Summary and Conclusions

Summary

Diarrheal diseases caused by multidrug resistant bacteria (such as *Vibrio* spp, *Shigella* spp., etc.) are a major health problem in the developing countries with poor hygiene and poor resources. Quinolone class of antibiotics has been widely used clinically to treat these diseases. Due to indiscriminate use of these antibiotics, pathogens became resistant to these drugs. As the etiological agents of diarrheal diseases such as *Vibrio* spp, *Shigella* spp., enterotoxigenic *E. coli* (ETEC), etc. are gaining resistance to quinolones, treatment of diarrheal infections is becoming complicated. Active surveillance of diseases and their etiological agents is an absolute requirement to keep pace with these continuously evolving pathogens. Therefore, this situation necessitates a thorough understanding of the mechanisms which are likely to be associated with quinolone resistance. Keeping in view the widespread use of quinolone class of drugs in clinical settings and the increasing resistance of various pathogens to these drugs, identification and characterization of the factors corresponding to quinolone resistance in these organisms is of fundamental interest and main focus of this doctoral research.

In this study, clinical isolates of *V. fluvialis* (n=18), *V. cholerae* (n=119), *V. parahaemolyticus* (n=58) and *Shigella* species (9 *S. flexneri*, 3 *S. dysenteriae*, 4 *S. boydii* and 2 *S. sonnei*), procured from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, were subjected for analysis of antibiotic resistance phenotypes. The isolates were tested with a panel of antibiotics consisting of representative drugs from each class of antibiotics. Nalidixic acid, norfloxacin and ciprofloxacin were included as the representatives of quinolone class of antibiotics. The antibiogram results revealed that these isolates were resistant to multiple drugs. Some of the representative quinolone resistant isolates were chosen for further studies. Hence, nine *V. cholerae*, eleven *V. fluvialis*, seven *V. parahaemolyticus* and ten *Shigella* isolates (seven *S. flexneri*, 2 *S. dysenteriae* and 1 *S. sonnei*) were selected for further analysis. Additionally, a quinolone sensitive strain from each of the above organism type was included in the study, to serve as a control. The selected quinolone resistant isolates were further subjected to antibiogram analysis for an extended panel of quinolones consisting of ofloxacin, gatifloxacin, levofloxacin, lomefloxacin and sparfloxacin. It was observed that *V.*

fluvialis and *Shigella* isolates showed resistance to most of the higher generation quinolones such as gatifloxacin and sparfloxacin. Lomefloxacin resistance was common among *V. cholerae* isolates whereas *V. parahaemolyticus* did not show any significant quinolone resistance phenotype.

Following the antibiogram results, to understand the extent of resistance quantitatively, MIC assay was carried out for the selected quinolone resistant isolates with some of the representative quinolones such as nalidixic acid, norfloxacin, ciprofloxacin and ofloxacin that are widely used clinically for diarrhea treatment. In this assay, the isolates showed varying levels of resistance to the tested quinolones. *V. fluvialis* and *Shigella* isolates presented a very interesting spectrum with different magnitudes of resistance clearly indicating the presence of multiple mechanisms of quinolone resistance and their interplay. *V. cholerae* isolates chiefly showed nalidixic acid resistance. A very low level of resistance to quinolones was found in *V. parahaemolyticus* isolates. In nutshell, though the quinolone resistance was widespread among these selected isolates, the degree of resistance to different quinolones varied.

Subsequent to qualitative as well as quantitative estimation of quinolone resistance phenotypes, work was carried out to analyse the genetic factors that could be responsible for the observed quinolone resistance phenotypes. The quinolone resistant isolates were screened for quinolone-resistance-determining genes like *qnrVC*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac (6') Ib-cr*, *qepA* and *oqxAB* using PCR. The results of the screening experiments showed the presence of *qnrVC5 gene* in three *V. fluvialis* isolates (BD146, L10734 and L9978) and the presence of *aac (6') Ib-cr* in BD146 only. The *V. cholerae* and *V. parahaemolyticus* isolates were devoid of any of the above mentioned quinolone resistance genes. The *qnrS* gene was found in one *Shigella* isolate, *S. flexneri* M11560. Reverse transcription PCR showed the expression of *qnrVC5* and *aac (6') Ib-cr* genes in their respective *V. fluvialis* hosts. Similarly, the expression of *qnrS* gene in its native *S. flexneri* M11560 was also confirmed.

The PCR amplifications were performed for quinolone-resistance-determining-regions (QRDRs) of genes for DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) from representative quinolone resistance phenotypes of *Vibrio* and *Shigella*

isolates. The sequences of these genes from resistant isolates were assembled and compared with the corresponding assembled sequences from sensitive isolates to detect the mutations. Four *V. cholerae* isolates (IDH2118, IDH2101, IDH1681 and BD81) were analysed for mutations and one point mutation each in GyrA (S83I) and ParC (S85L) was found in all the tested isolates. The same mutations were also found in six isolates of *V. fluvialis* (L10734, L15318, BD123, PL171b, PL78/6 and BD146). The two representative *V. parahaemolyticus* isolates (IDH02189 and IDH02191) were devoid of mutations in all the four genes. Similar analysis in quinolone resistant *Shigella* isolates showed a variety of mutational genotype. Two *S. flexneri* isolates NT4966 and NK05/08 showed GyrA (S83L, D87N) and ParC (S80I) mutations whereas *S. flexneri* B36, which showed the highest level of resistance to all the tested quinolones had GyrA (S83L, D87Y) and ParC (E84K) mutations. *S. dysenteriae* NK3898 and NK4771 were respectively found to have D87Y and S83L mutations in GyrA and *S.sonnei* NK2070 possessed GyrA (D87Y) mutation. The nucleotide sequences of these QRDR regions of topoisomerases from the selected isolates, and that of the detected PMQR genes were submitted to GenBank.

From the findings described above, synergy between various quinolone resistance determinants could be demonstrated. For example, *V. fluvialis* BD146, having four quinolone resistance determinants viz. mutations S83I in GyrA and S85L in ParC, *qnrVC5* gene and *aac (6') Ib-cr* gene, was found to have an elevated MIC of quinolone when compared to other isolates having only two or three above mentioned genetic factors.

As *qnrVC5* allele was first reported from this laboratory, it was of prime interest to decipher its role in conferring resistance to quinolones. In the present work, the new allele *qnrVC5* 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 to 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 *E. coli* transformants harbouring it by elevating the MIC towards various quinolones by 2- to 8-fold. In combination with other quinolone resistance factors such as topoisomerase mutations and *aac(6')-Ib-cr* gene, *qnrVC5* gene product contributed towards higher quinolone resistance displayed by *V. fluvialis* isolates. Silencing of the gene using antisense peptide nucleic acid sensitized the *V. fluvialis* parent isolates towards ciprofloxacin. Recombinant QnrVC5 vividly demonstrated its role in conferring quinolone resistance.

Conclusions

The conclusions drawn based on the work described here are as follows:

1. The clinical isolates of *Vibrio* and *Shigella* spp., obtained from the diarrhea patients from the Kolkata region of India showed resistance to multiple antibiotics (including quinolones) which is worrisome as it indicates that the quinolone drugs are also becoming ineffective for treatment of diarrhea-related diseases.

2. Topoisomerase mutations were found to play a major role in determining resistance to quinolones, chiefly nalidixic acid resistance.

3. Especially interesting was the case of *Shigella* isolates where each specific combination of mutations in topoisomerase genes conferred varying magnitude and spectrum of resistance to quinolones.

4. Different levels of quinolone resistance shown by the resistant isolates reflected the involvement of more than one kind of factor and their interplay.

5. QnrVC5 protein (produced from plasmid-borne gene) is effective in conferring resistance to all the generations of quinolones indicating the seriousness of spreading quinolone resistance and evolution of resistance mechanisms along with the introduction of new drugs.

6. A large array of genes studied here along with *qnrVC5* gene, their synergy and global dissemination should be perceived as a menace for quinolone-based therapies.