Septaplex PCR assay for rapid identification of *Vibrio cholerae* including detection of virulence and *int* SXT genes

Chinmay K. Mantri¹, Saswat S. Mohapatra¹, Thandavarayan Ramamurthy², Raikamal Ghosh², Rita R. Colwell² & Durg V. Singh¹

¹Institute of Life Sciences, Nalco Square, Bhubaneswar, India; ²National Institute of Cholera and Enteric Diseases, Kolkata, India; and ³Center for Bioinformatics and Computation Biology, University of Maryland Institute for Advanced Computer Studies, University of Maryland, MD, USA

Correspondence: Durg V. Singh, Institute of Life Sciences, Nalco Square, Bhubaneswar-751 023, India. Tel.: +091 674 230 2754; fax: +0091 674 230 0728; e-mail: durg_singh@yahoo.co.in

Received 21 August 2006; revised 28 September 2006; accepted 28 September 2006.

First published online 2 November 2006.

DOI:10.1111/j.1574-6968.2006.00491.x

Editor: Jeff Cole

Keywords

septaplex PCR; *Vibrio cholerae*; virulence; *int* SXT; detection.

Abstract

In this study, we describe a septaplex PCR assay for rapid identification of *Vibrio cholerae* including the detection of the virulence and *int* SXT genes. Conditions were optimized to amplify fragments of ISRrRNA (encoding for 16S–23S rRNA gene, Intergenic spacer regions), *O1rfb* (O1 serogroup specific rfb), *O139rfb* (O139 serogroup specific rfb), *ctxA* (cholera toxin subunit A), *tcpA* (toxin coregulated pilus), and *intSxt* (sxt integron) simultaneously in a single PCR. The septaplex PCR was evaluated using 211 strains of *V. cholerae* and six water samples for *in situ* testing. PCR results were correlated with genotype data obtained by individual PCR and slot-blot assays. The one-step PCR described here can be used to identify *V. cholerae* accurately and rapidly. Also, the virulence and *int*Sxt genes can be simultaneously detected, providing a useful method for monitoring pathogenic, *int*Sxt-positive and nonpathogenic, *int*Sxt-negative *V. cholerae* serogroups both in the environment and clinical settings.

Introduction

Cholera is a life-threatening disease caused by *Vibrio cholerae*, an autochthonous inhabitant of brackish water and estuarine systems (Colwell *et al.*, 1977). Toxigenic strains of *V. cholerae* belonging to the O1 and O139 serogroups are the causative agents of epidemic cholera, whereas non-O1 and non-O139 *V. cholerae* strains are associated only with occasional cholera-like diarrheal outbreaks (Kaper *et al.*, 1995). Conventional methods used to detect and classify vibrios isolated from clinical and environmental samples require several days to complete and involve enrichment culture in alkaline peptone water, followed by testing for growth on thiosulfate citrate bile salt-sucrose agar (TCBS), slide agglutination with specific antisera, and assay for cholera toxin (CT) (Sakazaki, 1992). This traditional procedure, however, is laborious, time consuming, and expensive for laboratories handling a large number of clinical and environmental samples. Furthermore, high similarity in the biochemical properties of *V. cholerae*, *Vibrio mimicus*, and other *Vibrio* spp., as well as with *Aeromonas*, renders unambiguous identification difficult.

*Vibrio cholerae* O1 or O139 can be detected rapidly using probes or primers that use the *rfb* region responsible for O-antigen biosynthesis (Albert *et al.*, 1997; Hoshino *et al.*, 1998). This might not be the case for non-O1, non-O139 strains diagnosed as the causative agent of diarrhea cases in the Indian subcontinent and elsewhere (Bagachi *et al.*, 1993; Dalsgaard *et al.*, 1995; Rudra *et al.*, 1996; Sharma *et al.*, 1998). From a diagnostic point of view, toxigenic—pathogenic and nontoxigenic—nontopathogenic strains of *V. cholerae* can be differentiated by the presence of CT and the toxin coregulated pilus genes. Recently, a PCR-based method targeted to the *toxR* gene was developed for species-specific identification of *Vibrio parahaemolyticus* (Kim *et al.*, 1999). We have used a hexaplex PCR assay, targeting the *toxR* gene (Miller *et al.*, 1987), for species-specific screening of *V. cholerae* including other virulence genes (Singh *et al.*, 2002). A PCR-based method targeted to the 16S–23S rRNA gene intergenic spacer regions (ISR) of *V. cholerae* and *V. mimicus* was developed for identification of *V. cholerae*, considering that the ISR rRNA gene is also present in non-O1, non-O139 strains of *V. cholerae* (Chun *et al.*, 1999). Although SXT, a 62-kb transposon, plays a role in acquisition and deletion of antibiotic resistance genes (Waldor *et al.*, 1996), it can serve as a useful marker, detecting the *intSxt* in *V. cholerae* by PCR (Bhanumathi *et al.*, 2003). Individually, none of these methods can be relied upon to
detect both serogroups and/or virulence genes and intX in V. cholerae in a one-step PCR.

Because of the serious epidemic potential of the V. cholerae O1 and O139, and occasionally, of V. cholerae non-O1, non-O139 serogroups, rapid diagnosis and identification of V. cholerae, including virulence and sxt genes, are important from a public health perspective. To this end, we have developed a rapid septaplex PCR assay for species-specific identification of virulent and sxt-positive strains of V. cholerae and report our results here.

Materials and methods

Bacterial strains

A total of 211 V. cholerae isolates were included in the study. One hundred four strains were O1 (nine from Alleppey, eight from Bhubaneswar, one from Delhi, seven from Kolkata, six from Kottayam, 36 from Trivandrum, two from Vaikom, and 36 from Varanasi). Of the 211, 68 were V. cholerae O139 (11 from Alleppey, nine from Kolkata, one from Madurai, eight from Trivandrum, 23 from Varanasi, and 16 from Wardha). Also included were 39 serogroup non-O1 and non-O139 strains (eight clinical: one each from Brazil, Kolkata and Trivandrum, and five from Varanasi, and 31 environmental: five from Kolkata and 26 from Varanasi). The 211 strains were obtained from laboratory stocks and identified using standard bacteriological methods (WHO, 1987). Three strains each of V. mimicus and V. parahaemolyticus, one strain each of enteropathogenic E. coli, enterohemorrhagic E. coli, and enteropathogenic E. coli, were also included as reference strains. Details of strains are given in Table 2.

Vibrio cholerae O1 biotype El Tor strain KO194 was used as the PCR-positive control for ctxA, tcpA (El Tor), toxR, ISRrRNA, and O1rfb and V. cholerae O1 biotype classical 569B was used as PCR-positive control for ctxA, tcpA (classical), toxR, ISRrRNA and O1rfb. Vibrio cholerae O139 strain ATCC51394 was similarly included for O139rfb, and intX, in addition to ctxA, tcpA (El Tor), toxR, and ISRrRNA genes. Nontoxigenic V. cholerae O1 strains X392 and 2740–80, and V. cholerae O139 CO788, and a toxR-negative V. cholerae O395–12 and tcpA-negative O395 RT110-112, were used as negative controls. All strains were stored in 20% glycerol-Luria–Bertani (LB) broth at −70 °C. Before use, identification of each isolate was confirmed by testing for selected biochemical tests and serology (WHO, 1987).

Table 1. Sequences of primers used for identification of Vibrio cholerae and detection of virulence and intX variant genes

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
<th>Gene accession number</th>
<th>Primer site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O139 rfb-F</td>
<td>AGCCCTTTTTATTACGGGTG</td>
<td>449</td>
<td>Y07786</td>
<td>12288–12307</td>
<td>Albert et al. (1997)</td>
</tr>
<tr>
<td>O139 rfb-R</td>
<td>GTCAACCCGACGTCGAAAG</td>
<td></td>
<td>Y07786</td>
<td>12717–12736</td>
<td></td>
</tr>
<tr>
<td>O1 rfb-F</td>
<td>GTCACGAAAGCATGAGG</td>
<td>192</td>
<td>X59554</td>
<td>13195–13213</td>
<td>Hoshino et al. (1998)</td>
</tr>
<tr>
<td>O1 rfb-R</td>
<td>GTCACTGTAAGTACAC</td>
<td></td>
<td>X59554</td>
<td>13368–13386</td>
<td></td>
</tr>
<tr>
<td>ISRrRNA VC-F</td>
<td>TTAAGCSTTTTTCTGAGAGAG</td>
<td>295–310</td>
<td>AF114723</td>
<td>227–248</td>
<td>Chun et al. (1999)</td>
</tr>
<tr>
<td>ISRrRNA VM-R</td>
<td>AGTCCTAAACCATACACCG</td>
<td></td>
<td>AF114723</td>
<td>501–522</td>
<td></td>
</tr>
<tr>
<td>ctxA</td>
<td>CCGGCGAGTTGCTGACCTCC</td>
<td>564</td>
<td>X00171</td>
<td>588–609</td>
<td>Singh et al. (2002)</td>
</tr>
<tr>
<td>toxR</td>
<td>CCGGGAACGAGCGAGTCG</td>
<td></td>
<td>X00171</td>
<td>1129–1151</td>
<td></td>
</tr>
<tr>
<td>tcpA</td>
<td>CCGGGAACGAGCGAGTCG</td>
<td>779</td>
<td>M21249</td>
<td>277–298</td>
<td>Singh et al. (2002)</td>
</tr>
<tr>
<td>tcpA-R Clas</td>
<td>CCGGGAACGAGCGAGTCG</td>
<td>620</td>
<td>M21249</td>
<td>1034–1055</td>
<td></td>
</tr>
<tr>
<td>tcpA-R El Tor</td>
<td>CCGGGAACGAGCGAGTCG</td>
<td></td>
<td>620</td>
<td>3379–3402</td>
<td></td>
</tr>
<tr>
<td>sxt-F</td>
<td>TCCTCCATGCGCCAAGGCA</td>
<td>823</td>
<td>X74730</td>
<td>3235–3256</td>
<td>This study</td>
</tr>
<tr>
<td>sxt-R</td>
<td>GCGAAGATCATGCAGCAACC</td>
<td>946</td>
<td>AF099172</td>
<td>90–109</td>
<td>Bhanumathi et al. (2003)</td>
</tr>
</tbody>
</table>

Clas, classical.

Septaplex PCR

Vibrio cholerae strains grown overnight at 37°C in LB broth (Difco, Becton-Dickinson) were boiled for 10 min and stored at −20°C until use. The bacterial cell lysate served as the source of template DNA. The sequences of the primers used in this study are given in Table 1.

PCR amplification of target DNA was carried out in a thermal cycler (MJ Research Inc. MA, Boston), with a 200 μL PCR tube containing a reaction mixture volume of 100 μL. Each reaction contained 10 μL of 10 × PCR amplification buffer [500 mM KCl, 100 mM Tris·HCl (pH 9.0), 1.0% Triton X-100] (Promega Madison, WI), 10 μL of MgCl2 (25 mM), 10 μL each of 2.5 mM dATP, dCTP, dGTP, and dTTP (Promega Madison, WI), 4.0 μL of the forward and reverse primers for ISRrRNA (20 ng μL⁻¹), 1.0 μL of Taq DNA polymerase at 5U μL⁻¹ (Promega Madison, WI), and Milli-Q water to a final volume of 97 μL and 3.0 μL of cell lysate (template DNA). 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°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min.
Septaplex PCR was carried out by simultaneous addition of primer pairs for O1rfb, O139rfb, ctxA, tcpA (El Tor and classical), toxR, and intstx in the same reaction mixture. In the initial experiments, O1rfb, O139rfb, ctxA, tcpA (El Tor/Classical), toxR, and intstx primer concentrations varied between 12 and 35 μM, keeping the ISRrRNA primer concentration fixed at either 40 μM or 50 μM in the PCR reaction mixture of 100 μL. Optimum results were obtained with a primer concentration of 12 μM for sxt, 15 μM for ctxA, tcpA, and toxR, 20 μM for O139rfb, and 35 μM for O1rfb genes. PCR conditions for amplification remained the same, except for the annealing temperature of template DNA at 60 °C for 1 min and extension temperature at 72 °C for 1 min for 20 cycles and 52 °C for 1.5 min and 72 °C for 2 min for 15 cycles. Amplified products were separated by agarose (2.5%, w/v) gel electrophoresis in Tris-borate-ethylene diamine tetra acetic acid (0.5 × TBE), stained in ethidium bromide, and visualized with a gel documentation system (Ultra-Lum, Inc., Claremont, CA). All strains of V. cholerae showing negative results by PCR either for O1rfb or O139rfb or both, ctxA, tcpA, toxR genes, and/or intstx were verified by slot-blot hybridization.

### Probes and hybridization

Slot-blots were prepared using nylon membranes (Hybond N²; Amersham International, London, UK) and processed by the method of Maniatis et al. (1982). Briefly, the cell lysates, with denaturing solution (0.5 M NaOH, 0.025 × TE), were transferred to nylon membranes using the Bio-Rad Slot-Blot apparatus. The DNA was fixed to the nylon membranes using a UV cross-linker (Bio-Rad) for 2 min.

PCR amplified products, obtained from V. cholerae O1 biotype El Tor strains KO194 for O1rfb, ctxA, tcpA (El Tor) and toxR, and V. cholerae O139 strain ATCC51394 for O139rfb and intstx, were used as probes and were randomly labeled (Feinberg & Vogelstein, 1984) with [α-32P] dATP (3000 Ci mmol⁻¹; BARC, Bombay, India) and hybridized at 65 °C in phosphate buffer containing 500 mM Na2HPO4 (pH 7.2), 7% (w/v) sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% (w/v) bovine serum albumin. Hybridized blots were washed once in 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min at room temperature, twice in 2 × SSC-0.1% SDS for 15 min at 65 °C, and...
Environmental sample analysis

Water samples from Bindusagar, Mausima temple pond and Daya River in Bhubaneswar, India, were collected aseptically in sterile bottles. Water was passed through Whatman No.1 filters and the filtrate was again passed through a 0.22 μm membrane (Millipore). The membrane was cut in to eight pieces, transferred to 10 mL alkaline peptone water, and incubated at 37 °C for 6 h. After enrichment, appropriate dilutions were prepared and spread on TCBS agar plates. Two-mL of enriched culture was boiled for 10 min and stored at −20 °C until further use and simultaneously used as template DNA in the septaplex PCR. Ten to 50 individual colonies from the TCBS agar plate were subjected to biochemical and serological tests and screened by septaplex PCR for identification.

Results and discussion

Of the 104 strains of *V. cholerae* O1 tested by the septaplex PCR for the presence of *O1rfb* or *O139rfb*, ISRrRNA, *toxR*, *ctxA*, *tcpA*, and/or *sxt* genes 103 strains of *V. cholerae* O1 gave positive results for *O1rfb*, ISRrRNA, and, except for three strains, the *toxR* genes. Fragment sizes were 192 bp for *O1rfb*, 295/310 bp for ISRrRNA, and 779 bp for *toxR* (Fig. 1a). Ninety five strains were positive for *ctxA* and *tcpA* genes, and 85 for *intsxt*. The fragment sizes were 564 bp for *ctxA*, 823 bp for El Tor specific *tcpA*, and 946 bp for *intsxt* (Fig. 1a, Table 2). All *ctxA* strains produced CT in the range of 20–6821 pg mL⁻¹ when tested by enzyme linked immunosorbent assay (data not shown). The strains that gave negative results by PCR for one or more genes were also negative in the corresponding slot-blot assay, except for two strains that were positive for *ctxA*, and one of these was also positive for *toxR*. A strain designated O1 serogroup gave positive results by septaplex PCR for *O139rfb*, *toxR*, ISRrRNA, *ctxA*, *tcpA* (El Tor), and *intsxt* (Table 2).

All *V. cholerae* O139 strains were positive by septaplex PCR for the genes encoding for *O139rfb*, ISRrRNA, *toxR*, *tcpA* (El Tor specific), and, except for three strains, for *ctxA* (Fig. 1a), and one strain, for *intsxt* (Table 2). All *ctxA* strains produced CT in the range of 10–8600 pg mL⁻¹ (data not shown). One *V. cholerae* strain O139 strain was positive for *O1rfb*, in addition to ISRrRNA, *toxR*, *tcpA* (El Tor specific), *ctxA*, and *intsxt* (Table 2).

Of the 39 *V. cholerae* non-O1, non-O139 strains isolated from clinical and environmental sources, all were positive by septaplex PCR for ISRrRNA and 34 strains, for *toxR*. Seven of the strains were also positive for *intsxt*. However, two strains were positive for *O1rfb*, one of which was *ctxA*.

**Fig. 1.** (a) Ethidium bromide-stained agarose gel electrophoresis of Septaplex PCR products from *Vibrio cholerae* strains. Lane M, 100 bp DNA ladder (Promega, Madison, WI); lane 1, *ctxA*, *tcpA* (El Tor), *toxR*, *sxt*, ISRrRNA, and *O1rfb* gene-positive *V. cholerae* O1 biotype El Tor strain K0194; lane 2, *ctxA*, *tcpA* (El Tor), and *sxt* gene-negative *V. cholerae* O1 biotype El Tor strain X-392; lane 3, *ctxA* and *sxt* gene-negative *V. cholerae* O1 biotype El Tor strain 2740–80; lane 4, *toxR*, and *sxt* gene-negative *V. cholerae* O1 biotype classical strain O939–12; lane 5, *tcpA* (classical), and *sxt* gene-negative *V. cholerae* O1 biotype classical strain O935 RT110-12; lane 6, *ctxA*, *tcpA* (classical), *toxR*, ISRrRNA, and *O1rfb* gene-positive *V. cholerae* O1 biotype classical strain 5698; lane 7, *ctxA*, *tcpA* (El Tor), *toxR*, *sxt*, ISRrRNA, and *O139rfb* gene-positive *V. cholerae* O139 strain ATCC51394; lane 8, *ctxA* gene-negative *V. cholerae* O139 strain CO788; and lane 9, *toxR*, and ISRrRNA gene-positive *V. cholerae* non-O1 and non-O139 strain 199. (b) Septaplex PCR products of representative *V. cholerae* O1, O139, and non-O1 and non-O139 strains in an ethidium bromide-stained agarose gel. Lane M, 100-bp DNA ladder (Promega, Madison, WI); lane 1, *ctxA*, *tcpA* (El Tor), *toxR*, ISRrRNA, and *O1rfb* gene-positive *V. cholerae* O1 biotype El Tor strain VO43 (Ganga water, 1986, Varanasi); lane 2, *ctxA*, *tcpA* (El Tor), *toxR*, ISRrRNA, and *O1rfb* gene-positive *V. cholerae* O1 biotype El Tor strain VO114 (Stool, 1996, Varanasi); lane 3, *ctxA* gene-negative *V. cholerae* O1 biotype El Tor strain CO38 (Stool, 1995, Kolkata); lane 4, *ctxA* and *sxt* gene-negative *V. cholerae* O1 biotype El Tor strain VO101 (Stool, 1975, Varanasi); lane 5, *ctxA*, *tcpA*, and *sxt* gene-negative *V. cholerae* O1 biotype El Tor strain VO94 (Ganga water, 1988, Varanasi); lane 6, *toxR* gene-negative *V. cholerae* O1 biotype El Tor strain AQ29 (Stool, 1996, Alappuzha); lane 7, *ctxA*, *tcpA* (El Tor), *toxR*, *sxt*, ISRrRNA, and *O139rfb* gene-positive *V. cholerae* O139 strain VO146 (Ganga water, 1992, Varanasi); lane 8, *ctxA* gene-negative *V. cholerae* O139 strain TO257 (Stool, 2002, Trivandrum); lane 9, *toxR*, *sxt*, and ISRrRNA gene-positive *V. cholerae* non-O1 and non-O139 strain VO222 (Sewage, 1987, Varanasi); lane 10, *toxR*, and ISRrRNA gene-positive *V. cholerae* non-O1 and non-O139 strain VO325A (Ganga water, 1986, Varanasi); lane 11, ISRrRNA gene-positive *Vibrio mimicus* strain VMCS0102; lane 12, *ctxA*, *tcpA* (El Tor/classical), *toxR*, ISRrRNA, *sxt*, *O1rfb* or *O139rfb* gene-negative *Vibrio parahaemolyticus* strain VPXV138.
positive and the other positive for O139rfb, in addition to ISRrRNA, toxR, ctxA, tcpA (El Tor specific), and intsxt (Table 2). In contrast, V. mimicus, V. parahaemolyticus, enterotoxigenic E. coli, enteropathogenic E. coli, enterohemorrhagic E. coli, and enterogauggerate E. coli were negative by PCR for all genes tested, except one strain of V. mimicus that amplified a portion of the ISRrRNA gene, where size was 310 bp. The septaplex PCR results for representative strains of V. cholerae O1, O139, and non-O1, non-O139 serogroups, V. mimicus, and V. parahaemolyticus possessing various combinations of genes are given in Fig. 1b.

All V. cholerae O1, O139, non-O1 and non-O139, and V. mimicus negative either for O1rfb or O139rfb, or both, were also negative for ctxA, tcpA (El Tor specific), toxR, and/or intsxt in the slot-blot assay (data not shown). These observations confirmed the absence of these genes in V. cholerae and V. mimicus strains analyzed in this study. These results also revealed a good correlation between negative results for O1rfb, or O139 rfb or both, with negative results for ctxA and tcpA.

When six water samples, concentrated and enriched in alkaline peptone water and boiled for 10 min, were used as template DNA in septaplex PCR, all gave positive results for the genes encoding for ISRrRNA and toxR. However, a water sample from Bindusagar Lake (sample site no. 2) also gave positive results by PCR for the El Tor-specific tcpA gene. Strains isolated from these water samples, when tested by standard biochemical reactions and serological tests, revealed that they were non-O1, non-O139 V. cholerae (Fig. 2a). Although the cell lysate prepared from the pure cultures of V. cholerae strains isolated from different water sampling sites including the strain isolated from Bindusagar Lake (sample site no. 2) failed to give positive results by PCR for tcpA, they were positive for ISRrRNA and toxR (Fig. 2b). The result of this study thus suggests that V. cholerae non-O1, non-O139 strains present in the water samples possessed the tcpA gene; however, the strains could not be cultured from the water samples even though they could be enriched, a common problem with environmental vibrios (Colwell & Huq, 1994). Septaplex PCR is useful in identifying pathogenic and intsxt-positive V. cholerae strains in the environment. Further study of this PCR method for both environmental and clinical samples is in progress.

The toxR gene is known to be involved in the regulation and expression of several V. cholerae genes (Ottemann & Mekalanos, 1994). The presence of toxR-related gene sequences in several Vibrio spp. has been reported, although the sequences varied significantly (Lin et al., 1993; Reich & Schoolnik, 1994; Osorio & Klose, 2000). However, toxR primers specific for V. cholerae can be used to differentiate it from other Vibrio spp., most likely because of the difference in the primer sequence of toxR compared with other vibrios (Nandi et al., 2000).

The rRNA gene nucleotide sequences have provided valuable information for identification and taxonomy of bacterial species. Unfortunately, the 16S rRNA gene sequences of Vibrio spp. reveal minimal differences, making species-specific identification difficult by this approach. Chun et al. (1999) designed primers based on subtle differences in the nucleotide sequence of a 16S–23S rRNA gene ISR of V. cholerae and V. mimicus. PCR amplification using the primer pair was able to generate amplicons, even though variable in size (295–310 bp), from several V. cholerae strains, including V. cholerae non-O1, non-O139.

In this study, the sequence similarity of the 16S–23S rRNA gene (ISRrRNA) ISR (Chun et al., 1999), and toxR gene (Ottemann & Mekalanos, 1994) of V. cholerae strains was exploited for species-specific identification and the tcpA gene for determining differences between classical and El Tor biotypes (Keasler & Hall, 1993; Taylor et al., 1987). We were able to differentiate strains belonging to V. cholerae serogroup O1 or O139 by targeting O1rfb- or O139rfb-specific primers by amplifying the O1- or O139-specific rfb genes (Albert et al., 1997; Hoshino et al., 1998). The toxigenic–pathogenic strains were differentiated from nontoxigenic–nonpathogenic strains on the basis of the presence of the ctxA and tcpA genes. In addition, the presence or absence of a 62-kb sxt gene responsible for acquisition or deletion of a...
number of antibiotic resistance genes (i.e. chloramphenicol, trimethoprim, sulfamethoxazole, streptomycin) was also determined (Waldor et al., 1996).

From the results presented here, it is concluded that V. cholerae strains belonging to serogroups O1, O139, and non-O1, non-O139 can be readily identified and differentiated. Furthermore, the presence of cholera toxin, toxin coregulated pilus genes, and intSXT could also be determined in the one-step PCR, a method that can be used for rapid identification of V. cholerae and simultaneously the detection of virulence genes and intSXT. The method offers a valuable procedure for identification of V. cholerae serogroups and for screening for both clinical and environmental pathogenic, intSXT-positive, and nonpathogenic, intSXT-negative V. cholerae.

Acknowledgements

This research was supported by Indian Council of Medical Research grant (Immuno. 18/11/17/2002-ECD-I) and Department of Science and Technology, New Delhi grant (SR/SO/HS-51/2002) to D.V.S. and funds contributed by the Department of Biotechnology, New Delhi to the Institute of Life Sciences, Bhubaneswar. Senior Research Fellowships awarded by the Council of Scientific and Industrial Research, New Delhi, India, to Chinmay K. Mantri and Saswat S. Mohapatra are gratefully acknowledged. We thank G.B. Nair of the International Centre for Diarrheal Diseases Research, Dhaka (ICDDB, B), Bangladesh, J.B. Kaper of the Center for Vaccine Development (CVD), University of Maryland School of Medicine, Baltimore, MD, J.J. Melakalos, Department of Microbiology and Molecular Genetics, Harvard School of Medicine, Boston, and A.C.P. Vicente of the Department of Genetics, Institute Oswaldo Cruz, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, B.B. Pal of Regional Medical Research Center, Bhubaneswar, and B.N. Shukla of the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, for providing nontoxigenic V. cholerae O139, E. coli, nontoxigenic O1, tcpA and toxR mutant strains, and non-O1, non-O139 strain199, V. cholerae O1, and O139 strains, respectively.

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