A large cholera outbreak due to a new cholera toxin variant of the *Vibrio cholerae* O1 El Tor biotype in Orissa, Eastern India

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A total of 32 *Vibrio cholerae* isolates were collected during a recent large cholera outbreak in Eastern India. Biochemical and serological studies revealed that all of the isolates belonged to serogroup O1, biotype El Tor, serotype Ogawa. Two multiplex PCR assays confirmed the presence of various toxigenic and pathogenic genes – *ace*, *ctxAB*, *hlyA*, *ompU*, *ompW*, *rfbO1*, *rtx*, *tcp*, *toxR* and *zot* – in all of the isolates. Sequencing of the *ctxB* gene from the isolates revealed a novel mutation in the gene. Sequencing also confirmed the presence of altered cholera toxin B of the classical biotype in all of the El Tor isolates, suggesting infection of isolates by classical CTXΦ. The molecular diversity of *V. cholerae* isolates studied by enterobacterial repetitive intergenic consensus sequence PCR, BOX-PCR and randomly amplified polymorphic DNA analysis uniformly showed the clonal relationship among the outbreak *V. cholerae* O1 isolates. The results of this study suggest that cholera-causing *V. cholerae* strains are constantly evolving in epidemic areas, highlighting the potential of the emergence of more virulent strains.

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INTRODUCTION

Cholera is still a major problem in countries with poor socio-economic conditions where access to safe water and adequate sanitation cannot be assured for all. According to a recent World Health Organization report, there was a dramatic increase in the number of notified cholera cases in 2006, and almost every developing country is facing either a cholera outbreak or the threat of an epidemic (WHO, 2007). Among the various serogroups of *Vibrio cholerae*, strains belonging to the O1 and O139 serogroups harness the epidemic and pandemic potential. The Indian subcontinent has been an important epicentre of cholera in most pandemics. There are two biotypes of *V. cholerae* O1, classical and El Tor, which are believed to have evolved from separate lineages (Kaper *et al.*, 1995). Among the two biotypes, strains belonging to the El Tor biotype have better adaptability to survive in the environment and in the human host (Finkelstein, 2006). Classical biotype strains are supposed to be more toxigenic than El Tor strains. The first six pandemics were caused by the classical biotype, but after 1961 the *V. cholerae* El Tor biotype displaced the

classical biotype (Kaper *et al.*, 1995). There are certain structural and functional peculiarities in the pathogenicity genes of El Tor vibrios that make the symptoms of cholera milder but longer lasting than the symptoms of the classical biotype strains (Smirnova *et al.*, 2004). However, in the recent past, evolution among El Tor strains by the emergence of hybrid biotype strains with altered cholera toxin (CT) has been noticed (Nair *et al.*, 2006).

Although several toxins have been reported from *V. cholerae*, CT is still the major one responsible for most of the manifestations of cholera (Kaper *et al.*, 1995). This is an A–B toxin, and on the basis of the B subunit, there are two immunologically related but not identical epitopes, CT1 and CT2, produced by the classical biotype and El Tor biotype strains, respectively. Three types of cholera toxin B subunit gene (*ctxB*) have been reported (Olsvik *et al.*, 1993). Genotype 1 has been found in strains of the classical biotype worldwide and in El Tor biotype strains associated with the US Gulf Coast, genotype 2 has been found in El Tor biotype strains from Australia, and genotype 3 has been found in El Tor biotype strains from the seventh pandemic and the recent Latin American epidemic. All base changes correspond to an amino acid substitution in the B subunit of CT.

In August–September 2007, a cholera outbreak hit many parts of Orissa in Eastern India, in the wake of massive

Abbreviations: CT, cholera toxin; ERIC, enterobacterial repetitive intergenic consensus sequence; mPCR, multiplex PCR.

The GenBank/EMBL/DDBJ accession numbers for the *ctxB* sequence of isolates VOC4, VOC6, VOC16, VOC22, VOC38 and VOC50 are EU496273–EU496278, respectively.

flooding following South Asia's worst monsoon season in living memory. The outbreak spread to many villages and thousands of tribal people, and was associated with hundreds of deaths, mainly from cholera, in the three tribal-dominated districts of Kalahandi (19.40° N 83.00° E), Koraput (18.49° N 82.43° E) and Rayagada (19.09° N 83.27° E). In this study, we present the genetic analysis and molecular typing of El Tor strains isolated from stool samples from this outbreak.

METHODS

Bacterial strains and clinical specimens. *V. cholerae* strains were isolated from randomly selected cholera patients. Samples were processed as described previously (Pourshafie *et al.*, 2007). Other bacterial strains used in the study were *V. cholerae* O1 (ATCC 14033), *V. cholerae* O139 (NICED), *Bacillus cereus* (ATCC 10876), *Escherichia coli* (ATCC 11775), *Salmonella paratyphi* A (MTCC 735), *Salmonella typhimurium* (MTCC 98), *Shigella dysenteriae* (NICED), *Shigella flexneri* (MTCC 1457), *Staphylococcus aureus* (ATCC 12600), *Vibrio fischeri* (MTCC 1738) and *Vibrio parahaemolyticus* (MTCC 451). These strains were obtained from the ATCC or MTCC (Microbial Type Culture Collection; Chandigarh, India) or from NICED (Kolkatta, India), as indicated.

Biochemical and serological characterization. All of the bacterial isolates were screened for the oxidase reaction followed by standard biochemical tests for presumptive identification of *V. cholerae* (Nair *et al.*, 1987; Tamrakar *et al.*, 2006). Serological identification of the isolates was carried out by slide agglutination using commercially available polyvalent antisera against *V. cholerae* O1 (Ogawa and Inaba) and O139 serogroups (Difco).

Multiplex PCR assays for virulence genes. Two multiplex PCR (mPCR) assays were developed to detect diverse traits of *V. cholerae* using the primers listed in Table 1. The first mPCR was designed to confirm the presence of *V. cholerae* and its toxigenicity, serogroup and pathogenicity using five sets of primers for genes encoding the outer-membrane protein OmpW (*ompW*), CT (*ctxAB*), zonula occludens protein (*zot*), O1 somatic antigen (*rfbO1*) and toxin-coregulated pilus (*tcp*). The second mPCR was designed to confirm the presence of other virulence and toxigenic genes encoding the accessory cholera enterotoxin (*ace*), haemolysin (*hlyA*), outer-membrane protein OmpU (*ompU*), repeat in toxin protein (*rtx*) and toxin regulator (*toxR*). The primers were selected to produce amplicons of different sizes for clear identification. Primers were selected from previous studies or were designed on the basis of GenBank gene sequences of *V. cholerae* using Oligo Explorer version 1.2 (Gene Link). The specificity of the mPCRs was determined using the other homologous bacterial strains described above. DNA was extracted and PCR was carried out in a reaction mixture of 25 µl containing *Taq* buffer, 200 nmol dNTPs ml⁻¹, 1.5 µmol MgCl₂ ml⁻¹, 1 U *Taq* polymerase, various concentrations of primers specific for each gene (7–12 pmol), 100 ng DNA as template and sterile distilled water up to 25 µl. The thermal cycling conditions were as follows: pre-incubation at 94 °C for 2 min; 30 cycles of 1 min at 94 °C for denaturation, 1 min at 59 °C for annealing and 2 min at 72 °C for extension; and incubation at 72 °C for 10 min for the final extension. In the control reaction, deionized water instead of bacterial cells was added to the reaction mixture.

Sequencing of the *ctxB* gene. The *ctxB* gene was amplified from the strains isolated from the outbreak areas using primers *ctxF* and *ctxR* as described previously (Olsvik *et al.*, 1993). The PCR product was purified and sequencing was carried out using the same primers on a 96-capillary model 3730xl system using a Big Dye Terminator kit (Applied Biosystems). The sequences of the *ctxB* gene for other *V.*

Table 1. PCR primers used in this study

Target	Nucleotide sequence (5′→3′)	Amplicon size (bp)	Reference
mPCR1			
<i>ompW</i> (F)	CACCAAGAAGGTGACTTTATTGTG	304	Nandi <i>et al.</i> (2000)
<i>ompW</i> (R)	GGTTTGTGCAATTAGCTTCACC		
<i>ctxAB</i> (F)	GCCGGGTTGTGGGAATGCTCCAAG	536	Goel <i>et al.</i> (2007)
<i>ctxAB</i> (R)	CATGCGATTGCCGCAATTAGTATGGC		
<i>rfbO1</i> (F)	TCTATGTGCTGCGATTGGTG	638	Goel <i>et al.</i> (2007)
<i>rfbO1</i> (R)	CCCCGAAAACCTAATGTGAG		
<i>tcp</i> (F)	CGTTGGCGGTCAGTCTTG	805	Goel <i>et al.</i> (2007)
<i>tcp</i> (R)	CGGGCTTTCTTCTTGTTTCG		
<i>zot</i> (F)	TCGCTTAACGATGGCGCGTTTT	947	Singh <i>et al.</i> (2001)
<i>zot</i> (R)	AACCCCGTTTCACTTCTACCCA		
mPCR2			
<i>rtxC</i> (F)	CGACGAAGATCATTGACGAC	265	Chow <i>et al.</i> (2001)
<i>rtxC</i> (R)	CATCGTCGTTATGTGGTTGC		
<i>ace</i> (F)	TAAGGATGTGCTTATGATGGACACCC	309	This study
<i>ace</i> (R)	CGTGATGAATAAAGATACTCATAGG		
<i>hlyA</i> (F)	GAGCCGGCATTCTGAAT	480	This study
<i>hlyA</i> (R)	CTCAGCGGGCTAATACGGTTTA		
<i>ompU</i> (F)	CCAAAGCGGTGACAAAGC	655	This study
<i>ompU</i> (R)	TTCCATGCGGTAAGAAGC		
<i>toxR</i> (F)	CCTTCGATCCCCTAAGCAATAC	779	Singh <i>et al.</i> (2001)
<i>toxR</i> (R)	AGGGTTAGCAACGATGCGTAAG

Fig. 1. Multiplex PCR analysis of different gene traits in outbreak isolates. The sizes of the fragments are given in parentheses (bp). Lane M, 100 bp ladder.

cholerae O1 El Tor and classical strains listed in Fig. 2 were retrieved from GenBank. The deduced amino acid sequences of the *ctxB* gene from all strains were aligned using CLUSTAL W.

Genomic fingerprinting by enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) and BOX-PCR. ERIC-PCR was performed as described previously with some modifications using the primers ERIC1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGTGAGCG-3') (Rivera *et al.*, 1995). The thermal cycler was programmed for 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 10 min at 68 °C, followed by a 20 min incubation at 70 °C. BOX-PCR (specific for the BOX element repeat sequences) was performed using a single nucleotide primer, BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3'; Versalovic *et al.*, 1994). The PCR program consisted of initial denaturation at 95 °C for 7 min, followed by 30 cycles of 94 °C for 1 min, 53 °C for 1 min and 65 °C for 8 min, with a final extension of 65 °C for 16 min.

Randomly amplified polymorphic DNA (RAPD) analysis. For RAPD analysis, one 10-mer random primer with a 50 mol% G + C content was chosen from the Operon 10-mer kit A (Operon Technologies). The reaction mix consisted of 20 ng DNA, 20 pmol primer in *Taq* buffer, 2.5 µmol MgCl₂ ml⁻¹, 200 nmol each dNTP ml⁻¹ and 1 U *Taq* polymerase in a final volume of 25 µl. The PCR cycling conditions were as follows: pre-incubation at 94 °C for 2 min; 40 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C; and a final incubation for 5 min at 72 °C.

RESULTS AND DISCUSSION

Strain identification

During the outbreak, a total of 32 *V. cholerae* isolates were obtained from the affected patients. All of the isolates were biochemically identified as *V. cholerae* and serologically confirmed as O1 Ogawa.

Detection of gene traits

Using two sets of mPCRs, all of the isolates were screened for the presence of various genes involved in toxigenicity and pathogenicity. The first mPCR analysis revealed that all of the isolates were positive for the *ompW*, *ctxAB*, *zot*, *tcpA* and *rfbO1* genes, whilst the second mPCR confirmed the presence of the *ace*, *hlyA*, *ompU*, *rtx* and *toxR* genes in all isolates (Fig. 1). The *ompW* gene is species specific for *V. cholerae* and its presence confirmed that the isolates were *V. cholerae*. The presence of the *ctxAB* and *rfbO1* genes confirmed the toxigenicity and O1 serogroup of all of the isolates, respectively. Determination of the classical and El Tor biotypes within the O1 serogroup relies on conventional biochemical and phage typing methods, which are

Fig. 2. Amino acid sequence alignment of the CTXB subunit of *V. cholerae* O1 El Tor strains from the Orissa outbreak with reference and other strains. Identical amino acid residues are indicated by dots. The amino acid sequences of *V. cholerae* CTXB used in the alignment were taken from GenBank.

tedious and time-consuming. However, on the basis of PCR assays developed for *rtxA* or *rtxC*, isolates can be differentiated into biotypes in the *V. cholerae* O1 serogroup (Chow *et al.*, 2001). The isolates belonged to the El Tor biotype on the basis of the *rtx* gene. The RTX toxins represent a family of important virulence factors which have become disseminated widely among Gram-negative bacteria (Coote, 1992). The RTX toxin gene cluster in *V. cholerae* encodes the presumptive cytotoxin (*rtxA*), an acyltransferase (*rtxC*) and an associated ATP-binding cassette transporter system and is physically linked to the core element in the *V. cholerae* genome (Lin *et al.*, 1999).

Cholera pathogenesis is a complex process and involves the synergetic action of several genes. CT is considered the most important epidemic marker among various toxins produced by *V. cholerae* (Kaper *et al.*, 1995). However, a number of other genes are also involved in the pathogenesis of *V. cholerae* in cholera, mainly the toxin co-regulated pilus (*tcp*) gene. The CT gene (*ctxAB*) is acquired from the genome of a filamentous CTX bacteriophage. The pilus colonization factor TCP acts as a receptor for CTX ϕ , which can infect non-toxigenic *V. cholerae*, leading to the emergence of new toxigenic strains (Waldor & Mekalanos, 1996). The zonula occludens (ZOT) toxin is another virulence factor. In this study, various other genes such as *ace*, *hly*, *toxR* and *zot* were also screened and all of the strains were found to be positive for these genes, suggesting the presence of the core toxin region in all isolates. These genes are found together and represent the genome of the filamentous bacteriophage CTX ϕ (Waldor & Mekalanos, 1996).

Sequence of the *ctxB* gene

The classical and El Tor biotypes have specific amino acid signature sequences for the CT-B subunit (Popovic *et al.*, 1994). The *ctxB* sequence from the isolates was amplified using specific primers and the deduced amino acid sequences were compared with existing amino acid sequences of classical and El Tor strains. We found that the amino acid sequence of CT-B from representative El Tor isolates of *V. cholerae* varied from that of the CT-B of reference El Tor strains at position 39 (histidine in place of tyrosine) and 68 (threonine in place of isoleucine) and this sequence was similar to the CT-B subunit of the reference classical strain (Fig. 2). However, at position 20, histidine, which was found in both the El Tor and classical strains, was replaced by asparagine in the outbreak strains.

Several studies have shown that there is continual change in the *ctxB* gene. Previously, changes in the amino acid sequence of El Tor strains at positions 39 and 69 have been reported and these sequences were similar to those of classical strains (Ansaruzzaman *et al.*, 2004). Subsequently, these El Tor strains producing classical CT were isolated from many parts of various Asian countries (Nair *et al.*, 2006). Biotype-specific CTX Φ is found in *V. cholerae* strains. El Tor biotype strains harbour CTX^{ET} Φ , whilst classical strains have CTX^{class} Φ (Ansaruzzaman *et al.*,

2004). However, in this study, all of the El Tor isolates were found to possess a *ctxB* sequence of the classical biotype, either by shedding their El Tor CTX prophage or by recombination within the phage. Moreover, considering the novel mutation at position 20, the present study showed the emergence of new El Tor strains with a modified classical CT. Thus it seems that the new El Tor isolates have themselves evolved from the El Tor isolates producing the classical CT. The occurrence of such changes in the *ctxB* gene is a novel genetic phenomenon that needs to be monitored carefully.

DNA fingerprinting

All of the *V. cholerae* isolates were characterized by ERIC-PCR, BOX-PCR and RAPD analysis to reveal their clonal

Fig. 3. DNA fingerprints of different outbreak isolates of *V. cholerae* O1 generated by ERIC-PCR (a), BOX-PCR (b) and RAPD analysis (c). Lanes: 1, 1 kb ladder; 2, *V. cholerae* O1 ATCC 14033; 3, *V. cholerae* O139; 4, VOC1; 5, VOC4; 6, VOC6; 7, VOC9; 8, VOC10; 9, VOC14; 10, VOC16; 11, VOC20; 12, VOC22; 13, VOC27; 14, VOC38; 15, VOC42; 16, VOC46; 17, VOC50; 18, VOC52; 19, VOC54; 20, VOC55.

relationships. ERIC-PCR with genomic DNA of various *V. cholerae* strains resulted in amplification of multiple fragments of DNA with sizes ranging from 0.6 to 1.8 kb (Fig. 3a). BOX-PCR of genomic DNA from various *V. cholerae* isolates also resulted in amplification fragments of DNA of varying length ranging from 0.65 to 6.0 kb (Fig. 3b). All of the fragments were of similar sizes in all of the outbreak *V. cholerae* isolates. Likewise, different DNA fragments of the same size were amplified by RAPD in all of the isolates (Fig. 3c).

Many methods have been developed for the typing of *V. cholerae* strains. Among the various methods, ribotyping and PFGE are the most widely used. However, ribotyping is cumbersome and costly, and PFGE takes several days to reveal the fingerprinting pattern (Currie *et al.*, 2007). Moreover, the facilities required are not available in many laboratories in developing countries. PCR-based methods of fingerprinting take advantage of the presence of repetitive sequences that are interspersed throughout the genome of diverse bacterial species. The fingerprinting methods used in this study are well established and have been applied to both clinical and environmental strains for their identification. Previous studies have shown that these methods are sufficient to differentiate outbreak strains (Radu *et al.*, 2002; Singh *et al.*, 2001). All three fingerprinting analyses revealed identical patterns among the Orissa outbreak isolates, suggesting that the outbreak was probably caused by a single clone of a *V. cholerae* strain. Thus, this study demonstrated the involvement of a single clone of modified El Tor strain with classical CT in a cholera outbreak in Eastern India.

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