# "Laboratory–based collaboration net work of infectious diseases in Asia"

**Funding Organization: National Institute of Infectious Diseases*, Tokyo, Japan***

**Indian Investigators :**

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**1. Title of the Research Project :**

**"**Laboratory –based collaboration net work of infectious diseases in Asia**"**

**2. Objectives**

The proposal has been developed with a broader objective to understand the evolution of pathogenesis of enteric pathogens and HIV that include phenotypic and genotypic characteristics of identified pathogens from diarrheal specimens as well as identification of novel factors for pathogenesis, to develop potential vaccine and to study mutations in HIV during infection which has an association with different genotypes and might be used as biomarkers; the study objectives will be covered though extensive work under the following areas and each of these areas will be covered by the Co-PIs of the project

*i) Retrospective analysis on the evolutionary aspects of Vibrio cholerae*

*ii) Differential pathogenesis of Giardia : Role of Giardia Virus*

*iii) Development of universal Shigella vaccine based on virulence gene expression.*

*iv) Analysis of HLA associated HIV-1 mutations in India and Japan.*

**3. Summary of the research for fiscal year 2013-14**

### *1. Retrospective analysis on the evolutionary aspects of Vibrio cholera*

# Third 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:**

In recent years, cholera has become endemic in an increasing number of geographical areas. Our previous study on *Vibrio cholerae* strains isolated from Kolkata over 17 years

from 1989 to 2005 depicted that in Kolkata, *V. cholerae* O1 strains with classical allele of *ctxB* have totally replaced seventh pandemic El Tor strains possessing El Tor allele of *ctxB* since 1995. Reports from other various groups revealed that the prototype El Tor strains of *Vibrio cholerae* O1 from the seventh pandemic have been replaced by variants of *V. cholerae* O1 El Tor which produces cholera toxin (CT) of classical type in many parts of the world. Shift of CT genotype 3 to genotype 1 in *V. cholerae* O1 strains and detection of diversity in the CTX phage repressor, *rstR* provided an impetus to analyze CT genotypes along with the CTX prophages in *V. cholerae* strains isolated from Zanzibar to understand whether emerging El Tor variant has disseminated in this isolated region. Detailed molecular analysis of representative strains showed that they are of El Tor biotype that produces classical CT and harboured single CTX prophage. In vitro CT production assay with representative strains showed that significant number of the variant strains produce higher amount of toxin in the highly sensitive beads-ELISA, which is almost equivalent to that of classical strains. To our knowledge, no such report has been made from this small island of Africa focusing the molecular epidemiology of cholera, although recurrent outbreaks have been documented since 1978. 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.

The toxin-coregulated-pilus (TCP) is a crucial determinant of the pathogenicity of *Vibrio cholerae*. TCP is essential for intestinal colonization and serves as a receptor for CTXΦ. Whole genome sequence analysis of *V. cholerae* strain isolated from the Haitian cholera outbreak revealed a unique mutation at the 89$^{th}$ amino acid position of the TcpA subunit. In this retrospective analysis, we investigated the emergence and dissemination of this Haitian TCP variant in Kolkata, India. Based on the sequencing analysis of *tcpA*, a new PCR technique was developed for the rapid identification of *V. cholerae* strains carrying Haitian, classical and El Tor alleles of *tcpA*. This assay was subjected to screen 251 *V. cholerae* O1 strains isolated during 2001-2012 in Kolkata for understanding the genesis and spread of the Haitian *tcpA* along with the bioinformatics analysis. Our results showed that Haitian *tcpA* first appeared in Kolkata during October, 2003, and interestingly soon after its appearance; this new variant *tcpA* displaced the canonical El Tor *tcpA* completely in the following years. Our bioinformatics analysis showed that among the three different mutations present in 89$^{th}$position, only Asparagine to Serine is positively selected. Moreover, this particular mutation is the result of a purine-purine transition, which is conserved natural selection. This finding indicated that Haitian *tcpA* might have originated in Kolkata and then

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 37ºC. 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).

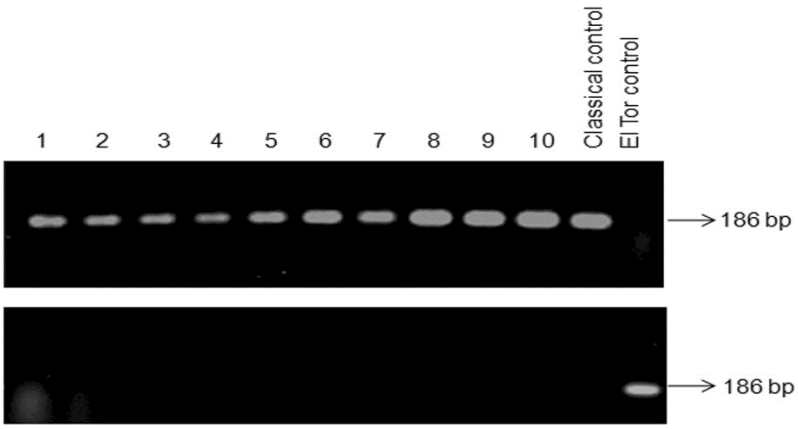



Figure 1

**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.

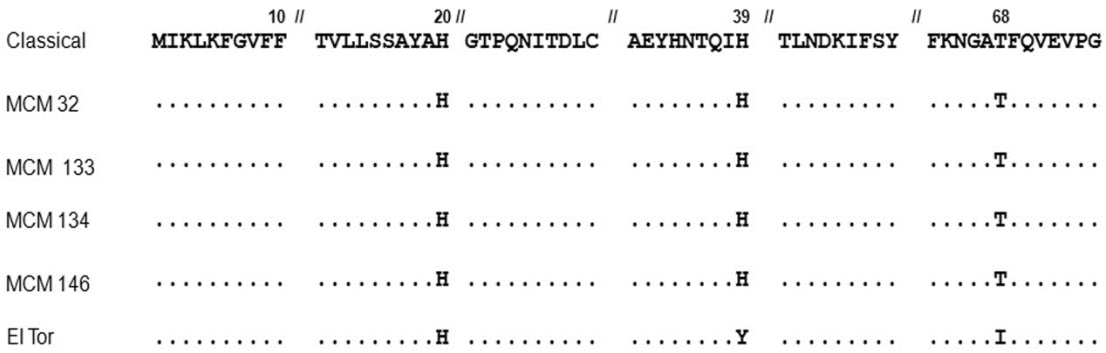



Figure 2

**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).

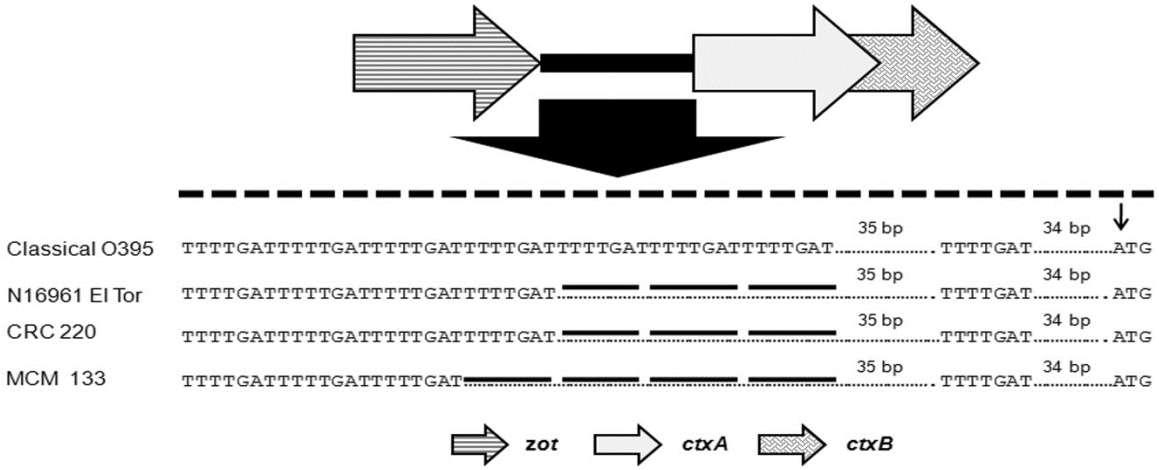
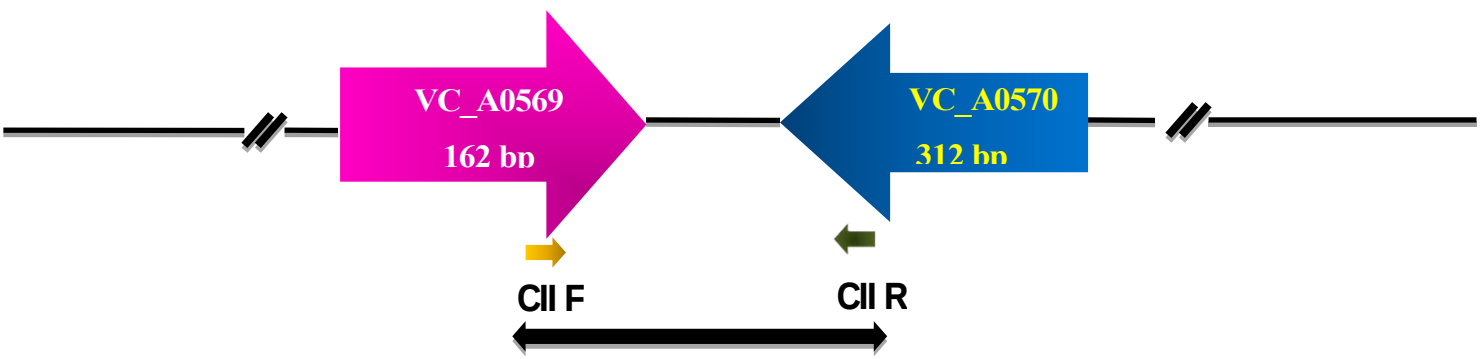



Figure 3

**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**

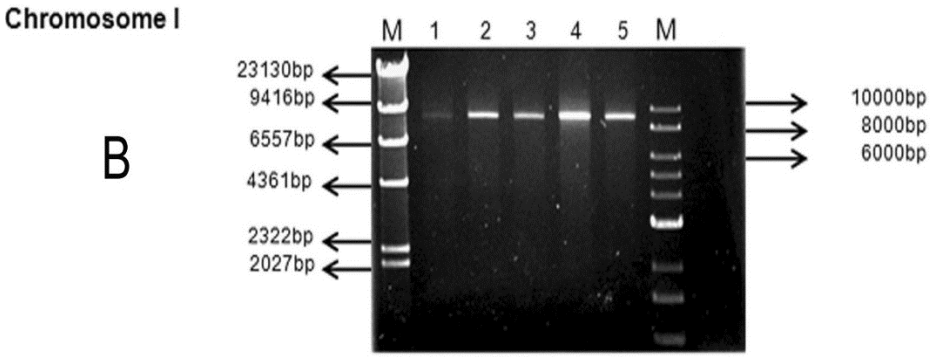
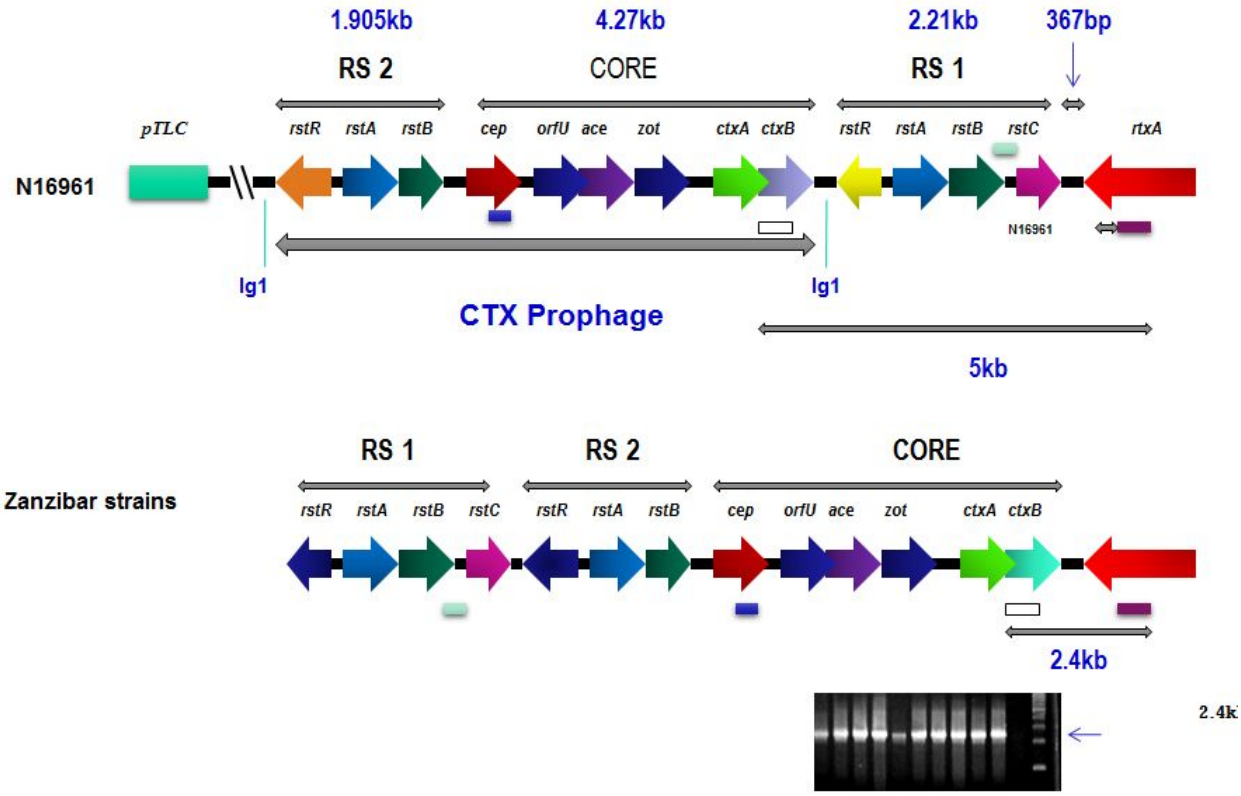



**Figure 4**

**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.

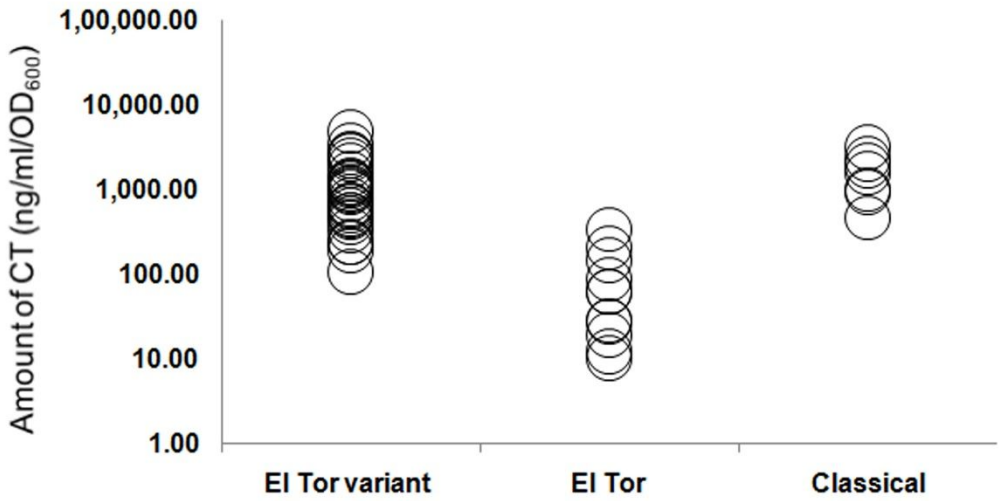



Figure 6

**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**.**

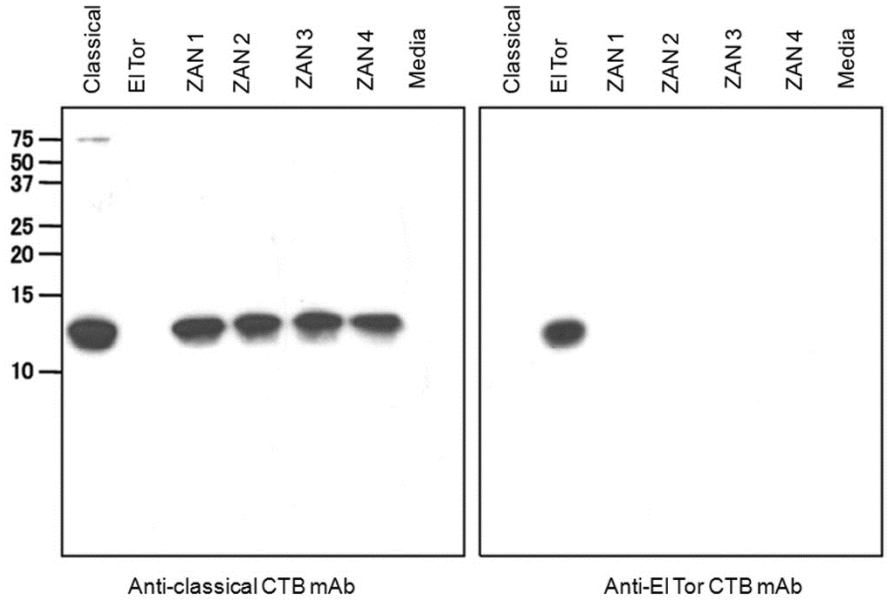



Figure 7

**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 265$^{th}$ 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*.

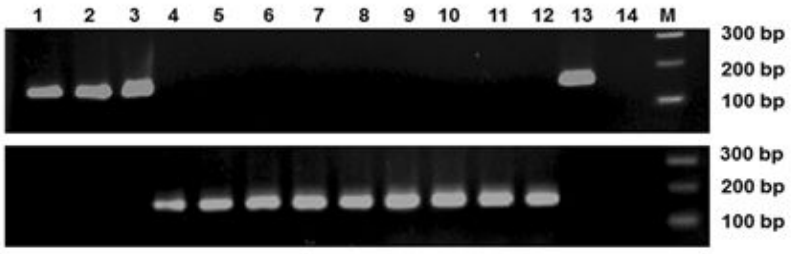



**Figure 8**

**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 64$^{th}$ position of the matured TcpA **(Fig 9).** Thus, the results from DNA sequencing of the *tcpA* gene confirmed the results of newly designed PCR.

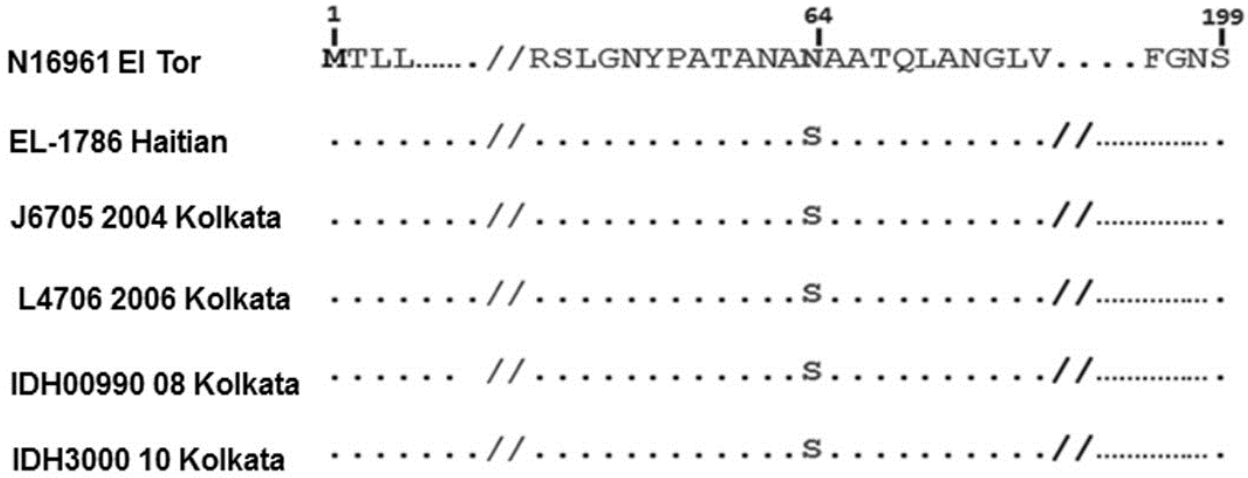



**Figure 9**

**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 64$^{th}$ 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).**

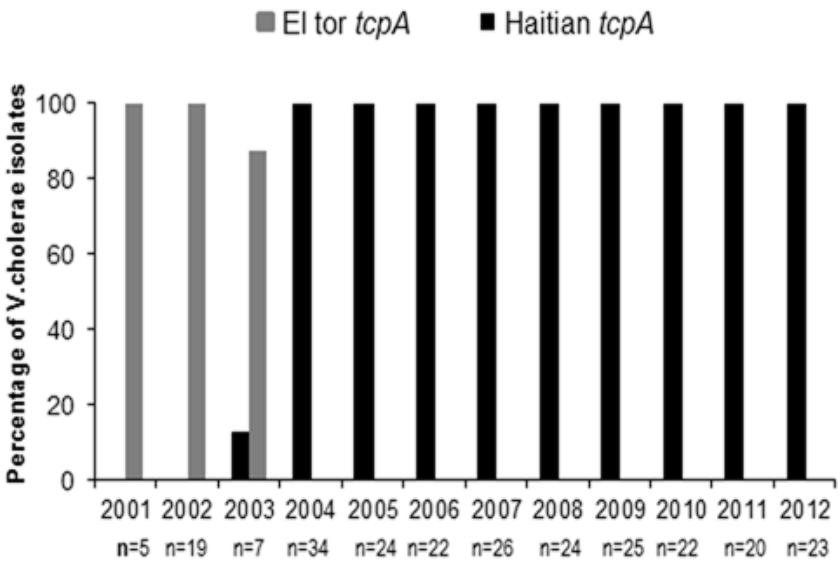



**Figure 10**

**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 89$^{th}$ 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 20$^{th}$ amino acid of CTB and at the 89$^{th}$ 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 89$^{th}$ amino acid of whole TcpA (or 64$^{th}$ 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

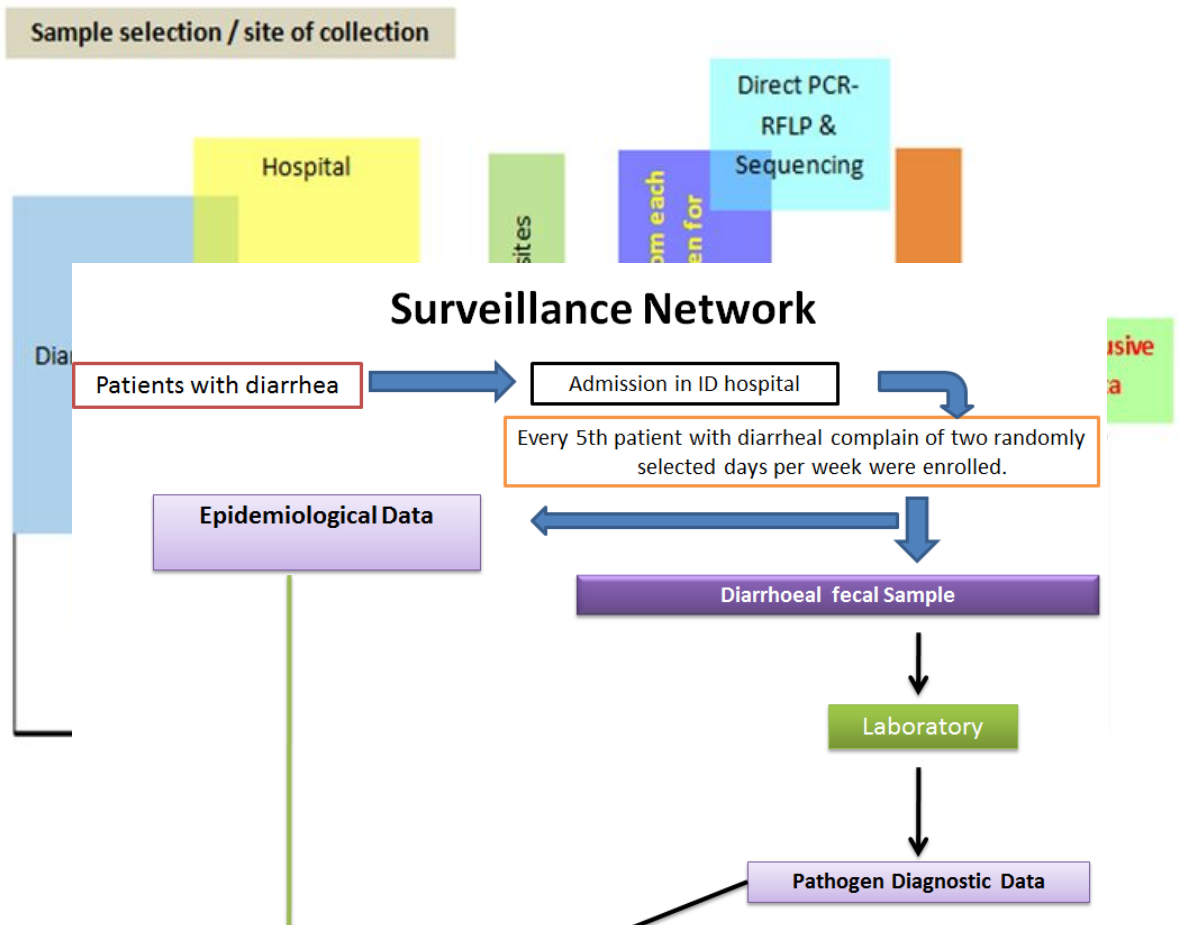
**iii)** **Title of the Project:** ***Differential pathogenesis of Giardia : Role of Giardia Virus***

**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.

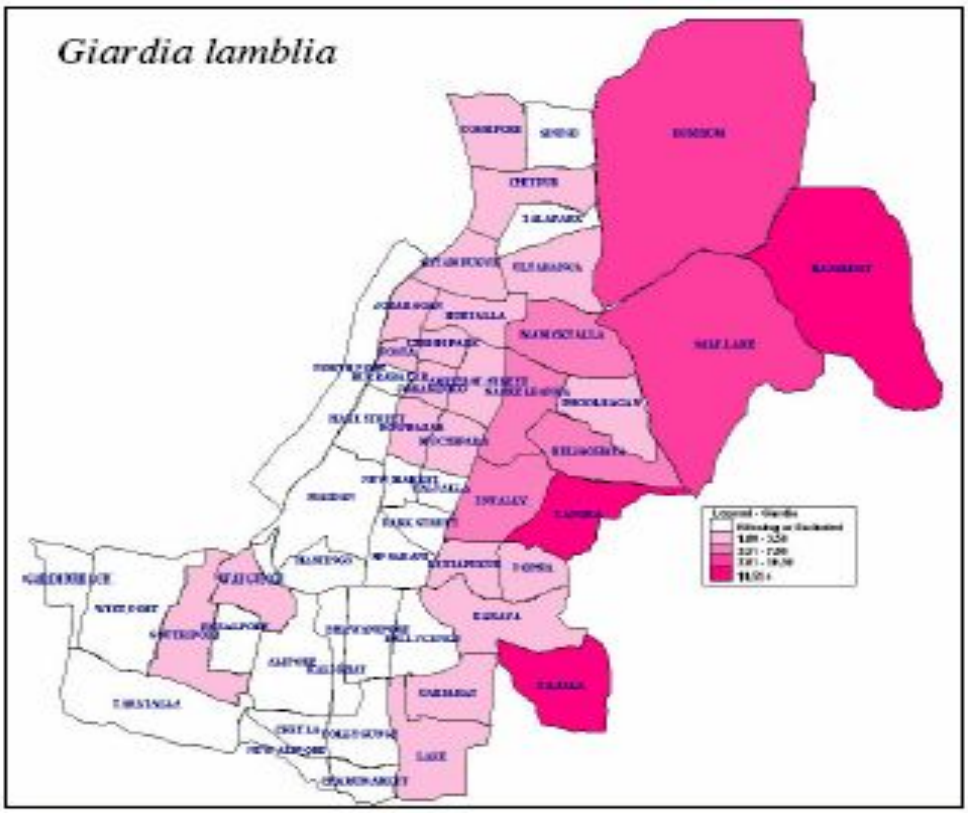
**v)** **Plan of work for fiscal year 2013-2014**

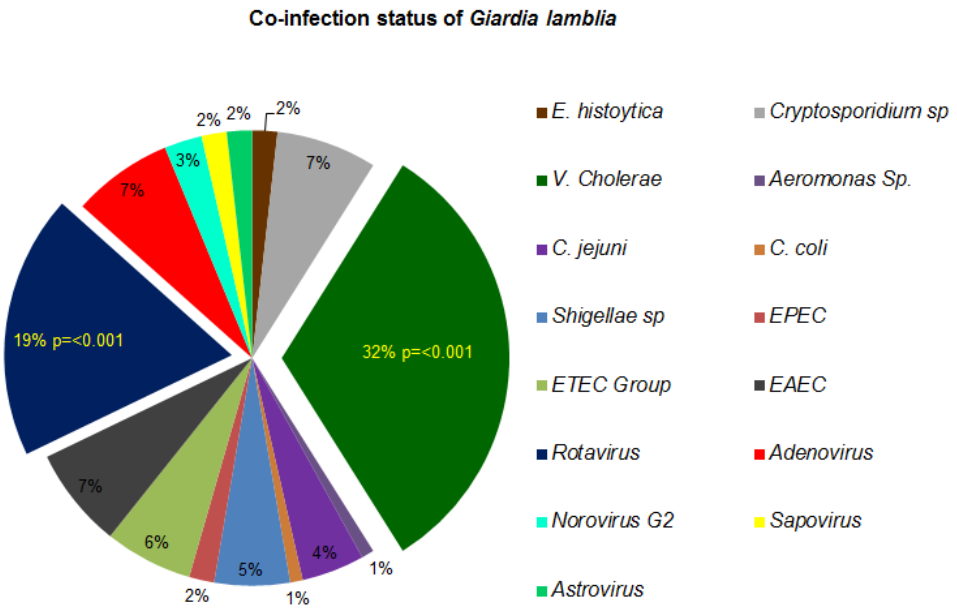
- **Accessing the genetic variability among local *Giardia* strain**
- **Detection of GLV**
- **Identification of new genes in Giardia inside human GUT**



## 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.



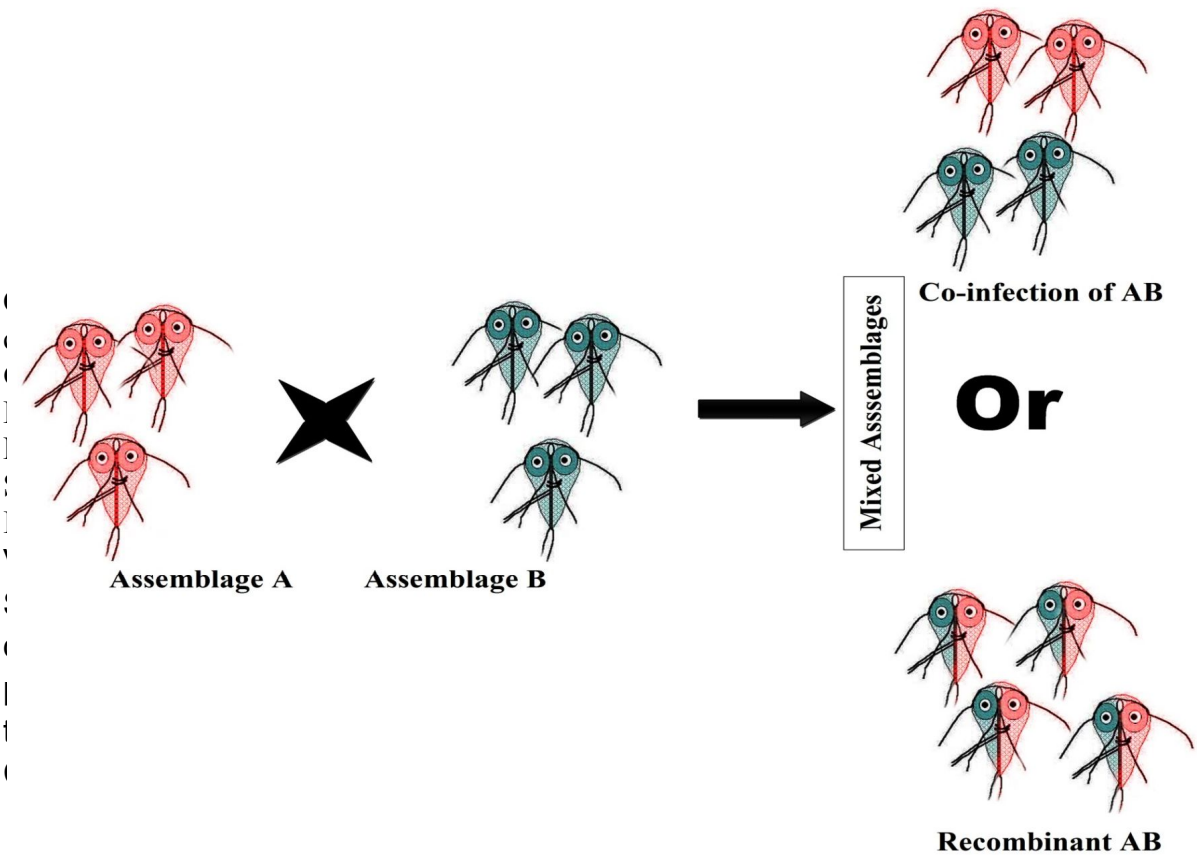



**Association between rainfall and Giardia prevalence:** Average seasonal rainfall in the study region (Indian Meteorological Department Database), average Giardia detection rates, and the percentage of Giardiasis among all diarrheal cases.

| **Season** | **Average Rain (mm)** | **Monthly average *G. duodenalis* positive cases** | **Total diarrhea cases** | **Monthly average *G. duodenalis* positive (%)** |
|---|---|---|---|---|
| Pre-Monsoon/ Summer 08 | 153.4 | 11 | 73 | 15.05 |
| Monsoon 08 | 1291.7 | 12.75 | 103.5 | 12.02 |
| Post-Monsoon 08 | 70.3 | 12 | 110.3 | 10.1 |
| Winter 09 | 3.4 | 4.5 | 91 | 4.8 |
| Pre-Monsoon/ Summer 09 | 251.8 | 11.7 | 123 | 9.26 |
| Monsoon 09 | 971.5 | 18.75 | 141 | 13.5 |
| Post-Monsoon 09 | 95.7 | 5.7 | 73.3 | 7.73 |
| Winter 10 | 16.6 | 2 | 34 | 6.3 |
| Pre-Monsoon/ Summer 10 | 143.7 | 7.3 | 67 | 10.83 |
| Monsoon 10 | 787.4 | 4 | 48.25 | 8.32 |
| Post-Monsoon 10 | 138.8 | 4.7 | 48 | 10.3 |
| Winter 11 | 5.4 | 4 | 37.5 | 10.7 |
| Pre-Monsoon/ Summer 11 | 245.2 | 5 | 51.7 | 10.03 |
| Monsoon 11 | 1391.6 | 1.75 | 35.5 | 4.87 |
| Post-Monsoon 11 | 29.5 | 2.7 | 32 | 9.6 |

Homology analysis showed that the sequences within each assemblage A and B clusters for all the loci are mostly homologous (data not shown) taking all the isolates in account. Also in the combined phylogenetic analysis, these 14 isolates doesn't make any separate cluster and moreover they are evenly distributed in the respective cluster either assemblage A or B similar to Fig. 1. Hence, in this case the outcome of assemblage was solely depended on the marker loci. Association of genotype outcome with other physical factors such as Age, Sex and co-infection status was also checked but no particular association was observed. From this finding it can be said that the differential taxon position of this 14 isolates could not be due to chance and it is a true example of mixed assemblage. Two major reasons can be placed in favor of this outcome i.e. presence of mixed infection or co-infection of two different strain & occurrence of genetic recombination through sexual reproduction. Previous reports of mixed assemblages relied on the presence of multiple peaks in a particular position in the chromatograms which lead to the ambiguous taxon positioning of the isolates in the phylogenetic tree which can be due to mixed infection but, in this case all the isolates with dual genotype are positioned perfectly in a particular cluster for a specific loci. Although, recent reports suggest towards considering this type of observations as mixed assemblage infection, in spite of a thin probability of inter-assemblage recombination, still detailed molecular epidemiological study is required to find out the exact reason behind this unique finding. However, detection of high percentage of mixed genotype is evident, whether it is due to mixed infection or genetic recombination.

**Possible hypothesis**



- Viral RNA was denatured in the presence of GLV-CF and GLV-CR primers (0.4 mM) by heating at 94 C for 3 min, quick-chilled in wet ice, and subjected to RT-PCR following procedures provided by the manufacturer (Invitrogen).
- [RT-PCR consisted of 1 hr incubation at 53°C, followed by 3 min denaturation at 94°C, and then 40 cycles of 94°C for 30 sec, 53°C for 30 sec, and 68°C for 1 min, followed by a final extension at 68°C for 5 min]
- RT-PCR products were analysed by polyacrylamide gel electrophoresis followed by ethidium bromide staining.

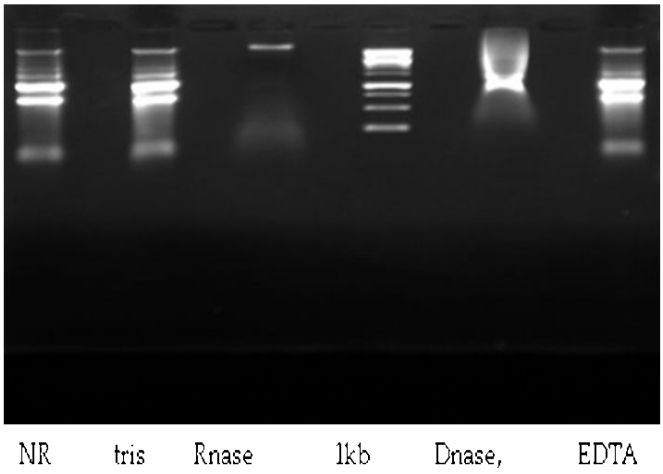



Fig: Isolated RNA with different treatment

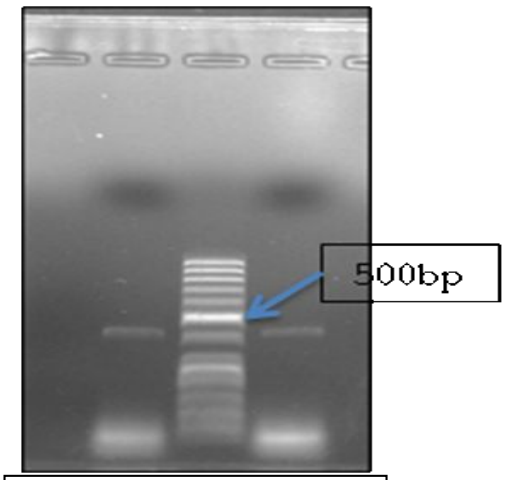



Fig: cDNA and PCR amplification using Superscript III one step RT PCR kit, Invitrogen (using Ref primer for GLV capsid protein.

**Sequencing Result:** The sequence obtained has no significant identity with reported cds of GLV capsid sequence. (alignment provided as FASTA file named GLV wg_cap_4F)
New PCR primers were designed targeting the conserved region of GLV capsid protein and PCR was done according to the reference protocol.

| Name of genes | sequence |
|---|---|
| Capv1F | 5'-CTGGTAATTGCTCACTTTCATC-3' |
| Capv1R | 5'-AACATATCCTTGTAAGCAGACC-3' |
| Capv2F | 5'-GCTCACTTTCATCGTCTATCTT-3' |
| Capv2R | 5'-CGGTGGAAACGTCGAGTG-3' |
| Capv3F | 5'-CTTCGAGAGCTCAATTCCACA-3' |
| Capv3R | 5'-GAAACGTCGAGTGAGGTGG-3' |
| Capv4F | 5'-CATGTCGAATAGAACGAGGTACT-3' |
| Capv4R | 5'-GTAACCATGGAAACATAGGG-3' |
| Capv5F | 5'-GGTCTGCTTACAAGGATATG-3' |
| Capv5R | 5'-AAGAACTGTGGGCCGCTCG-3' |

**PCR products**:

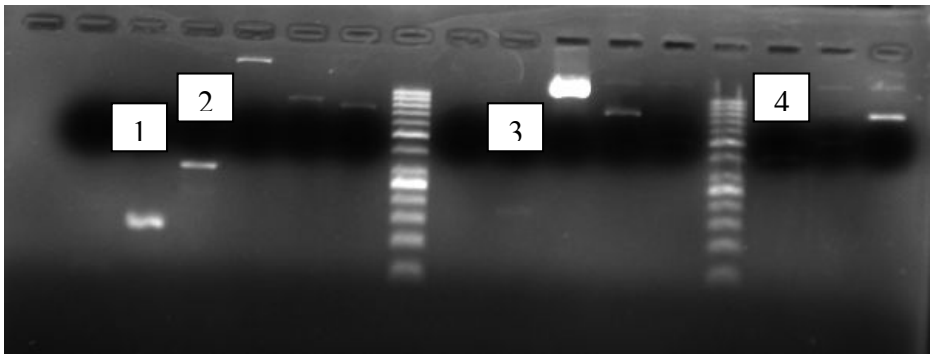



Fig: cDNA and PCR amplification using Superscript III one step RT PCR kit, Invitrogen (using new designed primer for GLV capsid protein.

**1 represents: >L21F (primer set 1F/1R)**
2 represents: >L31F (primer set
3 represents: >L45R
4 represents: >L51F

**Findings:**
Although the primers were designed against the GLV capsid protein but most of the PCR products with variety of PCR conditions were non-specific in nature. Few DNA bands from the desired base pair were purified and sequenced with the specific primers but the results obtained were not desirable (Table)

| **Primer Set** | **Identity with (Accession no.)** | **Score** | **E value** | **Query coverage** |
|---|---|---|---|---|
| L21F | XM_001705748.1 | 141 | 9e-31 | 95% |
| L31F | XM_001707957.1 | 326 | 7e-86 | 91% |
| L45R | XM_001706447.1 | 463 | 5.5e-16 | 90% |

| | | | | |
|---|---|---|---|---|
| L51F | XM_001706605.1 | 582 | 7e-163 | 99% |
| 4F | XM_001706802.1 | 129 | 0.78 | 59% |

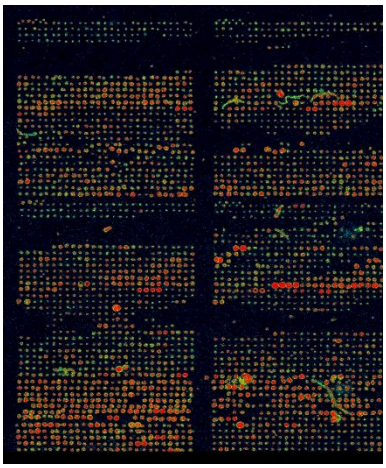
**Conclusion:**

- The Portland I strain of Giardia lacks GLV
- Local strains of Giardia also lacks GLV
- Thus, differential pathogenesis of Giardia is caused as per host system and genomic or better said transcriptomic and proteomic regulations.

- **Identification of new genes in Giardia inside human GUT (Microarrary hybridization)**

We have used different in vitro procedures for mimicking human GUT, like high oxygen tension etc. to find out what are the differentially regulated factors in Giardia that helps the parasite to live inside the human GUT even at very high oxygen tolerance level than they can withstand. We have used a genomic DNA microarrary for hybridization procedure for fishing out these particulat candidate regulators.

**Microarray analysis:** The hybridized microarray slides were scanned and more than 200 clones have been identified that show 5 folds or higher times upregulation or downregulation than the control set. The scanned picture (Fig.1) and the analysed result (Fig.2) have been shown below.



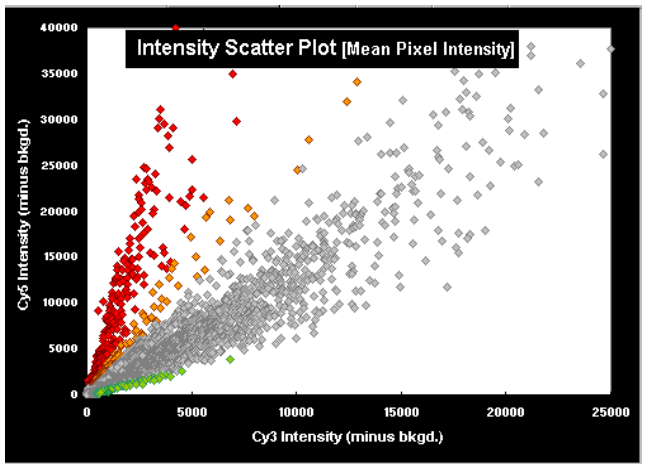
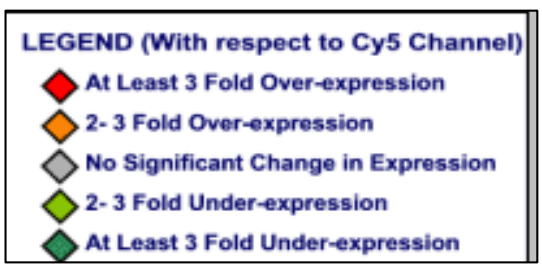



**Fig.1** DNA Array hybridization with stressed cell lines : some up-regulated (red) and down regulated (green) spots (genes) are highlighted here.

**Fig. 2**



**a. Sequencing result:** The result is shown in the following table.

| **Names of the genes** | **Gene_ID** |
|---|---|
| **Metabolic enzymes coding geness** | |
| NADH Oxidase | GL50803_9719 |
| NADH Ferrredoxin Oxidoreductase | GL50803_17151 |
| **Pyruvate Ferredoxin Oxidoreductase** | GL50803_114609 |
| Thioredoxin Reductase | GL50803_9827 |
| **Nitroreductase** | GL50803_15307 |
| **Arginine deiminase** | GL50803_112103 |
| **Malate dehydrogenase** | GL50803_3331 |
| Alcohol dehydrogenase | GL50803_13350 |
| **Phoaphatase and kinase coding genes** | |
| CAM Kinase | GL50803_16034 |
| Serine threonine protein phosphatase | Gl50803_21498 |
| **Transcriptional/translational and cell divisional protein coding genes** | |
| Small subunit rRNA | GL50803_r0019 |
| Large subunit rRNA | GL50803_r0013 |
| TAR RNA loop binding protein | GL50803_32741 |
| Nuclear LIM interactor interacting factor-I | GL50803_14905 |
| TMP 55 | GL50803_137641 |

| | |
|---|---|
| Protein 21.1 | GL50803_13590 |
| FtsJ cell division protein | GL50803_16993 |
| Spindle pole protein | GL50803_8512 |
| **Structural proteins coding genes** | |
| Beta Giardin | GL50803_4812 |
| Dynein light chain | GL50803_7578 |
| **Some other important protein coding genes** | |
| Hsp70B2 cytosolic form | GL50803_88765 |
| Hsp90 alpha | GL50803_98054 |
| Cysteine rich variant specific protein | GL50803_113297 |
| Sodium-hydrogen exchanger III | GL50803_102647 |
| Cathepsin B precursor | GL50803__17516 |
| **Hypothetical protein coding genes** | |
| Hypothetical protein | GL50803_17453 |
| ,, | GL50803_41258 |
| ,, | GL50803_9752 |
| ,, | GL50803_11772 |
| ,, | GL50803_15039 |
| ,, | GL50803_6464 |
| ,, | GL50803_16980 |

| | |
|---|---|
| ,, | GL50803_13274 |
| ,, | GL50803_3421 |
| ,, | GL50803_113722 |
| ,, | GL50803_8509 |

**b. Real time PCR validation:** Some of the important genes found from the sequencing result have been checked by Real time PCR. PCR result of Hsp90, nitroreductase and Pyruvate ferredoxin oxidoreductase have been shown below:

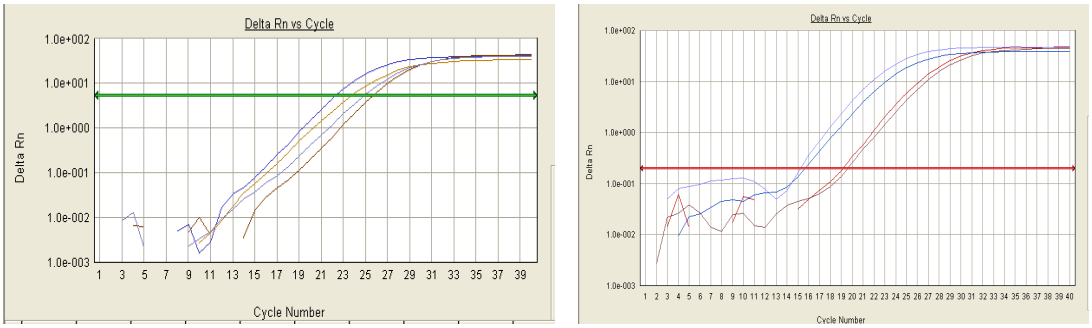



**Fig. 3** Differential expression of Hsp90, Nitroreductase (NR) and Pyruvate-ferredoxin oxidoreductase (PFOR) in control and stressed cells

**c. 2D analysis:** Differential transcription of some genes due to oxidative stress were further analysed in differential protein expression level using 2D gel electrophoresis. The gel picture after silver staining has been shown below:

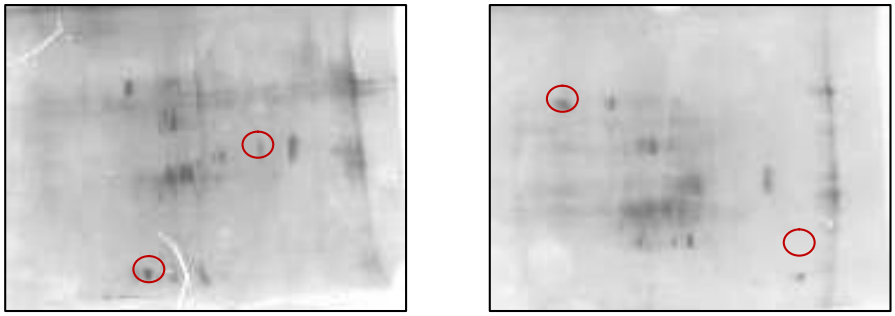


**Fig. 5** 2D gel electrophoresis. (a) Control set and (b) Oxidative stressed cell set.

**vi) Future goals and Plan for fiscal year 2014-2015**

1. Further validation of the results obtained in transcriptomic analysis.

2. To find out the regulatory mechanism for these candidate genes in differential pathogenic regulation.

3. To further extend the same in other pathogenic enteric protozoa like *Entamoeba histolytica*.

**Publications:**


1. Avik K Mukherjee, Punam Chowdhury, Mihir Bhattacharya, Krishnan Rajendran and Sandipan Ganguly. Role of Giardia in diarrhoeal disease regulation in an endemic region. 2014 (Communicated to *BMC Public Health*).

2. Dibyendu Raj, Esha Ghosh, Tomoyoshi Nozaki and Sandipan Ganguly. Differential Gene Expression in *Giardia lamblia* under Oxidative Stress: Significance in Drug Designing and Eukaryotic Evolution. *Gene* 535 (2014) 131–139.

3. Arjun Ghosh, Sumallya Karmakar, Avik K. Mukherjee, Dibyendu Raj, Koushik Das, Srimanti Sarkar, T. Nozaki, & Sandipan Ganguly. THE SPLICEOSOMAL PROTEIN SnRNP F BINDS TO BOTH U3 AND U14 CLASS OF snoRNA IN *Giardia lamblia*. *Global J Bio Agri & Health Sci.* Vol.2(3):178-184. 2013

4. Sumallya Karmakar, Dibyendu Raj and Sandipan Ganguly. Identification of the N-terminal Glycinearginine Rich (GAR) Domain in *Giardia lamblia* Fibrillarin and Evidence of its Essentiality for snoRNA Binding. *Int. J. Trop. Dis. Health* 3(4): 318-327, 2013.

5. Abhishek Sinha; Subhra Ghosh Dastidar; Sandipan Ganguly; Srimonti Sarkar. A unique variation in the evolutionarily conserved Sec61 protein translocon from the protist *Giardia lamblia*. 2013. (Communicated to *PLOS One*).

6. Avik Kumar Mukherjee, Sumallya Karmakar, Dibyendu Raj and Sandipan Ganguly. Multi-locus Genotyping Reveals High Occurrence of Mixed Assemblages in Giardia duodenalis within a Limited Geographical Boundary. *British Microbiology Research Journal*, 3(2): 190-197, 2013

3. ***Development of universal Shigella vaccine based on virulence gene expression.***

1. Title of Project

**Development of a universal *Shigella* vaccine based on virulence gene expression.**

2. Name of investigators

Hemanta Koley[a], JiroMitobe[b], GB Nair[a].

3. Division of Institute where work conducted

a) Division of Bacteriology, National Institute of cholera and Enteric Diseases, Kolkata, India

b) Department of Bacteriology, National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640, Japan.

4. Summary:

Enteric bacterial infection cause of diarrhea, among them, Shigella species cause of shigellosis as a result infant morbidity and mortality in developed as well as in developing countries. At present, only antibiotic therapy is available for treatment of shigellosis. Unfortunately, due to the global emergence of multidrug resistance, the choice of antimicrobial agents for treating shigellosis is very limited and we are approaching where the shigellosis can become an untreatable disease because of lake of an effective antibiotic. Therefore, the possibilities of other preventive measures such as anti-dysentery vaccines have attracted increasing attention in this field. Various trials of several candidates' vaccine are being done in different parts of the world, but till date no suitable Shigella vaccine is available for public health use. There are different serotypes of Shigella species and their distribution varies between endemic geographical regions. The immune response against Shigella species are serotype-specific, so current immunization strategies have required the administration of live vaccine strains to provide protection against multiple serotypes. In our study, we evaluated the protective efficacy and immune response live attenuated shigella in guineapig model. Constriction and preliminary protection work done by our Japanese scientist in Japan. In India, the protective efficacy after oral immunization with four doses (0, 14th & 28th Day) of Shigella strain was examined. In our protective efficacy studies, we have observed 100% protection (against *S. dysenteriae*) in the immunized group whereas the unimmunized group of animals, thre was noprotection (0%). Serum IgG and IgA showed exponential rise during oral immunization. Vaccine strain MF 4853 showed low reactogenic than wild type strain.

5**. Purpose:**

Current vaccines for bacterial diseases have a serotypic direction that limits the effect of vaccination to a narrow range of bacteria within the same species. An attempt to develop vaccine against broad serotype is worthwhile but difficult. This may result from powerful immunogenicity of serotypic polysaccharide antigen that could camouflage potential antigenicity of common virulence proteins. We have developed a candidate of broad *Shigella* vaccine based on molecular mechanism of virulence gene expression.

Keeping such ideas we started work with the salient objectives are:

a) To understand protective efficacy and immunogenicity of live genetically manipulated shigella vaccine strain against homologous as well as heterologous S*higella* strains in guinea pig model
b) To study the duration of protection offered by live genetically manipulated shigella vaccine strain.

## 6. Methods Materials:

**i) Animals**

Two-month-old English colored guinea-pigs of either sex, weighing between 250 and 300 g, were used in this study. Guinea-pigs were collected from the Animal Resource Department, National Institute of Cholera and Enteric Diseases, Kolkata. The study was conducted under dedicated biosafety level 2 conditions with the housing of animals in individually ventilated caging systems maintained at 24 1C with 65% humidity. The guinea-pigs were not starved during the experimental period and no gut sterilization with antibiotics was carried out before the experiment. Before the initiation of the study, the animals were tested *S. flexneri* 2a infections by ELISA against lipopolysaccharides of test pathogens. Institutional animal ethical committee granted approval to conduct this study.

**ii) Animal experimental design:**

One set of experiment will be conducted by three groups (A, B, C) of male Hartley guinea pig (more than 2 months old).

Protection Study for *S. dysenteriae* Type I ( total)

A) Immunization by vaccine strain MF4835--- six guinea pigs +2 for specimen

B) Immunization by wild-type 2457T: Control 1--- six guinea pigs +2 for specimen

C) Naïve, administration of saline : control 2--- six guinea pigs +2 for specimen

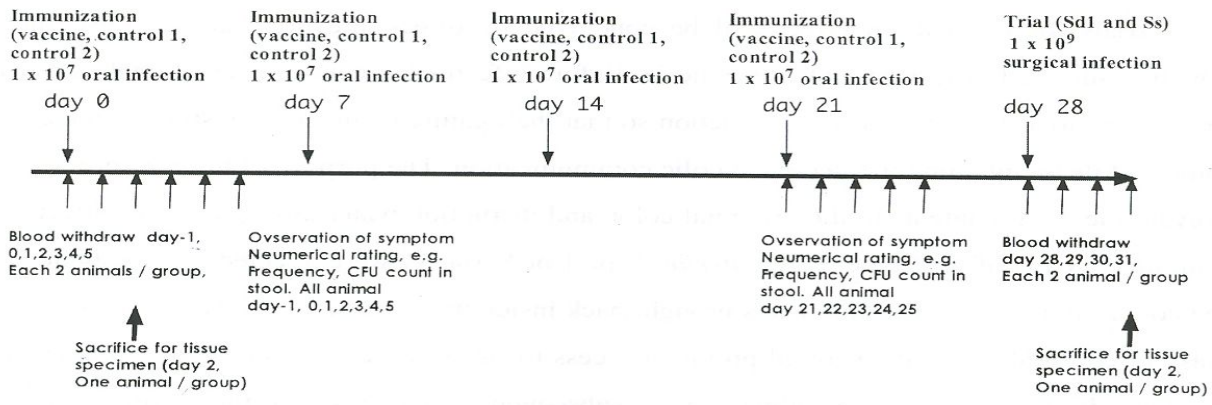
**iii) Immunogen preparation and immunization**

The overnight growth of *Shigella flexnari* 2a 2457T and Vaccine strain *Shigella flexanari* MF 4853 was scrapped off from TSA and suspended in PBS and centrifuged (10 min, 10 000 g). The resulting pellet was washed twice and resuspended in PBS. The bacterial suspension was adjusted to an OD 600 nm of 1.5. The suspension was adjusted again to OD 600nm 1.5 and was stored at $80^0$C till use for oral immunization. OD 1.5 corresponded to $10^7$ CFU/ml. Guinea-pigs were anesthetized using a mixture of ketamine (35 mg kg_1 of body weight) and xylazine (5 mg kg_1 of body weight). Guineapigs were orally immunized with $10^7$ CFU of **Shigellaflexnari 2a 2457T and Vaccine strain *Shigella flexanari* MF 4853** strains in lmL of PBS under anesthesia. Control guinea-pigs were treated with sterile PBS. The immunization schedule was followed on the 0, 7th, 14th and 21st day.

**iv) Flow chart for oral immunization:**

| Step | Time | Treatment |
|---|---|---|
| **I** | **-36 hours** | **Experimental Guinea pigs starved but given water *at libitum*** |
| **II** | **-18 hours** | **Intravenous injection of Ranitidine (2mg/kg body weight)** |
| **III** | **-35 minutes** | **Anaesthetized by Ketamine (35mg/ml) and Xylazine (5mg/kg body weight, intramuscular)** |
| **IV** | **-15 minutes** | **Neutralization of hydrochloric acid by 5%, 5 ml Sodium bicarbonate** |
| **V** | **0 minute** | **Again administered 5 ml of 5% Sodium bicarbonate along with 1ml ( $10^7$ ) WILD TYPE STRAIN *Shigella flexenari* 2a 2457T and VACCINE STRAIN MF 4831.** |

**v) Animal experimental Schedule**



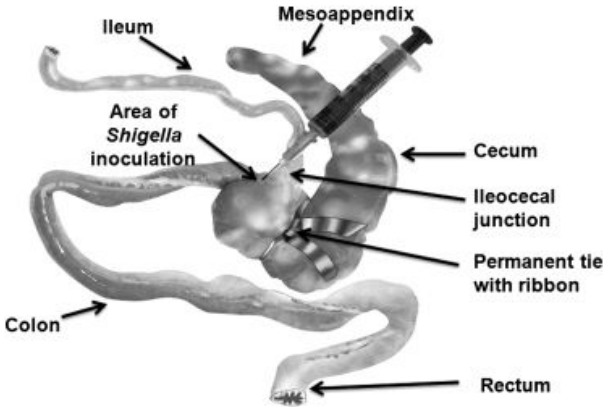

**vi) Surgical procedure:**

The test animal was sedated by an intramuscular injection of a mixture of ketamine (35 mg kg_1 body weight, Sterfil Laboratories Pvt Ltd, India) and xylazine (5 mg kg_1 body weight, AstraZeneca Pharma India Ltd, India). The cecum was brought out through a 3 cm midline incision without compromising the blood supply. A

permanent cecal tie was made 4 cm apart from the ileocecal junction so that the ligation completely obstructed the cecal lumen above this junction while maintaining the ileo–ceco–colic connection. The purpose of this ligation was to prevent the entry of cecal contents into the proximal colon and disruption of water absorption. During the surgery, hydration of the exposed intestine was maintained with sterile PBS. At the cecocolic junction, 1mL of test inoculum was injected into the lumen of the colon. The colon was placed back inside the abdominal cavity and the incision was closed. The incision site was checked twice a day for signs of infection, and each time, it was washed with a 1% chlorhexidine solution soaked with sterile gauze pads during the next 72 h. We did not find any wound infection in any of the guinea-pigs during the postsurgical period. After the surgery, the animals were allowed to consume food and water and were observed for the development of shigellosis for 48 h. Luminal inoculation with guinea-pigs without cecal bypass was also carried out with 2457T to assess the effects of cecal bypass on the development of shigellosis.

**vii) Challenge Efficacy study:**

| Step | Time | Treatment |
|---|---|---|
| **I** | **-36 hours** | **Experimental guinea pig was starved but water will be given *at libitum*** |
| **II** | **-15 minutes** | **Guinea pig will be anaesthetized through intramuscularly by ketamine (35 mg/kg) and xylazine (5 mg/kg body weight)** |
| **III** | **- 5minutes** | **Colon was brought out through a midline incision aseptically** |
| **IV** | **-3 minutes** | **Permanent tie was placed 2 cm apart from ileocecal junction** |
| **V** | **0 minute** | **1 ml of bacteria was injected to the lumen ileocecal junction of colon** |
| **VI** | **5 minutes** | **Animals were allowed to take food and water and observed for the development of shigellosis for 48 hours** |




**Surgical sketch of guinea-pig colon for the experimental shigellosis model**

**viii) Monitoring of challenged animal:**

The challenged animals were monitored for 48 hr after the rectal challenge. They were observed twice daily, every morning and evening, for general activity level, tenesmus, consistency of stools passed into the drop pan of their cages and the amount of blood or mucus observed in the feces (if any). Body weight and rectal body temperature were measured.

**ix) Collection of stool sample and quantification of bacteria (shedding)**

Stool samples of animals of both immunized and control group were collected from the drop pan. The samples were suspended to make 1 g stool/mL of PBS, 10-fold serially diluted and plated on Hekton enteric agar (HEA, Difco) and MacConkey agar plates (Difco) for bacterial counting. Representative colonies were subjected for the confirmation with appropriate typing sera (Denka Seiken, Tokyo, Japan).

**x) Collection of intestinal tissue and nature of colonization of bacteria**

After laparotomy, appropriate length (3–4 cm) of the intestine (distal colon) was excised. The excised intestinal tissues were minced, mixed with 3 mL PBS and homogenized with a pestle (Himedia, Delhi, India). The homogenized tissue was added with PBS up to 5 mL in volume. After 10-fold serial dilution of the sample, bacterial count was made on HEA (Difco) and MacConkey agar (Difco). Representative colonies were subjected for the confirmation with appropriate typing sera (Denka Seiken, Tokyo, Japan).

**xi) Collection of blood sample**

Blood samples of both the immunized and control groups were collected from the foot vein on days 0, 7, 14, 21, 28, 35, 42 and 63. Number of samples collected were 17 from each of the immunized and control group on days 0, 7, 14, 21 and 28, and were seven from each group on days 35, 42 and 63, since 10 each were challenged on day 28 and killed thereafter. Collected blood was allowed to clot at room temperature for 30 min and then kept at 4◦C for 24 hr. Serum was separated from the clotted blood by using a sterile Pasteur pipette and centrifuged at 1000 *g* for 10 min. The supernatant was collected and stored at –20◦C until use.

**xii) Immunological Assay :**

Analysis of immunogenicity of vaccine strain were performed using ELISA, essentially following the method developed by Keren (27). Disposable polystyrene (Nunc,Denmark) microtiter wells were coated with $10^9$ cells/ mL of vaccine strain and incubated for 18 h at 4 °C. Wells were washed three times with PBS (pH 7.4). Control wells were coated with 100 $\mu$L of PBS. After the plate was incubated at 4◦C for 18 hr, wells were washed three times with PBS with 0.5% Tween 20 (PBS-T). Non-specific binding sites were blocked by incubating thewellswith 200$\mu$Lof 5%non-fat dry milk (Bio-Rad, Hercules, CA, USA) at 37◦C for 2 hr. The wells were washed thrice with PBS-T and incubated with serially diluted samples at 37◦Cfor 1 hr. After washing with PBS-T, 100 $\mu$L of horseradish peroxidase-

conjugated goat anti-guinea pig IgG (Sigma, St. Louis, MO, USA) or horseradish peroxidase-conjugated sheep anti-guinea pig IgA (ICL, Portland, OR, USA) diluted 1:2000 times in PBS were added to each well and the plate was incubated at 37◦C for one hour. Following washing with PBS-T, 100 $\mu$L of OPD (1 mg/mL) dissolved in 100mMcitrate buffer (pH 4.5) containing 0.2% hydrogen peroxide were added to each well. The reaction was stopped after 10 min by adding 100 $\mu$L of 2 N sulfuric acid and the resulting color was read at 492 nm using an ELISA reader (Bio-Rad). The readings of PBS control wells were subtracted from those of the corresponding test wells to yield the net optical density. The endpoint ELISA titer was the highest reciprocal dilution yielding a net optical density of 0.100 or greater.

**xiii) Histology of intestinal tissue:**

After laparotomy, the segment of the intestine from the distal colon to the rectum was sectioned, opened and inspected for signs of mucosal edema, exudation, hemorrhage, ulceration, necrosis and perforation. Samples were fixed in 10% neutral buffered formalin, dehydrated with alcohol and embedded in paraffin. The samples were cut 3 $\mu$m in thickness and stained with hematoxylin and eosin

**7) Result:**

## i) Bacterial Shedding of immunizing strain from stool of animals:

Bacterial Shedding of the immunizing strains, wild *Shigella flexnari* 2a ( 2457T) and vaccine strain MF4853 were measured from stools of immunized animals. After each immunization time (day 0- 7), stools were collected for three consecutive days to identify shedding of both strains. Irrespective of the time of immunization, a quite significant number of the immunizing strain was recovered from stools collected at 24 hr after the immunization. However, at 48 hr after the immunization, the number of the immunizing strain recovered

decreased tremendously and no immunizing strain was recovered at 96 hr after the immunization.

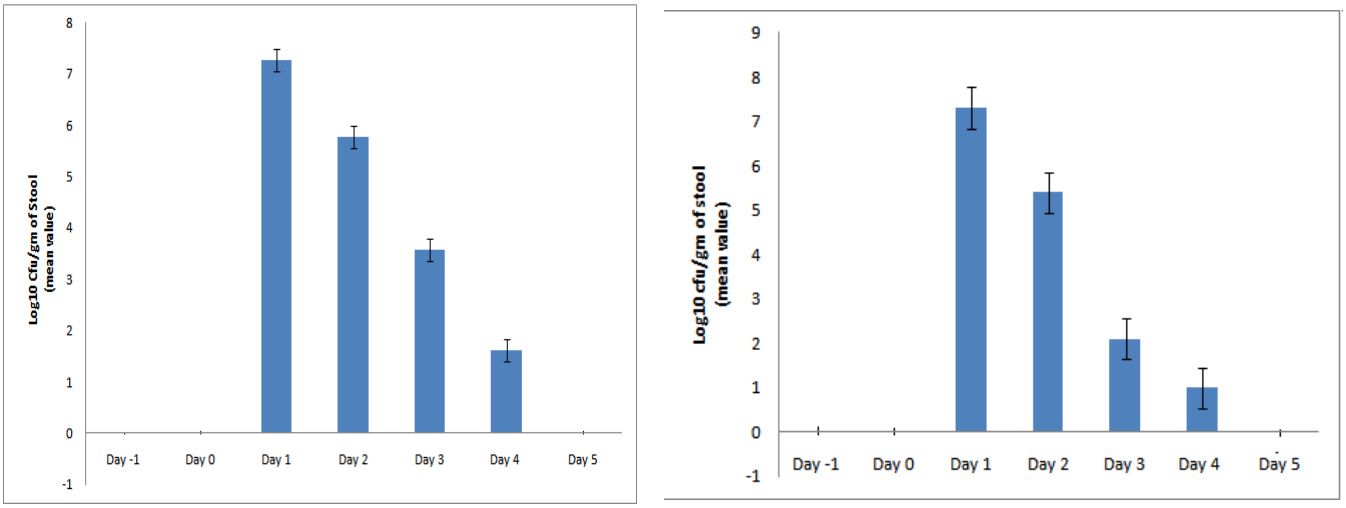



**Fig 1: Nature Of Bacterial Shedding of Wild type *Strain Shigella flexnari*2a (2457T) and Vaccine Strain *(* MF 4853) After 1st Oral Immunization.**

## ii) Protective efficacy in guinea-pigs

Table 1 shows protective efficacy of immunization of vaccine strain MF4853 against challenge with *S. dysenteriae* 1 NT4907. In the experiment, a total number of 6 animals of the immunized group were orally administered $10^8$ cfu of MF4853 in 1mL of PBS. Also 6 animals of the control group were administered 1mL of PBS .On day 28 after the initiation of the immunization (7 days after the last administration) 20 animals of each immunized and control group were challenged with $10^9$ cfu of *S. dysenteriae* 1 NT4907 in1mL of PBS.No dysentery symptoms were observed in any animals of the immunized group, which were challenged on day 28 (6 animals). Only one animals challenged on day 28 excreted semisolid stools within 24 hr after the challenge but recovered spontaneously within 48 hr. On the other hand, all animals of the control group challenged on day 28 developed symptoms of bacillary dysentery, such as tenesmus and mucoidal and bloody diarrhea. In all animals, tenesmus characterized by a sudden cramp that caused the body to rise up for an instant, frequently

began to occur at around 24 hr after the challenge and was ob servable during the subsequent 24 hr. Mucoidal bloody diarrhea and mucoidal diarrhea without blood

were observed within 24 hr after the challenge in some animals challenged on day 28. In the control group,

elevation of the rectal temperature by approximately 1.6◦C at 24 hr after the challenge on both day 28 and loss of body weight (approximately 11%) within 3 days after the challenge on both day 28 a were observed. No such observation was made in the immunized group.

**Table No.1 Protection Effacacy After Challenge With S. dysentriae type 1 NT4907**

| Experimental animal | Immunogen used | Challenged Strain used in intestine | Number of animal used | Disease symptoms | % of death with Shigellosis | % of protection against Shigellosis |
|---|---|---|---|---|---|---|
| **PBS Control Group** | **PBS** | **Wild type** *S. dysentriae* **type 1** ***(NT4907)*** | **6** | **Shigellosis** **100% ( 6/6)** | **100% (6/6)** | **0% ( 0/6)** |
| **Immunized Group** | **VACCINE STRAIN MF4831** | **Wild type** ***S. dysentriae 1(NT4907)*** | **6** | **16.3% ( 1/6)** | **0 % (0/6)** | **100%** |
| | **Wild Type Strain ( S. flexneri 2a 2457T )** | **Wild type** ***S. dysentriae* 1*(NT4907)*** | **6** | **32% ( 2/6)** | **0 % (0/6)** | **100%** |

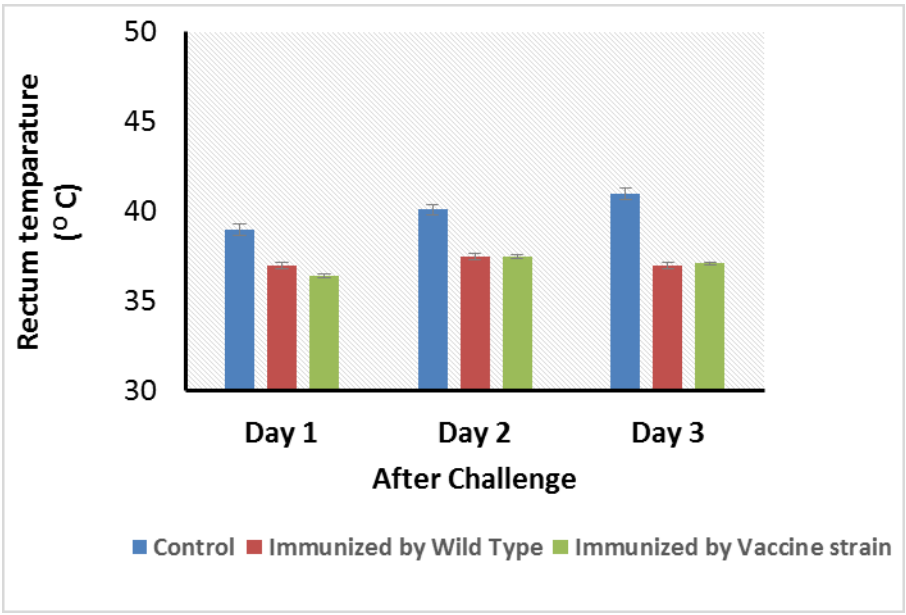



**Fig 2: Rectum temperature of Immunized and Non immunized Guinea pigs After Challenge by *Shigella dysentriae* 1.**

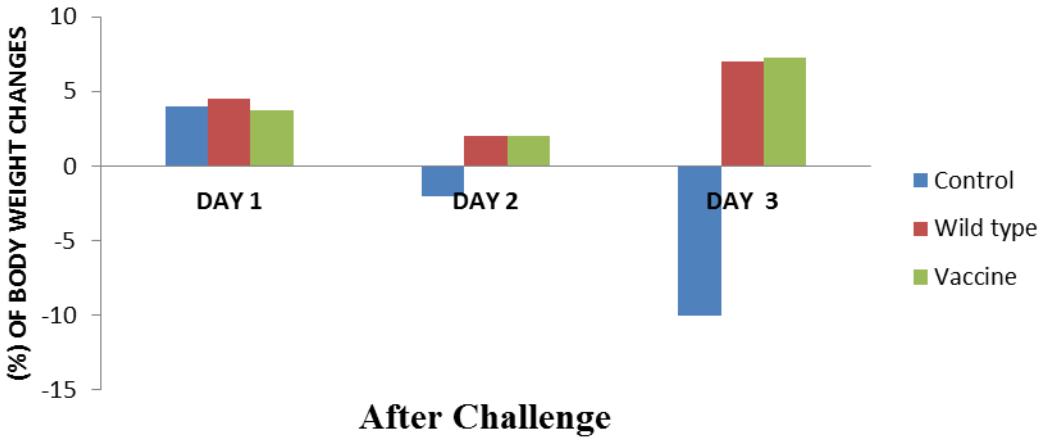



**Fig 3: Graphical Representation of Body weight of Animals after Challenge by *Shigella dysentriae* 1.**

### iii) Recovery of challenged strain from distal colon of animal:

Figure4 shows the results of recovery of challenged *S. dysenteriae* 1 NT4907 from the distal colon of animals of both the immunized and control groups. In animals challenged on day 28 after the initiation of the immunization, at 24 hr after the challenge, at 100± $2\times10^2$cfu per gram of tissue were recovered from animals of the immunized group, whereas 1.5±2.8 $\times10^9$ cfu per gram of tissue were recovered from animals of the control group.

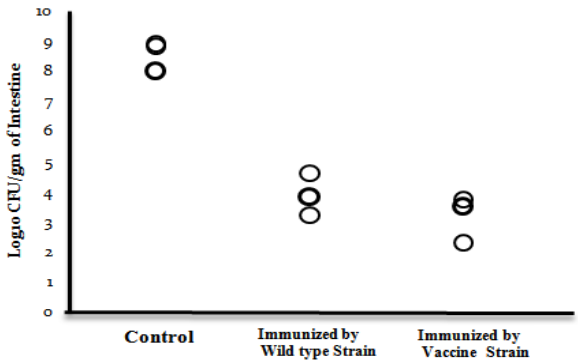



**Fig 4: Immunized and non immunized guinea pigs (n=3, each group) were challenged by (A) *Shigella dysentriae Type 1 NT4907* after 24 hrs animals were sacrificed for colonization.**

**iv) Recovery of challenged strain from the stool of animals:**

Results of recovery of the challenged strain NT4907 from the stool of animals of both the immunized and control groups are shown in Fig. From the control group, 24 hr after the challenge on day 28 , a significantly large number of challenged strain NT4907 were recovered.. On the other hand, from the immunized group, a quite significantly low number of the challenged bacteria were recovered both 24 and 48 hr after the challenge.

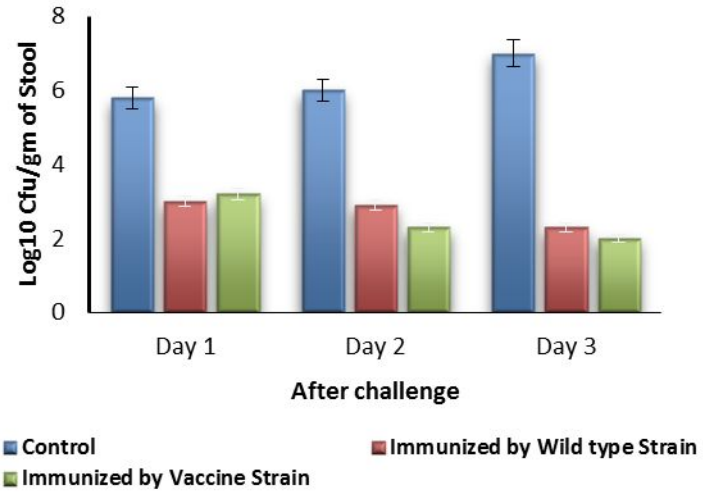



**Fig 5. Recovery of challenged strains from stool of immunized and non immunized guinea pigs (n=3, each group) successive three days after challenged by *Shigella dysentriae 1* ($1\times10^9$ cell) ,**

**v) Histology of colon of animals:**

Hemorrhage and inflammatory cells in the surface mucosa, mucosa and submucosal layers and widely dilated crypt lumen were observed at 48-h postinfection of *S. dysenteriae* 1 (NT4907) (Fig. 6,A1) and *S. sonnei* (Fig. 6,B1) in control animals. In immunized animal did not show any damage and inflammatory changes in the colonic mucosa. The surface epithelium including all the layers of the colonic mucosa remained normal.

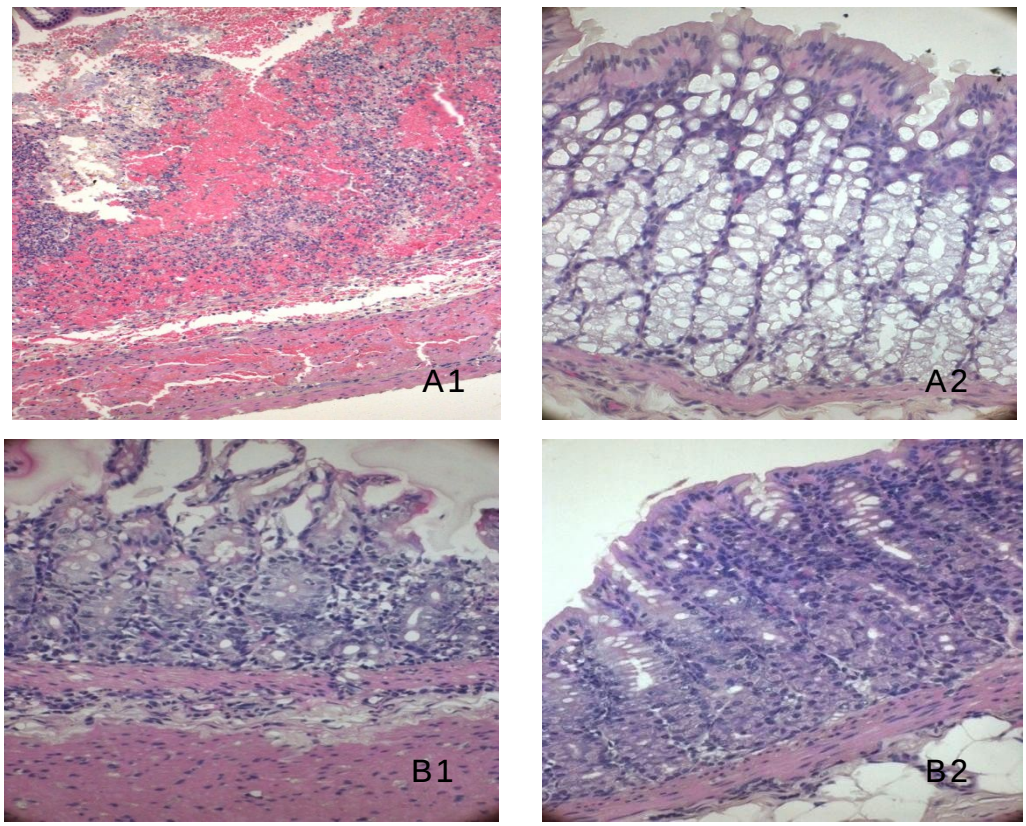



**Fig 6: Histological changes in the intestinal tissues.** Experimental procedures are as described in the text. Tissues were collected from animals of control group **(A1)** and immunized group **(A2)** challenged with *S. sonnei* on day 28 and killed at 48 hr. Control group **(B1)** and **(B2)** immunized group challenged on day 28 with *S. dysenteriae 1* and killed at 48 hr. Magnification: 40×.

**vi) Immunological Study:**

a) **Serum IgG and IgA against vaccine strain MF 4853:**

The anti-bacterial serum IgG and IgA titers of the animals during the course of the experiment were measured against vaccine strain MF 4853. As shown in Figure 2, the serum anti-bacterial IgG and IgA titers of the immunized group increased during the period of immunization, peaked on the 28th day after the initiation of the immunization and remained at the same level until the 35th day. The serum anti-bacterial IgG and IgA titers of the control group were below the limit of the detection during the entire period of the experiment.

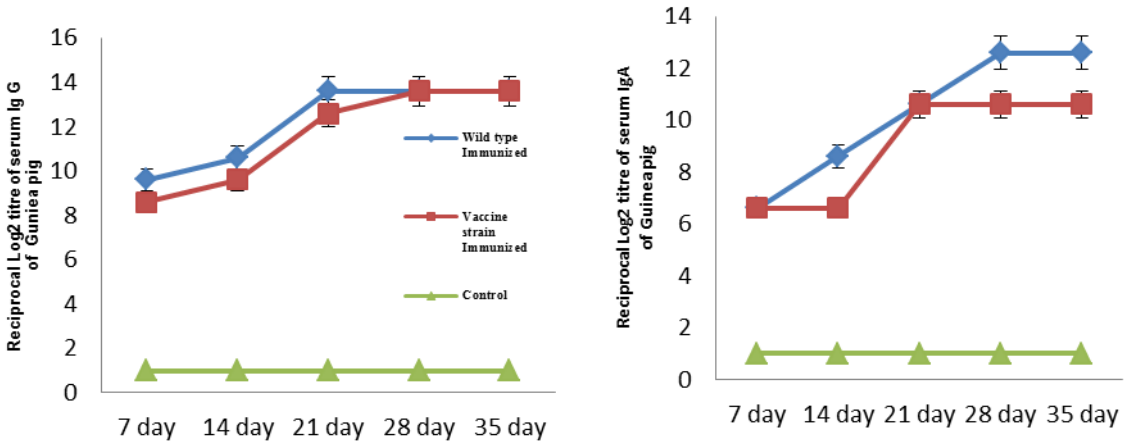



**Fig 7: Serum IgG and IgA response after oral immunization with wild type and vaccine strain against wild type Strain *Shigella flexenari 2a* 2457T in Orally Immunized Animals**

**b) IgA titer against vaccine strain MF 4853 in intestinal lavage:**

Anti-bacterial IgA titers of the intestinal lavage of the animals from immunized and control group were measured and the results are shown in fig . The titers of the immunized group challenged on both days 28 was significantly higher than those of the control group ($P$ <0.05).

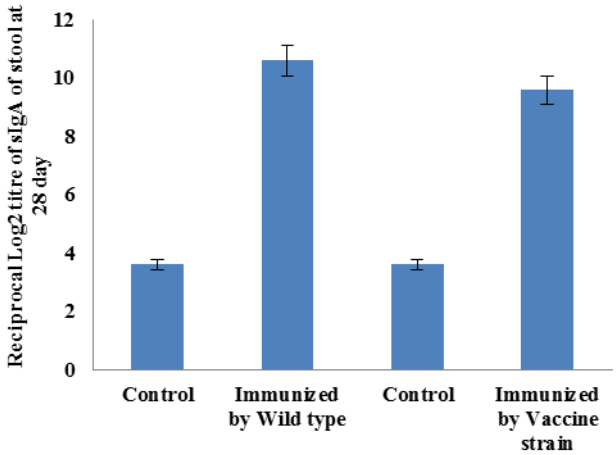



**Fig 8 : sIgA Response Against vaccine strain MF 4853 and *Shigella flexenari* 2457T in 28th day Stool of both control and Oral Immunized group animal.**

**C) Reactogenicity of Vaccine Strain MF 4853:**

IL-8 secretion (pg/ml) was noticed only after 4 h of incubation against Wild type Shigella flexenari 2a 2457T and Vaccine strain MF4853 . The concentration of live S. flexneri 2a (2457T) strain and MF4853 vaccine was equal to the infectious dose of the strain ($1\times10^8$ cells/100 µl). The results showed, after 4 h of incubation, wild type strains induced higher levels of IL-8 than Vaccine strain MF4853 ( Fig 9).

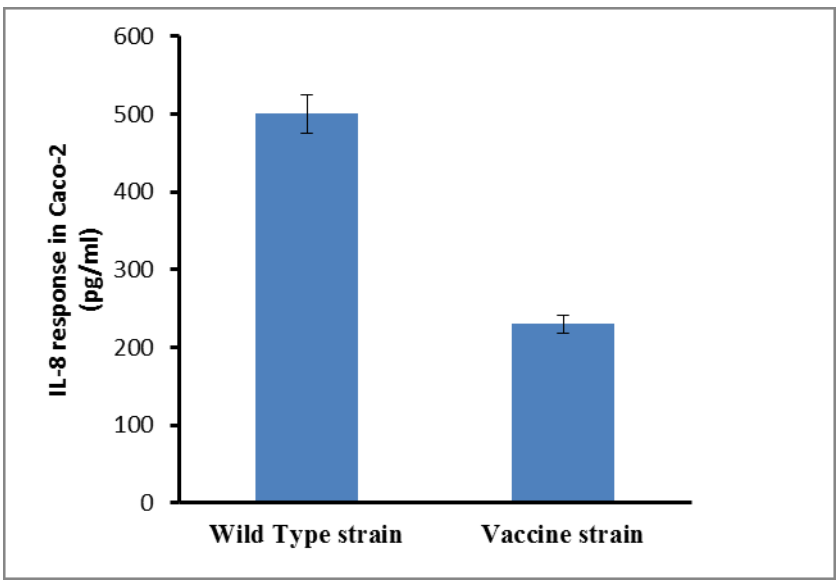



**Fig 9: Interleukin-8 expression after 4 hr in Caco-2 cell line with Wild type and Vaccine strain MF 4853.**

**Future Plan of work:**

1. Duration of protections

2. Passive protection studies
3. Heterologous cross protection against other 50 serotypes and subtypes
4. Single dose protection study
5. Heterologous protection other than Shigella (EIEC and other serotypes)

## *4. Analysis of HLA associated HIV-1 mutations in India and Japan.*

The transmission of HIV in Manipur, a north eastern state of India is primarily through Injecting Drug use sharing the needles and syringes. For the last decade, the Injecting drug users also transmit their infection to their non injecting spouses. In order to find out the dynamics of this transmission, a total of 500 IDUs and their spouses were included in the study. HIV seropositivity were determined by the HIV diagnostic kits approved by National AIDS Control Organization, Govt of India.

It was found that 199 IDU among the 500 were HIV positive ( 39.8%). 121 spouses of 500 IDUS were also HIV positive. Interestingly, 96 spouses out of 199 HIVseropositive IDUs were HIV positive, thus the HIV concordance became 48.2%.

Next, the genotyping of the HIV positive IDUs were undertaken as reported earlier. Although the majority of IDU samples showed subtype C, a substantial number of samples showed dual/multiple probe reactivity in the Multi region Hybridization Assay (MHA) as reported earlier. Attempt was made to amplify near full length genome of HIV from one such sample, MAN 40 by Reverse transcriptase polymerase chain reaction (RT PCR).

**Near full-length genome amplification**

Nearly 8.1 kb HIV-1 genome was successfully amplified from the cDNA of the sample MAN 40 with high fidelity proofreading polymerase (Roche, Inc) using nested PCRwith specific primers MSF1- 5 AAATCTCTAGCAGTGGCGCCCGAACAG 3, OFMR1- 5 TGAGGGATCT

CTAGTTACCAGAGTC 3 for the first round, and F2NST-

5 GCGGAGGCTAGAAGGAGAGAGATGG3, OFM19 5GCACTCAAGGCAAGCTTTATTGAGGCTTA 3 for the second round.

After enzymatic removal of the primers and dNTPs that remained in solution, purified

PCR products were sequenced directly in overlapping segments of 1,100 nt by primer walking using the ABI Prism BigDye Terminator Cycle Sequencing kit and

the ABI 377 Sequencer kit (Applied Biosystems).Sequences were corrected and assembled using the BioEdit program (Tom Hall, http://www.mbio.ncsu.edu/BioEdit/

bioedit.html). To exclude the possibility of PCR-mediated artifacts, breakpoints were confirmed in duplicate PCR amplifications that were carried out separately.

**Sequence and recombination analysis**

Sequences were screened for the presence of recombination patterns by the boot scanning method. The sequences were aligned using CLUSTAL X software with minor manual adjustments. Phylogenetic neighbor-joining trees were based on Kimura's two-parameter distance matrices with assessment of the consistency of tree topologies.Sites with a gap in any of the sequences were excluded from the analysis. Phylogenetic trees were constructed with MEGA 4.0 by the neighbor-joining method,under the Kimura two-parameter substitution model, with 1,000 bootstrap replications. To analyze the recombination of the MAN40 strain, the sequence was subjected to bootscan analysis as described using default parameters. To identify the mosaicism structure, bootscan analysis of MAN40 sequence was further analyzed using SimPlot (version 3.5.1; S. Ray, Johns Hopkins University,Baltimore, MD; http://sray.med.som.jhmi.edu/RaySoft/SimPlot/ ) within a gap-stripped, 300-bp sliding window moving in increment steps of 20 bp.

Analysis of the near full-length genome sequence revealed that MAN40 retained intact reading frames for a majority of its genes and no gross deletions or rearrangements were observed. After performing phylogenetic analysis of NFLG of MAN40 along with other global HIV-1 strains, it was observed that this HIV-1 strain formed a separate branch, which justified the strain to be newly evolving URF.

MAN40 exhibited a close relatedness to 07.BC.CN05 XJDC6441 and 07.BC.CN.97.97CN001, the CRFs of China. However, it was quite distantly related to the previously reported locally adapted B/C recombinant strains of India, namely BC.IN.2002.NARI 7-1 and BC.IN.2002.INDNARI 0218440. We endeavored to analyze whether this recombinant strain shared similarity with other BC recombinant strains from the neighboring countries (China, Thailand, and Myanmar). Hence, bootscan analysis was performed to compare the recombination breakpoints of MAN40 with native and global BC recombinant strains (Fig. 1). The bootscan analysis revealed that the breakpoints of MAN40 represented the area where majority of recombination had occurred in case of other BC recombinants as well. However, the simplot and phylogenetic analyses indicated that this recombinant HIV-1 strain seemed to be originated as a result of recombination between Indian subtype C strain (C.IN.93.93.IN905) and Thai-B strain B.TH.99.99TH_C1416.The results confirmed that the genomic structure of MAN40 consisted of subtypes B and C, with C as a backbone. The first and second breakpoints were witnessed in the upstream sequence of the polymerase gene (reverse transcriptase region, 3,026–3,259 bp) by Simplot analysis. The

sequence of MAN40 present in the region II (Fig. 2) clustered with B.TH.99.99TH_C1416 strain in the phylogenetic analysis (Fig. 3). The sequence present in region III clustered with C.IN.93.93.IN905 in phylogenetic analysis
(Fig. 3) . The third breakpoint was found at the downstream sequence of envelope gene (gp41 region, 8,183 bp). The sequence emerged in this region (III) spanned the gp41 and nef gene as shown in Fig.3. From the phylogenetic analysis
it was revealed that the MAN 40 sequence present in the region IV (Fig. 2 ) aligned with B.TH.99.99TH_C1416 strain. The Simplot analysis clearly demonstrated that the
recombinant HIV-1 strain MAN40 harbored recombination
sites in pol and env genes (Fig. 2). Information pertaining to the full-length genome
sequence of HIV-1 in Manipur lacks currently, and hence the present study attempted to address this gap by generating near full-length HIV-1 sequences from the local HIV- 1 isolate MAN40. Sharing of injecting equipments is the daily or the injecting drug users and drug traders as a part of their drug-purchasing behavior, thereby, leaving the doors open for the introduction of different HIV-1 subtypes into circulation. Similar observations were revealed from the studies of HIV-1 epidemic in
Thailand and Myanmar where different subtypes have taken the reins of infection owing to the path paved by heroin trafficking routes . Genetic diversity of
HIV-1 might also be related to cross-border migration and sharing practices of IDUs of different ethnicity. The findings suggest that an IDU posed to the risk behaviors, stands on the brink of getting exposed to different circulating subtypes of HIV-1 and thereby facilitating the development of recombinant strains. It is thus necessary to continuously monitor the evolution of strains in this region since they can be predictive of the phylogenetic nature of future dominating strains in the HIV
pandemic. Highly divergent HIV strains could affect HIV pathogenesis, ease of spread in a population, susceptibility to antiretroviral treatment, or vaccine development strategies. Studies of HIV-1 genetic diversity could have a potential
impact on the diagnosis of HIV infection and could provide useful reference reagents for standardization of assays. For these reasons, it is important to study the evolution
of an HIV-1 recombinant dwelling in these regions as it may potentially become circulating recombinant (CRF) in future.

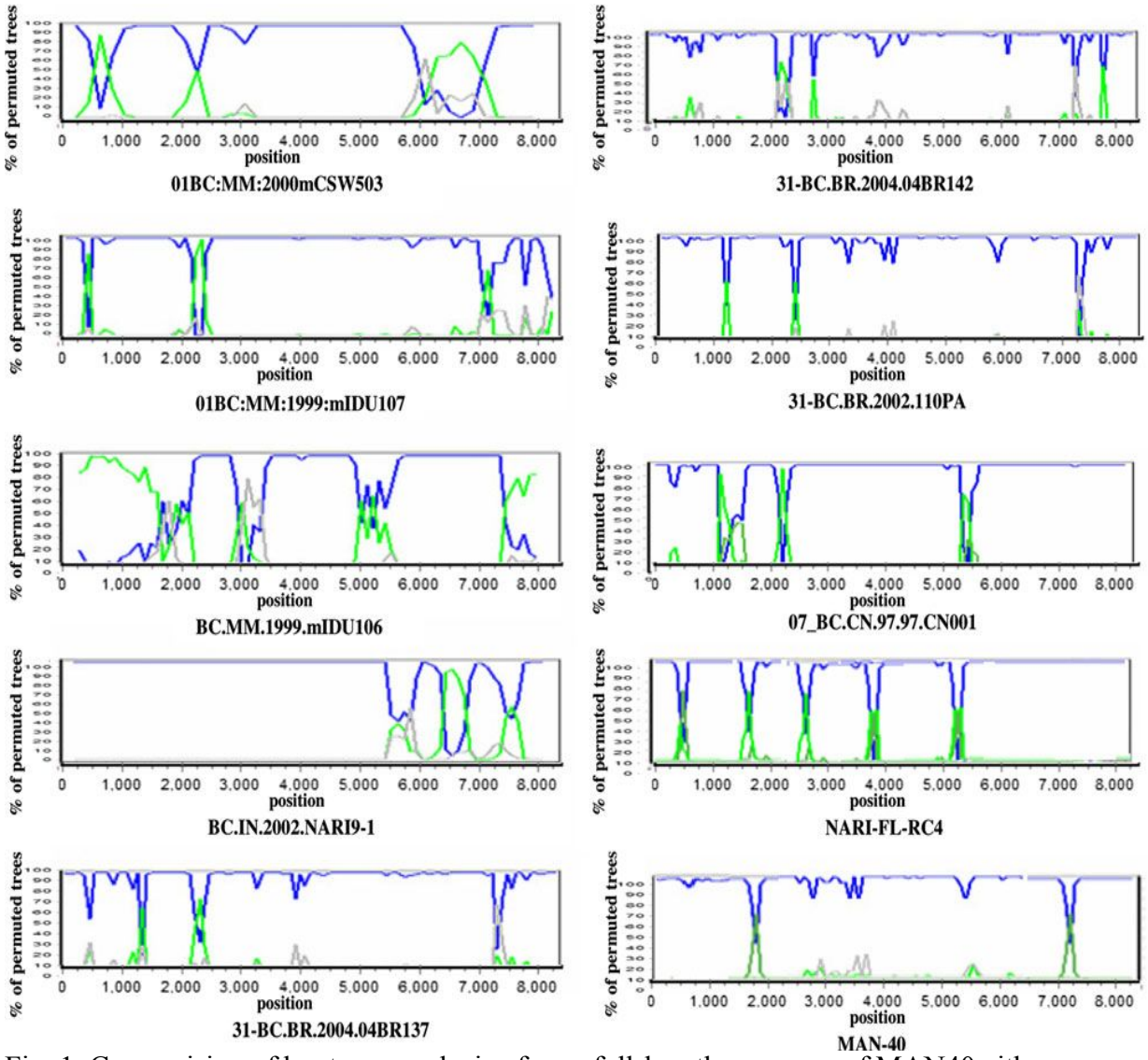



Fig. 1. Comparision of bootscan analysis of near full-length sequence of MAN40with otherBCrecombinant viruses-01BC:MM:2000mCSW503, 31-BC.BR.2004.04BR142, 01BC:MM:1999:mIDU107, 31-BC.BR.2002. 110PA,BC.MM.1999.mIDU106, 07_BC.CN.97.97.CN001, BC.IN.2002. NARI9-1, NARI-FL-RC4, and 31-BC.BR.2004.04BR137. The horizontal axis represents nucleotide distance of the midpoint of the window from the 5 end of the query sequence. The vertical axis represents the percentage of trees (using 100 bootstrap replicates) that support branching with the consensus subtype reference sequence. A 300 nt window advanced in 20 nt increments was used. Sequences were gap-stripped, transversion to transition ratio was set to 2 ± 0, distances were calculated according to Kimura's two-parameter model and trees were constructed with the neighbor-joining algorithm

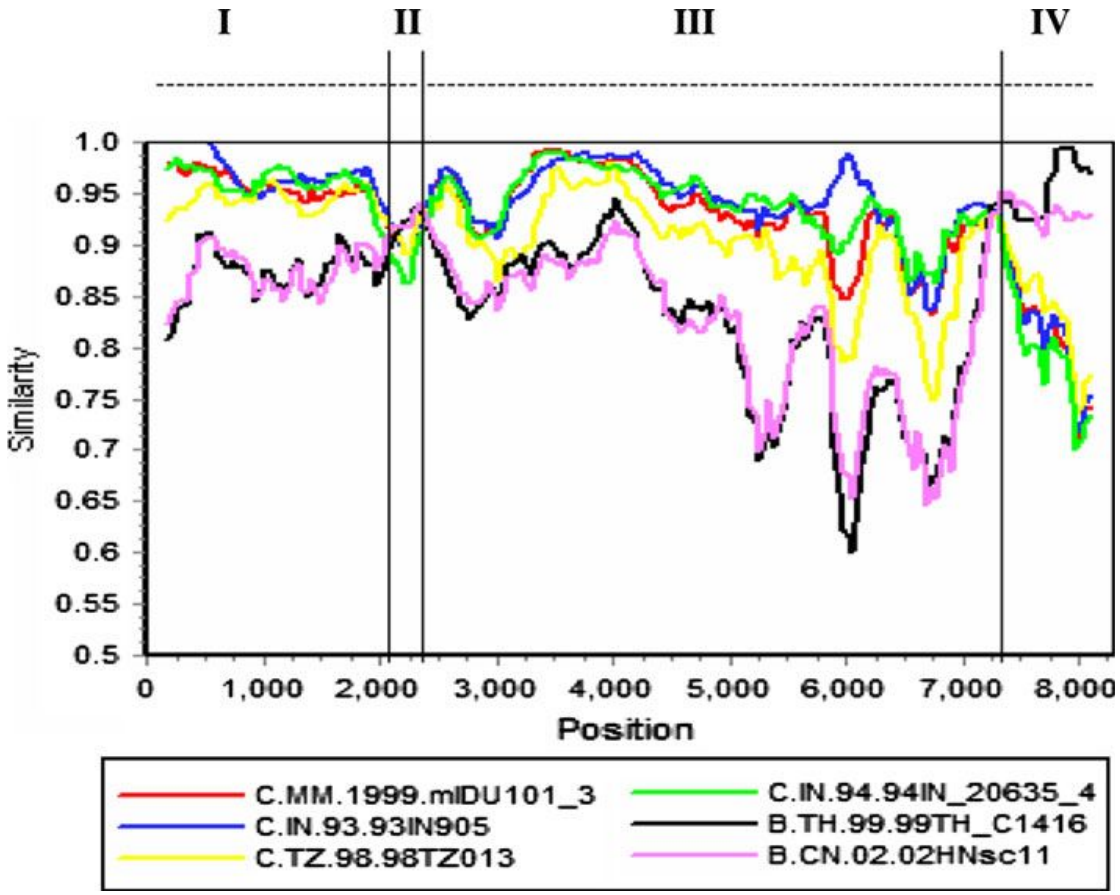



Fig. 2 Simplot analysis of (MAN40) HIV-1 strain isolated from an injecting drug user of Manipur. The HIV-1 strains included are C.IN.93.93IN905, C.IN.94.94IN_20635_4, B.TH.99.99TH_C1416, B.CN.02.02.HNsc11, C.MM.99.mIDU101_3, 07_BC.CN.97.97.CN001, 07_BC.CN.05XJDC6441, and BC.MM.1999.mIDU106. Recombinant viruses are indicated by the subtype designation followed by the name of the isolate. Bootstrap values 70 %, based on 100 replicates, of some key nodes are shown. The Breakpoint positions were obtained using Simplot 3.5.1 and numbered according to HXB2 references

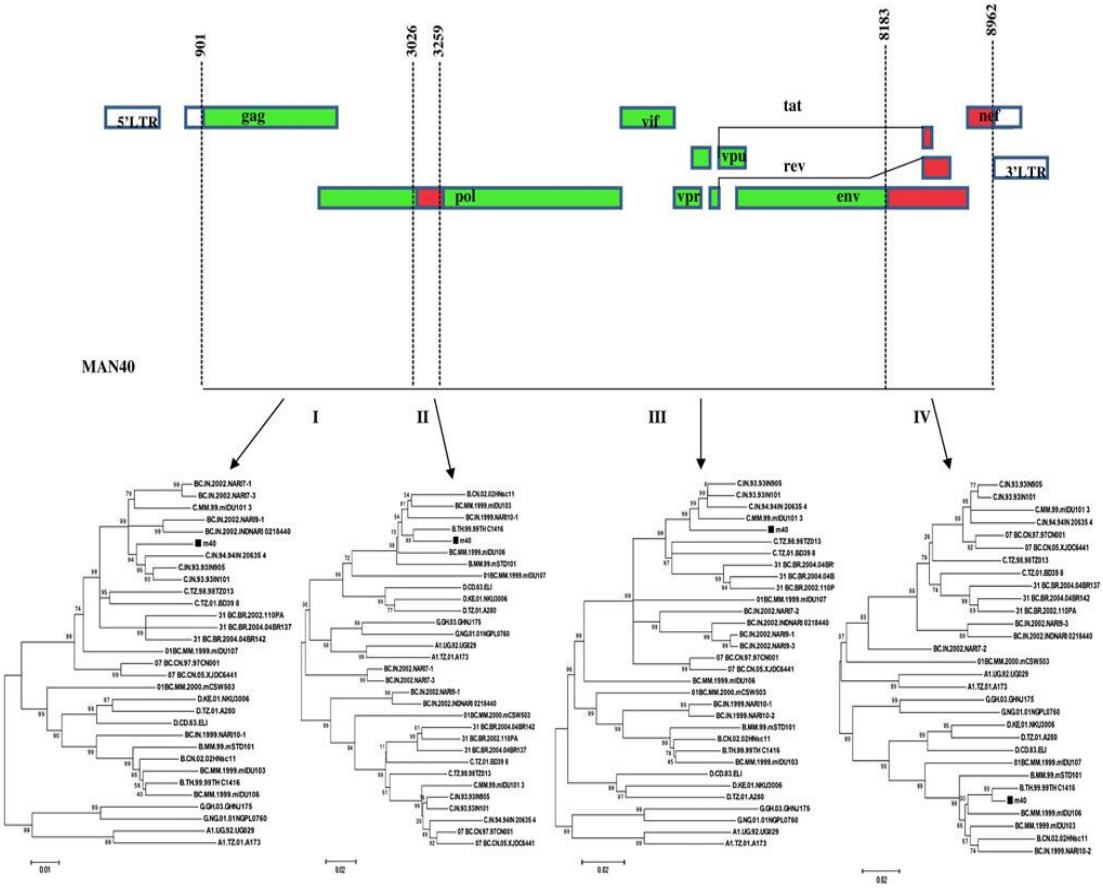



Fig. 3 The schematic drawing showing the mosaic recombination pattern of MAN40 HIV-1 genome. Subtype C and subtype B region are shown in green and red colors. The phylogenetic analysis of the Ist, IInd, IIIrd, and IVth fragments of MAN40 denoted as 'jm40' with HIV-1 sequences from other global HIV-1 strains are shown
below the recombinant structure indicated with arrows