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Transferable Quinolone Resistance in *Vibrio cholerae*▽

Hong Bin Kim,[1,2,3]† Minghua Wang,[3,4]† Sabeena Ahmed,[5] Chi Hye Park,[3] Regina C. LaRocque,[3] Abu S. G. Faruque,[5] Mohammed A. Salam,[5] Wasif A. Khan,[5] Firdausi Qadri,[5] Stephen B. Calderwood,[3] George A. Jacoby,[6] and David C. Hooper[3]*

Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, Republic of Korea[1]; Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Republic of Korea[2]; Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts[3]; Institute of Antibiotics, Huashan Hospital, Fudan University, Shanghai, China[4]; International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh[5]; and Lahey Clinic, Burlington, Massachusetts[6]

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Ciprofloxacin was introduced for treatment of patients with cholera in Bangladesh because of resistance to other agents, but its utility has been compromised by the decreasing ciprofloxacin susceptibility of *Vibrio cholerae* over time. We correlated levels of susceptibility and temporal patterns with the occurrence of mutation in *gyrA*, which encodes a subunit of DNA gyrase, followed by mutation in *parC*, which encodes a subunit of DNA topoisomerase IV. We found that ciprofloxacin activity was more recently further compromised in strains containing *qnrVC3*, which encodes a pentapeptide repeat protein of the Qnr subfamily, members of which protect topoisomerases from quinolone action. We show that *qnrVC3* confers transferable low-level quinolone resistance and is present within a member of the SXT integrating conjugative element family found commonly on the chromosomes of multidrug-resistant strains of *V. cholerae* and on the chromosomes of *Escherichia coli* transconjugants constructed in the laboratory. Thus, progressive increases in quinolone resistance in *V. cholerae* are linked to cumulative mutations in quinolone targets and most recently to a *qnr* gene on a mobile multidrug resistance element, resulting in further challenges for the antimicrobial therapy of cholera.

Cholera remains a major public health problem in many areas of the developing world. In addition to maintenance oral rehydration therapy, adjunctive antimicrobial therapy reduces the extent and duration of diarrhea, resulting in reduced fluid requirements and hospitalizations, reductions that are particularly important in resource-limited areas. Antimicrobial therapies have included tetracycline, azithromycin, and fluoroquinolones, such as ciprofloxacin, but the activity of fluoroquinolones has decreased in some areas, and this decreased activity has been associated with substantial reductions in the efficacy of ciprofloxacin relative to that of azithromycin (15, 20). To evaluate the evolution of ciprofloxacin resistance in *Vibrio cholerae*, we studied isolates from Bangladesh available over a 6-year period in which poor clinical responses of cholera patients to ciprofloxacin were recognized. We determined the presence of resistance mutations in genes encoding the subunits of the quinolone target enzymes DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*) and the presence of *qnr* and other acquired genes that confer additional resistance to quinolones (25). Some *qnr* gene products have been shown to protect gyrase and topoisomerase IV from quinolone action in enteric bacteria (26, 27). *qnr* genes are usually located on mobile genetic elements, such as plasmids, that can transfer between strains but have been found on the chromosomes of some *Vibrio* spp. (5, 18). In *V. cholerae*, a *qnr* homolog, *qnrVC1*, has been described for isolates from Brazil (9) but has not been shown to confer transferable quinolone resistance or to be linked to incremental quinolone resistance and poor response to ciprofloxacin therapy of cholera. We show here that progressively higher levels of resistance in *V. cholerae* in Bangladesh were driven by accumulating mutations in topoisomerase target enzymes and by the acquisition of a quinolone resistance determinant, *qnrVC3*, which we identified as part of an SXT integrating conjugative element (4, 10) that also carried genes conferring resistance to tetracycline, trimethoprim-sulfamethoxazole, and streptomycin and accounted for transferable multidrug resistance that included ciprofloxacin in isolates positive for *qnrVC3*.

MATERIALS AND METHODS

Bacterial isolates. Clinical isolates of *V. cholerae* O1 were recovered from patients seen at the Dhaka Hospital of the International Centre for Diarrhoeal Disease Research during the period from 2002 to 2008, as part of ongoing surveillance (24) and a prospective study of cholera patients and their household contacts (22).

Antibiotic susceptibility tests. MICs of ampicillin, ciprofloxacin, gentamicin, levofloxacin, nalidixic acid, streptomycin, tetracycline, trimethoprim, and trimethoprim-sulfamethoxazole were determined by Etest (AB Biodisk, Solna, Sweden). Susceptibility criteria were those of the Clinical and Laboratory Standards Institute (6).

Screening for QRDR mutations and the PMQR genes. The PCR amplifications for *gyrA*, *gyrB*, *parC*, and *parE* were carried out using the primers shown in Table 1, as previously described (1). Purified PCR products were sequenced on both strands, and quinolone resistance determining region (QRDR) DNA sequences were compared with the genome sequence of *V. cholerae* O1 strain N16961, available at http://www.jcvi.org/. In addition, we performed PCR screening for known plasmid-mediated quinolone resistance (PMQR) genes, including

* Corresponding author. Mailing address: Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114-2696. Phone: (617) 726-3812. Fax: (617) 726-7416. E-mail: dhooper@partners.org.

† These persons contributed equally to this work.

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TABLE 1. Primers used in this study

Gene	Primer	Sequence (5′→3′)	Size of PCR-amplified product (bp)
gyrA	Forward	AATGTGCTGGGCAACGA CTGG	239
	Reverse	GTGCGCGATTTTCGACA TACG	
gyrB	Forward	GGAAATGACTCGCCGTA AAGG	309
	Reverse	GTTGTGATAACGCAGTTTAT CTGGG	
parC	Forward	GTCTGAGTTGGGTCTCT CGGC	248
	Reverse	AGAATCTCGGCAAACTTTG ACAG	
parE	Forward	ATGCGTGCCAGCAAGAA AGTG	268
	Reverse	TTATCGCTGTCAGGGTCA ATCC	
qnrVC	Forward	AATTTTAAGCGCTCAAACC TCCG	521
	Reverse	TCCTGTTGCCACGAGCATA TTTT	

qnrA, *qnrB*, *qnrC*, *qnrS*, *aac(6′)-Ib-cr*, *qepA*, and *oqxAB*, using previously described primers (16).

Conjugation. In order to determine whether quinolone resistance was transferable in *V. cholerae*, conjugation experiments were carried out in Luria-Bertani (LB) broth, with *Escherichia coli* J53 resistant to azide (Az^r) as the recipient. Cultures of donor and recipient cells in logarithmic phase (0.5 ml of each) were added to 4 ml of fresh LB broth and incubated together overnight without shaking. Transconjugants were selected on Mueller-Hinton agar plates containing tetracycline (25 μg/ml; Sigma Chemical Co., St. Louis, MO) or sulfisoxazole (100 μg/ml) and sodium azide (100 μg/ml) for counterselection. To determine if quinolone resistance was cotransferred, MICs for the donor, recipient, and transconjugant strains were compared by Etest.

PCR amplification for the *qnrVC3* gene. We designed a pair of the primers (Table 1) based on the published sequences of the *qnrVC1* gene (9) (GenBank accession number EU436855) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). After detecting amplification products on the gel, we sequenced the purified PCR products on both strands. The purified PCR products were also used as the probe for Southern blotting.

Plasmid extraction and Southern hybridization for *qnrVC3*. Plasmid DNAs from the donor and transconjugant strains were obtained using a plasmid midi kit (Qiagen, Valencia, CA). DNAs were subjected to electrophoresis in 1.0% agarose gel with ethidium bromide at 90 V for 3 h. After depurination, denaturation, and neutralization of the gel, DNAs were transferred to Hybond-N^+ membrane by capillary blotting overnight. The membrane, which was fixed by UV exposure, was hybridized with the *qnrVC3* probe labeled with horseradish peroxidase (Amersham ECL kit; Amersham Biosciences Corp., GE Healthcare, Chalfont St. Giles, United Kingdom), and then the signals were detected by exposure of the membrane to Hyperfilm ECL film (Amersham Biosciences Corp., GE Healthcare, Chalfont St. Giles, United Kingdom).

Cloning of *qnrVC3*. The *qnrVC3* gene from *V. cholerae* O1 strain 59 was cloned into expression vector pQE-60 (Qiagen, Valencia, CA) at its NcoI and BamHI sites. The gene was amplified by PCR using primers with sequences 5′-GCCATGGAAAAATCAAAGCAAT and 5′-GCGGATCCGTCAGGAACAATGATTA and ligated after digestion with NcoI and BamHI into pQE-60. Proper construction was confirmed by sequencing, and the pQE60-QnrVC plasmid was transformed into *E. coli* J53 Az^r with selection on LB agar plates containing ampicillin.

Analysis of the *qnrVC3* structures. We sequenced the DNA adjacent to *qnrVC3* in transconjugant SXT1 with a series of outward-facing primers starting from both sides of the *qnrVC3* gene by the use of an inverse PCR strategy (9). Sequence analyses and comparison with known sequences were performed with the BLAST programs at the NCBI.

TABLE 2. Ciprofloxacin susceptibility of *V. cholerae* clinical isolates at the International Centre for Diarrhoeal Disease Research in Bangladesh

Yr	MIC (μg/ml)[a]			*n*
	Mean	Median	Range	
2003	0.23	0.25	0.19–0.25	47
2004	0.25	0.25	0.125–0.38	23
2005	0.43	0.38	0.19–1.50	19
2006	0.25	0.25	0.25	10
2007	0.33	0.38	0.25–0.50	20
2008	0.28	0.25	0.125–0.50	84

[a] Determined by Etest.

RESULTS AND DISCUSSION

Patterns of ciprofloxacin susceptibility. Between 2003 and 2008, the median MIC of ciprofloxacin was 0.25 to 0.38 μg/ml, without a change over this interval (Table 2). These values were, however, 100-fold and 10-fold higher than the median MICs of ciprofloxacin for isolates of *V. cholerae* from Bangladesh in 1996 and 2002, respectively (15, 21). The maximum MIC, however, increased between 2003 and 2005 from 0.25 to 1.5 μg/ml and then fell to 0.25 to 0.5 μg/ml from 2006 to 2008.

The progressive reduction in the quinolone susceptibility of *V. cholerae* has been associated with clinical failures. From 1993 to 1995, the MICs of ciprofloxacin were 0.003 μg/ml or less for all strains tested in one study that demonstrated 94% efficacy of a single 1-g dose of ciprofloxacin for treatment of cholera in adults (15). In 2001 and 2002, the MICs of ciprofloxacin were 10-fold higher (0.023 μg/ml and 0.047 μg/ml) and associated with 60% efficacy when a single dose of ciprofloxacin (20 mg/kg) was given to pediatric patients (21). By 2002 to 2004, a period that overlaps with that in which our isolates were collected, the median MIC of ciprofloxacin had risen another 10-fold increment to 0.25 μg/ml and was associated with a reduction in efficacy of a single 1-g dose of ciprofloxacin in adults to 27% (20). Discouragingly, despite the switch from ciprofloxacin to azithromycin as empirical therapy for cholera in 2005, reduced susceptibility has remained stable, and the low clinical efficacy of ciprofloxacin against strains with MICs below the clinical laboratory susceptibility breakpoint of 1.0 μg/ml raises questions about whether the breakpoint should be reevaluated. Such discrepancies between *in vitro* susceptibility and clinical efficacy remain mechanistically unexplained but have also been seen with doxycycline treatment of cholera patients (15) and ciprofloxacin treatment of patients with typhoid fever (7).

Evaluation of the mechanisms of reduced quinolone susceptibility. To evaluate the mechanisms of reduced susceptibility to ciprofloxacin, 16 clinical isolates from isolates obtained in a prior study (22) were chosen for study in detail to represent the range of ciprofloxacin MICs in the period from 2002 to 2008. All isolates were *V. cholerae* O1, and all were El Tor Ogawa biotype, except for five (strains 10, 11, 16, MDC-4, and CLS6) that were El Tor Inaba biotype. Based on ciprofloxacin MICs (Table 2), we divided strains into three groups, group I (MICs of 0.023 to 0.032 μg/ml), group II (MICs of 0.38 to 0.5 μg/ml), and group III (MICs of >0.5 μg/ml). Isolates from group I were all from 2002; isolates from group II were from 2002 to

TABLE 3. Characteristics of clinical isolates of *V. cholerae* and their *E. coli* transconjugants

Strain	Yr of isolation	MIC (μg/ml)[a]									QRDR mutation		*qnrVC* PCR
		CIP	LVX	NAL	TET	TMP	SXT	STR	AMP	GEN	*gyrA*	*parC*	
E. coli ATCC 25922		0.008	0.023	3	1.5	0.75	0.094	4	6	0.75			
E. coli J53 Az[r]		0.012	0.032	4	1.5	0.19	0.032	3	6	0.75			
V. cholerae N16961		0.015									Ser83	Ser85	
Clinical isolates													
Group I													
V. cholerae 10	2002	0.032			1.0		>32	256		1.5	Ile83	−	−
V. cholerae 11	2002	0.032			1.0		>32	256		1.5	Ile83	−	−
V. cholerae 12	2002	0.023			1.0		>32	384		1.5	Ile83	−	−
V. cholerae 13	2002	0.032			1.0		>32	512		1.5	Ile83	−	−
V. cholerae 16	2002	0.032			1.0		>32	192		1.0	Ile83	−	−
Group II													
V. cholerae 17	2002	0.38			1.0		>32	256		1.5	Ile83	Leu85	−
V. cholerae 19	2002	0.38			1.0		>32	384		1.5	Ile83	Leu85	−
V. cholerae 24	2004	0.5			12		>32	256		1.5	Ile83	Leu85	+
V. cholerae 57	2005	0.38			8		>32	192		1.5	Ile83	Leu85	−
V. cholerae CLS6	2006	0.38	0.38		0.75		>32	>1,024		0.75	Ile83	Leu85	−
V. cholerae MDC126	2006	0.38	0.38		8		>32	192		1.5	Ile83	Leu85	−
V. cholerae MDC4	2007	0.5	0.38		1.5	>32	>32	384		1.5	Ile83	Leu85	−
V. cholerae MDC125	2008	0.5	0.38		1.5	0.38	0.047	12		1.5	Ile83	Leu85	−
Group III													
V. cholerae 59	2005	0.75	0.75	>256	8	>32	>32	>128	3	1.5	Ile83	Leu85	+
V. cholerae CLS4	2006	0.75	0.75		8	>32	>32	128		1.5	Ile83	Leu85	+
V. cholerae MDC1	2007	0.75	0.75		8	>32	>32	96		1.0	Ile83	Leu85	+
E. coli J53 Az[r] transconjugants													
SXT1 (with strain 59)[b]		0.047	0.19	3	64	>32	>32	>192	4	0.75			+
SXT2 (with strain 59)[b]		0.012	0.032	3	64	>32	>32	>192	4	0.5			−
TET1 (with strain 59)[b]		0.012	0.032	4	64	>32	>32	>192	6	0.75			−
TET2 (with strain 59)[c]		0.064	0.125	4	64	>32	>32	>192	4	0.5			+
SXT13-1 (with strain 13)[c]		0.006			1.5		>32						
Cloned strains													
E. coli J53 (pQE60)		0.012	0.032										
E. coli J53 (pQE60-QnrVC3)		0.25	0.19–0.38										

[a] CIP, ciprofloxacin; AMP, ampicillin; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin; LVX, levofloxacin; NAL, nalidixic acid; SMZ, sulfisoxazole; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.
[b] Selected on Mueller-Hinton agar with sodium azide and sulfisoxazole.
[c] Selected on Mueller-Hinton agar with sodium azide and tetracycline.

2008; and isolates from group III were from 2005 to 2007. Strains of *V. cholerae* with differing levels of susceptibility have previously been shown to have similar ribotypes, highlighting the clonal nature of *V. cholerae* outbreaks and the additive nature of resistance determinants in this setting (8).

No strain possessed a mutation in the QRDR (28, 29) of the *gyrB* or *parE* gene, and the reference strain N16961 also had no mutations in the *gyrA* or *parC* gene (Table 3). All other strains, including those in group I, contained a mutation in *gyrA*, encoding Ser83Ile, which is within the QRDR and is known to cause increased MICs of ciprofloxacin (11). Group I strains had an increase of two- to fourfold in the MIC of ciprofloxacin relative to the reference strain. Strains in group II had additional mutations in *parC*, encoding Ser85Leu (Table 2). *qnrVC3* was not detected in group I. In contrast, one group II strain and all group III strains were positive for *qnrVC3* by PCR in addition to having *gyrA* and *parC* mutations. Strains positive for *qnrVC3* were found in each year of 2004 through 2007, and all were the El Tor Ogawa biotype. No other PMQR genes were found in isolates in any group. Thus, increasing MICs of ciprofloxacin correlated with sequential mutations in *gyrA* and *parC*, followed by acquisition of *qnrVC3*, which was present as early as 2004.

Mutations in *gyrA* and *parC* have previously been associated with increases in the MICs of ciprofloxacin for *V. cholerae* isolates from India (1), but the wide range of MICs associated with these strains made the contribution of these mutations to the MICs of ciprofloxacin difficult to determine. Other mobile quinolone resistance elements were not found in any of the isolates we studied, but the roles of other mechanisms of quinolone resistance, such as those related to expression of endogenous multidrug efflux pumps, remain to be defined (1, 12–14).

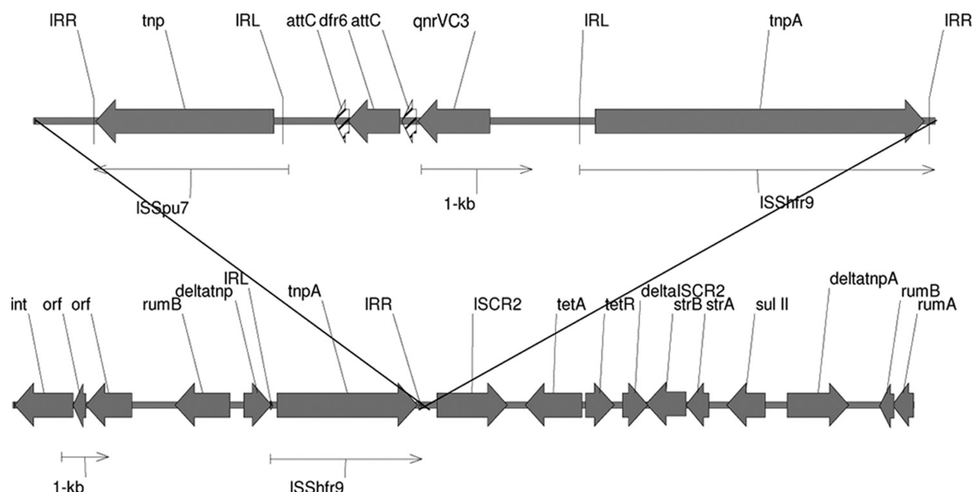

FIG. 1. Genetic environment of *qnrVC3*. Annotated map of genes flanking *qnrVC3* in *E. coli* transconjugant SXT1 from *V. cholerae* 59 in comparison to an SXT element from *V. cholerae* O1 strain HN1 (AB450045). IRR, inverted right repeat; IRL, inverted left repeat.

Transfer of reduced quinolone susceptibility. To investigate if an increased MIC of ciprofloxacin could be transferred to *E. coli* from a group III *qnrVC3*-positive isolate, *V. cholerae* 59 was used as a donor for conjugation, and transconjugants were selected with tetracycline or sulfisoxazole and then tested for changes in susceptibility to ciprofloxacin. Reduced susceptibility to ciprofloxacin was cotransferred with other antibiotic resistance determinants in transconjugants SXT1 (selected with sulfisoxazole) and TET2 (selected with tetracycline), resulting in a four- to sixfold increase in the ciprofloxacin MIC of the *E. coli* J53 Az^r recipient, and both transconjugants were positive for *qnrVC3* by PCR (Table 3). Two other transconjugants, SXT2 and TET1, which showed no increase in the ciprofloxacin MIC, were negative for *qnrVC3* by PCR. *V. cholerae* 13, which was negative for *qnrVC3* by PCR, transferred resistance to trimethoprim-sulfamethoxazole but not to ciprofloxacin (Table 3). Thus, transfer of ciprofloxacin resistance correlated with transfer of *qnrVC3*.

Characterization of *qnrVC3*. The nucleotide sequences of the PCR products obtained from *V. cholerae* 59 and its transconjugant strains showed 99% identity (654/657 nucleotides) with the nucleotide sequence of *qnrVC1* reported from a clinical strain from Brazil isolated in 1998 (EU436588) (9). Among the differences, the *qnrVC1* sequence had a nucleotide deletion at position 157 (relative to strain 59 reported here) and an insertion of an A at position 195, resulting in a frameshifted gene segment. The translated sequence for QnrVC3 predicted a 218-amino acid protein with two domains of 10 and 33 pentpeptide repeats and differed from QnrVC1 in 11 amino acids. Cloning of *qnrVC3* in plasmid pQE60 in *E. coli* J53 Az^r resulted in a 16- to 32-fold increase in the MIC of ciprofloxacin (0.19 to 0.38 μg/ml versus 0.012 μg/ml) relative to *E. coli* J53 Az^r containing the plasmid vector alone (Table 3). Thus, *qnrVC3* from *V. cholerae* 59 is itself capable of conferring substantial increases in the MIC of ciprofloxacin when expressed from a multicopy plasmid. The increase in the ciprofloxacin MIC for *E. coli* transconjugants of a donor *qnrVC3*-containing *V. cholerae* strain was less (four- or fivefold) than that conferred by plasmid-cloned *qnrVC3*, suggesting differences in copy number or expression mechanisms in the transferable element.

Characterization of the transferable element containing *qnrVC3*. Thus, to determine the nature of the transferable element that contained *qnrVC3*, we performed Southern blotting on whole-cell DNA from *V. cholerae* 59 and *E. coli* transconjugants SXT1, SXT2, TET1, and TET2, using the *qnrVC3* PCR product as a probe. A strong hybridization signal was found with the band corresponding to chromosomal DNA from *V. cholerae* 59 and *E. coli* SXT1 and TET2, which were positive for *qnrVC3* by PCR (data not shown). DNA from *E. coli* SXT2 and TET1, which were negative for *qnrVC* by PCR, and the *E. coli* recipient itself gave no hybridization signal. Additional experiments to identify plasmid DNA in transconjugant SXT1, using methods that can detect large plasmids were also negative (data not shown). Thus, *qnrVC3* was associated with chromosomal DNA, and no plasmid DNA could be detected. The absence of an identifiable plasmid that hybridized with *qnrVC3* in the *E. coli* recipient suggested that *qnrVC3* might be transferred by an integrated conjugative element (ICE), such as the SXT element that has been found in strains of *V. cholerae* (4). We thus determined the DNA sequences flanking *qnrV3* to evaluate its possible location within an ICE.

Using inverse PCR to detect flanking DNA sequences, we found (Fig. 1) that *qnrVC3* in transconjugant SXT1 was situated between *dfr6* and *tnpA*, which encodes the transposase of ISShfr9, and was linked to *tetA*, *tetR*, *strA*, *strB*, and *sul11*, which are identically represented in the core resistance determinants of the SXT ICE found in *V. cholerae* O1 El Tor strain HN1 (10). Near *dfr6* and *qnrVC3* is an insertion sequence (ISSpu7) first described for *Shewanella putrefaciens*. These three genes are bounded by directly repeated ISShfr9 units first characterized for *Shewanella frigidimarina* and suggest that *dfr6* and *qnrVC3* are located on a composite transposon, as has been found with other SXT ICEs (4, 10). A putative promoter sequence upstream of *qnrVC3* was identical to that found upstream of *qnrVC1* (9). Downstream from *qnrVC3* and *dfr6* were 126-base elements compatible with *attC* sites reported for mobile integrons and *V. cholerae* superintegrons (Fig. 1) (2, 3, 17,

19). The 126-bp element associated with *qnrVC1* (9) differs by four nucleotides from that of *qnrVC3* reported here. The sequence of the *int* gene, which encodes an integrase of the type found in mobile integrons and is distinct from the chromosomal *intV* gene of *V. cholerae* superintegrons, was identical to that found in the SXT element of *V. cholerae* HN1.

Thus, *qnrVC3* may have been acquired by integron gene capture mechanisms mediated by integrases such as that encoded by the *int* gene found to be transferred with the SXT element. The insertion sequences flanking *qnrVC3* also suggest a composite transposon-like structure, raising the additional possibility of mobilization of *qnrVC3* by transposition from a yet unknown source, although transposition of such an element has not been directly demonstrated. Other *Vibrio* spp., including *V. splendidus*, *V. parahaemolyticus*, and *V. vulnificus* (5, 18), have been found to have chromosomally encoded *qnr* genes, but these genes differ from *qnrVC3* by 35% or more and are not located on ICEs or linked to insertion sequences (ISs) (23). Other *qnr* genes have been found on the chromosomes of other aquatic organisms, such as *Shewanella algae*, and the presence in *V. cholerae* SXT elements of IS elements originally found in *Shewanella* spp. highlights the opportunities for gene exchange among bacteria living in aquatic environments.

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