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

Saswat S. Mohapatra,¹ Dhanya Ramachandran,² Chinmay K. Mantri¹ and Durg V. Singh¹

Correspondence

Durg V. Singh
durg_singh@yahoo.co.in

¹Institute of Life Sciences, Nalco Square, Bhubaneswar 751 023, India

²Rajiv Gandhi Centre for Biotechnology, Jagathy, Trivandrum 695 014, 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 were 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),

Abbreviation: CT, cholera toxin.

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.*, 2001). 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 MultiImager (Bio-Rad).

Assay for cholera toxin (CT) production. The ability of the Inaba isolates to produce CT *in vitro* was determined using a GM_1 -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-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, $0.025 \times \text{TE}$) 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 pKK3535 (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^{Glu}. The *ctxB* gene probe was a 1.4 kb *KpnI* fragment of pCVD621 (Kaper *et al.*, 1988). The probes were labelled by random priming (Feinberg & Vogelstein, 1984) using a random primer labelling kit (NEB) and [α -³²P]deoxycytidine 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 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

Strain	Serotype	Year and place of isolation	Antibiotic resistance pattern*	Presence of virulence and regulatory genes:											CT production
				<i>ctxA</i>	<i>zot</i>	<i>ace</i>	<i>tcpA</i>	<i>ompU</i>	<i>toxR</i>	<i>tcpI</i>	<i>acfB</i>	<i>rtxA</i>	<i>rtxC</i>	<i>sxt</i>	
VI	Inaba	1989, Kolkata	A, Co, Fr, S, Tr	+	+	+	+	+	+	+	+	+	+	+	+
VC20	Ogawa	1989, Kolkata	A	+	+	+	+	+	+	+	+	+	+	+	+
569B	Inaba	1948, India	A	+	+	+	+	+	+	+	+	+	+	+	+
DO183	Inaba	1998, Delhi	A, Co, Fr, S, Tr, Na	+	+	+	+	+	+	+	+	+	+	+	+
CO840	Ogawa	1995, Kolkata	A, Co, Fr, S, Tr, Na	—	—	—	+	+	+	+	+	+	+	+	—
Al11	Ogawa	1996, Alleppey	Co, Fr, S, Tr	+	+	+	+	+	—	+	+	+	+	+	+
Al40	Ogawa	1996, Alleppey	A, Co, Fr, S, Tr	+	+	+	+	+	+	+	+	+	+	+	+
TV277	Inaba	2000, Trivandrum	A, Co, Fr, S, Tr, Na	+	+	+	+	+	+	+	+	+	+	+	+
TV280	Inaba	2000, Trivandrum	A, Co, Fr, S, Tr, Na	+	+	+	+	+	+	+	+	+	+	+	+
TV281	Inaba	2000, Trivandrum	A, Co, Fr, S, Tr, Na	+	+	+	+	+	+	+	+	+	+	+	+
TV285	Inaba	2000, Trivandrum	A, Co, Fr, S, Tr, Na	+	+	+	+	+	+	+	+	+	+	+	+
TV289	Inaba	2000, Trivandrum	A, Co, Fr, S, Tr, Na	+	+	+	+	+	+	+	+	+	+	+	+

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.

Strain	Serotype	Year and place of isolation	Ribotype	Fragment size (kb) with the following probes and restriction enzymes								
				<i>ctxA</i>					RS1			
				<i>Hind</i> III	<i>Bgl</i> I	<i>Bgl</i> II	<i>Pst</i> I	<i>Xba</i> I	<i>Bgl</i> I	<i>Bgl</i> II	<i>Pst</i> I	
VI	Inaba	1989, Kolkata	B-II	23.0	4.8, 3.0	10.2, 7.5	12.0, 8.0	10.0, 8.0	10.8, 7.8, 5.8, 3.5	10.2, 7.5, 4.8, 2.7	12.0, 8.0, 6.0	
VC20	Ogawa	1989, Kolkata	B-II	23.0	4.8, 3.0	10.2, 7.5	12.0, 8.0	10.0, 8.0	14.0, 10.8, 7.8, 2.3	10.2, 7.5, 4.8, 3.6, 2.7	12.0, 8.0	
569B	Inaba	1948, India	B-I	22.0, 20.0	7.5, 3.0	10.2, 7.8	12.0, 7.5	10.0, 8.5	10.8, 7.8	10.2, 7.8, 4.8, 2.3	23.0, 12.0	
DO183	Inaba	1998, Delhi	B-III	23.0	8.4, 7.0	7.5	12.0, 11.0	8.0	12.0, 8.4	10.2, 7.5, 3.6, 2.7	23.0, 12.0, 11.0	
Al11	Ogawa	1996, Alleppey	B-IV	22.0	7.5, 4.8	7.8	7.5	8.0	8.4	7.8, 2.7	23.0	
Al40	Ogawa	1996, Alleppey	B-III	22.0	7.5, 4.8	7.8	7.5	8.0	8.4	7.8, 2.7	23.0	
TV277	Inaba	2000, Trivandrum	B-IV	22.0	7.5, 4.8	7.8	7.5	8.0	8.4	7.8, 2.7	23.0	
TV280	Inaba	2000, Trivandrum	B-IV	22.0	7.5, 4.8	7.8	7.5	8.0	8.4	7.8, 2.7	23.0	
TV281	Inaba	2000, Trivandrum	B-IV	22.0	7.5, 4.8	7.8	7.5	8.0	8.4	7.8, 2.7	23.0	
TV285	Inaba	2000, Trivandrum	B-IV	22.0	7.5, 4.8	7.8	7.5	8.0	8.4	7.8, 2.7	23.0	
TV289	Inaba	2000, Trivandrum	B-IV	22.0	7.5, 4.8	7.8	7.5	8.0	8.4	7.8, 2.7	23.0	

V. cholerae O1 El Tor Inaba strains isolated from Warangal have been reported to be non-toxicogenic, 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 *Bgl*I, *Bgl*II, *Pst*I and *Xba*I 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 *Bgl*I digest also produced a second band of 4.8 kb in all of the Inaba strains (Table 2). As *Bgl*I 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 (Mekalanos, 1983) using the enzymes *Bgl*I and *Pst*I, 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 *Bgl*II, 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 *Bgl*II-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.

Fig. 1. Schematic representation of the organization of the CTX prophage (not to scale) as deduced by Southern blot hybridization data for *Vibrio cholerae* O1 El Tor Inaba strains represented by TV285 (a) and *V. cholerae* O1 Inaba strain DO183 (b). Arrows and boxes represent the RS element and core region of the CTX prophage, respectively, with the hatched region of the box representing the *ctxAB* gene. Restriction sites: BI, *Bgl*I; BII, *Bgl*II; H, *Hind*III; P, *Pst*I; X, *Xba*I.

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 *Sul*III, *Tm* and *Sm* (data not shown).

Ribotyping of *V. cholerae* O1 Inaba strains

Ribotyping using *Bgl*I restriction digests 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

Fig. 2. Ribotype patterns of representative *V. cholerae* strains. Lanes: 1, classical Inaba strain 569B, pattern B-I; 2, El Tor Ogawa strain VC20, pattern B-II; 3, El Tor Inaba strain V1, pattern B-II; 4, O1 El Tor Ogawa strain CO840, pattern B-III; 5, El Tor Inaba strain DO183, pattern B-III; 6, El Tor Ogawa strain Al11, pattern B-IV; 7, El Tor Ogawa strain Al40, pattern B-III; 8–12, O1 El Tor Inaba strains TV281, TV285, TV289, TV280, TV277, pattern B-IV.

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 interconvert between Ogawa and Inaba forms (Colwell *et al.*, 1995; Gangarosa *et al.*, 1967; Garg *et al.*, 2000; Stroher *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 (Stroher *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.*, 1998b, 2005). Further studies on the influence of environmental factors on the prevalence of serotypes of pathogenic *V. cholerae* are necessary.

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