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Detection of Haitian ctxB7 & tcpA alleles in *Vibrio cholerae* O1 El Tor biotype in Puri, Odisha, India

Sir,

Vibrio cholerae O1, the causative agent of the majority of cholera outbreaks is classified into two biotypes, classical and El Tor[1] based on the assays such as chicken cell agglutination (CCA), Voges-Proskauer (VP) reaction, sheep erythrocyte lysis and polymyxin B susceptibility test[2]. Over the decades, three variants of *V. cholerae* O1 El Tor biotype have been reported globally such as Maltab variants in 2002, Mozambiqe variant in 2004-2005 and the altered El Tor type (El Tor variant) carrying classical cholera toxin since 2002[3-5].

The clinical manifestation of cholera is caused by cholera toxin, the major virulence factor encoded by *ctxAB* gene located on the CTX prophage integrated on the *V. cholerae* chromosome. Classical *V. cholerae* contains classical type *ctxB* and El Tor contains El Tor type *ctxB*. *V. cholerae* O1 with a typical El Tor phenotypes (resistant to 50 Units of polymyxin B and positive for CCA and VP test) but carrying *ctxB* classical (*ctxB1* genotype) is designated as El Tor variant[6]. On chromosome-1, the single-nucleotide polymorphism (SNP) at codon 19 results in replacement of the classical cholera toxin B histidine with asparagine residue resulting in a new *ctxB* genotype which is named as *ctxB7*[7,8].

El Tor variant carrying this new type variant *ctxB7* has been reported throughout the world but these were highlighted after the huge epidemic in Haiti in 2010 and designated as Haitian variant[9]. Genomic analysis of Haitian *V. cholerae* O1 strains revealed mutations in different segments of chromosome including *tcpA* (toxin co-regulated pilus) allele[10]. Haitian variant gradually spread throughout the world causing cholera outbreaks with predominance[11-13]. Here, we report the detection of *V. cholerae* O1 harbouring Haitian variant *ctxB* and *tcpA* associated with polymyxin B (pb, 50 U) susceptibility in Puri district, Odisha, India, during 2014-2015. This study was a part of the diarrhoea surveillance programme in Infectious Disease Hospital (IDH) Puri.

During the study, 170 rectal swab samples (72 in 2014 and 98 in 2015) were collected from hospitalized acute diarrhoea patients. The samples were transported to the ICMR-Regional Medical Research Center, Bhubaneswar for isolation of *V. cholerae*. The samples were incubated for six hours in alkaline peptone water and cultured on thiosulphate-citrate-bile-sucrose agar (TCBS, BD, USA) followed by biochemical analysis and serotyping[14] with polyvalent O1, O139 and mono-specific Ogawa and Inaba antisera (BD, USA). The study protocol was approved by the Institutional Ethics Committee. Bacteriological analysis of the rectal swabs revealed 20 samples positive for *V. cholerae* O1 Ogawa, El Tor biotype. All these 20 isolates were found positive for VP test and CCA. Antimicrobial susceptibility test was carried out by Kirby-Bauer disk diffusion method adhering to Clinical and Laboratory Standard Institute guidelines[15,16]. *V. cholerae* O1 isolates were found sensitive to drugs such as tetracycline, trimethoprim/sulphamethoxazole, chloramphenicol, neomycin, gentamicin, ciprofloxacin, norfloxacin, ofloxacin, doxycycline and azithromycin; and resistant to ampicillin, erythromycin, co-trimoxazole, nalidixic acid and furazolidone. Of the total 20 isolates, 15 (75%) were found sensitive to Pb (50 units). The genetic analysis of isolated *V. cholerae* isolates included PCR assays for detection of *wbe*, *ctxA* and *tcpA*. Haitian variants *ctxB* and *tcpA* were amplified using the primer pairs as described previously[10,17]. PCR analysis revealed that all the 20 isolates were positive for *ctxA*, *tcpA* (El Tor) and *rfb* genes. The presence of *ctxA* (301 bp) in all *V. cholerae* isolates confirmed their toxigenic potential, *wbe* (192 bp) provided the molecular evidence for O1 serogroup and *tcpA* El Tor (469 bp) determined the

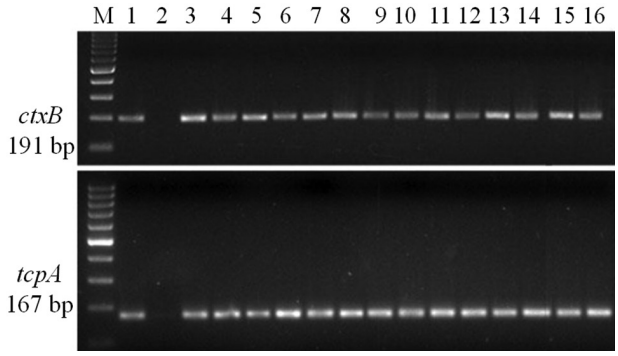

Figure. PCR analysis showing Haitian variant *ctxB* and *tcpA* allele in *V. cholerae* O1 isolates. Lane M, 100 bp size ladder; lane1, Haitian control 2010EL-1786; lane 2, classical control, O395; from lane 3 through lane 16, *V. cholerae* O1 isolates.

El Tor type. Further biotype-specific CTX prophage repressor *rstR* was amplified with El Tor-specific primers[18], indicating the presence of El Tor *rstR* in all these isolates. All *V. cholerae* isolates were found to carry Haitian variant *tcpA* allele [(167 bp) Figure] which confirmed that *tcpA* had a single base substitution at 266-nt position as was found in strains from Haiti[19]. Double mismatch-amplification-mutation assay (DMAMA)[17] detected Haitian variant *ctxB* allele (191 bp) in all 20 *V. cholerae* O1 isolates during the study (Figure).

A surveillance study conducted in three hospitals including IDH during 2004-2006 revealed the incidence rate of *V. cholerae* as 17.3 per cent where all *V. cholerae* O1 were El Tor biotype[20]. During 2011-2013, following a study on operational feasibility of an oral cholera vaccine in Satybadi Block of Puri district[21], the post-vaccination diarrhoea surveillance demonstrated the incidence rate of *V. cholerae* O1 as 3.4 per cent (unpublished data) and bio-typing of isolates revealed *V. cholerae* O1 Hybrid and El Tor variants[22]. In the present study, 11.8 per cent *V. cholerae* isolates were found to be Haitian variants.

The emergence of Haitian variant, tempted researchers to investigate its incidence and spread causing cholera outbreaks. Two cholera outbreaks in western and southern Odisha during 2013 and 2016 respectively presented the *ctxB7* genotype (unpublished data). Our previous study depicted that a large cholera outbreak occurred in western Odisha during 2014 was due to *V. cholerae* O1 with *ctxB7* and retrospective analysis of laboratory strains revealed that the origin of Haitian variant was since 1999 in Odisha[23]. Earlier studies in Kolkata reported *V. cholerae* with *tcpA* of Haitian allele, *ctxB7* allele and Pb susceptibility since 2003, 2006 and 2012, respectively[10,17,24]. In north India, including Delhi, Haryana and Uttar Pradesh, the combination of Haitian *ctxB* (*ctxB7*), classical *ctxB* (*ctxB1*) and *tcpA* of Haitian allele has been reported since 2008[25]. A cholera outbreak in Maharashtra during 2012 was due to *V. cholerae* O1 carrying Haitian variant *ctxB*[26]. The present study showed the combination of 100 per cent Haitian variants *ctxB* and *tcpA* associated with high (75%) susceptibility to Pb without the detection of *ctxB1* allele. This suggested that Haitian strains might have replaced the El Tor variant in this region as has been reported for El Tor being replaced by El Tor variant strains during 2008-2009 in Odisha[27]. Similar to the Kolkata strains[24], the high percentage (75%) of Pb susceptibility in our isolates was a unique phenotypic change contrast to the El Tor biotype of earlier decades and Haitian variants in Kerala, south India[28].

The cryptic change in the genetic backbone is the current evolutionary outcome in *V. cholerae* O1 that influences its virulence, transmission and spread. The presence of hypervirulent Haitian variant in Puri has significance in connection to the spread of cholera outbreaks. An effective surveillance acts as a resource to develop early warning to implement prevention and preparedness for disease outbreak. This report of Haitian variant in coastal Odisha warrants constant surveillance in other parts of Odisha and India.

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