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Molecular Characterization Reveals Involvement of Altered El Tor Biotype *Vibrio cholerae* O1 Strains in Cholera Outbreak at Hyderabad, India

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Thirty-four *Vibrio cholerae* isolates collected from a cholera outbreak in Hyderabad, South India were found to belong to serogroup O1 biotype El Tor serotype Ogawa. The genotype of all the isolates was confirmed by PCR assays. All the isolates were found PCR positive for *ctxAB*, *ompW*, *rfbO1*, *rtxC*, and *tcpA* genes. All the isolates but one harboured $rstR^{El\ Tor}$ allele. However, one isolate carried both $rstR^{El\ Tor}$ as well as $rstR^{Classical}$ alleles. Cholera toxin (*ctxB*) genotyping of the isolates confirmed the presence of altered cholera toxin B of classical biotype in all the isolates. All the isolates except VCH35 harboured an RS1-CTX prophage array on the large chromosome. The isolate VCH35 contained a tandem repeat of classical CTX prophage on the small chromosome. The clonal relationship among the *V. cholerae* isolates as carried out by enterobacterial repetitive intergenic consensus sequences PCR, BOX PCR and randomly amplified polymorphic DNA, uniformly showed a genetic relationship among the outbreak isolates. The results of this study suggest that altered El Tor biotype *V. cholerae* with the classical cholera toxin gene are involved in cholera outbreaks in India.

***Keywords*:** cholera, *Vibrio cholerae*, outbreak, cholera toxin, biotype, CTX prophage

Cholera, an acute secretory diarrhea, still remains one of the most feared infectious diseases in public health accounting for thousands of deaths annually (WHO, 2008). The disease is common in several developing countries and can be fatal in 50 percent of cases where treatment facilities are not available (Thiagarajah and Verkman, 2005). Cholera is caused by *Vibrio cholerae*, a Gram-negative bacterium that produces enterotoxins whose actions on the mucosal epithelium are responsible for the characteristic symptoms of the disease (Kaper *et al.*, 1995). In the most severe cases, cholera is one of the most rapidly fatal illnesses known, with death usually resulting from severe dehydration or water loss. A healthy person may become hypotensive within an hour of the onset of symptoms and may die within two to three hours although more usually death may come within a day or so.

V. cholerae has more than 200 serogroups. However, strains belonging to O1 and O139 serogroup only harness the epidemic and pandemic potential. Pathogenic strains harbor a cholera toxin prophage that carries the cholera toxin (CT) genes (Waldor and Mekalanos, 1996). *V. cholerae* O1 strains are classified into two biotypes: classical and El Tor (Kaper *et al.*, 1995). These two biotypes differ in their phenotypes, genotypes as well as their pathogenic potential (Safa *et al.*, 2006). Strains belonging to El Tor biotype are supposed to have better adaptability to survive in the environment and in the human host (Finkelstein, 2006). However, the clinical manifestation of the disease caused by classical biotype strains is more severe than that caused by the El Tor strains (Kaper *et al.*, 1995; Sack *et al.*, 2004). The first six pandemics were caused by classical biotype but after 1961 *V. cholerae* El Tor biotype displaced classical biotype (Kaper *et al.*, 1995). There are certain structural and functional peculiarities in the virulence genes of El Tor vibrios that make the symptoms of cholera milder but more long lasting than classical biotype strains (Smirnova *et al.*, 2004). However, in the recent past, *V. cholerae* strains having phenotypic and genotypic traits of both biotypes have been noticed (Nair *et al.*, 2006; Goel *et al.*, 2008; Goel and Jiang, 2010). These new variants which cannot be biotyped as classical or El Tor and harbour the classical type cholera toxin (*ctxB*) gene have been termed as "atypical El Tor" strains (Safa *et al.*, 2010).

In May 2009, hundreds of people were affected in a suddenly erupted cholera outbreak in the Bholakpur area, Hyderabad, South India. In this study, we report the genetic analysis and molecular typing of *V. cholerae* El Tor strains isolated from the patients of this outbreak.

Materials and Methods

Bacterial strains

A total of 34 *V. cholerae* O1 strains were analysed in this study. All the *V. cholerae* O1 El Tor strains were isolated from clinical cases during the cholera outbreak in Hyderabad as described earlier (Pourshafie *et al.*, 2007). Other bacterial strains used in the study were *V. cholerae* O1 (ATCC 11623), *V. cholerae* O1 (ATCC 14033) and *V. cholerae* O139.

Biochemical and serological characterization

All the bacterial isolates were screened for oxidase reaction followed

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Table 1. PCR primers used in this study

Target gene	Forward primer	Reference
ompWF	CACCAAGAAGGTGACTTTATTGTG	Nandi *et al.* (2000)
ompWR	GGTTTGTCGAATTAGCTTCACC	Nandi *et al.* (2000)
ctxABF	GCCGGGTTGTGGGAATGCTCCAAG	Goel *et al.* (2007)
ctxABR	CATGCGATTGCCGCAATTAGTATGGC	Goel *et al.* (2007)
rfbO1F	TCTATGTGCTGCGATTGGTG	Goel *et al.* (2007)
rfbO1R	CCCCGAAAACCTAATGTGAG	Goel *et al.* (2007)
tcpAF	CGTTGGCGGTCAGTCTTG	Goel *et al.* (2007)
tcpAR	CGGGCTTTCTTCTTGTTCG	Goel *et al.* (2007)
rtxCF	CGACGAAGATCATTGACGAC	Chow *et al.* (2001)
rtxCF	CATCGTCGTTATGTGGTTGC	Chow *et al.* (2001)
$rstR^{ElTor}$	CTTCTCATCAGCAAAGCCTCCATC	Bhattacharya *et al.* (2006)
$rstR^{Clasical}$	GCACCATGATTTAAGATGCTC	Bhattacharya *et al.* (2006)
$rstR^{Calcutta}$	CTGTAAATCTCTTCAATCCTAGG	Bhattacharya *et al.* (2006)
rstRR	TCGAGTTGTAATTCATCAAGAGTG	Bhattacharya *et al.* (2006)
rstCF	GATGTTTACGATAGCCTAGAAGACTT	Nguyen *et al.* (2009)
rstCR	TACAGTGATGGCTCAGTCAATGC	Nguyen *et al.* (2009)
ch1F	GACCACTCAGGCCGCTGAAAT	Nguyen *et al.* (2009)
ch1R	CCGCGCTCAAGTGGTTATCGG	Nguyen *et al.* (2009)
ctxBF	AGATATTTTCGTATACAGAATCTCTAG	Nguyen *et al.* (2009)
cepR	AAACAGCAAGAAAACCCCGAGT	Nguyen *et al.* (2009)
rstAR	CCGTGAAAGTCATCAACG	Nguyen *et al.* (2009)
ch2F	AACAACAGGTTGCAAGAGAGCATT	Nguyen *et al.* (2009)
ch2R	TATTGCTTTTTTAATGGCCGTT	Nguyen *et al.* (2009)

by standard biochemical tests for presumptive identification of *V. cholerae* (Nair *et al.*, 1987; Tamrakar *et al.*, 2006). Serological identification of the isolates was done by slide agglutination using commercially available polyvalent antiserum against *Vibrio cholerae* O1 (Ogawa and Inaba) and O139 serogroups (Difco, USA).

DNA preparation

DNA was extracted by using the Fermentas genomic DNA extraction kit as per the manufacturer instructions (MBI Fermentas, Lithuania). The amount and purity of the DNA was measured by spectrophotometer (NanoDrop ND-1000, Australia).

Gene detection by multiplex PCR

Various toxigenic and pathogenic genes were detected by PCR as described elsewhere (Kumar *et al.*, 2009). The genes encoding cholera toxin (*ctxAB*), an outer membrane protein (*ompW*), O1 somatic antigen (*rfbO1*), repeat in toxin (*rtxC*) and toxin coregulated pilus (*tcpA*) were detected by PCR assay. Isolates were subjected to *rstR* typing using the primers for $rstR^{El\ Tor}$, $rstR^{Classical}$, and $rstR^{Calcutta}$ as described elsewhere (Bhattacharya *et al.*, 2006). A list of all primers used in this study has been given in Table 1. The amplicons were electrophoresed through 1.2% agarose gel and then stained with ethidium bromide.

Sequence of *ctxB* gene

The cholera toxin B (*ctxB*) gene was amplified from the strains isolated from outbreak areas using the *ctxF* and *ctxR* primers as reported earlier (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 by using the Big Dye Terminator kit from Applied Biosystems. The sequences of *ctxB* gene for other *V. cholerae* O1 El Tor and classical strains were retrieved from the public domain (GenBank). The deduced amino acid sequences of *ctxB* gene from all the strains were aligned using the CLUSTAL W programme. The GenBank accession no. for the [*ctxB*] sequence of VCH2, VCH4, VCH5, VCH8, VCH10, VCH12. VCH16, VCH19, VCH22, VCH26, VCH31, and VCH34 are GU569396, GU569397, GU569398, GU 569399, GU569400, GU569401, GU569402, GU569403, GU569404, GU569405, GU569406, and GU569407, respectively.

Presence and location of RS1 and CTX prophage

The presence of the RS1 element was confirmed by PCR using the *rstC* specific primers *rstCF*/*rstCR* as described elsewhere (Nguyen *et al.*, 2009). The location of an RS1-CTX prophage array on the large chromosome was determined by using two sets of primers, *Ch1F*/*rstAR* and *ctxBF*/*Ch1R* (Nguyen *et al.*, 2009). The absence of CTX prophage or RS1 on the small chromosome was established using primers *ch2F*/*ch2R*. Another set of primers, *ch2F*/*rstAR* was used to determine the presence of tandem repeat of the CTX prophage on the small chromosome.

Genomic fingerprinting

Representative isolates were examined for clonal relationship using the enterobacterial repetitive intergenic consensus sequence (ERIC) PCR, BOX PCR, and RAPD. ERIC PCR was performed as described earlier with little modifications by using two primers ERIC1R 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2 5'-AAGTAAG TGACTGGGTGAGCG-3' (Rivera *et al.*, 1995). The thermal cycler was programmed for 35 cycles of 1 min at 94°C, 1 min at 52°C, 10 min at 68°C followed by 20 min incubation at 70°C. The BOX PCR was performed by using a single nucleotide primer BOX A1R (Versalovic *et al.*, 1994). BOX PCR was performed by using a single nucleotide primer BOX A1R 5'-CTACGGCAAGGCGACGCTGAC G-3' (Versalovic *et al.*, 1994). PCR program consisted of: initial dena-

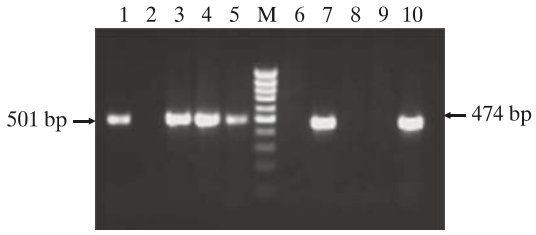

Fig. 1. *rstR* typing of *V. cholerae* outbreak isolates. Lanes: M, 100 bp ladder; 1-5, $rstR^{El\ Tor}$; 6-10, $rstR^{Classical}$. Lanes: 1 and 6, *V. cholerae* O1 ATCC 14033; 2 and 7, *V. cholerae* O1 ATCC 11623; 3 and 8, *V. cholerae* VCH4; 4 and 9, *V. cholerae* VCH20; 5 and 10, *V. cholerae* VCH35.

turation (95°C for 7 min), 30 cycles of 94°C for 1 min, 53°C for 1 min, 65°C for 8 min, and a final extension of 65°C for 16 min. RAPD was performed using 10-mer random primer from Operon 10-mer kit A (Operon Technologies, USA). The PCR reaction consisted of 20 ng of DNA, 20 pmol of primer in *Taq* buffer 2.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP and 1 U of *Taq* polymerase in a final volume of 25 μl. The PCR cycling conditions were as follows: preincubation 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

A total of 34 *V. cholerae* were isolated from the affected patients during the outbreak. All the isolates were biochemically identified as *V. cholerae* and serologically confirmed as O1 Ogawa.

Genotype by PCR

The PCR analysis of the isolates confirmed the presence of *ctxAB, ompW*, *rfbO1*, *rtxC*, and *tcpA* genes. The *ompW* gene is species-specific for *V. cholerae* and its presence in the isolates confirmed the *V. cholerae*. The presence of *ctxAB* and *rfbO1* genes confirmed the toxigenicity and group 1 antigen of the "O" side chain of the LPS of *V. cholerae*. Biotype of *V. cholerae* O1 serogroup isolates can be determined on the basis of PCR assays developed for *rtxA* or *rtxC* (Chow *et al.*, 2001). In this study, all the isolates belonged to the El Tor biotype on the basis of the repeat in toxin gene (*rtxC*).

Cholera toxin is one of the most important epidemic marker among various toxins produced by *V. cholerae* (Kaper *et al.*, 1995). The cholera toxin gene (*ctxAB*) is acquired from the genome of a filamentous CTX bacteriophage. The pilus colonization factor TCP acts as a receptor for CTXØ, which can infect non-toxigenic *V. cholerae*, leading to the emergence of new toxigenic strains (Waldor and Mekalanos, 1996).

RstR typing

PCR analysis for *rstR* allele in the isolates revealed that the isolates were of $rstR^{El\ Tor}$ type (Fig. 1). Only one isolate (VCH35) was found positive for $rstR^{El\ Tor}$ as well as $rstR^{Classical}$ genes indicating the presence of genes from both, the El Tor as well as classical CTX phage. The *rstR* alleles are believed to be biotype specific and are classified into $rstR^{Classical}$, $rstR^{El\ Tor}$, and $rstR^{Calcutta}$ for classical, El Tor, and O139 alleles, respectively (Kimsey *et al.*, 1998). However, presence of both $rstR^{El\ Tor}$ and $rstR^{Classical}$ allele in the same strain indicates the presence of two different copies of CTXΦ, either as a tandem array or located on a different chromosome (Nguyen *et al.*, 2009). Likewise, multiple *rstR* alleles in the same *V. cholerae* strain have been reported from other countries (Kimsey *et al.*, 1998; Nusrin *et al.*, 2004).

Sequence of *ctxB* gene

The alignment of *ctxB* sequences from outbreak strains with O1 El Tor and O1 classical reference strains revealed that the *ctxB* gene sequences of the isolates were identical and were aligned with that of the classical biotype of *ctxB*. The deduced amino acid sequences of CT-B from representative El Tor isolates of *V. cholerae* varied from CT-B of reference El Tor strains at position 39 (histidine in place of tyrosine) and 68 (threonine in place of isoleucine). Similar El Tor strains producing classical CT were isolated from many parts of Asian countries (Ansaruzzaman *et al.*, 2004; Nair *et al.*, 2006; Nguyen *et al.*, 2009; Goel and Jiang, 2010). Biotype specific CTXΦ is found in the *V. cholerae* strains. El Tor biotype strains harbour $CTX^{El\ Tor}\Phi$ and classical strains have $CTX^{Classical}\Phi$ (Ansaruzzaman *et al.*, 2004). However, in this study *V. cholerae* O1 El Tor isolates have shed their El Tor CTX prophage and acquired the classical prophage. Therefore, all the El Tor isolates were found to possess the *ctxB* sequence of the classical biotype. Classical biotype strains were replaced by the El Tor biotype in the seventh and current pandemic

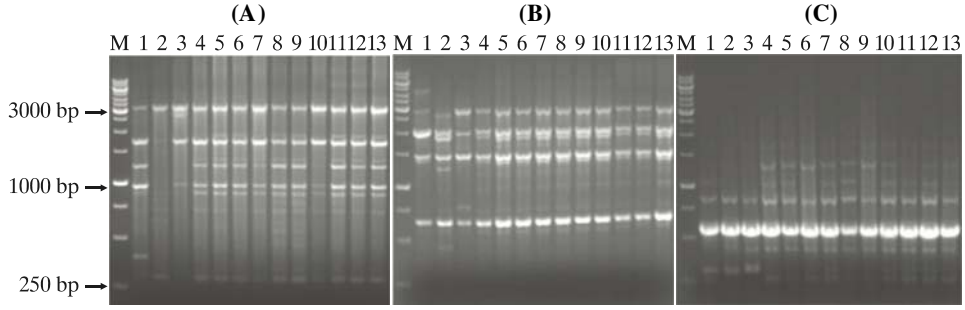

Fig. 2. DNA fingerprints of different outbreak isolates of *V. cholerae* O1 generated by ERIC PCR (A), Box PCR (B), and RAPD PCR (C). Lanes: M, Ladder; 1, *V. cholerae* O1 ATCC 11623; 2, *V. cholerae* O1 ATCC 14033; 3, *V. cholerae* O139; 4-13, outbreak isolates: 4, VCH2; 5, VCH4; 6, VCH5; 7, VCH8; 8, VCH10; 9, VCH12; 10, VCH16; 11, VCH19; 12, VCH22; 13, VCH34.

of cholera. Both biotypes of *V. cholerae* O1 are closely related in their O-antigen biosynthetic genes. However, the genomic structure of the CTXΦ, in which the cholera toxin genes are contained, differs between the classical and El Tor biotypes (Waldor and Mekalanos, 1996; Ansaruzzaman *et al.*, 2004).

RS1 element and CTX prophage array

The *rstC* specific primers amplified the DNA fragments from all the isolates indicating the presence of the *rstC* gene in the chromosome. The PCR amplified products using *ch1F*/*rstAR* and *ctxBF*/*ch1R* primers in all the isolates confirmed that the RS1-CTX prophage array is located on the large chromosome. All the isolates yielded PCR products using *ch2F*/*ch2R* primers indicating the absence of the CTX prophage or RS1 element on small chromosome. However, the isolate VCH35 was PCR negative for *ch2F*/*ch2R* primers indicating the presence of the CTX prophage or RS1 element on the small chromosome. Further, VCH35 was PCR positive using *ch2F*/*rstAR* primers confirming the presence of tandem repeat of the CTX prophage on the small chromosome. Atypical El Tor strains have been classified into two different groups on the basis of genetic structures of the CTX prophage and RS1 element on each chromosome (Lee *et al.*, 2009; Nguyen *et al.*, 2009). Group 1 strains harbor a tandem repeat of the classical CTX prophage on the small chromosome and Group II strains contain the RS1 and CTX prophage with El Tor type *rstR* and classical *ctxB* on the large chromosome (Lee *et al.*, 2009; Choi *et al.*, 2010). All the strains used in this study belonged to Group II strains. However, the isolate VCH35 seemed to belong to Group 1 strains.

DNA fingerprinting

Fingerprinting analysis by ERIC PCR, BOX PCR, and RAPD PCR revealed the clonal relationships among all the outbreak isolates. ERIC-PCR with genomic DNA of various *V. cholerae* strains resulted in amplification of multiple fragments of DNA in sizes ranging between 0.25 and 3.2 kb (Fig. 2A). The BOX PCR of genomic DNA from various *V. cholerae* isolates also resulted in amplification of different fragments of DNA of varying length ranging from 0.65 kb to 3.0 kb (Fig. 2B). Likewise, different DNA fragments of the same size were amplified by RAPD in all the isolates (Fig. 2C). However, in the entire outbreak *V. cholerae* isolates, fragments of similar sizes were found. PCR-based methods of fingerprinting take advantage of the presence of repetitive sequences that are interspersed throughout the genome of diverse bacterial species. The same genetic pattern was found among all the isolates using all three fingerprinting techniques suggesting the outbreak is likely caused by a single clone of *V. cholerae* O1 strain with altered biotype. Similar *V. cholerae* strains have been isolated from other cholera outbreaks in India (Kumar *et al.*, 2009; Goel *et al.*, 2010).

In conclusion, the *Vibrio cholerae* O1 'atypical El Tor' biotype strains with $rstR^{\text{El Tor}}$ allele were involved in the Hyderabad cholera outbreak. Most of the isolates contained an RS1-CTX prophage array on the large chromosome and harboured the classical cholera toxin (*ctxB*) gene. The results of this study showed that classical CT producing El Tor strains are now replacing the seventh pandemic El Tor strains. This is an evolutionary optimization of the El Tor biotype, which could represent a new and more significant emerging form of the El Tor biotype of *V. cholerae*.

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