source. The expression of B1 metallo-β-lactamase NDM-1 gene was significantly higher (upregulated; $\log_2$ fold change of 2.00088) than two other β-lactamases, class A extended-spectrum β-lactamase PME-1 and PDC (β-lactamase class C) respectively, in ampicillin-grown cells compared to glucose-grown cells. Two genes, coding for penicillin G amidase and penicillin acylase family protein respectively; and three genes coding for MBL fold metallo-hydrolases; and all paa genes of the phenylacetic acid catabolism were expressed in both conditions. On the basis of the comparative transcriptomics, it was revealed that there has been alteration of the expression of metabolic genes to meet growth requirements in minimal medium containing ampicillin as sole carbon and energy source.

5.3.5 Growth studies of MBL-positive strain in minimal-medium supplemented with ampicillin as sole source of carbon

Four MBL positive strains one from each genus, *Proteus* sp. KR01, *Escherichia* sp. KR23, *Pseudomonas mendocina*. KR25 and *Acinetobacter* sp. MR40, were selected to test viability and growth in minimal media supplemented with ampicillin as a sole source of carbon. Among these four strains, *Escherichia* sp. KR23 carry $bla_{\text{NDM-7}}$ genes, *Pseudomonas mendocina* KR25 carry $bla_{\text{VIM-2}}$ and other two strains carry $bla_{\text{NDM-1}}$ genes. The concentrations of ampicillin used in this study were 1.0 g/L, 2.0 g/L, and 4.0 g/L to support the growth of KR01, KR23 and KR25 and 0.5 g/L, 1.0 g/L, and 2.0 g/L ampicillin for MR40 (Figure 5.10). The cells of *Proteus* sp. KR01 has demonstrated the viability till 72 h followed by marginal cryptic growth (similar to growth that happens during stationary phase) but no visible growth showing persistent log phase (similar to a typical growth curve) took place (Figure 5.10a). In case of *Pseudomonas medocina* KR25 has shown the viability till 196 h followed by marginal cryptic growth at only 1 g/L ampicillin concentration but no growth was observed in any concentration (Fig. 5.10c). Of the different concentrations of ampicillin used as sole carbon and energy source to test growth, *Escherichia* sp. KR23, and *Acinetobacter* sp. MR40 demonstrated maximum growth in 4.0, and 1.0 g/L ampicillin respectively (Figure 5.10 b & d). Each strain grew in only one ampicillin concentrations (nearly half of the MIC value). These results confirm the validity of our hypothesis NDM-1 or NDM-7 role in ampicillin catabolism.

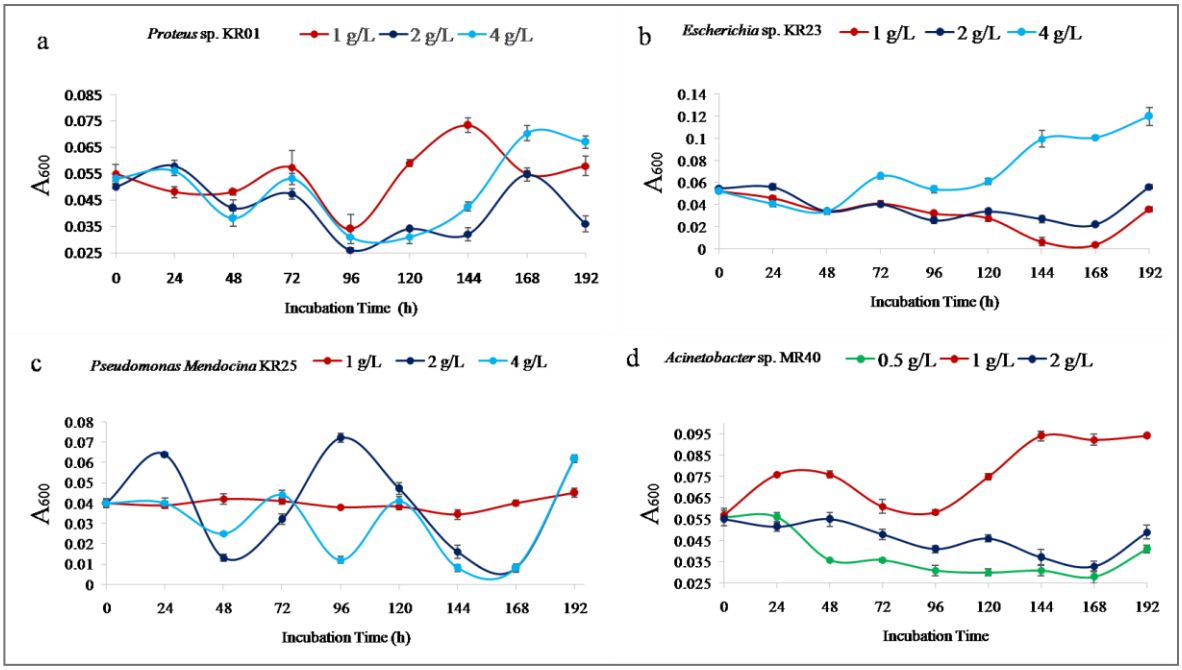


Figure 5.10: MBL positive strains catabolise ampicillin and use as sole carbon source a-d,

(a) growth of strain *Proteus* sp. KR01, (b) growth of strain *Escherichia* sp. KR23, (c) *Pseudomonas medocina* KR25, (d) *Acinetobacter* sp. MR40, in minimal media with ampicillin as sole carbon source. Data points are average of three experiments with standard error bars. OD_{600} Optical density at 600 nm.

5.4 Conclusion

Antibiotics resistance enzymes are known to be plentiful in soil habitats, and it is only because of their medical exploitation that as privileged molecules not bound by the carbon cycle. Gram-negative NDM-1 producers of the genera, *Acinetobacter*, *Escherichia*, and *Pseudomonas*, demonstrating significantly high MIC values against β- lactam antibiotics, endowed with the capacity of consuming ampicillin as a carbon-source has larger implications in cleaning antibiotic-stressed habitats.

General Discussion

Carbapenems are a broad range of β-lactam antibiotics currently considered to be the drug of 'last resort' for treating infections with multi-drug resistant bacteria. In the past two decades, the development and spread of strains resistant to carbapenem have been a growing concern (Meletis, 2016). Imipenem and meropenem are common carbapenem antimicrobial agents used to treat nosocomial and mixed bacterial infections. Resistance to these antimicrobial agents limits therapeutic options. Resistance to carbapenems occur either through bacterial production of metallo-β-lactamase enzymes that hydrolyze the carbapenems, and break down other β-lactam antibiotics as well (Queenan and Bush, 2007) or through porin channels or efflux pumps in the bacterial cell wall that reduce the permeability of the drug into the organism or efflux the drugs from organism (Fernandez and Hancock, 2012). In some bacteria like *Pseudomonas aeruginosa*, all these three mechanisms are present (Fernandez and Hancock, 2012). The most dangerous bacteria are known to produce metallo-β-lactamase enzymes and can pass it from organism to organism via plasmid or integron and a reservoir is required for horizontal gene transfer (Lutgring and Limbago, 2016; Meletis, 2016; Diene and Rolain, 2014). Human and animal gut, natural environment, including soil and water are the potential reservoir of antibiotic resistance genes. Antibiotic resistance is often focused around health sectors and the hospital environment has been shown to harbor and distribute antibiotic resistance including metallo- β-lactamase (Bush *et. al*., 2011). Metallo-β-lactamase producing bacterial strains is mainly reported in a hospital setting and these pathogenic bacterial strains release to environment from hospital sewage via WWTPs and waterways (Ray *et. al*., 2016; Zenati *et. al*., 2016).

The primary aim of this research was to investigate the presence and abundance of MBL producing bacteria in two city-waste polluted rivers. Since the resistance level of bacteria is settled on by the enzyme efficiency for hydrolyzing carbapenems and by the number of resistance mechanisms present in the bacteria, and by other probabilities through production of more than one hydrolyzing enzyme and may show modifications in more than one porin, producing high-level resistance to the carbapenemase as per German DIN 58940 standard (1998). The concentration of imipenem prescribed in media for isolation of imipenem-resistant bacteria is 8 μg/ml, we used 20 μg/ml imipenem in MHA

plates, in this study, to determine the density of imipenem-resistant bacteria in river water sample. We have also considered breakpoint criteria (S/R) changes for imipenem when testing *Enterobacteriaceae* while selecting 20 µg/ml (as in CLSI and EUCAST guidelines imipenem resistant is > 8 µg/ml) imipenem concentration to select isolates bearing MBLs (https://clsi.org/; http://www.eucast.org/clinicalbreakpoints/). Moreover, the level of resistance is determined by the efficiency of the enzyme for hydrolyzing the drug and by the number of resistance mechanisms present in the organism. Organisms can produce more than one hydrolyzing enzyme and may show modifications in more than one porin, producing high –level resistance to the carbapenems (MIC ≥ 16 µg/ml); while organisms with decreased susceptibility produced by pouring changes alone often have lower MICs (2 -8 µg/ml). Therefore, if we had used 8 µg/ml imipenem, we might have ended with an overwhelming high number of intrinsically imipenem resistant isolates. Earlier authors have also reported about the low prevalence of carbapenem-resistant bacteria in river water; intrinsic resistance being pre-dominant (Farzana *et.al*. 2013).

The water quality data of the two rivers, Mahananda and Karala, indicated that both of them are moderately polluted (unpolluted rivers typically have a BOD below 1 mg/L) (Appendix D1). The BOD was measured in the range of 1.6 – 2.1 mg /L in water samples collected from the rivers in the March and April (Pre-monsoon months), November and December (post monsoon months) of the year 2015. The dissolved oxygen (DO) is an important parameter to monitor the biological quality of water as it supports the aquatic life forms and regulates the biological degradation of organic impurities. In the pre-monsoon months, DO in the month of April, for both the rivers, was found to be lower than March data; while in the post-monsoon months, DO recorded in the month of November was lower than December (Table. D1). A slight decrease in DO at sampling location could be attributed to the addition of sewage upstream. The amounts of chloride in a water sample can give an indication of the amount of sewage effluent in river water. The chloride content of the Mahananda and Karala River water in the month of April was 1.9 and 2.7 times respectively, the content recorded in the March (Table. D1). Again, in the post-monsoon months, the chloride content in the November of both Mahananda and Karala was higher than the value obtained in the December. The main source of chlorides in the

waters is the discharge of domestic sewage; therefore, its concentration serves as an indicator of pollution by sewage.

The physiochemical data corroborated with the microbiological data on imipenem-resistant bacteria present in the water samples collected in March and April (pre-monsoon months) and, November and December (post-monsoon months). Data confirms maximum load of imipenem-resistant bacteria in the mid and downstream of both rivers in the April and November. Among water samples collected in December, from all the three sampling stations of the two rivers, imipenem-resistant bacteria were comparatively more in the downstream of Karala. The upstream of Karala River is situated in the less-populated rural region while it veins and bisects the town in two halves; the midstream is at the heart of the town very near to the District hospital, but in post-monsoon months maximum stagnation occurs in its downstream; this may explain the abundance of imipenem-resistant bacteria in the downstream water samples. Of the total number of imipenem-resistant bacteria retrieved from Mahananda River, maximum were derived from the midstream water sample followed by the downstream; least numbers were obtained from upstream water samples. This could be because all municipal waste channels carrying community and hospital waste-water are finally discharged in the mid-stream. The upstream and downstream of Mahananda drains predominantly less populated rural setting and also fast-flowing (due to persistent gradient) and less stagnating in pre-and post-monsoon months compared to Karala. Huge quantities of antibiotics are discharged into municipal waste because of the partial metabolism of humans or due to removal of idle antibiotics ensuing in the detection of antibiotic-resistant bacteria and ARGs (Zhang *et. al*., 2009, Selvaraj *et. al*., 2013, Rutgersson *et. al*., 2014, Chakraborty *et. al*, 2013). Hospital wastewaters contain fecal material from a large number of individuals, of which many are undergoing antibiotic therapy. It is, thus, conceivable that city-waste polluted river waters could provide opportunities to find novel carbapenemases and other resistance genes not yet described in clinical strains.

The most common metallo-β-lactamase detected in the both rivers isolates were bla_{NDM} (93.4%). The percentage occurrence of MBL-positive isolates among imipenem-resistant bacteria isolated from Mahananda and Karala River was 5.26% and 4.68% respectively. The gene, bla_{NDM}, was detected in 17.14% and 20.51 % of MBL-positive

strains isolated from Mahananda and Karala River respectively. Large number of imipenem-resistant bacteria, isolated from waters of the Mahananda and Karala river, which were not MBL-positive ones, could be those who have recruited other mechanisms like over expression of efflux pumps leading to carbapenem resistance associated with multi-drug resistance (Meletis *et. al*., 2012), or are able to prevent carbapenems reaching their PBPs by diminishing the permeability of their outer membrane by diminished expression or loss of specific porins, or mutation derived changes of their PBPs as in gram positive bacteria (Munita & Arias, 2016).

Bacterial resistance to antibiotic molecule, cover almost all clinically used antibiotic classes, and is leading the world towards a return to the condition of the pre antibiotic era (Fair and Tor, 2014). Antibiotic susceptibility testing and MIC results of all 15 MBL producing strains against 22 antibiotics (Table 1. 7 and Table 1.8) and 13 antibiotics (Table 1.9 and Table 1.10) respectively, showing that all MBL producing strain are multidrug-resistance and in this study we have established one MBL producing strain MR 02 as PDR (Pan-drug resistance) strain because both phenotypically and genotypically, it has shown resistance against all available clinically used antibiotics. In the current study, the MIC value vary between same bacterial species, Karala River MBL producing isolates KR07, KR22 and KR23 are belonging to same bacterial species but they are showing different MIC value against different antibiotics, similarly this type phenomenon happen with Mahananda River MBL producing isolates MR40 and MR41, both strain also belonging same bacterial species but MIC values are different. In this study we also characterised integron gene cassettes in MBL positive isolates, and found that the same species but different bacterial strain that was isolated from the same sampling site carried different antibiotics resistance gene cassettes. This result indicates that polluted river water is the hotspot of resistance evolution.

While identifying metallo-β-lactamase activity in this current study (Fig 1.10 and Fig 1.11) it was notated that isolates MR 02, MR15, MR40, MR41, KR01, KR04, KR07, KR22 and KR23 produced more than one class of β-lactamase. Same finding was also observed in some recent study and it was found that two different classes of β-lactamase gene MBL and CTX-M type are present in same *Klebsiella pneumoniae* strain (isolate from hospital in Italy) (Nucleo *et. al*., 2013). The production of different type's β-

lactamase in same bacterial strain makes it more resistant to antibiotic classes (Extensively drug-resistant or Pan-drug), and producing offspring of resistant bacteria with much more detrimental effect upon morbidity and mortality of human beings (Nucleo *et. al*., 2013).

The second observation is the detection of high frequencies of multidrug resistant pattern among the river water MBL producing isolates which may be attributable to intrinsic resistance. It has been claimed that water and soil bacteria are the most important source of antibiotic resistance (WHO 2020 https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance).

16S rRNA gene being universal, 16S rRNA sequences are widely used to ascertain the genus and species of the isolates (Srinivasan *et. al*., 2015). After the isolation and screening of MBL producing isolates from both the river water samples, all of these isolates were identified by 16S rRNA gene sequencing and variation in bla_{NDM} genes were detected by PCR amplification and sequencing of the complete ORF region. Thus, the amplification and sequencing results of bla_{NDM} genes among the both rivers isolates showed similarities with $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-7}}$ genes within GenBank. In this study, a large portion (07 out of 35, and 08 out of 39 from Mahananda and Karala respectively) of MBL-positive isolates, possessed NDM; and only one of the Karala isolates contained VIM-2 (Table 2.2). Fourteen bla_{NDM} possesing multidrug-resistant isolates belonged to five genera, *Pseudomonas*, *Myroides*, *Acinetobacter*, *Proteus*, and *Escherichia*; the only one isolate bearing $bla_{\text{VIM-2}}$ belonged to the genus *Pseudomonas* (Table 2.1). Three Karola river isolates, KR07, KR22, and KR23, bearing $bla_{\text{NDM-7}}$ belong to a novel species of *Escherichia* having closest phylogenetic relationship with *Escherichia albertii*. KR07 and KR22 contained one and two class 1 integrons respectively. Very recently, bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ were detected in *E. albertii*, an emerging member of the *Enterobacteriaceae* causing human and animal enteric infections (Li *et. al*., 2018). The first novel NDM-7 was also identified in 2013 in *E. coli* bacterial strain and it isolated from urine culture in France (Cuzon *et. al*., 2013).

NDM has been detected in the river water in Brazil (South Africa); in drinking water in Canada (North America) and in the Danube River water samples in Europe, (Sanchez *et. al*., 2018; Fernando *et. al*., 2016; Kittinger *et. al*., 2016). This indicates the importance of the river water and the water environment as it contains numerous bacteria,

some of which are metallo-β-lactamase and these bacteria in river water or drinking water can originate from hospital waste, aquaculture and WWTPs (Li *et. al*., 2014).

Overwhelming abundance (40%) of the *bla*$_{NDM}$ gene was found in the both river MBL-positive isolates belonging to the genus *Acinetobacter*. Two Mahananda river NDM-1 positive isolates MR26 and MR29 belonging to one novel species; and KR26 to another novel species of *Acinetobacter* may be ascertained from the phylogenetic tree; one Karala River isolates, KR29, were found close to *A. seohaensis*; and the remaining two isolates of the genus *Acinetobacter*, MR40 and MR41, were close to *A. variabilis*. The isolates, MR40 and MR41, contained two class 1 integrons (one containing two gene cassettes, *aad*B-*aac*(6')-Ib; the other contains *dfr*A5 in MR40; in MR41, *bla*$_{PSE-1}$ present in one class 1 integron and *dfr*A5 in the other one). The knowledge of the occurrence and pattern of antimicrobial susceptibility pattern of *Acinetobacter* spp. is significant as they have come out as a cause of ICUs infection (Rebic *et. al*., 2018). Among the six most important nosocomial MDR pathogens, *A. baumannii* has been reported to cause 2-10% of all hospital-borne infections caused by Gram negative bacteria (Antunes *et. al*., 2014). The first report from India about NDM-1 in *Acinetobacter* was in the year 2012 (Karthikeyan *et. al*., 2012). Earlier authors have indicated a higher propensity of isolation of NDM-1 producing *A. baumannii* from sewages of the hospitals, but could not recover any NDM-1 producing isolates from a river or fish pond water (Zhang *et. al*., 2013). We have estimated about the possession of *bla*$_{NDM-1}$ in diverse species of the genus *Acinetobacter* in waters of the Mahananda and Karala.

On examining the water quality of both the rivers, it cannot be concluded that bacterial communities within it easily grown in nutrient-rich environment, moderately they are subjected close to oligotrophic condition. All the multidrug-resistant MBL-positive isolates were facultatively oligotrophic in nature. Facultative oligotrophs are less reactive to abruptive resource availability; instead they switch on to exploit nutrient poor conditions.

The increase in gram-negative carbapenem antibiotic resistance is worrisome particularly as there are few, if any clinical trial antimicrobial agents possessing suitable activity against *Pseudomonas* spp., *Acinetobacter* spp. and *Escherichia* spp. and then these bacterial species increase own resistance by acquiring the metallo-β-lactamase (MBL)

gene, which can potentially confer broad-spectrum β-lactam resistance including carbapenem. In several studies, MBL genes are often associated with aminoglycoside resistance genes and thus bacteria that possess resistance against carbapenem antibiotics as well as aminoglycoside antibiotics, both types of genes can be found as gene cassettes and it carried by mobile genetic element like integron (Walsh, 2005). Integron consist of three regions and among these one are called variable regions (fig 3.1) and within the variable region they can carry more than one gene. Gene encoding MBL, IMP and VIM are found as gene cassettes in class 1 integrons (Poirel *et. al*., 2001; Senda *et. al*., 1996; Yan *et. al*., 2001). One among the three multi-drug resistant MBL-positive *Pseudomonas* spp., KR25, was found to bear two class 1 integrons which included five gene cassettes (*bla*$_{\text{VIM-2}}$, *aac*A4, *aad*A1, *aad*A2, and *aac*(6′)-Ib) (Table 3.1 and Figure 3.4) and phylogenetically it nearest to *P. mendocina*. Other two *Pseudomonas* strains, MR15 and MR20, possessing *bla*$_{\text{NDM-1}}$, belong to *P.putida* lineage, closest to *P. monteilii* and *P. plecoglossicida*. In an earlier study from China, a considerable prevalence of class 1 integrons in multidrug resistant clinical *P. aeruginosa* isolates was observed (Chen *et. al*., 2009).

In the recent past, aquatic sediments receiving partially untreated effluents from communities of two different countries, Congo and India of diverse climatic conditions, were investigated for the occurrence of MBL genes in *Pseudomonas* spp.; and revealed that *bla*$_{\text{VIM-1}}$ in congo isolates but to a lesser degree in Indian isolates, but incidence of *bla*$_{\text{NDM-1}}$ was highest among the Indian isolates and few from Switzerland isolates (Devarajan *et. al*., 2017).

Out of a total of 19 class 1 integron borne gene cassettes, the most abundant (09/19) was *aad*A family of genes which encode aminoglycoside-3-adenylyltransferases (AAD) which encodes streptomycin and spectinomycin resistance by adenylation (enzyme modification), and the second most common (04/19) was *aac* (6′) Ib gene which code for aminoglycoside 6′-N-acetyltransferase type I b, an enzyme of clinical importance that catalyzes the transfer of an acetyl group from acetyl CoA to the 6′-amino group of aminoglycoside molecules conferring amikacin and kanamycin resistance and it found in a wide variety of gram-negative pathogens.

The abundance of *aad*A genes in Mahananda and Karala rivers of northern West Bengal, India, could be due to the rampant use of streptomycin in agriculture and they

encode kanamycin, neomycin, amikacin and streptomycin resistance (Bennett, 1999). Paddy and tea are the main cash crops and amongst horticultural crops, citrus fruits, tomato, and potato are widely grown in this region. The formulation of streptomycin sulphate + tetracycline hydrochloride (9:1) (is one of the antimicrobials registered in India as fungicide and bactericide as per insecticide act, 1968; sold as 'Antibac' trade-name;) is sprayed in huge quantities (for example to protect paddy from bacterial leaf blight, 300 g/ha is sprayed) to control blister blight in tea, bacterial leaf blight of paddy, citrus canker in citrus, brown wilt, or ring or bangle disease of potato, and bacterial leaf spot in tomato to name a few (Sarkar *et. al*., 2018). Very recently, the state government of West Bengal had established fair price outlet for medicines where amikacin (250 / 500 mg vial injection) is included as important medicine under mandatory list (document entitled " Establishment of fair price outlet for medicines, consumables and implants at selected government hospitals through public private partnerships (PPP); http://www.wbhealth.gov.in> upload files > PPP > ppp_02) and sold from district and sadar hospitals in large quantities to treat diseases caused by gram-negative pathogens; unprecedented use of amikacin in hospitals and community may have been the reason for the widespread dissemination and enrichment of *aac* (6') Ib gene in integrons.

Two Karala river MBL positive *Proteus* spp., bearing class 1 integron having two gene cassettes, *aac* (6')-I-*aad*A2, and *aad*A2-*aad*A1 respectively and it also to bear $bla_{\text{NDM-1}}$ MBL gene. In a study carried out in the USA, two *P. mirabilis* isolates was found to contain $bla_{\text{IMP-27}}$ gene as the integral part of class 1 integron (Dixon *et. al*., 2016). In a hospital isolate, *P. mirabilis*, was reported to bear $bla_{\text{VIM-1}}$ and *aad*A2 cassettes in class 1 integron (Qin *et. al*., 2015).

The whole genome sequencing in the current study was done to explore the resistance pattern (intrinsic and adaptive antibiotic resistance genes) of Mahananda River MBL positive isolates *Pseudomonas* sp. MR 02 at molecular level. The resulting whole genome was to allow comparison between MR 02 genome and to other previously sequenced *Pseudomonas* species and also to help in the comparative analysis between phenotype and genotype characters (Figure 4.10).

The choice of *Pseudomonas* sp. MR 02 isolates for whole genome sequencing was based on antibiotic susceptibility pattern. It showed resistance against all antibiotics

categories which is defined for pan-drug resistance (PDR) strain by CLSI and EUCAST (antibiotic list for *Pseudomonas aeruginosa* described by Magiorakos *et. al*., 2012). In this study MR 02 isolates are genotypically and phenotypically characterised a PDR strain.

Two different bioinformatic tools, CARD and BLAST were applied in the antibiotic resistance gene analysis from whole genome sequence of *Pseudomonas* sp. MR 02 isolates. In draft genome of MR 02, 98 genes were identified as antibiotics resistance related genes and among this a total 64 genes have been predicted as antibiotic resistance efflux pumps. They cover all the five known efflux pump families of bacteria namely MFS, MATE, SMR, ABC and RND. Altogether eleven RND efflux pumps have been predicted in MR 02. One of the most relevant efflux pump families in the clinical context is the RND family, which has been characterized best in Gram-negative bacteria (Fernandez and Hancock, 2012). In general, RND efflux pumps consist of three components but in MR 02 single set of four component RND efflux pump TriABC-OpmH and MuxABC-OpmB has been found, same is the case with *P. aeruginosa* (Mima *et. al*., 2007 & 2009). One set of ABC type efflux pump Mac AB has been predicted in MR 02 and they show homology with *E. coli* Mac AB. Mac AB pump has involved in macrolide-specific resistance in *E. coli* (Kobayashi *et. al*., 2001). RND efflux pumps like MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexVW-OprM; MFS efflux pumps like TetG, EmrB, EmrAB and MdtA; MATE efflux pumps like NorM and SMR efflux pumps like emrE have identified from draft genome of MR 02.

Presence of mutated genes like *gyrA* and *parC* along with the abovementioned efflux channels might have played a role in development of resistance to fluoroquinolones in MR 02 (Yoshida *et. al*., 1990; Piddock *et. al*., 1999). 14 OprD porin channel genes were predicted. OprD is the major channel for entry of carbapenems. In *Pseudomonas aeruginosa* the most common mechanism of resistance to the cabapenems is loss or alteration of the outer membrane porin protein OprD (Atsuyuki *et. al*., 2009). Out of 14 porin channels annotated in MR 02 of which only 5 channels exhibited below 50% homology with *Pseudomonas aeruginosa* PA01 porin channels.

The resistance phenotype against the β-lactam antibiotics was corroborated with the genomic presence of three different β-lactamases, PME-1, NDM-1 and AMPC, of which PME-1 and NDM-1 exhibited 100% similarity with the reported sequences and only 52%

similarity with AMPC reported from *Pseudomonas aeruginosa* PA01 (PDC-1) sequence. PME-1 β-lactamase enzyme was first time detected in *Pseudomonas aeruginosa*, which is isolated from clinic sample. It was grouped in ambler class A extended-spectrum β-lactamase (ESBL) and conferred resistance to cephalosporin and aztreonam antibiotics but not to carbapenems (Tian *et. al*., 2011). Presence of NDM-1 in *Pseudomonas aeruginosa* was reported for the first time from Serbia (Jovcic *et. al*., 2011). AmpC enzymes are not carbapenemases, they posses however a low potential for carbapenem hydrolysis. But the combined effect of AmpC enzyme and the efflux pumps lead to carbapenem resistance in *P. aeruginosa* (Livermore, 1995 & 2002).

Active efflux of antibiotics is a major way by which bacteria can become multiple-antibiotic-resistant. Phenylalanine-arginine-β naphthylamide (PAβN) is a well-studied efflux pump inhibitor. The MICs of chloramphenicol and tetracycline were reduced to 2.0 % of their values shown in absence of PAβN, showing that the susceptibility changes were entirely due to efflux inhibition (Figure 4.). Similar significant reduction in MIC values for fluoroquinolone antibiotics, ciprofloxacin and levofloxacin, was observed in presence of PAβN. In *P. aeruginosa*, an extensive range of antibiotics groups (except polymixin B and imipenem) comprising of quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, most penicillins except carbenicillin and subbenicillin, most cephems except cefsulodin and ceftazidime, meropenem, and S-4661, are driven out of the cell by efflux systems (Masuda *et. al*., 2000; Lamers *et. al*., 2013). Besides Mex AB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, there are several additional chromosomally encoded efflux pumps like MexHI-OpmD, MexVW-OprM, the NorM ortholog, and mutations of certain genes (*gyrA*, *gyrB*, *parC*, and *parE*) were identified responsible for development of resistance to quinolone group of antibiotics in *Pseudomonas aeruginosa* (Yoshida *et. al*., 1990; Piddock *et. al*., 1999). To explain the molecular basis of MR 02's efflux-dependent resistance against chloramphenicol, tetracycline, ciprofloxacin, and levofloxacin, we were able to assign 8, 11, 5, and 5 efflux-system genes/gene-clusters annotated from its whole-genome-sequence. In addition to efflux genes, two mutated genes, *gyr*A and *par*C, were also assigned for imparting resistance to ciprofloxacin or levofloxacin.

At the end of this study, relevant role of *bla*$_{NDM-1}$ genes in PDR strain *Pseudomonas* sp. MR 02 was described for ampicillin catabolism. While determining the MIC of different antibiotics, very high MIC values were obtained against the ampicillin (10000 mg/L). This observation led us to re-think this issue from the bacterial perspective, with cleaved β-lactam ring of the antibiotic, the inactivated molecule is nontoxic and can be exploited as carbon and nitrogen sources, allowing the bacteria to grow in elevated concentration of the drug. The role of NDM-1 in ampicillin catabolism was established because the growth of MR 02 in the same ampicillin containing minimal media was inhibited, when we added 0.4 mM EDTA. NDM-1 is known to be more susceptible to EDTA than other metallo-β-lactamase (Li *et. al*., 2013). In this study, 0.4 mM EDTA was used to completely inhibit NDM-1 activity; which in turn did not allow MR 02 to grow on ampicillin. To rule out any untoward effect of 0.4 mM EDTA, uninhibited growth of MR 02 was demonstrated in minimal medium containing glucose and NH_4Cl (Figure A5.1).

Long back, in 1977, a *Pseudomonas fluorescence* strain with β-lactamase activity was reported to have the ability to use benzylpenicillin as a sole source of both carbon and nitrogen (Beckman & Lessie, 1979). Later in 1994, *Phormidium valderianum* BDU30501 strain was shown to utilize ampicillin as nitrogen source via a probable mechanism of splitting of 6-aminopenicillanic acid into cysteine and valine (Bosnic *et. al*., 1994). Taking the cue from a very recent report by Croft *et. al*., 2018 that the non-β-lactam region of pencillin, phenylacetamide is capable to support growth of bacteria via phenyl acetic acid catabolon, we hypothesized on the basis of earlier report ampicillin binds to the two zinc ions and various critical residues present in the active site of NDM-1 through number of coordination bonds and hydrogen bonds that might render the host bacterium an opportunity to utilize the ampicillin as sole source of carbon, nitrogen, and energy. We were successful to demonstrate growth of MR 02 on ampicillin not only as sole carbon source but both sources of carbon and nitrogen. Despite having a 6-aminopenicillanic acid core containing nitrogen in all β-lactam antibiotics, ampicillin possesses a side chain with an additional amino group. From the results, it was revealed that ampicillin is also being used by MR 02 as nitrogen source.

Moreover in support of the hypothesis, we assayed growth of the recombinant *E. coli* DH5α: pHSG398: *bla*$_{NDM-1}$ strain using ampicillin (100 mg/L) as the only carbon

source, we found that expression of bla_{NDM-1} was sufficient to confer a significant role in ampicillin catabolism. These results confirmed the validity of our conjecture as well as the role of NDM-1 in ampicillin catabolism.

The comparative transcriptome analysis was performed between minimal media with glucose as carbon source and minimal media with ampicillin as carbon source grown MR 02 cells. After RNA sequencing and data analysis, 3707 DEGs were identified between two samples. It was revealed that there has been alteration of the expression of metabolic genes to meet growth requirements in mineral medium containing ampicillin as sole carbon and energy source. MR 02 cells might have employed gluconeogenesis pathway to synthesize glucose-6-phosphate to make various cell macromolecules (as glucose was not available from the cell surroundings). The expression of *pck*, *gap*N, *pgm*, *adh*C, *gpm*I, and *tpi*A were higher along with significant upregulation of *ald*B (coding for aldehyde dehydrogenase) and *exa*A (coding for PQQ dependent dehydrogenase) genes of the gluconeogenesis pathway (Table A5.1). Besides gluconeogenesis pathway genes, three genes of the TCA cycle coding for NADP-dependent isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase E1 component (*suc*A), succinate dehydrogenase flavoprotein subunit (*sdk*A, *frd*A); and one gene coding for D-glycerate dehydrogenase (phosphor-gluconate 2-dehydrogenas) of pentose phosphate pathway were found to be significantly upregulated, suggestive of regulation of genes related to growth on ampicillin as sole carbon and energy source. On the other hand, two genes coding for NADPH dehydrogenase (quinone) of ubiquinone and terpenoid-quinone biosynthesis pathway was significantly downregulated (Table A5.2). MR 02 cells grown in both conditions (glucose or ampicillin) have expressed genes for PQQ-dependent dehydrogenases to use a PQQ-dependent ethanol oxidation system with exception of significant upregulation of two exaA genes (coding for PQQ-dependent dehydrogenase, methanol/ethanol family), and one exaC gene (coding for aldehyde dehydrogenase) in ampicillin-grown cells. Interestingly, significant differences in expression of genes coding for proteins essential for the biosynthesis of the cofactor PQQ, organised in an operon *pqq*ABCDE and *pqq*F (expressed singly) were not observed in ampicillin or glucose-grown cells. Four genes coding for different aminotransferase enzymes (02 for cysteine and methionine metabolism; 01 each for thiamine and propanoate metabolism respectively), one gene

coding for FAD-binding oxidoreductase (of the arginine and D-ornithine metabolism), two genes coding for asparaginase (of the alanine, aspartate and glutamate metabolism), one gene coding for acyloxyacyl hydrolase (of the lipopolysaccharide biosynthesis), and *hmg*L gene for hydroxymethylglutaryl-CoA lyase (for butanoate/ valine, leucine and isoleucine degradation/geraniol degradation pathway) were significantly upregulated; while two genes coding for dihydrofolate reductase (for folate biosynthesis) and 2-hydroxy-3-oxopropionate reductase (for glyoxylate and dicarboxylate metabolism) respectively were significantly downregulated in ampicillin-grown cells (Table A5.2). Two genes coding for peptidases were found to be significantly downregulated (M23 family metallopeptidase, LD-carboxypeptidase, and aminopeptidase) and upregulated (ATP –dependent Lon protease and S24 family peptidase) respectively in ampicillin grown cells.

Overall it was observed that, for ampicillin catabolism three components are required and according to my hypothesis, when any bacterial species carries all these three components they are able to grow in ampicillin by consuming ampicillin as carbon and nitrogen source. Again to validate this hypothesis, we selected four different MBL producing strains; *bla*$_{\text{NDM-1}}$ positive *Proteus* sp. KR01, *bla*$_{\text{NDM-7}}$ positive *Escherichia* sp. KR23, *bla*$_{\text{VIM-2}}$ positive *Pseudomonas mendocina* KR25 and *bla*$_{\text{NDM-1}}$ *Acinetobacter* sp. MR40 for ampicillin catabolism, which are isolated from both rivers. Among these four strains only two strains KR23 and MR40 were able to grow in ampicillin containing minimal media. When KR01 and KR25 bacterial strains were grown in minimal media with ampicillin as carbon source no significant growth was observed and this result also validated our hypothesis. On the basis of whole genome analysis of *Proteus mirabilis* phylogenetically nearest species of KR01, and we found that they did not have any PAA pathway related genes but in *Acinetobacter variabilis* nearest species of MR40 have other two component amidase and PAA genes which is required for ampicillin catabolism.

Summary

While a few reports give proof of the event of NDM-1-positive bacteria in tertiary healthcare centers of West Bengal, India, a finding of apparently more noteworthy for the general well-being has been given in the present study with water tests from two rivers bisecting two crowded towns, Siliguri and Jalpaiguri separately. No significant variation was detected in imipenem resistant bacteria counts between both river water samples. All the 15 MBL positive strains were isolated from the mid steam water sample. The most abundant and most common MBL in both river water samples were NDM (94%). The investigation has demonstrated that the issue of NDM-1 was not kept to clinical pathogenic strains, limited within the hospital environment but also has stretched its residence in the environmental bacteria. The spread of NDM has been perplexing the study of disease transmission, including the spread of an assortment of types of NDM-positive bacteria and the interstrain exchanges between species and between genera via the transmission of various mobile genetic platforms like plasmids and integrons containing bla_{NDM}, with the last instrument having assumed a progressively noticeable impact to date. Nearly 50% (8/15) MBL positive strains are carrying aminoglycosides resistance gene cassettes. The NDM gene was not evolved only for β-lactam antibiotic inactivation rather it also catabolized ampicillin and it provides carbon and nitrogen from ampicillin molecules to bacterial cell for growth and other energy sources. The spread of NDM shows that antimicrobial resistance is a general medical issue that rises above national fringes and will require worldwide collaboration between experts on the off chance that it is to be controlled.

Bibliography

Achari A, Somers D, Champness JN *et. al*. Crystal structure of the anti-bacterial sulfonamide drug target dihydropteroate synthase. *Nature structural biology*. 1997; 4(6):490-497.

Akinyemi K, Iwalokun B, Foli F, Oshodi K, Coker A. Prevalence of multiple drug resistance and screening of enterotoxin *stn* gene in *Salmonella enterica* serovars from water sources in Lagos, Nigeria. *Public Health*. 2011; 125(2):65-71.

Al-Agamy MH, Shibl AM, Tawfi AF, Radwan HH. High prevalence of metallo-β-lactamase-producing *Pseudomonas aeruginosa* from Saudi Arabia. *Journal of Chemotherapy*. 2009; 21:461-462.

Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics*. 2011; 12:402.

Almuzara M, Radice M, de Garate N, *et. al*. VIM-2-producing *Pseudomonas putida*, Buenos Aires. *Emerging Infectious Diseases*. 2010; 13(4):668-669.

Ambler R, Coulson A, Frere JM, Ghuysen JM, *et. al*. A standard numbering scheme for the class A β-lactamases. *Biochemical Journal*. 1991; 276(1):269-270.

Ambler RP. The structure of β-lactamases. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 1980; 289(1036):321-331.

Aminov RA. Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Frontiers in Microbiology*. 2010; 1(134).

Ankenbrand MJ, Keller A. bcg Tree: automatized phylogenetic tree building from bacterial core genomes. *Genome*. 2016; 59(10):783-791.

Antunes, L.C., P. Visca, and K.J. Towner. *Acinetobacter baumannii*: evolution of a global pathogen. *Pathogens and Disease*. 2014; 71:292-301.

Arakawa Y, Murakami M, Suzuki K, *et. al*. A novel integron-like element carrying the metallo-β-lactamase gene bla_{IMP}. *Antimicrobial Agents and Chemotherapy*. 1995; 39(7):1612-1615.

Arakawa Y, Murakami M, Suzuki K, Ito H, Wacharotayankun R, *et. al*. Classification of OprD sequence and correlation with antimicrobial activity of carbapenem agents in *Pseudomonas aeruginosa* clinical isolates collected in Japan. *Microbiology and immunology*. 2009; 53:361-367.

Auch AF, von Jan M, Klenk HP, Goker M. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Standards in Genomic Sciences*. 2010; 2(1):117-134.

Azim A, Dwivedi M, Rao PB. *et. al*. Epidemiology of bacterial colonization at intensive care unit admission with emphasis on extended-spectrum β-lactamase- and metallo-β-lactamase-producing Gram-negative bacteria an Indian experience. *Journal of Medical Microbiology.* 2010; 59:955-960.

Bankevich A. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*. 2012; 19:455-477.

Baruah B. Too precious to drink-an evaluation of the quality of drinking water sources in Delhi. *Water Aid*. 2009: 1-63.

Bassett EJ, Keith MS, Armelagos GJ, Martin DL, Villanueva AR. Tetracycline-labeled human bone from ancient Sudanese Nubia (A.D.350). *Science*. 1980; 209:1532-1534.

Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic Susceptibility Testing by a Standardized Single Disk Method. *American Journal of Clinical Pathology.*1966; 45:493-496.

Bebrone C. Metallo-β-lactamases (classification, activity, genetic organization, structure, zinc coordination) and their superfamily. *Biochemical Pharmacology*. 2007; 74(12):1686-1701.

Beckman W and Lessie TG. Response of *Pseudomonas cepacia* to β-Lactam antibiotics: utilization of penicillin G as the carbon source. *Journal of bacteriology*. 1979; 140(3):1126-1128.

Bellais S, Girlich D, Karim A, Nordmann P. EBR-1, a novel Ambler subclass B1 β-lactamase from *Empedobacter brevis*. *Antimicrobial Agents and Chemotherapy*. 2002; 46(10):3223-3227.

Bennett RE. Using new technology to improve assessment. *Educational Measurement: Issues and Practice*. 1999; 18(3):5-12.

Berendonk T, Manaia C, Merlin C. *et. al.* Tackling antibiotic resistance: the environmental framework. *Nature Reviews Microbiology*. 2015; 13:310-317.

Bharadwaj R, Joshi S, Dohe V *et. al*., Prevalence of New Delhi metallo-β-lactamase (NDM-1)-positive bacteria in a tertiary care centre in Pune, India. *International Journal* of *Antimicrobial Agents.* 2012; 39:265–266.

Boc A, Diallo AB, Makarenkov V. T-REX: a web server for inferring, validating and visualizing phylogenetic trees and networks. *Nucleic Acids Research.* 2012; 40:W573-W79.

Bosnic P, Gelineo A, Gajic B. Biogenez Biogenesis of 6-aminopenicillanic acid (6-APA) and penicillin in Penicillium chrysogenum: effect of the biocatalyst chrysin. *Antibiot Khimioter*. 1994; 39(9-10):6-11.

Bouki C, Venieri D, Diamadopoulos E. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. *Ecotoxicology and Environmental Safety*. 2013; 91:1–9.

Buehler B, Hogrefe HH, Scott G, *et. al*. Rapid quantification of DNA libraries for next generation sequencing. *Methods*. 2010.

Bush K, and Jacoby GA. Updated Functional Classification of β-Lactamases. *Antimicrobial Agents and Chemotherapy*, 2010; 54(3):969-976.

Bush K, Courvalin P, Dantas G, *et. al*. Tackling antibiotic resistance. *Nature Reviews Microbiology*. 2011; 9:894-896.

Bushnell G, Mitrani-Gold F, Mundy LM. Emergence of New Delhi metallo-β-lactamase type 1-producing *Enterobacteriaceae* and non-*Enterobacteriaceae*: global case detection and bacterial surveillance. *International Journal of Infectious Diseases*. 2013; 17(5):325-333.

Cairncross S. Sanitation in the developing world: current status and future solutions. *International Journal of Environmental Research and Public Health*.2003; 13 (suppl 1): S123-31.

Carfi A, Pares S, Duee E, *et. al*. The 3-Dstructure of a zinc metallo-β-lactamase from *Bacillus cereus* reveals a new type of protein fold. *EMBO* Journal. 1995; 14(20):4914-4921.

Castanheira M, Bell JM, Turnidge JD, *et. al*. Carbapenem resistance among *Pseudomonas aeruginosa* strains from India: evidence for nationwide endemicity of multiple metallo-β-lactamase clones (VIM-2, 5, 6, and 11 and the newly characterized VIM-18). *Antimicrobial Agents and Chemotherapy*. 2009; 53:1225-1227.

Castanheira M, Deshpande LM, Mathai D, *et. al*. Early dissemination of NDM-1 and OXA-181-producing *Enterobacteriaceae* in Indian hospitals: Report from the SENTRY antimicrobial surveillance program, 2006-2007. *Antimicrobial Agents and Chemotherapy*. 2011; 55:1274-1278.

Castanheira M, Toleman MA, Jones RN, *et. al*. Molecular characterization of a β-lactamase gene, $bla_{\text{GIM-1}}$, encoding a new subclass of metallo-β-lactamase. *Antimicrobial Agents and Chemotherapy*. 2004; 48: 4654–4661.

CDC (Centers for Disease Control and Prevention) Vital signs: carbapenem resistant *Enterobacteriaceae*. *Morbidity and Mortality Weekly Report.* 2015; 62:165–70 http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6209a3.htm

Chae SR, Yaffee AQ, Weng MK, *et. al*. Notes from the Field: Investigation of Carbapenemase-Producing Carbapenem-Resistant *Enterobacteriaceae* Among Patients at a Community Hospital-Kentucky, 2016. *MMWR Morbidity Mortality Weekly Report*. 2018; 66:51-52.

Chakraborty R, Kumar A, Bhowal SS, *et. al*. Diverse gene cassettes in class 1 integrons of facultative oligotrophic bacteria of River Mahananda,West Bengal, India. *PLoS One*. 2013; 8(8):e71753.

Chen J, Su Z, Liu Y, Wang S, Dai X. Identification and characterization of class 1 integrons among *Pseudomonas aeruginosa* isolates from patients in Zhenjiang, China. *The International Journal of Infectious Diseases*. 2009; 13:717-721.

Chen LR, Zhou HW, Cai JC, Zhang R, Chen GX. Detection of plasmid-mediated IMP-1 metallo-β-lactamase and quinolone resistance determinants in an ertapenem-resistant *Enterobacter cloacae* isolate. *Journal of Zhejiang University Science B*. 2009; 10:348-354.

Cheng X, Wang P, Wang Y, *et. al*. Identification and distribution of the clinical isolates of imipenem-resistant *Pseudomonas aeruginosa* carrying metallo-β-lactamase and/or class 1 integron genes. *J Huazhong Univ Sci Technolog Med Sci*. 2008; 28:235-238.

Chiou J, Leung TYC, Chen S. Molecular mechanisms of substrate recognition and specificity of New Delhi metallo-β- lactamase. *Antimicrobial Agents and Chemotherapy*. 2014; 58: 5372-5378.

Chu YW, Aal-Shah M, Houang ET, *et. al*. IMP-4, a novel metallo-β-lactamase from nosocomial *Acinetobacter* spp. collected in Hong Kong between 1994 and 1998. *Antimicrobial Agents and Chemotherapy*. 45: 710-714.

Chuanchuen R, Narasaki CT, Schweizer HP. The MexJK efflux pump of *Pseudomonas aeruginosa* requires OprM for antibiotic efflux but not for efflux of triclosan. *Journal of. Bacteriology*. 2002; 184:5036-5044.

Chun J, Oren A, Ventosa A, *et. al*. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *International Journal of Systematic and Evolutionary Microbiology.* 2018; 68:461-466.

Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new paradigm for antimicrobial therapy. *Nature Chemical Biology*. 2007; 3:541-548.

Codjoe FS, Donkor ES. 2017. Carbapenem Resistance: A Review. *Medical Sciences.* 2017; 6(1):1.

Concha NO, Rasmussen BA, Bush K, Herzberg O. Crystal structure of the wide-spectrum binuclear zinc β-lactamase from *Bacteroides fragilis*. *Structure*. 1996; 4:823-836.

Cornaglia G, Akova M, Amicosante G, *et. al*. Metallo-β-lactamases as emerging resistance determinants in Gram-negative pathogens: open issues. *International Journal* of *Antimicrobial Agents*. 2007; 29:380-388.

Cornaglia G, Riccio ML, Mazzariol A, *et. al*. Appearance of IMP-1 metallo-β-lactamase in Europe. *Lancet*. 1999; 353:899-900.

Costello A, Periyannan G, Yang KW. *et. al*. Site-selective binding of Zn(II) to metallo-β-lactamase L1 from *Stenotrophomonas maltophilia*. *Journal of Biological Inorganic Chemistry*. 2006; 11(3):351-358.

Crawford PA, Sharma N, Chandrasekar S. *et. al*. Over-expression, purification, and characterization of metallo-β-lactamase ImiS from *Aeromonas veronii* sobria. *Protein Expression and Purification*. 2004; 36(2):272-279.

Crofts TS. Wang B, Spivak A, *et. al*. Shared strategies for β-lactam catabolism in the soil microbiome. *Nature Chemical Biology.* 2018; 14:556–564.

Crowder MW, Spencer J, Vila AJ. Metallo-β-lactamases: novel weaponry for antibiotic resistance in bacteria. *Accounts of Chemical Research*. 2006; 39(10):721-728.

Cuzon G, Bonnin RA, Nordmann P. First Identification of Novel NDM Carbapenemase, NDM-7, in *Escherichia coli* in France. *PLoS One.* 2013; 8(4):e61322.

Da Silva GJ, Correia M, Vital C, *et. al*. Molecular characterization of bla_{IMP-5}, a new integron-borne metallo-β-lactamase gene from an *Acinetobacter baumannii* nosocomial isolate in Portugal. *FEMS Microbiology Letter*. 2002; 215:33–39.

Daiyasu H, Osaka K, Ishino Y, Toh H. Expansion of the zinc metallo-hydrolase family of the β-lactamase fold. *FEBS Letters*. 2001; 503(1):1-6.

Dalhoff A, Janjic N, Echols R. Redefining penems. *Biochemical Pharmacology*, 2006; 71(7):1085-1095.

Davies J, Davies D. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews*. 2010; 74(3):417–433.

Deshpande P, Rodrigues C, Shetty A, *et. al*. New Delhi metallo-β lactamase (NDM-1) in *Enterobacteriaceae:* treatment options with carbapenems compromised. *Journal* of the *Association* of *Physicians* of *India.* 2010; 58:147-149.

Devarajan N, Kohler T, Sivalingam P, *et. al*. Antibiotic resistant *Pseudomonas* spp. in the aquatic environment: A prevalence study under tropical and temperate climate conditions. *Water Research*. 2017; 115:256-265.

Devarajan N, Laffite A, Graham ND, *et. al*. Accumulation of clinically relevant antibiotic-resistance genes, bacterial load, and metals in freshwater lake sediments in central Europe. *Environmental Science & Technolog*y. 2015; 49(11):6528-6537.

Diene SM, Rolain JM. Carbapenemase genes and genetic platforms in Gram-negative bacilli: *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* species. *Clinical Microbiology &. Infection*. 2014; 20(9):831-838.

Dixon N, Fowler RC, Yoshizumi A, *et. al*. IMP-27, a unique metallo-- lactamase identified in geographically distinct isolates of Proteus mirabilis. *Antimicrobial Agents & Chemotherapy.* 2016; 60:6418-6421.

Docquier JD, Riccio ML, Mugnaioli C, *et. al*. IMP-12, a new plasmid-encoded metallo-β-lactamase from a *Pseudomonas putida* clinical isolate. *Antimicrobial Agents & Chemotherapy.* 2003; 47:1522-1528.

Drawz SM, Bonomo RA. Three decades of β-lactamase inhibitors. *Clinical Microbiology Review*. 2010; 23:160-201.

Ehrlich P, Hata S. *Die Experimentelle Chemotherapie der Spirilosen*. 1910.

Eichenberger EM, Thaden JT. Epidemiology and Mechanisms of Resistance of Extensively Drug Resistant Gram-Negative Bacteria. *Antibiotiotics*. 2019; 8:37.

Eleanor A, Howe, Sinha R, *et. al*. RNA-Seq analysis in MeV, *Bioinformatics*. 2011; 27(22):3209-3210.

Ellington MJ, Kistler J, Livermore DM, Woodford N. Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases NDM-1. *Journal of Antimicrobial Chemotherapy*. 2007; 59:321-322.

Ensink JH, Blumenthal UJ, Brooker S. Wastewater quality and the risk of intestinal nematode infection in sewage farming families in Hyderabad, India. *American Journal of Tropical Medicine and Hygiene*. 2008; 79:561-567.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Ver3.0, 2013. http://www.eucast.org/clinicalbreakpoints/

Fair RJ, Tor Y. Antibiotics and bacterial resistance in the 21st century. *Perspectives* in *Medicinal Chemistry*. 2014; 6:25-64.

Fair RJ, Tor Y. Antibiotics and bacterial resistance in the 21st century. *Perspectives* in *Medicinal Chemistry*. 2014; 6:25-64.

Fallah F, Karimi A, Goudarzi M, *et. al*. Determination of integron frequency by a polymerase chain reaction-restriction fragment length polymorphism method in multidrug-resistant Escherichia coli, which causes urinary tract infections. *Microbial Drug Resistance*. 2012; 18:546-549.

Farzana R, Shamsuzzaman S, Mamun KZ. Isolation and molecular characterization of New Delhi metallo-beta-lactamase-1 producing superbug in Bangladesh. *The Journal of Infection in Developing countries.* 2013; 7:161-168.

Fernandez L, Hancock REW. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clinical Microbiology Reviews*. 2012; 25;661-681.

Fernando DM, Tun HM, Poole J, *et. al*. Detection of antibiotic resistance genes in source and drinking water samples from a first Nations community in Canada. *Applied Environmental Microbiology*. 2016; 82(15):4767-4775.

Fluit AC, Schmitz FJ. Resistance integrons and super-integrons. *Clinical Microbiology Infection*. 2004; 10:272-288.

Furlong MA, Singleton DR, Coleman DC, Whitman WB. Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm Lumbricus rubellus. *Applied Environmental Microbiology*. 2002; 68:1265-1279.

Garau G, Garcia-Saez I, Bebrone C, *et. al*. Update of the standard numbering scheme for class B β-lactamases. *Antimicrobial Agents & Chemotherapy*. 2004; 48:2347-2349.

Garcia-Saez I, Mercuri P S, Papamicael C, *et. al*. Three-dimensional structure of FEZ-1, a monomeric subclass B3 metallo-β-lactamase from Fluoribacter gormanii, in native form and in complex with Dcaptopril. *Journal of Molecular Biology*. 2003; 325(4):651-660.

Ghafourian S, Sadeghifard N, Soheili S, Sekawi Z. Extended spectrum β-lactamases: definition, classification and epidemiology. *Current Issues Molecular Biology*. 2014; 17:11-22.

Ghuysen JM. Serine β-lactamases and penicillin-binding proteins. *Annual Reviews in Microbiology,* 1991; 45(1):37-67.

Gonzalez LJ, Meini MR, Tomatis PE. *et. al*. Metallo-β-lactamases withstand low Zn (II) conditions by tuning metal-ligand interactions. *Nature chemical biology*. 2012; 8(8):698-700.

Gonzalez LJ, Bahr G, Vila AJ. Lipidated lactamases: from bench to bedside. *Future Microbiology.* 2016; 11:1495-97.

Gould K. Antibiotics: from prehistory to the present day. *Journal of Antimicrobial Chemotherapy*, 2016; 71(3): 572-575.

Grebe T, Hakenbeck R. Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of β-lactam antibiotics. *Antimicrobial Agents & Chemotherapy*. 1996; 40(4):829-834.

Harmon DE, Miranda OA, McCarley A, *et. al*. Prevalence and characterization of carbapenem-resistant bacteria in water bodies in the Los Angeles–Southern California area. *Microbiology open*. 2019; 8(4):e00692.

Hawkey PM, Xiong J, Ye H, *et. al*. Occurrence of a new metallo-β-lactamase IMP-4 carried on a conjugative plasmid in *Citrobacter youngae* from the People's Republic of China. *FEMS Microbiology Letter*. 2001; 194:53-57.

Heinz U, Adolph HW. Metallo-β-lactamases: two binding sites for one catalytic metal ion?. *Cellular and Molecular Life Sciences.* 2004; 61(22):2827-2839.

Hu Z, Periyannan G, Bennett B, Crowder MW. Role of the Zn1 and Zn2 sites in metallo-beta-lactamase L1. *Journal of the American Chemical Society*. 2008; 130(43):14207-14216.

Hussain M, Garlin A, Madonna MJ, Lampen JO. Cloning and sequencing of the metallothioprotein β-lactamase II gene of *Bacillus cereus* 569/H in *E.coli*. *Journal* of *Bacteriology*, 1985; 164:223-229.

Hwang SH, Kim YJ, Meropenem-resistant bacteria in hospital effluents in Seoul, Korea. *Environmental Monitoring*. 2018; 190(11).

Iovleva A, Doi Y. Carbapenem-resistant enterobacteriaceae. *Clinics in Laboratory Medicine*. 2017; 37 (2):303-315.

Islam MA, Islam M, Hasan R, *et. al*. Environmental spread of New Delhi metallo-b-lactamase-1-producing multidrug-resistant bacteria in Dhaka, Bangladesh. *Applied Environmental Microbiology*. 2017; 83(15):11.

Isozumi R, Yoshimatsu K, Yamashiro T, *et. al*. $bla_{\text{NDM-1}}$ positive *Klebsiella pneumoniae* from environment, Vietnam. *Emerging Infectious Diseases.* 2012; 18:1383-1385.

Jain S, Panda A, Colson P, Raoult D, Pontarotti P. MimiLook: A Phylogenetic Workflow for Detection of Gene Acquisition in Major Orthologous Groups of Megavirales. *Viruses.* 2017;9(4):72(1-15).

Jamwal P, Mittal AK, Mouchel JM. Efficiency evaluation of sewage treatment plants with different technologies in Delhi (India). *Environmental Monitoring Assessment.* 2009; 153: 293-305.

Jamwal P, Mittal AK. Reuse of treated sewage in Delhi city: microbial evaluation of STPs and reuse options. *Resources, Conservation, and Recycling.* 2010; 54: 211-21.

Jia B, Amogelang R, Raphenya *et. al*. CARD expansion and model-centric curation of the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Research.* 2017; 45:566-573.

Jones CE, Schwerdt J, Bretag TA, *et. al*. GOSLING: a rule-based protein annotator using BLAST and GO. *Bioinformatics*. 2008; 24:2628-2629

Jones DS, Jones JH. Sir Edward Penley Abraham CBE. 10 June 1913-9 May 1999. *Biographical Memoirs of Fellows of the Royal Society*. 2014; 60:5-22.

Jovcic B, Lepsanovic Z, Suljagic V, *et. al*. Emergence of NDM-1 Metallo-β-Lactamase in *Pseudomonas aeruginosa* Clinical Isolates from Serbia. *Antimicrobial Agents & Chemotherapy. 2011; 55:3929-3931.*

Jury KL, Khan SJ, Vancov T, *et. al*. Are sewage treatment plants promoting antibiotic resistance? *Critical* Reviews in *Environmental Science and Technology*. 2011; 41(3):243-270.

Kadry A. Lack of Efflux Mechanism in a Clinical Isolate of *P. aeruginosa* Highly Resistant to Beta-Lactams and Imipenem," *Folia Microbiologica*, 2003; 48(4):529-533.

Karkman A, Do TT, Walsh F, Virta MPJ. Antibiotic-Resistance Genes in Waste Water. *Trends Microbiol*ogy. 2018; 26:220-228.

Karthikeyan K, Thirunarayan MA, Krishnan P. Coexistence of *bla*$_{\text{OXA-23}}$ with *bla*$_{\text{NDM-1}}$ and *arm*A in clinical isolates of *Acinetobacter baumannii* from India. *Journal of Antimicrobial Chemotherapy*. 2012; 65(10):2253-2254.

Karthikeyan K, Toleman M, Giske CG. *et al*. First report of the co-existence of *bla*$_{\text{OXA-48}}$ or *bla*$_{\text{OXA-48}}$ gene with *bla*$_{\text{NDM-1}}$ in *Enterobacteriaceae* from India. *Clinical Microbiol & Infection.* 2010; 2:S187.

Karthikeyan K,Thirunarayan MA, Krishnan P. Coexistence of *bla*$_{\text{OXA23}}$ with *bla*$_{\text{NDM-1}}$ and *arm*A in clinical isolates of *Acinetobacter baumannii* from India. *Antimicrobial Agents & Chemotherapy.* 2010; 65:2253-2254.

Keyes K, Lee MD, Maurer JJ. Antibiotics: mode of action, mechanisms of resistance and transfer. In: Torrance ME, Isaacson RE, eds. Microbial Food Safety in Animal Agriculture Current Topics. Ames, IA, USA: *Iowa State Press*, 2003; 45-56.

Khan AU, Maryam L, Zarrilli R. Structure, genetics and Worldwide spread of New Delhi Metallo-β-lactamase (NDM): a threat to public health. *BMC Microbiology.* 2017; 17:101.

Khosravi AD, Mihani F. Detection of metallo-β-lactamase-producing *Pseudomonas aeruginosa* strains isolated from burn patients in Ahwaz, Iran. *Diagnostic Microbiology and Infectious Disease.* 2008; 60:125-128.

Kittinger C, Lipp M, Folli B, *et. al*. *Enterobacteriaceae* isolated from the river Danube: antibiotic resistances, with a focus on the presence of ESBL and carbapenemases. *PLoS One*. 2016; 11(11).

Kobayashi N, Nishino K, Yamaguchi A. Novel macrolide-specific ABC-type efflux transporter in *Escherichia coli*. *Journal of Bacteriology*. 2001; 183:5639-5644.

Kohler T, Michea-Hamzehpour M, Henze U, *et al*. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Molecular Microbiology*. 1997; 23:345-354.

Konaklieva MI. Molecular targets of β-lactam-cased antimicrobials: beyond the usual suspects. *Antibiotics*, 2014; 3:128-142.

Kumarasamy K, Thirunarayan MA, Krishnan P. Coexistence of *bla*$_{\text{OXA-23}}$ with *bla*$_{\text{NDM-1}}$ and *armA* in clinical isolates of *Acinetobacter baumannii* from India. *Journal of Antimicrobial Chemotherapy*. 2010; 65:2253-2254.

Kumarasamy KK, Toleman MA, Walsh TR. *et. al*. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infectious Disease*. 2010; 10:597-602.

Kummerer K. Significance of antibiotics in the environment. *Journal of Antimicrobial Chemotherapy*. 2003; 52:5-7.

Kurtz S, Phillippy A, Delcher AL, *et. al*. Versatile and open software for comparing large genomes. *Genome Biology*. 2004; 5(2):R12.

Lamers RP, Cavallari JF, Burrows LL. The Efflux Inhibitor Phenylalanine-Arginine β-Naphthylamide (PAβN) Permeabilizes the Outer Membrane of Gram-Negative Bacteria. *PLoS One.* 2013; 8(3):e60666.

Lascols C, Hackel M, Marshal SH, *et. al*. Increasing prevalence and dissemination of NDM-1 metallo-β-lactamase in India: data from the SMART study (2009). *Journal of Antimicrobial Chemotherapy*. 2011; 66:1992-1997.

Lauretti L, Riccio ML, Mazzariol A, *et. al*. Cloning and characterization of *bla*$_{\text{VIM}}$, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. *Antimicrobial Agents and Chemotherapy*. 1999; 43(7):1584-1590.

Lee I, Kim YO, Park SC, Chun J. OrthoANI: An improved algorithm and software for calculating average nucleotide identity. *International Journal of Systematic and Evolutionary Microbiology*. 2016; 66(2):1100-1103.

Levine DP. Vancomycin: a history. *Clinical Infectious Diseases*. 2006; 42:5-12.

Levinson W. Antimicrobial drugs: mechanism of actions. In: Review of Medical Microbiology and Immunology, 10th edition. USA: *McGraw-Hill Companies*. 2008; 114-136.

Li N, Xu Y, Xia Q, *et. al*. Simplified captopril analogues as NDM-1 inhibitors. *Bioorganic & Medicinal Chemistry Letters*. 2014; 24(1):386-389.

Li T, Wang Q, Chen F, *et. al*. Biochemical characteristics of New Delhi metallo-β-lactamase-1 show unexpected difference to other MBLs. *PloS One*. 2013; 8(4), e61914.

Li XZ, Nikaido H, Poole K. Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrobial Agents & Chemotherapy*. 1995; 39:1948-1953.

Li Y, Mima T, Komori Y, *et. al*. A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy*. 2003; 52:572-575.

Livermore DM, Woodford N. The β-lactamase threat in *Enterobacteriaceae, Pseudomonas* and *Acinetobacter. Trends* in *Microbiology*. 2006; 14(9):413-420.

Livermore DM. beta-Lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews.* 1995; 8:557-584.

Livermore DM. Has the era of untreatable infections arrived? *Journal of Antimicrobial Chemotherapy.* 2009; 64:29-36.

Livermore DM. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? *Clinical Infectious Diseases.* 2002; 34:634-640.

Llano-Sotelo B, Azucena EF, Kotra LP, *et. al*. Aminoglycosides modified by resistance enzymes display diminished binding to the bacterial ribosomal aminoacyl-tRNA site. *Chemical Biology*. 2002; 9:455-463.

Lomovskaya O, Warren MS, Lee A, *et. al*. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrobial Agents & Chemotherapy*. 2001; 45:105–16.

Lutgring JD, Limbago BM, The problem of carbapenemase producing carbapenem-resistant *Enterobacteriaceae* detection. *Journal of Clinical Microbiology*. 2016; 54(3):529-534.

Magiorakos AP, Srinivasan A, Carey RB, *et. al*. Multidrug resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology & Infection*. 2012; 18(3): 268-281.

Mantilla CD, Jumat MR, Wang T, *et. al*. Isolation and characterization of NDM-positive Escherichia coli frommunicipal wastewater in Jeddah, Saudi Arabia. *Antimicrobial Agents & Chemotherapy*. 2016; 60(9):5223-5231.

Marchiaro P, Viale AM, Ballerini V, *et. al*. First report of a Tn402-like class 1 integron carrying bla_{VIM-2} in *Pseudomonas putida* from Argentina. *Journal of Infection in Developing Countries*. 2010; 4(6):412-416.

Masuda N, Sakagawa E, Ohya S, *et. al*. Substrate specificities of Mex AB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in Peudomonas aeruginosa. *Antimicrobial Agents & Chemotherapy*. 2000; 44:3322-3327.

Mazel D. Integrons: agents of bacterial evolution. *Nature Microbiology reviews.* 2006; 4:608-620.

Mazzariol A., Mammina C., Koncan R. *et. al*. A novel VIM-type metallo-β-lactamase (VIM-14) in a *Pseudomonas aeruginosa* clinical isolate from a neonatal intensive care unit. *Clinical Microbiology & Infection*. 2011; 17(5): 722-724.

Meletis G. Carbapenem resistance: overview of the problem and future perspectives. *Advances in Infectious Diseases*. 2016; 3(1):15-21.

Mendes RE, Bell JM, Turnidge JD, *et. al*. CRP isolates of *Klebsiella pneumoniae* in China and detection of a conjugative plasmid (bla_{KPC-2} + *qnrB4*) and a bla_{IMP-4} gene. *Antimicrobial Agents & Chemotherapy*. 2008; 52:798-799.

Meyers RA. Encyclopedia of molecular biology and molecular medicine. Weinheim, VCH. *Microbiological Reviews*. 1996; 20(3):440-458.

Mima T, Joshi S, Gomez-Escalada M, Schweizer HP. Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins. *Journal of Bacteriology*. 2007; 189;7600-7609.

Mima T, Kohira N, Li Y, *et. al*. Gene cloning and characteristics of the RND-type multidrug efflux pump MuxABC-OpmB possessing two RND components in *Pseudomonas aeruginosa*. *Microbiology*. 2009; 155:3509-3517.

Mima T, Kohira N, Li Y, *et. al*. Gene cloning and characteristics of the RND-type multidrug efflux pump MuxABC-OpmB possessing two RND components in *Pseudomonas aeruginosa*. *Microbiology*. 2009; 155:3509-3517.

Miriagou V, Cornaglia G, Edelstein M, *et. al*. Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. *Clinical Microbiology & Infection*. 2010; 16:112-122.

Mitic N, Smith SJ, Neves A. *et. al*. The catalytic mechanisms of binuclear metallohydrolases. *Chemical Reviews*. 2006; 106(8):3338-3363.

Moriya Y, *et. al.* KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic acids research*. 2007; 35(2):W182-W185.

Mukherjee S, Bhadra B, Chakraborty R, *et .al*. Unregulated use of antibiotics in Siliguri city vis-a-vis occurrence of MAR bacteria in community waste water and river Mahananda, and their potential for resistance gene transfer. *Journal of Environmental Biology.* 2005; 26:229-238.

Mukherjee S, Chakraborty R. Conjugation potential and class 1 integron carriage of resident plasmids in river water copiotrophs. *Acta Microbiologica et Immunologica Hungarica*. 2007; 254:379-397.

Mukherjee S, Chakraborty R. Incidence of class 1 integrons in multiple antibiotic-resistant Gram-negative copiotrophic bacteria from River Torsa in India. *Research in Microbiology*. 2006; 157:220-226.

Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. *Microbiology Spectrum*. 2016. 4:1-30.

Nalbantoglu O, Way S, Hinrichs S, *et. al*. RAIphy: phylogenetic classification of metagenomics samples using iterative refinement of relative abundance index profiles. *BMC Bioinformatics.* 2011; 12:41(1-14).

Nelson ML, Dinardo A, Hochberg J, *et. al*. Brief communication: mass spectroscopic characterization of tetracycline in the skeletal remains of an ancient population from Sudanese Nubia 350-550 CE. *American Journal of Physical Anthropology*. 2010; 143:15001-15410.

Neuwald AF, Liu JS, Lipman DJ. Extracting protein alignmentmodels from the sequence database. *Nucleic Acids Research*.1997; 25(9):1665-1677.

Nicolau DP. Carbapenems: a potent class of antibiotics. *Expert Opinion on Pharmacotherapy*. 2008; 9:23-37.

Nordman P, Poirel L, Toleman MA, Walsh TR. Does broad-spectrum β-lactam resistance due to NDM-1 herald the end of the antibiotic era for treatment of infections caused by Gram-negative bacteria? *Journal of Antimicrobial Chemotherapy*. 2011; 66: 689-692.

Nordmann P, Poirel L. Strategies for identification of carbapenemase-producing *Enterobacteriaceae*. *Journal of Antimicrobial Chemotherapy*. 2013; 68:487-489.

Novais A, Rodrigues C, Branquinho R, *et. al*. Spread of an OmpK36-modified ST15 *Klebsiella pneumoniae* variant during an outbreak involving multiple carbapenem-resistant *Enterobacteriaceae* species and clones. *European journal of clinical microbiology & infectious diseases*. 2012; 31:3057-3063.

Ochs MM, McCusker MP, Bains M, *et. al*. Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids, *Antimicrobial Agents & Chemotherapy*, 1999; 43:1085-1090.

Osano E, Arakawa Y, Wacharotayankun R, *et. al*. Molecular characterization of an enterobacterial metallo β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. *Antimicrobial Agents & Chemotherapy.* 1994; 38:71-78.

Pagani L, Colinon C, Migliavacca R, *et. al*. Nosocomial outbreak caused by multidrug-resistant *Pseudomonas aeruginosa* producing IMP-13 metallo-β-lactamase. *Journal of Clinical Microbiology*. 2005; 43:3824-3828.

Page MI, Badarau A. The mechanisms of catalysis by metallo-β-lactamases. *Bioinorganic Chemistry and Applications*. 2008; 57:62-97.

Papagiannitsis CC, Tzouvelekis LS, Miriagou V. Relative strength of the class 1 integron promoter hybrid 2 and the combinations of strong and hybrid 1 with an active P2 promoter. *Antimicrobial Agents & Chemotherapy.* 2009; 53:277-280.

Papp-Wallace KM, Edimiani A, Taracila MA, *et. al*. Carbapenems: past, present and future. *Antimicrobial Agents & Chemotherapy.* 2011; 55:4943–4960.

Park S, Lee KM, Yoo YS, *et. al*. Alterations of *gyr*A, *gyr*B, and *par*C and Activity of Efflux Pump in Fluoroquinolone-resistant *Acinetobacter baumannii*. *Osong Public Health* and *Research Perspectives*. 2011; 2(3):164-170.

Patzer J, Toleman MA, Deshpande LM, *et. al*. *Pseudomonas aeruginosa* strains harbouring an unusual $bla_{\text{VIM-4}}$ gene cassette isolated from hospitalized children in Poland (1998-2001). *Journal of Antimicrobial Chemotherapy*. 2004; 53(3):451-456.

Peden JF. CodonW PhD Thesis, University of Nottingham. 1999.

Perez F, Endimiani A, Hujer KM, Bonomo RA. The continuing challenge of ESBLs. *Current Opinion* in *Pharmacology*. 2007; 7:459-469.

Perry JD, Naqvi SH, Mirza IA, *et. al*. Prevalence of faecal carriage of *Enterobacteriaceae* with NDM-1 carbapenemase at military hospitals in Pakistan, and evaluation of two chromogenic media. *Journal of Antimicrobial Chemotherapy*. 2011; 66:2288-2294.

Piddock LJ. Mechanisms of fluoroquinolone resistance: an update 1994-1998. *Drugs*. 1999; 58:11-18.

Pitout JD, Nordmann P, Kevin B, *et. al*. Emergence of *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) in the community. *Journal of Antimicrobial Chemotherapy*. 2005; 5:52-59.

Poirel L, Al Maskari Z, Rashdi AF. *et. al*. NDM-1 producing *Klebsiella pneumoniae* isolated in the Sultanate of Oman. *Journal of Antimicrobial Chemotherapy*. 2010; 66(2):304-306.

Poirel L, Hombrouck-Alet C, Freneaux C, *et. al*. Global spread of New Delhi metallo-β-lactamase 1. *Lancet Infectious Disease.* 2010; 10(12):831-832.

Poirel L, Lambert T, Turkoglu S, *et. al*. Characterization of class 1 integrons from *Pseudomonas aeruginosa* that contain the $bla_{\text{VIM-2}}$ carbapenem hydrolyzing lactamase gene and of two novel aminoglycoside resistance gene cassettes. *Antimicrobial Agents & Chemotherapy*. 2001; 45:546-552.

Poirel L, Naas T, Nicolas D, *et. al*. Characterization of VIM-2, a carbapenem-hydrolyzing metallo-β-lactamase and its plasmid and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. *Antimicrobial Agents & Chemotherapy*. 2000; 44:891-897.

Poirel L, Nordmann P. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clinical Microbiology and Infectious Disease* 2006; 12:826-836.

Poirel L, Ros A, Carricajo A, *et. al*. Extremely drug-resistant *Citrobacter freundii* identified in a patient returning from India and producing NDM-1 and other carbapenemases. *Antimicrobial Agents & Chemotherapy*. 2010; 55:447-448.

Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. *Diagnostic Microbiology and Infectious Disease.* 2011; 70:119-125.

Poole K, Gotoh N, Tsujimoto H, *et. al*. Overexpression of the *mexC-mexD-oprJ* effux operon in *nfxB*-type multidrug resistant strains. *Molecular Microbiology*. 1996; 21:713-724.

Poole K, Krebes K, McNally C, Neshat S. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *Journal of Bacteriology*. 1993; 175:7363-7372.

Poole K, Tetro K, Zhao Q, *et. al*. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. *Antimicrobial Agents & Chemotherapy*. 1996; 40:2021-2028.

Qin S, Qi H, Zhang Q, *et. al*. Emergence of extensively drug-resistant *Proteus mirabilis* harboring a conjugative NDM-1 plasmid and a novel *Salmonella* Genomic Island 1 Variant, SGI1-Z. *Antimicrobial Agents & Chemotherapy*. 2015; 59:6601-6604.

Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. *Clinical Microbiology Reviews*. 2007; 20(3):440-458.

Ray MJ, Lin MY, Weinstein RA, Trick WE. Spread of carbapenem resistant enterobacteriaceae among Illinois healthcare facilities: the role of patient sharing. *Clinical Infectious Diseases*. 2016; 63(7):889-893.

Rebic V, Masic N, Teskeredzic S, *et. al*. The importance of *Acinetobacter* spp. in the hospital environment. *Medical Archives*. 2018; 72(5):330-334.

Rolain JM, Parola P, Cornaglia G. New Delhi metallo-β-lactamase (NDM-1): towards a new pandemia? *Clinical Microbiology & Infection*. 2010; 16(12):1699-1701.

Rowe-Magnus DA, Mazel D. The role of integrons in antibiotic resistance gene capture. *International Journal of Medical Microbiology*. 2002; 292:115-25.

Roy S, Singh AK, Viswanathan R, *et. al*. Transmission of imipenem resistance determinants during the course of an outbreak of NDM-1 *Escherichia coli* in a sick newborn care unit. *Journal of Antimicrobial Chemotherapy*. 2011; 66:2773-2780.

Russell AD. The antibacterial activity of a new cephalosporin, cefamandole. *Journal of Antimicrobial Chemotherapy*. 1957; 1:97-101.

Rutgersson C, Fick J, Marathe N, *et. al*. Fluoroquinolones and *qnr* genes in sediment, water, soil, and human fecal flora in an environment polluted by manufacturing discharges. *Environmental Science & Technology*. 2014; 48:7825-7832.

Sabath LD. Abraham EP. Zinc as a cofactor for a cephalosporinase from *Bacillus cereus* 569. *Biochemical Journal*. 1966; 98:11-13.

Saga T, Yamaguchi K. History of antimicrobial agents and resistant bacteria. *Japan Medical Association* journal. 2009; 52:103-108.

Sarkar DJ, Mukherjee I, Shakil NA, *et. al*. Antibiotics in agriculture: use and impact. *Indian Journal of Ethnophytop*. 2018; 4:4-19.

Sarma JB, Bhattacharya PK, Kalita D, *et. al*. Multi-drug resistant *Enterobacteriaceae* including metallo-β-lactamase producers are predominant pathogens of healthcare-associated infections in an Indian teaching hospital. *Indian Journal of Medical Microbiology*. 2011; 29:22-27.

Satlin MJ, Chen L, Patel G, *et. al*. Multicenter clinical and molecular epidemiological analysis of bacteremia due to carbapenem-resistant Enterobacteriaceae (CRE) in the CRE epicenter of the United States. *Antimicrobial Agents & Chemotherapy*. 2017; 61(4):e02349-16.

Schenk G, Mitic N, Gahan LR, *et. al*. Binuclear metallohydrolases:complex mechanistic strategies for a simple chemical reaction. *Accounts of Chemical Research*. 2012; 45(9):1593-1603.

Scholar EM. Pratt WB. *The Antimicrobial Drugs*. *Oxford University Press*, US. 2012; 3.

Schwarz S, Chaslus-Dancla E. Use of antimicrobials in veterinary medicine and mechanisms of resistance. *Veterinary Research*. 2001; 32:201-225.

Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*, 2014; 30:2068-2069.

Segatore B, Massidda O, Satta, G. *et. al*. High specificity of *cph*A encoded metallo-β-lactamase from *Aeromonas hydrophila* AE036 for carbapenems and its contribution to β-lactam resistance. *Antimicrobial Agents & Chemotherapy*. 1993; 37(6):1324-1328.

Selvaraj KK, Sivakumar S, Sampath S, *et. al*. Paraben resistance in bacteria from sewage treatment plant effluents in India. *Water Science & Technology*. 2013; 68:2067-2073.

Senda K, Arakawa Y, Ichiyama S, *et. al*. PCR detection of metallo-β- lactamase gene (*bla*$_{IMP}$) in gram-negative rods resistant to broad-spectrum β-lactams. *Journal of Clinical Microbiology*. 1996; 34:2909-2913.

Srikumar R, Paul CJ, Poole K. Influence of mutations in the *mexR* repressor gene on expression of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa*. *Journal of Bacteriology*. 2000; 182:1410-1414.

Srinivasan R, Karaoz U, Volegova M, *et. al*. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One.* 2015; 10:e0117617.

Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics.* 2014; 30(9):1312-1313.

Stover CK, Pham XQ, Erwin AL, *et. al*. Complete Genome Sequence of *Pseudomonas aeruginosa* PAO1, an Opportunistic Pathogen. *Nature*. 2000; 406(6799):959-964.

Sun Z, Hu L, Sankaran B, Prasad BVV, Palzkill T. Differential active site requirements for NDM-1 β-lactamase hydrolysis of carbapenem versus penicillin and cephalosporin antibiotics. *Nature Communication.* 2018; 9:4524(1-13).

Suzuki Y, Nazareno PJ, Nakano R, *et. al*., Environmental presence and genetic characteristics of carbapenemase-producing *Enterobacteriaceae* from hospital sewage and river water in the Philippines. *Applied Environmental Microbiology*. 2020; 86:1906-1919.

Tamames J, Abellan JJ, Pignatelli M, *et. al*. Environmental distribution of prokaryotic taxa. *BMC Microbiology*. 2010; 10:2180-2185.

Tan SY, Tatsumura Y. Alexander Fleming (1881-1955): Discoverer of penicillin. *Singapore Medical Journal*. 2015; 56(7):366-367.

Teufel, R. Mascaraque V, Ismail W, *et. al*. Bacterial phenylalanine and phenylacetate catabolic pathway revealed. *Proceedings of the National Academy of Sciences of the United States of America* 2010; 107:14390-14395.

Tian GB, Adams-Haduch JM, Bogdanovich T, *et. al*. PME-1, an extended-spectrum β-lactamase identified in *Pseudomonas aeruginosa*. *Antimicrobial Agents & Chemotherapy*. 2011; 55(6):2710-2713.

Torok E, Moran E, Cooke F. Oxford handbook of infectious diseases and microbiology. *Oxford University Press*. 2016.

Trapnell C, Hendrickson DG, Sauvageau M, *et. al*. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nature Biotechnology*. 2013; 3(1):46-53.

Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics*. 2009; 25(9):1105-1111.

Ullah JH, Walsh TR, Taylor IA, *et. al*. The crystal structure of the L1 metallo-β-lactamase from *Stenotrophomonas maltophilia* at 1.7 Aresolution. *Journal of Molecular Biol*ogy. 1998; 284:125-136.

Valenza G, Joseph B, Elias J, *et. al*. First survey of metallo-β-lactamases in clinical isolates of *Pseudomonas aeruginosa* in a German university hospital. *Antimicrobial Agents & Chemotherapy.* 2010; 54:3493-3497.

Van Bambeke F, Balzi E, Tulkens PM. Antibiotic Efflux Pumps. *Biochemical Pharmacology*. 2000; 60(4):457-470.

Van Duin D, Doi Y. The global epidemiology of carbapenemase-producing *Enterobacteriaceae*. *Virulence.* 2017; 8:460-469.

Vesth T, Lagesen K, Acar O, Ussery D. CMG-biotools, a free workbench for basic comparative microbial genomics. *PLoS One.* 2013; 8(4):e60120.

Waksman SA. What is an antibiotic or an antibiotic substance? *Mycologia*. 1947; 39(5):565-569.

Walsh TR, Toleman MA, Poirel L, *et. al*. Metallo-β-lactamases: the quiet before the storm? *Clinical Microbiology Reviews.* 2005; 18: 306-325.

Walsh TR, Toleman MA, Hryniewicz W, Bennett PM, Jones RN. Evolution of an integron carrying $bla_{\text{VIM-2}}$ in Eastern Europe: report from the SENTRY Antimicrobial Surveillance Program. *Journal of Antimicrobial Chemotherapy*. 2003; 52(1):116-119.

Walsh TR, Weeks J, Livermore DM, Toleman MA. 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*, 2011; 11(5):355-362.

Walsh TR. The emergence and implications of metallo-β-lactamases in Gram-negative bacteria. *Clinical Microbiology & Infection*. 2005; 11:2-9.

Wanda CR. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS microbiology*. 2018; 4(3);482-501.

Wang C, Wang J, Mi Z. *Pseudomonas aeruginosa* producing VIM-2 metallo-β-lactamases and carrying two aminoglycoside-modifying enzymes in China. *Journal of Hospital Infection.* 2006; 62:522-24.

Watanabe M, Iyobe S, Inoue M, Mitsuhashi S. Transferable imipenem resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents & Chemotherapy.* 1991; 35:147-151.

Wayne PA. M 100-S20. Performance standards for antimicrobial susceptibility testing: 20th informational supplement. *Clinical and Laboratory Standards Institute*. 2010.

Wayne PA. Performance standards for Antimicrobial Susceptibility Testing.27th ed. CLSI supplement M100. *Clinical and Laboratory Standards Institute.* 2017.

Webber MA, Piddock LJV. The Importance of Efflux Pumps in Bacterial Antibiotics Resistance. *Journal of Antimicrobial Chemotherapy,* 2003; 51(1):9-11.

Weile J, Rahmig H, Gfroer S, *et. al*. First detection of a VIM-1 metallo-β-lactamase in a carbapenemresistant *Citrobacter freundii* clinical isolate in an acute hospital in Germany. *Scandinavian Journal* of *Infectious Diseases*. 2007; 39:264-266.

Wilcox DE. Binuclear Metallohydrolases. *Chemical Reviews*. 1996; 96(7):2435-2458.

Wise R. Antimicrobial resistance: priorities for action. *J. Antimicrob. Chemother*. 2002; 49:585-586.

Witting K, Smith RD. Discovery of Antibacterials and Other Bioactive Compounds from Microorganisms-evaluating Methodologies for Discovery and Generation of Non-ribosomal Peptide Antibiotics. *Current Drug Targets*. 2011; 12(11):1547-1559.

Wozniak A, Villagra NA, Undabarrena A, *et. al*. Porin alterations present in noncarbapenemase-producing *Enterobacteriaceae* with high and intermediate levels of carbapenem resistance in Chile. *Journal of medical microbiology*. 2012; 61:1270-1279.

Wroblewska M. Novel Therapies of Multidrug-Resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp. Infections: The State of the Art. *Archivum Immunologiae et Therapia Experimentalis*, 2006; 54(2):113-120.

Wu G, Culley DE, Zhang W. Predicted highly expressed genes in the genomes of *Streptomyces coelicolor* and *Streptomyces avermitilis* and the implications for their metabolism. *Microbiology*. 2005; 151:2175-87.

Xiaofeng Hu, Xuebing Xu, Wang Xu, *et. al*. Diversity of NDM lactamases producing bacteria in China. *International journal of infectious diseases*. 2017; 55:92-95.

Yan JJ, Hsueh PR, Ko WC, *et. al*. Metallo-β-lactamases in clinical *Pseudomonas* isolates in Taiwan and identification of VIM-3, a novel variant of the VIM-2 enzyme. *Antimicrobial Agents & Chemotherapy*. 2001; 45:2224-2228.

Yang, F, Huang, L, Li, L, *et al*. Discharge of KPC-2 genes from the WWTPs contributed to their enriched abundance in the receiving river. *Science Total Environment*. 2017; 136-143.

Yong D, Toleman MA, Giske CG, *et. al*. Characterization of a new metallo-β-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. *Antimicrobial Agents & Chemotherapy*. 2009; 53(12):5046-5054.

Yoshida, H. Bogaki M, Nakamura M, *et al*. Quinolone resistance-determining region in the DNA gyrase *gyr*A gene of *Escherichia coli*. *Antimicrobial Agents & Chemotherapy*. 1990; 34:1271-1272.

Yum J. H., Yi K., Lee H., *et. al*. Molecular characterization of metallo-β-lactamase-producing *Acinetobacter baumannii* and *Acinetobacter* genome species 3 from Korea: identifi cation of two new integrons carrying the *bla*$_{\text{VIM-2}}$ gene cassettes. *Journal of Antimicrobial Chemotherapy*. 2002; 49:837-840.

Zenati K, Touati A, Bakour S, *et. al*. Characterization of NDM-1 and OXA-23-producing *Acinetobacter baumannii* isolates from inanimate surfaces in a hospital environment in Algeria. *Journal of Hospital Infection*. 2016; 92(1):19-26.

Zhang C, Qin S, Wang Y, *et. al*. Higher isolation of NDM-1 producing *Acinetobacter baumannii* from the sewage of the hospitals in Beijing. *Plos One*. 2013; (6):e64857.

Zhang H & Hao Q. Crystal structure of NDM-1 reveals a common β-lactam hydrolysis mechanism. *FASEB Journal*. 2011; 25(8):2574-2582.

Zhang, Y., C.F. Marrs, C. Simon, C. Xi. Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. *Science Total Environment*. 2009; 407:3702-3706.

Zheng B, Tan S, Gao J, *et. al*. An unexpected similarity between antibiotic-resistant NDM-1 and β-lactamase II from Erythrobacter litoralis. *Protein Cell*, 2011; 2:250-258.

Zhi-Wen Y, Yan-Li Z, Man Y, Wei-Jun F. Clinical treatment of pandrug-resistant bacterial infection consulted by clinical pharmacist. *Saudi Pharmaceutical Journal*. 2015; 23(4):377-380.

Zurfluh K, Bagutti C, Brodmann P, Wastewater is a reservoir for clinically relevant carba-penemase and 16s rRNA methylase-producing *Enterobacteriaceae*. *International Journal* of *Antimicrobial Agents.* 2017; 50(3):436-440.

Appendices

Appendix A1

A1.1 Isolation and enumeration of Imipenem resistant bacteria in Mahananda and Karala river of Northern West Bengal

After performing cultures for both river water samples from all sampling sites, using 100 µl from each dilution upon imipenem containing LB plates, and the plates were incubated for 24 hrs at 37°C, the resulting colonies were counted and reported (Table A1.1). No colony was observed in the 10^{-2} dilution or lower.

Table A1.1: Total number of culturable imipenem resistant bacterial colonies apperead on Luria agar plates containing imipenem (20µg/ml) and series dilutions

Sampling site	Dilution	1st sampling	2nd sampling	3rd sampling	4th sampling
Mahananda River (Siliguri)					
Sampling date		09-03-2015	07-04-2015	18-11-15	04-12-2015
SSM I	10^{-0}	31	26	22	4
	10^{-1}	5	2	3	0
	10^{-2}	0	0	0	0
SSM II	10^{-0}	206	278	135	36
	10^{-1}	22	34	12	2
	10^{-2}	0	0	0	0
SSM III	10^{-0}	180	205	87	27
	10^{-1}	20	19	7	2
	10^{-2}	0	0	0	0
Karala river (Jalpaiguri)					
Sampling date		18-03-2015	16-04-2015	15-11-2015	10-12-2015
SSK I	10^{-0}	20	25	20	22
	10^{-1}	1	2	0	0
	10^{-2}	0	0	0	0
SSK II	10^{-0}	120	161	118	48
	10^{-1}	14	15	13	5
	10^{-2}	0	0	0	0
SSK III	10^{-0}	292	368	317	82
	10^{-1}	32	33	31	9
	10^{-2}	0	0	0	0

Table A1.2: Mean numbers of culturable imipenem resistant bacteria colonies forming units/ml (CFU/ml) isolated from both river (Mahananda and Karala) water after incubation

		Mahananda River sampling site			**Karala River sampling site**		
Sampling number	**Dilution**	**SSM I**	**SSM II**	**SSM III**	**SSK I**	**SSK II**	**SSK III**
1	10^{-0}	310	2060	1800	200	1200	2920
2	10^{-0}	260	2780	2050	250	1610	3680
3	10^{-0}	220	1350	870	200	1180	3170
4	10^{-0}	40	360	270	220	480	820
Total		830	6550	4990	870	4470	10590
Mean		**207.5**	**1637.5**	**1247.5**	**217.5**	**1117.5**	**2647.5**
±SD		117.5798	1032.55	826.09	23.63	468.93	1258.71

Appendix A1.3: Numerical data on randomly picked colonies from imipenem containing plates (imipenem resistant bacteria from Mahananda River) having carriage of MBL coding genes

Sampling date and sampling points	Total no. of imipenem resistant bacteria [in agar plates (LB + imipenem) spreaded with undiluted river water samples]	No. of randomly picked colonies from imipenem plates	No. of colonies responded to inhibition in presence of EDTA	No. of colonies responded positively to Carba NP test	No. of Carba- NP positive-strains generated amplicons in Multiplex PCR experiment
First sampling (03-03-2015)					
SSM I	31	15	0	0	0
SSM II	206	102	3	1	1 MR 02
SSM III	180	100	0	0	0
Second sampling (07-04-2015)					
SSM I	26	13	0	0	0
SSM II	278	165	20	3	3 (MR15, MR24, MR26)
SSM III	205	105	0	0	0
Third sampling (18-11-15)					
SSM I	22	11	0	0	0
SSM II	135	67	16	3	3 (MR29, MR40, MR41)
SSM III	87	42	0	0	0
Fourth sampling (04-01-2016)					
SSM I	4	4	0	0	0
SSM II	36	18	0	0	0
SSM III	27	13	0	0	0
Total	**1,237**	**665**	**39**	**7**	**7**

Appendix A1.4: Numerical data on randomly picked colonies from imipenem containing plates (imipenem resistant bacteria from Karala River) having carriage of MBL coding genes

Sampling date and sampling points	Total no. of imipenem-resistant bacteria [in agar plates (LB + imipenem) spreaded with undiluted river water samples]	No. of randomly picked colonies from imipenem plates	No. of colonies responded to inhibition in presence of EDTA	No. of colonies responded positively to Carba NP test	No. of Carba-NP positive-strains generated amplicons in Multiplex PCR experiment
First sampling (18-03-2015)					
SSK I	20	10	0	0	0
SSK II	120	60	13	3	3 (KR01, KR04,KR07)
SSK III	292	150	0	0	0
Second sampling (16-04-2015)					
SSK I	25	10	0	0	0
SSK II	161	80	10	2	2 (KR22, KR23)
SSK III	368	160	0	0	0
Third sampling (15-11-2015)					
SSK I	20	10	0	0	0
SSK II	118	60	12	3	3 (KR25, KR26, KR29)
SSK III	317	150	0	0	0
Fourth sampling (10-12-2015)					
SSK I	22	10	0	0	0
SSK II	48	25	0	0	0
SSK III	82	40	0	0	0
Total	**1,593**	**765**	**35**	**8**	**8**

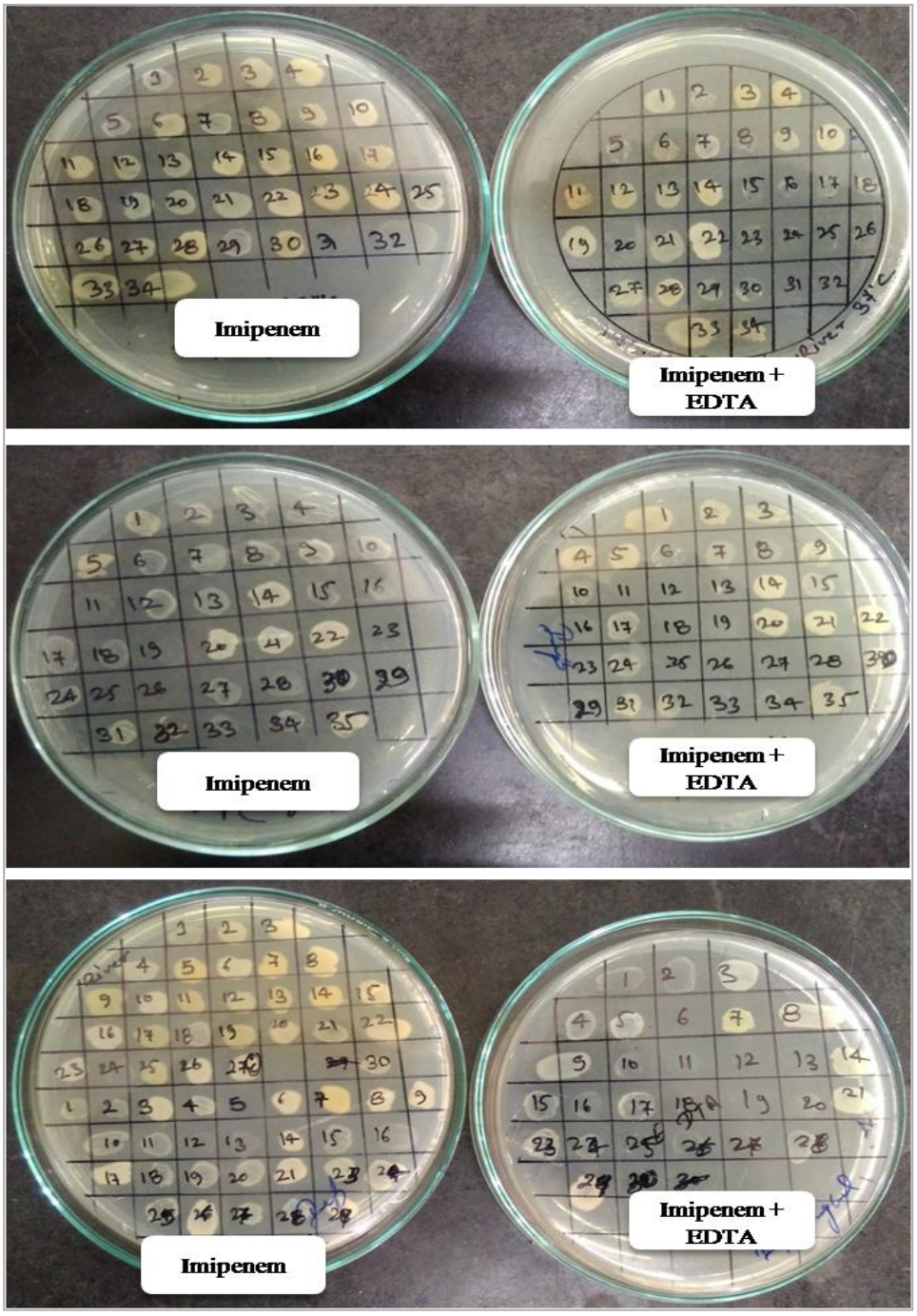


Figure A1.1: Screening of metallo-β-lactamase-producing strains from Mahananda River

The figure shows screening results of MBL positive strain from imipenem resistance bacteria which is isolated from Mahananda River using modified imipenem-EDTA combined synergy test. The isolates did not show any growth on EDTA containing plates after incubation were considered as putative MBL producers.

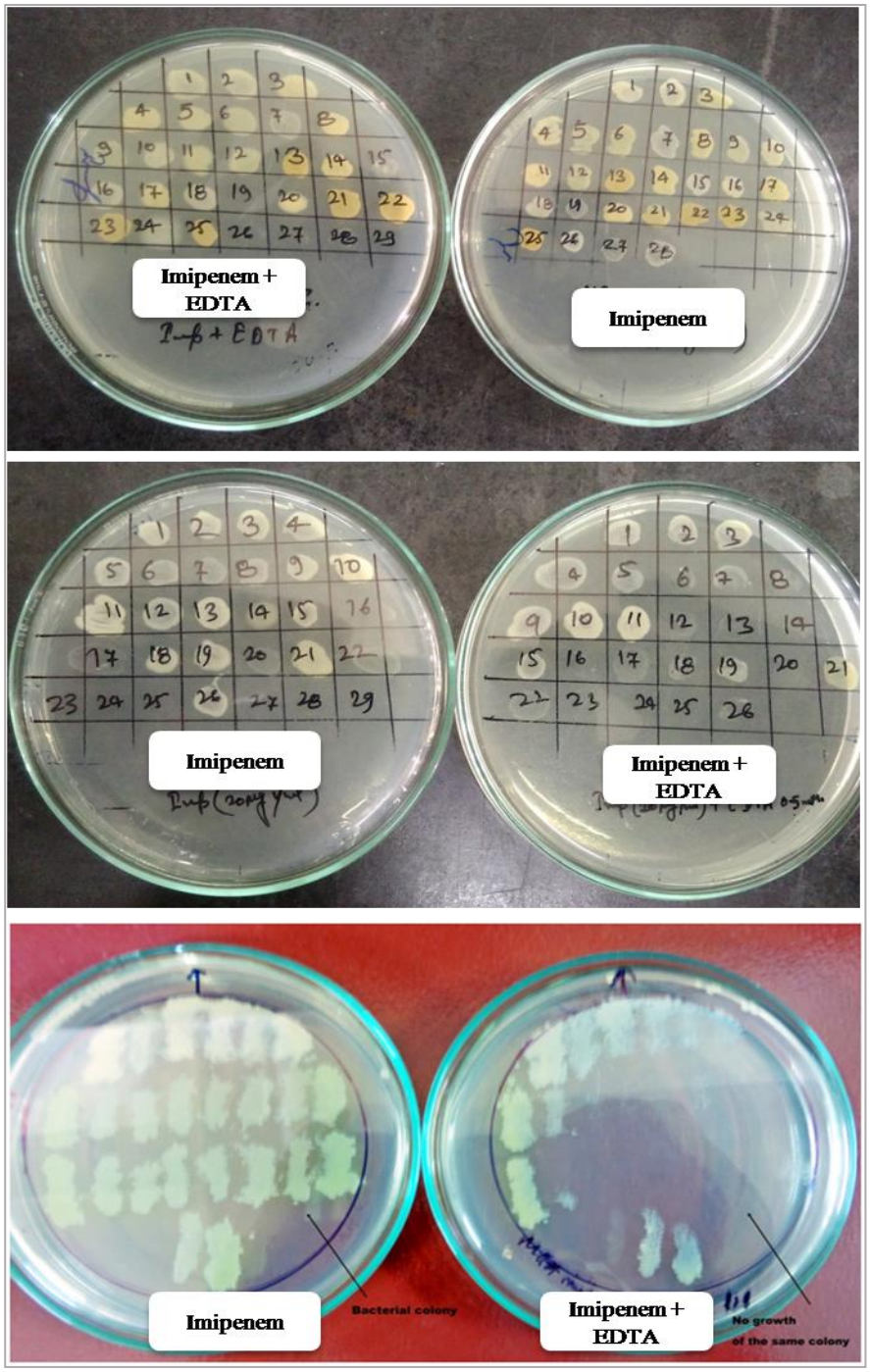
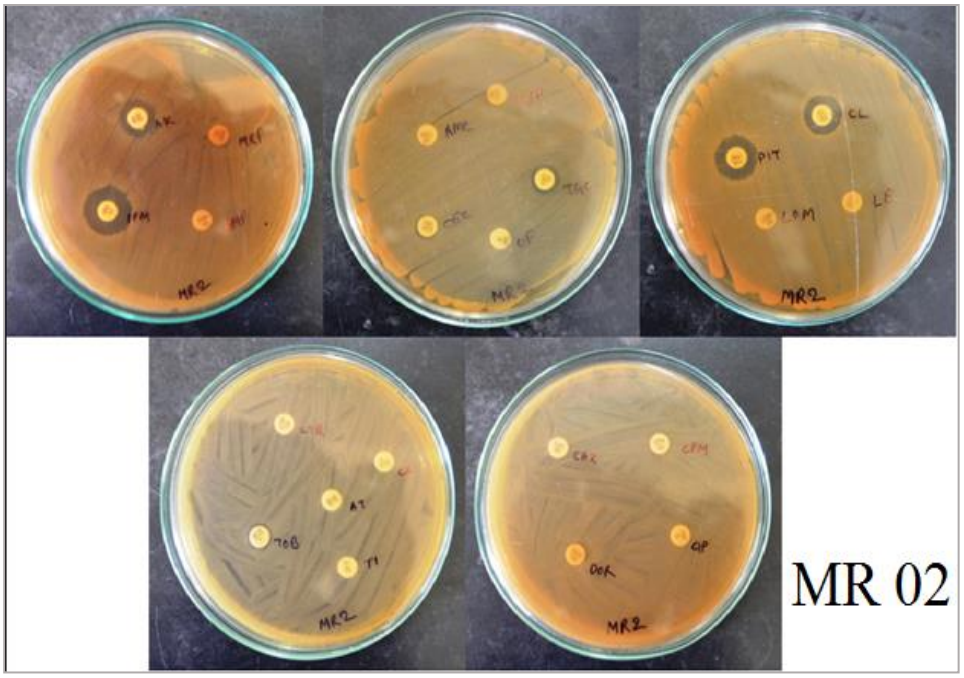


Figure A1.2: **Screening of Metallo-β-lactamase producing strain Karala River**

The figure shows screening results of MBL positive strain from imipenem resistance bacteria which is isolated from Mahananda River using modified imipenem-EDTA combined synergy test. The isolates did not show any growth on EDTA containing plates after incubation were considered as putative MBL producers.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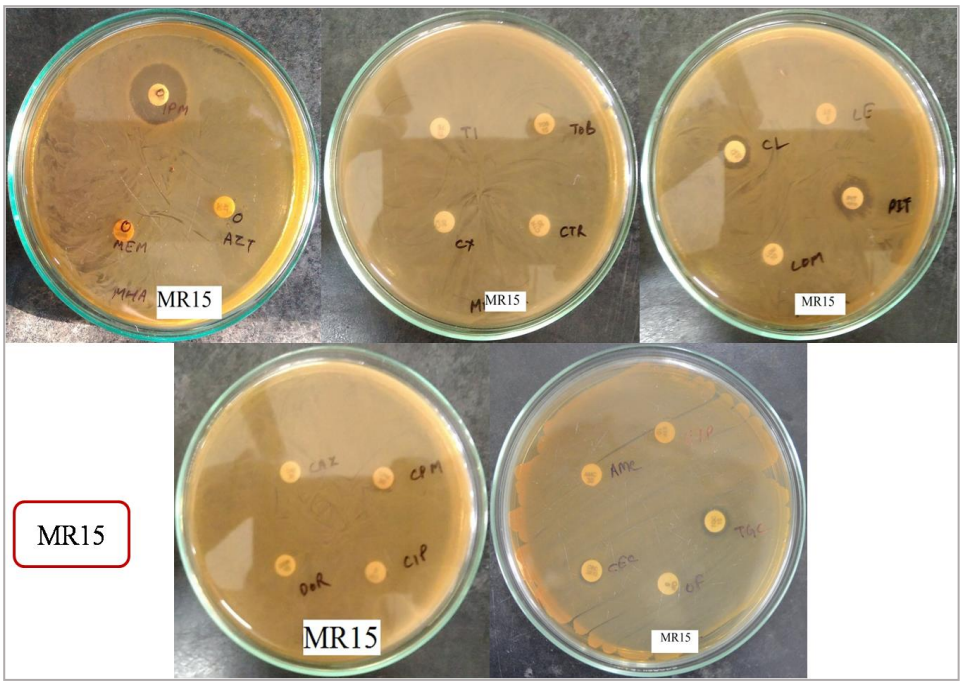
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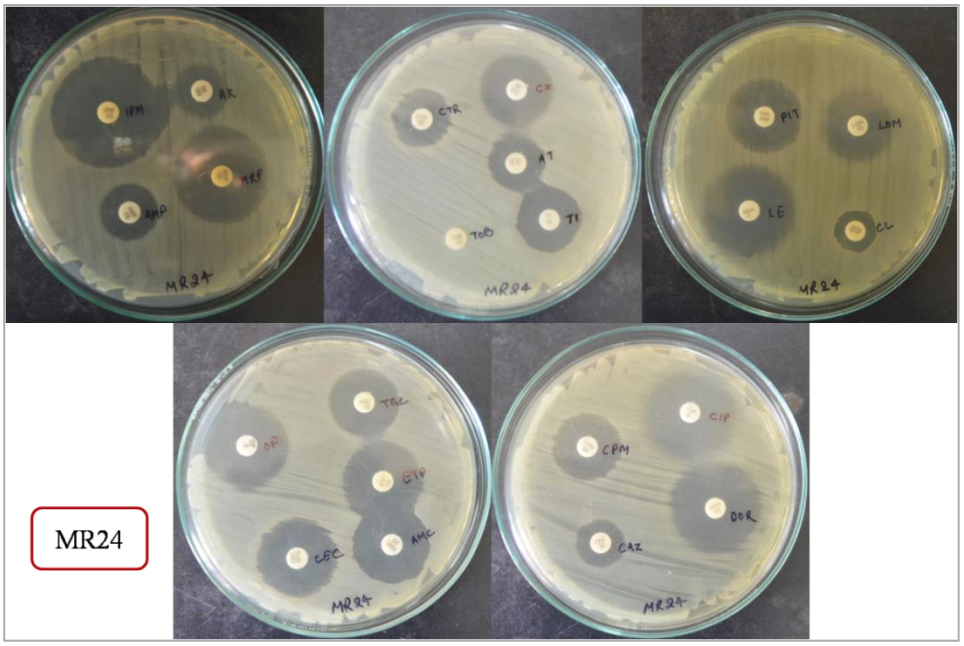
MR15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MRP
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CX
AT
TI
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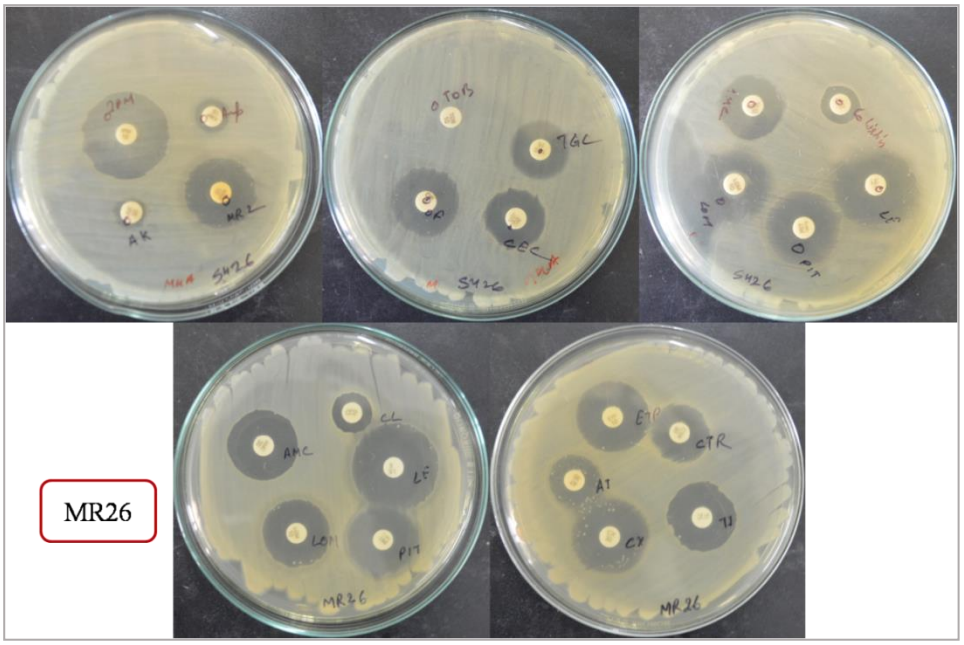
OF
ETP
CEC
AMC
MR24
CIP
CPM
DOR
CAZ
MR24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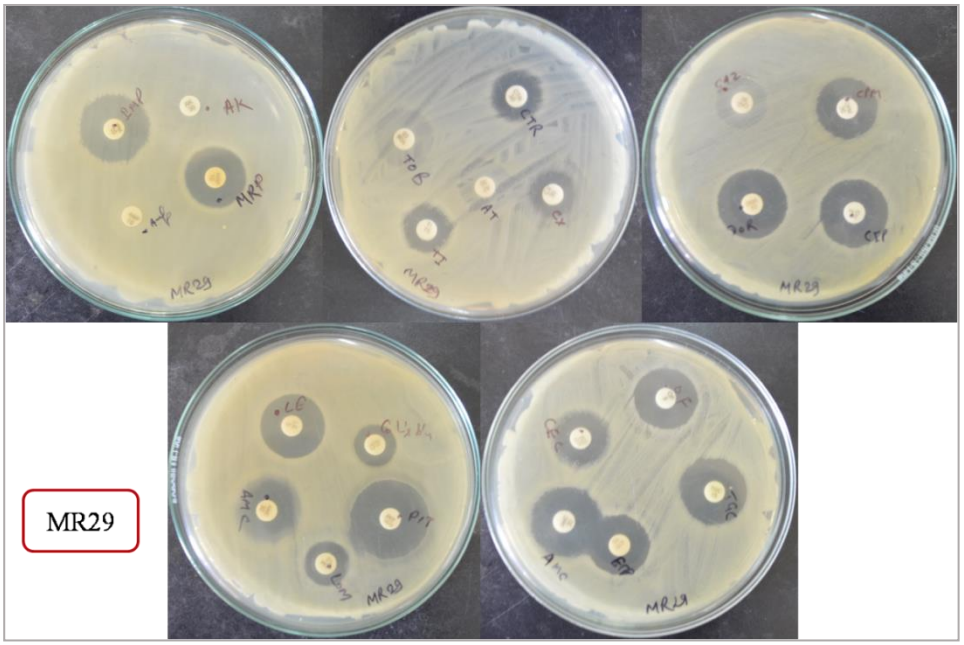
TI
MR26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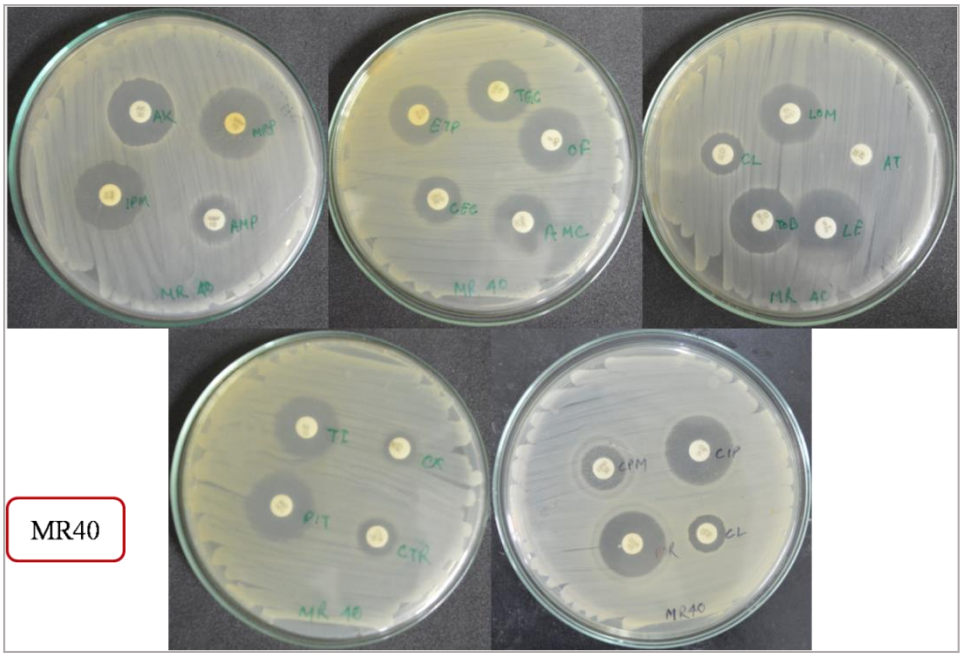

MR40

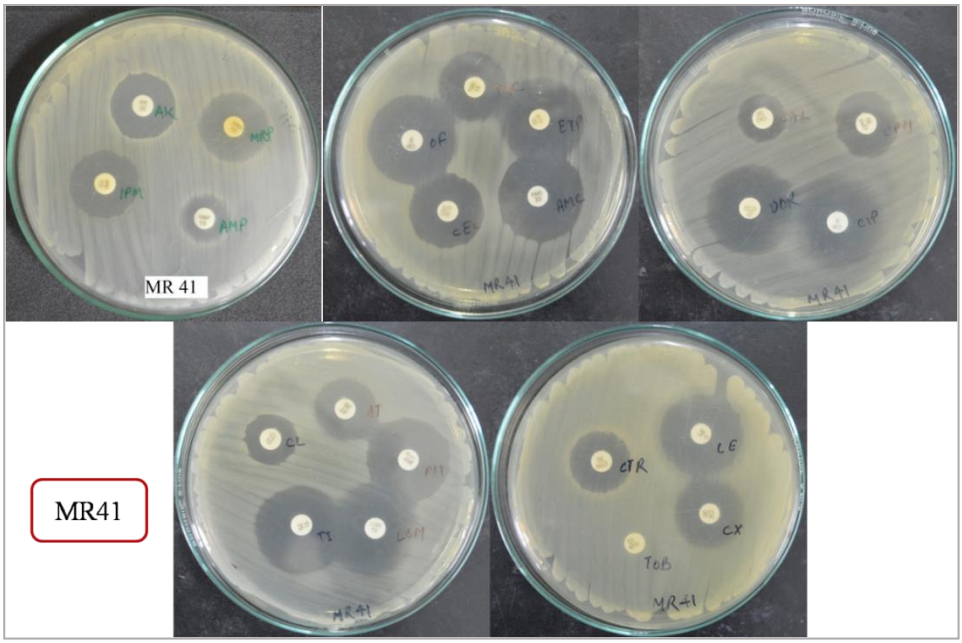
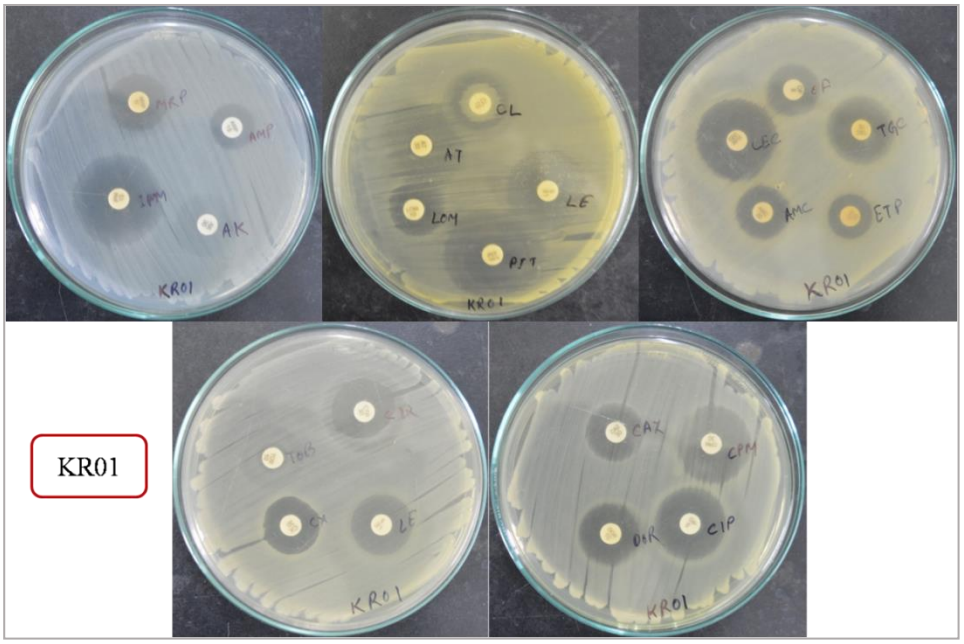


Figure A1.3: Kirby-Bauer disk diffusion assay results for MBL-positive strains isolated from Mahananda River

Antibiotic susceptibility testing of *Pseudomonas* sp. MR 02, *Pseudomonas* sp. MR15, *Myroides* sp. MR24, *Acinetobacter* sp. MR26, *Acinetobacter* sp. MR29, *Acinetobacter* sp. MR40 and *Acinetobacter* sp. MR41 against 22 different antibiotics including carbapenem antibiotics.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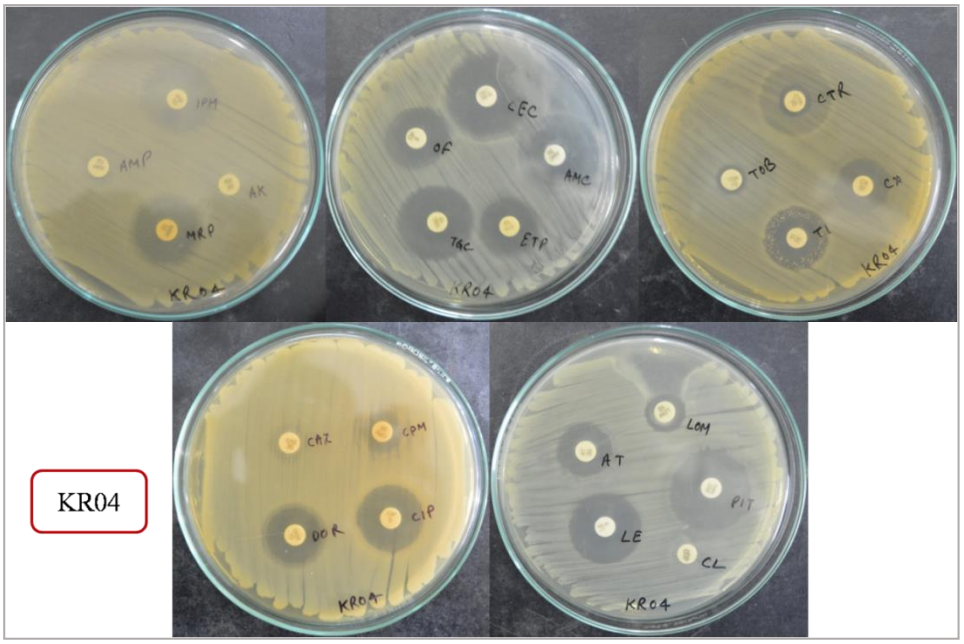
KR01
CAZ
CPM
DOR
CIP
KR01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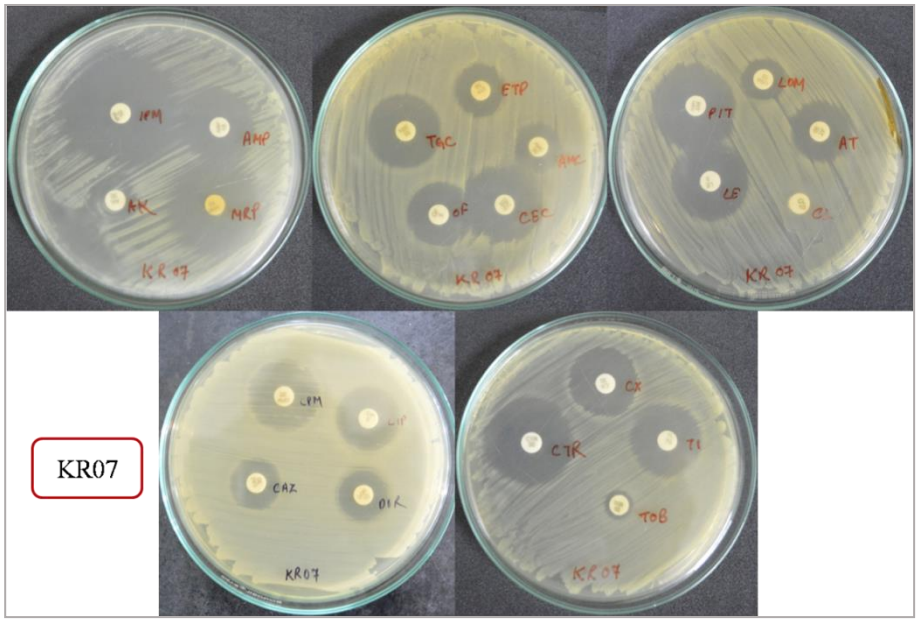
CL
KR04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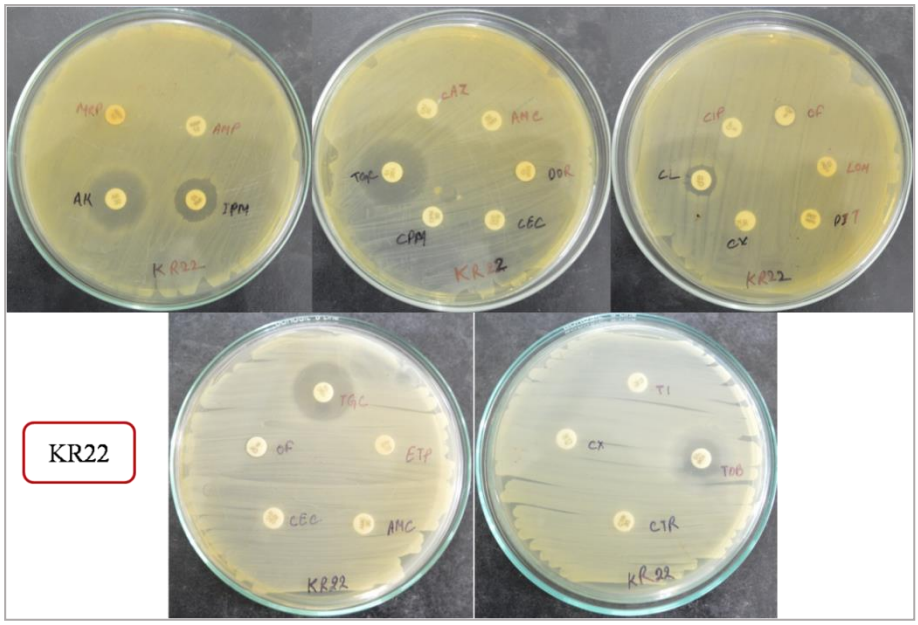
KR07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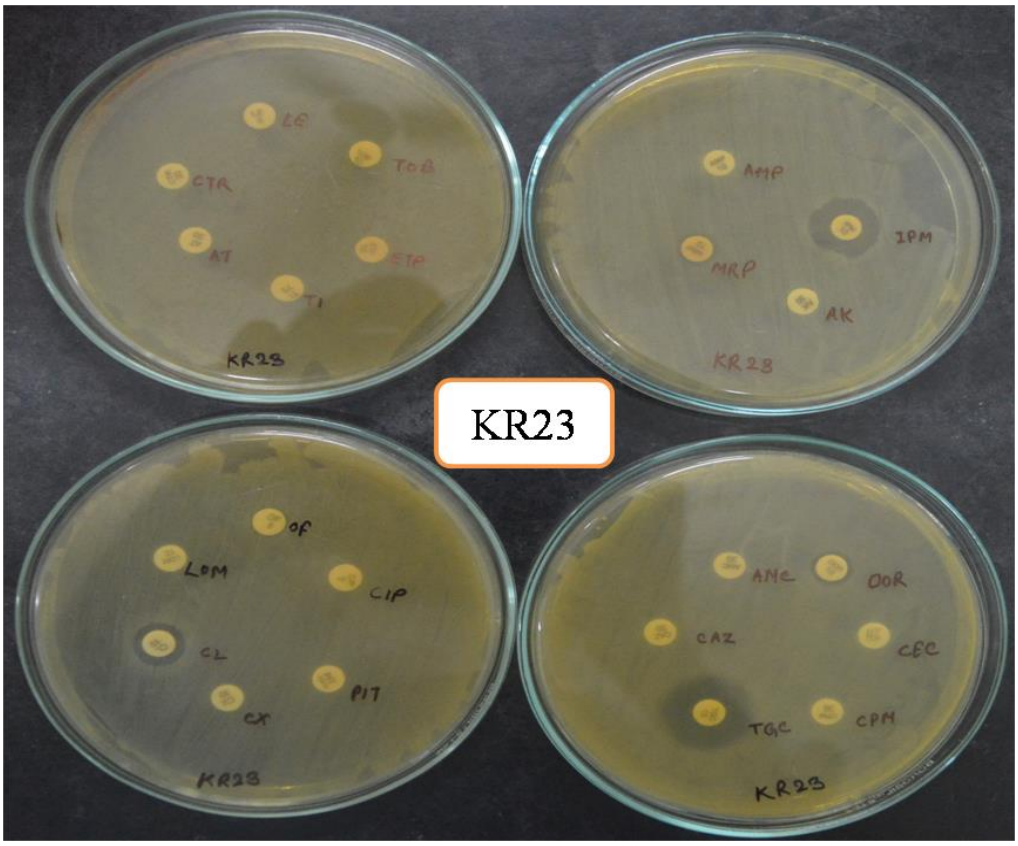
KR22

KR22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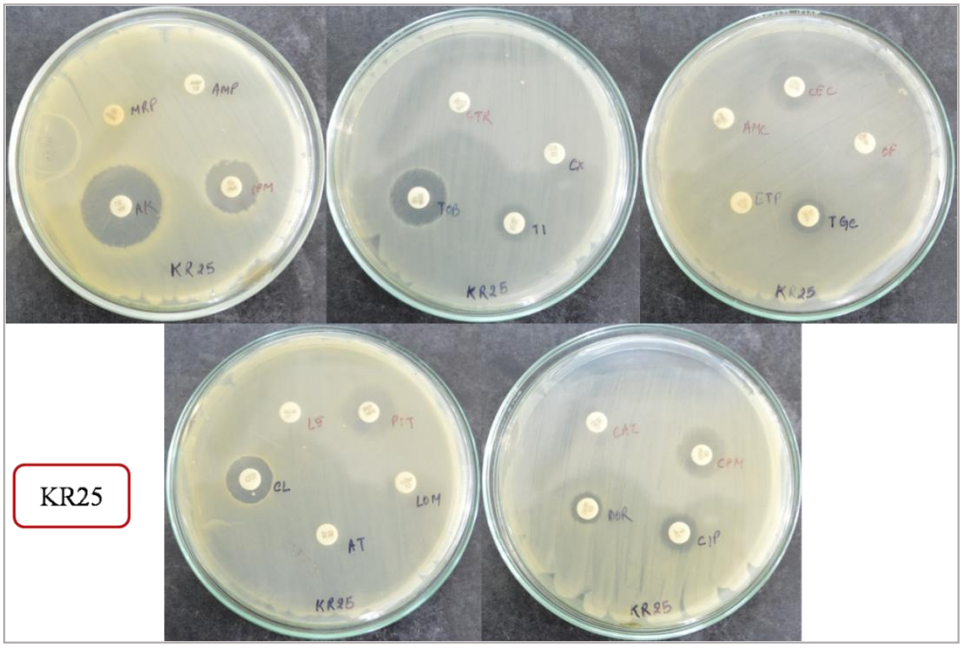
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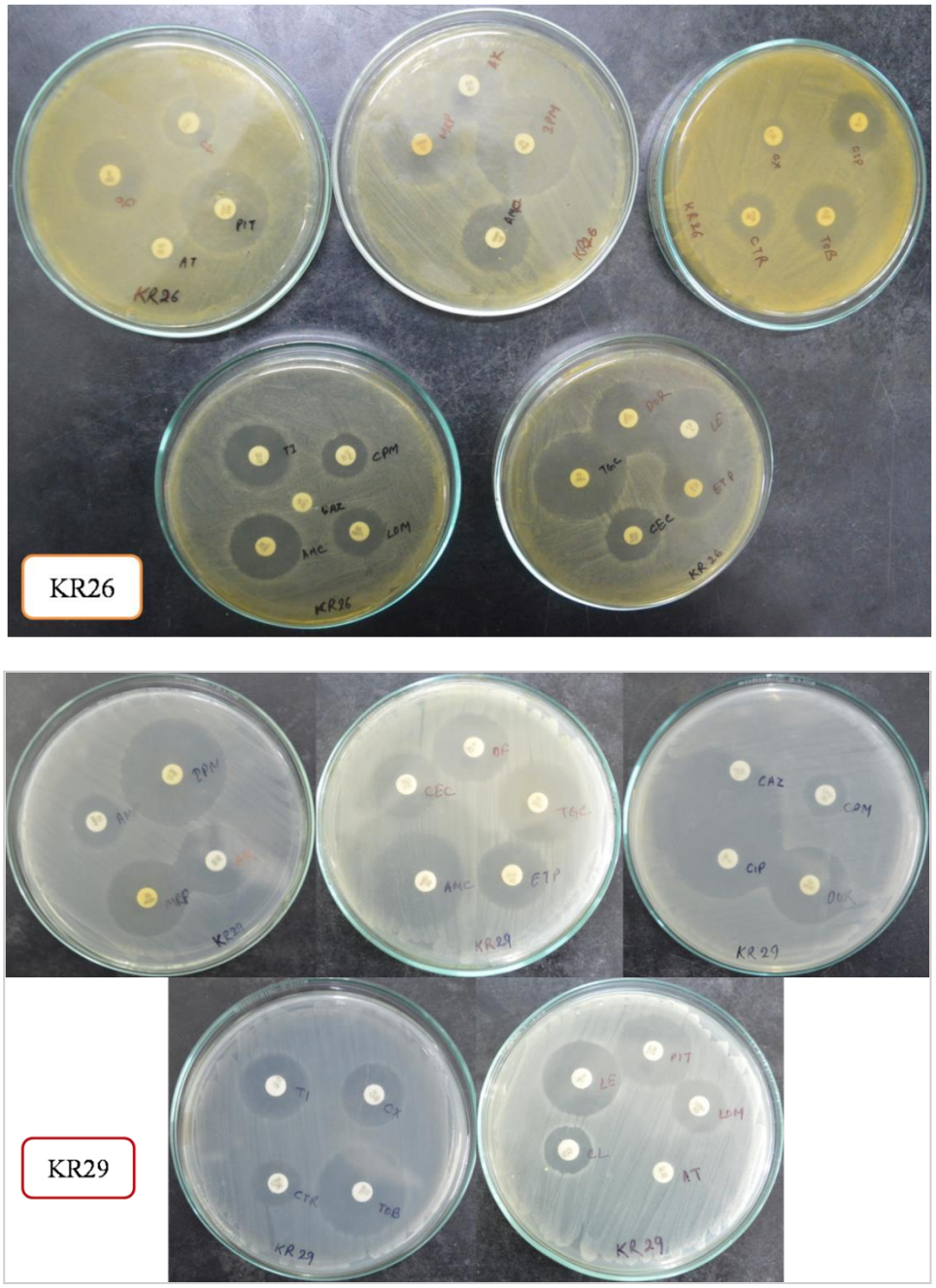

Figure A1.4: Kirby-Bauer disk diffusion assay results for MBL-positive strains isolated from Karala River

Antibiotic susceptibility testing of *Proteus* sp. KR01, *Proteus* sp. KR04, *Escherichia* sp. KR07/KR22/KR23, *Pseudomonas mendocina* KR25, *Acinetobacter* sp. KR26 and *Acinetobacter* sp. KR29 against 22 different antibiotics including carbapenem antibiotics

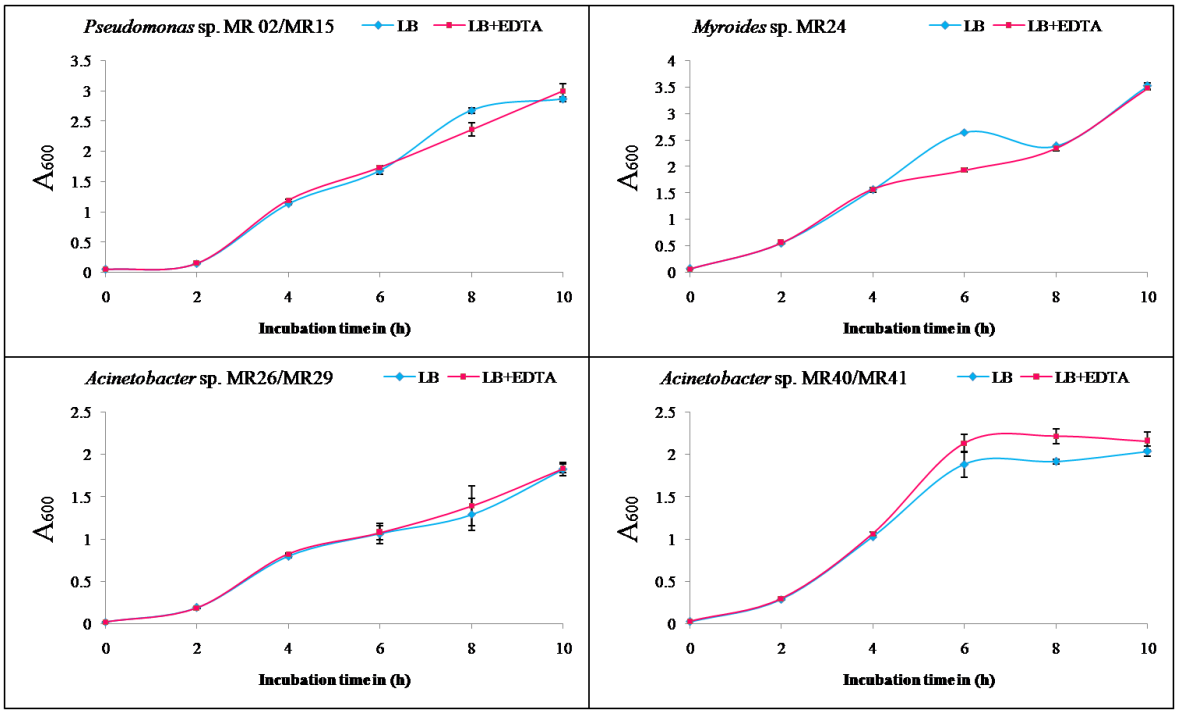

Figure A1.5: Growth of MBL-positive isolates (isolated from Mahananda River) in the presence or absence of EDTA

Growth curve of MBL positive strains *Pseudomonas* sp. MR 02/MR15, *Myroides* sp. MR24, *Acinetobacter* sp. MR26/MR29 and *Acinetobacter* sp. MR40/MR41 in Luria broth in the presence or absence of 0.4 mM EDTA over 10 hrs. $OD_{600=}$ optical density at 600 nm.

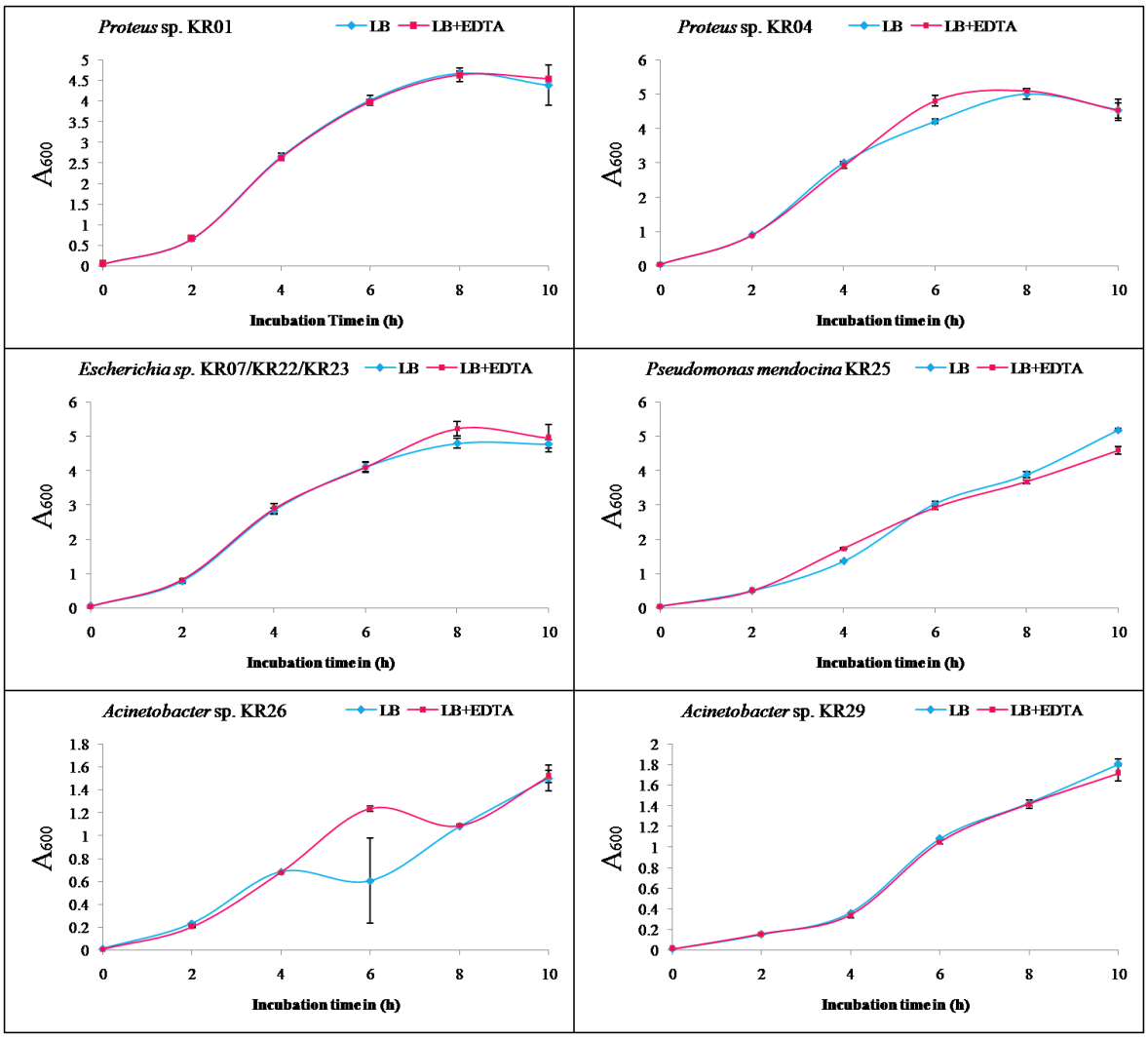

Figure A1.6: Growth of Karala River MBL-positive isolates (isolated from Karala River) in the presence or absence of EDTA

Growth curve of MBL positive strains *Proteus* sp. KR01, *Proteus* sp. KR04, *Escherichia* sp. KR07/KR22/KR23, *Pseudomonas mendocina* KR25, *Acinetobacter* sp. KR26 and *Acinetobacter* sp. KR29 in Luria broth in the presence or absence of 0.4 mM EDTA over 10 hrs. OD_{600}= optical density at 600 nm.

Appendix A2

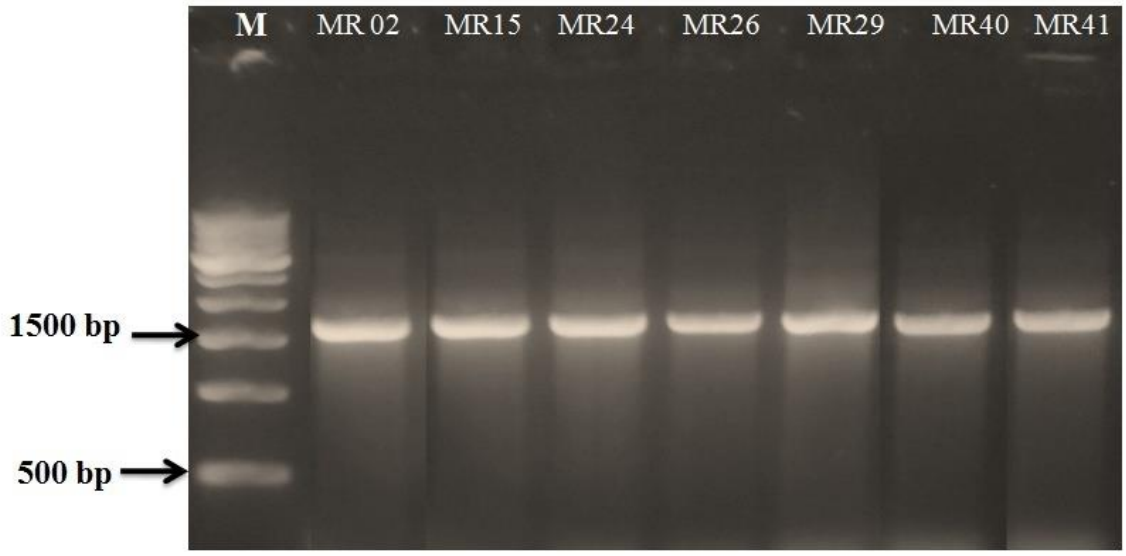

Figure A2.1: Agarose gel electrophoresis of PCR-amplified 16S rRNA genes in different MBL positive bacterial strains isolated from Mahananda River

Lane show positive products (nearly 1500 bp) of the 16S rRNA gene for isolates MR 02, MR15, MR24, MR26, MR29, MR40 and MR41, M-Marker 5kb.

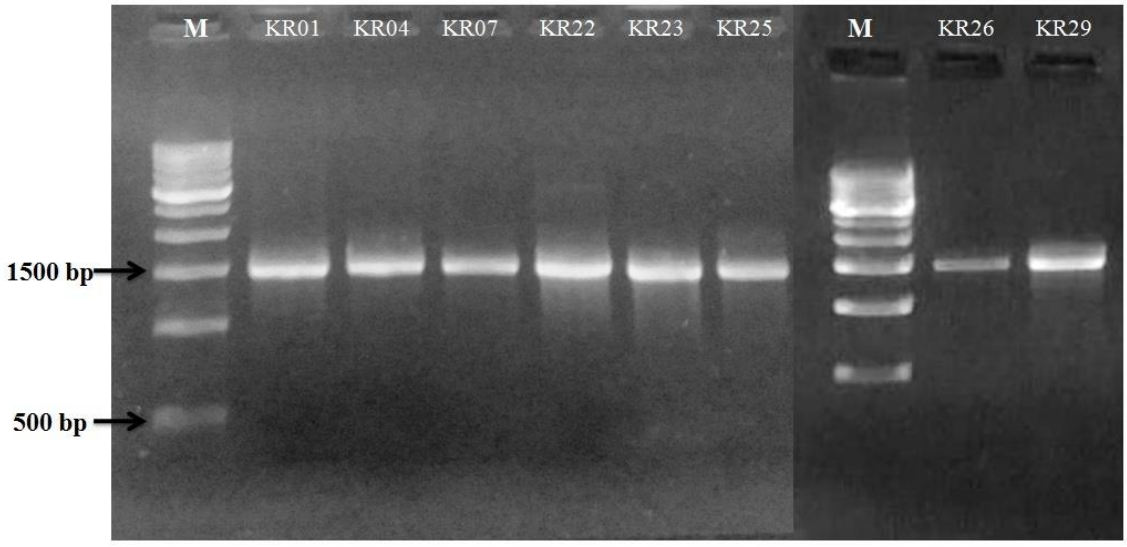

Figure A2.2: Agarose gel electrophoresis of PCR-amplified 16S rRNA genes in different MBL positive bacterial strains isolated from Karala River

Lane show positive products (nearly1500 bp) of the 16S rRNA gene for isolates KR01, KR04, KR07, KR22, KR23, KR25, KR26 and KR29, M-Marker 5kb.

Appendix A4

Figure A4.1: Agarose gel electrophoresis (0.7%) of genomic DNA isolated from *Pseudomonas* sp. MR 02 for whole genome sequencing

Table A4.1: Average Nucleotide Identity (ANI) (%) with MR02 vs other Pseudomonas spp.determined with the OrthoANI program

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	***Pseudomonas pyomelaninifaciens* MR02 ^{T}**		75.1	83.5	76.9	78.8	82.1	81.1	85.5	86.9	90.0	89.9	77.2	81.2	85.6	76.9	76.7	74.3	85.3	81.8	76.6	77.4	84.5	82.6	82.0	89.3	84.9	85.7	84.0
2	*Pseudomonas oryzihabitans* NBRC 102199 ^{T}	75.1		74.0	73.4	74.4	75.1	74.6	75.4	75.1	74.6	74.4	76.6	74.4	74.5	75.6	75.5	73.4	75.3	75.5	75.2	75.9	74.9	75.4	75.1	74.3	75.2	75.4	75.5
3	*Pseudomonas fulva* NBRC 16637 ^{T}	83.5	74.0		75.8	77.2	79.7	79.1	81.8	83.4	83.1	83.0	75.7	79.2	81.6	75.5	75.5	73.3	81.7	79.3	75.3	75.8	81.3	80.7	79.5	82.8	81.3	81.8	81.3
4	*Pseudomonas graminis* DSM 11363^{T}	76.9	73.4	75.8		78.1	77.9	77.6	76.8	76.8	76.7	76.6	75.1	77.5	76.3	75.4	75.2	73.5	76.8	77.8	75.0	75.7	76.7	76.8	77.9	76.3	77.1	77.0	76.9
5	*Pseudomonas asplenii* ATCC 23835^{T}	78.8	74.4	77.2	78.1		79.8	79.6	78.8	78.8	78.5	78.4	76.6	79.5	78.3	76.5	76.3	74.4	78.6	79.7	76.2	77.0	78.3	78.7	79.9	78.2	78.7	78.7	78.8
6	*Pseudomonas donghuensis* DSM 101685^{T}	82.1	75.1	79.7	77.9	79.8		86.3	82.1	82.0	81.5	81.5	77.1	85.7	81.1	77.2	76.9	74.6	81.7	82.9	76.7	77.5	81.4	81.2	93.1	81.3	82.1	82.1	81.5
7	*Pseudomonas alkylphenolica* KL28^{T}	81.1	74.6	79.1	77.6	79.6	86.3		81.4	81.1	80.8	80.7	76.8	86.6	80.5	76.7	76.2	74.4	81.3	82.2	76.2	77.1	80.8	80.6	86.4	80.5	81.5	81.4	81.1
8	*Pseudomonas entomophila* L48^{T}	85.5	75.4	81.8	76.8	78.8	82.1	81.4		85.5	84.9	85.0	77.7	81.5	84.5	77.2	76.9	74.6	87.9	81.9	76.7	77.5	84.5	82.7	82.0	84.6	84.8	88.5	83.8
9	*Pseudomonas plecoglossicida* DSM 15088^{T}	86.9	75.1	83.4	76.8	78.8	82.0	81.1	85.5		90.0	89.8	77.3	81.1	85.7	76.8	76.7	74.4	85.2	81.6	76.5	77.4	84.4	82.6	81.9	89.3	84.8	85.5	83.9
10	*Pseudomonas putida* NBRC14164^{T}	90.0	74.6	83.1	76.7	78.5	81.5	80.8	84.9	90.0		90.4	76.7	80.6	85.0	76.5	76.4	74.1	84.6	80.9	76.1	77.1	83.6	82.1	81.4	90.0	84.3	85.0	83.4
11	*Pseudomonas monteilii* NBRC 103158^{T}	89.9	74.4	83.0	76.6	78.4	81.5	80.7	85.0	89.8	90.4		76.4	80.7	85.1	76.5	76.2	74.0	84.6	80.9	76.0	77.1	83.8	82.0	81.4	90.2	84.2	85.5	83.4
12	*Pseudomonas aeruginosa* DSM 50071^{T}	77.2	76.6	75.7	75.1	76.6	77.1	76.8	77.7	77.3	76.7	76.4		76.5	76.4	77.2	77.4	75.1	77.4	78.0	77.4	78.1	76.9	77.2	77.1	76.3	77.2	77.8	77.5
13	*Pseudomonas vranovensis* DSM 16006^{T}	81.2	74.4	79.2	77.5	79.5	85.7	86.6	81.5	81.1	80.6	80.7	76.5		80.6	76.5	76.2	74.3	81.1	81.8	76.1	76.8	80.8	80.4	85.7	80.5	81.1	81.4	81.2
14	*Pseudomonas taiwanensis* DSM 21245 ^{T}	85.6	74.5	81.6	76.3	78.3	81.1	80.5	84.5	85.7	85.0	85.1	76.4	80.6		76.3	76.0	73.8	84.2	80.8	75.9	76.8	83.8	81.7	81.1	84.6	83.7	84.6	83.0
15	*Pseudomonas straminae* JCM 2783^{T}	76.9	75.6	75.5	75.4	76.5	77.2	76.7	77.2	76.8	76.5	76.5	77.2	76.5	76.3		79.0	75.3	77.1	77.3	79.1	79.5	76.7	77.2	77.1	76.3	77.0	77.2	77.3
16	*Pseudomonas toyotomiensis* DSM 26169^{T}	76.7	75.5	75.5	75.2	76.3	76.9	76.2	76.9	76.7	76.4	76.2	77.4	76.2	76.0	79.0		75.5	76.7	77.1	88.4	92.2	76.4	76.7	76.8	76.2	76.7	76.9	76.9
17	*Pseudomonas xanthomarina* DSM 18231^{T}	74.3	73.4	73.3	73.5	74.4	74.6	74.4	74.6	74.4	74.1	74.0	75.1	74.3	73.8	75.3	75.5		74.2	74.8	75.5	76.0	74.2	74.6	74.6	73.8	74.3	74.5	74.8
18	*Pseudomonas soli* LMG 27941^{T}	85.3	75.3	81.7	76.8	78.6	81.7	81.3	87.9	85.2	84.6	84.6	77.4	81.1	84.2	77.1	76.7	74.2		81.8	76.7	77.4	84.5	82.5	81.7	84.2	84.5	90.4	83.7
19	*Pseudomonas japonica* NBRC 103040^{T}	81.8	75.5	79.3	77.8	79.7	82.9	82.2	81.9	81.6	80.9	80.9	78.0	81.8	80.8	77.3	77.1	74.8	81.8		76.8	77.8	81.1	81.1	82.8	80.6	81.5	82.0	81.4
20	*Pseudomonas mendocina* NBRC 14162^{T}	76.6	75.2	75.3	75.0	76.2	76.7	76.2	76.7	76.5	76.1	76.0	77.4	76.1	75.9	79.1	88.4	75.5	76.7	76.8		88.7	76.3	76.7	76.6	75.8	76.5	76.9	76.8
21	*Pseudomonas oleovorans* subsp. Oleovorans DSM 1045	77.4	75.9	75.8	75.7	77.0	77.5	77.1	77.5	77.4	77.1	77.1	78.1	76.8	76.8	79.5	92.2	76.0	77.4	77.8	88.7		77.0	77.7	77.6	76.9	77.4	77.6	77.8
22	*Pseudomonas guariconensis* LMG 27394^{T}	84.5	74.9	81.3	76.7	78.3	81.4	80.8	84.5	84.4	83.6	83.8	76.9	80.8	83.8	76.7	76.4	74.2	84.5	81.1	76.3	77.0		82.2	81.5	83.6	84.1	84.6	83.4
23	*Pseudomonas cremoricolorata* ND07^{T}	82.6	75.4	80.7	76.8	78.7	81.2	80.6	82.7	82.6	82.1	82.0	77.2	80.4	81.7	77.2	76.7	74.6	82.5	81.1	76.7	77.7	82.2		81.1	81.8	82.8	82.8	81.8
24	*Pseudomonas wadenswilerensis* CCOS 864^{T}	82.0	75.1	79.5	77.9	79.9	93.1	86.4	82.0	81.9	81.4	81.4	77.1	85.7	81.1	77.1	76.8	74.6	81.7	82.8	76.6	77.6	81.5	81.1		81.1	82.1	82.0	81.5
25	*Pseudomonas hunanensis* P11^{T}	89.3	74.3	82.8	76.3	78.2	81.3	80.5	84.6	89.3	90.0	90.2	76.3	80.5	84.6	76.3	76.2	73.8	84.2	80.6	75.8	76.9	83.6	81.8	81.1		83.8	84.8	83.2
26	*Pseudomonas reidholzensis* CCOS 865^{T}	84.9	75.2	81.3	77.1	78.7	82.1	81.5	84.8	84.8	84.3	84.2	77.2	81.1	83.7	77.0	76.7	74.3	84.5	81.5	76.5	77.4	84.1	82.8	82.1	83.8		84.9	83.6
27	*Pseudomonas mosselii* DSM 17497^{T}	85.7	75.4	81.8	77.0	78.7	82.1	81.4	88.5	85.5	85.0	85.5	77.8	81.4	84.6	77.2	76.9	74.5	90.4	82.0	76.9	77.6	84.6	82.8	82.0	84.8	84.9		84.3
28	*Pseudomonas parafulva* CRS01-1^{T}	84.0	75.5	81.3	76.9	78.8	81.5	81.1	83.8	83.9	83.4	83.4	77.5	81.2	83.0	77.3	76.9	74.8	83.7	81.4	76.8	77.8	83.4	81.8	81.5	83.2	83.6	84.3	

Table A4.2: A matrix representing *in silco* DNA:DNA hybridization values as determined with GGDC v. 2.1.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	***Pseudomonas pyomelaninifaciens* MR02 ^{T}**		21	27.7	21.9	23.1	25.7	25.1	30.4	33.1	40.4	40.5	21.9	24.9	30.6	22.1	22.1	20.7	29.9	25.4	22.2	22.8	28.7	26.3	25.4	38.7	29.4	31.1	27.9
2	*Pseudomonas oryzihabitans* NBRC 102199 ^{T}	21		20.2	19.8	20.2	20.6	20.6	21.1	20.8	20.8	20.5	21.6	19.9	20.7	20.8	21.1	20.2	20.8	20.6	21.1	21.5	20.6	20.4	20.4	20.6	20.7	21	20.6
3	*Pseudomonas fulva* NBRC 16637 ^{T}	27.7	20.2		21.3	21.7	23.2	22.9	25.8	26.3	27.2	26.6	20.8	22.6	25.4	21.3	21	20.1	25.1	23.2	21.2	21.3	24.8	23.8	23.3	26.7	24.8	25.5	24.4
4	*Pseudomonas graminis* DSM 11363 ^{T}	21.9	19.8	21.3		22.8	22.5	22.3	22	21.9	22	21.5	20.2	22.2	21.7	21	20.7	19.9	21.7	22.4	20.5	20.9	21.7	21.4	22.3	21.6	22.1	21.8	21.6
5	*Pseudomonas asplenii* ATCC 23835 ^{T}	23.1	20.2	21.7	22.8		23.7	23.4	22.9	22.9	23.1	22.4	21.2	23.1	22.7	21.7	21.8	20.4	22.6	23.5	21.3	21.9	22.6	22.2	23.8	22.6	22.8	22.6	22.3
6	*Pseudomonas donghuensis* DSM 101685 ^{T}	25.7	20.6	23.2	22.5	23.7		31.5	25.4	25.3	25.1	24.6	21.4	30.7	24.4	21.8	21.7	20.6	24.8	26.6	21.6	22.2	24.6	23.8	51.4	24.8	25.7	25.2	23.9
7	*Pseudomonas alkylphenolica* KL28 ^{T}	25.1	20.6	22.9	22.3	23.4	31.5		24.7	24.5	24.7	24	21.3	32.1	23.9	21.5	21.9	20.5	24.2	25.8	21.7	22.5	24	23.5	32	24.2	24.7	24.6	23.9
8	*Pseudomonas entomophila* L48 ^{T}	30.4	21.1	25.8	22	22.9	25.4	24.7		30.1	29.5	29.6	22	24.5	28.7	22.1	21.9	21	34.2	25.2	21.9	22.3	28.7	26.3	25.1	29	29	36	27.9
9	*Pseudomonas plecoglossicida* DSM 15088 ^{T}	33.1	20.8	26.3	21.9	22.9	25.3	24.5	30.1		31.9	31.7	21.8	24.2	30.2	21.9	22	20.5	29.8	25.3	21.8	22.4	28.2	25.7	25.2	31.2	29.4	30.5	27.3
10	*Pseudomonas putida* NBRC14164 ^{T}	40.4	20.8	27.2	22	23.1	25.1	24.7	29.5	31.9		41.9	21.8	24.3	29.7	22.1	22.1	20.6	29.1	24.8	21.9	22.3	27.9	26	25	40	28.7	29.9	27
11	*Pseudomonas monteilii* NBRC 103158 ^{T}	40.5	20.5	26.6	21.5	22.4	24.6	24	29.6	31.7	41.9		21.2	23.7	29.5	21.8	21.8	20.3	28.7	24.4	21.5	22.6	27.4	24.9	24.5	40.9	28.3	30.6	26.2
12	*Pseudomonas aeruginosa* DSM 50071 ^{T}	21.9	21.6	20.8	20.2	21.2	21.4	21.3	22	21.8	21.8	21.2		20.9	21.1	21.8	21.6	20.4	21.6	22.1	21.5	22	21.7	21.1	21.3	21.2	21.5	21.8	21.4
13	*Pseudomonas vranovensis* DSM 16006 ^{T}	24.9	19.9	22.6	22.2	23.1	30.7	32.1	24.5	24.2	24.3	23.7	20.9		23.7	21.3	21.1	20.2	24	25.4	21.2	21.7	23.8	22.8	30.6	24.1	24.4	24.4	23.3
14	*Pseudomonas taiwanensis* DSM 21245 ^{T}	30.6	20.7	25.4	21.7	22.7	24.4	23.9	28.7	30.2	29.7	29.5	21.1	23.7		21.7	21.5	20.1	28	24.3	21.6	22.2	27.4	24.9	24.3	29.1	27.6	28.7	26
15	*Pseudomonas straminae* JCM 2783 ^{T}	22.1	20.8	21.3	21	21.7	21.8	21.5	22.1	21.9	22.1	21.8	21.8	21.3	21.7		23	20.9	21.9	22.2	23.3	23.5	21.8	21.6	21.8	21.6	21.8	21.9	21.7
16	*Pseudomonas toyotomiensis* DSM 26169 ^{T}	22.1	21.1	21	20.7	21.8	21.7	21.9	21.9	22	22.1	21.8	21.6	21.1	21.5	23		20.5	21.7	21.8	35.6	46.6	21.5	21.6	21.6	21.9	22.1	21.8	21.8
17	*Pseudomonas xanthomarina* DSM 18231 ^{T}	20.7	20.2	20.1	19.9	20.4	20.6	20.5	21	20.5	20.6	20.3	20.4	20.2	20.1	20.9	20.5		20.3	20.7	20.4	21	20.4	20.6	20.7	20.4	20.3	20.3	20.8
18	*Pseudomonas soli* LMG 27941 ^{T}	29.9	20.8	25.1	21.7	22.6	24.8	24.2	34.2	29.8	29.1	28.7	21.6	24	28	21.9	21.7	20.3		24.7	21.8	22.4	28	25.3	24.8	28.6	28.7	40.8	26.8
19	*Pseudomonas japonica* NBRC 103040 ^{T}	25.4	20.6	23.2	22.4	23.5	26.6	25.8	25.2	25.3	24.8	24.4	22.1	25.4	24.3	22.2	21.8	20.7	24.7		21.9	22.4	24.5	23.8	26.4	24.5	25	25.1	24
20	*Pseudomonas mendocina* NBRC 14162 ^{T}	22.2	21.1	21.2	20.5	21.3	21.6	21.7	21.9	21.8	21.9	21.5	21.5	21.2	21.6	23.3	35.6	20.4	21.8	21.9		36	21.6	21.5	21.6	21.6	21.9	21.9	21.7
21	*Pseudomonas oleovorans* subsp. Oleovorans DSM 1045 ^{T}	22.8	21.5	21.3	20.9	21.9	22.2	22.5	22.3	22.4	22.3	22.6	22	21.7	22.2	23.5	46.6	21	22.4	22.4	36		22.3	22	22.2	22.7	22.3	22.6	22.4
22	*Pseudomonas guariconensis* LMG 27394 ^{T}	28.7	20.6	24.8	21.7	22.6	24.6	24	28.7	28.2	27.9	27.4	21.7	23.8	27.4	21.8	21.5	20.4	28	24.5	21.6	22.3		25.1	24.5	27.3	27.8	28.7	26.5
23	*Pseudomonas cremoricolorata* ND07 ^{T}	26.3	20.4	23.8	21.4	22.2	23.8	23.5	26.3	25.7	26	24.9	21.1	22.8	24.9	21.6	21.6	20.6	25.3	23.8	21.5	22	25.1		23.6	25.2	25.7	25.8	24.9
24	*Pseudomonas wadenswilerensis* CCOS 864 ^{T}	25.4	20.4	23.3	22.3	23.8	51.4	32	25.1	25.2	25	24.5	21.3	30.6	24.3	21.8	21.6	20.7	24.8	26.4	21.6	22.2	24.5	23.6		24.6	25.7	25.1	23.9
25	*Pseudomonas hunanensis* P11 ^{T}	38.7	20.6	26.7	21.6	22.6	24.8	24.2	29	31.2	40	40.9	21.2	24.1	29.1	21.6	21.9	20.4	28.6	24.5	21.6	22.7	27.3	25.2	24.6		28	29.7	26.5
26	*Pseudomonas reidholzensis* CCOS 865 ^{T}	29.4	20.7	24.8	22.1	22.8	25.7	24.7	29	29.4	28.7	28.3	21.5	24.4	27.6	21.8	22.1	20.3	28.7	25	21.9	22.3	27.8	25.7	25.7	28		29.2	26.5
27	*Pseudomonas mosselii* DSM 17497 ^{T}	31.1	21	25.5	21.8	22.6	25.2	24.6	36	30.5	29.9	30.6	21.8	24.4	28.7	21.9	21.8	20.3	40.8	25.1	21.9	22.6	28.7	25.8	25.1	29.7	29.2		27.3
28	*Pseudomonas parafulva* CRS01-1 ^{T}	27.9	20.6	24.4	21.6	22.3	23.9	23.9	27.9	27.3	27	26.2	21.4	23.3	26	21.7	21.8	20.8	26.8	24	21.7	22.4	26.5	24.9	23.9	26.5	26.5	27.3	

Appendix 5

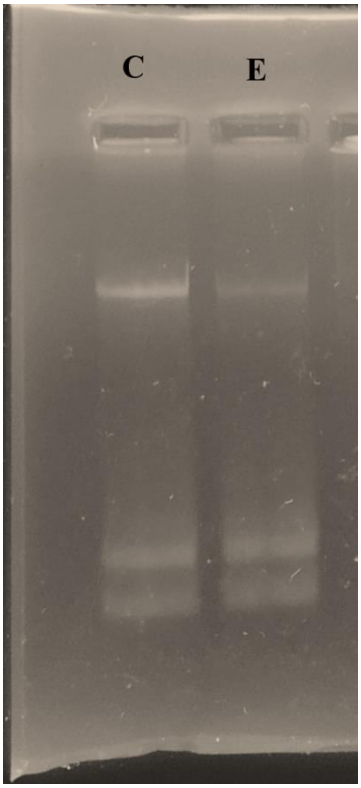

Figure A5.1: Agarose gel electrophoresis (1%) of total RNA extracts from *Pseudomonas* sp. MR 02 for transcriptome sequencing

Lane C: RNA sample from minimal media grown cell with glucose as carbon source, Lane E: RNA sample from minimal media grown MR 02 cell with ampicillin as carbon source

Table A5.1: Expression profile of upregulated genes in ampicillin-grown *Pseudomonas* sp. MR 02 cells

Gene Name/Locus Tag	Locus	FPKM_C	FPKM_E	Log2Fold change	P value	Significance	Gene Description	Pathway Annotation
CSW00_RS00090	NZ_PESJ01000001.1:20673-21789	135.253	239.757	0.82591	0.0332	Yes	branched-chain amino acid ABC transporter substrate-binding protein:WP_015271528.1	K01999 livK; branched-chain amino acid transport system substrate-binding protein
CSW00_RS00420	NZ_PESJ01000001.1:88592-89510	151.058	378.018	1.32336	0.00045	Yes	glutamate/aspartate ABC transporter substrate-binding protein:WP_015271573.1	K10001 gltI, aatJ; glutamate/aspartate transport system substrate-binding protein
CSW00_RS01565	NZ_PESJ01000001.1:328078-328513	112.739	465.496	2.04578	0.00135	Yes	DUF1043 family protein:WP_003257164.1	Pathway annotation not available
CSW00_RS01865	NZ_PESJ01000001.1:393016-393364	231.951	984.45	2.0855	0.0007	Yes	HPF/RaiA family ribosome-associated protein:WP_003260689.1	Pathway annotation not available
CSW00_RS00130	NZ_PESJ01000001.1:29740-30205	231.887	759.524	1.71167	0.001	Yes	glycine zipper 2TM domain-containing protein:WP_013973925.1	Pathway annotation not available
CSW00_RS00320	NZ_PESJ01000001.1:66837-68055	143.945	300.19	1.06036	0.00505	yes	argininosuccinate synthase:WP_013973956.1	K01940 argG, ASS1; argininosuccinate synthase [EC:6.3.4.5]
CSW00_RS00345	NZ_PESJ01000001.1:71136-71739	164.775	451.604	1.45456	0.001	yes	peroxiredoxin:WP_003254945.1	K03386 PRDX2_4, ahpC; peroxiredoxin (alkyl hydroperoxide reductase subunit C) [EC:1.11.1.15]
CSW00_RS00615	NZ_PESJ01000001.1:13643	177.704	421.172	1.24494	0.0066	yes	anti sigma-E protein%2C	Pathway annotation

	2-137023						RseA:WP_015271600.1	not available
CSW00_RS01390	NZ_PESJ01000001.1:293675-294587	151.131	268.579	0.829548	0.03065	yes	UDP-3-O-acyl-N-acetylglucosamine deacetylase:WP_003260771.1	K02535 lpxC; UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase [EC:3.5.1.108]
CSW00_RS03055	NZ_PESJ01000002.1:213889-214219	206.676	545.504	1.40022	0.02775	yes	hypothetical protein:WP_013974748.1	Pathway annotation not available
CSW00_RS03590	NZ_PESJ01000003.1:46928-47273	171.248	626.406	1.87101	0.00445	yes	hypothetical protein:WP_100412860.1	Pathway annotation not available
CSW00_RS03820	NZ_PESJ01000003.1:86423-87578	160.113	300.02	0.905965	0.0189	yes	aminotransferase class V-fold PLP-dependent enzyme:WP_054572084.1	K04487 iscS, NFS1; cysteine desulfurase [EC:2.8.1.7]
CSW00_RS04375	NZ_PESJ01000003.1:170461-172687	135.082	278.685	1.0448	0.009	yes	NADP-dependent isocitrate dehydrogenase:WP_013973293.1	K00031 IDH1, IDH2, icd; isocitrate dehydrogenase [EC:1.1.1.42]
CSW00_RS03420	NZ_PESJ01000003.1:10482-10695	225.64	1419.37	2.65315	0.0485	yes	cold-shock protein:WP_003257941.1	Pathway annotation not available
CSW00_RS04370	NZ_PESJ01000003.1:168777-170034	122.834	210.796	0.779131	0.0405	yes	NADP-dependent isocitrate dehydrogenase:WP_013973292.1	K00031 IDH1, IDH2, icd; isocitrate dehydrogenase [EC:1.1.1.42]
CSW00_RS05520	NZ_PESJ01000004.1:142696-144019	182.153	357.262	0.971832	0.0118	yes	flagellar hook protein FlgE:WP_023661398.1	K02390 flgE; flagellar hook protein FlgE
CSW00_RS05575	NZ_PESJ01000004.1:154505-156227	149.078	303.08	1.02363	0.00975	yes	flagellin:WP_100412935.1	K02406 fliC; flagellin

CSW00_RS05715,CSW00_RS05720	NZ_PESJ01000004.1:182762-184333	300.568	540.871	0.847591	0.02095	yes	please refer to the supplement_sheet_1	please refer to the supplement_sheet_2
CSW00_RS05790	NZ_PESJ01000004.1:199380-199956	187.707	423.462	1.17375	0.00885	yes	GNAT family N-acetyltransferase:WP_100412950.1	Pathway annotation not available
CSW00_RS05795	NZ_PESJ01000004.1:200029-200752	151.601	333.953	1.13937	0.0071	yes	hypothetical protein:WP_100412951.1	Pathway annotation not available
CSW00_RS05800	NZ_PESJ01000004.1:200775-202005	182.238	374.21	1.03802	0.0069	yes	MFS transporter:WP_003257315.1	Pathway annotation not available
CSW00_RS04915	NZ_PESJ01000004.1:19612-19993	129.255	333.389	1.36699	0.03105	yes	hypothetical protein:WP_013973727.1	Pathway annotation not available
CSW00_RS05205	NZ_PESJ01000004.1:81626-82043	225.784	568.295	1.3317	0.0118	yes	hypothetical protein:WP_013973674.1	Pathway annotation not available
CSW00_RS05925	NZ_PESJ01000005.1:9985-11272	153.023	396.981	1.37532	0.00065	yes	carbohydrate ABC transporter substrate-binding protein:WP_054573357.1	K17315 gtsA, glcE; glucose/mannose transport system substrate-binding protein
CSW00_RS05945	NZ_PESJ01000005.1:14399-15752	158.588	314.747	0.988911	0.01115	yes	porin:WP_100412956.1	Pathway annotation not available
CSW00_RS07955	NZ_PESJ01000007.1:36689-37676	206.664	533.983	1.3695	0.0003	yes	NAD(P)/FAD-dependent oxidoreductase:WP_015268950.1	Pathway annotation not available
CSW00_RS08065	NZ_PESJ01000007.1:56065-56659	133.474	262.385	0.975131	0.0384	yes	50S ribosomal protein L25/general stress protein Ctc:WP_013970896.1	K02897 RP-L25, rplY; large subunit ribosomal protein L25
CSW00_RS07780	NZ_PESJ01000007.1:3717-4504	166.519	411.782	1.30619	0.0139	yes	peptidylprolyl isomerase:WP_003259610.1	Pathway annotation not available
CSW00_RS09475	NZ_PESJ01000008.1:150844-151150	277.981	997.029	1.84265	0.0043	yes	STAS-like domain-containing protein:WP_080516231.1	Pathway annotation not available

CSW00_RS09995	NZ_PESJ01000009.1:76298-76781	236.959	623.546	1.39586	0.0047	yes	hypothetical protein:WP_015268607.1	Pathway annotation not available
CSW00_RS10625	NZ_PESJ01000010.1:23099-24188	133.415	231.958	0.797948	0.03825	yes	asparaginase:WP_015269784.1	K05597 aspQ, ansB, ansA; glutamin-(asparagin-)ase [EC:3.5.1.38]
CSW00_RS11360	NZ_PESJ01000010.1:158195-158849	133.73	279.788	1.06501	0.0168	yes	helix-turn-helix domain-containing protein:WP_041166538.1	Pathway annotation not available
CSW00_RS11095,CSW00_RS11100	NZ_PESJ01000010.1:119969-120595	427.125	1508.44	1.82033	0.0026	yes	please refer to the supplement_sheet_1	please refer to the supplement_sheet_2
CSW00_RS11730,CSW00_RS11735	NZ_PESJ01000011.1:56475-58448	322.304	1376.97	2.095	0.03465	yes	please refer to the supplement_sheet_1	please refer to the supplement_sheet_2
CSW00_RS11920	NZ_PESJ01000011.1:102503-103814	124.142	220.15	0.826495	0.0294	yes	nucleotide sugar dehydrogenase:WP_100413220.1	K13015 wbpA; UDP-N-acetyl-D-glucosamine dehydrogenase [EC:1.1.1.136]
CSW00_RS12165	NZ_PESJ01000011.1:150262-150535	236.954	804.637	1.76373	0.02405	yes	DUF3509 domain-containing protein:WP_013971511.1	Pathway annotation not available
CSW00_RS12560	NZ_PESJ01000012.1:61152-61695	242.96	501.665	1.046	0.0156	yes	glycine zipper 2TM domain-containing protein:WP_013971012.1	Pathway annotation not available
CSW00_RS12620	NZ_PESJ01000012.1:70223-70655	133.994	433.206	1.69289	0.00365	yes	nucleoside-diphosphate kinase:WP_013971021.1	K00940 ndk, NME; nucleoside-diphosphate kinase [EC:2.7.4.6]
CSW00_RS12950	NZ_PESJ01000012.1:146031-146340	247.853	2075.53	3.06592	0.00005	yes	ribosome-associated translation inhibitor RaiA:WP_003255135.1	Pathway annotation not available
CSW00_RS13935	NZ_PESJ01000014.1:23053-	159.473	734.871	2.20418	0.00005	yes	porin family	Pathway annotation

	23659						protein:WP_013973837.1	not available
CSW00_RS14050	NZ_PESJ01000014.1:47081-48350	153.557	349.256	1.18551	0.0031	yes	OprD family porin:WP_013973818.1	K18093 oprD; imipenem/basic amino acid-specific outer membrane pore [EC:3.4.21.-]
CSW00_RS14800	NZ_PESJ01000015.1:47491-47806	193.554	534.086	1.46433	0.03045	yes	hypothetical protein:WP_003257582.1	Pathway annotation not available
CSW00_RS15395	NZ_PESJ01000016.1:23049-24084	181.193	307.643	0.763728	0.0422	yes	ABC transporter substrate-binding protein:WP_015268730.1	K02055 ABC.SP.S; putative spermidine/putrescine transport system substrate-binding protein
CSW00_RS16300	NZ_PESJ01000017.1:97647-98685	173.834	1508.08	3.11693	0.00005	yes	OmpA family protein:WP_004376136.1	Pathway annotation not available
CSW00_RS16335	NZ_PESJ01000017.1:106046-106262	386.743	28896.9	6.2234	0.00225	yes	hypothetical protein:WP_003248687.1	Pathway annotation not available
CSW00_RS16355	NZ_PESJ01000017.1:113275-113620	169.531	1132.79	2.74025	0.0001	yes	YggL family protein:WP_004376110.1	Pathway annotation not available
CSW00_RS16525	NZ_PESJ01000018.1:19436-20255	108.69	219.071	1.01117	0.01765	yes	phosphate ABC transporter ATP-binding protein:WP_024086823.1	K02036 pstB; phosphate transport system ATP-binding protein [EC:7.3.2.1]
CSW00_RS16700	NZ_PESJ01000018.1:52411-53287	149.72	294.73	0.977124	0.0124	yes	transporter substrate-binding domain-containing protein:WP_015270051.1	Pathway annotation not available
CSW00_RS16715	NZ_PESJ01000018.1:55358-57143	128.309	216.642	0.755689	0.04915	yes	PQQ-dependent dehydrogenase%2C methanol/ethanol family:WP_024086834.1	Pathway annotation not available

CSW00_RS16720	NZ_PESJ01000018.1:57305-58826	126.894	368.556	1.53826	0.0001	yes	aldehyde dehydrogenase:WP_015270055.1	K00138 aldB; aldehyde dehydrogenase [EC:1.2.1.-]
CSW00_RS16605,CSW00_RS16610	NZ_PESJ01000018.1:33752-34924	304.875	847.632	1.47522	0.0018	yes	please refer to the supplement_sheet_1	please refer to the supplement_sheet_2
CSW00_RS16690	NZ_PESJ01000018.1:49794-51690	124.504	532.707	2.09715	0.00005	yes	PQQ-dependent dehydrogenase%2C methanol/ethanol family:WP_043935556.1	K00114 exaA; alcohol dehydrogenase (cytochrome c) [EC:1.1.2.8]
CSW00_RS17625	NZ_PESJ01000019.1:99295-100492	126.736	273.676	1.11064	0.0036	yes	aspartate/tyrosine/aromatic aminotransferase:WP_015270691.1	K00832 tyrB; aromatic-amino-acid transaminase [EC:2.6.1.57]
CSW00_RS17215	NZ_PESJ01000019.1:23160-24534	140.566	292.662	1.05799	0.00615	yes	LOG family protein:WP_100413456.1	K06966 ppnN; pyrimidine/purine-5'-nucleotide nucleosidase [EC:3.2.2.10 3.2.2.-]
CSW00_RS18400	NZ_PESJ01000021.1:27593-27926	160.616	434.273	1.43499	0.02735	yes	hypothetical protein:WP_003253895.1	Pathway annotation not available
CSW00_RS19105	NZ_PESJ01000022.1:51802-53506	127.186	220.516	0.793935	0.0388	yes	glutamine--tRNA ligase/YqeY domain fusion protein:WP_015270632.1	K01886 QARS, glnS; glutaminyl-tRNA synthetase [EC:6.1.1.18]
CSW00_RS18970	NZ_PESJ01000022.1:24597-25497	122.702	218.827	0.834632	0.0415	yes	hydroxymethylglutaryl-CoA lyase:WP_015270649.1	K01640 E4.1.3.4, HMGCL, hmgL; hydroxymethylglutaryl-CoA lyase [EC:4.1.3.4]

CSW00_RS19210	NZ_PESJ01000022.1:76950-78195	123.428	222.788	0.852004	0.0259	yes	saccharopine dehydrogenase family protein:WP_003248123.1	K00290 LYS1; saccharopine dehydrogenase (NAD+, L-lysine forming) [EC:1.5.1.7]
CSW00_RS20415	NZ_PESJ01000024.1:84741-86677	145.196	311.396	1.10074	0.0351	yes	site-specific integrase:WP_100413640.1	Pathway annotation not available
CSW00_RS29130	NZ_PESJ01000024.1:2009-2942	96.2771	170.382	0.823513	0.0378	yes	hypothetical protein:WP_137109010.1	Pathway annotation not available
CSW00_RS20040	NZ_PESJ01000024.1:2943-4431	94.1373	171.665	0.866758	0.0224	yes	ATP-binding protein:WP_137109011.1	Pathway annotation not available
CSW00_RS20820	NZ_PESJ01000025.1:54972-55650	129.243	250.006	0.951871	0.0372	yes	DUF3313 domain-containing protein:WP_085720250.1	Pathway annotation not available
CSW00_RS20765	NZ_PESJ01000025.1:42573-43095	139.446	331.605	1.24976	0.0159	yes	acyloxyacyl hydrolase:WP_015270391.1	K12976 pagL; lipid A 3-O-deacylase [EC:3.1.1.-]
CSW00_RS21705	NZ_PESJ01000027.1:38493-38862	108.469	290.352	1.42051	0.0319	yes	hypothetical protein:WP_015269978.1	Pathway annotation not available
CSW00_RS21865	NZ_PESJ01000027.1:83219-84083	123.981	235.284	0.924287	0.02135	yes	sensor domain-containing diguanylate cyclase:WP_100413707.1	Pathway annotation not available
CSW00_RS22025	NZ_PESJ01000028.1:11917-12937	139.409	308.122	1.14417	0.0042	yes	branched-chain amino acid aminotransferase:WP_013972500.1	K00826 E2.6.1.42, ilvE; branched-chain amino acid aminotransferase [EC:2.6.1.42]
CSW00_RS22620	NZ_PESJ01000029.1:53013-53307	142.613	468.081	1.71465	0.02395	yes	hypothetical protein:WP_015271093.1	Pathway annotation not available
CSW00_RS22655	NZ_PESJ01000029.1:61229-	128.865	270.685	1.07076	0.0108	yes	2-oxoglutarate dehydrogenase E1	K00164 OGDH, sucA; 2-oxoglutarate

	64061						component:WP_015271096.1	dehydrogenase E1 component [EC:1.2.4.2]
CSW00_RS22665	NZ_PESJ01000029.1:65022-66795	140.364	248.891	0.826339	0.0349	yes	succinate dehydrogenase flavoprotein subunit:WP_013973442.1	K00239 sdhA, frdA; succinate dehydrogenase / fumarate reductase, flavoprotein subunit [EC:1.3.5.1 1.3.5.4]
CSW00_RS22720	NZ_PESJ01000029.1:74685-75615	147.049	272.469	0.889796	0.0224	yes	electron transfer flavoprotein subunit alpha:WP_013973453.1	Pathway annotation not available
CSW00_RS22725	NZ_PESJ01000029.1:75615-76365	140.119	328.139	1.22765	0.00445	yes	electron transfer flavoprotein subunit beta/FixA family protein:WP_015271101.1	Pathway annotation not available
CSW00_RS23265	NZ_PESJ01000030.1:75820-76738	145.177	255.773	0.817052	0.03645	yes	DUF3577 domain-containing protein:WP_022580324.1	Pathway annotation not available
CSW00_RS23345	NZ_PESJ01000030.1:85255-86157	128.765	280.692	1.12424	0.00575	yes	please refer to the supplement_sheet_1	please refer to the supplement_sheet_2
CSW00_RS23395	NZ_PESJ01000031.1:8226-9363	147.173	270.51	0.878162	0.02145	yes	FAD-binding oxidoreductase:WP_015269674.1	K19746 dauA; D-arginine dehydrogenase [EC:1.4.99.6]
CSW00_RS23400	NZ_PESJ01000031.1:9375-9753	132.249	388.048	1.55298	0.0166	yes	RidA family protein:WP_015269675.1	Pathway annotation not available
CSW00_RS23560	NZ_PESJ01000031.1:39194-41591	136.031	244.638	0.84671	0.0329	yes	endopeptidase La:WP_015269697.1	K01338 lon; ATP-dependent Lon protease [EC:3.4.21.53]
CSW00_RS23565	NZ_PESJ01000031.1:41743-42016	273.111	791.385	1.53489	0.02935	yes	HU family DNA-binding protein:WP_003259401.1	Pathway annotation not available

CSW00_RS23600	NZ_PESJ01000031.1:47646-48015	174.18	468.027	1.42602	0.01455	yes	DUF962 domain-containing protein:WP_015269702.1	Pathway annotation not available
CSW00_RS24690	NZ_PESJ01000034.1:22144-22927	144.605	259.675	0.844589	0.04425	yes	AraC family transcriptional regulator:WP_013972825.1	Pathway annotation not available
CSW00_RS25170	NZ_PESJ01000035.1:44995-45943	117.048	205.31	0.810705	0.03635	yes	inovirus-type Gp2 protein:WP_100413902.1	Pathway annotation not available
CSW00_RS25395	NZ_PESJ01000036.1:24982-25588	144.672	304.088	1.07171	0.0217	yes	DUF4823 domain-containing protein:WP_015269417.1	Pathway annotation not available
CSW00_RS26085	NZ_PESJ01000038.1:32904-33747	119.609	226.935	0.923948	0.02565	yes	S24 family peptidase:WP_100413966.1	Pathway annotation not available
CSW00_RS26095	NZ_PESJ01000038.1:34375-34840	106.524	235.941	1.14725	0.0401	yes	hypothetical protein:WP_137108992.1	Pathway annotation not available
CSW00_RS27275	NZ_PESJ01000043.1:27922-28462	127.017	285.799	1.16997	0.02025	yes	gluconokinase:WP_100414074.1	K00851 E2.7.1.12, gntK, idnK; gluconokinase [EC:2.7.1.12]
CSW00_RS27910	NZ_PESJ01000049.1:1744-3091	169.184	376.423	1.15377	0.00305	yes	aspartate aminotransferase family protein:WP_054572853.1	K00822 E2.6.1.18; beta-alanine--pyruvate transaminase [EC:2.6.1.18]
CSW00_RS28460	NZ_PESJ01000056.1:3748-4711	139.002	240.94	0.793572	0.0429	yes	D-glycerate dehydrogenase:WP_015270505.1	K00032 E1.1.1.43; phosphogluconate 2-dehydrogenase [EC:1.1.1.43]
CSW00_RS28465	NZ_PESJ01000056.1:4722-6015	129.451	228.774	0.821524	0.0317	yes	MFS transporter:WP_015270506.1	Pathway annotation not available
CSW00_RS28470,CSW00_RS28475	NZ_PESJ01000056:6072-7804	275.452	484.942	0.816008	0.0285	yes	please refer to the supplement_sheet_1	please refer to the supplement_sheet_2

CSW00_RS28480	NZ_PESJ01000056.1:7871-8891	123.137	218.392	0.826655	0.0326	yes	LacI family DNA-binding transcriptional regulator:WP_013972769.1	Pathway annotation not available
CSW00_RS28580	NZ_PESJ01000058.1:3532-4990	105.045	179.719	0.774736	0.04165	yes	hypothetical protein:WP_100414182.1	Pathway annotation not available
CSW00_RS28735	NZ_PESJ01000062.1:4457-4886	157.453	598.772	1.92708	0.0012	yes	DUF3363 domain-containing protein:WP_077946514.1	Pathway annotation not available
CSW00_RS28765	NZ_PESJ01000063.1:3069-3882	138.156	552.734	2.00028	0.00005	yes	subclass B1 metallo-beta-lactamase NDM-1:WP_004201164.1	Pathway annotation not available
CSW00_RS28775	NZ_PESJ01000063.1:4259-4495	810.685	2138.72	1.39954	0.0435	yes	IS91 family transposase:.	Pathway annotation not available

Table A5.2: Expression profile of downregulated genes in ampicillin-grown *Pseudomonas* sp. MR 02 cells

Gene Name/Locus Tag	Locus	FPKM_C	FPKM_E	log2Foldchange	P value	Significance	Gene Description	Pathway Annotation
CSW00_RS00405	NZ_PESJ01000001.1:81447-82203	243.448	128.641	-0.920262	0.02305	Yes	DeoR/GlpR family transcriptional regulator:WP_015271570.1	Pathway annotation not available
CSW00_RS02450	NZ_PESJ01000002.1:91429-92098	190.185	48.7145	-1.96498	0.00005	Yes	NAD(P)H dehydrogenase (quinone):WP_015272171.1	Pathway annotation not available
CSW00_RS02600	NZ_PESJ01000002.1:123573-124062	210.223	77.2358	-1.44458	0.01065	Yes	transporter substrate-binding domain-containing protein:WP_100412811.1	Pathway annotation not available
CSW00_RS02925	NZ_PESJ01000002.1:188079-189192	263.349	148.963	-0.822022	0.02995	Yes	ABC transporter permease:WP_054573424.1	Pathway annotation not available
CSW00_RS03170	NZ_PESJ01000002.1:240633-242061	249.338	146.664	-0.765584	0.04815	Yes	mechanosensitive ion channel:WP_054572018.1	Pathway annotation not available
CSW00_RS02465	NZ_PESJ01000002.1:94926-95442	372.913	110.076	-1.76034	0.00095	Yes	dihydrofolate reductase:WP_039612552.1	K00287 DHFR, folA; dihydrofolate reductase [EC:1.5.1.3]
CSW00_RS02480	NZ_PESJ01000002.1:98370-98919	363.418	162.882	-1.1578	0.0172	Yes	HD domain-containing protein:WP_015272177.1	Pathway annotation not available
CSW00_RS02555	NZ_PESJ01000002.1:114185-114611	422.059	198.075	-1.0914	0.0409	Yes	DUF2269 domain-containing protein:WP_015272187.1	Pathway annotation not available
CSW00_RS04235	NZ_PESJ01000003.1:147423-148002	181.958	59.5475	-1.61149	0.0012	yes	cysteine hydrolase:WP_100412886.1	Pathway annotation not available

CSW00_RS06930	NZ_PESJ01000006.1:13270-13819	220.866	109.005	-1.01878	0.04265	Yes	hypothetical protein:WP_100412987.1	Pathway annotation not available
CSW00_RS07395	NZ_PESJ01000006.1:119330-120512	233.406	127.207	-0.875668	0.02425	Yes	NAD(P)/FAD-dependent oxidoreductase:WP_015272049.1	Pathway annotation not available
CSW00_RS08585	NZ_PESJ01000007.1:165795-167004	273.679	164.414	-0.735155	0.04935	Yes	type II secretion system F family protein:WP_015268864.1	Pathway annotation not available
CSW00_RS08695	NZ_PESJ01000007.1:182395-182911	302.039	100.496	-1.5876	0.001	Yes	type IV pili biogenesis protein FimT:WP_043935296.1	Pathway annotation not available
CSW00_RS08475	NZ_PESJ01000007.1:142616-143501	257.564	129.653	-0.990276	0.0099	Yes	LysR family transcriptional regulator:WP_061303411.1	K03566 gcvA; LysR family transcriptional regulator, glycine cleavage system transcriptional activator
CSW00_RS09615	NZ_PESJ01000008.1:180456-181131	269.469	132.469	-1.02447	0.01375	Yes	heavy metal response regulator transcription factor:WP_003253420.1	Pathway annotation not available
CSW00_RS08975	NZ_PESJ01000008.1:43479-44376	298.904	169.943	-0.814634	0.03245	Yes	M23 family metallopeptidase:WP_013974816.1	Pathway annotation not available
CSW00_RS09170	NZ_PESJ01000008.1:84621-85593	324.831	182.044	-0.835407	0.02805	Yes	GTP-binding protein:WP_100413076.1	Pathway annotation not available
CSW00_RS09190	NZ_PESJ01000008.1:87667-89084	242.109	61.8187	-1.96954	0.01605	Yes	DUF3617 domain-containing protein:WP_015272374.1	Pathway annotation not available
CSW00_RS09745	NZ_PESJ01000009.1:26182-26440	401.661	45.2978	-3.14847	0.01365	Yes	hypothetical protein:WP_003257487.1	Pathway annotation not available

CSW00_RS10425	NZ_PESJ01000009.1:167197-167653	284.353	124.445	-1.19218	0.03155	Yes	hypothetical protein:WP_015268533.1	Pathway annotation not available
CSW00_RS10470	NZ_PESJ01000009.1:174130-174925	243.565	141.767	-0.780788	0.0482	Yes	sel1 repeat family protein:WP_100413106.1	Pathway annotation not available
CSW00_RS11485	NZ_PESJ01000011.1:3700-3925	270.744	35.9367	-2.9134	0.0455	Yes	hypothetical protein:WP_003257337.1	Pathway annotation not available
CSW00_RS11615	NZ_PESJ01000011.1:30769-31738	226.907	124.262	-0.868718	0.02175	Yes	EAL domain-containing protein:WP_015269272.1	Pathway annotation not available
CSW00_RS12835	NZ_PESJ01000012.1:122646-123021	261.145	97.9017	-1.41544	0.0166	Yes	4-carboxymuconolactone decarboxylase:WP_013971059.1	K01607 pcaC; 4-carboxymuconolactone decarboxylase [EC:4.1.1.44]
CSW00_RS13450	NZ_PESJ01000013.1:86207-86651	384.036	130.017	-1.56254	0.00625	Yes	helix-turn-helix transcriptional regulator:WP_100413294.1	Pathway annotation not available
CSW00_RS13410	NZ_PESJ01000013.1:80449-80794	385.559	81.5268	-2.2416	0.0015	Yes	hypothetical protein:WP_043935270.1	Pathway annotation not available
CSW00_RS13985	NZ_PESJ01000014.1:30773-31100	685.344	284.64	-1.26769	0.028	Yes	hypothetical protein:.	Pathway annotation not available
CSW00_RS14835	NZ_PESJ01000015.1:54262-55201	264.559	147.956	-0.838419	0.02745	Yes	LD-carboxypeptidase:WP_100413356.1	Pathway annotation not available
CSW00_RS14970	NZ_PESJ01000015.1:82306-82936	246.175	122.677	-1.00483	0.0181	Yes	hypothetical protein:WP_061303515.1	Pathway annotation not available

CSW00_RS15330	NZ_PESJ01000016.1:9559-10600	269.089	152.358	-0.820617	0.0336	Yes	hypothetical protein:WP_015268737.1	Pathway annotation not available
CSW00_RS15550	NZ_PESJ01000016.1:59750-60041	454.429	157.825	-1.52573	0.04135	Yes	hypothetical protein:WP_015268711.1	Pathway annotation not available
CSW00_RS17080	NZ_PESJ01000018.1:126622-127888	125.536	12.3954	-3.34023	0.00005	Yes	MFS transporter:WP_039612867.1	Pathway annotation not available
CSW00_RS19500	NZ_PESJ01000023.1:15943-17602	232.613	122.539	-0.924694	0.01725	Yes	Na/Pi cotransporter family protein:WP_015268496.1	Pathway annotation not available
CSW00_RS19510	NZ_PESJ01000023.1:19162-20185	293.263	165.053	-0.829264	0.02955	Yes	DUF2914 domain-containing protein:WP_100413572.1	Pathway annotation not available
CSW00_RS19630	NZ_PESJ01000023.1:45532-46489	238.072	131.199	-0.859635	0.02415	Yes	zinc ABC transporter solute-binding protein:WP_043935186.1	K09815 znuA; zinc transport system substrate-binding protein
CSW00_RS19735	NZ_PESJ01000023.1:64217-65750	289.09	163.374	-0.823343	0.0349	Yes	SulP family inorganic anion transporter:WP_100413579.1	Pathway annotation not available
CSW00_RS19775	NZ_PESJ01000023.1:74209-75283	276.79	163.048	-0.763489	0.0463	Yes	aminopeptidase:WP_100413583.1	Pathway annotation not available
CSW00_RS19850	NZ_PESJ01000023.1:83632-84532	325.187	194.004	-0.745185	0.04865	Yes	LysR family transcriptional regulator:WP_015268447.1	Pathway annotation not available
CSW00_RS20630	NZ_PESJ01000025.1:14584-15235	356.527	197.862	-0.849515	0.03955	Yes	thiopurine S-methyltransferase:WP_100413648.1	K00569 TPMT, tpmT; thiopurine S-methyltransferase

								[EC:2.1.1.67]
CSW00_RS21440	NZ_PESJ01000026.1:87256-88150	149.288	44.1007	-1.75922	0.00005	Yes	2-hydroxy-3-oxopropionate reductase:WP_003259509.1	K00042 garR, glxR; 2-hydroxy-3-oxopropionate reductase [EC:1.1.1.60]
CSW00_RS23845	NZ_PESJ01000032.1:5580-5967	290.671	45.25	-2.68339	0.00005	Yes	hypothetical protein:WP_100413788.1	Pathway annotation not available
CSW00_RS27450	NZ_PESJ01000045.1:1787-2522	176.196	81.6009	-1.11052	0.01	Yes	secretin:WP_024087406.1	Pathway annotation not available
CSW00_RS27940	NZ_PESJ01000049.1:7594-8308	279.059	134.713	-1.05068	0.0098	Yes	helix-turn-helix transcriptional regulator:WP_013970739.1	Pathway annotation not available
CSW00_RS28215	NZ_PESJ01000052.1:2138-3002	252.055	129.262	-0.963439	0.01455	Yes	ABC transporter permease:WP_015268522.1	Pathway annotation not available
CSW00_RS28700	NZ_PESJ01000061.1:3457-4995	3928.59	269.425	-3.86606	0.02645	Yes	16S ribosomal RNA:.	Pathway annotation not available

Table D1: Physicochemical characteristics and bacteriological parameters of Mahananda and Karala River water samples

(sources: West Bengal Pollution Control Board, Siliguri Laboratory Paribesh Bhawan, Paribahan Nagar, P.O. Matigara, Siliguri, Darjeeling 734428. http://emis.wbpcb.gov.in/waterquality/viewsampledatacitizen.do).

Collection date →	Mahananda River sampling				Karala River				Unit
	09/03/2015	07/4/2015	18/11/2015	04/12/2015	18/03/2015	16/4/2015	15/11/2015	10/12/2015	
Parameter									
Ammonia-N	BDL	0.128	BDL	BDL	BDL	BDL	BDL	BDL	mg/L
BOD	2.80	1.50	2.10	1.60	2.10	1.80	2.10	1.00	mg/L
Boron	0.006	0.006	BDL	0.001	0.005	0.007	0.003	0.005	mg/L
Calcium	19.20	20.83	8.82	17.63	14.14	6.40	12.02	16.03	mg/L
Chloride	9.78	18.73	20.55	12.70	2.94	7.88	17.61	14.68	mg/L
COD	8.16	6.24	11.52	10.56	10.20	8.20	12.48	14.40	mg/L
Conductivity	185.00	192.00	151.60	146.60	114.50	127.60	89.94	98.24	µs/cm
Dissolved O2(DO)	8.90	8.20	6.30	6.50	8.10	7.80	5.60	6.50	mg/L
Fecal Coliform	4000	2600	9000	4000	1700	2700	7000	2700	MPN/100ml
Fluoride	0.203	0.246	0.129	0.167	0.150	0.332	0.118	0.401	mg/L
Magnesium	2.25	3.36	1.27	2.30	1.61	3.36	2.88	6.91	mg/L
Nitrate-N	0.02	0.92	0.50	0.15	0.20	0.10	0.22	006	mg/L
pH	7.46	6.89	7.31	6.92	7.68	7.20	7.53	7.61	Unit
Phenolpthalein Alkanity	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	mg/L
Phosphate-P	0.092	0.075	0.218	0.027	0.108	0.081	0.153	0.191	mg/L
Potassium	2.00	5.00	5.00	4.00	3.00	3.00	BDL	2.00	mg/L

Sodium	11.00	9.93	8.00	13.00	8.00	5.73	1	6.00	mg/L
Sulphate	17.94	20.00	16.90	25.78	5.24	4.50	7.47	7.81	mg/L
Temperature	25	22	20	20	28	34	28	20	°C
Total Alkalinity	64.00	74.00	76.00	48.00	60.00	36.00	32.0	56.00	mg/L
Total Coliform	17000	8000	33000	17000	11000	9000	17000	11000	MPN/100ml
Total Dissolved Solids(TDS)	136.00	108	85.00	126.00	115.00	67.00	84.0	123.00	mg/L
Total Fixed Solids(TFS)	74.00	12.00	90.00	102.00	74.00	42.00	11.60	68.00	mg/L
Total Hardness as CaCo3	56.00	66.00	66.00	52.00	41.00	30.00	40.00	64.00	mg/L
Total Suspended Solids(TSS)	30.00	270.00	34.00	30.00	10.00	12.00	20.00	8.00	mg/L
Turbidity	11.20	11.10	16.00	5.36	2.44	3.34	32.10	5.37	NTU

Publications and Conference papers

Ranjan VK, Saha T, Mukherjee S, Chakraborty R. Draft Genome Sequence of a Novel Bacterium, *Pseudomonas* sp. Strain MR 02, Capable of Pyomelanin Production, Isolated from the Mahananda River at Siliguri, West Bengal, India. *Genome Announcements*. 2018; 6(3):e01443-17; doi: 10.1128/genomeA.01443-17.

Ranjan VK, Mukherjee S, Thakur S, Gupta K, Chakraborty R. Pandrug-resistant *Pseudomonas* sp. expresses New Delhi metallo-β-lactamase-1 and consumes ampicillin as sole carbon source. *Clinical Microbiology and Infection*. 2020 Nov 4:S1198743X(20)30667-4. doi:10.1016/j.cmi.2020.10.032.

Ranjan VK, Tiwary BK, Chakraborty R. Quorum sensing dependent production of pyomelanin, in *Pseudomonas* sp. MR 02, offers an advantage to study mechanisms that alter biogeochemistry of the habitat. *Microbiology in the new Millennium: from Molecules to Communities*. 27-29 October 2017, Bose institute Kolkata, India (poster).

Ranjan VK, Mukherjee S, Thakur S, Chakraborty R. Super-superbug *Pseudomonas pyomelaninifaciens* MR 02, in water of River Mahananda at Siliguri, India. *2nd International Conference on Contemporary Antimicrobial Research*. 15-17 December 2018, Indian Institute Kharagpur, India (poster).

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PROKARYOTES

Draft Genome Sequence of a Novel Bacterium, *Pseudomonas* sp. Strain MR 02, Capable of Pyomelanin Production, Isolated from the Mahananda River at Siliguri, West Bengal, India

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ABSTRACT The draft genome sequence of a novel strain, *Pseudomonas* sp. MR 02, a pyomelanin-producing bacterium isolated from the Mahananda River at Siliguri, West Bengal, India, is reported here. This strain has a genome size of 5.94 Mb, with an overall G+C content of 62.6%. The draft genome reports 5,799 genes (mean gene length, 923 bp), among which 5,503 are protein-coding genes, including the genes required for the catabolism of tyrosine or phenylalanine for the characteristic production of homogentisic acid (HGA). Excess HGA, on excretion, auto-oxidizes and polymerizes to form pyomelanin.

The genome of *Pseudomonas* sp. strain MR 02 was sequenced using a NextSeq 500 system. Briefly, approximately 200 ng of DNA was fragmented by a Covaris M220 sonicator to generate ~400-bp segments. End-repaired products were size selected by AMPure XP beads, PCR amplified with index primers, and analyzed in a 4200 TapeStation system (Agilent Technologies). After obtaining the Qubit concentration, paired-end (PE) Illumina libraries, prepared using the Illumina TruSeq Nano DNA library preparation kit, were loaded onto the NextSeq 500 system for cluster generation and sequencing. The copied reverse strands were then used to sequence from the opposite end of the fragment. Thus, adapter-free data (1.1 Gb) were generated. The high-quality reads were then *de novo* assembled using the SPAdes genome assembler (1), and the quality of the assembly was evaluated using the QUAST software (2). The total size of the assembly (5,946,931 bp) was distributed in 90 scaffolds (average size of scaffolds, 66,077 bp; N_{50}, 144,752 bp). Genes were predicted from the assembled scaffolds using Prokka version 1.12 (3). Functional annotation of the genes was performed using the NCBI Prokaryotic Genome Annotation Pipeline, yielding a total of 5,503 protein-coding genes, of which 5,460 have shown BLAST hits. Gene Ontology (GO) annotations of the genes were determined by the Blast2GO platform. GO assignments were used to classify the functions of the predicted genes. A phylogenetic tree was constructed with an MR 02 scaffold fasta file and its closely related species using the Alignment and Assembly Free (AAF) phylogeny tool. The phylogenetic tree was later uploaded to MEGA 6 (http://www.megasoftware.net/web_help_7/helpfile.htm#). The neighbor-joining (NJ) tree with a bootstrap of 500 showed that the most closely related strains of MR 02 were *Pseudomonas putida* DLL-E4 and *Pseudomonas monteilii* strain SB3101.

Pyomelanin originates from the catabolism of tyrosine or phenylalanine (4). A complete breakdown of tyrosine to acetoacetate and fumarate requires the enzymes aromatic amino acid transaminase (TyrB), 4-hydroxyphenyl pyruvic acid dioxygenase (HppD), homogentisate dioxygenase (HmgA), maleylacetoacetate isomerase (MaiA), and fumarylacetoacetate hydrolase (FahA). Two copies each of *tyrB* (aromatic amino

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acid transaminase) and *hppD* (4-hydroxyphenylpyruvate dioxygenase) genes, along with the genes of the *hmg* (homogentisate dioxygenase) operon (*maiA*, *fahA*, and *hmgA*), were present in the MR 02 genome. Interestingly, the MR 02 *hmg* gene sequence produced 98% (1,279/1,302 bp) identities with the *hmg* gene of *P. putida* strain DLL-E4 and *hmg* operon of *P. monteilii* strain SB3101, indicating several point mutations. In the absence of functional HmgA, or if homogentisic acid (HGA) production exceeds that of HmgA activity, HGA is overproduced and excreted from the cells (5, 6). Consequently, pyomelanin will be formed nonenzymatically outside the cell. The excreted homogentisic acid will form benzoquinone acetic acid on chemical oxidation and undergo self-assembly, yielding pyomelanin polymers.

Accession number(s). The GenBank accession number for the 16S rRNA gene sequence of MR 02 is MF401548. This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number PESJ00000000. The version described in this paper is the first version, PESJ01000000. The sequence read data of assembled contigs have been deposited in the NCBI Sequence Read Archive under BioProject no. PRJNA415298. Strain MR 02 is currently available from the Korean Collection for Type Cultures (KCTC) under the accession number KCTC 62307.

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REFERENCES

1. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
2. Gurevich A, Saveliev V, Vyahhi N, Tesler G. 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics 29:1072–1075. https://doi.org/10.1093/bioinformatics/btt086.
3. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
4. Nelson DL, Cox MM. 2008. Amino acid oxidation and the production of urea, p 673–706. *In* Lehninger principles of biochemistry, 5th ed. W. H. Freeman and Company, New York, NY.
5. Yabuuchi E, Ohyama A. 1972. Characterization of "pyomelanin"-producing strains of *Pseudomonas aeruginosa*. Int J Syst Bacteriol 22:53–64. https://doi.org/10.1099/00207713-22-2-53.
6. Kotob SI, Coon SL, Quintero EJ, Weiner RM. 1995. Homogentisic acid is the primary precursor of melanin synthesis in *Vibrio cholerae*, a *Hyphomonas* strain, and *Shewanella colwelliana*. Appl Environ Microbiol 61:1620–1622.

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Research note

Pandrug-resistant *Pseudomonas* sp. expresses New Delhi metallo-β-lactamase-1 and consumes ampicillin as sole carbon source

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ABSTRACT

Objectives: This study aims to investigate ampicillin catabolism in a pandrug-resistant strain, *Pseudomonas* sp. MR 02 of *P. putida* lineage.
Methods: The characterization of carbapenem resistance was done following the standard protocol. The broth macrodilution method was used to determine the MIC values of antimicrobial agents both in the presence and in the absence of phenylalanine-β-naphthylamide. High MIC values (>10 000 mg/L) of ampicillin led to speculation that it may serve as a growth substrate, and thus minimal medium was used to evaluate ampicillin as a nutrient. The growth of MR 02 was measured in minimal medium in the presence or absence of 0.4 mM EDTA, supplemented with ampicillin as sole carbon, nitrogen and energy source. RNA-seq was used to generate expression profiles of genes in ampicillin or glucose-grown cells. The bla_{NDM-1} gene of MR 02 was cloned in the pHSG398 vector and expressed in *Escherichia coli* DH5α.
Results: Phenotypic analysis along with genome sequence data identifies *Pseudomonas* sp. MR 02 as a pandrug-resistant strain. Transcriptome data has revealed that bla_{NDM-1} was among the top 50 differentially expressed genes in ampicillin grown cells compared to the glucose grown cells in the minimal medium. Heterologous expression of bla_{NDM-1} gene in *E. coli* DH5α enabled its growth and subsistence on ampicillin as the sole source of carbon and energy.
Discussion: The ability of a pandrug-resistant *Pseudomonas* sp. MR 02 to consume ampicillin for growth has a huge implication in the bioremediation of β-lactam residues in the environment. **Vivek Kumar Ranjan, Clin Microbiol Infect 2020;▪:1**

Introduction

Antibiotic resistance is one of the major risks to global health. *Pseudomonas* spp., belonging to Gram-negative bacteria, known for their ubiquitous presence in soil and water, are also recognized to be associated with nosocomial infections by their multiple antibiotic-resistant members [1]. Hence, there is a rising concern in exploring the occurrence of antibiotic resistance in environmental *Pseudomonas* spp. The aquatic environment is seen as the hot spot for the acquisition and spread of antibiotic resistance. The presence of extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) in river water isolates has important implications for humans [2]. *Pseudomonas aeruginosa* is a well-known multidrug-resistant (MDR) pathogen [3,4], and also that environmental *Pseudomonas* such as *P. putida* and others are emerging with MDR profiles [5].

In the present study, an NDM-1 bearing river water isolate, *Pseudomonas* sp. MR 02, has shown a very high MIC value (>10 000 mg/L) of ampicillin. Earlier studies have found that hydrolysed ampicillin binds to the zinc ions and various critical residues present in the active site of NDM-1 through a number of coordination bonds and hydrogen bonds. Also, NDM-1 has a high catalytic efficiency (K_{cat}/K_m = 3.84 μM^{-1} s^{-1}) of ampicillin hydrolysis [6,7]. However, the mechanism of catalysis of ampicillin inactivation by NDM-1 remained obscure until combinational studies have proposed that active-site conformational fluctuation supports the enzymatic activity of NDM-1 which may lead additional studies

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about the catalytic mechanism [8]. As there was no certainty, but speculation (from extremely high MIC for ampicillin) that ampicillin may serve as any of the growth substrates, the minimal medium was used to evaluate ampicillin as a nutrient for MR 02. Subsequently, the growth curve of MR 02 in minimal medium devoid of glucose, but supplemented with ampicillin confirmed the utilization of ampicillin as the sole source of carbon and energy. In β-lactam antibiotic catabolism, amidases link resistance enzymes to central metabolism via phenylacetic acid catabolic pathway [9].

We hypothesized that possession and expression of NDM-1, in *Pseudomonas* sp. MR 02, would be the prime reason for its ability to tolerate high concentrations of ampicillin. It was also surmised that to catabolize ampicillin, innate support of active amidases and phenylacetic acid (*paa*) pathway genes should operate in the bacterium.

In this study, the molecular basis of ampicillin catabolism in an NDM-1-producing, pandrug-resistant (PDR) river water isolate, *Pseudomonas* sp. MR 02 of *P. putida* lineage, has been examined in detail. The data generated from whole-genome transcriptomics have provided evidence to support our hypothesis.

Materials and methods

Pseudomonas sp. MR 02 was isolated from a single sampling station on River Mahananda underneath the Mahananda bridge (26°43′11.52″N, 88°25′8.80″E) situated in the heart of Siliguri city, West Bengal, India.

Antibiotic susceptibility testing was performed by Kirby–Bauer disc diffusion method following CLSI guidelines and breakpoints [10]. MICs of antimicrobial agents under different antimicrobial categories (including the ones as per international expert proposal for interim standard definitions for acquired resistance in case of *Pseudomonas aeruginosa*) were determined by a standard broth macrodilution method, in Muller–Hinton broth, following BSAC and EUCAST guidelines [11–13]. The influence of efflux pump on MIC values was determined by the broth macrodilution method in the absence or presence of a broad-spectrum inhibitor of resistance-nodulation-cell division (RND) pumps, namely Phe-Arg-β-naphthylamide (PAβN) at a concentration of 30 mg/L. The $bla_{\text{NDM-1}}$ activity in MR 02 was determined by the broth macrodilution method in the presence of EDTA (0.4 mM).

For whole genome sequencing, paired-end libraries of total DNA (isolated by phenol: chloroform isolation method) were generated by using Illumina Traseq Nano DNA library preparation kit and then sequenced by Next Seq 500 system. The genome sequence of MR 02 was analysed for antibiotic-resistance genes (ARGs) using the CARD database [14], followed by validation with BLAST analysis against information in the COG, NCBI, CDD and Swiss-Prot databases. The MR 02 genome was compared with four other closely related *Pseudomonas* genomes by constructing the blast atlas using the BLAST Ring Image Generator (BRIG).

Growth of MR 02 was studied in minimal media containing ampicillin (6000 mg/L) as the sole source of carbon or both carbon and nitrogen. Whole transcriptome sequencing using Illumina Nextseq500 platform was done to compare the expression profile of genes in ampicillin-grown (test/treated) with glucose-grown (control) MR 02 cells. The *lac* promoter-enabled $bla_{\text{NDM-1}}$ (of MR 02) expression in *Escherichia coli* DH5α, using pHSG398 vector, was done to demonstrate growth and subsistence in minimal media containing ampicillin (100 mg/L) as the sole carbon and energy source (please see supplementary material).

Results

The MIC and disc diffusion test for strain MR 02 demonstrated resistance to 25 different antimicrobial agents belonging to 13 antimicrobial categories (Tables S1 and S2). In terms of the criteria for defining MDR, extensively drug-resistant (XDR) and PDR (as per international expert proposal for interim standard definitions for acquired resistance in case of *P. aeruginosa* [13]), it was found that *Pseudomonas* sp. MR02 was resistant to all agents in all antimicrobial categories (aminoglycosides, antipseudomonal carbapenems, antipseudomonal cephalosporins, antipseudomonal fluoroquinolones, antipseudomonal penicillins + β-lactamase inhibitors, monobactams, phosphonic acids and polymyxins). Therefore, MR 02 is a PDR strain.

In the presence of the efflux inhibitor, PAβN, fold reduction in the MICs of chloramphenicol, tetracycline, ciprofloxacin and levofloxacin were 50, 50, 75 and 6 respectively; thus demonstrating temporary reversion of the resistance to susceptible phenotype in the presence of PAβN. The MICs of imipenem, cefepime and ceftazidime, in the presence of PAβN, were reduced to a lesser degree (1.33-fold) and a 1.63-fold reduction in the MIC of meropenem. (Fig. S1a). It was observed that in the presence of metal chelator, EDTA, the fold reductions in MICs of imipenem, meropenem, cefepime and ceftazidime were 20.0, 5.26, 2.27 and 2.63 respectively (Fig. S1b).

The 16S rRNA gene sequence of *Pseudomonas* sp. MR 02 (Ac. No. MN252106) produced maximum identity (99.87%) with *P. monteilii* and *P. plecoglossicida*: 99.8% with *P. putida*; and 96.2% with *P. aeruginosa* in BlastN analysis. The overall similarity and the similarity of shared elements between two genomes were determined from the values obtained from *in silico* DNA: DNA hybridization (*is*DDH) and Average Nucleotide Identity (ANI), respectively. The *is*DDH and ANI values obtained for MR 02 with type strains, *P. monteilii* NBRC 103158^{T} or *P. plecoglossicida* DSM 15088^{T} or *P. putida* NBRC 14164^{T} or *P. aeruginosa* DSM 50071^{T} were 40.5% and 89.9% or 33.1% and 86.9% or 40.4% and 90.% or 28.7% and 84.5%, respectively. Hence, the distinctiveness of MR 02 from the other species was established (because an *is*DDH of ≥70% at the upper 95% confidence interval or an ANI value of ≥96% denote genomes belonging to the same species) [15]. A visual analysis system was used for gene characterization, functional assignment and function-based browsing of the five genomes, MR 02, *P. monteilii* NBRC 103158, *P. plecoglossicida* DSM 15088, *P. putida* NBRC 14164 and *P. aeruginosa* PA01 RefSeq genome (Ac. No. PRJNA57495). Non-homologous genomic regions present in the genomes of the four different species of *Pseudomonas*, compared with MR 02, have been presented where a sub-set of it was related to antibiotic resistance genes. Out of the predicted antibiotic resistance genes, 08 genes, extended-spectrum β-lactamase ($bla_{\text{PME-1}}$), β-lactamase (bla_{PDC}), tetracycline efflux MFS transporter Tet(G), TetR family transcriptional regulator, aminoglycoside 3-N-acetyltransferase (*aac*(3)-iiia), sulphonamide-resistant dihydropteroate synthase (*Sul*1), subclass B1 metallo-β-lactamase ($bla_{\text{NDM-1}}$) and aminoglycoside nucleotidyltransferase (*ant*(4)-IIb) were found unique to MR 02 (Fig. 1). Genome analyses identified a total of 98 genes associated with antibiotic resistance (Table S3). These genes are divided into two major groups coding for (a) the enzymes that conferred antibiotic resistance (Table S4a) and (b) the efflux pumps reported conferring antibiotic resistance (Table S4b). Sixty-four genes have been assigned to 21 efflux pump systems belonging to five different transporter families. Three different genes coding for β-lactamases in the MR 02 genome, belonging to class A (PME-1), class B (NDM-1) and class C (AMPC) β-lactamase family.

The strain MR 02 has shown its subsistence in minimal media supplemented with 6000 mg/L ampicillin as the sole source of carbon and nitrogen at 30°C (Fig. 2a); while it grew well in 6000 mg/L ampicillin as the sole source of carbon, demonstrating exponential growth from 120 hr to 168 hr of incubation, followed by the stationary phase (Fig. 2b). Under similar culture conditions, MR

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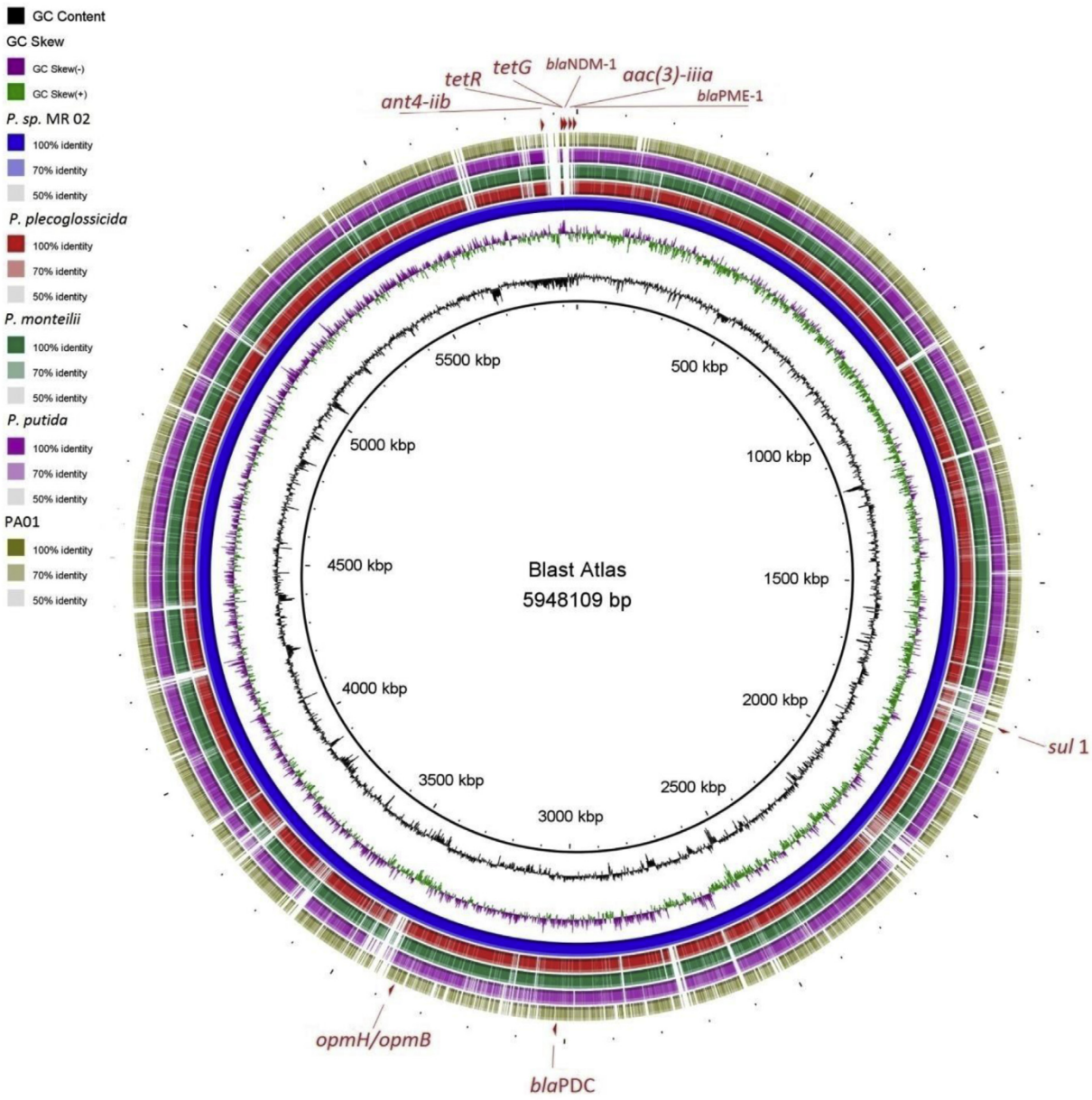

Fig. 1. Circular representation of the *Pseudomonas* sp. MR 02 draft genome in comparison with four reference genomes of *Pseudomonas* genus. Ring from inside to outside: (1) GC contents (black); (2) GC skew (purple and green); (3) coding sequences of *Pseudomonas* sp. MR 02 draft genome (dark blue); (4) BLAST comparison with *P. plecoglossicida* DSM 15088 (red); (5) BLAST comparison with *P. monteilii* NBRC 103158 (green); (6) BLAST comparison with *P. putida* NBRC14164 (violet); (7) BLAST comparison with *P. aeruginosa* PA01 (lime green); (8) Position of some important antibiotic resistance genes (red). Figure generated by BLAST Ring Image Generator v 0.95.

02 cells failed to subsist and grow in ampicillin as the sole carbon source in the presence of 0.4 mM EDTA (Fig. 2c); but its growth in minimal medium or rich medium, LB, remained almost unaffected in the presence of 0.4 mM EDTA (Fig. 2d, Fig. S2). Genomic analyses confirm the existence of the genes coding for enzymes and pathways utilized during β-lactam catabolism as per an earlier report [9]. Three chromosomal β-lactamase genes, belonging to the class A, subclass B1 and class C β-lactamase families, and genes for complete phenylacetic acid catabolic pathway, were annotated. Distribution of antibiotic resistance genes (known to confer resistance to 14 different antibiotics) in the MR 02 genome is shown in Fig. S3. Another figure has been constructed with the annotated genes and operons for catabolism of β-lactam antibiotic, ampicillin (Fig. S4). RNAseq data of MR 02 cells (grown in minimal media containing ampicillin or glucose as carbon source) were used to analyse the expression of genes responsible for the catabolism of ampicillin. The expression of the bla_{NDM-1} gene was significantly upregulated ($\log_2$ fold change of 2.00088), representing one of the top 50 differentially expressed genes (Fig. S5) while the expression of the other two β-lactamases, bla_{PME-1} and bla_{PDC}, in ampicillin-grown cells were similar to that of glucose-grown cells. Two genes, coding for penicillin G amidase and penicillin acylase family protein, and all *paa* genes of the phenylacetic acid catabolism were expressed in both conditions. On examining the expression profile of the *paa* genes, it was revealed that $\log_2$-fold changes in the case of *paa*B and *paa*G genes were 0.93659 and 0.528505, respectively (higher expression in ampicillin-grown cells compared to glucose-grown cells, but not 'upregulated') (Table S5a,b).

As both physiological and transcriptome data have indicated the pivotal role of NDM-1 in supporting the growth of MR 02

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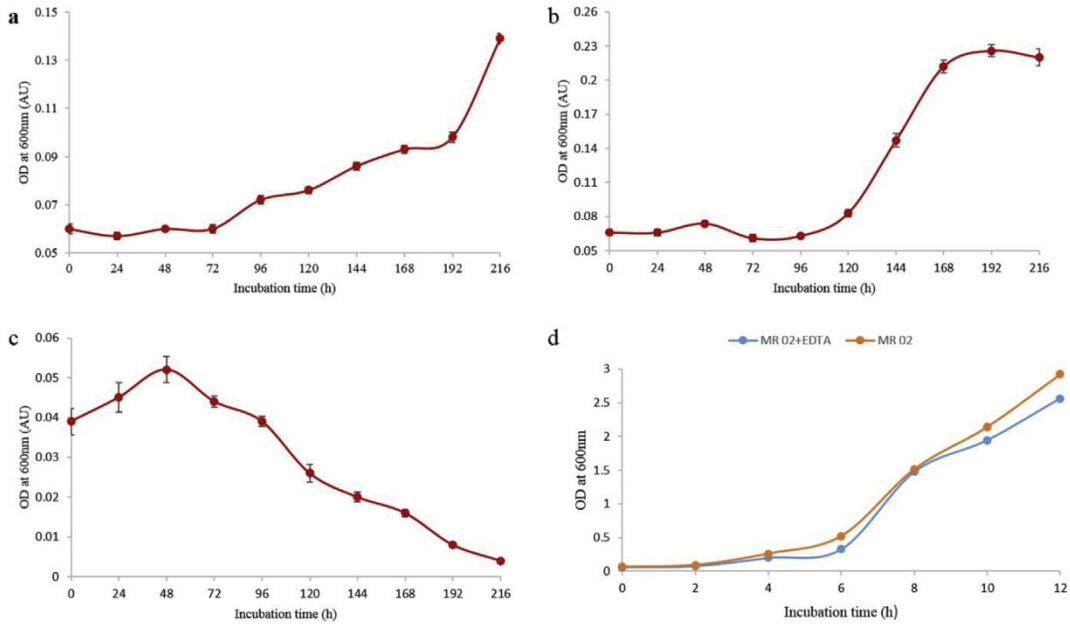

Fig. 2. (a–d). Consumption of ampicillin as growth substrate by *Pseudomonas* sp. MR 02. (a) Growth of MR 02 in ampicillin (6000 mg/L) as sole carbon, nitrogen, and energy source. (b) Growth of MR 02 in ampicillin (6000 mg/L) as sole carbon and energy source. (c) Growth of MR 02 in ampicillin (6000 mg/L) as sole carbon and energy source in the presence of metal chelator, EDTA (0.4 mM). (d) Growth of *Pseudomonas* sp. MR 02 in minimal media (containing glucose (6000 mg/L) as sole carbon and energy source and NH_4Cl (400 mg/L) as sole nitrogen source) in the presence or absence of EDTA (0.4 mM).

using ampicillin as sole carbon and energy source, it was hypothesized that this phenomenon should work in *E. coli* having similar metabolic pathways. Hence the NDM-1 gene of MR 02 was cloned and expressed in *E. coli* DH5α. The recombinant strain of *E. coli* DH5α was able to grow marginally in ampicillin (100 mg/L) as the sole carbon source (Fig. S6), thereby supporting the associated role of NDM-1 in empowering bacteria to consume β-lactams for subsistence.

Discussion

This study describes ampicillin catabolism in a PDR *Pseudomonas* sp. MR 02. While determining the MICs of different antibiotics, including different types of β-lactams, very high MIC values (mg/L) for cephalosporin (cefepime and ceftazidime, 1600), carbapenem (imipenem, 160; meropenem, 650) and ampicillin (>10 000) were obtained. The resistance phenotype against the β-lactam antibiotics was corroborated by the genomic presence of three different β-lactamases, PME-1, NDM-1 and AMPC. Indeed, MR 02 has surpassed *P. aeruginosa* PA01 in acquiring antimicrobial resistance genes with 08 more ARGs. To explain the genetic basis of MR 02's efflux-dependent resistance against chloramphenicol, tetracycline, ciprofloxacin and levofloxacin 8, 11, 5 and 5 efflux system genes/gene clusters were annotated from its whole-genome sequence (Fig. S3). Furthermore, 14 antibiotic-related genomic islands were identified using island viewer (Fig. S7).

We hypothesized that the possession of NDM-1 could be the primary reason for yielding high MIC values of ampicillin, and consumption of ampicillin as a sole source of carbon and energy. It was reported earlier that NDM-1 displays high catalytic efficiency of ampicillin hydrolysis (K_{cat}/K_m = 3.84 μM^{-1} S^{-1}), and, unlike other β-lactamases, localizes itself to the inner leaflet of the outer membrane and secreted via outer membrane vesicles [7,16,17]. Therefore, these properties of NDM-1 may have given a selective advantage to the bacterium to grow using ampicillin as the sole source of carbon and energy. Earlier studies have shown that soil bacteria can survive in minimal media containing antibiotic, either natural or artificial, as the carbon source [18]. Many of these antibiotics are produced by soil bacteria. The concentration of such antibiotics could be in milligram quantity per gram of soil [19]. Hence, it is predictable that certain groups of bacteria might have developed a mechanism for survival by consuming antibiotics as the carbon source. A report on β-lactam catabolism (penicillin) [9] has additionally supported the hypothesis. In fact, MR 02 has shown an extraordinary ability to grow in the high concentration of ampicillin. The role of NDM-1 in supporting the growth of MR 02 on ampicillin, as sole carbon and energy source, was established because the growth in the same medium was inhibited in the presence of a metal scavenger, EDTA. NDM-1 is known to be more susceptible to EDTA than other metallo-β-lactamases, and 50% inhibition of NDM-1 activity was reported in the presence of 412 nM EDTA [20]. It was observed that EDTA had no significant effect on the growth of MR 02 in unamended minimal medium or Luria broth. Despite having a 6-aminopenicillanic acid centre containing nitrogen in most β-lactam antibiotics, ampicillin owns a side chain having another amino group. It had been observed that ampicillin was also used by MR 02 as the only source of both nitrogen and carbon source. It is very likely that in a new growth environment, where no readily metabolizable carbon source such as sugar is available, MR 02 faces a survival challenge. Therefore, the bacterium might be requiring a protracted lag period to adapt and synthesize enzymes to utilize the alternate carbon source, ampicillin, with the assistance of NDM-1. Moreover, in support of the hypothesis, we have presented the results regarding log cfu/mL vs. time (0–48 hr) to show a rise in the viable cell number of *E. coli* DH5α:pHSG398:bla_{NDM-1} using ampicillin (100 mg/L) as the only source of carbon and energy.

Based on the comparative transcriptomics, it was demonstrated that besides the expression of genes coding for β-lactamases, amidases and *paa* pathway, there has been a change in the expression

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of metabolic enzymes to meet growth requirements in minimal medium containing ampicillin as sole carbon and energy source. MR 02 cells may have used the gluconeogenesis pathway to synthesize glucose-6-phosphate to create various cell macromolecules (as glucose was not available in the medium). The expression of *pck*, *gap*N, *pgm*, *adh*C, *gpm*I and *tpi*A was higher along with significant upregulation of *ald*B (coding for aldehyde dehydrogenase) and *exa*A (coding for PQQ-dependent dehydrogenase) genes of the gluconeogenesis pathway (Table S5a). Moreover, three genes of the TCA cycle coding for NADP-dependent isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase E1 component (*suc*A), succinate dehydrogenase flavoprotein subunit (*sdk*A, *frd*A) and one gene coding for D-glycerate dehydrogenase (phosphor-gluconate 2-dehydrogenase) of the pentose phosphate pathway were found to be significantly upregulated, suggestive of regulation of genes related to growth on ampicillin as sole carbon and energy source. Two genes coding for NAD(P)H dehydrogenase (quinone) of ubiquinone and terpenoid-quinone biosynthesis pathway were significantly downregulated (Table S5b).

The results presented up to now in this research have supported the hypothesis about the function of NDM-1 in the catabolism of ampicillin. However, there are limits of this study because no knockouts of these genes have been made to translate genome and transcriptome data in the context of ampicillin catabolism. Moreover, data from proteomics and metabolomics were not generated as a means to integrate them with genome and transcriptome data. Further research in this direction will elucidate novel bacterial strategies in repurposing metabolic pathways to derive nourishment from inactivated antibiotics.

Nucleotide sequence accession numbers

The GenBank accession number for the 16S rRNA gene sequence of MR 02 is MF401548.1. The whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number PESJ00000000. The transcriptome data have been deposited in NCBI under BioProject number PRJNA612657 with BioSample numbers SAMN14379771 and SAMN14379772.

Transparency declaration

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Author contribution

R.C. developed the study, designed the experiment, analysed the transcriptome data and wrote the manuscript. V.K.R. anchored the main study along with molecular biology work, performed the laboratory experiments with K.G. S.M. interpreted the results and took part in paper writing. S.T. performed all bioinformatic analyses. All authors gave feedback and participated in the manuscript editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.10.032.

References

[1] Obritsch MD, Fish DN, MacLaren R, Jung R. Nosocomial infections due to multidrug resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. Pharmacotherapy 2005;25:1353–64.
[2] Toleman MA, Bugert JJ, Nizam SA. Extensively drug-resistant New-Delhi Metallo-β-lactamase encoding bacteria in the environment, Dhaka, Bangladesh. Emerg Infect Dis 2012 2015;21:1027–30.
[3] Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 2000;406:959–64.
[4] Freeman L. Chronic general infection with the *Bacillus pyocyaneus*. Ann Surg 1916;64:195–202.
[5] Anaissie E, Fainstein E, Miller P, Kassamali H, Pitlik S, Bodey GP, et al. *Pseudomonas putida*: newly recognized pathogen in patients with cancer. Am J Med 1987;82:1191–4.
[6] Zhang H, Hao Q. Crystal structure of NDM-1 reveals a common β-lactam hydrolysis mechanism. FASEB J 2011;25:2574–82.
[7] Sun Z, Hu L, Sankaran B, Prasad BVV, Palzkill T. Differential active site requirements for NDM-1 β-lactamase hydrolysis of carbapenem versus penicillin and cephalosporin antibiotics. Nat Comm 2018;9:4524.
[8] Zhang H, Ma G, Zhu Y, Zeng L, Ahmad A, Wang C, et al. Active-site conformational fluctuations promote the enzymatic activity of NDM-1. Antimicrob Agents Chemother 2018;62. e01579-18.
[9] Croft TS, Wang B, Spivak A, Gianoulis TA, Forsberg KJ, Gibson MK, et al. Shared strategies for β-lactam catabolism in the soil microbiome. Nat Chem Biol 2018;14:556–65.
[10] Wayne PA. CLSI. Performance standards for antimicrobial susceptibility testing. 27 CLSI supplement M100. Clinical and Laboratory Standards Institute; 2017. http://www.clsi.org.
[11] Andrews JF. Determination of minimum inhibitory concentrations. J Antimicrobiol Chemother 2001;48:5–16.
[12] EUCAST discussion document E. Dis 5.1. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin Microbiol Infect 2003;9:1–7.
[13] Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012;18:268–81.
[14] Jia A, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res 2017;45:D566–73.
[15] Colston SM, Fullmer MS, Beka L, Lamy B, Gogarten JP, Graf J, et al. Bioinformatic genome comparisons for taxonomic and phylogenetic assignments using Aeromonas as a test case. M Bio 2014;5. e02136-14.
[16] Chiou J, Leung TYC, Chen S. Molecular mechanisms of substrate recognition and specificity of New Delhi metallo-β-lactamase. Antimicrob Agents Chemother 2014;58:5372–8.
[17] Li T, Wang Q, Chen F, Li X, Luo S, Fang H, et al. Biochemical characteristics of New Delhi metallo-β-lactamase-1 show unexpected difference to other MBLs. PLoS ONE 2013;8:e61914.
[18] Dantas G, Sommer MOA, Oluwasegun RD, Church GM, et al. Bacteria subsisting on antibiotics. Science 2008;320:100–3.
[19] Thiele-Bruhn S. Pharmaceutical antibiotic compounds in soils – a review. J Plant Nutr Soil Sci 2003;166:145–67.
[20] Meini MR, Gonsalez LJ, Vila AJ. Antibiotic resistance in Zn (II)-deficient environments: metallo-β-lactamase activation in the periplasm. Future Microbiol 2013;8:947–79.

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