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.

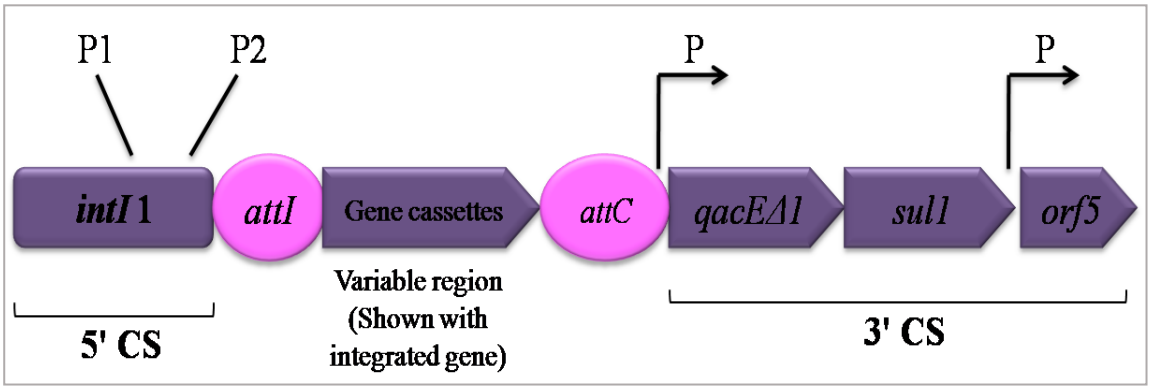

Figure 3.1: General structure of class 1 integron

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).

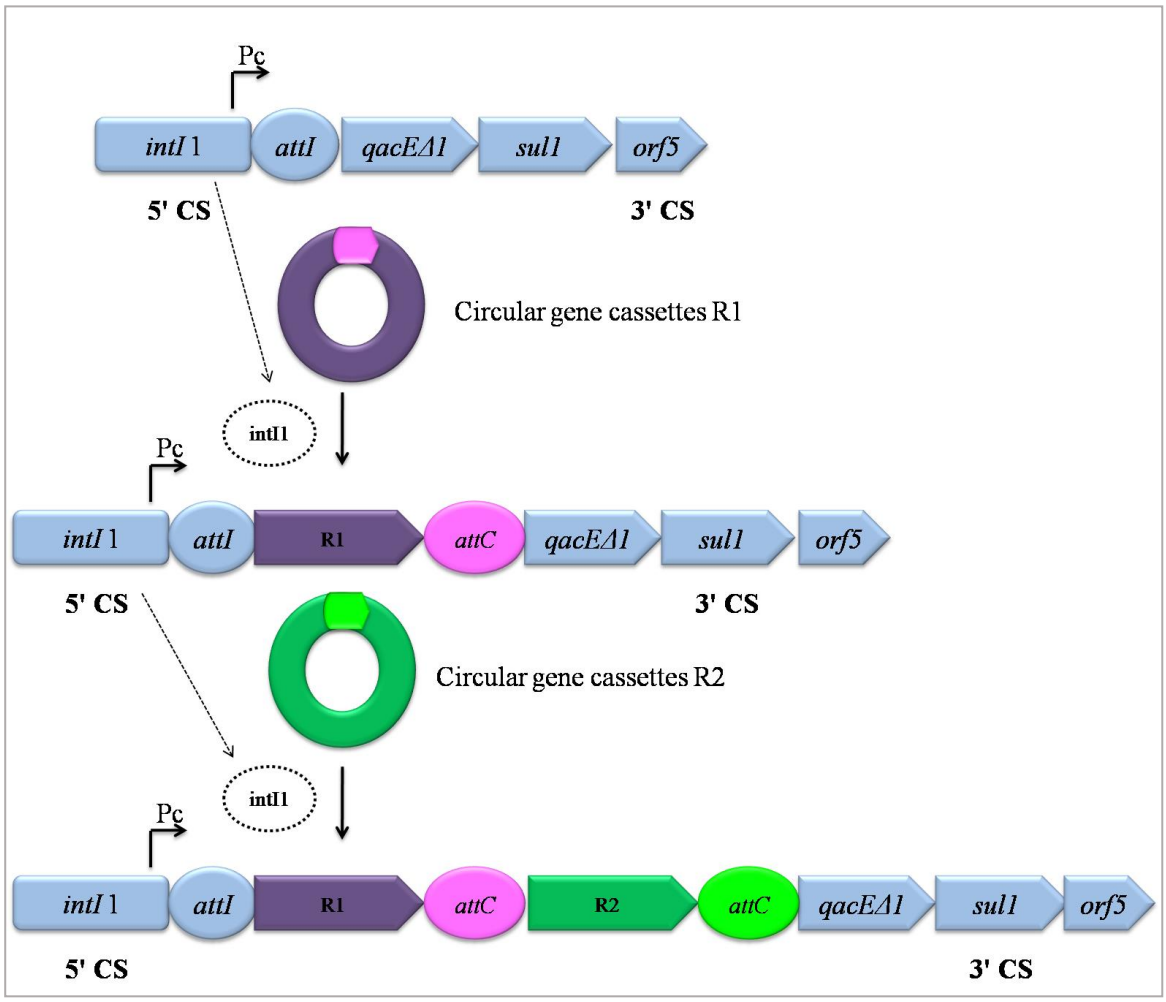

Figure 3.2: Integration of gene cassettes

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.

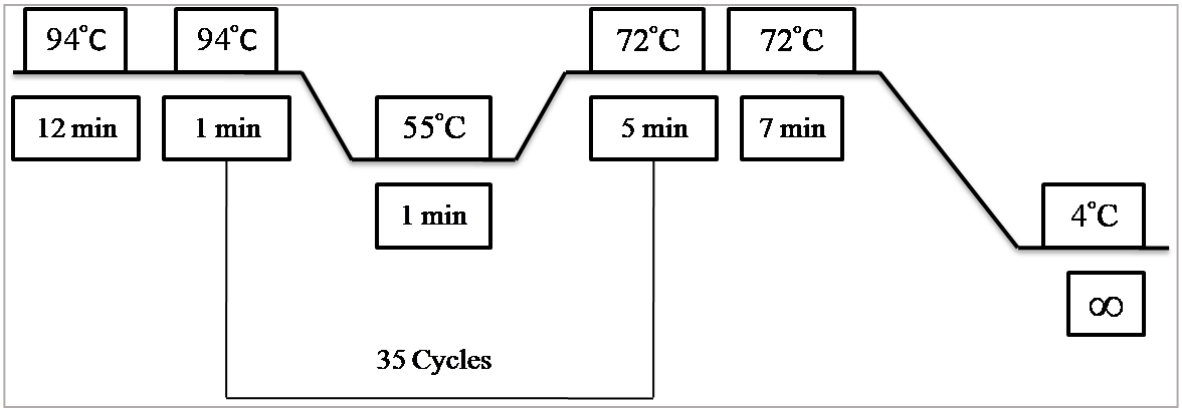

Figure 3.3: Polymerase chain reaction setup for variable region of class 1 integron

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)

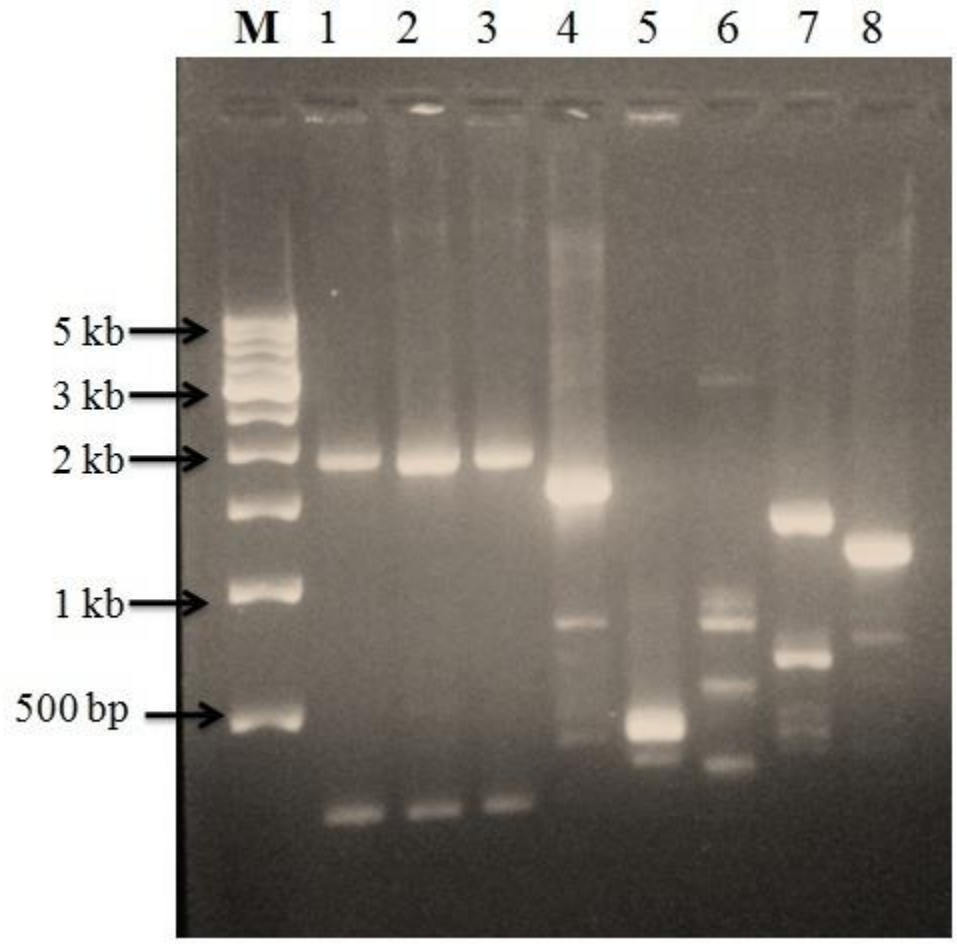

Figure 3.4: Agarose gel electrophoresis of PCR amplification products of different the 5' CS and 3' CS of variable regions of class 1 integrons from MBLs isolates from Mahananda and Karala Rivers

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 $bla_{\text{NDM-1}}$.

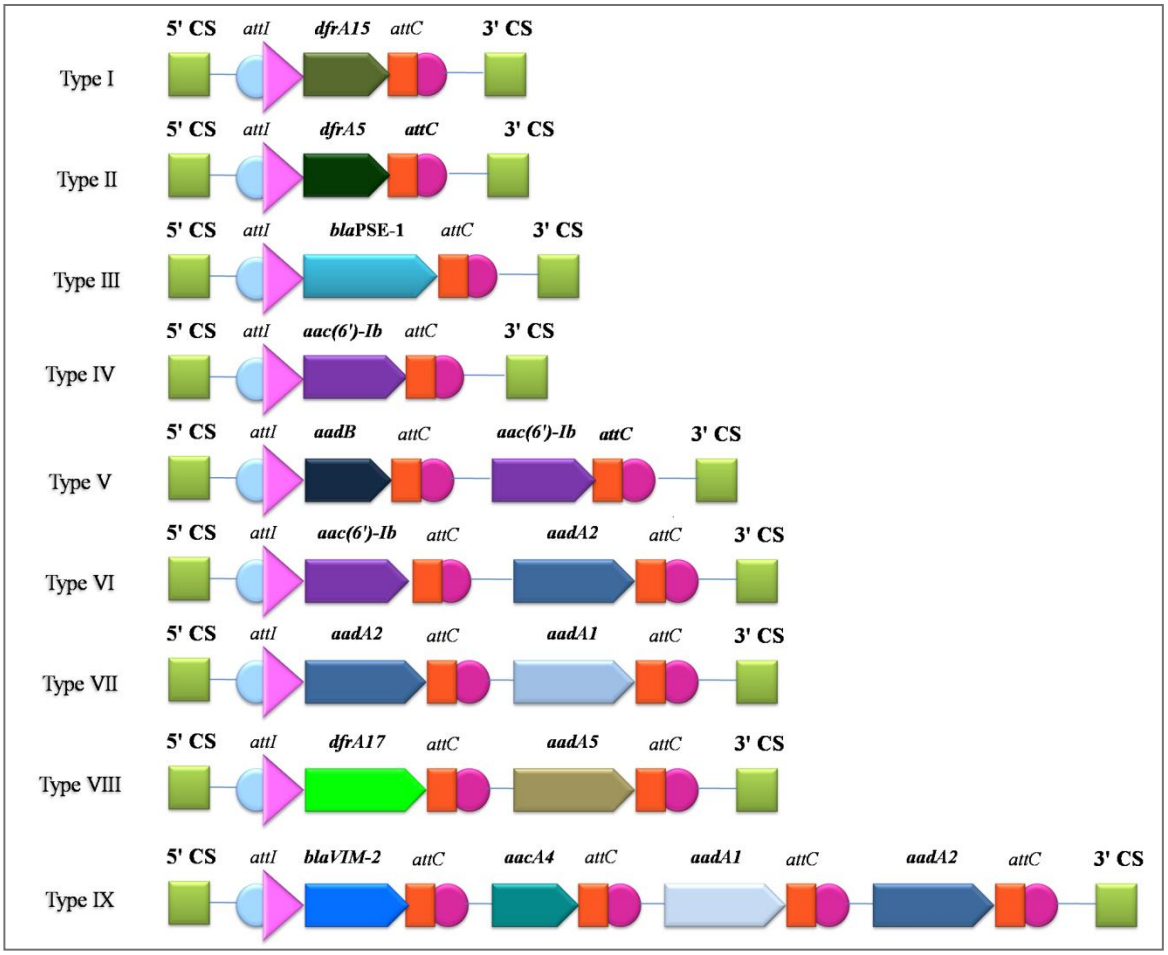

Figure 3.5: Schematic representation of the variable region of class 1 integron identified in MBLs positive isolates from Mahananda and Karala Rivers

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.