spread to other neighboring cholera endemic regions. Our study also revealed that Haitian variant strain may be result of the sequential event in the evolution of Indian subcontinent strain.

Purpose:

- To study the molecular organization of the CTX prophage of the *Vibrio cholerae* isolates from Zanzibar, Africa along with a focus to the production of cholera toxin by them
- Analysis of *tcpA* in *Vibrio cholerae* O1 El Tor strains of Kolkata, India

Materials and Methods:

Bacteriology and serology: Two hundred and sixty eight *V. cholerae* O1 strains were selected for this study. These strains were isolated between May and November, 2009 from nine study centres in Unguja and Pemba, two islands that constitute Zanzibar. All the strains were grown on TCBS agar () for 18 hrs and then streaked on Luria agar (Becton Dickinson, Sparks, MD, USA) plates. Identity of these strains was reconfirmed serologically by the slide agglutination with O1 specific polyclonal antiserum and serotype specific antisera (Becton Dickinson).

Biotype determination: Biotype determination of the *V. cholerae* strains was done by determining sensitivity to polymyxin B (50 U/ml) and by Voges -Proskauer (VP) test. Bacterial colony was inoculated in to Mueller Hinton broth (Becton Dickinson) and grown under aerobic condition until the growth equivalent to 0.5 McFarland standard. After that one loopful of this culture was streaked on Mueller Hinton agar (Becton Dickinson) plates containing polymyxin B sulphate (Sigma-Aldrich, St Louis, MO, USA). Results were taken after overnight incubation at 37°C. Voges-Proskauer (VP) test was done as described earlier. For both studies N16961 and 569 B were used as El Tor and classical control strains respectively.

Preparation of 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 mixing with mixture of phenol: chloroform: isoamyl alcohol (25:24:1) saturated with 10 mM Tris and 1mM EDTA. (Sigma-Aldrich, St Louis, MO, USA) Supernatant was collected carefully following centrifugation at 12,000 rpm for 15 min Any remaining traces of phenol was removed by mixing 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 appropriately diluted and used as template for PCR analysis.

PCR analysis: Mismatch amplification mutation assay (MAMA) PCR, which detects sequence polymorphisms between classical type CT and El Tor type CT based on nucleotide position 203 of the *ctxB* gene, has been used in this study with the *V. cholerae* isolates. The *rstR* and tcpA PCR was performed with biotype specific primers to determine the allele type of these two genes present in the Zanzibar isolates. Two primer pairs specific for the *tlc* element and *rtxC* gene were used to determine the presence of the gene. Chromosomal localization of the CTX prophages of *V. cholerae* strains was done using specific primer pair consisting of CIIF and CIIR, as described earlier (Maiti et al, 2006). Location of RS1 element was determined using two specific sets of primers ig1 F /rstC R and Fw-con/rtxA1. Another combination of primers rstC1 and rtxA1 were used to determine the copy number of CTX prophage. For this PCR assay, reaction mixture was set up according to the manufacturer's protocol (XT 20 PCR system, Bangalore Genei, Bangalore, India). For other PCR assays above mentioned, reaction mixture (20μl) was set up with 10 ng of template DNA, 200 nM of each primers , 200 μM of each dNTP (Roche Diagnostics, Mannheim, Germany) with 1 unit of Taq DNA Polymerase (Bangalore Genei, Bangalore, India) and 1.5mM $MgCl_2$ (Bangalore Genei, Bangalore, India). Reaction conditions and primer sequences used in this study have been depicted in table 1. The amplified DNA fragments were subjected to agarose gel electrophoresis, stained with ethidium bromide and digitally recorded.

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.

Bead enzyme linked immunosorbent assay (ELISA): In vitro toxin production by the *Vibrio* isolates was measured by the bead enzyme linked immunosorbent assay (ELISA). The *V. cholerae* strains were cultured in AKI medium for 20 h under static condition and then the optical density of the culture was measured at 600nm. Following centrifugation at 100000 rpm for 10 min at 4°C, the supernatant was collected and CT concentration was measured by bead ELISA as described previously. The amount of CT determined was expressed as ng/ml/OD_{600}.

Western blot: Sample from culture supernatant of each strain of *V. cholerae* containing more than 100 ng of CT was boiled with SDS-PAGE sample buffer and analyzed by SDS-PAGE, followed by transfer to PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked with PBS (pH 7.4) containing 5% skim milk for 1 h at room temperature and then it was incubated for 1.5 h with 1 □g/ml of monoclonal antibody against either classical CTB or El Tor CTB. CTB band was detected by incubation with anti-Rat IgG (H+L) horseradish peroxidase conjugate (Nacalai Tesque, Japan), and visualized with Super Signal West Pico Chemiluminascent Substrate (Thermo Fisher, Rockford, IL).

Results:

Determination of Serogroup and Biotype:

Agglutination results with O1 specific antiserum showed that 92 % of the *V. cholerae* strains included in this study belonged to O1 serogroup. Further phenotypic analysis showed that the *Vibrio cholerae* strains are of El Tor biotype.

Analysis of biotype specific *ctxB*:

The *ctxB* genes of the *V. cholerae* O1 strains were examined by the primers specific for classical and El Tor biotypes. All the strains yielded amplicon of 186 bp with classical specific primer pairs, but none of them produced any amplicon with El Tor specific primer pairs (Fig 1). To further reconfirm our PCR based result, 10 representative strains, which yielded positive bands for classical *ctxB* gene by MAMA-PCR, were selected for DNA sequencing of *ctxB* gene. Nucleotide sequence analysis of the *ctxB* genes of the representative strains revealed that the strains possessed DNA sequences identical to that of the classical

type of *ctxB*. The deduced amino acid sequences of all the strains were aligned with the CTB sequences of the reference strains N16961 (El Tor) and O395 (classical).

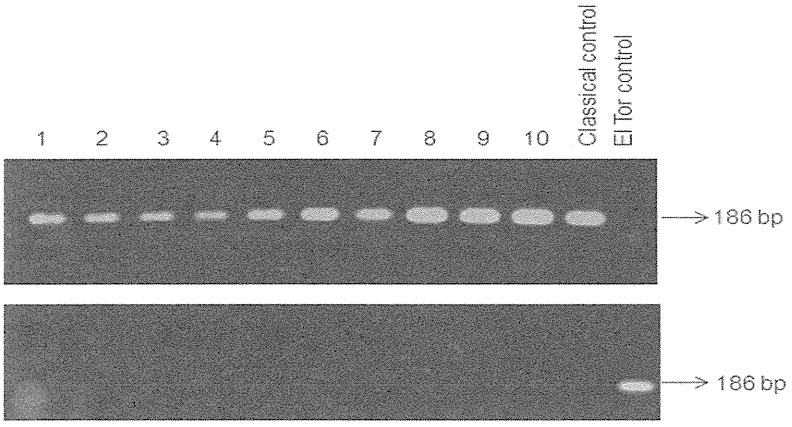


Figure 1

Figure 1: MAMA-PCR to detect the type of *ctxB* allele in representative *Vibrio cholerae* O1 strains isolated from Zanzibar, Africa, using primers (Fw-con/Rv-cla) for classical *ctxB* allele (Fig 1, upper panel) and Fw-con/Rv-elt for El Tor type *ctxB* allele (Fig 1, lower panel).. Lane 1: MCM 32, Lane 2: MCM 133, Lane 3: MCM 134, Lane 4: MCM 146, Lane 5: MCM 168, Lane 6: T1 Lane 7: MCF 084 Lane 8: MCF 001 Lane 9: WF 01 Lane 10: 210200, Lane 11: Classical control: 0395, Lane 12: El Tor control: N16961.

The deduced amino acid sequences of all the strains were found to be identical to the deduced amino acid sequence of the CT of the O395 classical reference strain, with a histidine at position 39 and a threonine at position 68 (Fig. 2). Thus, the result from DNA sequencing of *ctxB* gene confirmed the result of MAMA-PCR.

```
                    10 //          20 //          //          39 //          //      68
Classical       MIKLKFGVFF  TVLLSSAYAH GTPQNITDLC  AEYHNTQIH  TLNDKIFSY  FKNGATFQVEVPG
MCM 32          ..........  .........H ..........  ........H  .........  .....T.......
MCM 133         ..........  .........H ..........  ........H  .........  .....T.......
MCM 134         ..........  .........H ..........  ........H  .........  .....T.......
MCM 146         ..........  .........H ..........  ........H  .........  .....T.......
El Tor          ..........  .........H ..........  ........Y  .........  .....I.......
```

Figure 2

Figure 2: Amino acid sequence alignment of CTB subunit of *V. cholerae* Zanzibar isolates MCM 32, MCM 133, MCM134 and MCM 146. The amino acid sequences of the B subunit of *V. cholerae* O395 (classical) and N16961 (El Tor) were obtained from GenBank. Identical residues are indicated with dots. The sign (//) denotes the continuity of the amino acid sequences of CTB. Zanzibar strains with histidine at position 39 and threonine at position 68 confirmed its identity with the classical type CTB.

Studies of other biotype specific markers:

All of the tested strains yielded an amplicon of 501-bp with El Tor type *rstR*-specific primer set (Table 1), but not with the classical *rstR* specific one. Similarly, all the strains produced 472-bp amplicon with El Tor type *tcpA* specific primer pair whereas none of the strains had amplicon with the classical specific *tcpA* primers except the classical control strain O395. Earlier studies have reported that *rtxC*, which encodes the activator protein, is absent from strains of classical biotype and present in the El Tor biotype only. The presence of the *rtxC* gene grouped all the strains as El Tor biotype. The *rstC* gene acts as an antirepressor in the phage replication process. This gene is unique to the strains of the El Tor biotype and is absent in classical strains. Thus, we checked all the strains for the *rstC* gene and found that all test strains, including El Tor N16961, showed a positive amplification for the *rstC* whereas only classical O395 reference strain was negative for the same gene. Another PCR assay using primers ig1F/*rstC*-R amplified a 2.2 kb fragment confirming the presence of intact RS1 in the genome of the tested strains. Around 3-kb amplicon was received from a PCR using primers rstC1 and cep-R indicating the presence of RS1 element in the upstream of the CTX prophage. Similarly, 2.4-kb amplicon was yielded by PCR of the tested strains using primers *ctxB*-F and *rtxA*-R indicating absence of RS1 element in the downstream of the CTX prophage. Another gene representing 'toxin-linked cryptic element' or *tlc*, which is present adjacent to the CTX prophage, was positive in all the test strains.

Analysis of the *ctxA* promoter region: Sequencing analysis of the *ctxA* promoter region of representative *V. cholerae* O1 strains from Zanzibar revealed the presence of three tandem TTTTGAT heptanucleotide repeat. These repeat regions play an important role for ToxT binding and thereby influence the CT production. The *ctxA* promoter region of *V. cholerae* O1 isolates from Kolkata was also analyzed and it differs from that of the Zanzibar isolates (Fig 3).

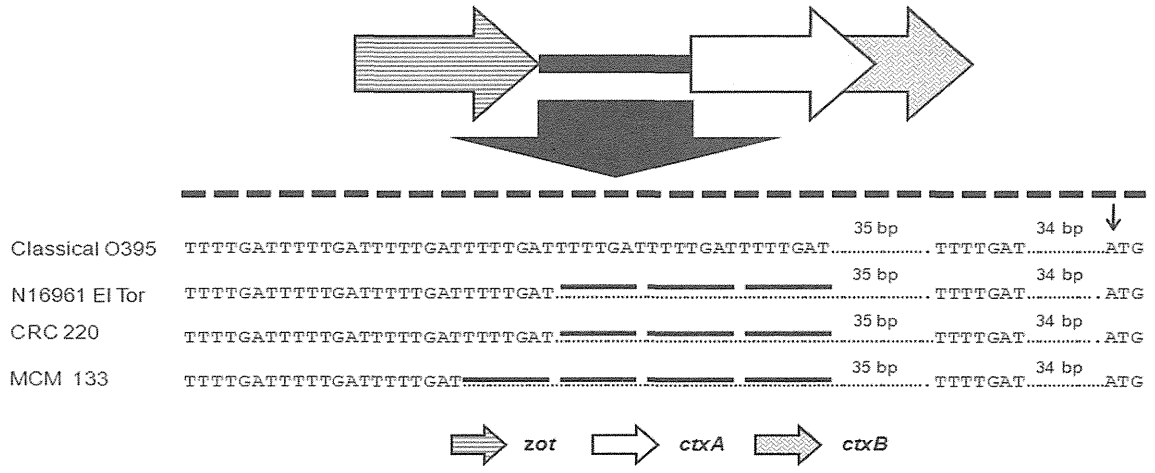
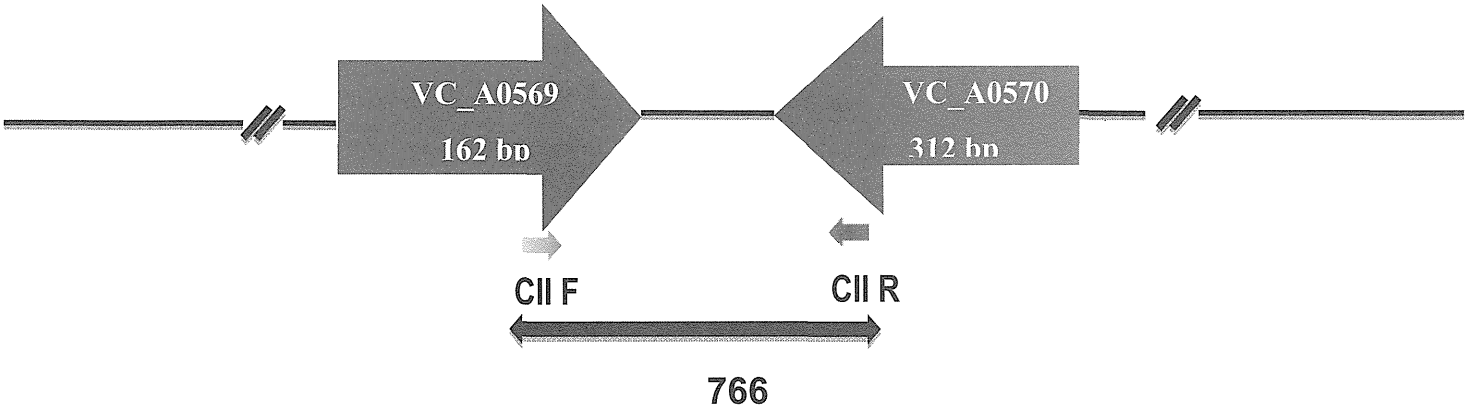


Figure 3

Figure 3: Comparative nucleotide sequence analysis of the promoter region the *ctxAB* operon (P_{ctxAB}) of Zanzibar isolate MCM 133 and Kolkata isolate CRC 220. The nucleotide sequences of P_{ctxAB} of O395 (classical control strain) and N16961 (El Tor control strain) were obtained from GenBank. Identical residues are indicated with dots. Each solid bar indicates the missing TTTTGAT heptads .The black arrow line represents the ATG start codon of *ctxA* gene. The Zanzibar isolate lacks a single heptad repeat in comparison with the Kolkata isolate.

Chromosomal localization of CTX prophage along with its organization: All the tested strains from Zanzibar yielded an amplicon of 766-bp in a PCR assay using CII-F and CII-R primers (Fig 4A). Presence of empty site amplicon indicated that small chromosome of the Zanzibar strains was devoid of any CTX prophage. We then carried out a long PCR designing primer from *rtxA* and *rstC*, respectively. This PCR assay yielded an amplicon of ~ 9 kb (Fig 4B) and therefore suggested that *V. cholerae* O1 isolates from Zanzibar probably harbors single copy of CTX prophage. **Fig. 5** shows a schematic diagram indicating how RS1 element of Zanzibar strain has shifted from upstream to downstream of the CTX prophage as compared to N16961 El Tor prototype strain.



A

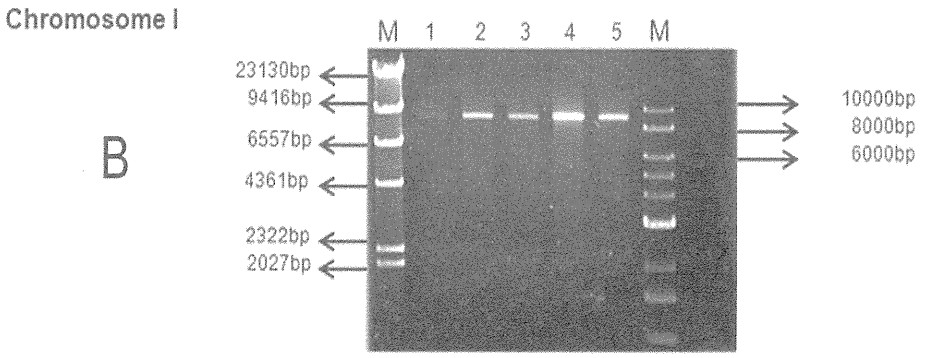


Figure 4

Figure 4: PCR results implicating the chromosomal organization of CTX Φ of *Vibrio cholerae* O1 Ogawa isolates from Zanzibar. (A). PCR results with primers CII F and CII R showing the absence of CTX prophage in chromosome II of Zanzibar isolates. The two black bars indicate the location of the two primers as shown in the figure. Extreme left include 100 bp ladder, 1: MCM 32, Lane 2: MCM 133, Lane 3: MCM 134, Lane 4: MCM 146, Lane 5: MCM 168, Lane 6: T1 Lane 7: MCF 084 Lane 8: MCF 001. El Tor control strain N16961 and classical control strain O395 were used as positive and negative controls, respectively. (B) Agarose gel electrophoresis showing the results of rstC1/rtxA1 PCR. Left M: lambda-Hind III ladder, Lane 1: MCM 133, Lane 2: MCM 168, Lane 3: KM 282, Lane 4: T1, Lane 5: WM 012: Right M: 1 kb DNA ladder.

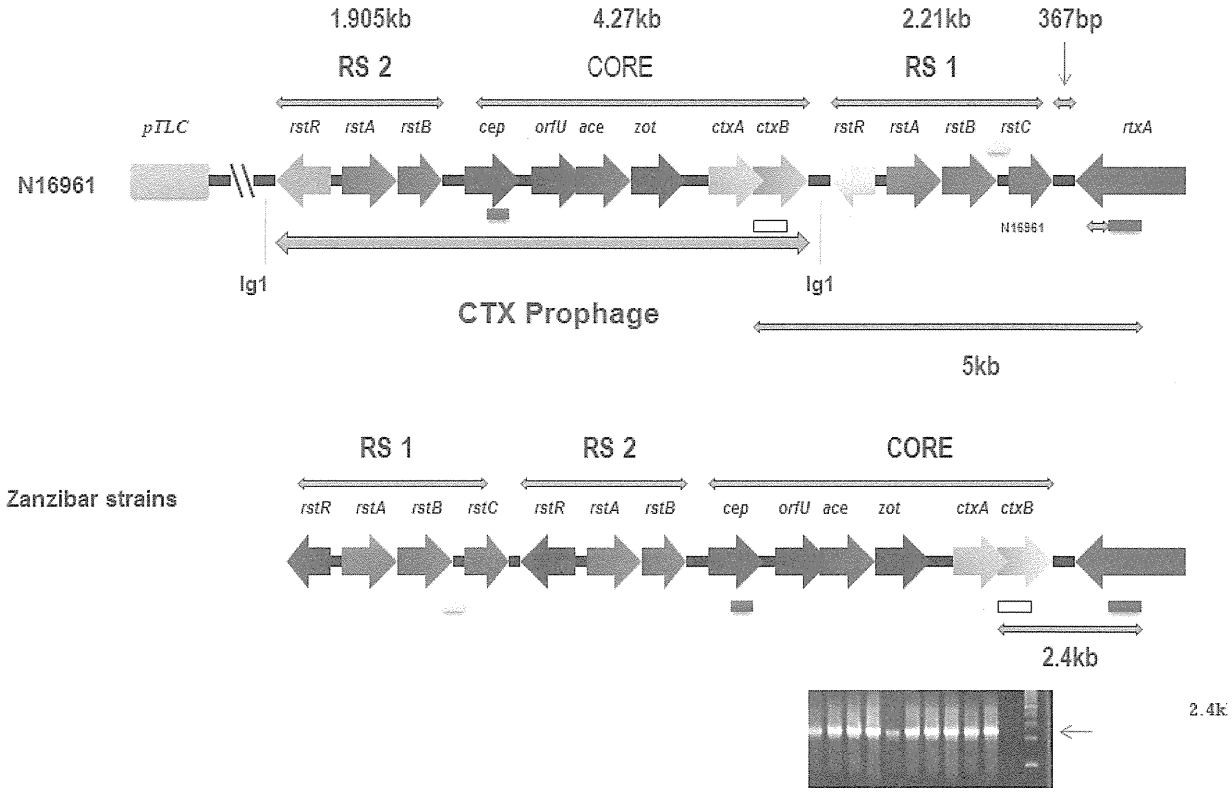


Figure 5

Figure 5: Comparative schematic diagram between N16961 and Zanzibar strain indicating shifting of the RS1 element.

Measurement of CT production by Beads ELISA and confirmation of production of classical CT by the Zanzibar strains:

The amount of CT produced was measured during the growth of the representative strains from Zanzibar in AKI medium and compared with the existing laboratory result using prototype El Tor and classical strains. It was found that all the El Tor variant stains from Zanzibar produced abundant CT in vitro than most strains of prototype El Tor (Fig 6). Fourteen of the 30 El Tor variant strains produced more than 1,000 ng/ml/OD_{600} of CT, and among them, 6 strains (MCM168, MCF001, WF01, MCF084, 210200 and 26NS051)

produced ≥2,500 ng/ml/OD_{600}, with the highest value of 4,957 ng/ml/OD600 by the 26NS001. Most of the El Tor strains produced <100 ng/ml/OD600 but all the classical strains produced >900 ng/ml/OD600. The CTB produced by the Zanzibar strains was confirmed to be the classical type with the Western blotting using monoclonal antibody against either classical CTB or El Tor CTB. All the Zanzibar strains reacted with monoclonal antibody specific to classical CTB, but not with that to El Tor CTB. The results of the Western blotting of the representative strains are shown in Fig 7.

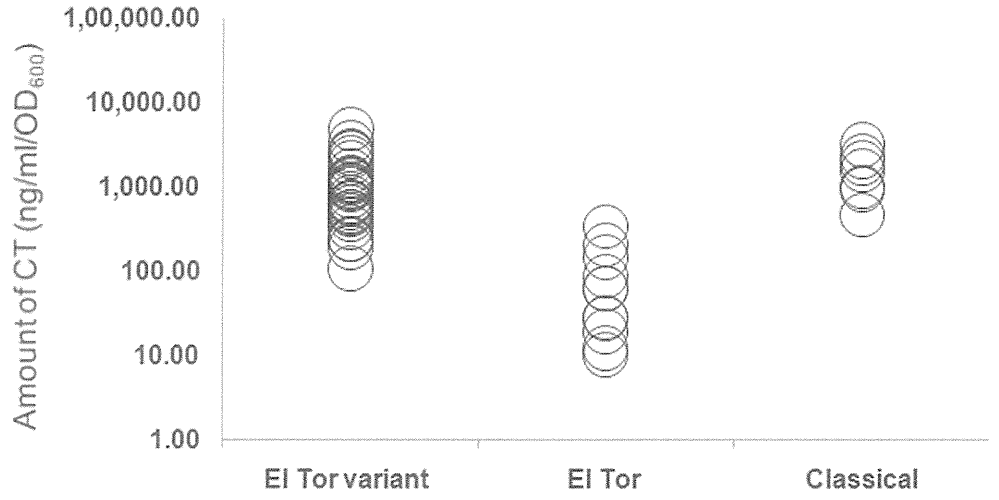


Figure 6

Figure 6: Amounts of cholera toxin production by Zanzibar variants, prototype El Tor strains and by classical strain. Each circle represents an average of three determinations.

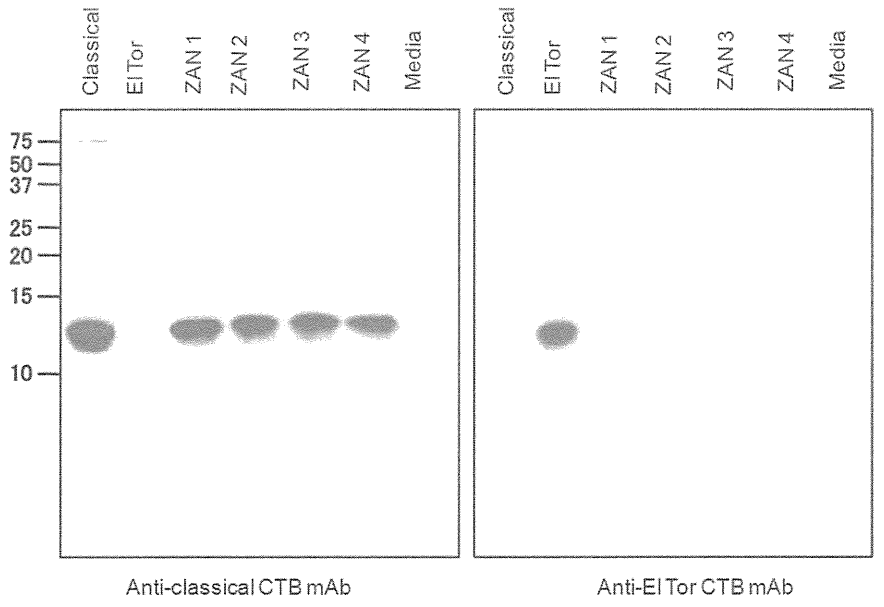


Figure 7

Figure 7: Western immunoblotting results of the culture supernatant of representative Zanzibar O1 isolates. 100 ng each of the purified classical CT (lane 1) and El Tor CT (lane 2) were used as positive controls for immunoblotting with the monoclonal antibody against classical and El Tor CTB, respectively. Lane 3: CF04, Lane 4: MCF147, Lane 5: MCF100, Lane 6: MCM79, Lane 7: media (negative control). Numbers at left are molecular masses in kilodaltons (X 1,000).

Discussion:

Cholera is mainly endemic in low-income countries in Africa, Asia, Central and South America. In recent years, it has become endemic in an increasing number of geographical areas, reflecting an unimproved socioeconomic infrastructure and difficulties in implementation of control measures in these settings. In 1970, the seventh cholera pandemic reached Africa after more than 100 years with devastating effects and since then the El Tor biotype has affected most parts of Africa including the sub-Saharan region. In Zanzibar, a cholera outbreak with 411 cases and 51 deaths was reported for the first time in 1978 from a fishermen village. Although, repeated outbreaks have been documented since 1978, we have very limited knowledge about the molecular epidemiology of *V. cholerae* isolated from these regions. To our knowledge, this is the first report elucidating the molecular epidemiology of cholera from this small island. Shifting of CT genotype 3 to 1 in *V. cholerae* O1 strains and its rapid spread to different parts of the world is one of the most enigmatic events in the history of cholera. This extraordinary and perhaps the most bewildering event forced us to

understand whether emerging variants of *V. cholerae* O1 have been disseminated in this isolated region.

Serotyping results have established that all the *V. cholerae* O1 strains isolated in this study belonged to Ogawa serotype. Interestingly, the neighbouring country Kenya has experienced around 40% isolation of *V. cholerae* O1, Inaba during the same period (Personal communication with Dr. Joseph Oundo). Our analysis found that the *ctxB* allele of all the tested strains were of classical type and Western blot analysis using monoclonal anti CTB specific for classical type confirmed this finding. Further genotypic and phenotypic characterizations of the tested strains fortified these strains as El Tor biotype. CT production assay showed that significant number of the Zanzibar strains produced much higher amount than the prototype El Tor, which was in agreement with our previous findings with the Kolkata strains. Growing number of unpublished data and two recently published reports revealed that significant number of El Tor variant strains produced more CT than the prototype El Tor strains. In addition, the amount of CT produced by El Tor variant strains was more or less equivalent to classical strains. It has also been shown that the difference of the amount of CT produced among these 3 biotypes were observed from the beginning of the growth (early logarithmic phase) till the late stationary phase. Although a definite evidence to explain this is still not available, it has been hypothesized that a significant difference between the amounts of CT produced by these two biotype strains may reflect severity of clinical manifestation. Another interesting observation is that the promoter region of *ctxA* of higher CT producing Zanzibar strains had lower number (3) of heptad repeats. This indicates lack of association of the repeat regions as the critical determinant for CT production. In another study, Siddique et al showed that during a clinical study, large number of patients were admitted with more severe dehydration in Bakerganj and Mathbaria, hospitals in southern Bangladesh and all the *V. cholerae* strains isolated from these patients produced classical CT. These reports clearly indicate that the emerging variants are environmentally sturdier than the prototype El Tor ones and dissemination of these strains can cause severe cholera in areas they spread.

Given that there are differences between the classical and El Tor biotypes, the selection of El Tor variant strain seems to signify an evolutionary optimization of the El Tor biotype and could represent a new, more virulent form of the El Tor biotype. It would be interesting to know the lineages of the Zanzibar strains as the specific change in *ctxB* of El Tor strains was first observed in Kolkata during 1990. These new *V. cholerae* O1 El Tor variant strains not only replaced the *V. cholerae* O1 El Tor prototype strains, but also turned

out to be genetically stable and spread rapidly even to remote islands in the east African continent as evidenced from this study. Moreover, the severity of the disease appears to be intensifying, and recent cholera outbreaks in various places, including Zimbabwe, have followed protracted period. An active holistic surveillance system should be in place for tracking the mode of the dissemination of the *V. cholerae* O1 El Tor variant strains in the population using latest molecular diagnostic assays, as these strains possess all the potentialities and foundation for a new pandemic.

Moreover, a recent study provided evidence from the temporal patterns of cholera cases reported between 2002 and 2008 in Zanzibar that rainfall and temperature, among various climate and ocean environmental factors s are the key drivers of cholera outbreaks. Such predictive models may help public health authorities to prepare medical equipment and mobilization of staff and mass oral cholera vaccination.

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

Development of a PCR assay based on single mutation: In this study, our first purpose was to establish a PCR based assay which can comprehensively discriminate *V. cholerae* strains carrying Haitian, classical and El Tor alleles of *tcpA* in a simple and rapid way and later on it can be used to understand the dissemination of the new variant in different parts of the world. Three separate primers, which include one reverse primer specific for both El Tor and Haitian type *tcpA* alleles (*tcpA* EL-Rev) and two forward primers (*tcpA*-F1 and *tcpA*-F2)specific for El Tor and Haitian type *tcpA* alleles, respectively were designed. These allele-specific primers each carry specific nucleotide, A and G, for El Tor and Haitian type, respectively, at the 3' end. Furthermore, we enhanced the 3' mismatch effect by introducing another nucleotide alternation G (rather than A) at the second nucleotide (i.e., the 265th nucleotide) from the 3'end of both the primers. We have standardized the PCR to optimize both the specificity and sensitivity. Our newly designed PCR successfully differentiated the three different allelic subtypes of *tcpA*. *V. cholerae*O1 strains having the *tcpA* allele of El-Tor type yielded a 167-bp fragment of DNA with the primer pair *tcpA*-F1/-tcpA el-rev but not with *tcpA*-F2/ tcpA el-rev (**Fig 8**). The Haitian control strain (EL-1786) produced just the reverse result with the same primer sets, and the classical strain (0395) did not show any amplicon in either PCR assay because irrespective of the 266 nucleotide position classical *tcpA* allele differs significantly from EL Tor *tcpA*.

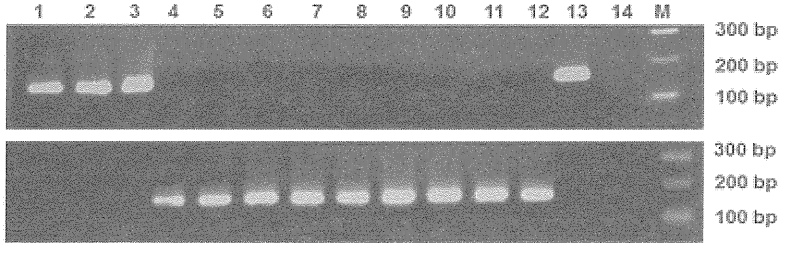


Figure 8

Figure 8: Standardized PCR assay to detect the type of *tcpA* allele in representative *V. cholerae* O1 strains of Kolkata using primers(*tcpA*F1/tcpA EL-Rev) for El Tor *tcpA* allele (Upper panel) and (*tcpA*F2/tcpA EL-Rev) for Haitian *tcpA* allele (Lower panel).

Sequencing analysis to evaluate the PCR based result: To further confirm our PCR based result, 16 representative strains, which yielded positive bands for Haitian *tcpA* gene using the newly developed PCR, were selected for DNA sequencing of *tcpA* gene. Nucleotide sequence analysis of the *tcpA* gene of *V. cholerae* O1 revealed that the strains possessed DNA sequences identical to that of the EL Tor type of *tcpA* but with a mutation at the 266th position (A to G). The deduced amino acid sequences of all 16 representative strains were aligned with the TcpA sequence of the El Tor reference strain N16961. The amino acid sequence of all strains were found to be identical to the deduced amino acid sequence of the TcpA of the El Tor reference strain N16961 except for an asparagine to serine substitution at the 64th position of the matured TcpA **(Fig 9).** Thus, the results from DNA sequencing of the *tcpA* gene confirmed the results of newly designed PCR.

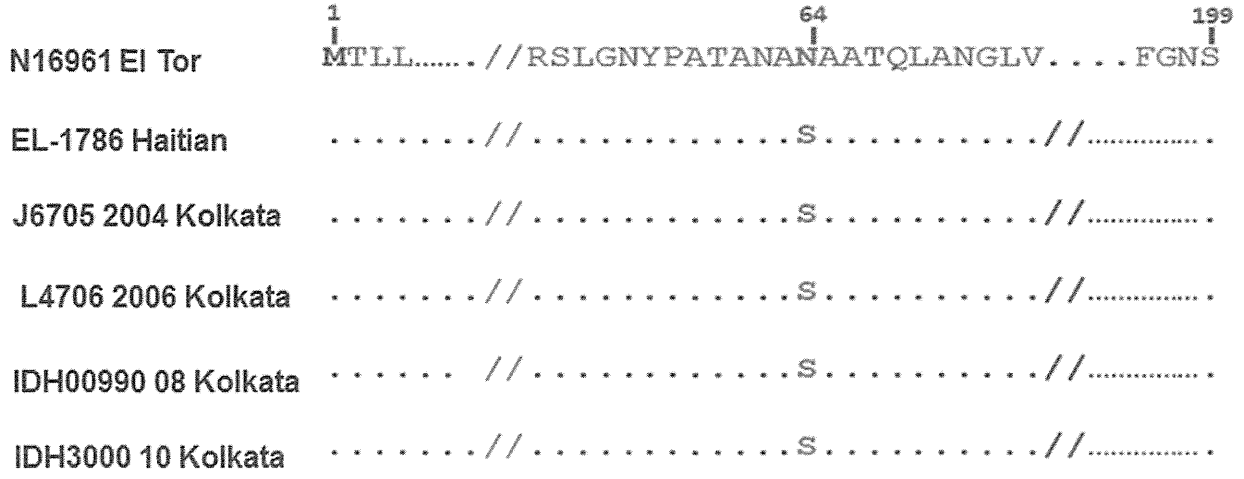


Figure 9

Figure 9: The deduced amino acid sequence of TcpA of representative Kolkata isolates were found to be identical to the amino acid sequence of the matured TcpA of the El Tor reference strain N16961 except for an asparagine to serine substitution at the 64th position of the sequence confirming its identity with the Haitian type TcpA.

Screening of Kolkata strains using the allele specific *tcpA* PCR: We screened 251.*V. cholerae* O1 strains isolated from Kolkata between 2001 and 2012. After standardizing the newly developed PCR, we used this assay extensively to investigate the emergence and dissemination of the Haitian *tcpA* in Kolkata. 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 noted in Kolkata in October, 2003 and interestingly soon after its appearance;

this new variant of *tcpA* displaced the canonical El Tor *tcpA* completely in the following years **(Fig 10).**

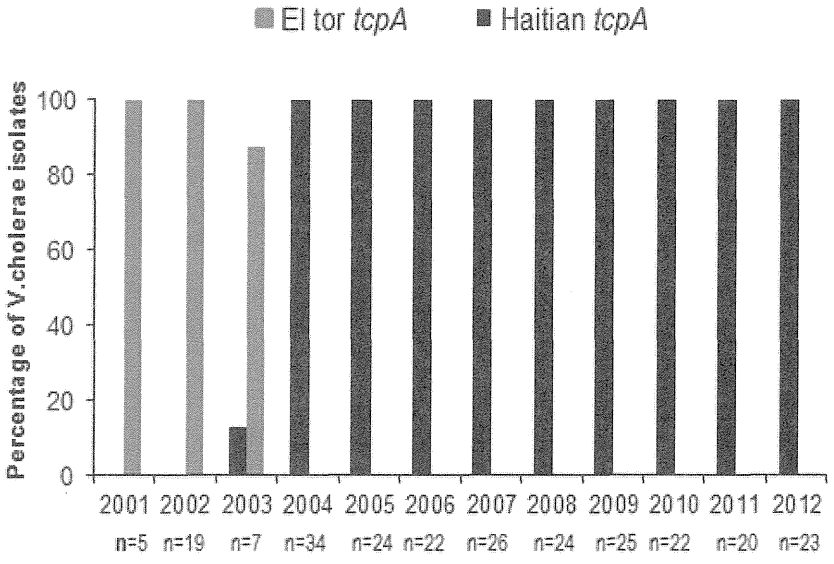


Figure 10

Figure 10: Isolation profile of *Vibrio cholerae*O1 strains with El Tor and Haitian type of *tcpA* in Kolkata. *V. cholerae* O1 strain with Haitian type *tcpA* was first time isolated in Kolkata during October 2003.

Bioinformatics based analysis: Considering that the evolutionary rate may vary depending on the functional constraints we have used SWAKK web server (http://oxytricha.princeton.edu/SWAKK/) to calculate evolutionary rate for each single amino acid residue in a protein sequence. 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 ω was measured individually. Here, ω is used as a measure of selection pressure. Out of these three mutations only Asn->Ser mutation has been found to be positively selected. Here the positive selection for the conversion of Asn->Ser of *tcpA* gene is a feature of antagonistic coevolution, which implies harmful effects on the host, but also mutualistic coevolution, which infers benefits.

Discussion:

TCP is one of the major virulence determinants of *V. cholerae.* Although TcpA distinctly does not mediate direct contact between *V. cholerae* cells and the intestinal epithelial cell surface, the strains deficient in TcpA are severely attenuated and unable to

colonize intestinal surfaces, indicating that microcolony formation is crucial for full bacterial adherence and pathogenesis. TCP is also the receptor for CTX Φ. The Type four pilus of the classical and El Tor biotypes of *V. cholerae* share 81% identity in their TcpA subunits, yet these filaments differ in pilus-pilus interaction patterns as assessed by electron microscopy. This bundle-forming pilus also appears to be involved in *V. cholerae* biofilm production on chitinous surfaces in aquatic environments.

Whole genome sequence analysis of *V. cholerae* strain isolated from the devastating Haitian cholera outbreak contained a unique mutation at the 20th amino acid of CTB and at the 89th amino acid position of the whole TcpA subunit. Our previous study indicated that the Haitian *ctxB* first appeared in Kolkata during April, 2006 and our newly developed PCR assay detected that Haitian variant *tcpA* first appeared in Kolkata during 2003. It means that all the *V. cholerae* strains in Kolkata had classical *ctxB* with El Tor *tcpA* up to September 2003. Then the combination changed to classical *ctxB* with Haitian *tcpA* from October 2003 onwards. Finally, certain percentage of *V. cholerae* strains in Kolkata acquired the combination of Haitian *ctxB* with Haitian *tcpA* from April 2006 onwards. Our study reveals that this Haitian variant strain may be result of the sequential genetic events in the evolution of the *V. Cholerae* in this region Indian subcontinent strain. These results not only signify a cryptic change in the epidemiology of cholera but also raise questions about the origin of these variants of *V. cholerae* O1 El Tor. We speculate that Haitian type of *tcpA* may have originated from Kolkata and then disseminated to the neighboring regions like Nepal, although conformation of this hypothesis requires several other epidemiological and experimental validations.

TcpA is made up of 224 amino acids. A proteolytic cleavage between amino acids at position 25 and 26 results in generation of a 25 amino acids atypical leader peptide and 199 amino acids mature TcpA. 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. Our result indicates that there is an underlying similarity in patterns of point mutation between El Tor and Haitian strain. 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. As purine concentrations are higher for intracellular than for extracellular fluids, these epithelium-associated bacteria may experience purine concentrations different from that of bacteria inhabiting the mucus. The present result thus provide us better understanding the ability of *V. cholerae* to transport and salvage purines from the environment, and the importance of purine salvage for virulence. It

has been hypothesized that the unique genetic composition of the new variants increases their relative fitness, perhaps as a consequence of increased pathogenicity. Moreover, positive selection in virulence factors might have different outcomes, including: adaptation of a species to optimize the process of infection, to escape host immune response, inhabit different environmental niches. Here the positive selection for the conversion of Asn->Ser of *tcpA* gene which may have the ability to successfully invade and colonize its host. It is a feature of antagonistic coevolution, which implies harmful effects on the host, but also mutualistic coevolution, which infers benefits.

Finally, our retrospective analysis using the newly developed PCR assay showed that new variant *V. cholerae* O1 with Haitian *tcpA* not only outfitted the prototype *V. cholerae* El Tor strains, but also turned out to be stable over the years as evidenced from our study. There exist a strong urgency to identify and tracking the mode of the spread of these new varieties of *V. cholerae* O1 strains in the population by strengthening and implementing an integrated, multisectoral holistic surveillance system to prevent and contain outbreaks among vulnerable populations living in high-risk areas. With the appearance of the new El Tor variant, there has been a subtle but distinct change in the epidemiology of cholera in recent years. These differences include an increase in the severity of the disease as compared to those caused by prototype El Tor, the tendency of recent epidemics being protracted and occurring over longer periods of time like the cholera outbreak in Zimbabwe and Haiti and the tendency of enhanced antibiotic resistance. El Tor strains currently associated with cholera in different parts of the world including the recent Haiti outbreak are the variant El Tor biotype and appear to represent the evolution of a more efficient emerging form that is capable of causing severe cholera. An active holistic surveillance using this newly developed PCR assay will help to track the dissemination mode of the new Haitian variant *V. cholerae* O1 strains in different endemic population, as these strains possess all the potentialities and foundation for a new pandemic.

Future plan of works:

- 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
- Comparative analysis of El Tor and Haitian TcpA containing strains in animal models

2. *Differential pathogenesis of Giardia : Role of Giardia Virus*

i) **Name of PI:** Dr. Sandipan Ganguly

ii) **Name of the Co-PI:** Prof. Tomoyoshi Nozaki

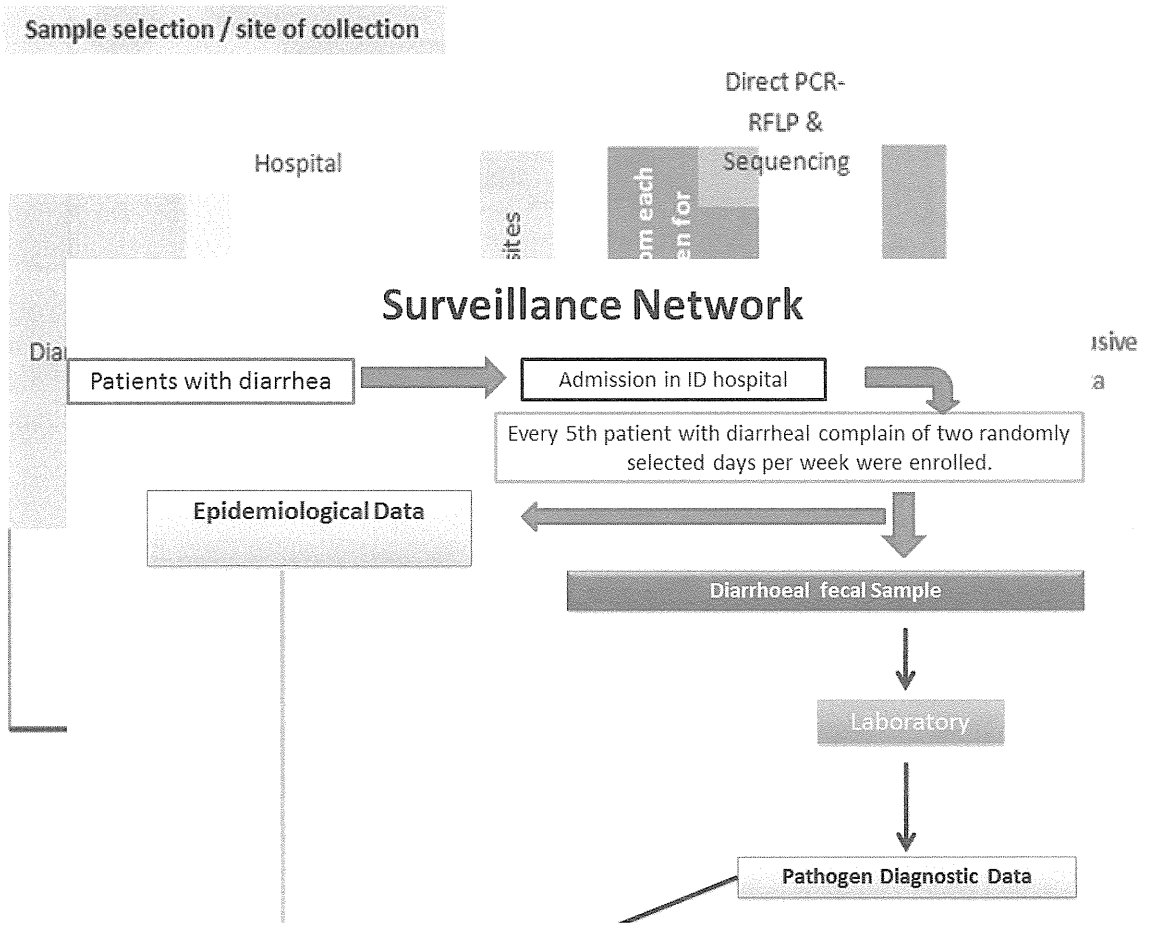
iii) **Title of the Project:** ***Differential pathogenesis of Giardia : Role of Giardia Virus***

iv) **Objectives of the Project:**

- To identify the infection of different GLV among different isolates of *Giardia lamblia* in patients with differential infection, i.e. with and without symptomatic *Giardia lamblia* infection and with multiple infections along with *Giardia lamblia* as a co-infection and also with asymptomatic *Giardia lamblia* infection.
- To understand if there is any genetic variability in housekeeping as well as pathogenic genes (*e.g.* the excretory secretory protein genes of *Giardia lamblia*) before and after infection of GLV or the outcome of differential pathogenesis is only a result of differential transcriptomic expression by PCR, RT PCR and Microarray hybridization.

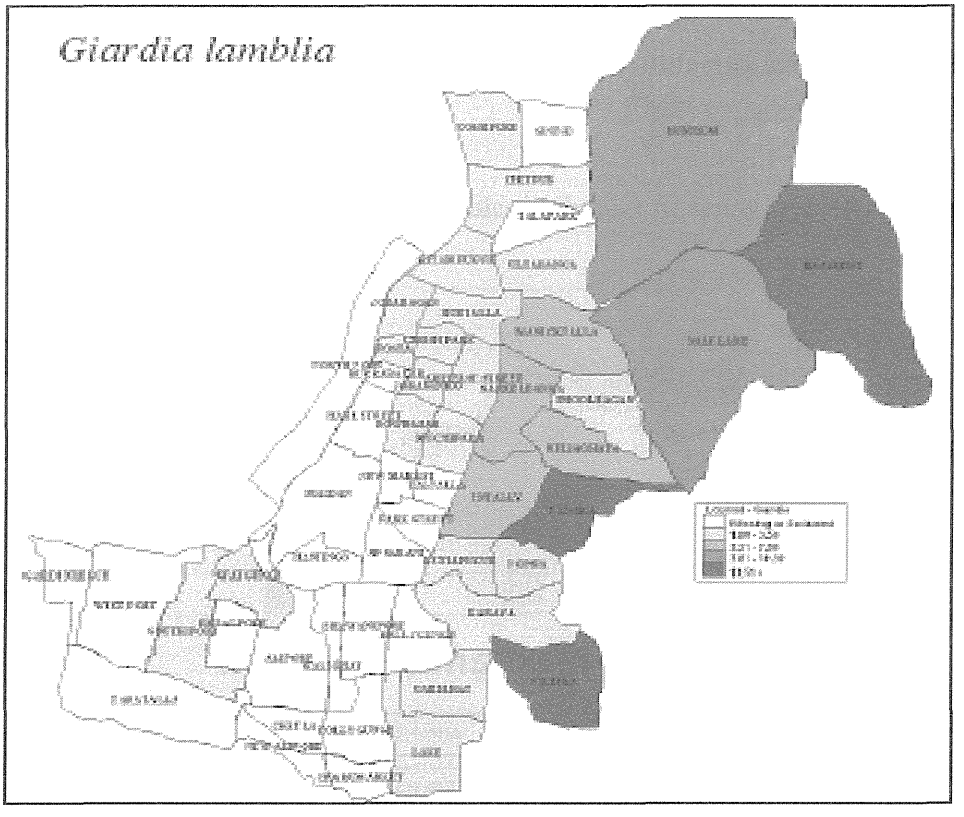
v) **Plan of work for fiscal year 2013-2014**

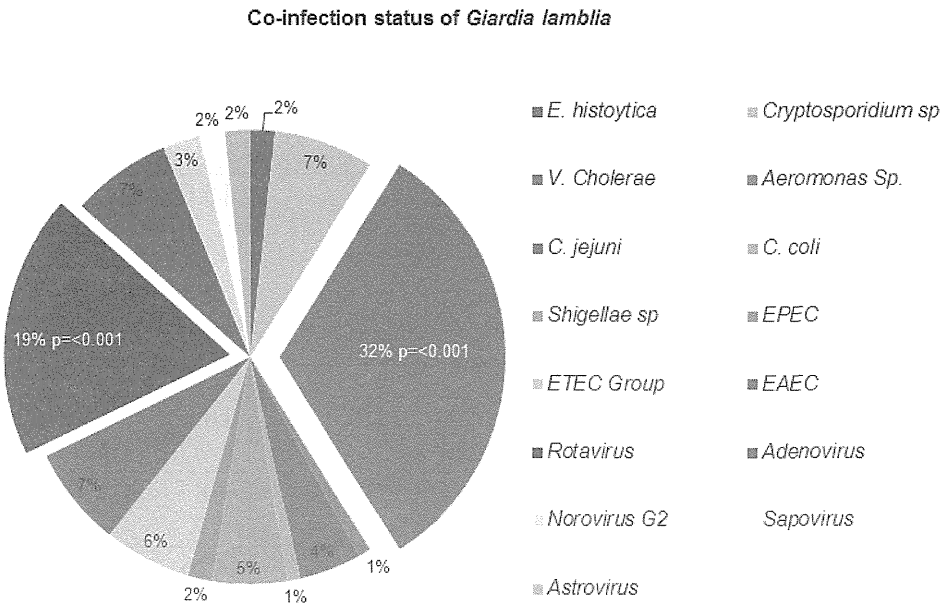
- **Accessing the genetic variability among local *Giardia* strain**
- **Detection of GLV**
- **Identification of new genes in Giardia inside human GUT**



GIS Mapping

A Geographical Information System (GIS) map was constructed using the Choropleth Mapping Analysis System (Frerichs et al. 2000) for all patients enrolled by the surveillance system in order to establish the catchment areas of the parasites by evaluating their spatial distributions in Kolkata. For this map, the different colors and patterns were combined to depict the different values of the attribute variable associated with each area. Each area is colored according to the category into which its corresponding attribute value had fallen. The positive cases were embedded on the thematic map by the GIS to visualize the infections.





Association between rainfall and Giardia prevalence: Average seasonal rainfall in the study region (Indian Meteorological Department Database), average Giardia detection rates, and the percentage of Giardiasis among all diarrheal cases.