Chapter 5

Characterization of Vibrio fluvialis qnrVC5 Gene in Native and Heterologous Hosts: Synergy of qnrVC5 with other Determinants in Conferring Quinolone Resistance

5 Characterization of *Vibrio fluvialis qnrVC5* gene in native and heterologous hosts: Synergy of *qnrVC5* with other determinants in conferring quinolone resistance

5.1. Introduction

Qnr proteins are pentapeptide repeat proteins (PRPs) which protect DNA gyrase from quinolone action [Tran and Jacoby, 2002]. *qnr*, a plasmid-mediated horizontally transferable gene conferring quinolone resistance, was first discovered in a plasmid from *Klebsiella pneumoniae* in 1998 [Martinez-Martinez et al. 1998]. Thereon, different variants of *qnr* genes such as *qnrA, qnrB, qnrC, qnrD* and *qnrS* were reported in different pathogens from different parts of the world [Strahilevitz et al. 2009]. Chromosomal *qnr*-like genes such as *qnrVC*, Pp*qnr*, Vp*qnr* and Sm*qnr* were also reported from *V. cholerae* [Fonseca et al. 2008], *Photobacterium profundum* [Poirel et al., 2005a], *V. parahaemolyticus* [Saga et al. 2005] and *Stenotrophomonas maltophilia* [Sanchez et al. 2008] respectively. Qnr proteins cause low-level resistance to quinolones, facilitating the emergence of resistant mutants. In combination with other mechanisms of quinolone resistance such as topoisomerase mutations and efflux action, Qnr proteins can help the pathogens to achieve clinical breakpoints of quinolone resistance [Martinez-Martinez et al. 2003; Jeong et al. 2008].

Till date, structures for PRPs such as MfpA, EfsQnr, QnrB1 and AhQnr have been solved [Hegde et al. 2005, 2011; Vetting et al. 2011; Xiong et al. 2011]. QnrB1 (from *K. pneumoniae*) and AhQnr (from *Aeromonas hydrophila)* possessed a right-handed quadrilateral β-helix, which is typical of PRPs and encompassed coils with two loops (loop A and loop B) extending outwards from the regular structure, interrupting the β-helix turn. Unlike the PRP structures of MfpA and EfsQnr from Gram-positive bacteria *Mycobacterium tuberculosis* and *Enterococcus faecalis* respectively, QnrB1 and AhQnr possessed these two loops. It was established that these two loops played a vital role in the interaction of Qnr and DNA gyrase subunits (GyrA and GyrB) [Xiong et al. 2011]. Loop A was

found to interact with GyrA "tower" whereas loop B was found to interact with GyrB TOPRIM (Topoisomerase-Primase) domains. This also indicated that the mechanisms of interaction of MfpA and EfsQnr with DNA gyrase were different from that of QnrB1 and AhQnr. These loops are found to be conserved among all plasmid-based Qnr variants and some chromosome-borne Qnr proteins [Vetting et al. 2011].

A former belief based on the mode of action of MfpA and EfsQnr explains that Qnr protein binds with DNA gyrase and prevents the formation of cleaved complex. Qnr protein does not interact with the quinolones and, therefore, renders resistance to drugs indirectly [Hegde et al. 2005, 2011]. As the structure of MfpA and EfsQnr varied from QnrB1 and AhQnr, their mode of action should also principally vary. The model proposed by Vetting et al. suggested that QnrB1 protein binds to and destabilizes the topoisomerase-quinolone-DNA cleavage complex, which eventually results in the release of quinolone and religation of DNA. After this process, the Qnr protein would be released and the active form of topoisomerase would be regenerated [Vetting et al. 2011].

The emergence of transferable *qnrVC* alleles in *Vibrionaceae* family and other bacterial species aggravated the hysteria on quinolone resistance [Fonseca and Vincente, 2013; Pons et al. 2013]. So far, seven *qnrVC* alleles (named as *qnrVC1* to *qnrVC7*) have been reported from different parts of the globe (http://www.lahey.org/qnrstudies/). These alleles are found as gene cassettes, equipped with all the elements necessary for their mobility, incorporation and expression such as *attC* sites and their own cassette-specific promoter [Fonseca and Vincente, 2013].

The presence of a *qnrVC*-like gene in the plasmids from three *V. fluvialis* strains BD146, L10734 and L9978 was reported earlier from this laboratory [Rajpara et al. 2009 and Singh et al. 2012]. This allele was later named *qnrVC5* by Fonseca and Vincente in 2013 [Fonseca and Vincente, 2013]. The plasmid that harboured *qnrVC5* in *V. fluvialis* BD146 also carried the gene encoding trimethoprim resistance (*dfrVI*) and showed 99% identity with pVN84 from *V. cholerae* O1

isolated from Vietnam [Rajpara et al. 2009] and a plasmid from *V. parahaemolyticus* V110 isolated from Hong Kong, China [Liu and Chen, 2013; Bhardwaj, 2015]. The plasmid from *V. parahaemolyticus* V110 possessed *qnrVC5* allele, whereas plasmid pVN84 carried *qnrVC2*, a nonfunctional form of *qnrVC5* due to the presence of several internal stop codons [Fonseca et al. 2008; Fonseca and Vincente, 2013]. From the above discussion and other reports, it is amply clear that *qnrVC* genes are disseminated globally and are likely to play a vital role in quinolone resistance due to their wide dispersal [Rajpara et al. 2009; Kim et al. 2010; Kumar and Thomas, 2011; Liu and Chen, 2013; Fonseca and Vincente, 2013; Pons et al. 2013; Bhardwaj, 2015]. Since *qnrVC5* allele was first reported from this laboratory, it was of prime interest to decipher its role in conferring resistance to quinolones. Therefore, this study was undertaken to understand the features of this gene/its product *in silico*. Another focus of the study was to functionally characterize QnrVC5 protein in native *V. fluvialis* host and heterologous *E. coli* host and also to study the synergy of this gene with other quinolone resistance determinants such as mutations in topoisomerase genes and *aac(6') Ib-cr* gene.

5.2. Results

5.2.1. Presence of *qnrVC5* gene in *V. fluvialis* isolates, characterization of the gene and its flanking genetic environment

The plasmid pBD146 (GenBank accession no. EU574928) obtained from a clinical isolate of *V. fluvialis* BD146, 2002, was earlier reported to harbour a *qnr* gene (GenBank accession no. JN408080) [Rajpara et al. 2009]. In another report, the same gene was also found in plasmid preparations from two *V. fluvialis* isolates L10734 (GenBank accession no. JN571550) and L9978 (GenBank accession no. JN571549) [Singh et al. 2012] and termed as *qnrVC*-like gene. Later, it was named *qnrVC5* allele [Fonseca and Vincente, 2013]. The sequence encoded a protein of 218 amino acids with two domains of 11 and 32 pentapeptide repeats bridged by a glycine residue.

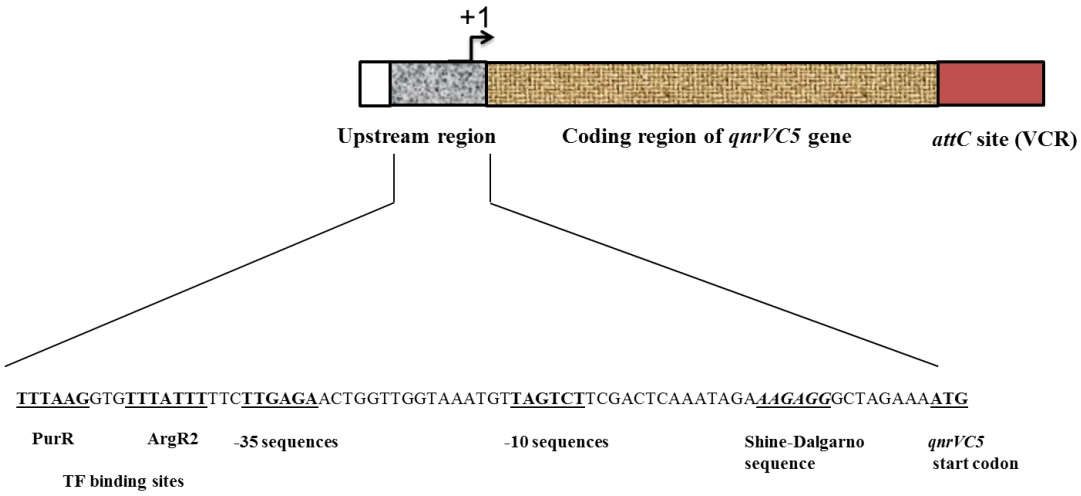

Figure 5.1. The genetic environment of *qnrVC5* gene from *V. fluvialis* isolate BD146. The structure of *qnrVC5* gene cassette in pBD146 with canonical promoter sequence and VCR recombination site, at the upstream and downstream positions respectively of the coding region.

Based on the sequence of pBD146, it was evident that *qnrVC5* gene in *V. fluvialis* BD146 was present in the form of a gene cassette where ORF was flanked with a downstream recombination site corresponding to *V. cholerae* repeat (VCR) region and an upstream internal promoter (P_{qnrVC}) with canonical sequence (Fig. 5.1). Most interestingly, upstream of -10 and -35 sequences of the P_{qnrVC} promoter, binding sites for PurR (purine metabolism repressor) and ArgR2 (arginine metabolism regulator) were also predicted in this cassette using Softberry – BPROM online tool (Fig. 5.1). To further characterize the gene sequence from other two isolates (L10734 and L9978), primers qnrVCcas-F and qnrVCcas-R were designed based on the sequence flanking *qnrVC5* in pBD146. PCR carried out using these primers revealed that the same length of gene cassette was also amplified from these two isolates suggestive of the similar gene organization in these three bacterial isolates.

5.2.2. Sequence analysis, homology and structure prediction for *qnrVC5* gene/protein

At nucleotide level, the ORF of *qnrVC5* allele shared 99% identity with *qnrVC4* and *qnrVC2*, and 97% identity with *qnrVC7*. *qnrVC5* sequence was found to have 76% identity with *qnrVC1* and 75% with both *qnrVC3* and *qnrVC6* alleles. The phylogenetic tree for all these *qnrVC* genes showed that *qnrVC1*, *qnrVC3* and *qnrVC6* belonged to the same group, whereas *qnrVC2*, *qnrVC4*, *qnrVC5* and *qnrVC7* formed another group (Fig. 5.2).

This clearly showed that *qnrVC5* was closely related to *qnrVC2* and *qnrVC4*, which corroborated the earlier findings [Fonseca and Vincente, 2013]. In addition to this, it was observed that among all the *qnrVC* alleles listed out by Fonseca and Vincente [Fonseca and Vincente, 2013], *qnrVC1*, *qnrVC3* and *qnrVC6* alleles were associated with *V. parahaemolyticus* repeats (VPRs) whereas *qnrVC2*, *qnrVC4* and *qnrVC5* alleles were associated with VCRs, indicating different lineage for these two groups of *qnrVC* alleles.

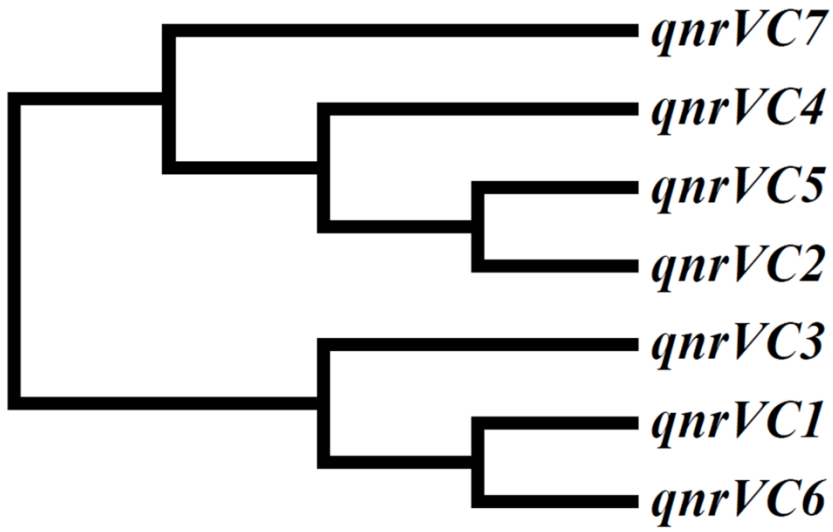

Figure 5.2. Homology of *qnrVC5* gene from *V. fluvialis* isolate BD146. Dendrogram of *qnrVC5* allele with other *qnrVC* alleles, showing the close relationship of *qnrVC5* with *qnrVC2*, *qnrVC4* and *qnrVC7*.

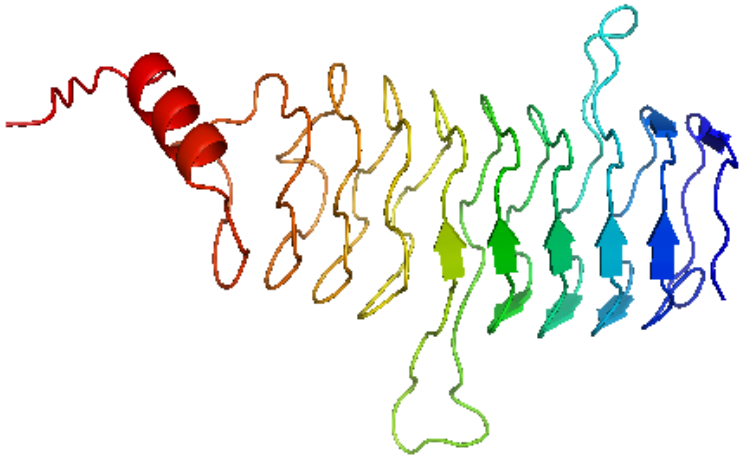

Figure 5.3. Structure prediction of QnrVC5 from *V. fluvialis* isolate BD146. The structure of QnrVC5 predicted using I-TASSER server, showing right-handed quadrilateral β- helix forming 10 coils, interrupted by two loops.

Three-dimensional structure prediction of QnrVC5 protein using I-TASSER server depicted the structure of a typical PRP, threading into β helical folds interrupted by two loops (Fig. 5.3). QnrVC5 protein encompassed 10 coils with two loops (loop A and loop B) extending outwards from the regular structure, interrupting the β-helix turn. Loop A and loop B from QnrVC5 shared 25% and 50% sequence homology respectively with the corresponding loops of QnrB1. Structure predicted from the threading experiments on I-TASSER server was based on the templates of O-methyl transferase (PDB ID: 3DUL) from *Bacillus cereus* and QnrB1 (PDB ID: 2XTY and 2XTW) from *K. pneumoniae*. The structural analogs of the predicted structure were QnrB1 (PDB ID: 2XTW), AhQnr (PDB ID: 3PSS) from *A. hydrophila* and O-methyl transferase (PDB ID: 3DUL).

5.2.3. Contribution of *qnrVC5* gene in quinolone resistance of their native *V. fluvialis* host

The effect of the *qnrVC5* gene in elevating the MIC and MPC of quinolones in *V. fluvialis* isolates was determined as described in sections 2.4.2.1 and 2.11 respectively. All the *V. fluvialis* isolates harbouring *qnrVC5* (BD146, L10734 and

L9978) were subjected to MIC assays with nalidixic acid, norfloxacin, ciprofloxacin and ofloxacin and MPC assays with ciprofloxacin. Among these three isolates, L9978 possessed *qnrVC5* as the only detected quinolone resistance determinant whereas rest two were having other factors along with *qnrVC5* gene (Table 5.1). *V. fluvialis* BD146 had GyrA S83I, ParC S85L mutations and *aac (6')-Ib-cr* gene as the quinolone resistance factors in addition to *qnrVC5* gene. *V. fluvialis* L10734 had GyrA S83I and ParC S85L mutations along with *qnrVC5* gene (Table 5.1). The quinolone resistant *V. fluvialis* isolate L15318 (having GyrA S83I and ParC S85L mutations and lacking *qnrVC5)* and quinolone sensitive *V. fluvialis* isolate L13828 were included as controls (Table 5.1). The MIC and MPC values of *V. fluvialis* isolates for quinolones have been mentioned in Table 5.1. It was observed that when compared to the sensitive isolate L13828, the *qnrVC5*-bearing isolate L9978 showed about 2- to 4-fold elevation in MIC of tested quinolones and 2-fold elevation in MPC of ciprofloxacin. When compared to L15318, BD146 showed 2- to 4-fold increase in MIC of ciprofloxacin and a slight increase (< 2-fold) in MIC of nalidixic acid, norfloxacin and ofloxacin presumably due to the additional presence of PMQR determinants *qnrVC5* gene and *aac (6')-Ib-cr* gene apart from GyrA and ParC mutations. This result also indicated that *qnrVC5* and *aac (6')-Ib-cr* determinants may chiefly contribute towards resistance to ciprofloxacin. As expected, the MPC of ciprofloxacin was also elevated 8-fold in BD146 when compared to L15318 and L10734. Though it may be difficult to compare MIC/MPC of non-isogenic strains, the synergy between different resistance factors was evident in the majority of cases except L10734 and L15318. Similarly, the lower MIC obtained for nalidixic acid in L10734 as compared to L15318 could not be explained.

5.2.4. Contribution of *qnrVC5* gene in quinolone resistance of *E. coli* transformants

In the previous studies from our laboratory, plasmid preparations from *V. fluvialis* isolate BD146 [Rajpara et al. 2009], L10734 and L9978 [Singh et al. 2012] were transformed into *E. coli* JM109 to elucidate their transferable traits. *E. coli* transformants of these three *V. fluvialis* plasmid preparations harbouring *qnrVC5*

gene were utilized in this study to find the effect of this gene in elevating the MIC of quinolones. For BD146, two types of *E. coli* transformants were observed on the basis of presence or absence of a 7.5 kb plasmid pBD146 harbouring *qnrVC5* gene. The transformants that possessed 7.5 kb plasmid as well as another low copy number plasmid (bearing *aac (6') Ib-cr* gene) were termed as 7.5 kb+. On the other hand, the transformants harbouring only low copy number plasmid but devoid of the 7.5 kb plasmid were termed as 7.5 kb- [Rajpara et al. 2009]. In other words, the transformants 7.5 kb+ possessed both *qnrVC5* and *aac (6') Ib-cr* genes whereas 7.5kb- transformants were positive for *aac (6') Ib-cr* gene only (Table 5.2). Therefore, in the present study, one 7.5kb+ transformant was selected to study the effect of the *qnrVC5* gene in combination with *aac (6') Ib-cr* and one 7.5kb- transformant was selected to study the effect of *aac (6') Ib-cr* alone in quinolone resistance. For similar reasons, transformants of L10734 and L9978 bearing *qnrVC5* gene [Singh et al. 2012] were included to study the effect of the *qnrVC5* gene alone. Untransformed *E. coli* JM109 was used as a negative control for the MIC assays. The MIC values for all the four transformants and *E. coli* JM109 are mentioned in Table 5.2. The transformants having *qnrVC5* alone showed 2- to 4-fold elevation in MIC of norfloxacin, ciprofloxacin and ofloxacin and 4- to 8-fold increase in the MIC of nalidixic acid when compared to *E. coli* JM109. The 7.5 kb+ transformant having *qnrVC5* along with *aac (6') Ib-cr* gene showed an 8-fold increase in MIC of nalidixic acid and 4-fold for norfloxacin and ciprofloxacin. 7.5kb+ transformant showed a 2-fold increase in the MIC of ofloxacin. The 7.5 kb- transformant having *aac (6') Ib-cr* gene alone, showed a 4-fold increase in MIC for nalidixic acid and 2-fold increase for norfloxacin and ciprofloxacin but did not show any effect against ofloxacin.

Table 5.1. Quinolone susceptibility of *Vibrio fluvialis* isolates

***V. fluvialis* Strain**	**Quinolone resistance determinants**	**MIC (µg/mL)**				**MPC of Ciprofloxacin (µg/mL)**
		Nalidixic acid	**Norfloxacin**	**Ciprofloxacin**	**Ofloxacin**	
BD146	GyrA S83I, parC S85L, *qnrVC5* and *aac(6')Ib-cr*	1500	17.5-20	10	10	320
L15318	GyrA S83I, parC S85L	1000	15	2.5-5	8	40
L10734	GyrA S83I, parC S85L and *qnrVC5*	125	10	2.5	8	40
L9978	*qnrVC5*	2	1.25	0.312	0.5	1.25-5
L13828	None	0.75	0.312	0.156	0.312	0.625-2.5

Table 5.2. Quinolone susceptibility of *E. coli* transformants

***E.coli* JM109 transformant**	**Quinolone resistance determinants**	**MIC (µg/mL)**			
		Nalidixic acid	**Norfloxacin**	**Ciprofloxacin**	**Ofloxacin**
BD146 transformant (7.5 kb+)	*qnrVC5* and *aac(6')Ib-cr*	400	4	0.5	1
BD146 transformant (7.5 kb-)	*aac(6')Ib-cr*	200	2	0.25	0.5
L10734 transformant	*qnrVC5*	200	2	0.25	1
L9978 transformant	*qnrVC5*	400	4	0.5	2
JM109 (untransformed)	None	50	1	0.125	0.5

5.2.5. Expression of *qnrVC5* gene in the native *V. fluvialis* host and *E. coli* transformants

RT-PCR was carried out to confirm the expression of the *qnrVC5* gene and the *aac (6') Ib-cr* gene in the native host and their *E. coli* transformants (Fig. 5.4).

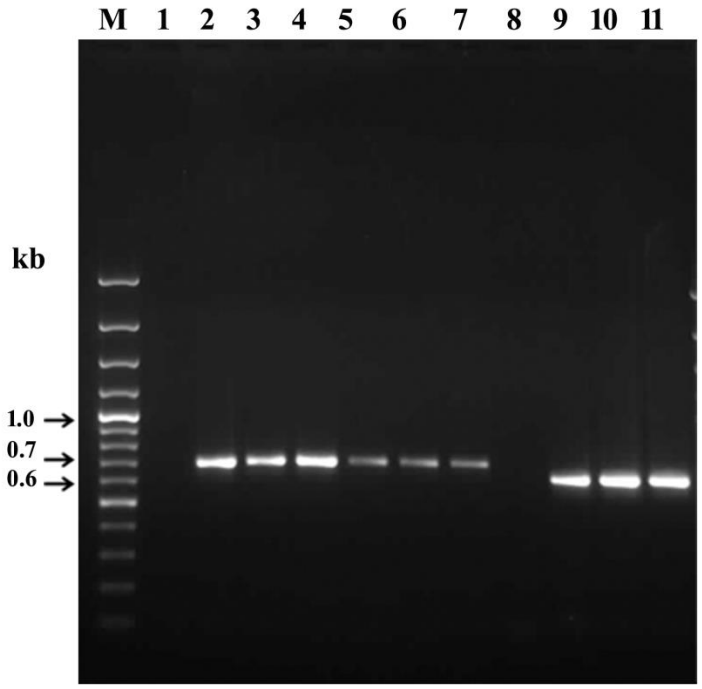

Figure 5.4. Expression of *qnrVC5* and *aac (6') Ib-cr* genes in the native *V. fluvialis* host and in *E. coli* JM109 transformants derived from the plasmids of native hosts. Lane 1: Negative control (without reverse transcription step) for RT-PCR product of *qnrVC5* transcript from *V. fluvialis* hosts BD146; Lanes 2 to 4: RT-PCR products of *qnrVC5* transcript (657 bp) from *V. fluvialis* hosts BD146, L10734 and L9978 respectively; Lanes 5 to 7: RT-PCR products of *qnrVC5* transcript from transformants of BD146 (7.5 kb +), L10734 and L9978; Lane 8: Negative control (without reverse transcription step) for RT-PCR product of *aac(6') Ib-cr* transcript from *V. fluvialis* hosts BD146; Lanes 9 to 11: RT-PCR products of *aac(6') Ib-cr* transcript (608 bp) from *V. fluvialis* BD146 and its transformants 7.5 kb + and 7.5 kb - respectively. 100 bp Gene ruler (Fermentas) was used as the marker (M).

In both the cases, genes were expressed with the expected band size of 657 bp for *qnrVC5* (Fig. 5.4, Lanes 2 to 7) and 608 bp for aac *(6') Ib-cr* (Fig. 5.4, Lanes 9 to 11). No bands corresponding to these genes were observed in the negative controls without reverse transcription step. This ensured the absence of DNA

contamination in RNA templates. Two such negative controls with BD146 RNA templates for RT-PCR of *qnrVC5* and *aac (6') Ib-cr* transcripts have been shown (Fig. 5.4, Lanes 1 and 8 respectively).

5.2.6. Effect of PNA for *qnrVC5* gene in the native *V. fluvialis* host

Once the presence of *qnrVC5* RNA was confirmed in the native host, a peptide-PNA against *qnrVC5* RNA was used to silence the expression of *qnrVC5* gene as described in the section 2.4.2.3. MIC of ciprofloxacin for *V. fluvialis* BD146 was determined in the presence of increasing concentrations of PNA (0 µM, 2 µM, 4 µM and 6 µM). The results showed a steady decrease in MIC values with increasing concentration of PNA with 4 µM PNA appearing as an effective dose for inhibition (Fig. 5.5).

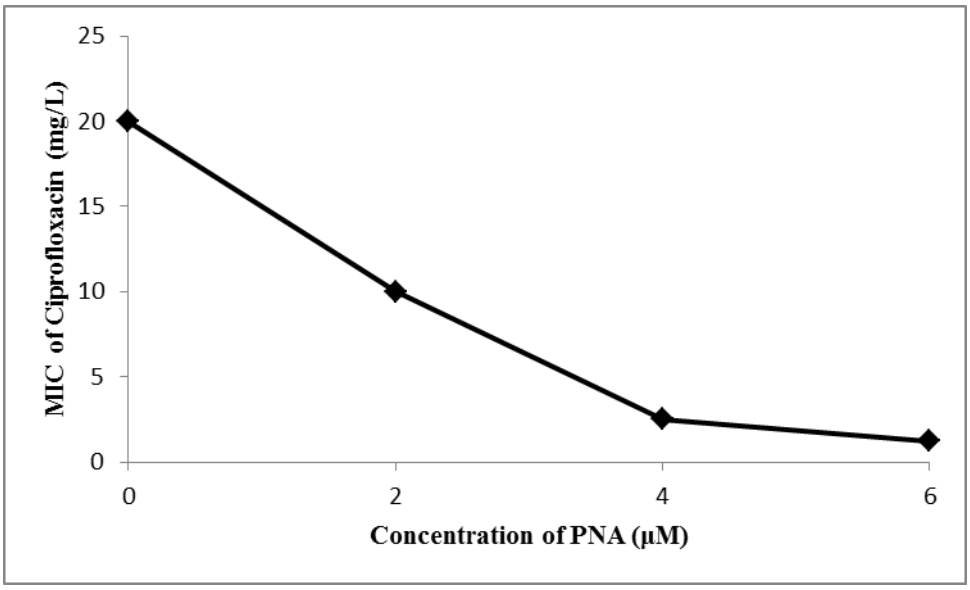

Figure 5.5. Effect of PNA on MIC of ciprofloxacin. MIC of ciprofloxacin for *V. fluvialis* BD146 was tested with increasing concentrations (0, 2, 4, 6 µM) of anti-*qnrVC* peptide PNA. A steady decrease in the MIC of ciprofloxacin for BD146 was observed as the concentration of PNA increased.

Subsequently, MIC of ciprofloxacin for all the three *qnrVC5*-bearing *V. fluvialis* isolates (BD146, L10734 and L9978) and one *qnrVC5*-lacking *V. fluvialis* isolate L15318 was tested at a final concentration of 4 µM of PNA. Two- to eight-fold decrease in MIC of ciprofloxacin in all the three isolates was observed in the presence of PNA when compared to the control with no PNA (Table 5.3). In *qnrVC5*-lacking isolate L15318, the MIC of ciprofloxacin was similar irrespective of the presence of PNA indicating that PNA did not have any non-specific inhibitory activity (Table 5.3).

Table 5.3. Silencing effect of *qnrVC5* gene in *Vibrio fluvialis* isolates

V. fluvialis	**MIC of Ciprofloxacin (µg /mL)**		**Fold reduction in MIC**
	PNA-	**PNA+**	
BD146	10-20	2.5-5	2-8
L10734	2.5	1.25	2
L9978	0.156	0.078	2
L15318	5	5	0

5.2.7. Gene cloning and recombinant protein expression

The *qnrVC5* gene was cloned in pET28a expression vector and the authenticity of the recombinants was confirmed as mentioned in section 2.8. A protein band of ~27 kDa was found to be overexpressed by the *E. coli* BL21(λDE3) recombinants on 1mM IPTG induction (Fig. 5.6). This protein band was excised from the SDS-PAGE gel and subjected to trypsin digestion. The peptide mass fingerprinting analysis confirmed it to be QnrVC5.

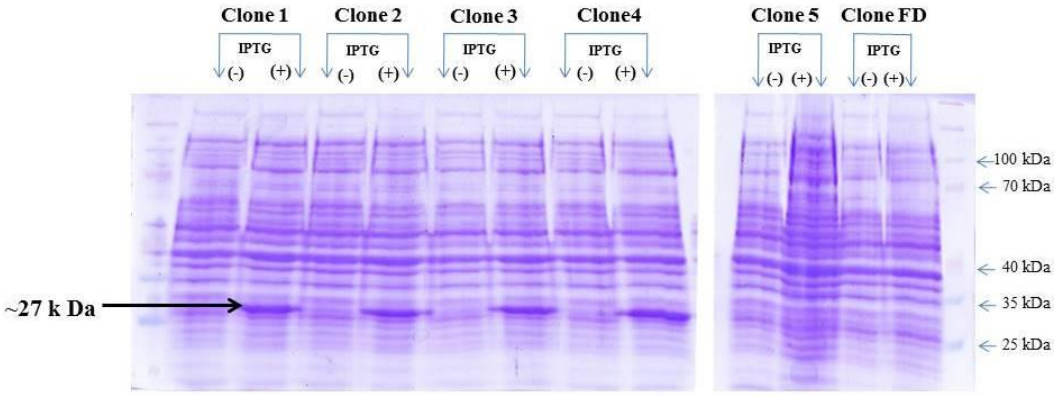

Figure 5.6. Expression of recombinant QnrVC5 protein. The expression of recombinant QnrVC5 protein was studied in the total cell lysates of *E. coli* BL21(λDE3) recombinants (clone 1, 2, 3 and 4) along with *E. coli* BL21(λDE3) non-recombinants (clone 5 and FD) in the presence and absence of IPTG. The QnrVC5 protein band of ~27 kDa was overexpressed by the *E. coli* BL21(λDE3) recombinants on 1mM IPTG induction for 2 h. The molecular marker (Fermentas) positions have been indicated in the right.

5.2.8. Elevation of MIC for quinolones in *qnrVC5* recombinants

MIC assays were carried out to study the functionality of recombinant QnrVC5 protein as described in the section 2.4.2.2. MIC was tested with different generations of quinolones with nalidixic acid representing first generation, norfloxacin, ciprofloxacin, ofloxacin and levofloxacin representing second generation, sparfloxacin for third generation and moxifloxacin as a representative of fourth generation. Interestingly, recombinant *E. coli* BL21(λDE3) was found to show 8-fold to more than 64-fold increase in MIC of different quinolones when compared to a control having pET28a alone (Table 5.4). QnrVC5 conferred higher resistance towards ciprofloxacin and sparfloxacin as the MICs of these two drugs were elevated to more than 64-fold. MIC of levofloxacin and moxifloxacin were elevated to 64-fold and 32-fold respectively. QnrVC5 elevated the MIC of both norfloxacin and ofloxacin to 16-fold and nalidixic acid to 8-fold (Table 5.4).

Table 5.4. MIC of quinolones for pET-qnrVC5 clone

Quinolones	**MIC (µg/mL)**		**Fold elevation in MIC**
	***E. coli* (BL21 λDE3) (pET-*qnrVC5* clone)**	***E. coli* (BL21 λDE3) (pET28a)**	
Nalidixic acid	5	0.625	8
Norfloxacin	0.125	0.0078	16
Ciprofloxacin	0.0156	< 0.000243	> 64
Ofloxacin	0.0625	0.0039	16
Levofloxacin	0.0312	0.000487	64
Sparfloxacin	0.125	< 0.00195	> 64
Moxifloxacin	0.125	0.0039	32

5.3. Discussion

In the studies aimed at unraveling the molecular mechanisms of drug resistance in the clinical isolates of *V. fluvialis*, a new allele named *qnrVC5* was reported from this laboratory [Rajpara et al. 2009; Singh et al. 2012]. The current study was intended to characterize this plasmid-associated gene from those clinical isolates, for its role in conferring protection/resistance towards quinolones. *qnrVC5* ORF was found as a gene cassette with its own promoter with canonical sequence and *attC* site which was identical to *V. cholerae* repeats (VCR). BLAST search indicated that this gene cassette showed 99% identity with *qnrVC2* gene cassette found in the plasmid pVN84 harboured by *V. cholerae* O1, isolated from Vietnam during 2004 as well as *qnrVC5* gene cassette found in another plasmid present in *V. parahaemolyticus* V110, isolated from Hong Kong during 2010. The *qnrVC5* gene cassette also showed 99% identity to *qnrVC4* gene cassettes carried in class I integrons of *Aeromonas caviae*, *A. hydrophila*, *E. coli* and *Salmonella enterica* and 98% identity to *qnrVC4* gene in class I integron of *A. punctata*. This indicated the evolutionary relationship among *qnrVC2*, *qnrVC5* and *qnrVC4* gene

cassettes and their dissemination in various species of bacteria through mobile genetic elements. *qnrVC5* gene cassette showed 97% homology with a *qnr* cassette found in super integron (SI) on the small chromosome of *V. cholerae* MS6 [Okada et al. 2014]. The VCR region downstream of *qnrVC5* ORF showed identity to the stretch of sequences present in the chromosome of various *Vibrio* and *Shewanella* species. This indicated the possible exchange of various resistance conferring genes or gene cassettes among these bacterial species of *Shewanellaceae* and *Vibrionaceae* families in their environmental vicinity. This observation supported the hypothesis by Poirel and his colleagues that these two families could be the reservoirs of *qnr* genes [Poirel et al. 2005a; 2005b]. This additionally supported the chromosomal origin of *qnr* alleles. The presence of regulatory motif for DNA-binding proteins PurR and ArgR indicated the possible involvement of these elements in controlling the expression of the *qnrVC5* gene. This was suggestive of the probable biological function of Qnr proteins in relation to purine and arginine metabolism or indicates that *qnrVC5* might be one of the potentially co-regulated set of genes interlinked with amino acid and nucleotide metabolism.

The antimicrobial susceptibility assays clearly showed the involvement of the *qnrVC5* gene in conferring resistance to different quinolones. Compared to the sensitive *V. fluvialis* strain (L13828), *qnrVC5*-bearing-isolates resisted the quinolone action by virtue of *qnrVC5* alone as in L9978 or in unison with other mechanisms as in BD146 and L10734. The *qnrVC5*-bearing-plasmids from the parent strain, transformed to the heterologous host *E. coli* JM109, proved the role played by the gene in reducing susceptibility towards quinolones. Hence, the *qnrVC5* gene could effectively express its traits in different bacterial hosts as these plasmids disseminated through horizontal gene transfer. Silencing of the gene increased the susceptibility of the parent strains towards ciprofloxacin from 2- to 8-fold, again confirming the role of *qnrVC5* in drug resistance.

The results of MIC and MPC assays were vivid representations of synergy between various quinolone resistance determinants (with some exceptions) with

BD146 as a carrier of all the three determinants and rest of the isolates as carriers of either one or two determinants. These results also clearly indicated the major role of topoisomerase mutations in susceptibility for quinolones with *qnrVC5* or *aac (6') Ib-cr,* playing the role of an apprentice. Though the level of resistance conferred by *qnrVC5* for ciprofloxacin was low, it apparently helped the pathogen in elevating the MIC and MPC of ciprofloxacin for BD146 and L9978 thus extending the mutant selection window for ciprofloxacin in these isolates. This is likely to help in the selection of more mutants at higher concentrations of the drug, by enhancing the survival ability of the pathogen [Strahilevitz et al. 2009]. However, as described in the results section, there were anomalies in this synergistic design and therefore, could not be explained. Perhaps, comparisons of susceptibility trends in non-isogenic strains may not be very straightforward and may reflect a complex interplay of many known and yet unknown genetic factors.

Recently, the role of *qnrVC1* gene located in a chromosomal integron in clinical and environmental *P. aeruginosa* isolates has been described in conferring resistance to quinolones [Belotti et al. 2015]. *qnrVC1* gene with its promoter was cloned in *E. coli* and *P. aeruginosa* and shown to confer variable resistance to quinolones. In the present study, T7 promoter-based overexpression of *qnrVC5* gene was utilized to study the gene function in isolation which was not possible in native *V. fluvialis* isolates. MIC study with the recombinant reflected that the potency of this gene in conferring resistance is significant and variable resistance was observed for different generations of quinolones.

Using I-TASSER server, structure of QnrVC5 protein was predicted and was found to be similar to that of QnrB1 [Vetting et al. 2011] and AhQnr [Xiong et al. 2011] implying that the function of QnrVC5 could also be similar to the above two proteins i.e. imparting quinolone resistance. The important feature in the structures of QnrB1 and AhQnr is the presence of two loops and their contribution in the protection of DNA gyrase from quinolones [Vetting et al. 2011; Xiong et al. 2011]. Therefore, the working model of Qnr proteins (PRPs with loops) in quinolone resistance was proposed, in which the Qnr proteins disturb the

quinolone-topoisomerase-DNA complex [Vetting et al. 2011; Xiong et al. 2011]. Our functional analysis of recombinant QnrVC5 protein also strengthened the above-mentioned model. QnrVC5 protein was shown to elevate the MIC of different quinolones by varying degrees. The possible reason for varied elevation in MIC would be the consequence of varying binding orientations of the different quinolones with the topoisomerase-DNA complex. For example, the interactions of nalidixic acid and ciprofloxacin with gyrase differ in drug binding orientation (perhaps reflected in differences in their potency) such that Qnr destabilizes the ciprofloxacin interaction to a greater extent than the nalidixic acid interaction.

To conclude, these findings proved that the carriage of *qnrVC* alleles on various mobile genetic elements such as plasmids, integrons and SXT elements in a variety of organisms of different genera has been disseminating quinolone resistance widely throughout the globe [Fonseca et al. 2008; Rajpara et al. 2009; Fonseca and Vincente, 2013; Kim et al. 2010; Kumar and Thomas, 2011). With the indiscriminate use of quinolones, this can turn out to be both a reason as well as a consequence of the serious problem of multidrug resistance. **This part of the study was published recently [Vinothkumar et al. 2016].**