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Vibrio cholerae non-O1, non-O139 strains isolated before 1992 from Varanasi, India are multiple drug resistant, contain *int*SXT, *dfr18* and *aadA5* genes

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Summary

In this study, we report the presence of the SXT element and Class I integron in *Vibrio cholerae* non-O1, non-O139 strains isolated from Varanasi, India. Isolates were resistant to cotrimoxazole, trimethoprim and/or streptomycin, furazolidone and ampicillin. None contained plasmids. Polymerase chain reaction (PCR) and DNA sequencing revealed the presence of antibiotic resistance gene cassettes, *aadA1*, *aadA2*, *aadA5* and *dfrA15*, in the Class I integron and SXT, an integrative conjugative element containing *dfr18*, *sulII* and *strAB*, in three and six of the isolates respectively. Conjugation experiments, followed by PCR analysis of transconjugants, provided evidence for the transferable nature of *int*SXT and associated antibiotic resistance gene cassettes. This is the first report of the occurrence of SXT ICE, *dfr18*, *sulII*, *strAB* and *aadA5* genes in environmental *V. cholerae* non-O1, non-O139 strains from Varanasi, India, that had been isolated before 1992.

Introduction

Mobile genetic elements play a crucial role in the horizontal transfer of genes in bacterial populations. These include a heterogeneous array of elements, such as plasmids, phages, transposons, integrons, etc. The term 'integrative conjugative element (ICE)' was coined to describe mobile genetic elements that transfer via conjugation and undergo replication by integrating with the host chromosome (Burrus *et al*., 2002). Unlike bacteriophages and plasmids, these elements do not have autonomous replication machinery and cannot undergo autonomous replication. Moreover, ICEs share a set of genes performing specific functions, such as excision, transfer and integration, but differ in genes that are not essential for transmissibility (Burrus *et al*., 2006a). In *V. cholerae*, antibiotic resistance determinants have been found associated with Class I integrons and an ICE, the SXT.

SXT^{MO10}, also referred to as SXT element, is a conjugative, self-transmissible, integrative element discovered in *V. cholerae* O139 strain MO10 isolated in 1992 from India (Waldor *et al*., 1996). SXT^{MO10}, ~100kb, was shown to harbour genes encoding resistance to several antibiotics, viz. sulfamethoxazole (SUL), trimethoprim (TMP), chloramphenicol and streptomycin (STR). The SXT element contains a gene encoding an integrase, *int*SXT, closely related to those found in lambdoid bacteriophages, responsible for integration and excision of the element (Beaber *et al*., 2002). SXT element can be conjugally transferred from *V. cholerae* O139 to *V. cholerae* O1 and to *Escherichia coli* strains in a *recA* independent manner, integrating in a site specific manner into the 5′ end of *prf C*- the gene encoding the peptide chain release factor 3, via action of a site specific recombinase (Hochhut and Waldor, 1999). Genetically, as well as functionally, SXT^{MO10} is closely related to R391, an integrating conjugative element derived from a South African strain of *Providencia rettgeri* (Hochhut *et al*., 2001). Besides SXT^{MO10} and R391, several other ICEs, such as R392, R397, R705, R706, R997, pMERPH, SXT^{Laos} and SXT^{ET}, that encode *int*SXT-like integrase and conjugally integrate into the *prfC* site are considered to belong to the SXT-related ICE family (Burrus *et al*., 2006a).

Apart from the SXT element, *V. cholerae* also harbours antibiotic resistant determinants in Class I integrons. Integrons are characterized, depending upon the class of integrase enzyme, into Class 1, 2, 3 or 4, the latter also referred to as the super integron (Mazel *et al*., 1998; 2006; Fluit and Schmitz, 2004). Characteristically, Class I integrons consist of the *intI* gene encoding an integrase, the attI recombination site and a promoter that directs the transcription of the captured gene cassettes. All integron-inserted gene cassettes generally contain an imperfect

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Table 1. Date, place and source of isolation of *V. cholerae* non-O1, non-O139 strains, antibiograms and results obtained by PCR for *int*SXT, antibiotic resistance genes and Class I integron.

				Strains showing presence of genes encoding for					
				SXT element-associated genes				Class I integron	
No	Strain No.	Date, Place and source of isolation	Antibiogram	*int*SXT	*sul*	*str*	*dfrA1/dfr18*	3′ CS	5′ CS
1.	VO190	1979, Varanasi, stool	AMP	–	–	–	–/–	–	–
2.	VO191	1979, Varanasi, stool	–	–	–	–	–/–	–	–
3.	VO193	1988, Varanasi, Gangawater	–	–	–	–	–/–	–	–
4.	VO194	1986, Varanasi, Gangawater	–	–	–	–	–/–	–	–
5.	VO195	1988, Varanasi, Gangawater	AMP	–	–	–	–/–	–	–
6.	VO219	1976, Varanasi, Stool	STR	–	–	–	–/–	+	+
7.	VO220	1979, Varanasi, stool	–	–	–	–	–/–	–	–
8.	VO222	1987, Varanasi, Sewage	STR	+	–	–	–/–	+	+
9.	VO223	1988, Varanasi, Gangawater	–	–	–	–	–/–	–	–
10.	VO224	1986, Varanasi, Gangawater	COT, STR, TMP	–	–	–	–/–	+	+
11.	VO226	1987, Varanasi, Gangawater	–	–	–	–	–/–	–	–
12.	VO321A	1986, Varanasi, *Gerris spinolae*	AMP	–	–	–	–/–	–	–
13.	VO322	1987, Varanasi, Gangawater	COT, TMP	+	–	–	–/–	–	–
14.	VO323	1986, Varanasi, Gangawater	–	–	–	–	–/–	–	–
15.	VO324	1987, Varanasi, Gangawater	COT, STR, TMP	+	+	+	–/–	–	–
16.	VO325	1986, Varanasi, Gangawater	–	–	–	–	–/–	–	–
17.	VO325A	1987, Varanasi, Gangawater	AMP	–	–	–	–/–	–	–
18.	VO327	1986, Varanasi, Gangawater	AMP	–	–	–	–/–	–	–
19.	VO327A	1986, Varanasi, Gangawater	AMP	–	–	–	–/–	–	–
20.	VO328	1987, Varanasi, Gangawater	COT, STR, TMP, FUR	+	+	+	–/–	–	–
21.	VO329	1986, Varanasi, Gangawater	AMP, COT, STR, TMP, FUR	+	+	+	–/+	–	–
22.	VO331	1984, Varanasi, Gangawater	AMP	–	–	–	–/–	–	–
23.	VO332	1986, Varanasi, Gangawater	COT, STR, TMP	+	+	+	–/+	–	–
24.	VO333	1987, Varanasi, Gangawater	AMP	–	–	–	–/–	–	–
25.	VO334	1987, Varanasi, Gangawater	AMP, FUR	–	–	–	–/–	–	–
26.	VC20	1989, Kolkata (Control strain)	AMP	–	–	–	–	+	+
27.	MO45	ATCC 51394 (Control strain)	COT, FUR, STR, SUL, TMP	+	+	+	–/+	–	–

repeat at the 3′ end of the gene, called attC site or 59 base element (Stokes *et al*., 1997). Often, but not always, Class I integrons at their 3′ end harbour genes encoding resistance to quaternary ammonium compounds and sulfonamides, *qacEΔ1* and *sul* (Recchia and Hall, 1995; Dalsgaard *et al*., 2000).

In this study, we discovered the presence of the SXT element and Class I integrons in *Vibrio cholerae* non-O1, non-O139 strains isolated from Varanasi, India, before 1992. This study has implications for comparative genetic analysis that will develop an improved understanding of the origin of the SXT element in *V. cholerae*.

Results and discussion

Most of the *V. cholerae* non-O1, non-O139 strains used in the study were multiply drug resistant (Table 1). Multiple antibiotic resistance and temporal and spatial changes in antibiotic resistance profiles of *V. cholerae* are well known (Mitra *et al*., 1996). Results of antibiotic susceptibility tests revealed that most of the isolates in this study were resistant to ampicillin (AMP), cotrimoxazole (COT), TMP and/or STR, and all were sensitive to tetracycline (TET), gentamycin, nalidixic acid (NAL) and/or ciprofloxacin (CIP). Our results corroborate the findings of Amita and colleagues (2003) who also observed widespread resistance to STR and TMP but not to TET, gentamycin and CIP in *V. cholerae* O1 El Tor strains isolated from Calcutta after 1992. Thungapathra and colleagues (2002) reported various combinations of multiple drug resistance among environmental and clinical non-O1, non-O139 strains, with multiple drug resistance more common among clinical isolates. However, we found that, except for strain VO219, all isolates showing multiple drug resistance were from environmental sources.

Three of 25 isolates, VO219, VO222 and VO224, possessed the ~800 bp amplicon, when tested using Class I integron primers targeted at 3′ CS and 5′ CS respectively. Polymerase chain reaction (PCR) with in-F and in-B primers yielded an amplicon of ~1000 bp from all three isolates and strain VO224 yielded an additional amplicon of ~750 bp (Fig. 1A), indicating that strain VO224 possessed two copies of Class I integron, confirmed by Southern hybridization of EcoRI digested genomic DNA, which does not cut within Class I integron, using inDS-F and inDS-B amplified product of VC20 as the probe (Fig. 1B). As was expected, the isolates also amplified ~1000 bp products with primers in-F and aadA-B. Sequencing results confirmed the presence of STR/spectinomycin resistance gene cassette *aadA5* (VO219), not reported in

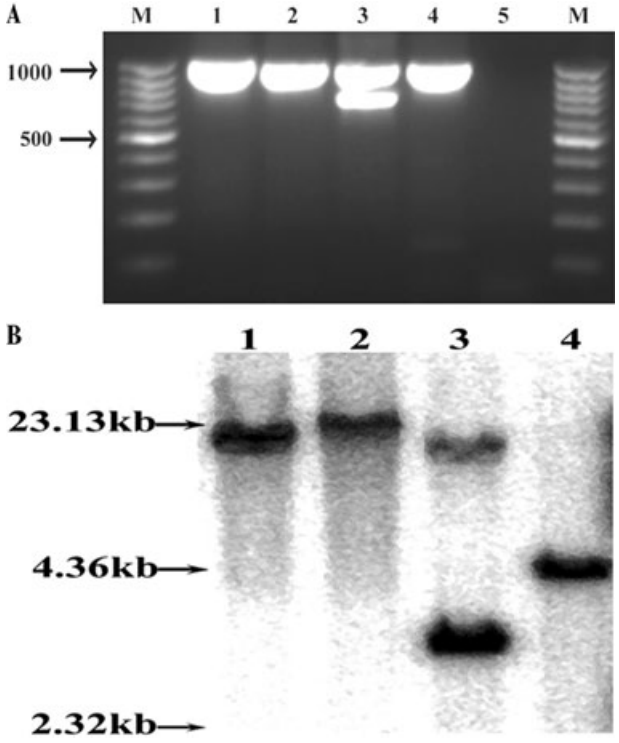

Fig. 1. A. Agarose gel of PCR products stained with ethidium bromide, obtained using in F and in B primers specific for the variable region of Class I integron in *V. cholerae* non-O1, non-O139 strains. Lanes M, 100 bp DNA ladder; lane 1, *V. cholerae* non-O1, non-O139 strain VO219; lane 2, *V. cholerae* non-O1, non-O139 strain VO222; lane 3, *V. cholerae* non-O1, non-O139 strain VO224; lane 4, *V. cholerae* O1 strain VC20 (positive control); lane 5, PCR mixture (negative control).
B. Southern hybridization of genomic DNA of *V. cholerae* non-O1, non-O139 strains digested with EcoRI and probed with *intI1* gene. Lane 1, *V. cholerae* non-O1, non-O139 strain VO219; lane 2, *V. cholerae* non-O1, non-O139 strain VO222; lane 3, *V. cholerae* non-O1, non-O139 strain VO224; lane 4, *V. cholerae* O1 strain VC20 (positive control).

V. cholerae, *aadA1* (VO222) and *aadA2* (VO224). These results are similar to those reporting *aadA* gene cassettes to be the most widely distributed gene cassettes in the Class I integrons of *V. cholerae* (Dalsgaard *et al*., 1999; 2001; Iwanaga *et al*., 2004; Ceccarelli *et al*., 2006; Shi *et al*., 2006). Subsequent sequencing of the additional amplicon of ~750 bp of strain VO224, confirmed the presence of TMP resistance gene cassette *dfrA15* (Figs 1A and 2). Thus, this observation suggests that strain VO224 also possessed the TMP resistance gene cassette *dfrA15*, along with *aadA2* gene cassette. Schematic representation of Class I integron and resistance gene cassette found in *V. cholerae* non-O1, non-O139 strains is shown in Fig. 2.

Four of the 25 strains of *V. cholerae* non-O1, non-O139 showed resistance to antibiotics COT, STR and TMP, but were devoid of Class I integron, determined by PCR. While screening isolates for the presence of *int*SXT and *sulII*, *strB* and *dfrA1*/*dfrA18* genes by multiplex PCR assay, six isolates; VO222, VO322, VO324, VO328, VO329 and VO332, yielded the expected amplicon of 946 bp for *int*SXT (Fig. 3A, Table 1). Two isolates, VO329 and VO332, yielded the expected amplicons of 626 bp for *sulII*, 515 bp for *strB* and 389 bp for *dfr18* genes, whereas two other isolates, VO324 and VO328, yielded expected amplicons of 626 bp for *sulII*, 515 bp for *strB*, but did not amplify products for the *dfr18/dfrA1* gene, although both showed phenotypic resistance to TMP. The remaining two isolates, VO222 and VO322, although positive for the *int*SXT gene, were negative by PCR for *sulII*, *strB*, *dfrA1*/*dfr18* genes (Fig. 3A, Table 1). However, strain VO222 also possessed the Class I integron. All four isolates, with the amplified fragment of *strB* gene, also had the expected amplicon of ~383 bp for *strA* gene, indicating the presence of a complete set of STR resistance genes. Furthermore, the self-transmissible and integrative nature of the element was confirmed by conjugation experiments using *E. coli* strain MTCC 1652 or DH5α as the recipients and representative *V. cholerae* strains VO324, VO328, VO329 and VO332, as donors. The conjugational transfer frequencies for these strains were 4.76×10^{-5}, 1.95×10^{-6}, 0.57×10^{-6} and 4.5×10^{-5} respectively. The transconjugants, when tested by PCR/multiplex PCR, gave positive results for the *int*SXT, *sulII*, *strAB* and/or *dfr18* genes identical to those found with donor strains (Fig. 3B), indicating that *int*SXT and associated genes were transferable. Overall, the antibiotic susceptibility of *V. cholerae* non-O1, non-O139 transconjugants indicated that the resistance gene contained in the SXT element was transferred and expressed in each of the transconjugants. DNA sequencing of the purified 946 bp PCR product, followed by sequence alignment and comparison of *int*SXT with other GenBank sequences, using BLAST software, showed 95–96% identity with five strains, VO322, VO324, VO328, VO329 and VO332, whereas strain VO222 showed 93% identity and the control strain, MO45, showed 97% sequence identity with the *int*SXT gene of *V. cholerae* O139 strain MO10 (Accession No. AF099172).

Based on these results, we investigated relationships, if any, between the *int*SXT of non-O1, non-O139 strains and *V. cholerae* MO10. The phylogenetic tree constructed using *int*SXT displayed two distinct clusters, one consisting of MO10, MO45, along with non-O1, non-O139 strains, except for VO222, which diverged and formed a separate cluster (Fig. 4). Strain MO45 clustered with MO10, while strains VO322, VO324, VO328, VO329 and VO332 formed a separate subcluster (Fig. 4). High bootstrap values were obtained at both cluster nodes, whereas the low branch length values obtained suggested less variation in the nucleotide sequences. DNA sequencing of the purified 389 bp PCR amplified product obtained for strains VO329 and VO332, followed by

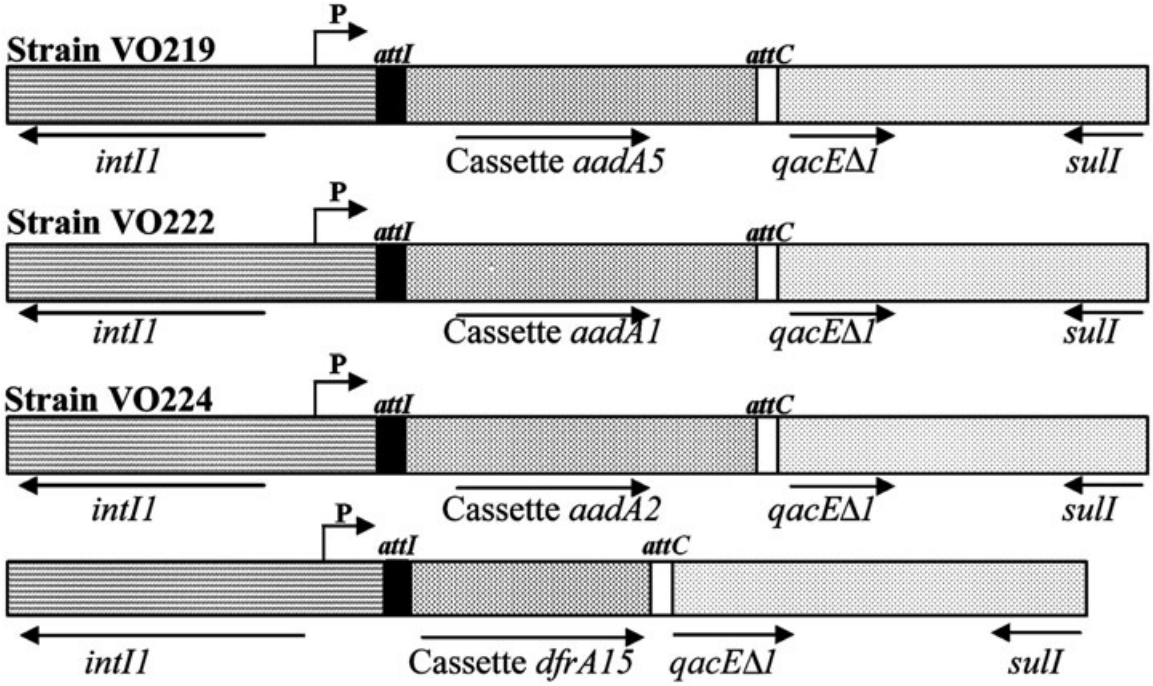

Fig. 2. Schematic representation of Class I integron structure and resistance gene cassettes found in *V. cholerae* non-O1, non-O139 strains, VO219, VO222 and VO224. ▨: 5′ CS (containing the *int*I gene); ▩: 3′ CS (containing the *qacEΔ1* and *sulI* genes); □: individual antibiotic resistance gene cassette with recombination site, 59 bp element.

sequence alignment and comparison with other GenBank sequences, showed 100% identity, while MO45 showed 99% identity with the *dfr18* gene of the *Vibrio cholerae* O139 strain MO10 (Accession No. AY034138).

Based on the results of the preceding sections, strains containing Class I integron and/or SXT element were tested for presence of plasmids. Repeated attempts to isolate plasmids did not yield plasmid DNA, when compared with the control strain *E. coli* V517. Thus, it is concluded that the strains containing either Class I integron and/or SXT element did not harbour plasmids.

Analysis of *V. cholerae* non-O1, non-O139 strains isolated from environmental and clinical sources between 1997 and 1998 showed the presence of *int*SXT (Thungapathra *et al*., 2002). However, there are no reports of the occurrence of *int*SXT in environmental non-O1, non-O139 strains isolated before 1992. Amita and colleagues (2003) showed that in *V. cholerae* there was a clear difference in strains isolated before, during and after 1992. About 80% of the strains in the sampling from before 1992 had integrons, while less than 20% had *int*SXT. This percentage dramatically changed in samples obtained during and after 1992. More than 90% of the strains harboured *int*SXT and about 10% harboured integrons. However, in the present study about 24% of the non-O1, non-O139 environmental strains isolated before 1992 had *int*SXT, while less than 12% harboured integrons.

SXT-associated TMP resistance genes viz. *dfr18*, *dfrA1* have been reported in *V. cholerae*. That is, in *V. cholerae* O139 strain MO10, resistance to TMP was encoded by the *dfr18* gene, located within the antibiotic gene cluster of SXT^{MO10}, while in the *V. cholerae* O1 El Tor strain, the TMP resistance gene *dfrA1* was located ~70 kbp away from the antibiotic resistance cluster of SXT^{ET} (Hochhut *et al*., 2001). It is known that *dfr18* is specific for *V. cholerae* O139 strains, while *dfrA1* is specific for *V. cholerae* O1 El Tor strains (Ramachandran *et al*., 2007). Two isolates showed presence of the *int*SXT, *str*B, *sulII* and *dfr18* genes, a first reporting of the occurrence of *int*SXT and *dfr18* genes, with *sulII* and *strAB* genes, identical to those of *V. cholerae* O139 strain MO10, in environmental *V. cholerae* non-O1, non-O139 strains isolated before 1992. SXT-related ICE from *V. cholerae* O1 El Tor strains isolated from Laos (SXT^{Laos}) contained ~3.34 kbp of an internal deletion in the *dfr18* region, but harboured the TMP resistance determinant elsewhere in the chromosome, which was co-transferrable during conjugational transfer of SXT (Iwanaga *et al*., 2004). Similar to the SXT^{Laos}, *V. cholerae* non-O1, non-O139 strains VO324 and VO328 showed phenotypic resistance to TMP, but failed to amplify either the *dfrA1* or *dfr18* gene. Unlike the Laotian strains, TMP resistance was not co-transferred *via* conjugation, suggesting at the presence of the TMP resistance determinant elsewhere in the chromosome,

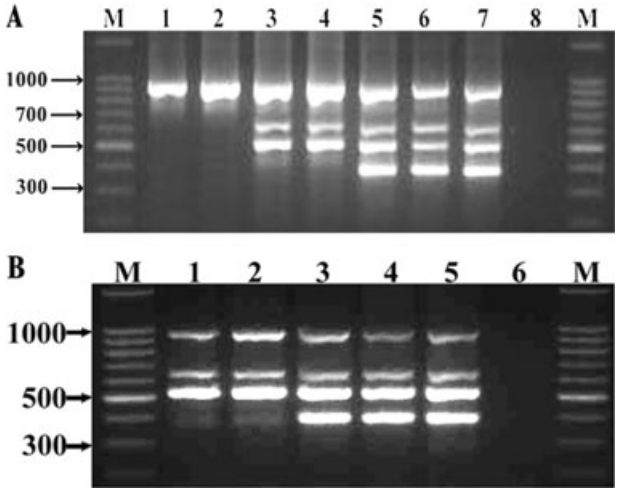

Fig. 3. A. Ethiduim bromide-stained agarose gel electrophoresis of multiplex PCR products from *V. cholerae* non-O1, non-O139 strains. Lanes M, 100 bp DNA ladder; lane 1, *V. cholerae* non-O1, non-O139 strain VO222; lane 2, *V. cholerae* non-O1, non-O139 strain VO322; lane 3, *V. cholerae* non-O1, non-O139 strain VO324; lane 4, *V. cholerae* non-O1, non-O139 strain VO328; lane 5, *V. cholerae* non-O1, non-O139 strain VO329; lane 6, *V. cholerae* non-O1, non-O139 strain VO332; lane 7, *V. cholerae* O139 strain ATCC 51394 MO45 (positive control); lane 8, PCR mixture (negative control).
B. Ethidium bromide stained agarose gel electrophoresis of multiplex PCR products obtained from transconjugants following the transfer of *intSXT* and associated antibiotic resistance gene(s) from donor *V. cholerae* non-O1 non-O139 strains to recipient *E. coli* strain. Lanes M, 100 bp DNA ladder; lane 1, *V. cholerae* non-O1, non-O139 strain VO324; lane 2, *V. cholerae* non-O1, non-O139 strain VO328; lane 3, *V. cholerae* non-O1, non-O139 strain VO329; lane 4, *V. cholerae* non-O1, non-O139 strain VO332; lane 5, *V. cholerae* O139 strain ATCC 51394 MO45 (positive control); lane 6, *E. coli* strain (negative control).

not co-transferable with the SXT element. Moreover, PCR amplified products could not be obtained with the SXT^{Laos} specific primers EXO-F and EXO-R (targeted at *orf1*) used to characterize *int*SXT in these strains (data not shown). The results of this study thus indicate that SXT-related ICEs of the two strains, VO324 and VO328, differ from SXT^{ET} as well as SXT^{Laos}, perhaps another interesting variant in the family of SXT-related ICEs. Further characterization of these SXT elements is in progress.

Presence of antibiotic resistance gene clusters is not a hallmark feature of the SXT related ICEs. As in case of strains; VO222 and VO322, the SXT-related ICEs, ICE*Vch*Mex1 in an environmental *V. cholerae* strain from Mexico and ICE*Vch*HKO1 in a *V. cholerae* O139 strain from Hong Kong, lack the antibiotic resistance genes (Hochhut *et al*., 2001; Burrus *et al*., 2006b). 'Empty SXT constins' have also been reported from many of the Laotian strains (Iwanaga *et al*., 2004).

In conclusion, the results obtained in the present study show existence of the SXT element and associated antibiotic resistance genes, including the *V. cholerae* O139 specific TMP resistance gene *dfr18*, in *V. cholerae* non-O1 non-O139 strains isolated before 1992 from the water of the Ganga river. It is not known whether SXT^{MO10} derived from, or is related to, these *V. cholerae* non-O1, non-O139 strains. Only a comprehensive analysis of the SXT element in non-pathogenic environmental isolates of *V. cholerae* can provide definitive answers to these questions.

Experimental procedures

Bacterial strains

A total of 25 *V. cholerae* non-O1, non-O139 strains from our laboratory stock collection that had been previously identified were included in the study. *Vibrio cholerae* O1 strain VC20, positive for Class I integron and O139 strain ATCC 51394 (MO45), positive for *int*SXT and associated genes encoding for SUL, STR and TMP, were used as controls. The year and source of isolation of the strains are presented in Table 1. All bacterial strains were maintained in LB broth supplemented with 30% glycerol at –80°C. Before use, the identity of cultures was confirmed by selected biochemical and serological tests (WHO, 1987).

Antibiotic susceptibility

Each of the strains was tested for susceptibility to 14 antibiotics, using the disc diffusion method described by Bauer and colleagues (1966). Commercial antibiotic disks (Hi Media, Mumbai, India) with the following potency were used: AMP (10 μg); cephotaxime (30 μg); cephalexin (30 μg);

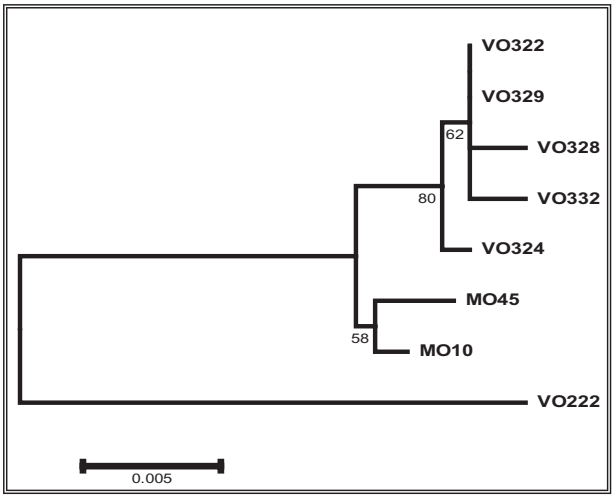

Fig. 4. Neighbour joining tree constructed by the modified Nei–Gojobori (p-distance) method, with the nucleotide sequence of *int*SXT gene fragment of *V. cholerae* non-O1, non-O139 strains and *V cholerae* O139 strains MO10 and MO45. Construction and bootstrapping of the trees were carried out using MEGA 3.0 suite programs. One thousand bootstrap replicates were performed for each analysis and bootstrap values are given at the nodes.

chloramphenicol (30 μg); CIP (5 μg); COT (25 μg); furazolidone (FUR, 50 μg); gentamicin (10 μg); neomycin (30 μg); NAL (30 μg); norfloxacin (10 μg); STR (10 μg); TET (30 μg); and TMP (5 μg). *Escherichia coli* strain ATCC 25922 was used as control for each assay. The diameter of the inhibition zones was recorded and interpreted following NCCLS standards proposed for the family *Enterobacteriaceae* (NCCLS, 1998).

Bacterial DNA isolation

Chromosomal DNA was extracted from overnight cultures grown at 37°C for each of the strains, using CTAB (cetyltrimethyammonium bromide), as described by Ausubel and colleagues (1995).

Plasmid DNA was isolated from the overnight cultures of each strain, following the alkaline lysis protocol of Birnboim and Doly (1979). The plasmid DNA was dissolved in 20 μl of 0.1× Tris-EDTA buffer, separated in 0.8% agarose gels, stained with ethidium bromide and visualized using the gel documentation system (Bio-Rad, USA). Plasmid sizes were estimated from the migration distance in agarose gel, relative to the migration distance of reference plasmids in *E. coli* V517 (Macrina *et al*., 1987; Dalsgaard *et al*., 1999).

PCR amplification

The presence of Class I integron in *V. cholerae* non-O1, non-O139 strains was determined sequentially by PCR using the method of Dalsgaard and colleagues (1999) with primers targeted at 5′ and 3′ conserved segments of the integron. In brief, PCR was performed with 25 μl of reaction mixture in a 200 μl PCR tube containing 100 ng of template DNA with initial denaturation of 94°C for 2 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 63.5° and 58°C, respectively, extension at 72°C for 1 min and final extension at 72°C for 10 min in a thermal cycler (Bio-Rad, USA). Isolates showing positive results with either of the primers were further amplified using primers targeted at the variable region of the integron at an annealing temperature of 55°C for 1 min.

The presence of *int*SXT and associated antibiotic resistance genes was determined by multiplex PCR following the method of Ramachandran and colleagues (2007), in 200 μl PCR tubes containing reaction mixture volumes of 30 μl. PCR was programmed as follows: an initial denaturation at 94°C for 2 min, followed by 35 cycles consisting of 94°C for 1 min, 60.5°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. Whereas a single PCR assay for detection of the *strA* gene was carried out in reaction mixture volumes of 25 μl, with 2.0 μl of forward and reverse primers at an annealing temperature of 65°C for 1 min, other conditions remained the same as for the multiplex PCR. Amplified products were separated by agarose (1.5%, w/v) gel electrophoresis in Tris-acetate-EDTA (1× TAE), stained in ethidium bromide and visualized using the Gel Doc-XR system (Bio-Rad). PCR negative and positive strains were verified by Southern blot hybridization. Following screening, primers targeted at the *int*SXT and *dfr18* genes, was used to amplify a segment of the i*nt*SXT and *dfr18* for sequencing. Primers used in PCR amplification are listed in Table 2.

DNA sequencing and analysis

Polymerase chain reaction products of variable regions of Class I integron, *int*SXT (946 bp) and *dfr18* (389 bp) genes were purified using GFX™ PCR DNA Gel Band purification kit (Amersham Biosciences, UK) and the nucleotide sequences were determined using the CEQ Dye Terminator Cycle Sequencing quick start reaction kit in an automated CEQ8000 Genetic Analysis System (Beckmann Coulter, CA,

Table 2. Sequences of primers used for detection of the *int*SXT, *intI1* and antibiotic resistance genes.

Primers	Sequence (5′ to 3′)	Locus (direction)[a]	Reference
qacEΔ1F	ATCGCAATAGTTGGCGAAGT	*qacE1* (+)	Dalsgaard *et al*. (1999)
sul1B	GCAAGGCGGAAACCCGCGCC	*sul1* (–)	Dalsgaard *et al*. (1999)
inDS-F	CGGAATGGCCGAGCAGATC	*intI1* (+)	Dalsgaard *et al*. (1999)
inDS-B	CAAGGTTCTGGACCAGTTGCG	*intI1* (–)	Dalsgaard *et al*. (1999)
inF	GGCATCCAAGCAGCAAGC	5′ CS	Dalsgaard *et al*. (1999)
inB	AAGCAGACTTGACCTGAT	3′ CS	Dalsgaard *et al*. (1999)
aadA-B	ATTGCCCAGTCGGCAGCG	*aadA* gene (–)	Dalsgaard *et al*. (1999)
SXT-F	TCGGGTATCGCCCAAGGGCA	*int*SXT (+)	Bhanumathi *et al*. (2003)
SXT-R	GCGAAGATCATGCATAGACC	*int*SXT (–)	Bhanumathi *et al*. (2003)
strA-F	TTGATGTGGTGTCCCGCAATGC	*strA* (+)	Hochhut *et al*. (2001)
strA-B	CCAATCGCAGATAGAAGGCAA	*strA* (–)	Hochhut *et al*. (2001)
strB-F	CCGCGATAGCTAGATCGCGTT	*strB* (+)	Ramachandran *et al*. (2007)
strB-R	CGACTACCAGGCGACCGAAAT	*strB* (–)	Ramachandran *et al*. (2007)
sul-F	AGGGGGCAGATGTGATCGAC	*sul* (+)	Hochhut *et al*. (2001)
sul -R	TGTGCGGATGAAGTCAGCTCC	*sul* (–)	Hochhut *et al*. (2001)
dfrA1DH1-F	CAAGTTTACATCTGACAATGAGAACGTAT	*dfrA1* (+)	Ramachandran *et al*. (2007)
dfrA1DH2-R	ACCCTTTTGCCAGATTTGGTA	*dfrA1* (–)	Ramachandran *et al*. (2007)
Tmp-F	TGGGTAAGACACTCGTCATGGG	*dfr18* (+)	Hochhut *et al*. (2001)
Tmp-B	ACTGCCGTTTTCGATAATGTGG	*dfr18* (–)	Hochhut *et al*. (2001)

a. +, oligonucleotide corresponding to the coding strand (forward primer); –, oligonucleotide corresponding to the non-coding strand (backward primer).

USA). Nucleotide sequences were analysed using BLAST and ClustalW programs. A phylogenetic tree for the SXT element was constructed using the MEGA 3.0 software package (Kumar *et al*., 2004) and the Neighbor joining method, including non-synonymous substitutions with 1000 bootstrap replicates. The pairwise distance was analysed using the Modified Nei-Gojobori (p-distance) model.

Conjugation analysis

Biparental conjugation experiments were carried out in LB broth using the NAL-resistant *E. coli* strain MTCC1652 or rifampicin-resistant *E. coli* DH5α as recipients, following the method of Anderson and Threlfall (1974). Briefly, donor *V. cholerae* and recipient *E. coli* strains were mixed in 1:1 ratio and incubated overnight at 37°C. The following day, *E. coli* transconjugants were selected against unmated recipients on LB agar plates containing STR (50 μg ml^{-1}), TMP (32 μg ml^{-1}), SUL (160 μg ml^{-1}) and rifampicin (100 μg ml^{-1}) or NAL (50 μg ml^{-1}). The frequency of conjugation was determined by dividing the number of transconjugants obtained by the total number of recipient cells. Transconjugants were analysed to determine if transfer of Class I integrons, *int*SXT, STR, SUL and TMP resistant determinants by PCR occurred as described above.

Probes and hybridization

For preparation of DNA blots, 4 μg of DNA was digested with appropriate enzyme (New England Biolabs, USA), separated by electrophoresis in 0.8% agarose gels and blotted onto nylon membrane (Hybond N$^+$, Amersham International, UK) by Southern blotting (Southern, 1975). The gene probe used to detect the Class I integron was an ~800 bp fragment amplified by PCR from *V. cholerae* strain VC20 using primers inDS-F and inDS-B. The rRNA probe was a 7.5 kb BamHI fragment of pKK3535 (Brosius *et al*., 1981), which is a pBR322-derived plasmid containing an *E. coli* rRNA operon consisting of one copy each of the genes encoding 5S rRNA, 16S rRNA, 23S rRNA and tRNA^{Glu}. The probes were labelled by random priming (Feinberg and Vogelstein, 1983) using a random primer labelling kit (New England Biolabs, USA) and [α-^{32P}] deoxyadenosine triphosphate (3000 Ci mmol^{-1}, Bhabha Atomic Research Centre, Mumbai, India). Southern blots were hybridized with labelled probes and autoradiographs were developed using a PhosphorImager (Fuji Photo Film, Tokyo, Japan).

Nucleotide sequence accession number

The nucleotide sequences of *aadA5*, *aadA1*, *aadA2*, *dfrA15*, SXT integrase and *dfr18* have been assigned accession numbers EF539212 to 539225.

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References

Amita, Chowdhury, S.R., Thungapathra, M., Ramamurthy, T., Nair, G.B., and Ghosh, A. (2003) Class I 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.

Anderson, E.S., and Threlfall, E.J. (1974) Characterization of plasmids in enterobacteria. *J Hyg* **72:** 471–487.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1995) *Short Protocol in Molecular Biology*, 3rd edn. New York, USA: Wiley.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., and Truck, M. (1966) Antibiotic susceptibility testing by a standardized single disk diffusion method. *Am J Clin Pathol* **45:** 493–496.

Beaber, J.W., Hochhut, B., and Waldor, M.K. (2002) Genomic and functional analyses of SXT, an integrating antibiotic resistance gene transfer element derived from *Vibrio cholerae*. *J Bacteriol* **184:** 4259–4269.

Bhanumathi, R., Sabeena, F., Isac, S.R., Shukla, B.N., and Singh, D.V. (2003) Molecular characterization of *Vibrio cholerae* O139 Bengal isolated from water and the aquatic plant *Eichhornia crassipes* in the river Ganga, Varanasi, India. *Appl Environ Microbiol* **69:** 2389–2394.

Birnboim, H.C., and Doly, J. (1979) Rapid alkaline extraction procedure for screening of recombinant plasmid DNA. *Nucleic Acid Res* **7:** 1513–1523.

Brosius, J., Ullrich, A., Raker, M.A., Gray, A., Dull, T.J., Gutell, R.R., and Noller, H.F. (1981) Construction and fine mapping of recombinant plasmids containing the rrnB ribosomal RNA operon of *E. coli*. *Plasmid* **6:** 112–118.

Burrus, V., Pavlovic, G., Decaris, B., and Gouedon, G. (2002) Conjugative transposons: the tip of the iceberg. *Mol Microbiol* **46:** 601–610.

Burrus, V., Marrero, J., and Waldor, M.K. (2006a) The current ICE age: biology and evolution of SXT-related integrating conjugative elements. *Plasmid* **55:** 173–183.

Burrus, V., Quezada-Calvillo, R., Marrero, J., and Waldor, M.K. (2006b) SXT-related integrating conjugative element in new world *Vibrio cholerae*. *Appl Environ Microbiol* **72:** 3054–3057.

Ceccarelli, D., Bani, S., Cappuccinelli, P., and Colombo, M.M. (2006) Prevalence of *aadA1* and *dfrA15* Class I integron cassettes and SXT circulation in *Vibrio cholerae* O1 isolates from Africa. *J Antimicrob Chemother* **58:** 1095–1097.

Dalsgaard, A., Forslund, A., Tam, N.V., Vinh, D.X., and Cam, P.D. (1999) 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., and Sandvang, D. (2000) 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., Sandvang, D., Arntzen, L., and 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.

Feinberg, A., and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132:** 6–13.

Fluit, A.C., and Schmitz, F.J. (2004) Resistance integrons and super integrons. *Eur Soc Clin Microbiol Infect Dis* **10:** 272–288.

Hochhut, B., and Waldor, M.K. (1999) Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prf C*. *Mol Microbiol* **32:** 99–110.

Hochhut, B., Lotfi, Y., Mazel, D., Faruque, S.M., Woodgate, R., and 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.

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

Kumar, S., Tamura, K., and Nei, M. (2004) MEGA 3: integrated software for molecular evolutionary genetics and sequence alignment. *Bioinformatics* **2:** 150–163.

Macrina, F.L., Kopecko, D.J., Jones, K.R., Ayers, D.F., and McCowen, S.K. (1987) A multiple plasmid containing *E. coli* strain: convenient source of size reference plasmid molecules. *Plasmid* **1:** 417–420.

Mazel, D. (2006) Integrons: agents of bacterial evolution. *Nature* **4:** 608–620.

Mazel, D., Dychinco, B., Webb, V.A., and Davies, J. (1998) A distinctive class of integron on the *Vibrio cholerae* genome. *Science* **280:** 605–608.

Mitra, R., Basu, A., Dutta, D., Nair, G.B., and Takeda, Y. (1996) Resurgence of *Vibrio cholerae* O139 Bengal with altered antibiogram in Calcutta, India. *Lancet* **348:** 181.

National Committee for Clinical Laboratory Standards (1998) *Performance Standards for Antimicrobial Susceptibility Testing.* Approved standard M100-S8, Vol. 18. Villanova, PA, USA: National Committee for Clinical Laboratory Standards.

Ramachandran, D., Bhanumathi, R., and Singh, D.V. (2007) Multiplex PCR for detection of antibiotic resistance genes and the SXT element: application in the characterization of *Vibrio cholerae*. *J Med Microbiol* **56:** 346–351.

Recchia, G.D., and Hall, R.M. (1995) Gene cassettes: a new class of mobile element. *Microbiology* **141:** 3015–3027.

Shi, L., Fujihara, K., Sato, T., Ito, H., Garg, P., Chakrabarty, R., *et al.* (2006) Distribution and characterization of integrons in various serogroups of *Vibrio cholerae* strains isolated from diarrheal patients between 1992 and 2000 in Kolkata, India. *J Med Microbiol* **55:** 575–583.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98:** 503–512.

Stokes, H.W., O'Gorman, D.B., Recchia, G.D., Parsekhiam, M., and Hall, R.M. (1997) Structure and function of 59-base element recombination sites associated with mobile gene cassettes. *Mol Microbiol* **26:** 731–745.

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

Waldor, M.K., Tschape, H., and 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.

World Health Organization. (1987) *Manual for Laboratory Investigation of Acute Enteric Infections*. Publication WHO CDD/83.3. *Program for Control of Diarrheal Diseases*. Geneva, Switzerland: World Health Organization.

