Revised Note: Ms. Ref. No.: JCM/06110-11

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[1], G.P. Pazhani[1], Mou Ganguly[1], Santanu Ghosh[1], T. Ramamurthy[1], Ranjan K. Nandy[1], G. Balakrish Nair[1], Yoshifumi. Takeda[2], Asish K. Mukhopadhyay[1*]

[1]National Institute of Cholera and Enteric Diseases, Kolkata-700010, India; [2]Collaborative Research Center of Okayama University for Infectious Diseases at NICED, Kolkata, India

***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: Haitian variant *ctxB* in Kolkata

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

Abstract: A PCR based assay was developed to discriminate the classical, El Tor and Haitian type *ctxB* alleles. Our retrospective study using this newly developed PCR showed that Haitian *ctxB* first appeared in Kolkata during April, 2006 and 93.3% strains during 2011 carried the new allele. Dendogram analysis showed a distinct PFGE pattern of the new variant strains isolated recently when compared with the strains carrying classical *ctxB* that closely matched with the 2006-07 variant strains.

Word Count: 75

Cholera still continues to be an important cause of human infection especially in developing countries those lacks 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 re-emergence of cholera as a significant global public health problem and called for 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). Till date, more than 200 serogroups of *V. cholerae* are known, but only serogroups O1 and O139 cause epidemic and pandemic cholera (7, 16). Till 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, emergence and dissemination of novel pathogenic variants of *V. cholerae* O1 throughout many Asian and African countries (1, 3, 5, 9, 10, 11, 15) indicated a cryptic change in the 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 having a mutation in the 58th nucleotide of *ctxB* (4) 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 mutation assay (DMAMA) to accurately discriminate the classical, El Tor and Haitian type *ctxB* allele through a rapid and simple PCR based assay. A total of 142 *V. cholerae* O1 strains were included for this study. These strains were selected from the repository of the National Institute of Cholera and Enteric Diseases, Kolkata 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 classical. El Tor and Haitian type, respectively.

Development of a double mismatch mutation assay (DMAMA) PCR:

All the 142 tested strains along with the control strains were grown in Luria Bertini broth (Becton Dickinson, Sparks, Maryland, USA) for 18 hrs and then streaked on Luria agar (LA) plates. In this study, we focused on the *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 CTB subunit of *V. cholerae* O1 necessitates MAMA-PCR with biotype specific primers, nucleotide sequencing of the *ctxB* allele or performing an ELISA using classical or El Tor CT specific monoclonal antibodies. Among these the first one has been the method of choice as it is simple and less time consuming. However, reports on influx of new variant strains of *V. cholerae* O1 with additional mutation at the 20th amino acid position (58th nucleotide position) clearly point out its limitation in discrimination of *ctxB* genotypes. Previously published MAMA-PCR (8) is based on two biotype specific reverse primers each bearing a mismatch at nucleotide position 203 and hence incapable 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 their 3$^{'}$ ends (Table 1). These allele-specific primers each carry specific nucleotide, A and C, for 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 the primers. We used the *ctxB* reverse

primer 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 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 both the PCR assays due to 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 Haitian *ctxB* gene by DMAMA-PCR, were selected for DNA sequencing. For sequencing, a separate pair of primer (*ctxB*-F and *ctxB*-R) was used to provide the sequences of whole *ctxB* gene. Nucleotide sequence analysis of the *ctxB* genes of 14 representative strains of *V. cholerae* O1 revealed that the strains possessed DNA sequences identical to that of the classical type of *ctxB* with an additional mutation at the 58^{th} 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 a histidine to asparagine substitution at 20^{th} position encompassing the signal peptide (GenBank accession number JN806157-59). Thus, the result from DNA sequencing of *ctxB* gene confirmed the results of DMAMA-PCR. We also sequenced *ctxB* gene from three representative strains that yielded amplicons with the classical specific primers (*ctxB*-F4/Rv-cla). The deduced amino acid sequences of all 3 strains were found to be identical to the classical reference strain, with a

histidine at position 39 and a threonine at position 68. Thus, the result from DNA sequencing of *ctxB* gene confirmed the result of DMAMA-PCR.

Screening of the Kolkata strains using the DMAMA-PCR: After standardizing the DMAMA-PCR, we extensively used this assay to investigate the emergence and dissemination of Haitian variant of *V. cholerae* strains in Kolkata. All the strains tested in 2004-05 were positive for classical type of *ctxB* indicating 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 Haitian *ctxB* allele (CTB genotype 7) during 2007and 2008. The percentage of the O1 isolates with CT B genotype 7 started to increase from 2009 (Fig 1B) and more than 93% Kolkata strains carried Haitian *ctxB* allele in 2011.

Phylogenetic analysis based on pulsed-field gel electrophoresis (PFGE): Results of DMAMA-PCR and the sequencing data clearly indicated appearance of novel variant strains of *V. cholerae* O1 in Kolkata since 2006, which and motivated us to take a closer look on the relatedness of these variants with the Haitian isolates. We also analyzed the *Not*I PFGE patterns with representative strains. The PFGE profiles of *V. cholerae* strains from Kolkata were compared using Bio Numeric 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 2006-07 were also found to be closely related to each other, with a similarity matrix of >97% (Fig 2, cluster B). Dendogram analysis showed that clusters A and B were closely related with the Haitian *V. cholerae* strains. Interestingly, all the tested strains with Haitian *ctxB* in 2008-2011

formed a distinct cluster (Fig 2, cluster C) suggesting considerable diversities in genomic content between strains containing Haitian *ctxB* in 2006-07 and 2008-11.

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 in *V. cholerae* O1 strains by Goel *et al* (5) isolated from a cholera outbreak in Kalahandi, Orissa in 2007. But our results clearly show that in Kolkata genotype 7 prevailed since April 2006. This finding tempted us to speculate that Haitian type of *ctxB* may have originated from Kolkata and then disseminated to the neighboring regions like Orissa and other places, although conformation of this hypothesis requires several other epidemiological and experimental validations, and then may have spread via Nepal to Haiti as reported in 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 non-endemic countries. 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 the cholera epidemiology around the globe.

Acknowledgement

The 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. **Chatterjee, S., T. Patra, K. Ghosh, A. Raychoudhuri, G. P. Pazhani, M. Das, B. L. 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.
3. **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.
4. **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.
5. **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.

6. **Hendriksen, R. S., L. B. Price, J. M. Schupp, J. D. Gillece, R. S. Kaas, D. M. Engelthaler, V. Bortolia, T. Pearson, A. E. Waters, B. P. Upadhyay, S. D. Shrestha, S. Adhikari, G. Shakya, P. S. Keim, and F. M. Aarestrup.** 2011. Population Genetics of *Vibrio cholerae* from Nepal in 2010: Evidence on the Origin of the Haitian Outbreak. M bio. **2:**doi:10.1128/mBio.00157-11

7. **Kaper, J. B., J.J. Morris Jr., and M. M. Levine**. 1995. Cholera. Clin. Microbiol. Rev. **8:**48–86.

8. **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.

9. **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.

10. **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.

11. **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.

12. **Olsvik, O.** *et al*. 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.
13. **Piarroux, R., R. Barrais, B. Faucher, R. Haus, M. Piarroux, J. Gaudart, R. Magloire, and D. Raoult.** Understanding the cholera epidemic, Haiti. Emerg Infect Dis.2011;17:1161-1167.
14. **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
15. **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
16. **Safa A, G. B. Nair, and R. Y. C. Kong**. 2010. Evolution of new variants of *Vibrio cholerae* O1. Trends Microbiol. **18:** 46–54.
17. **World Health Organization.** Cholera, 2010. 2011. Wkly. Epidemiol. Rec. **86:**325–340.

Table 1: Primer sequences, amplicon size and annealing temperature used in PCR assays.

Primer	Sequence (5′-3′)	Amplicon (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			

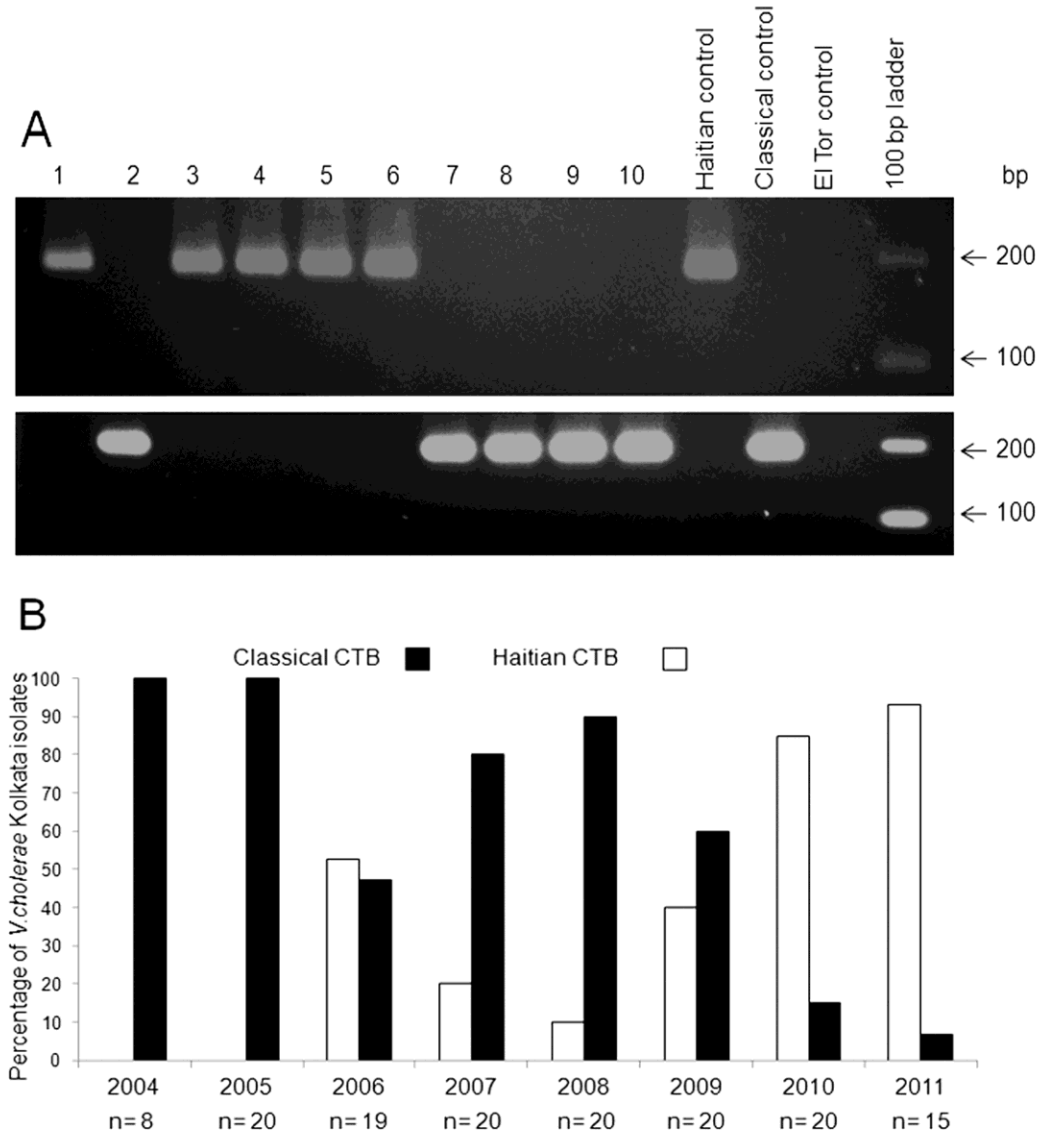

Figure 1

Figure 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 Haitian *ctxB* allele (upper panel) and (*ctxB*-F4/ Rv-cla) classical *ctxB* allele (Lower panel). Extreme right lane include 100-bp 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)** *ctxB* allele type in Kolkata *V. cholerae* O1 strains during 2004-11. Total 142 strains were tested during study period and "n" denotes the number strains tested in each year. *V. cholerae* O1 strain with Haitian type *ctxB* was first time isolated in Kolkata during April 2006.

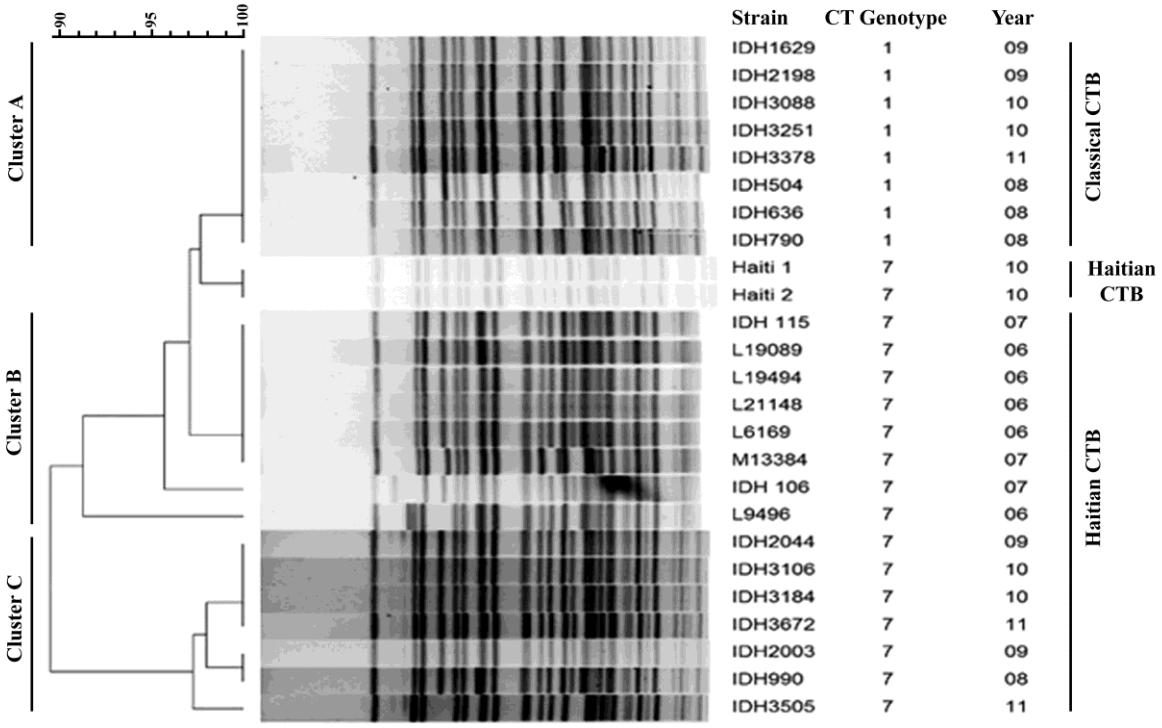

Figure 2

Figure 2: PFGE patterns of the *Not*I digested *V. cholerae* strains from Kolkata and Haitian control strains along with the dendogram analysis using Bionumeric 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) in 2006-07 and in 2008-2011 however, were found to form two distinct clusters suggesting considerable diversities in genomic content between them.