Fourth Year Report

1. Study Title:

Retrospective analysis on the evolutionary aspects of *Vibrio cholerae*

2. Study facility:

National Institute of Cholera and Enteric Diseases
Division of Bacteriology
Kolkata, India

National Institute of Infectious Diseases
Tokyo, Japan

3. PI from Indian Side for this report: Asish K. Mukhopadhyay

Summary:

The explosive life-threatening cholera outbreaks are still persisting as a trade mark of developing countries, especially in Asia and Africa. Poor sanitation and inadequate hygiene represent favorable environments for the survival of the facultative Gram negative bacterium *Vibrio cholerae*, which is the causative agent of dreadful, dehydrating diarrheal disease cholera. Prevention of the disastrous cholera outbreaks among vulnerable populations living in high-risk areas advocate strong urgency to trace the origin and dispersal of the *Vibrio cholerae* O1 strains that lead to the catastrophic cholera outbreak in Haiti (2010). Dissimilarities in the genes encoding toxin-co-regulated pilus (*tcpA*), cholera toxin B subunit (*ctxB)*, repeat in toxins (*rtxA*), quinolone resistance-determining region (QRDR) of gyrase A (*gyrA*), *rstB* of RS element along with the change in the number of repeat sequences at the promoter region of *ctxAB* was reported from the sequence analysis of the Haitian outbreak strain. This massive cholera outbreak in Haiti containing several unique genetic traits motivated us to investigate the emergence and dissemination of this new variant of *V. cholerae* O1 biotype El Tor strains, if any, in Delhi, India.

Our previous studies demonstrated that variant type of *tcpA, ctxB, rstB* and *gyrA* were first time isolated in Kolkata during 2003, 2006, 2004 and in the year 1994 respectively. On the other hand disparities were fingered between Kolkata and Haitian strains in respect to *rstB* and *ctxAB*

promoter repeat. Genomic traits of Kolkata strains further questioned the evolutionary genetic modulations of Haitian epidemic strains. To resolve this unresolved platform genotypic assessment of the 170 *V. cholerae* O1 Delhi strains isolated during 2004 to 2012 were analyzed in the present study.

Our study showed that all the tested strains carried Haitian *tcpA* (*tcpA* CIRS) and variant *gyrA* indicating their first appearance before 2004 in Delhi. The present study revealed that Haitian variant *rtxA* and *ctxB7* were first isolated in Delhi during 2004 and 2006, respectively. An interesting observation in this study was the failure of detecting single strain with the combination of El Tor *rtxA* and *ctxB*7. The Delhi strains carried four heptad repeats (TTTTGAT) in their CT promoter regions whereas Haitian strains carried 5 heptad repeats. Delhi strains did not have any deletion mutations in the *rstB* gene like Haitian strains. This study revealed the close proximity of the Delhi strains with the Kolkata *V. cholerae* strains isolated during the same period. We hypothesize that the sequential genetic events in the Indian subcontinent may have influence for the generation of the Haitian variant *V. cholerae* strains. An active holistic surveillance system should be in place for tracking the mode of dissemination of the *V. cholerae* O1 El Tor variant strains in naive population using molecular assays, as these strains possess all the potentialities for new pandemic.

This study revealed that there was a substitution from asparagine to Serine at 64^{th} position of the mature TcpA of the newly emerged *V. cholerae* strains in Delhi. Although both the amino acid resides at the Alpha-Helix, but the Haitian TcpA has lower thermal stability compared to the El Tor TcpA. It may be the reason for the Haitian TcpA having higher tendency to interact with other proteins (possibly involved in colonization) to become stabilized. The solvent accessibility for Haitian TcpA is 75.5% while that of El Tor TcpA is 92.5%. The reduced solvent accessibility of the Haitian tcpA may lead to the greater hydrophobic character of this protein which can eventually guide its interaction with other protein (of more hydrophobic character) and may influence the colonization process.

Purpose:

- Retrospective analysis of the *Vibrio cholerae* strains isolated from Delhi with special reference to the Haitian traits
- Elucidation of translocation mechanism of CTB and biochemical analysis of the signal sequence

Materials and Methods:

Bacteriology and serology:

A total of 170 representatives *V. cholerae* O1 strains isolated between 2004 and 2012 were gathered for the analysis of *tcpA, ctxB, rtxA, rstB,* and *gyrA* genes. All the *V. cholerae* strains used for this study were selected from the strain repository of 'Maharishi Valmiki Infectious Diseases Hospital', Delhi, India and were obtained from the hospitalized cholera patients. The strains were grown in Luria Bertani broth (Becton Dickinson, Sparks, MD, USA) for 18 hrs and then streaked on Luria agar (Becton Dickinson, Sparks) plates. Identity of these strains was reconfirmed serologically by the slide agglutination with O1 specific polyclonal antiserum and serotype specific antisera (Becton Dickinson, Sparks). *V. cholerae* O1 strains EL-1786 (Ogawa), N16961 (Inaba) and 0395 (Ogawa) were used as standard strains for the Haitian, El Tor and classical type, respectively.

Preparation of DNA template for PCR: One loopful of an overnight culture from LA plate was suspended in 200 µl of Tris-EDTA buffer (pH 8.0) and then lysed by vigorous vortexing with mixture of phenol: chloroform: isoamyl alcohol (25:24:1) saturated with 10 mMTris and 1mM EDTA. (Sigma-Aldrich, St Louis, MO, USA) Supernatant was collected carefully following centrifugation at 12,000 rpm for 15 min and was extracted once with 100 µl of mixture of chloroform: isoamyl alcohol (24:1) and centrifuged for 15 min at 12,000 rpm. The supernatant containing the DNA was used as template for PCR analysis.

Mismatch Amplification Mutation Assay: By exploiting the single base mutation, a simple PCR based method has been used in this study. We used our previously designed allele-specific either forward or reverse primer containing mismatch at the 3' ends (Table 1). Three separate primers, which include one reverse primer common for both El Tor and variant type of *tcpA* allele (tcpA El -Rev) and two forward primers (tcpAF1 and tcpA F'2) specific for El Tor and variant type, respectively were used (Table 1). Differentiation between variant and Classical *ctxB* allele was determined with the help of two forward (ctxBF3 and ctxBF4) and one common reverse primer (ctxB Cla-Rev) (Table 1). Further presence of different alleles of *rtxA* was assayed with one common forward (rtxAF) and two reverse primers (rtxA-R1 and rtxA-R2) specific for El Tor and *rtxA*-null mutant respectively (Table 1). Allele specific two reverse primers (gyrA-R1 and gyrA-R2) and one common forward primer (gyrA-F) was used for variant and El Tor type of *gyrA* allele

respectively (Table 1). Deletion in the *rstB* gene was determined by using rstBF1 and rstBR1 primer (Table 1).

Nucleotide Sequence of *ctxB* and *ctxA* promoter region: To determine the nucleotide sequence of the *ctxB*, PCR amplification of *ctxB* locus of six *V. cholerae* O1 isolates was performed in a 25µL reaction mixture. PCR primers and conditions used have been previously described ()PCR amplification of the *zot-ctxA* intergenic region encompassing the P_{ctxAB} of two El Tor variant strains along with two reference strains was performed using the primer pair zotF(S)/ctxAR(S) (Table). The resulting PCR amplicons were purified using the Qiaquick PCR purification kit (QIAGEN, GmbH, Hilden, Germany) and both the DNA strands were sequenced in an automated sequencer (ABI PRISM 3100 Genetic Analyser, Applied Biosystems, Foster city, CA, USA).The entire coding sequences of the *ctxB* gene of these six strains have been deposited in GenBank with accession numbers. The deduced amino acid sequences of CTB from these six strains were aligned with corresponding sequences from N16961 (GenBank accession number NC-002505) and O395 (GenBank accession number CP001235) by using the online server Clustal W.

Results:

Variant *tcpA* allele in *V. cholerae* Delhi isolates

Unique mutation in the 266th nucleotide position of the toxin-coregulated pilus (TCP) was reported in the Haitian cholera outbreak strains (Son MS *et al*, 2011). This variant *tcpA* was also reported in Kolkata isolates from the year 2003 onwards (Ghosh P *et al*, 2014).

In this present study, we investigated the emergence and dissemination of this variant *tcpA* allele in Delhi, India using previously developed allele specific primers which can broadly discriminate variant and El Tor *tcpA* carrying strains in a simple and rapid way. *V. cholerae* O1 clinical strains isolated during 2004 to 2012 in Delhi were screened for understanding the genesis and spread of the variant *tcpA* allele (**Fig. 1)**. Sequencing of the representative strains isolated from Delhi was performed to reconfirm the PCR assay. 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. Thus, the results from DNA sequencing of the *tcpA* gene confirmed our PCR result. Further our retrospective analysis demonstrated total replacement of El Tor *tcpA* in Delhi from the year 2004. Not a single strain was found with El Tor *tcpA* up to 2012.

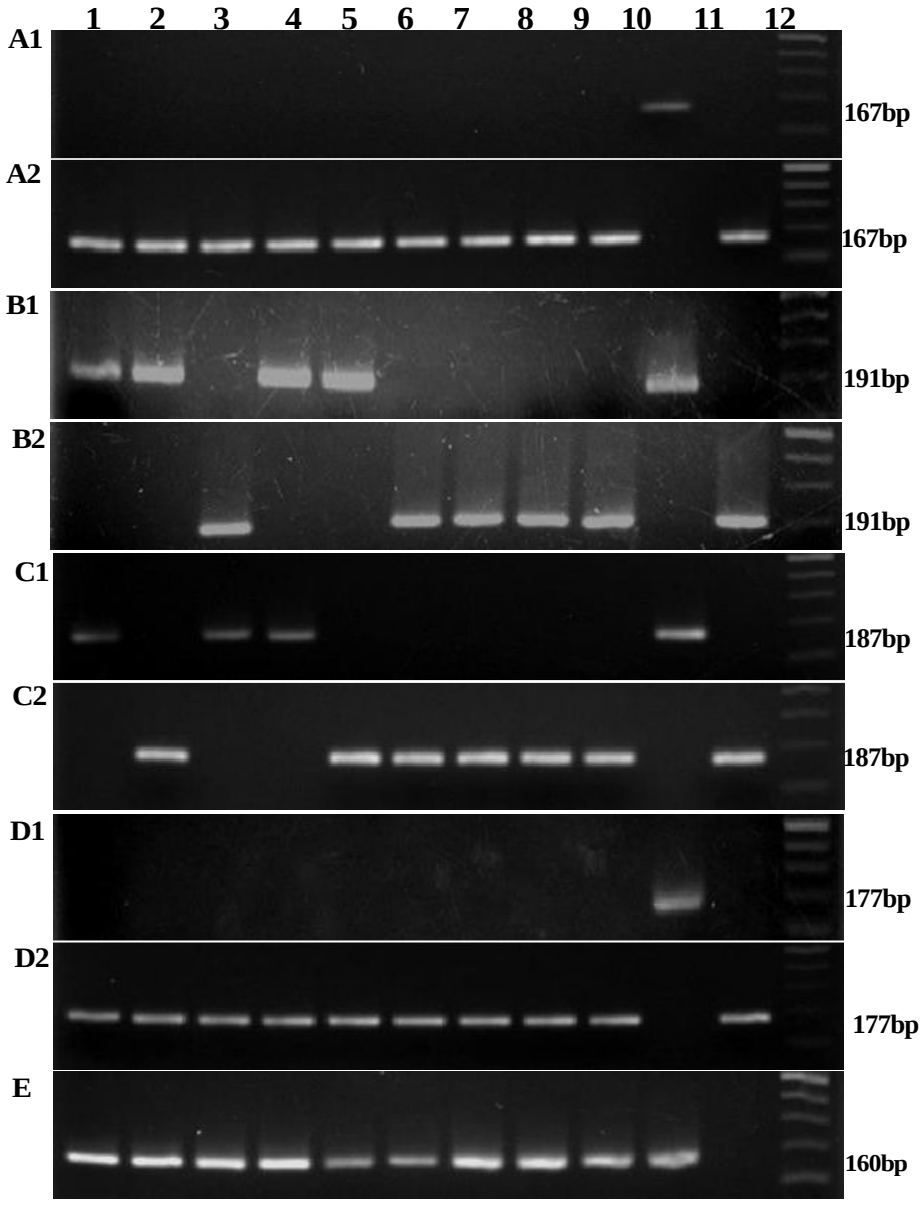
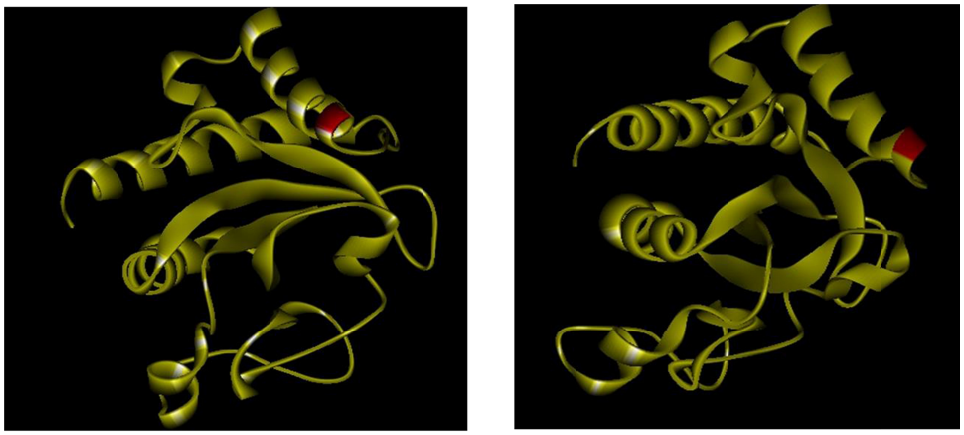


Figure 1: PCR based assay for *tcpA, ctxB, rtxA, gyrA* and *rstB* alleles in *V. cholerae* O1 Delhi isolates. MAMA-PCR to detect the type of *tcpA* allele in representative *Vibrio cholerae* O1 strains of Delhi using primers of (tcpAF1/tcpAR) for El Tor (A1) and (tcpAF'2/tcpAR) for Variant (A2). Lanes 1-9 represent 624 (2004), 463 (2005), 319 (2006), 3621 (2007), 6200 (2008), 15468 (2009), 23855 (2010), 27174 (2011), 31390 (2012), respectively. To detect the type of *ctxB* gene in

representative *Vibrio cholerae* O1 strains of Delhi using primers of (ctxBF4/Cla-Rv) for classical (B1) and (ctxBF3/Cla-Rv) for Haitian (B2). Lanes 1-9 represent 757 (2004), 684 (2005), 1854 (2007), 463 (2005), 7252 (2006), 1851 (2007), 13856 (2009), 23979 (2010), 27122 (2011), respectively. To detect the type of *rtxA* allele in representative *Vibrio cholerae* O1 strains of Delhi using primers (rtxAF/rtxAR1) for El Tor (C1) and (rtxAF/rtxAR2) for Variant (C2). Lanes 1-9 represent 831 (2004), 194 (2004), 6479 (2008), 27115 (2011), 22237 (2010), 499 (2005), 26976 (2011), 31267 (2012), 183 (2005), respectively. To detect the type of *gyrase A* allele in representative *Vibrio cholerae* O1 strains of Delhi using primers (gyrAF/gyrAR2) for El Tor (D1) and (gyrAF/gyrAR1) for Variant (D2). Lanes 1-9 represent 624 (2004), 463 (2005), 319 (2006), 3621 (2007), 6200 (2008), 15468 (2009), 23855 (2010), 27174 (2011), 31390 (2012) respectively. To detect the type of *rstB* allele in representative *Vibrio cholerae* O1 strains of Delhi using primers rstBF1-rstBR2 (E). Lanes 1-9 represent 624 (2004), 463 (2005), 319 (2006), 3621 (2007), 6200 (2008), 15468 (2009), 23855 (2010), 27174 (2011), 31390 (2012). In all cases expect *ctxB* genotype assessment N16961 (Lane 10) and El-1786 (Lane 11) were used as control for El Tor and variant strain respectively. But only in case of *ctxB* genotype O395 (Lane 10) and El-1786 (Lane 11) were used as control for Classical and variant strain. The extreme right lane contains a 100-bp size ladder (New England Biolab Inc., Beverly, MA, USA).

This study revealed that there was a substitution from asparagine to Serine at 64th position of the mature TcpA of the newly emerged *V. cholerae* strains in Delhi. Although both the amino acid resides at the Alpha-Helix, but the Haitian TcpA has lower thermal stability compared to the El Tor TcpA. It may be the reason for the Haitian TcpA having higher tendency to interact with other proteins (possibly involved in colonization) to become stabilized. The solvent accessibility for Haitian TcpA is 75.5% while that of El Tor TcpA is 92.5%. The reduced solvent accessibility of the Haitian tcpA may lead to the greater hydrophobic character of this protein which can eventually guide its interaction with other protein (of more hydrophobic character) and may influence the colonization process.



El Tor TcpA Haitian TcpA

Figure 2: There was a substitution of Asparagine to Serine substitution at 64th position of the mature TcpA, although both the AA resides at the Alpha-Helix

***ctxB7* allele in El Tor variant strains of Delhi:**

V. cholerae expresses and secretes cholera toxin upon colonization of the small intestine (Davis *et al*., 2000). This enterotoxin is an AB5 toxin consisting of one active (A) subunit and five identical binding (B) subunits (Lonnroth and Holmgren, 1973).

Presence of a SNP at the 58th nucleotide of the *ctxB* gene of the Haitian outbreak strain (Chin CS, *et al*. 2011) along with the emergence of this Haitian variant *ctxB* allele (*ctxB7*) in Kolkata isolates from the year 2006 (Naha A *et al*, 2012) was our prime inspiration for conducting the PCR based assay to track the presence of *ctxB7* allele if any in Delhi isolates. This allele specific PCR assay can successfully differentiated the two different alleles of *ctxB* **(Fig. 1)**. PCR result was further confirmed by sequencing analysis using different set of primers. We screened 170 *V. cholerae* O1 strains isolated from Delhi covering different months of each year from 2004 to 2012 using this PCR assay.

This analysis delineates the first appearance of variant type of *ctxB* in Delhi was in 2006 and interestingly there was a sudden increase in the isolation profile of *V. cholerae* O1 strains with *ctxB7* allele during the year 2007 encompassing a sudden decrease in the year 2008. After this unexpected decline of *ctxB7* allele, the percentage of the O1 isolates with *ctxB7* allele started to increase from 2009 and more than 83% Delhi strains carried *ctxB7* allele in the year 2012 **(Fig.3)**.

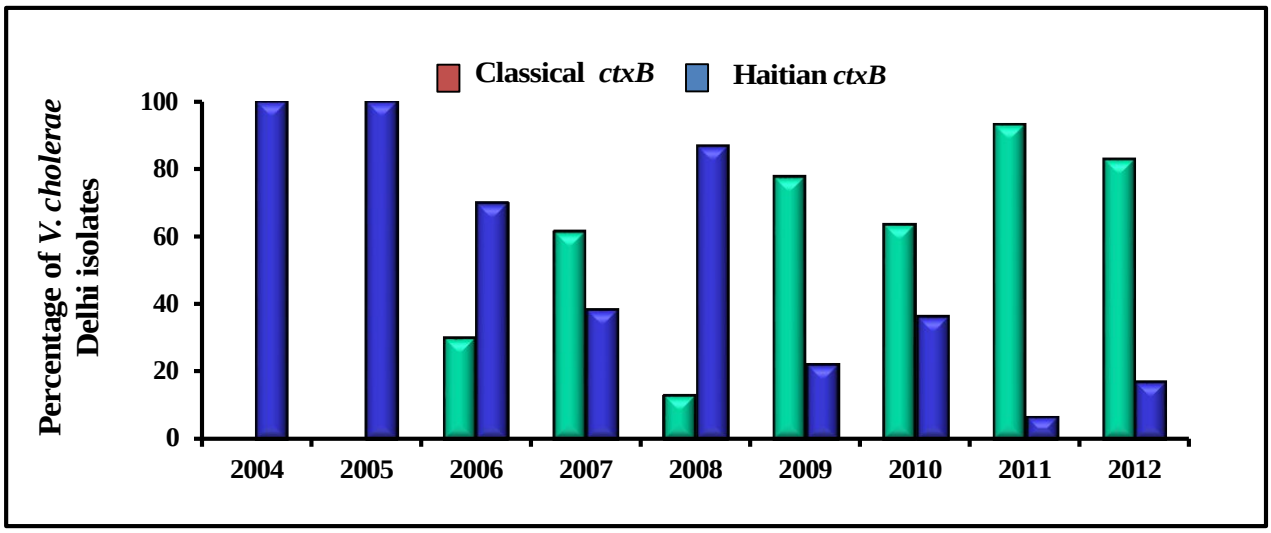


Figure 3: Retrospective analysis of *ctxB* allele in *V. cholerae* Delhi isolates. Occurrence of *ctxB* allele type in Delhi *Vibrio cholerae* O1 strains from 2004 to 2012. A total of 170 strains were tested during the study period. A *V. cholerae* O1 strains with Haitian type of *ctxB* was isolated in Delhi for the first time in the year 2006 and interestingly there was a sudden increase in the isolation profile of *V. cholerae* O1 strains with *ctxB7* allele during the year 2007 encompassing a sudden decrease in the year 2008. After this unexpected decline of *ctxB7* allele, the percentage of the O1 isolates with *ctxB7* allele started to increase from 2009 and more than 83% Delhi strains carried *ctxB7* allele in the year 2012.

Prevalence of *rxtA*-null mutants in Delhi

Dolores J *et al*. (2013) described occurrence of a single base mutation in the *rtxA* gene within the recently emerged Haitian outbreak strains which encodes a multifunctional autoprocessing RTX toxin. This mutation generates a premature stop codon which introduces a non-functional RTX toxin. Our recent studies also documented presence of *rtxA* null-mutation in Kolkata isolates from the year 2004 (Ghosh P *et al*, 2014). Not only that we were unable to detect a single strain with *ctxB7* allele in the El Tor *rtxA* background (Ghosh P *et al*, 2014).

A Mismatch Amplification Mutation based PCR assay was assayed to trace the appearance of the *rtxA*-null mutation among the *V. cholerae* Delhi isolates. This MAMA PCR assay successfully differentiated the two different alleles of *rtxA* **(Fig. 1)**.

Further confirmation was done by sequencing analysis. Retrospective analysis of *V. cholerae* O1 strains isolated from Delhi encompassing the year 2004 to 2012 was done. We found that an *rtxA*-null mutation (variant *rtxA*) in the Delhi strains were isolated during 2004. After the appearance and gradual upliftment of the *rtxA*-null- mutant up to 2007, El Tor kind of *rtxA* allele further became dominant in the year 2008. Then from 2010 onwards, the El Tor *rtxA* was replaced with a higher percentage by the *rxtA* variant **(Fig. 4)**. Our study also conveyed the emergence of *ctxb7* allele on *rtxA* null background.

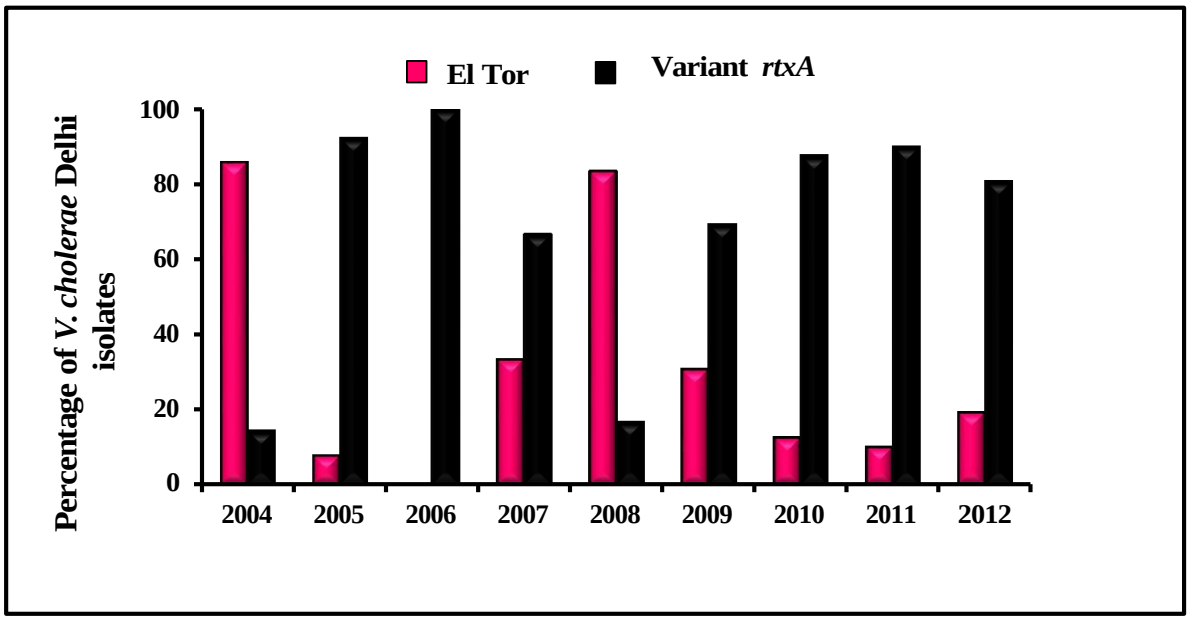


Figure 4: Isolation profile of variant *rtxA* in Delhi. Occurrence of *rtxA* allele type in Delhi *Vibrio cholerae* O1 strains from 2004 to 2012. A total of 170 strains were tested during the study period. A *V. cholerae* O1 strains with variant type of *rtxA* was isolated in Delhi for the first time in the year 2004. After the appearance and gradual upliftment of the *rtxA*-null- mutant up to 2007, El Tor kind of *rtxA* allele further became dominant in the year 2008. Then from 2010 onwards, the El Tor *rtxA* was replaced with a higher percentage by the *rxtA* variant.

Genetic features of *gyrA* gene within *V. cholerae* strains isolated from Delhi.

A Ser83→Ile substitution was documented in the *gryA* gene of the Haitian strains. This substitution is associated with quinolones resistance in clinical *V. cholerae* (Hasan NA *et al*., 2012). We also found a Ser83→Ile substitution in the *V. cholerae* O1 Kolkata isolates from the year 1994.

Occurrence of the variant type of *gyrase A* allele within the *V. cholerae* strains isolated from Delhi, India was determined with the help of allele specific primers. This allele specific primer based PCR assay successfully differentiated the two different alleles of *gyrA* **(Fig. 1).** After that sequencing of the representative strains isolated from Delhi was performed with another set of primers to reconfirm the PCR assay. Finally our study indicated that a variant type of *gyrA* allele was appeared and totally displaced the El Tor *gyrA* allele from the year 2004 in the *V. cholerae* O1 Delhi isolates.

Survey of *rstB* allele in Delhi isolates

Hasan NA *et al*. (2012) demonstrated a GTA deletion at nucleotide positions 77-79 in the *rstB* of the Haitian outbreak strain. We were unable to detect such deletion in the rstB gene of the *V. cholerae* O1 strains isolated from Kolkata, India (Ghosh P *et al*, 2014). In order to examine these deletions if any in Delhi strains, a PCR assay was done. All of the Delhi isolates from the year 2004-2012 gave amplicon at 160 bp region **(Fig. 5)**. Sequencing study reconfirmed the PCR based result.

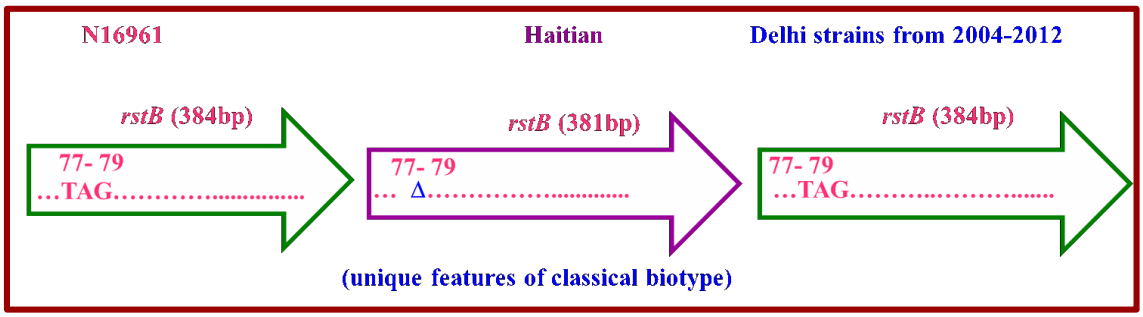


Figure 5: *Schematic Representation of different alleles of rstB* gene of *Vibrio cholerae* O1 strains

***ctxAB* promoter repeats in *V. cholerae* O1 Delhi isolates**

Variation in the number of *ctxAB* promoter repeats (TTTTGAT) has been reported earlier. Five copies of the heptads repeat in the ToxR binding region of the Haitian outbreak strain El-1786 was documented with the aid of whole genome sequence analysis (Son MS *et al*. 2011)**.** Previously we reported presence of four heptad repeats in the *V. cholerae* O1 strains isolated from Kolkata covering the year 2001-2012 (Ghosh P *et al*,) with the help of a sequence based study. Our present study was compacted on the sequence based analysis of the *ctxAB* promoter repeats in the *V. cholerae* O1 Delhi isolates. Presence of four heptad repeats in Delhi strains was narrated from our interpretation **(Fig. 6).**

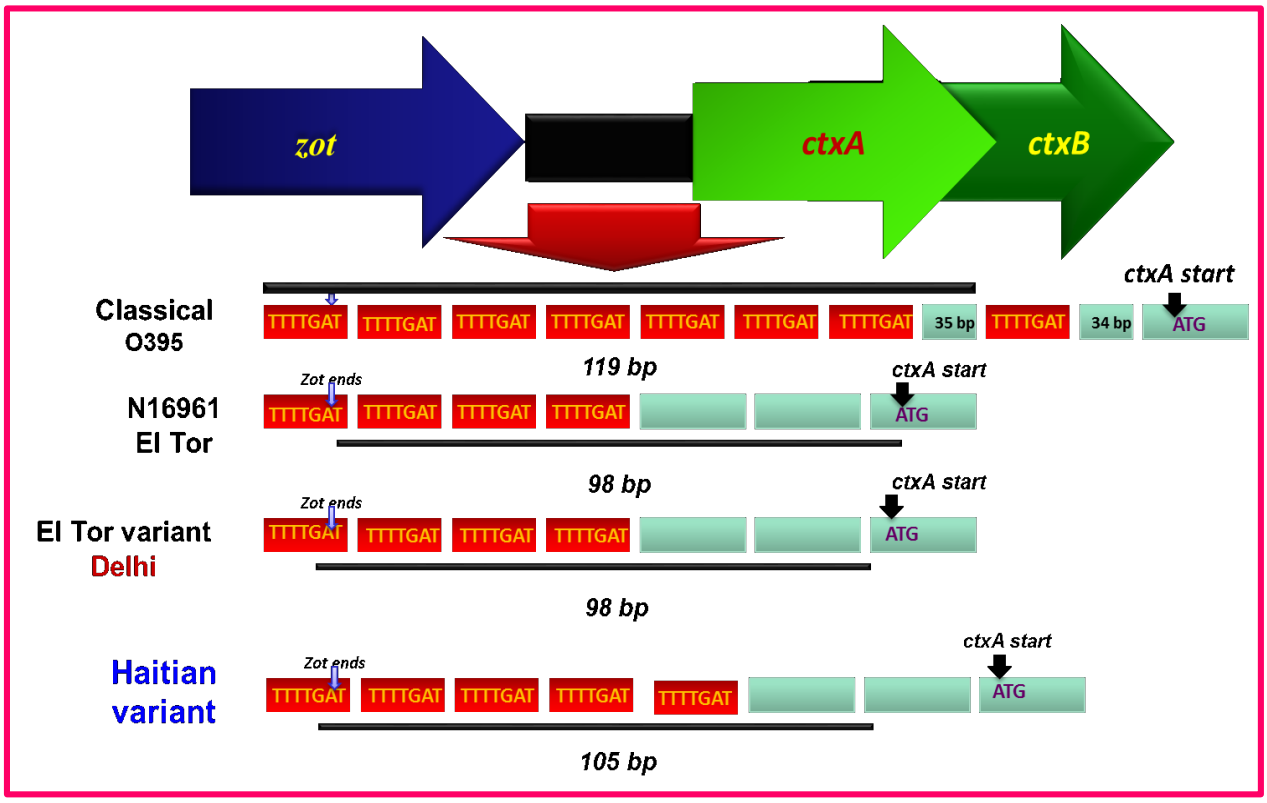


Figure 6. Schematic representation of the promoter region the *ctxAB* operon of *Vibrio cholerae* O1 El Tor variant strains isolated from Delhi and Haiti and its comparison with classical and El Tor strains. The Delhi strains contained four heptad repeats (TTTTGAT) in their CT promoter region whereas Haitian strain carried 5 heptad repeats.

Elucidation of translocation mechanism of CTB and biochemical analysis of the signal sequence

Cloning and mutagenesis of *ctxB* gene:

We amplified entire ORF of the *ctxB* gene from the *V. cholerae* strains O395 (Classical reference strain) and 2010-EL 1786 (Haitian reference strain). We digested the gel purified PCR products with EcoRI and HindIII (Fermentas) and ligated the double digested PCR amplified inserts with pBAD24 vector (EcoRI and HindIII digested). Restriction digestion with EcoRI and HindIII **(Fig. 7)** after colony PCR and finally DNA sequencing data confirmed the positive clones.

Using the QuikChange II Site-Directed Mutagenesis Kit (Agilent technologies), we have successfully created signal sequence mutants of cholera toxin B subunit. DNA sequencing data has confirmed occurrence of the desired mutations and we have electroporated these recombinant CTB constructs in to a *V. cholerae* El Tor biotype strain which has a chromosomal deletion in the *ctxAB* operon (*ctxA*$^{-}$*B*$^{-}$).

Expression of the clones and western immunoblot of CTB:

For effective expression of the ctxB gene cloned under an arabinose inducible promoter in pBAD24, we carefully altered the final dose of arabinose in the media and found an optimum of 0.2% gave satisfactory results (Fig 2). We prepared samples for SDS-PAGE from both the cell free media fractions as well as the whole cell lysates of the recombinant *V. cholerae* strains. Western immunobloting with monoclonal anti Classical CTB antibody successfully discriminated between precursor (unprocessed) and mature (processed) forms of CTB **(Fig 8).**

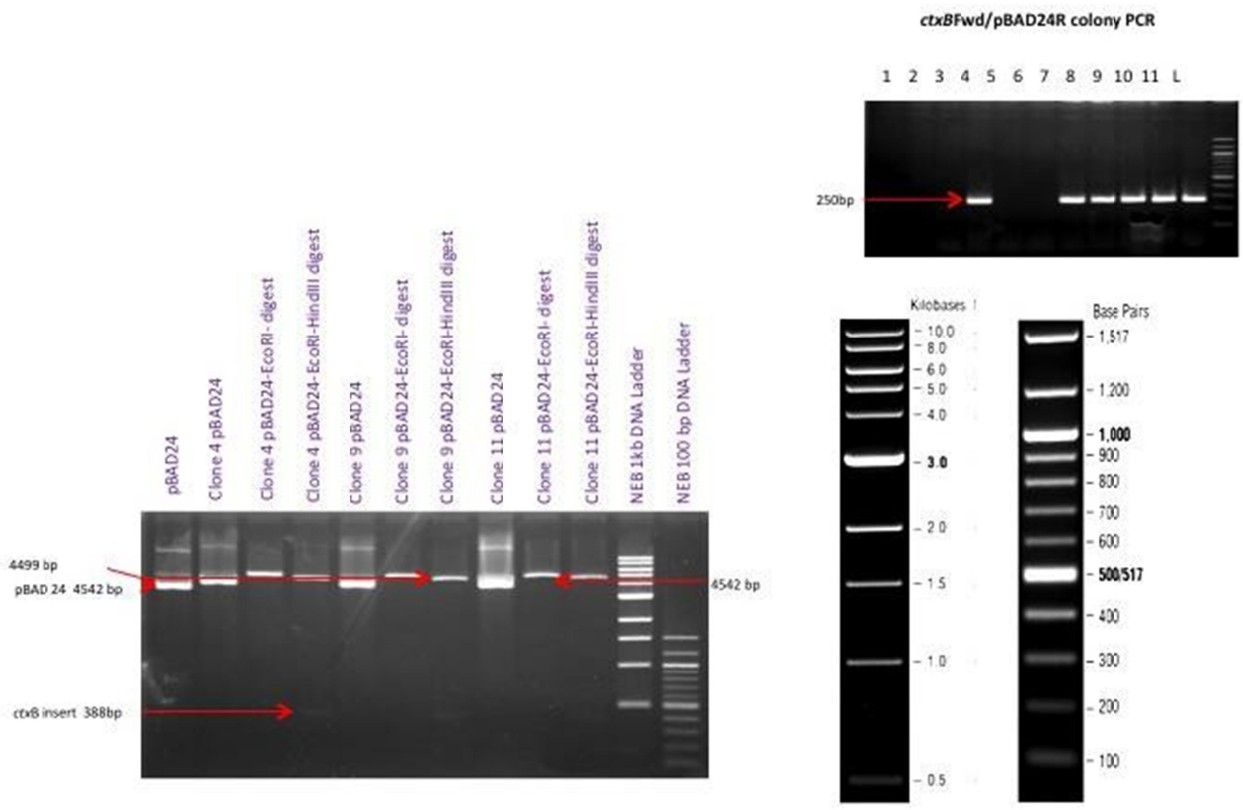


Fig. 7. Conformation of recombinant clones of CTB-pBAD:

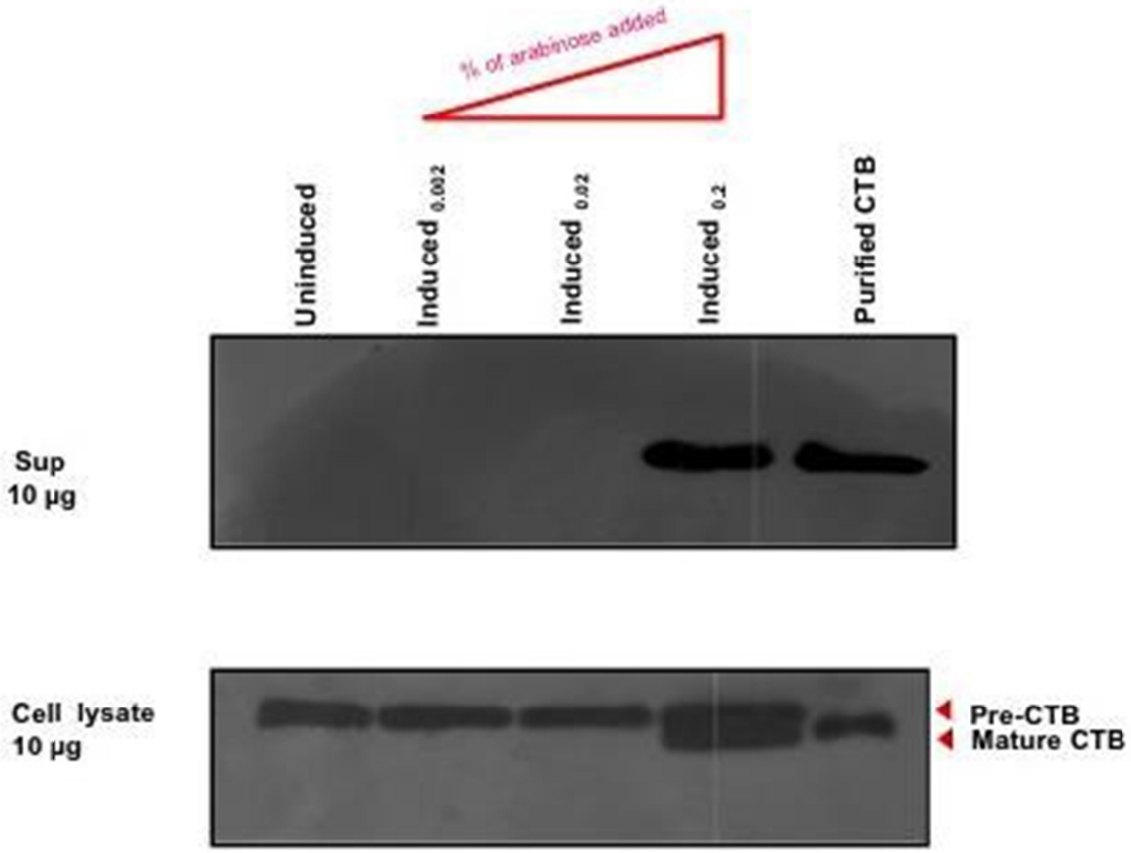


Fig. 8. Expression of unprocessed and mature CTB from the expression vector:

- **Localization of mature and precursor forms of CTB in N16961:**

By western immunoblotting, we were able to localize two different forms of the B subunit of cholera enterotoxin in the El Tor reference strain N16961. The cell free media fraction contains only the mature from of the protein whereas the whole cell extract sustains the unprocessed precursor as well as the mature form of the protein with the latter found in excess **(Fig 9).**

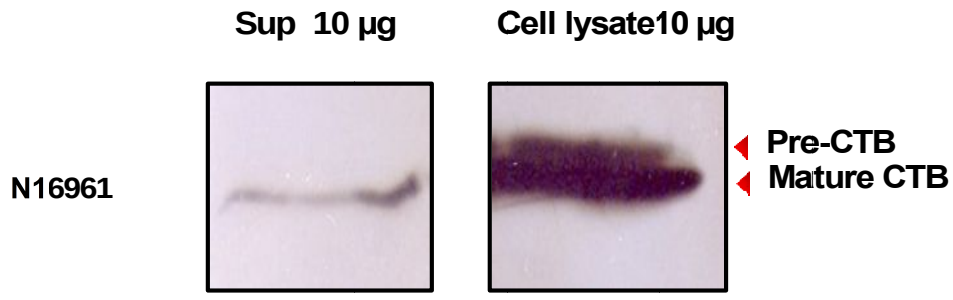


Fig. 9. Localization of different forms of CTB in N16961.

PCR detection of the genes consisting the sec translocation machinery:

Our PCR analysis data showed presence of the sec translocation system in both the wild type and recombinant strains **(Fig 10).**

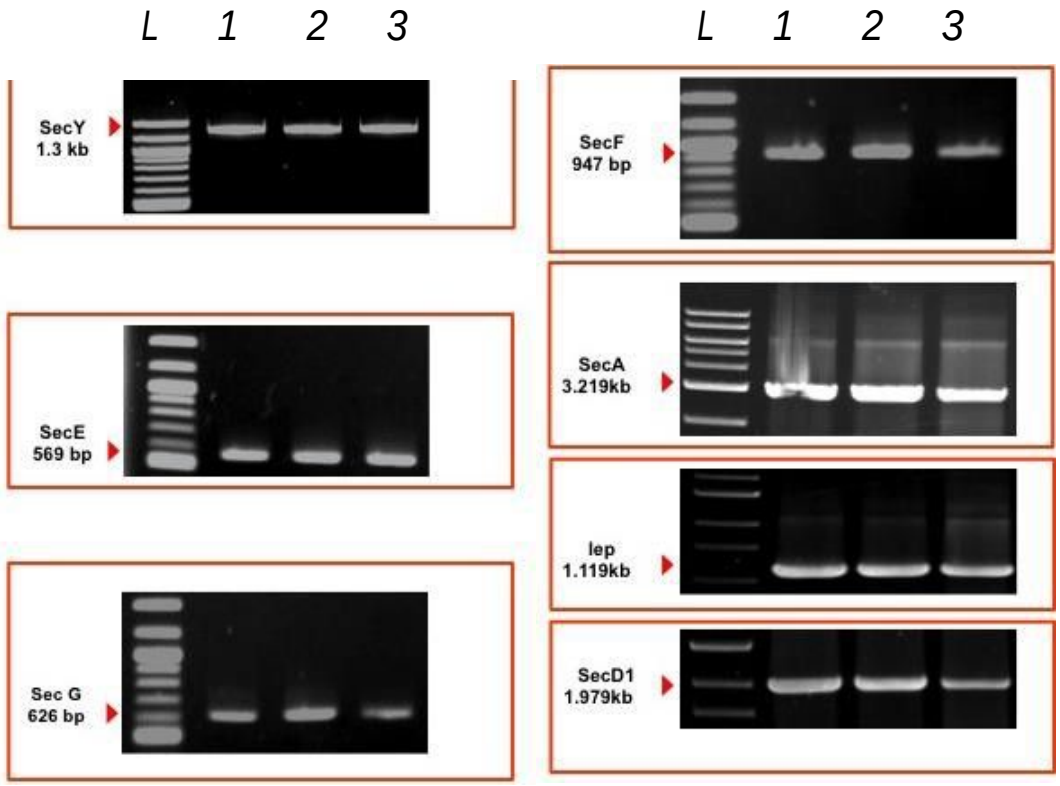


Fig. 10. PCR detection of the *sec* genes L- NEB DNA ladder, 1- JBAN1, 2- JBK70 and 3- N16961.

Gram-negative bacteria have evolved a number of methods to secrete proteins into the extracellular milieu, with at least six specific secretion systems currently described. Type II secretion (T2S), or the main terminal branch of the general secretory pathway is a feature of a number of proteobacteria and has been shown to be required for pathogenesis and maintenance of environmental niches in a large number of species. Secretion via the type II secretion pathway occurs in two membrane translocation steps that can be separated both genetically and biochemically. Initially, proteins to be secreted are produced with N-terminal signal peptides, which allows for **Sec-dependent translocation** across the cytoplasmic membrane. This is followed by removal of the signal peptide and folding and release of the mature proteins into the

periplasmic space.

Our PCR analysis data showed presence of the sec translocation system in both the wild type and recombinant strains. Furthermore, immunoblot analysis with CTB specific monoclonal antibody showed differential presence of precursor and mature form of CTB in the cell extract and cell free media system of both the wild type and recombinant strains. At present we are constructing different signal sequence mutants of CTB and we strongly belief that biophysical characterization of these mutants will help us to study the kinetic turnover of the precursor protein processing in the bacteria.

Conclusion: The present cholera global pandemic commenced by the diversification and rapid evolution of multiple descendants of a *Vibrio cholerae* El Tor ancestor mainly due to lateral gene transfer events via transduction, conjugation and transformation. The sequencing analysis of this diversified genome of *V. cholerae* strain in Haiti divulged some unique mutations in different segments of their chromosomes. Diversified genomic contents of the Haitian outbreak strains along with the genetic features of the newer variant *V. cholerae* Kolkata isolates further motivated us to track the emergence and dissemination of newer variant strains in Delhi, India. Contemplating several reports on Haitian cholera epidemic, our retrospective analysis of the *V. cholerae* O1 Delhi isolates further knocking the Indian subcontinent as the parentage of at least some of the genetic attributes of the Haitian variant strain. But, it must be highlighted here that the Haitian strains have its own genetic traits which is acquired mainly due to micro-evolution to adopt the pathogen to its local environment. Though these genomic events became spotlighted after Haitian outbreak but they prevailed erstwhile before the Haitian disaster. The unique genetic attributes of *rstB* and the *ctxAB* promoter repeat of the newer variant *V. cholerae* strains signify the continuously changing genomic features of *V. cholerae* O1. Finally similarity for some of the newer genetic traits of the Haitian isolates with the Kolkata and Delhi isolates foreground Asian countries as the parentage of Haitian outbreak and we can postulate that after originated in the Indian subcontinent they are disseminated to the neighbouring regions with several cryptic modifications.

Future plan of works:

- Completion of elucidation of translocation mechanism of CTB and biochemical analysis of the signal sequence
- Comparative analysis of El Tor and Haitian TcpA containing strains in animal models