Clonal recycling, dual peaks and off-season cholera outbreaks in Dhaka

Munirul Alam [1*]. H. Watanabe et al....

[1]International Center for Diarrheal Disease Research, Bangladesh, Dhaka, Bangladesh.

[2]National Institute of Infectious Diseases, Tokyo

Running Title: **Clonal recycling of hybrid El Tor, Dhaka**

[*]Corresponding author mailing address: Alejandro Cravioto

ICDDR,B
GPO Box 128
Dhaka 1000
Bangladesh

Tel. 880-2-8823031
Fax. 880-2-8823116

acravioto@icddrb.org

Abstract

Background: The seasonal dual-peak pattern of cholera often changes in Bangladesh. In August 2007, extreme climate-related flooding coincided with a big diarrhea outbreak causing record high severity of illness in Dhaka. Our aim was to understand the unusual peak and whether the hyper-virulence was related to circulation of a new clone.

Methods: Nineteen *Vibrio cholerae* strains isolated from 2007 outbreak were subjected to extensive phenotypic and molecular analyses, including multi-locus genetic screening by PCR, sequence-typing of *ctxB* gene, and compared by pulsed-field gel electrophoresis (PFGE) (*Not*I)-based dendrogram. Also, three years of diarrhea data were analyzed in Dhaka.

Results: According to microbiological and molecular data, the hyper-virulent bacterium was O1 El Tor (ET) that has recently acquired the cholera toxin of classical biotype. PFGE and clustering patterns in dendrograms showed that the bacterium was clonal and linked to the pre-2007 hybrid-ET occurring in Dhaka and Matlab, and resembled with one of the two distinct hybrid-ET clones of estuarine origin. Analysis of three subsequent years of diarrhea data suggests the off-season outbreak occurring in Dhaka, and not seen in other endemic sites, to be the sequel of flooding and related transmission of the same infectious clone being involved in the fecal-oral cycling of cholera that shows the unique dual peak pattern at Dhaka.

Conclusions: Unlike natural estuarine habitat where climate triggers non-culturable *V. cholerae* to be actively growing to initiate seasonal cholera, poor sanitation and dearth of safe drinking water allows the same infectious clone to circulate beyond the defined seasons facilitating off-season cholera outbreaks during flooding in Dhaka; a densely populated city bordering heavily polluted freshwater rivers and flood embankment. The disruption of infectious fecal-oral cycling of the hyper-virulent ET, which under the changing climate and growing population remains a growing concern, appears crucial to offset the non-stop cholera that invites instant outbreaks in Dhaka.

Introduction

Cholera is a rapidly dehydrating diarrhea that results from drinking water containing toxigenic *Vibrio cholerae*, serogroups O1 or O139. *V. cholerae* O1 has two biotypes, classical (CL) and El Tor (ET), which have been responsible for all of the seven cholera pandemics to date. Studies show that the 5th and 6th cholera pandemics were caused by the CL biotype, while the ET biotype is the cause of the current 7th pandemic by completely replacing the CL biotype, which was prominent in the early 1980's [1]. Despite the fact that the two biotypes share the same O polysaccharide genes [2,3], genetic studies show a high degree of gene conservation, including the *V. cholerae* seventh pandemic island (VSP)-I and -II, which are unique to this pandemic ET strain but not to CL biotype strains [4]. Apart from these phenotypic and genetic differences, there are also differences in the patterns of infection caused by these two biotypes. Although many studies revealed the ET strains to be better adapted to the environment, they have been shown to be less clinically virulent [5]. This was due to more asymptomatic than symptomatic carriers of ET strains, where asymptomatic patients outnumbered clinically symptomatic patients by a ratio of up to 50:1 [6]. Better survival in both the environment and the human host, together with a more efficient host-to-host transmission make the ET strains superior to CL strains [7].

Recent molecular analysis of ET strains isolated from patients with acute watery diarrhea in Bangladesh [8] shows that currently, the existing *V. cholerae* O1 ET strains are hybrids of both CL and ET. These hybrid strains were termed 'Matlab variants', since they were first isolated in Matlab, a rural, endemic cholera area 50 km south-east of Dhaka [8]. Further studies have shown that all circulating strains of the ET biotype isolated since 2001 were hybrids of both CL and ET biotypes, while those isolated before 2001 contained all the ET attributes associated with the 7th pandemic [9]. Although the consequence of such continued genetic shifts among

cholera bacteria is not fully understood, the severe dehydration caused by these hybrid- ET hybrid strains was shown to be increasingly significant in Bangladesh [10].

Cholera has been established as a seasonal disease that varies in the patterns of infections. The seasonal outbreaks of cholera occur in multiple endemic foci of Bangladesh, most showing a single annual peak like in other cholera affected countries of the world. Historically, most major epidemics have originated in coastal regions, including both the South American epidemic that began in the coastal regions of Peru spreading to 21 countries and the new O139 Bengal outbreaks in India and Bangladesh. However, cholera shows two distinct seasonal peaks pattern, one before (March – May) and the other after (September – November) the annual monsoon [6] in Dhaka and Matlab, Bangladesh ([1,14]**Glass** 1982). *V. cholerae* has been a brackish water flora [11] that survives in estuarine water making close association with plankton [12]. Although climate such as sea surface temperature has been shown to have a degree of correlation with the incidence of cholera in coastal villages of Bangladesh [13], little is understood as to how these factors contribute to the dual cholera peaks in Dhaka and Matlab, which are freshwater ecosystems 50km apart, and 350 km away from the coast of Bay of Bengal. In August 2007, Bangladesh suffered flooding, which was accompanied by a large diarrhea outbreak. During this outbreak, the ICDDR,B hospital in Dhaka treated a record number of cholera patients peaking at 1045 patients per day with 70% suffering from severe dehydration. This was more than double the number that had been seen in the past three years of flooding [15]. Although *V. cholerae* O1 ET was identified as the primary cause, detailed study was needed to understand the off-season outbreak, especially if the hyper-virulence was related to circulation of a new clone.

Materials and Methods

Bacterial strains. *V. cholerae* serogroup O1 strains characterized and compared in the present study with their source, place, and year of isolation are shown in Table 1 & 2.

Isolation of *V. cholerae* strains. *V. cholerae* O1 strains were isolated from rectal swabs of cholera patients admitted to the ICDDR,B hospital in Dhaka during the peak of the 2007 epidemic [15]. *V. cholerae* O1 strains used for comparison in the present study were isolated in Dhaka, Matlab, and Barisal between 2004 and 2007. Dhaka and Matlab are located 50km away from each other, and represent two major inland cholera endemic sites, each lying 350 km away from the cholera endemic coastal villages of Barisal, Bangladesh [16]. *V. cholerae* colonies were confirmed by standard culture methods and identified by a combination of biochemical, serological and molecular methods, as described previously [16,17].

Biotyping. Biotype determination involved a number of tests: chicken erythrocyte agglutination; hemolysis of sheep erythrocytes; the Voges–Proskauer reaction; sensitivity to polymyxin B; and Mukerjee classical (CL) phage IV and Mukerjee El Tor (ET) phage V tests [18]. To complement the biotype characterization by genetic traits, PCR assays targeted to detect *tcpA* (CL and ET variant) [19], and the type of *rstR* gene encoding the phage transcriptional regulator were carried out using previously described methods [20].

Genomic DNA preparation. For the extraction of the genomic DNA, harvested cells from 3ml of overnight LB broth (Miller) were subjected to alkaline lysis followed by phenol

chloroform extraction, as described elsewhere [21]. The DNA was stored at -20°C for subsequent PCR analysis.

Serogroup analysis by PCR. All the strains were reconfirmed using *V. cholerae*-species-specific *ompW* PCR as described previously [22]. The serogroup of these strains was further reconfirmed using polyvalent O1 and monovalent Inaba and Ogawa antisera, and by multiplex-PCR targeted to identify genes encoding O1 (*wbeO1*) and O139 (*wbfO139*)-specific O biosynthetic genes, as well as the cholera toxin gene (*ctxA*) as described previously [23].

MAMA-PCR for determination of *ctxB* gene type. A mismatch amplification mutation assay (MAMA)-PCR was performed to test for the presence of the *ctxB* genes specific for CL and ET biotypes according to previously described methods [24]. *V. cholerae* O1 isolates O395 CL and N16961 ET were used as standard reference strains.

DNA sequencing of *ctxB* gene. Nucleotide sequencing of the *ctxB* genes of six randomly selected strains of hyper-virulent *V. cholerae* O1 ET isolated during the peak of the 2007 Dhaka epidemic was carried out using an ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 310 automated sequencer as described previously [9]. The nucleotide sequences of the reference strains were compared with the corresponding sequences of ET strain N16961 (GenBank accession no. NC-002505), and the CL strain 569B (GenBank accession no. U25679), which were retrieved from GenBank by BLAST search.

DNA sequence and proteomic analysis. The chromatogram sequencing files were inspected using Chromas 2.23 (Technelysium). Nucleotide sequences of the test isolates were

compared with the corresponding sequences of the N16961 ET reference strain (NC_002505), and the 569B CL 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.

PFGE. Pulsed-field gel electrophoresis (PFGE) was carried out with a contour-clamped homogeneous electrical field (CHEF-DRII) apparatus (Bio-Rad) according to previously described methods [26]. Genomic DNA of test strains were digested by the *Not*I restriction enzyme (GIBCO-BRL, Gaithersburg, Md.) and *Salmonella braenderup* was digested by *Xba*I with the fragments being used as molecular size markers. The restriction fragments were separated in 1% pulsed-field-certified agarose in 0.5X 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 via the 1D Gel documentation systems (Bio-Rad). The images were then processed using Quantity One Software (version 4.4.1, Bio-Rad). The test fingerprint image was normalized according to the standard, and the molecular weights of the DNA fragments were determined using Quantity One Software (version 4.4.1, Bio-Rad). Digital images of PFGE fingerprint patterns were analyzed by Dice similarity coefficient and UPGMA clustering methods (Bio-Rad), as recommended by the manufacturer, and these were graphically represented as dendrograms.

Results

Flood related diarrheal outbreak 2007. In August 2007, Bangladesh suffered flooding, resulting from heavy monsoon rains, when an unusually big diarrheal outbreak ravaged Dhaka. The outbreak was remarkable because it occurred between the two annual seasonal diarrheal peaks setting a new daily record of extremely ill patients, most being severely dehydrated. In Dhaka where diarrhea occurs almost year-round, with a distinctive two-peaks pattern, one in spring and the other in autumn (Glass1982, 15, Samadi 1983, 33)As shown in Figure 1, the number of patients rose sharply in the first week of August 2007. In the second week, the epidemic peaked with daily admissions reaching a record numbers 1,045 patients [15]. During the month of August 2007, the ICDDR,B Dhaka hospital alone treated 21,401 diarrhea patients, which was almost three times the number seen over the same period in both 2006 and 2008 (Figure 1).

During this outbreak, which did not spread to any of the other endemic sites beyond Dhaka, analysis of a 2% random samples showed *Vibrio cholerae* to be the most commonly identified cause of diarrhea, followed by *Escherichia coli* and Rotavirus [15].

By analyzing diarrhea data for three subsequent years, we propose that the off-season outbreak was sequel of flooding amid the non-stop cholera circulating the same clone in Dhaka.

Biochemical and serological tests. All *V. cholerae* O1 strains included in the present study produced characteristic colonies when grown on selective agar media, which were then confirmed by biochemical, serological, and molecular methods [16,17].

Phenotypic and genotypic characteristics. The results obtained for the major phenotypic and related genetic characterizations of the hyper-virulent *V. cholerae* O1 (n=19) and their comparison with the pre-existing hybrid-ET (n=6) isolated from Dhaka and Matlab are

presented in Table 1. All of the 25 *V. cholerae* O1 strains, including the hyper-virulent strains, showed resistance to polymyxin B (50 U), an ability to agglutinate chicken erythrocytes, and sensitivity to ET-specific phage V, but exhibited non-sensitivity to CL phage IV (Manning 1994) (Table 1) suggest an ET ancestry.

In addition, all test strains amplified the primers for the *V. cholerae* species-specific *ompW* gene, the serogroup-specific *wbe* gene, cholera toxin (CT) encoding the *ctxA* gene, and biotype-specific genes such as *tcpA* (ET) and *rstR*2 (ET). This was confirmed by simplex and multiplex- PCR assays [6] (Table 1), and further showed that the hyper-virulent *V. cholerae* causing the 2007 Dhaka epidemic was toxigenic and belonged to serogroup O1 biotype ET.

MAMA-PCR. As shown in Table 1, all the hyper-virulent *V. cholerae* O1 strains tested, including the pre-existing hybrid-ET, amplified the primers specific for the *ctxB* gene of the CL biotype, as for the CL control. However, the ET control did not amplify these primers suggesting a CL trait of the *ctxB* gene. The ET control strain did amplify the primers specific for the *ctxB* gene of the ET biotype but not that of the CL strain or the test strains. These results confirmed the specificity of the mismatch amplification mutation assay (MAMA)-PCR used [24].

DNA sequencing and proteomic analysis of *ctxB* gene. Nucleotide sequence analysis of the *ctxB* genes and the deduced N'-terminal amino acid sequence revealed that all six strains tested had histidine at position 39, phenylalanine at position 46 and threonine at position 68, which is typical of the CL reference strain 569B. This confirmed the MAMA-PCR assay results (Table 1) in that the CT of the hyper-virulent strains was of a CL type.

Genome analysis by PFGE and cluster analysis. The hyper-virulent *V. cholerae* O1 ET strains causing the 2007 Dhaka outbreak were subjected to PFGE analysis and compared with the pre-existing hybrid-ET strains that had been isolated at Dhaka (2004 – 2006), Matlab (2004 – 2007), and Barisal (2004 – 2006), to determine their clonal origin and genetic relatedness. The *Not*I restriction enzyme digested the genomic DNA into between 20 and 23 fragments (Figure 2); the molecular sizes of the fragments being between 20 to 350 kb. Except for the Barisal strains, which varied significantly, the overall PFGE patterns of the hyper-virulent and the hybrid-ET of Dhaka and Matlab were more or less homogeneous (Figure 2), suggesting that they were clonally related.

The hyper-virulent and hybrid-ET strains from Dhaka and Matlab, including two of the four hybrid-ET from Barisal, shared a major cluster of closely related strains, suggesting that they belong to the same clonal lineage. The remaining two Barisal strains, which varied significantly in their PFGE patterns, formed a separate but distant cluster of clonally unrelated strains, confirming two different clones in Barisal of which one showed analogy to the hyper-virulent and hybrid-ET strains from Dhaka and Matlab. Although minor variations were also evident among the hyper-virulent and hybrid-ET strains sharing the major cluster, the similarity coefficient, as indicated in the dendogram, suggests them to be clonal, supporting the supposition of persistent recycling of the same clone and the view that the off-season outbreak in August 2007 was resulted by the infectious clone being recycled in the circular urban ecosystem of Dhaka (Figure 3), transmitted by flowing flood waters.

Discussion

Floods in 1988, 1998, and 2004 were shown to be associated with mass outbreaks of water-borne diseases in Bangladesh [27]. The latest flood-related diarrheal outbreak that struck Dhaka in August 2007 was remarkable firstly because of the record number of patients admitted to the ICDDR,B hospital in Dhaka, and secondly because more than 70% of the patients presented with high severity of dehydration [15,28]. Although the severity of dehydration was more than double compared with that seen in the previous three years of flooding [27], deaths were prevented among the hospitalized patients using aggressive rehydration therapy. The primary microbiological culture, together with biochemical and serological characterizations confirmed the hyper-virulent bacterium to be *Vibrio cholerae* O1, which possessed all the conventional phenotypic traits that are typical of the ET prototype [2,5]. This was also true for the pre-existing ET strains that were also included in this study. Genetic screening using simplex and multiplex-PCR assays [6] showed that all of the tested strains amplified the primers for *V. cholerae* species-specific gene *ompW* [27], *ctxA* gene encoding the sub unit 'A' of the CT, and surface antigen (serogroup O1-sepicific) encoding gene *wbe* [23] confirming initial results that the hyper-virulent *V. cholerae* was toxigenic and belonged to serogroup O1. Molecular data provided additional support showing that the strains had biotype-specific genes, such as *tcpA*-ET [19] and *rstR*2-ET [20], which are the genes that encode for major pilin and a repressor, respectively. All these results led to the conclusion that both the hyper-virulent ET causing the 2007 cholera outbreak and the pre-existing hybrid-ET isolated in Dhaka and Matlab shared the major phenotypic and genetic traits of the 7th pandemic ET prototype.

Acute dehydrating diarrhea is the hallmark of cholera and is caused by cholera toxin (CT) that is encoded by *ctxAB* genes, which are components of the novel filamentous bacteriophage, CTXΦ. The CTXΦ is bio-specific and lysogenizes *V. cholerae* genomes [29], mostly of the O1

and O139 cholera strains. In other words, ET strains are lysogenized by the 'ET-specific CTXΦ' while the CL biotype strains are lysogenized by 'CL-specific CTXΦ' [6]. Although CTXΦ is biotype specific, all ET isolated in Dhaka since 2001 was shown to have the *ctxB* gene of the CL biotype, while those isolated before 2001 had the *ctxB* gene of the ET biotype [8,9]. A *V. cholerae* O1 ET strain harboring CL type CTXΦ was also reported in Mozambique [30]. The CL nature of the *ctxB* gene of the hyper-virulent ET causing the 2007 Dhaka outbreak was confirmed by MAMA-PCR assay [24], followed by nucleotide sequencing and analysis of the *ctxB* genes, as reported recently in Dhaka and Matlab [6,9]. This raises a key question, was the hyper-virulence of hybrid-ET due to CL biotype CT under the ET biotype background? Although direct evidence is lacking, increasing severity of dehydration caused by the hybrid-ET was shown recently in a molecular epidemiological study carried out between 2004 -2006 in the coastal villages of Bangladesh [10].

The CL and ET biotype strains that differ from each other in their phenotypic and genotypic traits also differ significantly in the patterns of the infections that they cause [31-33]. Many studies have shown that ET is better able to adapt to the environment but clinically, it is less virulent [6]. Therefore, prolonged adaptations to different ecosystems and efficient host-to-host transmission may have made the hybrid-ET more robust [7]. However, if the increase in severe diarrhea and dehydration was attributed only to CL biotype CT under ET biotype background, the question would arise as to why the severity did not shoot-up immediately, since all ET strains isolated in Dhaka since 2001 have possessed the CL biotype CT [9]. Other explanations of the increase in severity of dehydration may include the fast-growing population of Dhaka ingesting higher numbers of infectious hybrid-ET cells during the August 2007

flooding, or the acquisition of more, as yet unknown, genetic elements through lateral gene transfer [32,33].

The overall results of PFGE, which is a reliable tool to determine clonal origin [34], and the clustering patterns in the dendrograms revealed the hyper-virulent and the pre-existing hybrid-ET strains isolated in Dhaka and Matlab, during 2005 and 2006, to be the same clone, which in turn resembled one of the two distinct clonal types of hybrid-ET confirmed in the estuarine ecosystem of Barisal. These results suggest persistent recycling of the same clone in Dhaka and Matlab, where cholera continues beyond the defined seasons. Although the genetic fidelity of *V. cholerae* causing recurrent cholera provides strong support for clonal recycling in Dhaka, the minor variations seen in the dendrograms suggest genetic re-assortment [9], presumably due to the selection pressures relating to the continued recycling in Dhaka's complex urban ecosystem. By contrast, the two distant clonal types of the hybrid-ET in Barisal suggest that unlike the urban ecosystem of Dhaka where year-round cholera involves recycling of the same clone, genetically divergent clones can arise in the natural estuarine ecosystem of *V. cholerae* [32,33].

V. cholerae is autochthonous to brackish and estuarine ecosystems, and remains in a non-culturable state during inter-epidemic periods of the year [11,12,16]. Many studies have shown that *V. cholerae* survives with plankton during these inter-epidemic periods and is stimulated to multiply when the plankton blooms leading to the on-set of seasonal cholera [13]. The actively growing state of the bacterium that initiates seasonal cholera is climate driven with signals such as temperature and conductivity playing a role in a series of naturally occurring events that still remain unclear [13,16]. Despite being a freshwater ecosystem, Dhaka, a heavily populated city, has the long history of endemic cholera that shows the patterns of same two seasonal peaks but

the disease does continue outside these periods [35]. Although the reason how the freshwater ecosystem supports the survival of *V. cholerae*, and why outbreaks continue even beyond the defined seasons in Dhaka are not well-understood, the bodies of water in and around the city (Figure 3) are heavily burdened with domestic sewage containing actively growing cells that allow their recycling in the non-exhaustive cholera in Dhaka. It is not in record exactly when toxigenic *V. cholerae* first entered into the infectious cycles of non-stop cholera in Dhaka, however, the poor sanitation and an increasing dearth of clean drinking water for growing millions living in the peripheral slums likely facilitate the continued fecal-oral transmission.

The August 2007 cholera outbreak in Dhaka was unique because it was off-season occurring between the two defined seasonal diarrhea peaks. Although the fundamental role of climate on the naturally occurring *V. cholerae* [6,8,9] cannot be ruled out, flooding clearly played a key role in both the early onset and severity of illness, likely by facilitating fecal-oral transmission of the highly virulent bacterium in large numbers. Under global climate change and the consequential rise in sea levels, frequent flooding and climate-related disasters are forcing many millions to migrate to urban areas. Evidence is provided here showing that although recurrent cholera is endemic in many remote villages of Bangladesh [15,16,27,28,35,36], *V. cholerae* is becoming more and more an urban problem; and thus, the newly emerged hybrid-ET showing increasing virulence remains a growing concern for Bangladesh and beyond [10].

Cholera has been suggested to start at a point source and then spread [16,37]. However, recent molecular data suggest that when cholera starts at different endemic locations, it is initiated by one or more clones that prevail locally [38]. Interestingly, the outbreak that was caused by the pre-existing hybrid-ET in Dhaka and its suburbs in August, 2007, did not spread beyond the city limits despite a major part of Bangladesh being under a constant flow of

floodwater [15]. It is presumed that cholera outbreaks, which characteristically begin by ingesting water that contains an infective dose of cholera bacteria [36], depend on the fecal-oral route of transmission. The infective dose, which is achieved by pre-epidemic enrichment presumably in the human host [37], and its transmission into drinking water sources, appears crucial for epidemics to begin. It is interesting that although the floodwater containing highly infectious *V. cholerae* from Dhaka, which has a faulty flood embankment around the city, should have escaped and reached villages lying downstream, the epidemic did not reach that far. This may have been due to continuous flushing of excessive floodwaters that may have diluted and/or prevented the infective dose from settling, thereby disrupting fecal-oral transmission.

Data and evidence provided here clearly suggest persistent recycling of the same clone in year-round cholera, and that, unlike the naturally occurring *V. cholerae*, which survives mostly in non-culturable state [11,12] and initiates the seasonal cholera in response to a cascade of climatic events [13], the infectious cholera bacterium proliferating rapidly under extreme weather conditions such as flooding allows instant outbreaks beyond the defined seasons in Dhaka. The hyper-virulence of hybrid-ET is of great concerns and requires further investigation, however, disruption of the fecal-oral transmission is crucial to offset the infectious cycles of *V. cholerae* and avert recurrent cholera outbreaks that continue to cause substantial morbidity and mortality, particularly during 'off-season' flooding in Dhaka.

ACKNOWLEDGEMENTS:

This research was supported in part by NIID, Tokyo, and the ICDDR,B. The ICDDR,B is supported by donor countries and agencies, which provide unrestricted support to the centre for its operation and research.

References:

1. Samadi AR, Huq MI, Shahid N, Khan MU, Eusof A, Rahman AS, Yunus M, Faruque AS. Classical *Vibrio cholerae* biotype displaces El tor in Bangladesh. Lancet. 1983;1(8328):805-7.

2. Manning PA, Clark CA, Focareta T. Gene capture in *Vibrio cholerae*. Trends Microbiol. 1999;7:93-95.

3. Manning PA, Stroeher UH, Morona R. Molecular basis of O-antigen biosynthesis of *Vibrio cholerae* O1: ogawa- Inaba switching. In K. Wachsmuth, P. A. Blake and O. Olsvik (eds.), *Vibrio cholerae* and cholera: Molecular to Global Perspective. ASM Press, Washington, D.C. 1994; P. 77-94.

4. Dziejman M, Balon E, Boyd D, Fraser CM, Heidelberg JF, Mekalanos JJ. Comparative genomic analysis of *Vibrio cholerae*: genes that correlate with cholera endemic and pandemic disease. Proc Natl Acad Sci USA. 2002;99:1556–1561.

5. Chanda PK, Chatterjee SN. Adsorption characteristic of a group I cholera phage. Bull Calcutta Sch Trop Med. 1973;21(1):7-8.

6. Safa A, Bhuyian NA, Nusrin S, Ansaruzzaman M, Alam M, Hamabata T, Takeda Y, Sack DA, Nair GB. Genetic characteristics of Matlab variants of *Vibrio cholerae* O1 that are hybrids between classical and El Tor biotypes. J Med Microbiol. 2006;55:1563–1569.

7. Finkelstein RA. 25 February 2006, posting date. Cholera, *Vibrio cholerae* O1 and O139, and other pathogenic vibrios. http://gsbs.utmb.edu/microbook/ ch024.htm.

8. Nair GB, Faruque SM, Bhuiyan NA, Kamruzzaman M, Siddique AK, Sack DA. 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. 2002:40:3296–3299.

9. Nair GB, Qadri F, Holmgren J, Svennerholm AM, Safa A, Bhuiyan NA, Ahmad QS, Faruque SM, Faruque AS, Takeda Y, Sack DA. Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. J Clin Microbiol. 2006;44:4211-4213.

10. Siddique AK, Nair GB, Alam M, Sack DA, Huq A, Nizam A, Longini IM, Qadri F, Faruque SM, Colwell RR, Ahmed S, Iqbal A, Bhuiyan NA, Sack RB. El Tor cholera with severe disease: a threat to Asia and beyond. Epidemiol. Infect. 2009; 1 - 6.

11. Colwell RR, Spira WM. The ecology of *Vibrio cholerae*, In Barua, D., and Greenough III, W.B. (eds), Cholera. Plenum Medical Book Co., New York. 1992;107-127.

12. Huq A, Colwell RR, Rahaman R, Ali A, Chowdhury MAR, Parveen S, Sack DA, Cohen ER. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent–monoclonal antibody and culture methods. Appl Environ Microbiol. 1990;56:2370-2373.

13. Colwell RR. Global Climate and infectious disease: the cholera paradigm. Science. 1996;274:2025-2031.

14. Glass RI, Huq MI, Stoll BJ, Khan MU, Merson MH, Leee JV, Black RE. Endemic cholera in rural Bangladesh, 1966-1980. Am J Epidemiol. 1982;116:959-970.

15. Responding to the 2007 floods: Record numbers of patients seek care at ICDDR,B's Dhaka Hospital. Health and Science Bulletin (English). 2007; 5:1-5.

16. Alam M., Hasan NA, Sadique A, Bhuiyan NA, Ahmed KU, Nusrin S, Nair GB, Siddique AK, Sack RB, Sack DA, Huq A, Colwell RR. Seasonal Cholera Caused by *Vibrio cholerae* Serogroups O1 and O139 in the Coastal Aquatic Environment of Bangladesh. *Appl. Environ Microbiol.* 2006;72:4096-4104.

18. Basu S, Mukerjee S. Bacteriophage typing of *Vibrio* eltor. Experientia. 1968;24(3):299-300.

19. Keasler SP, Hall RH. Detecting and biotyping *Vibrio cholerae* O1 with multiplex polymerase chain reaction. Lancet. 1993;341(8861):1661.

20. Kimsey HH, Nair GB, Ghosh A, Waldor MK. Diverse CTXΦ and evolution of new pathogenic *Vibrio cholerae*. Lancet. 1998;352:457–458.

21. Chowdhury NR, Chakraborty S, Ramamurthy T, Nishibuchi M, Yamasaki S, Takeda Y, Nair GB. Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. Emerg Infect Dis. 2000;6:631-636.

22. Nandi S, Maiti D, Shaha A, Bhadra RK. Genesis of variants of *V. cholerae* O1 biotype El Tor: role of the CTXΦ array and its position in the genome. Microbiology. 2003;149:89-97.

23. Hoshino K, Yamassaki S, Mukhodpadhyay AK, Chakraborty S, Basu A, Bhattacharya SK, Nair GB, Shimada T, Takeda Y. Development and evaluation of multiplex-PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. FEMS Immunol Med Microbiol. 1998;20:201-207.

24. Morita M, Ohnishi M, Arakawa E, Bhuiyan NA, Nusrin S, Alam M, Siddique AK, Qadri F, Izumiya H, Nair GB, Watanabe H. 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. Microbiol Immunol. 2008;52:314-7.

25. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25:3389–3402.

26. Cooper JE, Feil EJ. Multilocus sequence typing--what is resolved?. Trends Microbiol. 2004;12(8):373-7.

27. Schwartz BS, Harris JB, Khan AI, Larocque RC, Sack DA, Malek MA, et al. Diarrheal epidemics in Dhaka, Bangladesh, during three consecutive floods: 1988, 1998, and 2004. Am J Trop Med Hyg 2006;74:1067-73.

28. Alam NH, Ashraf H. Treatment of infectious diarrhea in children. Pediatr Drugs. 2003;5:151-65.

29. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science. 1996;272:1910–1914.

30. Ansaruzzaman M, Bhuiyan NA, Nair GB, Sack DA, Lucas M, Deen JL, Ampuero J, Chaignat CL. The Mozambique Cholera vaccine Demonstration Project Coordination Group. Cholera in Mozambique, variant of *Vibrio cholerae*. Emerg Infect Dis. 2004;10(11):2057-9.

31. Beltran P, Delgado G, Navarro A, Trujillo F, Selander RK, Cravioto A. Genetic diversity and population structure of *Vibrio cholerae*. J. Clin. Microbiol. 1999;37:581–590.

32. Karaolis DK, Lan R, Kaper JB, Reeves PR. Comparison of *Vibrio cholerae* pathogenicity islands in sixth and seventh pandemic strains. Infect Immun. 2001;69:1947–1952.

33. Karaolis DK, Lan R, Reeves PR. The sixth and seventh cholera pandemics are due to independent clones separately derived from environmental, nontoxigenic, non-O1 *Vibrio cholerae*; J. Bacteriol. 1995;177:3191–3198.

34. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33(9):2233-9.

34. Tison D. Vibrios,. In P. Murray, E. Baron, M. Pfaller, F. Tenover, and R. Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, D.C. 1999; p. 497-506.

35. Sack RB, Siddique AK, Longini IM, Nizam A, Yunus M, Islam MS, Moreris G, Ali A, Huq A, Nair GB, Qadri F, Faruque SM, Sack DA, Colwell RR. A 4-Year Study of the Epidemiology of *Vibrio cholerae* in Four Rural Areas of Bangladesh. J Infect Dis. 2003;187:96-101.

36. Sack DA, Sack RB, Nair GB, Siddique AK. Cholera. Lancet. 2004;363(9404):223-33.

37. Faruque SM, Islam MJ, Ahmed QS, Faruque ASG, Sack DA, Nair GB, Mekalanos JJ. Self-limiting nature of seasonal cholera epidemics: Role of host-mediated amplification of phage. Proc Natl Acad Science. 2005;102:6119-6124.

38. Steine C, Alam M, Tang L, Nair GB, Siddique AK, Faruque SM, Huq A, Colwell RR, Sack RB, Morris JG. Cholera epidemics in rural Bangladesh are the result of multiple small outbreaks. J Infect Dis. 2008:14(5):831-833.

Table 1: Phenotypic and molecular characteristics of *Vibrio cholerae* O1 strains isolated from flood-related Dhaka epidemic, 2007, and the pre-existing *V. cholerae* O1 strains isolated between 2004 and 2007 in Dhaka and Matlab. *V. cholerae* O1, classical and El Tor strains were also included in this study as controls.

Sl. No.	Location	Strain ID#	Serotype	*ompU*	*toxR*	*tcpA* Cla	*tcpA* ET	*ace*	*wbe* O1	*CtxA*	C.C.A	PolyB	*rstR* type	*CtxB* type
1	Dhaka	117514	INET	+	+	-	+	+	+	+	+	R	2	classical
2	Dhaka	117583	OGET	+	+	-	+	+	+	+	+	R	2	classical
3	Dhaka	117645	OGET	+	+	-	+	+	+	+	+	R	2	classical
4	Dhaka	117644	INET	+	+	-	+	+	+	+	+	R	2	classical
5	Dhaka	117685	OGET	+	+	-	+	+	+	+	+	R	2	classical
6	Dhaka	117773	OGET	+	+	-	+	+	+	+	+	R	2	classical
7	Dhaka	117633	INET	+	+	-	+	+	+	+	+	R	2	classical
8	Dhaka	117911	INET	+	+	-	+	+	+	+	+	R	2	classical
9	Dhaka	117935	OGET	-	-	-	-	-	+	+	+	R	2	classical
10	Dhaka	118012	INET	+	+	-	+	+	+	+	+	R	2	classical
11	Dhaka	118113	INET	+	+	-	+	+	+	+	+	R	2	classical
12	Dhaka	117793	OGET	+	+	-	+	+	+	+	+	R	2	classical
13	Dhaka	117902	INET	+	+	-	+	+	+	+	+	R	2	classical
14	Dhaka	117750	OGET	+	+	-	+	+	+	+	+	R	2	classical
15	Dhaka	117743	OGET	+	+	-	+	+	+	+	+	R	2	classical
16	Dhaka	117677	INET	+	+	-	+	+	+	+	+	R	2	classical
17	Dhaka	117675	INET	+	+	-	+	+	+	+	+	R	2	classical
18	Dhaka	117875	INET	+	+	-	+	+	+	+	+	R	2	classical
19	Dhaka	117865	OGET	+	+	-	+	+	+	+	+	R	2	classical
20	Matlab	L01-511	INET	+	+	-	+	+	+	+	+	R	2	classical
21	Matlab	L01-928	INET	+	+	-	+	+	+	+	+	R	2	classical
22	Matlab	L01-1786	INET	+	+	-	+	+	+	+	+	R	2	classical
23	Matlab	L01-1642	OGET	+	+	-	+	+	+	+	+	R	2	classical
24	Matlab	L01-1773	OGET	+	+	-	+	+	+	+	+	R	2	classical
25	Matlab	L01-1782	OGET	+	+	-	+	+	+	+	+	R	2	classical
26		569B	CL Cont.	+	+	+	-	+	+	+	-	S	1	Classical
27		N16961	ET Cont.	+	+	-	+	+	+	+	+	R	2	El Tor

Cca, chicken cell agglutination; Poly B, polymixixn B; R, resistant; S, sensitive; ET, El Tor; CL, classical; IN, Inaba; OG, Ogawa; *ctxB* type was confirmed by MAMA-PCR followed by DNA sequencing and analysis.