A story of *Shigella* vaccine development in ICMR-NICED involving multidimensional approaches

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Abstract

Enteric bacterial infection causes diarrhea throughout the world, especially in developing countries. *Shigella* is solely answerable to almost 1.1 million deaths annually in the pediatric population. Vaccine development against diarrheal diseases is always an encouraged concern. Our laboratory, dedicated to find a possible therapy against shigellosis, is working on a path of various potential methodologies and immunogens. Over the years, we have concentrated and reported different immunogens with their advantages and drawbacks, ultimately leading us to find the best possible vaccine candidate against bloody diarrhea.

The venture started with live attenuated vaccines that protect against multiple serotypes and subtypes of pathogens and found limited host-serotype specific immune responses. It was observed that introducing a lipopolysaccharide biosynthesis gene pPR 1347 in *Shigella dysenteriae* type 1, transformed it into an avirulent organism for candidate vaccine.

A mutant strain of *Shigella flexneri* 2a lacking the RNA-binding protein Hfq was made, leading to increased expression of the type III secretion system via loss of regulation, resulting in attenuation of cell viability through repression of stress response sigma factors. Such increased antigen production and simultaneous attenuation were expected to elicit protective immunity against homologous and a limited number of heterologous serotypes subtypes.

Although we formulated the live attenuated vaccine through the introduction of a lipopolysaccharide gene and a mutant lacking RNA binding protein Hfq, but due to lack of heterologous protective efficacy, these were not an ideal vaccine candidate to be made available in the market although they showed a significant amount of immunogenicity. Moreover, live attenuated strains always have a possibility to revert back to its virulent form.

Subsequently, monovalent and hexavalent heat-killed immunogens with single and six *Shigella* serotypes have shown significant protective efficacy in mice, and rabbit models. Recently we have shown the homologous as well some extent of heterologus protective efficacy of heat killed multi-serotype *Shigella* (HKMS) immunogens in a guinea pig colitis model.

A novel formulation for improved immunogen delivery system comprises substantially effective amounts of alginate chitosan nanoparticles with OmpA protein of *Shigella* species. Alginate chitosan nano formulations of OmpA consists essentially of OmpA protein as conserved active molecule, but efficacy study reveals partial protection efficacy against present circulating *Shigella*. Further improving the delivery system, we have also formulated a subunit-based vaccine by nanoformulation of ipaC protein of *Shigella*. The main drawback of OmpA and ipaC subunit based vaccines are they cannot provide a broad spectrum protection against 50 subtypes and serotypes of *Shigella,* although they act as a conserved protein in Enterobacteriaceae family, indicating single epitope cannot be the sole factor associated with the operational protective efficacy.

Eventually, our research moved a step ahead and found next-generation outer membrane vesicles (OMVs) based antigens from *Shigella*. Disruption of tolA, one of the genes of the Tol–Pal system of *Shigella* membrane, has increased the OMVs release rate by approximately 80% higher. Recently we have reported only four serotype-subtype cross-protection among 50 subtypes of circulating *Shigella* in mice models. Outer membrane vesicles based immunogen could be a potential cost-effective non-living, next-generation candidate vaccine against shigellosis for humans.

Keywords: *Shigella*, Animal Model, Immunogenicity, Protective efficacy, Vaccine

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Introduction

Children under the age of five and immunosuppressed people are at high risk of developing bacillary dysentery. The situation worsens in low- and medium-income countries (LMICs) due to poor sanitary measures and unhygienic practices.[1,2] Additionally, shigellosis has become prominent in developed countries and among travelers traveling to endemic regions.[3]

After transmission by the feco-oral route, *Shigella* enters through the micro fold cells of the colonic epithelial layer and is readily engulfed by the macrophages in gut-associated lymphoid tissue (GALT). *Shigella* induces macrophage pyroptosis, causing the release of inflammatory cytokines, which recruit polymorphonuclear neutrophils (PMN) in the infection area. This ultimately leads to damage of the epithelial lining and the dispersal of the colonized *Shigella*.[4] Though the primary symptom triggered by *Shigella* is watery stool with mucus and bloody stool, they are also accountable for Moderate to Severe Diarrhea (MSD) and other clinical complications such as fever, prolonged malnutrition, malaise, tenesmus, muscle cramp etc. Conventional therapy like oral rehydration not possible due to *Shigella* is an invasive organism The only available is antibiotic therapy, but it is now

troubled due to the global emergence of multi-drug resistant *Shigella* strains.[5] A prophylactic vaccine approach has become an ideal strategy to counter this issue. Several vaccine candidates are in different clinical and preclinical stages. These candidates range from conventional inactivated and/or killed whole bacterial immunogen to new-generation subunit candidates.[6] However, at present no licensed *Shigella* vaccine is available for public health use. outer membrane vesicles (OMVs) are nanoparticles primarily secreted from gram-negative bacteria. OMVs are being widely investigated as potent acellular vaccine candidates.[7] OMVs resemble the composition of bacterial outer membranes and some of their periplasmic contents, including membrane proteins, lipopolysaccharides, etc.[8] The presence of these innate pathogen-associated molecular patterns (PAMPs) can induce both host innate and adaptive immune responses, resulting in effective protection against their respective diseases.[9] An OMVs based vaccine MeNZB is already licensed for public use in several countries against meningitides serogroup B. MeNZB has proven to be a safe, cost-effective vaccine with more than 70% protective efficacy against meningococcal infection.[10] Another extracellular vesicle-based *Shigella* generalized modules for membrane antigens (GMMA) vaccine candidate, which successfully entered the clinical trial phase, showed high immunogenicity and protective efficacy against shigellosis.[11] Our laboratory has reported multiple immunogens along with monovalent and hexavalent *Shigella* OMVs to elicit mucosal immunity, conferring protection in different animal models.[12,13] However, based on the current epidemiological status provided by global enteric multicenter study (GEMS), most clinical isolates are *S. flexneri* and *S. sonnei*.[14] O-antigen moieties present in *S. flexneri 2a, S. flexneri 3a* and *S. flexneri 6* altogether cover up the entire serogroup of *S. flexneri*.[14] The negligible presence of *S. dysenteriae* and *S. boydii* clinically argue that these species do not require vaccines.[15] Consequently, a recipe with three *flexneri* and one *sonnei* strain with a broad-coverage should be adequate to provide protection against the vast majority of shigellosis.

Finally, we have developed an oral tetravalent *Shigella* OMVs immunogen and assessed the mucosal and systemic immune responses induced by it. Protective efficacy was then investigated in a non-surgical intra-peritoneal mouse challenge model. This study aims to provide preliminary data pointing to the possibility of the immunogen being a reliable and potent vaccine candidate conferring protection against major circulating serotypes of *Shigella*. We may have a number of different potential immunogens but due to the absence of a proper animal model to study the immunogenicity and protective efficacy of these vaccine candidates, to study of the efficacy of the vaccine, we have developed animal model based on host specificity and organ specificity.

Development of a new guinea-pig model to study shigellosis and Vaccine Efficacy Studies

Naturally, healthy rodents never show diarrhea. The stomach's highly acidic environment and rapid fluid absorption are the expected physiological barriers for diarrheal pathogens. To overcome these problems animal models could be developed. This study aimed to establish a new animal model for bacillary dysentery. Guinea pig is a host specific for invasive organisms especially *Shigella*. Scientists are using the guinea-pigs model kerotocongitivities test for *Shigella* virulence assay. A drawback to this model is keratoconjitivitis assay is not an intestinal assay model. As *Shigella* is an enteric organism, bacterial invasion of the colonic mucosa is associated with an intense inflammatory reaction, and experiments performed in rabbit ileal loops indicate that the inhibition of inflammation through the perfusion of IL-1 Ra, an antagonist of IL-1 receptor-mediated signaling, prevents not only tissue destruction but also bacterial dissemination in the large intestinal mucosa.[16] The direct luminal inoculation of virulent *S. dysenteriae type* 1 induced acute bacillary dysentery accompanied by loss of body weight, fever, elevated rectal temperature, severe damage to the colonic mucosa, mucus and occasional blood in stools. The isolation of the challenge organisms from colonic contents also reconfirmed colonization in colonic mucosa by Shigella. This model does not require any pretreatment of the animals, including starvation and gut treatment with antibiotics before the challenged colonization assay. Various *Shigella* vaccines have been developed and tested by several groups.[17] Controlled Human Infection Model (CHIM) studies to test the efficacy of *Shigella* vaccines are becoming harder to perform and testing of primates (the only animal model that mimics human shigellosis) has serious regulatory, ethical variability and cost constraints. Considering these difficulties, developing a small animal model is necessary to allow reliable protective efficacy and immunogenicity of potential vaccine strains.[18] This study evaluated the protective efficacy of orally administered heat-killed *S. dysenteriae* 1 (NT4907) and *S. flexneri 2a* (B294) against luminal inoculation with *Shigella*e of identical virulence features. We found that oral immunization

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following challenge with these *Shigella*e conferred more than 80% protective immunity. Thus, this simplified animal model would be useful for assessing shigellosis as well as the protective efficacy of *Shigella* vaccine candidates. The success of colonic infection in guinea pigs depends on several factors such as the route of inoculation of the bacteria. The direct inoculation of the organisms into the ceco-colic junction is more likely to yield successful colonization than the upper small intestine, which requires the organisms to survive and go down the entire length of the small bowel against a host of enteric defense mechanisms. In addition, motility in the colon is lower as compared with poor immunogenicity in humans. In this respect, the killed vaccines are getting importance and gaining confidence.[19]

The efficacy study showed complete protection against wild-type *S. dysenteriae* 1 (NT4907) and *S. flexneri 2a* (B294) after four doses of oral immunization with heat-killed *Shigella*e. Significantly higher levels of lipopolysaccharide specific IgG and IgA antibodies were detected in both serum and mucosal secretions of immunized guinea pigs. During oral immunization, an exponential increase of serum IgG was also observed. Serum IgG antibodies may confer the protective immunity to Shigellae to the O-specific polysaccharide of their lipopolysaccharide.[20] Although bacterial colonization was detected in the distal colon of immunized animals, their levels were far lower when compared with the control group. Histopathological features of the distal colon also revealed protection against homologous virulent live *Shigella*. Over the years, several approaches have been explored using mice, guinea pigs, rabbits, macaques and piglets as a suitable animal model for shigellosis. The mice model of pulmonary pneumonia with the intranasal inoculation of *Shigella*[19] was used to determine the virulence attenuation, immunization efficacy and protection against infection. However, this model lacked clinical relevance with respect to the infection site of the pathogen. Experimentally demonstrated a murine infection model with newborn mice in which inflammatory destruction of the mucosa and substantial infiltration of

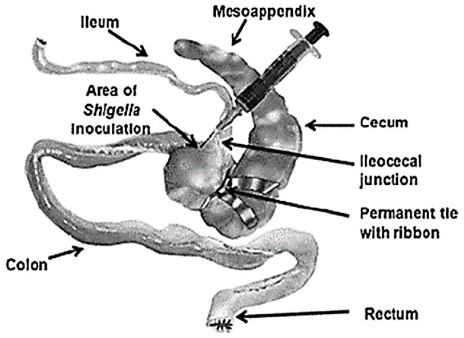

Figure 1 : Surgical sketch of guinea-pig colon for the experimental shigellosis model. This model requires the placement of a surgical tie above the ileocecal junction, followed by the inoculation of *Shigella* into the lumen of colon. A permanent cecal tie was placed 4 cm apart from the ileocecal junction so that the ligature completely obstructed the cecal lumen above the ileocecal junction while maintaining the ileo–ceco–colic communication.

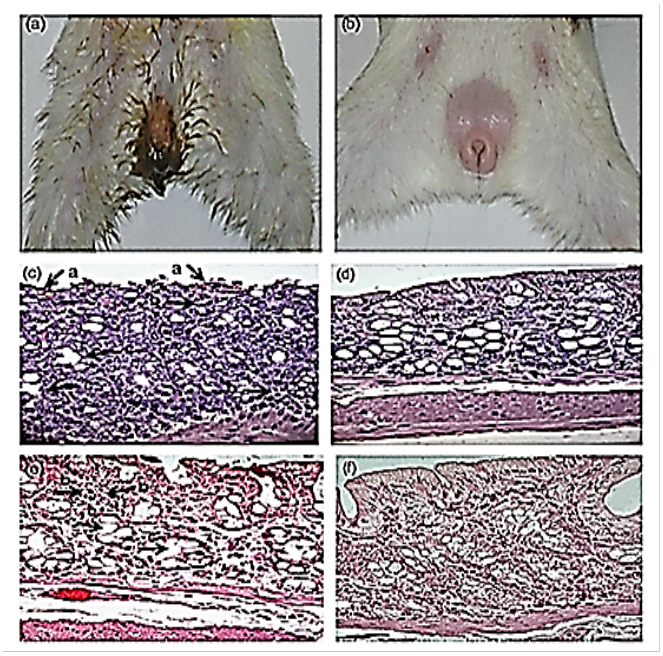

Figure 2 : Guinea-pigs infected with the invasive *Shigella dysenteriae* 1 (NT4907) and *Shigella flexneri* 2a (B294) strain developed bacillary dysentery characterized by weight loss, liquid stool mixed with mucus and blood. The perianal regions of the guinea-pigs that had developed dysentery remained constantly wet and soiled with feces (a). Noninvasive *S. dysenteriae* 1 (D1-vp) and *S. flexneri* 2a (SB11-vp) strains were used as a negative control (b). Each picture is taken at 24 h of postluminal inoculation. Histological features of the large intestine of the guinea-pig following luminal challenge with invasive *S. dysenteriae* 1 (NT4907) (c), invasive *S. flexneri* 2a (B294) (e), noninvasive *S. dysenteriae* 1 (D1-vp) (d) and noninvasive *S. flexneri* 2a (SB11-vp) (f). Samples of the distal colon were taken at 48 h after inoculation. Colonic mucosa after 48 h of infection with virulent *S. dysenteriae* 1 (c) and *S. flexneri* 2a (e) showing disruptedsurface epithelium with hemorrhage (arrow a),inflammatory cell infiltration in the mucosa (arrow b) and edematous mucosa with a dilated crypt lumen (arrow c) (magnification, The tissues of guinea-pigs challenged with noninvasive *Shigella*e (d, f) were within the normal limit as there was no characteristic damage and inflammatory changes in the colonic mucosa (magnification, 40X).

polymorphonuclear neutrophils into the gut were observed.[20] Because of the narrow window of time (3–4 days after birth), this model was not applicable for the evaluation of protective immunity. The keratoconjunctivitis assay is regarded as the gold standard for protective immunity in guinea pigs,[20-22] but its limitation lies in difficulty in the quantification of an inflammatory response and the irrelevance of the target organ. Guinea pigs that were administered wild-type *S. flexneri 2a* and treated with opium post 4 days of starvation developed fatal enteric infections.[23]

Because of the fatal effects at a relatively early stage of infection, this model was not ideal for the purpose of screening vaccine candidates. Although the rabbit shigellosis model was sensitive,[21] its suitability for measuring the protection is not known. Rhesus monkeys are the only animals in which typical bacillary dysentery can be induced by oral infection with *Shigella*e without starvation and/or pretreatment with antibiotics.[21] However, the use of

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this animal is a major constraint due to many reasons. A new guinea pig model has been described that represents typical bacillary dysentery and acute rectocolitis after rectal inoculation (Shim et al., 2007). In this model, the catheter does not reach the proximal colon, which is the specific site of *Shigella* colonization. In addition, the backflow of inoculum cannot be prevented while removing the catheter. Considering the difficulties in the several animal models and methods, luminal inoculation in guinea pigs is more reliable as this model allows *Shigella* to be retained in the proximal colon. Recently, successfully developed a model of intragastric infection in 1 to 3 day old piglets that induced symptoms and characteristic gut lesions similar to those of humans. The need for specialized isolators, environmentally controlled accommodation, competent animal handlers and labor-intensive systems are some of the issues that make this model unfavorable. The guinea-pig luminal model described in this study is ideal for studying bacillary dysentery in vivo as it covers several features such as the appropriate infection site, immune responsiveness and protective immunity. Thus, this model is ideal for the generation of preclinical information of *Shigella* vaccines before human volunteer studies. This model cannot entirely replace primate or human studies, but it can be used to generate preclinical information that should significantly reduce the number of studies in primates as well as in humans.[22]

An Experimental Adult Zebrafish Model for *Shigella* Pathogenesis, Transmission, and Vaccine Efficacy Studies

Water is an essential factor for the existence of life. Inadequate sanitary systems and unhygienic water usage lead to various waterborne acute microbial diarrheal diseases.[16,17] Although its transmission via solid food and other environmental means has been shown in the literature, the mode of spread in natural water bodies has not yet been evaluated in detail. *Shigella* is also one of the 12 priority pathogens listed by the WHO that requires urgent action.[23] We have developed an experimental adult zebrafish model to replicate the aquatic environment. Using this model, we further assessed the protective efficacy of a trivalent Shigella heat-killed immunogen. The potential of this model to be used to assess therapeutics was also evaluated. None of the animal models for *Shigella* pathogenesis[9] can assess the ecological spread of the organism.

Some of the models are specific for a single *Shigella* sp., and infection from other *Shigella* spp. cannot be assessed using that model. Moreover, trained personnel are a prerequisite to execute the models effectively. For instance, the surgical rabbit model for Sf2a needs the pathogen to be administered with precision into the colon following a cecal bypass. This model is effective but unsuitable to use as a natural host model.[24] The piglet model for *S. dysenteriae* 1 (Sd1) is a gnotobiotic animal model and, hence, is not a natural model of infection.[4] Due to animal ethical considerations, the rhesus monkey model for Sd1 is expensive and troublesome to execute.[5] Guinea pig models of *Shigella* also have limitations that keep them from being usable as natural infection models.[8,9] The suckling mouse model for Sf5a is another artificial model in an animal that lacks a mature immune response and significant intestinal microbiota.[6] The intraperitoneal mouse infection model is being heavily used presently due to its reproducibility and ease of handling.[7] However, it is not a natural infection model but an induced one. Last, an NAIP-NLRC4 oral infection model for *Shigella* has recently been described.[23] This model can be used in transmission experiments as a fecal-oral transmission model. It is evident from the literature that an animal model is needed to study *Shigella* transmission in the environment. Reduction in transmission would reduce the spread of the disease. Zebrafish are an excellent model organism due to their ease of maintenance, reproducibility of data, and anatomical and immunological aspects that are generally similar to human ones.[12] Zebrafish larvae have been used as a model for bacterial phagocytosis and autophagy due to their optical transparency, which helps in non-invasive real-time in vivo imaging techniques.[25] Although several studies have been carried out using zebrafish larvae, in the context of *Shigella* Testing a probiotic/commensal mixture as a potential therapeutic in zebrafish.[26]

Furthermore, mature intestinal microbiota and an adaptive immune system are absent in the larvae, limiting their use. Since adult fish are susceptible to Enterobacteriaceae (both *Shigella* and Salmonella),[26] it is worth trying to develop a model to study the pathogenesis and spread of these organisms. Spread of these diseases will naturally be high in low-income countries with less-developed sanitary systems and unhygienic use of water.

Dosage greater than 108 CFU/mL killed the adult fish within a short time. In a time course analysis, the infection ensued at 2 hpi, except for Sf2a, and it later reached 106 CFU/fish intestine for all *Shigella* strains tested. The observed difference in infection between *Shigella* species might result from variability in the infection's kinetics. Intriguing findings were noted while comparing the infection in fish and excretion into water. In almost all cases, the levels measured in water were found to be inversely proportional to the levels in the fish gut. This is notably different from the *Vibrio cholerae* adult zebrafish model, in which excreted bacteria in water paralleled the levels in fish intestine.[27]It is worth noting the infection curve of SsI, where the bacterial load rose over time, which was opposite for other *Shigella* species. Shigella strains were also inconsistent in colonization experiments at 24 h. Sf2a and SsI were found to be in the range of 6 to 7 log10, whereas Sb4 was only around 5 log10. This inconsistency might have been a result of fish physiology. Since they were kept at 37°C, which is not the optimum physiological temperature, the fish might have reacted differently to different *Shigella* strains, but the reasons remain unclear at present. For Sf2a, infection at 4 dpi showed

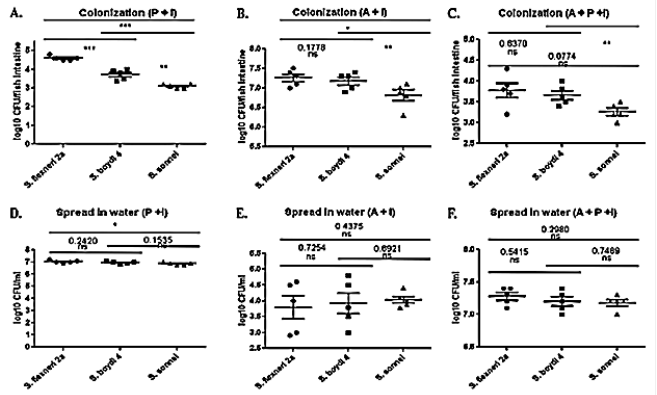

Figure 3: Testing a probiotic/commensal mixture as a potential therapeutic in zebrafish. The fish were (A) treated with probiotic/commensal mixture and then infected with *Shigella* sp. challenge strains (P+I), (B) treated with an antibiotic cocktail to damage gut microbiota before infection with *Shigella* sp. (A+I), or (C) treated with antibiotics, then treated with probiotic/commensal mixture, and then infected with *Shigella* sp. (A+P+I). Bacterial expulsion in water was also assessed and is shown in panels D, E, and F, respectively. Each dot represents the result from one fish. The horizontal bar represents the mean bacterial load. n = 5; *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$. p, probiotic treated; A, antibiotic-treated; I, infected with bacteria.

a significant increase over that at 3 dpi. This is an intriguing observation, but the mechanism is beyond this initial study's scope. This study also provides new evidence about the infection pattern of SsI. Being the main shigellosis-causing strain in developed countries,[15] it may link transmission of the bacteria via different water bodies. The huge amount of ballast water in cargo ships from different parts of the world may provide the inoculum. There is evidence that *Shigella* passaged strains from fish gut are more infectious in nature.[26] Therefore, infection from *Shigella* and its transmission via fish in water may act as a double-edged sword. Infections from intracellular pathogens in aquaculture and in natural water bodies have increased in the recent past, and the need for an effective vaccine has become profoundly apparent.[16,17] Various previous studies evaluated the Immune-stimulatory effects of heat-killed bacteria.[11,28] Heat-killed *Enterococcus faecalis* activates cell-mediated immunity in fish, which is essential against intracellular pathogens.[29] Effectiveness and usefulness of heat-killed bacteria over those of other immunogens have been assessed and often found to be more favorable.[30-32] Although live-attenuated bacteria provide a better immune-stimulatory effect, there is an added risk of reversion into the virulent wild-type form.[33] Several acellular vaccines are also limited, since they are more prone to degradation, and some are expensive to produce,[34] limiting their usage in low- and middle-income countries (LMIC). This study found that using a three-dose regimen of heat-killed *Shigella* provided significant protection to the fish and limited the transmission of *Shigella* in water. Systemic invasive infection was also found to be reduced in immunized fish. Based on the epidemiological evidence, selection of the strains was important in the study, and finding satisfactory protection against these three prevalent strains supports the relevance of the work. Pathogenesis is a complex phenomenon that decides the fate of an organism. Other than its plasmid-borne effector proteins,[35] *Shigella* relies on its O-antigen structure to cause host damage.[36] The TTSS is a needle-like complex that transfers effector proteins into the host intestinal epithelium and initiates the infection. Sf2a Dhfq lacks the RNA chaperone Hfq and has constitutive TTSS activity. hfq suppression also represses rpoE and rpoS response regulators, which are important during stress conditions and in the stationary phase of bacterial growth. upregulation of TTSS effector secretion and thus would theoretically damage its host more than the wild type does. In our experiments, secretion of TTSS effectors does not have sole control over *Shigella* virulence, since the Dhfq strain still cannot cause massive damage in the fish. *Shigella sonnei* phase II has a different O-antigen profile,[37] and we observed a 2- to 3-fold colonization decrease compared to that of the pathogenic SsI. Dhfq mutants have been used successfully in previous studies as an immunogen that confers cross-protection (14). Here, we observed moderate cross-protection against Sf2a, Sb4, and SsI. The observed difference in SsI could have been a result of other factors. Evolutionarily, SsI is different from Sf2a and Sb4. At the molecular level, SsI has an extra secretion system, namely, T6SS, whose genes are sparsely present in other *Shigella* strains. Increased fitness for interbacterial competition and niche occupancy may enhance fitness and lead to prolonged infection. Fish blood contains a complement system just as mammalian blood does. These complement factors are heat-labile and contain the same features as their mammalian counterparts, i.e., ability to lyse bacteria via classical pathway, among others.[37,38] Here, immunized fish heart extract caused lysis of wild-type bacteria, and as a result, fewer bacteria were recovered. These data suggest the involvement of the fish immune system to reduce the bacterial load via complement and immunoglobulins specific against *Shigella*. In-depth studies on fish immunology were beyond the scope of this preliminary study. The adult zebrafish was also tested for its potential use as a therapeutic model. As a proof of concept, it was hypothesized that antibiotic treatment will increase *Shigella* colonization, whereas probiotic/commensal treatment will help reduce and/or inhibit *Shigella* colonization in the fish gut. Antibiotic treatment significantly reduces the intestinal microbiota, and thus, *Shigella* can easily take up the available niche and exceed its "normal" colonization ability, as seen earlier. As a result of bacterial internalization, fewer bacteria were expelled into the water. On the other hand, probiotic/commensal treatment with or without prior antibiotic treatment reduced or inhibited the infection.

In summary, this new experimental model should be helpful for studies on *Shigella* pathogenesis, immune responses, and therapeutics.

Oral Live Transconjugant *Shigella* Vaccine

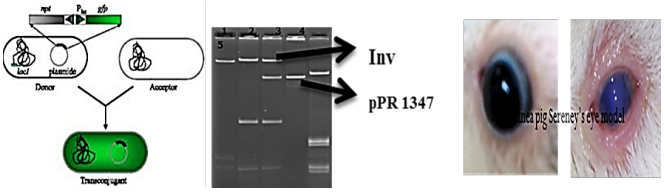

Figure 4: *S. dysenteriae* 1 strain isolated from an epidemic in West Bengal, India. After transconjugation *S. dysenteriae* 1 became noninvasive in guine pig Sereny's test and exhibited strong cross reactivity with both *S. dysenteriae* 1 and Salmonella typhimurium antisera. Plasmid profiles of the transconjugants were unaltered as compared with the wild type strain except for the presence of pPR1347. The transconjugants regained their invasive property after elimination (curing) of pPR1347.A 60 kDa IpaH protein was stop secreation into the culture supernatant by the transconjugants. Synthesis of lipopolysaccharides (LPS) of the hybrid strains was increased in comparison with the wild type *S. dysenteriae* 1 strains.

We have constructed a hybrid strain of *S. dysenteriae* type 1 by introducing a plasmid vector pPR 1347. After introduction of a lipopolysaccharide (LPS) biosynthesis gene, virulent *S. dysenteriae* type 1 strain became avirulent (Figure 5). We have evaluated the immune response and protective efficacy of avirulent live transconjugant *Shigella* hybrid (LTSH) strain against wild type *S. dysenteriae* type 1, after four doses of oral (rabbit) and intranasal (mouse) immunizations. Serum IgG titers showed exponential increase during immunization and peaking on the 28th day and remained at that level till the 35th day in both the rabbit and the mouse models. When tested, serum IgG titers persisted for 63 days in mice and relatively high for 150 days in case of rabbits. Homologous protection studies showed 100% protection against the challenge with wild type *S. dysenteriae* type 1 strain in rabbits and 80% protection in mice.

In guinea pigs, four successive oral administrations of LTSH stx showed exponential increase of the serum IgG and IgA titer against lipopolysaccharide of LTSH stx and peaked on day 28 and remained at that level until day 35 after the initiation of the immunization (Figure 6). In intestinal lavage of the immunized animals, significant increase of mucosal IgA titer against lipopolysaccharide of LTSH stx was also observed. Four successive doses of oral immunization also showed complete protection against rectal challenge with wild type *S. dysenteriae* 1 strain only.

Development of An attenuated *Shigella* mutant lacking the RNA-binding protein Hfq provides cross protection against *Shigella* strains of broad serotype

Deletion mutant lacked the RNA-binding protein Hfq leading to increased expression of the type III secretion system via loss of regulation, resulting in attenuation of cell viability through repression of stress response sigma factors. Such increased antigen production and simultaneous

attenuation were expected to elicit protective immunity against *Shigella* strains ofheterologous serotypes Few live attenuated vaccines protect against multiple serotypes of bacterial pathogen because host serotype-specific immune

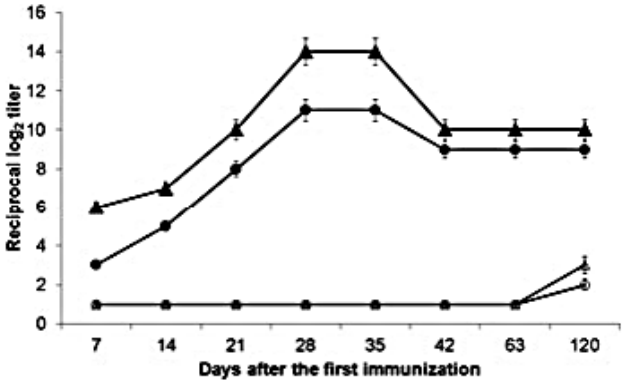

Figure 5: Anti-LPS serum IgG and IgA titers of the animals. Animals of the immunized group were immunized on day 0, 7, 14 and 21. Blood was collected from animals of both immunized and control groups on several days indicated in the X axis and anti-LPS IgG and IgA were titrated as described in the text. Each triangle and circle represents mean ± SD of the following determinants: Sample collected on days 7, 14, 21 and 28 (34 determinants, a total of two experiments) from each immunized and control group; sample collected on day 35, 42 and 63 (14 determinants, a total of two experiments) from each immunized and control group; sample collected on day 120 (two determinants, a total of two experiments). , IgG of immunized group; •, IgA of immunized group; •, IgG of control group; ◦, IgA of control group.

responses are limited to the serotype present in the vaccine strain. Here, immunization with a mutant of *Shigella flexneri* 2a protected guinea pigs against subsequent infection by *S. dysenteriae* type 1 and *S. sonnei* strains. Thus, the vaccine potential of this mutant was tested in two guinea pig models of shigellosis. Animals vaccinated in the left eye showed fewer symptoms upon subsequent challenge via the right eye, and even survived subsequent intestinal challenge.

In addition, oral vaccination effectively induced production of immunoglobulins without severe side effects, again protecting all animals against subsequent intestinal challenge with *S. dysenteriae* type 1 or *S. sonnei* strains. Antibodies against common virulence proteins and the O-antigen of *S. flexneri* 2a were detected by immunofluorescence microscopy. Reaction of antibodies with various strains, including enteroinvasive Escherichia coli, suggested that common virulence proteins induced protective immunity against a range of serotypes. Therefore, vaccination is expected to cover not only the most prevalent serotypes of *S. sonnei* and *S. flexneri* 2a, but also various *Shigella* strains, including *S. dysenteriae* type 1, which produces Shiga toxin.

In this study, animals immunized with a *S. flexneri* 2a-based Δhfq strain were protected from heterologous challenge with Sd1 and *S. sonnei*. The results provide strong evidence supporting cross protection against *Shigella* strains of heterologous serotypes; these results were replicated in independent guinea pig models. The amount of bacteria (5.0×108 cfu for ocular immunization and 1.0×106±107 cfu for oral immunization) was much higher than that usually required to cause diarrhea in humans (1×102 ~103 cfu).[36],

leading us to postulate that cross-protection was induced by administration of *Shigella* strains at excess amounts.

Exposure to a sufficient number of bacteria expressing common virulence proteins could induce immunity and broad protection, which may not be fully established during a natural infection cycle during which limited bacteria begin to propagate within intracellular spaces within the colon epithelium, thereby escaping from the host immune system. Indeed, several early studies of a keratoconjunctivitis model documented cross protection, albeit partial, which support this hypothesis. The colon loop model demonstrated effective attenuation of theΔhfq strain without loss of expression of virulence genes. Inoculation of the colon loop with excess amounts of bacteria (1.0×109 cfu) resulted in a greater number of locally invading bacteria without any symptoms, indicating that attenuation afforded by the Δhfq mutation was so effective that fewer side effects emerged, irrespective of the inoculation dose. This is a great advantage in terms of practical use. Experiments using two different breeds of guinea pig highlighted different responses against infection, although the use of two models arose because of difficulties with international transfer of materials. Hartley guinea pigs developed corneal lesions after inoculation with the Δhfq mutant , whereas non-albino guinea pigs were asymptomatic. After the challenge, Hartley guinea pigs immunized with the Wild type strain showed opaque changes in the cornea, which were not evident in the experiment involving non-albino guinea pigs. Different breeds of guinea pig show differing susceptibility to infection by pathogens.[41] Such differences would affect immunity against infection by microorganisms, and possibly by the highly-attenuated *Shigella* strain. Here, we developed a new guinea pig model based on achlorhydric treatment; this approach was first used to develop a rabbit model of enterohemorrhagic E. coli infection.[34] Preliminary experiments conducted with 1.0×107 cfu of both the Wt and Δhfq strains revealed a high efficacy of infection, resulting in the death of all six Wild type -immunized animals. As a challenge that induces lethal damage, an excess amount of bacteria (1.0×109 cfu) was directly inoculated into the colon of all animals.

Since the body weight of the two immunized groups remained constant, or even increased, at Day 28 ± 30, the surgical procedure used for the inoculation had a minimal effect on the condition of the animals. Expression of IFN-γ and its receptor increases in patients with shigellosis, and further increases during the convalescence period.[42] The IFN-γ and immunoglobulin responses in animals immunized with the Δhfq strain induced immunity comparable with that induced by the Wt strain. In the two independent experiments, the body weight of all Wt-immunized animals was at least 5% higher than that of the other groups at Day 28, reflecting the reproducibility of the two experiments. This increase may be due to the stress of severe diarrhea, which might encourage excess uptake of food. Immunological detection of induced antibodies was consistent with the protective effects observed in the challenge studies. Also, detection of antibodies against different strains, including EIEC, suggests positive responses against universal serotypes of *Shigella* strains. We did not conduct challenge experiments using *S. flexneri* 2a (which has a serotype identical to that of the immunizing strain) because the majority of studies, including one using the *S. Typhimurium* Δhfq vaccine,[32] report generation of immunity against strains of homologous serotype. Production of antibodies against homologous O-antigen was detected using the ΔinvE mutant of*S. flexneri* 2a strain 2457T (which has lost expression of all virulence proteins), as evidenced by the strongest signal upon immune analysis. Consistent with this, sera from immunized Cross protection by live attenuated vaccine candidate for *Shigella*, animals strongly agglutinated 2457T cells, but not *S. sonnei* or Sd1 cells. In addition, *Shigella* Specific proteins lacking InvE-dependent regulation could be considered potential antigens. However, if these proteins were common among *Shigella* species, detection of antibodies would be difficult in the experiment that required absorption of serum with the ΔinvE strain of *S. sonnei* to reduce non-specific signals generated by general bacterial proteins. A rough estimate suggests that vaccines that are effective against both serotypes of *S. sonnei* and*S. flexneri* 2a, 3a, 6 will cover about 60% of patients.[7,43] Detection of antibodies against these serotypes indicates a potentially broad effect for prevalent strains. In addition, the Oantigen of*S. flexneri* 2a provides cross protection against *S. flexneri* strains 1a, 2b, 3b, 4a, 5a, and Y, all of which possess group factor 3, 4 and type factor II.[44] This provides a *S. flexneri* 2a-based vaccine with a great advantage over other vaccine candidates that target a limited number of virulence proteins.

Immunization with common virulence proteins from an attenuated mutant is a new concept; fortunately, a single mutation attenuated the immunizing strain and increased antigen expression. These results suggest that such a strategy could be applied to other pathogens harboring common virulence machinery if one carefully selects the appropriate strain and mutation to provide effective attenuation without loss of antigen expression. Common virulence protein antigens expressed by the attenuated strain and acting as [a]live toxoids[o] are expected to elicit the same levels of host immunity against multiple pathogen serotypes as that obtained by conventional toxoids.

Heat-Killed *Shigella* Immunogen

It has previously been shown that acetone-killed *Shigella* is an effective mucosal immunogen (20). An orally administered formalin-inactivated whole-cell vaccine for *S. sonnei* was effective in a double-blind, placebo-controlled phase I trial (21). These reports are in contrast to an earlier study in which monkeys were protected by oral vaccination with attenuated organisms, but not by an acetone-inactivated vaccine (22). We have previously reported that heat-killed *S. flexneri* 2a protects against challenge with the homologous strain in

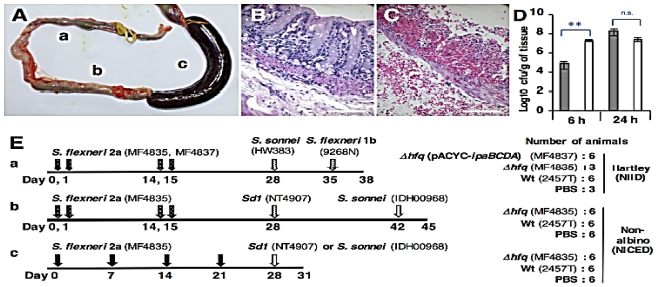

Figure 6: Characterization of the Δhfq mutant using a colon loop model, and the experimental schedules. (A) Colon segments were infected with (a) Δhfq (MF4835), (b) PBS, or (c) *S. flexneri* 2a Wt (2457T) for 24 h. Images of tissue infected with Δhfq (B) and Wt (C) for 24 h. Scale bars, 100 µm. (D) Bacterial counts within the tissue at 6 and 24 h post-inoculation. White and gray bars indicate Δhfq and Wt strains, respectively. Values are expressed as the mean ± SD; n = 3 animals. ** p<0.01; n.s., not significant. (E) Experimental schedules: (a) Ocular immunization/ocular challenge; (b) Ocular immunization/ocular/intestinal challenge; (c) Oral immunization/intestinal challenge. Black and white arrowheads denote immunization and challenge, respectively. Spotted arrowheads denote ocular inoculation.

a rabbit model (23). In that model, five doses of 1 X 10^11 bacteria were orally administered to rabbits at 7-day intervals and the immunized animals were challenged 7 days after the last dose of vaccine. Homologous protective efficacy was 100% and highly significant. A further study showed that protection could be achieved with a minimum of four doses.

In the present study, we used a guinea pig colitis model that has already been proven useful for assessing the protective efficacy of *Shigella* vaccine candidates (15, 16). Based on reported data concerning prevalence of serogroups/serotypes that cause shigellosis globally as well as locally in Kolkata, we selected *S. dysenteriae 1, S. flexneri 2a, S. flexneri 3a, S. flexneri 6, S. boydii 4 and S. sonnei* (1, 2, 4, 5, 8, 9). Heat-killed cells of single serogroup/serotype of *Shigella* strains as both monovalent and hexavalent immunogens induced significant protective immune responses; however, further studies are needed to elucidate the mechanisms underlying such protection. Several studies have shown that anti-LPS antibodies are elicited in response to *Shigella* infection, both locally as secretory IgA and systematically as serum IgG (25, 26). In our study, serum IgG and IgA titers against the LPS from each of the six serogroups/serotypes increased during four successive immunizations with heat-killed cell suspensions. Serum IgG can potentially neutralize pathogens that enter the mucosa and thus prevent systemic spread (27). The strong serum IgG responses in the immunized guinea pigs may have provided robust resistance to invasion by the challenge *Shigella*e by enhancing phagocytic clearance of the organisms; this would be a possible mechanism by which protection against bloody diarrhea is conferred. We also found high anti-LPS IgA titers against each of the six immunized strains in

intestinal lavage fluid from the and mucoidal diarrhea was excluded. Heat-killed *Shigella* vaccine guinea pigs. Gut-derived IgA responses are also thought to have a significant

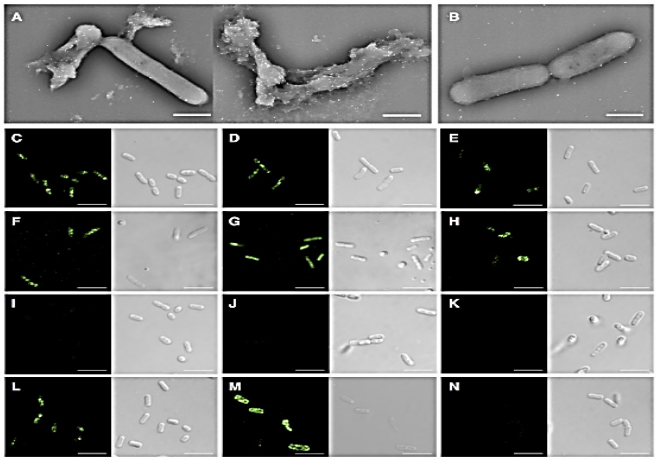

Figure 7: Detection of reactive antibodies. (A) Scanning electron microscopy images of *S. sonnei* (HW383) incubated with fresh serum and subsequently reacted with an anti-guinea pig gold-conjugate (white particles). (B) Negative control (unimmunized serum). Scale bars, 1 µm. Immunofluorescence- (left) and differential interference contrast- (right) based detection of antibodies against (C) *S. sonnei* (HW383), (D) Sd1 (TSH1669), (E) *S. flexneri* 1b (9268N), (F) *S. flexneri* 3a (GTC-01924) (G) *S. flexneri* 6 (GTC-01927), (H) EIEC (NIID1), (I) Sd1 (MD506), (J) *S. flexneri* 1b (9268N17-1) lacking the virulence plasmids, and (K) *S. sonnei* ΔT3SS (MS2834). (L) Detection of antibodies against HW383 in sera pre-adsorbed with MS2834. (M) *S. flexneri* 2a ΔinvE (MF1632) stained the sera used in A to K. (N) Control images of HW383 stained by pre-immune sera. Scale bars, 5 µm.

role in mucosal defense (1, 27). However, after immunization with the hexavalent immunogen mixture, one of eight guinea-pigs (12.5%) was not protected against challenge by a virulent strain of either *S. flexneri* 2a or *S. sonnei*. With the monovalent vaccination, 1*10^7 cfu of the *Shigella* strains were enough to induce protective immunity; however, the same amount of the bacteria may not be enough in hexavalent vaccination. This controversial finding needs to be further investigated. Despite the promising results of this study, several other issues remain to be addressed, including: (i) whether the current strategy of using a multivalent heat-killed vaccine can generate long-term protective efficacy; (ii) whether it will possible to induce high levels of immunogenicity with lower doses of immunogens; (iii) whether it will be possible to obtain sufficient cross-protective immunogenicity with an appropriate combination of different serogroups/serotypes; and (iv) whether there will be a limit to the number of serogroup/serotype that can be included in a multivalent immunogen. Further studies to elucidate these issues are in progress in our laboratory.

In our study, after three doses of immunization, higher levels of IgG, IgA and IgM in immunized rabbit sera and PBMC supernatants were detected against each serogroup and serotype specific antigen up to 180 days after first immunization. sIgA was found significantly higher amount in HKMS-immunized rabbits than in non-immunized ones. These indicated the induction of strong mucosal and systemic immune responses. *Shigella* O-antigen induced by wild-type infection correlated with protection data from

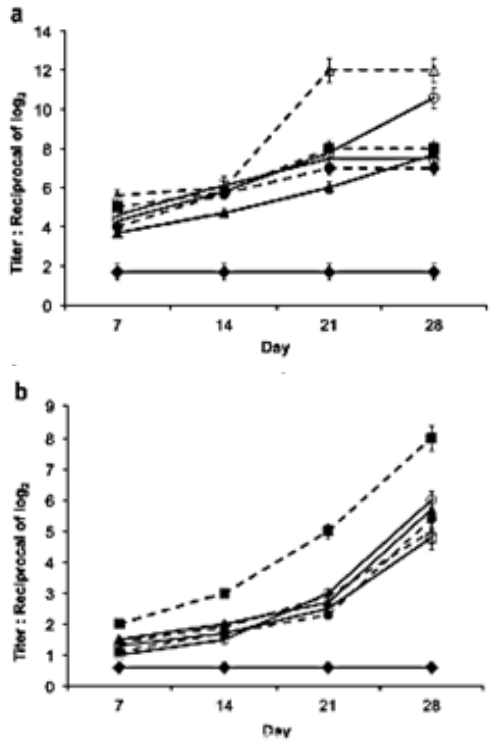

Figure 8: Anti-LPS serum IgG and IgA titers after hexavalent immunization. Serum (a) IgG titers and (b) IgA titers are shown. Each bar represents the mean_SD of four determinations except for the groups receiving *S. flexneri* 2a and *S. sonnei,* from each of which one animal with tenesmus

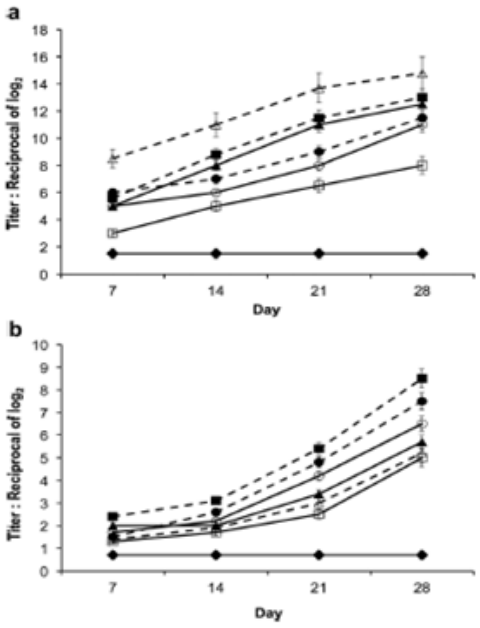

Figure 9: Anti-LPS serum IgG and IgA titers after monovalent immunization. Blood samples were collected from the animals' footpads on Days 7, 14, 21 and 28 and serum samples prepared. (a) Serum IgG titers and (b) IgA titers are shown. Each bar represents the mean_SD of five determinations. Immunized versus control groups. P<0.001. _, *S. dysenteriae 1; *, S. flexneri 2a; &, S. flexneri 3a; &, S. flexneri 6; D, S. boydii 4; ~, S. sonnei*; ^, control.

epidemiological and sero-epidemiological studies as well as non-human primate challenges. So, to determine the O-Antigen specific immune response, plotted LPS of each serogroup and serotype of challenge strains with immunized sera and found serogroup/serotype specific O-antigen specific bands.

At present, most of the few licensed enteric vaccines are unable to induce both humoral and cell mediated immune responses. Growing evidence suggests that not only B cells but also T cells are involved in protection against intracellular bacteria. An ideal vaccine should have some features that can generate T-cell mediated adaptive immune responses, antigen-specific memory B-cell responses, and provide long-term, broad-spectrum protective immune responses against circulating wild-type heterologous strains. Determination of *Shigella*-specific T cell responses and whether T cells contribute to the protection is the major concern to produce an anti-*Shigella* vaccine. Cytokines are proteins that play an integral role in the human immune response. The functions of these proteins are diverse and include roles in normal T-cell-mediated immunity, the inflammatory response, cancer, autoimmunity, and allergy (Borish and Rosenwasser, 1996). In our study, significantly higher levels of IL-12p35, IFN-gamma, and IL-10 expression at the mRNA level were observed after stimulation by HKMS immunogen on PBMC of immunized rabbits. These data indicate that our immunogen successfully induced Th1 mediated immune response.[44] IL-12 acts as a pro-inflammatory cytokine that promotes Th1 responses by enhancing IFN-gamma, increasing IL-2 receptor expression and inhibiting IL-4 production; IL-12 also supports the proliferation of antigen-stimulated T cells.[45] IFN-gamma, the key cytokine for the Th1 response, can also recruit and activate phagocytic and cytotoxic cells, which further enhance innate immunity to clear the infecting organisms.[46] IL-10 is expected to suppress macrophages and modulate T and B-cells. *Shigella*e invade macrophages and induce apoptosis; the released organisms from macrophages further spread through the mucosal epithelium.[47] IL-10 may help reduce the number of apoptotic macrophages, which would prevent further microbial dissemination. IL-10 also enhances IgG secretion. Therefore, IL-10 production may be important for vaccine-induced antibody responses. Therefore, induction of Th1 immunity could provide a therapeutic approach for preventing *Shigella* induced inflammation.[48] These sustainable B and T cell immune responses indicate a memory B cell response induction. In our study, memory Bcell stimulation in PBMC isolated from immunized rabbits long after the immunization was confirmed by production of much higher antigen-specific antibody responses in vitro upon stimulation with HKMS immunogen. The immunogen-induced antigen-specific memory B-cells through adaptive immunity. To determine broad spectrum protective efficacy of our formulated heat-killed vaccine, after three doses of immunization, we challenged the immunized rabbit luminally with heterologous wild type strains and found

complete protection against shigellosis in immunized rabbits as compared to non-immunized rabbits. The signs and symptoms of shigellosis were found in all of the non-immunized rabbits after a challenge experiment, whereas none of the immunized rabbits showed signs of shigellosis after the challenge. This indicates the power of this approach in generating protection against a group of pathogens.

Although we found significant immuogenicity, full proof protective efficacy was not observed as the antigenic epitopes were destroyed due to heat. This was also evident from immuoblot as some important immunogenic bands were missing.

Formulation of Chitosan Alginate Coated OmpA- a subunit vaccine

Despite the fact that while working with the OMVs , we have observed specificity of the anti-MOMVs response was verified against WCL and LPS of seven strains using immunized sera, collected on the 28th day (after first immunization), the day when maximum antibody titers were noticed. No bands were detected on the immunoblots of non-immune sera against both WCL and LPS. Immunoblot by anti-

MOMVs sera against WCL (Figure 4A) demonstrated numerous antigenic proteins of MOMVs. The most-reactive bands were in between the region of 210 and 32 kDa, correlated Figure 4. (A)

These bands were invisible against the noninvasive control strain *S. flexneri* 1a (NK4238), lacking the virulent plasmid; however a band at 34 kDa position was seen which corresponded to the chromosomally encoded OmpA protein, conserved among *S. flexneri* strains.

Polymeric nanoparticles have attracted much attention as delivery systems due to their ability to overcome physiological barriers and protect and target the loaded substances to specific cells. Naturally occurring polymers such

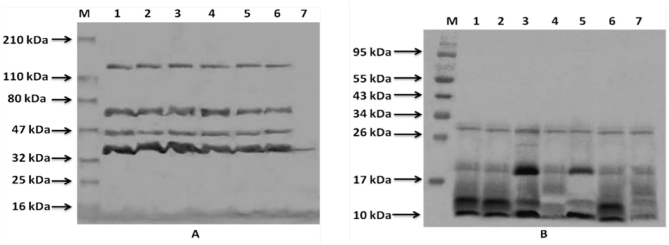

Figure 10: Representative Immunoblot against WCL of seven *Shigella* strains probed with 28th day's anti-MOMVs serum from a single mouse. Lane M: prestain molecular weight marker (Pierce, USA) Lane 1: *S. dysenteriae* 1 _stx (NT4907); Lane 2: *S. flexneri* 2a (B294); Lane 3: *S. flexneri* 3a (C519); Lane 4: *S. flexneri* 6 (C347); Lane 5: *S. boydii* type4 (BCH612); Lane 6: *S. sonnei* (IDH00968); and Lane 7: non-invasive strain *S. flexneri* 1a (NK4238). (B) Representative immunoblot against LPS of seven strains probed with 28th day's anti-MOMVs serum from a single mouse. Lane M: prestain molecular weight marker (Fermentas, USA) Lane 1: *S. dysenteriae* 1 _stx (NT4907); Lane 2: *S. flexneri* 2a (B294); Lane 3: *S. flexneri* 3a (C519); Lane 4: *S. flexneri* 6 (C347); Lane 5: *S. boydii* type4 (BCH612); Lane 6: *S. sonnei* (IDH00968); and Lane 7: noninvasive strain *S. flexneri* 1a (NK4238). with the area of the most abundant proteins found in OMVs; VirG (120 kDa); IpaB (62 kDa); IpaC (42 kDa) IpaD (38 kDa); OmpA(34 kDa).20,33

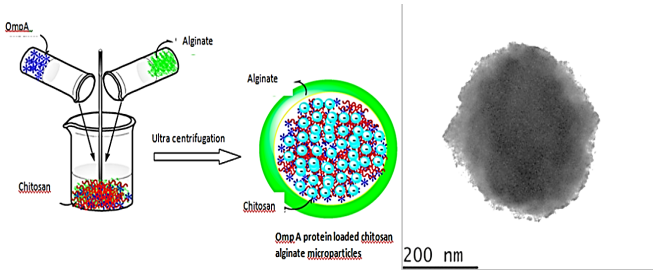

Figure 11: Chitosan nanoparticles are prepared by the ionic gelation of Chitosan Solution with anionic tripolyphosphate (TPP), Briefly, chitosan was dissolved in 1% (v/v) acetic acid aqueous solution at concentration of 2 mg/ml. Then, TPP was dissolved in distilled water at a concentration of 1-mg/mL. Subsequently, TPP solution was added dropwise into chitosan solution at 1:2 ratio. Chitosan colloid nanoparticles formed spontaneously under mild agitation at room temperature. Two hours later, chitosan colloid nanoparticles was centrifuged at 35,000 rpm for 1-hour. Then, the supernatant was discarded and the deposit was re-dispersed in distilled water. After the preparation of chitosan nanoparticle, it is loaded with ompA.

as chitosan (CS) have been studied to form nanoparticles. CS is a biodegradable polysaccharide, and it is derived from the deacetylation of chitin. Apart from its biocompatibility, the low toxicity, hemostatic, and bacteriostatic properties also contribute to its various applications in the pharmaceutical field. 'Chitosan Nanoparticle - A Drug Delivery System discloses that Chitosan Nanoparticles are good drug carriers because of their good biocompatibility and biodegradability and can be readily modified. Therefore, the principal object of the present invention is to design a vaccine comprising OmpA nanoformulations as active molecule vehicle upon chitosan nanoparticles.

The respective average diameters, measured by Zetasizer, of chitosan nanoparticles and OmpA-loaded nanoparticles were approximately 240 and 535 nm. The PDI value of chitosan nanoparticles was 0.256 while that of OmpA-loaded chitosan nanoparticles was 0.199, thus indicating a narrow and favorable particle size distribution (PDI<0.5).

BALB/c both mice, six weeks old, were taken from animal resource department of ICMR-NICED. All mice were caged separately and maintained at 25°C with 65% humidity and fed sterile food and water under the care of full time staff and in accordance with the rules of the institutional animal ethical committee (IAEC) (Apro/77/24/ 11/2O10, Reg. No. NICED/CPCSEA (AW) 215/2009-2015).

Female mice were immunized orally at days 0, 7, and 14 with 50 ug per 100 µl of purified OmpA coated with chitosan-alginate (CAOP) using the concentration. Fifteen minutes before the oral immunization, each mouse was anesthetized by 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). CAOP was introduced directly into the stomach through a mouse-feeding needle (Harvard Instrument, USA). The same volume of PBS was given by oral administration to the non-immunized group. All immunized and non-immunized group of mice were returned to their cages and

given limited amounts of sterile food and water. Protective efficacy of CAOP very was low although the reciprocal increase of serum IgG antibody titer was observed during the period of immunization.

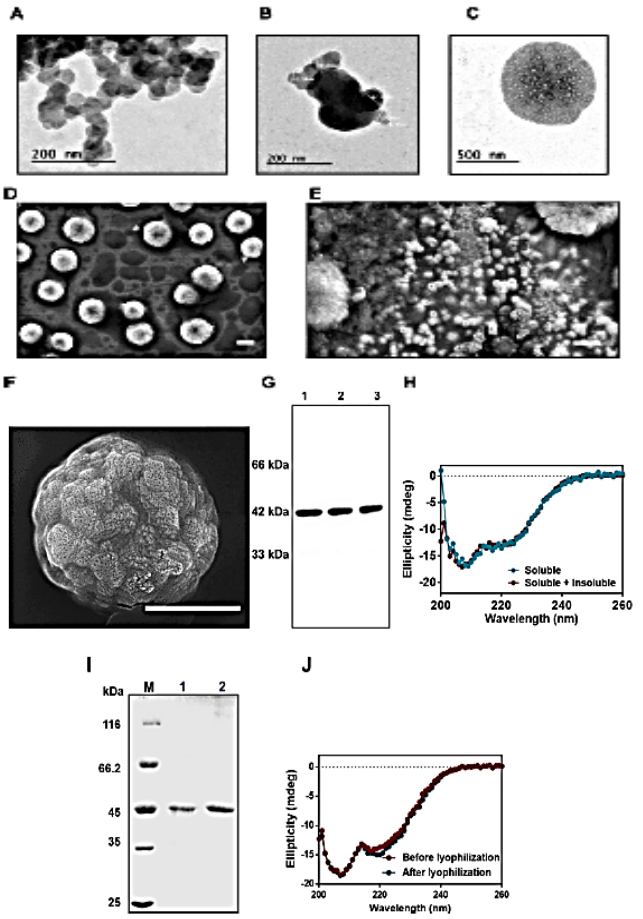

Figure 12: Characterization of stabilized IpaC. The morphology of the purified protein was visualized using electron microscopy, which showed that the protein in Tris buffer (containing 0.05% LDAO) is present in spherical complexes of variable size. (A–C) TEM images of pure IpaC solution. (D–F) FE-SEM images of pure IpaC solution (scale bar: 1-µm) in order of increasing magnification. (E) Small complexes in between larger ones. (F) Focused on a bigger complex. (G) Western blot of a few fractions of IpaC obtained from peak 2 during SEC (using anti-His antibody), stored at 4°C, shows distinct bands, indicating higher stability of the protein purified using the current protocol. (H) Secondary structures of the purified protein samples were analyzed using circular dichroism, which shows that the spectrograms of IpaC purified from both soluble and soluble + insoluble fractions have similar secondary structure indicating complete removal of urea used during purification from the insoluble fraction. (I) To increase the cost-effectiveness and convenience of storage/transportation, we explored lyophilization of stabilized IpaC. Lane M represents the molecular weight marker, lane 1 depicts purified protein before lyophilization, and lane 2 depicts the resuspended protein after lyophilization. The lyophilized IpaC reconstituted in ultrapure water showed an intact band without protein aggregation/degradation. (J) The secondary structure of the stabilized protein before and after lyophilization was found to be highly similar when analyzed using circular dichroism. This can aid in reducing the resource intensiveness of the process of manufacture and transportation.

Nanoformulation with IpaC subunit *Shigella* vaccine

The pursuit of an efficacious, cost-effective, noninvasive, cross-protective vaccine has resulted in multiple formulations including those involving broadly protective conserved Ipa proteins of *Shigella*. However, complete cross-protection has been difficult to achieve, especially against *S. dysenteriae* 1 (Sd1), the strain that causes the most severe form of the disease. Although outbreaks due to other strains such as *S.flexneri* 2a are on the rise, Sd1 have caused multiple pandemics/epidemics in the recent past. Therefore, a rational move to prepare for a possible pandemic would be to develop a broadly protective vaccine utilizing conserved proteins especially of Sd1 origin. Additionally, as most formulations require the presence of an adjuvant to provide sufficient protection, self-adjuvanting/single-antigen vaccines can lead to reduction in the resource intensiveness of the process. Despite multiple efforts, a licensed *Shigella* vaccine is still not available. The instability/aggregation of immunogenic conserved antigens such as IpaC further aggravates the situation. To obtain an improved cross-protective, noninvasive vaccine, we explored the stabilization of Sd1 IpaC, a highly immunogenic *Shigella* protein that has the potential to provide cross protection against species of *Shigella* due to its conserved nature.

Therefore, we explored stabilization of IpaC from *S. dysenteriae* 1 with a modified protocol and assessed its vaccine potential in BALB/c mice against both homologous and heterologous challenges of *Shigella*. Recombinant Sd1 IpaC expressed without the chaperone was stabilized with three simple processes, resulting in a high yield and a concentration of >8 mg/mL for SEC without aggregation. The first process involving purification of the soluble fraction can be completed in less than 10 h. However, if the yield required is higher and lacks a time constraint, the second or third process can be employed. Compared to the third process, the second process resulted in a slightly higher yield and, therefore, was utilized for all storage and vaccination studies. Interestingly, urea used in processes 2 and 3 could be completely removed as observed by a comparable CD spectrogram of the IpaC purified from the soluble + insoluble fraction with that of the soluble fraction (Figure 3H). Stabilized Sd1 IpaC can also be lyophilized and reconstituted in sterile water for transportation and usage at the point of care. The protein in solution could be stored for more than a year at −80, −20, and 4°C without degradation/aggregation improving cost-effectiveness of the process. It was also stable at 25°C for up to 1.25 months, suggesting that 4°C storage should suffice for maintaining functionality. The electron microscopic evaluation of the SEC-pure protein showed that spherical heterogenous complex structures can form as a result of the current protocol, which is speculated to enable higher immunogenicity, possibly even in the absence of an adjuvant, aiding the formulation of a self-adjuvanting vaccine.[51] Therefore, the self-adjuvanticity of the stabilized Sd1-IpaC was assessed in a mouse model of shigellosis.

To formulate a noninvasive vaccine, IpaC was intranasally administered in BALB/c mice.

Significant antibody and cytokine responses were observed in sera of the immunized animals. A low level of IL-6 along with an absence of coagulation in LAL assay confirms non contamination of endotoxin in the purified protein.[52] To assess the protective potential of the Sd1 IpaC, the treated animals were challenged with a high dose of either homologous Sd1 or heterologous *S. flexneri* 2a (most common strain in circulation worldwide). The fact that a protein originally from Sd1 provided 100% protection against a high dose of a heterologous species confirmed the cross-protective ability of stabilized Sd1-IpaC. For further testing of safety in higher organisms, we propose introducing a protease cleavage site in the gene to remove the poly-His tag from the protein.

Development of outer membrane vesicles based *Shigella* vaccine

Like most other gram-negative bacteria, *Shigella* releases outer membrane vesicles (OMVs) into the surrounding environment during growth. In this study, we have exploited OMVs of *Shigella* as a protective immunogen in a mice model against shigellosis. Distinctive vesicle secretion was noticed from different *Shigella* strains. Among them, *Shigella boydii* type 4 (BCH612) was secreting relatively higher amounts.

We immunized female adult mice orally with 32 ug of purified *Shigella boydii* type 4 (BCH612) OMVs four times at 1-week intervals. Antibodies against these vesicles were detected in immunized sera until 120 days, indicating a persistent immune response. To observe whether the passive immunity had been transferred to the neonates, the immunized female mice were mated and the offspring were challenged orally, with wild type homologous and heterologous *Shigella* strains. All offspring of immunized mothers survived the challenge with homologous strain BCH612 and up to 81% protective efficacy was noted against heterologous strains *S. dysenteriae* 1, *S. flexneri* 2a, *S. flexneri* 3a, *S. flexneri* 6 and *S. sonnei*. Our results exhibited for the first time that oral immunization of adult female mice with purified OMVs of *Shigella*, without any adjuvant, conferred passive protection to their offspring against shigellosis.

Then we forward-thinking our research by formulating multi-serotype outer membrane vesicles (MOMVs), mixing the OMVs of *S. dysenteriae* 1 , *S. flexneri* 2a, 3a and 6, *S. boydii* type 4 and *S. sonnei* to achieve a broad spectrum protection against shigellosis. Adult mice were immunized orally with 50 µg of MOMVs, four times at weekly intervals. In adult mice, immunological parameters were observed at various time points, before, during, and after immunization. Passive protection was examined in their offspring by measuring protective efficacy and studying intestinal colonization, after challenges with various *Shigella* strains. Immunized dams exhibited a consistent broad spectrum antibody response.

Development of a cost-effective vaccine candidate

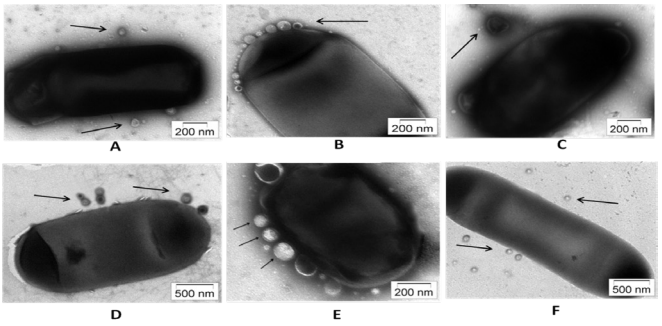

Figure 13 : Electron micrograph of outer membrane vesicles attached to the bacteria. Indicating with the arrow (A: *S. dysenteriae*1B: *S. flexneri* 2a, C: *S. flexneri* 3a , D: *S. flexneri* 6 , E: *S. boydii* type 4, F: *S. sonnei*). Supernatant from overnight grown culture was negatively stained and observed under transmission electron microscope (Bio Twin Transmission electron Microscope, FEI, the Netherlands) operating at 80 kV); 920 magnification.

with outer membrane vesicles of a tolA-disrupted *Shigella* boydii

While working on OMVs-based vaccine development,[12,13] we noticed that the rate and amount of OMVsreleased by *Shigella* would not be economical for GMP level production. To make it cost-effective, we targeted the Tol–Pal system which is crucial for maintaining outer membrane integrity in gram negative bacteria.[12] Furthermore, Neisseria, not naturally possessing tol–pal genes, have been shown to generate large amounts of OMVs during their growth.[28,29] The Tol–Pal system is composed of five proteins. TolA–TolQ–TolR proteins form a protein complex in the inner membrane. TolB is a periplasmic protein associated with Pal, lipoprotein anchored to the outer membrane which interacts with the peptidoglycan layer.[30] These two sub-complexes are linked through TolA–Pal and TolA–TolB interactions, suspected to participate in envelope cohesion.[31,32] TolA was targeted here to partially disrupt membrane integrity. Deletion of tolA rendered 60% increase in OMVs release compared to the wild type, without affecting the viability of the strain. 100% survival of adult BALB/c mice, against homologous lethal challenge, was observed, after 25 g of three oral doses of _tolA-OMVs, compared to only 60% in case of same doses of wt-OMVs in murine intranasal challenge model of *Shigella* infection.[22] Overall, after homologous lethal challenge, the mice immunized with_tolA-OMVs appeared healthier than those immunized with wt-OMVs. Surprisingly, tolA-OMVs showed greater OmpA level and LPS content compared towt-OMVs. OmpA is well-known for its protective immunogenicity in mice.[25] Our group has already showed LPS, a potential endotoxin, is one of the major immunogens for better protective efficacy 7. LPS can act as a powerful adjuvant for T cell responses to specific antigen. It has also been found that LPS can stimulate certain T cell clones and a small proportion (1–3%) of splenic T cells *in-vitro*.[33] Therefore complete detoxification by removing LPS from OMVs may be inappropriate for better performance of the vaccines *in-vivo*. However, the actual effective dose of LPS in the OMVs immunogen must be ascertained before

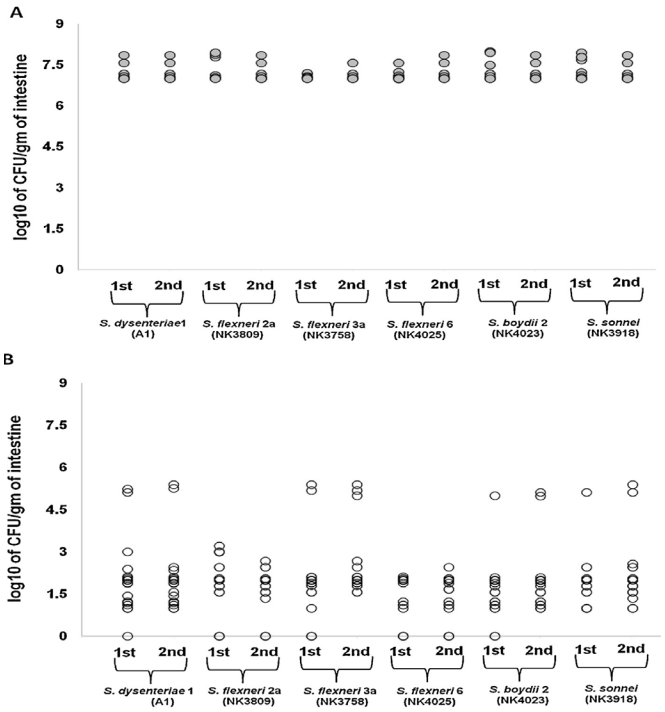

Figure 14: Comparative data of protective efficacies between control and immunized neonates, after challenging with six wild type virulent *Shigella* strains. (A) Colonization result of the neonates from a non-immunized group of dams and (B) colonization data of the neonates from immunized group of dams were plotted. Each circle represents the colonization data obtained from a single mouse. Data were expressed as log10 of recovered Colony Forming Unit (CFU)/gm of intestine of each mouse, presented on vertical axis. '1st' was referred to the first challenge study (mating between day 35 and 40, after first dose) and '2nd' was referred to the second challenge study (mating between day 84 and 91, after first dose). The differences in the rate of colonization between the immunized and non-immunized group were highly significant (*p-value* <0.005 for each of the six challenge strains). The immunized group showed long-term protection and less intestinal colonization than control group, against wild type invasive *Shigella* strains. Offspring of 3 to 4 day-old immunized dams showed significant long-term passive protection against wild type *S. flexneri* 2a, 3a, and 6, *S. boydii* type 2 and *S. dysenteriae* 1. Their stomach extracts containing mother's milk have also exhibited significant levels of anti-MOMVs immunoglobulins.

going for large scale animal studies with tolA-OMVs. Better immunogenicity manifested by tolA-OMVs, might result from the cumulative action of these factors without conferring any severe reactogenicity. Oral vaccines can elicit mucosal sIgA that prevents attachment and invasion and neutralize enterotoxins, while serum IgG controls mucosal and systemically invasive pathogens, inducing an array of cell-mediated immune responses against intracellular bacteria.[4] The better protective feature of _tolA-OMVs was manifested by two fold higher serum IgA and IgG levels than mice, immunized with wt-OMVs. Besides, sIgA, which is believed to be the hallmark of mucosal immune responsiveness, has also been assessed to be twofold higher in mucosal samples like lung and intestinal lavage fluids, collected from the mice immunized with tolA-OMVs, compared to wt-OMVs. These observations signify tolA-OMVs are more efficacious mucosal immunogen than wt-OMVs for the induction of antigen-specific systemic IgG and mucosal IgA productions. The induction of pro-inflammatory and anti-inflammatory cytokines are important in determining whether the immune system successfully protects against specific pathogenic organisms.[34,35] To gain deeper insight into the biological mechanisms responsible for the superior vaccine efficacy of tolA-OMVs immune responses in vivo were analyzed in the early and later stages of lung infection.

Rapid and high IFN-gamma response in the tolA-OMVs-immunized animals could serve to increase the microbicidal activity of infiltrating macrophages.[36] Such activated macrophages would have enhanced bactericidal activity[36] and this cell-mediated response, in conjunction with sIgA, may account for the better protective efficacy conferred by tolA-OMVs. IFN-gamma plays a pivotal role in determining the effectiveness of the immune response to pathogens. It has been shown that macrophages produce IFN-gamma in response to LPS.[37] During an ongoing microbial infection, immune system cells become activated directly or indirectly after encountering multiple PAMPs. LPS triggers a host innate immune response resulting in activation of various cell types and the production of multiple cytokines. There are evidence suggesting that bacterial LPS can activate innate immune cells in a bystander manner and that enhanced responsiveness of bystander-activated cells to PAMPs was mediated through IFN-gamma.[38] The late and persistent secretion of TNF-alpha and IL-6 in control mice (till 48 h) and wt-OMVs-immunized mice (till 24 h), correlated with the low survival rate in these groups of mice. Though It is already known that prolonged secretion of TNF-alpha and IL-6 by the infiltrating macrophages may elicit severe tissue damage leading to death, both of these cytokines are important for the early innate immune response in bacterial infection.[39]

Additionally, IL-6 activities are critical for resolving innate immunity and promoting acquired immune responses.[40] In contrast to the pro-inflammatory cytokines, IL-4 and IL-10

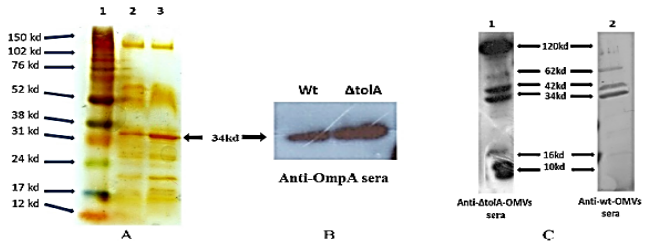

Figure 15: (A) SDS–PAGE of OMVs, stained with 0.1% silver nitrate solution. Lane 1, 2 and 3 are the molecular weight markers, wt-OMVs and tolA-OMVs, respectively. The band at 34 kDa position was very prominent in lane 3. To confirm this 34 kDa is OmpA protein, Western blot of these two types of OMVs was carried out against anti-OmpA sera.Part (B) shows a representative picture of this immunoblot. Lane Wt and tolA represented wt-OMVs and tolA-OMVs, respectively. It clearly showed the oversecretion of OmpA protein by tolA-OMVs. Part (C) showed the representative Western blots of the whole cell lysates of BCH612 wild type strain against anti-tolA-OMVssera (Panel 1) and anti-wt-OMVs sera (Panel 2), respectively.

responses were detected significantly only in tolA-OMVs immunized animals. The presence of both IL-4 and IL-10 at 24 and 48 h after challenge may have inhibited further production of TNF-alpha in tolA-OMVs immunized mice, thus curtailing the damaging effects of excessive productions of these cytokines.[22] IL-10, a potent anti-inflammatory cytokine, plays a central role in regulating host immune responses to pathogens.[41-43] *In-vitro* cytokine studies with mouse peritoneal macrophages and CD4+T cells showed similarity with the in-vivo cytokine profile of lung wash. Apart from the induction of TNF-alpha, IL-12p70 and IL-6, tolA-OMVs could also elicit significant levels of IL-10 in naïve mouse peritoneal macrophages. The level of iNOS mRNA expression has been significantly increased in these cells, in the presence of tolA-OMVs, suggesting that the induction of NO by the antigen occurs at the transcriptional level. iNOS is involved in immune response, producing NO, as an immune defense mechanism. Induction of NO production in naïve macrophages by bacterial products is important for host defense against intracellular bacteria.[44] The elevated level of NO production occurring in tolA-OMVstreated macrophages might act in combination with IL-12p70.

Next generation tetravalent *Shigella* Outer Membrane Vesicles based candidate vaccine offered cross-protection against 50 subtypes-serotypes of *Shigella*

A tetravalent Outer Membrane Vesicle (OMVs) based immunogen was formulated using the most commonly circulating *Shigella* strains, namely, *S. flexneri 2a, S. flexneri 3a, S. flexneri 6* and *S. sonnei I*, in a 1:1:1:1 ratio. Adult BALB/c

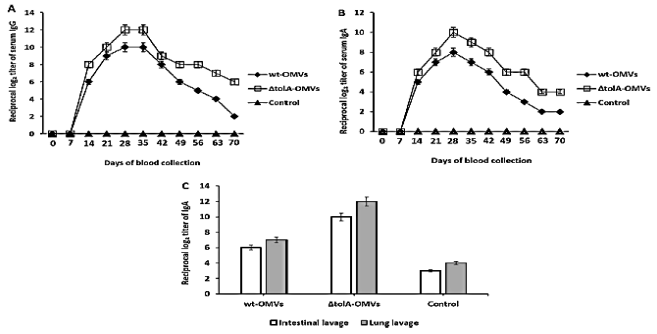

Figure 16: Graphical representation of the secretion of IgG (A) and IgA (B) in mouse sera. Blood was collected on the days indicated along the horizontal axis. Both the immunoglobulins started to respond from day 14 after first immunization, with a peak at day 28 and decreased gradually with time but remained above the level of detection till 10 weeks. Control sera showed a baseline response of these immunoglobulins and the difference between control and immunized on each day was found to be statistically significant (*p-value* < 0.005). The response in tolA-OMVs sera was significantly higher than wt-OMVs sera (*p-value* < 0.005). Data represented here are the mean ± SD of three independent experiments. (C) sIgA in mucosal fluids like lung and intestinal wash, was measured by ELISA before and after immunization. tolA-OMVs immunization helped to secrete significantly greater sIgA in both lung and intestine, compared to wt-OMVs immunized group (*p-value* < 0.005). Control group of mice showed basal level response. Data represented here are the mean ± SD of three independent experiments.

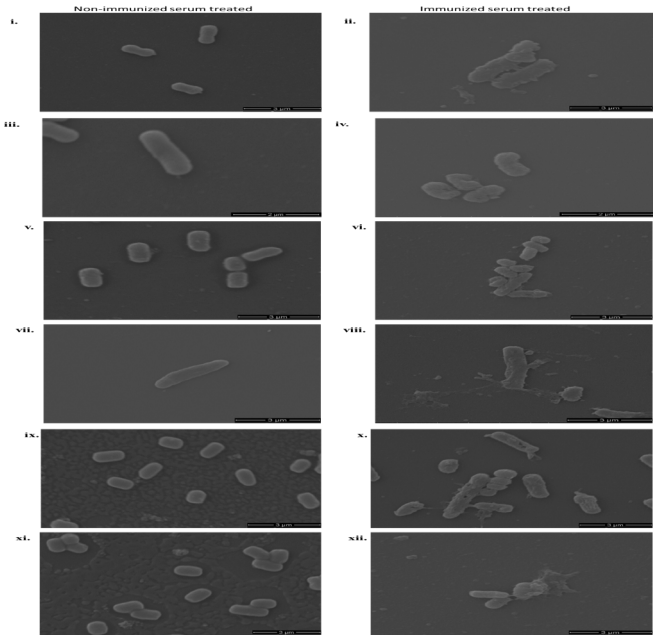

Figure 17: Scanning Electron Microscopic view of *Shigella* incubated with non-immunized sera on 35th day immunized sera. Non-immunized mice sera could not lyse bacterial cells (*i.-S. dysenteriae 1, iii- S. flexneri 2a, v-S. flexneri 3a, vii- S. flexneri 6, ix- S. boydii 2, xi- S. sonnei I)*. whereas immunized mice sera lyse bacterial cells (*ii- S. dysenteriae 1, iv- S. flexneri 2a, vi- S. flexneri 3a, viii- S. flexneri 6, x- S. boydii 2, xii- S. sonnei I*)

mice were orally immunized in a prime-boost-boost manner. Tetravalent *Shigella* OMVs immunogen induced significant and persistent serum and mucosal antibodies against OMVs, Outer Membrane Proteins (OMPs) and lipopolysaccharides(LPS).

Tetravalent OMVs also primed cell mediated immune response effectively. Protective efficacy against six heterologous *Shigella* strains was checked in an intra-peritoneal mouse model. Immunized mice survived lethal infection better than the non-immunized mice cohort with fewer replicating bacteria isolated from their gut. Here, we have developed an oral tetravalent *Shigella* OMVs immunogen and assessed the mucosal and systemic immune responses induced by it. Protective efficacy was then investigated in a non-surgical intra-peritoneal mouse challenge model. This work establishes the tetravalent OMVs-Based immunogen to be a reliable and potent vaccine candidate conferring protection against circulating 50 subtypes-serotypes of *Shigella*.

Discussion

Shigella remains a significant public health problem since its discovery. In light of the emergence of MDR and XDR strains, a prophylactic vaccine is much needed but is currently unavailable. Here, we demonstrate the immunogenicity and cross-reactivity of a tetravalent *Shigella* OMVs oral immunogen against all serotypes of circulating *Shigella* strains. The immunogen induced a Th1/Th17 biased immune response in BALB/c mice, protecting them from lethal challenge.

The literature suggests a number of candidate vaccines with limited success. Significant efforts are also going toward developing whole-cell bacterial vaccines, either inactivating by formalin treatment or by gathering multivalent heat-killed *Shigella* strains (HKMS).[22,23] Live attenuated strains have been devised by mutating genes, such as guaBA, virG, and genes important for LPS and O-antigen productions.[24-26] Development of vaccines against shigellosis continues with numerous conjugate candidates having different conserved proteins of the type three secretion system, such as IpaB, IpaC, IpaD, OmpA and O-antigen polysaccharides.[27-29] In the domain of new generation vaccines, Outer Membrane Vesicles (OMVs) already left their footprints under different names and forms.[7] Because of the presence of immunoreactive bacterial surface proteins and polysaccharides, OMVs have become desired molecules for vaccine formulation.[9] Generalized modules for membrane antigens (GMMA) derived from different gram-negative bacteria such as *Shigella*, Salmonella Enterica serovars *Typhimurium, Enteritidis*, and Paratyphi A, and *Neisseria meningitidis*, are reported as cost-effective, safe and protective candidate vaccines against respective bacterial infection.[30] GMMA derived from genetically modified *S. sonnei* entered clinical trial phase 2b and was reported to generate significant immune responses in adult human upon Intramuscular immunization.[31]

The reason behind diversified serotypes of *Shigella* is O-antigen present in the LPS.[32] To address the serotype specific cross-protection issue of *Shigella*, an oral multivalent OMVs vaccine candidate was formulated from *S. dysenteriae* type 1, *S. flexneri* 2a, *S. flexneri* 3a, *S. flexneri* 6, *S. boydii* type 4 and *S. sonnei* phase I which provided passive protection in neonatal mice against all the four serogroups of *Shigella*.[13] However, Global Enteric Multicenter Study (GEMS) in African and South Asian countries revealed that 89.6% of total clinical cases are *S. flexneri* and *S. sonnei*.[15] Thirteen of the fourteen serotypes of *S. flexneri* share a common backbone of tetrasaccharide domain with 3 rhamnose and N-acetylglucosamine. Only *S. flexneri* 6 has D-galactose as the 3rd sugar of tetrasaccharide and N- acetylgalactosamine as the terminal residue.[33-35] A hypothesis was thus proposed that a quadrivalent *Shigella* vaccine, with the antigens of *S. flexneri* 2a, *S. flexneri* 3a, *S. flexneri* 6 and *S. sonnei*, would provide broad coverage against most of the *Shigella* strains currently responsible for clinical cases.[15] Based on the current epidemiological situation, we have designed a tetravalent *Shigella* OMVs immunogen derived from most circulating strains i.e., *S. flexneri* 2a, *S. flexneri* 3a, *S. flexneri* 6 and *S. sonnei* phase I. At the same time, we have advanced our study by introducing three doses in the place of four doses used for hexavalent *Shigella* OMVs immunogen. In this study, oral immunization with three doses of tetravalent OMVs successfully induced humoral and T-cell mediated immune responses against homologous and heterologous strains.

Here, immunoblot analysis showed that anti-OMVs sera raised in tetravalent OMVs immunized mice could recognize a number of antigenic proteins present in whole bacterial cells, bacterial membranes and OMVs. Immune-dominant protein bands representing the immunogenic factors such as Ipa B (62 kDa), Ipa C (42 kDa), Ipa D (38 kDa), Omp A (34 kDa), Vir G (120 kDa) showed more prominence [13] These proteins present in *Shigella* are responsible for controlling T3SS machinery, invading the host cell and further inflammatory phenomena.[4] Hence, our tetravalent *Shigella* OMVs, possessing of these conserved immunogenic proteins, could be a potent immunomodulator in the host. In addition, IpaB and IpaD have proved their potential for a robust induction of mucosal and systemic immune responses against *S. flexneri* and *S. sonnei*.[36] In concert, OmpA protein of *S. flexneri* 2a exerted promising passive protection in a mouse model.[29] Immunized sera not only recognized the immunogenic proteins of homologous *Shigella* strains but also of the heterologous ones, i.e. *S. dysenteriae* 1 and *S. boydii* 2, indicating the evoked immunogenicity across all the serogroups of *Shigella*. Simultaneously, it was witnessed that a minimum amount of tetravalent *Shigella* OMVs immunogen (5 µg each) was remarkably successful in activating the humoral immune arm by eliciting both systemic and localized immunoglobulins against both the protein content and serotype specific LPS. Antibodies in immunized mice also recognized the OMVs, OMPs and LPS of heterologous strains, which ultimately crossed the barrier of serotype specific immunogenicity issues against *Shigella* infection. Serum IgG can effectively eliminate *Shigella* approaching mucosa, by opsonisation in the presence of complement.[37] Increased IgG3 was mainly due to the antigenic protein content of OMVs whereas IgG2a antibodies were secreted because of the carbohydrates present in OMVs.[38] Both of them play vital roles in eliminating invasive bacterial infections. Secretory IgA prevents bacteria from breaching the gut epithelial barrier either directly by neutralizing them or by encapsulating the dividing cells of bacteria.[39] Evoked secretion of sIgA in stool samples of immunized mice confirmed the activated mucosal immunity offered by tetravalent OMVs. Anti-O antigen sera antibodies are responsible for the cross immunogenicity across heterologous serotypes of *Shigella*, as reported earlier.[40] Serum bactericidal activity proved the ability of tetravalent OMV immunogen induced complement mediated killing of *Shigella* via antibodies. The observed effect is seen due to the opsonization where complements are binding with opsonized bacteria and eventually killing them. A number of studies already reported that antibodies against LPS and other antigens of *Shigella* are involved in bactericidal activity providing clinical protection.[41] Hence, persistent antibody production following a three-dose immunization, against all the representative six serotypes of *Shigella*, strongly suggests that tetravalent *Shigella* OMVs can purvey immunogenicity covering almost all the serotypes and sub-serotypes of *Shigella*.

In this study, mouse splenic cells incubated with tetravalent OMVs immunogen showed significantly increased

production of pro-inflammatory cytokines TNF- α, IL-6, IFN-γ and IL-17. IFN-γ is an indispensable tool inducing microphase death, leading to other inflammatory cytokines to eliminate *Shigella* infection. Upregulation of IFN- γ activates the Th1 immune pathway, which is most important for suppressing an intracellular bacterial infection.[42,43] IFN-γ also provokes Nitric oxide (NO) production, engaging more phagocytic killing of bacteria.[44] IL-6 not only bridges the innate and adaptive immune arms but also promotes differentiation of Th17 cells from naïve CD4+ cells.[45,46] IL-6 was also found to strongly suppress regulatory T cells.[47] The Th17 immune pathway was proven to play a prime role in reducing pathogen growth. However, it was also recorded that *Shigella*-specific Th17 cells were independent of cytokines IL-12/23p40 and IL-23. Hence, the primed IL-17/Th17 pathway is indicative of mucosal protection-inducing secondary immune responses.[48] This again strongly suggests that the tetravalent OMVs immunogen has a higher tendency to work through cell-mediated immune pathways, which is a must to fight an intracellular infection.

We chose an intra-peritoneal adult mouse model for the challenge study. This non-surgical model was preferred because intra-peritoneal administration of bacteria induces both mucosal inflammation, producing human like diarrhoea in mice, and systemic infection, resulting in death.[21] Hence, this model is apt for vaccine efficacy studies. However, natural infection is caused through the fecal-oral route in humans with a very small amount of bacterial inoculum i.e., 10-100 microorganisms. In the protection study, when mice were challenged with virulent homologous and heterologous *Shigella* strains, immunized mice showed no or mild diarrhoea. On the other hand, non-immunized mice developed human-like diarrhoea due to severe infections in mucosal compartments. Tetravalent *Shigella* OMVs reduced intracellular invasion and bacterial dissemination leading to several fold lesser bacterial colonization in tissues of large intestine and liver of immunized mice. We found non-immunized mice had prominent shortening of colon length, which is a marker of potent inflammation. On the contrary, immunized mice showed less or negligible colon shortening.[21] Our immunogen could effectively cut down the classical inflammatory signs, like reduction in goblet cells, hyperplasia and cellular destruction. Significantly higher survival rates in immunized mice than in non-immunized mice indicates protection exerted by the tetravalent OMVs immunogen against the systemic spread and sepsis caused by *Shigella*.[49,50] Research never stop, we are working on challenge of poor efficacy of oral vaccines in developing countries, we have evaluated two mucosal adjuvants, rCTB (recombinant cholera toxin B-subunit) and mmCT (multiple mutated cholera toxin) in animal immunogenicity and protective efficacy studies. Orally administered *Shigella* vaccine with either adjuvants, rCTB or mmCT, induced robust mucosal and serological immune response in mice and provided protection against *Shigella* challenge. The vaccine's protective efficacy and immune response with mmCT as adjuvant was fairly excellent.

In summary, the presence of bacterial surface proteins and LPS makes tetravalent *Shigella* OMVs immunogenic and self-adjuvant in nature. Highly accelerated and long-lasting antibodies in tetravalent OMVs immunized mice proved the capabilities of the immunogen to provoke both systemic and mucosal humoral immunity with a tendency biased towards the Th1 pathway, which is especially crucial for an intracellular pathogen like *Shigella*. An active protection study in a non-surgical and potent intra-peritoneal mouse model also revealed that oral immunization of tetravalent OMVs conferred protection against systemic infection and mucosal inflammation caused by *Shigella*. However, an investigation on the mechanism of protection observed in this study will be further required in primate preclinical trial. Protection study against other serotypes and sub-serotypes of *Shigella*, which are not used here, will also strengthen the cross-protective ability of tetravalent OMVs immunogen which will help in further clinical studies and future commercialization.

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