3. Results and Discussions

Role of mobile genetic elements in multidrug resistant *Vibrio fluvialis* isolates

3.1. Introduction

Vibrio fluvialis is a food-borne pathogen that causes diarrhoea, clinically indistinguishable from cholera [Chakraborty et al., 2005; Bellet et al., 1989; Lee et al., 1981; Huq et al., 1980; Furniss et al., 1977]. The molecular epidemiology of its infection and the mechanisms of its pathogenicity are not as well understood as *V. cholerae* and it was largely under-recognized as an enteric pathogen [Kolb et al., 1997]. *V. fluvialis* is implicated in sporadic cases of cholera-like diarrhoea and is often associated with pediatric diarrhoea [Bellet et al., 1989; Huq et al., 1980]. Its presence has also been reported in cases of AIDS, peritonitis, ocular infections, dental plaque, cellulitis and cerebritis [Huang and Hsu, 2005; Ratnaraja et al., 2005; Tamilselvan et al., 2004; Penland et al., 2000; Hodge et al., 1995]. There are few reports pertaining to multiple drug resistance (MDR) in *V. fluvialis* isolates [Chowdhury et al., 2016; Chowdhury et al., 2011; Srinivasan et al., 2006; Ahmed et al., 2005; Ahmed et al., 2004].

There are several different mechanisms by which bacteria are known to acquire drug resistance and bacteria often combine more than one mechanism to increase the efficacy of their defensive shield against an antibiotic. Often the genes responsible for these reside on mobile genetic elements for the easy dissemination of drug resistance to other organisms. Integrons, which are the gene capture systems that integrate gene cassettes harbouring a wide array of genes including antibiotic resistance genes, often reside on chromosomes or plasmids [Rowe-Magnus and Mazel, 1999; Stokes et al., 1997; Stokes and Hall, 1989]. The role of integrons in the capture and dissemination of antibiotic resistance genes has been well documented in gram-negative bacteria [Labbate et al., 2009; Correia et al., 2003; Goldstein et al., 2001]. Besides integrons, conjugative transposons, like the SXT elements [Beaber et al., 2002; Waldor et al., 1996], have been shown to be the vehicles for drug resistance markers for sulfamethoxazole, trimethoprim, chloramphenicol and streptomycin in many isolates of vibrios including *V. fluvialis* [Srinivasan et al., 2006; Ahmed et al., 2005; Amita et

al., 2003; Thungapathra et al., 2002; Dalsgaard et al., 2001]. There are few reports describing mechanisms contributing to the drug resistance in *V. fluvialis* [Chowdhury et al., 2016; Chowdhury et al., 2011; Srinivasan et al., 2006; Ahmed et al., 2005] as the occurrence of *V. fluvialis* is less as compared to *V. cholerae*. In this chapter, the role of plasmids and integrons in imparting drug resistance in clinical isolates of *V. fluvialis*, from Kolkata, India, has been described in two sections. The first section describes the results from a highly drug resistant clinical isolate of *V. fluvialis* BD146 (2002) while the second section describes the results from 12 clinical isolates of *V. fluvialis* from 2006.

3.2. Mechanisms of antibiotic resistance in a highly drug resistant *Vibrio fluvialis* isolate BD146 of 2002

3.2.1. Results

3.2.1.1. Characterisation of BD146

V. fluvialis BD146 isolated from a patient with acute cholera-like diarrhoea, admitted to the Infectious Diseases Hospital, Kolkata, India, in 2002, was procured from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. The antimicrobial resistance pattern of BD146 revealed that it was fully resistant to twelve out of fourteen antibiotics and showed intermediate resistance to the remaining two, chloramphenicol and tetracycline (Table 3.1). Agarose gel analysis of genomic DNA and plasmid DNA isolated from BD146 (Figure 3.1, lane gBD146 and pBD146) revealed that it contained a major plasmid band with the mobility between 6 kb and 8 kb bands of the DNA marker (later sequenced and submitted to GenBank as plasmid of 7.5 kb, **EU574928**). The plasmid was named pBD146.

3.2.1.2. Horizontal gene transfer of plasmids from *Vibrio fluvialis* BD146

The transferability of resistance markers carried by plasmid(s) were analysed by transformation and conjugation experiments.

3.2.1.2a Transformation

The plasmids preparation from *V. fluvialis* BD146 was electroporated in *E. coli* JM109. Transformants were selected on ampicillin plates. Two types of ampicillin resistant clones were obtained. The first type was found to contain 7.5 kb plasmid described above (Figure 3.1, lanes B2/JM109 and B5/JM109) while the other type did not have this 7.5 kb plasmid (Figure 3.1, lanes B44/JM109 and B51/JM109) even though the clones showed ampicillin resistance after several rounds of streaking on fresh ampicillin plates.

Both kinds of transformants (one carrying the 7.5 kb plasmid designated 7.5 kb+ and the other devoid of it named 7.5 kb-), were analysed for drug resistance. The 7.5 kb+ clones were resistant to ampicillin, chloramphenicol, gentamicin, trimethoprim, tetracycline, nalidixic acid and rifampicin, and showed intermediate resistance to neomycin and kanamycin (Table 3.1). The 7.5 kb- clones showed the similar resistance profile as 7.5 kb+ clones except that they did not carry trimethoprim resistance. The host used for the transformation reactions, JM109, was resistant to nalidixic acid and had intermediate resistance to neomycin. From these results, it appeared that the presence of 7.5 kb plasmid could confer resistance only to trimethoprim in JM109 and the resistance to other drugs was possibly due to another plasmid. The fact that a plasmid could not be visualized on agarose gel with the plasmid preparation from the 7.5 kb- transformants indicated that the plasmid was present in BD146 in a very low copy number. Resistance to nalidixic acid and intermediate resistance to neomycin were apparently JM109 derived while the trimethoprim resistance in 7.5 kb+ transformants was due to the 7.5 kb plasmid. It thus appeared that the 7.5 kb+ transformants in JM109 carried two plasmids; a very low copy number plasmid carrying the drug markers for ampicillin, chloramphenicol, gentamicin, kanamycin, tetracycline and rifampicin, and a more abundant 7.5 kb plasmid carrying trimethoprim resistance.

Table 3.1. Antibiotic susceptibility pattern for *V. fluvialis* BD146 and its transformants in *E. coli* host JM109

Antibiogram of			
JM109	**BD146**	**7.5 kb+ /JM109[a]**	**7.5 kb-/JM109 [a]**
NAL Intermediate: NEO	AMP, CIP, GEN, STR, SUL, TMP, NEO, NAL, NOR, KAN, CO-TRI, RIF Intermediate: CHL, TET	AMP, CHL, GEN, TMP, TET, NAL, RIF Intermediate: NEO, KAN	AMP, CHL, GEN, TET, NAL, RIF Intermediate: NEO, KAN

Drug names have been abbreviated: AMP, ampicillin (10 µg); CHL, chloramphenicol (30 µg); CO-TRI, co-trimoxazole (trimethoprim 1.25 µg/sulfamethoxazole 23.75 µg); CIP, ciprofloxacin (5 µg); GEN, gentamicin (10 µg); STR, streptomycin (10 µg); SUL, sulfafurazole (300 µg); TMP, trimethoprim (5 µg); TET, tetracycline (30 µg); NEO, neomycin (30 µg); NAL, nalidixic acid (30 µg); NOR, norfloxacin (10 µg); KAN, kanamycin (30 µg); RIF, rifampicin (5 µg). Interpretation of the results was done by using the criteria recommended by the CLSI. *E. coli* ATCC25922 was used for the quality control. The experiment was done in triplicates.

[a]Antibiograms of *E. coli* JM109 after transformation with plasmid preparations from *V. fluvialis* BD146

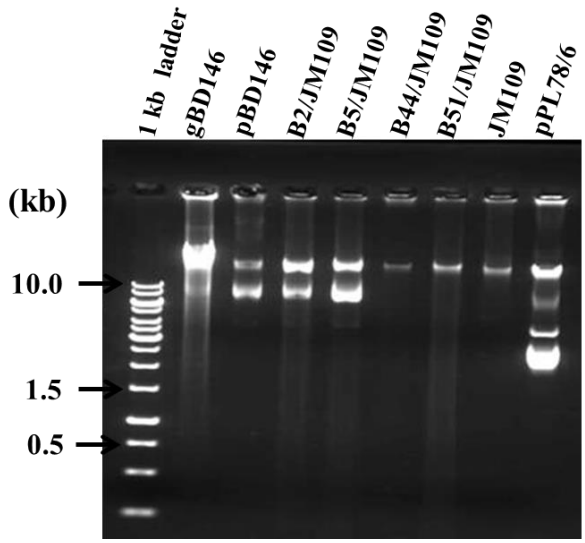


Figure 3.1. Agarose gel (1%) analysis of plasmid DNA samples from *V. fluvialis* BD146 and its transformants. The lanes from left are as follows: 1 kb molecular weight standard (Fermentas), the fragment sizes are indicated in left; gBD146, total genomic DNA from the strain BD146; pBD146, Qiagen-purified plasmid preparation from BD146; B2/JM109, clone B2 of JM109 transformant of pBD146; B5/JM109, clone B5 of JM109 transformant of pBD146; B44/JM109, clone B44 of JM109 transformant of pBD146; B51/JM109, clone B51 of JM109 transformant of pBD146; JM109, plasmid preparation from JM109 made exactly as other JM109 transformants; pPL78/6, Qiagen-purified plasmid preparation from *V. fluvialis* strain PL78/6.

The MIC values for the antibiotics tested for BD146 and its two types of JM109 transformants are depicted in Table 3.2. For most of the drugs tested, the MIC was in

the range of 0.25-10 µg for BD146. For drugs co-trimoxazole, nalidixic acid, sulfafurazole, sulfamethizole, trimethoprim and rifampicin, the MIC was very high (≥ 240 µg). Comparison of MIC values between the parent strain and its transformants clearly indicated that the majority of transferable resistance traits were contributed by the low copy number plasmid. The 7.5kb+ transformants showed an increase in MIC values for ciprofloxacin (25 fold), norfloxacin (2 fold), co-trimoxazole (5 fold) and trimethoprim (20 fold) when compared with 7.5kb- transformants. This clearly indicated that the 7.5 kb plasmid contributed resistance to these drugs which could be explained on the sequence analysis of this plasmid described later in section 3.2.1.8.

Table 3.2. Determination of MIC values for the *Vibrio fluvialis* strain BD146 and its JM109 transformants.

S. No.	Antibiotic	MIC values (µg)			
		BD146	**7.5kb+/JM109**	**7.5 kb-/JM109**	**JM109**
1	Chloramphenicol	1.0	30.0	30.0	5.0
2	Ciprofloxacin	10.0	0.25	0.01	0.08
3	Co-trimoxazole	> 240	0.5	0.1	0.1
4	Gentamicin	1.0-2.0	2.0-5.0	2.0	0.1
5	Kanamycin	3.0-7.5	3.0-7.5	3.0-7.5	0.1
6	Nalidixic acid	> 240	60.0-120.0	60.0-120.0	60.0-120.0
7	Neomycin	1.0	0.1	0.1	0.1
8	Norfloxacin	4.0	1.0	0.5	0.05
9	Streptomycin	10.0-30.0	3.0	3.0	0.1
10	Sulfafurazole	> 240	1.0-3.0	1.0-3.0	1.0-3.0
11	Sulfamethizole	> 240	1.0-3.0	1.0-3.0	1.0-3.0
12	Tetracycline	0.25-0.5	30.0-60.0	30.0-60.0	1.0-3.0
13	Trimethoprim	> 240	2.0	0.1	0.1
14	Rifampicin	120-240	> 240	> 240	8.0

MIC, minimal inhibitory concentration. The MIC values were determined using the Hicomb test from Himedia and *E. coli* ATCC25922 was included for quality control.

3.2.1.2b Conjugation

Conjugation experiment was carried out between *V. fluvialis* BD146 (donor) and *E.coli* XL1-Blue (recipient) to determine the horizontal gene transfer. Transconjugants were selected in two different combinations of antibiotics; ampicillin (50µg/ml) and tetracycline (120µg/ml) where transconjugants were denoted as amp_{50}+tet_{120} or trimethoprim (20µg/ml) and tetracycline (120 µg/ml) where

transconjugants were denoted as tri_{20}+tet_{120}. Plasmid analysis of both these transconjugants showed different profile (Figure 3.2). Transconjugants amp_{50}+tet_{120} conferred resistance to ampicillin, tetracycline, rifampicin and nalidixic acid while transconjugants tri_{20}+tet_{120} harbored all the above resistant traits and in addition, it also harbored trimethoprim resistance as shown in transformant analysis (Table 3.3).

Table 3.3. Antibiotic susceptibility profile of *Vibrio fluvialis* BD146 and its transconjugants.

	***Vibrio fluvialis* BD146**	**Transconjugants TRI_{20} + TET_{120}**	**Transconjugants AMP_{50} + TET_{120}**	***E. coli* XL1Blue**
Resistant	AMP, COT, CIP, SUL, NAL, TRI, RIF, CHL,GEN, STR, NOR, KAN, NEO, TET	AMP, TET, TRI, RIF, NAL	AMP, TET, RIF, NAL	TET, NAL

Intermediate resistance and complete resistance were together considered as a resistance trait.

AMP, Ampicillin; CHL, Chloramphenicol; CIP, Ciprofloxacin; COT, Co-Trimoxazole; GEN, Gentamicin; KAN, Kanamycin; NAL, Nalidixic Acid; NEO, Neomycin; NOR, Norfloxacin; STR, Streptomycin; SUL, Sulfisoxazole; TET, Tetracycline; TRI, Trimethoprim; RIF, Rifampicin; TRI_{20}, Trimethoprim (20µg/mL); TET_{120}, Tetracycline (120 µg/mL); AMP_{50}, Ampicillin (50 µg/mL)

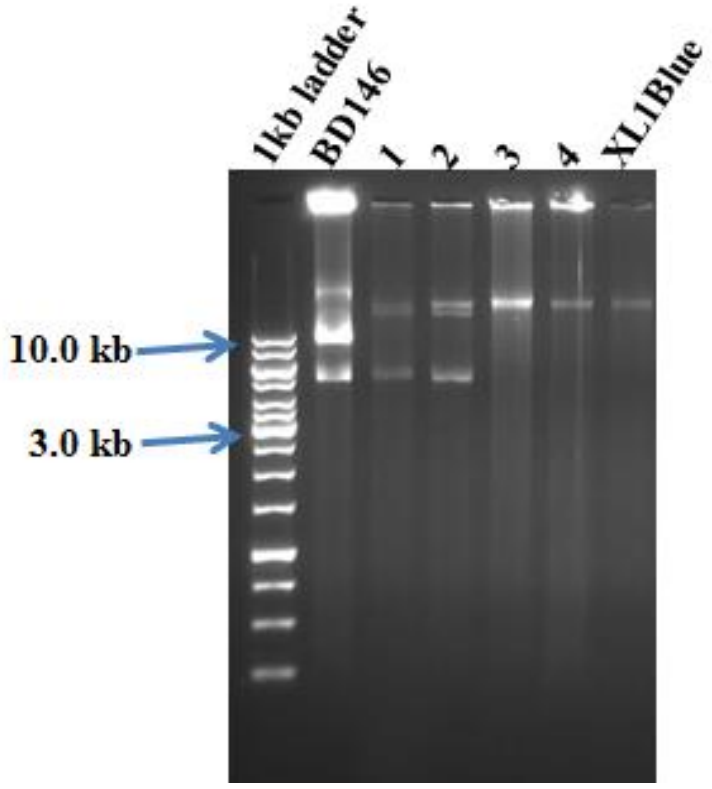


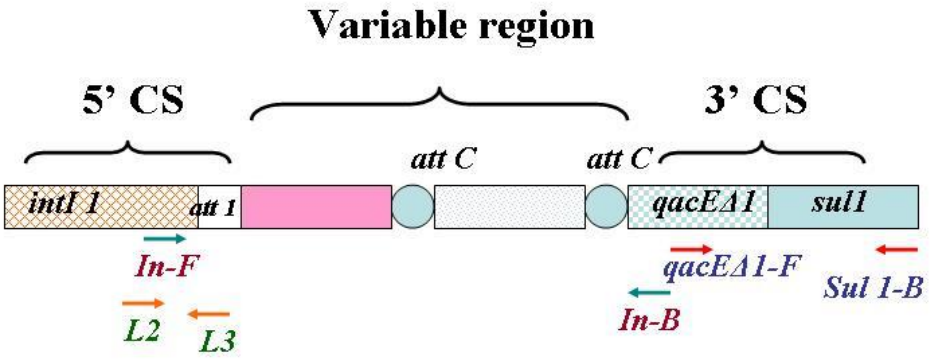
Figure 3.2. Agarose gel (1%) analysis of plasmid profiles for BD146 transconjugants Lanes 1 and 2: Plasmid DNA from transconjugants tri_{20} +tet_{120}; Lanes 3 and 4: Plasmid DNA from transconjugants amp_{50}+tet_{120}. 1 kb DNA ladder (Fermentas) were used as markers.

3.2.1.3. Presence of integrons and SXT elements in BD146

The presence of integrons and SXT elements as carriers of drug resistance genes were assessed by PCR with the primers specific for conserved segments of each kind of element. PCR analysis for integrases of class 1, class 2, class 3, class 4 integrons and SXT element revealed that all the elements except class 1 integron were absent in this isolate. For the PCR reactions, plasmid preparation from JM109 cells/ XL1-Blue were taken as a negative control and plasmid/genomic DNA preparation from *V. fluvialis* PL78/6 was taken as positive control for class 1 integron. Plasmid DNA obtained from parent isolate, transformants and transconjugants led to the amplification of a band of 0.3 kb from 5'CS region with L2/L3 primers (Figures 3.3 and 3.4a), and a band of 0.8 kb from 3'CS with qacEΔ1-F/sul1-B primer pair (Figures 3.3 and 3.4b) indicating the presence of class 1 integron in parent isolate, transformants and transconjugants. This clearly indicated that class 1 integron was resident on a plasmid that was transferable during the processes of transformation as well as conjugation. The attempt to amplify the variable region with in-F/in-B primers resulted in the amplification of a 4.0 kb and a 0.4 kb band (Figures 3.3 and 3.4c) in BD146. Interestingly, the transformants and transconjugants did not show the amplification of 0.4 kb band indicating that only 4.0 kb band was from the transferable plasmid. In addition, both kind of transformants and transconjugants carried class 1 integron with 4.0 kb variable region. This result indicated that the integron resided on low copy number plasmid.

3.2.1.4. Sequence analysis of Class 1 integron

Sequence analysis of 0.3 kb amplicons (GenBank accession no **GQ152140**) from 5'CS of this class 1 integron (using L2/L3 primer) confirmed the homology of this integrase with that from *Pseudomonas aeruginosa* (GenBank accession no **M73819**). Clearly, the integron segments having sequence similarity with 5' conserved segment (5'CS) and 3' conserved segment (3'CS) of pVS integron from *P. aeruginosa* resided on the low copy number plasmid [Bissonnette and Roy, 1992], as the 7.5 kb+ and 7.5 kb- transformants showed the amplification of this band.



Primer pair used		*Amplicon expected*
qacEΔ1-F and Sul 1-B	-	*0.8 kb*
In-F and In-B	-	*Variable*
L2 and L3	-	*0.3 kb*

Gene cassettes identified in this study

4.0 kb: *arr-3, cmlA, bla*$_{OXA10}$*, aadA1*

0.4 kb: putative exporter gene

Figure 3.3. Schematic representation of class 1 integron from BD146. 59-base elements are represented by solid circles. The primers based on the sequences of 5' conserved segment (5'CS) and 3' conserved segment (3'CS) and used for analysis of this integron in the present study have been shown.

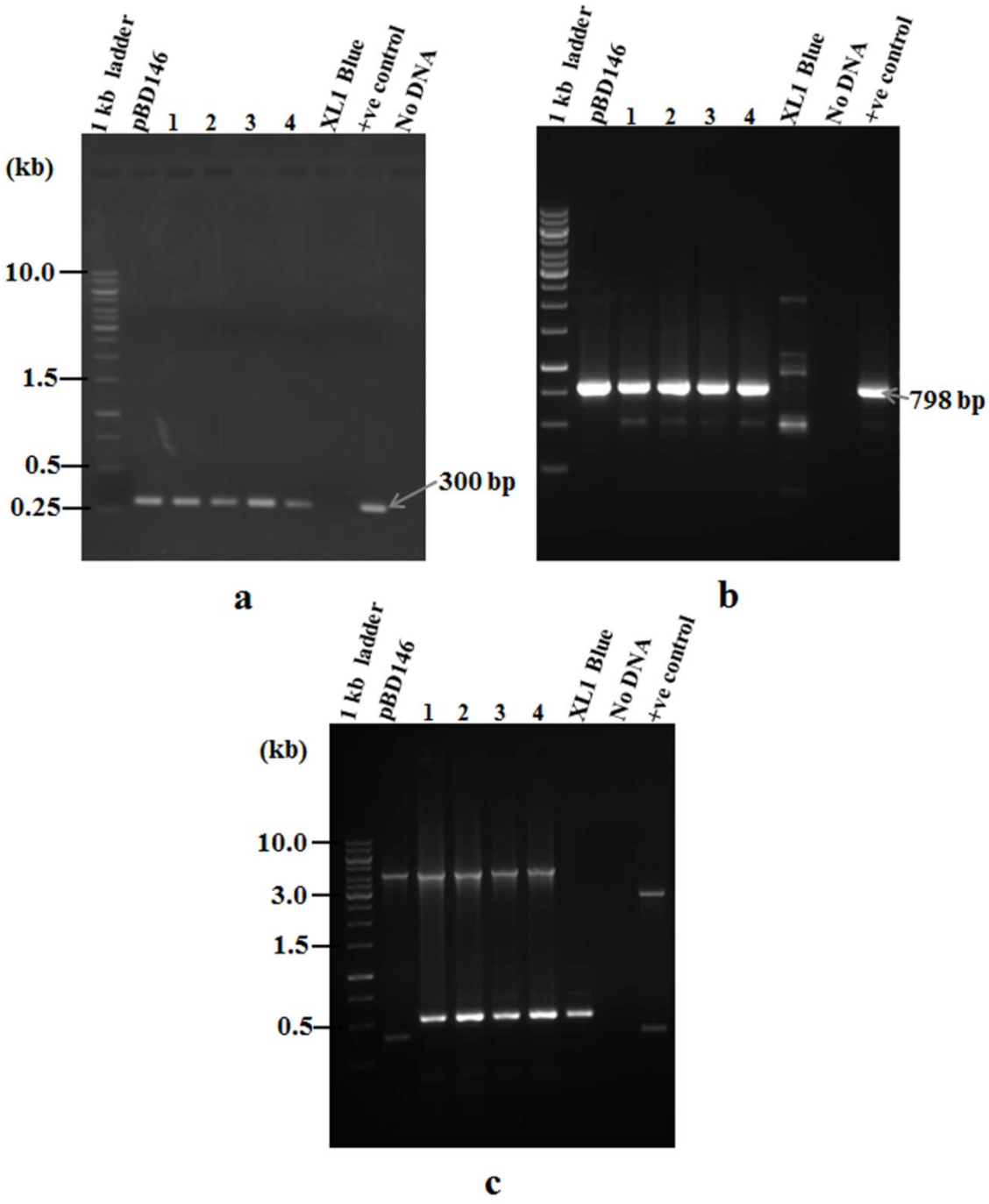


Figure 3.4. PCR amplification of class 1 integron/integrase-specific regions of BD146 and its transconjugants. Agarose gel analysis showing amplification with; (a) primer pair L2 /L3 for 5'CS; (b) primer pair qacEΔ-F/sul1-B for 3'CS; (c) primer pair in-F/in-B for amplifying variable region of class 1 integron. All the DNA samples used as templates have been indicated on top of each lane. Lanes 1 and 2: PCR amplicons from transconjugants tri_{20}+tet_{120}; Lanes 3 and 4: PCR amplicons from transconjugants amp_{50}+tet_{120}. *V. fluvialis* PL78/6 was used as positive control while XL1 Blue and no DNA were used as negative control. pBD146: plasmid preparation from BD146 isolate.

ORF analysis of the 4.0 kb variable region amplified from integron resident on low copy number plasmid revealed the presence of genes (Figure 3.4) for rifampin ADP-ribosylating transferase (GenBank accession no **FJ462717**), a hypothetical protein (GenBank accession no **FJ705852**), chloramphenicol efflux pump MFS transporter, extended spectrum beta-lactamase OXA-10 (GenBank accession no **FJ705851**) and aminoglycoside 3' adenyl transferase (GenBank accession no **FJ462718**). The 0.4 kb amplicon that could only be obtained from genomic/plasmid DNA of parent isolate coded for a putative exporter protein (GenBank accession no **FJ462719**).

BLAST analysis of 4.0 kb integron sequence showed 99% identity with segments of plasmid A from *E.coli* H3 (GenBank accession no **CP010168.1**), segments of plasmid pNDM15-1078 from *E.coli* N15-01078 (GenBank accession no **CP012902.1**), segments of plasmid pNDM-116-17 from *V. cholerae* 116-17a (GenBank accession no **LN831185.1**), class 1 integron from *Pseudomonas areuginosa* pae G18 (GenBank accession no **EU886979.1**) and part of *Enterobacter aerogenes* HN0711 plasmid pHN-NDM0711.

3.2.1.5. Quinolone resistance in *V. fluvialis* BD146

Mutation in topoisomerases was analysed in BD146 by PCR amplification and sequencing of Quinolone resistance determining region (QRDR) regions of topoisomerases. Sequence analysis of topoisomerases from BD146 carried mutations in GyrA (S_{83}→I) and ParC (S_{85}→L). Analysis of quinolone resistance genes in the parent as well as transformant/transconjugants revealed that two qnr genes i.e. *qnrVC5* and *aac(6')-Ib-cr* were detected (Figure 3.5). The *qnrVC5* was found on pBD146 while *aac(6')-Ib-cr* was probably found in low copy number plasmids as it was amplified in parent isolates and 7.5 kb- transformants and transconjugants amp_{50}+tet_{120}.

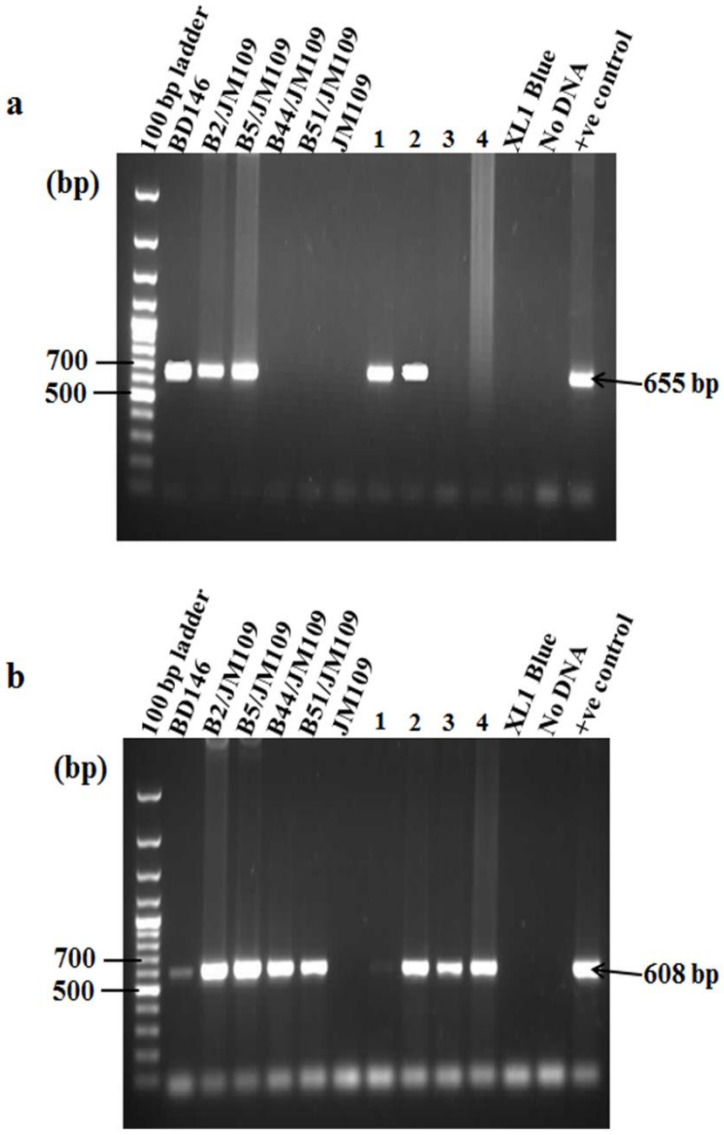


Figure 3.5. Agarose gel analysis of PCR amplicons of *qnrVC* (a) and *aac(6')-Ib-cr* (b) from BD146, and its transformants and transconjugants. The DNA samples used as templates are indicated on top of each lane. Lanes 1 and 2: PCR amplicons from transconjugants tri_{20}+tet_{120}; Lanes 3 and 4: PCR amplicons from transconjugants amp_{50}+$tet_{120.}$ 100 bp DNA ladder (Thermo Scientific) was used as a marker.

3.2.1.6. Extended spectrum beta lactamases (ESBL) in *V. fluvialis* BD146

ESBL activity in BD146 was analysed using the EzyMIC strip (HiMedia) for ESBL and ampC beta lactamase (Table 3.4). The MIC ratio BD146 for CAZ+/CAZ and CTX+/CTX was 42.67 and 64 respectively while the MIC ratio for MIX+/MIX was 64. These results revealed that it was ESBL and ampC beta lactamase positive isolate.

Table 3.4. Evaluation of extended spectrum beta lactamase activity in *V. fluvialis* BD146 isolate

ESBL	**ATCC 25922**	**BD146**
CTX (μg/mL)	0.38	>16
CTX+ (μg/mL)	0.125	0.25
Ratio CTX/CTX+	**3.04**	**64**
CAZ (μg/mL)	0.75	>32
CAZ+ (μg/mL)	0.50	0.75
Ratio CAZ/CAZ+	**1.5**	**42.67**
MIX (μg/mL)	0.25	>16
MIX+ (μg/mL)	0.125	0.25
Ratio MIX/MIX+	**2.0**	**64**

If the ratio is ≥ 8, the test is positive for lactamase activity

CTX: cefotaxime; CTX+: cefotaxime and clavulanic acid; CAZ: ceftazidime; CAZ+: ceftazidime and clavulanic acid; MIX: the mixture of ceftazidime, cefotaxime, cefepime and cloxacillin; MIX+: the mixture of ceftazidime, cefotaxime, cefepime and cloxacillin with clavulanic acid and tazobactam.

3.2.1.7. Role of efflux pumps in imparting drug resistance in *V. fluvialis* BD146

To check the synergy between efflux pump activity and other resistance genes, synergy tests were carried out to detect the efflux pump activity as described in materials and methods (section 2.11). The MIC of ampicillin, chloramphenicol, ciprofloxacin, kanamycin, streptomycin, tetracycline and trimethoprim were tested in the presence and absence of efflux pump inhibitor carbonyl cyanide-m-chlorophenyl hydrazone (CCCP). Fold decrease in MIC of BD146 for a particular drug in the presence of CCCP indicated the involvement of efflux pump activity in imparting resistance to that drug. Synergy tests revealed that efflux pumps were marginally involved in imparting the drug resistance phenotype (1.33 to 2 fold decrease in MIC value) for chloramphenicol, kanamycin, streptomycin and tetracycline, while they did not contribute in imparting drug resistance for ampicillin, ciprofloxacin and trimethoprim as no changes were observed in their MIC values (Table 3.5). Analysis of transformants and transconjugants with synergy test revealed that no change/ minimal change in MIC values were observed in chloramphenicol, tetracycline, kanamycin and streptomycin.

Table 3.5. Evaluation of efflux pump activity in *V. fluvialis* BD146 isolate

Antibiotics	**Antibiotics with CCCP (4 mg/L)**	**BD146 (µg/mL)**	**Fold change in MIC**	**Trans-formants (µg/mL)**	**Fold change in MIC**	**Trans-conjugants (µg/mL)**	**Fold change in MIC**
Ampicillin	-	>256	1	ND	-	ND	-
	+	>256		ND		ND	
Chloramphenicol	-	12	**2**	32	**1.33**	16	**1**
	+	6		24		16	
Ciprofloxacin	-	6	1	ND	-	ND	-
	+	6		ND		ND	
Trimethoprim	-	>32	1	ND	-	ND	-
	+	>32		ND		ND	
Tetracycline	-	16	**1.33**	>256	**1**	ND	**-**
	+	12		>256		ND	
Kanamycin	-	12	**1.5**	6	**1**	6	**1**
	+	8		6		6	
Streptomycin	-	64	**1.33**	4	**1**	12	**1**
	+	48		4		12	

MIC, minimal inhibitory concentration. The MIC values were determined using the EZyMIC test from Himedia. JM109 and XL1 Blue showed no change in MIC for chloramphenicol, tetracycline, kanamycin and streptomycin.

3.2.1.8. Analysis of 7.5 kb plasmid BD146

3.2.1.5a Sequence analysis of pBD146

Sequence of pBD146 (GenBank accession no **EU574928**) revealed that it carried genes encoding integrase (*intI*) named *BDint*, replicase (*repA*), trimethoprim resistance (*dfrVI*), quinolone resistance (*qnrVC5*), toxin-antitoxin (*parE/parD*) and some hypothetical proteins (Figure 3.6; Table 3.6). Interestingly, these hypothetical proteins seemed to have their origin chiefly in the members of *Vibrionaceae* family barring one protein which was derived from gamma proteobacterium. The hypothetical proteins corresponding to ORFs 3214 nt -3429 nt, 7192 nt-7428 nt, 1055 nt-1300 nt and 3557 nt- 3949 nt showed 84%- 99% identity with various hypothetical proteins from *Vibrio spp*. i.e. *Vibrio cholerae*, *V. parahaemolyticus*, *V. tasmaniensis*, *V. cyclitrophicus* and *V. fluvialis*.

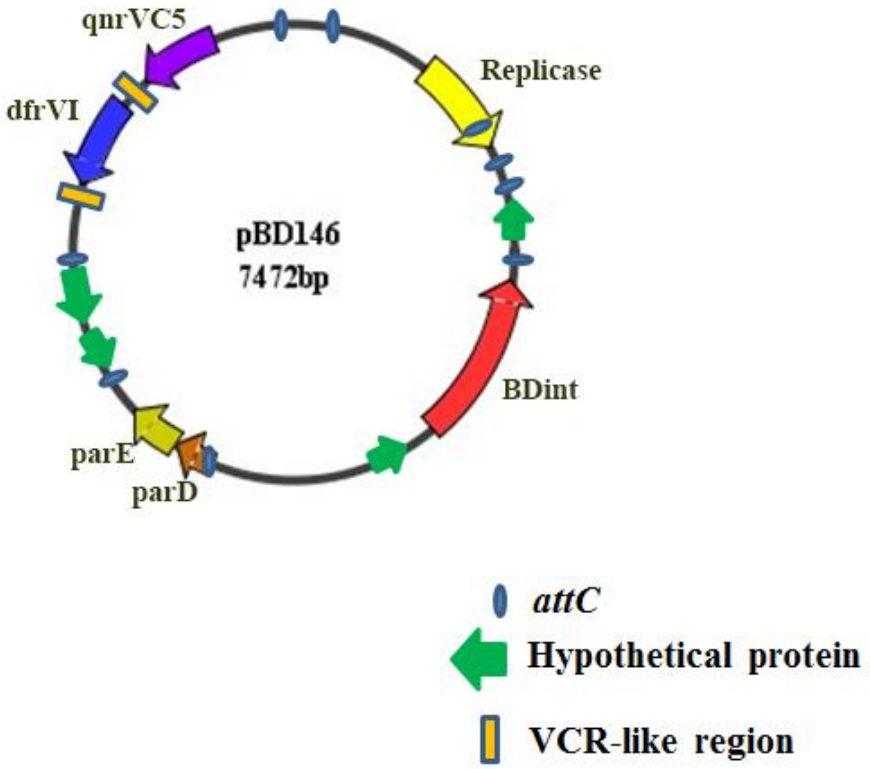


Figure 3.6. Schematic representation of plasmid pBD146. Protein coding regions present on the plasmid pBD146; integrase BDint (nt 154-1062), *parD/parE* genes (nt 2304-2546; 2539-2823), hypothetical proteins (nt 1055-1300; 3214-3429; 3557-3949;7192-7428), *dfrVI* (nt 4106-4579), *qnrVC5* (nt 4742-5173) and a replicase (6032-6922) on pBD146 with respect to 59-base elements and two VCR-like regions. The ORF finder tool was used to predict the ORFs in pBD146.

Table 3.6. Predicted ORFs of pBD146

Sr. no.	Predicted ORF of pBD146 (bp)	Length (amino acid)	Predicted Protein
1	154-1062	302	Integrase IntI, DNA BRE_C superfamily
2	6032-6922	296	Replicase *repA*
3	4106-4579	157	Dihydrofolate reductase *dfrVI*
4	4742-5173	143	Pentapeptide repeats *qnrVC5*
5	3557-3949	130	Hypothetical protein
6	2539-2823	94	ParE- plasmid stabilisation superfamily
7	5116-5397	93	Fluoroquinolone resistance protein
8	1055-1300	81	Hypothetical protein
9	2304-2546	80	ParD antitoxin to ParE; RHH-2 superfamily
10	7192-7428	78	Hypothetical protein
11	3214-3429	71	Hypothetical protein

NCBI ORF finder tool was used to predict the possible ORFs

3.2.1.5b BLAST analysis of pBD146 indicated horizontal gene transfer between three *Vibrio* species

The sequence of pBD146 showed 99% identity with a plasmid pVN84 of *V. cholerae* O1 El Tor (2004) from Vietnam (GenBank accession no **AB200915**) and another plasmid of *V. parahaemolyticus* v110 (2010) from Hong Kong (GenBank accession no **KC540630**). While the isolates BD146 and VN84 were clinical in origin, v110 was environmental in origin [Liu and Chen, 2013b]. This suggested horizontal transfer of a plasmid between three different *Vibrio* species at three different locations of Southeast Asia over a period of eight years. Alternately, this plasmid could have been present in these species for a much longer time but detected recently. Additionally, BLAST analysis of pBD146 also revealed that some part (integrase, toxin-antitoxin systems and hypothetical proteins) of this plasmid was present in several *V. tasmaniensis* isolates of 2014 from USA and *V. parahaemolyticus* clinical isolates from Canada (2001-2006).

3.2.1.5c BDint is a putative integrase

pBD146 encoded a putative integrase (BDint) that belonged to DNA_BRE_C superfamily consisting of DNA breaking-rejoining enzymes including tyrosine recombinase. BDint showed 86%, 85%, 81% and 75% identity with integrases from *V. alginolyticus* (**EMD77077**), *V. parahaemolyticus* (**KKX78391**), *V. cyclitrophicus* (**OEF33672**) and *Photobacterium* sp (**ELR64627**) respectively in a BLASTp analysis. The nucleotide sequence of *BDint* (**KT182072**) showed 99% identity with its counterparts in *V. cholerae* pVN84 and *V. parahaemolyticus* pv110 and 77% to 82% identity with the integrases from *V. tasmaniensis.*

The presence of this integrase was analysed in other *V. fluvialis* isolates from the laboratory and in transconjugants/transformants of BD146 isolate by PCR using VcintF/VcintR primers. PCR showed the presence of the 657 bp *BDint* amplicon in twelve out of eighteen *V. fluvialis* isolates from 1998-2006 (Figure 3.7a). Three of these 100% identical sequences were submitted to GenBank (**KT182073, KT182074** and **KT182075**). This BDint was also found in 7.5kb+ transformants and tri_{20}+tet_{120} transconjugants of BD146 (Figure 3.7b and Figure 3.7c) but not in 7.5 kb- transformants and amp_{50}+tet_{120} transconjugants. A 657 bp amplicon produced in

reverse transcription confirmed the presence of mRNA for BDint in BD146. Absence of this amplicon in a negative control without reverse transcription ensured the absence of DNA contamination in RNA templates.

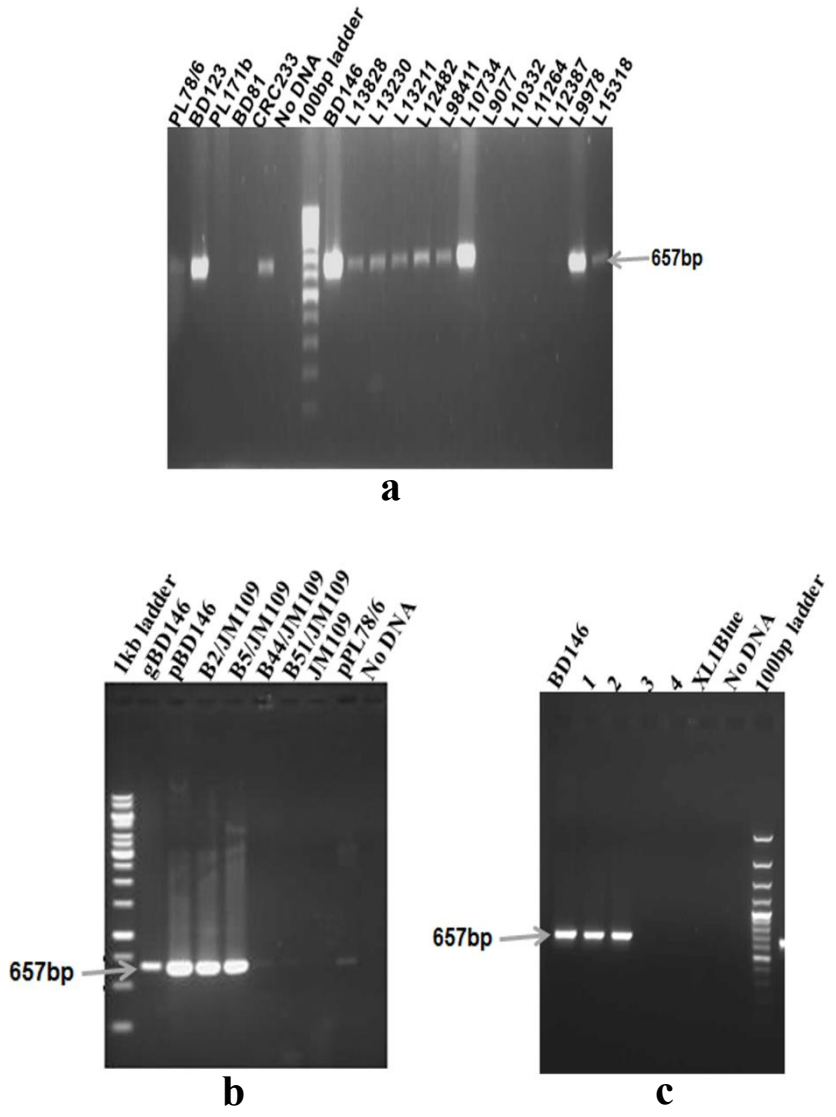


Figure 3.7. Agarose gel (1%) analysis of PCR amplicons of BDint from other clinical isolates of *V. fluvialis* and transformants and transconjugants of BD146 (a) PCR analysis of clinical isolates of *V. fluvialis* with VCint-F/VCint-R primer specific for BDint**;** (b) PCR analysis of BD146 and its transformants with VCint-F/VCint-R primer (c) PCR analysis of BD146 and its transconjugants with VCint-F/VCint-R primer**.** All the DNA samples used as templates are indicated on top of each lane. Lanes 1 and 2 (Panel c) BDint amplicons from transconjugants tri_{20} +tet_{120} respectively; Lanes 3 and 4 (Panel c) BDint amplicons from transconjugants amp_{50} +tet_{120} respectively. 100 bp DNA ladder (Fermentas) and 1 kb DNA ladder (Fermentas) were used as markers.

a

```
CLUSTAL O(1.2.4) multiple sequence alignment

int1shigella      ----------------MKTATAPLPPLRSVKVLDQLRERIRYLHYSLRTEQAYVHWVRAF
Int1M73819        ----------------MKTATAPLPPLRSVKVLDQLRERIRYLHYSLRTEQAYVHWVRAF
Bdint             MAKGHFAKSEKQAASVMKEMQGKGNAIESVGTA-------RNYEQALKTCCDY---LKEF
IntVC             ------------------------------MGTA-------RNYEQALKTCCDY---LKEF
IntVa             MAKGHFAKSEKQAASVMKEMQGKGNVIESVGTA-------RNYEQSLKTCCDY---LKEF
intVP             MAKGHFAKSEKQAASVMKEMQGKGNAIESVGTA-------RNYEQSLKTCCDY---LKEF
                                                 : .        *  . :*:*   *   :: *

int1shigella      IRFHGVRHPATLGSSEVEAFLSWLANER-KVSVSTHRQALAALLFFYGKVLCTDLPWL--
Int1M73819        IRFHGVRHPATLGSSEVEAFLSWLANER-KVSVSTHRQALAALLFFYGKVLCTDLPWL--
Bdint             K----LGSLRELTPEQAKNYIELRAHECKQSTIDMDRQAIQTMMQHVTHKLEPNQTLVPP
IntVC             K----LGSLRELTPEQAKNYIELRAHECKQSTIDMDRQAIQTMMQHVTHKLEPNQTLVPP
IntVa             K----LGSLREMTPEKANTYLELRAQECSQKTIDMDRQALQAMMQHVTHELKETDKLD--
intVP             K----LGSLREMTQEKANTYLELRAQECSQKTIDMDRQALQAMMQHVTHELKETEKLD--
                       :      :  .:.: ::.  *:*  : ::. .***: ::: .  : *

int1shigella      -QEIGRPRPSRRLPVVLTPDEVVRILGFLEGEHRLFAQLLYGTGMRISEGLQLRVKDL--
Int1M73819        -QEIGRPRPSRRLPVVLTPDEVVRILGFLEGEHRLFAQLLYGTGMRISEGLQLRVKDL--
Bdint             KELPTSKKETIENSRSYTPEQVNAIIQHQTEQHALSTQLCHEAGLRAHELYTLRPSGEVN
IntVC             KELPTSKKETIENSRSYTPEQVNAIIQHQTEQHALSTQLCHEAGLRAHELYTLRPSGEVN
IntVa             --VIKSSVETVESSRSYTPEQVNAIIQHQTEHHALSTQLCYESGLRAHELHTLRPSGEVN
intVP             --VIKSSVETIESSRSYTPEQVKAIIKHQTEHHALSTQLCHEAGLRAHELHTLRPFGEVS
                          : .     **::*  *: .   .* * :** : :*:*  *   **  .

int1shigella      ---------DFD----HGTIIVREGKGSKDRALMLPESLAPSLREQLSRARAWWLKDQAE
Int1M73819        ---------DFD----HGTIIVREGKGSKDRALMLPESLAPSLREQLSRARAWWLKDQAE
Bdint             PSPRDVHQDKFSQLPNDSKTYTVQGKGGLIREVQIPNHLAEKLEERRLD-----------
IntVC             PSPRDVHQDKFSQLPNDSKTYTVQGKGGLIREVQIPNHLAEKLEERRLD-----------
IntVa             PSPRDVHPNKFAHLPNESKTYTVNGKGGLIREVQIPNHLAEKLEERRLD-----------
intVP             PSPREVHQDKFSYFPKDSQTYTVEGKGGLIREVQIPNHLAEKLEERRLN-----------
                        .*     ..   . :***.  * : :*: ** .*.*:

int1shigella      GRSGVALPDALERKYPRAGHSWPWFWVFAQHTHSTDPRSGVVRRHHMYDQTFQRAFKRAV
Int1M73819        GRSGVALPDALERKYPRAGHSWPWFWVFAQHTHSTDPRSGVVRRHHMYDQTFQRAFKRAV
Bdint             ------TPQQL----SDRGVNYESHYNIA--------------GGHKFSDAFSKASSRA-
IntVC             ------TPQQL----SDRGVNYESHYNIA--------------GGHKFSDAFSKASSRA-
IntVa             ------MPQQI----SDRGVNYESHYNIA--------------GGHKFSDAFSKASSRA-
intVP             ------TPQKI----SDRGVNYESHYNIA--------------GGHKFSDAFSKASIRA-
                        *: :       * .:  .: :*               * :.::*.:*  **

int1shigella      EQAGITKPATPHTLRHSFATALLRS---GYD----IRTVQDLLGHSDVSTTMI-------
Int1M73819        EQAGITKPATPHTLRHSFATALLRS---GYD----IRTVQDLLGHSDVSTTMIYTHVLKV
Bdint             ----LGYSNGAHGLRHSYAQNRYEQLANHFERIDVMTIISQELGHFRPDITEVYLR----
IntVC             ----LGYSNGAHGLRHSYAQNRYEQLANHFERIDVMTIISQELGHFRPDITEVYLR----
IntVa             ----LGYSNGAHGLRHSYAQNRYEQLANHFERIDVMTIISQELGHFRPDITEVYLR----
intVP             ----LGYSNGAHGLRHSYAQNRYEQLANHFERLDVMTIISQELGHFRPDITEVYLR----
                      :      * ****:*    ..    ::    :  :.: ***   . * :

int1shigella      --------------------
Int1M73819        GGAGVRSPLDALPPLTSER
Bdint             --------------------
IntVC             --------------------
IntVa             --------------------
intVP             --------------------
```

b

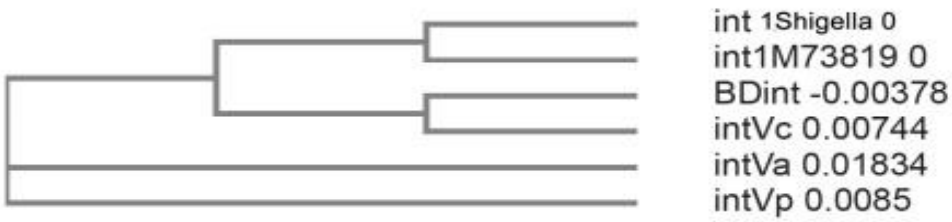


Figure 3.8. Comparison of BDint with other integrases. Multiple sequence alignment of BDint protein sequence with other integrase/recombinase sequences using Clustal O (1.2.4) program (a); and their phylogenetic analysis (b). Int1: class 1 integron integrases; intVc: integrase from *V. cholerae*; intVa: integrase from *V. alginolyticus*; intVp: integrase from *V. parahaemolyticus*

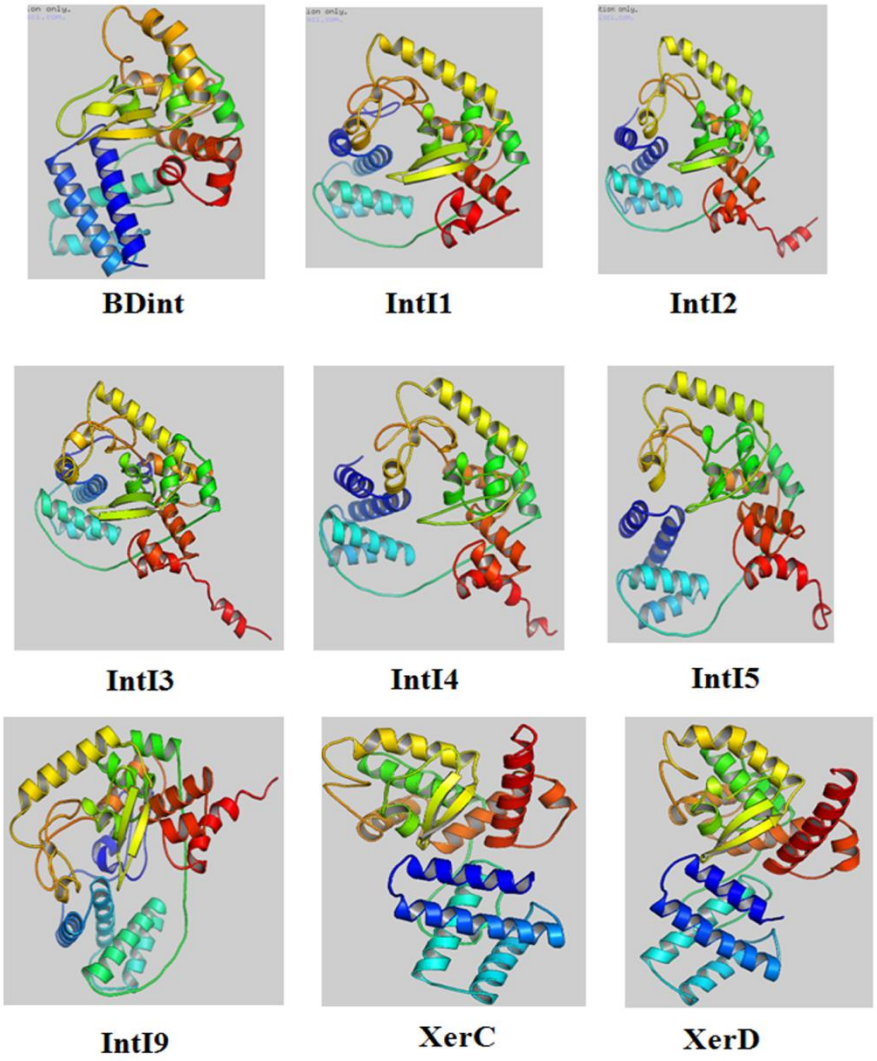


Figure 3.9. Schematic representations of the predicted 3D structures of various tyrosine recombinases. The structures were predicted based on the algorithm of I-TASSER [Yang et al., 2013; Roy et al., 2010; Zhang, 2008].

BDint was aligned pairwise separately to each of the integrases from class 1 to class 5 and class 9 integrons. These results along with those alignments to integrase from *V. cholerae, V. parahaemolyticus* and *V. alginolyticus* indicated BDint is more closely related to class 1 integron integrase which also included class 1 integrase from *Shigella* (Figure 3.8). The prediction of 3D structure of BDint by algorithm of I-TASSER [Yang et al., 2013; Roy et al., 2010; Zhang, 2008] showed different helices

each of which were partially overlapping with different integrases but no overall similarity was observed with any other integrase (Figure 3.9).

3.2.1.5d Toxin-Antitoxin system in *V. fluvialis*

The ORFs (nt 2539-2823 and nt 2304-2546) in pBD146 sequence corresponded to ParE (toxin) and ParD (antitoxin to ParE) respectively. ParE/ParD are addiction modules involved in plasmid stabilization. This is the first report of a putative toxin-antitoxin system in a plasmid from *V. fluvialis*. BLAST analysis indicated that this ParE/ParD system was co-existant with the integrase sequence in other *Vibrio* species mentioned in earlier.

3.2.1.5e *qnrVC5,* quinolone resistance gene for the first time reported from BD146

Seven *qnrVC* alleles encoding a family of pentapeptide repeat domain proteins (contributing to quinolone resistance) have been reported so far from *Vibrio spp*, *E. coli, Pseudomonas* spp. and *Aeromonas* spp. [Fonseca and Vicente, 2013; http://www.lahey.org./qnrstudies/].

In the present study, the ORF (nt 4742-5173) in pBD146 corresponded to the gene that encodes *qnrVC5*. There were two interesting aspects about this gene; it was for the first report from *V. fluvialis* and secondly, it was found associated with the plasmid. PCR experiments using primer specific to *qnrVC5* indicated the presence of this gene in *V. fluvialis* isolates L10734 and L9978 from 2006. The plasmid pV110 (Accession number KC540630) of *V. parahaemolyticus* (2010) from Hong Kong carried *qnrVC5* gene cassette [Fonseca and Vicente, 2013].

3.3.1.5f Cassettes for *dfrVI* and *qnrVC5* and their encompassing regions indicated their superintegron/transposon origin

Analysis of pBD146 sequence revealed the presence of *attC* sites (8-10) associated with various gene cassettes as shown in Figure 3.6. The *dfrVI* and *qnrVC5* genes were encompassed by *Vibrio cholerae* repeat regions (VCRs), the *attC*-like regions found on the superintegron of *V. cholerae*, indicating the superintegron origin of these cassettes. BLAST analysis of *qnrVC5* and *dfrVI* genes together revealed that it was

99% similar to their counterparts in *V. cholerae* and *V. parahaemolyticus* mentioned above and 86% similar to a transposon from *V. cholerae* MCV09 (HM015627) and a part of SXT element from *E. coli* J53 (FJ968160). Even the VCR regions were similar suggesting that these two genes have been moving together between different organisms either as part of integrons, plasmids or SXT elements, as also reported earlier [Fonseca and Vicente, 2013; Kumar and Thomas, 2011; Kumar and Thomas, 2009].

3.2.2. Discussion

In the scenario of emerging drug resistance and its spread between different genera, it becomes particularly interesting to study the acquisition and transfer of genes encoding drug resistance traits through mobile genetic elements. Such elements have been implicated in drug resistance phenotypes of many disease-causing bugs [Di Conza et al., 2005; Correia et al., 2003; Dalsgaard et al., 2001]. The studies aimed at unraveling the molecular mechanisms of multiple drug resistance in *V. fluvialis* are somewhat limited though a few studies have been carried out [Vinothkumar et al., 2016; Chowdhury et al., 2016; Singh et al., 2012; Chowdhury et al., 2011; Srinivasan et al., 2006; Ahmed et al., 2005; Ahmed et al., 2004] besides the cytotoxic and cell vacuolating potential of this bug [Chakraborty et al., 2005]. In this study, an attempt was made to analyse a clinical isolate of *V. fluvialis* BD146 of 2002 with respect to its resistance to various antibiotics and to elucidate the mechanisms underlying multidrug resistance with particular reference to the mobile genetic elements. The study was initiated with this isolate as it was found very promising due to its resistance to all the tested drugs. This isolate had shown complete or intermediate resistance to all the antibiotics tested seven of which could be transferred to *E. coli* JM109 through electroporation pointing to the possibility of the involvement of plasmids. A transformable plasmid pBD146 of 7.472 kb could be detected in this isolate. However, this plasmid sequence did not explain the transfer of resistance to six drugs as it contained only trimethoprim and quinolone resistance genes, which were not carried on an integron. pBD146 with the help of other conjugable plasmid was able to transfer in *E.coli* XL1-Blue through conjugation. This is reminiscent of an earlier observation, where only a fraction of the resistance markers, was located on integrons

in a 150 kb plasmid which harboured resistance markers to eight antibiotics [Dalsgaard et al., 2000].

As this strain showed resistance to streptomycin, trimethoprim and sulfamethoxazole, a drug resistance phenotype associated with SXT element, the SXT element could have been present in BD146. However, search for the presence of mobile genetic elements using PCR revealed the presence of class 1 integron and absence of SXT integrase as well as other classes of integrons in BD146. The 7.5kb-JM109 transformant showed resistance to ampicillin, chloramphenicol, gentamicin, tetracycline, nalidixic acid, kanamycin and rifampicin but not trimethoprim. This results indicated that integron(s) was located on a very low copy number plasmid which carried drug resistance markers mentioned above. Similar results were found in XL1-Blue transconjugants except for resistance phenotype for chloramphenicol and gentamicin. Comparison of the antibiograms of the parent strain and the transformants/transconjugants with the low copy number plasmid corroborated this observation that the majority of the resistance traits observed in the parent strain BD146 were contributed by the low copy number plasmid. Sequence analysis of 4.0 kb integron resident on this low copy number plasmid revealed a battery of drug resistance cassettes. The presence of resistance gene for rifampicin in this 4.0 kb variable region is especially noteworthy as this drug is not used for the treatment of cholera and has probably crept through the *Vibrio* genome from some other genus, probably mycobacterium/pseudomonas/klebsiella. Another interesting feature was the presence of efflux pump-like gene in the variable region of the integron that possibly resides on the chromosome. This kind of gene could account for resistance to many drugs. Carriage of this integron (with the battery of drug resistance genes) on a plasmid possibly aids in the faster dissemination of these traits in between the pathogenic bugs.

There are few reports on the plasmid characterization vis-à-vis multidrug resistance in literature for this *Vibrio*. A report has described a fluvialis plasmid isolated from salt marsh sediment [Hazen et al., 2007] which seems to indicate that *Vibrio* genomes are in a state of continuous flux due to genetic exchanges between *Vibrio* plasmids, phages and chromosomes. Our results presented in this section provide the first evidence that a plasmid exchange has taken place between the *V.*

fluvialis, V. cholerae O1 and *V. parahaemolyticus* strains. There was a high genetic relatedness between the three plasmids (pBD146, pVN84 and pV110) from clinical and environmental isolates derived from different species of *Vibrios* (*V. fluvialis*, *V. cholerae* and *V. parahaemolyticus* respectively). This was also suggestive of a high probability that these small plasmids are frequently exchanged and maintained in these *Vibrio* spp. from 2002 to 2010. There is also a distinct possibility of this transfer via an intermediate species or descent of this plasmid from a common ancestor. The 7.5 kb plasmid harboured many genes such as an integrase BDint*, dfrVI* (dihydrofolate reductase responsible for trimethoprim resistance), *qnr*VC5 (quinolone resistance gene), and genes for replicase, parE/parD toxin-antitoxin system and few hypothetical proteins. Additionally, pBD146 harboured two VCR regions (*Vibrio cholerae* Repeats) generally present on superintegrons and various *attC* (59-base elements) found associated with integrons. The presence of these *attC* and VCR regions encompassing some of the gene cassettes were indicative of their probable origin from superintegrons. It may be pertinent to mention here that in an earlier report, the presence of *qnrVC2* on class 1 integron of *V. cholerae* plasmid pVN84 had been reported [Fonseca et al., 2008] but the structure of a class 1 integron was not apparent from this plasmid sequence. Similar to pVN84, pBD146 did not appear to carry an integron though the recombination sites associated with some of the gene cassettes did indicate their provenance in a superintegron.

BLAST analysis of *parE* and *parD* genes revealed that these genes constituted the first toxin-antitoxin module reported from *V. fluvialis*. The toxin-antitoxin modules have been attributed multiple functions such as plasmid stability, stabilization of DNA segments, and protection against invading plasmids and phages and gene regulation [Unterhozner et al., 2013]. Hence, it appears that residence of this toxin-antitoxin module confers stabilization either to a pBD146 plasmid or some of the DNA segments of superintegron origin (*dfrVI* and *qnrVC*) in the vicinity of this module. Most interestingly, recent submissions in GenBank from mid-2015 to April 2016 have shown the presence of fragments of pBD146 harbouring the integrase, toxin-antitoxin module and a hypothetical protein in *V. parahaemolyticus* (2001-2006, Canada) and *V. tasmaniensis* (2014, USA). Therefore, there appears to be a very wide dispersal of these modules across the globe at least from 2001 to 2014. Hence, the results

presented here for pBD146 show the genesis of a mosaic of a variety of genes/cassettes derived from sources such as superintegrons and transposons. This plasmid has been maintained in different *Vibrio* spp. through the years 2002-2010, as a carrier of many novel modules capable of persistence in different bacteria and dissemination of drug resistance.

To summarize, in the present study multiple plasmids and integrons along with other genetic factors significantly contributed to the drug resistance phenotype of this clinical isolate (Table 3.7).

Table 3.7. Correlation of resistance to different antibiotics with corresponding genes/factors in clinical isolate of *V. fluvialis* BD146

Antibiotic	**Factor/genes responsible for resistance phenotype in *V. fluvialis* BD146**
Fluoroquinolone	Mutation in Topoisomerase, *qnrVC5*, *aac-6'-Ib-cr*
Aminoglycoside	*aadA1* (Aminoglycoside-3'-adenyltransferase) and efflux pump
Beta-lactam	*blaOXA-10* (extended spactrum β-lactamase OXA)
Rifampicin	*arr-3* (rifampicin ADP-ribosylating transferase)
Chloramphenicol	*cmlA* (MFS efflux pump)
Sulphonamide	*sul1* gene on Class 1 integron
Trimethoprim	*dfrVI* (dihydrofolate reductase)
Tetracycline	Efflux pump

3.3. Characterization of mobile genetic elements imparting drug resistance in *Vibrio fluvialis* isolates (2006)

3.3.1. Results

3.3.1.1. Antibiotic resistance profile of *Vibrio fluvialis* clinical isolates

Twelve clinical isolates of *V. fluvialis* from 2006 were analysed for their drug resistance profiles using 13 antibiotics. Antibiotic susceptibility tests revealed that all the isolates displayed drug resistance with varying antibiograms (Table 3.8). Resistance to ampicillin and neomycin was common to all. All the isolates except L12482 showed intermediate resistance to kanamycin. Three strains L12387, L9978 and L15318 displayed resistance to the majority (77-85%) of the antibiotics tested. 11 out of 12 were sensitive to tetracycline and 10 out of 12 to gentamicin.

Table 3.8. Antibiograms of clinical isolates of *Vibrio fluvialis* from 2006

Isolate	Resistance	Intermediate Resistance
L13828	AMP, NEO	KAN, GEN
L13230	AMP, NEO	KAN, STR, CIP
L13211	AMP, NEO	KAN
L12482	AMP	NEO
L98411	AMP, NEO	KAN, GEN, STR, CIP
L10734	NEO, CO-TRI, NAL, TRI	AMP, KAN, CIP
L9077	AMP	KAN, NEO, NAL
L10332	AMP, NEO	KAN
L11264	NEO	AMP, KAN
L12387	AMP,CO-TRI, NAL, NEO, STR, SUL, TRI	CHL, CIP, KAN, NOR
L9978	AMP, CO-TRI, NEO, STR, SUL, TRI	CHL, CIP, KAN
L15318	AMP, CO-TRI, NAL, NEO, SUL, TRI, TET	CHL, CIP, KAN, NOR

AMP, ampicillin; CHL, chloramphenicol; CO-TRI, co-trimoxazole; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; SUL, sulfisoxazole; TMP, trimethoprim; TET, tetracycline; NEO, neomycin; NAL, nalidixic acid; NOR, norfloxacin; KAN, kanamycin.

3.3.1.2. Relatedness between the twelve isolates

As all the isolates were obtained from the same location during the same year (2006), it was of interest to examine their relatedness. To this end, total genomic DNA from these strains was subjected to PFGE analysis (Figure 3.10). Gel showed that three isolates L13828, L10734 and L9978 contained plasmids (Figure 3.10a, Lanes 1, 6, 11). Plasmids from the isolates L10734 and L9978 (Figure 3.10a, Lanes 6, 11) had

similar molecular size, while the plasmid in L13828 (Figure 3.10a, Lane 1) was smaller with less than 0.225 Mb size. PFGE of the *Not* I-digested DNA (Figure 3.10b) revealed that the isolates L13230, L13211, L12482 (Lanes 2, 3, 4) had a similar pattern of the bands and therefore could be derived from the same clone. The rest of the nine isolates appeared to have different pulsotypes.

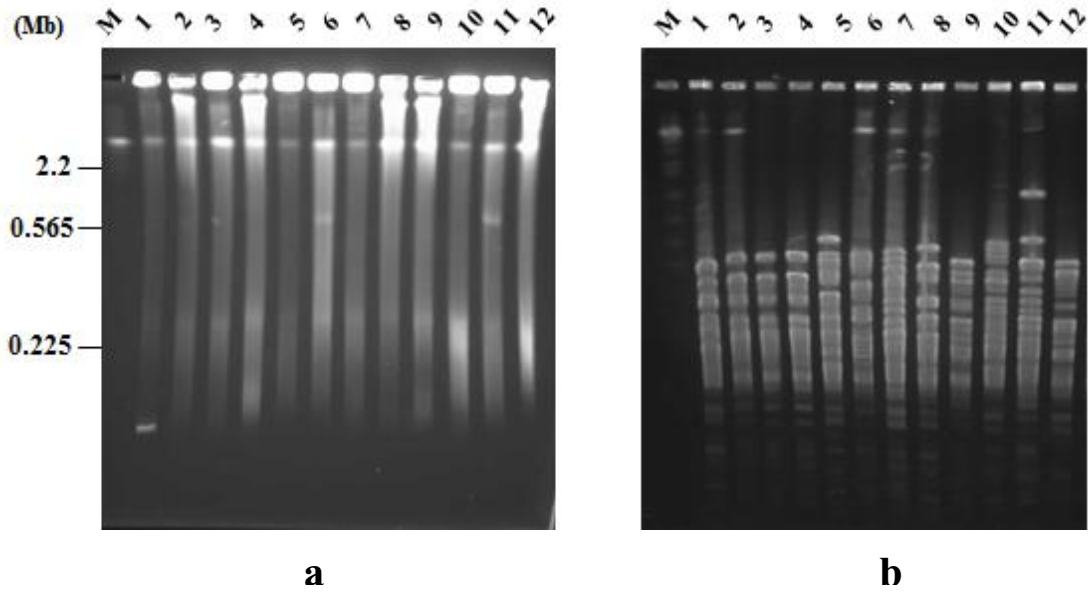


Figure 3.10. Total genomic DNA from twelve clinical isolates of *Vibrio fluvialis*. (a) PFGE (1%) analysis of the undigested and (b) *Not*I-digested genomic DNA. Lane M: CHEF (clamped homogeneous electric fields) DNA size standards (Bio-Rad) from *S. cerevisiae.* Positions of the marker bands have been indicated on left. Lanes 1 to 12, isolates L13828, L13230, L13211, L12482, L98411, L10734, L9077, L10332, L11264, L12387, L9978, L15318. Distinct plasmid bands can be seen in lanes 1 (L13828), 6 (L10734) and 11 (L9978) in panel a.

3.3.1.3. Analysis of integrons and SXT integrase

The presence of integrons and SXT elements as drug resistance carriers was analysed by PCR. Primers specific to the integrases from class 1, class 2, class 3 and class 4 integron and SXT integrase were used as described in section 2.7. Results revealed that these isolates carried neither any of the integrons nor SXT element, as they did not show any amplification in these PCR reactions.

3.3.1.4. Transfer of resistance traits by conjugation and transformation

In order to ascertain if the plasmids detected in the three isolates, namely L13828, L10734 and L9978 (Figure 3.10a and Figure 3.11) could transfer any drug resistance

genes to another bacterium, conjugation and transformation experiments were performed as described in section 2.3. The conjugation analysis was done with either *E. coli* DH5α or *V. cholerae* O1 El Tor N16961 and the transconjugants were examined for their drug resistance traits (Table 3.9). Transformation experiments were carried out with these three isolates by electroporation in *E. coli* JM109 and the transformants were analysed for their transferable resistance traits (Table 3.9). As depicted from the profiles of transconjugants/transformants in Table 3.9, L13828 isolate harbored non-conjugable plasmid carrying ampicillin and tetracycline resistance. As interpreted from Table 3.9 and Figure 3.12, L10734 carried conjugable plasmid (harboured resistance traits for neomycin, kanamycin, co-trimoxazole, trimethoprim and nalidixic acid) and non conjugable plasmids (harboured resistance traits for ampicillin, neomycin and tetracycline). The L9978 carried conjugable plasmid (harbored resistance traits for ampicillin, neomycin, kanamycin, co-trimoxazole, trimethoprim, ciprofloxacin, sulfisoxazole, chloramphenicol and streptomycin) and non conjugable plasmid that harboured tetracycline resistance (Table 3.9 and Figure 3.12).

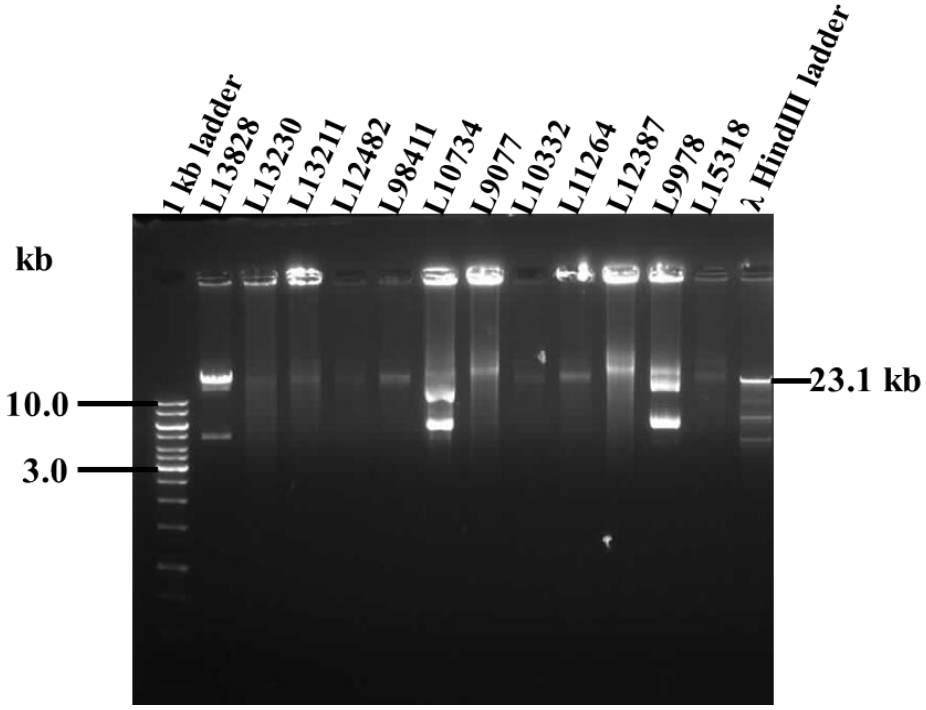


Figure 3.11. Agarose gel (1%) analysis of plasmid DNA from twelve clinical isolates of *Vibrio fluvialis*. All the DNA samples used as templates are indicated on top of each lane. Lambda *Hind*III DNA ladder (Sigma) and 1 kb DNA ladder (Fermentas) were used as markers.

Table 3.9. Antibiotic susceptibility patterns of *Vibrio fluvialis* parent isolates, their transformants and transconjugants

Isolates	**L13828**	**L10734**	**L9978**
Parent strain	AMP, NEO, KAN, GEN	AMP, NEO, KAN, COT, TRI, CIP, NAL	AMP, NEO, KAN, COT, TRI, CIP, SUL, STR, CHL
Transconjugants with recipients *E. coli* DH5α (NAL^r) or *V. cholerae* O1 El Tor N16961 (STR^r)	No transconjugants	NEO, KAN, COT, TRI, NAL, STR	AMP, NEO, KAN, COT, TRI, CIP, SUL, STR, CHL, NAL
Transformant with *E. coli* JM109 (NAL^r)	AMP, TET, NAL	AMP, NEO, TRI, TET, NAL	AMP, NEO, TRI, TET, NAL

r- Resistance

No distinction was made for full and intermediate resistance. In transformants, resistance to NAL was derived from host JM109 cells. In L10734 transconjugants, STR and L9978 transconjugants, NAL resistance were host-derived.

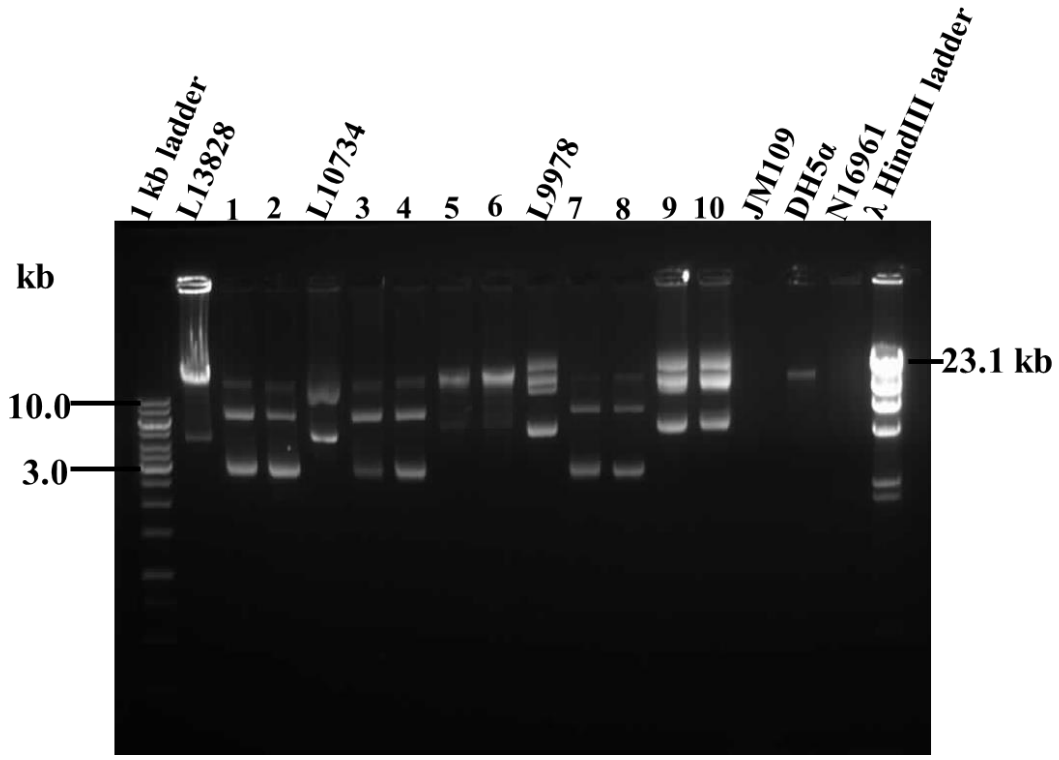


Figure 3.12. 1% agarose gel analysis of plasmid DNA from *Vibrio fluvialis* isolates their transformants, transconjugants, and recipients *E. coli* JM109, DH5α and *V. cholerae* O1 El Tor N16961. The sample identity has been indicated on the top of each lane. Lanes 1 and 2: plasmid preparations of transformants from *V. fluvialis* L13828; Lanes 3 and 4: plasmid preparations of transformants from *V. fluvialis* L10734; Lanes 5 and 6: plasmid preparations of transconjugants from *V. fluvialis* L10734; Lanes 7 and 8: plasmid preparations of transformants from *V. fluvialis* L9978; Lanes 9 and 10: plasmid preparations of transconjugants from *V. fluvialis* L9978.

3.3.1.5. Quinolone resistance in *Vibrio fluvialis* isolates

Out of twelve clinical isolates, seven showed resistance to one or more quinolones (Table 3.8). Four isolates were resistant to nalidixic acid and six were resistant to different degrees (intermediate or complete resistance) to ciprofloxacin and/or norfloxacin (ciprofloxacin resistant isolates L12387, L15318, L13230, L98411, L10734 and L9978; norfloxacin resistant isolates L12387, L15318).

The plasmid-mediated quinolone resistance (PMQR) has been described with three mechanisms: A) Qnr proteins protecting topoisomerases from quinolone action; B) modification of antibiotics by enzymes; C) export of antibiotics through efflux pumps [Yamane et al., 2007; Perichon et al., 2007; Robicsek et al., 2006]. Analysis of PMQR genes in quinolone resistant isolates by PCR (Section 2.7) revealed that *qnrVC* gene was present only in two isolates L10734 and L9978 (quinolone-resistant) whereas other PMQR genes were not detected in the rest of isolates. In addition, it was observed that this resistance was plasmid-mediated as the transconjugants derived from L10734 and L9978 confirmed the presence of *qnrVC* allele by PCR (Figure 3.13).

Those isolates where the PMQR genes were absent, were analysed for mutations in topoisomerase gene. Quinolone Resistance Determining Regions (QRDR) of the four topoisomerase genes (*gyrA*, *gyrB*, *parC* and *parE*) were amplified and sequenced from L15318, L13828 and *V. cholerae* N16961. The results revealed that serine 83 in gyrase A of the sensitive strain was replaced by isoleucine, and serine 85 in parC of the sensitive strain was replaced by leucine in resistant strain L15318 (Table 3.10). No other mutations were detected in the other two topoisomerase genes *gyrB* and *parE*.

Table 3.10 Topoisomerase mutations in quinolone resistance determining regions of *Vibrio fluvialis* isolates

Isolate	Quinolone resistance	MIC (µg)			Mutations in topoisomerases
		NAL	CIP	NOR	
L15318	NAL, CIP, NOR	>240	2.0	2.0	GyrA (S_{83}→I); ParC (S_{85}→L)
L13828	Sensitive to all	0.1	0.125	0.05	No mutation

NAL: nalidixic acid; CIP: ciprofloxacin; NOR: norfloxacin

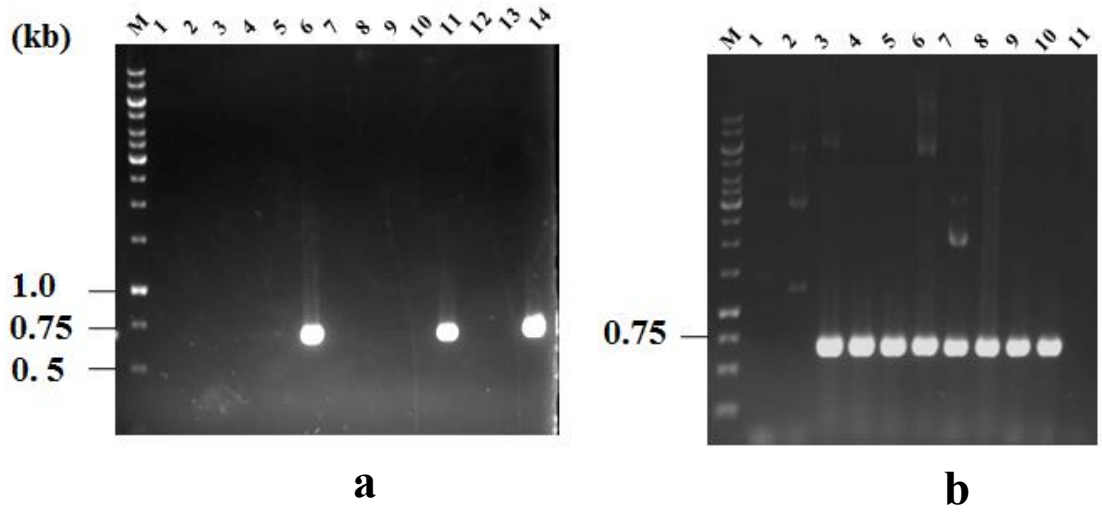


Figure 3.13 Plasmid-mediated quinolone resistance. PCR was carried out with the primers based on *qnrVC* allele. (a) Agarose gel analysis (1%) of amplicons obtained from genomic DNA of twelve isolates of the year 2006. Lanes 1 to 14: isolates L13828, L13230, L13211, L12482, L98411, L10734, L9077, L10332, L11264, L12387, L9978, L15318, no template control, positive control. Lane M: 1 kb DNA ladder (Fermentas). Positions of the marker bands have been indicated on left. (b) Transfer of *qnr* during transformation and conjugation. PCR products from the following samples have been loaded in Lanes: 1: plasmid from L13828; 2: transformant from L13828; 3: plasmid from L10734; 4: transformant from L10734; 5: transconjugant from L10734; 6: plasmid from L9978; 7: transformant from L9978; 8: transconjugant from L9978; 9: plasmid from BD146; 10: transformant from BD146; 11: No template control. Lane M: 1 kb DNA ladder (Fermentas). Positions of the marker bands have been indicated on left.

3.3.1.6. Role of efflux pumps in MDR of *Vibrio fluvialis* isolates

The role of efflux pump towards various antibiotics was confirmed using synergy test. The MIC was analysed in two representative isolates L15318 and L13828 against various antibiotics in presence and absence of efflux pump inhibitor CCCP (4 mg/L). Out of 13 antibiotics tested for antibiotic susceptibility (Section 3.3.1.1), one representative antibiotic from each group of antibiotics was taken for synergy test. These antibiotics were ampicillin (beta-lactum), chloramphenicol, ciprofloxacin (quinolone), trimethoprim (dhfr inhibitor), tetracycline (tetracyclines) and kanamycin (aminoglycosides) to which the isolates were resistant (Table 3.11). As shown in Table 3.11, 2.0 to 5.3 fold difference in MIC was observed towards ampicillin and tetracycline. Therefore, these results revealed that efflux pumps were contributed in conferring resistance to these antibiotics and active in both the isolates.

Table 3.11. Synergy test for efflux pump activity in *Vibrio fluvialis* isolates

Antibiotics	**Antibiotics with CCCP (4 mg/L)**	**L13828**	**Fold change in MIC**	**L15318**	**Fold change in MIC**
Ampicillin	-	24	**1.5**	>256	**4 to 5.33**
	+	16		48-64	
Chloramphenicol	-	1	1	12	**1.5**
	+	1		8	
Ciprofloxacin	-	0.125	1	2	**1.33**
	+	0.125		1.5	
Trimethoprim	-	1.5	**1.5**	>32	1
	+	1		>32	
Tetracycline	-	1.5	**2**	48	**3**
	+	0.75		16	
Kanamycin	-	1.5	1	3	**1.5**
	+	1.5		2	

3.3.1.7. Role of extended spectrum beta lactamases in *Vibrio fluvialis* isolates

As all the isolates showed resistance to ampicillin, they were further analysed for the presence of extended spectrum beta lactamases (ESBL) in two representative sensitive L13828 isolate and resistant L15318 isolate (Table 3.12). For phenotypic ESBL analysis, cefotaxime (CTX)/cefotaxime and clavulanic acid (CTX+), and ceftazidime (CAZ)/ceftazidime and clavulanic acid (CAZ+) EzyMIC strips were used, to evaluate MIC of this beta lactam antibiotic in presence or absence of β-lactamase inhibitor. It was also analysed for AmpC beta lactamases with the mixture (MIX) of ceftazidime, cefotaxime, cefepime and cloxacillin and MIX with clavulanic acid and tazobactam. Results revealed that L15318 was ESBL positive, as the MIC ratio of CTX/CTX+ was 16 and CAZ/CAZ+ was 32. L13828 was ESBL sensitive isolate as the MIC ratio was below 8 for CTX/CTX+ and CAZ/CAZ+. Further, both the isolates L15318 and L13828 were sensitive for the AmpC beta lactamase.

Table 3.12. Evaluation of extended spectrum beta lactamase activity in *V. fluvialis* isolates

ESBL	**ATCC 25922**	**L13828**	**L15318**
CTX	0.38	0.50	>16
CTX+	0.125	0.125	1.0
Ratio CTX/CTX+	**3.04**	**4.0**	**16**
CAZ	0.75	1.0	>32
CAZ+	0.50	0.75	1.0
Ratio CAZ/CAZ+	**1.5**	**1.33**	**32**
MIX	0.25	0.50	1-1.5
MIX+	0.125	0.19	0.38
Ratio MIX/MIX+	**2.0**	**2.6**	**2.63-3.94**

If the ratio is ≥ 8, the test is positive for lactamase activity

3.3.2. Discussion

With the dismal scenario of an alarming increase in drug resistance of various infectious pathogens, it becomes imperative to understand the mechanisms of drug resistance in these pathogens and their changing drug resistance profiles for an effective treatment. The present study was undertaken to elucidate the drug resistance mechanisms operative in twelve clinical isolates of *V. fluvialis* obtained from Kolkata, India in the year 2006. These isolates were resistant to two or more antibiotics out of 13 antibiotics tested. There are reports on multiple antibiotic resistance in *V. fluvialis* isolates around the world [Chowdhury et al., 2016; Vinothkumar et al., 2016; Chowdhury et al., 2011; Lagana et al., 2011; Srinivasan et al., 2006; Ahmed et al., 2005].

Three isolates out of twelve showed the presence of plasmids that had the capability to transfer drug resistance traits through horizontal gene transfer. The drug resistance markers were present on the plasmids and two of the strains, L10734 and L9978, contained besides the common plasmid carrying ampicillin and tetracycline resistance markers, other self-transmissible plasmid(s), harbouring some more resistance genes. One apparently puzzling finding was the presence of tetracycline resistance in all the three types of transformants even though the donor strains were susceptible to this antibiotic. There are reports where the cryptic tetracycline resistance genes get expressed upon their transfer to recipient [Zhao et al., 2001; Park et al., 1987] and it is likely that the same thing was happening in the present study. Quinolone resistance was observed in 7 out of 12 isolates and appeared to be primarily due to mutations in *gyrA* and *parC* genes. In addition, qnr pentapeptide repeat protein *qnrVC* also played a role in governing quinolone resistance in two isolates and it could get transferred from one bacterium to another through conjugation and transformation experiments indicating of the presence of this gene on a plasmid.

Qnr family of pentapeptide repeat proteins has been shown to confer protection to DNA gyrases thus rendering quinolone resistance. From India, there is a report describing the emergence of *qnrA1* from Indian isolates of *V. fluvialis*, of 2009 [Chowdhury et al., 2011]. Though efflux pumps govern quinolone resistance phenotype as has been observed earlier [Srinivasan et al., 2006; Baranwal et al. 2002],

it did not seem to be the case for the isolates studied here. However, resistance phenotype for ampicillin and tetracycline, seemed to be associated with the efflux pump activity.

To summarise, the study has indicated the interplay of a large number of genetic factors such as plasmids, mutations, efflux pumps, ESBLs and qnr proteins for the drug resistance found in *V. fluvialis* isolates from Kolkata, India.