

Genetic characteristics of drug-resistant *Vibrio cholerae* O1 causing endemic cholera in Dhaka, 2006–2011

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Vibrio cholerae O1 biotype El Tor (ET), causing the seventh cholera pandemic, was recently replaced in Bangladesh by an altered ET possessing *ctxB* of the Classical (CL) biotype, which caused the first six cholera pandemics. In the present study, *V. cholerae* O1 strains associated with endemic cholera in Dhaka between 2006 and 2011 were analysed for major phenotypic and genetic characteristics. Of 54 representative *V. cholerae* isolates tested, all were phenotypically ET and showed uniform resistance to trimethoprim/sulfamethoxazole (SXT) and furazolidone (FR). Resistance to tetracycline (TE) and erythromycin (E) showed temporal fluctuation, varying from year to year, while all isolates were susceptible to gentamicin (CN) and ciprofloxacin (CIP). Year-wise data revealed erythromycin resistance to be 33.3% in 2006 and 11% in 2011, while tetracycline resistance accounted for 33, 78, 0, 100 and 27% in 2006, 2007, 2008, 2009 and 2010, respectively; interestingly, all isolates tested were sensitive to TE in 2011, as observed in 2008. All *V. cholerae* isolates tested possessed genetic elements such as SXT, *ctxAB*, *tcpA*^{ET}, *rstR*^{ET} and *rtxC*; none had *IntI* (Integron I). Double mismatch amplification mutation assay (DMAMA)-PCR followed by DNA sequencing and analysis of the *ctxB* gene revealed a point mutation at position 58 (C→A), which has resulted in an amino acid substitution from histidine (H) to asparagine (N) at position 20 (genotype 7) since 2008. Although the multi-resistant strains having tetracycline resistance showed minor genetic divergence, *V. cholerae* strains were clonal, as determined by a PFGE (*NotI*)-based dendrogram. This study shows 2008–2010 to be the time of transition from *ctxB* genotype 1 to genotype 7 in *V. cholerae* ET causing endemic cholera in Dhaka, Bangladesh.

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INTRODUCTION

Cholera is a life-threatening form of dehydrating diarrhoeal disease caused by the toxigenic serogroup strains of *Vibrio cholerae*. Of more than 200 O serogroups of *V. cholerae*, epidemics of cholera were caused by *V. cholerae* serogroup O1 until 1992. *V. cholerae* O1 has two biological variants or

designated biotypes, namely Classical (CL) and El Tor (ET), which differ from each other in both phenotypic and genetic characteristics, as well as by the type of cholera toxin (CT) that they harbour (Dziejman *et al.*, 2002; Kaper *et al.*, 1995; Olsvik *et al.*, 1993). In addition, the two biotypes differ in terms of infection patterns of disease; for example, the CL biotype strains cause more severe disease, while ET strains are more efficient in host-to-host transmission than their CL counterparts. The CL biotype is believed to have caused the first six cholera pandemics, which began in the Indian subcontinent, and subsequently appeared in other areas of the world between 1817 and 1923 (Poltzner, 1959). *V. cholerae* O1 biotype ET, which was first reported in 1905 (Poltzner, 1959) initiated the

Abbreviations: AMP, ampicillin; CCA, chicken cell agglutination; CIP, ciprofloxacin; CL, Classical; CN, gentamicin; CT, cholera toxin; E, erythromycin; ET, El Tor; FR, furazolidone; icddr, International Centre for Diarrhoeal Disease Research, Bangladesh; KN, kanamycin; MDR, multidrug resistant; NOR, norfloxacin; RTX, repeat in toxin; SM, streptomycin; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline; UPGMA, unweighted-pair group method.

seventh cholera pandemic in the early 1960s by displacing the CL biotype (Kaper *et al.*, 1995). In 1992, a *V. cholerae* non-O1 serovar, designated *V. cholerae* O139 synonym Bengal, appeared as the cause of epidemic cholera in Bangladesh (Cholera Working Group, 1993) and India (Ramamurthy *et al.*, 1993). *V. cholerae* O139 Bengal emerged by displacing *V. cholerae* O1 ET, an occurrence that was considered to be significant in the history of cholera. *V. cholerae* O1 ET is still the major pathogen of cholera, although O139 Bengal continues to co-exist by sharing a niche with O1 ET in the estuarine ecosystem of the Bay of Bengal (Alam *et al.*, 2006).

In spite of significant advances in our understanding of diarrhoeal diseases, including *V. cholerae* pathogenesis, endemic cholera still kills many people in the Ganges delta of the Bay of Bengal. In Bangladesh, the pattern of cholera disease shows two seasonal peaks, the first during spring (March–May), and the second in autumn (September–November) (Alam *et al.*, 2006; Sack *et al.*, 2004). The treatment of cholera, which includes appropriate oral or intravenous rehydration therapy together with a 3-day course of effective antibiotics, can significantly shorten the duration of diarrhoea (Sack *et al.*, 2004), disease severity and hospitalization (Lindenbaum *et al.*, 1967). However, in recent years antibiotic therapy has faced difficulties, with the rapid emergence of multiple antibiotic-resistant strains of *V. cholerae* being reported in Africa, Asia and America (Jesudason & John, 1990; Maimone *et al.*, 1986). During the past two decades, several cholera-endemic countries including India and Bangladesh have reported *V. cholerae* serogroup O1 resistance to tetracycline (TE), ampicillin (AMP), kanamycin (KN), sulfonamides, streptomycin (SM), trimethoprim/sulfamethoxazole (SXT), norfloxacin (NOR), gentamicin (CN), furazolidone (FR), ciprofloxacin (CIP) and erythromycin (E) (Faruque *et al.*, 2007; Sack *et al.*, 2004; Jain *et al.*, 2011). The increasing trend of multi-drug resistance of *V. cholerae* associated with severe disease is becoming a serious public health concern globally (Jain *et al.*, 2011; Kumar *et al.*, 2010; Quilici *et al.*, 2010).

In Asia and Africa, *V. cholerae* O1 biotype ET, which is the cause of the current seventh pandemic, has recently been replaced by an altered ET possessing the CT of the CL biotype. Over the past few years, ET causing Asiatic cholera has shown remarkable changes in its phenotypic and genetic characteristics (Nair *et al.*, 2002). Recent molecular analysis of ET strains causing acute watery diarrhoea in Bangladesh shows them to be hybrids because they possess phenotypic and genotypic traits of the CL biotype against an ET background (Nair *et al.*, 2002). Subsequent retrospective studies showed that all of the O1 ET strains isolated in Bangladesh since 2001 were hybrids of both CL and ET biotypes, while those isolated before 2001 contained all the ET attributes of the seventh pandemic *V. cholerae* O1 (Nair *et al.*, 2006). *V. cholerae* hybrid ET continues to be routinely isolated from clinical cholera cases in Asia and Africa (Safa *et al.*, 2008), and has been reported to be a new pandemic pathogen capable of causing more severe disease (Siddique *et al.*, 2010), which

is spreading globally (Chin *et al.*, 2011). A recent study in India reported that a new CT variant of *V. cholerae* O1 ET with an amino acid substitution at position 20 caused a large cholera outbreak in Orissa, Eastern India (Kumar *et al.*, 2009). Subsequently, the same CT variant was found to have been associated with cholera in Kolkata, India, since 2006 (Naha *et al.*, 2012). Considering these recent changes, the current study was undertaken in order to understand the phenotypic and genetic traits of contemporary *V. cholerae* causing endemic cholera in Dhaka, Bangladesh.

METHODS

Bacterial strains. A set of 54 randomly selected strains isolated between 2006 and 2011, as part of the 2% surveillance of cholera patients seeking treatment at the hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), were included in the study. Rectal swabs were collected from suspected cholera patients and transported to the laboratory within 3 h using Cary Blair transport media. The isolation of *V. cholerae* began by enriching the rectal swab in alkaline peptone water (APW) (pH 8.4) at 37 °C for 4–6 h, and the resulting solution was cultured on selective media as described previously (Alam *et al.*, 2007). *V. cholerae* was identified and confirmed using standard cultural, biochemical and molecular methods (Alam *et al.*, 2007).

Serogrouping. The serogroups of the *V. cholerae* strains identified by biochemical and molecular methods were confirmed serologically by a slide agglutination test using specific polyvalent antisera for *V. cholerae* O1 and O139, followed by a monoclonal antibody that is serotype-specific (Alam *et al.*, 2007).

Biotyping. Biotyping involved a number of phenotypic tests: chicken erythrocyte agglutination (chicken cell agglutination; CCA), sensitivity to polymyxin B, and Mukerjee CL phage IV and Mukerjee ET phage V tests (Kaper *et al.*, 1995). To complement the biotype characterization by phenotypic traits, PCR assays were carried out using previously described procedures that were targeted to detect the *tcpA* allele (CL and ET) (Alam *et al.*, 2010), the type of the *rstR* gene encoding the phage transcriptional regulator (Kimsey *et al.*, 1998), and the *rtxC* gene of RTX (repeat in toxin) (Chow *et al.*, 2001).

Genomic DNA preparation. Genomic DNA extraction was carried out following previously described methods (Alam *et al.*, 2010).

Confirmation of serogrouping by PCR assay. All strains that were preliminarily identified as *V. cholerae* were reconfirmed using a *V. cholerae* species-specific *ompW* PCR. The serogroups of these strains were reconfirmed using polyvalent O1 and monovalent Inaba and Ogawa antisera, and by multiplex PCR targeted to identify genes encoding O1- (*wbe*) and O139- (*wbf*) specific O biosynthetic genes, as well as the CT gene (*ctxA*) (Hoshino *et al.*, 1998).

Antimicrobial susceptibility. Bacterial susceptibility to antimicrobial agents was determined by standard disc diffusion methods (Bauer *et al.*, 1966; CLSI, 2010) using commercially available discs (Oxoid International). A total of six antibiotics, E (15 µg), TE (30 µg), CN (10 µg), CIP (5 µg), SXT (30 µg) and FR (100 µg), were used.

PCR assay for the detection of SXT and class 1 integron. Using PCR assays, all antibiotic-resistant *V. cholerae* O1 strains were examined for the presence of the SXT element and class 1 integron. The detection of SXT and *intI1* was performed using primers and procedures described elsewhere (Thungapathra *et al.*, 2002).

Double mismatch amplification mutation assay (DMAMA)-PCR for determination of *ctxB* genotype. DMAMA-PCR was recently developed to discriminate the CL (*ctxB* genotype 1), ET (*ctxB* genotype 3) and Haitian types (*ctxB* genotype 7) of *ctxB* alleles by focusing on nucleotide positions 58 and 203 of the *ctxB* gene (Naha *et al.*, 2012). DMAMA-PCR was performed in this study to detect the *ctxB* genotype using the primers and conditions described elsewhere (Naha *et al.*, 2012). *V. cholerae* O1 strains O395 (CL), N16961 (ET) and 2010EL-1786 (Haiti variant, genotype 7) were used as control strains for DMAMA-PCR analysis.

Nucleotide sequence analysis of the *ctxB* gene. To complement the results of DMAMA-PCR, the *ctxB* gene of representative *V. cholerae* O1 strains was sequenced using primers and conditions as described elsewhere (Olsvik *et al.*, 1993). PCR amplification of the *ctxB* gene was performed in a 25 µl reaction mixture in an automated Peltier thermal cycler (PTC-200, MJ Research). Subsequently, PCR products were purified with a Microcon centrifugal filter device (Millipore Corporation) and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems) on an ABI PRISM 310 automated sequencer (Applied Biosystems). The sequences of the *ctxB* gene for other *V. cholerae* O1 ET and CL strains listed in Fig. 1 were retrieved from GenBank (accession nos NC_002505, U25679, EU496278). The deduced amino acid sequences of the *ctxB* gene from all strains were aligned using CLUSTAL W.

PFGE. The whole agarose-embedded genomic DNA for *V. cholerae* was prepared. PFGE was carried out with a contour-clamped homogeneous electrical field (CHEF-DR II) apparatus (Bio-Rad), according to procedures described elsewhere (Cooper *et al.*, 2006). The conditions used for separation were as follows: 2–10 s for 13 h, followed by 20–25 s for 6 h. An electrical field of 6 V cm⁻¹ was applied at an included field angle of 120°. Genomic DNAs of the test strains were digested by the *NotI* restriction enzyme (Gibco-BRL), and *Salmonella enterica* serovar Braenderup was digested by *XbaI*, with the fragments being used as molecular size markers. The restriction fragments were separated in 1% pulsed-field-certified

agarose in 0.5 × TBE (Tris/borate-EDTA) buffer. In the post-electrophoresis gel treatment step, the gel was stained and de-stained. The DNA was visualized using a UV transilluminator, and images were digitized by a 1D gel documentation system (Bio-Rad).

Image analysis. The fingerprint pattern in the gel was analysed using the software package Bionumeric (Applied Maths). After background subtraction and gel normalization, the fingerprint patterns were subjected to typing on the basis of banding similarity and dissimilarity by the Dice similarity coefficient and unweighted-pair group method (UPGMA), using average linkage clustering methods, as recommended by the manufacturer; these were graphically represented as dendrograms.

RESULTS

Microbiological and serological tests

All tested strains ($n=54$) produced characteristic colonies typical of *V. cholerae* when they were grown on the selective medium, taurocholate tellurite gelatin agar (TTGA). The characteristic colonies gave biochemical reactions typical of *V. cholerae*, and all strains reacted to polyvalent antibody specific for *V. cholerae* serogroup O1 followed by positive agglutination with monovalent Ogawa antisera, suggesting that all belonged to *V. cholerae* O1 of Ogawa serotype (Table 1).

Amplification of primers specific for *V. cholerae* serogroup O1 and *ctxA* by PCR assay

All 54 strains amplified the primers for the *V. cholerae* species-specific gene *ompW*, and O-antigen biosynthetic gene *wbeO1*, but failed to amplify the primers specific for the *wbfO139* gene. In addition, all of the strains serologically identified to be O1 amplified the primers for the CT gene *ctxA*, confirming that all tested *V. cholerae* O1 strains harboured the toxigenic CTX prophage in their genome (Table 1).

Phenotypic and related genetic characteristics

The phenotypic and related genetic characteristics of the *V. cholerae* serogroup O1 strains are presented in Table 1. All of the *V. cholerae* O1 strains were primarily identified as ET biotype based on specific phenotypic characteristics such as positive CCA, sensitivity to ET-specific phage V, and resistance to both polymyxin B (50 U) and CL-specific phage IV. All phenotypically confirmed ET strains amplified the primers for the *rtxC* gene, which is unique to the ET biotype, thereby confirming their ET traits. In addition, all ET strains amplified the primers for the *tcpA*^{ET} and *rstR*^{ET} genes, but not for the *tcpA*^{CL} and *rstR*^{CL} genes, further confirming that the strains belong to the ET biotype.

Antibiotic susceptibility testing

The response of *V. cholerae* O1 strains towards six different antibiotics revealed that multidrug resistant (MDR) strains

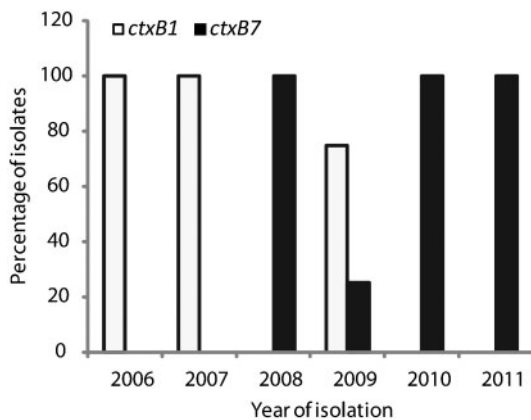


Fig. 1. Results of DMAMA-PCR performed to determine the *ctxB* genotype of tested *V. cholerae* O1 strains ($n=54$) from Dhaka, Bangladesh. All representative strains from 2006 and 2007 possessed *ctxB* genotype 1 only (*ctxB1*), while *ctxB* genotype 7 (*ctxB7*) was predominant during 2008, 2010 and 2011. Although *ctxB1* and *ctxB7* genotypes co-existed during 2009, none of the strains that were isolated during 2010 and thereafter possessed *ctxB1*, suggesting that the transition from genotype 1 to genotype 7 took place during 2008–2010.

Table 1. Phenotypic and genotypic traits of *V. cholerae* O1 ET causing endemic cholera in Dhaka, 2006–2011

Abbreviations: Clin, clinical; Poly B, polymyxin B; R, resistant; S, sensitive.

Year of isolation	No. of strains or accession no.	Source	Serotype	<i>wbeO1</i>	<i>ctxA</i>	Phenotypic trait				Genotypic trait				Antibiotic resistance profile	<i>sxt</i>	<i>intI1</i>
						Poly B (50 U)	CCA	Phage IV	Phage V	<i>tcpA</i> type	<i>rstR</i> type	RTX	<i>ctxB</i> genotype*			
2006	6	Clin†	Ogawa	+	+	R	S	ET	ET	ET	B1	SXT, FR	+	-		
	3	Clin	Ogawa	+	+	R	S	ET	ET	ET	B1	SXT, FR, TE, E	+	-		
2007	2	Clin	Ogawa	+	+	R	S	ET	ET	ET	B1	SXT, FR	+	-		
	7	Clin	Ogawa	+	+	R	S	ET	ET	ET	B1	SXT, FR, TE	+	-		
2008	8	Clin	Ogawa	+	+	R	S	ET	ET	ET	B7	SXT, FR	+	-		
2009	6	Clin	Ogawa	+	+	R	S	ET	ET	ET	B1	SXT, FR, TE	+	-		
	2	Clin	Ogawa	+	+	R	S	ET	ET	ET	B7	SXT, FR, TE	+	-		
2010	8	Clin	Ogawa	+	+	R	S	ET	ET	ET	B7	SXT, FR	+	-		
	3	Clin	Ogawa	+	+	R	S	ET	ET	ET	B7	SXT, FR, TE	+	-		
2011	8	Clin	Ogawa	+	+	R	S	ET	ET	ET	B7	SXT, FR	+	-		
	1	Clin	Ogawa	+	+	R	S	ET	ET	ET	B7	SXT, FR, E	+	-		
1971	N16961	Clin	Inaba	+	+	R	S	ET	ET	ET	B3	All sensitive	-	-		
1965	O395	Clin	Ogawa	+	+	S	R	CL	CL	CL	B1	All sensitive	-	-		

*Determined by DMAMA-PCR (Naha *et al.*, 2012).

having resistance to 3-4 antibiotics were circulating between 2006 and 2011 (Table 1). The drugs to which all of the 54 tested *V. cholerae* O1 strains showed resistance throughout the study period were SXT and FR, while resistance against TE and E was highly unstable, varying between years. All of the tested strains were uniformly susceptible to CN and CIP. Three of the nine *V. cholerae* O1 strains (33.3%) showing resistance to four drugs, namely SXT, FR, TE and E, were found in 2006 only. Year-wise data revealed that none of the tested strains showed resistance to E during 2007–2010, although one of nine (11%) *V. cholerae* strains was resistant to E in 2011. The year-wise data also revealed that TE resistance in MDR *V. cholerae* occurred in 33.3, 77.8, 0, 100 and 27.3% of the strains in 2006, 2007, 2008, 2009 and 2010, respectively, whereas all tested strains were sensitive to TE in 2011. The overall data suggest that continuous monitoring of the drug susceptibility of *V. cholerae* is important, considering that the response can change temporarily, making an effective drug ineffective.

PCR assay for detecting SXT and class 1 integron

All drug-resistant *V. cholerae* strains ($n=54$) supported the amplification of the primers for the SXT gene, a mobile genetic element carrying multidrug resistance gene cassettes. None of the strains in the present study except the positive control amplified the primers targeted to the genetic element *intI1*, confirming the absence of the class 1 integron among the tested *V. cholerae* O1 strains.

ctxB typing by DMAMA-PCR

All *V. cholerae* O1 strains ($n=54$), including the O395 (CL), N16961 (ET) and 2010EL-1786 (Haiti variant, *ctxB* genotype 7), were analysed by the DMAMA-PCR technique to determine the CTX-B genotype. As shown in Table 1, 24 *V. cholerae* O1 ET strains isolated between 2006 and 2009 amplified only the CL biotype-specific *ctxB* allele (*ctxB* genotype 1), confirming them to be the altered ET that replaced the prototype seventh pandemic ET (*ctxB* genotype 3) in Dhaka from 2001 onwards. The remaining ET strains isolated between 2008 and 2011 amplified the primers specific for *ctxB* genotype 7, which was reported recently from *V. cholerae* O1 strains in India and Haiti. Fig. 1 shows the time of transition from *ctxB* genotype 1 to genotype 7 that took place in Dhaka, Bangladesh. Although genotype 1 and genotype 7 coexisted in 2009, all *V. cholerae* O1 strains tested in 2010 and thereafter were of *ctxB* genotype 7 alone.

Sequencing of the *ctxB* gene

PCR-amplified *ctxB* genes (460 bp) of representative *V. cholerae* O1 strains ($n=12$) from each year between 2006 and 2011 were sequenced, and the amino acid sequences were determined by employing bioinformatics tools. As shown in Fig. 2, *V. cholerae* O1 isolated before 2008 showed an amino acid sequence identical to that of the CL biotype

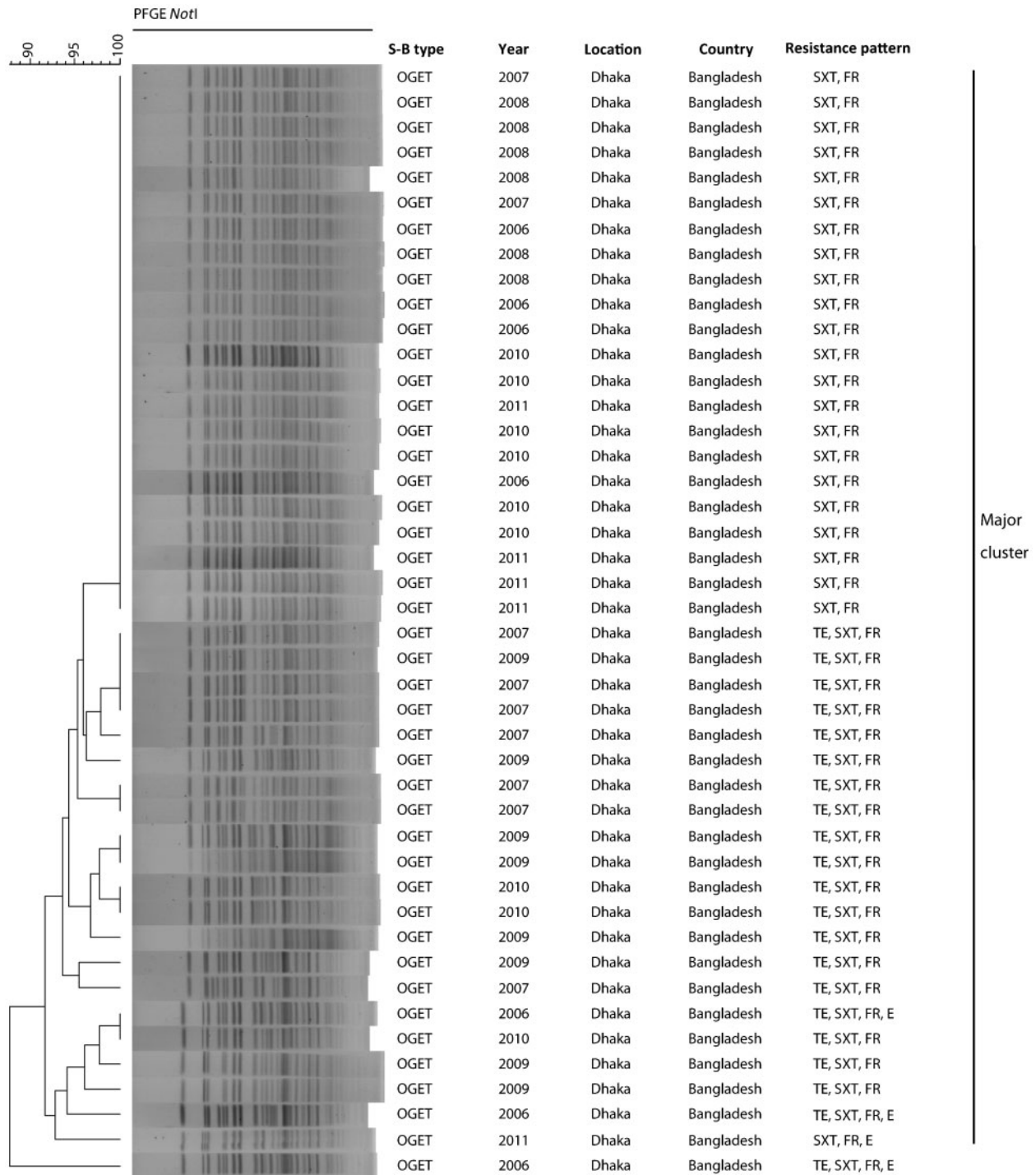


Fig. 3. Genomic fingerprinting of drug-resistant *V. cholerae* O1 ET strains isolated in Dhaka between 2006 and 2011. The dendrogram was constructed by Dice similarity coefficient and UPGMA clustering using PFGE images of *NotI*-digested genomic DNA. The scale bar at the top left indicates the correlation coefficient (range: 90–100%). The banding patterns and the similarity coefficient, which is within 90% for all *V. cholerae* strains included in the dendrogram, suggest that they all belong to the same clonal lineage. Cluster analysis shows that all *V. cholerae* strains resistant to SXT and FR, irrespective of their year of isolation, shared a major cluster with an identical banding pattern, while MDR strains having TE resistance showed genetic divergence in their patterns and formed several closely related subclusters.

resulting in a switch of the *ctxB* gene from genotype 1 (CL) to genotype 7 in eastern India in 2007 (Kumar *et al.*, 2009). Here we present data on the antibiotic resistance and related phenotypic and genetic characteristics of *V. cholerae* O1 associated with epidemic and endemic cholera in Dhaka, between 2006 and 2011, showing 2008–2010 to be the time of a genetic transition from *ctxB* genotype 1 to genotype 7. During this period, *V. cholerae* exhibited temporal variation in drug resistance in Dhaka, rendering *V. cholerae* strains sensitive to TE, which was the drug of choice for treatment of cholera in the 1960s (Greenough *et al.*, 1964).

The results of microbiological culture, biochemical and serological tests preliminarily confirmed that all the tested *V. cholerae* strains belonged to serogroup O1. These microbiological test results were complemented by simplex and multiplex PCR assays for *V. cholerae* species-specific gene *ompW*, the virulence-associated gene *ctxA* encoding the subunit A of the CT, and the *wbe* gene encoding a serogroup O1-specific marker (Hoshino *et al.*, 1998). All of these assays further confirmed the *V. cholerae* strains to be toxigenic and as belonging to serogroup O1 (Alam *et al.*, 2010). The presence of biotype-specific marker genes, such as *rtxC*, *tcpA^{ET}*, *rstR^{ET}* and *ctxB^{CL}*, confirmed that the *V. cholerae* O1 strains causing endemic cholera in Dhaka between 2006 and 2011 were ET but contained the *ctxB* marker gene of the CL biotype, as reported previously (Nair *et al.*, 2006; Alam *et al.*, 2010).

Ever since effective antimicrobial agents were employed as successful therapeutic options in the treatment of cholera (Greenough *et al.*, 1964; Lindenbaum *et al.*, 1967), TE was the drug of choice worldwide, except for young children and pregnant women (Greenough *et al.*, 1964; Sack *et al.*, 2004). Other effective drugs included FR, E, SXT and chloramphenicol (C) (Greenough *et al.*, 1964). Following a few years of successful use of these antimicrobial agents for the treatment of cholera (Lindenbaum *et al.*, 1967), a rapid emergence of *V. cholerae* strains resistant to antimicrobial agents was reported in Africa (Mhalu *et al.*, 1979). Likewise, in December 1979, *V. cholerae* O1 that was resistant to TE, AMP, KN, SM and SXT emerged as the causal agent of cholera in Bangladesh (Glass *et al.*, 1980). Subsequent studies reported *V. cholerae* resistant to nalidixic acid (NA), and most recently to E and CIP. Higher numbers of MIC and clinical failures forced clinicians to stop using CIP, and to start administering azithromycin (AZM) as the only effective drug against cholera at the Dhaka Hospital of icddr,b. Recent studies in southwestern India have shown *V. cholerae* to be resistant to several antibiotics, including TE, FR, NOR and CIP (Jain *et al.*, 2011). In the present study, *V. cholerae* strains isolated at the icddr,b Dhaka Hospital between 2006 and 2011 were found to be susceptible to CIP. Although all tested *V. cholerae* strains were resistant to SXT and FR, and the resistance to TE and E varied between 2006 and 2010, all of the tested strains were sensitive to TE in 2011, once again supporting its use as an effective drug for treating cholera, as reported many years ago (Greenough *et al.*, 1964).

After MDR *V. cholerae* first emerged (Mhalu *et al.*, 1979; Glass *et al.*, 1980, 1983), the antibiotic susceptibility patterns of epidemic strains have changed frequently. Likewise, the emergence of *V. cholerae* O1 or O139 having resistance towards different drugs has been reported in Bangladesh (Faruque *et al.*, 1998). The genetic basis for such fluctuation in drug resistance was shown to be due to lateral acquisition of the self-transmissible genetic element designated SXT, which carries multiple antibiotic resistance markers (Waldor, *et al.*, 1996). In the present study, a significant proportion of the tested *V. cholerae* strains associated with the cholera epidemic in Dhaka between 2006 and 2011 were found to be MDR. Although four different antibiotic resistance profiles were found, all of the tested *V. cholerae* strains had the SXT element, presumably in their genome, a fact that appears to be in line with their consistent resistance towards SXT and FR. Temporal variation in TE resistance was observed, as none of the tested strains isolated in the years 2008 and 2011 showed resistance to TE. TE resistance in *V. cholerae* is known to be plasmid-mediated, although plasmids have been shown to be highly unstable in vibrios (Taneja *et al.*, 2010). This could be a reason for the temporal fluctuation in TE resistance observed in the present study. Given that antibiotic susceptibility patterns of epidemic strains change frequently, continuous monitoring of *V. cholerae* drug resistance is crucial for choosing an effective drug for the treatment of cholera.

CT, encoded by the *ctxAB* genes, is the major virulence factor of *V. cholerae* that is responsible for causing severe cholera disease. Divergence within the *ctxB* gene and the corresponding amino acid sequence was first reported in the early 1990s. Based on amino acid substitutions at positions 39, 46 and 68, three different *ctxB* genotypes of *V. cholerae* O1 strains have been identified (Olsvik *et al.*, 1993). Genotype 1 is associated with strains of the CL biotype worldwide and along the USA gulf coast, genotype 2 is found in the pre-seventh pandemic ET biotype strains from Australia, and genotype 3 is found in ET biotype strains from the seventh pandemic and the Latin American epidemic (Olsvik *et al.*, 1993). However, subsequent investigation of the *ctxB* gene sequence revealed the presence of 11 distinct genotypes in different serogroups of *V. cholerae* (Marin & Vicente, 2012). Genotypes 1, 2, 3, 7, 10 and 11 were found in serogroup O1 strains, genotypes 3, 4, 5 and 6 were found in serogroup O139 strains, and genotypes 8 and 9 were found only in serogroups O27 and O37, respectively (Marin & Vicente, 2012). A significant and recent incident in the history of cholera has been the emergence of *V. cholerae* altered ET, which has the *ctxB* gene of *V. cholerae* CL biotype (Nair *et al.*, 2006), and which has been recognized as a new pandemic pathogen with the capacity to spread globally (Chin *et al.*, 2011; Nair *et al.*, 2002, 2006), causing more severe disease (Siddique *et al.*, 2010). According to a recent study, a new *ctxB* variant of *V. cholerae* with an amino acid substitution at position 20 [histidine (H)→asparagine (N)], designated genotype 7, was found to be associated with a large cholera

outbreak in India (Kumar *et al.*, 2009). Results from the present study confirm the emergence of genotype 7 in Dhaka, and 2008–2010 was the time of transition from *ctxB* genotype 1 to genotype 7. *V. cholerae* O1 ET possessing *ctxB* genotype 7 and showing reduced susceptibility to CIP, as reported in India (Kumar *et al.*, 2009), has been reported recently in western Africa (Quilici *et al.*, 2010). *V. cholerae* strains with *ctxB* genotype 7 in Dhaka were uniformly sensitive to CIP; however, switching from *ctxB* genotype 1 to genotype 7 may be yet another major turning point, especially if the altered ET with the same *ctxB* genotype 7 was responsible for the severe cholera epidemic that continues in Haiti (Chin *et al.*, 2011).

PFGE has been a reliable molecular tool for typing enteric pathogens, including *V. cholerae*. Results from contemporary literature reviews on comparative genomic studies concur with PFGE-based conclusions that suggest circulation of very closely related clones of *V. cholerae* in the global wave of the seventh pandemic cholera worldwide (Chun *et al.*, 2009; Chin *et al.*, 2011; Mutreja *et al.*, 2011). The overall PFGE results presented in this study, including the clustering of strains observed in the resulting dendrograms, demonstrated high genetic relatedness between *V. cholerae* O1 strains that have been associated with cholera in Dhaka between 2006 and 2011. This clustering of *V. cholerae* O1 strains correlated highly with the antibiotic resistance markers, not to the genetic change observed in the *ctxB* genotype, which is simply a base substitution and unlikely to be reflected at the PFGE level. Nonetheless, the data presented in this study showed the transmission of varying antibiotic resistant, but genetically very closely related, *V. cholerae* O1 ET in Dhaka city. The minor divergence observed in the strains showing resistance to SXT, FR and TE and/or E is presumed to be due to the acquisition of mobile genetic elements (Chun *et al.*, 2009; Chin *et al.*, 2011; Mutreja *et al.*, 2011) carrying a variable number of antibiotic resistance gene cassettes (Thungapathra *et al.*, 2002; Jesudason & John, 1990).

Cholera has been established as a climate-driven disease that continues to be a growing concern for Dhaka city, where population density is already very high and people living in slums do not have access to safe drinking water. While the changing climate and a sea-level rise will result in major cities like Dhaka becoming even more densely populated, severe infection caused by MDR-altered *V. cholerae* ET (Nair *et al.*, 2006; Chin *et al.*, 2011) could lead to higher case fatality rates, prolonged hospitalization, increased secondary infections, and increased healthcare costs for the growing population. In Bangladesh, the ET biotype of *V. cholerae*, the most prolific pandemic strain to date, has switched its CT gene from ET type to CL type (altered ET) since 2001 (Nair *et al.*, 2006). Evidence provided here shows that the ET biotype has been undergoing yet another change in its *ctxB* gene in Dhaka since 2008. Although the change is subtle and the epidemiological significance of such a genetic change is not fully understood, it is presumed that this may be a factor in the increasing severity of disease caused by altered ET in

recent outbreaks (Siddique *et al.*, 2010; Goel & Jiang, 2010; Chin *et al.*, 2011). Finally, the data presented in this study underscore the need for close monitoring of *V. cholerae* causing endemic cholera in Bangladesh. This is important not only in Bangladesh to ensure that the correct antibiotic is chosen according to resistance variations, but also around the world, considering the increasing global burden of cholera, and the emergence and spread of new variants that will significantly influence the clinical management of cholera and its prevention.

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REFERENCE

- Alam, M., Hasan, N. A., Sadique, A., Bhuiyan, N. A., Ahmed, K. U., Nusrin, S., Nair, G. B., Siddique, A. K., Sack, R. B. & other authors (2006). Seasonal cholera caused by *Vibrio cholerae* serogroups O1 and O139 in the coastal aquatic environment of Bangladesh. *Appl Environ Microbiol* **72**, 4096–4104.
- Alam, M., Sultana, M., Nair, G. B., Siddique, A. K., Hasan, N. A., Sack, R. B., Sack, D. A., Ahmed, K. U., Sadique, A. & other authors (2007). Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proc Natl Acad Sci U S A* **104**, 17801–17806.
- Alam, M., Nusrin, S., Islam, A., Bhuiyan, N. A., Rahim, N., Delgado, G., Morales, R., Mendez, J. L., Navarro, A. & other authors (2010). Cholera between 1991 and 1997 in Mexico was associated with infection by classical, El Tor, and El Tor variants of *Vibrio cholerae*. *J Clin Microbiol* **48**, 3666–3674.
- Bauer, A. W., Kirby, W. M., Sherris, J. C. & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* **45**, 493–496.
- Chin, C. S., Sorenson, J., Harris, J. B., Robins, W. P., Charles, R. C., Jean-Charles, R. R., Bullard, J., Webster, D. R., Kasarskis, A. & other authors (2011). The origin of the Haitian cholera outbreak strain. *N Engl J Med* **364**, 33–42.
- Cholera working group (1993). Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. *Lancet* **342**, 387–390.
- Chow, K. H., Ng, T. K., Yuen, K. Y. & Yam, W. C. (2001). Detection of RTX toxin gene in *Vibrio cholerae* by PCR. *J Clin Microbiol* **39**, 2594–2597.
- Chun, J., Grim, C. J., Hasan, N. A., Lee, J. H., Choi, S. Y., Haley, B. J., Taviani, E., Jeon, Y. S., Kim, D. W. & other authors (2009). Comparative genomics reveals mechanism for short-term and long-term clonal

- transitions in pandemic *Vibrio cholerae*. *Proc Natl Acad Sci U S A* **106**, 15442–15447.
- CLSI (2010)**. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. *Approved Guideline 2nd edn, document M45-A2* (ISBN 1-56238-732-4). Wayne, PA: Clinical and Laboratory Standards Institute.
- Cooper, K. L., Luey, C. K., Bird, M., Terajima, J., Nair, G. B., Kam, K. M., Arakawa, E., Safa, A., Cheung, D. T. & other authors (2006)**. Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae*. *Foodborne Pathog Dis* **3**, 51–58.
- Dziejman, M., Balon, E., Boyd, D., Fraser, C. M., Heidelberg, J. F. & Mekalanos, J. J. (2002)**. Comparative genomic analysis of *Vibrio cholerae*: genes that correlate with cholera endemic and pandemic disease. *Proc Natl Acad Sci U S A* **99**, 1556–1561.
- Faruque, A. S. G., Salam, M. A., Faruque, S. M. & Fuchs, G. J. (1998)**. Aetiological, clinical and epidemiological characteristics of a seasonal peak of diarrhoea in Dhaka, Bangladesh. *Scand J Infect Dis* **30**, 393–396.
- Faruque, A. S. G., Alam, K., Malek, M. A., Khan, M. G., Ahmed, S., Saha, D., Khan, W. A., Nair, G. B., Salam, M. A. & other authors (2007)**. Emergence of multidrug-resistant strain of *Vibrio cholerae* O1 in Bangladesh and reversal of their susceptibility to tetracycline after two years. *J Health Popul Nutr* **25**, 241–243.
- Glass, R. I., Huq, I., Alim, A. R. M. A. & Yunus, M. (1980)**. Emergence of multiply antibiotic-resistant *Vibrio cholerae* in Bangladesh. *J Infect Dis* **142**, 939–942.
- Glass, R. I., Huq, M. I., Lee, J. V., Threlfall, E. J., Khan, M. R., Alim, A. R., Rowe, B. & Gross, R. J. (1983)**. Plasmid-borne multiple drug resistance in *Vibrio cholerae* serogroup O1, biotype El Tor: evidence for a point-source outbreak in Bangladesh. *J Infect Dis* **147**, 204–209.
- Goel, A. K. & Jiang, S. C. (2010)**. Genetic determinants of virulence, antibiogram and altered biotype among the *Vibrio cholerae* O1 isolates from different cholera outbreaks in India. *Infect Genet Evol* **10**, 815–819.
- Greenough, W. B., III, Gordon, R. S., Jr, Rosenberg, I. S., Davies, B. I. & Benenson, A. S. (1964)**. Tetracycline in the treatment of cholera. *Lancet* **1**, 355–357.
- Hoshino, K., Yamasaki, S., Mukhopadhyay, A. K., Chakraborty, S., Basu, A., Bhattacharya, S. K., Nair, G. B., Shimada, T. & Takeda, Y. (1998)**. Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. *FEMS Immunol Med Microbiol* **20**, 201–207.
- Jain, M., Goel, A. K., Bhattacharya, P., Ghatole, M. & Kamboj, D. V. (2011)**. Multidrug resistant *Vibrio cholerae* O1 El Tor carrying classical *ctxB* allele involved in a cholera outbreak in South Western India. *Acta Trop* **117**, 152–156.
- Jesudason, M. V. & John, T. J. (1990)**. Transferable trimethoprim resistance of *Vibrio cholerae* O1 encountered in southern India. *Trans R Soc Trop Med Hyg* **84**, 136–137.
- Kaper, J. B., Morris, J. G., Jr & Levine, M. M. (1995)**. Cholera. *Clin Microbiol Rev* **8**, 48–86.
- Kimsey, H. H., Nair, G. B., Ghosh, A. & Waldor, M. K. (1998)**. Diverse CTX ϕ s and evolution of new pathogenic *Vibrio cholerae*. *Lancet* **352**, 457–458.
- Kumar, P., Jain, M., Goel, A. K., Bhaduria, S., Sharma, S. K., Kamboj, D. V., Singh, L., Ramamurthy, T. & Nair, G. B. (2009)**. A large cholera outbreak due to a new cholera toxin variant of the *Vibrio cholerae* O1 El Tor biotype in Orissa, Eastern India. *J Med Microbiol* **58**, 234–238.
- Kumar, P., Wilson, P. A., Bhai, R. & Thomas, S. (2010)**. Characterization of an SXT variant *Vibrio cholerae* O1 Ogawa isolated from a patient in Trivandrum, India. *FEMS Microbiol Lett* **303**, 132–136.
- Lindenbaum, J., Greenough, W. B. & Islam, M. R. (1967)**. Antibiotic therapy of cholera. *Bull World Health Organ* **36**, 871–883.
- Maimone, F., Coppo, A., Pazzani, C., Ismail, S. O., Guerra, R., Procacci, P., Rotigliano, G. & Omar, K. H. (1986)**. Clonal spread of multiply resistant strains of *Vibrio cholerae* O1 in Somalia. *J Infect Dis* **153**, 802–803.
- Marin, M. A. & Vicente, A. C. (2012)**. Variants of *Vibrio cholerae* O1 El Tor from Zambia showed new genotypes of *ctxB*. *Epidemiol Infect* **140**, 1386–1387, author reply 1387–1388.
- Mhalu, F. S., Mmari, P. W. & Ijumba, J. (1979)**. Rapid emergence of El Tor *Vibrio cholerae* resistant to antimicrobial agents during first six months of fourth cholera epidemic in Tanzania. *Lancet* **1**, 345–347.
- Mutreja, A., Kim, D. W., Thomson, N. R., Connor, T. R., Lee, J. H., Kariuki, S., Croucher, N. J., Choi, S. Y., Harris, S. R. & other authors (2011)**. Evidence for several waves of global transmission in the seventh cholera pandemic. *Nature* **477**, 462–465.
- Naha, A., Pazhani, G. P., Ganguly, M., Ghosh, S., Ramamurthy, T., Nandy, R. K., Nair, G. B., Takeda, Y. & Mukhopadhyay, A. K. (2012)**. Development and evaluation of a PCR assay for tracking the emergence and dissemination of Haitian variant *ctxB* in *Vibrio cholerae* O1 strains isolated from Kolkata, India. *J Clin Microbiol* **50**, 1733–1736.
- Nair, G. B., Faruque, S. M., Bhuiyan, N. A., Kamruzzaman, M., Siddique, A. K. & Sack, D. A. (2002)**. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *J Clin Microbiol* **40**, 3296–3299.
- Nair, G. B., Qadri, F., Holmgren, J., Svennerholm, A. M., Safa, A., Bhuiyan, N. A., Ahmad, Q. S., Faruque, S. M., Faruque, A. S. & other authors (2006)**. Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. *J Clin Microbiol* **44**, 4211–4213.
- Olsvik, O., Wahlberg, J., Petterson, B., Uhlén, M., Popovic, T., Wachsmuth, I. K. & Fields, P. I. (1993)**. Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. *J Clin Microbiol* **31**, 22–25.
- Politzer, R. (1959)**. Cholera, pp. 11–50. Geneva, Switzerland: World Health Organization.
- Quilici, M. L., Massenot, D., Gake, B., Bwalki, B. & Olson, D. M. (2010)**. *Vibrio cholerae* O1 variant with reduced susceptibility to ciprofloxacin, Western Africa. *Emerg Infect Dis* **16**, 1804–1805.
- Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya, S. K., Nair, G. B., Shimada, T., Takeda, T., Karasawa, T., Kurazano, H. & other authors (1993)**. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. *Lancet* **341**, 703–704.
- Sack, D. A., Sack, R. B., Nair, G. B. & Siddique, A. K. (2004)**. Cholera. *Lancet* **363**, 223–233.
- Safa, A., Sultana, J., Dac Cam, P., Mwansa, J. C. & Kong, R. Y. (2008)**. *Vibrio cholerae* O1 hybrid El Tor strains, Asia and Africa. *Emerg Infect Dis* **14**, 987–988.
- Saha, D., Karim, M. M., Khan, W. A., Ahmed, S., Salam, M. A. & Bennish, M. L. (2006)**. Single-dose azithromycin for the treatment of cholera in adults. *N Engl J Med* **354**, 2452–2462.
- Siddique, A. K., Nair, G. B., Alam, M., Sack, D. A., Huq, A., Nizam, A., Longini, I. M., Jr, Qadri, F., Faruque, S. M. & other authors (2010)**. El Tor cholera with severe disease: a new threat to Asia and beyond. *Epidemiol Infect* **138**, 347–352.

Taneja, N., Samanta, P., Mishra, A. & Sharma, M. (2010). Emergence of tetracycline resistance in *Vibrio cholerae* O1 biotype El Tor serotype Ogawa from north India. *Indian J Pathol Microbiol* **53**, 865–866.

Thungapathra, M., Amita, Sinha, K. K., Chaudhuri, S. R., Garg, P., Ramamurthy, T., Nair, G. B. & Ghosh, A. (2002). Occurrence of antibiotic resistance gene cassettes *aac(6′)-Ib*, *dfrA5*, *dfrA12*, and

ereA2 in class I integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrob Agents Chemother* **46**, 2948–2955.

Waldor, M. K., Tschäpe, H. & Mekalanos, J. J. (1996). A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. *J Bacteriol* **178**, 4157–4165.