RESEARCH LETTER

Genetic analysis of CTX prophages with special reference to ctxB and rstR alleles of Vibrio cholerae O139 strains isolated from Kolkata over a decade

Amit Raychoudhuri1, Piyali Mukherjee1, Thandavarayan Ramamurthy1, Ranjan K. Nandy1, Yoshifumi Takeda2, Gopinath B. Nair1 & Asish K. Mukhopadhyay1

1Division of Bacteriology, National Institute of Cholera and Enteric Diseases, Kolkata, West Bengal, India; and 2Collaborative Research Center of Okayama University for Infectious Diseases at NICED, Kolkata, West Bengal, India

Correspondence: Asish K. Mukhopadhyay, Division of Bacteriology, National Institute of Cholera and Enteric Diseases, P 33, CIT Road, Scheme XM, Beliaghata, Kolkata 700010, India. Tel.: +91 33 2353 7469; fax: +91 33 2370 5066; e-mail: asish_mukhopadhyay@yahoo.com

Received 1 July 2009; accepted 22 October 2009. Final version published online 16 December 2009.

DOI:10.1111/j.1574-6968.2009.01856.x

Editor: Craig Winstanley

Keywords
Vibrio cholerae; CTX prophages; rstR alleles; ctxB; cholera toxin genotype.

Abstract
Chronological analysis of 125 Vibrio cholerae O139 strains isolated during 1993–2005 in Kolkata revealed the prevalence of two new genotypes of cholera toxin (CT) and novel combinations of ctxB and rstR alleles resulting in variant CTX prophages. One of the new genotypes of ctxB, which first appeared in 1996 with the re-emerged V. cholerae O139 strains that had CTX Calcutta phage, was designated as genotype 4. In 1998, another new genotype, designated as genotype 5, was detected that prevailed mostly in CTX phages with El T or rstR. The prototype El T or CTX phage with genotype 3 gradually disappeared in O139, and since 2002 the predominant CTX prophages in O139 are Calcutta phages with genotype 4 and El Tor phages with genotype 5. Results showed that V. cholerae O139 strains of Kolkata, isolated over a decade, harboured CTX prophages in the large chromosome having no RS1 downstream of CTX prophage. During the course of its intermittent incidence over a decade, five types of O139 strains were detected based on CT genotypes. Such abrupt genetic changes in O139 strains might not favour its continued prevalence in human cases in Kolkata, India.

Introduction
The emergence of Vibrio cholerae serogroup O139 in 1992 in south India and its quick spread to different cholera endemic regions of India, Bangladesh and neighbouring countries is considered an unprecedented event in the history of cholera (Cholera Working Group, 1993; Chongsa-Nguan et al., 1993; Fisher-Hoch et al., 1993; Ramamurthy et al., 1993; Nair et al., 1994). The genesis of V. cholerae O139 attracted worldwide attention, particularly because this was the first non-O1 serogroup associated with widespread epidemics of cholera. Ever since, O139 strains have undergone various alterations in both phenotypic and genetic characteristics, for example changing patterns of antimicrobial resistance, restriction fragment-length polymorphisms in conserved rRNA genes (ribotype), rearrangement of the CTX prophage and acquisition of new CTX prophages (Mitra et al., 1996; Sharma et al., 1997; Basu et al., 1998; Mukhopadhyay et al., 1998; Faruque et al., 2000). Molecular evolutionary studies have also recorded temporal variations in the prevalence of O139 and O1 serogroups over the years in India along with the emergence of new clones within the O139 serogroup. Soon after the complete replacement of the O1 serogroup by the new serogroup O139, in September 1993 the O1 serogroup reappeared and in February 1994 completely replaced the O139 serogroup in Kolkata (Mukhopadhyay et al., 1996). Subsequent studies revealed that the O1 serogroup, which replaced the O139, was a new clone of the O1 El Tor biotype (Faruque et al., 1997; Sharma et al., 1997; Yamasaki et al., 1997).

Due to the quiescent period in the incidence of V. cholerae O139, it was thought that the appearance of O139 was a one-time event. But a resurgence of O139 was recorded in August 1996 in Kolkata (Mitra et al., 1996) and this serogroup remained dominant until 1997 (Fig. 1). Between December 1999 and December 2000, escalating association of V. cholerae O139 with outbreaks of cholera were recorded in many parts of India, including Kolkata (Sinha et al., 1997;
After this period, V. cholerae O1 continued to be a dominant serogroup in Kolkata, and the incidence of O139 gradually decreased over the years (Raychoudhuri et al., 2007) (Fig. 1).

Cholera toxin (CT) is the principal toxin of epidemic-causing V. cholerae serogroup O1 and O139 and is encoded by ctxA and ctxB, the major enzymatic subunit and the binding subunit, respectively. Generally, ctxB is polymorphic in nature and exists in three major genotypes, namely genotype 1, found among strains of the classical biotype worldwide and the US Gulf Coast, genotype 2, found among El Tor biotype strains from Australia and genotype 3, found in El Tor biotype strains from the seventh pandemic and the Latin American epidemic (Olsvik et al., 1993). Previous studies have shown that the V. cholerae serogroup O139 originated from the seventh pandemic El Tor biotype by horizontal transfer of novel O antigen genes (Bik et al., 1995; Comstock et al., 1996).

A recent study revealed that the prototype seventh pandemic El Tor biotype of V. cholerae O1 was completely replaced in 1995 by El Tor strains that had classical type ctxB in Kolkata (Raychoudhuri et al., 2009). This shift of CT from genotype 3 to genotype 1 in V. cholerae O1 strains of Kolkata and detection of diversity in the CTX phage repressor, rstR (Kimsey et al., 1998; Davis et al., 1999; Nusrin et al., 2004), has formed the impetus for a retrospective analysis of CT genotypes along with rstR of CTX prophages in O139 strains isolated from Kolkata over a period of 13 years.

**Materials and methods**

**Bacteriology and serology**

A total of 125 O139 strains were selected for this study from the strain repository of the National Institute of Cholera and Enteric Diseases, Kolkata, and were isolated in different time frames between 1993 and 2005. All the strains were grown in Luria–Bertani (LB) broth (Difco) for 18 h and then streaked on Luria agar plates. These strains were confirmed serologically by slide agglutination with O139-specific antiserum.

**Preparation of template for PCR**

A 1-mL aliquot of overnight LB broth culture was taken into a sterile 1.5-mL microfuge tube, and the cells collected as a pellet following centrifugation at 3421 g (Biofuge, Heraeus, Germany) for 5 min. The cell pellet was then resuspended in 300 µL of sterile distilled water and boiled for 10 min in a water bath. The boiled sample was snap-cooled on ice and centrifuged at 13 684 g for 10 min. The supernatant was collected in a sterile microfuge and 5 µL of this supernatant was used as template for PCR analysis.

**PCR analysis**

Mismatch amplification mutation assay (MAMA) PCR, which detects sequence polymorphisms between CT genotype 1 (classical type CT) and genotype 3 (El Tor type CT) based on the nucleotide position 203 of the ctxB gene (Morita et al., 2008), has been utilized in this study with O139 strains. The rstR PCR was performed to determine the allele type of rstR (regulatory region for phage lysogeny) of CTX phages present in the O139 strains of Kolkata (Kimsey et al., 1998; Nusrin et al., 2004). The primers used in this study are given in the Table 1. Vibrio cholerae O1 strains O395 and N16961 were used as standard reference strains for classical and El Tor biotypes, respectively.

**Nucleotide sequence of ctxB**

To determine the nucleotide sequence of the ctxB, PCR amplification of ctxB locus of 22 strains of V. cholerae O139 was performed in a 25-µL reaction mixture using Ex Taq™ polymerase (Takara, Japan) with proofreading activity. The
PCR primers and conditions used have been described previously (Olsvik et al., 1993). PCR products were purified with the QIAquick PCR purification kit (Qiagen GmBH, Germany) and both strands were sequenced in an automated sequencer (ABI PRISM 3100 Genetic Analyser, Applied Biosystems).

Nucleotide sequence accession number

The sequences obtained here were deposited in GenBank with the accession numbers FJ999956–FJ999988.

Nested PCR assay

Amplicons of ~3, 6.3 and 6 kb were obtained by PCR with primer pairs ctxA (F) and rtxA1, rstr2F and ctxB (R), and rstr3F and ctxB (R), respectively, using XT-20 PCR system (Bangalore Genei), and the products were separated by electrophoresis using 1% agarose gel in TAE buffer followed by staining with ethidium bromide. A λ-HindIII molecular size ladder (Takara) was run with the gel. The desired DNA fragments were excised from agarose gels and purified using a Gel Extraction kit (Qiagen GmBH). The purified DNA of ~3, 6.3 and 6 kb thus obtained were used as template for nested PCR using ctxB (F) and ctxB (R) primers (Olsvik et al., 1993). Nucleotide sequencing of ctxB genes was performed with the resulting 460-bp amplicon.

Chromosomal localization of CTX prophage

Chromosomal localization of the CTX prophages of V. cholerae O139 strains was performed using two sets of primers followed by Southern blot hybridization. The specific primer pair consisting of CIIF and CIIR, as described earlier (Maiti et al., 2006), was used to confirm the CTX prophage in the small chromosome. Strains that do not have CTX prophage in the small chromosome will give an expected PCR amplicon of 766 bp. The strains that have CTX prophage integrated between these regions in the small chromosome will not yield any amplicon in the assay due to the large size (around 8 kb) of the target gene. A set of primers consisting of ctxB common (F) (Morita et al., 2008) and ctxB (R) was used to determine the location of CTX prophage in the large chromosome (Colombo et al., 1994; O'Shea et al., 2004). The rtxA gene encodes a presumptive cytotoxin that is a part of the RTX (repeats in toxin) gene cluster containing GD-rich repeated motifs, which represent a family of toxin well disseminated in Gram-negative bacteria and has been reported to be present in the large chromosome adjacent to ctx genes (Lin et al., 1999; Sheahan et al., 2004). Vibrio cholerae O1 strains devoid of a CTX prophage in the small chromosome but possessed of the same in the large chromosome without any direct repeat sequence (RS) element connecting the core downstream of ctx genes will yield an amplicon of nearly 2.4 kb. Another combination of primers zotF and rtxA1 was used to determine the presence of CTX prophages lacking the ctxAB operon and lying downstream of the RS1 element adjacent to rtx genes, which will produce an expected amplicons of ~2.35 kb.

DNA probes and hybridization

Purified genomic DNA was treated with suitable restriction endonuclease enzymes and separated by electrophoresis in
0.8% agarose gels. DNA fragments were denatured by treatment with alkali and subsequently transferred to a nylon membrane (Hybond-N\textsuperscript{1}; Amersham Pharmacia Biotech), according to the procedure of De et al. (2005), and hybridized with a DNA probe. CTX typing was performed by digesting the genomic DNA with HindIII, PstI, AvaI and BglII (Takara). A 540-bp XbaI–ClaI fragment of ctxA was ligated with the EcoRI linker and subsequently the ligated product was cloned into the EcoRI site of pKTN901 that served as a probe for ctxA (Kaper et al., 1988). The specific probes of cep (core-encoded pilus) encoding a putative colonization factor present in the core (Pearson et al., 1993), rst\textsuperscript{E1}R and rst\textsuperscript{calc}, which are cloned in the plasmids pSC01, pSC06 and pSC10, respectively, were obtained by digesting the plasmids individually with EcoRI (Chatterjee et al., 2007). DNA probes were labelled with chemiluminescent dye (Amersham Biosciences) and hybridization reactions were developed following the manufacturer’s protocol and recognition patterns recorded on X-ray film.

### Results

**Analysis of ctxB by MAMA PCR**

The results of MAMA PCR showed that all *V. cholerae* O139 strains isolated up to 1995 yielded amplicons with El Tor allelic primer pair of ctxB only. But 54% and 18% of the *V. cholerae* O139 strains isolated during 1996 produced amplicons with El Tor or classical specific ctxB primer pairs, respectively, while 28% of the tested strains yielded amplicon with both classical and El Tor primer pairs of ctxB (Table 2). The same trend was continued among *V. cholerae* O139 strains isolated in 1997. Strains isolated during 1998 did not produce amplicons using only the El Tor ctxB primer pair, but 68% produced amplicon with classical specific ctxB primers and 32% yielded amplicons with both classical and El Tor-specific ctxB primer pairs. Between 1999 and 2005, the O139 strains yielded amplicon only with the classical allelic primer pair of ctxB. Detailed results are shown in Table 2.

**Nucleotide sequence analysis of ctxB gene**

To confirm the results of MAMA PCR, 22 representative *V. cholerae* O139 strains isolated from 1993 to 2005 were selected for sequencing of ctxB. The results (Table 3) showed that two *V. cholerae* O139 strains isolated from 1993 to 1995 produced amplicon for El Tor-specific primers of ctxB that had identical sequence to El Tor genotype of ctxB, i.e. genotype 3. Four strains isolated from 1996 to 1998 yielded amplicons for both classical and El Tor ctxB, producing overlapping sequence peaks of C/A, C/T and C/T at nucleotide positions 83, 115 and 203, respectively. A likely scenario for the presence of overlapping peaks is that the polymerase introduced nucleotide substitutions during the amplification process. But by addressing the chromosomal localization and subsequent resequencing of the associated ctxB alleles, it was shown that these substitutions were not amplification artifacts. Four strains isolated during 1996–1998 yielded amplicons similar to classical ctxB, but are associated with a new genotype, with nucleotide ‘C’ at positions 83, 115 and 203 corresponding to amino acid changes with alanine, histidine and threonine at positions 28, 39 and 68, respectively. This allele of ctxB has been designated as a new genotype, ‘genotype 4’. Five strains isolated from 1998 to 2001, which yielded amplicons similar

---

**Table 2.** PCR-based analysis regarding the distribution of different types of ctxB and rstR among *Vibrio cholerae* O139 strains isolated from Kolkata over the period 1993–2005

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>No. of strains</th>
<th>Type of ctxB by MAMA PCR</th>
<th>Type of rstR by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Only El Tor type</td>
<td>Only Classical type</td>
</tr>
<tr>
<td>1993</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1994</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1995</td>
<td>14</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>1996</td>
<td>11</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>1997</td>
<td>12</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>1998</td>
<td>25</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>1999</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>2000</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>2001</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>2002</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>2003</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2005</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^{I}\)These strains yielded amplicons of ctxB gene for El Tor as well as classical ctxB.
to classical ctxB, showed another new genotype with nucleotides A, T and C at positions 83, 115 and 203, respectively, corresponding to amino acid changes with aspartic acid, tyrosine and threonine at positions 28, 39 and 68, respectively. This sequence differed from genotype 3 or El Tor allele of ctxB by having a 'C' nucleotide at position 203, similar to genotype 1 or the classical allele, instead of an 'A' and hence has been designated as 'genotype 5'. Thus, genotype 5 is a hybrid between genotypes 1 and 3. Seven strains isolated from 1998 to 2005, which yielded amplicons similar to classical ctxB, produced overlapping peaks of A/C and T/C at nucleotide positions 83 and 115, respectively, and nucleotide C at position 203.

### Nested PCR and subsequent nucleotide sequence analysis

To isolate a single copy of ctxB with non-overlapping peaks of nucleotides adjacent to rtxA gene from *V. cholerae* O139 strains isolated from 1996 to 1998 (which had overlapping nucleotide sequences), a PCR was performed with primers ctxA (F) and rtxA1. An amplicon of ∼3 kb was obtained and was used as template in the nested PCR using ctxB primers. An amplicon of 460 bp obtained from this nested PCR was used for the nucleotide sequencing. The subsequent sequencing analysis at ctxB loci depicted the prevalence of CT genotype 4.

To separately isolate the copies of CTX prophage with *rstR<sup>ET</sup>* and *rstR<sup>ET</sup>calc* possessing non-overlapping peaks of nucleotides from the *V. cholerae* O139 strains isolated from 2000 to 2005, PCRs were performed with primers *rstR2F*/ *rstR<sup>ET</sup>* and ctxB (R) and primers *rstR3F*/ *rstR<sup>ET</sup>calc* and ctxB (R), respectively. The amplicons of 460 bp thus obtained were used for nucleotide sequencing. The subsequent sequencing at ctxB loci revealed the presence of genotype 5 of ctxB in CTX prophage with *rstR<sup>ET</sup>* and genotype 4 of ctxB in CTX prophage with *rstR<sup>ET</sup>calc*.

The prominent events in the changing profile of CTX prophages with respect to CT genotypes and *rstR* alleles among O139 strains from January 1993 to December 2005 are shown in Fig. 1 along with the isolation status of *V. cholerae* O139 strains from patients hospitalized due to acute secretory diarrhoea at the Infectious Diseases Hospital, Kolkata. Nested PCR results depicted the schematic representation (Fig. 2) of variable combinations of CT genotypes, and *rstR* alleles prevailed among O139 strains in Kolkata. Since its first appearance in 1993, five types of O139 strains have been detected successively with the following important changes: (1) strains with CT genotype 3 only; (2) strains with CT genotype 4 only; (3) strains with CT genotype 5 only; (4) strains with CT genotypes 3 and 4; and (5) strains with CT genotypes 4 and 5.

### Chromosomal localization of CTX prophage

All the O139 strains yielded an amplicon of 766 bp, when a PCR was performed using CIIF and CIIR primers, which indicated lack of the CTX element in the small chromosome. All the O139 strains isolated from 1993 to 2000 and 40% of O139 strains of 2001 yielded an amplicon of nearly 2.4 kb with ctxB forward (F) and rtxA1 primers. Strain N16961, which possessed RS1 downstream of CTX prophage, and O395, which lacked RS1, were used as controls considering the fact that N16961 has CTX prophage only in the large chromosome, whereas the other strain O395 possessed CTX...

---

**Table 3.** Different combinations of ctxB and *rstR* of O139 strains isolated from Kolkata over a decade after sequencing

<table>
<thead>
<tr>
<th>Year</th>
<th>Nucleotide positions</th>
<th>Amino acid positions</th>
<th>CT genotype</th>
<th><em>rstR</em> type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1993–1995</td>
<td>A T T</td>
<td>Asp Tyr Ile</td>
<td>Genotype 3</td>
<td>El Tor</td>
</tr>
<tr>
<td>1996–1998*</td>
<td>A T T</td>
<td>Asp Tyr Ile</td>
<td>Genotype 3</td>
<td>El Tor</td>
</tr>
<tr>
<td>1996–1997</td>
<td>C C C</td>
<td>Ala His Thr</td>
<td>Genotype 4</td>
<td>El Tor</td>
</tr>
<tr>
<td>1998</td>
<td>C C C</td>
<td>Ala His Thr</td>
<td>Genotype 4</td>
<td>Calcutta</td>
</tr>
<tr>
<td>1998–2001</td>
<td>A T C</td>
<td>Asp Tyr Thr</td>
<td>Genotype 5</td>
<td>El Tor</td>
</tr>
<tr>
<td>2000–2005*</td>
<td>C C C</td>
<td>Ala His Thr</td>
<td>Genotype 4</td>
<td>Calcutta</td>
</tr>
<tr>
<td></td>
<td>A T C</td>
<td>Asp Tyr Thr</td>
<td>Genotype 5</td>
<td>El Tor</td>
</tr>
</tbody>
</table>

*Indicates that a single strain contains two types of ctxB with two different *rstR* types.

**Fig. 2.** Schematic diagram showing the four types of combinations of *rstR* and ctxB alleles prevailing in different time frames among *Vibrio cholerae* O139 strains of Kolkata isolated between 1993 and 2005.
prophage in both the large and the small chromosome. N16961 yielded a product of > 5 kb with ctxB common forward (F) and rtxA1 primers and 766-bp amplicons with CIIF and CIIR primers. O395 yielded a product of 2.4 kb with ctxB forward (F) and rtxA1 primer pairs but no product with CIIF and CIIR primer sets. About 60% of the O139 strains of 2001 and all the tested strains isolated from 2002 to 2005 did not produce any amplicon using ctxB forward (F) and rtxA1 primer pairs. But an amplicon of ~2.35 kb was obtained from these strains using another primer pair, zotF and rtxA1. Thus, our results depicted that V. cholerae O139 strains isolated over the period of 2001–2005 harboured CTX prophage in the large chromosome having no RS1 downstream of CTX prophage and with an empty site in the small chromosome. Some of the strains of 2001 and most of the strains isolated during 2002–2005 had a truncated CTX prophage adjacent to rtx gene cluster. These strains were further analyzed for the detection of RS1 and TLC (toxin-linked cryptic) element to understand the upstream region of CTX prophage. Detection of RS1 was carried out by PCR assay using the primers rstC1 and rstC2. All the tested strains along with the reference El Tor strain N16961 yielded an amplicon ~245 bp in size, whereas the classical O395 failed to generate any amplicon as it is devoid of the RS1 element. The RS1 element has been shown to be linked with the CTX prophage of V. cholerae O1 El Tor, and O139 strains in general, but the existence of free RS1 in V. cholerae is not uncommon. Similarly, all the tested strains yielded an amplicon of ~2 kb for pTLC using primers tlcF and tlcR. A schematic genetic map displaying the chromosomal localization of CTX prophage among re-emerged V. cholerae O139 strains between 1996 and 2003 is shown in Fig. 3.

**Detection of copy number and arrangement of CTX prophage**

Southern hybridization (detailed results not shown) showed that the O139 strains that re-emerged in 1996 had three copies of the CTX prophage, the first one with rstR<sup>ET</sup>, followed by two rstR<sup>calc</sup>. The 2003 strains had one CTX prophage with rstR<sup>ET</sup>, followed by one intact copy of CTX prophage with rstR<sup>calc</sup> and one truncated CTX prophage (ctxAB gene absent) with rstR<sup>calc</sup>. Figure 3a and b shows a schematic diagram of the copy number of CTX prophages with the probable combination of rstR and ctxB alleles in the re-emerged O139 in 1996 and recent O139 of Kolkata.

**Analysis of rstR alleles**

The nucleotide sequence variations in the repressor region rstR formed the basis of the distinct alleles, namely CTX<sup>C1</sup>, CTX<sup>ET</sup> and CTX<sup>calc</sup> (Kimsey et al., 1998; Davis et al., 1999). Determination of rstR alleles revealed that V. cholerae O139 strains isolated during 1993–1995 possessed only the rstR<sup>ET</sup> allele (Table 2). However, 65% of the O139 strains isolated from 1996 to 2001 yielded an amplicon of the rstR<sup>ET</sup> allele only and 35% of the strains yielded amplicons for both the rstR<sup>ET</sup> and rstR<sup>calc</sup> alleles. Strains isolated from 2002 to 2005 yielded amplicons for both rstR<sup>ET</sup> and rstR<sup>calc</sup> alleles.

**Discussion**

The lack of evidence on the nature of ctxB alleles among V. cholerae O139 strains and the emergence of V. cholerae O1 El Tor variants in Kolkata with classical ctxB formed the impetus to undertake this study. We found two new CT genotypes in V. cholerae O139 strains isolated from Kolkata apart from genotype 3, with different allelic combinations of rstR resulting in CTX prophage variants. Vibrio cholerae O139 isolated before 1996, i.e. from its first appearance in Kolkata during 1993–1995, was found to possess genotype 3, similar to the prototype El Tor strains. The new genotype 4, which had nucleotide C at positions 83, 115 and 203 in the ctxB gene, first appeared among re-emerged O139 strains during August 1996 in Kolkata after a hiatus of 2½ years. Interestingly, these V. cholerae O139 strains harboured a new rstR allele, rstR<sup>calc</sup> (Kimsey et al., 1998; Davis et al., 1999). In
addition, strains that yielded amplicons for both classical as well as El Tor ctxB during this period also possessed both types of rstR alleles, $rstR^{ET}$ and $rstR^{calc}$. The nested PCR results showed that the new single genotype of ctxB was present in a CTX prophage residing just adjacent to rtxA gene and possessing $rstR^{calc}$. One V. cholerae O139 strain isolated during 1998 possessed only one CTX prophage containing CT genotype 4 and $rstR^{calc}$. V. cholerae O139 strains isolated during 1996–1998 yielded amplicons of classical ctxB and $rstR^{ET}$ and displayed CT genotype 4 as determined by sequencing of ctxB. Most of the newly emerged CTX Calcutta phage and few El Tor CTX prophage residing in the re-emerged V. cholerae O139 strains possessed the new CT genotype 4. Interestingly, this genotype had closest homology to CT genotype 1 (classical ctxB genotype), with a difference of only single nucleotide (nucleotide cytosine instead of adenine) at position 83. It is possible that this new CT genotype originated from a single mutation at CT genotype 1 and was subsequently acquired by the re-emerged O139 strains during 1996. Another new CT genotype, genotype 5, was detected for the first time during 1998 among V. cholerae O139 strains in Kolkata. The strains of genotype 5 had $rstR^{ET}$ only. The strains isolated in 2000 and 2001 had two combinations of ctxB and rstR alleles: one with only CT genotype 4 along with only $rstR^{ET}$ and another with genotype 5 along with both $rstR^{ET}$ and $rstR^{calc}$. Strains isolated from 2002 onwards displayed a ctxB nucleotide sequence with overlapping peaks of A/C and T/C at positions 83 and 115, respectively, and nucleotide C at position 203. These strains harboured more than one copy of CTX prophage and had $rstR^{ET}$ and $rstR^{calc}$. We have already shown that V. cholerae O139 strains of Kolkata isolated in 2003 had more than one copy of the CTX prophage (Chatterjee et al., 2007). Our Southern hybridization results also reconfirmed the presence of more than one copy number of CTX prophage and their arrangement in recent O139, which was similar to our previous findings (Sharma et al., 1997; Chatterjee et al., 2007). The nested PCR result and subsequent sequencing indicated that most O139 strains isolated since 2002 and some strains isolated in 2000 and 2001 possessed CTX prophage containing $rstR^{ET}$ and CT genotype 5, along with combination of $rstR^{calc}$ and CT genotype 4. Thus, from 1999 onwards most of the El Tor phages had CT genotype 5 replacing the genotype 3 that prevailed from the time of its genesis in 1993 until 1998. Conversely, most Calcutta CTX phages displayed CT genotype 4 since its first appearance in 1996. Thus, this study revealed the occurrence of different allelic combinations of ctxB and rstR resulting from the integration of diverse CTX phages among O139 strains in Kolkata.

This study also confirms that MAMA PCR is more suitable for determining ctxB alleles (Morita et al., 2008) for serogroup O1, as indicated by several reports (Saña et al., 2008; Raychoudhuri et al., 2009) than O139, especially those isolated after 1995. This was due to the fact that MAMA PCR was based on the differences of nucleotides at position 203 in the ctxB gene that differentiate CT genotypes 3 and 1. Any additional change apart from this nucleotide position could not be detected using this PCR. In the case of O1 strains, differences between genotype 1 and genotype 3 at nucleotide position 203 was concurrent with the difference of nucleotides at position 115 and thus could be detected by this PCR. In the case of O139 strains, due to the additional mutations at positions 83 and 115, the MAMA PCR may not be useful in detecting such changes. This study also revealed that, similar to the O1 serogroup, the ctxB allele of O139 strains had been changed over years (Raychoudhuri et al., 2009). These changes in O139 ctxB occurred at multiple sites as compared with the O1 serogroup. Our results also showed distinct sequential correlation between prevalence of O139 and diversification among ctxB and rstR allelic combination in Kolkata. The resurgence of O139 in 1996 in Kolkata coincided with the appearance of CT genotype 4 along with $rstR^{calc}$, whereas the sudden escalation of O139 during 1999–2000 and its subsequent declination overlapped the emergence of CT genotype 5 with a $rstR^{ET}$ arrangement. The effect of the diverse changes in the genotypes of ctxB as well as rstR alleles along with the variations in other genetic segments of O139 strains have not been ascertained as yet. We assume that such genetic changes are the consequences of temporal variation in the incidence of O139. The structural and functional aspects of these new CT genotypes will be interesting areas to be explored in future, which may reveal vital information regarding phasing-in and phasing-out phenomena in the epidemiology of V. cholerae O139. The frequent mutations and other genetic changes of V. cholerae O139 might not be supported by its persistent incidence in Kolkata. This observation should be explored further with the collection of strains from other cholera endemic regions as well.

Acknowledgements

The work was supported in part by the Indian Council of Medical Research (ICMR), Government of India, and Program of Founding Research Center for Emerging and Reemerging Infectious Diseases, Ministry of Education, Culture, Sports, Science and Technology of Japan. A.R. is the recipient of Senior Research Fellowship from ICMR.

References

Basu A, Mukhopadhyay AK, Sharma C et al. (1998) Heterogeneity in the organization of the CTX genetic element in strains of Vibrio cholerae O139 Bengal isolated from Calcutta, India and Dhaka, Bangladesh and its possible link to


Raychoudhuri A, Chatterjee S, Pazhani GP, Nandy RK, Bhattacharya MK, Bhattacharya SK, Ramamurthy T & Mukhopadhyay AK (2007) Molecular characterization of...
recent *Vibrio cholerae* O1, El Tor, Inaba strains isolated from hospitalized patients in Kolkata, India. *J Infect* **55**: 431–438.


