Manuscript # JCM03042-13R1

Haitian Variant *tcpA* in *Vibrio cholerae* O1 El Tor strains of Kolkata, India

Priyanka Ghosh[1], Arindam Naha[1], Surajit Basak[2, 3], Santanu Ghosh[1], T. Ramamurthy[1], Hemanta Koley[1], Ranjan K. Nandy[1], Sumio Shinoda[4], HaruoWatanabe[5], Asish K. Mukhopadhyay[1*]

[1]Division of Bacteriology, National Institute of Cholera and Enteric Diseases (NICED), Kolkata; [2]Department of Molecular Biology & Bioinformatics, [3]Bioinformatics Centre , Tripura University, Tripura; [4]Collaborative Research Center of Okayama University for Infectious Diseases at NICED, Kolkata, India; [5]National Institute of Infectious Diseases, Tokyo, Japan

***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 *tcpA* in Kolkata

Key words: Cholera, *Vibrio cholerae, tcpA*, El Tor

The toxin-coregulated-pilus (TCP) is a crucial determinant of the pathogenicity of *Vibrio cholerae* (1-3). TCP is essential for intestinal colonization and serves as a receptor for CTX prophage. (4). Whole genome sequence analysis of *V. cholerae* strain isolated from the Haitian cholera outbreak revealed a novel single nucleotide polymorphism (SNP) at nucleotide position 266 (amino acid 89) of the *tcpA* gene uniquely associated with this variant (5-7). This finding together with novel genetic variations in the Haitian strains motivated us to further investigate the emergence and dissemination of El Tor variant strains carrying this novel mutant of *tcpA* allele if any, in Kolkata, India. We developed a PCR based assay which can broadly discriminate *V. cholerae* strains carrying Haitian, classical and El Tor alleles of *tcpA* in a simple and rapid way and it can be used to understand the dissemination of the new variant in different cholera endemic regions. Three separate primers, which include one common reverse primer for both El Tor and Haitian type *tcpA* alleles [*tcpA* EL-Rev (5'-CCGACTGTAATTGCGAATGC-3') and two forward primers [*tcpA*-F1 (5'-CCAGCTACCGCAAACGCAGA-3') and *tcpA*-F'2 (5'-CCAGCTACCGCAAACGCAGG-3')] specific for El Tor and Haitian type *tcpA* alleles, respectively were designed. Our newly designed PCR was standardized to optimize both the specificity and sensitivity using the annealing temperature at 56°C for 25 sec. with 25 cycles which successfully differentiated the three different *tcpA* allelic subtypes. *V. cholerae* O1 control strain (N16961, which is O1 Inaba El Tor biotype and was isolated during 1971 in Bangladesh) having the *tcpA* allele of El-Tor type yielded a 167-bp amplicon with the El Tor *tcpA* specific primer pair but not with the Haitian *tcpA* specific primers**.** The Haitian control strain (EL-1786, O1 Ogawa El Tor biotype was isolated from a patient in Artibonite Department, Haiti during October 2010) produced just the reverse result with the same set of primer, and the classical strain (O395, which is O1 Ogawa, classical biotype strain and was isolated from a patient in India) did not

yield any amplicon in any of the PCR assay due to the significant difference in the classical *tcpA* from El Tor *tcpA*. To further confirm our PCR based result, 16 representative strains, (Table 1) which yielded positive amplicons for Haitian *tcpA* gene using the newly developed PCR, were selected for DNA sequencing of whole *tcpA* gene with separate primers. The amino acid sequences of all strains were found to be identical to the deduced amino acid sequence of the whole TcpA of the El Tor reference strain N16961 except for an asparagine to serine substitution at the 89th position of the sequence encompassing the signal peptide (GenBank accession number: KC918809–KC918816). Thus, the results from DNA sequencing of the *tcpA* gene confirmed our PCR results. This newly developed PCR assay was used to screen 251 *V. cholerae* O1 clinical strains isolated during 2001-2012 in Kolkata for understanding the genesis and spread of the Haitian *tcpA*. All the tested strains from 2001 through September, 2003 were positive for the El Tor type of *tcpA*. The first appearance of Haitian type *tcpA* was noticed in Kolkata during October, 2003. Soon after its appearance; this new variant *tcpA* containing strain displaced the canonical El Tor *tcpA* containing strains completely in the succeeding years (Figure 1). A set of orthologues of *tcpA* genes from *V. cholerae* strains downloaded from GenBank were aligned with the 16 isolates using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Evolutionary rate of each individual residue for a given *tcpA* gene was calculated using SWAKK server (http://oxytricha.princeton.edu/SWAKK/) (8). It estimates the ratio of non-synonymous (Ka) to synonymous substitution rates (Ks) between a pair of protein-coding DNA sequences, by sliding a 3D window. If Ka/Ks>1 for an aligned residue it indicates positively selected site. We observed three different mutations present in the 89th position of the matured TcpA from the multiple sequence alignment of a set of orthologues of TcpA. These three mutations are: Asn->Ser, Asn->Thr and Asn->Ala. For each of these three mutations Ka/Ks was measured individually. Here, Ka/Ks is used as a measure of selection pressure. Out of these three mutations only Asn->Ser mutation has been found to be positively selected. The particular mutation (Asn->Ser) at the 89th amino acid of whole TcpA (or 64th amino acid of mature TcpA) is the result of

transition, i.e., purine-purine conversion. This pattern is conserved natural selection, since a transition bias (i.e., purine-purine conversion) is expected to reduce the incidence of potentially harmful mutations and thus evolutionarily preferred. Our previous study indicated that the Haitian *ctxB* first appeared in Kolkata during April, 2006 (9). Therefore, a certain proportion of *V. cholerae* strains in Kolkata acquired the combination of Haitian *ctxB* along with Haitian *tcpA* from April 2006 onwards. It should be noted however that this occurrence (acquisition of Haitian *ctxB* and *tcpA*) does not always occur in tandem. This Haitian variant strain may be the result of the sequential genetic events in the evolution of *V. cholerae* strain in the Indian subcontinent. Our results highlight a significant event in the evolution of recent variants of *V. cholerae*. Finally, this finding not only shows a cryptic change in the epidemiology of cholera but also raises questions about the origin of this variant of *V. cholerae* O1 El Tor.

Acknowledgements:

This study 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, National Institute of Infectious Diseases Japan and by the Indian Council of Medical Research, Government of India. PG would like to acknowledge for the Inspire fellowship (IF110706) received from the Department of Science & Technology (DST), India.

Reference:

1. **Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM.** 1988. Toxin, toxin-coregulated pili, and the toxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. J Exp Med.**168:**1487-1492.
2. **Attridge SR, Voss E, Manning PA.** 1993. The role of toxin-coregulated pili in the pathogenesis of *Vibrio cholerae* O1 El Tor. Microb Pathog.**15:**421-431.
3. **Kaufman MR,Shaw CE,Jones ID,Taylor RK.**1993. Biogenesis and regulation of the *Vibrio cholerae* toxin-coregulated pilus: analogies to other virulence factor secretory systems. Gene.**126:**43-49.
4. **Waldor MK, Mekalanos JJ.** 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science.**272:**1910-1914.
5. **Chin CS, Sorenson J, Harris JB, Robins WP, Charles RC, Jean-Charles RR, Bullard J, Webster DR, Kasarskis A, Peluso P, Paxinos EE, Yamaichi Y, Calderwood SB, Mekalanos JJ, Schadt EE, Waldor MK.** 2011. The origin of the Haitian cholera outbreak strain. **N Engl J Med.364:33-42.**
6. **Talkington D, Bopp C, Tarr C, Parsons MB, Dahourou G, Freeman M, et al.** 2011. Characterization of toxigenic *Vibrio cholerae* from Haiti, 2010-2011. **Emerg Infect Dis. 17(11):2122-9.**
7. **Grim CJ, Hasan NA, Taviani E, Haley B, Chun J, Brettin TS.** 2010. Genome sequence of hybrid *Vibrio cholerae* O1 MJ-1236, B33, and CIRS101 and comparative genomics with V. cholerae. **J. Bacteriol. 192:3524-33.**
8. **Liang H, Zhou W, Landweber LF .**2006 SWAKK: a web server for detecting positive selection using a sliding window substitution rate analysis. **Nucleic Acids Res; 34(Web Server issue):W382-4**
9. **Naha A, Pazhani GP, Ganguly M, Ghosh S, Ramamurthy T, Nandy RK, Nair GB, Takeda Y, Mukhopadhyay AK**.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**

Legend to Figure 1:

Isolation profile of *Vibrio cholerae* O1 strains with El Tor and Haitian type of *tcpA* in Kolkata during 2001-2012. *V. cholerae* O1 strain with Haitian *tcpA* was first isolated in Kolkata during October 2003 and the "n" denotes the number of strains studied during that particular year.

Table 1:

The list of clinical *Vibrio cholerae* strains, which were sequenced to validate our PCR based study, isolated from diarrheal patients in Kolkata,.

Strain ID	**Year of Isolation**	***tcpA***	***ctxB***
J6705	2004	Haitian	Classical
J26075	2004	Haitian	Classical
K8833	2005	Haitian	Classical
K16207	2005	Haitian	Classical
L4706	2006	Haitian	Classical
L17378	2006	Haitian	Haitian
M15175	2007	Haitian	Classical
M15953	2007	Haitian	Classical
IDH00990	2008	Haitian	Classical
IDH01629	2009	Haitian	Classical
IDH03000	2009	Haitian	Haitian
IDH03251	2010	Haitian	Classical
IDH03532	2011	Haitian	Haitian
IDH03378	2011	Haitian	Classical
IDH04543	2012	Haitian	Classical
IDH04021	2012	Haitian	Haitian

147

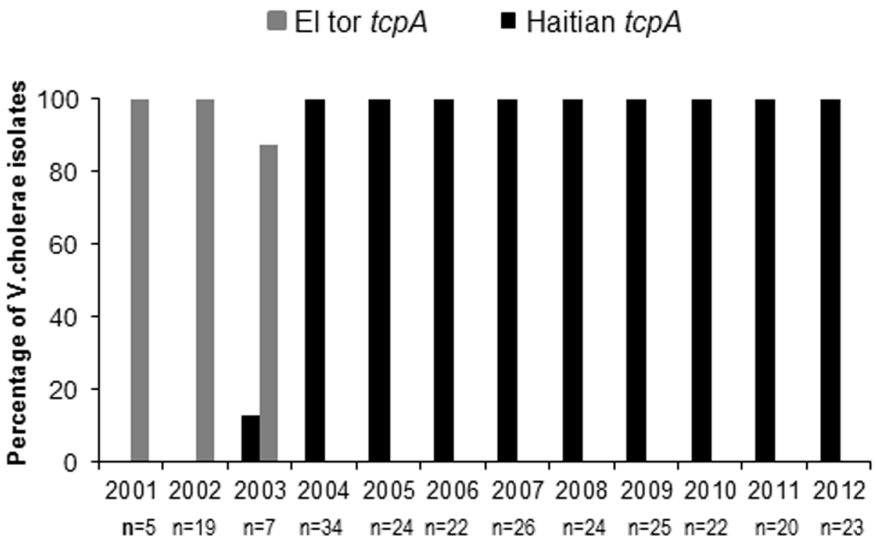

Figure 1

159