Characterization of the genetic background of *Vibrio cholerae* O1 biotype El Tor serotype Inaba strains isolated in Trivandrum, southern India

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Isolates of *Vibrio cholerae* O1 biotype El Tor serotype Inaba associated with an outbreak of cholera in Trivandrum, southern India, were characterized. PCR testing revealed that all five isolates examined carried the TCP pathogenicity island, the CTX genetic element and the RTX toxin, and produced cholera toxin (CT). RFLP analysis revealed that these Inaba isolates possessed a single copy of the CTX element flanked by two tandemly arranged copies of the RS element upstream of the core region. The isolates were resistant to ampicillin, nalidixic acid, trimethoprim, sulfamethoxazole, streptomycin and the vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (O/129). Ribotyping of these Inaba isolates revealed a hybridization profile similar to a strain of serotype Ogawa prevalent in southern India.

INTRODUCTION

*Vibrio cholerae* is a natural inhabitant of the aquatic environment and water plays an important role in disease transmission (Colwell et al., 1977; Islam et al., 1994). *V. cholerae* O1 and O139 are currently believed to be the only serogroups causing epidemic cholera, characterized by severe watery diarrhoea. *V. cholerae* O1 is further classified into two biotypes, classical and El Tor, and into two major serotypes, Inaba and Ogawa. *V. cholerae* O1 strains may exhibit serotype conversion or switching between Inaba and Ogawa serotypes (Colwell et al., 1995; Gangarosa et al., 1967; Stroher et al., 1992). Extensive analysis of epidemic O1 strains that caused cholera outbreaks in Latin America in 1991 revealed that the El Tor Inaba strains were unique to Latin America (Manning et al., 1994; Salazar-Lindo et al., 1991). However, Ogawa isolates that are identical to epidemic strains in all respects began to appear in approximately month 7 of the epidemic, suggesting that the epidemic strains had undergone serotype conversion, possibly because of immune pressure in the population (Manning et al., 1994).

In 1992, *V. cholerae* O139 serogroup replaced the Inaba serotype of *V. cholerae* O1 in Kolkata and other parts of India (Mukhopadhyay et al., 1996), and the last predominance of Inaba was observed in 1989 (Ramamurthy et al., 1992). Isolation of *V. cholerae* O1 belonging to Inaba became rare, but isolations were reported from Warangal (Saha et al., 1996), Delhi and Sewagram (Garg et al., 2000). Extensive characterization of these Inaba isolates indicated that they were remarkably different from the earlier Inaba strains, but were very similar to the prevailing *V. cholerae* O1 Ogawa strains (Garg et al., 2000). In May 1996, a large outbreak of cholera caused by *V. cholerae* O1 El Tor Ogawa occurred in the Alleppey district of Kerala, India, and subsequently spread to the neighbouring Palghat district (Radhakutty et al., 1997). Moreover, in 2000, *V. cholerae* O1 Ogawa strains caused cholera outbreaks in the Kottayam district, and subsequently spread to the Alleppey and Trivandrum districts of Kerala, India (Sabeena et al., 2001). During this period, a toxigenic *V. cholerae* O139 strain was also isolated from a diarrhoeal patient (Bhanumathi et al., 2002).

In this study, we used molecular techniques to characterize *V. cholerae* O1 El Tor Inaba isolates from the recent outbreak in Trivandrum and compared them with *V. cholerae* O1 El Tor Inaba strains isolated from other parts of India prior to 2000 with respect to clonality, presence of virulence and regulatory genes and pathogenicity islands, and antibiotic resistance.

METHODS

Bacterial strains. We received a set of representative *V. cholerae* strains isolated from diarrhoeal patients admitted to the Government Medical College and Hospital, Trivandrum, India, on 9 October 2000, five of which were identified as *V. cholerae* O1 El Tor Inaba. Reference strains of *V. cholerae* 569B (O1 classical Inaba), VC20 (O1 El Tor Ogawa), V1 (O1 El Tor Inaba), CO840 (O1 El Tor Ogawa), DO183 (O1 El Tor Inaba), Al11 (O1 El Tor Ogawa) and Al40 (O1 El Tor Ogawa), provided by Dr T. Ramamurthy of the National Institute of Cholera and Enteric Diseases (NICED),
Kolkata, India, were used as controls. All strains were maintained in tryptic soy broth (Difco) supplemented with 30% glycerol at −70°C, or in Luria–Bertani agar stab cultures at room temperature. Before use, the identity of the cultures was confirmed by selected biochemical tests and serology (World Health Organization, 1987).

**PCR assays.** The presence of the virulence and regulatory genes ctxA, zot, ace, tcpA, ompU and toxR was determined using a hexaplex PCR assay (Singh et al., 2002). Single PCR assays were used to detect the tcpI (Rivera et al., 2001), acfB (Faruque et al., 1998a) and RTX toxin genes (Chow et al., 2003). Oligonucleotide primer sequences were as described by Rivera et al. (2001) and Singh et al. (2001), and were purchased from GCC. Amplified products were separated in agarose gel, stained with ethidium bromide and visualized using a Fluor-S MultilImager (Bio-Rad).

**Assay for cholera toxin (CT) production.** The ability of the Inaba isolates to produce CT in vitro was determined using a GM1-ganglioside-dependent ELISA, as described by Svennerholm & Holmgren (1978).

**Antibiotic resistance.** Isolates were tested for antimicrobial resistance by the method of Bauer et al. (1966) using antibiotics discs (Hi-Media Laboratories) containing ampicillin (10 μg), chloramphenicol (30 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), cephalixin (30 μg), co-trimoxazole (25 μg), furazolidone (100 μg), gentamicin (10 μg), neomycin (30 μg), norfloxacin (10 μg), polymyxin B (50 U), streptomycin (30 μg), nalidixic acid (30 μg), tetracycline (30 μg) or the vibriostatic agent 2,4-diamino-6,7-diisopropylpterdine (O/129) (10 and 150 μg).

**Detection of the SXT element by PCR and RFLP.** The presence of the SXT genetic element (a self-transmissible transposon-like element) was determined using a PCR assay (Bhanumathi et al., 2003) and the amplicon was digested with HindIII or Msel (NEB). Following electrophoresis in 3% agarose, the sizes of the fragments were determined and compared with the expected sizes based on the published sequence of the sxt gene (Hochhut et al., 2001).

**Probes and hybridization.** Slot blots were prepared using Hybond-N+ nylon membranes (Amersham International) and processed using standard methods (Sambrook & Russell, 2001). Briefly, cell lysates in denaturing solution (0.5 M NaOH, 0.025× TE) were transferred to nylon membrane using the Bio-Rad slot-blot apparatus and the DNA was fixed by exposure to UV light for 2 min, in accordance with the manufacturer’s instructions. For preparation of DNA blots, total cellular DNA was isolated from overnight cultures as described by Ausubel et al. (1995). Aliquots of DNA (5 μg) were digested with appropriate restriction enzymes (NEB), separated by electrophoresis in 0.8% agarose gels and blotted onto nylon membranes by Southern blotting (Southern, 1975).

The gene probe used to detect the CTX genetic element was a 0.54 kb fragment amplified by PCR from *V. cholerae* strain 569B (Fields et al., 1992) and the RS1 probe was a 0.9 kb fragment amplified by PCR from the same strain (Qu et al., 2003). The rRNA gene probe was a 7.5 kb BamHI fragment of pKK3555 (Brosius et al., 1981), which is a pBR322-derived plasmid containing an *Escherichia coli* rRNA operon consisting of one copy each of the genes encoding 5S rRNA, 16S rRNA, 23S rRNA and tRNA

**RESULTS AND DISCUSSION**

**Analysis of virulence and regulatory genes**

Hexaplex PCR studies revealed that each of the five isolates of *V. cholerae* O1 Inaba from Trivandrum harboured the *ctxA*, *zot* and *ace* genes and the El Tor *tcpA*, *ompU* and *toxR* genes (Table 1). However, O1 El Tor Ogawa strain CO840 and Al11 were negative for the *ctxA* and *toxR* genes, respectively, but carried *tcpI* and *acfB* in addition to the *rtxA* and *rtxC* genes (Table 1). All strains tested, except for strain CO840, carried the *ctxB* gene (data not shown).

### Table 1. Antibiotic resistance patterns, virulence profiles and CT production by the *V. cholerae* O1 strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Year and place of isolation</th>
<th>Antibiotic resistance pattern*</th>
<th>Presence of virulence and regulatory genes:</th>
<th>CT production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ctxA</td>
<td>zot</td>
<td>ace</td>
</tr>
<tr>
<td>VI</td>
<td>Inaba</td>
<td>1989, Kolkata</td>
<td>A</td>
<td>Co</td>
<td>Fr</td>
</tr>
<tr>
<td>VC20</td>
<td>Ogawa</td>
<td>1989, Kolkata</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>569B</td>
<td>Inaba</td>
<td>1948, India</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DO183</td>
<td>Inaba</td>
<td>1998, Delhi</td>
<td>A</td>
<td>Co</td>
<td>Fr</td>
</tr>
<tr>
<td>CO840</td>
<td>Ogawa</td>
<td>1995, Kolkata</td>
<td>A</td>
<td>Co</td>
<td>Fr</td>
</tr>
<tr>
<td>Al11</td>
<td>Ogawa</td>
<td>1996, Alapp erfahren</td>
<td>A</td>
<td>Co</td>
<td>Fr</td>
</tr>
<tr>
<td>Al40</td>
<td>Ogawa</td>
<td>1996, Alapp erfahren</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TV277</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TV280</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TV281</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TV285</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TV289</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

A, Ampicillin; Co, co-trimoxazole; Fr, furazolidone; S, streptomycin; Tr, trimethoprim; Na, nalidixic acid.

*All of the *V. cholerae* O1 strains were resistant to the vibriostatic agent 2,4-diamino-6,7-diisopropylpterdine (O/129).
**Table 2.** Ribotype, CTX and RS1 RFLP patterns of the *V. cholerae* O1 strains used in this study

*V. cholerae* O1 El Tor Ogawa strain CO840 isolated from Kolkata in 1995 belonging to ribotype B-III does not have CTX prophage genes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Year and place of isolation</th>
<th>Ribotype</th>
<th>Fragment size (kb) with the following probes and restriction enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ctxA (HindIII, BglII, BglI, PstI, Xbal)</td>
</tr>
<tr>
<td>VI Inaba</td>
<td>1989, Kolkata</td>
<td>B-II</td>
<td>23.0</td>
<td>4.8, 3.0, 10.2, 7.5, 12.0, 8.0, 10.0, 8.0</td>
</tr>
<tr>
<td>VC20 Ogawa</td>
<td>1989, Kolkata</td>
<td>B-II</td>
<td>23.0</td>
<td>4.8, 3.0, 10.2, 7.5, 12.0, 8.0, 10.0, 8.0</td>
</tr>
<tr>
<td>569B Inaba</td>
<td>1948, India</td>
<td>B-I</td>
<td>22.0, 20.0</td>
<td>7.5, 3.0, 10.2, 7.8, 12.0, 7.5, 10.0, 8.5</td>
</tr>
<tr>
<td>DO183 Inaba</td>
<td>1998, Delhi</td>
<td>B-III</td>
<td>23.0</td>
<td>8.4, 7.0, 7.5, 12.0, 11.0, 8.0</td>
</tr>
<tr>
<td>Al11 Ogawa</td>
<td>1996, Alleppey</td>
<td>B-IV</td>
<td>22.0</td>
<td>7.5, 4.8, 7.8, 7.5, 8.0</td>
</tr>
<tr>
<td>Al40 Ogawa</td>
<td>1996, Alleppey</td>
<td>B-III</td>
<td>22.0</td>
<td>7.5, 4.8, 7.8, 7.5, 8.0</td>
</tr>
<tr>
<td>TV277 Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</td>
<td>7.5, 4.8, 7.8, 7.5, 8.0</td>
</tr>
<tr>
<td>TV280 Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</td>
<td>7.5, 4.8, 7.8, 7.5, 8.0</td>
</tr>
<tr>
<td>TV281 Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</td>
<td>7.5, 4.8, 7.8, 7.5, 8.0</td>
</tr>
<tr>
<td>TV285 Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</td>
<td>7.5, 4.8, 7.8, 7.5, 8.0</td>
</tr>
<tr>
<td>TV289 Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</td>
<td>7.5, 4.8, 7.8, 7.5, 8.0</td>
</tr>
</tbody>
</table>

*V. cholerae* O1 El Tor Inaba strains isolated from Warangal have been reported to be non-toxigenic, i.e. they lacked the core of the CTX genetic element and did not produce CT (Saha et al., 1996). However, the *V. cholerae* O1 Inaba isolates from Trivandrum possessed the core of the CTX genetic element, the TCP pathogenicity island and the RTX genetic element and all produced CT at a concentration of between 20 and 330 pg ml⁻¹. Thus, these strains had all of the characteristics of epidemic cholera in their ability to adhere, colonize and produce CT in the small intestine.

Studies were conducted to examine differences in the location, number and arrangement of the CTX prophage among the *V. cholerae* O1 Inaba isolates from Trivandrum. Hybridization of chromosomal DNA digested with *Hind*III, which cleaves DNA sequences outside the CTX prophage, with the *ctxA* probe revealed a single band of 22 kb (Table 2), indicating that the CTX prophage is located at a single site. The arrangement and number of copies of the CTX prophage were investigated using the same probe and other enzymes that cut within the CTX prophage but not in *ctxA*. RFLP patterns generated with *BglII, BglI*, *PstI* and *Xbal* showed a single band in all of the recent Inaba strains (Table 2), suggesting the presence of a single copy of the CTX prophage. Southern blotting of the *BglII* digest also produced a second band of 4.8 kb in all of the Inaba strains (Table 2). As *BglII* cuts within the *zot* gene, the number of bands obtained with Inaba strains after probing with *ctxA* indicated that the downstream sequences of the CTX prophage differed in these Inaba strains from O1 strains reported by Garg et al. (2000). The CTX prophage has two regions: a 4.5 kb core region that includes *ctxAB* and a 2.4 kb region termed RS2. The integrated CTX prophage is frequently flanked by an element known as RS1, which is related to RS2 (Waldor et al., 1997). These related elements contain three nearly identical ORFs. Southern blot hybridization with an RS1 probe was carried out to determine the organization of the RS sequences upstream and downstream of the core region (Mekalans, 1983) using the enzymes *BglII* and *PstI*, which cut within the core region; the RFLP patterns generated showed the presence of single bands of 8.4 and 23 kb, respectively, for each enzyme, indicating that all of the Inaba strains from Trivandrum contained at least one copy of the RS1 element. Digestion of DNA from these strains with *BglII*, which cuts within the RS1 region, revealed two bands of 7.8 and 2.7 kb on hybridization with the RS1 probe; one of these was the size of the CTX prophage (4.5 + 2.7 kb). This result confirmed the presence of the RS1 element upstream of the CTX prophage. Comparison of hybridization results of *BglII*-digested DNA with *ctxA* and RS1 probes showed a common band of 7.8 kb, indicating the presence of a single copy of the CTX prophage. The presence of a 2.7 kb band in all of the Inaba strains was consistent with a tandemly arranged RS element upstream of the CTX prophage. This organization (a single copy of the CTX prophage with two tandemly arranged RS elements upstream) is very similar to O1 Ogawa strains isolated in Alleppey in 1996, but different from the other O1 El Tor Inaba strains isolated prior to 2000 elsewhere in India. Fig. 1 gives a schematic representation, based on RFLP analysis, of the CTX genetic element of Inaba strain TV285 isolated in Trivandrum in 2000 and DO183 isolated from Delhi in 1998.
Antibiotic resistance

*V. cholerae* O1 El Tor Ogawa, which emerged in 1996 in Alleppey, was sensitive to tetracycline and showed resistance to sulfamethoxazole, trimethoprim and streptomycin. Waldor et al. (1996) reported the presence of a 62 kb SXT element encoding resistance to sulfamethoxazole, trimethoprim and streptomycin in *V. cholerae* O1 strains isolated after the O139 epidemic. All of the recent *V. cholerae* O1 El Tor Inaba isolates, like *V. cholerae* O1 Ogawa, were resistant not only to sulfamethoxazole, trimethoprim and streptomycin, but also to ampicillin and nalidixic acid. All of the recent O1 Inaba strains were positive by PCR for the *sxt* gene and amplified a 946 bp portion of the SXT genetic element (Table 1). Subsequent restriction analysis with *Hind*III and *Mse*I produced sets of fragments whose sizes agreed with the expected sizes based on the published sequence of SXT (data not shown). This indicated that *V. cholerae* O1 El Tor Inaba, like *V. cholerae* O139 strains, possesses the SXT genetic element encoding sulfamethoxazole, trimethoprim and streptomycin resistance. This was further confirmed by multiplex PCR for the respective genes *SulII*, *Tm* and *Sm* (data not shown).

Ribotyping of *V. cholerae* O1 Inaba strains

Ribotyping using *Bgl*II restriction digest has been used successfully to study genomic diversity among *V. cholerae* O1 strains (Dalsgaard et al., 1997). We utilized ribotyping to ascertain whether Inaba strains isolated from the recent outbreak in Trivandrum had emerged from the prevailing *V. cholerae* O1 Ogawa strains. The ribotype patterns of reference strains consisted of seven to nine bands of between 1.5 and 18.9 kb and were reproducible in repeated assays. Fig. 2 shows the patterns obtained with the reference strains of *V. cholerae* O1 strain and those of the strains from Trivandrum. The ribotype designations (patterns I to III) followed those of Dalsgaard et al. (1997) and other investigators (Faruque et al., 1997; Garg et al., 2000). However, *V. cholerae* O1 strain Al11, isolated in Alleppey in 1996, was classified as ribotype pattern IV as it lacked two bands of approximately 1.5 and 16.5 kb. The Trivandrum strains also showed pattern IV (Table 2 and Fig. 2).
Emergence of a new clone of V. cholerae O1 El Tor Inaba strains

Three ribotypes have been demonstrated in V. cholerae O1 El Tor Inaba strains (Dalsgaard et al., 1997; Garg et al., 2000). Seasonal epidemics of cholera are often associated with the emergence of new epidemic clones, which replace existing clones of V. cholerae. The mechanisms controlling the temporal shifts of clones within the epidemic serogroups are not well understood. Our data show that recent isolates of V. cholerae O1 El Tor Inaba share the ribotype pattern of the Ogawa strain and carry the known virulence gene cluster including the TCP pathogenicity island (Faruque et al., 2004) and the CTX prophage (Siddique et al., 1992). It is known that V. cholerae O1 strains interconvert between Ogawa and Inaba forms (Colwell et al., 1995; Gangerosa et al., 1967; Garg et al., 2000; Strother et al., 1992). The frequency of conversion of Ogawa to Inaba is approximately 10^{-3} (Bhaskaran & Gorrill, 1957), whereas conversion from Inaba to Ogawa is a rare phenomenon and may be strain dependent (Manning et al., 1994). It was reported that a point mutation within the wbeT gene (Strother et al., 1992) of the lipopolysaccharide gene cluster of V. cholerae is responsible for the serotype conversion and this may occur as a result of selection due to the pressure of lytic phages and immune response during cholera infection (Faruque et al., 1998b, 2005; Mukhopadhyay et al., 1996). It is therefore possible that this mechanism was responsible for the seroconversion of recent Inaba strains from the prevailing Ogawa strain in southern India.

Faruque et al. (2006) recently demonstrated in real-time the replacement of epidemic Inaba serotype strains with a newly emerged strain of Ogawa serotype by synchronized clinical and environmental monitoring. However, the transmission of cholera epidemics is determined by several biological factors, such as resistance to predation of phages, resistance to prevailing immune mechanisms in human populations and probably resistance to antibiotics used in the treatment of human infections. These selective pressures may provide an explanation for the periodic shift in the prevalence of serogroups and serotypes of V. cholerae (Faruque et al., 1999b, 2005). Further studies on the influence of environmental factors on the prevalence of serotypes of pathogenic V. cholerae are necessary.

ACKNOWLEDGEMENTS

This research was supported by grant SR/SO/HS-51/2002 to D.V.S. from the Department of Science and Technology, New Delhi, India, and funds contributed by the Institute of Life Sciences, Bhubaneswar, India. Senior Research Fellowships awarded by the Council of Scientific and Industrial Research, New Delhi, India, to S.S.M., D.R. and C. K. M. are gratefully acknowledged. The authors thank Dr P. Indu of the Department of Microbiology, Government Medical College, Trivandrum, India, for providing strains.

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