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

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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.

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 (Cecarelli 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 spectrophotometer (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

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**Table 1. Primers used in the study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>GenBank accession no.</th>
<th>Primer position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GyrA-F</td>
<td>TACACCAGCGCTACTGT</td>
<td>AE003852</td>
<td>131–148</td>
</tr>
<tr>
<td>GyrA-R</td>
<td>TCGATCAGGCCAAAAGTTA</td>
<td>AE003852</td>
<td>321–338</td>
</tr>
<tr>
<td>ParE-F</td>
<td>CAGCAAGAAAGTTGGGCTGA</td>
<td>AE003852</td>
<td>724–744</td>
</tr>
<tr>
<td>ParE-R</td>
<td>AGACTTTGCGTAACCGACTA</td>
<td>AE003852</td>
<td>426–445</td>
</tr>
<tr>
<td>QNR-F</td>
<td>ATGGATCAGCTATGAAAAACAGGACCG</td>
<td>EU574928</td>
<td>5380–5397</td>
</tr>
<tr>
<td>QNR-R</td>
<td>ATCTCGAGTATTAGCAGAACTACTAT</td>
<td>EU574928</td>
<td>4742–4759</td>
</tr>
</tbody>
</table>
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,

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Resistant to</th>
<th>Intermediate resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>L13828</td>
<td>AMP, NEO</td>
<td>KAN, GEN</td>
</tr>
<tr>
<td>L13230</td>
<td>AMP, NEO</td>
<td>KAN, STR, CIP</td>
</tr>
<tr>
<td>L13211</td>
<td>AMP, NEO</td>
<td>KAN</td>
</tr>
<tr>
<td>L12482</td>
<td>AMP</td>
<td>NEO</td>
</tr>
<tr>
<td>L98411</td>
<td>AMP, NEO</td>
<td>KAN, GEN, STR, CIP</td>
</tr>
<tr>
<td>L10734</td>
<td>NEO, CO-TRI, NAL, TRI</td>
<td>AMP, KAN, CIP</td>
</tr>
<tr>
<td>L9077</td>
<td>AMP</td>
<td>KAN, NEO, NAL</td>
</tr>
<tr>
<td>L10332</td>
<td>AMP, NEO</td>
<td>KAN</td>
</tr>
<tr>
<td>L11264</td>
<td>NEO</td>
<td>AMP, KAN</td>
</tr>
<tr>
<td>L12387</td>
<td>AMP, CO-TRI, NAL, NEO, STR, SUL, TRI</td>
<td>CHL, CIP, KAN, NOR</td>
</tr>
<tr>
<td>L9978</td>
<td>AMP</td>
<td>CHL, CIP, KAN</td>
</tr>
<tr>
<td>L15318</td>
<td>AMP, CO-TRI, NEO, SUL, TRI</td>
<td>CHL, CIP, KAN, NOR</td>
</tr>
</tbody>
</table>

**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 *Hind*III-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 N16961 and 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 and norfloxacin (L9077) showed intermediate resistance to this drug. Whilst 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>
<thead>
<tr>
<th>Strain</th>
<th>L13828</th>
<th>L10734</th>
<th>L9978</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>AMP, NEO, KAN, GEN</td>
<td>AMP, NEO, KAN, CO-TRI, TRI, CIP, NAL</td>
<td>AMP, NEO, KAN, CO-TRI, TRI, CIP, SUL, STR, CHL</td>
</tr>
<tr>
<td>Transconjugant</td>
<td>Non-conjugable strain</td>
<td>NEO, KAN, CO-TRI, TRI, NAL, STR</td>
<td>AMP, NEO, KAN, CO-TRI, TRI, CIP, SUL, STR, CHL, NAL</td>
</tr>
<tr>
<td>Transformant</td>
<td>AMP, TET, NAL</td>
<td>AMP, NEO, TET, NAL</td>
<td>AMP, NEO, TRI, TET, NAL</td>
</tr>
</tbody>
</table>

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.
by PCR. The \( \text{aac}(6’)-\text{Ib-cr} \) 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 chromosomal-borne (mutations in topoisomerases and efflux pumps) or plasmid-mediated (\( \text{qnr} \)) (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 gyrase, resulting in quinolone resistance. From India, there is only a single report recently describing the emergence of \( \text{qnr} \)A1 from Indian isolates of \( V. \) fluvialis in 2009 (Chowdhury et al., 2011). In this study, the presence of \( \text{qnr} \)B1 in clinical isolates of \( V. \) fluvialis from 2002 and 2006 indicates that \( \text{qnr} \) has been circulating in Indian isolates from 2002 to 2009.

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