JOURNAL OF CLINICAL MICROBIOLOGY, May 2009, p. 1568–1571 Vol. 47, No. 5
0095-1137/09/$08.00+0 doi:10.1128/JCM.02040-08

Cholera Outbreaks Caused by an Altered *Vibrio cholerae* O1 El Tor Biotype Strain Producing Classical Cholera Toxin B in Vietnam in 2007 to 2008▽

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Received 21 October 2008/Returned for modification 22 January 2009/Accepted 5 March 2009

***Vibrio cholerae* O1 isolates collected during cholera outbreaks occurring from late 2007 to early 2008 in northern Vietnam were revealed to represent an altered strain containing the RS1 element followed by a CTX prophage harboring El Tor type *rstR* and classical *ctxB* on the large chromosome.**

Cholera continues to impose a major toll of both epidemic and endemic proportions worldwide, accounting for an estimated 120,000 deaths annually in spite of the advances in rehydration therapy and disease management (12). Cholera is caused by the gram-negative toxigenic bacterium *Vibrio cholerae*. More than 200 serogroups of *V. cholerae* are known, but only serogroups O1 and O139 cause epidemic and pandemic cholera (12). The O1 serogroup of *V. cholerae* is categorized into one of two biotypes, classical and El Tor, based on the results of assays such as agglutination of chicken red blood cells, Voges-Proskauer reaction, and polymyxin B resistance tests (7). Cholera toxin (CT) is encoded by the *ctxAB* gene, which is located on the CTX prophage integrated on the *V. cholerae* chromosome (7). The classical toxin (epitype CT1) is produced by classical biotype strains and U.S. Gulf Coast strains, while the El Tor type toxin (CT2) is generated by El Tor biotype strains and O139 strains (10). These two toxins differ in the CT B subunit by 2 out of 124 amino acids, and a PCR-based method to discriminate the classical type *ctxB* and El Tor type *ctxB* was recently described elsewhere (9).

Beginning in the late 1990s, El Tor biotype strains with the classical biotype traits (hybrid strains or Matlab variants) and El Tor strains producing classical toxin (altered strains) have been described previously (10, 13). Among the altered strains, two types of CTX prophages containing classical *ctxB* have been reported. One is the CTX prophage harboring classical *rstR* and classical *ctxB* genes, as seen in the *V. cholerae* isolates collected in Mozambique (4, 8), and the other is the CTX prophage containing El Tor *rstR* and classical *ctxB*. The Mozambican strain contains a tandem repeat of the classical CTX prophage on the small chromosome. In contrast, the altered strains vary by their CTX prophage type and presence of the RS1 element, and the exact array of the CTX prophage and the RS1 element on the genome of these strains has yet to be clarified (13).

Before 2005, only a few cases of cholera were reported in the northern part of Vietnam. However, by the end of 2007, major outbreaks of cholera were occurring in this region (14). The first case of cholera was reported on 23 October 2007 in Hanoi, Vietnam, and up until 11 April 2008, a cumulative total number of 3,271 cases were reported from 18 northern provinces in Vietnam.

In this study, we analyzed 70 *V. cholerae* isolates which were collected from patients and the physical environment in northern Vietnam during three cholera outbreaks between November 2007 and February 2008. Of the 70 isolates, 38 were collected in 2007 and 32 were collected in 2008. Isolates were collected as follows: 50 from patients, 10 from people who were in contact with patients, 5 from water samples, 3 from food, and 2 from vegetables. For comparison, 12 isolates collected from the same area of Vietnam between 1995 and 2004 were also analyzed (one collected in 1995, two collected in 2002, three collected in 2003, and six collected in 2004). All of the 70 isolates were identified as being of the *V. cholerae* O1 Ogawa serotype. By use of the polymyxin B sensitivity and Voges-Proskauer tests, all of the isolates were classified as being of the El Tor biotype (Table 1).

The genetic structures of the CTX prophage and RS1 element on the genome of Vietnamese isolates were determined by a number of PCR and sequencing. Primers used in this study and their sequences are listed in Table 2, and their locations are shown in Fig. 1.

***rstR* type.** Only the El Tor type-specific primer set (rstRETF/rstAR) amplified a DNA fragment from the Vietnamese isolates. The DNA sequences of the amplified fragments from the Vietnamese isolates and from the El Tor biotype reference strain N16961 were found to be identical. No DNA fragment was amplified from the Vietnamese isolates with the classical *rstR*-specific primer set (rstRclaF/rstAR). For additional confirmation of the absence of other types of *rstR*, namely, $rstR^{Cal}$ (O139 type) and $rstR^{env}$ (environmental type), we used PCR

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▽ Published ahead of print on 18 March 2009.

TABLE 1. Biotype characterization of *V. cholerae* O1 isolates from Vietnam

Strain(s)	Voges-Proskauer test result[a]	Sensitivity to polymyxin B (50 IU)[b]	*rstR* type	*ctxB* type	MLST type[c]
Vietnamese strains (n = 70)	+	R	El Tor	Classical	1,1,1,2,1,1,1,1,1
O395 (classical)	−	S	Classical	Classical	1,1,12,2,3,1,1,6,1
N16961 (El Tor)	+	R	El Tor	El Tor	1,1,1,2,1,1,1,1,1
B33 strain	+	R	Classical	Classical	1,1,1,2,1,1,1,1,1

[a] +, positive; −, negative.
[b] R and S indicate resistance and sensitivity, respectively.
[c] MLST loci in the order *dnaE*, *lap*, *rstA*, *gmd*, *recA*, *pgm*, *gry*, *chi*, and *cat*.

primers rstRCalF/rstRCalR and rstREnvF/rstREnvR, and no PCR product was identified from the Vietnamese isolates (11).

Presence and the location of *rstC* (RS1). A DNA fragment was amplified from the Vietnamese isolates with the *rstC*-specific primer set rstCF/rstCR, which implies that there is a *rstC* gene(s) in the genome. With the primer pair ctxBF/rstCR, a CTX prophage-RS1 array can be determined and, similarly, an RS1-CTX prophage array can be identified with the rstCF/cepR primer set. A DNA fragment was amplified from the Vietnamese isolates only by the rstCF/cepR primer pair, which suggests that the RS1-CTX prophage array exists on the genome of the Vietnamese isolates. The same primer pair, rstCF/cepR, produced identical DNA fragments from all of the 70 Vietnamese isolates, which indicates that all of the isolates contain the same RS1-CTX prophage array on the genome.

Absence of the tandem repeat of CTX prophage and RS1. The presence or absence of a tandem repeat of CTX prophage can be determined by using the primer pair ctxBF/cepR. No DNA fragment was amplified from the Vietnamese isolates, indicating that a tandem repeat of the CTX prophage does not exist on the genome. A tandem repeat of RS1 element can be verified by the PCR primer set rstCF4/rstCR4; however, no DNA fragment was amplified from the Vietnamese isolates, implying that they do not have a tandem repeat of RS1 on their genome.

Location of RS1-CTX prophage array. Two primer pairs, Ch1F/rstAR and ctxBF/Ch1R, produced corresponding DNA fragments from the Vietnamese isolates, indicating that the RS1-CTX prophage array is located on the large chromosome. The sequences of the DNA fragment encompassing the RS1 element and the DNA fragment encompassing the *ctxB* and downstream integration site were deposited in GenBank (Fig. 1). The absence of CTX prophage or RS1 on the small chromosome was confirmed by the PCR product of primer pair Ch2F/Ch2R, and the DNA sequence of this fragment was deposited in GenBank.

TABLE 2. Primer sequences used in this study[a]

Primer	Sequence	Reference(s)
Ch1F	GACCACTCAGGCCGCTGAAAT	6
Ch1R	CCGCGCTCAAGTGGTTATCGG	6
Ch2F	AACAACAGGTTGCAAGAGAGCATT	6
Ch2R	TATTGCTTTTTTAATGGCCGTT	6
rstRclaF	TTTGCTACTTCTTCTTGGTT	11
rstRETF	TGAGCATAAGCTCTTGATTT	6, 11
rstAR	CCGTGAAAGTCATCAACG	6
rstCF	GATGTTTACGATAGCCTAGAAGACTT	6
rstCR	TACAGTGATGGCTCAGTCAATGC	6
rstCF4	AAATCCGCAACTCAAGGCATTGA	6
rstCR4	TAAGCGCCTGAACGCAGATATAAAG	6
ctxBF	AGATATTTTCGTATACAGAATCTCTAG	6
cepR	AAACAGCAAGAAAACCCCGAGT	6
rstRCalF	TCAAGCTTTTTTTTGCTTTATCTTA	2, 11
rstRCalR	TGGCAACAAAGCACATTAAAGA	2, 11
rstREnvF	GCTTCATTTGTGTATTGGTCTATTAGGT AGTTA	11
rstREnvR	TCGAGTTGTAATTCATCAAGAGTGAAAA	11

[a] Primers were designed in this study based on the sequences described in the references.

Toxin type determination. From the DNA sequence of the *ctxB* fragment amplified with primer set ctxBF/Ch1R, we identified His and Thr at the 39th and 68th amino acid positions, which shows that this *ctxB* gene is of the classical type (10).

Multilocus sequence typing (MLST) analysis. The allele profile for the nine loci (*dnaE*, *lap*, *rstA*, *gmd*, *recA*, *pgm*, *gyrB*, *cat*, and *chi*) of the Vietnamese isolates was found to be the same as that for the seventh pandemic cholera strain, N16961, and for the Mozambican strain (Table 1) (5, 8).

We also analyzed *V. cholerae* O1 strains from countries neighboring Vietnam, namely, four clinical isolates collected from the cholera patients during a recent outbreak in the Lao People's Democratic Republic (February 2008) and two altered strains from Bangladesh, AR-32732 and MQ-1194, isolated in 1999 and 2001, respectively (1). They were found to contain the same RS1-CTX prophage array (El Tor *rstR* and classical *ctxB*) as seen in the isolates from the Vietnamese outbreaks in 2007 and 2008. We also collected and analyzed 400 *V. cholerae* isolates from Kolkata, India, between 2003 and 2007 and observed that these Indian isolates also contain the same RS1-CTX prophage array (data not shown). An analysis of their genetic relatedness is currently being conducted.

Interestingly, 12 isolates that were collected between 1995 and 2004 in northern Vietnam were identified being of as serotype O1 Inaba and biotype El Tor, and they contained a tandem repeat of the classical CTX prophage on the small chromosome, similar to the Mozambican isolates (8). A few of the isolates have an additional classical CTX prophage on the large chromosome (data not shown). This suggests that the isolates responsible for the 2007 and 2008 epidemics in Vietnam were recently introduced into the area. The isolates collected between 1995 and 2004 in Vietnam were not responsible for any major cholera outbreak in recent decades, while the similar *V. cholerae* strains were isolated during a series of outbreaks in Mozambique in 2003. The differences among the altered strains with respect to their ability to cause epidemics, as well as their genetic backgrounds, should be further elucidated.

Our findings indicate that the altered strains are prevalent in

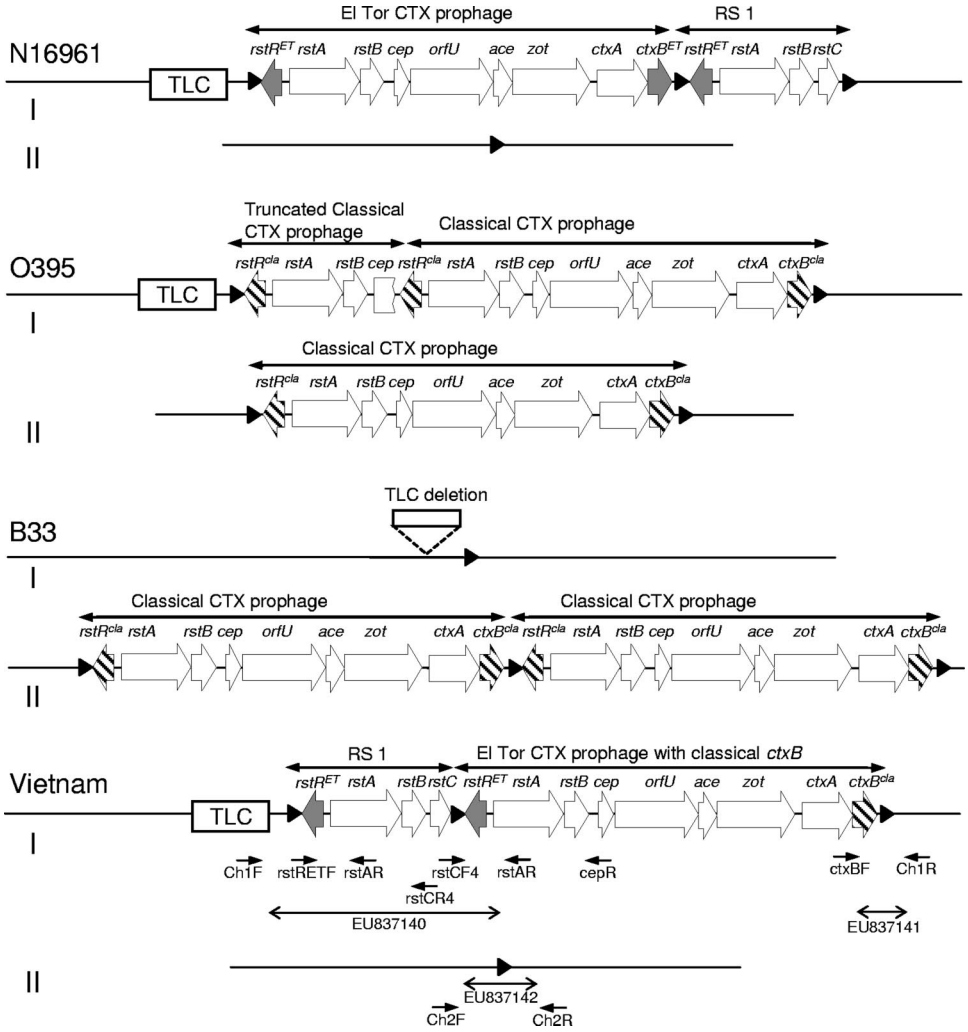

FIG. 1. Genetic map of the CTX prophage and RS1 element in *V. cholerae* O1 strains. For comparison, the CTX prophage and the RS1 element on each chromosome of the El Tor reference strain N16961 (6), the classical biotype reference strain O395 (3), the Mozambican strain B33 (8), and the presumed genetic map of Vietnamese strain are shown. Locations of primers and the sequenced regions with GenBank accession numbers are shown on the map of the Vietnamese strain. Black triangles represents the CTX phage integration site. Superscript terms *cla* and *ET* refer to the classical and El Tor type genes, respectively. TLC, toxin-linked cryptic plasmid.

south and southeast Asia, and they may be gradually spreading globally. More information about strains from other continents should be investigated in order to monitor the global distribution of the hybrid and altered strains.

Nucleotide sequence accession numbers. The DNA sequences for the fragment encompassing the RS1 element and the fragment encompassing *ctxB* and the downstream integration site were deposited in GenBank under accession numbers EU837140 and EU837141, respectively. The DNA sequence of the Ch2F/Ch2R primer pair PCR product was deposited in GenBank under accession number EU837142.

This work was supported by the Cholera Vaccine Initiative, funded by the Bill and Melinda Gates Foundation. The International Vaccine Institute is supported by the governments of Korea, Sweden, and Kuwait. J.H.L., S.Y.C., and D.W.K. were supported by grant number RTI05-01-01 from the Regional Technology Innovation Program of the Ministry of Knowledge and Economy and grant R01-2006-000-10255-0 from the Basic Research Program of the Korea Science and Engineering Foundation.

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