Distribution and characterization of integrons in various serogroups of *Vibrio cholerae* strains isolated from diarrhoeal patients between 1992 and 2000 in Kolkata, India

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A total of 133 clinical strains of *Vibrio cholerae* comprising 44 strains of O1, 45 strains of O139 and 44 strains of non-O1, non-O139 serogroups isolated from hospitalized patients in Kolkata, India, from 1992 to 2000 was examined for the presence of class 1, 2 and 4 integrons. By PCR and DNA sequencing, seven strains of O1, one strain of O139 and six strains of non-O1, non-O139 serogroups were found to contain class 1 integron-harbouring genes *aadA1*, *aadA2* (encoding resistance to streptomycin and spectinomycin), *blaP1* (resistance to β -lactams), *aar-3* (resistance to rifampicin), *aacA4* (resistance to kanamycin and gentamicin), and *dfrA1* and *dfrA15* (resistance to trimethoprim). Most strains produced one or two bands using primers specific for the amplification of the variable region where the antibiotic-resistance genes are located, and their sizes ranged from 700 to 1250 bp. However, one strain of *V. cholerae* O1 isolated in 1994 gave a 2483 bp fragment, the largest fragment so far found in a class 1 integron of *V. cholerae* O1. No strain was positive for the *int2* gene. All *V. cholerae* strains, regardless of serogroup, were positive for the *int4* gene by PCR and using a colony hybridization test. Amplification of the *int4* gene by PCR yielded a 2200 bp fragment (1260 bp larger than the expected size) from three *V. cholerae* O139 strains isolated in 1999. Sequence analysis of this amplicon revealed an insertion of IS1359 in the middle of the *int4* gene. These data indicate that a class 1 integron is present in some clinical strains of *V. cholerae* isolated in Kolkata, India, and that a class 4 integron is ubiquitously distributed among *V. cholerae* strains regardless of serogroup.

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INTRODUCTION

Cholera is a life-threatening illness, particularly among children, in countries with poor sanitation. Before 1992,

only the O1 serogroup of *Vibrio cholerae* was responsible for causing cholera. However, in 1992, large outbreaks of cholera-like diseases caused by a clonal strain of non-O1 *V. cholerae*, later designated *V. cholerae* O139 synonym Bengal,

Abbreviation: CS, conserved segment.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the genes *aacA1c* (O1: VC2), *aar-3-aacA4-dfrA1-orfC* (O1: CO747), *dfrA1-orfC* (O139: CO406), *dfrA1-orfC* (non-O1 non-O139: SG6), *dfrA15* (non-O1 non-O139: CO248), *aadA2* (non-O1 non-O139: CO248), *dfrA1-orfC* (non-O1 non-O139: CO775), *dfrA1-orfC* (non-O1 non-O139: PG92), *dfrA15* (non-O1 non-O139: NLC35), *blaP1* (non-O1 non-O139: NLC35), *aadA2* (non-O1 non-O139: NLC38), *blaP1* (non-O1 non-O139: NLC38), *int4-IS1358-int4* (O139: NLC31), *int4-IS1358-int4* (O139: NLC40) and *int4-IS1358-int4* (O139: NLC50) are AB219433, AB219436, AB219437, AB219453, AB219434, AB219435, AB219451, AB219452 and AB219454–AB219460, respectively.

emerged from Madras and spread rapidly, not only in India but also to several neighbouring countries (Nair *et al.*, 1996; Ramamurthy *et al.*, 2003). This strain caused an acute secretory disease indistinguishable from that caused by *V. cholerae* O1, and is now also considered to be a causative agent of classic cholera. It is clearly differentiated from non-O1, non-O139 strains, which are associated with sporadic cases of diarrhoeal diseases. The National Institute of Cholera and Enteric Diseases in Kolkata has conducted identification and molecular characterization of *V. cholerae* from severe diarrhoeal patients admitted to the Infectious Diseases Hospital since the discovery of O139. It was observed that, from time to time, the prevalent serogroup of *V. cholerae* changed from O1 to O139 and vice versa (Basu *et al.*, 2000; Mitra *et al.*, 1996; Mukhopadhyay *et al.*, 1995b; Yamasaki *et al.*, 1997). In addition, non-O1, non-O139 strains are sometimes associated with severe diarrhoeal cases indistinguishable from those caused by *V. cholerae* O1 and O139 serogroups (Mukhopadhyay *et al.*, 1995a; Sharma *et al.*, 1998b).

The use of antibiotics for the treatment of severe cholera is recommended, as it significantly shortens the duration of diarrhoea, reduces the volume of rehydration and maintenance fluids required and decreases the time-length of faecal excretion of *V. cholerae*, which, in turn, reduces the transmission of infection to other contacts (Sack *et al.*, 2004). Antimicrobial agents such as tetracyclines and fluoroquinolones are often used to treat cholera and, in the past, most strains were susceptible to these and other antibiotics used for the treatment of diarrhoeal diseases (O'Grady *et al.*, 1976). However, reports of toxigenic *V. cholerae* strains resistant to the commonly used antibiotics are appearing with increasing frequency in India, as well as in other countries (Basu *et al.*, 2000; Chakraborty *et al.*, 2001; Dalsgaard *et al.*, 1999a; Garg *et al.*, 2000, 2001; Mitra *et al.*, 1996; Mukhopadhyay *et al.*, 1995b; Ramamurthy *et al.*, 2000; Yamamoto *et al.*, 1995a, b).

Bacteria become resistant to antibiotics by mutation and/or by the acquisition of foreign DNA in the form of plasmids and transposons. In addition, elements known as integrons have been recognized as a mechanism for the acquisition and spread of drug-resistance genes (Hall & Collis, 1995; Mazel & Davis, 1999; Ploy *et al.*, 2000; Recchia & Hall, 1995). An integron consists of three elements, the *intI* gene encoding an integrase, a recombination site, *attI*, and a promoter gene. Several classes of integrons have been characterized based on the integrase gene. Classes 1, 2 and 3 represent the so-called multi-resistance integrons, and have largely been implicated in the acquisition and dissemination of antibiotic-resistance genes (Ploy *et al.*, 2000). A second type of integron, initially called a class 4 integron, but now termed a super-integron, was identified in a *V. cholerae* O1 strain (Mazel *et al.*, 1998), but later was found among other members of the family *Vibrionaceae* (Rowe-Magnus *et al.*, 2001, 2003). This super-integron contains 179 gene cassettes in a 126 kb region, which corresponds to 3% of the total

genome, indicating that it may have a role in genome evolution rather than antibiotic resistance (Mazel *et al.*, 1998).

Some information is available about the distribution and importance of class 1 integrons in encoding antibiotic resistance in clinical strains of *V. cholerae* isolated in various geographic areas (Dalsgaard *et al.*, 1999b, 2000a, b, 2001; Ehara *et al.*, 2004; Falbo *et al.*, 1999; Iwanaga *et al.*, 2004; Thungapathra *et al.*, 2002). However, little is known about the distribution and role of integrons in multidrug-resistant *V. cholerae* strains in India (Thungapathra *et al.*, 2002). In this study, we conducted a comprehensive analysis of the distribution of class 1, 2 and 4 integrons, and the relationship to antibiotic resistance in a series of *V. cholerae* strains isolated over a period of time from a single geographical area.

METHODS

Bacterial strains. A total of 133 clinical strains of *V. cholerae* isolated from acute diarrhoeal patients from 1992 to 2000 in Kolkata, India, was used in this study (Table 1). Three to six strains each of O1, O139 and non-O1, non-O139 serogroups were randomly selected to represent each year from 1992 to 2000. The serogroup of toxigenic *V. cholerae* O1 or O139 was reconfirmed by using the multiplex PCR described by Hoshino *et al.* (1998) using the primer sets described in Table 2. *V. cholerae* O1 strain SK-10 (for the *intI1* gene and 3'-CS) (Dalsgaard *et al.*, 2000a), *Escherichia coli* carrying the R483::Tn7 plasmid (*intI2*) (Falbo *et al.*, 1999) and *V. cholerae* O1 strain N16961 (*intI4*) were used for preparation of the DNA probes and as a positive control for each gene. *E. coli* C600 was used as a negative control in all experiments.

Chemicals, media, enzymes and kits. Chemicals were purchased from Nakalai Tesque, Wako Pure Chemicals or Sigma. Proteinase K was from Nakalai Tesque. Restriction enzymes and *Taq* DNA polymerase were purchased from Takara Shuzo or New England Biolabs. [α - 32 P]dCTP (111 TBq mmol $^{-1}$) was from Perkin Elmer. The Multi-prime DNA Labelling System was from Amersham Biosciences and the DNA Labelling and Detection kit was from Roche Diagnostics. The PCR Purification kit was purchased from Qiagen and the Big Dye Terminator Sequencing kit was from Applied Biosystems. NuSieve GTG agarose for the analysis of DNA fragments was from Takara Shuzo. Molecular mass markers for electrophoresis were purchased from Takara Shuzo or Gibco-BRL. Tryptone and yeast extract for L-broth and L-agar were from Difco Laboratories. Thiosulfate-citrate-bile salts-sucrose agar was from Eiken Kagaku.

Antimicrobial susceptibility testing. Antimicrobial susceptibility analysis of *V. cholerae* was performed by a disc diffusion method using commercial discs (Hi Media) with ampicillin (10 μ g), chloramphenicol (30 μ g), ciprofloxacin (5 μ g), co-trimoxazole (25 μ g), furazolidone (50 μ g), gentamicin (10 μ g), nalidixic acid (30 μ g), neomycin (30 μ g), norfloxacin (10 μ g), streptomycin (10 μ g) and tetracycline (30 μ g) as described previously (Garg *et al.*, 2000). *E. coli* ATCC 25922 was used for the quality control of each assay.

PCR amplification. DNA from *V. cholerae* strains used for the PCR template was prepared from overnight L-broth cultures at 37°C. The culture was diluted tenfold in 10 mM Tris/HCl (pH 8.0) containing 1 mM EDTA and the suspension was boiled for 10 min. After centrifugation at 12 000 g for 3 min, the supernatant was stored at -20°C. PCR was carried out in 50 μ l vols containing 5 μ l template DNA, 5 μ l 10 $\times$ concentrated PCR buffer [100 mM Tris/HCl

Table 1. Distribution of the *int1*, *int2* and *int4* genes in clinical strains of *V. cholerae* isolated from 1992 to 2000 in Kolkata, India

Serogroup	Year of isolation	No. of strains	No. of strains positive for:			
			<i>int1</i>	<i>int2</i>	<i>int4</i>	3'-CS
O1	1992	5	4	0	5	4
	1993	4	2	0	4	2
	1994	5	1	0	5	1
	1995	5	0	0	5	0
	1996	5	0	0	5	0
	1997	5	0	0	5	0
	1998	5	0	0	5	0
	1999	5	0	0	5	0
	2000	5	0	0	5	0
Total		44	7	0	44	7
O139	1992	6	1	0	6	1
	1993	5	0	0	5	0
	1994	5	0	0	5	0
	1995	5	0	0	5	0
	1996	4	0	0	4	0
	1997	5	0	0	5	0
	1998	5	0	0	5	0
	1999	5	0	0	5*	0
	2000	5	0	0	5	0
Total		45	1	0	45	1
Non-O1, non-O139	1992	6	1	0	6	1
	1993	5	1	0	5	1
	1994	5	1	0	5	1
	1995	5	0	0	5	0
	1996	3	0	0	3	0
	1997	5	0	0	5	0
	1998	5	1	0	5	1
	1999	5	2	0	5	2
	2000	5	0	0	5	0
Total		44	6	0	44	6
Overall total		133	14	0	133	14

*Three of the five strains contained IS1359 in the *int4* gene.

(pH 8.3), 500 mM KCl, 15 mM MgCl₂, 5 µl (10 pmol µl⁻¹) each of appropriate primers, 4 µl dNTP mix (2.5 mM each dNTP), 0.25 µl (5 U µl⁻¹) *Taq* DNA polymerase and 25.75 µl sterilized distilled water. The PCR primers and conditions are given in Table 2. All PCR assays were performed using an automated thermal cycler (GeneAmp PCR System 2400; Applied Biosystems). Primer sets INT-1U/INT-1D, INT-2U/INT-2D and INT-4U/INT-4D were used to detect the conserved regions of the integron-encoded integrase genes *int1*, *int2* and *int4*, respectively. The primer set qacEA1-F and sul1-B was used to determine whether the class 1 integron contained the 3'-conserved segment (CS) (Dalsgaard *et al.*, 2000a). Strains yielding PCR products with the primer sets INT-1U/INT-1D and qacEA1-F/sul1-B were amplified further using the integron primer set in-F/in-B (Dalsgaard *et al.*, 2000a), which amplified the region between the 5'-CS and 3'-CS (Fig. 1). PCR products were analysed by electrophoresis in 1.5 or 3% agarose gels, stained with ethidium bromide, visualized under UV light and recorded with the aid of a gel documentation system (Bio-Rad).

DNA sequencing and analysis. PCR products were purified using the QIAquick PCR Purification kit (Qiagen) and their nucleotide

sequences determined using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit on an ABI PRISM 310 Genetic Analyser (Perkin-Elmer Japan Applied Biosystems). Nucleotide and amino acid sequences were analysed and compared using GenBank and DDBJ sequences and DNASIS software (Hitachi Software Engineering).

Colony hybridization test. A colony hybridization test was carried out as described previously (Mosely *et al.*, 1980) using nitrocellulose membranes (Schleicher & Schuell) or Hybond-N⁺ (Amersham Biosciences) under high-stringency conditions. DNA probes for *int1*, *int2*, *int4* and 3'-CS were prepared by PCR using the primers described in Table 2, with *V. cholerae* O1 SK-10, *E. coli* with the R483::Tn7 plasmid or *V. cholerae* O1 N16961 as template. PCR products were purified using the QIAquick PCR Purification kit according to the manufacturer's instructions and used as DNA probes. DNA probes were labelled either by random priming using the Multiprime DNA Labelling System and [α -³²P]dCTP or with the DNA Labelling and Detection kit.

Preparation of genomic DNA. Genomic DNA from the strains was prepared using the method described by Wilson (1987).

Table 2. PCR primers used in this study

Primer	Sequence (5'→3')	Target	PCR conditions			Amplicon size (bp)	Reference
			Denaturation	Annealing	Extension		
INT-1U	GTTCGGTCAAGGTTCTG	<i>intI1</i>	94 °C, 30 s	50 °C, 30 s	72 °C, 90 s	923	This study
INT-1D	GCCAACTTTCAGCACATG						
INT-2U	ATGTCTAACAGTCCATTTT	<i>intI2</i>	94 °C, 30 s	50 °C, 30 s	72 °C, 60 s	450	Falbo <i>et al.</i> (1999)
INT-2D	AAATCTTTAACCCGCAAAC						
INT-4U	GTGTTTCGCGAATTTATGC	<i>intI4</i>	94 °C, 30 s	50 °C, 30 s	72 °C, 90 s	936	This study
INT-4D	ACGGGATAATGGGCTTAA						
in-F	GGCATACAAGCAGCAAGC	Gene cassette	94 °C, 30 s	52 °C, 30 s	72 °C, 120 s	Variable	Dalsgaard <i>et al.</i> (2000a)
in-B	AAGCAGACTTGACCTGAT						
qacEΔ1-F	ATCGCAATAGTTGGCGAAGT	3'-CS	94 °C, 30 s	56 °C, 30 s	72 °C, 60 s	800	Dalsgaard <i>et al.</i> (2000a)
sul1-B	GCAAGGCGGAAACCCGCGCC	O1 <i>rfb</i>	94 °C, 60 s	55 °C, 60 s	72 °C, 60 s	308	Hoshino <i>et al.</i> (1998)
O1F2-1	GTTTCACTGAACAGATGGG						
O1R2-2	GGTCATCTGTAAGTACAAC	O139 <i>rfb</i>	94 °C, 60 s	55 °C, 60 s	72 °C, 60 s	449	Hoshino <i>et al.</i> (1998)
O139F2	AGCCTCTTTATTACGGGTGG						
O139R2	GTCAAACCCGATCGTAAAGG	<i>ctxA</i>	94 °C, 60 s	55 °C, 60 s	72 °C, 60 s	192	Hoshino <i>et al.</i> (1998)
VCT1	ACAGAGTGAGTACTTTGACC						
VCT2	ATACCATCCATATATTTGGGAG						

Southern blot hybridization test. Genomic DNA was digested by *Eco*RI or *Eco*RV and separated by electrophoresis in a 0.7% agarose gel. DNA fragments were transferred to a Hybond-N⁺ membrane (Amersham Biosciences) and hybridized under high-stringency conditions with the *intI1* gene probe. Radioactivity was analysed using BAS FLA-3000 (Fujifilm).

RESULTS AND DISCUSSION

The panel of 133 strains of *V. cholerae* O1, O139 and non-O1, non-O139 serogroups was analysed for the distribution of class 1, 2 and 4 integrons (Table 1) and for resistance to antimicrobial agents (data not shown). Most of the strains

were multidrug resistant (three or more drugs) with several combinations of resistance. In *V. cholerae* O1, the rate of multidrug resistance increased year by year: the six strains isolated before 1993 were resistant to only one or two antimicrobial agents tested, whilst the 38 strains isolated after 1993 were multidrug resistant. However, antimicrobial profiles of *V. cholerae* O139 and non-O1, non-O139 serogroups varied irrespective of the year of isolation. Nineteen and 28 strains of O139 and non-O1, non-O139, respectively, were resistant to one or two antimicrobial agents tested, whilst 26 and 16 were resistant to three to five drugs, respectively.

Fig. 1. Class 1 integron structure and resistance gene cassettes found in *V. cholerae* O1, O139 and non-O1, non-O139 strains. The 5'-CS (containing the *intI1* gene) and the 3'-CS (containing the *qacEΔ1* and *sul1* genes) are shown by open boxes. Att1 sites are indicated by a filled bar at the end of the integrase 1 (*intI1*) gene. The individual gene cassettes are shown together with their recombination sites, a 59 bp element indicated by a circle.

A correlation between antimicrobial resistance and the presence of integrons was explored by PCR and/or colony hybridization tests for the *intI1* and *intI2* genes. Although none of the strains produced an amplicon of the *intI2* gene, a fragment of ~900 bp corresponding to the *intI1* gene was amplified from 14 strains using the INT-IU and INT-ID primers (data not shown). Table 1 shows that, among 44 strains of the O1 serogroup, four strains isolated in 1992, two strains isolated in 1993 and one strain from 1994 were positive for the *intI1* gene. Although O139 strains have not been reported in the literature to be positive for class 1 integrons (Dalsgaard *et al.*, 2000a), one of the 45 O139 strains tested in this series, which was isolated in 1992, tested positive for the presence of the *intI1* gene. This is the first report, to our knowledge, of this class of integron in this serogroup. Six out of 44 non-O1, non-O139 strains harboured the *intI1* gene (Table 1). The 900 bp fragment of the *intI1* gene obtained from the 14 strains was sequenced and confirmed to be identical to the sequence of the *intI1* gene reported previously (data not shown). To confirm whether a complete class 1 integron exists in *intI1*-positive *V. cholerae* strains, 3'-CS, which contains the *qacEA1* and *sul1* genes, was also amplified using the primer set indicated in Table 2. Each of the 14 strains positive for the *intI1* gene gave a fragment of ~800 bp, which corresponds to the expected size of 3'-CS (data not shown). A colony hybridization test with *intI1* and 3'-CS gene probes also confirmed the presence of the integron in these strains. Although a class 1 integron may not always contain the entire 3'-CS segment, these 14 *V. cholerae* strains isolated in India possessed a complete class 1 integron.

The presence of a class 1 integron varied among serogroups. For example, seven of the *V. cholerae* O1 strains isolated between 1992 and 1994 were positive for class 1 integron, corresponding to 50 % of the isolates recovered in this period. Our findings are in agreement with Amita *et al.* (2003), who reported that the class 1 integron was widely distributed among O1 strains isolated in India before the initial O139 outbreak in 1992, but was less frequent in O1 strains isolated after that year. Similar observations have also been reported from other Asian countries for isolates from 1992 to 1996, including Laos (1993–1996) (Iwanaga *et al.*, 2004), Albania and Italy (1994) (Falbo *et al.*, 1999), Vietnam (1994–1996) (Dalsgaard *et al.*, 1999b; Ehara *et al.*, 2004) and Thailand (1992–1996) (Dalsgaard *et al.*, 2000a). None of the *V. cholerae* O1 strains isolated after 1998 in Vietnam and Laos was positive for the class 1 integron (Ehara *et al.*, 2004; Iwanaga *et al.*, 2004). However, *V. cholerae* O1 strains isolated in Guinea-Bissau (1996–1997) (Dalsgaard *et al.*, 2000b) and in 1998 in Mozambique and South Africa (Sharma *et al.*, 1998a) contained a class 1 integron. These data indicate that *V. cholerae* O1 strains carrying this integron in Asian countries before 1996 may have spread to Africa, as has been suggested by others (Dalsgaard *et al.*, 2001; Sharma *et al.*, 1998b).

A class 1 integron was also detected in 14 % of *V. cholerae* non-O1, non-O139 strains in this study. Thungapathra *et al.*

(2002) reported that 23 % (22/94) of non-O1, non-O139 strains isolated in India between 1997 and 1998 contained a class 1 integron. In Thailand, 30 % of isolates of these serogroups isolated from children between 1993 and 1995 were positive compared with 58 % of the strains from adult patients in 1990 (Dalsgaard *et al.*, 2000a). However, in the last study, 45 environmental strains from water, sediment and seafood samples did not contain a class 1 integron, and only one strain isolated from a seafood sample was positive for the element (Dalsgaard *et al.*, 2000a). No environmental strains isolated in India were examined for a class 1 integron in this study.

Since all of the 14 strains carried a complete class 1 integron, we attempted to amplify the variable region to examine the nature of antimicrobial-resistance genes located in the region by using the in-F/in-B primer set shown in Table 2. Amplification of the integron gene cassette of seven *intI1*-positive O1 strains yielded a fragment of approximately 1.0 kb (VC2, VC12, VC18, VC19, CO361 and CO371) or 2.5 kb (CO747), the largest amplicon so far reported in *V. cholerae* (Fig. 2a). Nucleotide sequence analysis revealed that the 1.0 kb fragment contained 1009 bp harbouring the *aadA1* gene homologue, which is responsible for streptomycin and spectinomycin resistance, and that the 2.5 kb fragment contained 2483 bp comprising the *aar-3*, *aacA4*, *dfrA1* and *orfC* genes responsible, respectively, for rifampicin, kanamycin and gentamicin, and trimethoprim resistance, and an unknown ORF (Table 3, Fig. 1). Since the *aadA1* gene homologue differed by 6 and 10 nt from *aadA1a* (GenBank accession no. X12870) and *aadA1b* (GenBank accession no. M95287), respectively, the *aadA1* gene homologue from this study was designated *aadA1c*. Similar amplification of the gene cassette of the *intI1*-positive strain of *V. cholerae* O139 gave a fragment of ~1.2 kb (Fig. 2a), which on sequence analysis was revealed to be 1242 bp and harboured the *dfrA1* and *orfC* genes responsible for trimethoprim resistance and an unknown ORF, respectively (Table 3). Three of the six strains of non-O1, non-O139 serogroup also gave an amplicon of approximately 1.2 kb, and the remainder were characterized by two amplicons in different combinations and of different sizes of approximately 0.7, 1.0 and 1.2 kb (Fig. 2b). The nucleotide sequence of the 1.2 kb fragment confirmed that three strains (SG6, CO775 and PG92) harboured the *dfrA1* and *orfC* genes identical to those of the O139 strain (Table 3, Fig. 2b). Sequence analysis of the other three strains showed that the 0.7 kb fragment (739 bp) contained the *dfrA15* gene (trimethoprim resistance), the 1.0 kb fragment (1009 bp) equated to the *aadA2* gene (streptomycin and spectinomycin resistance) and the 1.2 kb fragment (1197 bp) equated to the *blaP1* gene, which encodes β -lactam resistance. In general, there was good correlation between the antibiotic-resistant phenotype and the presence of drug-resistance genes in the class 1 integron. However, the *aacA4* gene, which encodes kanamycin and gentamicin resistance and was present in the integron of O1 strain CO747, did not show phenotypic resistance to these antibiotics. The detailed arrangement of

Fig. 2. PCR amplification using the in-F and in-B primers specific for the variable region in the class 1 integron in *V. cholerae* O1 and O139 (a), and non-O1, non-O139 serogroups (b). (a) Lanes: M, 500 bp ladder; 1, O1 VC2 (1992); 2, O1 VC12 (1992); 3, O1 VC18 (1992); 4, O1 VC19 (1992); 5, O1 CO361 (1993); 6, O1 CO371 (1993); 7, O1 CO747 (1994); 8, O139 CO406 (1992); 9, O1 SK-10; 10, *E. coli* C600. (b) Lanes: M, 500 bp ladder; 1, SG6 (1992); 2, CO248 (1993); 3, CO775 (1994); 4, PG92 (1998); 5, NLC35 (1999); 6, NLC38 (1999); 7, O1 SK-10; 8, *E. coli* C600.

the class I integrons revealed by this study is shown in Fig. 1 and Table 3.

The *aadA1c* gene, which confers resistance to streptomycin and spectinomycin, was predominant in *V. cholerae* O1 strains isolated in India. This gene, together with *aadA2*, has also been reported in a class 1 integron found in O1 strains in Laos (Iwanaga *et al.*, 2004), Vietnam (Dalsgaard *et al.*, 1999b; Ehara *et al.*, 2004) and Thailand (Dalsgaard *et al.*,

2000a). However, the class 1 integron found in O1 strains from Guinea-Bissau contained *dfrA1* and *orfC* genes (Dalsgaard *et al.*, 2000b), which were more frequent in non-O1 strains in India. Although all 44 O1 strains investigated were resistant to streptomycin, 37 did not contain a class 1 integron, indicating that other mechanisms also contribute to this resistance. Since all of the O1 strains isolated after 1993 were resistant to trimethoprim, a possible explanation is that an SXT constin, a conjugative,

Table 3. Resistance gene cassettes found in *V. cholerae* strains containing class 1 integron

Serogroup	Strain	Year of isolation	Antibiogram	Copy no. of <i>intI1</i> gene	Size (bp) of PCR product(s) obtained with in-F and in-B	Inserted
O1	VC2	1992	FzS	1	1009	<i>aadA1c</i>
	VC12	1992	S	1	1009	<i>aadA1c</i>
	VC18	1992	S	1	1009	<i>aadA1c</i>
	VC19	1992	S	ND	1009	<i>aadA1c</i>
	CO361	1993	ACoS	1	1009	<i>aadA1c</i>
	CO371	1993	S	1	1009	<i>aadA1c</i>
	CO747	1994	CoFzS	2	2483	<i>aar-3-aacA4-dfrA1-orfC</i>
O139	CO406	1992	ACoNS	1	1242	<i>dfrA1-orfC</i>
Non-O1, non-O139	SG6	1992	ACoFzS	3	1242	<i>dfrA1-orfC</i>
	CO248	1993	ACoFzS	2	739, 1009	<i>dfrA15, aadA2</i>
	CO775	1994	CoFz	2	1242	<i>dfrA1-orfC</i>
	PG92	1998	CoNa	1	1242	<i>dfrA1-orfC</i>
	NLC35	1999	ACoFzNa	2	739, 1197	<i>dfrA15, blaP1</i>
	NLC38	1999	ACoFzNaS	2	1009, 1197	<i>aadA2, blaP1</i>

A, Ampicillin; C, chloramphenicol; Cf, ciprofloxacin; Co, co-trimoxazole; Fz, furazolidone; G, gentamicin; Na, nalidixic acid; N, neomycin; Nx, norfloxacin; S, streptomycin; T, tetracycline; ND, not determined.

self-transmissible, integrating element harbouring resistance determinants to trimethoprim, streptomycin, sulfamethoxazole and chloramphenicol (Hochhut *et al.*, 2001; Waldor *et al.*, 1996), may have contributed to streptomycin and trimethoprim resistance in strains lacking the integron. These SXT elements have been reported to be frequent in O1 strains isolated after the initial O139 epidemic (Amita *et al.*, 2003).

Two amplicons were obtained with the in-F/in-B primer set for three non-O1, non-O139 strains. Since two copies of the *intI1* gene have been found in Thai non-O1, non-O139 strains (Dalsgaard *et al.*, 2000a), hybridization with the *intI1* gene probe was performed with 14 *intI1*-positive *V. cholerae* strains. As no *EcoRI* and *EcoRV* sites are present in the sequence of the *intI1* gene, the number of reactive bands obtained by *EcoRI* or *EcoRV* digestion is a good indicator of the copy number of this gene. Two *intI1* genes were detected in a single O1 strain (CO747; Table 3, Fig. 3) and in four out of five non-O1, non-O139 strains, and one strain of the latter serogroup carried three copies (Table 3). These data indicated that two amplicons obtained by the in-F/in-B primer set were derived from a class 1 integron and that two or more copies of the integron are present in some *V. cholerae* strains. However, these results may indicate that the same resistance gene or no gene cassette was present in some class 1 integrons. The *dfrA15*, *aadA2* and *blaP1* genes found in Indian strains have also been reported in Thai non-O1, non-O139 strains (Dalsgaard *et al.*, 2000a), which is supportive of a similar strain in these countries. Thungapathra *et al.* (2002) reported that additional

resistance gene cassettes, such as *aadA1*, *aac(6')*-Ib, *dfrA5* and *ereA2*, and *dfrA12*, *orfC* and *aadA2* genes were present among *V. cholerae* non-O1, non-O139 strains isolated in India between 1997 and 1998.

Analysis of the *V. cholerae* O1 El Tor strain N16961 genome sequence showed that a super-integron structure with 179 gene cassettes upstream of an integrase gene and of ~130 kb corresponds to about 3 % of the genome. Mazel *et al.* (1998) speculated that this super-integron might be responsible for genome evolution rather than the capture of antibiotic-resistance genes. The presence of the *intI4* gene, which is associated with the super-integron, was examined by PCR to establish its frequency in different *V. cholerae* serogroups. Most of the strains gave a fragment of ~900 bp, which is the expected size of *intI4* (Table 2). However, three O139 strains isolated in 1999 produced a fragment of ~2200 bp (Fig. 4), which is 1.3 kb larger than the expected size. These results were confirmed by colony hybridization, which showed that all strains were positive for the gene, regardless of serogroup, suggesting that the *intI4* gene is ubiquitous in the species. Nucleotide sequence analysis of both fragments revealed that the 936 bp fragment was identical to the *intI4* gene. The large fragment contained 2196 nt with an insertion of IS1359 in the *intI4* gene, as shown in Fig. 5, indicating that the active form of integrase 4 cannot be translated. However, the exact function of the super-integron is not yet known and these strains may prove useful for further structure and function studies.

In conclusion, we have demonstrated that class 1 integrons containing different antibiotic-resistance gene cassettes are distributed among clinical strains of *V. cholerae* O1, O139 and non-O1, non-O139 isolated in India. The role of these

Fig. 3. Southern blot hybridization test for the detection of the *intI1* gene after restriction digestion of genomic DNA of *V. cholerae* strains. Lanes: 1–3, *EcoRI* digest of CO747, CO775 and SG6, respectively; 4–6, *EcoRV* digest of CO747, CO775 and SG6, respectively.

Fig. 4. Detection of *intI4* genes among *V. cholerae* strains by PCR. Lanes: 1, 100 bp ladder; 2, O1 VC2 (1992); 3, O139 CO406 (1992); 4, non-O1, non-O139 CO248 (1993); 5, O139 NLC50 (1999); 6, O139 NLC31 (1999); 7, O139 NLC40 (1999); 8, O1 N16961; 9, *E. coli* C600; 10, λ HindIII digest.

Fig. 5. Schematic representation of IS1359 inserted in the middle of the integrase 4 (*intI4*) gene of *V. cholerae* O139.

integrons in the dissemination of antibiotic-resistance genes appears to be less significant for O1 and O139 serogroups and their presence accounted for resistance to relatively few drugs. This suggests that other determinants of antibiotic resistance are functional in these strains. PCR mapping of integrons and DNA sequencing of their genetic content may be a useful epidemiological tool to study the dissemination of antibiotic-resistance genes within *V. cholerae*.

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REFERENCES

- Amita, Chowdhury, S. R., Thungapathra, M., Ramamurthy, T., Nair, G. B. & Ghosh, A. (2003). Class 1 integrons and SXT elements in El Tor strains isolated before and after 1992 *Vibrio cholerae* O139 outbreak, Calcutta, India. *Emerg Infect Dis* 9, 500–502.
- Basu, A., Garg, P., Datta, S. & 8 other authors (2000). *Vibrio cholerae* O139 in Calcutta, 1992–1998: incidence, antibiograms, and genotypes. *Emerg Infect Dis* 6, 139–147.
- Chakraborty, S., Garg, P., Ramamurthy, T. & 9 other authors (2001). Comparison of antibiogram, virulence genes, ribotypes and DNA fingerprints of *Vibrio cholerae* of matching serogroups isolated from hospitalised diarrhoea cases and from the environment during 1997–1998 in Calcutta, India. *J Med Microbiol* 50, 879–888.
- Dalsgaard, A., Forslund, A., Bodhidatta, L., Serichantalergs, O., Pitarangsi, C., Pang, L., Shimada, T. & Echeverria, P. (1999a). A high proportion of *Vibrio cholerae* strains isolated from children with diarrhoea in Bangkok, Thailand are multiple antibiotic resistant and belong to heterogenous non-O1, non-O139 O-serotypes. *Epidemiol Infect* 122, 217–226.
- Dalsgaard, A., Forslund, A., Tam, N. V., Vinh, D. X. & Cam, P. D. (1999b). Cholera in Vietnam: changes in genotypes and emergence of class I integrons containing aminoglycoside resistance gene cassettes in *Vibrio cholerae* O1 strains isolated from 1979 to 1996. *J Clin Microbiol* 37, 734–741.
- Dalsgaard, A., Forslund, A., Serichantalergs, O. & Sandvang, D. (2000a). Distribution and content of class 1 integrons in different

Vibrio cholerae O-serotype strains isolated in Thailand. *Antimicrob Agents Chemother* 44, 1315–1321.

Dalsgaard, A., Forslund, A., Petersen, A. & 7 other authors (2000b). Class 1 integron-borne, multiple-antibiotic resistance encoded by a 150-kilobase conjugative plasmid in epidemic *Vibrio cholerae* O1 strains isolated in Guinea-Bissau. *J Clin Microbiol* 38, 3774–3779.

Dalsgaard, A., Forslund, A., Sandvang, D., Arntzen, L. & Keddy, K. (2001). *Vibrio cholerae* O1 outbreak isolates in Mozambique and South Africa in 1998 are multiple-drug resistant, contain the SXT element and the *aadA2* gene located on class 1 integrons. *J Antimicrob Chemother* 48, 827–838.

Ehara, M., Nguyen, B. M., Nguyen, D. T., Toma, C., Higa, N. & Iwanaga, M. (2004). Drug susceptibility and its genetic basis in epidemic *Vibrio cholerae* O1 in Vietnam. *Epidemiol Infect* 132, 595–600.

Falbo, V., Carattoli, A., Tosini, F., Pezzella, C., Dionisi, A. M. & Luzzi, I. (1999). Antibiotic resistance conferred by a conjugative plasmid and a class I integron in *Vibrio cholerae* O1 El Tor strains isolated in Albania and Italy. *Antimicrob Agents Chemother* 43, 693–696.

Garg, P., Chakraborty, S., Basu, I. & 8 other authors (2000). Expanding multiple antibiotic resistance among clinical strains of *Vibrio cholerae* isolated from 1992–7 in Calcutta, India. *Epidemiol Infect* 124, 393–399.

Garg, P., Sinha, S., Chakraborty, R., Bhattacharya, S. K., Nair, G. B., Ramamurthy, T. & Takeda, Y. (2001). Emergence of fluoroquinolone-resistant strains of *Vibrio cholerae* O1 biotype El Tor among hospitalized patients with cholera in Calcutta, India. *Antimicrob Agents Chemother* 45, 1605–1606.

Hall, R. M. & Collis, C. M. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol* 15, 593–600.

Hochhut, B., Lotfi, Y., Mazel, D., Faruque, S. M., Woodgate, R. & Waldor, M. K. (2001). Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrob Agents Chemother* 45, 2991–3000.

Hoshino, K., Yamasaki, S., Mukhopadhyay, A. K., Chakraborty, S., Basu, A., Bhattacharya, S. K., Nair, G. B., Shimada, T. & Takeda, Y. (1998). Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. *FEMS Immunol Med Microbiol* 20, 201–207.

Iwanaga, M., Toma, C., Miyazato, T., Insisiengmay, S., Nakasone, N. & Ehara, M. (2004). Antibiotic resistance conferred by a class 1 integron and SXT constin in *Vibrio cholerae* O1 strains isolated in Laos. *Antimicrob Agents Chemother* 48, 2364–2369.

Mazel, D. & Davis, J. (1999). Antibiotic resistance in microbes. *Cell Mol Life Sci* 56, 742–754.

Mazel, D., Dychinco, B., Webb, V. A. & Davis, J. (1998). A distinctive class of integron in the *Vibrio cholerae* genome. *Science* 280, 605–608.

Mitra, R., Basu, A., Dutta, D., Nair, G. B. & Takeda, Y. (1996). Resurgence of *Vibrio cholerae* O139 Bengal with altered antibiogram in Calcutta, India. *Lancet* 348, 1181.

Mosely, S. L., Huq, I., Alim, A. R. M. A., So, M., Samadpour-Motalebi, M. & Falkow, S. (1980). Detection of enterotoxigenic *Escherichia coli* by DNA colony hybridization. *J Infect Dis* 142, 892–898.

Mukhopadhyay, A. K., Saha, P. K., Garg, S., Bhattacharya, S. K., Shimada, T., Takeda, T., Takeda, Y. & Nair, G. B. (1995a). Distribution and virulence of *Vibrio cholerae* belonging to serogroups other than O1 and O139: a nationwide survey. *Epidemiol Infect* 114, 65–70.

Mukhopadhyay, A. K., Garg, S., Nair, G. B. & 7 other authors (1995b). Biotype traits and antibiotic susceptibility of *Vibrio cholerae* serogroup O1 before, during and after the emergence of the O139 serogroup. *Epidemiol Infect* 115, 427–434.

- Nair, G. B., Albert, M. J., Shimada, T. & Takeda, Y. (1996). *Vibrio cholerae* O139 Bengal: the new serogroup causing cholera. *Rev Med Microbiol* 7, 43–51.
- O'Grady, F., Lewis, M. J. & Perarson, N. J. (1976). Global surveillance of antibiotic sensitivity of *Vibrio cholerae*. *Bull W H O* 54, 181–185.
- Ploy, M.-C., Lambert, T., Couty, J.-P. & Denis, F. (2000). Integrans: an antibiotic resistance gene capture and expression. *Clin Chem Lab Med* 38, 483–487.
- Ramamurthy, T., Rajendran, K., Garg, P. & 8 other authors (2000). Cluster-analysis and patterns of dissemination of multidrug resistance among clinical strains of *Vibrio cholerae* in Calcutta, India. *Indian J Med Res* 112, 78–85.
- Ramamurthy, T., Yamasaki, S., Takeda, Y. & Nair, G. B. (2003). *Vibrio cholerae* O139 Bengal: odyssey of a fortuitous variant. *Microbes Infect* 5, 329–344.
- Recchia, G. D. & Hall, R. M. (1995). Gene cassettes: a new class of mobile element. *Microbiology* 141, 3015–3027.
- Rowe-Magnus, D. A., Guerout, A.-M., Ploncard, P., Dychinco, B., Davies, J. & Mazel, D. (2001). The evolutionary history of chromosomal super-integrans provides an ancestry for multiresistant integrans. *Proc Natl Acad Sci U S A* 98, 652–657.
- Rowe-Magnus, D. A., Guerout, A.-M., Biskri, L., Bouige, P. & Mazel, D. (2003). Comparative analysis of superintegrans: engineering extensive genetic diversity in the *Vibrionaceae*. *Genome Res* 13, 428–442.
- Sack, D. A., Sack, R. B., Nair, G. B. & Siddique, A. K. (2004). Cholera. *Lancet* 363, 223–233.
- Sharma, C., Thungapathra, M., Ghosh, A. & 11 other authors (1998a). Molecular analysis of non-O1, non-O139 *Vibrio cholerae* associated with an unusual upsurge in the incidence of cholera-like disease in Calcutta, India. *J Clin Microbiol* 36, 756–763.
- Sharma, C., Ghosh, A., Dalsgaard, A., Forslund, A., Ghosh, R. K., Bhattacharya, S. K. & Nair, G. B. (1998b). Molecular evidence that a distinct *Vibrio cholerae* O1 biotype El Tor strain in Calcutta may have spread to the African continent. *J Clin Microbiol* 36, 843–844.
- 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 integrans in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrob Agents Chemother* 46, 2948–2955.
- Waldor, M. K., Tschaeppe, H. & Mekalanos, J. J. (1996). A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. *J Bacteriol* 178, 4157–4165.
- Wilson, K. (1987). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1–2.4.5. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Wiley Interscience.
- Yamamoto, T., Nair, G. B. & Takeda, Y. (1995a). Emergence of tetracycline resistance due to a multiple drug resistance plasmid in *Vibrio cholerae* O139. *FEMS Immunol Med Microbiol* 11, 131–136.
- Yamamoto, T., Nair, G. B., Albert, M. J., Parodi, C. C. & Takeda, Y. (1995b). Survey of *in vitro* susceptibilities of *Vibrio cholerae* O1 and O139 to antimicrobial agents. *Antimicrob Agents Chemother* 39, 241–244.
- Yamasaki, S., Nair, G. B., Bhattacharya, S. K., Yamamoto, S., Kurazono, H. & Takeda, Y. (1997). Cryptic appearance of a new clone of *Vibrio cholerae* serogroup O1 biotype El Tor in Calcutta, India. *Microbiol Immunol* 41, 1–6.