Vibrio cholerae O1 clinical strains isolated in 1992 in Kolkata with progenitor traits of the 2004 Mozambique variant

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Retrospective analysis led to the detection of two *Vibrio cholerae* variant O1 strains (VC51 and VC53), which were isolated in 1992 in Kolkata from clinical cases, with identical traits to 2004 Mozambique variant O1 strains. The Mozambique O1 strains that caused a huge outbreak in 2004 have been shown to have phenotypic traits of both classical and El Tor biotypes, and thereby have been reported as variant. Our study demonstrated that two O1 strains isolated in Kolkata during 1992 were of the El Tor background as evidenced by polymyxin B (50 U ml⁻¹) resistance, positivity in Voges–Proskauer reactions and sensitivity to biotype-specific vibrio phages. With the features of classical CTX prophage, localization in the small chromosome, and an absence of RS1 and pTLC, both Mozambique and Kolkata strains appeared to be identical. Furthermore, two Kolkata strains exhibited an identical ribotype to that of the Mozambique variant, displaying ribotype pattern RI that had been assigned to Kolkata *V. cholerae* O1 strains isolated on or before 1992. *NotI* pulsotype analysis indicated that these 1992 Kolkata strains along with the Mozambique variant O1 belonged to very closely related clones. Considering the chronological events, and the typical identity at the phenotypic and the genotypic level between the two O1 strains isolated during 1992 from Kolkata and during 2004 from Mozambique, we propose that some of the 1992 Kolkata O1 strains might have acted as progenitors for Mozambique variant O1 strains.

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INTRODUCTION

Vibrio cholerae O1 and O139 strains are responsible for causing the disease cholera that afflicts million of people every year. To date, human civilization has witnessed seven pandemics of cholera and the seventh pandemic is still ongoing from its beginning in 1961 (Kaper *et al.*, 1995). The members of the serogroup O1 are classified into two biotypes, classical and El Tor, and two major serotypes, Ogawa and Inaba (Kaper *et al.*, 1995). While immunological specificity of the ‘O’ antigenic polysaccharides forms the basis of serotype differentiation, biotype differentiation is based on the phenotypic traits that include the typical resistance phenotype of the El Tor

strains against 50 U polymyxin B, positivity for Voges–Proskauer reactions, agglutination with chicken erythrocytes, haemolysis of sheep erythrocytes and sensitivity to El Tor group V vibrio phages (Kaper *et al.*, 1995). However, the test for the lysis of sheep erythrocytes is of limited use as most of the El Tor strains after the 1960s became non-haemolytic (Barrett & Blake, 1981). In addition to conventional biotyping methods, nowadays, molecular methods are widely in use that target biotype-specific allelic gene loci. These include biotype-specific alleles of *tcpA* and CTX prophages (Davis *et al.*, 1999; Keasler & Hall, 1993).

The aetiological agent of the seventh cholera pandemic is *V. cholerae* O1 biotype El Tor, which has replaced classical biotype strains over a period of time. In a recent study, strains with hybrid biotype traits (Matlab variants) have been reported from Bangladesh (Nair *et al.*, 2002). These

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Abbreviations: CHEF, contour-clamped homogeneous electric field; NICED, National Institute of Cholera and Enteric Diseases.

Matlab variants were negative for the Voges–Proskauer reaction, a trait typical for the classical biotype. Interestingly, these strains were resistant to lysis by the classical-biotype-specific vibrio phages. In fact, these strains displayed phenotypes that were not specific for either of the two biotypes and therefore they were considered as hybrids. The latest addition is the emergence and progression of a unique variant of *V. cholerae* O1 from Mozambique in 2004 (Ansaruzzaman *et al.*, 2004, 2007). The Mozambique O1 strains have been reported to possess traits of both classical and El Tor biotypes. In contrast to the Matlab variants, the background of Mozambique variants is typical El Tor as it displayed resistance to classical vibrio phages and polymyxin B (50 U ml⁻¹), but was sensitive to El Tor group V vibrio phages and positive for Voges–Proskauer reactions. All these traits still remain as the most reliable biotype differentiation markers for *V. cholerae* O1. The classical biotype properties of the Mozambique variant included detection of classical CTX prophage (CTX^{Cl}) localized in the small chromosome and absence of RS1 (Das *et al.*, 2007; Faruque *et al.*, 2007). In addition, these strains were also devoid of the toxin linked cryptic plasmid (pTLC) (Rubin *et al.*, 1998). Comparative analysis of the PFGE profiles of Matlab and Mozambique variants revealed that these two groups of strains belonged to different clones (Ansaruzzaman *et al.*, 2007).

The identification and rapid spread of the Mozambique variant led researchers to consider whether there had been an emergence of a more virulent variant of the El Tor biotype that possibly had the added advantages of the El Tor background with virulence phenotypes of classical origin. Previously, we have reported on the variant traits of *V. cholerae* O1 El Tor strain VC20 that possessed both classical and El Tor alleles of *rstR*, one of the constituent genes of CTX prophage (Bhattacharya *et al.*, 2006); the strain was isolated in 1992 from clinical cases. Detection of such a variant prompted us to revive archived *V. cholerae* O1 strains to screen them for traits similar to that of the Mozambique variant isolated previously. Detailed characterization of 15 O1 strains isolated during 1992 in Kolkata led to the identification of two strains with progenitor traits of the 2004 Mozambique variant.

METHODS

Bacterial strains. Clinical *V. cholerae* O1 strains isolated during 1992 were revived from cultures maintained at the National Institute of Cholera and Enteric Diseases (NICED), Kolkata. The bacterial strains were maintained in nutrient agar slabs and stored in the dark. Fifteen *V. cholerae* O1 strains including VC20 (Bhattacharya *et al.*, 2006), were considered in this study. Reference *V. cholerae* O1 strains of classical (569B, O395) and El Tor (N16961) biotypes were included as a comparison. The O1 variant strain B33 isolated from the Mozambique outbreak was also included (Ansaruzzaman *et al.*, 2004).

Detection of biotype traits. Biotype-specific traits were assayed by determining the susceptibilities to polymyxin B and to biotype-specific vibrio phages. In addition, Voges–Proskauer reaction patterns were also determined for *V. cholerae* strains by growing the cells in

MR-VP (methyl red–Voges–Proskauer) medium supplemented with 1% NaCl (Kelly *et al.*, 1992). Susceptibility to polymyxin B was determined by plate assay using Mueller–Hinton (MH) agar containing 50 U polymyxin B ml⁻¹ (Sigma). For this, cells were grown in 5 ml MH broth for 4–5 h (OD₅₄₀ ~0.3) at 37 °C and 10 µl culture was carefully placed on an MH/polymyxin B (50 U ml⁻¹) plate and allowed 30 min for absorption. The polymyxin B resistance phenotype was assigned to those strains that grew on the plate after 18 h incubation at 37 °C. Susceptibility patterns of these strains towards El Tor group V or classical group IV phages were determined (Chakrabarti *et al.*, 2000). Briefly, 3–4 h statically grown *V. cholerae* cells were mixed with molten (~45 °C) 0.8% soft agar, poured onto a nutrient agar plate and 20–30 min allowed for solidification. Next, each of the biotype-specific phages was spotted (from routine test dilutions) separately onto these plates. Next, plates were kept at room temperature for 10 min to allow the drops to dry. Following an incubation of 18 h at 37 °C, each reaction was recorded as positive if there was detection of a halo zone or five or more plaques.

PCR assay and nucleotide sequencing. Genomic DNA free from RNA and proteinaceous material was isolated from *V. cholerae* strains grown in Luria–Bertani 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. Next, cells were lysed with SDS followed by digestion with proteinase K (20 µg ml⁻¹) for 2 h at 60 °C. Purified genomic DNA free from proteinaceous materials and RNA was obtained by treatment with *N*-cetyl-*N,N,N*-trimethylammonium bromide (10% CTAB in 0.5 M NaCl) at 65 °C for 10 min, and sequential extraction with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and chloroform. Purified genomic DNA was subjected to ethanol precipitation, air-dried, reconstituted in sterile water and stored at 4 °C. The DNA was then used in the PCR assay to detect the *rstR* alleles (Bhattacharya *et al.*, 2006). Detection of 474 or 501 bp amplicons corresponded to the presence of *rstR* alleles of classical (*rstR*^{Cl}) or El Tor (*rstR*^{El}) types, respectively. Details of the PCR primers used in this study are presented in Table 1. In the multiplex assay, toxigenic traits and the presence of the biotype-specific *tcpA* allele among these *V. cholerae* strains were determined (Keasler & Hall, 1993). PCR amplicon of 301 bp corresponded to the presence of *ctxA*, while 472 bp or 618 bp amplicons represented the presence of the *tcpA* allele of El Tor or classical biotypes, respectively. Presence or absence of the RS1 element among these strains was determined using a combination of PCR primers Ig1F and RstCR. Detection of an amplicon of 2270 bp indicated the presence of RS1 among these strains. A simplex PCR assay using the primers Tlc-F and Tlc-R generated a 2032 bp amplicon for the pTLC positive strains. Amplification of the CII region (small chromosome) that spans either side of the CTX prophage when localized in the small chromosome was also carried out (Das *et al.*, 2007; Maiti *et al.*, 2006). For this, combinations of PCR primers CII-F/RstA-3R and CtxAF/CII-R were used. Localization of the CTX prophage in the small chromosome results in 1.8 and 1.4 kb amplicons in the PCR assays with CII-F/RstA-3R and CtxAF/CII-R primers, respectively. Detection of a 760 bp amplicon in the PCR assays with CII-F/CII-R was interpreted as detection of empty site in the small chromosome (Das *et al.*, 2007). PCR amplicons obtained for the alleles of the gene loci *rstR* and *tcpA* were sequenced directly using a BigDye terminator cycle sequencing kit (Applied Biosystems) and alignment analysis made with the respective nucleotide sequences available in a public database (www.ncbi.nlm.nih.gov) to confirm their identity as respective allele. The *ctxB* gene of O1 *V. cholerae* strains belonging to classical and El Tor biotypes differs at nucleotide positions 115 and 203, where the first base of the start codon is considered as nucleotide 1 (Popovic *et al.*, 1994). An amplicon of 465 bp generated through PCR assay using primers CtxBF/CtxBR (Olsvik *et al.*, 1993) was therefore sequenced directly to determine the nature of *ctxB* present among these strains.

Table 1. List of PCR primers and amplicon sizes of target genes

rstR, *igl*, *rstC*, *ctxA* and *ctxB* are the genes in the CTX prophage; *tcpA* represents a gene in the *V. cholerae* pathogenicity island.

Target gene/region	Primer ID	Sequence (5'–3')	Amplicon size (bp)	Reference
<i>rstR</i> ^{Cl}	RstR-1F	CTTCTCATCAGCAAAGCCTCCATC	474	Bhattacharya <i>et al.</i> (2006)
	RstA-3R	TCGAGTTGTAATTCATCAAGAGTG		
<i>rstR</i> ^{El}	RstR-2F	GCACCATGATTTAAGATGCTC	501	Bhattacharya <i>et al.</i> (2006)
	RstA-3R	TCGAGTTGTAATTCATCAAGAGTG		
<i>Igl</i> to <i>rstC</i>	Ig1-F	GAGCCTGTGACACTCACCTTGAT	2249	Bhattacharya <i>et al.</i> (2006)
	RstC-R	GCTCAGTCAATGCCTTGAGTTG		
<i>ctxA</i>	CtxA-F	CTCAGACGGGATTGTAGGCACG	301	Keasler & Hall (1993)
	CtxA-R	TCTATCTCTGTAGCCCCTATTACG		
<i>tcpA</i> (El)	TcpA(El)-F	GAAGAAGTTTGTAAGAAGAAGAACAC	472	Keasler & Hall (1993)
	TcpA(El)-R	GAAAGCACCTTCTTTCACGTTG		
<i>tcpA</i> (Cl)	TcpA(Cl)-F	CACGATAAGAAAACCGGTCAAGAG	618	Keasler & Hall (1993)
	TcpA(Cl)-R	ACCAAATGCAACGCCGAATGGAGC		
IS1004	IS1004-F	ATTGTCATCCCTAAACCACC	603	This study
	IS1004-R	AGGCGGTTTTAATATAAGCC		
pTLC	Tlc-F	GATTGTGCGTCTTGCAATTTAGG	2011	Maiti <i>et al.</i> (2006)
	Tlc-R	GTGAATAAATCAGGTGTAATGTGCG		
CII	CII-F	CTCACGCTGAACAGCAAGTC	747	Maiti <i>et al.</i> (2006)
	CII-R	TTGCTTGAATCGAAAGGACA		
<i>ctxB</i>	CtxB-F	GGTTGCTTCTCATCATCGAACCAC	465	Olsvik <i>et al.</i> (1993)
	CtxB-R	GATACACATAATAGAATTAAGGAT		

Cl, Classical allele; El, El Tor allele.

DNA probes and molecular typing. A PCR amplicon of 603 bp generated with IS1004F/IS1004R primers from O1 strain O395 was cloned into pGEM-T EASY vector (Promega) to generate pKG506. Similarly, a 2032 bp amplicon of pTLC generated from the O1 strain O395 was cloned into pCR2.1 (Invitrogen) to generate pSC20. The identity of the cloned fragments compared to IS1004 and pTLC in pKG506 and pSC20, respectively, was confirmed by nucleotide sequencing using M13 universal primers (Takara Shuzo). The *EcoRI* fragments of pKG506 and pSC20 served as specific DNA probes for IS1004 and pTLC, respectively. The 7.5 kb *BamHI* fragment of pKK3535 served as a specific DNA probe for rDNA and was used in ribotype analysis (Brosius *et al.*, 1981). The IS1004 typing and ribotype analysis was carried out with *BglII*-digested genomic DNA. The digested DNA was electrophoresed in agarose gels, transferred to nylon membrane (Hybond-N⁺; Amersham Pharmacia Biotech) and allowed to hybridize with a specific DNA probe. The hybridized membrane was developed using a non-radioactive labelling kit using the substrate CDP-Star following the procedures described by the manufacturer (Amersham Pharmacia Biotech). Results were recorded on X-ray film, which was subsequently processed digitally and analysed using a Bio-Rad documentation system. Dot-blot analysis was carried out to determine the presence or absence of pTLC among these strains. For this, 2 µl purified genomic DNA (50 ng µl⁻¹) was denatured and spotted onto nylon membrane (Hybond-N⁺) to form an array. The DNA was immobilized on the membrane and allowed to hybridize with labelled DNA probe specific to pTLC. The membrane was developed, and results were recorded on X-ray film and digitalized with the Bio-Rad documentation system.

PFGE. Agarose-embedded genomic DNA from *V. cholerae* strains was treated with 50 U *NotI* (Takara) and the DNA fragments were separated electrophoretically under the contour-clamped homogeneous electric field (CHEF) method on a CHEF Mapper system (Bio-Rad) (Chatterjee *et al.*, 2007). Run conditions were generated by the

autoalgorithm mode of the CHEF Mapper PFGE system using a size range of 20 to 300 kb that was typically followed an electrophoresis time of 40 h 24 min with 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. Following electrophoresis, the gels were stained for 30 min in Elix MilliQ water (Millipore) containing 1.0 µg ethidium bromide ml⁻¹, destained in Elix MilliQ water for 15 min and photographed under UV light using the Gel Doc 2000 gel documentation system (Bio-Rad).

RESULTS AND DISCUSSION

Classical CTX prophage in the 1992 Kolkata O1 strains VC51 and VC53

V. cholerae O1 strains that were isolated in 1992 from clinical cases and archived in the repository maintained by NICED were revived and the identity of these strains was reconfirmed by conventional microbiological techniques: serogroup analysis through slide agglutination tests using antisera specific to the O1 serogroup followed by serotyping with antisera specific to Ogawa and Inaba serotypes (Denka Seiken). An analysis was carried out to detect strains with genotypic traits resembling classical biotype of *V. cholerae* O1. These traits included the presence of biotype-specific alleles of CTX prophages and the *tcpA* gene that encodes a major subunit protein of toxin co-regulated pilus (TCP) (Davis *et al.*, 1999; Keasler & Hall, 1993). Among the constituent genes of the CTX prophages, heterogeneity at the nucleotide level of *rstR*, the phage

repressor located on the RS2 region of CTX prophage, formed the basis of differentiation between CTX prophages of classical (CTX^{Cl}) and El Tor (CTX^{El}) types (Davis *et al.*, 1999; Kimsey & Waldor, 1998). A PCR-based assay has been developed by exploiting the variations that exist between the *rstR* loci of the CTX^{Cl} and CTX^{El} phages (Bhattacharya *et al.*, 2006). PCR-based screening was carried out using allele-specific *rstR* against clinical *V. cholerae* strains isolated in 1992 in Kolkata. Analysis revealed only two strains, VC51 and VC53, that produced the 474 bp amplicon specific to *rstR*^{Cl} but failed to generate the 501 bp amplicon specific for *rstR*^{El} (Fig. 1a, b; lanes 1, 2). Excepting these two strains, none of the remaining strains (*n*=13) produced only *rstR*^{Cl} amplicon. However, six strains gave amplicons specific to both *rstR*^{Cl} and *rstR*^{El} along with the O1 strain VC20. Results generated with representative strains are presented in Fig. 1. The 1992 O1 strains VC49 (Fig. 1, lane 3) and VC20 (Fig. 1, lane 6) possessed both alleles of *rstR* as they produced amplicons of 474 bp and 501 bp when assayed under identical conditions. The reference O1 variant strain isolated from the Mozambique outbreak (B33) produced only *rstR*^{Cl} (Fig. 1; lane 5). The reference strains of O1 El Tor (N16961) (Fig. 1, lane 7) and classical (O395, 569B) (Fig. 1, lanes 8, 9) biotypes gave *rstR*-specific amplicons for the

respective biotypes. The *rstR*-based screening was also carried out with clinical *V. cholerae* strains that were isolated between 1989–1991 and 1993–1994, at least 10 strains were randomly selected for each of the 5 years. However, none of these strains produced only an *rstR*^{Cl}-specific amplicon (data not shown). Excepting the strains VC51 and VC53, all other strains showed either both types of *rstR* alleles (*rstR*^{Cl} and *rstR*^{El}) or only the El Tor allele (*rstR*^{El}). These data indicated that the 1992 Kolkata O1 strains VC51 and VC53 possessed only classical CTX prophage. The results were extended further by determining the nucleotide sequence of the *rstR*. A PCR amplicon spanning the entire *rstR* region was generated from 1992 Kolkata O1 strains VC51 and VC53, and sequenced directly. Nucleotide sequence analysis revealed complete identity of the *rstR* gene to that of the classical allele (GenBank accession no. AF055890) and to the Mozambique variant strain B33 as well. These data corroborated well with the PCR-based detection of *rstR*^{Cl} alone (Fig. 1a). The presence of classical CTX prophage in the 1992 Kolkata O1 strains (VC51 and VC53) was further confirmed by the analysis of *ctxB* locus. Alignment analysis of the *ctxB* nucleotide sequences derived from the 1992 Kolkata O1 strains revealed complete identity among themselves and to the *ctxB* gene of strain B33 (GenBank accession no. EF158842), as well as the reported sequence of a classical O1 strain 569B (GenBank accession no. U25679).

Fig. 1. Agarose gel electrophoresis patterns of PCR amplicons obtained with primers specific to *rstR* of classical (*rstR*^{Cl}) (a) and El Tor (*rstR*^{El}) (b) types with *V. cholerae* strains. The *V. cholerae* strains included were isolated in Kolkata during 1992 (lanes: 1, VC51; 2, VC53; 3, VC49; 4, VC61; 6, VC20). Reference *V. cholerae* O1 strains biotype El Tor (lane 7, N16961) and classical (lanes 8, O395; lane 9, 569B) and 2004 Mozambique O1 variant (lane 5, B33) were included as controls. Amplicon sizes of 474 and 501 bp specific for *rstR*^{Cl} and *rstR*^{El}, respectively, are indicated. The band positions of the 100 bp ladder (Takara) run on the same gel are also indicated.

Small chromosomal localization of the classical CTX prophage in the 1992 Kolkata O1 strains (VC51 and VC53) that resembled the Mozambique variant

The Mozambique variant O1 strains were reported as unique in that the CTX prophage genome was classical while the background of the strain remained typical El Tor (Ansaruzzaman *et al.*, 2007; Das *et al.*, 2007; Faruque *et al.*, 2007). The filamentous phage was reported to be present in the small chromosome, a trait similar to that of the classical biotype. Detection of classical CTX prophage in the 1992 Kolkata O1 strains prompted us to characterize its chromosomal localization. For this, PCR-based analysis was carried out using primers specific to the region (CII) that spans either side of the CTX prophage localized in the small chromosome (Maiti *et al.*, 2006). The PCR assay was carried out with primer combination of CII-F/RstA-3R and CtxAF/CII-R (Maiti *et al.*, 2006). Data on representative strains are presented in Fig. 2. It is evident from Fig. 2 that 1992 Kolkata O1 strains (VC51, VC53) (lanes 1, 2) as well as the Mozambique O1 variant strain B33 (lane 5), produced amplicons of 1.8 and 1.4 kb in size for CII-F/RstA-3R and CtxAF/CII-R PCR assays, respectively. Identical amplicons were also obtained with reference O1 classical strains O395 and 569B (lanes 8, 9). The reference O1 El Tor strain N16961 (lane 7) failed to generate any band due to the absence of the CTX prophage in the small chromosome. The presence of an empty CTX prophage site in the small chromosome of N16961 was further confirmed by the detection of a 747 bp amplicon generated with

Fig. 2. Agarose gel electrophoresis patterns of PCR amplicons obtained with primers CII-F/RstA-3R (a), CtxA-F/CII-R (b) and CII-F/CII-R (c) with *V. cholerae* strains. The *V. cholerae* strains included were isolated in Kolkata during 1992 (lanes: 1, VC51; 2, VC53; 3, VC49; 4, VC61; 6, VC20). Reference *V. cholerae* O1 strains biotype El Tor (lane 7, N16961) and classical (lanes 8, O395; 9, 569B) and 2004 Mozambique O1 variant (lane 5, B33) were included as controls. Amplicon sizes of 1.8 kb, 1.4 kb and 747 bp specific for CII-F/RstA-3R, CtxA-F/CII-R and CII-F/CII-R, respectively, are indicated. The band positions of the 1 kb (a, b) and 100 bp (c) ladders (Takara) run on the same gel are also indicated.

CII-F/CII-R primers (Fig. 3c, lane 7). The 1992 O1 strains VC51 and VC53, along with B33 and classical strains (O395, 569B), failed to give any band in the PCR assay with CII-F/CII-R due to the presence of CTX prophage in this region. Detection of classical CTX prophage and its localization in the small chromosome of 1992 O1 strains is a testimony of the close similarity between the 1992 Kolkata O1 strains (VC51 and VC53) to that of the Mozambique O1 variant.

El Tor biotype traits of Kolkata O1 strains and genotypic identity to Mozambique O1 variant

The 1992 Kolkata O1 strains VC51 and VC53 displayed typical identity to the Mozambique variant O1 strain B33 as these strains possessed classical CTX prophage localized

Fig. 3. Agarose gel electrophoresis patterns of PCR amplicons obtained with primers Ig1-F/RstC-R (a) and pTLC (b) with *V. cholerae* strains. The *V. cholerae* strains included were isolated in Kolkata during 1992 (lanes: 1, VC51; 2, VC53; 3, VC49; 4, VC61; 6, VC20). Reference *V. cholerae* O1 strains biotype El Tor (lane 7, N16961) and classical (lanes: 8, O395; 9, 569B) and 2004 Mozambique O1 variant (lane 5, B33) were included as controls. Amplicon sizes of 2249 bp and 2011 bp specific for the region spanning *ig1F* to *rstC* and pTLC, respectively, are indicated. The band positions of the 1 kb ladder (Takara) run on the same gel are also indicated.

in the small chromosome, a trait similar to the classical biotype but not the El Tor biotype. Therefore, detailed analysis was carried out to help elucidate the biotype background of the 1992 Kolkata O1 strains VC51 and VC53. Analysis revealed that these strains were resistant against 50 U polymyxin B, a stable phenotype typically associated with the El Tor biotype. Although the 1992 O1 strains (VC51, VC53) displayed the classical trait of the presence of the classical CTX prophage (*rstR^{Cl}*, *ctxB^{Cl}*), the background of these strains was considered as El Tor on the basis of their polymyxin B resistance phenotype. The lineage of the El Tor biotype for these strains was further confirmed through positive reactions to the Voges-Proskauer test and their sensitivity to El Tor group V vibrio phage (Table 2). The strain B33 displayed an identical phenotype to that of these two 1992 strains (Table 2). The analysis was extended further to reveal presence or absence of genes that were reported to act as markers for the detection/ differentiation of Matlab and Mozambique variants of *V. cholerae* O1 (Ansaruzzaman *et al.*, 2007; Safa *et al.*, 2006). Multiplex-PCR-based assay revealed that two 1992 strains (VC51 and VC53) possessed both *ctxA* and *tcpA* (Table 2). The *tcpA* of these two strains was El Tor type, a phenotype common for 2004 Mozambique variant but not for the polymyxin B-resistant Matlab hybrid that was identified in Bangladesh

Table 2. Phenotypic and genotypic traits of *V. cholerae* O1 strains included in this study

Strain	Place of isolation	Year	Phenotype			Genotype					Reference		
			Polymyxin B resistance	Voges-Proskauer reaction	Lysis by classical group IV phage	Lysis by Tor group V phage	RS1 *	rstR type*	ctxA *	ctxB type†		tcpA *	pTLC‡
VC20	India	1992	+	+	—	+	+	Cl, El	+	Cl	El	+	Bhattacharya <i>et al.</i> (2006)
VC49	India	1992	+	+	—	+	+	Cl, El	+	El	El	+	This study
VC51	India	1992	+	+	—	+	—	Cl	+	Cl	El	—	This study
VC53	India	1992	+	+	—	+	+	Cl	+	Cl	El	—	This study
VC61	India	1992	+	+	—	+	+	El	—	—	El	+	This study
B33	Mozambique	2004	+	+	—	+	+	Cl	+	Cl	El	—	Safa <i>et al.</i> (2006)
N16961	Bangladesh	1971	+	+	—	+	+	El	+	El	El	+	Safa <i>et al.</i> (2006)
O395	India	1964	—	—	+	—	—	Cl	+	Cl	Cl	+	Trucksis <i>et al.</i> (1998)
569B	India	1948	—	—	+	—	—	Cl	+	Cl	Cl	+	Safa <i>et al.</i> (2006)

Cl, Classical allele; El, El Tor allele; +, assay positive; -, assay negative.

*Assayed by PCR.

†Confirmed by nucleotide sequencing analysis.

‡Assayed by dot-blot as well as by PCR.

(Ansaruzzaman *et al.*, 2007; Nair *et al.*, 2002). Considering the striking similarity to that of the Mozambique variant O1 strain, these 1992 strains (VC51 and VC53) were analysed for the detection of RS1 and pTLC. Detection of RS1 was carried out by PCR assay using the primers Ig1F and RstCR (Table 1). The reference O1 El Tor strain N16961 gave an amplicon of ~2.2 kb in size (Fig. 3a, lane 7), while the two 1992 O1 strains (VC51 and VC53) failed to generate any amplicons (Fig. 3a, lanes 1, 2). The PCR negativity was also evident against Mozambique variant strain B33 and reference O1 classical strains 569B and O395 (Fig. 3a, lanes 5, 8, 9). Excepting the strains VC51 and VC53, other strains that were also isolated in 1992 (VC49, VC61 and VC20) gave a positive amplicon of ~2.2 kb under identical conditions and thereby confirmed the presence of RS1 among these strains (Fig. 3a, lanes 3, 4, 6). 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 existence of free RS1 in *V. cholerae* is not rare. Interestingly, the O1 classical strains are known to be devoid of RS1 (Davis *et al.*, 2000). The absence of RS1 among the 1992 Kolkata O1 strains (VC51 and VC53) pointed towards the existence of a genotype known for the classical biotype despite the fact that these strains displayed certain stable El Tor phenotypes. Similar phenotypes were reported for the 2004 Mozambique variant O1 strains B33 (Faruque *et al.*, 2007). The identity at the genetic level between the 1992 Kolkata variant O1 strains (VC51 and VC53) and 2004 Mozambique variant O1 strain B33 was further confirmed through PCR negativity with primers specific to pTLC (Fig. 3b, lanes 1, 2, 5) and the data were further substantiated through dot-blot analysis with a DNA probe specific to pTLC. No recognition by the pTLC probe was evident against 1992 Kolkata strains (VC51 and VC53) or B33. Excepting these three, reference *V. cholerae* O1 El Tor and classical biotypes strains gave the expected amplicon and were recognized by the pTLC probe. Therefore, the strains VC51 and VC53 displayed traits that are known to be unique for Mozambique variant O1 strain B33.

Macro restriction analysis of Kolkata and Mozambique variant strains

DNA fingerprinting analysis has been widely used to demonstrate clonal relationships among bacterial strains. The RFLP associated with the presence of insertion (IS) elements has been used successfully for the differentiation of the strains of a variety of bacterial species. An IS element, *IS1004*, was identified among *V. cholerae* strains of O1 and O139 origin (Bik *et al.*, 1996) and it was successfully used to demonstrate that O139 *V. cholerae* evolved from O1 El Tor strains. Southern hybridization analysis, carried out with *Bgl*I-digested genomic DNA and DNA probes specific to *IS1004*, revealed identical patterns among the El Tor background strains that diverged from classical strain. Representative profiles generated with *V. cholerae* strains are presented in Fig. 4(a). It is evident that

Fig. 4. The *Bgl*I IS1004 typing (a) and ribotype (b) patterns obtained for *V. cholerae* strains. The *V. cholerae* O1 strains included were classical 569B (lane 1), 1992 Kolkata variant VC51 (lane 2), 2004 Mozambique variant B33 (lane 3), El Tor reference strain N16961 (lane 4) and 2003 Kolkata strain CO366 (lane 5). The band positions of the λ -HindIII markers run on the same gel are also indicated.

1992 Kolkata O1 strains (lanes 2) displayed a profile identical to that of the reference O1 El Tor strain N16961 (lane 4) and Mozambique variant O1 strain B33 (lane 3). However, the profile obtained with classical strain 569B (lane 1) was very different. These data confirmed the lineage of the 1992 Kolkata O1 strains to be that of the El Tor biotype at the genetic level. The analysis was extended further by generating ribotype profiles for these strains, a technique that has been used successfully to demonstrate the emergence and progression of different clones of *V. cholerae* strains that have been circulating in the Indian subcontinent over a period of time. It has already been reported that *V. cholerae* O1 strains that were isolated before the O139 outbreak in late 1992 had a typical *Bgl*I ribotype profile named RI (Sharma *et al.*, 1997). The RII and RIII *Bgl*I ribotype profiles were assigned to *V. cholerae* O1 strains isolated during and after the O139 outbreaks. As expected, the 1992 Kolkata O1 strains displayed a profile identical to that of RI, which was identical to that of the 2004 Mozambique variant O1 strain B33 (Fig. 4b, lanes 2, 3) but differed from that of the classical strain 569B (Fig. 4b, lane 1). The RI profile obtained with the 1992 Kolkata O1 strain had diverged from the profile obtained with O1 El Tor strains N16961 as well as reference O1 strain for RIII profile CO366 (Sharma *et al.*, 1997). Apart from ribotyping, PFGE analysis is also a very useful technique to illustrate the clonal relatedness among

different strains of the same species. *Not*I pulsotype patterns were reported to have the most discriminatory power when applied to *V. cholerae* strains. The *Not*I pulsotype patterns of the 1992 Kolkata O1 strains (VC51 and VC53) with hybrid traits of classical and El Tor biotypes were generated and compared to the pattern of B33. It is evident that the two O1 strains of Kolkata differed from each other by two bands of sizes ~ 80 and ~ 100 kb (Fig. 5, lanes 2, 3). Though both the strains were isolated in Kolkata during 1992, variations in the profile indicated that these two strains did not belong to a single clone but belonged to closely related clones. Comparative analysis also showed variations with 2004 Mozambique variant O1 strains. The 1992 Kolkata variant O1 strains (VC51 and VC53), and the 2004 Mozambique variant O1 strain B33, displayed almost identical phenotypes and genotypes excepting pulsotype profile. Considering chronological events, we hypothesized that the 1992 Kolkata O1 variant strains might have acted as precursors to the 2004 Mozambique variant O1 strains. Considering the 12 year span between the isolation of Kolkata and Mozambique variant strains, and their different geographical locations, it is quite possible that strains had undergone changes at the genetic level that are only evident through PFGE analysis and not with other molecular tools.

Fig. 5. PFGE profiles generated with *Not*I-digested genomic DNA obtained from *V. cholerae* O1 strains. The *V. cholerae* O1 strains included were classical 569B (lane 1), 1992 Kolkata variant VC51 and VC53 (lanes 2, 3), 2004 Mozambique variant B33 (lane 4), El Tor reference strain N16961 (lane 5) and 2003 Kolkata strain CO366 (lane 6). The band positions of the PFGE λ ladder are indicated.

Conclusion

Detailed analysis of 1992 *V. cholerae* variant O1 strains VC51 and VC53 revealed almost complete identity to the 2004 Mozambique variant O1 strains and is a testimony that the variant strains existed in Kolkata during 1992. Considering the time span between the isolation periods of Kolkata variant strains to that of the Mozambique variant strains, there is an immense possibility that Mozambique variants acquired certain genetic alterations, as evident in the PFGE.

The overall analysis indicated that 1992 Kolkata variant O1 strains VC51 and VC53 were built on the El Tor-like backbone but typically acquired the classical CTX prophage. Such genetic reassortment between the classical and El Tor traits represents genetic variations. It is well recognized that genetic replacement between divergent bacterial lineages can provide certain strains with an increased fitness in the environment or in the host. It is known that the El Tor biotype is more infective and sustainable but less virulent than the classical biotypes (Kaper *et al.*, 1995). Therefore, the El Tor *V. cholerae* that produces the classical cholera toxin appears to be an altered strain that emerged causing a more severe disease. The year 1992 is a landmark in the history of cholera as O139 vibrios emerged and subsequently caused large epidemics. Since the emergence of O139 strains in late 1992, isolation of O1 El Tor vibrios from cholera cases has been virtually non-existent. However, a resurgence of O1 vibrios occurred within a span of a year and both serogroups co-existed for a long period of time. Interestingly, the resurgent O1 vibrios in the post-O139 era were reported to belong to newer clones as compared to the pre-O139 era (Sharma *et al.*, 1997). To date, the factors that played a crucial role in the disappearance of pre-O139 O1 clones are incomprehensible. Retrospective analysis of the O1 strains indicated that biotype variants of *V. cholerae* O1 existed in Kolkata during 1992. Most probably the variant disappeared with the epidemic caused by the O139 vibrios. Our analysis of 1992 Kolkata variant O1 strains indicated that they were progenitor strains of the 2004 Mozambique variant O1 strains. Therefore, we presume that the 2004 Mozambique variant O1 strains of *V. cholerae* are a case of resurgence of certain clones of the O1 strains that were isolated in Kolkata during 1992.

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