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

Saswat S. Mohapatra, Dhanya Ramachandran, Chinmay K. Mantri and Durg V. Singh

1Institute of Life Sciences, Nalco Square, Bhubaneswar 751 023, India
2Rajiv Gandhi Centre for Biotechnology, Jagathy, Trivandrum 695 014, India

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 (O1/129). 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 diarrhea. *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; Stroerher 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 diarrheal 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 diarrheal 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), AI11 (O1 El Tor Ogawa) and AI40 (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 tcpL (Rivera et al., 2001), acfB (Faruque et al., 1998a) and RTX toxin genes (Chow et al., 2001), and were purchased from GCC. Amplified products were processed using standard methods (Sambrook & Russell, 2001). Briefly, sequences were as described by Rivera 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), cephalexin (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-diisopropylpteridine (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 MseI (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 pKK355 (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 rRNA
g. The ctxB gene probe was a 1.4 kb KpnI fragment of pCDV621 (Kaper et al., 1988). The probes were labelled by random priming (Feinberg & Vogelstein, 1984) using a random primer labelling kit (NEB) and [α-32P]deoxyctydine triphosphate (3000 Ci mmol−1; Bhabha Atomic Research Center, Bombay, India). Southern and slot blots were hybridized with the labelled probes and autoradiographs were developed using a PhosphorImager (Fuji Photo Film).

**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 strains CO840 and AI11 were negative for the ctxA and toxR genes, respectively, but carried tcpL 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>
<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>
</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-diisopropylpteridine (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><strong>ctxA</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>HindIII</strong></td>
<td><strong>BglI</strong></td>
</tr>
<tr>
<td>VI</td>
<td>Inaba</td>
<td>1989, Kolkata</td>
<td>B-II</td>
<td>23.0</td>
</tr>
<tr>
<td>VC20</td>
<td>Ogawa</td>
<td>1989, Kolkata</td>
<td>B-II</td>
<td>23.0</td>
</tr>
<tr>
<td>569B</td>
<td>Inaba</td>
<td>1948, India</td>
<td>B-I</td>
<td>22.0</td>
</tr>
<tr>
<td>DO183</td>
<td>Inaba</td>
<td>1998, Delhi</td>
<td>B-III</td>
<td>23.0</td>
</tr>
<tr>
<td>Al11</td>
<td>Ogawa</td>
<td>1996, Alleppey</td>
<td>B-IV</td>
<td>22.0</td>
</tr>
<tr>
<td>Al40</td>
<td>Ogawa</td>
<td>1996, Alleppey</td>
<td>B-III</td>
<td>22.0</td>
</tr>
<tr>
<td>TV277</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</td>
</tr>
<tr>
<td>TV280</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</td>
</tr>
<tr>
<td>TV281</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</td>
</tr>
<tr>
<td>TV285</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</td>
</tr>
<tr>
<td>TV289</td>
<td>Inaba</td>
<td>2000, Trivandrum</td>
<td>B-IV</td>
<td>22.0</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 HindIII, 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 BglI, BglII, PstI and XbaI 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 BglI digest also 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 HindIII and MseI 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 BglII 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). This
finding suggested that the latter strains of Inaba serotype may have emerged from the prevalent Ogawa strain in southern India.

**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 interchange between Ogawa and Inaba forms (Colwell et al., 1995; Gangerosa et al., 1967; Garg et al., 2000; Stroeher et al., 1992). The frequency of conversion of Ogawa to Inaba is approximately $10^{-3}$ (Bhaskaran & Gorill, 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 (Stroeher 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 and environmental 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., 1998b, 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.

**REFERENCES**


