

Genetic characterization of *Vibrio cholerae* O1 strains isolated in Zambia during 1996–2004 possessing the unique VSP-II region of El Tor variant

N. A. BHUIYAN¹, S. NUSRIN¹, M. ANSARUZZAMAN^{1*}, A. ISLAM¹,
M. SULTANA¹, M. ALAM¹, M. A. ISLAM¹, A. CRAVIOTO¹,
A. K. MUKHOPADHYAY², G. B. NAIR², J. C. L. MWASNA³ AND H. P. ENDTZ^{1,4}

¹ Enteric and Food Microbiology Unit, Laboratory Sciences Division, ICDDR,B, Bangladesh

² National Institute of Cholera and Enteric Diseases, Beliaghata, Kolkata, India

³ University Teaching Hospital, Department of Pathology and Microbiology, Lusaka, Zambia

⁴ Department of Medical Microbiology and Infectious Diseases Erasmus MC, University Medical Center Rotterdam, The Netherlands

(Accepted 1 May 2011; first published online 31 May 2011)

SUMMARY

New variants of *Vibrio cholerae* O1 have appeared in different time-frames in various endemic regions, especially in Asia and Africa. Sixty-nine strains of *V. cholerae* O1 isolated in Zambia between 1996 and 2004 were investigated by various genotypic techniques to determine the lineage of virulence signatures and clonality. All strains were positive for *Vibrio* seventh pandemic Islands (VSP)-I and VSP-II and repeat toxin (RTX) gene clusters attesting their El Tor lineage. Interestingly, strains isolated in recent times (2003–2004) were identified as an altered variant (El Tor biotype that harbours El Tor type *rstR* but produce classical *ctxB*) that replaced completely the progenitor El Tor strains prevalent in 1996–1997. Recent altered variant strains differed from prototype El Tor strains isolated earlier in that these strains lacked two ORFs, VC0493 and VC0498, in the VSP-II region. PFGE analysis revealed two major clonal lineages in the strains; cluster A represented the strains isolated before 2003 and cluster B the altered strains isolated in 2003–2004. Cluster A was closely related to prototype El Tor reference strain isolated in Bangladesh in 1971. Cluster B was found to be matched with Bangladeshi altered strains but was different from the hybrid strains isolated from Mozambique and Bangladesh. This report provides important information on the genesis of altered strains of *V. cholerae* O1 isolated in Zambia and emphasizes the need for further studies to follow the trends of evolutionary changes.

Key words: Cholera, classical *ctxB*, El Tor, *V. cholerae*.

INTRODUCTION

Cholera remains an important public health problem in Africa and Asia. Several countries in both these continents are known to be endemic for cholera. At

the beginning of the seventh pandemic during the 1960s, cholera had a strong presence in Asian countries. In the early 1970s, the pandemic spread to sub-Saharan Africa and caused explosive outbreaks with a high case fatality, mainly because of a lack of background immunity in the population. During this pandemic, cholera spread along the coast and into the interior through waterways and further spread into the interior of the Sahelian states by land travel

* Author for correspondence: M. Ansaruzzaman, Enteric and Food Microbiology, Laboratory Sciences Division, ICDDR,B, Mohakhali, Dhaka 1212, Bangladesh.
(Email: ansar@icddr.org)

fostered by nomadic tribes [1, 2]. Based on cholera reports covering a period of more than 10 years (1995–2005), it is evident that about 88% of cholera originated in sub-Saharan Africa and the rest from South East Asia [3], and a 5.4% case-fatality rate (CFR) was reported between 2000 and 2005 in the eastern provinces of Congo [4]. Thirty-one (78%) of the 40 countries which reported cholera cases to WHO in 2005 were from sub-Saharan Africa with an incidence rate of 166 cases/million population and recently, cholera outbreaks have spread to newer African countries like Namibia [5].

The cholera statistics released recently by WHO paint a gloomy picture with both the number of cases and the number of countries reporting cases to WHO increasing during 2008, reaching the level of the late 1990s [6]. The overall number of notified cases of cholera to WHO during the most recent 5-year period (2004–2008) showed a 24% increase in the number of cases compared to cases between 2000 and 2004 [6]. Globally, there was a 27.5% increase in the number of deaths, representing an overall CFR of 2.7%. Of the total number of deaths, 98% were reported from the African continent. In vulnerable groups living in areas at high risk for cholera, the CFR reached as high as 50% [6]. During 2004, major outbreaks of cholera occurred in Cameroon, Chad, Guinea, Mali, Niger, Senegal and Zambia [7]. Cholera surveillance in Beira, Mozambique, in 2004 [8] revealed the presence of a distinct El Tor strain that displayed most of the typical traits of the El Tor biotype but resident CTX prophage in the strain was of the classical type (CTX^{class} ϕ). Since Zambia shares a common border with Mozambique, cholera surveillance in Zambia between 1996 and 2004 [9] showed that Zambian *V. cholerae* O1 El Tor strains are not hybrids based on the repressor gene (*rstR*) of the CTX prophage and showed typical seventh pandemic strains of the El Tor biotype [10].

The pathogenesis of cholera is mainly related to the production of cholera toxin (CT) encoded by the *ctxAB* gene, which is located on the CTX prophage integrated on the *V. cholerae* chromosome which has two epitopes or immunological forms: CT1 displayed by classical biotype strains and by US Gulf Coast strains, and CT2 demonstrated by the El Tor biotype and O139 strains [10]. These two toxins differ in the CT B subunit by 2/124 amino acids, and a polymerase chain reaction (PCR)-based method to discriminate classical-type *ctxB* and El Tor-type *ctxB* has recently been described [11]. Another classification recognizes

three genotypes based on the *ctxB* gene: genotype 1 is observed in classical strains and US Gulf Coast strains, genotype 2 is found in El Tor biotype strains from Australia, and genotype 3 is observed in El Tor biotype strains from the seventh pandemic and the Latin American epidemic [12]. The DNA sequences of the *ctxB* gene of Zambian *V. cholerae* have been deposited in GenBank under accession numbers EU932878–EU932884.

Recently, genetic hybrids of *V. cholerae* O1 strains possessing both the properties of classical and El Tor biotypes were found in Bangladesh [13, 14] and studies also showed that the CT genotype of the El Tor strains currently associated with cholera in Bangladesh have shifted from genotype 3 to genotype 1. It has been shown that the seventh pandemic prototype El Tor strains had been replaced by El Tor variant strains in Kolkata and Bangladesh from 1995 and 2002, respectively [15, 16]. The genetic analysis results showed that these altered strains vary by CTX prophage type and presence of the RS1 element, although the exact array of the CTX Φ and RS1 element and their location on the genome of these strains remain to be clarified. Comparative genomic analysis using a DNA microarray showed differences in gene content between the sixth (classical biotype) and current seventh (El Tor biotype) pandemic strains of *V. cholerae* O1 and identified two genomic regions designated as the *Vibrio* seventh pandemic island-I (VSP-I) and VSP-II that are unique to seventh pandemic El Tor strains [17].

In view of rapidly growing genetic diversity in toxigenic *V. cholerae* strains with epidemic potential, we conducted detailed genetic analysis on the Zambian *V. cholerae* O1 strains isolated between 1996 and 2004 to better understand the genetic organization of Zambian strains.

MATERIALS AND METHODS

Bacterial strains

Sixty-nine strains of *V. cholerae* O1 isolated between 1996 and 2004 in Zambia were included in this study. Of the 69 strains, 27 were isolated in 1996/1997 and 42 strains were isolated in 2003/2004. Three *V. cholerae* O1 strains isolated between 1991 and 1994, designated as Matlab type I (MJ-1236), type II (MG-116226) and type III (MG-116955) [14] and two more strains, one (B-33) isolated in Beira, Mozambique in 2004 [9], one *V. cholerae* O1 isolated in Dhaka, Bangladesh in 2001

[17] were also included in this study for comparison. We used *V. cholerae* O1 strain N16961 of El Tor biotype and O395 classical biotype as reference strains. All strains were grown in Luria Bertani broth (LB) and stored frozen (-80°C) in LB broth containing 25% (v/v) glycerol.

Genomic DNA isolation

For extraction of genomic DNA, cells were harvested from 3 ml of overnight culture in LB broth (Miller). The harvested cells were subjected to alkaline lysis by 10% SDS in the presence of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The cells were then treated with freshly prepared Proteinase K (final concentration 100 $\mu\text{g}/\text{ml}$ in 0.5% SDS) and incubated at 37°C for 1 h. After incubation, 1.0% CTAB/NaCl (cetyl trimethyl ammonium bromide in 0.7 M NaCl) was added followed by incubation for 10 min at 65°C . RNA was removed by treating with RNaseA (Sigma, USA, code no. R6513) at 37°C for 1 h. This was followed by phenol chloroform extraction and precipitation of the nucleic acid in the presence of isopropanol [18]. Excess salt was removed by 70% alcohol wash and the nucleic acid was air-dried, re-suspended in sterile TE buffer and the purity assayed using a spectrophotometer (Gene Quant, UK) that calculates the ratio of optical densities at 260 nm and 280 nm. The DNA was stored at -20°C for subsequent PCR analysis.

Mismatch amplification mutation PCR assay (MAMA)-PCR analysis

PCR assays were performed to test for the presence of *ctxB* genes specific for classical and El Tor biotypes. A conserved forward primer (Fw-con, 5'-actatctcagc atagcactgg-3') and two allele-specific polymorphism detection primers, Rv-cla (5'-cctggtacttctactgaaacg-3') and Rv-elt (5'-cctggtacttctactgaaaca-3') [19] were used in this study. PCR conditions were as follows: initial denaturation at 96°C for 2 min, 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 10 s, and extension at 72°C for 30 s, with a final extension at 72°C for 2 min. *V. cholerae* O1 isolates, O395 classical and N16961 El Tor were used as standard reference strains.

Multilocus virulence gene profiling

We used a multilocus virulence gene profiling strategy that scanned for nine virulence-associated genes

and/or gene clusters in the genome of 68 representative Zambian *V. cholerae* O1 isolates by year (1996–2004) using 33 sets of PCR primers and conditions as described previously [14, 20–23]. PCR was performed in 20 μl reaction mixture as follows: initial denaturation step at 96°C for 1 min followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at $45\text{--}58^{\circ}\text{C}$ for 30 s, 1–4 min of primer extension at 72°C and 7 min of final extension at 72°C for one cycle. The PCR products were analysed by electrophoresis in 1% agarose gels, stained with ethidium bromide, visualized under UV light and recorded by a gel documentation system (Gel DocTM 2000, Bio-Rad, USA). The PCR products were sized with standard molecular-weight markers and documented.

Nucleotide sequence of *ctxB* subunit

To determine the nucleotide sequence of the *ctxB* subunit of CT, PCR amplification of *ctxB* genes of eight representative strains of *V. cholerae* O1 based on VSP-II pattern resulting from MAMA-PCR, that were isolated in Zambia during 1996/1997 and 2003/2004, was performed in a 25 μl reaction mixture in an automated Peltier thermal cycler (PTC-200, MJ Research, USA). PCR primers and conditions were as previously described [24]. PCR products were purified with a Microcon centrifugal filter device (Millipore Corporation, USA) and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (PerkinElmer Applied Biosystems, USA) on an ABI PRISM 310 genetic analyser (Applied Biosystems).

DNA sequence analysis

The chromatogram sequencing files were inspected using Chromas 2.23 (Technelysium, Australia). Nucleotide sequences of the test isolates were compared with the corresponding sequences of the N16961 El Tor reference strain (NC_002505), the 569B classical reference strain (U25679), retrieved from GenBank using Basic Local Alignment Search Tool (BLAST) [25]. Multiple sequence alignments were developed using CLUSTALX 1.81.13, and DNA sequences were translated using GeneDoc version 2.6.002 alignment editor (<http://www.psc.edu/biomed/genedoc>).

Pulsed-field gel electrophoresis (PFGE)

Intact agarose-embedded chromosomal DNA from *V. cholerae* isolates was prepared and PFGE was

Table 1. Results of *ctxB* of *Vibrio cholerae* O1 strains from Zambia

Strain	No. of strains	Year of isolation	<i>tcpA</i>	<i>rstR</i> type	<i>ctxA</i>	<i>ctxB</i>	
						El Tor	Classical
<i>V. cholerae</i> O1	27	1996/1997	El Tor	<i>rstR2</i>	26	26	0
	42	2003/2004	El Tor	<i>rstR2</i>	42	1	41

performed using a contour-clamped homogeneous electric field apparatus (CHEF Mapper, Bio-Rad) following the standardized PFGE protocol [26]. Genomic DNA was digested with *NotI* enzyme (10 U/μl, Invitrogen, USA). The restriction fragments were separated in 1% SeaKem Gold (USA) agarose in 0.5 × Tris-borate-EDTA buffer. *V. cholerae* N16961 (El Tor) and 569B (classical) were used as reference strains to which the test strains were compared. *Salmonella enterica* serotype Braenderup strain H9812 was used as the molecular size marker. Following electrophoresis, the gels were stained in ethidium bromide solution (0.5 μg/ml) for 20–30 min and destained with reagent grade water. Images were captured using Gel Doc 2000 and Gel Doc XR systems (Bio-Rad). The test fingerprint image was normalized with Quantity One software (Bio-Rad), and the molecular weights of DNA fragments were determined. The degree of genetic diversity between the new variants and the classical and El Tor strains was determined by using Diversity Database software (version 2.2; Bio-Rad) and BioNumeric software (Applied Maths, Belgium). The similarity between the strains was determined using the Dice coefficient, and cluster analysis was performed using the unweighted pair-group method using average linkages (UPGMA).

RESULTS AND DISCUSSION

All 69 *V. cholerae* O1 strains were serologically confirmed as serogroup O1 and Ogawa serotype. Genetic screening by PCR revealed that all strains except two had important epidemic virulence markers namely *ctxA*, *rstR2* and *tcpA* El Tor. One strain isolated in 1996 lacked *ctxA*.

The *ctxB* genes of the 69 *V. cholerae* O1 strains isolated between 1996 and 2004 were examined by the primers specific for classical and El Tor biotypes. All strains, except one (*ctxA* negative) isolated in 1996 were positive for the *ctxB* El Tor gene while those isolated in 2003 and 2004 were positive for *ctxB*

classical gene, except for one (Table 1) which was positive for *ctxB* El Tor gene isolated in 2003.

All 69 *V. cholerae* O1 strains were selected for multilocus virulence gene profiling that involved examining for nine virulence-associated genes and/or gene clusters. All strains showed the presence of all genes comprising VSP-I, MSHA, and RTX gene clusters (Table 2). All the strains isolated in 1996 and 1997 showed the presence of all ORFs in the VSP-II gene clusters whereas all strains isolated in 2003 and 2004 showed the presence of all ORFs in the VSP-II region except the two ORFs, i.e. VC0493 and VC0498 (Table 2). Two individual loci, *hlyA* and *pileE*, were also present in all the test strains and the classical and El Tor reference strains examined (Table 2). In the VPI-I gene cluster the major virulence-associated genes; *toxT*, *tcpA* and *acfB* were present in all strains. All the strains were positive for *rstA*, *orfU*, *zot* and *ctxAB* genes (Table 2). The recognized allele, *rstR2* of the repressor gene *rstR* was present in all the strains (Table 1). Apart from this, two other genes, namely *tlc*, which is present adjacent to the CTX prophage and integron (*int14*), were present in all the strains (Table 2). The overall results of the virulence genetic screening indicated that all the *V. cholerae* O1 strains isolated in 1996–1997 were similar to the reference El Tor strain N16961. The strains isolated in 2003–2004 carried *rstR* of El Tor type (*rstR^{ET}*) but produced CTXB of classical type. Interestingly, these strains isolated during 2003–2004 in Zambia and lacking two ORFs, i.e. VC0493 and VC0498 in the VSP-II region, completely replaced the seventh pandemic progenitor strains and produced outbreaks with full virulence fitness. Nusrin *et al.* reported that strains isolated between 1991 and 2003 in Peru were negative for ORFs VC0512–VC0515 in the VSP-II gene cluster and concluded that the Peruvian strains had independently evolved new variants [27]. It has also been reported that *V. cholerae* O1 El Tor strain CIRS101 [15] was isolated in 2002 in Bangladesh, another variant of VSP-II, with deletion of ORFs VC0495–VC0512 [28]. The El Tor strains isolated between 2003 and 2004 in

Table 3. Genotypes of *Vibrio cholerae* O1 strains of Zambia based on the DNA sequence of the cholera toxin B subunit genes

Strain	Nucleotide at position			Amino acid at position			<i>ctxB</i> genotype
	115	138	203	39	46	68	
<i>V. cholerae</i> O1 classical, 569B	C	T	C	H	F	T	1
<i>V. cholerae</i> O1 El Tor, Australia	C	G	C	H	L	T	2
<i>V. cholerae</i> O1 El Tor, N16961	T	T	T	Y	F	I	3
<i>V. cholerae</i> O1, Zambia (1996–1997)	T	T	T	Y	F	I	3
<i>V. cholerae</i> O1, Zambia (2003–2004)	C	T	C	H	F	T	1

two ORFs, VC0493 and VC0498 in the VSP-II region. How these two ORFs were deleted and what functional impact it has at this point is still unknown and requires further characterization.

Nucleotide sequence analysis of the *ctxB* genes of *V. cholerae* O1 strains isolated between 1996 and 2004 using specific primers [24] was performed and showed that the strains isolated in 1996 and 1997 had tyrosine at position 39, phenylalanine at position 46 and isoleucine at position 68 which is identical to the El Tor reference strain N16961, whereas the strains isolated in 2003 and 2004 had histidine at position 39, phenylalanine at position 46 and threonine at position 68, which is identical to the classical reference strain 569B and indicated its relationship with classical biotype.

On the basis of DNA and protein sequencing, heterogeneity within the B subunit was first reported in the early 1990s [1, 35], and since then *ctxB* typing has been used as a tool for differentiating *V. cholerae* strains. To date, three *ctxB* genotypes of *V. cholerae* O1 strains of both biotypes and serotypes have been identified globally [13, 36]. Based on the amino-acid residue substitution at the three positions 39, 46 and 68, all classical and El Tor strains from the Gulf Coast of the USA are categorized as *ctxB* of genotype 1, El Tor strains associated with the Australian environmental reservoir are genotype 2, and El Tor strains of the seventh pandemic and the recent Latin American epidemic are classified as *ctxB* genotype 3. In this study, all strains isolated in 1996–1997 belonged to genotype 3 and all strains, except one, isolated in 2003–2004 grouped into genotype 1 (Table 3).

Thirty-eight *V. cholerae* O1 strains, which include 32 from Zambia, one El Tor variant strain and three clinical hybrid strains (Matlab types I, II, III) from Bangladesh [13], one hybrid strain from Mozambique and reference strains of classical and El Tor biotypes

were analysed by PFGE using the *NotI* restriction enzyme. The *NotI* restriction enzyme restricted the chromosomal genome to 15–27 fragments (Fig. 1) and the fragments ranged from 22.5 to 398 kb. Two major pulsotypes among the Zambian isolates, namely A and B, were observed.

Dendrogram analysis showed that two major clusters, A and B, were produced by all strains isolated in Zambia. The El Tor variant strains from Bangladesh clustered together with strains isolated in Zambia in 2003 and 2004 (cluster B) with a similarity level of >0.98% indicating the same clonal origin (Fig. 1). The strains isolated in Zambia in 1996/1997 also produced a second cluster A with a similarity level of >0.98.5% but closely related to El Tor reference strain N16961, a Bangladeshi seventh-pandemic El Tor strain isolated in 1971 (Fig. 1). Second, hybrid El Tor strains from Matlab, Bangladesh (MJ-1236) [13] and Mozambique (B-33) carrying the classical CTX prophage [8] clustered closely (similarity level >0.90%). Finally, the Matlab variant strains MG-116226 (Matlab type II) and MG-116955 (Matlab type III), which are phenotypically different from conventional El Tor biotype of reference strain N16961, clustered together but quite differently from classical and El Tor reference strain O395 (similarity level <0.75%) and N16961 (similarity level <0.85%). The PFGE dendrogram analysis showed that the strains isolated in Zambia between 2003 and 2004 were a different clonal cluster than that of strains isolated between 1996 and 1997. This result indicates that the new strains that emerged in 2003–2004 with a deletion of two ORFs, VC0493 and VC0498 of the VSP-II gene cluster was a recent occasion that may be clonally originated from the progenitor strain *V. cholerae* O1 El Tor strain. It is important to perform whole genome sequencing to understand the evolutionary changes of these newly

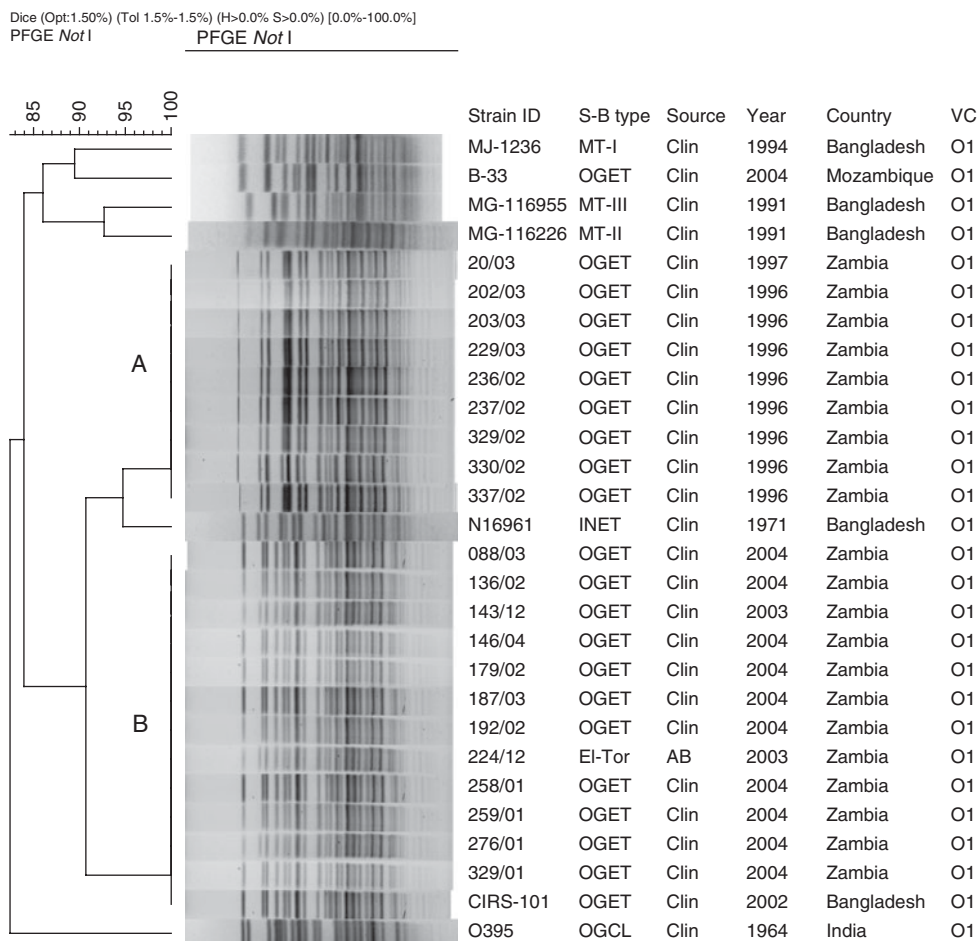


Fig. 1. Dendrogram constructed from the PFGE profiles generated by *NotI*-digested genomic DNA of representative strains of *V. cholerae* O1 isolated in Zambia, hybrid strains from Bangladesh and Mozambique, and classical, El Tor reference strains.

emerging strains that appeared globally. Therefore, discovering how all these changes occurred in the Zambian *V. cholerae* O1 strains isolated between 1996 and 2004, and fitted into environmental niches needs to be elucidated.

Given that there are differences between the classical and El Tor biotypes, the selection of this El Tor strain seems to indicate an evolutionary optimization of the El Tor biotype and could represent a new, more efficient emerging form of the El Tor biotype of *V. cholerae* O1. As this El Tor variant biotype strain cannot be differentiated from other El Tor strains by currently used bacteriological methods, a revision of methods is needed to track the spread of such strains. The implication of heterogeneity in the B subunit of CTs of altered El Tor strains also needs to be assessed from a vaccine and diagnostic perspective. Perhaps the new type of *V. cholerae* strains have arisen from *V. cholerae* O1 strains having typical

seventh-pandemic El Tor backgrounds with the replacement of their *ctxB* gene driven by the selective pressure to survive and adapt better in the host's intestine and other environmental niches. These new El Tor variant strains of *V. cholerae* O1, not only outfitted the conventional *V. cholerae* El Tor strains, but were also stable in nature. Our molecular characterization showed the emergence of a new variant strain of *V. cholerae* O1 in Zambia during 2003–2004 and how this strains was related to other variant strains isolated in Bangladesh. A continually changing pattern of *V. cholerae* O1 has been observed during the last decade in epidemic-prone areas especially in Asia and Africa.

There exists a definite need to identify and track the mode of spread of these new varieties of *V. cholerae* O1 strains in the population by vigilant surveillance systems, as these strains possess great potential for a new pandemic.

ACKNOWLEDGEMENTS

The work was supported by the Program of Founding Research Center for Emerging and Reemerging Infectious Diseases, Ministry of Education, Culture, Sports, Science and Technology of Japan and International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). ICDDR,B acknowledges the following donors which provided unrestricted support to the Centre's research efforts: Australian Agency for International Development (AusAID), Government of the People's Republic of Bangladesh, Canadian International Development Agency (CIDA), Swedish International Development Cooperation Agency (Sida), Swiss Agency for Development and Cooperation (SDC) and the Department for International Development, UK (DFID).

DECLARATION OF INTEREST

None.

REFERENCES

1. **Kaper JB, et al.** Cholera. *Journal of Clinical Microbiology Review* 1995; **8**: 48–86.
2. **Faruque SM, Albert MJ, Mekalanos JJ.** Epidemiology, genetics and ecology of toxigenic *Vibrio cholerae*. *Microbiology and Molecular Biology Reviews* 1998; **62**: 1301–1314.
3. **Griffith DC, et al.** Review of reported cholera outbreaks worldwide, 1995–2005. *American Journal of Tropical Medicine and Hygiene* 2006; **75**: 973–977.
4. **Bompangue D, et al.** Lakes as source of cholera outbreaks, Democratic Republic of Congo. *Emerging Infectious Diseases* 2008; **14**: 798–800.
5. **Smith AM, et al.** Characterization of cholera outbreak isolates from Namibia, December 2006 to February 2007. *Epidemiology and Infection* 2007; **29**: 1–3.
6. **World Health Organization.** Cholera annual report. *Weekly Epidemiological Record* 2009; **84**: 309–329.
7. **World Health Organization.** Cholera in 2004. *Weekly Epidemiological Record* 2005; **80**: 261–268.
8. **Ansaruzzaman M, et al.** Cholera in Mozambique, variant of *Vibrio cholerae*. *Emerging Infectious Diseases* 2004; **10**: 2057–2059.
9. **Mwansa JC, et al.** Multiply antibiotic-resistant *Vibrio cholerae* O1 biotype El Tor strains emerges during cholera outbreaks in Zambia. *Epidemiology and Infection* 2006; **23**: 1–7.
10. **Finkelstein R A, et al.** Antigenic determinants of the cholera/coli family of enterotoxins. *Review of Infectious Diseases* 1987; **9**: 490–502.
11. **Morita M, et al.** Development and validation of a mismatch amplification mutation PCR assay to monitor the dissemination of an emerging variant of *Vibrio cholerae* O1 biotype El Tor. *Microbiology and Immunology* 2008; **52**: 314–317.
12. **Olsvik Ø, et al.** Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. *Journal of Clinical Microbiology* 1993; **31**: 22–25.
13. **Nair GB, et al.** New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *Journal of Clinical Microbiology* 2002; **40**: 3296–3299.
14. **Nusrin S, et al.** Diverse ctx phages among toxigenic *Vibrio cholerae* O1 and O139 strains isolated between 1994 and 2002 in an area where cholera is endemic in Bangladesh. *Journal of Clinical Microbiology* 2004; **42**: 5854–5856.
15. **Nair GB, et al.** Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. *Journal of Clinical Microbiology* 2006; **44**: 4211–4213.
16. **Raychoudhuri A, et al.** Classical ctxB in *Vibrio cholerae* O1, Kolkata, India. *Emerging Infectious Diseases* 2009; **15**: 131–132.
17. **Dziejman M, et al.** Comparative genomic analysis of *Vibrio cholerae*: genes that correlate with cholera endemic and pandemic disease. *Proceedings of the National Academy of Sciences USA* 2002; **99**: 1556–1561.
18. **Chowdhury NR, et al.** Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. *Emerging Infectious Diseases* 2000; **6**: 631–636.
19. **Morita M, et al.** Development and validation of a mismatch amplification mutation PCR assay to monitor the dissemination of an emerging variant of *Vibrio cholerae* O1 biotype El Tor. *Microbiology and Immunology* 2008; **52**: 314–317.
20. **Chow KH, et al.** Detection of RTX toxin gene in *Vibrio cholerae* by PCR. *Journal of Clinical Microbiology* 2001; **39**: 2594–2597.
21. **O'Shea YA, et al.** Evolutionary genetic analysis of the emergence of epidemic *Vibrio cholerae* isolates based on comparative nucleotide sequence analysis and multi-locus virulence gene profiles. *Journal of Clinical Microbiology* 2004; **42**: 4657–4671.
22. **Rivera IN, et al.** Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. *Applied Environmental Microbiology* 2001; **67**: 2421–2429.
23. **Keasler SP, Hall RH.** Detecting and biotyping *V. cholerae* O1 with multiplex polymerase chain reaction. *Lancet* 1993; **341**, 1661.
24. **Mitra RK, et al.** Molecular characterization of rough variants of *Vibrio cholerae* isolated from hospitalised patients with diarrhoea. *Journal of Medical Microbiology* 2001; **50**: 268–276.
25. **Altschul SF, et al.** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 1997; **25**: 3389–3402.
26. **Cooper KL, et al.** Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae*. *Foodborne Pathogens and Disease* 2006; **3**: 51–58.

27. **Nusrin S, et al.** Peruvian *Vibrio cholerae* O1 El Tor strains possess a distinct region in the *Vibrio* seventh pandemic island-II that differentiates them from the prototype seventh pandemic El Tor strains. *Journal of Medical Microbiology* 2009; **58**: 342–254.
28. **Taviani E, et al.** Discovery of novel *Vibrio cholerae* VSP-II genomic islands using comparative genomic analysis. 2010. *FEMS Microbiology Letters* **308**: 130–137.
29. **Herrington DA, et al.** Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. *Journal of Experimental Medicine* 1988; **168**: 1487–1492.
30. **Kovach ME, Shaffer MD, Peterson KM.** A putative integrase gene defines the distal end of a large cluster of Tox-regulated colonization genes in *Vibrio cholerae*. *Microbiology* 1996; **142**: 2165–2174.
31. **DiRita VJ, et al.** Differential expression of *ToxR* regulon in classical and El Tor biotypes of *Vibrio cholerae* is due to biotype-specific control over *ToxT* expression. *Proceedings of the National Academy of Sciences USA* 1996; **93**: 7991–7995.
32. **Jonson G, Holmgren J, Svennerholm AM.** Identification of a mannose-binding pilus on *Vibrio cholerae* El Tor. *Microbial Pathogenesis* 1991; **11**: 433–441.
33. **Brown MH, Manning PA.** Haemolysin genes of *Vibrio cholerae*: presence of homologous DNA in non-haemolytic O1 and haemolytic non-O1 strains. *FEMS Microbiology Letters* 1985; **30**: 197–201.
34. **Davis BM, Waldor MK.** Filamentous phages linked to virulence of *Vibrio cholerae*. *Current Opinion in Microbiology* 2003; **6**: 35–42.
35. **Brickman TJ, et al.** Molecular cloning and nucleotide sequence analysis of cholera toxin genes of the CtxA2 *Vibrio cholerae* strains Texas Star-SR. *Infection and Immunity* 1990; **58**: 4142–4144.
36. **Mekalanos JJ, et al.** Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. *Nature* 1993; **306**: 551–557.