Multiplex PCR for detection of antibiotic resistance genes and the SXT element: application in the characterization of *Vibrio cholerae*

Dhanya Ramachandran,¹ R. Bhanumathi^{1†} and Durg V. Singh²

Correspondence

Durg V. Singh

durg_singh@yahoo.co.in

¹Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram-695 014, India

²Institute of Life Sciences, Nalco Square, Bhubaneswar-751 023, India

This study describes a multiplex PCR assay for the detection of antibiotic resistance genes and the SXT element in *Vibrio cholerae*. Conditions were optimized to amplify fragments of *sulIII* (encoding sulfamethoxazole resistance), *dfrA1* (O1-specific trimethoprim resistance), *dfr18* (O139-specific trimethoprim resistance), *strB* (streptomycin B resistance) and the SXT element simultaneously in one PCR. This multiplex PCR was evaluated on 142 *V. cholerae* isolates and the results correlated with the phenotypic antibiotic data obtained using a disc diffusion assay and a colony blot assay. Thus this one-step PCR can be used as a simple, rapid and accurate method for identification of antibiotic resistance profiles and could be used for the surveillance of the spread of antibiotic resistance determinants in epidemiological and environmental studies.

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INTRODUCTION

Vibrio cholerae is the causative agent of cholera, a severe and sometimes lethal disease. Multiple drug resistance in *V. cholerae* has been reported frequently, usually after acquisition by strains of a conjugative plasmid (Falbo *et al.*, 1999; Glass *et al.*, 1983; Tabtieng *et al.*, 1989). Other genetic elements, such as the class 1 integron and SXT element, have also been reported to carry genetic determinants for antimicrobial resistance (Dalsgaard *et al.*, 2000, 2001; Hochhut *et al.*, 2000, 2001; Iwanaga *et al.*, 2004; Thungapathra *et al.*, 2002). Historically, only the O1 serogroup of *V. cholerae* has been the causative agent of epidemic cholera. However, in late 1992, in India and Bangladesh, a novel serogroup designated *V. cholerae* O139 emerged, replacing *V. cholerae* O1, and gave rise to major cholera outbreaks (Ramamurthy *et al.*, 1993). These strains were characteristically resistant to the antibiotics furazolidone (Fr), sulfamethoxazole (Sul), trimethoprim (Tmp), chloramphenicol (Cm) and low concentrations of streptomycin (Str). Genes encoding resistance to these antibiotics were found to be located on a novel transmissible genetic element, designated SXT (Waldor *et al.*, 1996).

After the extensive cholera outbreaks caused by *V. cholerae* O139, *V. cholerae* O1 El Tor strains re-emerged in 1994 as the predominant cause of cholera on the Indian continent. In contrast to El Tor strains isolated before the O139

outbreak, the re-emerged El Tor strains, like the initial O139 isolates, were resistant to Fr, Sul, Tmp, Cm and Str (Yamamoto *et al.*, 1994). The corresponding genes were found to be located in an integrating conjugative element that was closely related but not identical to the O139 SXT element (Hochhut & Waldor, 1999; Waldor *et al.*, 1996). Variations were noted in recent *V. cholerae* O139 isolates from India, i.e. they were generally no longer resistant to Sul and Tmp, and showed varying resistance to Str (Mitra *et al.*, 1996; Sinha *et al.*, 2002; Yam *et al.*, 1994).

As the SXT genetic element plays a role in the acquisition of antibiotic resistance, it is important to look for the presence of the *sulIII* (encoding Sul resistance), *dfrA1* (O1-specific Tmp resistance), *dfr18* (O139-specific Tmp resistance) and *strB* (Str B resistance) genes and the SXT element (Bhanumathi *et al.*, 2003; Falbo *et al.*, 1999; Hochhut *et al.*, 2001) in *V. cholerae* strains. To trace the changes in antibiotic resistance, we developed a multiplex PCR for the detection of antibiotic resistance genes and the SXT element and used it to characterize *V. cholerae* strains.

METHODS

Bacterial strains. A total of 142 strains of *V. cholerae* were included in this study. Of these, 72 were serogroup O1, 62 were serogroup O139 and eight were non-O1, non-O139 serogroup obtained from laboratory stocks identified previously using standard bacteriological methods (World Health Organization, 1987). All isolates were examined for their oxidase reaction, and the identities of the *V. cholerae* O1 strains were confirmed by serogrouping using growth from triple-sugar iron agar slants with polyclonal O1 and monospecific Inaba and Ogawa antisera. *V. cholerae* strains that did not agglutinate with O1 antiserum were checked with O139

†Present address: Patent Office, Chennai Branch, IPR Building, GST Road, Chennai-600 032, India.

Abbreviations: Cm, chloramphenicol; Fr, furazolidone; Str, streptomycin; Sul, sulfamethoxazole; Tmp, trimethoprim.

Fig. 1. Map showing the different cities in India from where the *Vibrio cholerae* strains were obtained. Not to scale.

monoclonal antibody supplied by the WHO Regional Office of South East Asia, New Delhi, India. *V. cholerae* strains that did not agglutinate with either O1 or O139 antisera were assumed to belong to non-O1, non-O139 serogroups. *V. cholerae* O1 strains were further bityped using multiplex PCR by exploiting sequence differences between classical and El Tor biotype *tcpA* genes to distinguish the two biotypes (Keasler & Hall, 1993). The cities in India from which the *V. cholerae* strains were obtained are shown in Fig. 1.

V. cholerae O139 strains ATCC 51394, CO594 and VO143 were used as the PCR positive controls for *sulII*, *dfr18*, *strB* and SXT; and O1 biotype El Tor strains KO194 for *sulII*, *dfrA1*, *strB* and SXT and TO267 for *dfrA1* and SXT, respectively. *V. cholerae* O1 biotype El Tor strain VO101 was used as a negative control. All strains were maintained in tryptic soy broth (Difco) supplemented with 30% glycerol and stored at -70°C . Before use, the identification of each culture was confirmed by selected biochemical tests and serology (World Health Organization, 1987).

Multiplex PCR. *V. cholerae* strains grown overnight at 37°C in Luria–Bertani broth (USB, Amersham Life Technology) were boiled and stored at -20°C until use. The bacterial cell lysate served as the source of template DNA. Primer sequences for the antibiotic resistance genes are shown in Table 1.

PCR amplification of target DNA was carried out in a thermal cycler (Bio-Rad) in a 200 μl PCR tube containing a reaction mixture volume of 30 μl . Each reaction contained 2.5 μl $10\times$ amplification buffer [500 mM KCl, 100 mM Tris/HCl (pH 9.0), 1.0% Triton X-100; Promega], 2.0 μl 25 mM MgCl_2 , 2.5 μl each 2.5 mM dATP, dCTP, dGTP and dTTP (Amersham Pharmacia Biotech), 0.9 μl 3% DMSO (Sigma), 1.2 μl forward and reverse primers for SXT (20 ng μl^{-1}), 0.25 μl *Taq* DNA polymerase (5 U μl^{-1} ; Promega), Milli-Q water to a final volume of 27.5 μl , and 2.5 μl cell lysate (template DNA). PCR cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 60.5°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min.

Multiplex PCR was carried out by the simultaneous addition of primer pairs for *sulII*, *dfr18*, *dfrA1* and *strB* in the same reaction mixture. In initial experiments, *sulII*, *dfr18*, *dfrA1* and *strB* primer concentrations varied between 6 and 25 μM , keeping the SXT primer concentration fixed at either 12 or 15 μM in the reaction mixture of 30 μl . Optimum results were obtained with primer concentrations of 6 μM for *sulII*, 9 μM for *dfr18*, 10 μM for *strB* and 25 μM for *dfrA1* and 12 μM for SXT. PCR conditions for amplification were maintained as described above. Amplified products were separated by agarose (1.5%) gel electrophoresis in $0.5\times$ TBE, stained in ethidium bromide and visualized with a Fluoro-S-MultiImager (Bio-Rad). PCR-negative and -positive strains were verified by Southern blot hybridization.

Probes and hybridization. Colony blots were prepared using nylon membranes (Hybond-N⁺; Amersham International) and processed using a standard method (Sambrook & Russell, 2001). Briefly, colony blots were denatured in denaturing solution (0.5 M NaOH, 1.5 M NaCl), neutralized in neutralizing solution (0.5 M Tris/HCl, pH 8.0; 1.5 M NaCl) and the DNA was fixed on to nylon membranes by exposure to UV light for 2 min, in accordance with the manufacturer's instructions.

PCR-amplified products obtained from *V. cholerae* O139 strain ATCC 51394 for the *sulII*, *dfr18* and *strB* genes and SXT and from *V. cholerae* O1 strain KO194 for the *dfrA1* gene were used as probes and were randomly labelled (Feinberg & Vogelstein, 1984) with [α - ^{32}P]dCTP (3000 Ci mmol⁻¹; BARC) and hybridized at 65°C in phosphate buffer containing 500 mM Na_2HPO_4 (pH 7.2), 7% SDS, 1 mM EDTA and

Table 1. Sequences of primers used for detection of antibiotic resistance genes and the SXT element

Primer	Sequence (5'→3')	Amplicon size (bp)	GenBank no.	Primer site (nt)	Reference
<i>sulII</i> -F	TGTGCGGATGAAGTCAGCTCC	626	AY034138	16 101–16 121	Hochhut <i>et al.</i> (2001)
<i>sulII</i> -R	AGGGGGCAGATGTGATCGAC		AY034138	16 707–16 726	Hochhut <i>et al.</i> (2001)
<i>dfr18</i> -F	ACTGCCGTTTTTCGATAATGTGG	389	AY034138	7443–7464	Hochhut <i>et al.</i> (2001)
<i>dfr18</i> -R	TGGGTAAGACACTCGTCATGGG		AY034138	7810–7831	Hochhut <i>et al.</i> (2001)
<i>strB</i> -F	CCGCGATAGCTAGATCGCGTT	515	AY034138	14 364–14 384	This study
<i>strB</i> -R	CGACTACCAGGCGACCGAAAT		AY034138	14 858–14 878	This study
SXT-F	ATGGCGTTATCAGTTAGCTGGC	1035	AF099172	1–22	Bhanumathi <i>et al.</i> (2003)
SXT-R	GCGAAGATCATGCATAGACC		AF099172	1016–1035	Bhanumathi <i>et al.</i> (2003)
<i>dfrA1</i> -F	CAAGTTTACATCTGACAATGAGAACGTAT	278	X00926	429–457	Falbo <i>et al.</i> (1999)
<i>dfrA1</i> -R	ACCCTTTTGCCAGATTTGGTA		X00926	706–686	Falbo <i>et al.</i> (1999)

1% BSA. Hybridized blots were washed once in $2 \times$ SSC for 5 min at room temperature, twice in $2 \times$ SSC/0.1% SDS for 15 min at 65 °C and once in $0.1 \times$ SSC/0.1% SDS for 15 min at 65 °C. Autoradiographs were developed from the hybridized filters with the Bio-Rad PhosphorImager screen and visualized in a PhosphorImager (Bio-Rad).

Antibiotic susceptibility testing. *V. cholerae* strains were tested for antibiotic susceptibility using the method of Bauer *et al.* (1966) using the following antibiotics (Hi-Media Laboratories): ampicillin (10 µg), Cm (30 µg), ciprofloxacin (5 µg), Fr (100 µg), gentamicin (10 µg), neomycin (30 µg), norfloxacin (10 µg), nalidixic acid (30 µg), polymyxin B (50 U), Str (30 µg), Tmp-Sul (25 µg), Tmp (5 µg), tetracycline (30 µg) and the vibriostatic agent O/129 (10 and 150 µg). A 3 h culture of *V. cholerae* in Mueller–Hinton broth (Difco) was spread plated on well-dried Mueller–Hinton agar (Difco). Plates were incubated for 24 h at 37 °C. Characterization of strains as susceptible or resistant was based on the size of inhibition zone around each disc, according to the manufacturer's instructions, which matched the interpretive criteria recommended by the World Health Organization (1993).

RESULTS

V. cholerae strains belonging to serogroups O1, O139 and non-O1, non-O139 were examined for the presence of antibiotic resistance genes and the SXT element. Two strains of *V. cholerae* O1, one each isolated from Varanasi in 1976 and 1992, gave negative PCR results for the *sulII*, *dfr18*, *dfrA1* and *strB* genes and the SXT element (Fig. 2a, b, and Table 2). These strains, when tested for antibiotic susceptibility, were sensitive to all of the antibiotics tested, whereas 51 strains of *V. cholerae* O1, of which six were isolated from Kottayam in 2000, 26 from Trivandrum in 2000 and 2003, and 19 from Varanasi in 1994, 1995, 1996, 1998 and 1999, gave positive results by PCR and amplified a 626 bp fragment of *sulII*, a 278 bp fragment of *dfrA1*, a 515 bp

fragment of *strB* and a 1035 bp fragment of the SXT element (Fig. 2a, b). All of these strains showed resistance to ampicillin, Fr, nalidixic acid, Str, Tmp-Sul and Tmp (Table 2). Furthermore, 19 strains, of which seven were isolated from Alleppey in 2000 and 12 from Trivandrum in 2002, when tested by multiplex PCR, gave positive results for the *dfrA1* gene and the SXT element. They were resistant to ampicillin, Fr and Tmp, but not to Tmp-Sul (Table 2). All of them were resistant to the vibriostatic agent O/129.

Twenty-one *V. cholerae* O139 strains, of which one was isolated from Alleppey in 2000, two from Kolkata in 1992, and 18 from Varanasi in 1992, 1993 and 1994, gave positive results by PCR and amplified a 626 bp fragment of *sulII*, a 389 bp fragment of *dfr18*, a 515 bp fragment of *strB* and a 1035 bp fragment of the SXT element (Fig. 2a, b). These strains were resistant to ampicillin, Fr, Str, Tmp-Sul and Tmp and to vibriostatic agent O/129 (Table 2). However, 41 strains of *V. cholerae* O139, comprising 14 from Alleppey, 11 from Trivandrum and 16 from Wardha, were SXT positive when tested by PCR. They were resistant to ampicillin, Fr and nalidixic acid and sensitive to all of the other antibiotics tested, including the vibriostatic agent O/129 (Table 2).

One of eight *V. cholerae* non-O1, non-O139 strains was SXT positive. The other strains were negative for the *sulII*, *dfr18*, *dfrA1* and *strB* genes and for the SXT element (Fig. 2b). All eight strains were resistant to ampicillin (Table 2).

All *V. cholerae* O1, O139 and non-O1, non-O139 strains negative for the *sulII*, *strB* and/or *dfr18/dfrA1* genes and for the SXT element by PCR were also negative in the colony blot assay, whereas representative *V. cholerae* O1, O139 and non-O1, non-O139 strains positive for the *sulII*, *strB* and/or

Fig. 2. Agarose gels of multiplex PCR products stained with ethidium bromide. (a) Lanes: M, 100 bp DNA ladder; 1–3, *V. cholerae* O139 strains ATCC 51394 (isolated in Madras, 1992), CO594 (Kolkata, 1992) and VO143 (Varanasi, 1992) (SXT⁺ *sulII*⁺ *dfr18*⁺ *strB*⁺); 4, *V. cholerae* O1 strain VO101 (Varanasi, 1975) (SXT[−] *sulII*[−] *dfrA1*[−] *strB*[−]); 5, *V. cholerae* O1 El Tor strain KO194 (Kottayam, 2000) (SXT⁺ *sulII*⁺ *dfrA1*⁺ *strB*⁺); 6, *V. cholerae* O1 El Tor strain TO267 (Trivandrum, 2003) (SXT⁺ *sulII*[−] *dfrA1*⁺ *strB*[−]). (b) Multiplex PCR products of representative *V. cholerae* O1, O139 and non-O1, non-O139 strains. Lanes: M, 100 bp DNA ladder; 1–4, *V. cholerae* O139 strains CO129 and CO130 (Kolkata, 1992), and VO135 and VO144 (Varanasi, 1992) (SXT⁺ *sulII*⁺ *dfr18*⁺ *strB*⁺); 5 and 6, *V. cholerae* O139 strains TO255 (Trivandrum, 2002) and WO167 (Wardha, 1998) (SXT⁺ *sulII*[−] *dfr18*[−] *strB*[−]); 7, *V. cholerae* non-O1, non-O139 strain VO222 (Varanasi, 1987) (SXT⁺ *sulII*[−] *dfr18*[−] *strB*[−]); 8, 10 and 12, *V. cholerae* O1 El Tor strains KO52 (Kottayam, 2000), TO69 (Trivandrum, 2000) and TO289 (Trivandrum, 2003) (SXT⁺ *sulII*⁺ *dfrA1*⁺ *strB*⁺); 9 and 11, *V. cholerae* O1 El Tor strains AO60 (Alleppey, 2000) and TO274 (Trivandrum, 2002) (SXT⁺ *sulII*[−] *dfrA1*⁺ *strB*[−]); 13, *V. cholerae* O1 El Tor strain VO102 (Varanasi, 1976) (SXT[−] *sulII*[−] *dfrA1*[−] *strB*[−]).

Table 2. Results of analysis employing multiplex PCR to study *Vibrio cholerae* O1, O139 and non-O1, non-O139 strains isolated from different geographical locations in India

	Species and serotype	No. of strains tested	Place and year of isolation (no. of strains)	Antibiotic resistance pattern*	Strain(s) showing presence of genes encoding:				
					<i>strB</i>	<i>sulII</i>	<i>dfrA1</i>	<i>dfr18</i>	SXT
Test strains	<i>Vibrio cholerae</i> O1	72	Varanasi, 1976 (1), 1992 (1)	A	—	—	—	—	—
			Kottayam, 2000 (6); Trivandrum, 2000 (12), 2000 (5†), 2003 (9); Varanasi, 1994 (6), 1995 (1), 1996 (6), 1998 (4), 1999 (2)	A, Tm–Sul, F, Na, S, Tm, P	+	+	+	—	+
	<i>Vibrio cholerae</i> O139	62	Alleppey, 2000 (7); Trivandrum, 2002 (12)	A, F, Na, Tm, P	—	—	+	—	+
			Alleppey, 2000 (1); Kolkata, 1992 (2); Varanasi, 1992 (6), 1993 (8), 1994 (4)	A, Tm–Sul, F, S, Tm, P	+	+	—	+	+
			Alleppey, 2002 (14); Trivandrum, 2002 (11); Wardha, 1997 (14), 1998 (1), 1999 (1)	A, F, Na	—	—	—	—	+
	<i>Vibrio cholerae</i> non-O1, non-O139	8	Varanasi, 1976 (1), 1979 (2), 1986 (1), 1988 (2); Trivandrum, 2000 (1)	A	—	—	—	—	—
Varanasi, 1987 (1)			A	—	—	—	—	+	
Positive controls	<i>Vibrio cholerae</i> O139 strain ATCC 51394	—		A, Tm–Sul, F, S, Tm, P	+	+	—	+	+
	<i>Vibrio cholerae</i> O139 strain CO594	—		A, Tm–Sul, F, S, Tm, P	+	+	—	+	+
	<i>Vibrio cholerae</i> O139 strain VO143	—		A, Tm–Sul, F, S, Tm, P	+	+	—	+	+
	<i>Vibrio cholerae</i> O1 strain KO194	—		A, Tm–Sul, F, S, Tm, P	+	+	+	—	+
	<i>Vibrio cholerae</i> O1 strain TO267	—		A, F, Tm, P	—	—	+	—	+
	Negative control	<i>Vibrio cholerae</i> O1 strain VO101	—		—	—	—	—	—

*A, ampicillin; Tm-Sul, trimethoprim-sulfamethoxazole; F, furazolidone; Na, nalidixic acid; S, streptomycin; Tm, trimethoprim; P, vibriostatic agent O/129.

†*V. cholerae* O1 biotype El Tor Inaba strains.

dfr18/dfrA1 genes and for the SXT element by PCR were also positive in the colony blot assay (data not shown).

DISCUSSION

Antibiotic therapy shortens the duration of diarrhoea caused by cholera (Lindenbaum *et al.*, 1967), but the use of antibiotics has contributed to resistance in *V. cholerae*. The SXT element is a 62 kb, conjugative, self-transmissible, integrating element encoding resistance to Sul, Tmp, Cm and Str (Waldor *et al.*, 1996). Furthermore, SXT encodes an integrase at its 5' end required for SXT transfer. The SXT^{MO10} element was first detected in the newly emerged O139 serogroup of *V. cholerae* in 1992. Since 1994, *V. cholerae* O1 strains isolated from India, Bangladesh, Mozambique and Laos have been found to contain the SXT element (Amita *et al.*, 2003; Dalsgaard *et al.*, 2001; Hochhut *et al.*, 2001; Iwanaga *et al.*, 2004). In El Tor *V. cholerae* strains, the resistance genes are located in SXT^{ET}.

The results obtained in this study showed that *V. cholerae* O1 El Tor strains isolated from Alleppey, Kottayam and Trivandrum were resistant to Tmp and vibriostatic agent O/129 and positive by multiplex PCR for the *dfrA1* gene, indicating that genes encoding resistance to Tmp and O/129 are linked, a finding further supported by the fact that O139 *V. cholerae* strains isolated up to 1994 were resistant to Tmp and vibriostatic agent and possessed the *dfr18* gene, whereas strains isolated after 1996 from different regions of India were susceptible to Tmp and vibriostatic agent and lacked *dfr18*. However, further study is needed to confirm the presence of Tmp and vibriostatic agent O/129 genes in the SXT element.

In conclusion, there is a difference in the antibiotic resistance gene clusters in the SXT element in *V. cholerae* O1 and O139, corroborating the results of earlier workers (Hochhut *et al.*, 2001) who reported differences in antibiotic resistance and suggested that these genes were not intrinsic features of this family of integrase, but rather appeared to have been inserted into these elements, becoming transmissible bacterial populations. At least one non-O1, non-O139 *V. cholerae* strain possessed the SXT element, suggesting a role in the acquisition of antibiotic resistance genes encoding Sul, Tmp and/or Str resistance. Thus one-step multiplex PCR can be used effectively to detect antibiotic resistance genes and could be used in surveillance of the spread of antibiotic resistance in epidemiological and environmental studies.

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REFERENCES

- Amita, Choudhury, 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* outbreak, Calcutta, India. *Emerg Infect Dis* **9**, 500–502.
- Bauer, A. W., Kirby, W. M. M., Sherris, J. C. & Truck, M. (1966). Antibiotic susceptibility testing by a standard single diffusion method. *Am J Clin Pathol* **45**, 493–496.
- Bhanumathi, R., Sabeena, F., Isac, S. R., Shukla, B. N. & 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.
- Dalsgaard, A., Forslund, A., Petersen, A., Brown, D. J., Dias, F., Monteiro, S., Molbak, K., Aaby, P., Rodrigues, A. & Sandström, A. (2000). 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.
- Falbo, V., Carattoli, A., Tosini, F., Pezzella, C., Dionisi, A. M. & Luzzi, I. (1999). Antibiotic resistance conferred by a conjugative plasmid and a class 1 integron in *Vibrio cholerae* O1 El Tor strains isolated in Albania and Italy. *Antimicrob Agents Chemother* **43**, 693–696.
- Feinberg, A. & Vogelstein, B. (1984). A technique for rapid labeling of DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **137**, 266–267.
- Glass, R. I., Huq, M. I., Lee, J. V., Threlfall, E. J., Khan, M. R., Alim, A. R., Rowe, B. & Gross, R. J. (1983). Plasmid-borne multiple drug resistance in *Vibrio cholerae* serogroup O1, biotype El Tor: evidence for a point-source outbreak in Bangladesh. *J Infect Dis* **147**, 204–209.
- Hochhut, B. & Waldor, M. K. (1999). Site specific integration of the non-conjugal *Vibrio cholerae* SXT element into *prfC*. *Mol Microbiol* **32**, 99–110.
- Hochhut, B., Marrero, J. & Waldor, M. K. (2000). Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a constin found in *Vibrio cholerae* O139. *J Bacteriol* **182**, 2043–2047.
- 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.
- 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.
- Keasler, S. P. & Hall, R. H. (1993). Detecting and biotyping *V. cholerae* O1 with multiplex polymerase chain reaction. *Lancet* **341**, 1661.

- Lindenbaum, J., Greenough, W. B. & Islam, M. R. (1967). Antibiotic therapy of cholera. *Bull World Health Organ* **36**, 871–883.
- Mitra, R., Basu, A., Datta, D., Nair, G. B. & Takeda, Y. (1996). Resurgence of *Vibrio cholerae* O139 Bengal with altered antibiogram in Calcutta, India. *Lancet* **348**, 1181.
- Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya, S. K., Nair, G. B., Shimada, T., Takeda, T., Karasawa, T., Kurazano, H. & other authors (1993). Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. *Lancet* **341**, 703–704.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sinha, S., Chakraborty, R., De, K., Khan, A., Datta, S., Ramamurthy, T., Bhattacharya, S. K., Takeda, Y. & Nair, G. B. (2002). Escalating association of *Vibrio cholerae* O139 with cholera outbreaks in India. *J Clin Microbiol* **40**, 2635–2637.
- Tabtieng, R., Wattanasri, S., Echeverria, P., Seriwatana, J., Bodhidatta, L., Chatkaemorakot, A. & Rowe, B. (1989). An epidemic of *Vibrio cholerae* El Tor Inaba resistant to several antibiotics with a conjugative group C plasmid coding for type II dihydrofolate reductase in Thailand. *Am J Trop Med Hyg* **41**, 680–686.
- Thungapathra, M., Amita, Sinha, K. K., Choudhury, 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 1 integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrob Agents Chemother* **46**, 2948–2955.
- Waldor, M. K., Tschape, 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.
- World Health Organization (1987). Program for control of diarrhoeal diseases. In *Manual for Laboratory Investigation of Acute Infection*, CDD/83.3. Geneva: World Health Organization.
- World Health Organization (1993). *Guidelines for Cholera Control*. Geneva: World Health Organization.
- Yam, W.-C., Yuen, K.-Y., Wong, S.-S.-Y. & Que, T.-L. (1994). *Vibrio cholerae* O139 susceptible to vibriostatic agent O/129 and co-trimoxazole. *Lancet* **344**, 404–405.
- Yamamoto, T., Nair, G. B., Albert, M. J., Parodi, C. & Takeda, Y. (1994). In vitro susceptibility of *Vibrio cholerae* O1 and O139 to antimicrobial agents and appearance of drug resistance plasmids in *Vibrio cholerae*. In *Proceedings of the 30th US–Japan Joint Conference on Cholera*, pp. 52–57. US–Japan Cooperative Medical Science Program for Cholera and Related Diarrheal Diseases Panel: Fukuoka, Japan.