Project Report (2015)

1. Title of Project

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

2. Name of investigators

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4. 4.Summary:

Enteric pathogens are also associated with growth faltering, which can lead to impaired cognitive development as well as other long-term health problems. Enteric and diarrheal diseases kill about 550,000 children under age 5 each year, primarily in the developing world. Among them *Shigella* infection is a major cause of infant morbidity and mortality in developed as well as developing countries. Global,regional and national estimates clearly place diarrhoealdiseases as a major threat, although this public health problem has been neglected. Child mortality (<5 years of age) owing to diarrhoea was estimated to be 1.87 million at the global level, which is approximately 19% of all child deaths. Population-based surveillance studies in developing countries showed that Shigella flexneri2a and Shigella dysenteriae are predominant causes of infection, but Chile and Thailand showed a notable proportion of cases due to Shigella sonnei. 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 passive protective efficacy of live attenuated shigella in suckling mice 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. Moreover, this vaccine showed above 80% protective efficacy against *Shigella flexenari*

6. Only 75% suckling mice were protected against *Shigella flexenari* 3a ,*Shigella boydii type* 4 and Enteroinvasive *E.coli*. The above results suggested a satisfactory 80-75 % protective efficacies induced by vaccine strain immunization against different Shigella strains studied here.

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 study invasion ability and virulency of vaccine strain *Shigella flexenari 2a* Δ*hfq (*MF 4853) in guinea pig cecal loop model.
b) To understand passive protective efficacy of live genetically manipulated Shigella vaccine strain against heterologous wild type S*higella* strains and Enteroinvasive *E.coli* in suckling mice model.
c) To study the duration of protection offered by live genetically manipulated Shigella vaccine strain.

6. Methods:

A) Bacterial strains and culture condition:

Shigella flexenari 2a Δ*hfq (*MF 4853) were used for oral immunization. For the protective efficacy study, we challenged with outbreak strain *Shigella flexenari* 6, *Shigella flexenari* 3a, *Shigella boydii type* 4 and Enteroinvasive *E. coli.* All wild type strains were routinely grown in LB lenox and *Shigella flexenari* Δ*hfq* MF4853 was grown in LB lenox with chloramphenical (12.5µg/ml) at 37◦C. and all those strains stocked under 15% glycerol at -80^{O} C.

B) Characterization of invasion ability and virulency of Vaccine strain *Shigella flexenari 2a* Δ*hfq (*MF 4853) in guinea pig cecal loop model:

i) Animal

Two-month old English colored guinea pigs of either sex, weighing 250–300 g, were used. All guinea pigs were collected from the animal resource department, National Institute of Cholera and Enteric Diseases, Kolkata. All animals were confirmed serologically negative for *Shigella* antibodies by ELISA and gave *Shigella* species negative fecal cultures. Study animals were housed in standard individually ventilated caging systems with individual drop pans and maintained at

24◦C with 75% humidity. Approval of the institutional animal ethical committee was taken before the animal experiments were performed.

ii) Guinea pig cecal loop model:

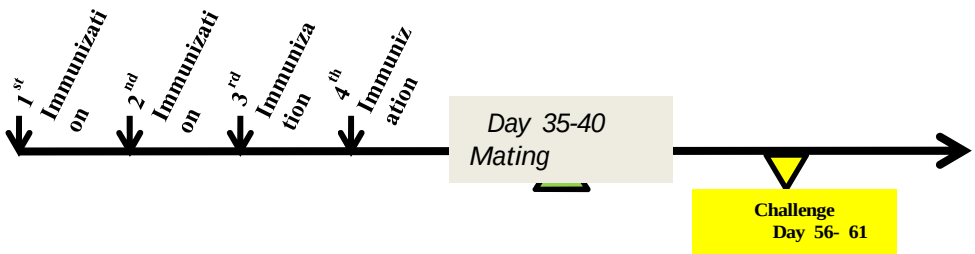
Guinea pig cecal loop studies were performed with healthy pathogen free male guinea pigs. Guinea pig were subjected to fasting for 24 h prior to surgery and were provided glucose (2%) water. Aseptic surgical technique was employed under anesthesia, induced with a mixture of ketamine (100 mg) and xylazine (16 mg)), administered intramuscularly. Each loop in the distal colon was about 5 cm in length and tied at each end, with a 1-cm spacing between two loops. A 1-ml inoculum (10^8 CFU/ ml) of cells, grown in TSB, was injected into the lumen of each loop. Guinea pig were killed after 6h and 18 h.Infected loops were then opened and washed with PBS containing gentamycin for killing of extracellular bacteria. Then tissue were homogenized. Then homogenized soup were serially diluted and plated on HEA for enumerate number of invasive bacteria. Some portions were fixed in 10% formalin. Samples were processed by standard procedures and 3^4 Wm thick sections were cut by a Leica rotary microtome 2145 and stained with hematoxylin-eosin stain and examined under a Leica compound trinocular microscope (DMLB). Photographs were taken with an MPS 60 camera.

B) Passive Protection Study in Suckling Mice:

i) Oral Immunization:

Oral immunization Seven-week-old female mice were immunized orally at days 0, 7, 14 and 21 with 1×108 cfu/ml vaccine strain in 200 µL of PBS. Fifteen minutes before the oral immunization, each mouse was anaesthetized by intramuscular injection of a mixture of ketamine (35 mg kg1 body weight; Sterfil Laboratories Pvt. Ltd, India) and xylazine (5 mg kg1 body weight, AstraZeneca Pharma India Ltd, India). Two boluses of sodium bicarbonate (500 µL of a 5% solution; SRL, India) at 5-min intervals were introduced directly into the stomach through a mouse feeding needle (Harvard Apparatus). The second bolus was immediately followed by oral administration of vaccine strain for the experimental mice and the same volume of PBS for the non-immunized group. All immunized and nonimmunized group of mice were returned to their cages and given limited amounts of sterile food and water.

Immunization Schedule



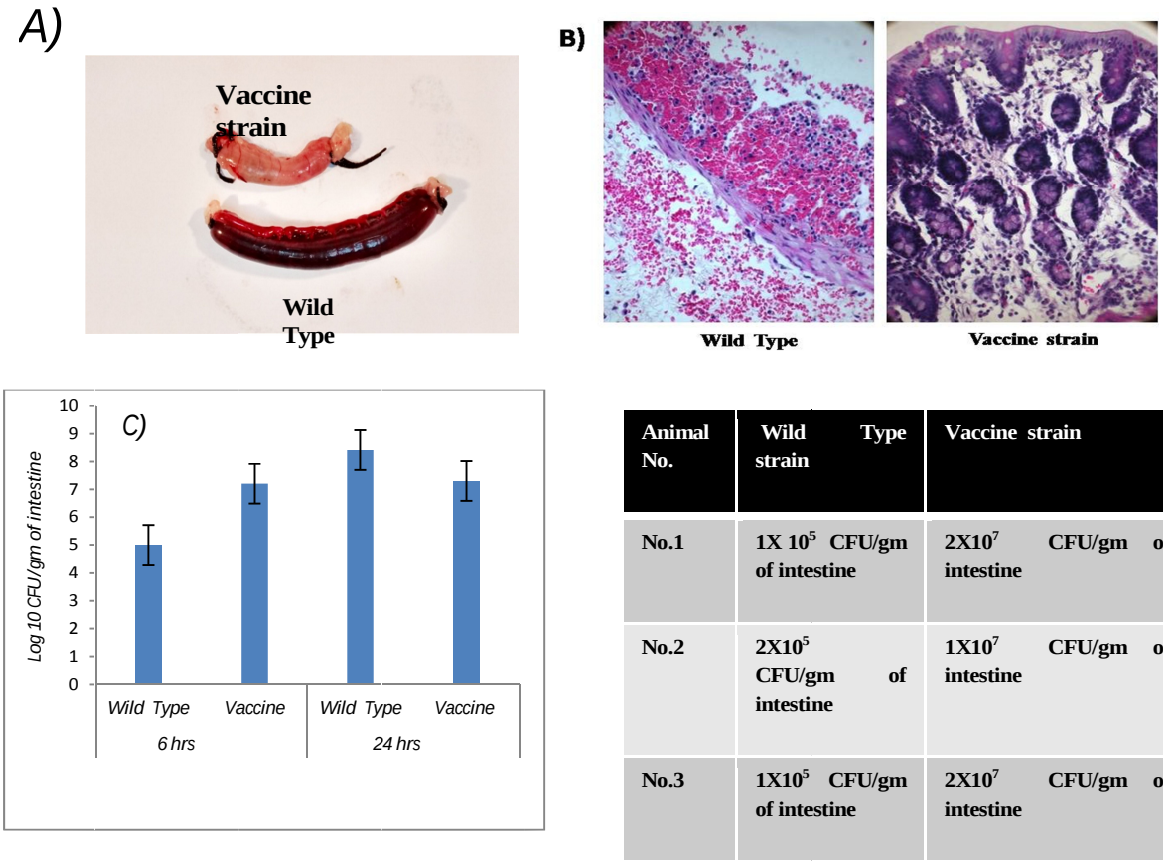
ii) Protective efficacy study:

Protective efficacy study Adult mice were immunized and their pups were challenged with live strains as described by Schild et al.. Briefly, immunized and non-immunized groups of female mice were mated with age-matched males at a 2 : 1 ratio from day 35 to 40 (after first immunization). Neonatal mice 3–4 days old (Fernandez et al., 2003), of both the immunized and non-immunized group of dams, were challenged with an inoculum size that was 50-fold higher than the calculated ID50 of each strain. After oral inoculation, the neonatal mice were monitored for next 18 h. Animals were sacrificed and their intestines were removed by dissection, weighed and mechanically homogenized in 1 mL PBS (pH 7.4). Aliquots of 100 lL of appropriate dilutions of the homogenate in PBS were plated on TSA plates with appropriate antibiotic, in duplicate and incubated overnight at 37 °C. Next day, the colonies on the plates were counted and the CFU /gm of intestine of each mouse calculated.

7. Result:

i) Guinea pig cecal loop model:

Using guinea pig cecal loop model we determine invasion ability of vaccine strain. We quantified colony forming units (cfu) using a gentamicin protection assay of infected loops. Our result showed that vaccine strain were more invasive than wild type strain at initial infection stage (6hrs). But invasion ability of vaccine strain were decreased after 24 hrs than wild type strain. The loops were opened longitudinally for examination of the intestinal mucosa. Grossly the loops treated with *S. flexenari* 2a 2457T strain were diluted and filled with hemorrhagic thick fluid whereas the loops with the vaccine strain showed minimum fluid accumulation like the control loops treated with phosphate-buffered saline(Fig. 1,A).. Sections from tissue infected with *S. flexenari* 2a 2457T showed a complete loss of villous architecture with denudation of surface epithelium in places. Massive hemorrhage with neutrophilic infiltration in the lamina propria extending up to the submucosa with congested and dilated crypts was seen (Fig. 1,B). Sections from tissues treated with the vaccine strain did not reveal such changes and maintained normal villous contour.



Animal No.	Wild Type strain	Vaccine strain
No.1	1X 10^5 CFU/gm of intestine	2X10^7 CFU/gm of intestine
No.2	2X10^5 CFU/gm of intestine	1X10^7 CFU/gm of intestine
No.3	1X10^5 CFU/gm of intestine	2X10^7 CFU/gm of intestine

Fig1: Guinea pig cecal loop model: Wild type *Shigella flexenari 2a,* 2467T and vaccine strain, MF4853 (1X10^9 cell/ml) were injected into cecal loop, after 6 hrs of inoculation, we measured number of invasive bacteria in cecal portion by gentamicin treatment expressed in log of CFU/gm of intestine . A) Pictorial picture showed nature of fluid accumulation with haemarage among two strains. B) Nature of histopathology of infected cecal portion. C) and D) Invasive potential among wild and vaccine strains.

Passive protection study:

Protection against colonization of Shigella To determine whether immunization with OMVs was protective against shigellosis, we used a modified version of the infant mouse model wherein the offspring of immunized dams were challenged and the level of protection, i.e. protective efficacy, was calculated on the basis of the death rate of neonatal mice, obtained from both immunized and nonimmunized group, following the formula {[(percent deaths of controls) (percent deaths of immunized mice)]/[percent deaths of controls]} 9 100. The degree of colonization of Shigella sp. in the intestine of suckling mice was also measured (Fig. 4). Offspring 3–4 days old, from both immunized and nonimmunized groups were challenged with a dose that was 50-fold higher than the infectious dose (Table 1) and were monitored for next 18 h. Most of the suckling mice from nonimmunized mothers became sick and died between 10 and 16 h of incubation due to the bacterial load in their intestine. Neonates from immunized groups, both dead and alive, showed a lowerdegree of intestinal colonization by three virulent Shigella strains and one Enteroinvasive *E. coli* strain(Fig. 2). Moreover, this vaccine showed above 80% protective efficacy against *Shigella flexenari* 6.

Only 75% suckling mice were protected against *Shigella flexenari* 3a ,*Shigella boydii type* 4 and Enteroinvasive *E.coli*. The above results suggested 80-15 % protective efficacies induced by vaccine strain immunization against different Shigella strains studied here.

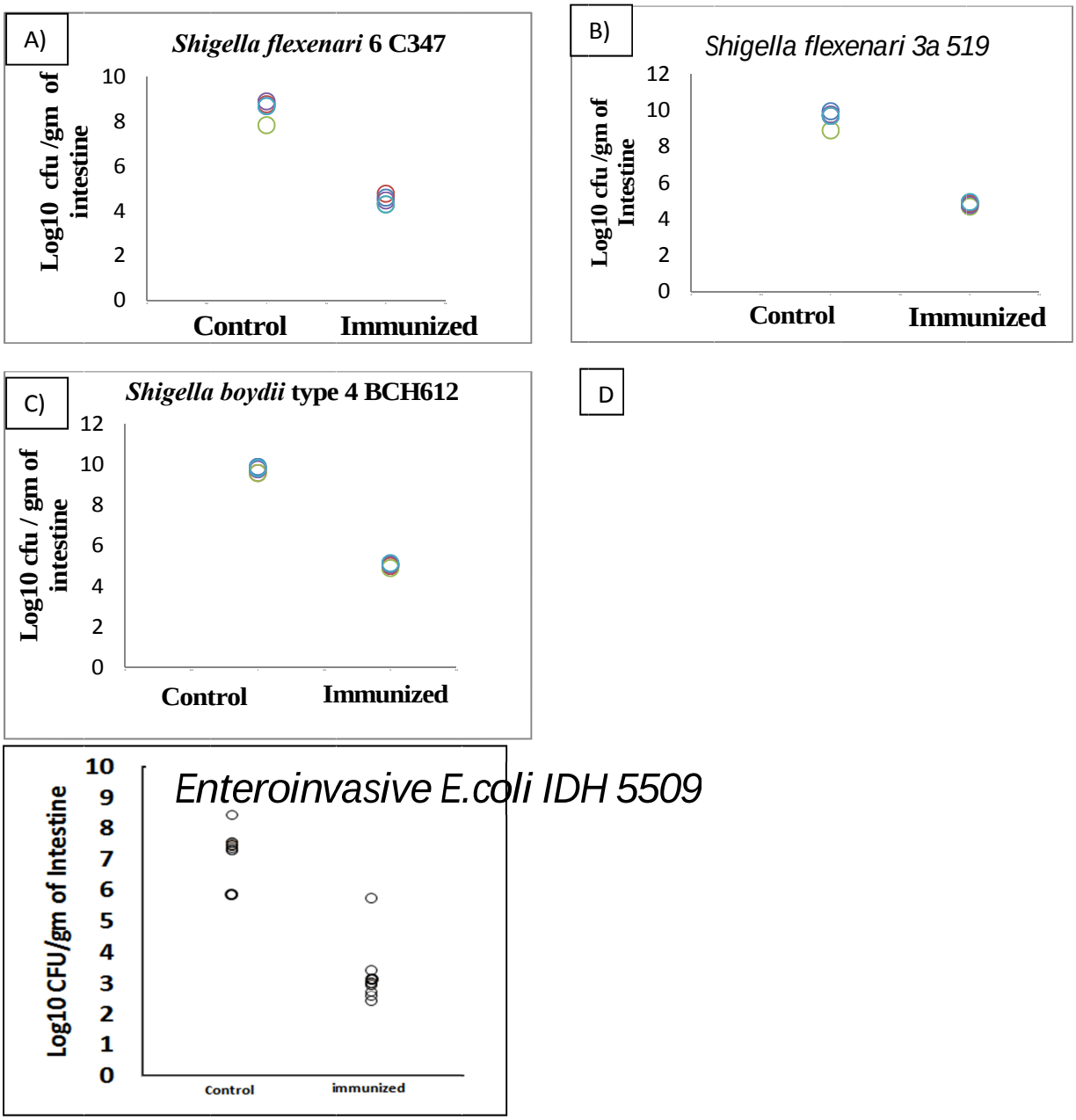


Fig 2: Comparative data of protective efficacies between control and immunized neonates, after challenging with wild type Shigella strains : Each circle represents the colonization data obtained from a single suckling mouse. Data were expressed as Log10 of recovered colony forming unit (CFU)/gm of intestine of each mouse, Pups were challenged according to the challenge dose ~10^9.

Table No.1:

Challenge strain	Challenge dose	Experimental group	Experimental neonatal	% of survival after first challenge	Protective efficacy (%) [a]
Shigella flexenari 6	2±1×10^9/ml	Control	5	0(0/5)	80
		Immunized	5	80(4/5)	
Shigella flexenari 3a	3±1×10^9/ml	Control	5	20 (1/5)	75
		Immunized	5	80 (4/5)	
Shigella boydii type 4	2±1×10^9/ml	Control	5	20 (1/5)	75
		Immunized	5	80 (4/5)	
EIEC	2±1×10^9 /ml	Control	5	20 (1/5)	75
		Immunized	5	80(4/5)	

Protective efficacy was calculated as {[(percent deaths of non-immunized mice) − (percent deaths of immunized mice)]/ [percent deaths of non-immunized mice]} ×100

8. DISCUSSION:

Knowledge of the process involved in the invasion of *S. flexenari* into the gastrointestinal tract is the major theme to understand the pathogenesis of infections. The key steps in the invasion of *Shigella flexenari 2a,* 2467T are epithelium proliferation, invasion and movement to the tissues. Interestingly our vaccine strain were more invasive than wild type strain at initial infection stage but fluid accumulation and inflammation were less than wild type strain. From these study vaccine strain were safe and non reactogenic.

In another study, we have been able to demonstrate that our vaccine strain exhibited protective passive immunity in immunized dams against different heterologous wild type strains A. After four successive oral immunizations, most of the suckling mice from non-immunized mother became sick and eventually died between 10 and 16h of incubation due to higher rate of intestinal colonization ($\sim 10^{7}$ CFU/gm of intestine) by the challenge strains. Immunized groups showed $\sim 10^{2}$ CFU/gm of intestine. There were few dead mice in immunized groups that yielded $\sim 10^{5}$ CFU/gm of intestine. Undoubtedly, our vaccine strain has the potential to become an ideal non-living vaccine candidate against human shigellosis.

9. References:

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