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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

Arindam Naha,[a] G. P. Pazhani,[a] Mou Ganguly,[a] Santanu Ghosh,[a] T. Ramamurthy,[a] Ranjan K. Nandy,[a] G. Balakrish Nair,[a] Yoshifumi Takeda,[b] and Asish K. Mukhopadhyay[a]

National Institute of Cholera and Enteric Diseases, Kolkata, India,[a] and Collaborative Research Center of Okayama University for Infectious Diseases at NICED, Kolkata, India[b]

A PCR-based assay was developed to discriminate the classical, El Tor, and Haitian types of *ctxB* alleles. Our retrospective study using this newly developed PCR showed that Haitian *ctxB* first appeared in Kolkata during April 2006, and 93.3% of strains isolated during 2011 carried the new allele. Dendrogram analysis showed a pulsed-field gel electrophoresis (PFGE) pattern of the new variant strains isolated recently that was distinct from the PFGE pattern of the strains carrying classical *ctxB* that closely matched the 2006 to 2007 variant strains.

Cholera still continues to be an important cause of human infection, especially in developing countries that lack access to safe drinking water and proper sanitation. The recent devastating cholera outbreak in Haiti (13), for the first time in almost a century, placed this ancient disease at the forefront of the global public health agenda. In May 2011, the World Health Assembly recognized the reemergence of cholera as a significant global public health problem and called for the implementation of an integrated and comprehensive global approach to cholera control (17). This dreadful diarrheal disease is caused by the Gram-negative toxigenic bacterium *Vibrio cholerae* (7). To date, more than 200 serogroups of *V. cholerae* are known, but only serogroups O1 and O139 cause epidemic and pandemic cholera (7, 16). To date, the world has experienced seven pandemics of cholera. Among these, the first six were caused by the classical biotype strains, whereas the ongoing seventh pandemic has been caused by the El Tor biotype (16). In recent years, the emergence and dissemination of novel pathogenic variants of *V. cholerae* O1 throughout many Asian and African countries (1, 2, 3, 5, 9, 10, 11, 14, 15) indicated a cryptic change in cholera epidemiology. Our recent study showed that the El Tor variant strains of *V. cholerae* O1 have replaced the prototype El Tor biotype strains in Kolkata, India, since 1995 (15). This report, together with the recent massive cholera outbreak in Haiti, caused by *V. cholerae* organisms with a mutation in the 58th nucleotide of *ctxB* (3), motivated us to investigate the emergence and dissemination of this new variant of *V. cholerae* O1 biotype El Tor strains, if any, in Kolkata.

In this study, we have developed a double-mismatch-amplification mutation assay (DMAMA) to accurately discriminate the classical, El Tor, and Haitian type *ctxB* alleles through a rapid and simple PCR-based assay. A total of 142 *V. cholerae* O1 strains were included in this study. These strains were selected from the repository of the National Institute of Cholera and Enteric Diseases, Kolkata, India, covering different months of each year from 2004 to 2011. *V. cholerae* O1 strains O395 (serotype Ogawa), N16961 (serotype Inaba), and EL-1786 (Ogawa, El Tor) were used as standard strains for the classical, El Tor, and Haitian type, respectively.

Development of the DMAMA-PCR. All 142 tested strains, along with the control strains, were grown in Luria-Bertani broth (Becton Dickinson, Sparks, MD) for 18 h and then streaked on Luria agar (LA) plates. In this study, we focused on *ctxB* in *V. cholerae* O1 strains to confirm the strains carrying Haitian, classical, and El Tor alleles in a simple PCR-based assay. Current methods for differentiating the biotype-specific cholera toxin B (CTB) subunit of *V. cholerae* O1 necessitate MAMA-PCR with biotype-specific primers, nucleotide sequencing of the *ctxB* allele, or an enzyme-linked immunosorbent assay (ELISA) using classical or El Tor CT-specific monoclonal antibodies. Among these, the first has been the method of choice as it is simple and less time consuming. However, reports on influxes of new variant strains of *V. cholerae* O1 with an additional mutation at the 20th amino acid position (58th nucleotide position) clearly point out its limitation in the discrimination of *ctxB* genotypes. The previously published MAMA-PCR (8) is based on two biotype-specific reverse primers, each bearing a mismatch at nucleotide position 203 and, hence, incapable of identifying the Haitian type *ctxB* allele. Therefore, for discriminating the classical, El Tor, and Haitian type *ctxB* alleles, DMAMA-PCR was designed and validated in this study. We designed two allele-specific polymorphism detection forward primers, *ctxB*-F3 and *ctxB*-F4, each bearing a mismatch at its 3′ end (Table 1). These allele-specific primers each carry specific nucleotides, A and C, for the Haitian and classical allele, respectively, at the 3′ end. Furthermore, we enhanced the 3′ mismatch effect by introducing another nucleotide, G (instead of A), at the second nucleotide position (i.e., the 57th nucleotide) from the 3′ end of both primers. We used the *ctxB* reverse primer that is specific for the classical biotype (Rv-cla), as described by Morita et al. (8), as the conserved reverse primer. As shown in Fig. 1A, the DMAMA-PCR successfully discriminated the three different allelic subtypes

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Address correspondence to Asish K. Mukhopadhyay, asish_mukhopadhyay@yahoo.com.

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TABLE 1 Primer sequences, amplicon size, and annealing temperature used in PCR assays

Primer	Sequence (5′–3′)	Amplicon size (bp)	Annealing (°C)	Reference
Rv-cla	CCTGGTACTTCTACTTGAAACG			8
ctxB-F3	GTTTTACTATCTTCAGCATATGCGA	191	56	This study
ctxB-F4	GTTTTACTATCTTCAGCATATGCGC	191	60	This study
ctxB-F	GGTTGCTTCTCATCATCGAACCAC	460	55	12
ctxB-R	GATACACATAATAGAATTAAGGAT			

of *ctxB*. *V. cholerae* O1 strains having the *ctxB* allele of genotype 7 yielded a 191-bp fragment of DNA with the primer pair *ctxB*-F3/Rv-cla but not with *ctxB*-F4/Rv-cla. The classical control strain (O395) produced just the opposite result with the same primer sets, and the El Tor strain (N16961) did not show any amplicon in either PCR assay due to the double mismatch in the forward and reverse primers (Fig. 1A).

Sequencing analysis to evaluate the PCR-based result. To further confirm our PCR results, 14 representative strains, which yielded positive bands for the Haitian *ctxB* gene by DMAMA-PCR, were selected for DNA sequencing. For sequencing, a separate pair of primers (*ctxB*-F and *ctxB*-R) was used to provide the sequences of the whole *ctxB* genes. Nucleotide sequence analysis of the *ctxB* genes of the 14 representative strains of *V. cholerae* O1

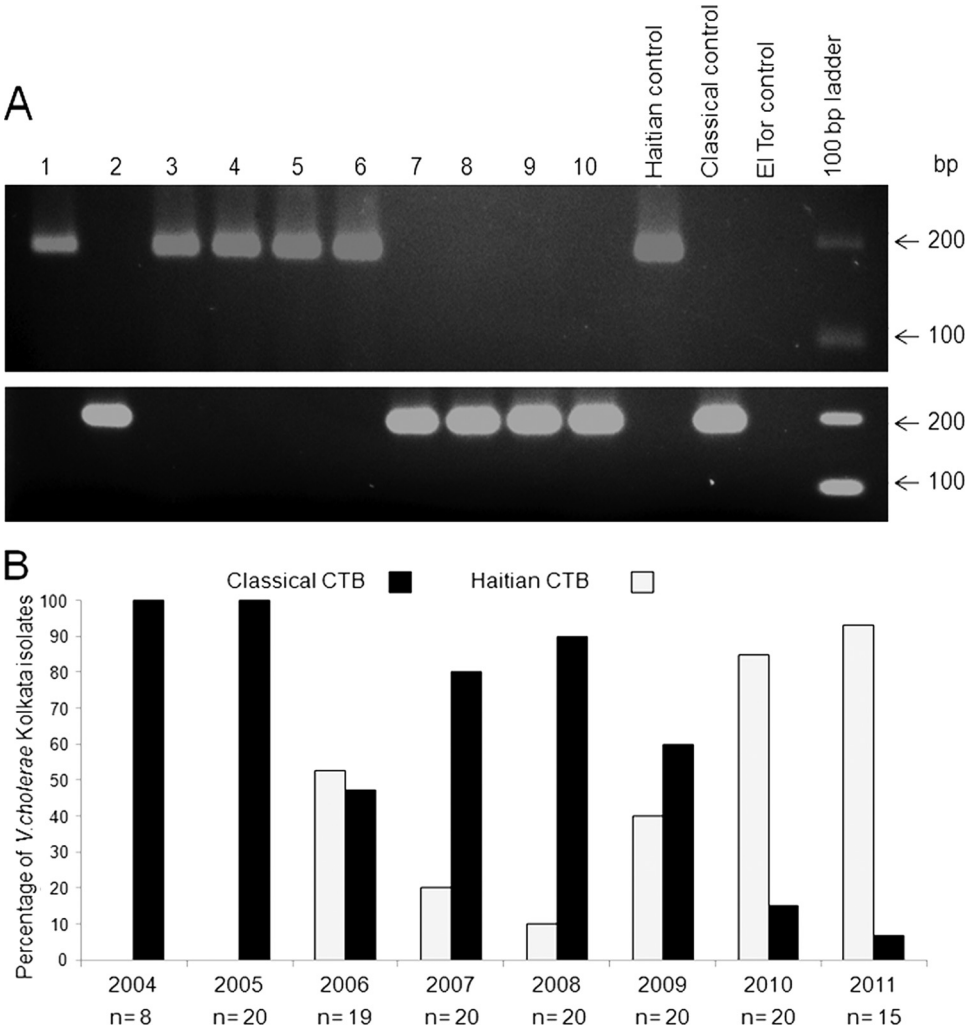

FIG 1 (A) DMAMA-PCR to detect the type of *ctxB* allele in representative *Vibrio cholerae* O1 strains of Kolkata using primers (*ctxB*-F3/Rv-cla) for the Haitian *ctxB* allele (top) and (*ctxB*-F4/Rv-cla) the classical *ctxB* allele (bottom). The extreme right lane contains a 100-bp size ladder. Lane 1, L19089 (*V. cholerae* O1, 2006); lane 2, L4706 (*V. cholerae* O1, 2006); lane 3, M12821(*V. cholerae* O1, 2007); lane 4, IDH00990 (*V. cholerae* O1, 2008); lane 5, IDH02003 (*V. cholerae* O1, 2009); lane 6, IDH03106 (*V. cholerae* O1, 2010); lane 7, IDH00504 (*V. cholerae* O1, 2008); lane 8, K16492 (*V. cholerae* O1, 2005); lane 9, J25916 (*V. cholerae* O1, 2004); lane 10, IDH03378 (*V. cholerae* O1, 2011); Haitian control, 2010EL-1786; Classical control, 0395; El Tor control, N16961. (B) Occurrence of *ctxB* allele type in Kolkata *V. cholerae* O1 strains from 2004 to 2011. A total of 142 strains were tested during the study period; "n" denotes the number of strains tested in each year. A *V. cholerae* O1 strain with Haitian type *ctxB* was isolated in Kolkata for the first time during April 2006.

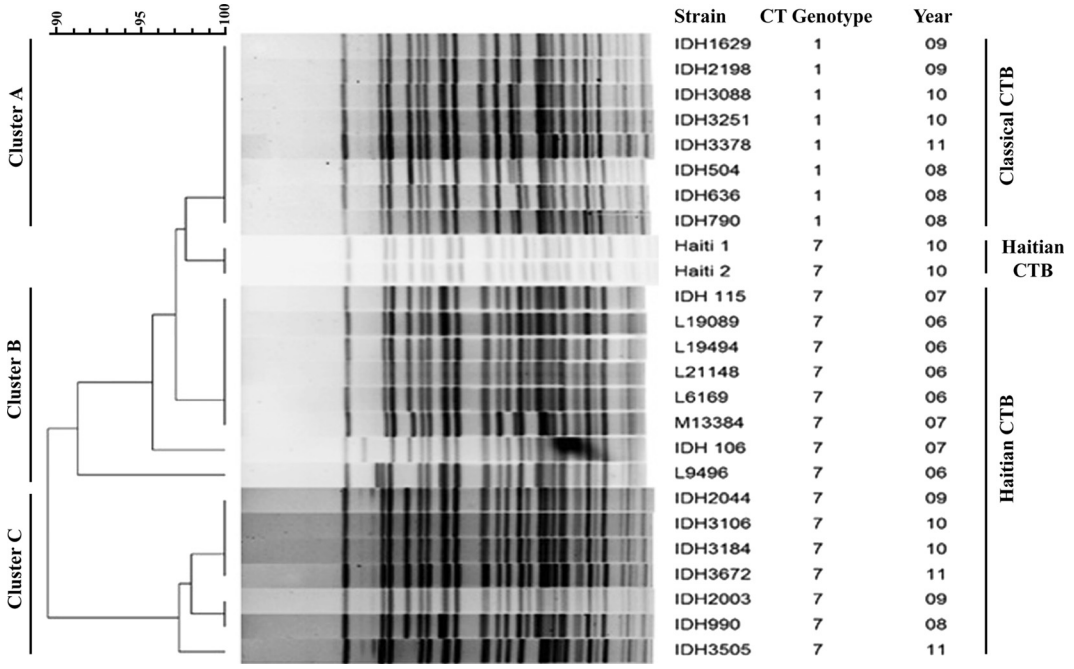

FIG 2 PFGE patterns of the NotI-digested *V. cholerae* strains from Kolkata and Haitian control strains along with the dendrogram analysis using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Analysis showed 3 distinct clusters, with all the *V. cholerae* strains having classical *ctxB* (genotype 1) clustered together. All the tested isolates with Haitian *ctxB* (genotype 7) from 2006 through 2007 and in 2008 through 2011, however, were found to form two distinct clusters, suggesting considerable diversities in genomic content between them.

revealed that the strains possessed DNA sequences identical to that of the classical type of *ctxB* but with an additional mutation at the 58th position (C to A). The deduced amino acid sequences of all 14 representative strains were aligned with the CTB sequences of the reference strains N16961 (El Tor) and O395 (classical). The amino acid sequences of all strains were found to be identical to the deduced amino acid sequence of the CTB of the O395 classical reference strain except for a histidine-to-asparagine substitution at the 20th position of the sequence encompassing the signal peptide (GenBank accession number JN806157-59). Thus, the result from DNA sequencing of the *ctxB* gene confirmed the results of DMAMA-PCR. We also sequenced the *ctxB* genes from three representative strains that yielded amplicons with the classical specific primers (*ctxB*-F4/Rv-cla). The deduced amino acid sequences of all three strains were found to be identical to that of the classical reference strain, with a histidine at position 39 and a threonine at position 68. Thus, the results from DNA sequencing of *ctxB* genes confirmed the results of DMAMA-PCR.

Screening of the Kolkata strains using the DMAMA-PCR. After standardizing the DMAMA-PCR, we used this assay extensively to investigate the emergence and dissemination of the Haitian variant of *V. cholerae* strains in Kolkata. All the tested strains from 2004 through 2005 were positive for the classical type of *ctxB*, indicating that they are El Tor variant strains. The first appearance of Haitian type *ctxB* was noted in Kolkata during April 2006. There was an abrupt decrease in the isolation profile of *V. cholerae* O1 strains with the Haitian *ctxB* allele (CTB genotype 7) during 2007 and 2008. The percentage of the O1 isolates with CTB genotype 7 started to increase from 2009 (Fig. 1B), and more than 93% of Kolkata strains carried the Haitian *ctxB* allele in 2011.

Phylogenetic analysis based on PFGE. The results of DMAMA-PCR and the sequencing data clearly indicated the appearance of novel variant strains of *V. cholerae* O1 in Kolkata since 2006, which motivated us to take a closer look at the relatedness of these variants with the Haitian isolates. We also analyzed the NotI pulsed-field gel electrophoresis (PFGE) patterns with representative strains. The PFGE profiles of *V. cholerae* strains from Kolkata were compared using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) (Fig. 2). The similarity between strains was determined using the Dice coefficient, and cluster analysis was carried out using the unweighted-pair group method using average linkages (UPGMA). All the tested *V. cholerae* strains with classical *ctxB* (genotype 1) clustered together (Fig. 2, cluster A), with a similarity matrix of >98%. All the tested strains with Haitian *ctxB* (genotype 7) in 2006 and 2007 were also found to be closely related to each other, with a similarity matrix of >97% (Fig. 2, cluster B). Dendrogram analysis showed that clusters A and B were closely related to the Haitian *V. cholerae* strains. Interestingly, all the tested strains with Haitian *ctxB* in 2008 to 2011 formed a distinct cluster (Fig. 2, cluster C), suggesting considerable diversities in genomic content between strains containing Haitian *ctxB* in 2006 through 2007 and 2008 through 2011.

Our results not only signify a cryptic change in the circulating strains in Kolkata but also raise questions about the origin of these variants of *V. cholerae* O1 El Tor. This new type of *ctxB* (genotype 7) was first reported by Goel et al. (5) in *V. cholerae* O1 strains isolated from a cholera outbreak in Kalahandi, Orissa, India, in 2007. But our results clearly show that in Kolkata, genotype 7 prevailed since April 2006. This finding tempted us to speculate that the Haitian type of *ctxB* may have originated from Kolkata and then disseminated to the neighboring regions like Orissa and other places, although confirmation of this hypothesis requires several other epidemiological and experimental validations, and then may have spread via Nepal to Haiti as reported from many

investigations (6, 13). It has been hypothesized that the unique genetic composition of the variant type of strains increases their relative fitness, perhaps as a consequence of increased pathogenicity (4).

Recent reports by several research groups showed a putative link between the strains associated with cholera in Haiti and in Nepal (6, 13), underscoring the speed at which infectious diseases can be transferred globally even to other countries where they are not endemic. Implementing a coordinated, integrated multidisciplinary approach is the only effective way to prevent and contain outbreaks among vulnerable populations living in high-risk areas. Prevention, preparedness, and response depend upon an effective and holistic surveillance system and are linked and interdependent. We strongly believe that the DMAMA-PCR will be an easy and accurate tool for tracking the emergence and dissemination of Haitian variant *ctxB* in *V. cholerae* O1 isolates and, therefore, will help in understanding cholera epidemiology around the globe.

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