frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 February 2016
doi: 10.3389/fmicb.2016.00146

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

Kittappa Vinothkumar[1,2], *G. N. Kumar*[2] *and Ashima K. Bhardwaj*[1]*

[1] *Molecular Biology of Diseases, Department of Human Health and Diseases, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Gandhinagar, India,* [2] *Department of Bio-Chemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, India*

OPEN ACCESS

Edited by:
Yuji Morita,
Aichi Gakuin University, Japan

Reviewed by:
Xian-Zhi Li,
Health Canada, Canada
José Manuel Rodriguez-Martínez,
University of Seville, Spain
Veronique Dubois,
University of Bordeaux, France

***Correspondence:**
Ashima K. Bhardwaj
ashima.bhardwaj@gmail.com

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *14 September 2015*
Accepted: *26 January 2016*
Published: *15 February 2016*

Citation:
Vinothkumar K, Kumar GN and Bhardwaj AK (2016) Characterization of Vibrio fluvialis qnrVC5 Gene in Native and Heterologous Hosts: Synergy of qnrVC5 with other Determinants in Conferring Quinolone Resistance. Front. Microbiol. 7:146. doi: 10.3389/fmicb.2016.00146

Resistance of various pathogens toward quinolones has emerged as a serious threat to combat infections. Analysis of plethora of genes and resistance mechanisms associated with quinolone resistance reveals chromosome-borne and transferable determinants. *qnr* genes have been found to be responsible for transferable quinolone resistance. In the present work, a new allele *qnrVC5* earlier reported in *Vibrio fluvialis* from this laboratory was characterized in detail for its sequence, genetic context and propensity to decrease the susceptibility for quinolones. The study has revealed persistence of *qnrVC5* in clinical isolates of *V. fluvialis* from Kolkata region through the years 2002–2006. *qnrVC5* existed in the form of a gene cassette with the open reading frame being flanked by an upstream promoter and a downstream *V. cholerae* repeat region suggestive of its superintegron origin. Sequence analysis of different *qnrVC* alleles showed that *qnrVC5* was closely related to *qnrVC2* and *qnrVC4* and these alleles were associated with *V. cholerae* repeats. In contrast, *qnrVC1*, *qnrVC3*, and *qnrVC6* belonging to another group were associated with *V. parahaemolyticus* repeats. The gene manifested its activity in native *V. fluvialis* host as well as in *Escherichia coli* transformants harboring it by elevating the MIC toward various quinolones by twofold to eightfold. In combination with other quinolone resistance factors such as topoisomerase mutations and *aac(6')-Ib-cr* gene, *qnrVC5* gene product contributed toward higher quinolone resistance displayed by *V. fluvialis* isolates. Silencing of the gene using antisense peptide nucleic acid sensitized the *V. fluvialis* parent isolates toward ciprofloxacin. Recombinant QnrVC5 vividly demonstrated its role in conferring quinolone resistance. *qnrVC5* gene, its synergistic effect and global dissemination should be perceived as a menace for quinolone-based therapies.

Keywords: gene cassettes, plasmids, superintegron, quinolone susceptibility

INTRODUCTION

Vibrio fluvialis is known to cause severe cholera-like diarrhea in humans and has been perceived as an emerging pathogen (Bhattacharjee et al., 2010; Ramamurthy et al., 2014). Diarrheal illnesses caused by this kind of bacteria are generally treated using quinolone class of antibiotics. Increase in the reports of emergence of multi drug resistant (MDR) *V. fluvialis*, showing considerable resistance to quinolones has been a public health concern (Srinivasan et al., 2006; Singh et al., 2012; Ramamurthy et al., 2014). Reduced susceptibility to quinolones in bacteria is mediated by factors such as mutations in the genes for DNA gyrase and topoisomerase IV (the drug targets), efflux activity, Qnr proteins and the inactivation of drug by quinolone-modifying enzyme [AAC (6') Ib-Cr; Bhardwaj et al., 2014; Kim and Hooper, 2014]. The above mentioned mechanisms may work alone or in combination. The synergistic action of these mechanisms helps the pathogen to achieve higher-level of resistance toward quinolones (Baranwal et al., 2002; Srinivasan et al., 2006; Rushdy et al., 2013; Zhu et al., 2013).

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 outward 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).

Emergence of transferable *qnrVC* alleles in *Vibrionaceae* family and other bacterial species aggravated the hysteria on quinolone resistance (Fonseca and Vicente, 2013; Pons et al., 2013). So far, seven *qnrVC* alleles (named as *qnrVC1* to *qnrVC7*) have been reported from different parts of the globe[1]. 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 Vicente, 2013).

Previous studies from this laboratory have revealed the presence of a *qnrVC*-like gene in the plasmids of three *V. fluvialis* clinical isolates, BD146, L10734, and L9978 (Rajpara et al., 2009; Singh et al., 2012) and this allele was named *qnrVC5* by Fonseca and Vicente in 2013 (Fonseca and Vicente, 2013). The plasmid that harbored *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 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 non-functional form of *qnrVC5* due to the presence of several internal stop codons (Fonseca et al., 2008; Fonseca and Vicente, 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; Fonseca and Vicente, 2013; Liu and Chen, 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, the present 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. Results reflected the role of *qnrVC5* in conferring resistance to quinolones and its synergy with other quinolone resistance factors such as mutations in topoisomerase genes and *aac(6') Ib-cr* gene.

[1]http://www.lahey.org/qnrstudies/

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Clinical isolates of *V. fluvialis* BD146, L13828, L10734, L9978, and L15318 were obtained from patients suffering from acute cholera-like diarrhea admitted to The Infectious Diseases Hospital, Kolkata during 2002–2006 (Kind gift from Dr. T. Ramamurthy, National Institute of Cholera and Enteric Diseases, Kolkata), and have been used in earlier studies (Rajpara et al., 2009; Singh et al., 2012). Plasmid pET 28a (Novagen) was used for expression of *qnrVC5* gene. *E. coli* JM109 was used for transformation experiments and *E. coli* BL21 (λDE3) was used for recombinant protein expression studies.

In Silico Analysis

The DNA sequence analyses were done using BLAST tool available at NCBI site[2] and phylogenetic tree was constructed using maximum-likelihood method in MEGA6 (Tamura et al., 2013). Softberry-BPROM, a promoter prediction tool was used to find the promoters and other regulatory elements in *qnrVC5* gene cassette[3]. 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 approach based on structural templates from PDB (Zhang, 2008; Roy et al., 2010, 2012).

Peptide Nucleic Acid (PNA)

The anti-*qnrVC* peptide-PNA [H-$(KFF)_3$K-O-ccattttctagccct-NH_2] complementary to the region encompassing ribosomal binding site and start codon of *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 (Daejeon, South Korea) and the lyophilized PNA oligomer was dissolved in sterile water as per manufacturer's instructions.

DNA Preparations

The preparations of genomic and plasmid DNA of *V. fluvialis* were done as described earlier (Thungapathra et al., 2002). Plasmid Mini kit or Maxi kit (Qiagen) was used for plasmid DNA isolation according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR) and Reverse Transcription PCR (RT-PCR)

Genomic DNA (200 ng) or plasmid DNA (10–50 ng) were used as templates for PCR reactions. PCR reactions were performed using the protocol described earlier (Singh et al., 2012). Primers used for PCR experiments were qnrVC-F 5′-CGC**GGATCC**ATGGATAAAACAGACCAG-3′ and qnrVC-R 5′-CCG**CTCGAG**TTAGTCAGGAACTACTAT-3′. These primers incorporated sites (in bold) for *Bam*HI and *Xho*I restriction enzymes that were used for cloning in the expression vector pET28a. Primers used to amplify the entire *qnrVC5* gene cassette, encompassing the upstream and downstream sequences along with coding region, were qnrVCcas-F 5′-CGTATAGAAAGCGTTATGTG-3′ and qnrVCcas-R 5′-CTGCTGCCATAATGGATAT-3′. Each PCR consisted of an initial denaturation at 94°C for 5 min, followed by 30 amplification cycles, each involving an initial denaturation at 94°C for 0.5 min followed by annealing and extension steps. Annealing condition for qnrVC-F and qnrVC-R primer pair was 65°C for 0.5 min, and for qnrVCcas-F and qnrVCcas-R primer pair was 60°C for 0.5 min. For both the above reactions, extension was performed at 72°C for 1 min and final polymerization was carried out at 72°C for 10 min. Taq polymerase (Fermentas) was used and reactions were performed in T100 thermal cycler (Bio-Rad Laboratories). Purification of PCR products was performed using QIA-quick PCR purification kit (Qiagen) as per the manufacturer's instructions.

RT-PCR was carried out to confirm the expression of *qnrVC5* gene and *aac(6') Ib-cr* gene in the native isolates of *V. fluvialis* and their *E. coli* JM109 transformants. Total RNA was isolated from *V. fluvialis* isolates and their *E. coli* transformants, using RNeasy bacteria mini kit (Qiagen) as per the manufacturer's instructions. The protocol consisted of growing the cultures in LB medium followed by treatment with lysozyme and proteinase K for cell lysis and RNA was purified from the lysate using RNeasy mini spin columns. The RNA preparations were subsequently treated with DNaseI (Fermentas) to remove the genomic DNA contamination. 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. The 1.0 μl template RNA (0.1 μg μl^{-1}) was added to make the final reaction volume of 50.0 μl. The primer pair qnrVC-F and qnrVC-R, mentioned above, were used for RT-PCR of *qnrVC5* transcripts, whereas AG-F 5′- TGACCAACTGCAACGATTCC -3′ and AG-R 5′- ACCCATAGAGCATCGCAAGGT -3′ were used for RT-PCR of *aac(6')Ib-cr* transcripts. 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 consisting of a denaturation step at 94°C for 0.5 min followed by annealing and extension steps. The annealing condition for *qnrVC5* amplification was 60°C for 0.5 min and for *aac(6') Ib-cr* amplification was 64°C for 0.5 min. Extension was performed at 72°C for 1 min and final polymerization was carried out at 72°C for 10 min. The reactions were performed in T100 thermal cycler (Bio-Rad Laboratories).

Gene Cloning and Protein Expression

Vector pET28a and the amplicon of *qnrVC5* (from *V. fluvialis* BD146) each were separately double-digested with *Bam*HI and *Xho*I. Subsequent to digestion, the dephosphorylated vector and *qnrVC5* insert were ligated, the ligation mixture was electroporated in *E. coli* JM109 cells and the transformed cells

[2]http://blast.ncbi.nlm.nih.gov/Blast.cgi

[3]http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb

were plated on LB plates containing kanamycin (50 μg ml^{-1}) to obtain the recombinants. Expression of QnrVC5 protein was studied by induction of *E. coli* BL21 (λDE3) cells harboring the recombinant clones with 1mM IPTG for 2 h at 37°C, followed by SDS-PAGE analysis of total cell lysates. The protein band corresponding to QnrVC5 was excised from the gel and subjected to trypsin digestion. The authenticity of this protein was then confirmed by peptide mass fingerprinting on Bruker Ultraflex III MALDI instrument. The protein identification was done through Mascot software.

Minimum Inhibitory Concentration (MIC) Assays

Twofold dilution method was used to determine the MIC of various quinolones for native *V. fluvialis* clinical isolates, *E. coli* transformants and QnrVC5 recombinant as described previously with minor modifications described below (Mohanty et al., 2012).

(i) MIC assay for native *V. fluvialis* clinical 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 optical density at 600 nm reached 0.1. This culture was used as inoculum for MIC assay. Concentration of the test drug was diluted twofold in Muller Hinton Broth (MHB). Fifty microliters 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. 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^{-1}) before inoculating into the MIC assay plate.

(ii) MIC assay for QnrVC5 recombinants

The recombinant plasmid was transformed into *E. coli* BL21 (λDE3) cells and the transformants were selected on LB agar containing kanamycin (50 μg ml^{-1}). *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 optical density of 0.1 at 600 nm and used as inoculum for MIC assays. The concentration of the test drug was diluted twofold in MHB containing kanamycin (50 μg ml^{-1}) and IPTG (1 mM). Fifty microliters 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.

(iii) MIC assay for *V. fluvialis* isolates in presence of PNA

MIC of ciprofloxacin was determined for *V. fluvialis* isolates (BD146, L10734, L9978, and L15318) using the same twofold dilution method described above, but in a 96-well polypropylene plate in a 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 on MIC with increasing concentrations of PNA (0, 2, 4, and 6 μM). Subsequent to that, assays were performed without and with PNA (4 μM) for all the above mentioned *V. fluvialis* isolates.

Mutant Prevention Concentration (MPC) Assay

Mutant prevention concentration assay was performed as described previously with minor modifications (Marcusson et al., 2005). Hundred microliters of overnight grown culture of each of the *V. fluvialis* isolates (BD146, L15318, L10734, L9978, and L13828) was inoculated in 25 ml LB and grown at 37°C until the optical density at 600 nm reached 1.0 ($\sim 10^9$ cells/ml). The culture was centrifuged at 8000 rpm for 5 min and the pellet was resuspended in LB to contain $\geq 10^{10}$ cells/ml. Subsequently, 200 μl of resuspended culture was spread on the Muller Hinton Agar (MHA) plates containing defined concentrations of ciprofloxacin. Each strain was tested with six concentrations of ciprofloxacin starting from their MIC (i.e., 1X, 2X, 4X, 8X, 16X, 32X MIC). The plates were incubated at 37°C for 96 h and MPC was recorded as the lowest concentration of ciprofloxacin that prevented the emergence of mutant colonies. The assays were individually performed three times.

RESULTS

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 harbor 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 Vicente, 2013). The sequence encoded a protein of 218 amino acids with two domains of 11 and 32 pentapeptide repeats bridged by a glycine residue.

Based on the sequence of pBD146, it was evident that *qnrVC5* gene in *V. fluvialis* isolate 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 (**Figure 1A**). 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 (**Figure 1A**). 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

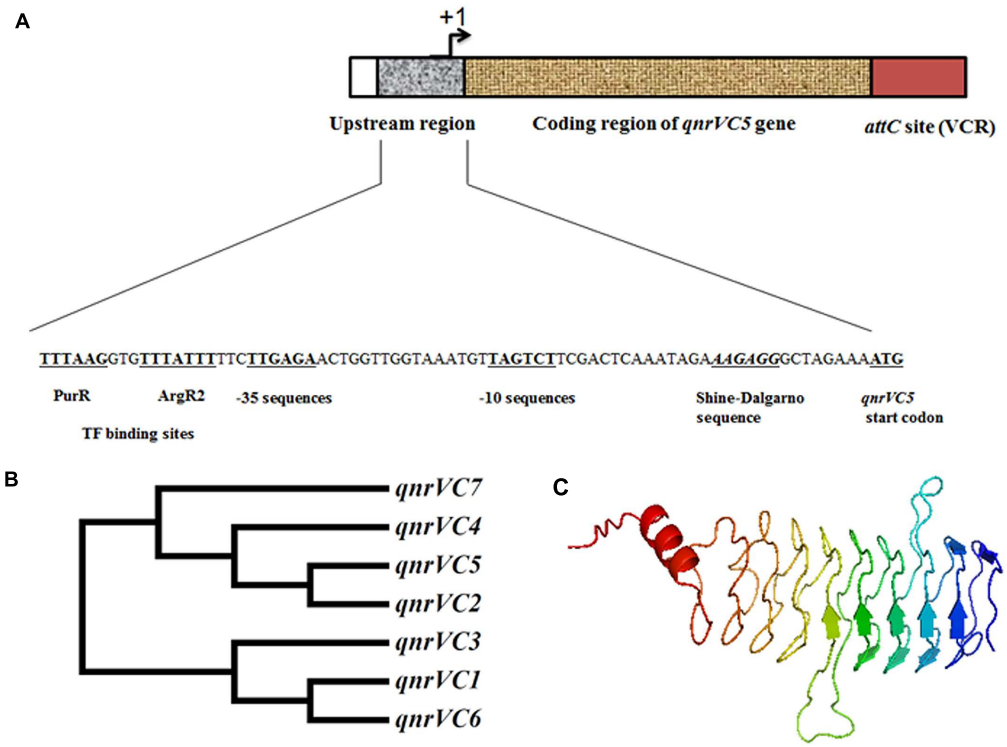


FIGURE 1 | The genetic environment, homology and structure prediction of the gene/gene product of *qnrVC5* gene from *V. fluvialis* isolate BD146. **(A)** The structure of *qnrVC5* gene cassette in pBD146 with canonical promoter sequence and VCR recombination site, at the upstream and downstream positions of the coding region, respectively. **(B)** Dendrogram of *qnrVC5* allele with other *qnrVC* alleles, showing the close relationship of *qnrVC5* with *qnrVC2* and *qnrVC4*. **(C)** Structure prediction of QnrVC5 using I-TASSER server, showing right handed quadrilateral β-helix forming 10 coils, interrupted by two loops.

cassette was also amplified from these two isolates suggestive of the similar gene organization in these three bacterial isolates.

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 (**Figure 1B**). This clearly showed that *qnrVC5* was closely related to *qnrVC2* and *qnrVC4*, which corroborated the earlier findings (Fonseca and Vicente, 2013). In addition to this, it was observed that among all the *qnrVC* alleles listed out by Fonseca and Vicente, (Fonseca and Vicente, 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. 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 (**Figure 1C**). QnrVC5 protein encompassed 10 coils with two loops (loop A and loop B) extending outward 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).

Contribution of *qnrVC5* Gene in Quinolone Resistance of their Native *V. fluvialis* Host

The effect of *qnrVC5* gene in elevating the MIC and MPC of quinolones in *V. fluvialis* isolates was determined. All the *V. fluvialis* isolates harboring *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 strains, L9978 possessed *qnrVC5* as the only detected quinolone resistance determinant whereas rest two were having other factors along with *qnrVC5* gene (**Table 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

TABLE 1 | Quinolone susceptibility of *V. fluvialis* strains and their corresponding *E. coli* transformants.

Strain/transformant	Quinolone resistance determinants	MIC (μg ml^{-1})				MPC of Ciprofloxacin (μg ml^{-1})
		Nalidixic acid	Norfloxacin	Ciprofloxacin	Ofloxacin	
V. fluvialis						
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
***E. coli* JM109**						
BD146 transformant (7.5 kb+)	*qnrVC5* and *aac(6')Ib-cr*	400	4	0.5	1	ND
BD146 transformant (7.5 kb–)	*aac(6')Ib-cr*	200	2	0.25	0.5	ND
L10734 transformant	*qnrVC5*	200	2	0.25	1	ND
L9978 transformant	*qnrVC5*	400	4	0.5	2	ND
JM109 (non-transformant)	None	50	1	0.125	0.5	ND

ND, Not done.

S83I and ParC S85L mutations along with *qnrVC5* gene (**Table 1**). The quinolone resistant *V. fluvialis* strain L15318 (having GyrA S83I and ParC S85L mutations and lacking *qnrVC5)* and quinolone sensitive *V. fluvialis* strain L13828 were included as controls (**Table 1**). The MIC and MPC values of *V. fluvialis* isolates for quinolones have been mentioned in **Table 1**. It was observed that when compared to the sensitive strain L13828, the *qnrVC5*-bearing strain L9978 showed about twofold to fourfold elevation in MIC of tested quinolones and twofold elevation in MPC of ciprofloxacin. When compared to L15318, BD146 showed twofold to fourfold increase in MIC of ciprofloxacin and slight increase (<twofold) in MIC of nalidixic acid, norfloxacin, and ofloxacin presumably due to 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 toward resistance to ciprofloxacin. As expected, the MPC of ciprofloxacin was also elevated eightfold in *V. fluvialis* BD146 when compared to L15318 and L10734. Though it may be difficult to compare MIC/MPC of non-isogenic strains, synergy between different resistance factors was evident in 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.

Contribution of *qnrVC5* Gene in Quinolone Resistance of *E. coli* Transformants

In the previous studies from our laboratory, plasmid preparations from *V. fluvialis* isolates 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 harboring *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 harboring *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 harboring 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 7.5 kb- transformants were positive for *aac (6') Ib-cr* gene only (**Table 1**). Therefore, in the present study, one 7.5 kb+ transformant was selected to study the effect of *qnrVC5* gene in combination with *aac (6') Ib-cr* and one 7.5 kb- 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 *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 1**. The transformants having *qnrVC5* alone showed twofold to fourfold elevation in MIC of norfloxacin, ciprofloxacin and ofloxacin and fourfold to eightfold 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 eightfold increase in MIC of nalidixic acid and fourfold for norfloxacin and ciprofloxacin. The 7.5 kb+ transformant showed twofold increase in the MIC of ofloxacin. The 7.5 kb- transformant having *aac (6') Ib-cr* gene alone, showed fourfold increase in MIC for nalidixic acid and twofold for norfloxacin and ciprofloxacin but did not show any effect against ofloxacin.

Expression of *qnrVC5* Gene in Native *V. fluvialis* Host and *E. coli* Transformants

RT-PCR was carried out to confirm the expression of *qnrVC5* gene and *aac(6') Ib-cr* gene in the native host and their *E. coli* transformants (**Figure 2**). In both the cases, genes were expressed

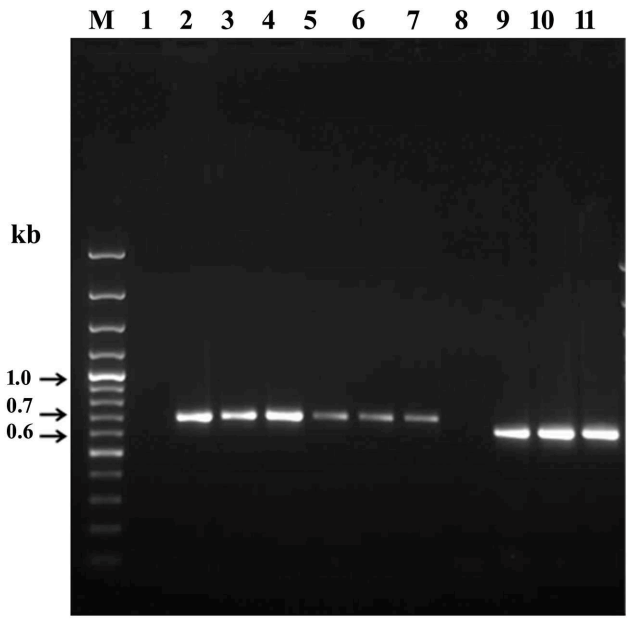


FIGURE 2 | Expression of *qnrVC5* and *aac(6') Ib-cr* genes in the native *V. fluvialis* host and in *E. coli* JM109 transformants derived from the plasmids of native hosts. 100 bp Gene ruler (Fermentas) was used as the marker (M). Lane 1: Negative control (without reverse transcription step) for RT-PCR product of *qnrVC5* transcript (657 bp) 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 (657 bp) 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 (657 bp) 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.

with the expected band size of 657 bp for *qnrVC5* (**Figure 2**, Lanes 2 to 7) and 608 bp for *aac(6') Ib-cr* (**Figure 2**, Lanes 9 to 11). The negative controls without reverse transcription step that were included to ensure the absence of DNA contamination in RNA templates, did not show any band corresponding to the expressed genes. Two such negative controls with BD146 RNA templates for RT-PCR of *qnrVC5* and *aac(6') Ib-cr* transcripts have been shown (**Figure 2**, Lanes 1 and 8, respectively).

Effect of PNA Against *qnrVC5* Gene in 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 its expression. MIC of ciprofloxacin for *V. fluvialis* BD146 was determined in the presence of increasing concentrations of PNA (0, 2, 4, and 6 μM). The results showed a steady decrease of MIC values with increasing concentration of PNA with 4 μM of PNA appearing as an effective dose for inhibition. 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. Twofold to eightfold 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 2**). In *qnrVC5*-lacking *V. fluvialis* 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 2**).

Gene Cloning and Recombinant Protein Expression

The *qnrVC5* gene was cloned in pET28a expression vector and the authenticity of the recombinant clones was confirmed. A protein band of ∼27 kDa was found to be overexpressed by the recombinants on 1 mM IPTG induction. 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.

Elevation of MIC for quinolones in *qnrVC5* Recombinants

Minimum inhibitory concentration assays were carried out to study the functionality of recombinant QnrVC5 protein. 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) cells were found to show eightfold to more than 64-fold increase in MIC of different quinolones when compared to control having pET28a alone (**Table 3**). QnrVC5 conferred higher resistance toward 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 eightfold (**Table 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 toward quinolones. *qnrVC5* ORF was found as a gene cassette with its

TABLE 2 | Silencing effect of *qnrVC5* gene in *V. fluvialis* strains.

V. fluvialis	MIC of Ciprofloxacin (μg ml^{-1})		Fold in reduction of 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

TABLE 3 | MIC of quinolones for pET-qnrVC5 clone.

Quinolones	MIC ($\mu g\ ml^{-1}$)		Fold in elevation of 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

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 harbored 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 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 et al. (2005a,b) that these two families could be the reservoirs of *qnr* genes. 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 *qnrVC5* gene. This was suggestive of the probable biological function of Qnr proteins in relation to purine and arginine metabolism or indicate 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 *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 toward 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 toward ciprofloxacin from twofold to eightfold, 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 *V. fluvialis* strains (BD146 and L9978) thus extending the mutant selection window for ciprofloxacin in these strains. This is likely to help in the selection of more mutants at higher concentrations of 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 straight-forward 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 *Pseudomonas aeruginosa* isolates, in conferring resistance to quinolones, has been described (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 structure (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 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.

CONCLUSION

These findings prove 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 have been disseminating quinolone resistance widely throughout the globe (Fonseca et al., 2008; Rajpara et al., 2009; Kim et al., 2010; Kumar and Thomas, 2011; Fonseca and Vicente, 2013). 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 multi-drug resistance.

AUTHORS CONTRIBUTIONS

Conceived and designed the experiments: AB and KV. Performed the experiments: KV. Analyzed the data: AB, GK, and KV. Contributed reagents/materials/analysis tools: AB. Wrote the paper: AB, GK, and KV.

FUNDING

This work was supported by the grants from Gujarat State Biotechnology Mission (GSBTM; No. GSBTM/MD/PROJECTS/SSA/3386/2012-13), Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India (BT/PR/11634/INF/22/104/2008) and Indian Council of Medical Research (ICMR; No.80/845/2013-ECD-I). Authors thankfully acknowledge The Puri Foundation for Education in India for providing infrastructure facilities. KV is the recipient of Senior Research Fellowship from ICMR grant mentioned above.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Amit Ghosh, Dr. T. Ramamurthy and Dr. S. K. Niyogi, National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India, for their support and advice. The authors thank Ms. Neha Rajpara, Mr. Priyabrata Mohanty, Mr. Braj M. N. R. S. Kutar, Ms. Aneri Shah, and Mr. Shailesh Bhalara for their suggestions and technical support.

REFERENCES

Baranwal, S., Dey, K., Ramamurthy, T., Nair, G. B., and Kundu, M. (2002). Role of active efflux in association with target gene mutations in fluoroquinolone resistance in clinical isolates of *Vibrio cholerae*. *Antimicrob. Agents Chemother.* 46, 2676–2678. doi: 10.1128/AAC.46.8.2676-2678.2002

Belotti, P. T., Thabet, L., Laffargue, A., Andre, C., Coulange-Mayonnove, L., Arpin, C., et al. (2015). Description of an integron encompassing blaVIM-2, qnrVC1 and genes encoding bacterial group II intron proteins in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 70, 2237–2240. doi: 10.1093/jac/dkv103

Bhardwaj, A. K. (2015). "Multi-drug resisance in vibrios: never underestimate bacteria," in *Advances in Biotechnology and Allied Areas*, eds N. N. Nawani, M. Khetmalas, P. M. Razdan, A. Pandey, and K. M. Powar (New Delhi: I. K. International Publishing House Pvt. Ltd.), 91–105.

Bhardwaj, A. K., Vinothkumar, K., Rajpara, N., Mohanty, P., and Kutar, B. M. R. N. S. (2014). "Therapeutic limitations due to antibiotic drug resistance: road to alternate therapies," in *Frontiers in Anti-Infective Drug Discovery*, Vol. 3, eds A. Rahman and M. I. Choudhary (Sharjah: Bentham Science Publiser), 72–141.

Bhattacharjee, S., Bal, B., Pal, R., Niyogi, S. K., and Sarkar, K. (2010). Is *Vibrio fluvialis* emerging as a pathogen with epidemic potential in coastal region of eastern India following cyclone Aila? *J. Health Popul. Nutr.* 28, 311–317.

Fonseca, E. L., Freitas, F., Vieira, V. V., and Vicente, A. C. (2008). New qnr gene cassettes associated with superintegron repeats in *Vibrio cholerae* O1. *Emerg. Infect. Dis.* 14, 1129–1131. doi: 10.3201/eid1407.080132

Fonseca, E. L., and Vicente, A. C. (2013). Epidemiology of qnrVC alleles and emergence out of the Vibrionaceae family. *J. Med. Microbiol.* 62, 1628–1630. doi: 10.1099/jmm.0.062661-0

Good, L., Awasthi, S. K., Dryselius, R., Larsson, O., and Nielsen, P. E. (2001). Bactericidal antisense effects of peptide-PNA conjugates. *Nat. Biotechnol.* 19, 360–364. doi: 10.1038/86753

Hegde, S. S., Vetting, M. W., Mitchenall, L. A., Maxwell, A., and Blanchard, J. S. (2011). Structural and biochemical analysis of the pentapeptide repeat protein EfsQnr, a potent DNA gyrase inhibitor. *Antimicrob. Agents Chemother.* 55, 110–117. doi: 10.1128/AAC.01158-10

Hegde, S. S., Vetting, M. W., Roderick, S. L., Mitchenall, L. A., Maxwell, A., Takiff, H. E., et al. (2005). A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. *Science* 308, 1480–1483. doi: 10.1126/science.1110699

Jeon, B., and Zhang, Q. (2009). Sensitization of *Campylobacter jejuni* to fluoroquinolone and macrolide antibiotics by antisense inhibition of the CmeABC multidrug efflux transporter. *J. Antimicrob. Chemother.* 63, 946–948. doi: 10.1093/jac/dkp067

Jeong, J. Y., Kim, E. S., Choi, S. H., Kwon, H. H., Lee, S. R., Lee, S. O., et al. (2008). Effects of a plasmid-encoded qnrA1 determinant in *Escherichia coli* strains carrying chromosomal mutations in the acrAB efflux pump genes. *Diagn. Microbiol. Infect. Dis.* 60, 105–107. doi: 10.1016/j.diagmicrobio.2007.07.015

Kim, E. S., and Hooper, D. C. (2014). Clinical importance and epidemiology of quinolone resistance. *Infect. Chemother.* 46, 226–238. doi: 10.3947/ic.2014.46.4.226

Kim, H. B., Wang, M., Ahmed, S., Park, C. H., Larocque, R. C., Faruque, A. S., et al. (2010). Transferable quinolone resistance in *Vibrio cholerae*. *Antimicrob. Agents Chemother.* 54, 799–803. doi: 10.1128/AAC.01045-09

Kumar, P., and Thomas, S. (2011). Presence of qnrVC3 gene cassette in SXT and class 1 integrons of *Vibrio cholerae*. *Int. J. Antimicrob. Agents* 37, 280–281. doi: 10.1016/j.ijantimicag.2010.12.006

Liu, M., and Chen, S. (2013). Draft genome sequence of vibrio parahaemolyticus V110, Isolated from Shrimp in Hong Kong. *Genome Announc.* 1:e300–e313. doi: 10.1128/genomeA.00300-13

Marcusson, L. L., Olofsson, S. K., Komp Lindgren, P., Cars, O., and Hughes, D. (2005). Mutant prevention concentrations of ciprofloxacin for urinary tract

infection isolates of *Escherichia coli*. *J. Antimicrob. Chemother.* 55, 938–943. doi: 10.1093/jac/dki136

Martinez-Martinez, L., Pascual, A., Garcia, I., Tran, J., and Jacoby, G. A. (2003). Interaction of plasmid and host quinolone resistance. *J. Antimicrob. Chemother.* 51, 1037–1039. doi: 10.1093/jac/dkg157

Martinez-Martinez, L., Pascual, A., and Jacoby, G. A. (1998). Quinolone resistance from a transferable plasmid. *Lancet* 351, 797–799. doi: 10.1016/S0140-6736(97)07322-4

Mohanty, P., Patel, A., and Kushwaha Bhardwaj, A. (2012). Role of H- and D-MATE-type transporters from multidrug resistant clinical isolates of Vibrio fluvialis in conferring fluoroquinolone resistance. *PLoS ONE* 7:e35752. doi: 10.1371/journal.pone.0035752

Okada, K., Na-Ubol, M., Natakuathung, W., Roobthaisong, A., Maruyama, F., Nakagawa, I., et al. (2014). Comparative genomic characterization of a Thailand-Myanmar isolate, MS6, of *Vibrio cholerae* O1 El Tor, which is phylogenetically related to a "US Gulf Coast" clone. *PLoS ONE* 9:e98120. doi: 10.1371/journal.pone.0098120

Poirel, L., Liard, A., Rodriguez-Martinez, J. M., and Nordmann, P. (2005a). Vibrionaceae as a possible source of Qnr-like quinolone resistance determinants. *J. Antimicrob. Chemother.* 56, 1118–1121. doi: 10.1093/jac/dki371

Poirel, L., Rodriguez-Martinez, J. M., Mammeri, H., Liard, A., and Nordmann, P. (2005b). Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob. Agents Chemother.* 49, 3523–3525. doi: 10.1128/AAC.49.8.3523-3525.2005

Pons, M. J., Gomes, C., and Ruiz, J. (2013). QnrVC, a new transferable Qnr-like family. *Enferm. Infecc. Microbiol. Clin.* 31, 191–192. doi: 10.1016/j.eimc.2012.09.008

Rajpara, N., Patel, A., Tiwari, N., Bahuguna, J., Antony, A., Choudhury, I., et al. (2009). Mechanism of drug resistance in a clinical isolate of *Vibrio fluvialis*: involvement of multiple plasmids and integrons. *Int. J. Antimicrob. Agents* 34, 220–225. doi: 10.1016/j.ijantimicag.2009.03.020

Ramamurthy, T., Chowdhury, G., Pazhani, G. P., and Shinoda, S. (2014). *Vibrio fluvialis*: an emerging human pathogen. *Front. Microbiol.* 5:91. doi: 10.3389/fmicb.2014.00091

Roy, A., Kucukural, A., and Zhang, Y. (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nat. Protoc.* 5, 725–738. doi: 10.1038/nprot.2010.5

Roy, A., Yang, J., and Zhang, Y. (2012). COFACTOR: an accurate comparative algorithm for structure-based protein function annotation. *Nucleic Acids Res.* 40, W471–W477. doi: 10.1093/nar/gks372

Rushdy, A. A., Mabrouk, M. I., Abu-Sef, F. A., Kheiralla, Z. H., Mohamed Abdel-All, S., and Saleh, N. M. (2013). Contribution of different mechanisms to the resistance to fluoroquinolones in clinical isolates of *Salmonella enterica*. *Braz. J. Infect. Dis.* 17, 431–437. doi: 10.1016/j.bjid.2012.11.012

Saga, T., Kaku, M., Onodera, Y., Yamachika, S., Sato, K., and Takase, H. (2005). *Vibrio parahaemolyticus* chromosomal qnr homologue VPA0095: demonstration by transformation with a mutated gene of its potential to reduce quinolone susceptibility in *Escherichia coli*. *Antimicrob. Agents Chemother.* 49, 2144–2145. doi: 10.1128/AAC.49.5.2144-2145.2005

Sanchez, M. B., Hernandez, A., Rodriguez-Martinez, J. M., Martinez-Martinez, L., and Martinez, J. L. (2008). Predictive analysis of transmissible quinolone resistance indicates *Stenotrophomonas* maltophilia as a potential source of a novel family of Qnr determinants. *BMC Microbiol.* 8:148. doi: 10.1186/1471-2180-8-148

Singh, R., Rajpara, N., Tak, J., Patel, A., Mohanty, P., Vinothkumar, K., et al. (2012). Clinical isolates of *Vibrio fluvialis* from Kolkata, India, obtained during 2006: plasmids, the qnr gene and a mutation in gyrase A as mechanisms of multidrug resistance. *J. Med. Microbiol.* 61, 369–374. doi: 10.1099/jmm.0.037226-0

Srinivasan, V. B., Virk, R. K., Kaundal, A., Chakraborty, R., Datta, B., Ramamurthy, T., et al. (2006). Mechanism of drug resistance in clonally related clinical isolates of *Vibrio fluvialis* isolated in Kolkata, India. *Antimicrob. Agents Chemother.* 50, 2428–2432. doi: 10.1128/AAC.01561-05

Strahilevitz, J., Jacoby, G. A., Hooper, D. C., and Robicsek, A. (2009). Plasmid-mediated quinolone resistance: a multifaceted threat. *Clin. Microbiol. Rev.* 22, 664–689. doi: 10.1128/CMR.00016-09

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Thungapathra, M., Sinha, K. K., Chaudhuri, S. R., Garg, P., Ramamurthy, T., Nair, G. B., et al. (2002). Occurrence of antibiotic resistance gene cassettes aac(6')-Ib, dfrA5, dfrA12, and ereA2 in class I integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrob. Agents Chemother.* 46, 2948–2955. doi: 10.1128/AAC.46.9.2948-2955.2002

Tran, J. H., and Jacoby, G. A. (2002). Mechanism of plasmid-mediated quinolone resistance. *Proc. Natl. Acad. Sci. U.S.A.* 99, 5638–5642. doi: 10.1073/pnas.082092899

Vetting, M. W., Hegde, S. S., Wang, M., Jacoby, G. A., Hooper, D. C., and Blanchard, J. S. (2011). Structure of QnrB1, a plasmid-mediated fluoroquinolone resistance factor. *J. Biol. Chem.* 286, 25265–25273. doi: 10.1074/jbc.M111.226936

Xiong, X., Bromley, E. H., Oelschlaeger, P., Woolfson, D. N., and Spencer, J. (2011). Structural insights into quinolone antibiotic resistance mediated by pentapeptide repeat proteins: conserved surface loops direct the activity of a Qnr protein from a gram-negative bacterium. *Nucleic Acids Res.* 39, 3917–3927. doi: 10.1093/nar/gkq1296

Zhang, Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* 9:40. doi: 10.1186/1471-2105-9-40

Zhu, J. M., Jiang, R. J., Kong, H. S., Zhang, R., Lu, H. X., Sun, C. G., et al. (2013). [Emergence of novel variants of gyrA, parC, qnrS genes in multi-drug resistant *Klebsiella caused* pneumonia]. *Zhonghua Liu Xing Bing Xue Za Zhi.* 34, 61–66.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Diagnostic Microbiology and Infectious Disease 76 (2013) 526–528

Contents lists available at SciVerse ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Triplex PCR assay for the rapid identification of 3 major *Vibrio* species, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio fluvialis*

Kittappa Vinothkumar [a], Ashima Kushwaha Bhardwaj [a,*], Thandavarayan Ramamurthy [b], Swapan Kumar Niyogi [b]

[a] Department of Human Health and Diseases, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Gandhinagar, Gujarat 382 007, India
[b] National Institute of Cholera and Enteric Diseases, P-33, Beliaghata, Kolkata 700 010, India

ARTICLE INFO

Article history:
Received 18 October 2012
Received in revised form 7 March 2013
Accepted 14 April 2013
Available online 22 May 2013

Keywords:
Haemolysin gene
Diagnostics
Epidemiology
Haem-utilising gene
Toxin-regulatory gene

ABSTRACT

A triplex PCR assay was developed for the identification of 3 major *Vibrio* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio fluvialis* by targeting their haemolysin, haem-utilizing, and central regulatory genes, respectively. This simple, rapid, sensitive, and specific assay using cell lysates from 227 samples established its usefulness in research and epidemiology.

Vibrio spp. have been associated with several outbreaks of diarrhea and sporadic infections throughout the world (Faruque et al., 1998; Huq et al., 1980; Thompson et al., 2004). The genus *Vibrio* includes several species like *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Vibrio vulnificus*, *Vibrio mimicus*, etc., that have been reported to cause diarrheal illnesses in humans. Mixed infections due to 2 or more *Vibrio* spp. have also been reported (Hoge et al., 1989; Lesmana et al., 2002). Rapid identification of the causative organisms up to the species level is very useful for research and epidemiological studies as it helps in determining the exact source of any outbreak and in devising strategies to reduce the severity of the disease. However, the conventional identification techniques involving a series of biochemical tests and agglutination with specific antisera are time consuming and ambiguous (Sakazaki, 1992). Therefore, several monoplex and multiplex PCR methods have been developed for species-specific identification of vibrios by targeting various housekeeping, virulence, and regulatory genes (Bauer and Rorvik, 2007; Chakraborty et al., 2006; Dalmasso et al., 2009; Fields et al., 1992; Izumiya et al., 2011; Khuntia et al., 2008; Kim and Bang, 2008; Olsvik et al., 1993; Tarr et al., 2007; Yu et al., 2010). Cholera is endemic in India, and the presence of *V. cholerae*, *V. parahaemolyticus*, and *V. fluvialis* has been frequently reported from India as enteric/food-poisoning organisms (Bhattacharjee et al., 2010; Chowdhury et al., 2012; Kanungo et al., 2012; Nair et al., 2007; Okuda et al., 1997; Rajpara et al., 2009; Sarkar et al., 2012; Sen et al., 2007; Singh et al., 2012; Srinivasan et al., 2006; Thekdi et al., 1982). *V. fluvialis* was especially included in the present study because it has been reported as an emerging pathogen with epidemic potential and thus is an increasing public health concern (Ahmed et al., 2004; Ahmed et al., 2005; Bhattacharjee et al., 2010; Chakraborty et al., 2005; Chowdhury et al., 2011; Igbinosa and Okoh, 2010; Rajpara et al., 2009; Singh et al., 2012; Srinivasan et al., 2006). Hence, we report here the development of a multiplex PCR assay to identify these 3 species.

The target genes selected for the identification were the central regulatory factor gene (*toxR*) for *V. fluvialis*, the haemolysin gene (*hlyA*) for *V. cholerae*, and the haem-utilizing gene (*hutA*) for *V. parahaemolyticus*. For identification of *V. fluvialis*, the primers based on membrane tether region of the *toxR* gene were included in this study as this part of the gene has already been established as a species-specific marker for *V. fluvialis* (Chakraborty et al., 2006). Although the genes used for this study were present in all the 3 species tested, the primers were designed by exploiting the unique sequences of each species after alignment of these chromosome-borne gene sequences from the 3 *Vibrio* spp. The specificity of each primer pair was also confirmed by Primer-BLAST search at the National Centre for Biotechnology Information site. The nucleotide sequences of the primer pairs are given in Table 1.

As a first step, monoplex PCR was carried out using the genomic DNA as template for all the reference strains (Table 2) to check the specificity of each primer. Genomic DNA was prepared according to

* Corresponding author. Tel.: +91-79-30514235.
E-mail address: ashima.bhardwaj@gmail.com (A.K. Bhardwaj).

0732-8893/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.diagmicrobio.2013.04.005

Table 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			

the published protocols (Rajpara et al., 2009). 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 (Hi Media, Mumbai, India) broth at 37 °C until the OD_{600} reached 1.0. DNA was extracted by boiling the samples for 10 min, centrifuging them at high speed followed by their storage at −20 °C until use. Multiplex PCRs were carried out with these cell lysates after confirming the specificity of the primers in monoplex PCRs described above. Once these PCRs were standardized with reference strains, test strains were evaluated following the same PCR protocol (Table 2). Each multiplex PCR reaction mixture consisted of 2.5 μL of 10× PCR amplification buffer, 2.0 μL of 25 mmol/L magnesium chloride, 2.0 μL of dNTP mix containing 2.5 mmol/L of each dNTP, 50.0 pmol of each primer, 1.5 U of recombinant *Taq* DNA polymerase (Fermentas International, Inc., Burlington, Ontario, Canada), 4.0 μL of the cell lysate as template DNA, and sterile water to make the final reaction volume to 25.0 μL. Each multiplex PCR involved an initial denaturation at 94 °C for 5 min followed by 30 amplification cycles, each consisting of an initial denaturation at 94 °C for 0.5 min, annealing at 62 °C for 0.75 min and extension at 72 °C for 1 min. The final polymerization was carried out at 72 °C for 10 min.

The multiplex PCR assay carried out with the reference strains (n = 13) yielded fragments of the expected size from *toxR* (217 bp), *hlyA* (427 bp), and *hutA* (330 bp) as shown in Fig. 1, lanes 1, 4, and 8, respectively. Subsequently, the multiplex PCR assay was evaluated with a total of 214 *Vibrio* and non-*Vibrio* test strains (Table 2) that were identified earlier with standard biochemical techniques (Colwell et al., 1981; Huq et al., 1984). These included 120 clinical *V. cholerae* strains, 8 environmental *V. cholerae* non-O1/non-O139 strains, 15 clinical *V. fluvialis* strains, 57 clinical *V. parahaemolyticus* strains, and 14 clinical *Shigella* strains. The reference strains *Aeromonas caviae* (MTCC 6832), *Aeromonas culicicula* (MTCC 3249), *Aeromonas hydrophila* sub spp. *hydrophila* (MTCC 1739 and MTCC 646), *Aeromonas liquefaciens* (MTCC 2654), *Aeromonas salmonicida* (MTCC 1522), and *V. vulnificus* (MTCC 1145 and MTCC 1146) were included for the evaluation of PCR as *Aeromonas* spp. and *V. vulnificus* share close similarity with *V. fluvialis.* All the *V. cholerae* test strains showed the expected band of 427 bp, and *V. fluvialis* and *V. parahaemolyticus* showed bands of 217 bp and 330 bp, respectively, as expected. PCR amplicons for some of the test strains have been shown in Fig. 1. All the other strains of *Shigella*, *Aeromonas*, *V. vulnificus*, and *Vibrio alginolyticus* (MTCC 4439) showed no amplification which proved the specificity of this multiplex PCR. The multiplex was also used to detect the presence of *V. cholerae* in 2 cases of mixed infections. One was a mixed infection of *V. cholerae* and *V. fluvialis* in a clinical sample where the PCR showed 2 bands corresponding to both the species (Fig. 1, lane 5). In the second case, a mixed infection of *V. cholerae* with *Providencia* spp. specifically detected only *V. cholerae* and not the *Providencia* strain (data not shown), which further confirmed that this multiplex did not show cross-reactivity with either *Shigella* spp., *Aeromonas* spp., *Providencia* spp., *V. vulnificus*, or *V. alginolyticus*. The multiplex PCR yielded results 100% consistent with the conventional identification techniques (Table 2). The whole assay starting from a colony on the agar plate to the detection of PCR amplicons on agarose gel could be completed within 6 hours, whereas from the stool samples, the whole assay could be completed in less than 24 hours. Using this multiplex PCR, amplicons could be detected in cell lysates

Table 2
Analysis of specificity of the triplex PCR using a variety of strains from the family of *Vibrionaceae* and *Enterobacteriaceae*.

S. no	Species	Source	No. of strains	Consistency (%)[a]
	Test strains (n = 214)			
1	*V. cholerae* O1 ElTor	Clinical	120	100
2	*V. cholerae* non-O1/non-O139	Environmental	08	100
3	*V. parahaemolyticus*	Clinical	57	100
4	*V. fluvialis*	Clinical	15	100
5	*Shigella* spp.	Clinical	14	100
	Reference strains (n=13)			
6	*V. vulnificus*	MTCC	02	
7	*V. alginolyticus*	MTCC	01	
8	*V. cholerae* O1 ElTor N16961	NICED	01	
9	*V. cholerae* classical 569B	NICED	01	
10	*V. cholerae* O139 MO10	NICED	01	
11	*V. fluvialis* BD123	NICED	01	
12	*V. parahaemolyticus*	NICED	01	
13	*Aeromonas* spp.	MTCC	05	

MTCC = Microbial Type Culture Collection and Gene Bank (Institute of Microbial Technology, Chandigarh, India); NICED = National Institute of Cholera and Enteric Diseases (Kolkata, India).

[a] Consistency of results from triplex PCR assay with conventional identification techniques.

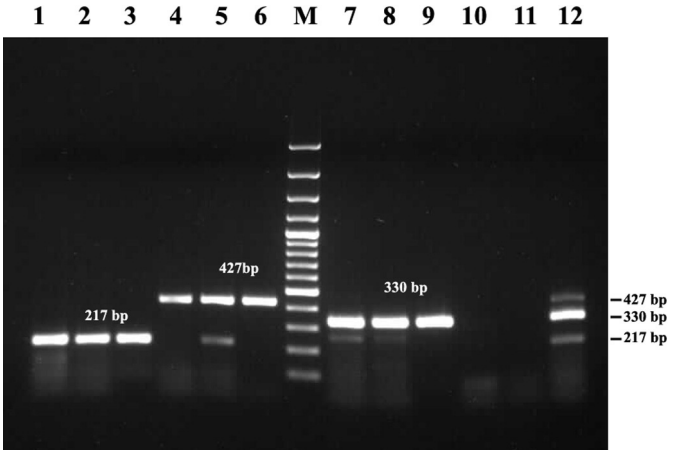


Fig. 1. Agarose gel electrophoresis (1.2%) of triplex PCR discriminates *V. fluvialis*, *V. cholerae*, and *V. parahaemolyticus* from each other by producing specific amplicons of 217 bp, 427 bp, and 330 bp, respectively. Lane 1, Triplex PCR product of *V. fluvialis* BD123; lane 2, triplex PCR product of *V. fluvialis* L12482; lane 3, monoplex PCR for *toxR* gene in *V. fluvialis* BD123; lane 4, triplex PCR product of *V. cholerae* N16961; lane 5, triplex PCR product of a clinical specimen with mixed infections of *V. cholerae* IDH01426 and *V. fluvialis*; lane 6, monoplex PCR for *hlyA* gene in *V. cholerae* 569B; lane M, 100-bp DNA ladder (Fermentas); lane 7, triplex PCR product of *V. parahaemolyticus* IDH02127; lane 8, triplex PCR product of *V. parahaemolyticus* IDH02033; lane 9, monoplex PCR for *hutA* gene in *V. parahaemolyticus* IDH02033; lane 10, triplex PCR product of *Shigella flexneri* type 2a NT4966; lane 11, triplex PCR product of *A. culicicula* MTCC 3249; lane 12, triplex PCR product of the cell lysates from the mixed cultures of *V. fluvialis*, *V. cholerae* and *V. parahaemolyticus*.

diluted up to 10000× (10 CFU), which further underlined the applicability of this multiplex as an assay with high detection power in addition to being specific, consistent, and rapid.

Most of the research carried out to develop PCR-based molecular identification techniques has either aimed at their utility in detection of pathogenic vibrios in food industry or in research and clinical diagnostics for the identification of vibrios and detection of their serotypes, biotypes, and regulatory genes. All these reports have used a single gene like *rpoA*, *rpoB*, *toxR*, *atpA*, or 16S-23S intergenic spacer region (Chakraborty et al., 2006; Dalmasso et al., 2009; Izumiya et al., 2011; Kim and Bang, 2008; Tarr et al., 2007), and some of them involved techniques like real-time PCR or sequencing of the amplified fragments (Dalmasso et al., 2009; Olsvik et al., 1993; Tarr et al., 2007; Tracz et al., 2007) that increases the cost as well as time for the final detection. A recent report has also described a highly sensitive and specific multiplex PCR for the detection of *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* using *toxR* gene for *V. cholerae* and *V. parahaemolyticus* and *vvhA* (*V. vulnificus* haemolysin A) gene for *V. vulnificus* (Neogi et al., 2010). The triplex reported in the present study also describes a reliable, sensitive, specific, rapid, and cost-effective method based on 3 different genes to unambiguously detect a different combination of vibrios, *V. cholerae*, *V. fluvialis*, and *V. parahaemolyticus* from a pool of pathogens. Such tests could be appropriate for research and epidemiology in the Indian settings as well as in global scenario considering the emergence and dissemination of pathogens at different geographical locations at different time points. To the best of our knowledge, this is the first report describing the identification of the aforementioned *Vibrio* spp. based on 3 different genes. The direct detection of these 3 species from stool samples and larger number of target genes and target organisms should be aimed to extend the practical utility of this assay and development of this assay for commercial utility. This assay should find wide applications in study of environmental and clinical samples in case of natural calamities, outbreaks, and industries related to food and aquaculture.

Acknowledgments

This work was partially supported by the grant (BT/PR/11634/INF/22/104/2008) from Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India. The authors thank The Puri Foundation for Education in India for the partial funding provided by them. Mr. K. Vinothkumar is the recipient of Junior Research Fellowship from the DBT grant mentioned above. The authors wish to thank Mr. Braj Mohan, Ms. Neha Rajpara, and Mr. Priyabrata Mohanty for their technical support.

References

Ahmed AM, Nakagawa T, Arakawa E, Ramamurthy T, Shinoda S, Shimamoto T. New aminoglycoside acetyl transferase gene, aac(3)-Id, in a class 1 integron from a multiresistant strain of *Vibrio fluvialis* isolated from an infant aged 6 months. J Antimicrob Chemother 2004;53:947–51.

Ahmed AM, Shinoda S, Shimamoto T. A variant type of *Vibrio cholerae* SXT element in a multidrug-resistant strain of *Vibrio fluvialis*. FEMS Microbiol Lett 2005;242:241–7.

Bauer A, Rorvik LM. A novel multiplex PCR for the identification of *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*. Lett Appl Microbiol 2007;45:371–5.

Bhattacharjee S, Bal B, Pal R, Niyogi SK, Sarkar K. Is *Vibrio fluvialis* emerging as a pathogen with epidemic potential in coastal region of eastern India following cyclone Aila? J Health Popul Nutr 2010;28:311–7.

Chakraborty R, Sinha S, Mukhopadhyay AK, Asakura M, Yamasaki S, Bhattacharya SK, et al. Species-specific identification of *Vibrio fluvialis* by PCR targeted to the conserved transcriptional activation and variable membrane tether regions of the *toxR* gene. J Med Microbiol 2006;55:805–8.

Chakraborty R, Chakraborty S, De K, Sinha S, Mukhopadhyay AK, Khanam J, et al. Cytotoxic and cell vacuolating activity of *Vibrio fluvialis* isolated from paediatric patients with diarrhoea. J Med Microbiol 2005;54:707–16.

Chowdhury G, Pazhani GP, Dutta D, Guin S, Dutta S, Ghosh S, et al. *Vibrio fluvialis* in patients with diarrhea, Kolkata, India. Emerg Infect Dis 2012;18:1868–71.

Chowdhury G, Pazhani GP, Nair GB, Ghosh A, Ramamurthy T. Transferable plasmid-mediated quinolone resistance in association with extended-spectrum β-lactamases and fluoroquinolone-acetylating aminoglycoside-6'-N-acetyltransferase in clinical isolates of *Vibrio fluvialis*. Int J Antimicrob Agents 2011;38:169–73.

Colwell RR, Seidler RJ, Kaper J, Joseph SW, Garges S, Lockman H, et al. Occurrence of *Vibrio cholerae* Serotype O1 in Maryland and Louisiana Estuaries. Appl Environ Microbiol 1981;41:555–8.

Dalmasso A, Civera T, Bottero MT. Multiplex primer-extension assay for identification of six pathogenic vibrios. Int J Food Microbiol 2009;129:21–5.

Faruque S, Albert M, Mekalanos J. Epidemiology, genetics and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol Rev 1998;62:1301–14.

Fields PI, Popovic T, Wachsmuth K, Olsvik O. Use of polymerase chain reaction for detection of toxigenic *Vibrio cholerae* O1 strains from the Latin American cholera epidemic. J Clin Microbiol 1992;30:2118–21.

Hoge CW, Watsky D, Peeler RN, Libonati JB, Israel E, Morris JG, et al. Epidemiology and spectrum of Vibrio infections in a Chesapeake Bay community. J Infect Dis 1989;160:985–93.

Huq A, West PA, Small EB, Huq MI, Colwell RR. Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* serovar 01 associated with live copepods in laboratory microcosms. Appl Environ Microbiol 1984;48:420–4.

Huq MI, Alam AKMJ, Brenner DJ, Morris GK. Isolation of *Vibrio*-like group EF-6 from Patients with diarrhea. J Clin Microbiol 1980;11:621–4.

Igbinosa EO, Okoh AI. *Vibrio fluvialis*: an unusual enteric pathogen of increasing public health concern. Int J Environ Res Public Health 2010;7:3628–43.

Izumiya H, Matsumoto K, Yahiro S, Lee J, Morita M, Yamamoto S, et al. Multiplex PCR assay for identification of three major pathogenic *Vibrio* spp., *Vibrio cholera*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Mol Cell Probes 2011;25:174–6.

Kanungo S, Sur D, Ali M, You YA, Pal D, Manna B, et al. Clinical, epidemiological, and spatial characteristics of *Vibrio parahaemolyticus* diarrhea and cholera in the urban slums of Kolkata India. BMC Public Health 2012;12:830.

Khuntia HK, Pal BB, Chhotray GP. Quadruplex PCR for simultaneous detection of serotype, biotype, toxigenic potential, and central regulating factor of *Vibrio cholerae*. J Clin Microbiol 2008;46:2399–401.

Kim MN, Bang HJ. Detection of marine pathogenic bacterial *Vibrio* species by multiplex polymerase chain reaction (PCR). J Environ Biol 2008;29:543–6.

Lesmana M, Subekti DS, Tjaniadi P, Simanjuntak CH, Punjabi NH, Campbell JR, et al. Spectrum of vibrio species associated with acute diarrhea in North Jakarta, Indonesia. Diagn Microbiol Infect Dis 2002;43:91–7.

Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y, Sack DA. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clin Microbiol Rev 2007;20:39–48.

Neogi SB, Chowdhury N, Asakura M, Hinenoya A, Haldar S, Saidi SM, et al. A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Lett Appl Microbiol 2010;51:293–300.

Okuda J, Ishibashi M, Hayakawa E, Nishino T, Takeda Y, Mukhopadhyay AK, et al. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. J Clin Microbiol 1997;35:3150–5.

Olsvik O, Wahlberg J, Petterson B, Uhlen M, Popovic T, Wachsmuth IK, et al. Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. J Clin Microbiol 1993;31:22–5.

Rajpara N, Patel A, Tiwari N, Bahuguna J, Antony A, Choudhury I, et al. Mechanism of drug resistance in a clinical isolate of *Vibrio fluvialis*: involvement of multiple plasmids and integrons. Int J Antimicrob Agents 2009;34:220–5.

Sakazaki R. Bacteriology of *Vibrio* and related organisms. In: Barua D, Greenough WB, editors. Cholera. 3rd ed. New York: Plenum Medical Book Company; 1992. p. 37–55.

Sarkar BL, Kanungo S, Nair GB. How endemic is cholera in India? Indian J Med Res 2012;135:246–8.

Sen B, Dutta B, Chatterjee S, Bhattacharya MK, Nandy RK, Mukhopadhyay AK, et al. The first outbreak of acute diarrhea due to a pandemic strain of *Vibrio parahaemolyticus* O3:K6 in Kolkata, India. Int J Infect Dis 2007;11:185–7.

Singh R, Rajpara N, Tak J, Patel A, Mohanty P, Vinothkumar K, et al. Clinical isolates of *Vibrio fluvialis* from Kolkata, India, obtained during 2006: plasmids, the *qnr* gene and a mutation in gyrase A as mechanisms of multidrug resistance. J Med Microbiol 2012;61:369–74.

Srinivasan VB, Virk RK, Kaundal A, Chakraborty R, Datta B, Ramamurthy T, et al. Mechanism of drug resistance in clonally related clinical isolates of *Vibrio fluvialis* isolated in Kolkata, India. Antimicrob Agents Chemother 2006;50:2428–32.

Tarr CL, Patel JS, Puhr ND, Sowers EG, Bopp CA, Strockbine NA. Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. J Clin Microbiol 2007;45:134–40.

Thekdi R, Lakhani AG, Vachha SM, Chandrakapure MR. *Vibrio fluvialis* (group F vibrio) in Maharashtra. Indian J Med Res 1982;76:80–5.

Thompson FL, Iida T, Swings J. Biodiversity of vibrios. Microbiol Mol Biol Rev 2004;68:403–31.

Tracz DM, Backhouse PG, Olson AB, McCrea JK, Walsh JA, Ng LK, et al. Rapid detection of *Vibrio* species using liquid microsphere arrays and real-time PCR targeting the *ftsZ* locus. J Med Microbiol 2007;56:56–65.

Yu S, Chen W, Wang D, He X, Zhu X, Shi X. Species-specific PCR detection of the food-borne pathogen *Vibrio parahaemolyticus* using the *irgB* gene identified by comparative genomic analysis. FEMS Microbiol Lett 2010;307:65–71.

Journal of Medical Microbiology (2012), **61**, 369–374 DOI 10.1099/jmm.0.037226-0

Clinical isolates of *Vibrio fluvialis* from Kolkata, India, obtained during 2006: plasmids, the *qnr* gene and a mutation in gyrase A as mechanisms of multidrug resistance

Rochika Singh,[1] Neha Rajpara,[1] Jyoti Tak,[1] Arati Patel,[1] Priyabrata Mohanty,[1] Kittappa Vinothkumar,[1] Goutam Chowdhury,[2] Thandavarayan Ramamurthy,[2] Amit Ghosh[2] and Ashima Kushwaha Bhardwaj[1]

Correspondence
Ashima Kushwaha Bhardwaj
ashima.bhardwaj@gmail.com

[1]Department of Human Health and Diseases, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar, Gujarat, India

[2]National Institute of Cholera and Enteric Diseases, P-33, CIT Road, Beliaghata, Kolkata, India

Received 11 August 2011
Accepted 27 October 2011

Resistance profiles and their correlation with genetic factors were investigated in 12 isolates of *Vibrio fluvialis* obtained from hospitalized patients in Kolkata, India, in 2006. All the strains displayed drug resistance with varying antibiograms. However, resistance to ampicillin and neomycin was common to all of them. Three isolates harboured plasmids carrying drug-resistance genes that could be transferred to recipient strains by conjugation and transformation. PCR results indicated the absence of class 1 integrons and SXT elements in these isolates. A mutation in gyrase A (serine 83→isoleucine) and the presence of the *qnrB1* gene were found to contribute towards quinolone resistance. In the 12 isolates, the *qnrB1* gene was associated only with two plasmid-bearing isolates, L10734 and L9978, which displayed resistance to quinolones. The gene was transferable during transformation and conjugation, indicating that it was plasmid-borne. Taken together, these data indicate that plasmids, the *qnrB1* gene and a mutation in gyrase A were responsible for the observed drug resistance in these strains. To the best of our knowledge, this is the first report of the presence of the *qnrB1* allele in *V. fluvialis* isolates from India.

INTRODUCTION

Vibrio fluvialis is implicated in sporadic cases of cholera-like diarrhoea and is often associated with paediatric diarrhoea (Huq *et al.*, 1980; Bellet *et al.*, 1989). Its presence has also been reported recently in cases of AIDS, peritonitis, ocular infection, dental plaque, cellulitis and cerebritis (Hodge *et al.*, 1995; Penland *et al.*, 2000; Tamilselvan *et al.*, 2004; Huang & Hsu, 2005; Ratnaraja *et al.*, 2005). The molecular epidemiology of infection and the mechanisms of pathogenicity are not as well understood as for *Vibrio cholerae*, and it has been largely under-recognized as an enteric pathogen (Kolb *et al.*, 1997). There are a few reports pertaining to multiple drug resistance in *V. fluvialis* strains and the putative mechanisms of pathogenicity, indicating that this pathogen needs to be studied in greater detail in order to understand its epidemic-causing potential (Ahmed *et al.*, 2004, 2005; Chakraborty *et al.*, 2005; Srinivasan *et al.*, 2006; Rajpara *et al.*, 2009; Chowdhury *et al.*, 2011).

In a recent report from our laboratory (Rajpara *et al.*, 2009), it was shown that drug-resistance genes harboured by plasmids and integrons were responsible for the multiple drug-resistance phenotype exhibited by a *V. fluvialis* isolate from 2002 in Kolkata, India. To see whether this was true for other strains isolated subsequently, we analysed the antibiotic susceptibility patterns of *V. fluvialis* isolates collected from the same place in 2006. The results described in this paper show that plasmids, the presence of the *qnrB1* gene and a mutation in gyrase A all contribute to the drug-resistance phenotype displayed by these strains.

Abbreviation: CLSI, Clinical and Laboratory Standards Institute.

The GenBank/EMBL/DDBJ accession numbers for the new *gyrA*, *gyrB*, *parC* and *parE* gene sequences are GQ466187–GQ466195 and GU326332–GU326334.

METHODS

Bacterial strains. Twelve *V. fluvialis* isolates were obtained from patients with acute cholera-like diarrhoea admitted to the Infectious Diseases Hospital, Kolkata, India, in 2006. *V. fluvialis* isolate BD146 obtained from the same place in 2002 was used as a control in some

experiments. *Escherichia coli* JM109 was used for electroporation experiments. *E. coli* DH5α and *V. cholerae* O1 El Tor N16961 were used as recipients in conjugation experiments.

Antimicrobial susceptibility testing and MIC determination. The *V. fluvialis* isolates were tested for their susceptibility to ampicillin (10 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) and kanamycin (30 μg) by the disc diffusion method using commercial discs (HiMedia) in accordance with the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2010). When no interpretive criteria for *V. cholerae* were available based on CLSI guidelines, breakpoints for *Enterobacteriaceae* were applied. MIC determination was carried out using a HiComb MIC test (HiMedia) following the manufacturer's instructions. Interpretation of the results used the criteria recommended by the CLSI (2010). *E. coli* ATCC 25922 was used for quality control. All experiments were performed in triplicate.

Bacterial genomic and plasmid DNA extraction. Genomic and plasmid DNA extraction was performed as described previously (Thungapathra *et al.*, 2002). For large-scale purification of DNA, a Plasmid Maxi kit (Qiagen) was used as described by the manufacturer.

Bacterial transformation. Transformation of *E. coli* JM109 was carried out by electroporation (Gene Pulser XCell; Bio-Rad Laboratories) with 150–500 ng purified DNA from *V. fluvialis*. Transformants were selected on Luria–Bertani (LB) plates containing ampicillin (25 μg ml^{-1}).

Bacterial conjugation. Three plasmid-bearing strains (L13828, L10734 and L9978) were tested for their ability to transfer resistance traits to a recipient strain in conjugation experiments. These experiments were carried out according to a published protocol (Ceccarelli *et al.*, 2006). For strain L13828, which was sensitive to nalidixic acid as well as to streptomycin, both *E. coli* DH5α and *V. cholerae* O1 El Tor were used as recipients. For strain L9978 (sulfamethoxazole-resistant), *E. coli* DH5α (nalidixic acid-resistant and sulfamethoxazole-sensitive) was used as recipient, and for strain L10734 (nalidixic acid-resistant and streptomycin-sensitive), *V. cholerae* O1 El Tor N16961 (streptomycin-resistant) was used as recipient. Briefly, the recipient and donor strains were mixed in a 1 : 1 ratio on a sterile 0.45 μm nylon membrane (Nytran N; Whatman) and incubated overnight for mating on LB agar at 37 °C. The transconjugants were selected on LB agar plates containing the appropriate antibiotics. For conjugation between L13828 and *V. cholerae* O1 El Tor, ampicillin (25 μg ml^{-1}) and streptomycin (20 μg ml^{-1}) were used, and for conjugation between L10734 and *V. cholerae* O1 El Tor, nalidixic acid (30 μg ml^{-1}) and streptomycin (20 μg ml^{-1}) were used for the selection of transconjugants. For conjugation between L9978 and *E. coli* DH5α, sulfamethoxazole (160 μg ml^{-1}) and nalidixic acid (30 μg ml^{-1}) were used.

PFGE. PFGE was carried out as described previously (Parsons *et al.*, 2007). For gel electrophoresis, a 1% agarose gel (Pulsed Field Certified Agarose; Bio-Rad) was prepared in 0.5× TBE and run in a CHEF Mapper (Bio-Rad Laboratories) using the autoalgorithm mode (molecular mass range 6–600K and a run time of 12 h). The gel was stained with 0.05 mg ethidium bromide ml^{-1} for 30 min and destained with sterile water for 1 h.

PCR. Genomic DNA (200 ng) or plasmid DNA (10–50 ng) was used as template in PCRs with the primers described in Table 1. Each PCR involved an initial denaturation at 95 °C for 4 min, followed by 25–30 amplification cycles, each consisting of an initial denaturation at 95 °C for 0.5 min followed by annealing and extension steps. The final polymerization was carried out at 72 °C for 10 min. PCRs for analysis of SXT elements and class 1 integrons were carried out according to published protocols (Rajpara *et al.*, 2009). For the amplification of quinolone resistance-determining regions of four topoisomerase genes (*gyrA*, *gyrB*, *parC* and *parE*), primer pairs and conditions were as described previously (Srinivasan *et al.*, 2006) or as in Table 1, except that different annealing conditions were used (*gyrA*: 44 °C for 45 s; *gyrB*: 50 °C for 1 min; *parC*: 52 °C for 45 s; *parE*: 52 °C for 1 min). Extension was carried out at 72 °C for 1 min. For amplification of *qnrB1*, annealing was carried out at 62 °C for 1 min. PCRs were performed using a PTC-225 DNA Engine Tetrad Cycler (MJ Research). Recombinant *Taq* or *Pfu* DNA polymerase (Fermentas) was used along with appropriate buffers.

DNA sequencing and analysis. DNA segments amplified from the topoisomerase genes or *qnrB1* were sequenced. The assembled sequences were analysed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Alignment of the topoisomerase sequences was carried out using Expasy (http://www.expasy.ch).

Efflux pumps. The accumulation of ethidium bromide, nalidixic acid and ciprofloxacin was determined following the protocol of Long *et al.* (2008). Supernatants collected after the final cell lysis using glycine/HCl were transferred to new tubes and measured at excitation and emission wavelengths of 500 and 580 nm, respectively, for assays with ethidium bromide (used at a concentration of 20 μg ml^{-1}), using an RF 5701 PC series spectrofluorophotometer (Shimadzu). For nalidixic acid and ciprofloxacin accumulation, the drugs were added at a concentration of 100 μM. The excitation and emission wavelengths for nalidixic acid were 330 and 417 nm, and for ciprofloxacin were 275 and 440 nm, respectively.

RESULTS AND DISCUSSION

Antibiotic resistance profile of *V. fluvialis* clinical isolates

Antibiograms of the 12 clinical isolates showed that all displayed multiple drug resistance (Table 2). Resistance to

Table 1. Primers used in the study

Primer	Sequence (5′→3′)	GenBank accession no.	Primer position
GyrA-F	TACACCGACGCGTACTGT	AE003852	131–148
GyrA-R	TCGATCGAGCCAAAGTTA	AE003852	321–338
ParE-F	CAGCAAGAAAGTGGTGCGTA	AE003852	724–744
ParE-R	AGACTTTGCCGTAACGCAGT	AE003852	426–445
QNR-F	ATGGATCCATGGATAAAACAGACCAG	EU574928	5380–5397
QNR-R	ATCTCGAGTTAGTCAGGAACTACTAT	EU574928	4742–4759

Table 2. Antibiograms of *V. fluvialis* clinical isolates from 2006

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

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

ampicillin and neomycin was common to all isolates. All the isolates except L12482 showed intermediate resistance to kanamycin. Three strains, L12387, L9978 and L15318, displayed resistance to the majority (77–85 %) of antibiotics tested. Almost all the strains were sensitive to tetracycline (11/12) and gentamicin (10/12).

Relatedness among the 12 isolates

As all the isolates were obtained from the same location during the same year, it was of interest to examine their relatedness. To this end, total genomic DNA from these strains was subjected to PFGE analysis (Fig. 1a, b). PFGE of the *Not*I-digested DNA revealed that isolates L13230, L13211 and L12482 (Fig. 1b, lanes 2–4) had a similar band pattern and therefore could be derived from the same clone. The remaining nine isolates appeared to have different pulsotypes.

Transfer of resistance traits by conjugation and electroporation

Repeated analysis of the isolates for plasmid carriage using agarose gel and PFGE revealed that only three of them,

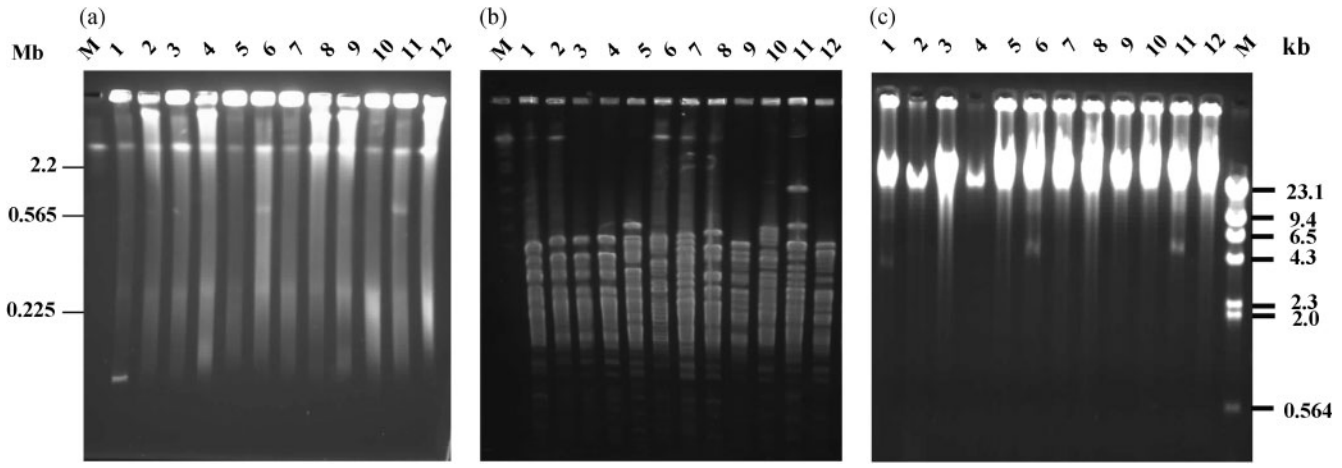


Fig. 1. Analysis of total genomic DNA from the 12 clinical isolates of *V. fluvialis*. (a, b) PFGE (1 %) analysis of undigested (a) and *Not*I-digested (b) genomic DNA. Lane M, CHEF DNA size standards (Bio-Rad) from *Saccharomyces cerevisiae*. The positions of the marker bands (Mb) are indicated on the left. (c) Agarose gel (1 %) analysis of genomic DNA preparations. Lanes 1–12, isolates L13828, L13230, L13211, L12482, L98411, L10734, L9077, L10332, L11264, L12387, L9978 and L15318, respectively. A *Hin*dIII-digested λ DNA marker (Sigma) was used, with fragment sizes (kb) indicated on the right. Distinct plasmid bands can be seen in lanes 1 (L13828), 6 (L10734) and 11 (L9978) in (a) and (c).

namely L13828, L10734 and L9978, harboured plasmids (Fig. 1a, c). To see whether these plasmids carried any drug-resistance genes, these three isolates were mated with *E. coli* DH5α or *V. cholerae* O1 El Tor N16961and the transconjugants were examined for drug resistance. The results showed that the majority of the drug-resistance genes in isolates L9978 and L10734 were indeed plasmid-borne (Table 3). The same conclusion, however, could not be drawn for L13828, as it failed to conjugate in repeated attempts. However, when the plasmid preparation from this isolate was electroporated in JM109 cells, it was found to be able to confer resistance to ampicillin and tetracycline on the recipient. At first sight, this was a surprising result, as strain L13828 was not resistant to tetracycline. A review of the literature showed that such a phenomenon has indeed been observed in the past (Park *et al.*, 1987; Zhao *et al.*, 2001). This may be due to the fact that, for some reason, the gene for tetracycline resistance is not expressed in the original host but is able to be expressed in the new host. When similar electroporation experiments were carried out with plasmid preparations from the two other isolates, L10734 and L9978, the recipient *E. coli* acquired resistance to ampicillin and tetracycline as well as other drugs, the genes for which could also be transferred through conjugation. Thus, it appeared from these results that a plasmid bearing resistance genes for ampicillin and tetracycline was common to all three isolates, and that L10734 and L9978 also contained additional plasmids harbouring resistance genes to the other drugs listed in Table 3.

Resistance to quinolones: mutation in a topoisomerase gene, involvement of efflux pumps and the presence of *qnr*

Of the 12 clinical isolates, seven (58 %) showed resistance to one or more quinolones. Of these, three were fully resistant to nalidixic acid (L10734, L12387 and L15318) and one (L9077) showed intermediate resistance to this drug. Whilst intermediate resistance to both ciprofloxacin and norfloxacin was observed in two isolates, namely L12387 and L15318, four others (L13230, L98411, L10734 and L9978) displayed intermediate resistance only to ciprofloxacin (Table 2).

To study the factors governing resistance to quinolones, two representative isolates, L13828 (quinolone-sensitive) and L15318 (quinolone-resistant), were chosen. The MIC values of nalidixic acid, ciprofloxacin and norfloxacin were 0.1, 0.008 and 0.05 μg ml^{-1}, respectively for L13828 and >240, 0.5 and 2.0 μg ml^{-1}, respectively for L15318. The quinolone resistance-determining regions of four topoisomerase genes (*gyrA*, *gyrB*, *parC* and *parE*) were amplified from these two isolates and for *V. cholerae* N16961 and sequenced. The results revealed that serine 83 in the *gyrA* gene of the sensitive strain L13828 was replaced by isoleucine in the resistant strain L15318. No other mutations were detected in the other three topoisomerase genes.

As efflux mechanisms are also known to contribute to quinolone resistance, accumulation studies were carried out using ethidium bromide, nalidixic acid and ciprofloxacin. It was found that accumulation of these compounds increased after the addition of reserpine, a decoupler of membrane proton gradients (Fig. 2). However, when glucose was added after 15 min to reverse the effect of reserpine, a decrease in accumulation of all compounds was observed. Furthermore, it was seen that the levels of accumulation of the compounds tested were almost similar in both the sensitive and the resistant isolates (Fig. 2), suggesting that, although the efflux mechanisms were operative for quinolones in both isolates, they did not contribute to the quinolone resistance of L15318 and it was the mutation in the *gyrA* gene that determined quinolone resistance.

In addition to these two factors, it was of interest to look for the presence of the plasmid-borne quinolone resistance (*qnr*) gene in these 12 isolates. In a recent study from our laboratory (Rajpara *et al.*, 2009), plasmid pBD146 (GenBank accession no. EU574928) from *V. fluvialis* isolate BD146 was found to harbour a *qnr* gene (GenBank accession no. JN408080). Primers were designed based on this gene and PCR experiments with the 12 isolates revealed the presence of the *qnrB1* gene only in the two plasmid-bearing isolates L10734 and L9978 (quinolone-resistant), whereas in the third plasmid-bearing isolate, L13828 (quinolone-sensitive), this gene was not detected. This result indicated that the Qnr pentapeptide repeat protein also played a role in governing the quinolone resistance of these two isolates. In addition, it was observed that this resistance was plasmid mediated, as both the transformants and transconjugants derived from L10734 and L9978 confirmed the presence of the *qnrB1* allele

Table 3. Antibiotic susceptibility patterns of *V. fluvialis* parental isolates and their transformants and transconjugants

No distinction was made between full and intermediate resistance. In transformants, resistance to nalidixic acid was derived from host JM109 cells. In the L10734 and L9978 transconjugants, streptomycin and nalidixic acid resistance, respectively, were host-derived. See Table 2 for abbreviations.

Strain	L13828	L10734	L9978
Parent	AMP, NEO, KAN, GEN	AMP, NEO, KAN, CO-TRI, TRI, CIP, NAL	AMP, NEO, KAN, CO-TRI, TRI, CIP, SUL, STR, CHL
Transconjugant	Non-conjugable strain	NEO, KAN, CO-TRI, TRI, NAL, STR	AMP, NEO, KAN, CO-TRI, TRI, CIP, SUL, STR, CHL, NAL
Transformant	AMP, TET, NAL	AMP, NEO, TRI, TET, NAL	AMP, NEO, TRI, TET, NAL

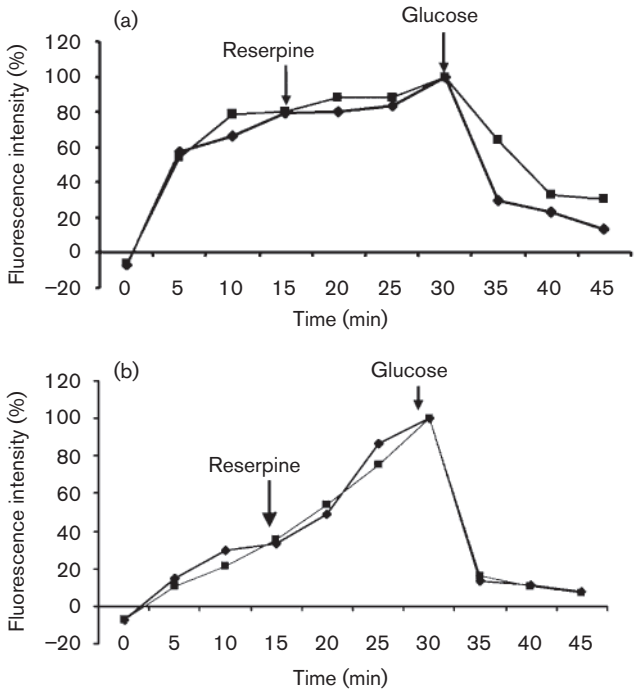


Fig. 2. Involvement of efflux pumps in the transport of quinolones. Accumulation of 100 μM nalidixic acid (a) and 100 μM ciprofloxacin (b) in a quinolone-sensitive (L13828; ◆) and a quinolone-resistant (L15318; ■) isolate. Accumulation was studied in the presence of 20 μg reserpine ml^{-1}, an efflux pump inhibitor. Experiments were performed in triplicate and results are expressed as the percentage fluorescence compared with the maximum fluorescence intensity obtained during the experiment. A similar trend was observed for ethidium bromide (data not shown).

by PCR. The *aac(6′)-Ib-cr* gene, which is responsible for promiscuous drug resistance towards aminoglycosides as well as the quinolone ciprofloxacin, was not detected in any of the 12 isolates when tested in PCR using the primers described by Park *et al.* (2006) (data not shown). These results again corroborated the earlier findings by several groups that resistance to quinolones can be chromosome-borne (mutations in topoisomerases and efflux pumps) or plasmid-mediated (*qnr*) (Baranwal *et al.*, 2002; Tran & Jacoby, 2002; Chowdhury *et al.*, 2011). The Qnr family of pentapeptide repeat proteins has been shown to confer protection to DNA gyrases, resulting in quinolone resistance. From India, there is only a single report recently describing the emergence of *qnrA1* from Indian isolates of *V. fluvialis* in 2009 (Chowdhury *et al.*, 2011). In this study, the presence of *qnrB1* in clinical isolates of *V. fluvialis* from 2002 and 2006 indicates that *qnr* has been circulating in Indian isolates from 2002 to 2009.

ACKNOWLEDGEMENTS

This work was partially supported by the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India (grants BT/PR10304/GBD/27/95/2008 and BT/PR/11634/INF/22/104/2008). The authors thankfully acknowledge the partial funding received from Puri Foundation for Education in India. P. M. and K. V. are recipients of Junior Research Fellowships from the DBT grants mentioned above. The authors also thank Mr Praful Dargi for his technical support.

REFERENCES

Ahmed, A. M., Nakagawa, T., Arakawa, E., Ramamurthy, T., Shinoda, S. & Shimamoto, T. (2004). New aminoglycoside acetyltransferase gene, *aac(3)-Id*, in a class 1 integron from a multiresistant strain of *Vibrio fluvialis* isolated from an infant aged 6 months. *J Antimicrob Chemother* **53**, 947–951.

Ahmed, A. M., Shinoda, S. & Shimamoto, T. (2005). A variant type of *Vibrio cholerae* SXT element in a multidrug-resistant strain of *Vibrio fluvialis*. *FEMS Microbiol Lett* **242**, 241–247.

Baranwal, S., Dey, K., Ramamurthy, T., Nair, G. B. & Kundu, M. (2002). Role of active efflux in association with target gene mutations in fluoroquinolone resistance in clinical isolates of *Vibrio cholerae*. *Antimicrob Agents Chemother* **46**, 2676–2678.

Bellet, J., Klein, B., Altieri, M. & Ochsenschlager, D. (1989). *Vibrio fluvialis*, an unusual pediatric enteric pathogen. *Pediatr Emerg Care* **5**, 27–28.

Ceccarelli, D., Salvia, A. M., Sami, J., Cappuccinelli, P. & Colombo, M. M. (2006). New cluster of plasmid-located class 1 integrons in *Vibrio cholerae* O1 and a *dfrA15* cassette-containing integron in *Vibrio parahaemolyticus* isolated in Angola. *Antimicrob Agents Chemother* **50**, 2493–2499.

Chakraborty, R., Chakraborty, S., De, K., Sinha, S., Mukhopadhyay, A. K., Khanam, J., Ramamurthy, T., Takeda, Y., Bhattacharya, S. K. & Nair, G. B. (2005). Cytotoxic and cell vacuolating activity of *Vibrio fluvialis* isolated from paediatric patients with diarrhoea. *J Med Microbiol* **54**, 707–716.

Chowdhury, G., Pazhani, G. P., Nair, G. B., Ghosh, A. & Ramamurthy, T. (2011). Transferable plasmid-mediated quinolone resistance in association with extended-spectrum β-lactamases and fluoroquinolone-acetylating aminoglycoside-6′-*N*-acetyltransferase in clinical isolates of *Vibrio fluvialis*. *Int J Antimicrob Agents* **38**, 169–173.

CLSI (2010). *Performance Standards for Antimicrobial Susceptibility Testing*; 20th Informational Supplement. M100-S20. Wayne, PA: Clinical and Laboratory Standards Institute.

Hodge, T. W., Jr, Levy, C. S. & Smith, M. A. (1995). Diarrhea associated with *Vibrio fluvialis* infection in a patient with AIDS. *Clin Infect Dis* **21**, 237–238.

Huang, K.-C. & Hsu, R. W.-W. (2005). *Vibrio fluvialis* hemorrhagic cellulitis and cerebritis. *Clin Infect Dis* **40**, 75–77.

Huq, M. I., Alam, A. K. M. J., Brenner, D. J. & Morris, G. K. (1980). Isolation of *Vibrio*-like group, EF-6, from patients with diarrhea. *J Clin Microbiol* **11**, 621–624.

Kolb, E. A., Eppes, S. C. & Klein, J. D. (1997). *Vibrio fluvialis*: an underrecognized enteric pathogen in infants? *South Med J* **90**, 544–545.

Long, F., Rouquette-Loughlin, C., Shafer, W. M. & Yu, E. W. (2008). Functional cloning and characterization of the multidrug efflux pumps NorM from *Neisseria gonorrhoeae* and YdhE from *Escherichia coli*. *Antimicrob Agents Chemother* **52**, 3052–3060.

Park, B. H., Hendricks, M., Malamy, M. H., Tally, F. P. & Levy, S. B. (1987). Cryptic tetracycline resistance determinant (class F) from *Bacteroides fragilis* mediates resistance in *Escherichia coli* by actively reducing tetracycline accumulation. *Antimicrob Agents Chemother* **31**, 1739–1743.

Park, C. H., Robicsek, A., Jacoby, G. A., Sahm, D. & Hooper, D. C. (2006). Prevalence in the United States of *aac(6′)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob Agents Chemother* **50**, 3953–3955.

Parsons, M. B., Cooper, K. L., Kubota, K. A., Puhr, N., Simington, S., Calimlim, P. S., Schoonmaker-Bopp, D., Bopp, C., Swaminathan, B. & other authors (2007). PulseNet USA standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio parahaemolyticus*. *Foodborne Pathog Dis* **4**, 285–292.

Penland, R. L., Boniuk, M. & Wilhelmus, K. R. (2000). *Vibrio* ocular infections on the U.S. Gulf Coast. *Cornea* **19**, 26–29.

Rajpara, N., Patel, A., Tiwari, N., Bahuguna, J., Antony, A., Choudhury, I., Ghosh, A., Jain, R., Ghosh, A. & Bhardwaj, A. K. (2009). Mechanism of drug resistance in a clinical isolate of *Vibrio fluvialis*: involvement of multiple plasmids and integrons. *Int J Antimicrob Agents* **34**, 220–225.

Ratnaraja, N., Blackmore, T., Byrne, J. & Shi, S. (2005). *Vibrio fluvialis* peritonitis in a patient receiving continuous ambulatory peritoneal dialysis. *J Clin Microbiol* **43**, 514–515.

Srinivasan, V. B., Virk, R. K., Kaundal, A., Chakraborty, R., Datta, B., Ramamurthy, T., Mukhopadhyay, A. K. & Ghosh, A. (2006). Mechanism of drug resistance in clonally related clinical isolates of *Vibrio fluvialis* isolated in Kolkata, India. *Antimicrob Agents Chemother* **50**, 2428–2432.

Tamilselvan, S., Srikanth, P., Balaji, T. M., Vijayalakshmi, T. & Suresh, R. (2004). Isolation of rare species *Vibrio fluvialis* in dental plaque. *Indian J Pathol Microbiol* **47**, 448–449.

Thungapathra, M., Amita, Sinha, K. K., Chaudhuri, S. R., Garg, P., Ramamurthy, T., Nair, G. B. & Ghosh, A. (2002). Occurrence of antibiotic resistance gene cassettes *aac(6′)-Ib*, *dfrA5*, *dfrA12*, and *ereA2* in class I integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrob Agents Chemother* **46**, 2948–2955.

Tran, J. H. & Jacoby, G. A. (2002). Mechanism of plasmid-mediated quinolone resistance. *Proc Natl Acad Sci U S A* **99**, 5638–5642.

Zhao, S., White, D. G., Ge, B., Ayers, S., Friedman, S., English, L., Wagner, D., Gaines, S. & Meng, J. (2001). Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. *Appl Environ Microbiol* **67**, 1558–1564.

PubMed

Abstract

Full text links

J Med Microbiol. 2012 Mar;61(Pt 3):369-74. doi: 10.1099/jmm.0.037226-0. Epub 2011 Oct 27.

Clinical isolates of Vibrio fluvialis from Kolkata, India, obtained during 2006: plasmids, the qnr gene and a mutation in gyrase A as mechanisms of multidrug resistance.

Singh R[1], Rajpara N, Tak J, Patel A, Mohanty P, Vinothkumar K, Chowdhury G, Ramamurthy T, Ghosh A, Bhardwaj AK.

Author information

Erratum in
J Med Microbiol. 2013 Mar;62(Pt 3):498.

Abstract

Resistance profiles and their correlation with genetic factors were investigated in 12 isolates of Vibrio fluvialis obtained from hospitalized patients in Kolkata, India, in 2006. All the strains displayed drug resistance with varying antibiograms. However, resistance to ampicillin and neomycin was common to all of them. Three isolates harboured plasmids carrying drug-resistance genes that could be transferred to recipient strains by conjugation and transformation. PCR results indicated the absence of class 1 integrons and SXT elements in these isolates. A mutation in gyrase A (serine 83→isoleucine) and the presence of the qnrVC-like [corrected] gene were found to contribute towards quinolone resistance. In the 12 isolates, the qnrVC-like [corrected] gene was associated only with two plasmid-bearing isolates, L10734 and L9978, which displayed resistance to quinolones. The gene was transferable during transformation and conjugation, indicating that it was plasmid-borne. Taken together, these data indicate that plasmids, the qnrVC-like [corrected] gene and a mutation in gyrase A were responsible for the observed drug resistance in these strains. To the best of our knowledge, this is the first report of the presence of the qnrVC-like [corrected] allele in V. fluvialis isolates from India.

PMID: 22034163 [PubMed - indexed for MEDLINE]

Publication Types, MeSH Terms, Substances, Secondary Source ID

LinkOut - more resources

Synergistic Effect of Various Virulence Factors Leading to High Toxicity of Environmental *V. cholerae* Non-O1/ Non-O139 Isolates Lacking *ctx* Gene : Comparative Study with Clinical Strains

Neha Rajpara[1☯], Kittappa Vinothkumar[1☯], Priyabrata Mohanty[1], Arun Kumar Singh[2], Rajesh Singh[2], Ritam Sinha[3], Dhrubajyoti Nag[3], Hemanta Koley[3], Ashima Kushwaha Bhardwaj[1*]

1 Department of Human Health and Diseases, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Gandhinagar, Gujarat, India, **2** Department of Cell Biology, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Gandhinagar, Gujarat, India, **3** National Institute of Cholera and Enteric Diseases, Beliaghata, Kolkata, India

Abstract

Background: *Vibrio cholerae* non-O1/ non-O139 serogroups have been reported to cause sporadic diarrhoea in humans. Cholera toxins have been mostly implicated for hypersecretion of ions and water into the small intestine. Though most of the *V. cholerae* non-O1/ non-O139 strains lack these cholera toxins, several other innate virulence factors contribute towards their pathogenicity. The environmental isolates may thus act as reservoirs for potential spreading of these virulence genes in the natural environment which may cause the emergence of epidemic-causing organisms.

Results: The environmental isolates of *vibrios* were obtained from water samples, zooplanktons and phytoplanktons, from a village pond in Gandhinagar, Gujarat, India. They were confirmed as *Vibrio cholerae* non-O1/ non-O139 using standard biochemical and serotyping tests. PCR experiments revealed that the isolates lacked *ctxA, ctxB, tcpA, zot* and *ace* genes whereas other pathogenicity genes like *toxR*, *rtxC*, *hlyA*, *hapA* and *prtV* were detected in these isolates. Compared with epidemic strain *V. cholerae* O1 El Tor N16961, culture supernatants from most of these isolates caused higher cytotoxicity to HT29 cells and higher hemolytic, hemagglutinin and protease activities. In rabbit ileal loop assays, the environmental isolates showed only 2-4 folds lesser fluid accumulation in comparison to N16961 and a *V. cholerae* clinical isolate IDH02365 of 2009. Pulsed Field Gel electrophoresis and Random amplification of Polymorphic DNA indicated that these isolates showed considerable diversity and did not share the same clonal lineage even though they were derived from the same water source. All the isolates showed resistance to one or more antibiotics.

Conclusion: Though these environmental isolates lacked the cholera toxins, they seem to have adopted other survival strategies by optimally utilising a diverse array of several other toxins. The current findings indicate the possibility that these isolates could cause some gastroenteric inflammation when ingested and may serve as progenitors for overt disease-causing organisms.

Citation: Rajpara N, Vinothkumar K, Mohanty P, Singh AK, Singh R, et al. (2013) Synergistic Effect of Various Virulence Factors Leading to High Toxicity of Environmental *V. cholerae* Non-O1/ Non-O139 Isolates Lacking *ctx* Gene : Comparative Study with Clinical Strains. PLoS ONE 8(9): e76200. doi: 10.1371/journal.pone.0076200

Editor: Michael Hensel, University of Osnabrueck, Germany

Received February 1, 2013; **Accepted** August 21, 2013; **Published** September 23, 2013

Funding: This work was partially supported by the grant BT/PR/11634/INF/22/104/2008 from the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India. The authors thankfully acknowledge the partial funding received from The Puri Foundation for Education in India. N.R. and P.M. are the recipients of Senior Research Fellowships from Indian Council of Medical Research, New Delhi, India with grant numbers AMR/49/11-ECDI and F/815/2011-ECD-II respectively. K.V. is recipient of Junior Research Fellowship from Gujarat State Biotechnology Mission GSBTM/MD/PROJECTS/SSA/3386/2012-13. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

* E-mail: ashima.bhardwaj@gmail.com

☯ These authors contributed equally to this work.

Send Orders of Reprints at reprints@benthamscience.net

Bacterial Quorum Sensing Inhibitors: Attractive Alternatives for Control of Infectious Pathogens Showing Multiple Drug Resistance

Ashima K. Bhardwaj*, Kittappa Vinothkumar and Neha Rajpara

Department of Human Health and Diseases, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar 382 007, Gujarat, India

Received: December 13, 2012; Revised: January 31, 2013; Accepted: January 31, 2013

Abstract: Quorum sensing (QS) is a bacterial communication process that depends on the bacterial population density. It involves small diffusible signaling molecules which activate the expression of myriad genes that control diverse array of functions like bioluminescence, virulence, biofilm formation, sporulation, to name a few. Since QS is responsible for virulence in the clinically relevant bacteria, inhibition of QS appears to be a promising strategy to control these pathogenic bacteria. With indiscriminate use of antibiotics, there has been an alarming increase in the number of antibiotic resistant pathogens. Antibiotics are no longer the magic bullets they were once thought to be and therefore there is a need for development of new antibiotics and/or other novel strategies to combat the infections caused by multidrug resistant organisms. Quorum sensing inhibition or quorum quenching has been pursued as one of such novel strategies. While antibiotics kill or slow down the growth of bacteria, quorum sensing inhibitors (QSIs) or quorum quenchers (QQs) attenuate bacterial virulence. A large body of work on QS has been carried out in deadly pathogens like *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio fischeri*, *V. harveyi*, *Escherichia coli* and *V. cholera*e etc to unravel the mechanisms of QS as well as identify and study QSIs. This review describes various aspects of QS, QSI, different model systems to study these phenomena and recent patents on various QSIs. It suggests QSIs as attractive alternatives for controlling human, animal and plant pathogens and their utility in agriculture and other industries.

Keywords: Biofilms, multidrug resistance, patents, *Pseudomonas aeruginosa*, quorum sensing, quorum sensing inhibitors, *Staphylococcus aureus*, *Vibrio cholerae*.

INTRODUCTION

There has been an increase in the reports of treatment complications and failures due to multiple drug resistance in all the known human, animal and plant pathogens. In this scenario, there is an urgent need for the alternatives to replace antibiotics for combating bacterial infections or adjunct therapies to be used in combination with antibiotics so that lower doses of the conventional antibiotics are required for treatment [1, 2]. Use of antibiotics puts selection pressures on bacteria by interfering with their vital housekeeping functions of protein synthesis, RNA synthesis and DNA synthesis. Inhibitors of quorum sensing (QS) process, also called quorum quenchers (QQs) or quorum sensing inhibitors (QSIs), provide attractive alternatives for the antimicrobials. QSIs do not threaten bacteria with life-or-death situations and therefore are less likely to yield resistant phenotype in bacteria. As QS is not found in humans and is vital for the expression of bacterial virulence, QS is a highly specific antibacterial target. The present review is an attempt to summarise the research pertaining to various aspects of QS, QSI and the tremendous patent activity in these fields.

*Address correspondence to this author at the Department of Human Health and Diseases, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar 382 007, Gujarat, India; Tel: + 91 -079- 30514235; Fax: + 91 -079-30514110; E mail: ashima.bhardwaj@gmail.com

QUORUM SENSING: BACTERIA ALSO COMMUNICATE, LIVE IN COMMUNITIES, EAVESDROP AND CHEAT OTHER BACTERIA

QS is a mechanism that bacteria use to ensure that sufficient number of those bacterial cells are present for eliciting a biological response to an external stimulus. It includes expression of large number of genes that allow bacteria to work in unison to avert any kind of catastrophe during microbe-microbe or host-microbe interactions [3, 4]. QS process involves generation of a signal molecule, accumulation of the signal molecule in medium to certain threshold concentration, recognition of the signal molecule by a receptor and expression of a large array of genes in response to the concentration of the signal-receptor complex [5]. With the help of this process, bacteria regulate myriad activities such as virulence, biofilm formation, luminescence, DNA transfer, to name a few (Fig. (**1**)) [2, 5]. QS is known to overcome the host defense barriers by affecting the human transcriptional programs, detecting the human cytokines and stress hormones and capture when the host is most vulnerable [2]. QS signals can transgress the interspecies and interkingdom barriers [2, 6]. When the bacterial population densities are low, the expression of virulence genes is not activated so as to avoid the detection of pathogen and immune stimulation against the pathogenicity factors. This gives ample time to bacteria for colonization and establishment in the host.

2212-4071/13 $100.00+.00

Evolution of MDRs

Ashima Kushwaha Bhardwaj and Kittappa Vinothkumar

Introduction

With the evolution of multidrug-resistant bacteria at threatening rates, the mankind has witnessed the glorious rise and fall of antibiotics. Antibiotics, the miracle drugs and the magic bullets that appeared to have marked the end of the infectious diseases, are fast losing their charm and effectiveness in human medicine. The swift and untimely demise of these wonder molecules has been attributed chiefly to the resistance mounted by bacteria against them. The phenomenon of antibiotic resistance is inevitable and was something that was cautioned in the Noble Prize lecture by Sir Alexander Fleming in 1945. Dr. Joshua Lederberg very accurately fathomed the seriousness of these resistant bacteria whom he considered much more dangerous a threat as compared to Ebola and West Nile virus. Resistance to any molecule or drug intended to kill a target organism is a very natural phenomenon for the survival of that organism; a cancer cell being subjected to chemotherapeutic treatment, a fungal cell subjected to anti-fungals and, similarly, anti-parasitic and antibacterial compounds are all likely to face resistance from their target cells. Thus, all the popular drugs including antimalarials, anti-tuberculosis, anti-parasitic, antivirals, anti-fungals and antibacterial drugs are facing the risk of becoming obsolete. Consequently, the human race faces the risk of an apocalypse in the hands of these invincible bugs that no drug is able to kill. This chapter describes various genetic and some of the non-genetic factors such as environmental, social and political factors that have led to the evolution of a phenomenon called multidrug resistance (MDR). The threat of antibiotic resistance now spans a wide range of infectious agents including Gram-positive and Gram-negative bacteria, all the infectious diseases and all the geographical locations on this planet.

There has been an evolution of a myriad of resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), vancomycin-resistant *Staphylococcus aureus* (VRSA), extremely drug-resistant tuberculosis (XDR), totally drug-resistant tuberculosis (TDR), New Delhi metallo-β-lactamases (NDM)-carrying superbugs, extended spectrum β-lactamases (ESBLs)-carrying bugs and carbapenem-resistant *Klebsiella pneumoniae* (CRKP) to name a few. Having thrived in hospital settings at operation theatres and intensive care units or in community settings, these superbugs have wreaked havoc and led to the number of deaths spiralling high. This exhaustive list also deserves the mention of major threats posed by *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in

A.K. Bhardwaj (✉) • K. Vinothkumar
Department of Human Health and Disease, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar 382007, Gujarat, India
e-mail: ashima.bhardwaj@gmail.com

V.C. Kalia (ed.), *Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight*,
DOI 10.1007/978-81-322-1982-8_2, © Springer India 2014

CHAPTER 2

Therapeutic Limitations due to Antibiotic Drug Resistance: Road to Alternate Therapies

Ashima Kushwaha Bhardwaj*, Kittappa Vinothkumar, Neha Rajpara, Priyabrata Mohanty and Braj Mohan Ram Narayan Singh Kutar

Department of Human Health and Diseases, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar 382 007, Gujarat, India

Abstract: The antibiotics are destined for obsolescence as microbes would find a way to deal with them either by innate or by acquired genes. It is truly said that the power of bacteria should never be underestimated. There is a constant race between the humans for design and use of new drugs and the acquisition of genes by bacteria to render these novel drugs harmless. Situation has worsened with the indiscriminate use of antibiotics in human and animal health, agriculture, aquaculture and poultry. There have been reports of extremely drug resistant (XDR), totally drug resistant (TDR) bacteria and superbugs that have complicated the treatment of infectious diseases. Methicillin–resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) recognized as the bane of hospitals are some of the most dreaded bugs. This chapter discusses various mechanisms of multiple drug resistance (MDR) in bacteria and the limitations of antibacterial chemotherapy due to MDR. Various innate and acquired mechanisms of drug resistance like integrons, SXT elements, efflux pumps and quinolone resistance mechanisms are described in details. Some of the important databases related to these genetic factors have also been described here. The possibility of attacking the virulence of bacteria rather than the bug itself in order to circumvent the crisis of MDR has been discussed. It further highlights some of the novel strategies such as efflux pump inhibition and quorum sensing inhibition as anti-virulence strategies. It is advocated that this never-ending war with bacteria would probably require multifaceted approach combining antibacterial, antivirulent regimes in addition to the constant search for novel drug targets and newer drugs by the pharmaceutical companies. Success of these strategies would involve cumulative and strenuous efforts from public, policy makers, research community, clinicians and pharmaceutical companies.

Keywords: Databases, efflux pumps, government policies, inhibitors, integrons, multidrug resistance, phage therapy, plasmids, quinolone resistance, quorum sensing, SXT elements, virulence, vaccines.

***Author's address:** Department of Human Health and Diseases, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar 382 007, Gujarat, India; Tel: +91-079-30514235; Fax: +91-079-30514110; Email: ashima.bhardwaj@gmail.com

Atta-ur-Rahman / M. Iqbal Choudhary (Eds.)

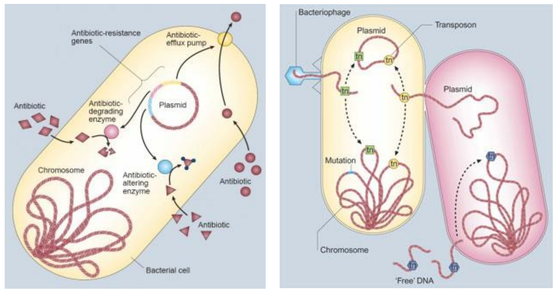
Role of QnrVC protein in conferring quinolone resistance to the multi-drug resistant clinical isolates of *Vibrio fluvialis*

Kittappa Vinothkumar and Ashima Kushwaha Bhardwaj*

Indian Institute of Advanced Research, Koba, Gandhinagar, Gujarat - 382007

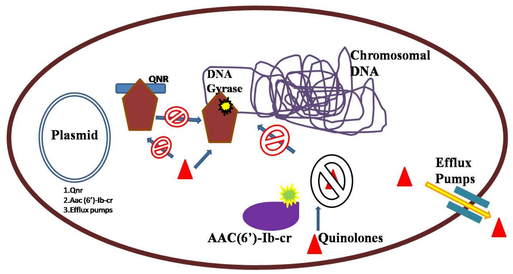
Introduction

The diarrhoeal diseases caused by multidrug resistant *Vibrio* species are major health problems in the developing countries. *Vibrio fluvialis* is a lesser known *Vibrio* species that causes diarrhoea-like illness which is clinically indistinguishable from cholera.



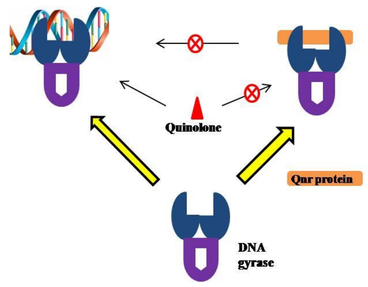
Mechanisms of drug resistance in bacteria

Nowadays, quinolone group of antibiotics are widely used clinically to treat the diarrhoeal diseases. In the decades that have elapsed since the introduction of quinolones, resistance of the bacteria to these agents has become common and widespread. As the consequence of which, treating the infection caused by quinolone resistant bug has become complicated. Quinolone resistance could be mediated by either chromosome-borne (mutation in topoisomerases and efflux pumps) or plasmid-borne (Qnr protein, Aac (6') Ib-cr enzyme and efflux pumps) genetic elements. Generally, the plasmid-borne resistance factors like Qnr protein, Aac (6') Ib-cr confer low-level resistance towards quinolones but in combination with chromosome-borne factors, they help the pathogen to achieve higher-level resistance towards quinolones.



Various mechanisms of quinolone resistance

The Qnr protein is a pentapeptide repeat protein which binds to DNA gyrase, the primary target of quinolones and protects it from the antibiotic action. Several *qnr* genes have been reported so far in *Vibrio* spp. of both plasmid (*qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*) and chromosomal (*qnrVC*) origin.



Action of Qnr protein

In our study, the *qnrVC* gene of chromosomal origin was found to be associated with a transferrable plasmid for the first time in the clinical isolate of *V. fluvialis* BD146 isolated from Kolkata, India. Here, we present our results pertaining to characterization of this gene in conferring quinolone resistance.

Methods

- Screening of quinolone resistance determining genes in the quinolone resistant isolates of *V. fluvialis*
- Antibiotic susceptibility test and MIC assay for quinolones in *V. fluvialis* BD146
- PCR amplification and sequencing of *qnrVC* gene from *V.fluvialis* BD146
- Cloning of *qnrVC* gene in expression vector pET28a
- Structure prediction of recombinant QnrVC protein
- MIC of quinolones for recombinant bacteria

Results

- Quinolone resistance genetic determinants found in the *V. fluvialis* isolates BD146 and L15318

Strain	Quinolone resistance determinants
BD146	Mutation S_{83} to I in *gyrA*, *norM*, *qnrVC* & *aac (6') Ib-cr*
L15318	Mutation S_{83} to I in *gyrA* and *norM*

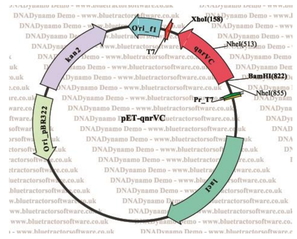
- Four fold elevation in minimum inhibitory concentration (MIC) of ciprofloxacin in *V. fluvialis* BD146 due to the presence of plasmid-borne quinolone resistance genes (*qnrVC* and *aac(6')Ib-cr* genes)

Antibiotic	BD146	L15318
Ciprofloxacin	10µg/ml	2.5µg/ml

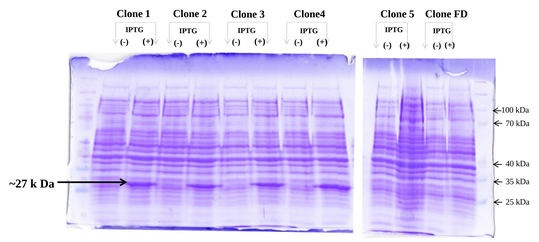
- *V. fluvialis* BD146 showed resistance to newer generation quinolones also

Antibiotics	Suceptibility
Ofloxacin	Intermediate resistant
Gatifloxacin	Intermediate resistant
Levofloxacin	Intermediate resistant
Lomefloxacin	Resistant
Sparofloxacin	Resistant

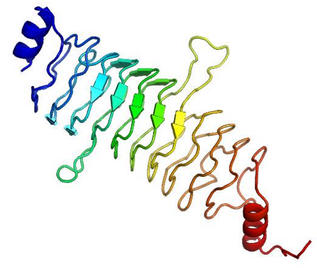
- *qnr VC* gene was amplified and sequenced from *V. fluvialis* BD146. The sequence was submitted to GenBank (accession no. JN408080)
- *qnrVC* gene was cloned in pET28a expression vector



- *qnrVC*-pET28a recombinants expressed QnrVC protein under IPTG induction



- The structure of recombinant QnrVC protein was predicted using I-TASSER server



- *qnrVC*-pET28a recombinant showed increased elevation in MIC of quinolones

Antibiotic	qnrVC-pET28a in *E.coli* BL21 (λDE3)	pET28a in *E.coli* BL21 (λDE3)	Elevation in MIC
Nalidixic acid	5 µg/ml	0.625 µg/ml	8 fold
Norfloxacin	0.125 µg/ml	0.0078 µg/ml	16 fold
Ciprofloxacin	0.0156 µg/ml	< 0.000243 µg/ml	> 64 fold
Ofloxacin	0.0625 µg/ml	0.0039 µg/ml	16 fold

Conclusion

The role of QnrVC protein in quinolone resistance was investigated here and it was found to be responsible for resistance towards mainly ciprofloxacin followed by norfloxacin, ofloxacin and nalidixic acid. The structure of QnrVC was found to be similar to that of other well-studied Qnr proteins like QnrB. As these *qnr* genes are generally associated with plasmids and other mobile genetic elements, the possibilities of dissemination of these genes among the bacterial community is more. The recipient bacteria of this gene could attain an initial basal level resistance to ciprofloxacin and other quinolones. The carriage of such genes on plasmids could have serious consequences in human health.

Acknowledgement

- **Gujarat State Biotechnology Mission, Government of Gujarat, grant no. (GSBTM/MD/PROJECTS/SSA/3386/2012-13)**
- **The Puri Foundation for Education in India**
- **Authors gratefully thank Dr. Amit Ghosh and Dr. T. Ramamurty, NICED, Kolkata, for providing *V. fluvialis* strains**

References

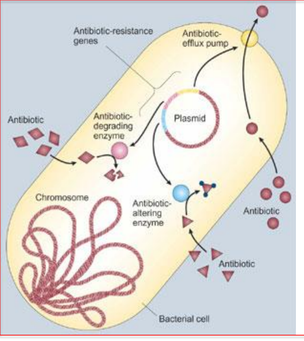
- **Strahilevitz J, Jacoby G A, Hooper D C and Robicsek A.** Plasmid-Mediated Quinolone Resistance: a Multifaceted Threat. Clin Microbiol Rev. 2009. **22,** 664-689.
- **Singh R, Rajapara N, Tak J, Patel A, Mohanty P, Vinothkumar K, Chowdhury G, Ramamurthy T, Ghosh A and Bhardwaj A K.** Clinical isolates of *Vibrio fluvialis* from Kolkata, India, obtained during 2006: plasmids, qnr and mutation in gyrase A as mechanism of multidrug resistance. J Med Microbiol. 2012. **61,** 369-374.

Contact information

***Ashima Kushwaha Bhardwaj**

Email. ashimab@iiar.res.in

Website: www.iiar.res.in



Invincible Microbes and Their Antibiotic Resistance Mechanisms: *Our Wits Versus Their Genes*

Ashima Kushwaha Bhardwaj*, Neha Rajpara, Priyabrata Mohanty, Braj Mohan Ram Narayan Singh Kutar, Kittappa Vinothkumar and Aneri Shah

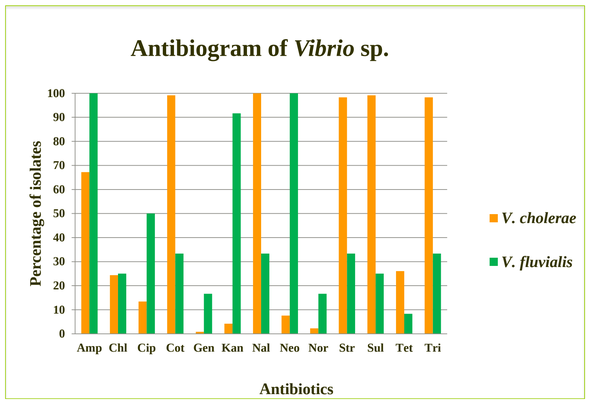
Department of Human Health and Disease, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar 382007, Gujarat, India **Correspondence:** *ashimab@iiar.res.in*

Introduction

Pervasiveness of antimicrobial resistance through all the microbes and through all the geographical locations has been a matter of concern globally. The glorious rise and subsequent untimely demise of the antibiotics has led to the brewing of a public health crisis. Antibiotics are destined for obsolescence as bacteria would use various mechanisms to render these drugs ineffective. Though the appearance of antimicrobial resistance is a natural phenomenon for evolution of bacteria growing in the antibiotic-laden environment, situation has worsened with indiscriminate use of antibiotics in human and animal health, agriculture, aquaculture and poultry. Bacteria often employ a large battery of genes either borne on chromosomes or harboured by various mobile genetic elements to wage a war against antibiotics. Therefore, to overcome the problem of MDR, it becomes imperative to understand the reasons for the acquisition and dissemination of these antibiotic resistance genes that cross the genus and species barriers. Here, we describe the work carried out to unravel various resistance mechanisms in vibrios such as *V. cholerae* and *V. fluvialis* that causes sporadic cases of diarrhoea clinically similar to that of cholera.

Results

Pervasiveness of MDR in Vibrios



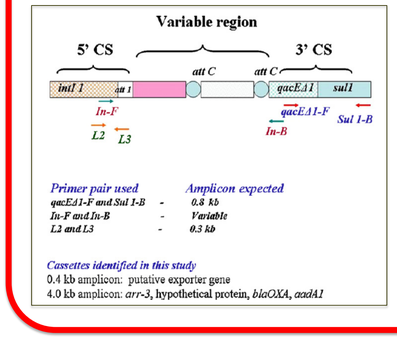
Both the Vibrio species, *V. cholerae* (n=119) and *V. fluvialis* (n=12) show high level of multi drug resistance

Dissemination of resistance genes through mobile genetic elements

Plasmids

Antibiogram of *V.fluvialis* BD146 and its transformants			
JM109	**BD146**	**7.5 kb+ /JM109**	**7.5 kb-/JM109**
NAL **Intermediate: NEO**	**AMP, CIP, GEN, STR, SUL, TMP, NEO, NAL, NOR, KAN, CO-TRI, RIF** **Intermediate: CHL, TET**	**AMP, CHL, GEN, TMP, TET, NAL, RIF** **Intermediate: NEO, KAN**	**AMP, CHL, GEN, TET, NAL, RIF** **Intermediate: NEO, KAN**

Integrons



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

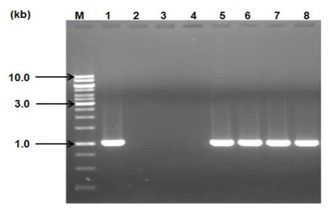
Presence of integrons carrying antibiotic resistance genes in *V. fluvialis* BD146

SXT Elements

Presence of SXT element in the clinical isolates of *V. cholerae* (2009, Kolkata) and transfer of its resistance traits by conjugation

Isolates	Resistant	Intermediate resistant
IDH01572	AMP, COT, NAL, PB, STR, SUL, TRI	CHL, NEO
IDH01572 transconjugant	CHL, COT, NAL, SUL, **TET**, TRI	STR
IDH01738	COT, NAL, PB, STR, SUL, TRI	CIP
IDH01738 transconjugant	COT, NAL, SUL, **TET**, TRI	STR

Bold face indicates the resistance trait from recipient XL-1Blue cells.



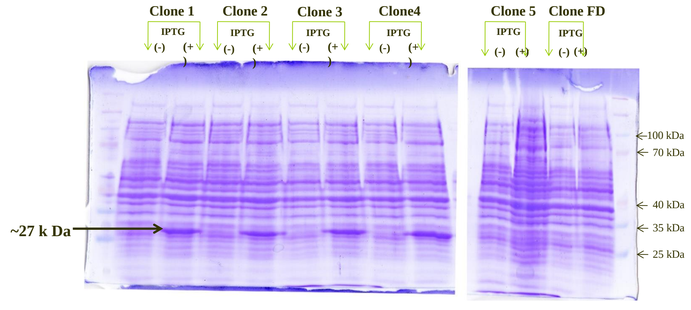
Agarose gel (1%) analysis of PCR product of SXT integrase from IDH isolates and their transconjugants. Lane M : 1 kb ladder (Fermentas); Lane 1: Positive control *V.cholerae* O139 MO10 ; Lane 2: Recipient *E. coli* XL-1 Blue; Lanes 3 and 4: Negative controls of no DNA template and SXT-negative IDH02095 isolate respectively; Lanes 5 and 6 : IDH01572 (SXT-positive) isolate and its transconjugant respectively; Lanes 7 and 8 : IDH01738 (SXT-positive) isolate and its transconjugant respectively.

Unraveling the quinolone resistance mechanisms

Strain	Quinolone resistance determinants	MIC for Ciprofloxacin (µg/ml)
V. fluvialis BD146	Mutation S 83 I in gyrA, qnrVC & aac (6') Ib-cr	10
V. fluvialis L15318	Mutation S 83 I in gyrA	2.5

***V. fluvialis* BD146 showed high resistance to ciprofloxacin due to mutation in DNA gyrase and PMQR factors (*qnrVC and aac (6') Ib-cr*)**

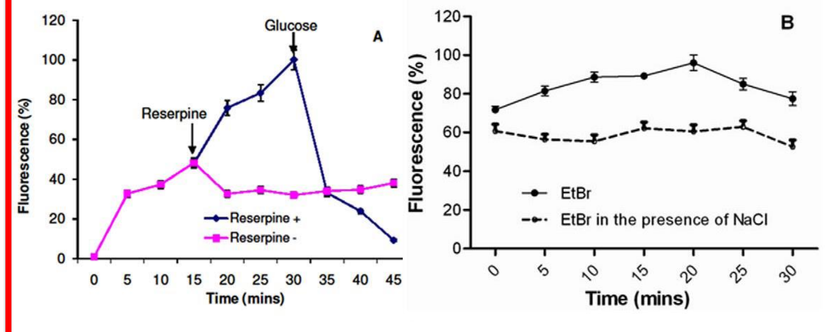
Cloning and expression of *qnrVC* gene using pET28a Vector



pET28a recombinants expressing QnrVC protein confer elevation in MIC for Ciprofloxacin

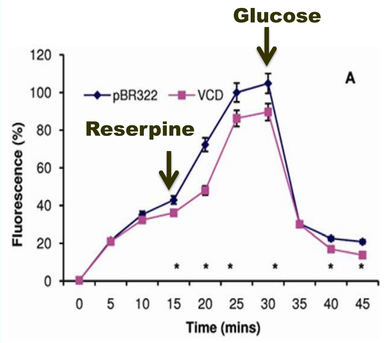
Antibiotic	qnrVC-pET28a in *E.coli* BL21 (λDE3)	pET28a in *E.coli* BL21 (λDE3)	Elevation in MIC
Ciprofloxacin	0.0156 µg/ml	< 0.00195 µg/ml	> 8 fold

Cloning and characterization of MATE efflux pumps

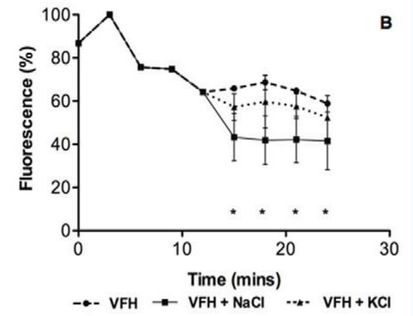


Presence of energy-dependent and Sodium-driven transporters in parent strain of *V. fluvialis*

H-and D- MATE-type efflux pumps isolated from clinical isolates of *V. fluvialis,* cloned in pBR322 and expressed in *E.coli* KAM32 showed activity in transport assays using fluorimeter



Involvement of recombinant efflux pumps in the transport of Norfloxacin



Sodium-ion dependence of recombinant efflux pumps

Elevation in MICs for recombinant clones carrying MATE-type efflux pump genes from *V. fluvialis* and *V. cholerae* implicating their role in MDR.

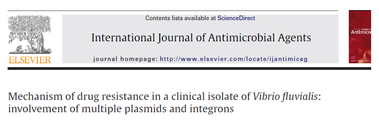
Drug in µg/mL	pBR322	VCH	VFH	VCD	VFD
Ciprofloxacin	0.0045	0.009	0.009	0.009	0.009
Norfloxacin	0.019	0.078	0.039	0.039	0.039

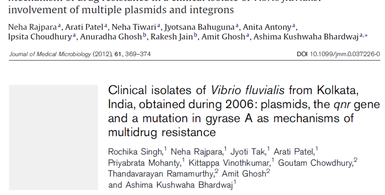
Conclusions and Perspectives

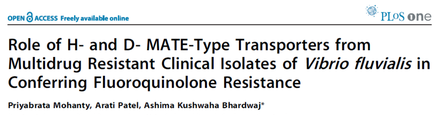
- Our studies have revealed that bacteria are fast becoming multidrug resistant
- Mobile Genetic Elements such as integrons, plasmids and SXT elements play a vital role in acquisition and dissemination of genes for MDR
- Quinolone resistance is assuming dangerous proportions and it is mainly due to mutations and some plasmid-borne genes. Our work with *qnrVC5* reported for the first time from our lab has shown its role in resistance to quinolones
- Efflux pumps play an important role in resistance to variety of drugs
- With additional studies all over the globe, it becomes clear that this never-ending war of mankind with bacteria would require antibacterial as well as antivirulent regimes in addition to the constant search for novel drug targets and newer drugs by the pharmaceutical companies. Alternatives such as efflux pump inhibition, quorum sensing inhibition and phage therapy need to be explored more seriously.

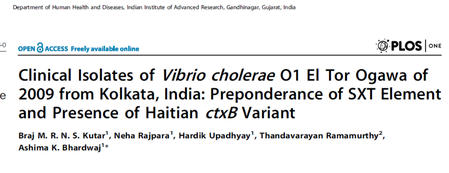
Acknowledgements

- **Indian Council of Medical Research (ICMR), New Delhi**
- **Council of Scientific and Industrial Research (CSIR), New Delhi**
- **Department of Biotechnology (DBT), Ministry of Science and Technology, New Delhi**
- **Gujarat State Biotechnology Mission (GSBTM) , Government of Gujarat**
- **The Puri Foundation for Education in India, Gandhinagar, Gujarat**
- **Dr. T. Ramamurthy, Dr. Amit Ghosh, NICED, Kolkata, Prof. V. K. Chaudhary, UDSC, New Delhi**


Contents lists available at ScienceDirect
International Journal of Antimicrobial Agents
journal homepage: http://www.elsevier.com/locate/ijantimicag
Mechanism of drug resistance in a clinical isolate of *Vibrio fluvialis*: involvement of multiple plasmids and integrons
Neha Rajpara[a], Arati Patel[a], Neha Tiwari[a], Jyotsana Bahuguna[a], Anita Antony[a], Ipsita Choudhury[a], Anuradha Ghosh[b], Rakesh Jain[b], Amit Ghosh[a], Ashima Kushwaha Bhardwaj[a,*]
Journal of Medical Microbiology (2012), 61, 369–374
DOI 10.1099/jmm.0.037226-0


Clinical isolates of *Vibrio fluvialis* from Kolkata, India, obtained during 2006: plasmids, the *qnr* gene and a mutation in gyrase A as mechanisms of multidrug resistance
Rochika Singh,[1] Neha Rajpara,[1] Jyoti Tak,[1] Arati Patel,[1] Priyabrata Mohanty,[1] Kittappa Vinothkumar,[1] Goutam Chowdhury,[2] Thandavarayan Ramamurthy,[2] Amit Ghosh[2] and Ashima Kushwaha Bhardwaj[1]


OPEN ACCESS Freely available online
PLoS one
Role of H- and D- MATE-Type Transporters from Multidrug Resistant Clinical Isolates of *Vibrio fluvialis* in Conferring Fluoroquinolone Resistance
Priyabrata Mohanty, Arati Patel, Ashima Kushwaha Bhardwaj*
Department of Human Health and Diseases, Indian Institute of Advanced Research, Gandhinagar, Gujarat, India


OPEN ACCESS Freely available online
PLOS ONE
Clinical Isolates of *Vibrio cholerae* O1 El Tor Ogawa of 2009 from Kolkata, India: Preponderance of SXT Element and Presence of Haitian *ctxB* Variant
Braj M. R. N. S. Kutar[1], Neha Rajpara[1], Hardik Upadhyay[1], Thandavarayan Ramamurthy[2], Ashima K. Bhardwaj[1]*

Study on toxic potential of environmental *Vibrio cholerae* isolates from a village of Gujarat

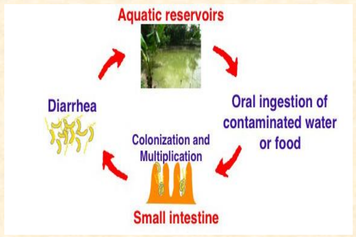
Neha Rajpara, Kittappa Vinothkumar, Priyabrata Mohanty and Ashima Kushwaha Bhardwaj*

Human Health and Diseases, Indian Institute of Advanced Research, Koba,
Gandhinagar -382007

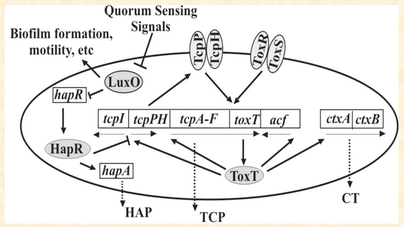
Introduction

- Cholera and cholera-like diarrhea are caused by *Vibrio cholera*e and other *Vibrio* spp. The disease generally spreads through faeco-oral route.
- Pathogenic *Vibrio* strains harbour key virulence factors that include cholera toxins and toxin corregulated pilus (*tcp*). Cholera secretes enterotoxin, which causes rice water stool.
- Many other virulence factors are also involved which enhance the severity of the disease for example zonula occludens toxin, accessory toxin, haemolysin, heat stable entrotoxin, etc.
- The organism's life cycle is completed in two niches i.e aquatic environment and secondly in the human intestinal environment.
- Toxigenic strains have evolved from environmental nonpathogenic strains by acquisition of virulence genes from *ctx* Φ phage.
- Cholera has been reported from various regions of Gujarat like Ahmedabad, Vadodara, Surat, Rajkot, Jamnagar etc. but there have been no studies pertaining to the molecular epidemiology of environmental isolates of Gujarat.
- Hence, this study intends to investigate about epidemic potential of environmental *V. cholerae* isolated from Gujarat region.

Life cycle of *Vibrio cholerae*



Regulation of toxic and virulence genes



Methods

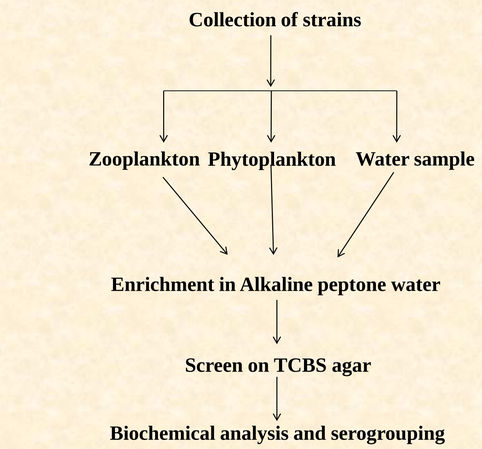
- Collection of Strains

Sample collection site

Pond at Lawarpur village, Gandhinagar, Gujarat

Methods

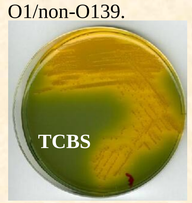
- Isolation and identification of strains

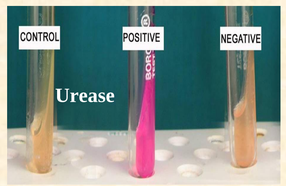


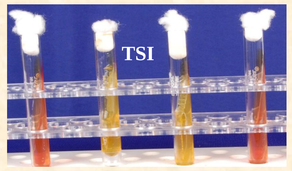
- Genomic and Plasmid DNA preparations
- PFGE and RAPD
- PCR of toxigenic and regulatory genes
- Haemolytic activity

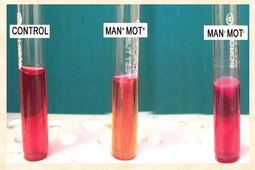
Results

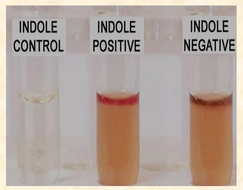
- *Vibrio cholerae* isolates were obtained from zooplankton, phytoplankton and water samples from the pond of Lawarpur village, Gandhinagar, Gujarat, in 2009. Biochemical analysis and serogrouping results revealed that these were *V. cholerae* non-O1/non-O139.

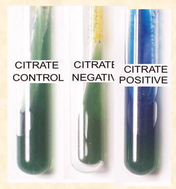


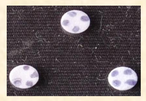










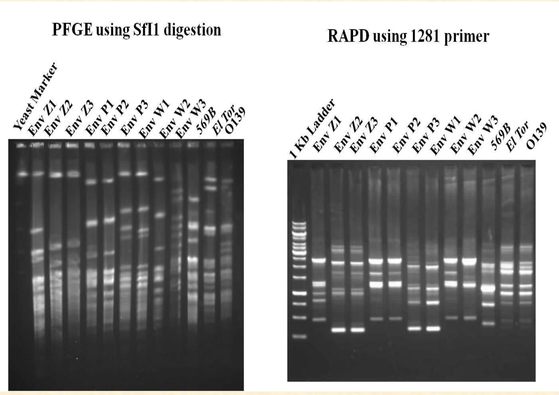


Serotyping

Polyvalent O1 — Monovalent O139

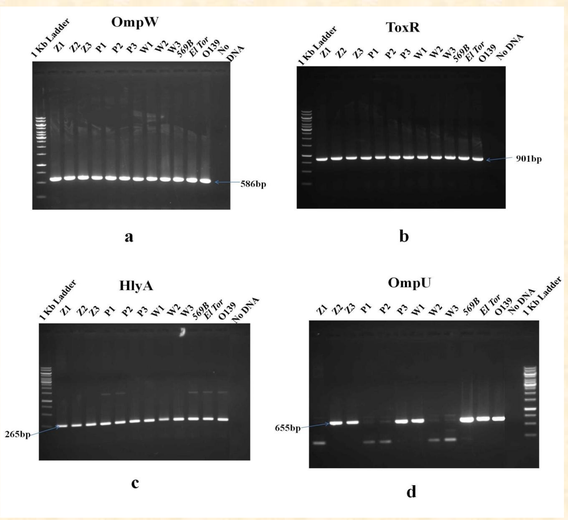
Ogawa — Inaba

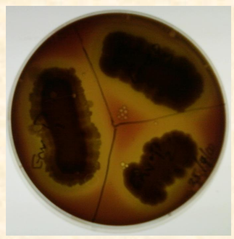
Biochemical analysis and serogrouping



- Five clonal patterns were found among the nine strains
1. Group A : Z1
2. Group B : Z2 and Z3
3. Group C : P1, P2 and W2
4. Group D : P3 and W1
5. Group E : W3

PCR of toxin-coding and regulatory genes





- These strains showed haemolytic activity on Soyabean Casein digest medium contained 5% sheep blood RBC.

Strains	Toxin coding and regulatory genes											Hemolytic activity
	toxR	*omp W*	*omp U*	*tcp A* (ElTor)	*tcp A* (class)	*tcp I*	*rtx c*	*hly A*	*ctx A* and *ctx B*	*ace*	*zot*	
Env Z1	+	+	-	-	-	+	+	+	-	-	-	+
Env Z2	+	+	+	-	-	+	+	+	-	-	-	+
Env Z3	+	+	+	-	-	+	+	+	-	-	-	+
Env P1	+	+	-	-	-	+	+	+	-	-	-	+++
Env P2	+	+	-	-	-	+	+	+	-	-	-	+++
Env P3	+	+	+	-	-	+	+	+	-	-	-	+++
Env W1	+	+	+	-	-	+	+	+	-	-	-	+++
Env W2	+	+	-	-	-	-	+	+	-	-	-	+++
Env W3	+	+	-	-	-	+	+	+	-	-	-	-
569B	+	+	+	-	+	+	-	+	+	+	+	-
O1 El Tor	+	+	+	+	-	+	+	+	+	+	+	ND
O139	+	+	+	+	-	+	+	+	+	+	+	ND

Conclusion

- The *Vibrio cholerae* non-O1/ non-O139 were found in the environmental water reservoir of Gandhinagar, Gujarat.
- Though these environmental *Vibrios* were isolated from the same water source, they showed different clonal relationship.
- These isolates lacked toxin genes like *ctxA*, *ctx B*, *zot* and *ace* but have capacity to lyse RBC.
- To the best of our knowledge this is the first report regarding the environmental *Vibrio cholerae* isolate form this region, which is a non-endemic region of cholera.
- Although these are non toxigenic, there are possibilities for these isolates to evolve as epidemic-causing strains through the production of other toxins and acquisition of the toxigenic genes from *ctx* Φ phage.

Acknowledgement

- Department of Biotechnology, Ministry of Science and Technology, Government of India (Grant No. BT/PR/11634/INF/22/104/2008)
- We kindly acknowledge the technical help provided by Ms. Jyoti Tak and Mr. Praful Dargi for the collection of strains.
- The Puri Foundation for education in India

References

- Bidinost C, Saka HA, Aliendro O, Sola C, Panzetta-Duttari G, Carranza P, Echenique J, Patrito E, Bocco JL (2004) Virulence factors of non-O1 non-O139 *Vibrio cholerae* isolated in Cordoba, Argentina. Rev Argent Microbiol 36 (4):158-163
- Faruque SM, Albert MJ, Mekalanos JJ (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol Rev 62 (4):1301-1314

Contact information

Ashima Kushwaha Bhardwaj
Email: ashimab@iiar.res.in
Website: www.iiar.res.in