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

A. Naha,a G. Chowdhury,a J. Ghosh-Banerjee,a M. Senoh,b T. Takahashi,b B. Ley,c K. Thriemer,c J. Deen,c L. V. Seidlein,d S. M. Ali,a,f A. Khatib,a T. Ramamurthy,a R. K. Nandy,a G. B. Nair,a Y. Takeda,b A. K. Mukhopadhyay,a

National Institute of Cholera and Enteric Diseases, Kolkata, Indiaa; Collaborative Research Center of Okayama University for Infectious Diseases at NICED, Kolkata, Indiab; The International Vaccine Institute, Seoul, Republic of Koreac; Menzies School of Health Research, Casuarina, Northern Territory, Australiad; Ministry of Health and Social Welfare, Zanzibar, Tanzaniae; Public Health Laboratory, Pemba, Zanzibar, Tanzaniaf

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 that they belonged to the El Tor biotype. Genetic analysis of ctxB of these strains revealed that they were classical type, and production of classical cholera toxin B (CTB) was confirmed by Western blotting. These strains produced more CT than the prototype El Tor and formed a separate cluster by pulsed-field gel electrophoresis (PFGE) analysis.

Cholera infection 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. The incidence of cholera increased steadily from 2010 to 2011, and the number of deaths increased by 3.5%. Cholera statistics released recently by the WHO show an 85% increase in the number of reported cholera cases in 2011 compared to the previous year (1). Recent cholerat outbreaks in Cameroon, Haiti, and Zimbabwe (2–4) provide an indication of the alarming increase in the incidence of cholera, making it a major disease 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 across 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 (5). Reports of new variant strains of V. cholerae, which have characteristics of both the El Tor and classical biotypes, appeared first in 2002 (6) and then in 2004 (7). Studies from Asia and Africa revealed the emergence and dissemination of classical ctxB in El Tor biotype strains, which replaced the seventh-pandemic El Tor prototype strains in most of the areas where cholera is endemic (8–15).

Zanzibar, an archipelago off the coast of east Africa, consists of two major islands, Unguja (also named Zanzibar) and Pemba. They are situated in the Indian Ocean about 40 to 60 km off the coast of mainland Tanzania and have a population of about 1.1 million. During 2009, an increased number of cases occurred in the United Republic of Tanzania, with 7,700 cases being reported, compared with 2,911 in the previous year (15a). Cholera’s incursion into Haiti after an absence of almost 100 years (16) 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 cholera toxin (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.

(Part of this article was presented at the 45th Annual Joint Panel Meeting on Cholera and Other Bacterial Enteric Infections, organized by the United States-Japan Cooperative Medical Science Program, at Kyoto, Japan, 6 to 8 December 2010.)

This study was part of a surveillance program of mass oral cholera vaccination in high-risk populations in Zanzibar supported by the International Vaccine Institute of South Korea, the WHO, and the Zanzibari Ministry of Health and Social Welfare. Stool samples were collected from patients with acute watery diarrhea from March to November 2009 at four health care centers on Unguja (Chumbuni, Akbar, Kundi, and Mnaazi Moja Hospitals), five centers on Pemba (Shamiani, Kengeja, Mwambe, Mtambili, and Mkoani), and 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, monospecific Ogawa, and Inaba antisera (each from Difco Laboratories, Detroit, MI) and monoclonal O139 antiserum (16a) established that 247 of the total V. cholerae isolates belonged to the Ogawa serotype and the remaining 21 isolates were non-O1 non-O139. An isolation profile organized according to month showed that there was a sudden increase in the isolation of V. cholerae O1 in July and September. We restricted our study to the O1 strains obtained in this study. All strains tested were resistant...
to polymyxin B and positive in the 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, was examined with biotype-specific primers as described elsewhere (17). Results from mismatch amplification mutation assay (MAMA)-PCR showed that all the strains (Fig. 1) had the 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 (9, 18, 19) 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 (19), we used a double mismatch amplification mutation assay (DMAMA) for this study. Our DMAMA results together with DNA sequence analysis data also confirmed our initial MAMA PCR results. The deduced amino acid sequences of the strains were found to be identical to the classical CTB (GenBank accession numbers JQ683131 to JQ683136), with a histidine at position 39 and a threonine at position 68. N16961 and O395 were considered to be identical to the classical CTB (9, 18, 19) and 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 (5, 14, 20–24) with primers specific for genes encoding the RS1 element antirepressor RstC, the transcriptional repressor Rsr, toxin coregulated pilus subunit A, and repeat in toxin C (rstC, rsr, tcpA, and rtxC, respectively) was employed to confirm the biotype of the Zanzibar isolates. Table 1 summarizes our PCR results, which genetically characterize all of the 247 O1 isolates as 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 positive for the toxin-like cryptic element (*tlc*). All of the primers used in this study have been enlisted in Table 2.

**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 repeats. These repeat regions play an important role for binding the transcriptional activators ToxR (28, 29) and ToxT (30, 31). The analysis of the ctxA promoter region of *V. cholerae* O1 isolates from Kolkata showed 4 repeat units (Fig. 2).

**Chromosomal localization of CTX prophage along with its organization.** All tested strains from Zanzibar yielded an amplion of 766-bp in a 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* (27). The presence of a 766-bp amplicon indicated that the small chromosome of the Zanzibar strains was devoid of any CTX prophage in the usual position. The primers would have failed to amplify a DNA segment of around 7.8 kb under the PCR conditions used if there had been a single copy of CTX prophage in this region, as was the case with O395. Analysis of these 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. Strains which lack a CTX prophage in their small chromosomes (e.g., 2010EL-1786, M66-2, and IEC224) shared 99 to 100% sequence identity in this specific region with the Zanzibar strains. The primers rstC1 and rtxA1 yielded an ~9-kb amplicon (using the XT 20 PCR system; Bangalore Genei, Bangalore, India) DNA fragment (Fig. 3B), suggesting that *V. cholerae* O1 isolates from Zanzibar probably had a single copy of the CTX prophage. Figure 3C shows a schematic diagram of the copy number of CTX prophages with probable combinations of rstR and ctxB alleles in the Zanzibar strains.

**Measurement of CT production by bead ELISA and confirmation of production of classical CT by the Zanzibar strains.** The amount of CT produced was measured as described previously (30, 31), and the analysis of the promoter region of representative *V. cholerae* O1 strains isolated from Zanzibar revealed the presence of three tandem TTTTGAT heptanucleotide repeats. These repeat regions play an important role for binding the transcriptional activators ToxR (28, 29) and ToxT (30, 31). The analysis of the ctxA promoter region of *V. cholerae* O1 isolates from Kolkata showed 4 repeat units (Fig. 2).

**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 repeats. These repeat regions play an important role for binding the transcriptional activators ToxR (28, 29) and ToxT (30, 31). The analysis of the ctxA promoter region of *V. cholerae* O1 isolates from Kolkata showed 4 repeat units (Fig. 2).

**Chromosomal localization of CTX prophage along with its organization.** All tested strains from Zanzibar yielded an amplion of 766-bp in a 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* (27). The presence of a 766-bp amplicon indicated that the small chromosome of the Zanzibar strains was devoid of any CTX prophage in the usual position. The primers would have failed to amplify a DNA segment of around 7.8 kb under the PCR conditions used if there had been a single copy of CTX prophage in this region, as was the case with O395. Analysis of these 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. Strains which lack a CTX prophage in their small chromosomes (e.g., 2010EL-1786, M66-2, and IEC224) shared 99 to 100% sequence identity in this specific region with the Zanzibar strains. The primers rstC1 and rtxA1 yielded an ~9-kb amplicon (using the XT 20 PCR system; Bangalore Genei, Bangalore, India) DNA fragment (Fig. 3B), suggesting that *V. cholerae* O1 isolates from Zanzibar probably had a single copy of the CTX prophage. Figure 3C shows a schematic diagram of the copy number of CTX prophages with probable combinations of rstR and ctxB alleles in the Zanzibar strains.

**Measurement of CT production by bead ELISA and confirmation of production of classical CT by the Zanzibar strains.** The amount of CT produced was measured as described previously (32, 33) 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 strains from Zanzibar produced significantly larger amounts of CT in *vitro* than most strains of prototype El Tor (P < 0.001, Mann-Whitney U test) (Fig. 4A). Most of the El Tor strains produced <100 ng/ml/unit of optical density at 600 nm (OD600), while all the classical strains produced >900 ng/ml/OD600 unit. Western blot analysis using a CTB-specific monoclonal antibody also

---

**TABLE 1 Genetic characterization of the *V. cholerae* O1 strains isolated from samples from Zanzibar**

<table>
<thead>
<tr>
<th>Strain tested</th>
<th>Serogroup</th>
<th>Serotype</th>
<th>Biotype</th>
<th>PCR result for target gene&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>N16961</td>
<td>O1</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>C, E, E, C, C, E, E, E, C, C, C, C</td>
</tr>
</tbody>
</table>

<sup>a</sup> C, classical; E, El Tor.

---
showed that the Zanzibar isolates produced classical CTB (Fig. 4B).

**Molecular typing by pulsed-field gel electrophoresis (PFGE).**
PFGE analysis of 16 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 recently (Fig. 5). Dendrogram analysis using Bionumeric software (Applied Maths, Belgium) showed that the Zanzibar strains formed a separate cluster, indicating their different lineage (Fig. 5).

Cholera is endemic mainly in low-income countries in Africa, Asia, and 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 fishing village (34). Before the present 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 study at the molecular level of cholera epidemiology in the archipelago. A growing number of published articles indicate that the *V. cholerae* O1 El Tor variant strains have replaced the seventh-pandemic El Tor biotype strains in many areas in Africa and Asia. Siddique et al. found in a clinical study that large numbers of patients were admitted with severe dehydration in Bakerganj and Mathbaria hospitals in southern Bangladesh and that all the *V. cholerae* O1 El Tor strains isolated from these patients produced classical CT (35). Two recently published reports (32,33) also motivated us to speculate that a significant difference between the amounts of CT produced by strains of

**TABLE 2** Primer sequences, amplicon sizes, and annealing conditions used in PCR assays

<table>
<thead>
<tr>
<th>Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primer sequence (5'–3')</th>
<th>Amplicon size (bp)</th>
<th>Annealing temp (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rtxA1</td>
<td>GGATTTTCCTAAGAGATGCG</td>
<td>~2,400</td>
<td>54</td>
<td>24</td>
</tr>
<tr>
<td>Fw-con</td>
<td>ACTACCTTCAGCATATGCAATGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv-elt</td>
<td>CTCGTTACTCTACTGGAAAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv-cla</td>
<td>CTCGTTACTCTACTGGAAAAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctxB-F3</td>
<td>GTTTTTATATCTCATGATGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctxB-F4</td>
<td>GTTTTTATATCTCATGATGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctxB (F)</td>
<td>GATGTGCTTGCATGCGACCAC</td>
<td>~460</td>
<td>54</td>
<td>25</td>
</tr>
<tr>
<td>ctxB (R)</td>
<td>GATACACAATAAGAATTAAAGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rstR&lt;sup&gt;class&lt;/sup&gt;-F</td>
<td>CTTCTCATTGCAAGGCGTTCATC</td>
<td>~55</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>rstR&lt;sup&gt;ET&lt;/sup&gt;-F</td>
<td>GACACATGATTTAAGATGCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rstA3R</td>
<td>TGGAGTIGTAATCTGCAAGGGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIIIF</td>
<td>CTGCCCTAAGCGAAAGTCA</td>
<td>~766</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>CIIR</td>
<td>TGCTTGAATCGGTTAAGAGCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tlc (F)</td>
<td>GATGTTGCGTTTGCTGATTAGG</td>
<td>~2,011</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>tlc (R)</td>
<td>GTAAATTACAGTTGTTAAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cep (R)</td>
<td>TTTGCCCTTACGGAATTAAAG</td>
<td>~3,047</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>rstC1</td>
<td>AAACGTAGGCGTTATCAC</td>
<td>245</td>
<td>55</td>
<td>24</td>
</tr>
<tr>
<td>rstC2</td>
<td>GAAGGTCCGAAAGGACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zotF(S)</td>
<td>CGAGCTAGGCTAAGAGGGTCTA</td>
<td>~470</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>ctxAR(S)</td>
<td>GTGCCTAAACACACGTGTGAG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> F, forward; R, reverse.

**FIG 2** Comparative nucleotide sequence analysis of the promoter region the *ctxAB* operon (P<sub>ctxAB</sub>) of Zanzibar isolate MCM 133 and Kolkata isolate CRC 220. The nucleotide sequences of P<sub>ctxAB</sub> 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 one more heptad repeat than the Kolkata isolate.
these two biotypes may reflect the severity of clinical manifestation.

The selection of the 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 (13). 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 re-

**FIG 3** PCR results implicating the chromosomal organization of the CTX prophage of Vibrio cholerae O1 Ogawa isolates from Zanzibar. (A) PCR results with primers CII F and CII R showing the absence of the CTX prophage in chromosome II of Zanzibar isolates. The two black bars indicate the locations of the two primers. Lane M, 100-bp DNA ladder; 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. El Tor control strain N16961 and classical control strain O395 were used as positive and negative controls, respectively. (B) Agarose gel electrophoresis showing the results of PCR with primers rstC1 and rtxA1. Lane L, lambda-HindIII DNA ladder; lane 1, MCM 133; lane 2, MCM 168; lane 3, KM 282; lane 4, T1; lane 5, WM 012; lane M, 1-kb DNA ladder. (C) Predicted molecular organization of the CTX prophage of V. cholerae isolates from Zanzibar with a probable combination of rstR and ctxB in their large chromosome. The black bars indicate the locations of the two primers rstC1 and rtxA1.

**FIG 4** (A) Amounts of cholera toxin production by Zanzibar variants, prototype El Tor strains, and the classical strain. Error bars show standard errors for results of tests done in triplicate. (B) Western immunoblotting results of the culture supernatant of representative Zanzibar O1 isolates. Samples of 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, medium only (negative control). Numbers at left are molecular masses, in kilodaltons.
mote islands off the eastern coast of Africa, as evidenced by this study. Moreover, the severity of the disease appears to be intensifying, and recent cholera outbreaks in various places, including Zimbabwe and Haiti, have been protracted (3, 36). An active holistic surveillance system should be in place in order to track the dissemination of the \textit{V. cholerae} O1 El Tor variant strains in the population using the latest molecular diagnostic assays, as these strains possess all the necessary characteristics for a new pandemic. Moreover, a recent study by Reyburn et al. (4) 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 and distribute mass oral cholera vaccines.

\textbf{Nucleotide sequence accession numbers.} Nucleotide sequences of the \textit{rstR} gene from a representative strain have been deposited in GenBank under the accession numbers JX312666 to JX312670. The nucleotide sequences of the \textit{ctxA} promoter region of five Zanzibar isolates have been deposited in GenBank under the accession numbers JX144324 to JX144328. The nucleotide sequences of the 766-bp region from five Zanzibar isolates have been deposited in GenBank under the accession numbers JX255488 to JX255492.

\textbf{ACKNOWLEDGMENTS}

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, South Korea. Additional funding was provided by the Swedish International Development Cooperation Agency and the Republic of Korea. The laboratory work was supported in part by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) of the Ministry of Education, Culture, Sports, Science and Technology of Japan and by the Indian Council of Medical Research, Government of India.

\textbf{REFERENCES}

6. Nair GB, Faruque SM, Bhuiyan NA, Kamruzzaman M, Siddique AK, Sack DA. 2002. New Variants of \textit{Vibrio cholerae} O1 biotype El Tor with...


