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 2012-2013**

- **Accessing the genetic variability among local Giardia strain**

- **Standardization of GLV detection procedure**

Pilot survey for assessment of genetic variations among local Giardia isolates

Significance of study area

Kolkata (N 22°577242 E 88°398743) is a densely populated city of India, where the giardiasis in human caused by infection of *G. duodenalis* is a serious problem of public health. It is one of the major metro cities of eastern India and Southeast Asia with plenty of immigration and emigration of multi cultured people with varied socio economic condition. It has a favorable climatic condition for the growth of enteric pathogens. So, it is very much likely to find plenty of opportunistic enteric parasites in this city and also a possibility of genetic variations among them.

Procedure

68 Giardia positive stool samples were randomly taken from the surveillance program of IDBG hospital and were subjected to multi-locus genotyping. The DNA was extracted directly from the positive stools using StoolDNAMiniKit (QIAGEN, USA) according to the manufacturer's protocol. A portion of *ß*-giardin (*ßg*) [511 bp] on 90 kb long contig ctg02-35, *Glutamate dehydrogenase* (*gdh*) [434 bp] on 231 kb long contig ctg02-15 and *Triose phosphate isomerase* (*tpi*) [530 bp] on 200 kb long contig ctg02-19 (www.*Giardia*db.org), were individually amplified according to the previously described nested PCR protocols [10-12]. The nested PCR products were separated in 1.5% (w/v) agarose gel and purified by gel cut purification process using High Pure PCR purification Kit (Roche, Germany) as per the manufacturer's protocol. Bi-directional sequencing was performed with the respective purified products and nested PCR primers on an ABI 3100 automated sequencer by using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem, USA). The

sequences obtained from all three loci (*ßg, gdh* and *tpi*) were validated using the database BLAST search (i.e. NCBI and *Giardia*db) and were submitted to NCBI GenBank (ACC no. JF918436 – JF918523 & JN647526 – JN647641). The sequences from each locus were separately aligned by using 'MEGA Version 4' software [13,14] and were manually checked and edited. Previously reported sequences of the respective loci representing different *G. duodenalis* assemblages were included in the analysis to get a better resolution of the assemblage distribution. The extent of sequence diversity among the wild isolates based on the target loci was determined using the 'Maximum Composite Likelihood' method through the MEGA4 software.

Findings

Based on the cumulative sequence data of all three loci, 41 samples could be assigned as assemblage 'B' (60.2%) and 13 as assemblage 'A' (19.1%), while 14 (20.5%) isolates showed multiple assemblages depending on the marker loci.

Sequence analysis showed higher degree of diversity in the '*tpi*' loci in compare to other two **(Table 1).**

Table 1. Estimation of average sequence diversity

Target locus					
ß giardin (*ßg*)		**Glutamate dehydrogenase (*gdh*)**		**Triose phosphate isomerase (*tpi*)**	
N	*D ± SE*	*N*	*D ± SE*	*N*	*D ± SE*
68	0.001 ±0.004	67	0.053 ±0.011	64	0.106 ±0.019

The number of base substitutions per site from averaging over all sequence pairs is shown. All results are based on the pairwise analysis of 'N' number of sequences. Standard error (SE) estimate(s) are shown besides the diversity (D) estimates and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Maximum Composite Likelihood method in MEGA4. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). The tpi loci showed highest diversity in compare to the other two.

Detection of Assemblage 'B' and assemblage 'A' overlaps the results obtained from previous studies in southern India using single locus PCR-RFLP method [18]. However, non-specific sequence heterogeneity among same loci of identical assemblages led to the difficulty in assigning sub-assemblages [19]. In the previous reports from the same country, presence of mixed assemblages may be evident but, those were usually found through multiple assemblage specific bands in RFLP analysis of a particular locus [18]. This may occur due to presence of overlapping DNA sequence derived from two or more different isolates, although it was not proved. The unique finding of our study was, 14 (20.5%) isolates showed multiple assemblages depending on the marker loci. Clustal distribution with these 14 isolates in the NJ tree revealed a better picture where isolates were placed in distinct clusters of assemblage A and B (marked with red and blue bars respectively) supported by high boostrap values (Fig. 1) but, the cluster selection (i.e. A or B) of the isolates were different for each loci. For example the isolate number GLI11 is positioned in assemblage A cluster in '*ßg*' and '*gdh*' tree (Fig. 1A & B) but falls in the other cluster in '*tpi*' tree (Fig. 1C). Again isolate GLI23 is positioned in assemblage A cluster in *ßg* and '*tpi*' tree (Fig. 1A & C) but is present in the assemblage B cluster in '*gdh*' tree (Fig. 1B).

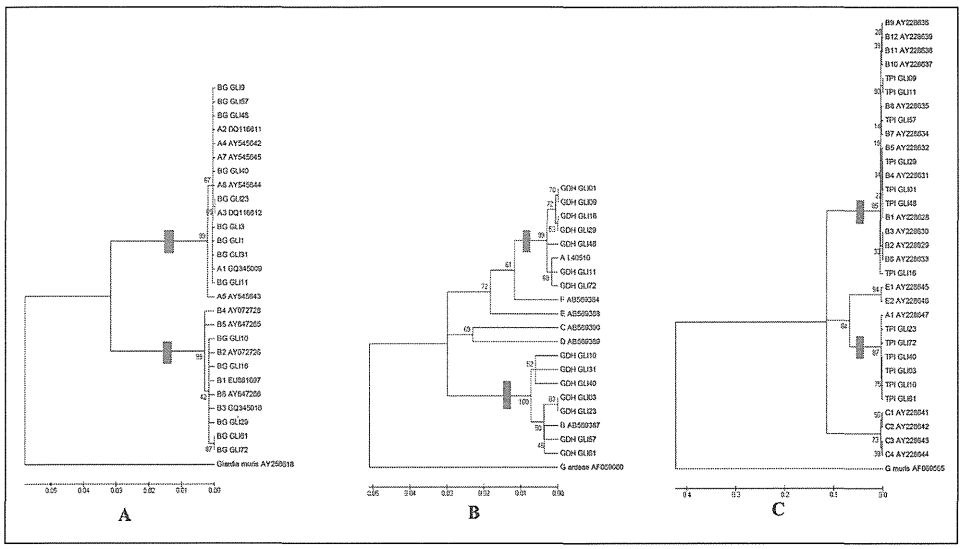

Fig. 1. Phylogenetic analysis of *ß-giardin, Glutamate dehydrogenase and Triose phosphate isomerase loci* using the 'MEGA version 4 program'

The evolutionary history was inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (1000 replicates) are shown next to the branches. The evolutionary distances have been computed using the Maximum Composite Likelihood method and are shown in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Assemblage A clusters are marked with RED bars and Assemblage B clusters are marked with BLUE bars. (1. A) Phylogenetic analysis of ß-giardin locus. (1. B) Phylogenetic analysis of Glutamate dehydrogenase locus. (1. C) Phylogenetic analysis of Triose phosphate isomerase locus.

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.

Other than sexual reproduction, genetic recombination can also occur through some extra-chromosomal genetic material which is capable of incorporating the host genome. Here comes the concept of GLV.

GLV

In 1985, the abundant presence of a 7-kb linear double-stranded RNA (ds RNA) species in the trophozoites of a *G. lamblia* strain, Portland I, which turned out to be the genome of a non-segmented, ds RNA virus was named as *Giardia lamblia virus* (GLV) (Ching C.Wang *et. al*). *Giardiavirus* is a small, icosahedron, nonenveloped virus comprising a monopartite double-stranded RNA genome, a major protein of 100 kDa, and a less abundant polypeptide of 190 kDa. It can be Isolated from the culture supernatant of *Giardia lamblia,* a parasitic flagellate in human and other mammals, and efficiently infects other virus-free *Giardia lamblia. (*Miller,R.L., *et. al*). Unlike the uninfectious *yeast killer virus* (ScV), *Leishmania RNA virus* (LRV), or other fungal viruses, GLV is shed into the culture supernatant and is highly infectious in its purified form. (Furfine, E.S., *et. al*). Virus particles, released by the transfected cells into the culture medium, were capable of infecting the virus-sensitive *Giardia lamblia* WB strain. Two aspects of GLV distinguish it from the rest of the totiviruses: i) its ability to infect *Giardia* trophozoite. ii)The fact that a single-stranded transcript of the GLV dsRNA genome can be isolated and introduced into *Giardia* trophozoites *by* electroporation to initiate *GLV* infection and replication. Moreover, GLV can possibly incorporate into host genome.

GLV Detection procedure and outcomes:

RNA isolation and PCR amplification: Viral RNA was isolated by using Viral RNA Minikit, Qiagen. cDNA preparation and PCR amplification was performed by using Superscript-III One Step RT-PCR kit, Invitrogen.

Protocol

- Various amounts (1–2 ng) of eluted RNA were subjected to RT-PCR using the SuperScriptIII One-Step RT-PCR kit (Invitrogen Cat no. 12574-030) and primers directed to the GLV-capsid protein sequence (GenBank L13218). [The forward primer sequence (GLV-CF) was 5'-GCCAGGATCTGGTAATTGCT-3' corresponding to nt 1251–1270; the reverse primer sequence (GLV-CR) was 5'-CTAGCGTCCTTTGAATACA-3' corresponding nt 1541–1569.]
- 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.

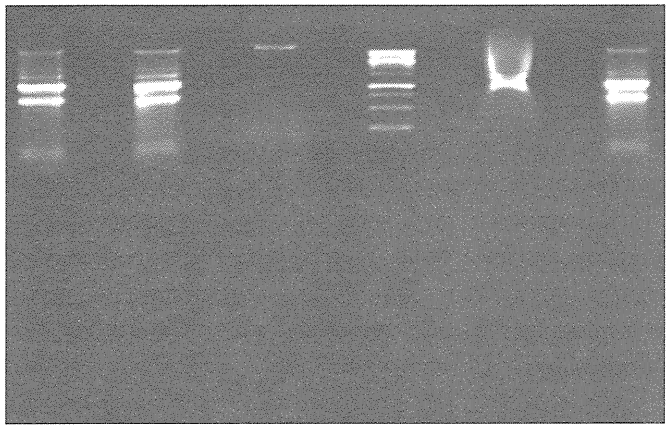

Fig: Isolated RNA with different treatment

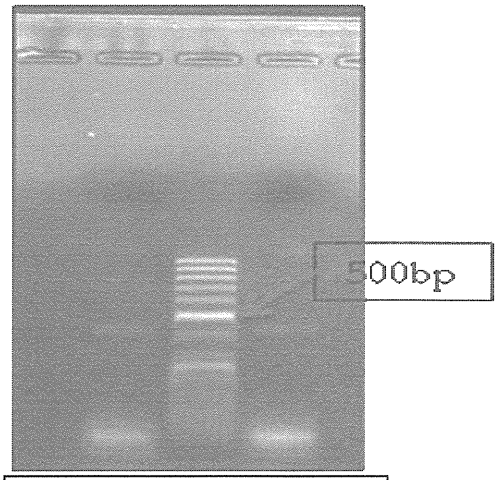

Fig: cDNA and PCR amplification using Superscript III one step RT PCR kit, Invitrogen (using Ref primer for GLV capsid protein.

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:

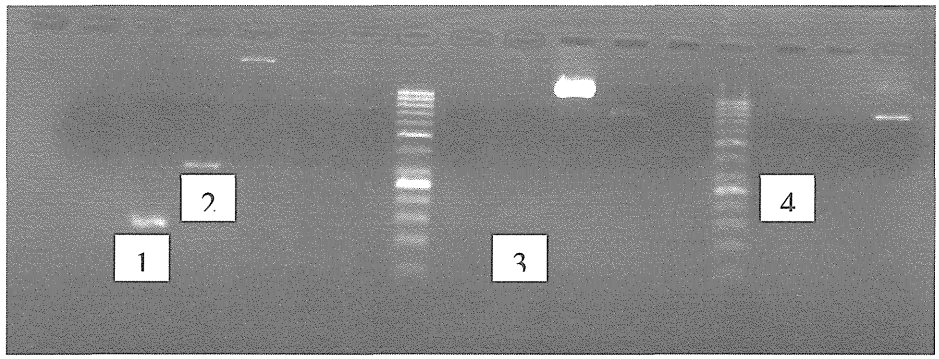

Fig: cDNA and PCR amplification using Superscript III one step RT PCR kit, Invitrogen (using new designed primer for GLV capsid protein.

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%

Possible reasons for negative results:

- The lab Portland I lacks GLV
- We should look for some better protocol

vi) Future goals and Plan for fiscal year 2013-2014

1. Establishing better protocol for GLV identification.
2. Identification of infection of GLV among different *G. l.* isolates in patients with differential infection, e.g. with sole *G. l.* infection and with multiple infections.

3. Identification of infection of GLV among non symptomatic *G. l.* isolates (if any) with sole and multiple infections.

vii) Publications:

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. 2013 ***British Microbiology Research Journal***. (In press).

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, Jiro Mitobe, GB Nair.**

3. Name of Student: **Ritam Sinha**

5 .Objective of Our Work:

Current vaccines for bacterial diseases have a serotype 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 it is really difficult. This may result from powerful immunogenicity of serotype specific polysaccharide antigen that could camouflage potential antigenicity of common virulence proteins. We have developed a candidate of broad range of Shigella vaccine based on molecular mechanism of virulence gene expression.

Keeping above ideas we have started work with these salient objectives:

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

6) Brief Report:

Bacillary dysentery caused by Shigella species, is a major cause of 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. *Shigella sonnei*, the most frequent serogroup in developed countries, accounted for 40–57% of shigellosis infections reported between 1994 and 2002 . In India and Bangladesh, *S. dysenteriae* 1 epidemics spread to adjoining areas, had high attack rates and showed extremely high fatality rates among children and adults. Sanitation, and improved hygiene and water would help to control the disease but progress is slow in the poor communities where the disease is most prevalent. Therefore, the only hope to control *S. dysenteriae* 1 and *Shigella sonnei* infection is to develop a vaccine. 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 guinea pig model. Constriction and preliminary protection work done by our Japanese scientist.

The protective efficacy after eye immunization with four doses (0, 1st, 14th, 15th Day) of Shigella strain was examined. The protection following challenge was 100% protection (against *Shigella dysenteriae*) in the immunized group whereas the non immunized group of animals developed keratoconjunctivitis mean zero protection (Fig 1).

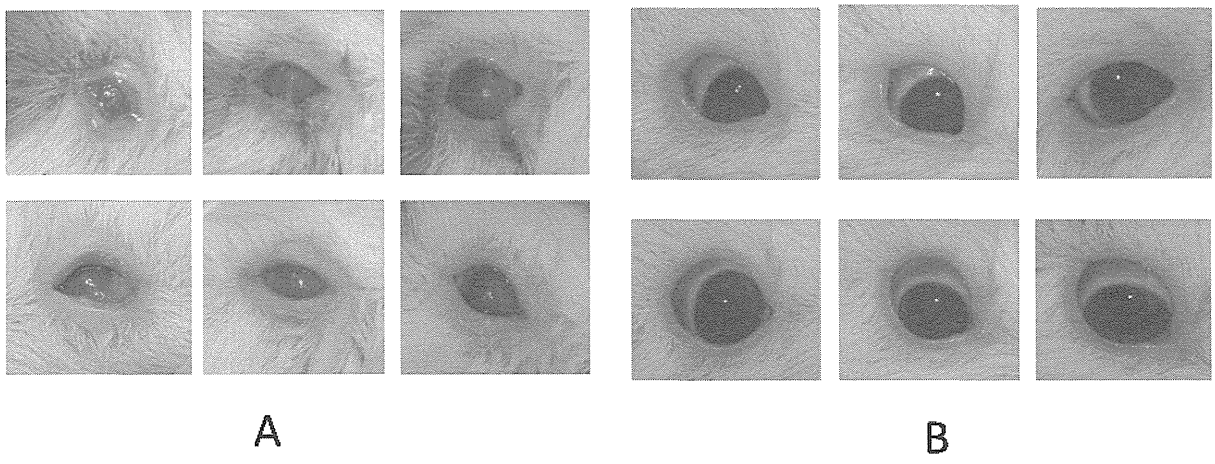

Fig 1: Keratoconjunctisvitis developed after challenged with *Shigella dysenteriae* 1 to the Non - immunized guinea pigs (A). No effect to the immunized animals (B)

Nature of colonization ability after challenged with *S. sonnei* from the distal colon of animals of both the immunized and control groups was observed. In animals challenged on day 42 after the first day of the immunization, at 72 hrs after the challenge, at most $5\pm2.8\times10^3$cfu per gram of tissue were recovered

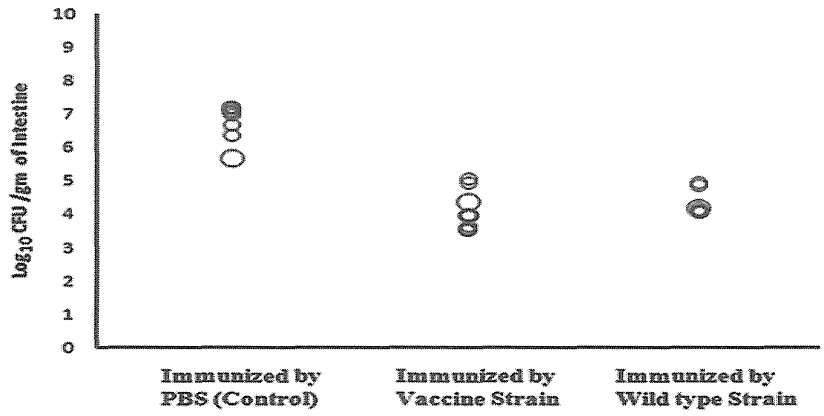

Fig 2: . Nature of colonization ability of challenge strain *S. sonnei* in the distal colon among challenged immunized and non-immunized animals.

from animals of the non-immunized group, whereas $1.8\pm 2.8\times10^9$ cfu per gram of tissue were recovered from animals of immunized group of animal (Fig: 2).

Table 1: Protective efficacy of the immunization with Vaccine strain MF 4831 (hfq mutant) and wild type strain.

Experimental animal	Immunogen used in Eye.	Challenged Strain used in intestine	Number of animal used	Disease symptoms	% of death with Shigellosis	% of protection against Shigellosis
PBS Control Group	Nil	Wild type S. sonnei	6	Shigellosis 100% (6/6)	100% (6/6)	0% (0/6)
Immunized Group	VACCINE STRAIN MF4831	Wild type S. sonnei	6	33.3% (2/6)	0 % (0/6)	100%
	Wild Type Strain (S. flexneri 2a 2457T)	Wild type S. sonnei	5	40% (2/5)	0 % (0/5)	100%

After 42th day of first immunization, luminal challenge was performed with *Shigella sonnei* to observed ectopic effect. We have observed that immunized groups of animals showed 100% protection against *Shigella sonnei* challenged. Among four animals of immunized group excreted semisolid stools within 24 hours after the challenge but recovered spontaneously within 48 hours. On the other hand, all animals of the control group challenged developed symptoms of bacillary dysentery, such as tenesmus and mucoidal and bloody diarrhea and died within 72 hrs. (Table1).

The results of the current study might suggest us that **this MF 4831 (hfq mutant)** vaccine strain could be a promising future vaccine candidate, to prove, we need to work more detailed in animal models.

7) Reference:

1. Mitobe, J., Morita-Ishihara, T., Ishihama, A., and Watanabe, H. Involvement of RNA-binding Protein Hfq in the Post-transcriptional Regulation of *invE* Gene Expression in *Shigella sonnei*(2008) *J BiolChem* 283, 5738-5747
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3. Sereny, B. Experimental keratoconjunctivitis shigellosa. 1957. *ActaMicrobiol. Acad. Sci. Hung.*(4:367-376.
4. Sack RB, Kline RL &SpiraWM Oral immunization of rabbits with enterotoxigenic Escherichia coli protects against intraintestinal challenge (1988). *Infect Immun* 56: 387-394.
5. Barman S, Saha DR, Ramamurthy T, Koley H. Development of a new guinea-pig model of shigellosis. (2011), *FEMS Immunol Med Microbiol*.. 62(3):304-14.

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

Initial few months were utilized for recruitment and training of field staffs, collaborating with NGOs of Manipur and West Bengal etc. Sample collection started from August 2012. Eligible HIV positive patients with and without receiving ART were approached for the study by our social/field workers to explain the purpose of this study and to seek their voluntary participation. A team from NICED visits these states every month to monitor and supervise sample and data collection procedure. So, far 42 blood samples have been collected from eligible candidates by trained laboratory technicians along with data related to their socio-demography, risk behavior and history of ART compliance. Written informed consent has been obtained from all eligible and willing candidates. Blood samples were transferred to NICED laboratory on same day of the collection. Data editing and entry are being carried out at present.

Viral Load estimation

Viral load is estimated using the Cobas Amplicor HIV-1 monitor test version 1.5 (Roche) by quantitaing HIV-1 RNA in human plasma on the cobas amplicor analyser .

1. 200μl of blood plasma is taken for RNA extraction and finally eluted in 400 μl of diluent medium.
2. 50 μl of RNA is mixed with 50 μl of provided master mix for the reverse transcription of target RNA to generate cDNA.
3. It is followed by PCR amplification of target cDNA by specific complementary primers.
4. The amplified products are then hybridized to target specific oligonucleotide probes.
5. Finally, the probe bound amplified product is detected by colorimetric method indicating the viral load estimated by RNA copies / ml of plasma.

The viral load results of the 42 samples have been shown in **Table 1**.

SERIAL NO.	SAMPLE I.D.	CD4 COUNT	PLASMA VIRAL LOAD (HIV-1 RNA copies/ml)
1	JK-RI-000-4615	36	16800
2	RPC-DEC-K3747/12	80	575
3	RG-MCH-000-517	58	18800
4	DURG/CG/0684	40	35700
5	WB/MR/000/170	140	585
6	WB/TM/00/2964	37	49
7	JK/MG/00881	96	41000
8	WB-RPC-40	373	90
9	WB-MLD-73	118	35
10	WB/STM/00/1047	273	26600
11	WB-TM-00-6720	48	156000
12	WB-RPC-592	89	83
13	RGKAR-MCH-000-415	176	27200
14	RGKAR-MCH-001066	136	165000
15	JK-DHN-017	96	43000
16	MDH-000-638	1120	4700
17	DURG/0653	189	165000
18	WB/MR/000/107	198	142000
19	AS/GMC/1175	137	53100
20	JK/RI/00/130	32	31500
21	WB/STM/0000/77	12	14000
22	RAIPUR/CG/08/1058	64	133000
23	WB/MMCH/000/106	511	239
24	JR/RI/000/2431	36	15300
25	BMCH/0000/53/2010	46	143000
26	JK/DHN/009	279	165
27	WB/MR/000/723	357	105
28	WB/TM/2660	51	1640000
29	WB/NB/32	226	399000
30	WB/RPC/001214	82	162000
31	DURG/CG/1145	135	862000
32	WB/TM/2883	84	4420
33	WB/STM/00/5598	401	72900
34	WB/TM/005011	171	10600
35	WB/TM/9604	264	84500

36	AS/GMC/1228	55	199000
37	RPC/K-677	307	11000
38	WB/RPC/K-192	135	61700
39	OR/CTC/87/07	80	5590
40	WB/TM/00/3674	96	76000
41	WB/BMCH/000/016	230	92
42	WB/TM/00/5299	162	48

HIV-1 sub-typing and genetic characterization of the HIV-1 seropositive samples

In continuation to the study of the molecular characterization of HIV-1 strains, till date 42 samples were analysed. Multi Region Hybridization Assay were performed with these samples. The blood samples were collected in Na-citrate solution after pre- and post-test counseling. HIV-1 sero-positivity was determined by rapid spot test (Immunocomb HIV-1/2 Bi-spot, Orgenics, Israel), followed by ELISA (Immunogenetics, Belgium) and line immunoassay (Inno-LIA, HIV-1/HIV-2). Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by Ficoll-Hypaque gradient centrifugation.

A) Multi-region Hybridization Assay (MHA) :

An advanced genotyping method multi-region hybridization assay (MHA) was commenced for sub-typing analysis and determination of the recombination break points, among the HIV-1 positive samples. MHA was performed on the basis of 8 different genomic regions of HIV-1. The principle of the MHA was to amplify multiple short fragments throughout the HIV-1 genome and to assess the hybridization of clade specific fluorescent probes in real time PCR. Almost all the genomic regions of 40 samples were tested each with probes for subtype B, C and AE. The amplified regions were positioned in areas of the genome that would tend to maximize discrimination among different strains of interest. Viral RNA was extracted by Viral RNA Mini Kit (QIAGEN, Germany). The RT-PCR and first round PCR was done following proper PCR conditions. All the genomic regions of HIV-1 genome viz. *p17*, pro, reverse transcriptase (*rt*), *int*, *tat*, gp120, gp41 and *nef* were amplified in separate 1st round PCRs. The second round real time PCR was done using TaqMan 2x universal PCR master mix (ABI), 400 µM of each inner primer, 250 µM of probe and 1 µl of 1st round PCR product. Real time PCR amplification was performed in a 96 well ABI PRISM 7900HT sequence detection system (Applied Biosystem) with following routine: hold at 95°C-10 mins and 95°C-10s, 60°C-1 min and in case of gp120 region, the extension step was performed at

60°C-2 mins. Fluorescence intensity was measured during the reaction and was analysed by using the SDS v2.1 software (Applied Biosystem).

MHA analysis showed that in case of 35 samples, all the genomic regions (*p17*, *pro*, *rt*, *int*, *tat*, gp120 and *nef*) of HIV-1 reacted with the probes belonged to subtype C whereas 5 of the remaining samples showed probe reactivity for subtype C (*p17*, *pro*, *int*, *tat*, gp120 and *nef*) genes but the *rt* region of the *pol* gene showed dual probe reactivity.

Table 3

(a) Results for multiregion hybridization assay (MHA):

Subtype	Total no. of samples	No. of samples	Genomic regions
C	35	35	p17, pro, rt, int, tat, gp120, gp41, nef
B		-	p17, pro, rt, int, tat, gp120, gp41, nef
Dual reactivity		5	pol
Multigenomic variants		-	-

The AIDS epidemic in Manipur, India, manifests unique features, having co-circulation of B and C HIV-1 subtypes along with recombinant forms. Manipur has the highest incidence of HIV-1 infection compared to the other states of India, but limited information is available regarding the full-length sequence of HIV-1 recombinants.

Viral RNA, extracted from the plasma of a male injecting drug user diagnosed with HIV-1 infection. Near full-length genome was amplified by polymerase chain reaction using primer walking approach. Phylogenetic relationships were determined with neighbor-joining trees. The recombination break points were detected using boot scan and Simplot analyses.

B) Near full length genome sequencing:

This recombinant predominantly had subtype C genome and exhibited mosaic structures with subtype B insertions at three different positions of HIV-1 genome. Simplot analysis of near full-length genome sequence from the recombinant HIV-1 strain, MAN86 exhibited similarity with the sequence of C.IN.93.93IN905 in its subtype C backbone, while the subtype B insertions showed resemblance with the sequence of B.TH.99.99TH_C1416. This study confirms the presence of a unique recombinant HIV-1 strain, emerging as a result of

recombination between HIV-1 strains from India and Thailand (Fig.1). The Simplot analysis was shown in (Fig. 2) for sample no. MAN-86 (HIV-1 strain).

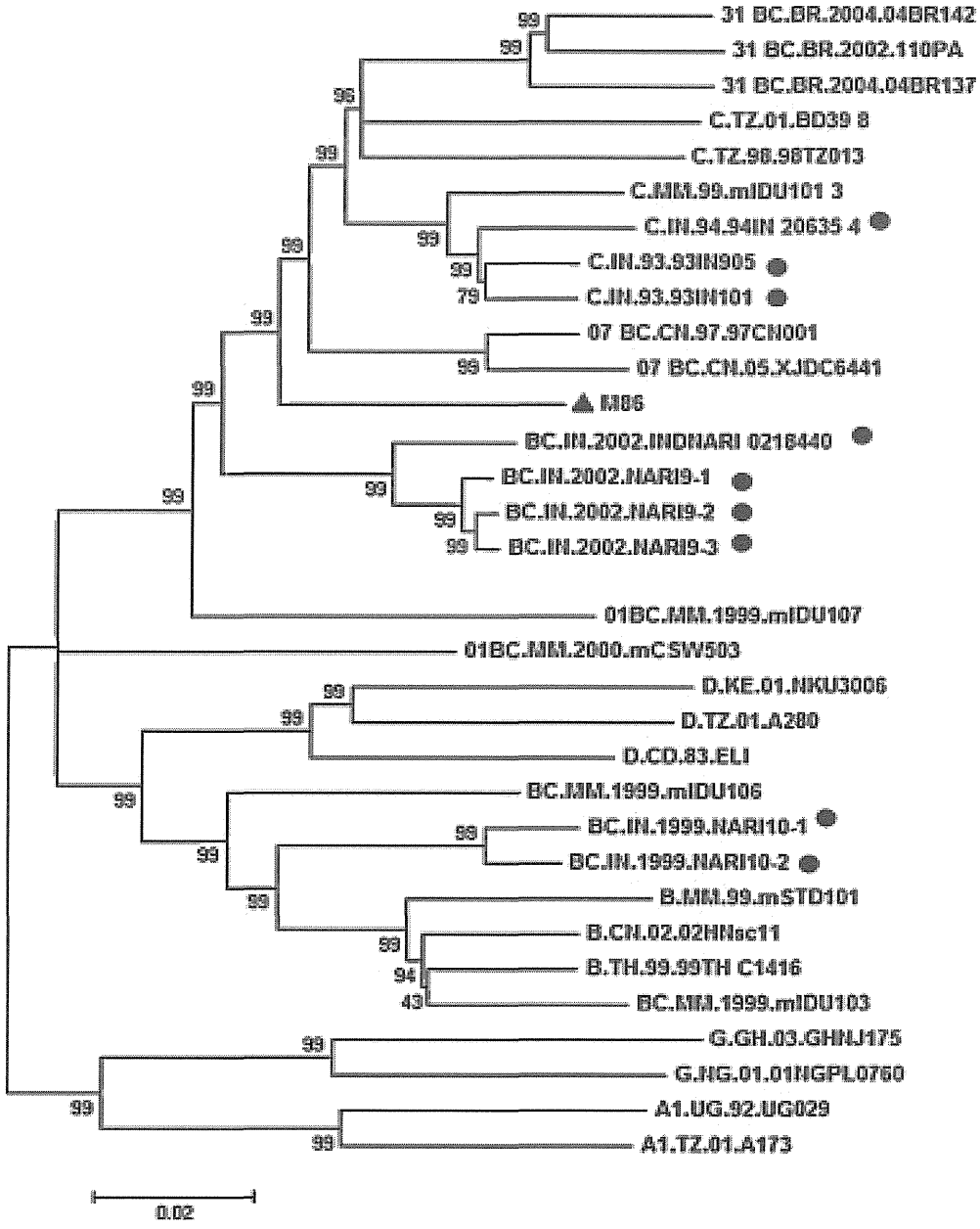

Fig. 1. Phylogenetic analysis of virtually full-length genome sequences. The tree was constructed by the neighbor-joining method. The Manipur sequence (MAN86) is shown as '▲', Indian strains are shown as '●' and the unmarked ones represent other global strains. The scale bar represents 10% difference. Interpatient HIV-1 diversity across the entire genome or individual genes among Manipur isolate from IDU compared with HIV-1 sequences from other global HIV-1 strains. The interpatient genetic distances were calculated using the Kimura two-parameter distance measurement.

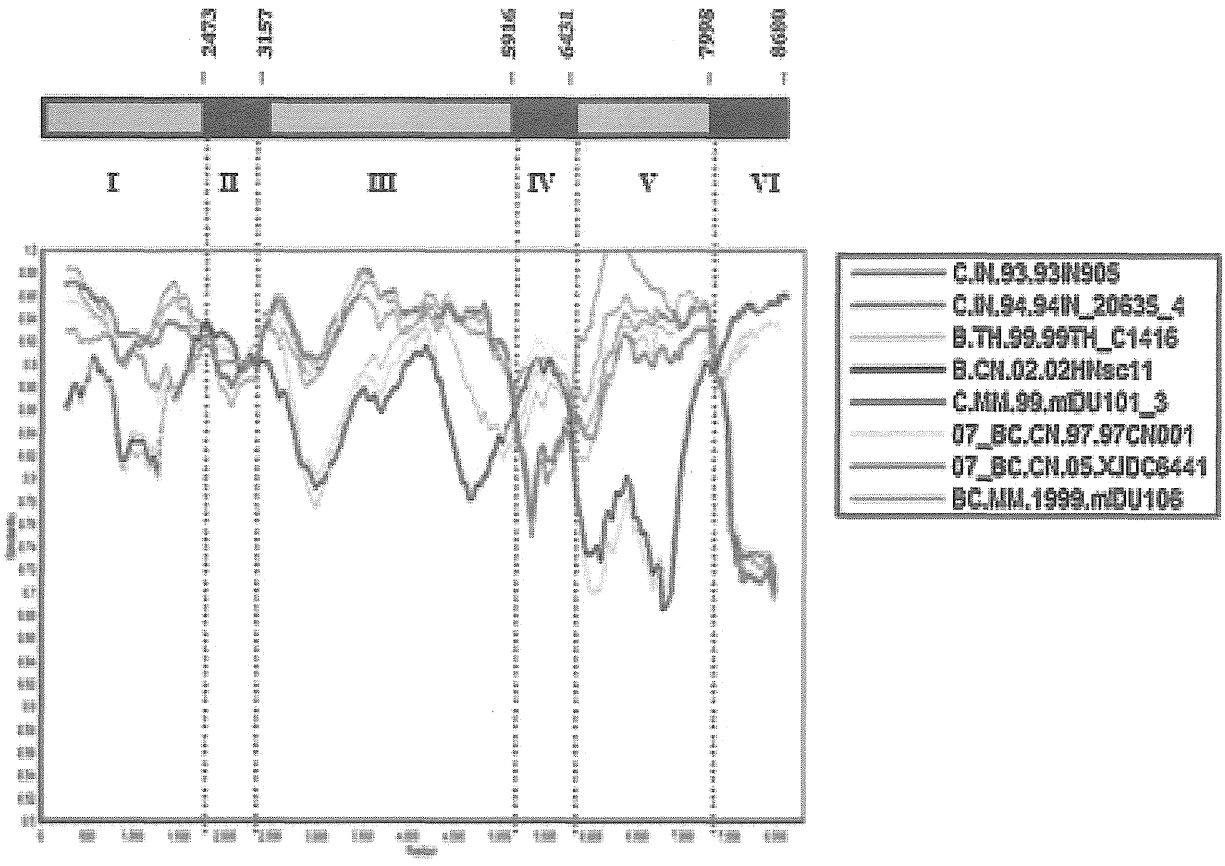

Fig. 2. Simplot analysis of a HIV-1 strain (MAN86) isolated from an IDU of Manipur. The HIV-1 strains included are C.IN.93.93IN905, C.IN.94.94IN_20635_4, B.TH.99.99TH_C1416, B.CN.02.02.HNsc11, C.MM.99.mIDU101_3, 07_BC.CN.97.97.CN001, 07_BC.CN.05XJDC6441, BC.MM.1999.mIDU106. Recombinant viruses are indicated 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 reference. The schematic drawing was performed to divide the HIV-1 genome of MAN86 into six different fragments based on mosaic recombination pattern. Subtype C and subtype B regions were shown in blue and black colors.

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 reference.

Publications:

1. **Roni Sarkar**, Kamalesh Sarkar, N. Brajachand Singh, Y. Manihar Singh, Debashis Mitra, Sekhar Chakrabarti. **Emergence of a unique recombinant form of HIV-1 from Manipur, India.** ***Journal of Clinical Virology***, **(2012). 55: 274- 277.**

プロジェクト４：ベトナム

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平成24年度　分担研究報告書

研究課題名：「腸内細菌の molecular typing に関する研究」

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研究要旨　　本研究は、わが国をはじめアジア各国で発生する種々の細菌感染症に対応するため、主として食水系由来腸管感染症を対象に遺伝子解析をベースとした疫学指標、診断法の開発、ならびにそれらの有用性についての検討を主眼としている。本年度はベトナム National Institute of Hygiene and Epidemiology (NIHE) の細菌部門とコンタクトを持ち、コレラ菌を中心とした対象菌種の設定、解析手法の検討などを行った。

A. 研究目的

コレラ菌、赤痢菌、サルモネラといった、食水系腸管感染症起因菌を中心に研究を行う。当該感染症の流行地域であるアジア各国において、流行菌種あるいは菌型を把握するための分子タイピング法の検討を行う。また、必要に応じて、当該国の能力向上を図ることを目的とする。

B. 研究方法

分子タイピング法としてパルスフィールドゲル電気泳動法 (PFGE) および multilocus variable number tandem repeat analysis (MLVA) を主として活用する。

C. 研究結果および考察

コレラはコレラ菌（コレラ毒素産生性 *Vibrio cholerae* O1/O139）によって発生する経口感染症である。上下水道等、いわゆるインフラ整備が不十分な途上国では、コレラの流行は公衆衛生上の脅威である。本研究のカウンターパートの一つであるベトナムでは、2007年ごろから比較的頻繁にコレラの流行が発生している。コレラの流行は当該国において非常な脅威であり、当該国の感染症対策において上位に位置づけられている。また、ベトナムは現在、我が国との交易も盛んな国であることから、当該国でのコレラの流行はわが国にとってもリスクとなりえる。こうした背景から、本研究においてはベトナムにおけるコレラ流行の把握と制御に向けた共同研究を遂行している。

具体的にはベトナム国立衛生疫学研究所（National Institute of Hygiene and Epidemiology; NIHE）の細菌部門・コレラセンターと共同して、コレラ菌のサーベイランスシステムの構築を検討している。

本研究においては2つの活動を基点とする。一つは NIHE の能力向上であり、今一つは感染研との共同研究である。ベトナムで流行した、もしくは流行しているコレラ菌の特徴づけを行うべく、感染研と NIHE とで材料およ