Phenotypic and genotypic traits and epidemiological implication of *Vibrio cholerae* O1 and O139 strains in India during 2003

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During 2003, *Vibrio cholerae* O1 Ogawa was the predominant serotype among diarrhoeal patients admitted to different hospitals in India. With the exception of 3 strains from Kolkata, none of 172 strains examined exhibited resistance to tetracycline, but 45.7% showed reduced susceptibility to ciprofloxacin. Extensive molecular characterization using randomly amplified polymorphic DNA analysis, ribotyping and PFGE revealed that almost all the strains within a serogroup were clonally related. Along with the H pulsotype, a newly described L pulsotype of recently emerged O1 Inaba strains was detected among the O1 Ogawa strains from 2003. The striking similarity in their molecular properties and antibiograms indicated that at least certain clones of recently emerged Inaba strains from 2004 may have evolved from O1 Ogawa strains. This view was further supported by the detection of a nearly identical *wbeT* region among the O1 Ogawa and recently emerged Inaba strains, the latter differing only by a single point mutation. Since 2003, a hiatus in the isolation of serogroup O139 was observed and these strains share the same PFGE profiles as those isolated during 2000. Organization of tandemly arranged CTX^{El}, CTX^{Cal} and truncated CTX^{Cal} (devoid of *ctxAB*) prophages was unique among the majority of these O139 strains.

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INTRODUCTION

Vibrio cholerae strains belonging to serogroups O1 and O139 are responsible for the vast majority of epidemic cholera in humans (Bennish, 1994), which continues to be a growing concern in most developing countries. *V. cholerae* O1 is further divided into two biotypes, Classical, and El Tor and two major serotypes, Inaba and Ogawa. Since its emergence in 1961 as a causative agent of the seventh cholera pandemic, *V. cholerae* O1 El Tor has spread to almost 75 countries in Asia, Africa and South America. In 2005, a sharp increase of cholera cases with increased mortality was reported from 52 countries, and identified 131 943 cases with 2272 deaths (World Health Organization) (WHO, 2006). The changing trends in the epidemiology of pathogenic *V. cholerae* has presented problems for the control of the disease. A good example is the sudden emergence of *V. cholerae* O139 strains in the Indian subcontinent during late 1992 and its rapid spread to neighbouring countries (Ramamurthy *et al.*, 1993). The clinical picture of diarrhoea caused by either *V. cholerae* O1 or O139 strains is indistinguishable and the similar

propensity to spread lead both to be classified as the agents of epidemic cholera (Bhattacharya *et al.*, 1993). It has also been suggested that *V. cholerae* O139 strains possibly evolved from the seventh pandemic clone of *V. cholerae* O1 El Tor (Berche *et al.*, 1994; Popovic *et al.*, 1995).

The last decade has witnessed periodic switching between *V. cholerae* O1 and O139 strains as agents of cholera in India and Bangladesh (Basu *et al.*, 2000; Faruque *et al.*, 2003). Extensive use has been made of molecular tools to index changes occurring among circulating strains, and a number of ribotypes and DNA macrorestriction pattern types have been identified in strains of both serotypes (Dutta *et al.*, 2006; Faruque *et al.*, 2000; Garg *et al.*, 2000; Nair *et al.*, 2002; Popovic *et al.*, 1993; Safa *et al.*, 2005; Sharma *et al.*, 1997; Yamasaki *et al.*, 1997).

During 2004 in many parts of India, the re-emergence and progression of *V. cholerae* O1 serotype Inaba causing outbreaks and sporadic infections was documented by Taneja *et al.* (2005). This prompted us to analyse *V. cholerae* O1 and O139 strains isolated from acute diarrhoeal patients admitted to hospitals in six different parts of India prior to the spread of the Inaba serotype.

Abbreviations: RAPD, randomly amplified polymorphic DNA; TET, tetracycline.

METHODS

Study design and bacteriology. *V. cholerae* strains isolated from cholera patients admitted to different hospitals during 2003 were selected from the archive collection of the National Institute of Cholera and Enteric Diseases, which is the national reference centre. During 2003, 247 clinical strains were identified as *V. cholerae* and comprised 133 strains from patients in different parts of India [37 from Chennai, 4 from Manipal (southern India), 9 from Mumbai, 21 from Ahmedabad (western India), 10 from Raygaunge (eastern India), 34 from Aligarh and 18 from Haryana (northern India)] and 114 strains from patients admitted to the Infectious Diseases Hospital, Kolkata.

Stools or rectal swabs were plated on thiosulfate/citrate/bile salts/sucrose agar. Presumptive *V. cholerae* isolates formed sucrose-fermenting yellow-pigmented colonies, which were oxidase-positive and produced an alkaline slant and acid butt (K/A) in Kaper's multitest medium (Nair *et al.*, 1987). *V. cholerae* strains were typed using antisera specific for O1 and O139 serogroups that were raised in our institute. Strains of O1 serogroup were further classified with monospecific antisera to the Ogawa and Inaba serotypes.

Antibiotic susceptibility assay. The susceptibility of strains to 11 antimicrobial agents was determined by a disc diffusion assay (Bauer *et al.*, 1966) with commercially available discs (HiMedia) containing ampicillin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), furazolidone (100 µg), gentamicin (10 µg), neomycin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), streptomycin (10 µg) and tetracycline (TET) (30 µg). The inhibition zone diameters were recorded, and strains were classified as susceptible, reduced susceptible or resistant to a particular antimicrobial agent according to the published criteria (NCCLS, 2002). *E. coli* strain ATCC 25922 was used for quality control.

PCR and randomly amplified polymorphic DNA (RAPD) analysis. Purified genomic DNA was isolated from the test strains grown in LB medium at 37 °C overnight (Murray & Thompson, 1980). Briefly, cells were harvested, washed and resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA) pH 8.0 and then lysed by treatment with SDS, followed by digestion with proteinase K (20 µg ml⁻¹) for 2 h at 60 °C. Genomic DNA was further purified by treatment with 10% *N*-cetyl-*N,N,N*-trimethylammonium bromide in 0.7 M NaCl at 65 °C for 10 min and sequential extraction with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) and chloroform. The DNA was precipitated with ethanol, air-dried, reconstituted in sterile water and stored at 4 °C. This DNA was used in the PCR assay with primers specific for *ctxA* and allele-specific *tcpA* (De *et al.*, 2001; Keasler & Hall, 1993). In separate assays, allele-specific *rstR* primers were used for the detection of allelic types of *rstR* among different strains (Bhattacharya *et al.*, 2006). The PCR assays were carried out in a final reaction volume of 25 µl, and the concentration of primers and dNTPs adjusted to 1 pmole µl⁻¹ and 0.25 µM, respectively. The *ctxA*-*tcpA* multiplex PCR assay consisted of initial denaturation at 94 °C for 2 min, followed by 30 cycles comprising 1.5 min each at 94, 60 and 72 °C, and a final extension at 72 °C for 7 min. The *rstR* allele-specific PCR assays consisted of initial denaturation at 94 °C for 2 min, followed by 30 cycles of 40 s at 94 °C, 40 s at 50 °C and 1.5 min at 72 °C, and a final extension for 7 min at 72 °C. For RAPD-PCR, about 20 ng purified genomic DNA was used in each assay with primer 1281 (Sinha *et al.*, 2002). Cycling conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C (Sinha *et al.*, 2002). Amplification products were separated in agarose gels, stained with ethidium bromide, viewed under UV and recorded digitally using a gel documentation system (GelDoc 2000, Bio-Rad).

DNA probes and Southern blot hybridization. Purified genomic DNA was treated with restriction endonuclease enzymes and separated by electrophoresis in 0.8% agarose gels. DNA fragments were denatured by treatment with alkali and subsequently transferred to nylon membrane (Hybond-N⁺; Amersham Pharmacia Biotech), according to the procedure of De *et al.* (2005), and hybridized with a DNA probe. Ribotyping analysis was carried out with *Bgl*I digested genomic DNA using a 7.5 kb *Bam*HI fragment (from the plasmid pKK3535) as a probe for *rrn* genes (Brosius *et al.*, 1981). For CTX typing, genomic DNA was digested individually with *Bgl*II, *Bgl*III, *Pst*I, *Xba*I, *Mlu*I, *Hind*III and *Ava*I (Takara, Shuzo). A 540 bp *Xba*I-*Cla*I fragment of *ctxA*, cloned in pKTN901 using the *Eco*RI linker, served as a probe for *ctxA* (Kaper *et al.*, 1988). The 251 bp PCR amplicon of *cep* was cloned into PCR 2.1 (Invitrogen) to generate clone pSC01. This clone was sequenced using M13 primers to confirm the presence of *cep*. Similarly, the clones pSC02, pSC06 and pSC10 were generated by the cloning of allele-specific *rstR* amplicons of Classical, El Tor and Calcutta types, respectively, and their identities were confirmed by nucleotide sequencing. *Eco*RI digested products of pSC clones served as specific probes for allele-specific *rstRs* and *cep*. DNA probes were labelled with chemiluminescent dye (Gene Images AlkPhos direct labelling and detection system; Amersham Biosciences) and hybridization reactions were developed following the manufacturer's protocol and recognition patterns recorded on X-ray film.

PFGE. Agarose embedded genomic DNA of the *V. cholerae* strains was cleaved with 50 U *Not*I (Takara) and the fragments were separated in a contour-clamped homogeneous electric field (CHEF Mapper; Bio-Rad) by the procedure of Garg *et al.* (2000). Run conditions were generated by the autoalgorithm mode of the CHEF Mapper system using a size range of 20 to 300 kb with an electrophoresis time of 40 h, 24 min in 1% PFGE grade agarose gel in 0.5× TBE [44.5 mM Tris/HCl, 44.5 mM boric acid, 1.0 mM EDTA (pH 8.0)] at 14 °C. The gels were stained for 30 min in Elix Milli-Q water (Millipore) containing 1.0 µg ethidium bromide ml⁻¹, destained in Elix Milli-Q water for 15 min and photographed under UV light using the Gel Doc 2000 gel documentation system (Bio-Rad).

Nucleotide sequencing and analysis of *wbeT*. A 902 bp region of *wbeT* was amplified by PCR using primers VFBT-F and VFBT-R (Rijpkema *et al.*, 2004). The amplified product was purified (PCR purification kit; Qiagen) and used directly as a template for nucleotide sequencing. Both strands of DNA were sequenced with the BigDye terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions, using an ABI PRISM 310 DNA sequencer (Applied Biosystems). The nucleotide and deduced amino acid sequences were compared to published sequences available in GenBank with DNASTAR (DNA Star).

RESULTS AND DISCUSSION

Phenotypic traits of *V. cholerae* O1 and O139 strains in 2003

Serogroup analysis assigned 240 strains to O1 and 7 strains to O139 serogroups. A total of 1107 diarrhoeal specimens were collected from the Infectious Diseases Hospital, Kolkata: 112 were serogroup O1 Ogawa and 2 were serogroup O139 (Table 1). No serotype Inaba strains were found. A periodic shift between *V. cholerae* O1 and O139 from cholera patients was observed in India and Bangladesh (Basu *et al.*, 2000), but despite the re-emergence of *V. cholerae* O139 in 2002 in Bangladesh

Table 1. Antimicrobial percentage resistance patterns exhibited by *V. cholerae* O1 and O139 strains from different parts of India during 2003

Apart from Kolkata and Royguange strains, strains were received from the King Institute of Preventive Medicine, Chennai; the Kasturba Medical College, Manipal; Seth G. S. Medical College & KEM Hospital, Mumbai; Sheth V. S. General Hospital, Ahmedabad; Aligarh Muslim University, Aligarh; and the Post Graduate Institute of Medical Science, Haryana. SP, Sporadic case; O, outbreak case; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CO, co-trimoxazole; FZ, furazolidone; GEN, gentamicin; NEO, neomycin; NAL, nalidixic acid; NOR, norfloxacin; STR, streptomycin.

Area and place	Serogroup	Status	No. of strains tested	Percentage of resistant strains									
				AMP	CHL	CIP	CO	FZ	GEN	NEO	NAL	NOR	STR
Southern India													
Chennai	O1	O	16	87.5	0	0	93.7	100	0	44	100	0	100
Chennai	O139	O	2	100	0	0	0	100	0	0	100	0	50
Manipal	O1	SP	2	100	0	0	100	100	0	0	100	0	100
Western India													
Mumbai	O1	O	9	100	0	22.2	100	100	11	0	100	0	66.6
Ahmedabad	O1	SP	10	80	0	0	100	100	0	0	100	0	80
Northern India													
Aligarh	O1	SP	12	100	0	0	83.3	100	0	8	91.6	0	8.3
Haryana	O1	SP	9	100	0	33.3	77.7	100	0	0	100	55.5	66.6
Haryana	O139	SP	2	100	0	50	0	100	0	0	100	33.3	0
Eastern India													
Royguange	O1	O	4	100	25	0	25	100	0	0	75	0	0
Kolkata	O1	SP	104	85	0	1.7	98	99	0	0	99	0	97
Kolkata	O139	SP	2	100	0	0	0	100	0	0	100	0	0

(Faruque *et al.*, 2003), this serogroup appears to be mostly restricted to certain areas in China (WHO, 2005, 2006). Indeed, since 2003 serogroup O139 has been rarely isolated from Kolkata and *V. cholerae* O1 serotype Ogawa remains predominant. The absence of the Inaba serotype accords with reported patterns of *V. cholerae* O1 strains in the Indian subcontinent. With the exception of the recently reported increase in its prevalence (Dutta *et al.*, 2006), serotype Inaba was last predominant during 1989 (Ramamurthy *et al.*, 1992), and was then isolated sporadically during 1998–1999 (Garg *et al.*, 2000). Moreover, O1 Inaba strains from the latter period were reported to have evolved from the then circulating Ogawa strains (Garg *et al.*, 2000). The fact that switching between Inaba and Ogawa serotypes was demonstrated to occur with relative ease (Manning *et al.*, 1994), lead us to speculate whether the O1 Ogawa strains of 2003 were the precursors of the O1 Inaba strains that started to appear since mid 2004.

Approximately half (66) of the *V. cholerae* clinical isolates from different parts of India and 106 isolates from Kolkata were tested for antimicrobial susceptibility. Table 1 shows that the great majority of isolates showed resistance to ampicillin, furazolidone and nalidixic acid, and many were resistant to co-trimoxazole and streptomycin. Resistance profiles were variable and not associated with particular strains or serogroups. Interestingly, 45.7 % Ogawa strains showed reduced susceptibility to ciprofloxacin, while 91 %

of Inaba strains recovered in 2004–2005 were also reported to be less susceptible to this drug (Dutta *et al.*, 2006). All isolates, except one, were susceptible to chloramphenicol and gentamicin, and apart from seven isolates from Chennai and one from Aligarh, neomycin susceptibility was universal. With the exception of three serogroup O1 Ogawa strains from Kolkata, none exhibited resistance to TET. Resistance to TET among O1 Inaba strains that were isolated in India during 2005 has already been reported (Jesudason, 2006).

Virulence genes and genotypes

All strains, except one from Aligarh, showed the presence of an identical El Tor *tcpA* allele, and all harboured the *ctxA* encoding A subunit of the cholera toxin. Sixty-six *V. cholerae* O1 and six O139 strains were selected for molecular analysis. The serogroup O1 strains were all indistinguishable by RAPD-PCR, but the O139 strains were separated into two groups based on the presence of an additional amplification band (Fig. 1). This single difference may not be significant in terms of strain discrimination and suggests that isolates within each serogroup formed single or very closely related clones. It is noteworthy that the RAPD profile of the O1 strains resembled the reported profiles of O1 Ogawa strains that caused an outbreak of cholera in Orissa during 1999 (Chhotray *et al.*, 2002). Furthermore, the O139 strains

Fig. 1. RAPD-PCR profiles of *V. cholerae* strains of serogroups (a) O1 and (b) O139 with primer 1281. (a) Lanes: 1, Ahmedabad, Ahm6; 2, Royguange, WD15; 3, Kolkata, SC11; 4, Kolkata, SC213; 5, Aligarh, AMU12/03; 6, Mumbai, MUM21; 7, Haryana, HAR5B; 8, Chennai, VCH156; 9, Manipal, VCA. (b) Lanes: 1, Haryana, HAR3A; 2, Haryana, HAR6B; 3, Chennai, VCH133; 4, Chennai, VCH209; 5, Kolkata, SC185; 6, Kolkata, SC199. The size marker was 1 kb ladder.

lacking the additional band (Fig. 1b, lanes 2, 3, 6) displayed a profile identical to that reported for O139 strains from an outbreak in Ahmedabad in 2000 (Chakraborty *et al.*, 2001).

All of 32 serogroup O1 Ogawa strains (two random strains from each region) analysed by ribotyping were identical in hybridization profile (Fig. 2) and this corresponded to pattern RIII, which was first described for O1 strains that emerged in 1993 after the spread of the O139 serogroup subsided in many cholera endemic regions (Sharma *et al.*, 1997). The six O139 strains were also indistinguishable from each other and the profile was classified as the pattern BII (Faruque *et al.*, 2000) (Fig. 2). However, newly emerged Inaba strains during 2004–2005 displayed in addition to RIII, a new ribotype pattern designated type RIV (Dutta *et al.*, 2006). Furthermore, the ribotypes of the O1 strains circulating in India during 2003 are different from O1 strains, with attributes of both Classical and El Tor biotypes (Matlab variant) described from Bangladesh (Nair *et al.*, 2002).

Segregation of multiple clones of *V. cholerae* O1 and O139

PFGE analysis has been widely used for analysing the clonal nature of several groups of bacteria, and has been applied for the distinction of multiple clones of *V. cholerae* that have emerged and declined in India and Bangladesh (Bag *et al.*, 1998; Cooper *et al.*, 2006; Safa *et al.*, 2005; Yamasaki *et al.*, 1997). Indeed, six new pulsotypes, in addition to the five established types, have been reported recently for *V. cholerae* O1 strains that appeared during 2004–2005 in India (Dutta *et al.*, 2006). In this study, PFGE of *V. cholerae* O1 Ogawa strains from different parts of India were grouped into two types, coded types H (25 strains; Fig. 3a, lanes 1, 3, 8, 9) and L (8 strains; Fig. 3a, lanes 2, 4–7). The latter represents a new pulsotype first detected in O1 Inaba strains during 2004–2005 (Dutta *et al.*, 2006), but pulsotype H has prevailed among O1 strains for the last decade. The predominant pulsotype pattern of the O139 strains was comparable to that of strains isolated during

Fig. 2. *Bgl*I ribotype patterns of *V. cholerae* strains of serogroups (a) O1 and (b) O139. (a) Lanes: 1, Ahmedabad, Ahm6; 2, Royguange, WD15; 3, Kolkata, SC11; 4, Kolkata, SC213; 5, Aligarh, AMU12/03; 6, Mumbai, MUM21; 7, Haryana, HAR5B; 8, Chennai, VCH156; 9, Manipal, VCA. (b) Lanes: 1, Chennai, VCH133; 2, Chennai, VCH209; 3, Haryana, HAR3A; 4, Haryana, HAR6B; 5, Kolkata, SC185; 6, Kolkata, SC199. The size marker was *Hind*III-digested λ DNA.

Fig. 3. *NotI* PFGE profiles of *V. cholerae* strains of serogroups (a) O1 and (b) O139. (a) Lanes: 1, Ahmedabad, Ahm6; 2, Royguange, WD15; 3, Kolkata, SC11; 4, Kolkata, SC213; 5, Aligarh, AMU12/03; 6, Mumbai, MUM21; 7, Haryana, HAR5B; 8, Chennai, VCH156; 9, Manipal, VCA. (b) Lanes: 1, Chennai, VCH133; 2, Chennai, VCH209; 3, Haryana, HAR3A; 4, Haryana, HAR6B; 5, Kolkata, SC185; 6, Kolkata, SC199. The size marker was λ ladder.

2000 from an outbreak in Ahmedabad (Fig. 3b, lanes 1, 2, 5, 6) (Chakraborty *et al.*, 2001). Two new pulsotype profiles were given by two strains isolated from Haryana, but the widely distributed pulsotypes F and G described by Yamasaki *et al.* (1997) were not found, suggesting that a new clone of O139 may have already displaced older pulsotype F and G clones.

The copy number and organization of the CTX genetic element (CTX prophage) in *V. cholerae* strains was investigated. It has already been established that toxigenic traits of *V. cholerae* strains are due to lysogenic conversion of CTX ϕ carrying *ctxAB* (Waldor & Mekalanos, 1996). Interestingly, the genes encoding cholera toxin (*ctxAB*) were also shown to be acquired by the pre-CTX ϕ , which was originally devoid of *ctxAB*. Furthermore, detailed studies established that primarily three allelic types of CTX prophages (chromosomally integrated form of CTX ϕ) exist in toxigenic *V. cholerae* O1 and O139 (Davis *et al.*, 1999; Kimsey & Waldor, 1998). The nucleotide sequence variations in the repressor region *rstR* formed the basis of the distinct alleles, namely CTX^{Cl}, CTX^{El} and CTX^{Cal} (Davis *et al.*, 1999; Kimsey & Waldor, 1998). Initially, CTX^{Cl} and CTX^{El} types were detected in O1 strains of Classical and El Tor biotypes, respectively, and accordingly named, and CTX^{Cal} and CTX^{El} also have been identified in O139 strains (Davis *et al.*, 1999). Nevertheless, Matlab variants with attributes of both biotypes have caused cholera in both Bangladesh (Nair *et al.*, 2002) and Mozambique (Lee *et al.*, 2006). We screened the *V. cholerae* O1 strains by PCR assays with allele-specific *rstR* (Bhattacharya *et al.*, 2006) and found that, with the exception of one strain from Chennai (VCH18), all strains produced amplicons specific to El Tor type of *rstR* (Fig. 4). It is evident from Fig. 4(b, c) that strain VCH18 (lane 1) possessed both the 'El Tor' as well as the 'Cal' type of *rstR*, and this may have been acquired by lysogenic conversion of CTX ϕ ^{Cal} into the O1 strain. This is an unusual finding as lysogenic conversion of CTX ϕ ^{Cal} had been reported for O139 *V. cholerae* strains

(Davis *et al.*, 1999). PCR-based analysis revealed all six O139 strains had both the El Tor and the Cal type of *rstR* (data not shown). These strains might therefore have acted as a precursor of CTX ϕ ^{Cal} that lysogenized into certain O1 strains in the same region during the study period.

To determine the copy number of the CTX genetic element, DNA from *V. cholerae* strains was digested with different restriction enzymes with unique recognition sites

Fig. 4. PCR amplicon profiles of *V. cholerae* strains obtained with primers specific to (a) *rstR* of Classical biotype (*rstR*^{Cl}), (b) El Tor biotype (*rstR*^{El}) and (c) Calcutta biotype (*rstR*^{Cal}). Lanes: 1, Chennai, VCH18; 2, Aligarh, AMU22/03; 3, Royguange, WD15; 4, Manipal, VCA; 5, Kolkata, SC32; 6, reference strain *V. cholerae* O1 Classical, O395; 7, El Tor, CO366; 8, O139, AS207. Amplicon sizes of 474, 501 and 313 bp specific for *rstR*^{Cl}, *rstR*^{El} and *rstR*^{Cal}, respectively, are indicated.

Fig. 5. Southern blot hybridization patterns of *Hind*III-digested genomic DNA of *V. cholerae* O139 strains with DNA probes specific to (a) *ctxA*, (b) *rstR*^{Cal} and (c) *cep*. Lanes: 1, Haryana, HAR3A; 2, Haryana, HAR6B; 3, Chennai, VCH133; 4, Chennai, VCH209; 5, Kolkata, SC185; 6, Kolkata, SC199; 7, reference *V. cholerae* O139 strain AS207. The size marker was *Hind*III-digested λ DNA.

within the CTX element. Southern blot hybridization with a *ctxA* probe revealed the presence of one copy of CTX in serogroup O1 Ogawa strains, except for VCH18. The complete organization of CTX genetic elements in the strain VCH18 could not be ascertained and further studies on this aspect are in progress. On the other hand, DNA from O139 strains produced multiple bands on digestion with *Hind*III and separate probing with *ctxA*, *rstR*^{Cal} and *cep* (Fig. 5); the results generated with different enzymes are summarized in Table 2. Based on these data and allele-specific *rstR*, the organization of copies of the CTX genetic element in O139 strains was postulated as presented in Fig. 6. The detection of a single DNA fragment, with an estimated size of 7.2 kb, generated in the *ctxA* RFLP assays using most of the enzymes (except for *Hind*III) can be explained by the presence of one additional copy of CTX

prophage devoid of *ctxAB* (truncated CTX prophage) downstream to that of two tandemly arranged CTX^{El} and CTX^{Cal} prophages (Fig. 6b). The predicted three copies of the prophages were further confirmed by hybridization analysis with *cep* and allele-specific probes. The O139 strain isolated from Chennai (VCH209) displayed an altered profile as compared to the other O139 strains. Studies on the organization of the CTX genetic element in VCH209 are under way. The finding of a new CTX prophage profile in most of the O139 strains to the best of our knowledge is novel and has not been reported before.

The analysis was extended further by determining the nucleotide sequence of the *wbeT* region of the O1 Ogawa strains. Sequence data revealed complete identity of the *wbeT* region to that of the O1 Ogawa strains (GenBank

Table 2. Southern hybridization patterns generated with probes specific to *ctxA* and *rstR* alleles among *V. cholerae* O139 strains isolated during 2003 in India

Place of isolation	Strain	Size of hybridized fragment (kb) obtained with DNA probe specific to:								
		<i>ctxA</i>				<i>rstR</i> ^{El}			<i>rstR</i> ^{Cal}	
		<i>Bgl</i> II	<i>Ava</i> I	<i>Pst</i> I	<i>Hind</i> III	<i>Bgl</i> II	<i>Ava</i> I	<i>Hind</i> III	<i>Ava</i> I	<i>Hind</i> III
Haryana	HAR3A	23.0	7.2	7.2	7.2, 18	2.8, 21.0, 23.1	23.0	18.0	7.2	5.0, 7.2
Haryana	HAR6B	23.0	7.2	7.2	7.2, 18	2.8, 21.0, 23.1	23.0	18.0	7.2	5.0, 7.2
Chennai	VCH209	23.0	7.2, 14	7.2, 14	3.5, 18	2.8, 21.0, 23.1	23.0	18.0	6, 7.2, 14	4.6, 5.6
Kolkata	SC185	23.0	7.2	7.2	7.2, 18	2.8, 21.0, 23.1	23.0	18.0	7.2	5.0, 7.2
Kolkata	SC199	23.0	7.2	7.2	7.2, 18	2.8, 21.0, 23.1	23.0	18.0	7.2	5.0, 7.2
Kolkata	AS207	23.0	7.2, 20	5.8, 7.2	7.2, 8.6, 18.0	2.8, 21.0, 23.1	23.0	18.0	7.2	7.2, 9.4

Fig. 6. Schematic diagram showing the genomic organization of CTX prophages (not to scale) in (a) *V. cholerae* O1 and (b) O139 strains, as predicted from the data obtained in the Southern blot hybridization assay using DNA probes specific to *ctxA*, allele-specific *rstR* and *cep*. *ctxAB*, RS1 and RS2, comprising either *rstR^{El}* or *rstR^{Cal}*, are indicated. The DNA probe and corresponding hybridized fragments generated by restriction enzymes are shown. A, *Ava*I; BI, *Bgl*I; BII, *Bgl*II; H, *Hind*III; P, *Pst*I.

accession nos AY458622, AY458623, AY458624), but the region differed from those of the previously isolated O1 Inaba strains of 1989 and 1998–1999 in India (GenBank accession nos AY458621, AY458625, AY458626) (Garg *et al.*, 2000; Rijpkema *et al.*, 2004). However, comparative analysis revealed *wbeT* of 2003 O1 Ogawa differed from that of the recently emerged Inaba by a single point mutation, and that mutation has already been reported as unique for the newly emerged Inaba serotype.

In conclusion, the phenotypic and the genotypic analysis of *V. cholerae* strains isolated in 2003 has an added epidemiological importance considering the emergence and spread of the Inaba serotype in 2004, which gradually replaced the Ogawa serotype in several cholera endemic regions of India (Dutta *et al.*, 2006; Taneja *et al.*, 2005). The O1 and O139 strains studied here exhibited resistance to multiple drugs in keeping with previous reports, but it is noteworthy that except for a few strains from Kolkata none were TET resistant, although reduced susceptibilities to this drug were observed, which might result in spontaneous selection of TET resistance. A comprehensive molecular analysis gives support to the hypothesis that O1 Ogawa strains of 2003 may have been precursors for recently emerged epidemic Inaba strains. Indeed, the nucleotide sequence of *wbeT* was identical except for a unique point mutation reported earlier for the newly emerged Inaba

strain (GenBank accession no. DQ401028) (Dutta *et al.*, 2006); this mutation might play a role in the switching of Ogawa to Inaba serotype. Serogroup O139 strains have continued to be uncommon in India since 2003 to the present date. The continued prevalence of serogroup O1 for many years might act as a selective pressure for O139 strains to undergo slight genetic changes to ensure that they remain a significant agent in the cause of cholera. Interestingly, the O139 strains harboured two copies of complete CTX prophages, along with tandemly arranged truncated prophage characterized by an absence of *ctxAB*. Considering the epidemic potential of O1 and O139 strains, it is imperative to maintain molecular epidemiological surveillance of cholera infections to document subtle evolutionary changes in the constitution of *V. cholerae*.

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REFERENCES

- Bag, P. K., Maiti, S., Sharma, C., Ghosh, A., Basu, A., Mitra, R., Bhattacharya, S. K., Nakamura, S., Yamasaki, S. & other authors (1998). Rapid spread of the new clone of *Vibrio cholerae* O1 biotype El Tor in cholera endemic areas in India. *Epidemiol Infect* **121**, 245–251.
- Basu, A., Garg, P., Datta, S., Chakraborty, S., Bhattacharya, T., Khan, A., Ramamurthy, T., Bhattacharya, S. K., Yamasaki, S. & other authors (2000). *Vibrio cholerae* O139 in Calcutta, 1992–1998: incidence, antibiograms, and genotypes. *Emerg Infect Dis* **6**, 139–147.
- Bauer, A. W., Kirby, W. M., Sherris, J. C. & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* **45**, 493–496.
- Bennish, M. L. (1994). Cholera: pathophysiology, clinical features, and treatment. In *Vibrio cholerae and Cholera: Molecular to Global Perspectives*, pp. 229–255. Edited by I. K. Wachsmuth, P. A. Blake & O. Olsvik. Washington, DC: American Society for Microbiology.
- Berche, P., Poyart, C., Abachin, E., Lelievre, H., Vandepitte, J., Dodin, A. & Fournier, J. M. (1994). The novel epidemic strain O139 is closely related to the pandemic strain O1 of *Vibrio cholerae*. *J Infect Dis* **170**, 701–704.
- Bhattacharya, S. K., Bhattacharya, M. K., Nair, G. B., Dutta, D., Deb, A., Ramamurthy, T., Garg, S., Saha, P. K., Dutta, P. & other authors (1993). Clinical profile of acute diarrhoea cases infected with the new epidemic strain of *Vibrio cholerae* O139: designation of the disease as cholera. *J Infect* **27**, 11–15.
- Bhattacharya, T., Chatterjee, S., Maiti, D., Bhadra, R. K., Takeda, Y., Nair, G. B. & Nandy, R. K. (2006). Molecular analysis of the *rstR* and *orfU* genes of the CTX prophages integrated in the small chromosomes of environmental *Vibrio cholerae* non-O1, non-O139 strains. *Environ Microbiol* **8**, 526–634.
- Brosius, J., Ullrich, A., Raker, M. A., Gray, A., Dull, T. J., Gutell, R. R. & Noller, H. F. (1981). Construction and fine mapping of recombinant plasmids containing the *rrnB* ribosomal RNA operon of *E. coli*. *Plasmid* **6**, 112–118.
- Chakraborty, S., Deokule, J. S., Garg, P., Bhattacharya, S. K., Nandy, R. K., Nair, G. B., Yamasaki, S., Takeda, Y. & Ramamurthy, T. (2001). Concomitant infection of enterotoxigenic *Escherichia coli* in an outbreak of cholera caused by *Vibrio cholerae* O1 and O139 in Ahmedabad, India. *J Clin Microbiol* **39**, 3241–3246.
- Chhotray, G. P., Pal, B. B., Khuntia, H. K., Chowdhury, N. R., Chakraborty, S., Yamasaki, S., Ramamurthy, T., Takeda, Y., Bhattacharya, S. K. & Nair, G. B. (2002). Incidence and molecular analysis of *Vibrio cholerae* associated with cholera outbreak subsequent to the super cyclone in Orissa, India. *Epidemiol Infect* **128**, 131–138.
- Cooper, K. L., Luey, C. K., Bird, M., Terajima, J., Nair, G. B., Kam, K. M., Arakawa, E., Safa, A., Cheung, D. T. & other authors (2006). Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae*. *Foodborne Pathog Dis* **3**, 51–58.
- Davis, B. M., Kimsey, H. H., Chang, W. & Waldor, M. K. (1999). The *Vibrio cholerae* O139 Calcutta bacteriophage CTX ϕ is infectious and encodes a novel repressor. *J Bacteriol* **181**, 6779–6787.
- De, K., Ramamurthy, T., Ghose, A. C., Islam, M. S., Takeda, Y., Nair, G. B. & Nandy, R. K. (2001). Modification of the multiplex PCR for unambiguous differentiation of the El Tor and classical biotypes of *Vibrio cholerae* O1. *Indian J Med Res* **114**, 77–82.
- De, K., Nandy, R. K. & Nair, G. B. (2005). Microbiological and molecular methods to identify and characterize toxigenic *Vibrio cholerae* from food samples. In *Food-Borne Pathogens: Methods and Protocols (Methods in Biotechnology, vol. 21)*, pp. 99–124. Edited by C. C. Adley. Totowa, NJ: Humana Press.
- Dutta, B., Ghosh, R., Sharma, N. C., Pazhani, G. P., Taneja, N., Raychowdhuri, A., Sarkar, B. L., Mondal, S. K., Mukhopadhyay, A. K. & other authors (2006). Spread of cholera with newer clones of *Vibrio cholerae* O1 El Tor, serotype Inaba, in India. *J Clin Microbiol* **44**, 3391–3393.
- Faruque, S. M., Saha, M. N., Asadulghani, Bag, P. K., Bhadra, R. K., Bhattacharya, S. K., Sack, R. B., Takeda, Y. & Nair, G. B. (2000). Genomic diversity among *Vibrio cholerae* O139 strains isolated in Bangladesh and India between 1992 and 1998. *FEMS Microbiol Lett* **184**, 279–284.
- Faruque, S. M., Chowdhury, N., Kamruzzaman, M., Ahmad, Q. S., Faruque, A. S., Salam, M. A., Ramamurthy, T., Nair, G. B., Weintraub, A. & Sack, D. A. (2003). Reemergence of epidemic *Vibrio cholerae* O139, Bangladesh. *Emerg Infect Dis* **9**, 1116–1122.
- Garg, P., Nandy, R. K., Chaudhury, P., Chowdhury, N. R., De, K., Ramamurthy, T., Yamasaki, S., Bhattacharya, S. K., Takeda, Y. & Nair, G. B. (2000). Emergence of *Vibrio cholerae* O1 biotype El Tor serotype Inaba from the prevailing O1 Ogawa serotype strains in India. *J Clin Microbiol* **38**, 4249–4253.
- Jesudason, M. V. (2006). Change in serotype and appearance of tetracycline resistance in *V. cholerae* O1 in Vellore, South India. *Indian J Med Microbiol* **24**, 152–153.
- Kaper, J. B., Morris, J. G., Jr & Nishibuchi, M. (1988). DNA probes for pathogenic *Vibrio* species. In *DNA Probes for Infectious Diseases*, pp. 65–77. Edited by F. C. Tenover. Boca Raton, FL: CRC Press.
- Keasler, S. P. & Hall, R. H. (1993). Detecting and biotyping *Vibrio cholerae* O1 with multiplex polymerase chain reaction. *Lancet* **341**, 1661.
- Kimsey, H. H. & Waldor, M. K. (1998). CTX ϕ immunity: application in the development of cholera vaccines. *Proc Natl Acad Sci U S A* **95**, 7035–7039.
- Lee, J. H., Han, K. H., Choi, S. Y., Lucas, M. E., Mondlane, C., Ansaruzzaman, M., Nair, G. B., Sack, D. A., von Seidlein, L. & other authors (2006). Multilocus sequence typing (MLST) analysis of *Vibrio cholerae* O1 El Tor isolates from Mozambique that harbour the classical CTX prophage. *J Med Microbiol* **55**, 165–170.
- Manning, P. A., Stroehrer, U. H. & Morona, R. (1994). Molecular basis for O-antigen biosynthesis in *Vibrio cholerae* O1: Ogawa-Inaba switching. In *Vibrio cholerae and Cholera: Molecular to Global Perspectives*, pp. 77–94. Edited by I. K. Wachsmuth, P. A. Blake & O. Olsvik. Washington, DC: American Society for Microbiology.
- Murray, M. G. & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* **8**, 4321–4325.
- Nair, G. B., Misra, S., Bhadra, R. K. & Pal, S. C. (1987). Evaluation of the multitest medium for rapid presumptive identification of *Vibrio cholerae* from environmental sources. *Appl Environ Microbiol* **53**, 1203–1205.
- Nair, G. B., Faruque, S. M., Bhuiyan, N. A., Kamruzzaman, M., Siddique, A. K. & Sack, D. A. (2002). New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *J Clin Microbiol* **40**, 3296–3299.
- NCCLS (2002). *Performance Standards for Antimicrobial Susceptibility Testing*, 12th informational supplement, approved standard M100–S12. Wayne, PA: National Committee for Clinical Laboratory Standard.

- Popovic, T., Bopp, C., Olsvik, O. & Wachsmuth, K. (1993). Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae* O1. *J Clin Microbiol* **31**, 2474–2482.
- Popovic, T., Fields, P. I., Olsvik, O., Wells, J. G., Evins, G. M., Cameron, D. N., Farmer, J. J., III, Bopp, C. A., Wachsmuth, K. & other authors (1995). Molecular subtyping of toxigenic *Vibrio cholerae* O139 causing epidemic cholera in India and Bangladesh, 1992–1993. *J Infect Dis* **171**, 122–127.
- Ramamurthy, T., Pal, A., Bhattacharya, M. K., Bhattacharya, S. K., Chowdhury, A. S., Takeda, Y., Takeda, T., Pal, S. C. & Nair, G. B. (1992). Serovar, biotype, phage type, toxigenicity & antibiotic susceptibility patterns of *Vibrio cholerae* isolated during two consecutive cholera seasons (1989–90) in Calcutta. *Indian J Med Res* **95**, 125–129.
- 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 novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. *Lancet* **341**, 703–704.
- Rijpkema, S. G., Durrani, Z., Ramamurthy, T. & Nair, G. B. (2004). Assessing clonality of *Vibrio cholerae* Inaba isolates by characterization of nonsense mutations in *wbeT*. *J Med Microbiol* **53**, 1105–1107.
- Safa, A., Bhuiyan, N. A., Alam, M., Sack, D. A. & Nair, G. B. (2005). Genomic relatedness of the new Matlab variants of *Vibrio cholerae* O1 to the classical and El Tor biotypes as determined by pulsed-field gel electrophoresis. *J Clin Microbiol* **43**, 1401–1404.
- Sharma, C., Nair, G. B., Mukhopadhyay, A. K., Bhattacharya, S. K., Ghosh, R. K. & Ghosh, A. (1997). Molecular characterization of *Vibrio cholerae* O1 biotype El Tor strains isolated between 1992 and 1995 in Calcutta, India: evidence for the emergence of a new clone of the El Tor biotype. *J Infect Dis* **175**, 1134–1141.
- 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.
- Taneja, N., Biswal, M., Tarai, B. & Sharma, M. (2005). Emergence of *Vibrio cholerae* O1 Biotype El Tor serotype Inaba in north India. *Jpn J Infect Dis* **58**, 238–240.
- Waldor, M. K. & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**, 1910–1914.
- WHO (2005). Cholera 2004. *Wkly Epidemiol Rec* **80**, 261–268.
- WHO (2006). Cholera 2005. *Wkly Epidemiol Rec* **81**, 297–308.
- Yamasaki, S., Nair, G. B., Bhattacharya, S. K., Yamamoto, S., Kurazono, H. & Takeda, Y. (1997). Cryptic appearance of a new clone of *Vibrio cholerae* serogroup O1 biotype El Tor in Calcutta, India. *Microbiol Immunol* **41**, 1–6.