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.

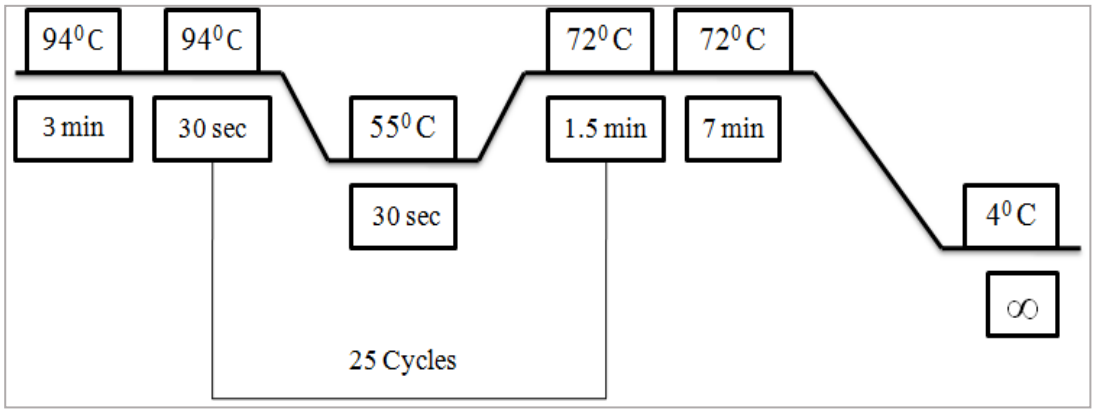

Figure 2.1 Polymerase chain reaction setup for 16S rRNA.

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.

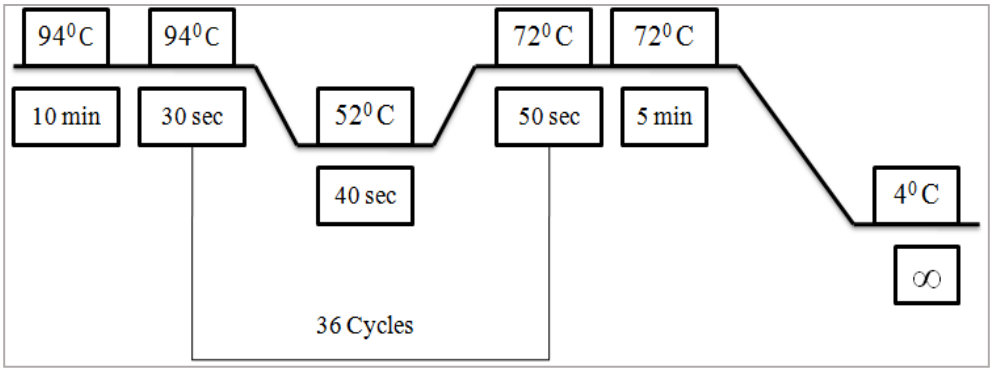

Figure 2.2 Polymerase chain reaction setup for full length ORF of *bla*$_{NDM}$ genes

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.

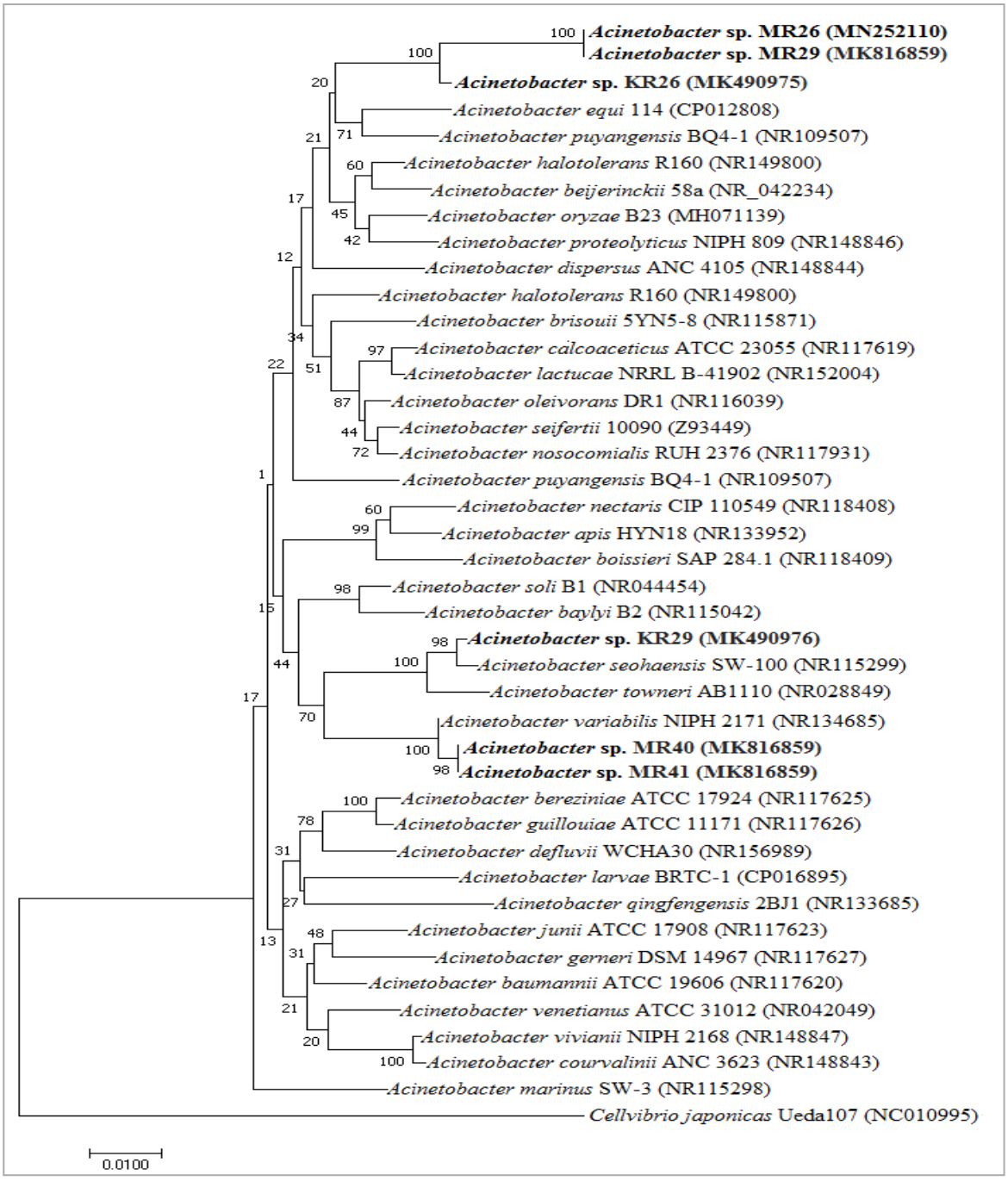

Figure 2.3: Phylogenetic tree of MBL-producing strains MR26, MR29, MR40, MR41, KR26 and KR29

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.

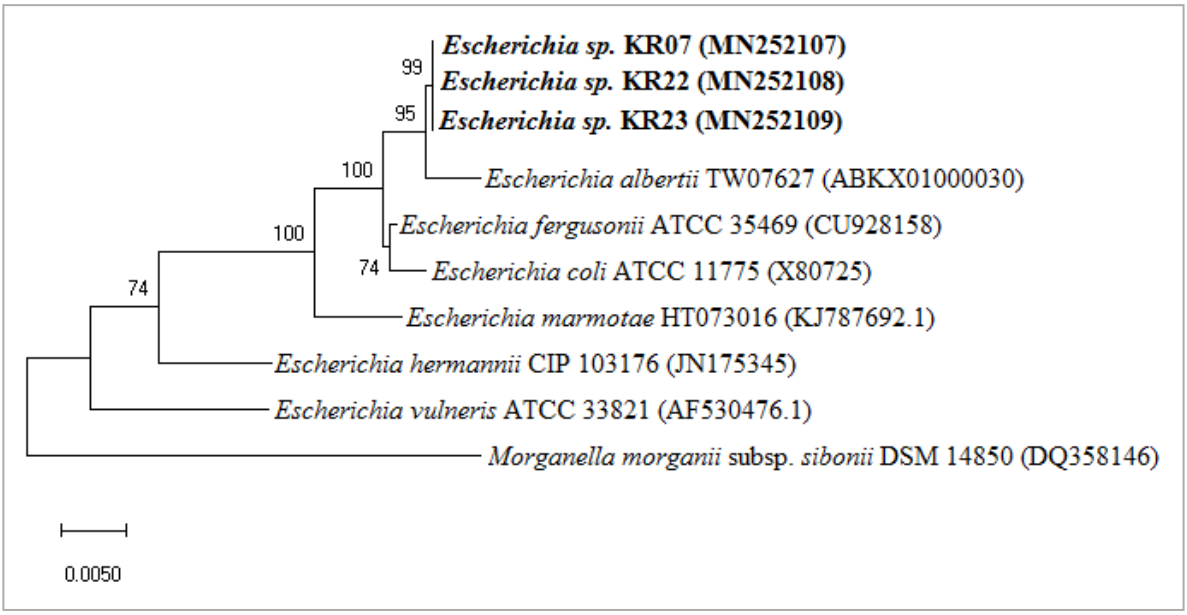

Figure 2.4: Phylogenetic tree of MBL-producing strains KR07, KR22 and KR23

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.

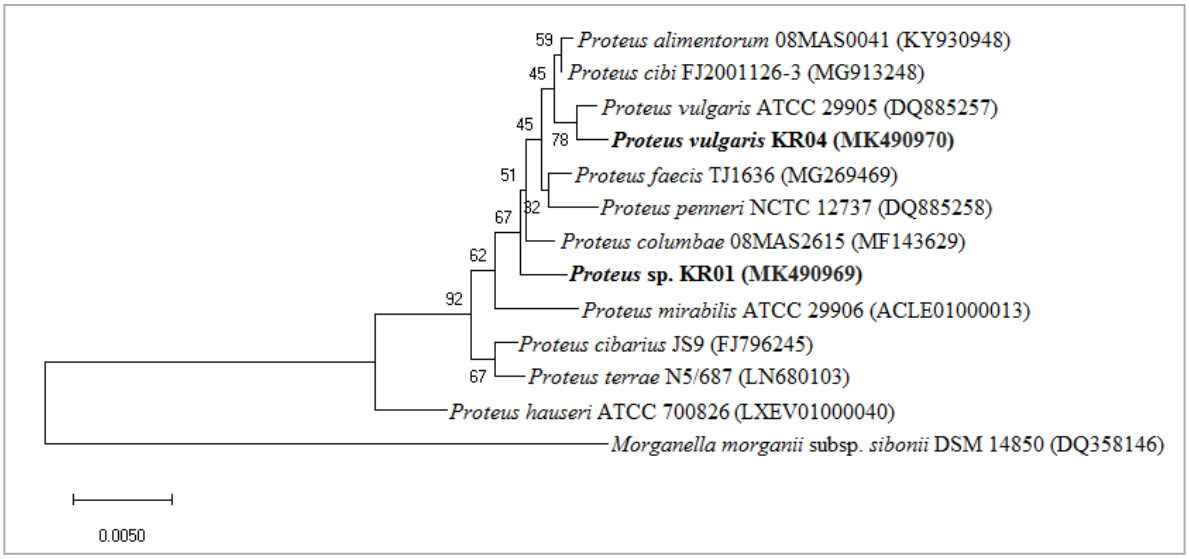

Figure 2.5: Phylogenetic tree of MBL-producing strains KR01 and KR04

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).

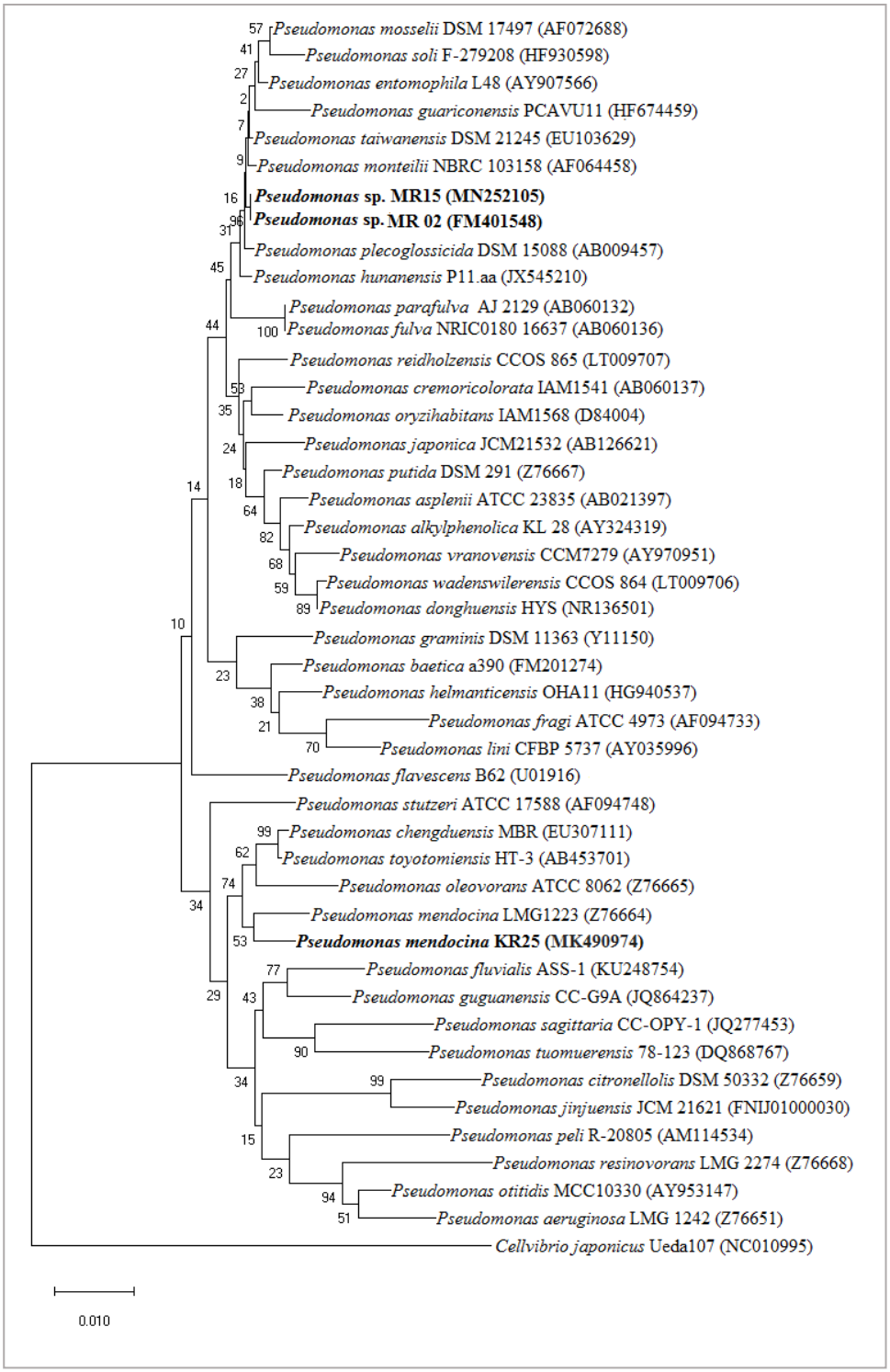

Figure 2.6: Phylogenetic tree of MBL-producing strains MR 02, MR15 and KR25

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.

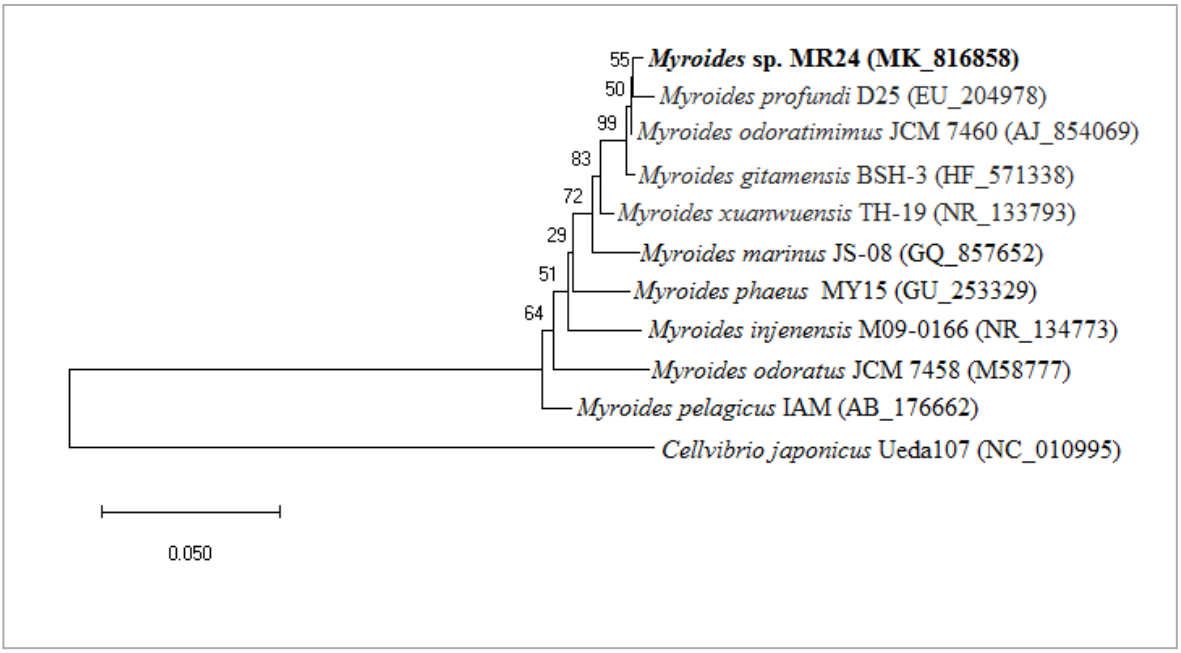

Figure 2.7: Phylogenetic tree of MBL-producing strain MR24

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.