Ms. Ref. No.: JCM00557-12

Molecular Characterization of High-Level-Cholera Toxin-Producing El Tor Variant *Vibrio cholerae* Strains in the Zanzibar Archipelago of Tanzania

A. Naha[1], G. Chowdhury[1], J. Ghosh-Banerjee[1], M. Senoh[2], T. Takahashi[2], B. Ley[3], K. Thriemer[3], J. Deen[3], L. V. Seidlein[4], S. M. Ali[5, 6], A. Khatib[5], T. Ramamurthy[1], R.K. Nandy[1], G.B. Nair[1], Y. Takeda[2] and A.K. Mukhopadhyay[1*]

[1]National Institute of Cholera and Enteric Diseases, Kolkata, India; [2]Collaborative Research Center of Okayama University for Infectious Diseases at NICED, Kolkata, India; [3]The International Vaccine Institute, Seoul, Korea; [4]Menzies School of Health Research, Casuarina, Northern Territory, Australia; [5]Ministry of Health and Social Welfare, Zanzibar, Tanzania, [6]Public Health Laboratory, Pemba, Zanzibar

***Corresponding Author: Dr. Asish K. Mukhopadhyay**
Division of Bacteriology
National Institute of Cholera and Enteric Diseases,
P 33, CIT Road, Scheme XM, Beliaghata
Kolkata 700010
India
E-mail: asish_mukhopadhyay@yahoo.com
FAX: 91-33-2370-5066

Running Title: Analysis of *V. cholerae* in Zanzibar

Key words: *Vibrio cholerae, ctxB*, Cholera, PFGE

Abstract:

Analysis of 1,180 diarrheal stool samples in Zanzibar detected 247 *Vibrio cholerae* O1, Ogawa strains in 2009. Phenotypic traits and PCR based detection of *rstR, rtxC* and *tcpA* alleles showed them as El Tor biotype. Genetic analysis of *ctxB* of these strains revealed as classical type and production of classical CTB was confirmed by Western blotting. These strains produced higher amount of CT than the prototype El Tor and formed separate cluster by PFGE analysis.

Word count: 75

Introduction

Cholera infection still continues to be a substantial health burden in developing countries, due to lack of proper hygiene and sanitation infrastructure, especially in Africa and Asia. There was no published report of cholera in Africa for more than a century until the disease struck western regions in 1970. It quickly spread and became endemic across much of the continent, killing hundreds of people each year. Since 2000, the incidence of cholera has increased steadily, from 2010 to 2011 and the number of deaths increased by 3.5%. Cholera statistics released recently by the WHO have shown an 85% increase in the number of reported cholera cases in 2011 compared to the previous year (37). Recent cholera outbreaks in Cameroon, Haiti and Zimbabwe (20, 28, 31) provide an indication of alarmingly increasing propensity of cholera making it one of the major diseases in the global public health scenario.

Cholera is caused by the Gram-negative bacterium *Vibrio cholerae. V. cholerae* strains are classified into over 200 serogroups. The O1 serogroup is further classified into two biotypes, namely, classical and El Tor. Seven times since 1817, cholera has spread into the world in the form of pandemics. There is firm evidence that the fifth and sixth pandemics of cholera were caused by the classical biotype while the most extensive and ongoing seventh pandemic which started in 1961 is caused by the El Tor biotype (15). The report of new variant strains of *V. cholerae*, which had the characteristic of both El Tor and Classical biotypes, first appeared in 2002 (24) and then in 2004 (2), Studies from Asia and Africa revealed the emergence and dissemination of classical *ctxB* in El Tor biotype strains replacing the seventh pandemic El Tor prototype strains in most of the cholera endemic areas (1, 6, 23, 25, 29, 30, 32, 33).

Zanzibar, an archipelago, consists of two major islands, Unguja (also named Zanzibar) and Pemba. They are situated in the Indian Ocean about 40–60 km off the eastern coast of mainland Tanzania having population of about 1.1 million. During 2008, an increased number of cases occurred in the United Republic of Tanzania, with 7700 cases reported compared with 2911 in the previous year (WHO 2009). Cholera's new global incursion in Haiti after its absence of almost 100 years (4) and the rapidly growing genetic diversity among toxigenic *V. cholerae* strains with epidemic potential provided the impetus for molecular characterization of strains collected in Zanzibar in 2009. We put a special emphasis on CT genotypes along with the CTX prophages of the *V. cholerae* strains isolated from Zanzibar to understand whether the emerging El Tor variant has disseminated in this isolated region.

This study is part a surveillance program of Mass oral cholera vaccination in high-risk populations in Zanzibar supported by the International Vaccine Institute, Korea, the WHO and the Zanzibari Ministry of Health and Social Welfare. Stool samples were collected from patients with acute watery diarrhea cases during March to November, 2009 at four health care centers in Unguja (Chumbuni, Akbar, Kundi and Mnazi Moja Hospital), five centers from Pemba (Shamiani, Kengeja, Mwambe, Mtambili and Mkoani), and from a number of temporary cholera camps set up by the government in response to suspected outbreaks. Among the 1,180 samples collected from patients with acute diarrhea, 268 samples were positive for *V. cholerae*. Serotyping results with polyvalent O1, mono-specific Ogawa and Inaba antisera (Difco, USA) and monoclonal O139 antiserum (developed at NICED) established that 247 of the total *V. cholerae* isolates belonged to Ogawa serotype and the remaining 21 isolates were non-O1 non-O139. Month wise isolation profile showed

that there was a sudden increase in the isolation of *V. cholerae* O1 in July and September. We restricted our study with the O1 strains only in this study. All strains tested were resistant to polymyxin B and positive for Voges-Proskauer test suggesting that they were phenotypically El Tor.

Analysis of biotype specific *ctxB*: The *ctxB* gene of the *V. cholerae* O1 strains, which encodes the cholera enterotoxin B subunit were examined by the biotype specific primers as described elsewhere (21). Results from Mismatch amplification mutation assay (MAMA) PCR showed that all the strains (Fig 1) had classical *ctxB* allele in their CTX prophage. Reports of the emergence of novel variants of *V. cholerae* O1 El Tor strains with an additionally mutated CTB (6, 13, 22) prompted us to further characterize the *ctxB* allele of 50 representative strains which yielded positive amplicons for classical *ctxB* gene in MAMA-PCR. As described in our last report (22), we used Double mismatch amplification mutation assay (DMAMA) for this study. Our DMAMA results together with DNA sequence analysis data also reconfirmed our initial MAMA PCR results. The deduced amino acid sequences of the strains were found to be identical to the classical CTB (GenBank accession number JQ683131-36), with a histidine at position 39 and a threonine at position 68. N16961 and O395 were used as El Tor and classical reference strains in all cases.

Studies of other biotype specific markers: Further genetic characterization based on earlier studies (7, 8, 9, 15, 17, 27, 32) with primers specific for genes encoding RS1 element antirepressor rstC, transcriptional repressor rstR, toxin co-regulated pilus subunit A, and repeat in toxin C subunit (*rstC, rstR, tcpA* and *rtxC* respectively) was employed to reconfirm the biotype of the Zanzibar isolates. Table 1 summarizes our polymerase chain reaction (PCR) results which genetically characterize all of the 247 O1 isolates as of El Tor biotype.

Further PCR analysis with primers from different genetic segments of the CTX prophage and its downstream region confirmed the presence of intact an RS1 element upstream of the CTX prophage. All of the tested strains were found positive for the toxin like cryptic element (*tlc*). All of the primers used in this study have been enlisted in Table 2. Nucleotide sequences of the *rstR* gene from representative strain have been deposited in to GenBank under the accession numbers **JX312666-70.**

Analysis of the *ctxA* promoter region: Sequence analysis of the *ctxA* promoter region of representative *V. cholerae* O1 strains from Zanzibar revealed the presence of three tandem TTTTGAT heptanucleotide repeat. These repeat regions play an important role for binding the transcriptional activators ToxR (**16, 19**) and ToxT (**3, 38**). The analysis of the *ctxA* promoter region of *V. cholerae* O1 isolates from Kolkata showed 4 repeat units (Fig 2). The nucleotide sequence of the *ctxA* promoter region of five Zanibar isolates have been deposited into the GenBank under the accession numbers **JX144324-328**.

Chromosomal localization of CTX prophage along with its organization: All tested strains from Zanzibar yielded an amplicon of 766-bp in a Polymerase chain reaction (PCR) using CII-F and CII-R primers (Fig 3A). CII-F and CII-R primers flank the predicted CTX prophage integration site in the small chromosome of *V. cholerae*. **(18).** Presence of 766 bp amplicon indicated that the small chromosome of the Zanzibar strains was devoid of any CTX prophage in the specific position**.** The primers would have failed to amplify a DNA segment of around 7.8 kb under the provided PCR conditions if there had been a single copy of CTX prophage in this region, as with the case of O395. Nucleotide sequence of 766 bp region from 5 Zanzibar isolates have been deposited to the GenBank under the accession numbers **JX255488-92**. Analysis of this sequencing data revealed that there are neither any

remnants of CTX prophage nor any indication of mobility in this site. Furthermore, it also showed the precise location of CTX prophage insertion in the small chromosome of classical reference strain O395. Those strains, which lack CTX prophage in their small chromosomes (e.g. 2010EL-1786, M66-2 and IEC224), shared 99-100% sequence identity in this specific region with the Kolkata strains. The primer rstC1 and rtxA1 yielded ~ 9 kb amplicon (using XT 20 PCR system, Bangalore Genei, Bangalore, India) DNA fragment (Fig 3B) and suggested that *V. cholerae* O1 isolates from Zanzibar probably had single copy of CTX prophage. Fig 3C showed a schematic diagram of the copy number of CTX prophages with probable combination of *rstR* and *ctxB* alleles in the Zanzibar strains.

Measurement of CT production by Beads ELISA and confirmation of production of classical CT by the Zanzibar strains: The amount of CT produced was measured as described previously (**12, 36**) during the growth of the representative strains from Zanzibar in AKI medium and compared with prototype El Tor and classical strains. It was found that all the El Tor variant stains from Zanzibar produced significantly higher amounts of CT in vitro than most strains of prototype El Tor (using Mann-Whitney U test method $P<0.001$) (**Fig 4A**). Most of the El Tor strains produced <100 ng/ml/OD600 while all the classical strains produced >900 ng/ml/OD600. Western blot study using CTB specific monoclonal antibody also showed that the Zanzibar isolates produced classical CTB (**Fig 4B**).

Molecular typing by Pulsed-field gel electrophoresis (PFGE): PFGE analysis of sixteen representative strains from Zanzibar along with several reference strains from other parts of the world showed that the Zanzibar strains formed a homogeneous banding pattern (except one strain) and this pattern is different from Indian and other African strains isolated in recent times (**Fig 5**). Dendogram analysis using Bionumeric software (Applied Maths,

Belgium) showed that the Zanzibar strains formed a separate cluster indicating its different lineage (**Fig 5**).

Cholera is mainly endemic in low-income countries in Africa, Asia, Central and South America. In recent years, it has become endemic in an increasing number of geographical areas. In Zanzibar, a cholera outbreak with 411 cases and 51 deaths was reported for the first time in 1978 from a fishermen village (**34**). Before the recent study, we had very limited knowledge about the molecular epidemiology of *V. cholerae* isolated from these regions although recurrent outbreaks have been documented since 1978. To our knowledge, this is the first report elucidating the molecular characterization of cholera epidemiology from the archipelago. A growing number of published articles indicates that the *V. cholerae* O1 El Tor variant strains have replaced the seventh pandemic El Tor biotype strains in many parts around Africa and Asia. Siddique *et al* reported from a clinical study that large numbers of patients were admitted with more severe dehydration in Bakerganj and Mathbaria, hospitals in southern Bangladesh and all the *V. cholerae* O1 El Tor strains isolated from these patients produced classical CT (**35**). Two recently published reports (**12, 36**) also motivated us to speculate that a significant difference between the amounts of CT produced by these two biotype strains may reflect severity of clinical manifestation.

The selection of El Tor variant strain seems to be an evolutionary optimization of the El Tor biotype and could represent a new, more virulent form of the El Tor biotype. It would be interesting to know the lineages of the Zanzibar strains as the specific change in *ctxB* of El Tor strains was first observed in Kolkata during 1990 (**30**). These new *V. cholerae* O1 El Tor variant strains not only replaced the *V. cholerae* O1 El Tor prototype strains, but also turned out to be genetically stable and spread rapidly even to remote islands in the east African

continent as evidenced from this study. Moreover, the severity of the disease appears to be intensifying, and recent cholera outbreaks in various places, including Zimbabwe and Haiti, have followed protracted period (14, 28). An active holistic surveillance system should be in place in order to track the dissemination mode of the *V. cholerae* O1 El Tor variant strains in the population using latest molecular diagnostic assays, as these strains possess all the potentialities and foundation for a new pandemic. Moreover, a recent study by Reyburn et al (31) provided evidence from the temporal patterns of cholera cases reported between 2002 and 2008 in Zanzibar that rainfall and temperature, among various climate and ocean environmental factors are the key drivers of cholera outbreaks. Such predictive models may help public health authorities to prepare medical equipment, mobilize staff and stock / distribute mass oral cholera vaccination.

Acknowledgement:

The cholera project in Zanzibar received financial support from the Bill & Melinda Gates Foundation and was coordinated by the WHO Initiative for Vaccine Research, Geneva, Switzerland and the International Vaccine Institute, Seoul, Korea. Additional funding was provided by the Swedish International Development Cooperation Agency and the Republic of Korea. Part of this article has been published at the 45th Annual Joint Panel Meeting on Cholera and other Bacterial Enteric Infections Panel organized by United States-Japan Cooperative Medical Science Program at Kyoto, Japan during December 6-8, 2010. The laboratory work was supported in part by the Japan Initiative for Global

Research Network on Infectious Diseases (J-GRID) Ministry of Education, Culture, Sports, Science and Technology of Japan; and Indian Council of Medical Research, Government of India.

References:

1. **Ang, G. Y., Y. Y. Choo, B. Kamarudin, H. T. Elina, A. Hussin, M. H. Hani, and C. Y. Yean**. 2010. Molecular Evidence of Cholera Outbreak Caused by a Toxigenic *Vibrio cholerae* O1 El Tor Variant Strain in Kelantan, Malaysia. J. Clin. Microbiol. **48:**3963-3969.
2. **Ansaruzzaman, M., N. A. Bhuiyan, G. B Nair, D. A. Sack, M. Lucas, J. L. Deen, J. Ampuero, C. L. Chaignat, and The Mozambique Cholera Vaccine Demonstration Project Coordination Group** 2004. Cholera in Mozambique. Emerg. Infect. Dis. **10:**2057-2059.
3. **Champion, G. A., M. N. Neely, M. A. Brennan, and V. J. DiRita.** 1997. A branch in the ToxR regulatory cascade of *Vibrio cholerae* revealed by characterization of *toxT* mutant strains. Mol. Microbiol. **23:**323–331.
4. **Chao, D. L., M. E. Hallorana, and I. M. Longini, Jr. 2011**. **Vaccination strategies for epidemic cholera in Haiti with implications for the developing world.** Proc. Natl. Acad. Sci. U. S. A. **108**:7081–7085
5. **Chatterjee, S., T. Patra, K. Ghosh, A. Raychoudhuri, G. P. Pazhani, M. Das, B. Sarkar, R. K. Bhadra, A. K. Mukhopadhyay, Y. Takeda, G. B. Nair, T. Ramamurthy and R. K. Nandy.** 2009. *Vibrio cholerae* O1 clinical strains isolated in 1992 in Kolkata with progenitor traits of the 2004 Mozambique variant. J Med Microbiol. **58**:239–247. doi: 10.1099/jmm.0.003780-0.
6. **Chin, C. S., J. Sorenson, J. B. Harris, W. P. Robins, R. P. Charles, R. R. Jean-Charles, J. Bullard, D. R. Webster, A. Kasarskis, P. Peluso, E. E. Paxinos, Y. Yamaichi, S. B. Calderwood, J. J. Mekalanos, E. E. Schadt, and M. K. Waldor**. 2011. The Origin of the Haitian Cholera Outbreak Strain. The New England journal of medicine. **364:**33-42.
7. **Davis, B. M., K. E. Moyer, E. F. Boyd. &, M. K. Waldor.** 2009**.** CTX prophages in classical biotype Vibrio cholerae: functional phage genes but dysfunctional phage genomes. J Bacteriol **182**:6992–6998**.**
8. **Davis, B. M., and M. K Waldor.** 2003. Filamentous phages linked to virulence of *Vibrio cholerae*. Curr. Opin. Microbiol. **6**:35–42.

9. **Dziejman, M., E. Balon, D. Boyd, C. M. Fraser, J. F. Heidelberg, and J. J. Mekalanos**. 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.
10. **Finkelstein, R, A., M. F. Burks, A. Zupan, W. S. Dallas, C. O. Jacob, and D. S. Ludwig.** 1987. Epitopes of the cholera family of enterotoxins. Clin Infect Dis. **9**:544-56.1.
11. **Garg, S., T. Ramamurthy, A. K. Mukhopadhyay, B. C. Deb, G. B. Nair, T. Shimada, T. Takeda, A. Huq, R. R. Colwell, & Y. Takeda.** 1994**.** Production and cross reactivity pattern of panel of high affinity monoclonal antibodies to *Vibrio cholerae* O139 Bengal. FEMS Immunol Med Microbiol. **8**:293–298.
12. **Ghosh-Banerjee, J., M. Senoh, T. Takahashi, T. Hamabata, S. Barman, H. Koley, A. K. Mukhopadhyay, T. Ramamurthy, S. Chatterjee, M. Asakura, S. Yamasaki, G. B. Nair, and Y. Takeda.** 2010**.** Cholera Toxin Production by the El Tor Variant of *Vibrio cholera* O1 Compared to Prototype El Tor and Classical Biotypes. J. Clin. Microbiol. **48:**4283-4286.
13. **Goel, A. K., M. Jain, P. Kumar, S. Bhadauria, D. V. Kmboj, and L. Singh**. 2008. A new variant of *Vibrio cholerae* O1 El Tor causing cholera in India. J. Infect. **57:**280-281.
14. **Kanungo, S., B. K. Sah, A. L Lopez, J. S. Sung, A. M. Paisley, D. Sur, J. D. Clemens, and G. B. Nair. 2010**. Cholera in India: an analysis of reports, 1997–2006. Bulletin of the World Health Organization. **88**:185-191.
15. **Kaper, J. B., J.J. Morris Jr., and M. M. Levine**. 1995. Cholera. Clin. Microbiol. Rev. **8:**48–86.
16. **Li, C. C, J. A. Crawford, V. J. DiRita, and J. B. Kaper**. 2000. Molecular cloning and transcriptional regulation of ompT, a ToxR-repressed gene in *Vibrio cholerae*. Mol. Microbiol. **35**:189-203.
17. **Lin, W., K. J. Fullner, R. Clayton, J. A. Sexton, M. B. Rogers, K. E. Calia, S. B. Calderwood, C. Fraser, and J. J. Mekalanos.** 1999**.** Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. Proc. Natl. Acad. Sci. U. S. A. **96**:1071–1076.

18. **Maiti, D., B. Das, A. Saha, R. K. Nandy, G. B. Nair, and R. K. Bhadra. 2006.** Genetic organization of pre-CTX and CTX prophages in the genome of an environmental *Vibrio cholerae* non-O1, non-O139 strain. Microbiology. **152**:3633–3641.

19. Miller, V. L., R. K. Taylor, and J. J. Mekalanos. 1987. Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell **48:**271–279.

20. **Mintz, E. D, and R. L. Guerrant. 2009.** A Lion in Our Village — The Unconscionable Tragedy of Cholera in Africa. The New England journal of medicine. **360:**1060-1063.

21. **Morita, M., M. Ohnishi, E. Arakawa, N. A. Bhuiyan, S. Nusrin, M. Alam, A. K. Siddique, F. Qadri, H. Izumiya, G. B. Nair, and H. Watanabe.** 2008. 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. **52:**314–317.

22. **Naha. A., G. P. Pazhani, M. Ganguly, S. Ghosh, T. Ramamurthy, R. K. Nandy, G. B. Nair, Y. Takeda and A K. Mukhopadhyay.** 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.

23. **Nair, G. B., F. Qadri, J. Holmgren, A. M. Svennerholm, A. Safa, N. A. Bhuiyan, Q. S. Ahmad, S. M. Faruque, A. S. G. Faruque, Y. Takeda, and D. A. Sack.** 2006. Cholera Due to Altered El Tor Strains of *Vibrio cholerae* O1 in Bangladesh. J. Clin. Microbiol. **44:**4211-4213.

24. **Nair, G. B., S. M. Faruque, N. A. Bhuiyan, M. Kamruzzaman, A. K. Siddique, and D. A. Sack**. 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.

25. **Nguyen, B. M., J. H. Lee, N. T. Cuong, S. Y. Choi, N. T. Hien, D. D. Anh, H. R. Lee, M. Ansaruzzaman, H. P. Endtz, J. Chun, A. L. Lopez, C. Czerkinsky, J. D. Clemens, and D. W. Kim.** 2009. Cholera Outbreaks Caused by an Altered *Vibrio*

cholerae O1 El Tor Strain Producing Classical Cholera Toxin B in Vietnam in 2007 to 2008. J. Clin. Microbiol. **47:**1568-1571.

26. **Olsvik, O., J. Wahlberg, B. Petterson, M. Uhlen, T. Popovic, I. K. Wachsmuth, and P. I. Fields.** 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.
27. **O'Shea, A. Y., J. F. Reen, A. M. Quirke, & E. F. Boyd.** 2004. Evolutionary genetic analysis of the emergence of epidemic *Vibrio cholerae* isolates on the basis of comparative nucleotide sequence analysis and multilocus virulence gene profiles. J Clin Microbiol **42**, 4657–4671.
28. **Piarroux, R., R. Barrais, B. Faucher, R. Haus, M. Piarroux, J. Gaudart, R. Magloire, and D. Raoult.** 2011. Understanding the cholera epidemic, Haiti. Emerg Infect Dis.;**17**:1161-1167.
29. **Raychoudhuri, A., A. K. Mukhopadhyay, T. Ramamurthy, R. K. Nandy, Y. Takeda, and G. B. Nair.** 2008. Biotyping of *Vibrio cholerae* O1: Time to redefine the scheme. Indian J. Med. Res. **128:**695-698
30. **Raychoudhuri, A., T. Patra, K. Ghosh, T. Ramamurthy, R. K. Nandy, Y. Takeda, G. B. Nair, and A. K. Mukhopadhyay.** 2009. Classical *ctxB* in *Vibrio cholerae* O1, Kolkata, India. Emerg. Infect. Dis. **15:**131-132.
31. **Reyburn, R., J. L. Deen, R. F. Grais2, S. K. Bhattacharya, D. Sur, A. L. Lopez, M. S. Jiddawi, J. D. Clemens, and L. V. Seidlein**. 2011. The Case for Reactive Mass Oral Cholera Vaccinations. PLoS Negl Trop Dis. **5:**1-10.
32. **Safa A, G. B. Nair, and R. Y. C. Kong**. 2010. Evolution of new variants of *Vibrio cholerae* O1. Trends Microbiol. **18:** 46–54.
33. **Safa, A, J. Sultana, P. D. Cam, J. C. Mwansa, and R. Y.C. Kong. 2008.** *Vibrio cholerae* O1 Hybrid El Tor Strains, Asia and Africa. Emerg. Infect. Dis. **14:**987-988.
34. **Schaetti, C, R. Hutubessy, S. M Ali, A. Pach, M. G. Weiss, C.-L. Chaignat and A. M Khatib.** Oral cholera vaccine use in Zanzibar: socioeconomic and behavioural features affecting demand and acceptance. 2009. BMC Public Health. **9**:99. doi:10.1186/1471-2458-9-99.

35. **Siddique, A. K, G. B. Nair, M. Alam, D. A. Sack, A. Huq, A. Nizam, I. M. Longini JR., F. Qadri, S. M. Faruque, R. R. Colwell, S. Ahmed, A. Iqbal, N. A. Bhuiyan, and R. B. Sack**. 2010 El Tor cholera with severe disease: a new threat to Asia and beyond. Epidemiol Infect. **138**:347-52.

36. **Son, M., S., C. J. Megli, G. Kovacikova, F. Qadri, and R. K. Taylor. 2011.** Characterization of *Vibrio cholerae* O1 El Tor biotype variant clinical isolates from Bangladesh and Haiti, including a molecular genetic analysis of virulence genes. J. Clin. Microbiol. **49:**3739-3749.

37. **World Health Organization.** Cholera, 2011. 2012. Wkly. Epidemiol. Rec. **87:**289–304.

38. **Yu, R. R., and V. J. DiRita.** Regulation of gene expression in *Vibrio cholerae* by ToxT involves both antirepression and RNA polymerase stimulation. 2002. Mol. Microbiol. **43:**119–134.

39. **Zuckerman, J. N., L. Rombo, and A. Fisch**. 2007. The true burden and risk of cholera: implications for prevention and control. Lancet Infect Dis. **7:**521-530.

1

2 **Table 1**: Genetic characterization of the *V. cholerae* O1 strains isolated from Zanzibar.

Tested Strain	Bacteriology			Target genes and PCR results					
	Serogroup	**Serotype**	**Biotype**	***ctxB***	***rstR***	***tcpA***	***rstC***	***rtxC***	***tlc***
V. cholerae Zanzibar	O1	Ogawa	El Tor	C*	E*	E*	+	+	+
N16961	O1	Inaba	El Tor	E*	E*	E*	+	+	+
O395	O1	Ogawa	Classical	C*	C*	C*	-	-	+

3 C*: Classical type, E*: El Tor type

4 **Table 2**: Primer sequences, amplicons size and annealing conditions used in PCR assays

Primer	Primer Sequence 5'-3'	Amplicon size(bp)	Anneling(°C)	Reference
rtxA1	GCGATTCTCAAAGAGATGC	~2400[1]	54	**(27)**
ctxB common(F)	ACTATCTTCAGCATATGCACATGG			**(21**
Re-elt	CCTGGTACTTCTACTTGAAACA		55	
Rv-cla	CCTGGTACTTCTACTTGAAACG	191		
ctxB-F3	GTTTTACTATCTTCAGCATATGCGA		56	**(22)**
ctxB-F4	GTTTTACTATCTTCAGCATATGCGC		60	
ctxB (F)	GGTTGCTTCTCATCATCGAACCAC	460		**(26)**
ctxB (R)	GATACACATAATAGAATTAAGGAT		55	
rstR^{class}(F)	CTTCTCATCAGCAAAGCCTCCATC	474	50	**(5)**
rstR^{ET}(F)	GCACCATGATTTAAGATGCTC	501		
rstA3R	TCGAGTTGTAATTCATCAAGAGTG			
CIIF	CTCACGCTGAACAGCAAGTC	766	55	**(18)**
CIIR	TTGCTTGAATCGAAAGGACA			
tlcF	GATTGTGCG TCTTGCATTTAGG	2011	55	**(18)**
tlcR	GTGAATAAATCAGGTGTAATGTCG			
cep R	TTTAGCCTTACGAATTAAGCC	~3047[2]		
RstC1	AAC AGC TAC GGG CTT ATT C	245	55	**(27)**
RstC2	TGAGTTGCGGATTTAGGC			
zotF(S)	CGAGCTACCGCTACAAGGTGCTA	470	55	**This study**
ctxAR(S)	CGTGCCTAACAAATCCCGTCTGAG			

1

2

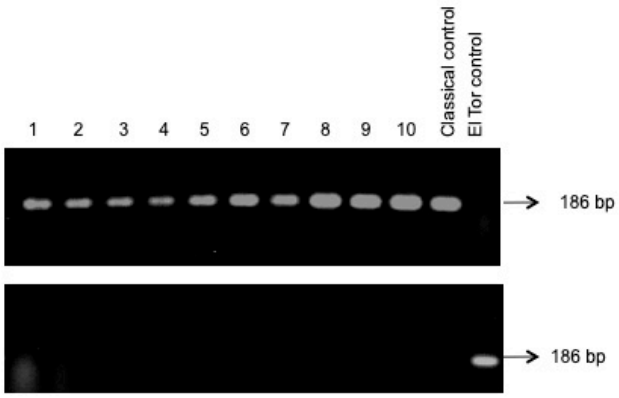

Figure 1

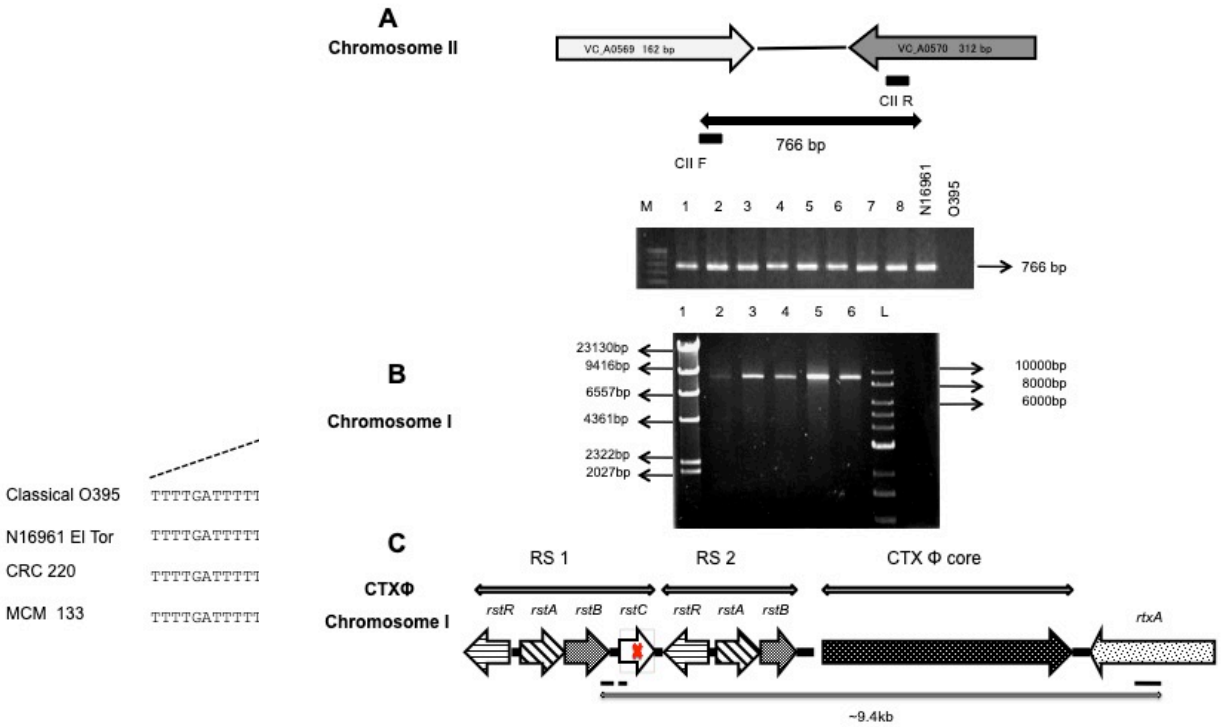

Figure 3

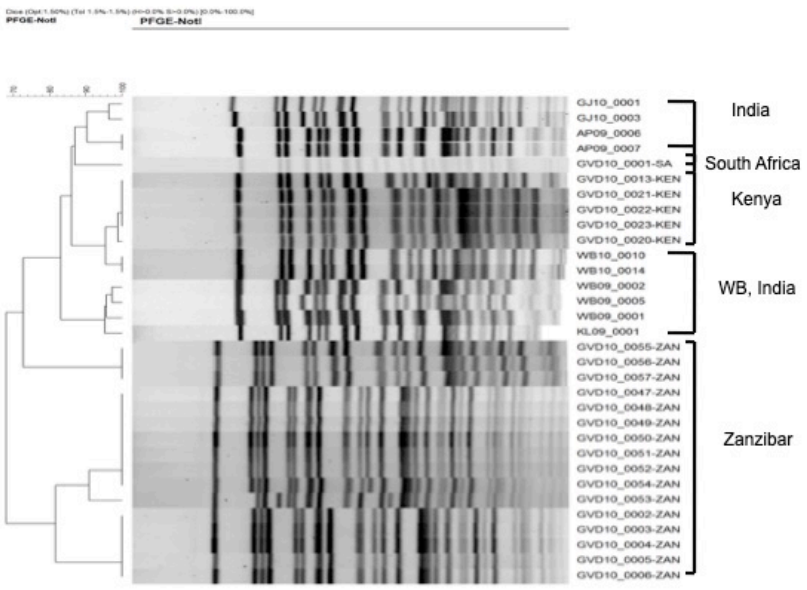
PFGE-NotI
PFGE-NotI
GJ10_0001
GJ10_0003
AP09_0006
AP09_0007
GVD10_0001-SA
GVD10_0013-KEN
GVD10_0021-KEN
GVD10_0022-KEN
GVD10_0023-KEN
GVD10_0020-KEN
WB10_0010
WB10_0014
WB09_0002
WB09_0005
WB09_0001
KL09_0001
GVD10_0055-ZAN
GVD10_0056-ZAN
GVD10_0057-ZAN
GVD10_0047-ZAN
GVD10_0048-ZAN
GVD10_0049-ZAN
GVD10_0050-ZAN
GVD10_0051-ZAN
GVD10_0052-ZAN
GVD10_0054-ZAN
GVD10_0053-ZAN
GVD10_0002-ZAN
GVD10_0003-ZAN
GVD10_0004-ZAN
GVD10_0005-ZAN
GVD10_0006-ZAN
India
South Africa
Kenya
WB, India
Zanzibar

Figure 5

Legends to Figures:

Figure 1: MAMA-PCR to detect the type of *ctxB* allele in representative *Vibrio cholerae* O1 strains isolated from Zanzibar, Africa, using primers (Fw-con/Rv-cla) for classical *ctxB* allele (Fig 1, upper panel) and Fw-con/Rv-elt for El Tor type *ctxB* allele (Fig 1, lower panel).. Lane 1: MCM 32, Lane 2: MCM 133, Lane 3: MCM 134, Lane 4: MCM 146, Lane 5: MCM 168, Lane 6: T1 Lane 7: MCF 084 Lane 8: MCF 001 Lane 9: WF 01 Lane 10: 210200, Lane 11: Classical control: 0395, Lane 12: El Tor control: N16961.

Figure 2: Comparative nucleotide sequence analysis of the promoter region the *ctxAB* operon (P_{ctxAB}) of Zanzibar isolate MCM 133 and Kolkata isolate CRC 220. The nucleotide sequences of P_{ctxAB} of O395 (classical control strain) and N16961 (El Tor control strain) were obtained from GenBank. Identical residues are indicated with dots. Each solid bar indicates the missing TTTTGAT heptads .The black arrow line represents the ATG start codon of *ctxA* gene. The Zanzibar isolate lacks a single heptad repeat in comparison with the Kolkata isolate.

Figure 3: PCR results implicating the chromosomal organization of CTX Φ of *Vibrio cholerae* O1 Ogawa isolates from Zanzibar. (A) Agarose gel electrophoresis showing the results of rstC1/rtxA1 PCR. Left M: lambda-Hind III ladder, Lane 1: MCM 133, Lane 2: MCM 168, Lane 3: KM 282, Lane 4: T1, Lane 5: WM 012: Right M: 1 kb DNA ladder.

(B). PCR results with primers CII F and CII R showing the absence of CTX prophage in chromosome II of Zanzibar isolates. The two black bars indicate the location of the two primers as shown in the figure. Extreme left include 100 bp ladder, 1: MCM 32, Lane 2: MCM 133, Lane 3: MCM 134, Lane 4: MCM 146, Lane 5: MCM 168, Lane 6: T1 Lane 7: MCF 084 Lane 8: MCF 001. El Tor control strain N16961 and classical control strain O395 were used as positive and negative controls, respectively.

(C) Predicted molecular organization of the CTX prophage of V. cholera Zanzibar isolates with probable combination of *rstR* and *ctxB* in their large chromosome. The solid and dotted bars indicate the location of the two primers.

Figure 4: (A) Amounts of cholera toxin production by Zanzibar variants, prototype El Tor strains and by classical strain. Error bars denote the standard error in taking each data in triplicate. (B) Western immunoblotting results of the culture supernatant of

representative Zanzibar O1 isolates. 100 ng each of the purified classical CT (lane 1) and El Tor CT (lane 2) were used as positive controls for immunoblotting with the monoclonal antibody against classical and El Tor CTB, respectively. Lane 3: CF04, Lane 4: MCF147, Lane 5: MCF100, Lane 6: MCM79, Lane 7: media (negative control). Numbers at left are molecular masses in kilodaltons.

Figure 5: PFGE patterns of the *Not*I digested *V. cholerae* strains from Zanzibar strains Dendogram analysis using Bionumeric software (Applied Maths, Sint-Martens-Latem, Belgium) shows three distinct clusters among the Zanzibar isolates tested. Sixteen representative strains were used for the study.