(O'Handley et al., 2000; Huetink et al., 2001; Trout et al., 2004, 2005; Itagaki et al., 2005; Mendonca et al., 2007; Langkjaer et al., 2007).

Results of this study provide useful molecular evidence on the zoonotic transmission of *G. duodenalis* between cattle and dairy farm workers. DNA sequencing of nested PCR products of the β-giardin gene genotyped Assemblage A isolates from all calves and most dairy farm workers as subtype A1. It is interesting to note here that the two most common subtypes of Assemblage A, A1 and A2, differ significantly in host preference; animals are mostly infected with A1 whereas humans are mostly infected with A2 (Xiao and Fayer, 2008). Therefore, a relatively higher prevalence of A1 in comparison to A2 found in humans in this study is of zoonotic importance. In addition, mixed *G. duodenalis* Assemblages A and E infections were also detected in three calves in the present study which suggests that calves, although primarily infected with *G. duodenalis* Assemblage E, are frequently hosts of the zoonotic *G. duodenalis* Assemblage A.

In conclusion, results of this study provide new insights into the epidemiology and genetic diversity of *G. duodenalis* in Indian dairy cattle. Additionally, results also point towards a possible zoonotic transmission of this parasite between cattle and human workers on dairy farms in India. This is of potential public health significance since calves infected with *Giardia* normally shed a large number of cysts in their faeces (O'Handley et al., 1999) and could thus act as a potential zoonotic reservoir for human giardiasis especially on the dairy farm premises.

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分担研究報告書

エイズの流行とウイルス変異に関する研究

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研究要旨

HIV 感染症は世界三大感染症の一つであり、その克服は国際的最重要課題の一つである。細胞傷害性 T リンパ球（CTL）反応は HIV 複製抑制に中心的な役割を担っており、CTL 逃避変異を有するウイルスの選択は感染病態に大きく影響しうる。このような CTL 逃避変異の伝播状況の把握は、HIV 感染症のコントロールに極めて重要である。しかし、多数の検体において免疫学的解析により HIV 逃避変異を網羅的に同定していくことは困難であるため、これらの逃避変異と相関する HLA 関連変異を同定することが一つの戦略として重要視されている。各 HLA アリルの頻度は人種間で大きく異なるため、世界の各地域における流行 HIV 株の HLA 関連変異を同定することは非常に重要であり、特にインドはアジアにおける HIV 感染流行地域の一つとして重要な対象地域である。そこで本研究では、インド国立コレラ腸管感染症研究所（NICED）との共同研究で、インド国 HIV 感染者における HLA 遺伝子型同定と HIV ゲノム gag 変異同定を進め、HLA 関連変異同定に結びつけることとした。平成 23 年度は共同研究計画を作成するとともに倫理委員会の承認を得た。さらに、インドの感染者の HLA 遺伝子型同定を行い、方法論を含めて研究体制を確立した。

A. 研究目的

世界三大感染症の一つである HIV 感染症は、宿主免疫によるウイルス複製抑制が困難で慢性持続感染を呈し、エイズ発症に至る致死感染症である。その克服は国際的最重要課題の一つであり、HIV 伝播状況の把握は HIV 感染症のコントロールに向けた基盤情報となる。特にゲノム塩基配列の解析研究は以下のように進められてきた。

(1) 1980年代からの主にenv領域を中心とする解析に基づく多様性に関する研究：各種サブタイプが同定され、HIVの各地域への経時的な伝播についての知見が得られた。

(2) 1990年代後半の抗レトロウイルス薬治療導入以降の主にpol領域を中心とする解析に基づく薬剤耐性に関する研究：逆転写酵素阻害剤やプロテアーゼ阻害剤等の抗レトロウイルス薬に対する耐性変異の同定が進められてきた。

(3) 近年の主に gag 領域を中心とする解析に基づく HLA 関連変異に関する研究：HIV 感染病態に最も影響の大きい宿主因子として知られている HLA（クラス I）の遺伝子型を各々の HIV 感染者について決定し、HIV ゲノム変異の解析結果とあわせて HLA 関連変異を同定するものである。細胞傷害性 T リンパ球（CTL）の標的抗原エピトープの提示に関与する HLA 分子の遺伝子型の違いは、CTL 標的の違いに直結するので、HLA 関連変異はある程度 CTL 逃避変異を反映するものとして、その解析研究が進められている。特に最近、Gag 抗原特異的 CTL の有効性が示唆されていることから、gag 領域の解析が先行している。

本研究はこの(3)の HLA 関連変異に着目するものである。CTL 反応は HIV 複製抑制に中心的な役割を担っており、CTL 逃避変異を有するウイルスの選択は感染病態に大きく影響しうる。近年、

抗 HIV 薬投与により HIV 複製のコントロールが可能となったが、その複製抑制には CTL 反応が不可欠であることも知られている。このような CTL 逃避変異の伝播状況の把握は、HIV 感染症のコントロールに極めて重要である。

しかし、免疫学的手法により数多くの検体について逃避変異を網羅的に同定していくことは容易ではないため、新たなアプローチとして、これらの逃避変異と相関する HLA 関連変異の同定が重要視されている。各 HLA アリルの頻度は人種間で大きく異なっているため、世界各地域における流行 HIV 株の HLA 関連変異を同定することは非常に重要であり、特にインドはアジアにおける HIV 感染流行地域の一つとして重要な対象地域である。そこで本研究では、インド国立コレラ腸管感染症研究所（NICED）との共同研究で、インド国 HIV 感染者における HLA 遺伝子型同定と HIV ゲノム gag 変異同定を進め、HLA 関連変異同定に結びつけることとした。平成 23 年度は共同研究計画を作成し、方法論を含めて研究体制の確立を進めた。

B. 研究方法

本研究は、インドNICEDとの共同研究により、インド国のHIV感染者検体を用い、HLAタイピング（遺伝子型決定）と血漿中ウイルスゲノムの遺伝子配列決定を行い、統計学的手法を用いてHIVゲノムにおけるHLA関連変異の同定を進めるものである。有効なCTL標的抗原として有力なGag蛋白に焦点をおき、HIV gagの塩基配列解析を進める。インドNICEDのSekhar Chakrabarti博士との討論により、以下のような共同研究計画を作成した。

インドNICEDにおいて、まずは約200名のHIV感染者の血液検体を収集して、血清およびリンパ球を分離し、ウイルス学的解析を行う。一方、日本国の国立感染症研究所（NIID）にて、HLA遺伝子型決定を進め、両国で得られたデータをもとにHLA関連変異を同定する。

(A) 血清中ウイルス量の定量、末梢血CD4陽性Tリンパ球数等測定（インドNICED担当）：HIV感染者の病態を把握する。

(B) 血清中HIVゲノムの遺伝子配列決定（インドNICED担当）：血清よりウイルスRNAを抽出しHIV gag領域の塩基配列解析を行う。

(C) HIV感染者のHLAタイピング（日本NIID担当）：末梢血リンパ球より宿主ゲノムDNAを抽出しHLA遺伝子型を決定する。

(D) HLA関連変異の同定（日本NIID担当）：上記(B)と(C)で得られる情報をもとに統計学的解析によりHLA関連HIV変異を同定する。

平成23年度は、特にHLAタイピングシステムを確立し、方法論も含めた研究体制構築に努めた。

（倫理面への配慮）

ヒトサンプルを用いる研究については、ヒトゲノム・遺伝子解析研究に関する倫理指針に従い進める予定である。平成 23 年度に、所属機関である国立感染症研究所の倫理委員会に申請し承認済みであり、また、インドの共同研究者から所属機関である NICED の倫理委員会に申請し承認済みである。

C. 研究結果

インド NICED の Sekhar Chakrabarti 博士との討論により共同研究計画を作成するとともに、倫理委員会への申請を行い承認をえた。

インド国の HIV 感染者 15 名の末梢血リンパ球より得られた DNA を用い、東京医科歯科大学難治疾患研究所の木村先生・成瀬先生の協力のもと HLA-A、HLA-B、HLA-C 遺伝子型を同定した（表 1）。

D. 考察

研究計画作成、倫理委員会承認等、順調に進展した。HLA タイピングについては、アリル頻度が人種間で大きく異なっていることをふまえ慎重な判断が必要となるが、方法論も含め研究体制を確立することができた。今後、本体制にて HLA 関連変異同定を進める予定である。

E. 結論

インドNICEDとの共同研究で、インド国HIV感染者におけるHLA遺伝子型同定とHIVゲノムgag変異同定を進め、HLA関連変異同定に結びつけることとし、共同研究計画を作成するとともに倫理委員会の承認を得た。さらに、インドの感染者のHLA遺伝子型同定を行い、方法論を含めて研究体制を確立した。

F. 研究発表

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G. 知的財産権の出願・登録状況

無し。

Sample #	HLA-A		HLA-B		HLA-C	
1	A*11:01/02	A*24:02	B*40:06	B*57:01	C*06:02	C*15:02
2	A*11:01/02	A*33:01	B*39:01	B*58:01	C*03:02	C*12:03
3	A*11:01/02	A*68:01	B*40:06	B*40:06	C*15:02	C*15:02
4	A*11:01/02	A*31:01	B*13:01	B*44:03	C*04:03	C*07:01
5	A*01:01	A*03:01	B*07:02	B*55:01/03	C*01:02	C*07:02
7	A*01:01	A*24:02	B*07:02	B*37:01	C*06:02	C*07:02
8	A*33:03	A*33:03	B*44:03	B*44:03	C*07:01	C*07:01
9	A*31:01	A*33:03	B*07:02	B*44:03	C*07:01	C*07:02
10	A*11:01	A*68:01	B*48:04	B*52:01	C*07:01	C*08:01
11	A*24:02	A*68:01	B*52:01	B*52:01	C*07:02	C*12:02
12	A*11:01/02	A*68:01	B*15:32	B*58:01	C*03:02	C*12:03
13	A*02:03	A*33:03	B*27:07	B*44:03	C*07:01	C*15:02
14	A*31:01	A*33:03	B*44:03	B*51:01	C*07:01	C*16:02
15	A*01:01	A*11:01/02	B*40:06	B*57:01	C*06:02	C*15:02

表１．インド HIV 感染者の HLA 遺伝子型
15 名の HLA-A、HLA-B、HLA-C 解析結果を示す。

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. Name of Student

Ritam Sinha[a]

4. 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.

5. Summary:

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. 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. The protection following challenge was 100% protection (against *Shigellasonnei*) in the immunized group whereas the unimmunized group of animals developed dysentery, (protection 0%). Serum IgG showed exponential rise during oral immunization.

6. 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.

7. Methods:

7. 1 Animal experimental design

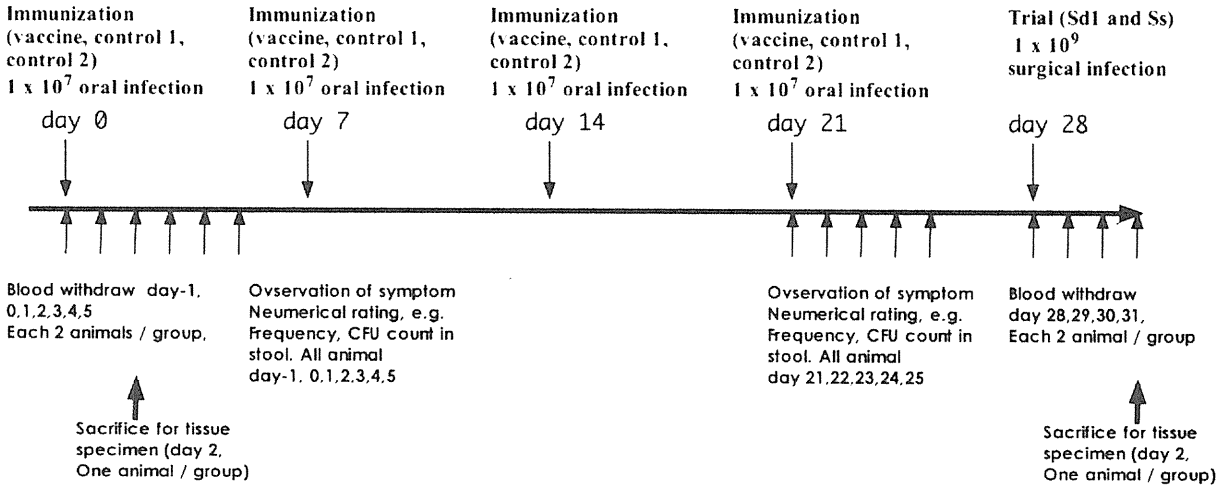
One set of experiment will be conducted by three groups (A, B, C) of male Hartley guinea pig (more than 2 months old).

SET- I: Protection Study for *S. dysenteriae* Type I (total 24 animals)
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

SET-II: Protection Study for *S. sonnei* (total 21 animals, three tissue specimens for vaccination effects are omitted from this set)

A) Immunization by vaccine strain MF4835--- six guinea pigs +1 for specimen
B) Immunization by wild-type 2457T: Control 1--- six guinea pigs +1 for specimen
C) Naïve, administration of saline : control 2--- six guinea pigs +1 for specimen
cf: 2457T (*S. flexneri* 2a) , MF4835 (2457T *hfq*::Km)

7.2 Schedule



c) Observations: Inspection of the samples will be done in NIID Japan

1. Numerical rating of diarrhea (frequency and amount). Daily graph of CFU for 2457T and MF4835, Sd1 and *S. sonnei* (in a constant amount of stool).

2. Serum; day 1 (For control), day 28 (For detection of vaccine antigen). 250-500 micro L pre animal with individual discrimination. (two animals in one group).

3. Histology-tissue specimen for colon, ileum, jejunum (immunization : vaccine / wild-type / naïve),(Trial : vaccine / wild-type / naïve) will be done.

4. Inflammation markers in serum. Inflammation markers will be detected by RT-PCR from blood RNA. 0.5 ml blood sample will be treated and stored in Mouse RiboPure™-Blood RNA Isolation Kit (ABI/Ambion cat#AM1951).

Remarks for animal welfare

All procedures accompanying discomfort to animal will be alleviated by anesthesia with mixture of Ketamine (35 mg/kg of body weight, Sterfil Laboratories Pvt. Ltd., India) and Xylocane (5 mg/kg body weight, AstraZeneca Pharma India Ltd., India). All experiments were conducted in compliance with the Animal Welfare Act, and adhered to the principles stated in the Guide for Care and Use of Laboratory animals .

7.3 Immunogen preparation and immunization

The overnight growth of Shigellaflexnari 2a 2457T and Vaccine strain *Shigellaflexanari*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 OD600 nm of 1.5. Organisms were heat-killed at 100 1C for 1 h, washed twice after centrifugation and resuspended in PBS. The suspension was adjusted again to OD600nm 1.5 and was stored at_80 1C till use for oral immunization. OD 1.5 corresponded to 107 CFUmL_1.

Two groups (4 animals in each) were used for oral immunization with heat-killed S. dysenteriae 1 and S. flexneri 2a. Oral immunization was performed according to the method of Sack et al. (1988). 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 *Shigellaflexanari*MF 4853** strains in lmL of PBS under anesthesia. Control guinea-pigs were treated with sterile PBS instead of heatkilled immunogens. The immunization schedule was followed on the 0, 7th, 14th and 21st day

ORAL IMMUNIZATION followed accordingly:

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 *Shigellaflexenari* 2a 2457T and VACCINE STRAIN MF 4831.

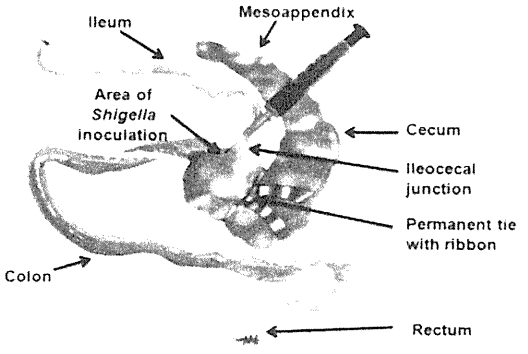
Step	Time	Treatment
I	-36 hours	Experimental guinea pig was starved but water will be given *at libitum*
II	-15 minutes	Guinea pig will be anaesthetized through intramuscularly by ketamine (35 mg/kg) and xylazine (5 mg/kg body weight)
III	- 5minutes	Colon was brought out through a midline incision aseptically
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

CHALLENGE EXPERIMENT done accordingly:

7.4 CHALLENGE EXPERIMENT

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.



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

8. Results:

To determine protective efficacy of vaccine candidates, two groups of guinea-pigs were immunized with vaccine by an oral route. After 1st immunization by wild type strain (10^7 CFUmL_1.) . We obtain highest cfu count in stool at Day 1and cfu count gradually decreased on Day2 and Day 3. (Fig 1).

On 1st immunization by vaccine strain, we obtain highest cfu count in stool at Day1, cfu count gradually decreases on Day 2 and 3 days. All animals were lived. After 3rd immunization, we obtained highest cfu count in stool on Day 22 (Fig 3).

Body weight of wild type vaccinated animals decreased after 24 hr immunization. Whereas in case of vaccinated by *Shigella flexanari* MF 4853 VACCINE STRAIN animals increases body weight after 48 hr. (fig 4). Compared with the immunized group by vaccine strain group of animal, the rectal

temperatures were increased by 1.5^{0}C within 24 h after infection in the immunized group by wild type strain. Group (Fig 5).

To determine the protective efficacy of vaccine candidates, two groups of guinea-pigs were immunized with *Shigella flexanari* MF 4853 VACCINE STRAIN by an oral route. After 24 h of luminal inoculation of wildtype *S.dysenteriae* Type 1 (SB1) and *S. Sonnei* strains, most of the unimmunized guinea-pigs had typical developed mucoidal diarrhea within 24 h, with the occasional presence of blood. However, such symptoms were not observed among the guinea-pigs immunized with vaccine strain against Shigella sonnei . (Table 2 & 3) In this study, 100% protection was observed in the immunized groups of guinea-pigs against S. sonnei

9) DISCUSSION:

After four successive oral immunizations, both immunized and PBS control guinea-pigs were challenged on the 28th day with wild-type strains. The challenge experiment was performed with the direct introduction of live virulent shigellae (1mL of 10^9 CFU) into the cecocolic junction after ligation of the distal cecum. The protection following challenge was 100% protection (against Shigella sonnei) in the immunized group whereas the unimmunized group of animals developed dysentery, (protection 0%). Serum IgG showed exponential rise during oral immunization.

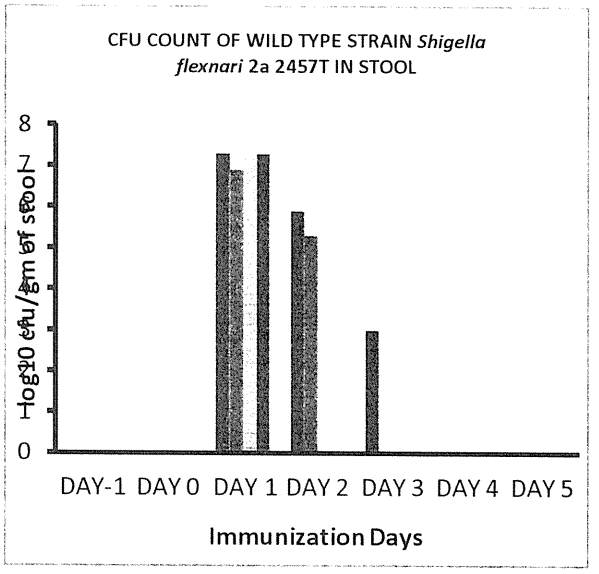


Fig 1: CFU count of wild type strain Shigellaflexnari 2a 2457T in stool after 1st oral immunization

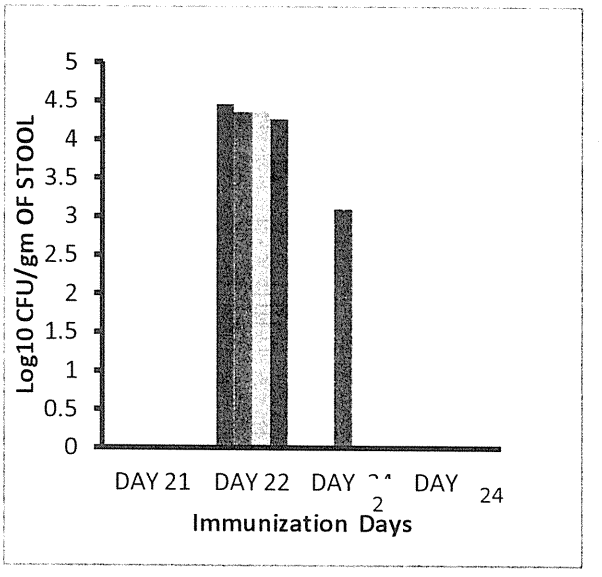


Fig: 3. CFU count of Vaccine strain *Shigella flexanari*MF 4853 in stool after 3rd oral immunization.

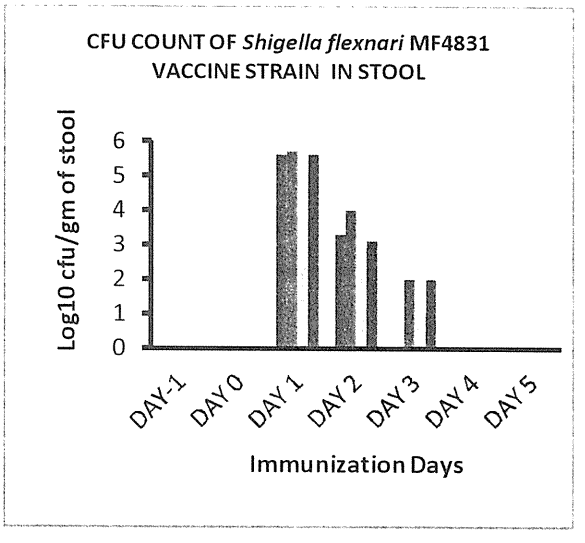


Fig: 2 CFU count of Vaccine strain *Shigellaflexanai* MF 4853 in stool after 1st oral immunization.

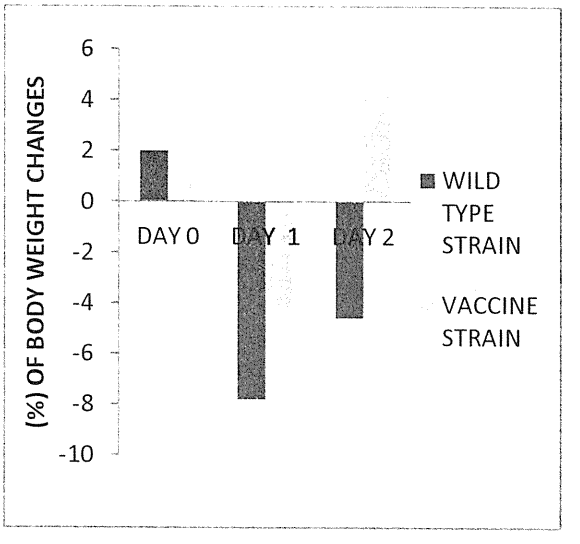


Figure -4 : Body temperature and body of wild type strain Shigellaflexnari 2a 2457T and Vaccine strain *Shigellaflexanari*MF 4853 during oral immunization.Mean percentage change in body weight compared with the base line weight after inoculation or immunization. Base line weight was taken 3 days before immunization

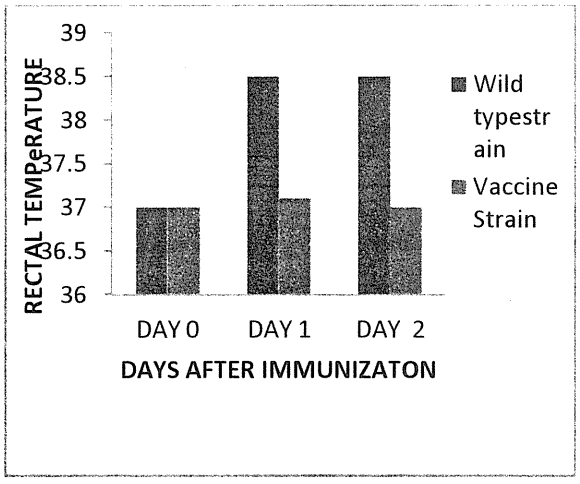


Figure:5 - : Mean rectal temperature of luminally inoculated animals.

Experimental animal	Immunogen used	Challenged Strain used	Number of animal used	Disease symptoms	% of death with Shigellosis	% of protection against Shigellosis
PBS Control Group	**Nil**	**Wild type Shigella dysenteriae**	**4**	**Shigellosis 100% (4/4)**	**75% (3/4)**	**0% (0/4)**
Immunized Group	**VACCINE STRAIN MF4831**	**Wild type Shigella dysenteriae**	**4**	**100% (4/4)**	**50% (2/4)**	**0% (2/2)**

Table 1; Challenge with *S.dysenteriae* against immunized vaccine strain MF 4853

Experimental animal	Immunogen used	Challenged S	Number of animal used	Disease symptoms	% of death with Shigellosis	% of protection against Shigellosis
PBS Control Group	**Nil**	**Wild type S. sonnei**	**4**	**Shigellosis 100% (4/4)**	**50% (2/4)**	**0% (0/4)**
Immunized Group	VACCINE STRAIN MF 4831	Wild type S. sonnei	4	0% (0/2)	0% (0/4)	100% (4/4)

Table:2. **Challenge with *S. Sonnei* against immunized vaccine strain MF 4831**

10. References:

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 Shigellasonnei* (2008) J BiolChem 283, 5738-5747

2. Mitobe, J., Morita-Ishihara, T., Ishihama, A., and Watanabe, H. Involvement of RNA-binding protein Hfq in the osmotic-response regulation of invE gene expression in Shigellasonnei (2009) BMC Microbiol 9, 110

3. Sereny, B. Experimental keratoconjunctivitisshigellosa. 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), FEMSImmunol Med Microbiol.. 62(3):304-14.

11 Publication list for this work

1) Jiro Mitobe, Hemanta Koley, RitamSinha, Ken Shimuta, Nobou Koizumi, JuTerajima, Makoto Ohnishi, G. Balakrish Nair, Haruo Watanabe.An attempt to develop experimental *Shigella* Vaccine based on virulence gene expression: Abstract presented at 46th Annual joint panel meeting : Cholerae and Other Bacterial Enteric Infectionss, United States- Japan, Cooperative Medical Science Program ..December 13-15,2011, Kolkata

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

Summary:

Our recent 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. Our study also showed that these El Tor variant strains produced higher amount of cholera toxin than prototype El Tor strains. Keeping in mind the unpredictable and ever changing nature of *V. cholerae*, the analysis of the *V. cholerae* O1, El Tor strains with classical *ctxB* isolated from Kolkata that completely replaced seventh pandemic El Tor strains have been initiated in the following study to assess the different biotype determining traits as well epidemic causing prospective.

In this study we found that these *V. cholerae* O1 strains possessed characteristics of El Tor backbone as evidenced from phenotypic tests like polymixinB sensitivity test and Voges Proskauer test and genotypic analysis like possession of *rstC* and *rtxC* genes besides possessing El tor allele of *rstR* and *tcpA*. They also possessed all other essential virulence genetic segments like *rtxA, hapA* and *hlyA*. They also have borne intact VSPI region but truncated VSPII region. These strains also possessed a single copy of CTX prophage in large chromosome with empty site in small chromosome similar to prototype El Tor strain N16961. But unlike N16961 the circulating O1 strains of Kolkata possessed RS1 genetic element only in upstream instead of downstream of CTX prophage The nucleotide sequencing of *tcpPH* promoter region of these strains and subsequent analysis revealed that they had El Tor like *tcpPH* promoter region with identical nucleotide at -66 position as that of El Tor indicating *tcpPH* promoter seemed to have identical regulatory effect on these El Tor variant strains compare to that of prototype El Tor

strains. It is also reported that repeats sequence (TTTTGAT) at the *ctxA* promoter region was correlated with the amount of enterotoxin protein produced by various *V. cholerae* isolates. Analysis of nucleotide sequence of *ctxA* promoter region of O1 strains isolated from kolkata since 1995 showed the presence of four contiguous repeats of the sequence TTTTGAT similar to El Tor strains.

So, in spite of possessing classical type of *ctxB* and showing higher cholera toxin production like classical, O1 strains of Kolkata displayed the regulatory mechanisms similar to El Tor. So, some unknown and yet undiscovered factors seems to be responsible for higher CT production similar to classical strains by O1 strains circulating in Kolkata since 1995.

Purpose:

Genetic analysis of *Vibrio cholerae* O1 strains (1995-2005) harboring classical *ctxB* isolated from Kolkata that completely replaced seventh pandemic El Tor strains

Materials and methods:

Bacterial strains: Fifty five *V. cholerae* O1 strains isolated from Kolkata with classical *ctxB* between 1995 and 2005 were selected for this study. *V. cholerae* O1 strain N16961 of El Tor biotype and O395 of classical biotype have been included in the study for comparison. All the strains were grown in Luria Bertani broth (LB) for 18 hours and then streaked in Luria agar plate. Later all the strains were checked serologically by slide agglutination with O1 polyvalent antiserum for conformation. All the strains identified as *V. cholerae* O1 strains were preserved in 1% nutrient agar stabs.

Biotyping: Biotyping of the selected O1 strains isolated from Kolkata was performed using polymyxin B susceptibility (50 U) test and Voges Proskauer (VP) test.

Preparation of template for PCR: 1 ml of overnight culture from 3 ml LB broth culture was taken in a sterile 1.5 ml microfuge tube and the cells were collected as pellet following centrifugation at 6,000 rpm (Biofuge, Heraeus, Germany) for 5 mins. Then the cell pellet was resuspended in 300 µl of sterile distilled water. The cell suspension was boiled for 10 mins on a boiling water bath. The boiled sample was immediately transferred to ice and kept for 10 mins. Then it was centrifuged at 12,000 rpm for 10 mins. The supernatant was collected in a sterile microfuge and 5µl of this supernatant was used as template for PCR assay.

Confirmation of genetic identity of strains: The biotype of the strains were further confirmed by genetic traits using PCR assays targeting the *tcpA* (classical or El Tor variant) (Keasler *et al.*, 1993) and by the type of *rstR* gene that regulates the replication and integration of the CTXΦ in the *V. cholerae* genome (Davis *et al.*, 1999). These PCRs were performed using the primers and procedures described previously (Nusrin *et al.*, 2004).

Multilocus virulence gene profiling: Of the 55 *V. cholerae* O1 strains, 22 were selected for the multilocus virulence gene profiling that involved examining for *rtxA*, *rtxC*, *cep*, *rstC*, *tlc*, *hlyA* using primer pairs and conditions described in Table 1. Same set of strains were also tested for the prevalence of genes of VSPI regions and genes of VSPII regions using primer pairs and

conditions described previously (O'Shea *et al.*, 2004). PCR was performed in 20 µl reaction mixture as follows: an initial denaturation step at 94 °C for 4 min followed by 30 cycles of denaturation at 94°C for 30s, primer annealing at 45 to 58°C for 30s, 1 to 4 min of primer extension at 72°C and 7 min of final extension at 72°C for one cycle. Amplicons were separated by agarose gel electrophoresis (1%) in 1X Tris-acetate EDTA buffer. The PCR products were stained with ethidium bromide, visualized under UV light and recorded by a gel documentation system (Gel Doc™ 2000, BioRad). The PCR products were sized with standard molecular weight markers and documented.

Determination of position and arrangement of RS1 and CTXΦ: To determine whether RS1 is present upstream of CTXΦ i.e.-whether RS1-CTXΦ array is present we used primer pair rstC1 and rstA3R for amplification. If RS1-CTXΦ array exist in *V. cholerae* O1 strains from Kolkata, an amplified product of ~1.2kb will be obtained. Similarly, to determine whether tandem repeat of CTXΦ is present we used the primer pair ctxB(F) and cep(R). If tandem repeat of CTXΦ exist *V. cholerae* O1 strains from Kolkata, an amplified product of~3.2 kb will be obtained.

Southern hybridization for determining the copy number of CTX prophage: Purified genomic DNA was treated with suitable restriction endonuclease enzymes and separated by electrophoresis in 0.8% agarose gels. DNA fragments were denatured by treatment with alkali and subsequently transferred to a nylon membrane (Hybond-N1; Amersham Pharmacia Biotech), according to the procedure of De et al. (2005), and hybridized with a DNA probe. CTX typing was performed by digesting the genomic DNA with HindIII, PstI, AvaI and BglII (Takara). A 540-bp XbaI–ClaI fragment of *ctxA* was ligated with the EcoRI linker and subsequently the ligated product was cloned into the EcoRI site of pKTN901 that served as a probe for *ctxA* (Kaper et al., 1988). The specific probes of cep (core-encoded pilus) encoding a putative colonization factor present in the core (Pearson et al., 1993), $rstR^{ET}$ and $rstR^{calc}$, which are cloned in the plasmids pSC01, pSC06 and pSC10, respectively, were obtained by digesting the plasmids individually with *EcoR*I (Chatterjee et al., 2007). DNA probes were labelled with chemiluminescent dye Amersham Biosciences) and hybridization reactions were developed following the manufacturer's protocol and recognition patterns recorded on X-ray film.

Chromosomal localization of CTX prophage: Chromosomal localization of CTX prophage of *V. cholerae* O1 strains isolated from Kolkata was determined by PCR using two sets of primers. One set of primers consisting of CIIF and CIIR as described elsewhere was used to determine whether the CTX prophage is located in small chromosome (Maiti *et al.*, 2006). The strains that have CTX prophage integrated between these regions in small chromosome will not yield any amplicon. Another set of primers consisting of *ctxB* (F) (Morita *et al.*, 2008) and rtxA1 (O'Shea *et al.*, 2004) was used to determine whether the CTX prophage is located in large chromosome. The *rtxA* gene has been reported to be present in large chromosome adjacent to *ctx* genes (Lin *et al.*, 1999; Sheahan *et al.*, 2004). The *V. cholerae* O1 strains which does not possesses CTX prophage in small chromosome but possess the same in large chromosome without any RS element downstream of *ctx* genes will yield an amplicon of nearly 2.4 kb. The strains without CTX prophage in large chromosome will not yield any amplicon and the strains possessing CTX

prophage in large chromosome but having RS1 element downstream of *ctx* genes will also yield no amplicon as the expected amplicon size of more than 5 kb will be not achieved in the present PCR conditions implemented. The PCR condition for the second set of PCR reaction are as follows: Initial denaturation at 94°C for 4 mins followed by denaturation at 94°C for 30 sec, primer annealing at 54°C for 30 sec and primer extension at 72°C for 3 mins repeated for 30 cycles, followed by final primer extension at 72°C for 7 min.

Nucleotide sequence of *tcpPH* promoter region: Eight representatives *V. cholerae* O1 strains were selected for nucleotide sequencing at promoter region of *tcpPH* which have differences in nucleotides of at least 16 positions between classical and El Tor biotypes. Among them difference in nucleotide of single base pair at positions -66 and -65 of El Tor and classical *tcpPH* promoter, respectively, is responsible for the differential regulation of virulence gene expression in these two biotypes (Kovacikova and Skorupski, 2000). To determine the nucleotide sequence of *tcpPH* promoter region, PCR amplification of *tcpPH* promoter region were performed in a 25μL reaction mixture in an automated Peltier thermal cycler (PTC-200, M. J. Research) with primer pairs *tcpPH* promoter (F): GAAAGATAATGTAACCAAGTTAAT and *tcpPH* promoter(R): GGATGATATTCACAGAGTATT using PCR conditions as follows: initial denaturation at 94°C for 4 mins followed by denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec and primer extension at 72°C for 1 mins repeated for 30 cycles, followed by final primer extension at 72°C for 7 min. PCR products were purified with Qiaquick PCR purification kit (Qiagen, GmBH, Germany) and sequenced using BigDye Terminator v3.1 kit (Applied Biosystems, USA) on ABI PRISM 3100 automated sequencer.

Results:

Biotyping: All the *V. cholerae* O1 strains tested were found to be resistance to polymixinB and yield positive Voges Proskauer (VP) test (**Figure 1**).

Confirmation of genetic identity of strains: All the *V.cholerae* O1 strains yielded positive amplicons for El Tor allele of *tcpA* and also for $rstR^{ET}$ allele (**Figure 2**).

Multilocus virulence gene profiling: All the strains tested yielded amplicons for *rtxA, rtxC, cep, rstC, tlc, hlyA*. All the strains yielded amplicons with primer pairs for the genes tested for *Vibrio* seventh pandemic island I (VSPI) region, while some primer pairs targetting VSPII failed to yield amplicons and thus there seemed to be truncations in different genetic segments of *Vibrio* seventh pandemic island II (VSPII) regions in different strains that didn't follow any particular trend in terms of time period.

Determination of position and arrangement of RS1 and CTXΦ: All the strains tested yielded amplicons of ~1.2kb with rstC1 and rstA3R primer pairs, while no amplicons were obtained using ctxB(F) and cep(R) primer pairs.

Southern hybridization (detailed data not shown) showed that the O1 strains of Kolkata from 1995 onwards has single copy of the CTX prophage with atleast one RS1 located upstream of CTX prophage. Figure XX showed schematic diagram of the copy number of CTX prophage and arrangement of RS1.

Chromosomal localization of CTX prophage: The entire representative *V. cholerae* O1 strains yielded 766 bp amplicon with primer pairs CIIF and CIIR (**Figure 3**). The same strains also yielded amplicon of ~2.4 kb with ctxB(F) and rtxA1 primer pairs. Thus, the *V. cholerae* O1 strains of Kolkata isolated over the period of 1995 to 2005 have harboured CTX prophage in large chromosome with an empty site in small chromosome. The schematic diagram displaying the chromosomal localization of CTX prophage among *V. cholerae* O1 strains of Kolkata isolated during 1995 and 2005 were presented in **Figure 4**.

Analysis of promoter region of *ctxA*: Nucleotide sequencing of *ctxA* promoter region of 6 representatives *V. cholerae* O1 strains isolated from Kolkata showed the presence of four contiguous repeats of the sequence TTTTGAT **(Figure 5).**

Nucleotide sequence of *tcpPH* promoter region: Sequencing of eight representatives *V. cholerae* O1 strains isolated from Kolkata with classical *ctxB* allele at *tcpPH* promoter region revealed that these *V. cholerae* strains possessed Guanine at nucleotide position -66 like El Tor strains instead of Adenine in nucleotide position -65 as in classical strains **(Figure 6).** The sequence analysis also revealed that *V. cholerae* O1 strains of Kolkata had identical nucleotides to that of El Tor strains at all the other nucleotide positions of *tcpPH* promoter **(Figure 6).**

Discussion:

V. cholerae O1 strains from 1995 onwards were found to carry classical type *ctxB*, totally replacing El Tor type *ctxB* allele (Raychoudhuri *et al.*, 2009). In this study we found that these *V. cholerae* O1 strains possessed characteristics of El Tor backbone as evidenced from phenotypic tests like polymixinB sensitivity test and Voges Proskauer test and genotypic analysis like possession of *rstC* and *rtxC* genes besides possessing El tor allele of *rstR* and *tcpA*. They also possessed all other essential virulence genetic segments like *rtxA, hapA* and *hlyA*. They also have borne intact VSPI region but truncated VSPII region. There was a recent report of possession of VSPII differing from that of prototype seventh pandemic El Tor strain among *V. cholerae* O1 strains in Peru (Nusrin *et al.*, 2009). The proper reason as well as functional aspect of such changes occurring among *V. cholerae* O1 strains has been not ascertained yet and can be explored in the future studies. The constant mingling of classical strains with the El Tor strains in the environment belonging to the same niche might have given the *V. cholerae* strains the opportunity for the inter-exchange of the beneficiary genes among themselves. Perhaps the new type of *V. cholerae* strains have appeared from *V. cholerae* strains having seventh pandemic El Tor backgrounds or from some pre-seventh pandemic strain with the replacement of their *ctxB* gene driven by the selective pressure to survive and adopt better in host intestine. The nucleotide sequencing of *tcpPH* promoter region of these strains and subsequent analysis revealed that they had El Tor like *tcpPH* promoter region with identical nucleotide at -66 position as that of El Tor (as shown in fig 6) indicating *tcpPH* promoter seemed to have identical regulatory effect on these El Tor variant strains compare to that of prototype El Tor strains. These strains also possessed a single copy of CTX prophage in large chromosome with empty site in small chromosome similar to prototype El Tor strain N16961. But unlike N16961 the circulating O1

strains of Kolkata possessed RS1 genetic element only in upstream instead of downstream of CTX prophage (as shown in fig 4). It has been shown earlier that repeats of the sequence TTTTGAT was correlated with the amount of enterotoxin protein produced by various *V. cholerae* isolates (Mekalanos et al., 1983). Classical O1 strain have seven of such repeat sequence, while El tor have four of them in their respective *ctxA* promoter region that correlates to higher cholera toxin production by classical strains. Analysis of nucleotide sequence of *ctxA* promoter region of O1 strains isolated from kolkata since 1995 showed the presence of four contiguous repeats of the sequence TTTTGAT similar to El Tor strains.

So, in spite of possessing classical type of *ctxB* (Raychoudhuri et al., 2009) and showing higher cholera toxin production like classical (Ghosh-Banerjee et al., 2010), O1 strains of Kolkata displayed the regulatory mechanisms similar to El Tor. So, some unknown and yet undiscovered factors seems to be responsible for higher CT production similar to classical strains by O1 strains circulating in Kolkata since 1995. Considering the increase in the global burden of cholera (WHO, 2011), and recent reports of isolation of *V. cholerae* O1 with El Tor phenotypes but with classical cholera toxin from various parts of the world, systematic cholera surveillance should be in place in all cholera endemic regions of the world.

Studies in progress:

We are presently perusing for expression study along with some animal model assay to make this work into a publication format.