Chapter 2

Materials and Methods

2 Materials and Methods

The composition and recipes of the media and reagents, mentioned in this chapter are described in the appendices.

2.1. Bacterial isolates/strains

The clinical isolates of *Vibrio* and *Shigella* species were procured from National Institute of Cholera and Enteric Diseases (NICED) (kind courtesy Dr. Amit Ghosh, Dr. T. Ramamurthy and Dr. S. K. Niyogi) in the form of stab cultures. The initial microbiological identifications were carried out in the NICED laboratory before dispatching the strains. In this study, 18 strains of *V. fluvialis*, 119 strains of *V. cholerae*, 58 strains of *V. parahaemolyticus* and 18 strains of *Shigella* species (9 *S. flexneri*, 3 *S. dysenteriae*, 4 *S. boydii* and 2 *S. sonnei*) were included. Among these, the strains showing resistance towards quinolones were selected for detailed characterization of quinolone resistance in these isolates. Various strains of *E. coli* were used for the transformation, sequencing and functional characterization of the genes according to the need of the experiment and vector specification. *E. coli* JM109 was used for transformation/electroporation experiments. *E. coli* BL21 (λDE3) strain was utilized for pET vector-based recombinant protein expression. *E. coli* ATCC 25922 was included as a quality control strain for antimicrobial susceptibility tests.

2.2. Triplex PCR assay for rapid identification of *Vibrio* species

A triplex PCR assay was developed for the identification of three major *Vibrio* spp., *V. cholerae*, *V. parahaemolyticus* and *V. fluvialis* by targeting their haemolysin (*hlyA*), haem-utilizing (*hutA)* and central regulatory (*toxR*) genes respectively. For identification of *V. fluvialis*, the published primers based on membrane tether region of the *toxR* gene were included in this study [Chakraborty et al. 2006]. The primers for the genes *hlyA* of *V. cholerae* and *hutA* of *V. parahaemolyticus* were designed by exploiting their unique sequences after

alignment of these chromosome-borne gene sequences from the three *Vibrio* species. The specificity of each primer pair was also confirmed by Primer-BLAST search at National Centre for Biotechnology Information site (www.ncbi.nlm.nih.gov/tools/primer-blast/). The nucleotide sequences of the primer pairs are given in Table 2.1.

As a first step, monoplex PCR was carried out using the genomic DNA as template for some reference strains to check the specificity of each primer. Subsequently, the same assays were performed using cell lysates as the templates. The cell lysates were prepared by growing the isolated colonies in Luria-Bertani broth (Himedia) (10 g/L casein enzyme hydrolysate, 5 g/L yeast extract, 10 g/L sodium chloride, pH 7.5) at 37°C until the OD_{600} reached 1.0. DNA was then extracted by boiling the samples (1 mL) for 10 min, centrifuging them at high speed followed by storage of the supernatants at -20°C until use. After confirming the specificity of the primers in these lysates as described above, the multiplex PCRs were carried out with these cell lysates. Once these PCRs were standardized with reference strains, test strains were also evaluated in the same reactions. Each multiplex 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 (Fermentas) and sterile water to a final volume of 21.0 µL. 4.0 µL of the cell lysate was added as template DNA to make the final reaction volume of 25.0 µL. Each multiplex PCR involved an initial denaturation at 94ºC for 5 min. Subsequent to this, 30 amplification cycles were carried out, each consisting of an initial denaturation at 94ºC for 0.5 min followed by annealing at 62°C for 0.75 min and extension at 72°C for 1 min. The final polymerization was carried out at 72ºC for 10 min. PCR reactions were performed using a PTC-225 DNA Engine Tetrad^{TM} Cycler (MJ Research Inc).

Table 2.1. Primers used in the triplex PCR assay, their sequences and their product lengths

S. No.	Primers	Primer sequence 5'→ 3'	Product length (bp)	Accession no. of the gene	Reference
1	Vf toxR-F	GACCAGGGCTTTGAGGTGGACGAC	217	AF170885	Chakraborty et al. 2006
	Vf toxR-R	AGGATACGGCACTTGAGTAAGACTC			
2	Vc hlyA- F	CAATCGTTGCGCAATCGCG	427	X51746	This Study
	Vc hlyA- R	TAATAAGCGAGCGGTACG			
3	Vp hutA-F	GTCTCTGGCACTTGGACG	330	VP0882	This Study
	Vp hutA-R	CAGCAGAGATAGGCAACACT			

Vc- *V. cholerae*; Vf- *V. fluvialis*; Vp- *V. parahaemolyticus*

2.3. Antibiogram tests for clinical isolates

The antibiograms for each isolate were determined using the already published protocols [Bauer et al. 1966; Thungapathra et al. 2002; Rajpara et al. 2009] in accordance with Clinical and Laboratory Standards Institute guidelines [CLSI, 2010]. The cultures of the bacterial isolates containing 10^5-10^6 cells/mL were streaked on the LB agar, to obtain a confluent growth and were tested for their susceptibility to Ampicillin (10 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Cotrimoxazole (25 µg), Gentamicin (10 µg), Kanamycin (30 µg), Nalidixic acid (30 µg), Norfloxacin (10 µg), Streptomycin (10 µg), Tetracycline (30 µg) and Trimethoprim (5 µg). The commercial disks (HiMedia) were used for this experiment using Mueller-Hinton agar (MHA) (HiMedia) (17.5 g/L casein acid hydrolysate, 3 g/L beef extract, 1.5 g/L starch, 17 g/L agar, pH 7.3). 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. Based on the antibiograms obtained with the isolates, quinolone resistant isolates were selected for further study and subjected to antibiogram analysis for an extended panel of quinolones including some higher generation quinolones such as Gatifloxacin (5 µg), Levofloxacin (5 µg), Lomefloxacin (10 µg), Ofloxacin (5 µg) and Sparfloxacin (5 µg). The quinolone susceptible isolates were selected to serve as a control. *E. coli* ATCC 25922 was included as a quality control for all the antibiogram tests.

2.4. Minimum inhibitory concentration (MIC) assays

2.4.1. MIC assays using Ezy MIC strip method

MIC determination of all the selected quinolone resistant isolates of *Vibrio* and *Shigella* spp.was carried out for nalidixic acid, norfloxacin, ciprofloxacin and ofloxacin using Ezy MIC strips (HiMedia) following the manufacturer's instructions. Interpretation of the results used the criteria recommended by the

Clinical and Laboratory Standards Institute [CLSI, 2011]. The cultures of the isolates containing 10^5-10^6 cells/mL (OD_{600} = 0.08 to 0.13) were streaked on the LB agar to obtain a confluent growth and the strips 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.

2.4.2. MIC assay using two-fold dilution method

Two-fold dilution method was used to detect the MIC of various quinolones for native *V. fluvialis* isolates, *E. coli* transformants and QnrVC5 recombinant as described previously [Mohanty et al. 2012] with minor modifications and have been described below.

2.4.2.1. MIC assay of native *V. fluvialis* isolates and their *E. coli* JM109 transformants

Overnight grown colonies of *V. fluvialis* were inoculated in 5 mL LB and grown at 37°C till the OD_{600} reached 0.1 (10^5-10^6 cells/mL). This culture was used as inoculum for the MIC assay. The concentration of the test drug was diluted two-fold in Mueller Hinton Broth (MHB, HiMedia, 17.5 g/L casein acid hydrolysate, 3 g/L beef extract, 1.5 g/L starch, pH 7.3). 50 µL of inoculum was added to 2 mL of each drug concentration in a 24-well cell culture plate and incubated at 37°C for 18 h. The MIC was read as the lowest concentration of the drug where no growth was observed. The assays were repeated at least three times. The MIC of *E. coli* JM109 transformants (obtained from the plasmid preparation derived from the native *V. fluvialis* host) was determined by the same method except that the transformants were selected in LB medium containing ampicillin (25 µg/mL) before inoculating into the MIC assay plate.

2.4.2.2. MIC assay of QnrVC5 recombinants

The recombinant plasmid pET28a-qnrVC5 was transformed into *E. coli* BL21 (λDE3) cells and the transformants were selected on LB agar containing kanamycin (50 µg/mL). *E. coli* BL21 (λDE3) cells carrying vector pET28a was used as a control. Both the recombinant and the control were induced with 1mM IPTG for 2 h. Subsequently, the induced cultures were adjusted to the OD_{600} of 0.1 and used as inoculum for MIC assays. The concentration of the test drug was diluted two-fold in MHB containing kanamycin (50 µg/mL) and IPTG (1mM). 50 µL of inoculum was added to 2 mL of each drug concentration in a 24-well cell culture plate and incubated at 37°C for 18 h to determine the MIC value as described above. The assays were repeated at least three times.

2.4.2.3. MIC assay of *V. fluvialis* isolates in the presence of peptide nucleic acid (PNA)

The anti-*qnrVC* peptide-PNA ($H-(KFF)_3K-O-ccattttctagccct-NH_2$) complementary to the region encompassing ribosomal binding site and start codon of the *qnrVC5* gene was designed to silence the *qnrVC5* gene at RNA level. The PNA was conjugated with cell penetrating peptide (KFF_3) to enhance the permeability of this antisense oligonucleotide across the cell membrane of bacteria [Good et al. 2001]. The peptide-PNA was synthesized by PANAGENE (Taejeon, South Korea) and the lyophilized PNA oligomer was dissolved in sterile water as per the manufacturer's instructions.

MIC of ciprofloxacin was determined for *V. fluvialis* isolates (BD146, L10734, L9978 and L15318) using the same two-fold dilution method described in the 2.4.2.1 section, but in a 96-well plate in the total assay volume of 100 µL with 5 µL of cultures as inoculum. The effect of PNA in reducing the MIC of ciprofloxacin was tested as described previously [Jeon and Zhang, 2009]. The assay was initially done with *V. fluvialis* BD146 to check the effect of increasing concentrations of PNA (0 µM, 2 µM, 4 µM and 6 µM) on MIC. Subsequent to

that, assays were performed without and with PNA (4 μM) for all the above mentioned *V. fluvialis* isolates.

2.5. Isolation and analysis of the genomic and plasmid DNA from the clinical isolates

2.5.1. Genomic DNA isolation from the clinical isolates

The genomic DNA from the clinical isolates was isolated by the protocols described previously [Murray and Thompson, 1980; Thungapathra et al. 2002]. A colony of cells from which the genomic DNA was to be isolated, was inoculated into 5 mL LB medium in a culture tube and the tubes were incubated at 37°C, 200 rpm (Orbitek, Model: LE) for overnight. 1.5 mL of overnight grown culture was harvested in a microfuge tube by spinning 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). Thirty microliters of 10% SDS and 0.6 μL of proteinase K (20 mg/mL) was added and mixed with cells by pipetting. The tube was then incubated at 37°C for 1 h. Subsequently, 100 μL of 5M NaCl and 80 μL of 10% CTAB (prepared in 0.7 M NaCl) were added and incubated at 65°C for 10 min in a heat block (Labnet International, Inc). Subsequently, equal volume of chloroform:isoamyl alcohol (24:1) was added to the tube and mixed by vortexing. The mixture was spun at 13000 rpm (Force Micro, Model: Force 1624) for 15 min at RT. The aqueous layer was then transferred to a fresh tube. Equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed by vortexing. Subsequently, the tube was centrifuged at maximum speed at 13000 rpm (Force Micro, Model: Force 1624) for 15 min at RT. From the centrifuged sample, the supernatant was transferred to a fresh microfuge tube and equal volume of isopropanol was added and mixed by gentle inversion. The mixture was incubated at RT for 5 min and spun at maximum speed for 15 min at RT to pellet the DNA. The supernatant was decanted and the remaining supernatant was removed by tapping on the tissue paper. The DNA pellet was washed with 750 μL

of 70% ethanol followed by centrifugation at maximum speed for 10 min at RT. After washing, ethanol was drained off and the tube was inverted on a tissue paper. The tube was left open for air drying. The DNA was then suspended in 40 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0). The genomic DNA preparation was treated with RNAse (final concentration of 100 µg/mL) for 30 min at 37°C. The isolated DNA samples were electrophoresed in 1.0% agarose gel prepared in 1X TAE using 1X TAE running buffer at 10V/cm. A 1kb ladder (Fermentas) and λ *Hin*dIII ladder (Sigma) was used as the molecular size marker. DNA was visualized by ethidium bromide (EtBr) staining at 0.1 µg/mL EtBr concentration.

2.5.2. Plasmid DNA isolation from the clinical isolates

Alkaline-lysis method was used for the preparation of plasmid DNA [Birnboim and Doly, 1979]. A colony of cells from the LB plate was inoculated into 5 mL LB medium in a culture tube and incubated at 37°C, 200 rpm (Orbitek, Model: LE) for overnight. Overnight grown culture (3 mL = 2 X 1.5 mL) was harvested in a 1.5 mL microfuge tube by spinning at 5000 rpm at RT for 5 min. 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) using pipette. This was followed by addition of 250 µL of solution II (0.2 N NaOH, 1% SDS), gentle mixing by inversion and incubation at RT for 1 min. 250 µL of chilled solution III (2.5 M potassium acetate, pH 4.8) was added to the tube and mixed well by inversion followed by incubation in ice for 15 min. The tube was centrifuged at maximum speed (13000 rpm) (Force Micro, Model: Force 1624) at 4°C for 15 min. From the centrifuged sample, 700 µL of supernatant was transferred to a fresh microfuge tube and 0.7 volume (490 µL) of isopropanol was added and mixed by gentle inversion. The mixture was spun at maximum speed for 15 min at RT to pellet the DNA. The supernatant was decanted and the remaining supernatant was removed by tapping on the tissue paper. 750 µL of 70% ethanol was then added to wash the DNA pellet and centrifuged at maximum speed for 5 min at RT. After washing, ethanol was drained off and the tube was inverted on a tissue paper. The DNA was air dried

and suspended in 40 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The plasmid DNA samples were electrophoresed in 1.0% agarose gel prepared in 1X TAE using 1X TAE running buffer at 10 V/cm. A 1kb ladder (Fermentas) was used as the molecular size marker.

2.5.3. Plasmid DNA isolation using QIAGEN plasmid purification kit

Plasmid purification kit (Qiagen) was used for the midi-scale preparation of plasmid DNA from the recombinants and 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. 100 mL of overnight grown bacterial culture was pelleted down by centrifugation at 8000 rpm (Plasto crafts, Model: Rota 4R-V/FA, No.9) for 15 min at 4°C. The pellet was resuspended in 4 mL of cold Buffer P1 with RNase (100 µg/mL). Subsequently, 4 mL of Buffer P2 was added and mixed by vigorously inverting the tube. The mixture was incubated at RT for 5 min. 4 mL of chilled Buffer P3 was added and mixed vigorously. The tubes were incubated in the ice for 15 min and then centrifuged at 11000 rpm (Plasto crafts, Model: Rota 4R-V/FA, No.9) for 15 min at 4°C. The supernatant was re-centrifuged at 11000 rpm for 20 min at 4°C. The supernatant was applied to the QIAGEN-tip 100 and allowed to enter the resin by gravity flow. Before applying the supernatant to the column, it was equilibrated with 4 mL Buffer QBT. The column was washed twice with 10 mL of Buffer QC and then the DNA was eluted with 5 mL of Buffer QF. The DNA was concentrated by adding 0.7 volumes (3.5 mL) of isopropanol and centrifuged at 11000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet was washed with 2 mL of 70% ethanol by centrifugation at 11000 rpm for 20 min. The pellet was air dried for 10 min and dissolved in 30 µL of 1X TE buffer.

2.6. Polymerase chain reaction (PCR)

The PCRs to screen the presence of quinolone resistance genes and to amplify quinolone-resistance-determining region (QRDR) of topoisomerase genes were carried out using the methods as described previously [Rajpara et al. 2009]. The details are given below.

2.6.1. PCR screening of quinolone resistance genes

Genomic DNA (200 ng) or plasmid DNA (10–50 ng) was used as template in PCRs with the primers described in Table 2.2. 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 Fermentas) 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. 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 and extension conditions for each PCR varied depending on the Tm of the primer pairs and the length of the amplicons respectively as mentioned in Table 2.2. The final polymerization was carried out at 72°C for 10 min. The reactions were performed in a T100 thermal cycler (BioRad Laboratories).

2.6.2. PCR amplification of QRDR regions of topoisomerase genes

PCR amplification of DNA segments encoding QRDR regions of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) was done as mentioned in the section 2.6.1. with minor modifications. Genomic DNA (200 ng) was used as template in PCRs with the primers described in Table 2.3. Each PCR reaction mixture consisted of 5 µL of 10X PCR amplification buffer, 3 µL of 25 mM magnesium chloride, 4.0 µL of dNTP mix containing 2.5 mM of each dNTP, 5 to 10 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 respectively as mentioned in Table 2.3 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- and VP- were used for amplifying topoisomerase genes in *Shigella* and *V. parahaemolyticus* species. The primers prefixed with VC- were used for amplifying all the topoisomerase genes in both *V. cholerae* and *V. fluvialis* species except VF-parC primer pair which was specifically used for amplification of *parC* gene in *V. fluvialis* species (Table 2.3).

2.7. DNA sequencing, sequence analysis and GenBank submissions

DNA sequencing of the PCR amplified gene products was carried out using facility at University of Delhi South Campus (UDSC) (Courtesy Prof. Vijay K. Chaudhury and Ms. Shilpi) by Sanger's chain termination method using DNA sequencer (Applied Biosystems; 3730/3730xl DNA analyzer). The genes were assembled and the sequences were analyzed by DNA dynamo (Blue Tractor Software Ltd.) and NCBI-BLAST tool. Alignment of these sequences with that of already known antibiotic resistance genes sequences were done at Expasy (www.expasy.ch). The assembled and analyzed gene sequences were submitted to GenBank.

2.8. Cloning and expression of *qnrVC5* gene in heterologous *E. coli* host

The *qnrVC5* gene amplified from *V. fluvialis* BD146 was cloned in pET28a expression vector to express the gene in heterologous *E. coli* host and to study the

function of the gene in isolation. The methods involved in this process are described below.

2.8.1. PCR amplification of *qnrVC5* gene

PCR amplification was performed according to the method described in section 2.6.1. The *qnrVC5* gene (encoding a pentapeptide repeat protein) was amplified from genomic DNA of *V. fluvialis* BD146 as template using the primers based on the sequence from pBD146 (Accession no. EU574928) (Table 2.4). These primers introduced restriction sites *Bam*HI at 5' end and *Xho*I at 3' end of the ORF for cloning into *Bam*HI and *Xho*I sites of pET28a vector (Novagen). The conditions used for amplifications and constitution of the reaction mixture are mentioned in Tables 2.5 and 2.6 respectively. For isolation of higher amount of DNA to be used in cloning, preparatory PCR was carried out in 200 µL (4 X 50 µL) volume. For electrophoresis of PCR products, 1.0% agarose gel was electrophoresed in 1X TAE at 10 V/cm. A 1kb ladder (Fermentas) was used as the molecular size marker during electrophoresis.

2.8.2. Purification of PCR product (*qnrVC5*) using Qiagen QIAquick PCR purification kit

5 volumes of buffer PB were added to 1 volume of the PCR sample and mixed well. The QIAquick spin column was placed in a 2 mL collection tube. The mixed sample was applied to the column for DNA to bind and centrifuged for 30-60 seconds. The flow-through was discarded and the column was placed back in the same tube. 0.75 mL of Buffer PE was added to the column for washing and centrifuged for 30-60 seconds. Then flow-through was discarded and the column was placed back in the same tube. The column was centrifuged for additional 1 min to remove excess Buffer PE and placed in a clean 1.5 mL microcentrifuge tube. To elute DNA, 50 µL buffer EB (10 mM Tris-HCl, pH 8.5) was added to the center of the QIAquick membrane and the column was centrifuged for 1 min. The eluted DNA sample was electrophoresed in agarose gel as mentioned above.

Table 2.2. Primers used to screen quinolone resistance conferring genes and the PCR conditions for their amplification

Sr. No.	Primer ID	Primer sequences	Annealing temp. and time	Extension temp. and time	Amplicon length (bp)
1	qnrA-F*	5'- ATGGATATTATYGATAAAG-3'	40°C for 0.5 min	72°C for 1.0 min	657
2	qnrA-R*	5'- ATCCGGMAGCACTATKAYYC-3'			
3	qnrB-F*	5'- ATGRCWCTRGYRYTMRTBRGCG-3'	60°C for 0.5 min	72°C for 1.0 min	645
4	qnrB-R*	5'- ACCMATNAYMGCGATRCCAAG-3'			
5	qnrC-F	5'- ATGGGTTGTACATTTATTGAATCG-3'	57°C for 0.5 min	72°C for 1.0 min	537
6	qnrC-R	5'- AAACACTTTGTCTGGAATAACAATCACC-3'			
7	qnrD-F	5'- ATGGAAAAGCACTTTATC-3'	56°C for 0.5 min	72°C for 1.0 min	645
8	qnrD-R	5'- TCGGTGAACAATAACACC-3'			
9	qnrS-F*	5'-ATGGAAAYCTACMRTCAYACATATCG -3'	56°C for 0.5 min	72°C for 1.0 min	657
10	qnrS-R*	5'- GTCAGGAWAAACAACAATACC-3'			
11	qnrVC-F	5'-CGCGGATCCATGGATAAAACAGACCAG -3'	65°C for 0.5 min	72°C for 1.0 min	657
12	qnrVC-R	5'- CCGCTCGAGTTAGTCAGGAACTACTAT-3'			
13	oqxA-F	5'- CGCGGATCCATGAGCCTGCAAAAAACC -3'	59°C for 1.0 min	72°C for 1.5 min	1175
14	oqxA-R	5'- CCGCTCGAGTCAGTTAAGGGTGGCGCT-3'			
15	oqxB-F	5'- CGCGGATCCATGGACTTTTCCCGCTTT-3'	62°C for 1.0 min	72°C for 2.0 min	3172
16	oqxB-R	5'- CCGCTCGAGCTAGGCGGGCAGATCCTC-3'			
17	qepA-F	5'- CGCGGATCCATGTCCGCCACGCTCCAC-3'	63°C for 0.75 min	72°C for 1.5 min	1100
18	qepA-R	5'- CCGCTCGAGTCAACCAGATGCGAGCGC-3'			
19	aac(6') Ib-cr-F	5'- TGACCAACTGCAACGATTCC -3'	64°C for 0.5 min	72°C for 1.0 min	608
20	aac(6') Ib-cr-R	5'- ACCCATAGAGCATCGCAAGGT-3'			

*Degenerate primers (**B**-C/G/ T; **K**-G/T; **M**-A/C; **N**-any base; **R**-A/G; **Y**-C/T; **W**- A/T)

Table 2.3. Primers used to amplify the QRDR regions of topoisomerase genes and the PCR conditions for their amplification

Sr. No.	Primer ID*	Primer sequences	Annealing temp. (ºC) and time	Extension temp. (ºC) and time	Amplicon length (bp)
1	S- gyrA-F	5'- TACACCGGTCAACATTGAGG- 3'	64°C for 0.5 min	72°C for 1.0 min	648
2	S- gyrA-R	5'- TTAATGATTGCCGCCGTCGG -3'			
3	S- gyrB-F	5'- TGAAATGACCCGCCGTAAAGG- 3'	60.7°C for 0.5 min	72°C for 1.0 min	310
4	S- gyrB-R	5'- GCTGTGATAACGCAGTTTGTCCGGG -3'			
5	S-parC-F	5'- GTCTGAACTGGGCCTGAATGC- 3'	68.6°C for 0.5 min	72°C for 1.0 min	249
6	S-parC-R	5'- AGCAGCTCGGAATATTTCGACAA-3'			
7	S-parE-F	5'- ATGCGTGCGGCTAAAAAAGTG- 3'	63°C for 0.5 min	72°C for 1.0 min	290
8	S-parE-R	5'- TCGTCGCTGTCAGGATCGATAC-3'			
9	VC- gyrA-F	5'- TACACCGACGCGTACTGT-3'	50°C for 0.5 min	72°C for 1.0 min	208
10	VC -gyrA-R	5'- TCGATCGAGCCAAAGTTA-3'			
11	VC -gyrB-F	5'- GGAAATGACTCGCCGTAAAGG-3'	50°C for 0.5 min	72°C for 1.0 min	310
12	VC -gyrB-R	5'- GTTGTGATAACGCAGTTTATCTGGG-3'			
13	VC -parC-F	5'- GTCTGAGTTGGGTCTCTCGGC-3'	50°C for 0.5 min	72°C for 1.0 min	249
14	VC -parC-R	5'- AGAATCTCGGCAAACTTTGAC-3'			
15	VC -parE-F	5'- CAGCAAGAAAGTGGTGCGTA-3'	50°C for 0.5 min	72°C for 1.0 min	311
16	VC -parE-R	5'- AGACTTTGCCGTAACGCAGT-3'			
17	VP- gyrA-F	5'- GTGCGTGATGGCCTAAAACC-3'	60°C for 0.5 min	72°C for 1.0 min	267
18	VP -gyrA-R	5'- TACTTCGGTGTAACGCATTGCCGC-3'			
19	VP -gyrB-F	5'- GCACGTGAAGCCGCACGTAAAGCG-3'	60°C for 0.5 min	72°C for 1.0 min	291
20	VP -gyrB-R	5'- ACCACAGCCAAGTGCTGTAATAAGC-3'			
21	VP -parC-F	5'- ATGTACGTAATCATGGACCGTGC-3'	60°C for 0.5 min	72°C for 1.0 min	333
22	VP -parC-R	5'- CAGCAATACTTCTGCAAACTTCGAC -3'			
23	VP -parE-F	5'- TTCAGTCTGTGGCTAAACGAAAAGCC -3'	60°C for 0.5 min	72°C for 1.0 min	447
24	VP -parE-R	5'- CAATAGCGTTGCGATGTGAAGACC-3'			
25	VF-parC-F	5'- GTCTGAGCTGGGTCTTTCTGCCTC-3'	50°C for 0.5 min	72°C for 1.0 min	249
26	VF-parC-R	5'- CAGCACTTCCGCGAACTTAGACAG-3'			

VC- *V. cholerae*; VF- *V. fluvialis*; VP- *V. parahaemolyticus*; S- *Shigella* spp.

Table 2.4. Primers used for *qnrVC5* gene amplification

S.No	Primer	Sequence (5’ to 3’)
1	qnrVC5-F	5’-CGC**GGATCC**ATGGATAAAACAGACCAG-3’
2	qnrVC5-R	5’-CCG**CTCGAG**TTAGTCAGGAACTACTAT-3’

Table 2.5. PCR conditions for amplification of *qnrVC5* gene

S. No	Steps	Temperature	Duration
1	Initial Denaturation	94°C	5 min
2	Denaturation	95°C	30 seconds
3	Annealing	65°C	45 seconds
4	Extension	72°C	1.5 min
5	The steps 2 to 4 were repeated 29 times		
6	Final extension	72°C	10 min
7	Cooling	4°C	Forever

Table 2.6. PCR reaction mixture for amplification of *qnrVC5* gene

S.No	Component	Final concentration	Volume for 50 μL reaction mixture
1	Forward primer (0.05nmol/ μL)	0.1nmol	2 μL
2	Reverse primer (0.05nmol/ μL)	0.1nmol	2 μL
3	dNTPs (2.5mM each dNTP)	200 μM of each dNTPs	4 μL
4	Taq DNA polymerase (Fermentas)	1.5 units	1.5 μL
5	10X buffer	1X	5 μL
6	25mM MgCl2	2 mM	4 μL
7	Template DNA	200 ng	2 μL
8	Autoclaved type I water	----------	29.5 μL

2.8.3. Restriction digestion of DNA samples

The restriction digestion of the vector (pET28a) and the insert (*qnrVC5*) DNA was carried out according to the manufacturer's (Fermentas) instructions as described below. The digested vector was treated with 2 μL of calf intestinal alkaline phosphatase (1U/ μL) (Fermentas) at 37°C for 30 min to prevent reannealing of the singly-digested vector.

Table 2.7. *Bam*HI and *Xho*I digestion of the vector and the insert

S.No	**Component**	**Insert *qnrVC5*** (200 µL total)	**Vector pET 28a** (200 µL total)
1	DNA	40.0 µL	10.0 µL (Approximately 27 µg)
2	10X Tango Buffer (Fermentas)	40.0 µL	40.0 µL
3	*Bam*HI (10 U/ µL) (Fermentas)	4.0 µL	4.0 µL
4	*Xho*I (10 U/ µL) (Fermentas)	2.0 µL	2.0 µL
5	Autoclaved type I water	114.0 µL	144.0 µL
Incubation at 37ºC for 2 h			

2.8.4. Phenol chloroform purification of restriction digested samples of pET28a vector and *qnrVC5* insert

The digested mixtures for the vector and the insert were mixed with an equal volume of Phenol:Chloroform:Isoamylalcohol (25:24:1) mix and the mixture was spun at 13000 rpm (Force Micro, Model: Force 1624) for 5 min. After centrifugation, the aqueous layer was transferred to a fresh microfuge tube and an equal volume of Chloroform:Isoamyl alcohol (24:1) was added. The mixture was spun at 13000 rpm (Force Micro, Model: Force 1624) for 3 min and then the aqueous layer was transferred to a fresh microfuge tube. Subsequent to that, the aqueous layer was mixed with a $1/10^{th}$ volume of 3M sodium acetate (pH 5.2) and twice the volume of chilled ethanol. The mixture was kept at -70°C for 30 min and centrifuged at 13000 rpm (Plasto Crafts, Model: Rota 4R-V/FA, No.3) for 30 min at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol at 13000 rpm for 15 min at 4°C. The ethanol was drained off and the tubes were kept for air drying at 37°C for 10 min. The DNA pellet was then

dissolved in an appropriate volume of 1X TE at RT. The purity and concentration of the purified vector and insert DNA were evaluated by agarose gel analysis.

2.8.5. Ligation of *qnrVC5* and pET 28a

Two microlitres of purified pET28a (5.4 kb) was mixed with 9 µl of purified *qnrVC5* (657 bp). To this, 2 µl of 10X T4 ligation buffer (In 1X concentration- 50mM Tris-HCl, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, pH 7.5 at 25ºC) and 1 µL of 400 U/µL T4 ligase enzyme (NEB) was added. The final volume was made up to 20 µL using sterile water. The ligation mix was incubated at RT for 2 h, followed by incubation at 37ºC for 30 min. After incubation, the ligation mix was heat inactivated at 70ºC for 10 min.

2.8.6. Preparation of Electrocompetent (EC) cells

E. coli JM109 cells were streaked on LB plate and incubated at 37ºC overnight. Next day, a fresh colony was inoculated into a 50 mL SOB (without Mg^{2+}) in a 500 mL flask. The cells were grown with vigorous aeration overnight at 37ºC, 200 rpm (Orbitek, Model: LE). Next day, 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 37ºC, 200 rpm. The flask was chilled in ice for 20 min. The cells were then harvested by centrifugation at 5000 rpm (Kubota, Model: 6500, Rotor: AG2506) for 10 min at 4ºC. After centrifugation, the supernatant was poured off. 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. Then the supernatant was poured off. The cell pellet was again washed in 250 mL sterile ice-cold wash buffer as described above. Finally, the pellet was resuspended in a volume of 0.5 mL of wash buffer and stored at -70ºC in smaller aliquots.

2.8.7. Electroporation in *E. coli*

Forty microlitres of *E. coli* JM109 EC cells were taken in a sterile, pre-chilled microfuge tube. 2 µL of ligation mixture was added to the EC cells and was 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, BioRad). The electroporator (Gene Pulser Xcell, BioRad laboratories Inc.) was used for this process. The machine was made ready by selecting the pre-set protocol, Bacterial, *E. coli* 2 mm: 2.5 kV. The electroporation cuvettes were placed in the shock pod and pulsed once for 5 milliseconds and 25 µF capacitance. The cuvettes were placed on ice immediately after the shock and 1 mL SOC was added into the cuvette, the cells were collected and transferred to the microfuge tubes for further 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 having kanamycin (50 µg/mL) and incubated at 37°C overnight.

Prior to the electroporation of the ligation mixtures, 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.

2.8.8. Preparation of master plate for transformants

Colonies obtained from the ligation mix were streaked on another LB plate having same antibiotic. The plate was incubated overnight at 37°C and next day, it was stored at 4°C as the master plate of the clones.

2.8.9. Small scale preparation (Miniprep) of plasmid DNA from recombinant clones

The cells from each streak of the master plate were picked by the sterile toothpicks and suspended in 5 mL LB medium containing kanamycin (50 µg/mL). The cultures were grown overnight and plasmid DNA was isolated from them by alkaline lysis method as mentioned in the section 2.5.2.

2.8.10. Confirming the authenticity of recombinant pET28a-qnrVC5 clones

2.8.10.1. PCR of *qnrVC5* gene

The PCR analysis of pET-qnrVC5 clones were performed exactly as mentioned in the section 2.8.1. The miniprep DNA were used as templates.

2.8.10.2. Restriction analysis of clones using *Nhe*I enzyme

The restriction analysis of miniprep DNA of pET-qnrVC5 clones was performed using *Nhe*I enzyme, the restriction sites of which were present in both the insert and the vector (Table 2.8).

Table 2.8. *Nhe*I digestion analysis of pET-qnrVC5 clones

S.No	Component	Final concentration	Volume for 20 µL reaction mixture
1	pET-qnrVC5 clone	Approximately 2 – 4 µg	8.0 µL
2	10X Tango Buffer	1X	2.0 µL
3	*Nhe*I (10 U/ µL) (Fermentas)	5 U	0.5 µL
4	Autoclaved type I water		9.5 µL
Incubation at 37ºC for 2 h			

2.8.10.3. DNA sequence analysis of clones

Sequence analysis for three of the pET-qnrVC5 clones was performed as mentioned in section 2.7. The miniprep DNA of clones was used as templates and pET28a backbone specific sequencing primers (T7 promoter and terminator primers) were used.

2.8.11. Preparation of calcium chloride ($CaCl_2$) chemically competent cells

E. coli BL21 (λDE3) cells were streaked on LB plate and incubated at 37ºC overnight. Next day, a fresh colony of cells was inoculated into 5 ml LB medium in a 50 ml flask. Cells were grown with vigorous aeration overnight at 37ºC, 200 rpm. Next day, 1.0 mL of cells from the overnight grown culture was diluted in 100 mL LB in a 1 liter flask and grown at 37ºC, 200 rpm till the OD_{600} of 0.3. The flask was chilled in ice for 30 min and the cells were harvested by centrifugation at 5000 rpm (Kubota, Model: 6500, Rotor: AG2506) for 10 min at 4ºC. The tubes were then inverted to drain off the medium. The cells were suspended gently in 0.25 volumes (25 mL) of chilled sterile 0.1 M $MgCl_2$ using sterile pipettes by gently moving the suspension up and down. The suspension was centrifuged at 5000 rpm, 4ºC for 10 min. After centrifugation, the excess liquid was drained off and the pellet was very gently resuspended in 0.25 volumes (25 mL) of ice cold sterile 0.1 M $CaCl_2$. The resuspended solution was incubated on ice for 1 h with intermitten mixing and centrifuged at 3000 rpm, 4ºC for 20 min. The supernatant was drained off and the pellet was resuspended in 4 mL ice-cold 0.1 M $CaCl_2$, 20% glycerol solution and stored at -70ºC in smaller aliquots of 400 µL each.

2.8.12. Transformation of *E. coli* in chemically competent cells

One microlitre of pET-qnrVC5 plasmid DNA was mixed with 20 µL of $CaCl_2$ competent cells in a prechilled microfuge tube through gentle pipetting. Then the tubes were incubated on ice for 30 min. After that, a heat shock was given to the cells at 42°C for 45 seconds and the tubes were quickly chilled. 200 µL of LB/ SOC broth was then added to these cells. The suspended cells were incubated at

37°C, 200 rpm for 45 min to 1 h. Then the cells were plated on the LB agar plates having appropriate antibiotics. Plates were incubated at 37°C overnight.

The transformation efficiency of chemically competent cells was checked by transforming 10 ng of pBS into the cells by $CaCl_2$ transformation method described above and the cells were plated on LB having ampicillin (100 µg/mL). The plates were incubated at 37°C overnight and next day the number of colonies was counted to calculate transformation efficiency as described in section 2.8.7.

2.8.13. Expression of QnrVC5 protein

Once the authenticity of recombinant clones was confirmed, few clones were selected for studying the expression of the recombinant *qnrVC5* protein. The recombinant plasmids were transformed into BL21 (λDE3) $CaCl_2$ competent cells, plated on LB kanamycin (50 µg/mL) plates and incubated at 37ºC overnight. Next day, a loopful of colonies were inoculated from the overnight grown plate to 5 mL LB kanamycin (50 µg/mL). The cultures were grown at 37ºC, 200 rpm until the OD_{600} reached ≥0.7. One mL culture was transferred to a microcentrifuge tube as –IPTG (uninduced) control and was chilled in ice. Subsequently, IPTG was added to a final concentration of 1mM to the remaining 4 mL culture to induce the expression of the gene. The tube was incubated at 37ºC, 200 rpm for 2 h. 1 mL of sample was taken after 2 h as + IPTG sample (induced) and chilled in ice. Both – IPTG and + IPTG cultures were spun at 8000 rpm for 10 min. The total cell pellet was resuspended in 150 µL 1X SDS-PAGE sample buffer, heated at 100°C for 10 min and spun at 15000 rpm, 15 min at RT to pellet down the insoluble fraction.

The SDS-Polyacrylamide gel (SDS–PAGE) was made as described previously [Laemmli, 1970] and shown in Table 2.9. Both resolving and stacking gels were cast in the BioRad SDS-PAGE assembly. The supernatants from the above treated total cell cultres were loaded on the gel and electrophoresed using 1X SDS-PAGE Running buffer at 100 volts. The electrophoresed gel was stained using

Coomassie blue R-250 stain for 20 min and destained in destaining solution till the bands became visible.

The protein band corresponding to QnrVC5 was excised from the gel and subjected to trypsin digestion. The authenticity of the protein was then confirmed by peptide mass finger printing on Bruker Ultraflex III MALDI instrument. The protein identification was done through Mascot software.

Table 2.9. Constituents of resolving gel (10%) and stacking gel (5%) for SDS-PAGE

S.No.	**Compound**	**Resolving gel (10%) 10 mL**	**Stacking gel (5%) 5 mL**
1	30% Acrylamide	3.33 mL	0.85 mL
2	1.5 M Tris-HCl pH 8.9	2.5 mL	-
3	1.0 M Tris-HCl pH 6.8	-	0.625 mL
4	0.25 M EDTA	0.32 mL	0.02 mL
5	10% SDS	0.4 mL	0.05 mL
6	Water	3.54 mL	3.4 mL
7	TEMED	0.008 mL	0.004 mL
8	10% APS	0.1 mL	0.50

2.9. Total RNA isolation from the bacteria using QIAGEN RNeasy Mini Kit

The protocol and the solutions used here were exactly as provided by the manufacturer (Qiagen). Overnight grown culture of bacterial cells was inoculated in 10 mL of LB and grown at 37°C, 200 rpm until the OD_{600} reached 0.8. 1.5 mL of culture was added to 15 mL sterile centrifuge tube having 3 mL of RNA protect bacteria reagent. The contents were mixed by vortexing for 5 seconds and incubated at RT for 5 min. The tubes were centrifuged at 8000 rpm (Plasto crafts, Model: Rota 4R-V/FA, No.9) for 10 min. Subsequent to that, the supernatant was decanted and the tube was left inverted on a tissue paper for 10 seconds. 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 vortexed for 10 seconds. The suspension was incubated at RT for 10 min with vortexing for 10 seconds after every 2 min. Subsequently, 700 µL of Buffer RLT (with β-mercaptoethanol) was added and vortexed vigorously. 500 µL of ethanol (100%) was added and mixed by pipetting. The lysate (700 µL) was transferred to RNeasy Mini spin column placed in a 2 mL collection tube. The column was spun at 13000 rpm (Force Micro, Model: Force 1624) for 15 seconds. The flow through was discarded and the last step was repeated using the remaining lysates. After that, 350 µL of wash Buffer RW1 was added to the column and spun at 13000 rpm for 15 seconds. The flow through was discarded and on-column DNase treatment was carried out. 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) was added to the column membrane and incubated at RT for 15 min. Subsequent to that, 350 µl of Buffer RW1 was added to the column and incubated for 5 min. The column was spun at maximum speed for 15 seconds and the flow through was discarded. After that, the column was placed in a new 2 mL collection tube. 500 µL of Buffer RPE (containing ethanol) was added to the column and spun at maximum speed for 15 seconds. The flow through was discarded and 500 µL of Buffer RPE was added again and spun at maximum speed for 2 min. The column was then placed in a new 1.5 mL microfuge tube and the RNA was eluted by adding the appropriate volume of RNase-free water

followed by centrifugation at maximum speed for 1min. The RNA preparations were subsequently treated with DNaseI (Fermentas) to remove the residual genomic DNA contamination if required. The RNA was stored at -20°C in smaller aliquots till further use.

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. The mixture was heated to melt agarose and cooled to 65°C. 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 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).

2.10. Expression of the putative resistance genes in the native isolates and their transformants

To confirm the expression of putative resistance-conferring genes in the native isolates (*Vibrio* spp. and *Shigella* spp.) /their transformants, 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. Each 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 30 amplification cycles, each involving an initial denaturation at 94°C for 0.5 min followed by annealing step. The extension step was carried out at 72°C for 1 min. The annealing condition of each PCR varied depending on the Tm of the primer pairs and the length of the amplicons respectively as mentioned in Table 2.10. The final polymerization was carried out at 72°C for 10 min. The reactions were performed in a T100 thermal cycler (BioRad Laboratories). The presence of mRNA corresponding to the gene could be confirmed by electrophoresing the RT-PCR samples on agarose gel and looking for the amplicon of the expected size as described in the Table 2.10.

Table. 2.10. Primers used for reverse transcription PCR

Primer	**Sequence (5' to 3')**	**Annealing temp. and time**	**Amplicon length (bp)**
qnrVC5-F	CGCGGATCCATGGATAAAACAGACCAG	64ºC for 0.5 min	657
qnrVC5-R	CCGCTCGAGTTAGTCAGGAACTACTAT		
aac(6') Ib-cr-F	TGACCAACTGCAACGATTCC	60 ºC for 0.5 min	608
aac(6') Ib-cr-R	ACCCATAGAGCATCGCAAGGT		
qnrS-F*	ATGGAAAYCTACMRTCAYACATATCG	56 ºC for 0.5 min	657
qnrS-R*	GTCAGGAWAAACAACAATACC		

*Degenerate primers (**M**-A/C; **R**-A/G; **Y**-C/T; **W**- A/T)

2.11. Mutant prevention concentration (MPC) assay

MPC assay was performed as described previously with minor modifications [Marcusson et al. 2005]. Overnight grown culture (100µL) of *V. fluvialis* isolates were inoculated in 25 mL LB and grown at 37°C until the OD_{600} reached 1.0 (~ 10^9cells/mL). The culture was then centrifuged at 8000 rpm (Plasto crafts, Model: Rota 4R-V/FA, No.9), RT for 5 minutes and the pellet was resuspended in

LB to contain ≥ 10^{10} cells/mL. Subsequently, 200 µL of resuspended culture was spread on MHA plates containing defined concentrations of ciprofloxacin. Each strain was tested with six concentrations of ciprofloxacin starting from their MIC (i.e 1, 2, 4, 8, 16, 32 X MIC). The plates were incubated at 37°C for 96 h and the MPC was recorded as the lowest concentration that prevented the emergence of mutant colonies. The assays were individually performed at least three times.

2.12. i*n silico* analysis

The DNA sequence analysis were done using BLAST tool available at NCBI site (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic tree was constructed using the maximum-likelihood method in MEGA6 [Tamura et al., 2013]. The structure of QnrVC5 was predicted by I-TASSER server using automated mode, as it employs hierarchical method for protein structure and function prediction using multiple threading approaches based on structural templates from PDB [Zhang, 2008; Roy et al. 2010; Roy et al. 2012]. Softberry-BPROM, a promoter prediction tool was used to find the promoters and other regulatory elements in *qnrVC5* gene cassette (http://liux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb).