2. Materials and Methods

Recipes and preparation methods for media and reagents mentioned in this chapter are described in the appendices.

2.1. Bacterial isolates

The clinical isolates of *Vibrio fluvialis* (n=13) and *Shigella* (n=95) were procured from National Institute of Cholera and Enteric Diseases [NICED]; (Kind courtesy Dr. Amit Ghosh, Dr. Thandavarayan Ramamurthy and Dr. Swapan Kumar Niyogi) in the form of pure culture stabs. This study included, *V. fluvialis* (n=13*)* from 2002 and 2006, and *Shigella* (n=95) from 2001 to 2010 i.e. *S. flexneri* (n= 42), *S. sonnei* (n=42), *S. boydii* (n=6) and *S. dysenteriae* (n=5) (Table 2.1). These isolates were collected from patients with acute cholera-like diarrhoea and bloody diarrhoea admitted to the Infectious Diseases Hospital, Kolkata, India. The study was carried out after the formal consent of patients as required by the Institutional Ethical Clearance Committee of NICED.

The isolation and microbiological identification was carried out at NICED laboratory before dispatching the strains and their identity was reconfirmed at IIAR by streaking them on various selective media such as Thiosulfate Citrate Bile salts Sucrose (TCBS) agar, Xylose Lysine Deoxycholate (XLD) agar, Hektoen Enteric Agar (HEA), Luria-Bertani (LB) agar and MacConkey agar. Subsequently, the cultures were grown in LB broth and cultures were suspended in 25% glycerol and stored at -70 °C as glycerol stocks for long term. Stabs were also prepared for the long term use.

Escherichia coli JM109, *E. coli* DH5α, *E. coli* XL1Blue, *E. coli* J53, and *V. cholerae* O1 El Tor N16961 were used in transformation and conjugation experiments (Table 2.1). *Salmonella enterica* serotype *Braenderup* H9812 and CHEF DNA size marker *Saccharomyces cerevisiae* (catalogue no.170-3605, Bio-Rad) were used as standards in pulsed-field gel electrophoresis (PFGE) experiments (Table 2.1). *E. coli* ATCC25922 was utilized as a quality control for the antibiotic susceptibility tests and minimal inhibitory concentration determination (Table 2.1).

Table 2.1. List of bacterial strains used.

Organism	Description	Reference
V. fluvialis (n= 13)	Clinical sample	In this study
S. flexneri (n= 42)	Clinical sample	In this study
S. sonnei (n=42)	Clinical sample	In this study
S. boydii (n=6)	Clinical sample	In this study
S. dysenteriae (n=5)	Clinical sample	In this study
E. coli ATCC25922	Reference strain	CLSI, 2010
Salmonella enterica serotype *Braenderup* H9812	Reference strain	Hunter et al., 2005
E. coli DH5α	Reference strain nalidixic acid resistant	Taylor et al.,1993
E. coli XL1Blue	Reference strain nalidixic acid resistant and tetracycline resistant	Casali, 2003
E. coli J53	Reference strain Sodium azide resistant	Yi et al., 2012
E. coli JM109	Reference strain nalidixic acid resistant	Casali, 2003
V. cholerae O1 El Tor N16961	Clinical sample	Heidelberg et al., 2000

2.2. Determination of antibiotic susceptibility

2.2.1. Disc diffusion method

Antibiotic susceptibility test was carried out using disk diffusion method [Bauer et al. 1966] in accordance with Clinical and Laboratory Standards Institute guidelines [CLSI, 2010; CLSI, 2015]. 3-5 overnight grown colonies were inoculated in 10 mL LB broth and incubated at 37°C, 200 rpm till OD_{620}=0.08-0.13 (~10^5-10^6 cells/mL). Then, the cultures were spread on the Muller-Hinton agar (MHA) (HiMedia) with the cotton swab, to obtain a confluent growth. The antibiotic discs were placed on the plate for their susceptibility to the antibiotics (HiMedia). After incubation of plates at 37°C for 18 hours, the diameter of the zone of inhibition was measured and interpreted with the interpretative chart based on CLSI guidelines to reveal the

resistance phenotype of each isolate. Experiments were performed individually at least three times. *E. coli* ATCC 25922 was included as a quality control for all the antibiogram tests.

2.2.1.1. Antibiotic susceptibility tests for *Vibrio fluvialis* and *Shigella* isolates

V. fluvialis and *Shigella* isolates were analysed by disc diffusion method for their susceptibility to various antibiotics as described in Table 2.2. The test was carried out in accordance with the criteria recommended by Clinical and Laboratory Standards Institute (CLSI) standards (CLSI, 2010; CLSI, 2015). When no interpretive criteria for *V. cholerae* were available based on CLSI guidelines, breakpoints for *Enterobacteriaceae* were applied.

Table 2.2. List of antibiotics used for the analysis of antibiogram of *V. fluvialis* and *Shigella* isolates.

Antibiotics	***V. fluvialis* isolates**	***Shigella* isolates**
ampicillin (10 µg)	√	√
azithromycin (15 µg)	-	√
ceftriaxone (30 µg)	-	√
cefuroxime (30 µg)	-	√
chloramphenicol (30 µg)	√	√
co-trimoxazole (1.25 µg trimethoprim/23.75 µg sulfamethoxazole)	√	√
ciprofloxacin (5 µg)	√	√
gentamicin (10 µg)	√	√
streptomycin (10 µg)	√	√
sulfisoxazole (300 µg)	√	-
trimethoprim (5 µg)	√	√
tetracycline (30 µg)	√	√
neomycin (30 µg)	√	-
nalidixic acid (30 µg)	√	√
norfloxacin (10 µg)	√	√
kanamycin (30 µg)	√	√
ofloxacin (5µg)	-	√
rifampicin (5 µg)	√	-

- symbol indicates that these antibiotics were not used for these isolates

2.2.2. Minimal Inhibitory concentration (MIC) assays

MIC determination of *Vibrio* and *Shigella* spp. was carried out using Ezy MIC strips (HiMedia) or HiComb MIC test (HiMedia) as per the manufacturer's instructions. Interpretation of the results used the criteria recommended by CLSI [CLSI, 2010; CLSI, 2015]. The cultures of the isolates containing 10^5-10^6 cells/mL (OD_{600} = 0.08 to 0.13) were streaked on the MHA plates to obtain a confluent growth and the strips/combs were placed on the agar plate using the applicator. Subsequently, MHA plates were incubated at 37 °C for 18 h. The concentration of the antibiotic, where the edge of the inhibition ellipse intersected the sides of the strip was taken as the MIC value of the antibiotic for that bacterial strain. *E. coli* ATCC 25922 was used as quality control. All experiments were independently performed at least three times.

2.3. Transferability of mobile genetic elements

To study the transfer of antibiotic resistance traits within/from bacterial isolates, two types of experiments were carried out: transformation and conjugation. They have been described below.

2.3.1. Transformation

Transformation of *E. coli* JM109 was carried out by electroporation (GenePulser Xcell, Bio-Rad Laboratories) with 150 ng of Qiagen purified plasmid preparations from *V. fluvialis* isolates. The transformants were selected on LB plates containing appropriate antibiotics. A single colony of the transformants from LB plates with antibiotics, or the parent Vibrio strain from LB plate, was grown in the liquid medium and tested for the antibiotic resistance profile and the presence of transferable mobile genetic elements as described in section 2.7. The detailed procedure for electroporation is described in the following sections.

2.3.1.1. Preparation of electrocompetent (EC) cells

A fresh colony of *E. coli* JM109 or DH5α was inoculated into a 50 mL SOB (without Mg^{2+}) in a 500 mL flask. The cells were grown with vigorous aeration overnight at 37 ºC, 200 rpm (Orbitek, Model: LE). 0.5 mL of cells from the overnight grown culture were diluted in 250 mL SOB in a 2 L flask and it was grown until the OD_{550} = 0.8 at

37 ºC, 200 rpm. The flask was chilled in ice for 20 min. The cells were harvested at 5000 rpm (Kubota, Model: 6500, Rotor: AG2506) for 10 min at 4 ºC. The cell pellet was washed in 250 mL sterile ice-cold wash buffer (10% [v/v] glycerol in water) by pipetting the suspension up and down several times using sterile chilled pipettes. The suspension was centrifuged at 5000 rpm for 15 min. The cell pellet was again washed in 250 mL sterile ice-cold wash buffer and finally, resuspended in a volume of 0.5 mL of wash buffer and stored at -70ºC in smaller aliquots.

2.3.1.2. Electroporation in *E. coli*

Forty microliters of *E. coli* JM109 EC cells (~10^9) were taken in a sterile, pre-chilled microfuge tube. 1 µL of plasmid DNA (150 ng) was added to the EC cells and mixed well through gentle pipetting and the tubes were incubated on ice for 1 min. The mixture was added into pre-chilled electroporation cuvettes (2 mm, Bio-Rad). The electroporation was carried out at 2.5 kV and 25 µF capacitance for 5 milliseconds (Gene Pulser Xcell, Bio-Rad Laboratories Inc.). The cuvettes were placed on ice immediately after the shock and 1 mL SOC/LB was added into the cuvette, the cells were collected and transferred to the microfuge tubes for incubation at 37 °C, 200 rpm for 1 h. After incubation, the cultures were pelleted down, 800 µL of supernatant was decanted from each tube and the pellet was resuspended in remaining 200 µL supernatants. The cultures were spread on LB agar plates containing appropriate antibiotic (example ampicillin 25 µg/mL) and incubated at 37 °C overnight.

Prior to the electroporation of the plasmid DNA, the transformation efficiency of the EC cells was checked by transforming 2 pg and 10 pg of pBS plasmid into the cells by the electroporation method described above. The cells were plated on LB ampicillin (100 µg/mL), incubated at 37 °C overnight and the colonies were counted. The transformation efficiency was calculated as the number of transformants obtained per microgram of the DNA.

2.3.2. Bacterial conjugation

These experiments were carried out according to a published protocol [Ceccarelli et al., 2006]. Overnight culture (0.1 mL) from donor and recipient strains grown with appropriate antibiotics were diluted in 10 mL LB and grown until OD_{600} 0.3-0.4 (~

10^7 cells). The flask containing donor culture was kept at 37 °C for 30 min while the recipient culture was kept in a shaker at 37 ºC, 200 rpm. The recipient and donor strains were mixed in the ratio of 1:1 on a sterile 0.45 µm nylon membrane (Nytran N, Whatman or PALL Life Sciences) and incubated overnight for mating on LB agar at 37 ºC. The membrane was transferred to 10 mL LB and incubated at 37 ºC, 200 rpm for 1 h. 1 mL culture was pelleted down and resuspended in 200 µL. The cultures were spread on LB agar/MacConkey agar plates containing appropriate antibiotics and incubated at 37 °C overnight. The transconjugants were tested for transferable resistance traits by determination of their antibiotic susceptibility profiles and presence of mobile genetic elements.

2.3.2.1. Conjugation of *Vibrio fluvialis* isolates of 2006

The three plasmid-bearing strains (L13828, L10734 and L9978) were tested for conjugation experiments. For the strain L13828 which is sensitive to nalidixic acid as well as streptomycin, both *E. coli* DH5α and *V. cholerae* O1 El Tor were used as recipients. For the strain L9978 (sulfamethoxazole resistant), *E. coli* DH5α (nalidixic acid resistant and sulfamethoxazole sensitive) was used as recipient whereas for strain L10734 (nalidixic acid resistant and streptomycin sensitive), *V. cholerae* O1 El Tor N16961 (streptomycin resistant) was used as a recipient. The transconjugants were selected on LB agar plates containing appropriate antibiotics. For conjugation between L13828 and *V. cholerae* O1 El Tor, ampicillin (25 µg/mL) and streptomycin (20 µg/mL) and for conjugation between L10734 and *V. cholerae* O1 El Tor, nalidixic acid (30 µg/mL) and streptomycin (20 µg /mL) were used for selection of transconjugants. For conjugation between L9978 and *E. coli* DH5α, selection of sulfamethoxazole (160 µg/mL) and nalidixic acid (30 µg/mL) was used.

2.3.2.2. Conjugation of *Vibrio fluvialis* isolate BD146 of 2002

BD146 donor harboured resistance to ampicillin (MIC >256 µg/mL) and trimethoprim (MIC >32 µg/mL) and intermediate resistance to tetracycline (MIC ≤16 µg/mL). The recipient *E. coli* XL1-Blue was sensitive to ampicillin (MIC = 4 µg/mL) and trimethoprim (MIC = 0.25 µg/mL) and highly resistant to tetracycline (MIC > 256 µg/mL). The transconjugants were selected on LB agar plates containing two

antibiotic combinations; ampicillin (50 µg/mL) and tetracycline (120 µg/mL) or trimethoprim (20 µg/mL) and tetracycline (120 µg/mL).

2.3.2.3. Conjugation in *Shigella* isolates

Six *Shigella* isolates were tested in the conjugation experiments. *E. coli* XL1-Blue (tetracycline and nalidixic acid resistant) or *E. coli* J53 (sodium azide resistant) was used as recipient. The transconjugants were selected on MacConkey (for *E. coli* J53 recipient) containing trimethoprim (20 µg/mL) and sodium azide (150 µg/mL) or streptomycin (20 µg/mL) and sodium azide (150 µg/mL). While for *E. coli* XL1-Blue recipient, the transconjugants were selected on LB agar plates containing trimethoprim (20 µg/mL) and tetracycline (60 µg/mL) or streptomycin (20 µg/mL) and tetracycline (60 µg/mL).

2.4. Pulsed-field gel electrophoresis (PFGE)

2.4.1. Agarose plug preparation for PFGE

PFGE was carried out as described earlier [Parsons et al., 2007; Ribot et al., 2006]. The swab from 16 to 18 hour culture was taken for the PFGE plug preparation. The culture was suspended in 2 mL cell suspension buffer till the OD reached 1.3 to 1.4 for *Vibrio* or 1.0 for *Shigella* at 600 nm. After that 100 µL (~10^7-10^8) of cell suspension was mixed with 5 µl proteinase K (20 mg/mL) and preheated (50 °C) 100 µL 1.2% PFGE grade agarose. This mixture was dispensed in PFGE plug mold and allowed to solidify. These plugs were removed from the mold and washed in 5 mL cell lysis buffer with 25 µL 20 mg/mL proteinase K contained in 50 mL falcon tube by shaking at 150 rpm and 55°C for 1.5 to 2 h. After cell lysis, the plugs were washed with the sterile ultrapure water at 150 rpm and 55°C for 10-15 min. Then water was decanted and washing of the plugs was repeated for 10-15 min. Subsequently, the plugs were washed four times with TE buffer under similar conditions with 10-15 min for each washing. After washing, the TE buffer was removed and plugs were stored at 4°C in fresh sterile TE buffer.

2.4.2. Restriction Digestion of DNA in agarose plugs

DNA plugs were digested with *Not*I (for *V. fluvialis*), *Xba*I (for *S. sonnei, S. dysenteriae* and *S. boydii*) and *Not*I (for *S. flexneri*) as recommended by the Pulsenet International protocol (https://www.cdc.gov/pulsenet/pathogens/pfge.html). The details for each step are described below:

A. Pre-restriction incubation step: A master mixture of appropriate 10X restriction buffer was prepared with sterile water and 200 µL of this 1:10 diluted restriction buffer was distributed in 1.5 mL microcentrifuge tubes (Table 2.3). 2.0 to 2.5 mm wide slice from each test sample plug and the appropriate number of *S. enterica* serotype *Braenderup* H9812 plugs or CHEF DNA size marker *S. cerevisiae* were sliced and transferred to a tube containing diluted restriction buffer. These plugs were incubated at 37°C water bath for 5 to 10 min. After incubation, diluted restriction buffer from plug slice was carefully removed by micropipette.

Table 2.3. Mixture for Pre-restriction incubation

Reagent	**µL/Plug slice**
Sterile water	180
10X Restriction buffer	20
Total volume	200

B. Restriction digestion of plug slice: The restriction enzyme master mixture was prepared according to the Table 2.4 and this mixture was distributed in the micro-centrifuge tubes containing plug slices earlier incubated with diluted restriction buffer. Plug slices were then incubated in 37°C water bath for 1.5- 2 h.

Table 2.4. Mixture for restriction digestion

Reagent	**µL/Plug slice**
Sterile water	175
10X Restriction buffer with BSA	20
Enzyme (10 U/µL)	5
Total volume	200

2.4.3. Agarose gel electrophoresis for PFGE

For gel electrophoresis, 1% agarose gel (Bio-Rad Pulsed Field Certified Agarose) was made in 0.5 X TBE and casted in gel casting assembly of PFGE. After solidification of gel, restriction digested plug slices were loaded in wells of gel and then all the wells covered with the 1% PFGE agarose gel. The gel was then kept in the electrophoretic chamber of CHEF MAPPER (Bio-Rad) and gels were run with electrophoresis conditions described in Table 2.5. After the completion of the electrophoretic run, the gel was stained with 0.05 mg/mL ethidium bromide for 30 min and destained with sterile water for 1 h.

Table 2.5. PFGE conditions for *Vibrio fluvialis* and *Shigella* spp. restricted with *Not*I or *Xba*I enzyme.

Strains	**Electrophoresis conditions**
Vibrio fluvialis (*Not*I digested)	Auto Algorithm mode; 6 kb- Low MW 600 kb-High MW Selected default values Initial switch time: 2.16 s; Final switch time: 17.33 s; Runtime: 19 h
Shigella sonnei, S. dysenteriae and *S. boydii* (*Xba*I digested)	Auto Algorithm mode; 30 kb- Low MW 600 kb-High MW Selected default values Initial switch time: 2.16 s; Final switch time: 54.17 s; Runtime: 20 h
Shigella flexneri (*Not*I digested)	Auto Algorithm mode; 50 kb- Low MW 400 kb-High MW Selected default values Initial switch time: 5 s; Final switch time: 35 s; Runtime: 20 h

2.4.4. PFGE gel analysis

PFGE images were captured by using a gel documentation system (Vilber Lourmat, France). PFGE profiles were analysed using the BioNumerics version 4.0 software (Applied Maths, Belgium). The tagged image file formats were normalized by using the universal *Salmonella enterica* serotype Braenderup (H9812) size standard on each gel against the reference in the database. In the dendrogram analysis, the PFGE profiles were matched using the Dice coefficient and unweighted pair group method using arithmetic averages (UPGMA). Clustering of PFGE profiles was made using 1.5% band position tolerance window and 1.5% optimization.

2.5. DNA isolation from the clinical isolates

2.5.1. Genomic DNA isolation

Genomic DNA from the clinical isolates was prepared as described previously [Thungapathra et al., 2002; Murray and Thompson, 1980]. 1.5 mL or 3 mL of overnight grown pure culture was harvested in a microfuge tube at 8000 rpm (Force Micro, Model: Force 1624) at RT for 10 min. The pellet was resuspended in 567 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Then, 30 µL of 10% SDS and 3 µL of proteinase K (20 mg/mL) was added, mixed and incubated at 37 °C for 1 h. Subsequently, 100 µL of 5 M NaCl and 80 µL of 10% CTAB (Cetyl tri-methylammonium bromide) were added and incubated at 65 °C for 10 min in a heat block (Labnet International, Inc). Subsequently, samples were treated with phenol: chloroform:isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1). DNA was precipitated from the supernatant by adding an equal volume of isopropanol and centrifugation. The DNA pellet was washed with 750 µL of chilled 70% ethanol followed by centrifugation, air drying and resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0). This genomic DNA preparation was treated with RNAse (final concentration of 100 µg/mL) for 30 min at 37 °C.

2.5.2. Plasmid DNA isolation from the clinical isolates/transformants /transconjugants

2.5.2.1. Plasmid preparation by alkaline-lysis method

Alkaline-lysis method was used for the preparation of plasmid DNA [Birnboim and Doly, 1979]. Overnight grown culture (3 mL) was harvested at 5000 rpm at RT for 5 min and the pellet was resuspended in 250 µL of Solution I (100 µg/mL RNAse A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) followed by addition of 250 µL of solution II (0.2 N NaOH, 1% SDS) at RT for 1 min. 250 µL of chilled solution III (2.5 M potassium acetate) was mixed and incubated on ice for 15 min and then centrifuged at maximum speed (13000 rpm) in 4 °C for 15 min. The DNA was precipitated from supernatent with isopropanol and washed with chilled 70% ethanol as described above. The DNA was air dried and suspended in 40 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.5.2.2. Plasmid DNA isolation using Qiagen plasmid purification kit

Plasmid purification kit (Qiagen) was used for the midi-scale preparation of plasmid DNA from the clinical isolates using manufacturer's instructions. The protocol was based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to Qiagen anion-exchange resin under appropriate low salt and pH conditions and elution in a high-salt buffer. Plasmid DNA from 100 mL culture was purified as per instructions in the kit. The pellet was air dried and dissolved in 30 µL of 1X TE buffer.

2.6. Agarose gel analysis

The DNA samples were electrophoresed in 0.8% to 2.0% agarose gel prepared in 1X TAE using 1X TAE running buffer at 6-7 V/cm. A 1kb ladder (Thermo Scientific), 100 bp DNA ladder (Thermo Scientific) and λ *Hin*d III ladder (Sigma) were used as the molecular size markers. DNA was visualized by ethidium bromide (EtBr) staining at 0.1 µg/mL EtBr concentration.

2.7. PCR screening of mobile genetic elements

The presence of various factors such as integrons, SXT elements, and quinolone resistance genes in *Vibrio* and *Shigella* isolates was established by PCR amplification using primers specific for each element. Genomic DNA (100 ng) or plasmid DNA (10-50 ng) were used as templates in PCR with the primers and conditions described in Table 2.6 for the screening of class 1, class 2, class 3, class 4 integrons, VCint and SXT element. Each PCR reaction mixture consisted of 2.5 µL of 10X PCR amplification buffer, 2.0 µL of 25 mM magnesium chloride, 2.0 µL of dNTP mix containing 2.5 mM of each dNTP, 50.0 pmol of each primer, 1.5 U of recombinant *Taq* DNA polymerase (PCR reagents from Thermo Scientific) and sterile water to a final volume of 24.0 µL. 1.0 µL of template DNA was added to make the final reaction volume of 25.0 µL. PCR was carried out with an initial denaturation at 95 ºC for 4 min. Subsequent to this, 25-30 amplification cycles were performed, each consisting of an initial denaturation at 95 ºC for 0.5 min followed by annealing and extension steps (Table 2.6). The final polymerization was carried out at 72 ºC for 10 min.

The quinolone resistant isolates were analysed for the known plasmid-mediated quinolone resistance genes (*qnrA*, *qnrD*, *qnrB*, *qnrS*, *oqxA*, *aac-(6')-Ib-cr* and *qnrC*) by multiplex PCR (Table 2.6). Multiplex PCRs were carried out using the conditions described above except that annealing was carried out at 63 ºC for 1min and extension at 72 ºC for 1.5 min.

Table 2.6. Primers used in the present study

Sr. No	Primers name	Description of the gene	Sequence 5'→3'	Annealing temp/time (°C/min)	Extension at 72°C (min)	Amplicon size (bp)	Reference
1	L2	5' CS-Class 1 integron	GACGATGCGTGGAGACC	55/0.5	1	300	Maguire et al., 2001
	L3		CTTGCTGCTTGGATGCC				
2	qac EΔ1	3' CS-Class 1 integron	ATCGCAATAGTTGGCGAAGT	58/2	1	798	Dalsgaard et al., 2000
	Sul1B		GCAAGGCGGAAACCCGCC				
3	In F	Variable region - Class 1 integron	GGCATCCAAGCAGCAAGC	60/1	1	Variable	Dalsgaard et al., 2000
	In B		AAGCAGACTTGACCTGAT				

4	Int1CA F IS1CA R	Variable region - atypical Class 1 integron	CGTAGAAGAACAGCAAGG AGTGAGAGCAGAGATAGC	52/1	3	Variable	Pan et al., 2006
5	Int 2 F Int 2 R	Integrase-Class 2 integron	GTAGCAAACGAGTGACGAAATG CACGGATATGCGACAAAAAGGT	66.2/1	1	789	This study
6	Int 2 VA F Int 2 VA R	Variable region - Class 2 integron	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	55/1	3	Variable	Dubois et al., 2007
7	Int3 F Int3 R	Integrase-Class 3 integron	AGTGGGTGGCGAATGAGTG TGTTCTTGTATCGGCAGGTG	59/1	1	600	Goldstein et al. 2001
8	IntI4 F IntI4 R	Integrase-Class 4 integron	CGGTATGTCTAATTGCTCTTG TGGCCACAAAGACTCAATCAC	50/1	1	696	Goldstein et al. 2001
9	VcIntF VcIntR	Integrase-pBD146	CCTAGCTCTTGAGAAATAATCG CTCACGAATGTAAACAAAGC	55/1	1	657	This study
10	SXT F SXT R	Integrase-SXT element	ATGGCGTTATGAGTTAGCTC GCGAAGATCATGCATAGAC	57/0.5	1	1000	This study
11	S- gyrA F S- gyrA R	QRDR region-GyrA-*Shigella* spp.	TACACCGGTCAACATTGAGG TTAATGATTGCCGCCGTCGG	64/0.5	1	648	Dutta et al., 2005
12	S- gyrB F S- gyrB R	QRDR region-GyrB-*Shigella* spp.	TGAAATGACCCGCCGTAAAGG GCTGTGATAACGCAGTTTGTCCGGG	60.7/0.5	1	310	Dutta et al., 2005
13	S-parC F S-parC R	QRDR region-ParC - *Shigella* spp.	GTCTGAACTGGGCCTGAATGC AGCAGCTCGGAATATTTCGACAA	68.6/0.5	1	249	Dutta et al., 2005
14	S-parE F S-parE R	QRDR region-ParE - *Shigella* spp.	ATGCGTGCGGCTAAAAAAGTG TCGTCGCTGTCAGGATCGATAC	63/0.5	1	290	Dutta et al., 2005
15	gyrA F gyrA R	QRDR region-GyrA -*V. fluvialis*	TACACCGACGCGTACTGT TCGATCGAGCCAAAGTTA	50/0.5	1	207	This study
16	gyrB F gyrB R	QRDR region-GyrB-*V. fluvialis*	GGAAATGACTCGCCGTAAAGG GTTGTGATAACGCAGTTTATCTGGG	50/0.5	1	310	This study

17	parC F	QRDR region-ParC-*V. fluvialis*	GTCTGAGTTGGGTCTCTCGGC	50/0.5	1	249	This study
	parC R		AGAATCTCGGCAAACTTTGAC				
18	parE F	QRDR region-ParE-*V. fluvialis*	CAGCAAGAAAGTGGTGCGTA	50/0.5	1	318	This study
	parE R		AGACTTTGCCGTAACGCAGT				
19	qnrA F	QnrA protein	CAGCAAGAGGATTTCTCACG	63/1	1.5	630	Ciesielczuk et al., 2013
	qnrA R		AATCCGGCAGCACTATTACTC				
20	qnrD F	QnrD protein	CGAGATCAATTTACGGGGAATA	63/1	1.5	581	Ciesielczuk et al., 2013
	qnrD R		AACAAGCTGAAGCGCCTG				
21	qnrB F	QnrB protein	GGCTGTCAGTTCTATGATCG	63/1	1.5	488	Ciesielczuk et al., 2013
	qnrB R		GAGCAACGATGCCTGGTAG				
	qnrB R(D)		SAKCAACGATGCCTGGTAG				
22	qnrS F	QnrS protein	GCAAGTTCATTGAACAGGGT	63/1	1.5	518	This study
	qnrS R		GTCAGGAWAAACAACAATACC				
23	oqxA F	OqxA efflux pump	CCGCACCGATAAATTAGTCC	63/1	1.5	313	Ciesielczuk et al., 2013
	oqxA R		GGCGAGGTTTTGATAGTGGA				
24	aac(6')-Ib-cr F	Aac(6')-Ib-cr enzyme	TTGGAAGCGGGGACGGAM	63/1	1.5	260	Ciesielczuk et al., 2013
	aac(6')-Ib-cr R		ACACGGCTGGACCATA				
25	aac(6')-Ib-cr F	Aac(6')-Ib-cr enzyme	TGACCAACTGCAACGATTCC	64/0.5	1.0	608	This study
	aac(6')-Ib-cr R		ACCCATAGAGCATCGCAAGGT				
26	qnrC F	QnrC protein	GCAGAATTCAGGGGTGTGAT	63/1	1.5	118	Ciesielczuk et al., 2013
	qnrC R		AACTGCTCCAAAAGCTGCTC				
27	qnrF	QnrVC protein-pBD146	ATGGATCCATGGATAAAACAGACCAG	62/1	1	655	This study
	qnrR		ATCTCGAGTTAGTCAGGAACTACTAT				

2.8. Amplification of topoisomerases and mutation analysis

PCR amplification of DNA segments encoding Quinolone Resistance Determining Regions (QRDRs) of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) was done as mentioned in section 2.7. with minor modifications. Genomic DNA (100 ng) was used as template in PCRs with the primers described in Table 2.6. Each PCR reaction mixture consisted of 5 µL of 10X PCR amplification buffer, 4.0 µL of 25 mM magnesium chloride, 4.0 µL of dNTP mix containing 2.5 mM of

each dNTP, 20 pmol of each primer, 1.5 U of recombinant *Taq* DNA polymerase and sterile water to a final volume of 49.0 µL. 1.0 µL of template DNA was added to make the final reaction volume of 50 µL. Each PCR consisted of an initial denaturation at 94°C for 4 min, followed by 30 amplification cycles, each involving an initial denaturation at 94°C for 0.5 min followed by annealing and extension steps. The annealing condition of each PCR varied depending on the Tm of the primer pairs and the length of the amplicons as mentioned in Table 2.6 and extension steps were carried out at 72°C for 1 min. The final polymerization was carried out at 72°C for 10 min. The primers prefixed with S- were used for amplifying *Shigella*-topoisomerase genes and other primers for topoisomerases were used for amplifying from *V. fluvialis* isolates. PCR amplicons were sequenced on both strands and the sequences were assembled. The sequences were compared with topoisomerase sequences from sensitive isolates or corresponding GenBank sequences to detect the mutations.

2.9. Preparation of DNA fragments for sequencing

For sequencing of large PCR amplicons ≥ 1.0 kb, they were cloned in TA cloning vectors for facilitating multiple primer walking reactions to cover the entire DNA sequence. Small PCR amplicons (<1.0 kb) were directly sequenced without cloning in a vector. Various steps taken for preparation of DNA from PCR amplicons and then cloning are described in the following sections.

2.9.1. Gel extraction of DNA using Qiagen gel extraction kit

In the case of amplification of multiple band, each amplicon was retrieved by resolving them on low melting agarose and cutting gel slice containing each DNA band. Three volumes of buffer QG were added to 1 volume of the gel slice and dissolved at 50°C for 10 min, after which 1 gel volume of isopropanol was added. This mixture was transferred to the QIAquick column and centrifuged at maximum speed for 1 min. The column was treated with buffer QG and buffer PE to remove agarose traces and salts respectively. The DNA was eluted with 50 µL of buffer EB and electrophoresed.

2.9.2. Purification of PCR product using Qiagen Qiaquick PCR purification kit

In case the amplifications yielded a single major band, the gel extraction was not required for purification of amplicons. Therefore, DNA was directly purified using Qiagen PCR purification kit. Five volumes of buffer PB were mixed to 1 volume of the PCR sample, applied to the column for DNA to bind and centrifuged for 30-60 seconds. 0.75 mL of Buffer PE was added to the column for washing. The column was centrifuged for additional 1 min to remove excess Buffer PE. The DNA was eluted with 50 µL of buffer EB (10 mM Tris-HCl, pH 8.5) and electrophoresed.

2.9.3. TA cloning of variable regions of integrons

The PCR purified or gel extracted fragments of the variable region of integrons were cloned either in pDrive (Qiagen) or pBAD TOPO (Invitrogen) TA cloning vectors.

For pDrive TA cloning, PCR product was mixed with 1X ligation mix and pDrive vector (Table 2.7). The mixture was incubated at 16 °C for 30 min and transformed in *E. coli* DH5α by electroporation. Transformants were selected on 100 µg/mL ampicillin, 60 µL of 40 mg/mL Xgal and 15 µL 100 mM IPTG. The plates were incubated at 37 °C overnight. White colonies were selected from the mixture of blue/white colonies and analysed for recombinants.

Table 2.7. Ligation mixture for pDrive TA cloning

Reagent	**Volume in µL/ reaction**
pDrive vector (50 ng/µL)	1.0
PCR product	Variable
Sterile water	Variable
Ligation Mix (2X)	5.0
Total Volume	**10.0**

For pBAD-TOPO-TA cloning, PCR product was mixed with the salt solution and pBAD Topo vector (Table 2.8). The reaction mixture was incubated at 22 °C for 30 min. This mixture was transformed in *E. coli* DH5α by electroporation. Transformants were spread on LB agar plates containing 100 µg/mL ampicillin and incubated at 37 °C overnight.

Table 2.8. Ligation mixture for pBAD TOPO TA cloning

Reagent	**Volume in µL/ reaction**
pBAD Topo vector	1.0
PCR product	Variable
Sterile water	Variable
Salt solution	1.0
Total Volume	**6.0**

2.9.4. Analysis of TA clone

Recombinants were confirmed by isolation of plasmids followed by PCR and restriction analyses.

2.10. DNA sequencing, sequence analysis and GenBank submissions

DNA sequencing was carried out by Sanger's chain termination method using DNA sequencer (Applied Biosystems; 3730/3730xl DNA analyzer) at University of Delhi South Campus (UDSC) (Courtesy Prof. Vijay K. Chaudhury and Ms. Shilpi).

DNA of three different catagories were sequenced.

1. The native plasmid from BD146 (pBD146) was sequenced by subcloning in the pBluescript vector at *Kpn*I site as a single insert. Primer walking was carried out to cover the whole sequence and the sequence was assembled.
2. The variable regions of integrons were cloned in the TA cloning vector (pDrive TA cloning vector [Qiagen] or pBAD TOPO TA vector [Invitrogen]) as described in section 2.9 and sequenced by primer walking.
3. The conserved segments of integrons, topoisomerase gene sequences and small amplicons of drug resistance genes were directly sequenced.

The genes were assembled and the sequences were analyzed by DNA Dynamo (Blue Tractor Software Ltd.) and NCBI-BLAST tool. ORF finder tool at the NCBI site (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to predict all the possible ORFs in integron and plasmid sequences. These ORFs were further analysed by BLAST search. I-TASSER (Iterative Threading ASSEmbly Refinement) server was used for 3D protein structure prediction of integrases [Yang et al., 2013b; Roy et al., 2010; Zhang, 2008]. It employs hierarchical approach for the prediction of structure

and function of a protein using multiple threading methods based on structural templates from PDB. Full atomic models are then constructed by iterative template fragment assembly simulations and finally, functional aspects of the target are derived by threading into BioLip database. Multiple sequence alignments were carried out using CLUSTAL Omega (1.2.1) at Expasy (http://www.expasy.org/genomics/sequence_alignment). The assembled and analyzed gene sequences were submitted to GenBank and described in Table 2.9.

Table 2.9. GenBank submissions made from the present study

S. No.	Accession No.	Description
1	EU574928	Plasmid pBD146
2	FJ462717	*arr-3* gene from BD146
3	FJ462718	*aadA1* gene from BD146
4	FJ462719	Exporter from BD146
5	FJ705851	blaOXA from BD146
6	FJ705852	hypothetical protein from BD146
7	KY883670	Variable region of class 1 integron of *V. fluvialis* BD146
8	GQ152140	*IntI*1 from BD146
9	GQ152139	*IntI*1 from L12387
10	GQ452010	Integron cassette with pectin metabolism proteins
11	GQ452011	Integron cassette with methyl-accepting chemotaxis protein
12	GQ466187	QRDR, gyrA of Vc
13	GQ466188	QRDR, gyrA of 2552
14	GQ466189	QRDR, gyrA of 2563
15	GQ466190	QRDR, gyrB of Vc
16	GQ466191	QRDR, gyrB of 2552
17	GQ466192	QRDR, gyrB of 2563
18	GQ466193	QRDR, parE of Vc
19	GQ466194	QRDR, parE of 2552
20	GQ466195	QRDR, parE of 2563
21	GQ466196	*arr-3* gene from 2562
22	GU326332	QRDR, parC of Vc
23	GU326333	QRDR, parC of 2552
24	GU326334	QRDR, parC of 2563
25	JN408080	qnrB1 from BD146
26	JN571549	qnrB1 from L10734 (2557)
27	JN571550	qnrB1 from L9978 (2562)
28	JQ013420	gyrB from BD146
29	JQ013421	parC from BD146
30	JQ013422	parE from BD146
31	JQ013425	Aac(6')-Ib-cr from BD146
32	KT182072	Integrase from BD146
33	KT182073	Integrase from CRC233
34	KT182074	Integrase from L13230 (2553)
35	KT182075	Integrase from L10734 (2557)
36	KX817772	QRDR of gyrA of *S. dysenteriae* NK4036
37	KX817774	QRDR of gyrB of *S. dysenteriae* NK4036
38	KX817776	QRDR of ParC of *S. dysenteriae* NK4036

39	KX817778	QRDR of ParE of *S. dysenteriae* NK4036
40	KX817773	QRDR of gyrA of *S. dysenteriae* 1244
41	KX817775	QRDR of gyrB of *S. dysenteriae* 1244
42	KX817777	QRDR of ParC of *S. dysenteriae* 1244
43	KX817779	QRDR of ParE of *S. dysenteriae* 1244
44	KX817788	QRDR of gyrA of *S. flexneri 102*
45	KX817790	QRDR of gyrB of *S. flexneri 102*
46	KX817792	QRDR of ParC of *S. flexneri 102*
47	KX817794	QRDR of ParE of *S. flexneri 102*
48	KX817789	QRDR of gyrA of *S. flexneri* NK2640
49	KX817791	QRDR of gyrB of *S. flexneri* NK2640
50	KX817793	QRDR of ParC of *S. flexneri* NK2640
51	KX817795	QRDR of ParE of *S. flexneri* NK2640
52	KX583660	QRDR of gyrA of *S.sonnei* NK4219
53	KX583664	QRDR of gyrB of *S.sonnei* NK4219
54	KX583668	QRDR of ParC of *S.sonnei* NK4219
55	KX583672	QRDR of ParE of *S.sonnei* NK4219
56	KX583661	QRDR of gyrA of *S.sonnei* IDH1694
57	KX583665	QRDR of gyrB of *S.sonnei* IDH1694
58	KX583669	QRDR of ParC of *S.sonnei* IDH1694
59	KX583673	QRDR of ParE of *S.sonnei* IDH1694
60	KX583662	QRDR of gyrA of *S.sonnei* IDH0734
61	KX583666	QRDR of gyrB of *S.sonnei* IDH0734
62	KX583670	QRDR of ParC of *S.sonnei* IDH0734
63	KX583674	QRDR of ParE of *S.sonnei* IDH0734
64	KX583663	QRDR of gyrA of *S.sonnei* NK4846
65	KX583667	QRDR of gyrB of *S.sonnei* NK4846
66	KX583671	QRDR of ParC of *S.sonnei* NK4846
67	KX583675	QRDR of ParE of *S.sonnei* NK4846
68	KX817780	QRDR of gyrA of *S.boydii* NK1919
69	KX817782	QRDR of gyrB of *S.boydii* NK1919
70	KX817784	QRDR of ParC of *S.boydii* NK1919
71	KX817786	QRDR of ParE of *S.boydii* NK1919
72	KX817781	QRDR of gyrA of *S.boydii* 442
73	KX817783	QRDR of gyrB of *S.boydii* 442
74	KX817785	QRDR of ParC of *S.boydii* 442
75	KX817787	QRDR of ParE of *S.boydii* 442
76	KX768278	Integrase of class 1 integron from *S. sonnei* IDH0734
77	KX768279	Integrase of class 1 integron from *S. flexneri* H20145
78	KX768280	Integrase of class 1 integron from *S. flexneri* M11560
79	KX768281	Integrase of class 1 integron from *S. flexneri* 102
80	KX768282	Integrase of class 1 integron from *S. dysenteriaei* NK4771
81	KX768283	Integrase of class 1 integron from *S. dysenteriaei* 1244
82	KX536825	Integrase of class 2 integron from *S. sonnei* NK4846
83	KX463270	Integrase of class 2 integron from *S. flexneri* H20145
84	KX463271	Integrase of class 2 integron from *S. flexneri* 102
85	KX536827	Integrase of class 2 integron from *S. dysenteriaei* NK4036
86	KX536826	Integrase of class 2 integron from *S. dysenteriaei* 1244
87	KX536824	Integrase of class 2 integron from *S. boydii* 442
88	KX777251	Variable region of class 1 integron from *S. sonnei* IDH0734
89	KX777252	3'CS of class 1 integron from *S. sonnei* IDH0734
90	KX817769	Variable region of Atypical class 1 integron from *S. flexneri* 102
91	KX863674	Variable region of Atypical class 1 integron from *S. flexneri* NK2640
92	KX817770	Variable region of Atypical class 1 integron from *S. flexneri* 593
93	KX863676	Variable region of Atypical class 1 integron from *S. flexneri* NK2293

94	KX863675	Variable region of Atypical class 1 integron from *S. flexneri* 452
95	KX951422	Variable region of Atypical class 1 integron from *S. dysenteriae* 1244
96	KX817771	Variable region of Atypical class 1 integron from *S.boydii* NK1919
97	KX817767	Variable region of class 2 integron from *S. flexneri* 102
98	KX817766	Variable region of class 2 integron from *S. flexneri* H20145
99	KX781233	Variable region of class 2 integron from *S. sonnei* L1137
100	KX781232	Variable region of class 2 integron from *S. sonnei* NK4846
101	KX792556	Variable region of class 2 integron from *S. dysenteriae* 1244
102	KX817768	Variable region of class 2 integron from *S.boydii* 442

2.11. Efflux pump assay

To ascertain the role of efflux pumps in imparting resistance to drugs, synergy test was carried out as described earlier [Taneza et al., 2015; Azmi et al., 2014; Kim et al., 2008]. The test was performed using various antibiotics i.e. ampicillin, azithromycin, chloramphenicol, ciprofloxacin to which a particular clinical isolate was resistant. The efflux pump inhibitor carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) was added on MHA at 4 mg/L concentration. Susceptibility testing for antibiotics by MIC strip was performed as described in the earlier section, both in the presence and absence of CCCP. Lowering in MIC of the isolates in the presence of CCCP indicated the role of efflux pumps in reducing the concentration of that drug inside the cell by throwing it out.

2.12. Expression of the putative integrase gene in *Vibrio fluvialis* BD146

To check the expression of putative integrase in *V. fluvialis* BD146, RNA was isolated by RNeasy kit (Qiagen) as per manufacturer's protocol involving lysis with lysozyme and proteinase K digestion followed by the reverse transcription assay. Details of the procedure are described below.

2.12.1. Total RNA isolation from bacteria using Qiagen RNeasy Mini Kit

The overnight grown primary culture of bacterial cells was inoculated in 10 mL of LB for a secondary culture and grown at 37°C, 200 rpm until the OD_{600} reached 0.8. 1.5 mL of culture was mixed with 3 mL of RNA protect bacteria reagent and incubated at RT for 5 min and centrifuged at 8000 rpm for 10 min. The pellet was resuspended using the enzyme mixture (200 µL of TE buffer containing 15 mg/mL lysozyme and 10 µL of Qiagen proteinase K) and incubated at RT for 10 min with intermittent vortexing. Subsequently, 700 µL of Buffer RLT (with β-mercaptoethanol) followed

by 500 µL of 100% ethanol was mixed. The lysate (700 µL) was centrifuged at 13000 rpm for 15 seconds in a RNeasy Mini spin column. The column was washed with Wash Buffer RW1 and on-column DNase treatment was carried out at RT for 15 min using 300 µL DNase I incubation mix (consisting of 30 µL DNase I, 30 µL 10X DNase I incubation buffer with $MgCl_2$ and 240 µL of RNase-free water). Subsequently, the column was again washed with Buffer RW1 and Buffer RPE (containing ethanol) to remove the DNase and salts. The RNA was eluted by RNase-free water and treated with DNaseI (Fermentas) to remove the residual genomic DNA contamination if required.

2.12.2. RNA gel electrophoresis

The isolated RNA samples were visualized under UV by formaldehyde agarose (FA) gel electrophoresis. 1.2% FA gel was prepared by adding 1.2 g agarose in 10 mL of 10X FA gel buffer and the volume was made up to 100 mL using RNase-free water. Subsequently, 1.8 mL of 37% formaldehyde and 1 µL of a 10 mg/mL stock solution of EtBr was added before pouring the gel. Prior to running the gel, it was equilibrated in 1X FA gel running buffer for 30 min. RNA samples for electrophoresis were prepared by adding 1 volume of 5X RNA loading buffer to 4 volumes of RNA sample and the mixture was incubated at 65°C for 5 min. The prepared RNA samples were loaded onto the equilibrated FA gel and the gel was run at 7 V/cm in 1X FA gel running buffer. The concentration of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer and the purity of the RNA was estimated using the ratio of the absorbance of the RNA sample at 260 nm and 280 nm (OD_{260} / OD_{280} > 1.8 for a pure RNA sample).

2.12.3. Reverse transcriptase PCR

To confirm the expression of putative integrase gene in the *V. fluvialis* BD146, one step RT-PCRs (Reverse transcription PCR) were carried out using total RNA from the exponential phase cells. RT-PCR was carried out using Qiagen one-step RT-PCR kit following manufacturer's instructions. Each RT- PCR reaction mixture consisted of 10 µL of 5X Qiagen 1-step RT-PCR buffer, 2.0 µL of dNTP mix containing 2.5 mM of each dNTP, 50.0 pmol of each primer, 2.0 µL of Qiagen 1-step RT-PCR enzyme

mix and RNase-free water to a final volume of 49.0 µL. 1.0 µL template RNA (0.1 µg/µL) was added to make the final reaction volume of 50.0 µL. RT-PCR experiment consisted of reverse transcription step at 50 °C for 30 min and an initial denaturation at 95 °C for 15 min, followed by 25 amplification cycles, each involving an initial denaturation at 95 °C for 0.5 min followed by annealing at appropriate Tm for 1 min. The extension step was carried out at 72 °C for 1 min. The final polymerization was carried out at 72 °C for 7 min. The reactions were performed in a T100 thermal cycler (BioRad Laboratories). The presence of mRNA corresponding to the gene was confirmed by electrophoresing the RT-PCR samples.