Immunogenicity and efficacy studies of typhoidal Salmonella immunogen in animal models

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by

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"Immunogenicity and efficacy studies of typhoidal Salmonella immunogen in animal models"** Submitted by **Sri** / ~~Smt.~~ **Prolay Halder** who got his / ~~her~~ name registered on **2nd September on 2019, (Index No.: 58/19/Life Sc./26, Registration No. SLSBT1205819 Dated: 02.09.2019)** for the award of Ph. D. (Science) Degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Hemanta Koley** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

06/05/2026

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Dedication

I dedicate my thesis to almighty, my grand parents', parents and family.

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Contents

Abstract

Title of Thesis: "Immunogenicity and efficacy studies of typhoidal Salmonella immunogen in animal models"

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The severe intestinal disease typhoid, along with the growing paratyphoid fever, has a high rate of morbidity and mortality globally. There are licensed typhoid vaccines on the market, but there isn't one as of yet for paratyphoid. In the current work, we used a bacterial ghost platform to create a bivalent vaccination against *Salmonella* Typhi and *Salmonella* Paratyphi A. Bacterial ghost cells (BGs) are cell membranes generated from bacteria that are devoid of cytoplasm and yet maintain their whole cellular shape, including all surface characteristics. Moreover, the intrinsic adjuvant qualities of BGs stimulate a stronger humoral and cellular immunological response to the target antigen. *Salmonella* Typhi and *Salmonella* Paratyphi A ghost cells were prepared using sodium hydroxide. Electron microscopy was used to characterize the bacterial ghost cells. BALB/c mice were immunized intraperitoneally with the bivalent typhoidal bacterial ghost cells on the 0^{th}, 14^{th}, and 28^{th} day of the immunization schedule. An investigation of the hematopathology of adult mice during the immunization period revealed that the immunogen was safe and had no negative effects on the animals' health. Significant amount of increased, serum and secretory antibody titers against the outer membrane protein, lipopolysaccharide, whole cell lysate of both bacteria and Vi-polysaccharides of *Salmonella* Typhi were detected after complete immunization. In comparison to non-immunized control mice, bivalent typhoidal ghost cell-immunized animals exhibited improved survival, reduced bacterial colonization in systemic organs, and less inflammation and/or tissue degradation in histopathological investigation. Serum antibodies from animals that have received vaccinations have superior killing capabilities against *Salmonella* Typhi and *Salmonella* Paratyphi A, and they can dramatically reduce bacterial motility and mucus penetration ability. Significant passive protection was also seen when immunized animals' lymphocytes and serum antibodies were adopted and transferred to naïve animals following bacterial infection. Result from the ex-vivo studies revealed that, splenic cell of immunized animals showed an increase in $CD4^+$, $CD8a^+$, and $CD19^+$ cell populations. We also observed a dominant IgG2a antibody response over IgG1 in serum of immunized animals. On the other hand, there is a strong Th1-Th17 response in immunized mice was obtained from dendritic cell and splenic cell restimulation, and co-culture study. For the long-term memory response following vaccination, we have evaluated T cell memory response. Significant induction of T effector memory response suggested that the proposed vaccine was effective over the long run. Overall, Bivalent Typhoidal Bacterial Ghost cells (BTBGs) are safe and augmented protective humoral and mucosal antibody response, induces cellular immunine response as well as stimulate memory responses. The newly formulated bacterial ghost cell-based vaccine is protective against both *Salmonella* Typhi and *Salmonella* Paratyphi A infections as well. This vaccine formulation acts as bivalent vaccine candidate and can be used to prevent typhoidal and paratyphoidal infectons near future.

Hemanta Koley 06/05/2026

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Chapter 1:

Introduction

1. Introduction: One of the largest genera in the bacterial kingdom, *Salmonella* has more than 2,500 serovars [1]. It stores contaminated food and unclean water, both from natural sources and from human usage. The two primary anthropogenic factors contributing to the infection are unclean food handling practices and inappropriate use of sanitary water. Both host-specific and host-adapted specialists as well as their generalist counterparts are found in these genera. A more severe acute gastrointestinal illness is caused by generalists, primarily by Non-Typhoidal *Salmonella* (NTS), while systemic disease is produced in hosts by specialists, primarily by Typhoidal *Salmonella* (TS). Specialized human host-restricted obligate bacteria, *Salmonella* Typhi (referred to as *S.* Typhi) and *Salmonella* Paratyphi A, B, and C (referred to as *S.* Paratyphi A, B, and C), cause enteric fever [2]. The World Health Organization (WHO) has designated Typhi as the 8th most significant pathogen that needs priority investigation, based on studies on antimicrobial drug resistance patterns [3]. *S.* Typhi is more endemic and various modelling studies have assessed the worldwide estimated risk burden was 20.6 million (17.5–24.2) cases and 223,000 (131,000–344,000) deaths [4]. A pooled assessment of local area-based reports in India proposed a paratyphoid event of 105 for every 100,000 people yearly, signifying the importance of S. Paratyphi (A, B and C) [5]. As of right now, *S.* Typhi patients can choose from three licensed vaccines that exactly target Typhi; however, none of them protect against *S.* Paratyphi A, B, or C [6]. Live attenuated Ty21a, or the galE mutant of the mother strain Ty2, was the first typhoidal vaccine to be licensed. For seven long years, it provided moderate protection (≤ 70%), but if it reverted to its wild type form, it could wreak havoc. Before becoming the vaccine of choice, other licensed vaccinations, such as the tetanus toxoid conjugated vaccine, Vi-conjugate and the Vi-polysaccharide (ViPS) vaccine, must overcome certain disadvantages. Although Vi vaccinations are ineffective against *S.* Paratyphi A, B because they lack the outer Vi-polysaccharide (ViPS) layer. Due to financial constraints, these subunit vaccinations are not as commonly used in developing nations [6]. There appears

to be an increase in paratyphoid infections in Asian and Sub-Saharan nations, according to recent research [7]. In order to prevent infection from both typhoid and paratyphoid, a more bivalent strategy is needed.

It is difficult in many ways to work on a rigorous human host-restricted pathogen. Human models could not be used due to strict ethical issues. In order to replicate the disease pattern in humans, an infection must first be developed in the model animal before using it. A number of pseudogenes that are highly active in their NTS counterparts are present in TS, which causes host-restriction [2]. These genes result in a strong immune response in humans after NTS infection, but not in TS infection. Because of this, several animal hosts all of which were artificial were employed in the past to test their effectiveness. Although the iron overload adult mouse model closely resembles the biology of TS infection in humans, NTS is actually employed to guarantee systemic infection in mice [8]. As *S.* Typhi and Paratyphi A are human-restricted pathogens, which is a major obstacle, we need to work with humanized mice model. But some important limitations of this model should be recognized. The individual engraftment of new-born mice is an expensive and labour-intensive process, significant subject to subject variation maybe seen because of the genetic heterogeneity of donors and degrees of engraftment. Finally, a chimeric immune system in which murine (epithelial and stromal cells) and human hematopoietic cells co-exist may create artefactual interactions [9-11]. Other hand, after adult mice receive an intraperitoneal (IP) injection of TS, the systemic character of the infection can also be replicated in the mice [12]. Along with that, there is a strong association of dose-dependent response with colonization rate as well. In the time-dependent approach, for both typhoidal *Salmonella* strains to be present in the spleen and liver after 2–3 days. Overall vaccine safety, the vaccinated mice showed excellent health post challenge with a heterologous *Salmonella* strains with minimal weight loss [13,14].

In this study, Bacterial ghost was prepared from *Salmonella* Typhi and *Salmonella* Paratyphi A by minimal inhibitory concentration of chemical treatment. Scanning electron microscopy (SEM), SDS-PAGE, and western blot were performed to characterize the immunogen. Cytotoxicity assay was performed in murine macrophage cell line by lactate dehydrogenase assay. The bivalent vaccine was administered intraperitoneally into the white albino mice and serum antibody titers against anti-outer membrane protein, anti-lipopolysaccharide and anti-Vi-polysaccharide (ViPS) were measured by enzyme-linked immunosorbent assay (ELISA). Protective efficacy of the vaccine was evaluated by the challenge with heterologous wild type strain in intraperitoneal mouse model. I have also checked the immunogenicity of the said immunogen using different cytokine assays, surface marker expression by FACS, Western blotting, and ex vivo, in vitro and in vivo animal studies. I have showed that, three doses of the said immunogen protect the mice from wild type bacterial infection [14]. The passive immunization of naïve animals with immunized serum and splenocytes have a very good impact on in-vivo bacterial challenged against both *Salmonella* Typhi and Paratyphi. The long-term efficacy of the bacterial ghost cells (BGs) also evaluated against *Salmonella* Typhi and Paratyphi. Bactericidal test of immunised serum in presence of baby rabbit complement have a significant effect that would indicate the usefulness of bivalent typhoidal bacterial ghost cells (BGs) immunogen to combat the Typhoidal *Salmonella* induce health burden globally.

Chapter 2:

Review of literature

2.1. Background: Infectious diseases have been one of the worst sources of morbidity and mortality in humans since the Middle Ages [1]. Typhoidal *Salmonella* was known to cause infection and death in the same historical period (the 5th to the 15th century), and it continued into the early modern period (15th to 18th century). As early as 430 BC, some historians assume that this illness caused a massive pandemic in Athens [1]. Three regions have been found worldwide: low-, medium-, and high-incidence areas. According to another study, *Salmonella* has been around since the 1200s. It was discovered that the remains of a young woman's buried body had *Salmonella* genome remnants. One of the deadliest bacterial outbreaks in Mexican history during the 16th century was triggered by *Salmonella* [2]. The buried bodies' DNA revealed the presence of *Salmonella* Paratyphi C. It is also possible to trace the existence of *Salmonella* Paratyphi C back to Norway in the thirteenth century. Currently, if treatment is not received, enteric fever caused by *Salmonella* Paratyphi C results in 10–15% mortality [3]. Despite being named for Daniel Elmer Salmon of the United States Department of Agriculture (USDA), Karl Joseph Eberth was the first person to identify the genus in 1879 [4]. The presence of the bacterium that causes typhoid fever in the afflicted individuals was shown by Dr. Willium Bud. When this infectious agent is transmitted, infection spreads to the naïve population. During this period, hog cholera was referred to as *Salmonella*. Subsequently, in the 1900s, a chef by the name of Mary Mallon—a chronic carrier—caused seven epidemics in a mere six years prior to being imprisoned in her own home until her death [5].

2.2. Nomenclature and taxonomical classification of *Salmonella*:

One example of the most intricate nomenclature scheme in the bacterial kingdom is seen in the genus *Salmonella* (**Fig. 1**). It has more than 2500 serovars, comprising both non-typhoidal *Salmonella* (NTS) and Typhoidal *Salmonella* (TS), as was previously stated. The fundamental categorization relies on Kauffmann's (1953) one serotype-one species theory, which is based

on the serological identification of the flagellar (H) and somatic (O) antigens. Each serotype was regarded as a distinct species in this instance [6].

Repeated oligosaccharide units found in Gram-negative bacteria's outer membrane make up the O antigen, which is a component of lipopolysaccharide (LPS). ABC transporter, various synthases, and proteins from the Wzx/Wzy pathway are all involved in the production of O antigens. In all, 37 of the 46 O serogroup structures in the *Salmonella* genus are known. The O units typically include three to five sugar residues and can have a linear, branched, or double branched structure. The most prevalent residues are L-rhamnose, D-glycerol, D-mannose, and D-galactose. fliC and fljB, which express the phase 1 and phase 2 antigens of the bacterial flagella, respectively, are the two primary genes involved in H or flagellar antigen synthesis. A single gene product, or phase, is present in a given bacteria at a given moment due to the regulation mechanisms of these two genes. According to the Kauffmann-White scheme [6], there are currently 114 recognized kinds of H antigens.

A different method for classifying *Salmonella* was employed by Crosa in 1973, and it involved the use of DNA hybridization. Le Minor and Popof recommended in 1987 that *S.* Typhimurium LT2 should be the type strain of *Salmonella*, and that *S.* enterica should be the primary type species rather than *S.* cholerasuis [6]. The genus *Salmonella* was categorized as follows following a plethora of further suggestions and deliberation:

Domain: Bacteria,

Phylum: Proteobacteria,

Class: Gammaproteobacteria,

Order: Enterobacteriales,

Family: Enterobacteriaceae,

Genus: *Salmonella*,

Species and subspecies: *Salmonella* enterica

- *Salmonella* bongori.
- *Salmonella* enterica –

S. enterica subsp. arizonae

S. enterica subsp. diarizonae

S. enterica subsp. enterica

S. enterica subsp. houtenae

S. enterica subsp. indicaS. enterica subsp. Salamae

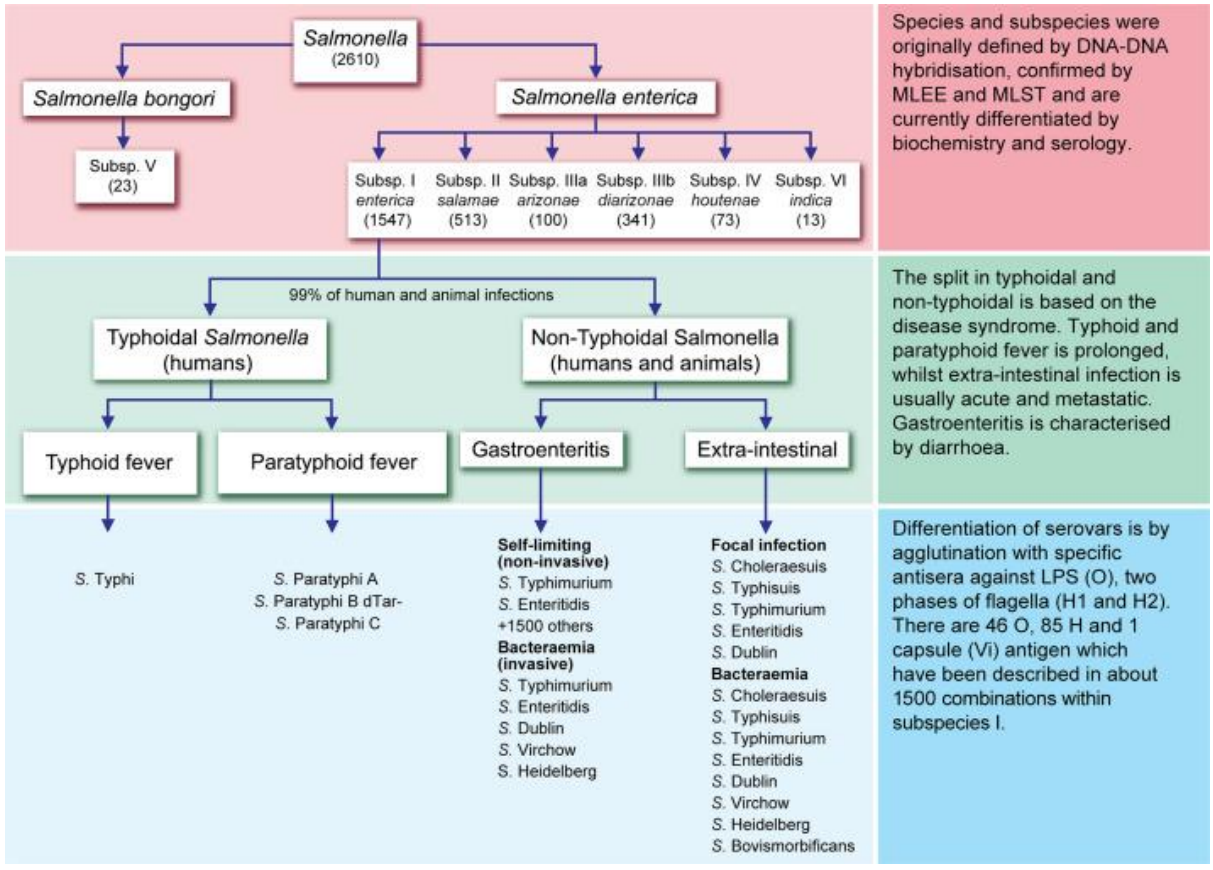


Figure 1. Classification of *Salmonella*. With more than 2500 serovars, *Salmonella* constitutes the largest genera in bacterial kingdom. With both NTS and TS, this group can cause a range of physiological distress from gastroenteritis to enteric fever. (Image adapted from "Achtman, Mark et al. "Multilocus sequence typing as a replacement for serotyping in Salmonella enterica." PLoS pathogens vol. 8,6 (2012): e1002776. doi: 10.1371/journal.ppat.1002776")

2.3. Epidemiology:

The number of new cases per population at a given moment is referred to as the disease's incidence. Conventionally, it is reported as cases per 100,000 inhabitants annually and classified as low, medium, high, and very high, which correspond to incidence bands of less than 10, 10–100, > 100–<500, and more than 500 per 100,000 populations annually, respectively (**Fig. 2**). In order to calculate the burden of disease, blood culture is used to estimate the population-based incidence of disease [7].

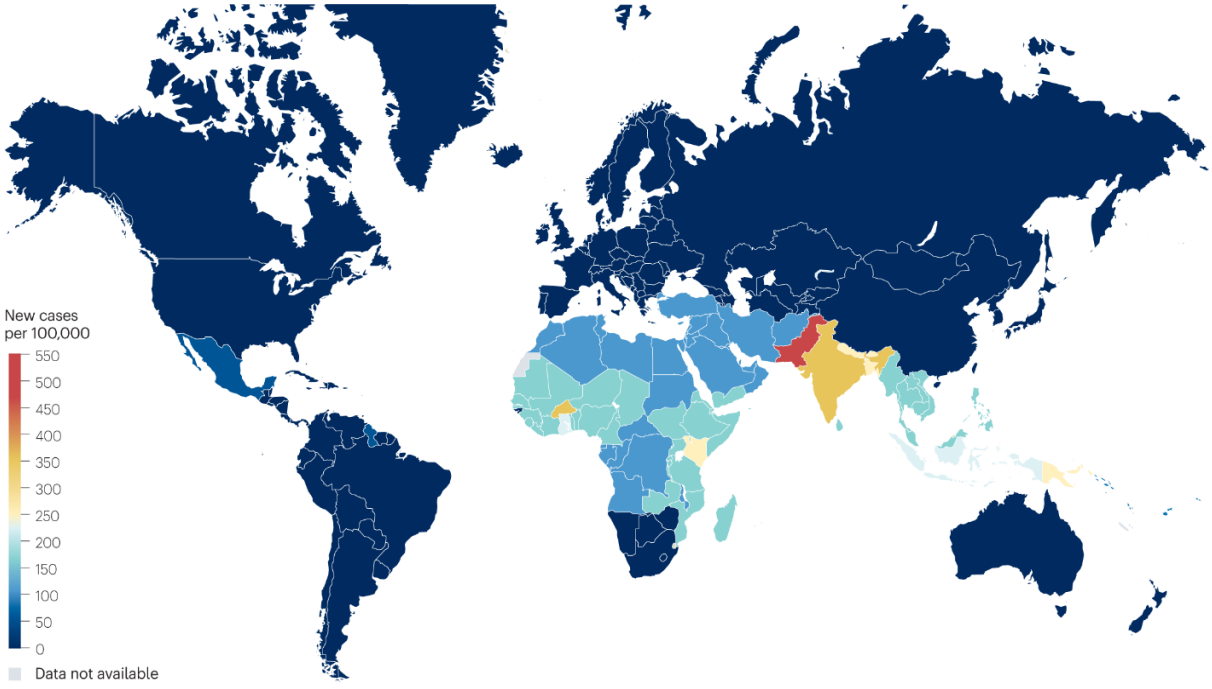


Figure 2. Current epidemiological scenario of typhoidal *Salmonella* globally. The presence of typhoidal *Salmonella* has been documented over the whole world with high incidence of disease occurring in Asian and African countries. Middle incidence zones are found in South-American countries with low incidence happening in the developed world. (Image adapted from "Meiring, J.E., Khanam, F., Basnyat, B. et al. Typhoid fever. Nat Rev Dis Primers 9, 71 (2023). https://doi.org/10.1038/s41572-023-00480-z")

WHO estimates from recently indicate that the incidence of typhoid fever was between 11 and 20 million cases, with approximately 161000 deaths reported worldwide [8]. Enteric fever is expected to affect 17.8 million out of the 5.5 billion people who live in low- to middle-income countries (LMIC) annually. Typhoidal fever is far less common in Europe (<0.1 per 100,000 people), although it can occur in South Asian and Sub-Saharan African nations (up to 724.4 per 100,000 people) [7].

The basic pattern of *Salmonella* Typhi being the primary cause of enteric fever has changed during the last few decades (**Fig. 3**). *Salmonella* Paratyphi A was reported to have emerged in India in 2003, however the tendency reversed itself in 2005. South Asian nations continued to report similar trends with *Salmonella* Paratyphi A infection findings [9]. Approximately half of the isolates were sometimes *Salmonella* Paratyphi A, which would have been unanticipated a few years ago. However, due to economic hardship and adequate hygienic conditions, typhoidal illnesses are declining in certain Latin American nations. Moreover, knowledge of the origins, trends, and mechanisms of *Salmonella* transmission in various contexts is emphasized by sanitation improvements, appropriate water use, and the introduction of vaccinations. Furthermore, as was previously mentioned, there is a possibility that the H58 haplotype will spread in the future, which would complicate the treatment plan.

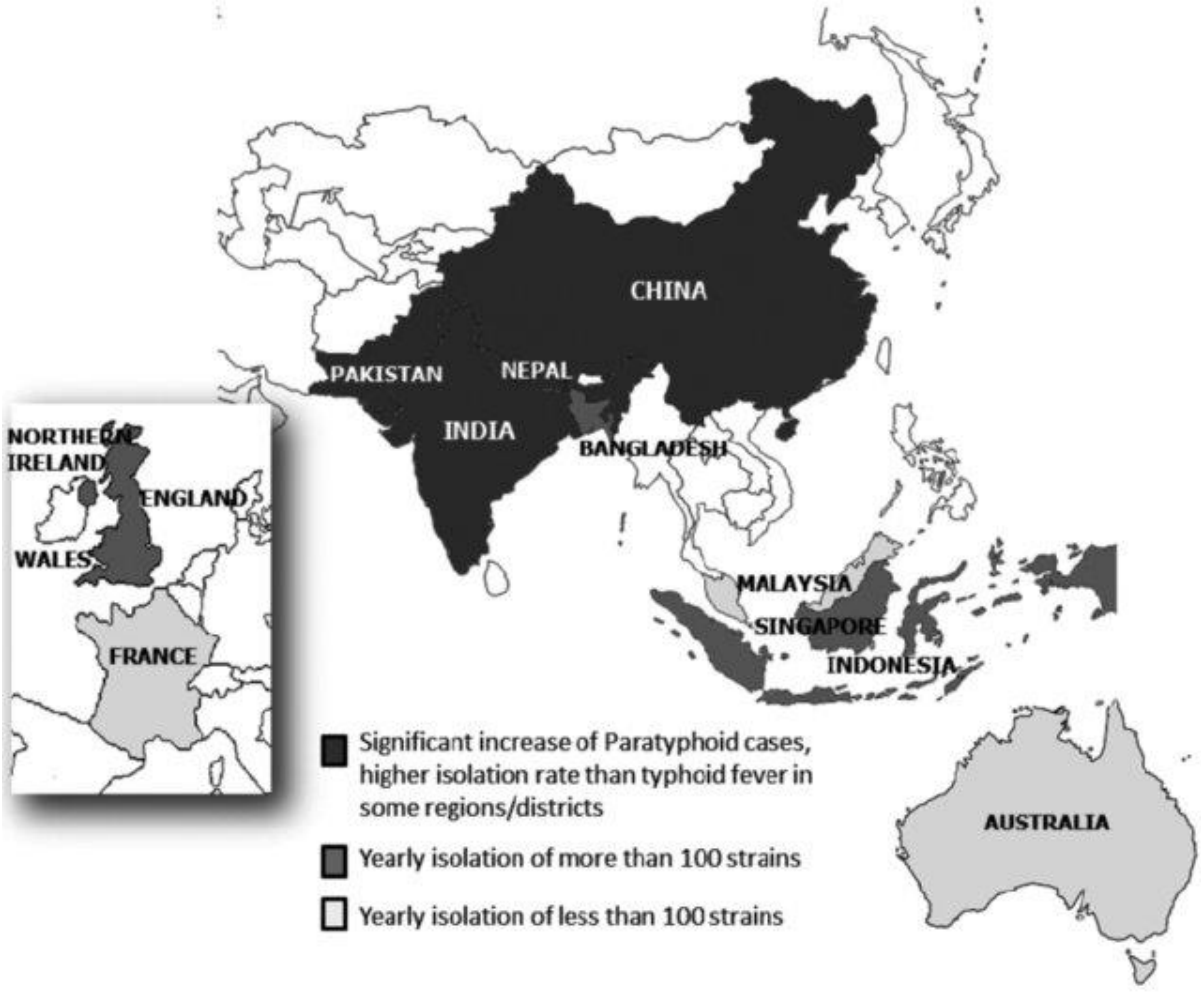


Figure 3. Presence of Paratyphi A in the South-East Asian countries –shift of etiological agent: Enteric fever has long been attributed to the infection from S. Typhi, but recent reports suggest a shift in this trend. The main etiological agent for enteric was S. Paratyphi A, but not S. Typhi in South-East Asian countries. (Image adapted from "Teh, C.S.J.; Chua, K.H.; Thong, K.L. Paratyphoid Fever: Splicing the Global Analyses. Int. J. Med. Sci. 2014, 11 (7), 732-741. DOI: 10.7150/ijms.7768.")

2.4. *Salmonella* Typhi specific virulence factors:

Salmonella Typhi's virulence factors are involved in the generation of vi-antigen, typhoid toxin (toxins (LPS) endotoxin, enterotoxin, and cytotoxin), flagella, Type Three Secretion Systems as well as colonization, adhesion, and penetration of host cells, during the different stages of infection (**Fig. 4**)

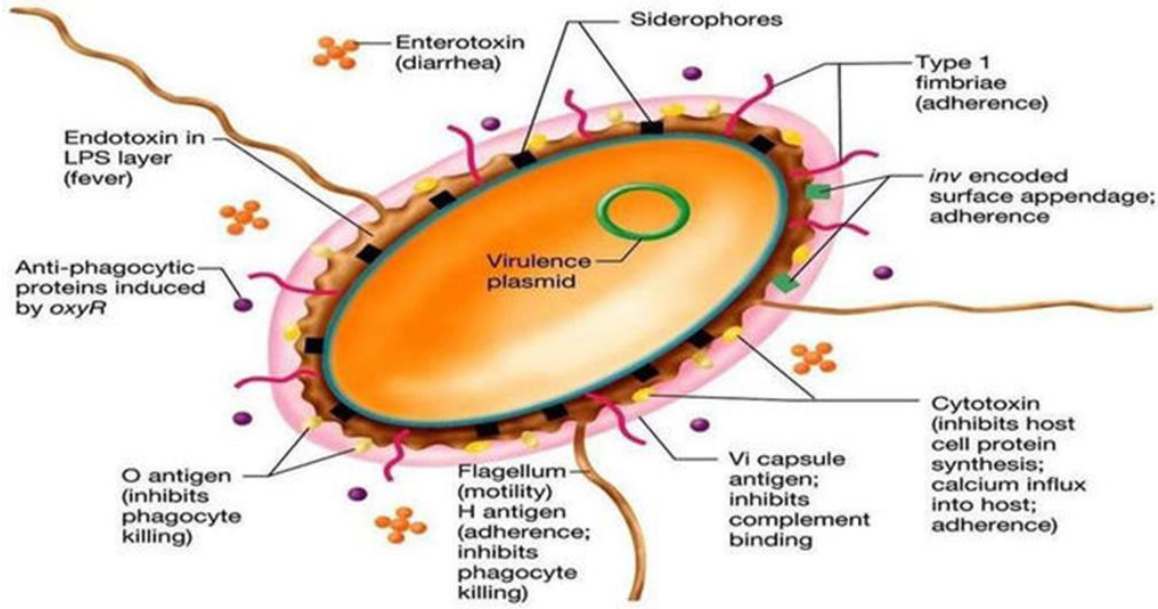


Figure 4. ***Salmonella*** **virulence factors.** (Image adapted from "Madigan M., Martinko J. (2007). Biologie Des Micro-Organismes. 11 Ème Édition. Pearson, Paris. P: 731-735, 790-792, 943, 947-948").

2.4.1. The Vi antigen. The existence of a polysaccharide capsule known as Vi polysaccharide is the primary morphological distinction between *Salmonella* Typhi and other *Salmonella* strains. This is the outer layer of *Salmonella* Typhi that prevents the bacterial phagocytic cells from consuming the encasement and shielding of the O-antigen. Within *Salmonella* Pathogenicity Island 7 (SPI-7), the viaB locus contains the genes that produce this capsule. The genes tviA, tviB, and vexABCDE, which are in charge of exporting and retaining Vi on the surface of the bacteria, are all encoded by the viaB locus [10].

The viaB locus is not found in *Salmonella* Paratyphi A or other non-typhoidal *Salmonella* (NTS), however it is found in *Salmonella* Dublin and *Salmonella* Paratyphi C. In addition to downregulating the expression of the SPI-1 and flagellar genes, the regulator tviA also increases the expression of the viaB locus. *Salmonella* Typhi exhibits a notable variation in the Vi polysaccharide expression. Vi expression is lacking in the intestine, where flagellar and SPI-

1 genes are necessary for colonization and disease progression in the early stages. However, in the case of a systemic infection, both the SPI-1 gene expression and the presence of the Vi are missing. This aids the bacteria in continuing to elude the host immune system by remaining covert. As a result, pyroptosis takes place and the cells cease the macrophages' production of IL-1β. Vi binds to prohibitin, which inhibits the release of MAPK and IL-8 and reduces inflammation. The characteristic lack of neutrophil inflow observed in typhoidal *Salmonella* infections is also highly prevalent in non-typhoidal *Salmonella* (NTS) infections. However, because the viaB locus is lacking in *Salmonella* Paratyphi A, this Vi polysaccharide is absent as well. Their LPS layer's long O chain is elaborated to prevent antibody binding and enhance complement activation [10].

2.4.2. Typhoid toxin. *Salmonella* Typhi activates SPI-11 genes to begin expressing its only toxin while it is still within SCVs. The AB type typhoid toxin is made up of one homo-pentamer binding subunit (PltB) and two enzymatically active A subunits (CdtB and PltA). While PltA has ADP-ribosyltransferase activity, the CdtB toxin is identical to the cytolethal distending toxin A component (16). Due to its DNase I activity, CdtB damages DNA in host cells and triggers G2/M cell cycle arrest. *Salmonella* Typhimurium and *Salmonella* Enteritidis lack a homolog of this toxin, although *Salmonella* Paratyphi A and some non-typhoidal *Salmonella* (NTS) do. This toxin, as previously stated, is released when the bacteria remain inside SCV and, upon export, it intoxicates nearby infected and uninfected cells via autocrine and paracrine pathways. The most likely mechanism for its binding and entrance into the cells is through PltB-mediated binding to glycans that ends with N-acetylneuraminic acid (Neu5Ac). Typhoid toxin receptors have been found on human epithelial cells that have glycosylated podocalyxin like protein 1 (PODXL) and immune cells that carry CD45. Typhoid infection host specificity is determined by the presence of Neu5Ac, which is exclusively found in humans. There is a

positive correlation between chronic carriers of *Salmonella* Typhi and gall bladder cancer, thus it would be interesting to investigate if the typhoid toxin has any influence in this disease.

2.4.3. Flagella. Recognition of flagella by the host's innate immunity is a significant pathogenicity component. Type H antigen-producing *Salmonella* are primarily monophasic, expressing just FliC, whereas the majority of non-typhoidal *Salmonella* (NTS) are biphasic, expressing both FliC and FljB. A fliC gene in-frame mutation causes some *Salmonella* Typhi of Indonesian origins to produce H: j. Some strains also exhibit H: z66, another antigenic variant, as a result of evolution in this highly endemic region, which results in the development of a plasmid-encoded fljB analogue [10]. This mutation modifies the way *Salmonella* Typhi interacts with macrophages and epithelial cells, aiding in the bacteria's ability to evade the immune system. In *Salmonella* Paratyphi A, flagella not only aid in adhesion and invasion but also in the production of SPI-1.

2.4.4. Type Three Secretion Systems (T3SS). SPI-1 and SPI-2 are two crucial Salmonella TTSS mechanisms. SPI-1 is necessary for epithelial cell invasion in both non-typhoidal *Salmonella* (NTS) and typhoidal *Salmonella* (TS), and SPI-2 is necessary for intracellular survival and replication in non-typhoidal *Salmonella* (NTS). However, the necessity of SPI-2 in typhoidal *Salmonella* (TS) is questionable because *Salmonella* Typhi was reported to be surviving in THP-1 and human monocyte-derived macrophages without SPI-2. It's interesting to note that disruption of ssaQ, ssaP, or ssaNvia transposon insertion has a negative effect on human macrophage competitive growth characteristics. Apart from these, TTSS control in non-typhoidal *Salmonella* (NTS) and typhoidal *Salmonella* (TS) differs significantly. The regulation of SPI-1 genes varies between non-typhoidal *Salmonella* (NTS) and typhoidal *Salmonella* (TS). Bile induces the existence of the regulator HilD, whose activity is three times more in typhoidal *Salmonella* (TS) than in non-typhoidal *Salmonella* (NTS). This TTSS regulator differential expression helps to explain why typhoidal *Salmonella* (TS) is present in

the gall bladder but not in non-typhoidal *Salmonella* (NTS). The presence of gall bladders and variations in their expression when exposed to bile salts suggest that typhoidal *Salmonella* (TS) behaves in a controlled manner when intestinal inflammation occurs. Another element that influences TTSS expression is the presence of oxygen. While SopE expression is completely necessary for typhoidal *Salmonella* (TS) growth in the microaerophilic state, it is less necessary in the aerophilic state. Due to the intestinal lumen's microaerobic nature and the epithelium's greater aerobic content, this plays a significant role in the regulation of intestinal inflammation. The host's body temperature influences the expression of many TTSS effectors. While non-typhoidal *Salmonella* (NTS) is unaffected, typhoidal *Salmonella* (TS) invasion and motility are decreased at fever-like temperatures (39–42°C). At 42°C, the expression of Paratyphi A SPI-1 decreases, whereas its SPI-2 expression rises [10]. This is because intracellular survival takes precedence over SPI-1 once fever has started. This genus is ideal for stealth infections because of all these rules (**Fig. 5**).

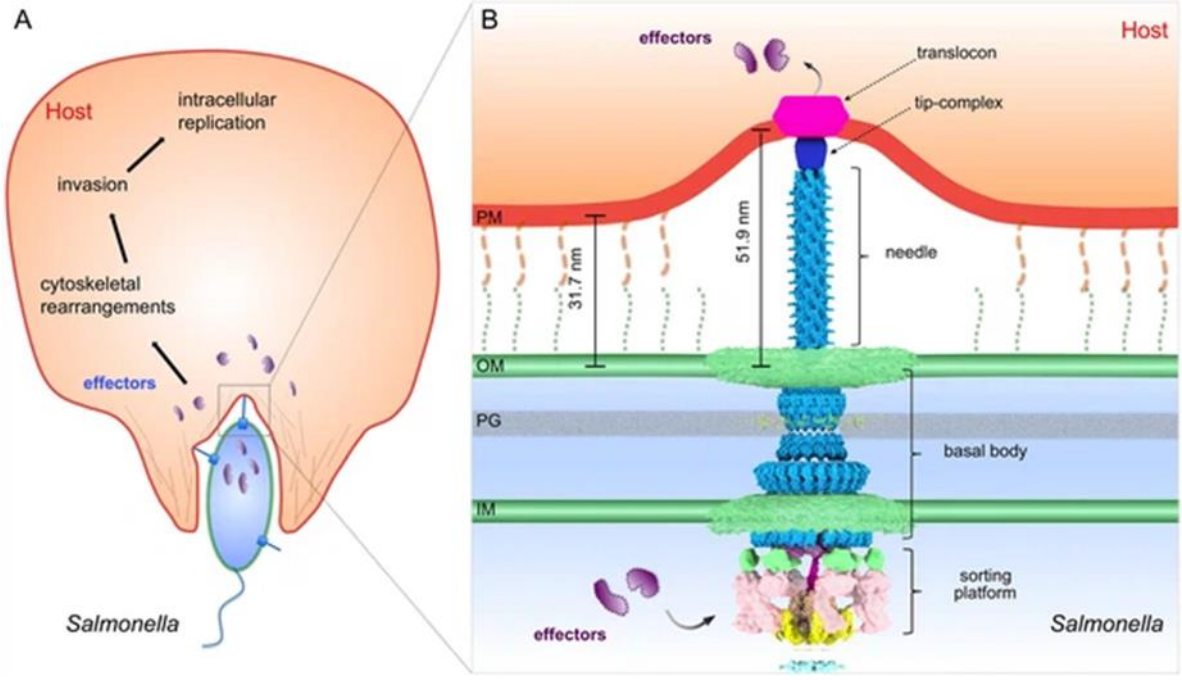


Figure 5. An illustration of how the injectosome of *S.* Typhimurium interacts with the host cell membrane. (A) A schematic illustration of the interactions between S. Typhimurium

and the host cell. (B) Molecular model of the Salmonella-host cell interaction T3SS injectisome. (Image adapted from "Donghyun ParkMaria Lara-TejeroM Neal WaxhamWenwei LiBo HuJorge E GalánJun Liu (2018) Visualization of the type III secretion mediated Salmonella–host cell interface using cryo-electron tomography eLife 7:e39514.")

2.5. Variables affecting the risk of enteric infection:

With its almost 200-fold bigger size than skin, the mucosa is the greatest place in humans where various infections can colonize the respiratory, digestive, and urogenital tracts. It has a big surface area that might be open to the outside world. The front-line barriers, which include tuft cells, Paneth cells, enterocytes, goblet cells, lamina propria, tight junctions, and epithelium, prevent any invasion from happening, whether it comes from a pathogenic microbe or commensal flora [11]. However, an additional layer of defence is offered by a mucus layer with varying densities. In biology and medicine, research on gut ecology has become a fascinating area of study. The following is a description of the different elements that can affect an enteric infection in this context:

2.5.1. Mucus. Mucus, which is secreted by the epithelium, is essential factor of host defence. Mucus layer, which is primarily responsible for shielding the host's epithelial cells from incoming microorganisms and retaining them on the luminal side of the intestine, is represented by the abundant presence of mucin glycoprotein together with other host defence molecules. The digestive systems are coated in this thick, intricate layer of mucus. Mucus secretion is modulated by immune cells through the release of cytokines and other stimuli, although mucus-secreting cells almost constantly renew the mucus layer. The main components that remain vigilant in the event of an external attack are a variety of antimicrobial components, such as α-defensins, cathlecidins, lysozymes, angiogenin 4, secretory phospholipase A2, etc., in addition to cell surface mucins, such as MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15,

MUC16, and MUC17, etc. [12, 13]. Enteric infections have developed a variety of defence mechanisms to effortlessly evade this barrier in spite of all these precautions [13, 14].

2.5.2. The intestinal epithelial cells (IECs) line surface monolayer. Simple columnar epithelial cells with polarization line the gastrointestinal system. The basement membrane and neighbouring cells communicate with each other through the basolateral side, which acts as a protective barrier against opportunists and invasive pathogens [12,15]. These host cells create a strong barrier that stops the intruder from penetrating at the beginning, posing a challenge to the invader. Intestinal epithelial cells protect the underlying mucosa while producing a variety of chemicals, including chemokines and cytokines, that aid the host's defence mechanism against the intruders. When a bacterial pathogen is first introduced into the host epithelium, it exhibits a few defensive characteristics, including prostaglandin synthesis, pro-inflammatory cytokine up- or down-regulation, and modulation of mucus formation, among others [16].

Given the little-known fact that prostaglandin E2 (PGE2) production is linked to diarrhea, it may appear a little unclear at first. However, they do produce more after contracting Shigella or NTS infections. Selectively, they function as synergistic agents of adhesion molecules, chemokines, and proinflammatory cytokines. Reducing IL-1 production while boosting TNF-α, GM-CSF, IL-8, MCP-1, and PGE2 synthesis aids in eliminating the invader microorganism with minimal damage to the host. Prostaglandin H synthase 2 (PGHS2) induction brought on by the bacterial infection would rather act as a barrier of protection than cause an increase in PGE2 output. Additionally, PGE2 indirectly controls mucosa formation, which acts as an additional barrier of defence during infection (**Fig. 6**) [16].

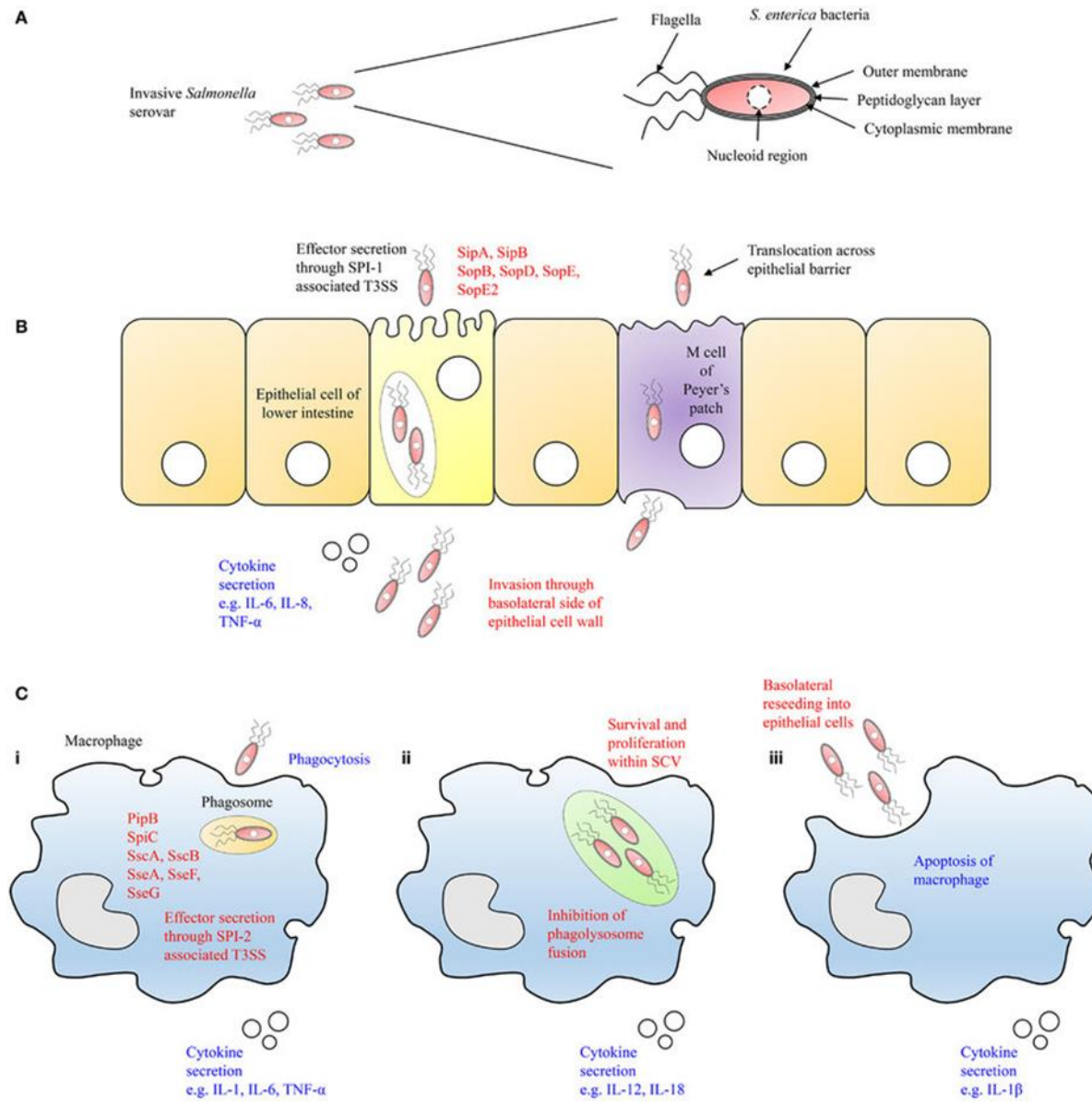


Figure 6: A schematic representation of the Salmonella infection of macrophages and lower intestine epithelial cells is provided. (A) Salmonella can live until it reaches the host's epithelial cell wall in the lower intestine thanks to its intricate membrane structure. (B) Salmonella then uses the T3SS-1 gene encoded by SPI-1 to secrete effector proteins that aggressively penetrate epithelial cells or translocate across M cells of Peyer's patches. (C) (i) Proximal macrophages consume Salmonella after they have crossed the epithelial barrier. These macrophages then discharge effector proteins into the cell's cytosol via the T3SS-2 encoded by SPI-2, preventing the phagosome from fusing with the lysosome. (ii) Salmonella

will multiply within the SCV, causing the macrophage to secrete cytokines. (iii) At last, the macrophage will die, allowing Salmonella to break free and re-invade the host innate immune system by basolaterally penetrating epithelial cells or other phagocytic cells. (Image adapted from "Hurley, Daniel et al. "Salmonella-host interactions - modulation of the host innate immune system." Frontiers in immunology vol. 5 481. 7 Oct. 2014, doi:10.3389/fimmu.2014.00481")

2.5.3. Tight junction. Intestinal polarized epithelial cells were found at the junction of lumen and the deep tissues of the body. As they are arranged in a typical manner side by side, they form a complex, also called the apical junction complex, consisting of tight junctions, adherens junctions and desmosomes [17]. Interaction between cytoskeletal elements (in the form of attachment between cytoplasmic adaptor proteins and actin filaments) with the apical junction complex strengthens and stabilizes the interaction. Apical junctions, regulated by cytokine response, often allow very small molecules, such as inulin, mannitoletc to pass through them but majority of "interested candidates" to delve inside the tight junction niche are being stopped from entering. Tight junction trans-membrane proteins, such as claudins, Tight junction Associated MARVEL proteins (TAMPs), Junctional Adhesion Molecules (JAMs) interacts with peripheral membrane proteins such as zonula occluding (ZO), Membrane Associated Guanylate kinase (MAGI) proteins and cingulin [18, 19].

Immunological and/or cellular events might harm the integrity of these closely spaced proteinaceous attachment particles. When this integrity breaks, an aberrant tissue exposure to luminal antigens and pathogens via the leaky gut might give response towards diseases such as Inflammatory Bowel Disease, Ulcerative colitis [20]. The same disruptive response of tight junctions in the course of a TS breach has the capability of sampling the invaders via the host intestinal dendritic cells (CX3CR1+ CD103-), which spreads their "arms" or trans-epithelial dendrites through these junctions [21]. Moreover, a leaky gut may also be able to pass the

invaders through them to the underlying soft tissues and cells, increasing the chance of actually causing harm to the host. Paracellular permeability is also dependent on IFN-γ and TNF-α, which in the course of infection, tends to rise high [22]. The modifications include altered actin structure, down-regulation of various tight junction proteins, among others [18, 19, 20]. Involvement of myosin motor is another important factor. This may change during infection with TS because of their “stealthy” nature which is described in detail in latter part of this review. It has also been seen for attaching and effacing pathogen to change the junctional stability and disrupt the junctions without any inflammation [23].

2.5.4. The "inhospitable" intestinal microenvironment: niche occupation and survival tactics. The physicochemical makeup and the availability of food sources in the gut determine the density and make-up of the microbiota. Together, these bacteria and the compounds they produce safeguard, preserve, and maintain the "pristine" state of the intestinal milieu. The barrier function, sometimes referred to as the protective function, makes sure that no further bacteria can adhere. Additionally, rivalry for available nutrients guarantees that harmful bacteria won't have any food [24]. These colonic bacteria's primary metabolic job is to break down big, indigestible carbohydrate molecules. The end products, or short-chain fatty acids, or SCFAs, are a vital source of energy for the colon. In vivo, the significant SCFAs acetate, butyrate, and propionate (3:1:1) promote both the proliferation and differentiation of epithelial cells. Additionally, produced are formate, lactate, and caproate, all of which support the "good gut" environment. Additionally, the presence of bacteria such as Firmicutes, Proteobacteria, and Bacteroides in this unique niche ensures the development of oral tolerance in the developing baby's gut, lowering the risk of autoimmune disorders [25, 26].

The delicate balance between commensal and potentially pathogenic bacterial communities is essential to human health. Despite the immune systems that are in existence, the pathogenic gut bacteria manage to breach the barrier created by the commensals and infect its hosts. Once

a pathogen is consumed by its host, it reacts with the stomach's hydrochloric acid and the gall bladder's bile salts, stabilizing itself through the bacterial acid resistance and efflux mechanism, respectively. To further lessen the impact, pathogenic bacteria subsequently alter the permeability of their membranes. The LPS layer was modified to protect host antimicrobial peptides (AMPs) [27]. Furthermore, the pathogen will proliferate more vigorously over commensals if aerobiosis is replaced with anaerobiosis and/or microaerobiosis (**Fig. 7**).

The location of the gut bacteria varies based on the immunochemical composition of the surrounding milieu. A healthy gut bacterial community is characterized by a preponderance of Bacteroides, Prevotella, Fusobacterium, and very few Proteobacteria [28].

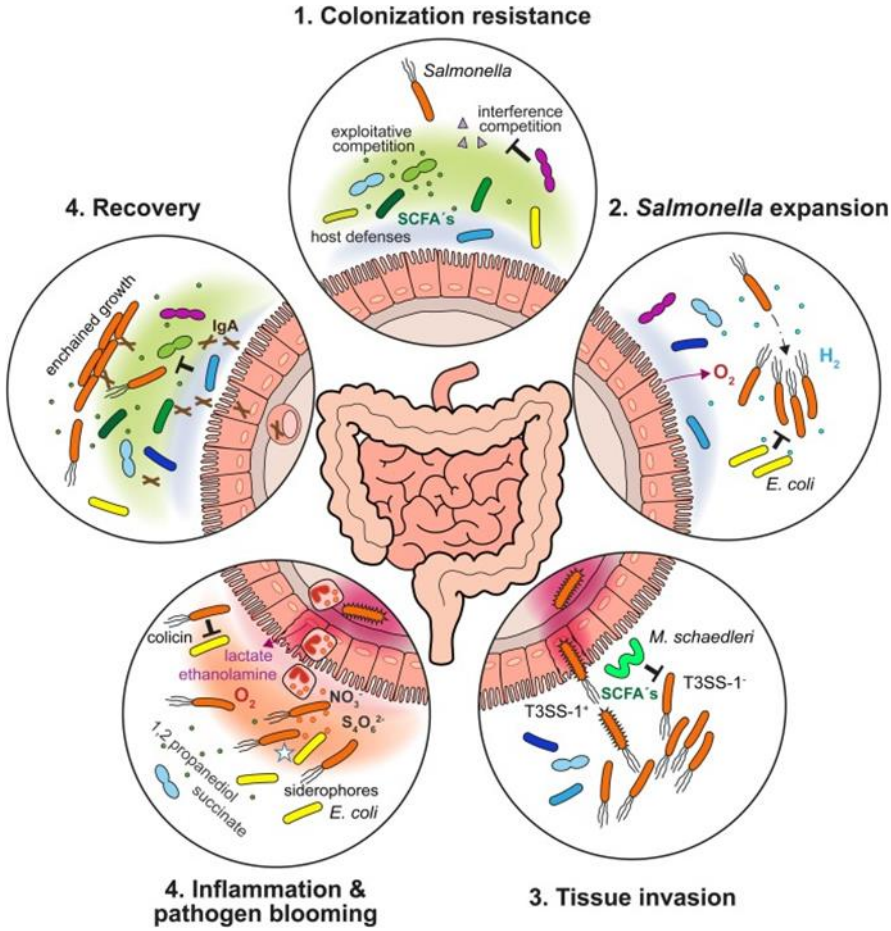


Figure 7. Five stages of *Salmonella* infection in the intestines. The stages are displayed one after the other in a clockwise manner. 1. Colonization resistance: Through interference, exploitative competition with the virus, modification of host immunological systems, and metabolite profiles, a diverse microbial community prevents S. Typhimurium invasion. 2. Salmonella expansion: S. Typhimurium may be able to reach high concentrations in specific conditions, such as when the microbiota is disturbed by antibiotics or nutrition, and it may use H2 as an electron donor to carry out both aerobic and anaerobic respiration. 3. Tissue invasion: S. Typhimurium uses its invasion machinery to dock onto the epithelium, break through the mucus layer, and infiltrate the mucosa through flagella-mediated chemotaxis. 4. Inflammation and Pathogen Blooming: Acute mucosal inflammation is brought on by S. Typhimurium invasion, intracellular replication, and particular virulence factors. This changes the gut luminal environment and encourages pathogen blooming at the expense of the anaerobic microbiota. 5. Recovery: The host's immune responses trigger the production of IgA specific to the pathogen, reduce inflammation, restore the microbiota, and drive the pathogen out of the body. (Image adapted from "techer, Bärbel. "Establishing causality in Salmonella-microbiota-host interaction: The use of gnotobiotic mouse models and synthetic microbial communities." International journal of medical microbiology: IJMM vol. 311,3 (2021): 151484. doi: 10.1016/j.ijmm.2021.151484")

2.5.5. Additional factors. Diet, gut microbiota, and epigenetics are all influenced by an invisible thread that influences the other three. Once food particles have been absorbed and partially digested in the stomach, microbes in our bodies start the real breakdown process. Final products of the microbiological breakdown of the food act as substrates for modifications in the epigenetic state. Generations to generation, epigenetic changes are inherited even though they are subtle. One of the repetitive epigenetic alterations that takes place inside a cell is the methylation of arginine and lysine, which initiates transcription.

Dietary changes can affect how an infant's microbiota develops. As a result of increased indigestible roughage introduced by a high-fiber diet, there is less contact between hazardous compounds and the gut epithelia. An increase in stool volume brought on by a high-fiber diet reduces the retention of stool and the transit time of microbial metabolic end products in the rectum, resulting in less interaction and a lower likelihood of any host alterations. Thus, bowel rest and faecal diversion are quite beneficial for patients with Chron's Disease (CD). Conversely, in a mouse model, a high-fat diet results in colitis. In mice, dietary histidine can lessen immune-mediated colitis. The correlation between intestinal diseases and food is evident from all of these observations [29, 30].

2.6. Pattern and mode of infection.

When the bacteria enter a human host, the infection chain is set in motion. The bacteria are primarily found in humans, where they can proliferate, exhibit virulence, and potentially develop a chronic illness. The bacteria can live for a very long time in the environment, despite their limited ability to reproduce and replicate outside of their human reservoirs. Along with pee, the main exit point is represented by faeces. *Salmonella* Typhi acute infections may resolve on their own, but if they are not treated, they can lead to bacteraemia, neuropsychiatric problems, and other consequences. Conversely, there exist two kind of carrier states: the convalescent carrier state and the chronic carrier state [31]. If a person sheds the bacilli between three and twelve months after an acute infection, they are considered convalescent carriers. After an acute infection, chronic carriers can occasionally have no symptoms and continue to release bacteria for up to 12 months [31]. Through food or water, typhoidal *Salmonella* can spread. Both short-cycle transmission and long-cycle transmission are possible varieties. Food and/or water can get contaminated in short-cycle transmission when faeces are shed nearby. Contaminated water is used in crop fields by long-cycle transmission; that is, the long-cycle transmission guarantees the involvement of more environmental elements. In low-incidence

nations, short-cycle transmission is typically observed in local infections, whereas long-cycle transmission is typically observed in high-incidence countries where polluted water serves as the primary source of infection [31]. This bacterium genus uses the host's mouth as a portal of entrance once the infection takes hold, and the risk of infection and illness rises with increasing inoculum dose.

2.7. Recognizing the distinction between feverish and diarrheagenic infections: In the context of enteric bacterial pathogenesis.

Salmonella that causes diarrhea is distinct from *Salmonella* that causes enteric fever in a number of aspects, including the acquisition of pseudogenes, clinical presentation, and altered immune regulation in the host organism. The duration of the infection varies as well; feverish infections often take 14 days to fully manifest and last for 3 weeks, whereas diarrheagenic infections only last a few days and require significantly less time to fully manifest (6–12 hours). Acute gastroenteritis and watery diarrhea are the only symptoms of the self-limiting (up to 10 day) non-typhoidal *Salmonella* (NTS) infection-induced gastroenteritis. These common symptoms can occasionally be accompanied by nausea, vomiting, and a fever. Conversely, the primary sign of enteric fever is a gradually developing persistent fever accompanied by a dry cough, splenomegaly and hepatomegaly, skin patches, diarrhea, or constipation [32]. The following lists the causes and mechanisms that distinguish these two types of illnesses from one another.

2.7.1. Genomes and Pseudogenes: Similar disease symptoms are caused by *Salmonella* Typhi and *Salmonella* Paratyphi A in humans, their host. In the past, around 20% of all infections caused by typhoidal infection were attributed to *Salmonella* Paratyphi A infection. However, using vaccines against *Salmonella* Typhi alone, which provides only a slight protection against *Salmonella* Paratyphi A infection, is crucial due to a number of factors, including the growth

in antibiotic resistance. *Salmonella* Paratyphi A infections have increased recently, primarily in South-East Asian nations, as a result of these two pathogens sharing the same bacterial habitat and host-specificity [33, 34]. Pseudogenes in the genomes of these two-human host-restricted pathogens are a major factor in their shared bacterial habitat. The apparent similarity between their genomes is ascribed to minimal sequence divergence in a particular region, specifically 23% of their genomes (mean of 0.18%). Their remaining genomes have a mean divergence of 1.2%, which is the same as that of the other organisms in the *Salmonella* enterica group. These two genomes are remarkably similar to one another due to a relatively recent convergence. It might not be mutually exclusive for these two infections to cause sickness in the same host at the same time, but it would be very challenging to determine if they were both responsible. These two species share a set of pseudogenes that are active in *S.* enterica strains that cause gastroenteritis but inert in typhoidal strains, in addition to the genome-wide similarities that have been noted. These pseudogenes originate via deletion, rearrangement, frameshifting, nonsensical substitutions, etc., either alone or in combination. In non-typhoidal *Salmonella* (NTS), the coding sequences stay active after genetic drift, while in typhoidal *Salmonella* (TS), they become dormant. The two primary processes regulated by the existence of these dormant coding sequences are host limitation and immune evasion. The clinical isolate CT18 has been sequenced, however the *S.* Typhi Ty2 strain exhibits a large-scale phage variation and half of its genome is inverted between two rRNA operons [35, 36, 37]. Events of insertion, deletion, and/or substitution were also noted in *S.* Paratyphi A. As a result of insertion/deletion and/or substitution, several pseudo-forming and pseudogenes, including aidB, asnB, ccmH, nmpC, pduF, pduG, proQ, rbsC, rbsR, rhlB, SSPA3202, SSPA4008a, etc., were found to be present [37].

It is discovered that clinical isolate CT18 has pseudogenes, including ybaD, fimI, ttrS, sopE2, wcaA, and torC. These pseudogenes alter the bacterial pathway(s) and produce new diseases

for the bacterium in addition to lowering the energy consumption of the cell. A few of the major alterations brought about by these tiny, subtle changes in the bacteria are listed below:

YbaD is a ribonucleotide reductase found in bacteria [37]. Microorganisms attach to epithelial cells less when this gene product is overexpressed. It was discovered that in the instance of Escherichia coli, active nrdR reduced bacterial growth. Additionally, over-expression affects the expression of proteins globally; a reduction was observed after nrdR over-expression. The clinical isolates of typhoidal Salmonella are therefore better suited to cause the infection when these genes are deactivated. Regarding piliation in bacteria, a different pseudogene called fimI results in a negative phenotype [38]. rpoS+ (stationary phase-specific σ-factor) bacterial genes are discovered to be suppressed in the presence of FimI. Fimbriae production in *Salmonella* remains unaffected by deletion of fimI. Additionally, it results in the overproduction of longer-than-normal fimbria. Fimbria aid in the adherence of epithelial cells, further damaging their host. Together with ttrR, another pseudogene, ttrS, is the sensor protein of a ttrSRBCA operon and together they constitute a two-component regulatory network. When ttrS is inactivated in clinical isolates of typhoidal *Salmonella* (TS), a strain that is metabolically deficient is created, making it unable to use tetrathionate [39]. For non-typhoidal *Salmonella* (NTS) strains that cause gastroenteritidis, tetrathionate metabolism is a characteristic metabolic pathway; however, TS lacks this process. SopE2 [40] is maybe one of the most significant pseudogenes discovered in typhoidal strains. It triggers the inflammatory response in non-typhoidal *Salmonella* (NTS) strains through the caspase 1 pathway, which results in the generation of pro-inflammatory cytokines such as IL-1β and IL-18 [41]. Because of their inactivity, typhoidal *Salmonella* (TS) are unable to initiate the neutrophil transmigration pathway. This effectively increases the bacterium's stealth and prevents the host's immune system from identifying the breach. Because of this, the infection spreads throughout the host's body without their knowing until the immune system uses all of its resources to fight off the bacterial invasion. Other genes

that are identified as pseudogenes in Ty2, but not in CT18, include astA, stcC, gabP, narV, narW, stbC, and aroM [42].

2.7.2. Host specificity: *Salmonella* is a genus that is known as a "universal pathogen" due to its ability to adapt to different hosts. Some strains of the bacteria exhibit a high degree of host adaptability, known as "Host Restricted" while others show varying degrees of host restrictiveness, known as "Host Restricted". *Salmonella* separated from *Escherichia coli* through three stages of diversifying selections, culminating in the acquisition of *Salmonella* Pathogenicity Islands (SPIs) 1 and 2, which allowed them to invade host cells and grow inside macrophages, respectively. The separation of Salmonella from *Escherichia coli* occurred through these stages of diversifying selections [43, 44].

Salmonella Typhi, which has a limited host range, is an illustration of a within-host evolution in which a unique pathovar causing a widespread illness has appeared. As a result of other hosts' decline in fitness, Typhoidal *Salmonella* (TS) exhibits restricted access to them, which exacerbates its impact on human hosts. The enhanced transmission capacity balances this host limitation [49]. *Salmonella* strains that are host-adapted or host-restricted enter their host and attempt to suppress the immune system. The *Salmonella* Typhi strain that is human; host restricted (HR) starts its infection process by utilizing the physiological processes of its host, including antigen uptake by M cells, engulfment of the Payer's Patches (PPs) by macrophages and lymphocytes, and apoptosis. What sets them apart is their acquired capacity to proliferate and divide exclusively within human macrophages, not in mice or other animals. Another element that aids in the development of host-adapted or host-restricted processes inside their hosts is the necessity for amino acids. Furthermore, the illness pattern is altered from other sources by the acquisition of pseudogenes. Point mutations, convergent horizontal gene transfer, and genome degradation all contribute to the development of host-adapted or host-restricted organisms. The bacterium's translocon protein triad, SipD, SseC, and SseD, produces

the protein SptP, which inhibits the host cell's mitogen-activated protein kinase. The proteins SseF and SifA additionally aid in host specificity. Additional research revealed that mannose-specific FimH is an additional element in host adaptability. These strains become more pathogenic and host-restricted due to convergent evolution through genome shortening and other genetic mechanisms [45].

2.7.3. Immunity of the host against infection: Following ingestion, *Salmonella* passes through the small intestine's epithelial cells. They are now ingested by macrophages and other cells, which then distribute themselves in a methodical manner. The IL-12/IL-23-IFN-γ axis is a crucial mechanism that makes a major contribution to the infection [46]. Macrophages and dendritic cells, which interact with NK-cells, T-cells, and NKT-cells, generate IL-12 and IL-23 in response to stimulation by *Salmonella* [46, 47]. This reaction functions in tandem with IFN-γ secretion from NK-cells, T-cells, and NKT-cells. Each of these reactions activates the host's defence mechanisms against the pathogen. *Salmonella* contributes to host immune evasion when this axis is absent, or more specifically, when none of the proteins—that is, β1 from IL-12R/IL-23R, p40 from IL-12/IL-23, chains 1 and 2 from IFN-ηR, and STAT1—are present [48].

A functional IL-12/IL-23 arm is essential for resistance to *Salmonella* infection, even though the lack of the IFN-γ arm may be partially restored by the actions of TNF-α and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF). The outermost layer of a polysaccharide coating, specifically Vi-polysaccharide, is present in the majority of typhoidal *Salmonella* (TS) strains. Their polysaccharide coating makes them a stealthy bacterium that avoids the need for neutrophils and infects the host without the host being aware of the breach location. Research has demonstrated that the presence of Vi-polysaccharide inhibits complement-deposition on the surface of the bacteria and prevents the host complement C5a and C5a receptor (C5aR) from identifying the pathogen breach. This leads to a bloodstream infection and discriminates

between a mild and heightened immune response against non-typhoidal *Salmonella* (NTS) and typhoidal *Salmonella* (TS), respectively [49].

FepE expression also plays a role in distinguishing these two illnesses from one another. Complement C3b deposition is less likely to occur when non-typhoidal *Salmonella* (NTS) infection is present because typhoidal *Salmonella* (TS) lacks the ability to produce very lengthy O-antigen due to fepE's involvement in its creation. This is because a stop codon in the typhoidal genome prevents typhoidal *Salmonella* (TS) from expressing fepE. The reason for this effect is that Vi-polysaccharide lacks any free hydroxyl groups that may be the surface for complement deposition [50]. Other alterations in flagellar gene expression impact the illness pattern in addition to the mechanisms previously outlined. Accordingly, a typhoidal *Salmonella* (TS) infection compromises the mucosal barrier, which facilitates the rapid and easy systemic dissemination.

2.7.4. Organ specificity: Every invasive infection prefers a certain organ within its host. The spleen and liver show signs of increased cellularity and expansion after infection with *Salmonella*. The spleen is a vital organ in terms of haematology. The balance of erythroid development is disturbed by persistent infection [51]. This results in erythroid cell up-regulation, which induces splenomegaly and extra-medullary erythropoiesis in the spleen. Nitric oxide production from activated macrophages and the production of inflammatory cytokines are subsequently inhibited by EPO when serum levels of this protein are high. Moreover, bacterial persistence is brought on by EPO signalling in non-erythroid cells. Consequently, an increased erythroid cell count makes mice more prone to infection, which further corroborates the observation that splenomegaly is exhibited by SIRP-α defective mice. When there is a *Salmonella* infection, the bacteria live in the spleen due to an increase in the population of CD71+ Ter119+ reticulocytes and a subsequent decrease in the number of mature

erythrocytes. Since the kidney and liver also secrete EPO, there is a noticeable increase in the number of bacteria that recover after contracting *Salmonella* [52].

2.8. Transmission and systemic expansion:

Bacterial departure following the host's epithelium and immune cells' absorption (mostly by macrophages), typhoidal *Salmonella* (TS) transforms the cell into a *Salmonella*-Containing Vacuole (SCV) and multiplies inside the cell [53]. The Early Endosome associated Antigen (EEA1), Rab5, Transferring Receptor (TfnR), and other early indicators identify these early endosomal compartments. Generally speaking, early endosomes change into late endosomes [54]. They then contain indicators of late endosomes, namely Vacuolar proton pump (V-ATPase), Rab7, LAMP2, LAMP3, and Lysosomal Associated Membrane Protein 1 (LAMP1). When *Salmonella* is present, this endolysosomal event in epithelial cells is delayed, giving the resident bacteria ample opportunity to settle in and multiply Moreover, in the preformed SCVs, an endosome-lysosome content acquisition that was Rab7-dependent was noted. Experiments have shown that, despite Salmonella's ability to effectively neutralize an acidic environment, the cytosol is a more conducive growth medium for the bacteria than a SCV [53, 54]. Within the comparatively "hostile" SCVs, *Salmonella* grows, replicates, and matures using the Type-III Secretion System 2 (T3SS-2), and its cytosolic form should contain active T3SS-1 and flagellar genes [45]. A study on various *Salmonella* gene constructs revealed that in a tiny percentage of bacterial cells identified in a population known as hyper-replicating bacteria in the cytosol, the expression of T3SS-1 and flagellar genes was still "switched on." However, the T3SS-2 gene expression (LAMP1) of the bacteria inside the SCVs was distinctive. Consequently, after infection, there would be two transcriptionally different subsets of typhoidal *Salmonella*: one with cells expressing the marked T3SS-2 gene (in the SCVs) and another with cells expressing the marked T3SS-1 and flagellar genes (in the cytosol). The quick turnover of epithelial cells affects cell extrusion in a small way [55]. In systemic locations like

the gallbladder, the activity of T3SS-1-induced cells aids in the extrusion and dissemination of germs [56]. Instead of causing an acute illness, this aids the germs in gaining a firm grip. From the epithelium's apical side, hyper replicating cells protrude. A vacuolar membrane was absent from these bacteria. In addition to all of the above, it is important to note that physiologic homoeostatic extrusion and *Salmonella*-induced extrusion are distinct processes rather than mutually exclusive ones (**Fig. 7**). In the first, the apical side of the epithelial cell monolayer produces IL-18 in response to Caspase-1 and -3, rupturing the plasma membrane; in the second, crowding-mediated cell extrusion gives rise to the same outcome. The bacteria exploit both of these channels as a method of escape [57].

2.9. The mechanism behind the environmental occurrence of typhoidal *Salmonella*:

It's fascinating to learn how different strains of *Salmonella* survive and propagate in the environment, even if their pathophysiology is well established. Birds, wild animals, reptiles, sewage water, and seasonal variations in an area are among the sites where salmonella can be discovered [58, 59, 60]. whereas *Salmonella* was shown to be associated with summer in the USA, where every 1°F increase in temperature resulted in an increase in 4 instances of Salmonella infection. In Scotland, the presence of non-typhoidal *Salmonella* (NTS) was linked to the presence of gulls throughout the winter. According to recent findings, they are able to thrive inside Acanthamoeba polyphaga amoebas thanks to the SPI-2 sseC gene, which is part of the bacteria's protective secretive system. Acanthamoeba castellani and TS can coexist and flourish [61, 62, 63]. Research findings indicate that A. castellani enhances the growth of the co-culture relative to the bacterial culture in isolation. It functions as a buffering agent and is unrelated to *Salmonella* strains' intracellular survival strategy. When amoeba is co-cultured, they are known to produce cysts instead of remaining trophozoites in pure culture. The bacteria were also demonstrated to be spread by fish. To make the process of identifying the *Salmonella*

infection pattern in the environment simple and efficient, a strong instrument or process must be developed.

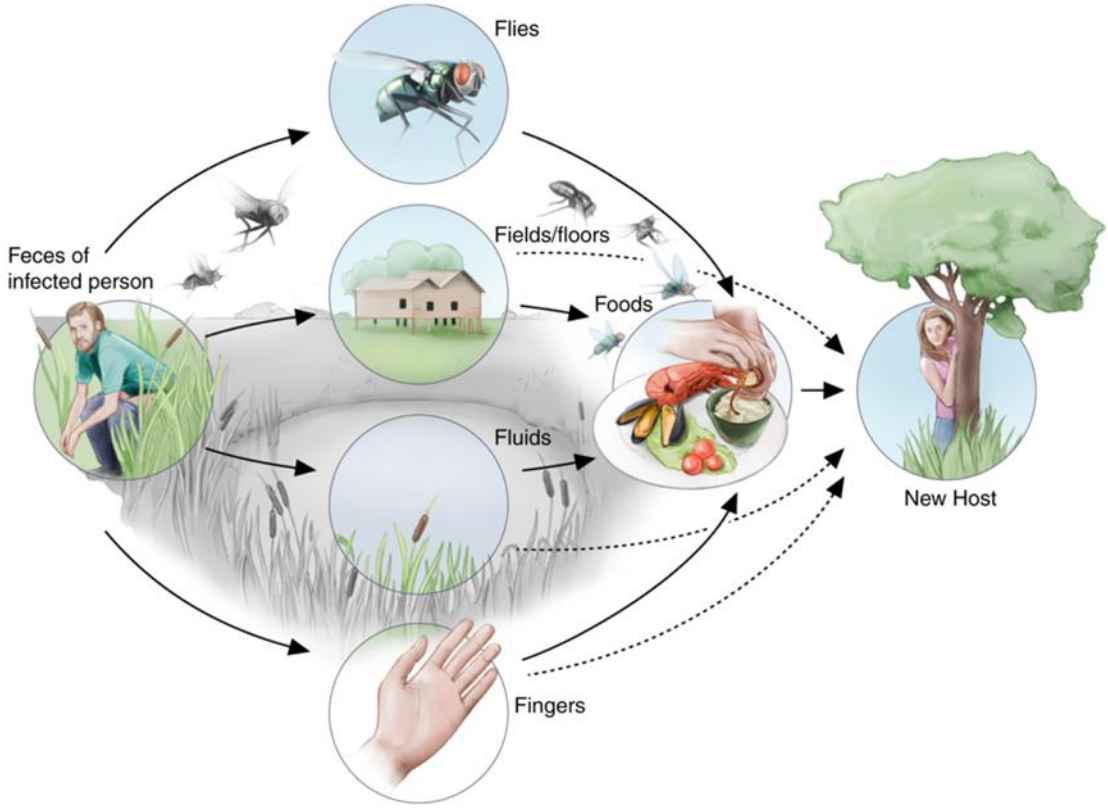


Figure 8. The Incidence of *Salmonella* Infections

2.10. Traditional animal models to study typhoidal *Salmonella* infection's mechanism.

Both *Salmonella* Typhi and *Salmonella* Paratyphi A can cause systemic illnesses such as enteric fever. When they come into contact with their host, they integrate themselves into particular body parts and exhibit pathogenicity.

2.10.1. Traditional animal models. Animal models have been employed in the past in order to gain a detailed understanding of the pathogenicity. Despite the fact that Typhoidal

Salmonella are only allowed to infect human hosts, some animals have been modified to simulate the way these creatures naturally infect human hosts. They are as follows:

2.10.1.1. Acute iron-overload model. For many reasons, normal serum prevents the growth of microorganisms. The relatively modest levels of free iron present in the serum were thought to be the cause of the usual bacteriostatic effect. The iron-binding substances of the infectious pathogen and the host compete with one another for available iron. Two important iron-binding molecules involved in non-specific host resistance are transferrin and lactoferrin. On the other hand, the pathogen's virulence potential is linked to the bacteria's ability to sequester iron through siderophores. Mice were given intraperitoneal (i.p.) injections of iron compounds (ferric ammonium sulfate, or FAS), FAS with citrate, and FAS with 2, 3-dihydrobenzoic acid, or 2,3-DHB) in order to simulate acute iron overload. Next, 0.5 milliliters of 0.9% NaCl solution containing the bacteria *Salmonella* Typhimurium is added. An increase in local iron concentration encourages overgrowth and the eventual death of the animal, therefore a systemic iron inoculation is preferable [64]. For both high and low pathogenicity bacteria, an increased rate of bacterial multiplication was noted under iron overload circumstances. Thus, in our experiments, the variations in virulence were reduced when iron supply increased. Furthermore, iron excess slows down the removal of microorganisms from the bloodstream.

2.10.1.2. TLR11$^{-/-}$ mice model. Pathogen Associated Molecular Patterns (PAMPs) are recognized by Toll-Like Receptors (TLRs), which are inbuilt Pattern Recognition Receptors (PRRs). The host's innate and adaptive immune responses are triggered by these ligand binding sites, which are the first steps towards pathogen elimination. The kidney, heart, gut, liver, and spleen all have TLR11. It was discovered that the small intestine's epithelial layer expressed it substantially. The location of TLR11 in the gut raised the possibility that it could identify entero-pathogens. While TLR11 expression is absent in humans, it is present in mice. Infection is possible in TLR11-/-mice. This may explain why wild-type TLR11+/+ mice exhibit greater

resistance to S. Typhi infection. The ill animal displayed a profile like a cytokine storm. In addition, tlr11-/-mice showed substantial colony forming unit (CFU) burden and bacterial spread to many organs. It is plausible that TLR11 functions to enhance barrier responses against *Salmonella* sp., since it is also expressed in dendritic cells and small intestine epithelial cells [65].

2.10.1.3. Mouse intraperitoneal (i.p.) infection model. To answer certain research questions, such as whether higher systemic levels of *Salmonella* are caused by increased extraintestinal growth/survival or are a result of gut-associated factors, it is desirable to as certain whether an observed phenotype depends on the intestinal or extraintestinal stage of infection. An easy way to test this hypothesis is to inject *Salmonella* intraperitoneally (i.p.), which allows the infection to spread throughout the body without being affected by the gut's selective forces. In the event that the phenotype disappears after intraperitoneal infection, the intestinal stage of infection is thought to be the mechanism causing the phenotype [66].

2.10.2. Molecular machinery of infection. Typhoidal *Salmonella* enter the gastrointestinal (GI) tract and proceed towards the small intestine following the ingestion of tainted food or water [67]. The progression of the disease is significantly influenced by gastric pH since low pH makes the patient more prone to infection. There are two components to the entire illness process. These are as follows:

2.10.2.1. Initial line of defense: the intestinal mucosa. After the bacteria are consumed, they come into touch with the gastrointestinal (GI) tract. The inoculum travels to the terminal ileum portion of the small intestine after successfully passing over the stomach acid barrier. One or more of the mechanisms listed below absorb them at this point. They may enter the body directly through the Microfold Cell (M-cells) on top of the Payer's Patches (PPs) or the intestinal epithelial cells. Bacterial cells are internalized, neutralized, and digested by

phagocytic cells (also known as antigen-presenting cells, or APCs). Nevertheless, if the original inoculum is sufficiently massive to avoid the macrophages' and dendritic cells' abilities to neutralize it, it then lives inside the cells, complicating the entire process. Then, with normal cells surrounding them, these infected phagocytes form particular foci within a diseased lesion. It takes a complex process involving the involvement of adhesion molecules like Vascular Cell Adhesion Molecule-1 (VCAM-1) and Inter-Cellular Adhesion Molecule-1 (ICAM-1), as well as cytokines like Tumor Necrosis Factor-α (TNF-α), Interleukin-12 (IL-12), IL-18, IL-14, and IL-15. The bacterial growth and spread in the diseased tissue are aberrant when this lesion fails to occur. By getting to the mature lymphoid follicles produced by T lymphocytes, DCs, etc., certain escape artists trigger an immunological response mediated by B and T cells [67].

2.10.2.2. Transmission from the mucosa of the intestine. From the lymphatic nodules, the T and B lymphocytes carrying the germs travel via the reticuloendothelial system to the liver and spleen. Within the mononuclear phagocytic cells, some bacteria are phagocytosed while the remainder survive. Following their sequestration in the bloodstream, the bacteria are liberated from their intracellular environment, based on the host immunological competence, initial bacterial load, and pathogenicity. Following the onset of bacteraemia, the bacteria can now develop secondary infections in the bone marrow, liver, spleen, and gallbladder. From the basolateral side, it can potentially enter the PPs. The phagocytic activity of the Kupffer cells kills the bacteria present in the liver [67]. The remainder invades hepatocytes and induces apoptosis to destroy them (**Fig. 9)**.

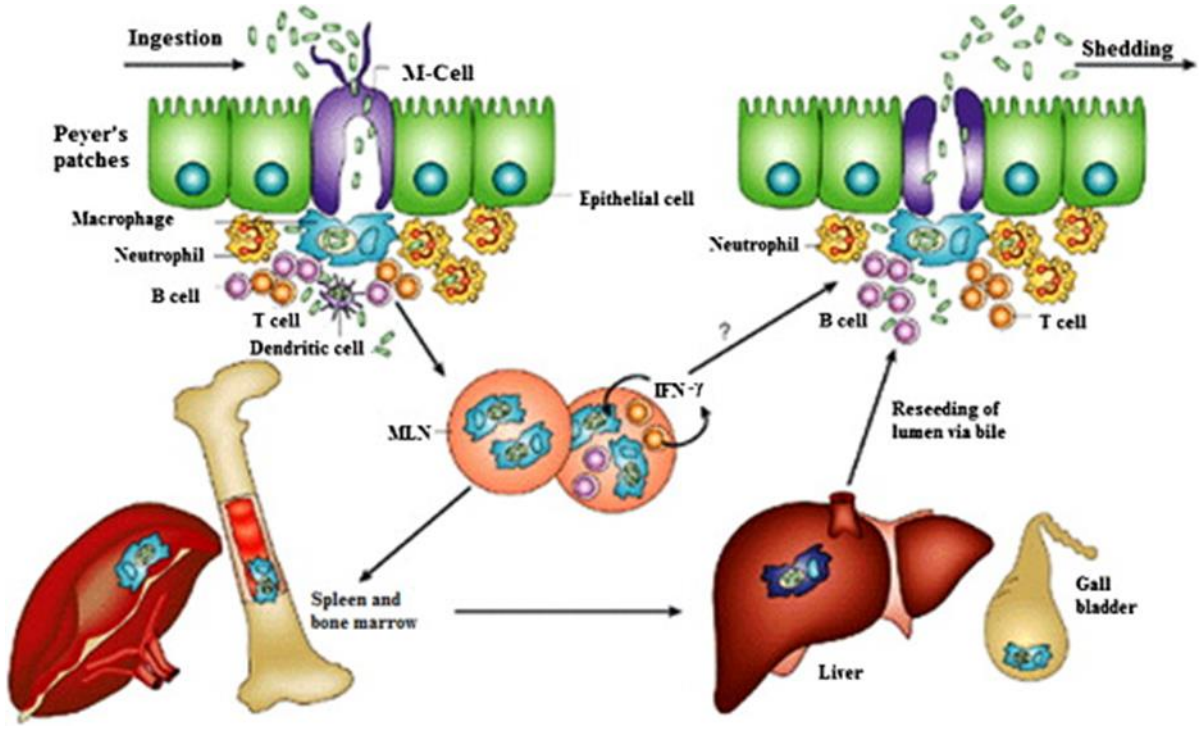


Figure 9. Typhoidal *Salmonella* pathogenesis. Fecal-oral transmission is the method by which typhoidal Salmonella infects its host. The immune cells that live inside the dome beneath the PP in the ileam absorb the germs after they have been consumed. There has been a textual description of the pathogenesis. (image adapted from "Kaur, Jasmine, and S K Jain. "Role of antigens and virulence factors of Salmonella enterica serovar Typhi in its pathogenesis." Microbiological research vol. 167,4 (2012): 199-210. doi:10.1016/j.micres.2011.08.001")

2.11. Host immune responses to prevent *Salmonella* infection:

Host immune responses that are generated to combat infection (**Fig. 10**) are described as follows:

2.11.1. Humoral immunity against *Salmonella* infection: Serum antibodies against the O antigen of lipopolysaccharide (LPS), the Vi antigen, and the H or flagellar antigen have been

reported to be produced by S. Typhi infection or immunisation, and these antibodies have been identified as markers of immunogenicity after immunisation [68, 69, 70, 71, 72, 73, 74]. It is unclear how antibodies help defend against S. Typhi. There have been reports of typhoid fever relapse in people with increased serum titers against S. Typhi [75, 76]. Pre-challenge levels of anti-H and Vi antibodies did not confer protection in human challenge with S. Typhi, confirming earlier observations [77].

It was observed that, those who had the illness showed elevated levels of IgM, IgG, and IgA against the O and H antigen, but those who did not showed any alterations of this kind. It's interesting to note that levels of antibodies against Vi stayed constant over the investigation [76]. This finding is corroborated by the finding that vaccinations with Vi polysaccharide can produce protection against typhoid, suggesting that antibodies against Vi are protective. According to some theories [77, 78], Vi evades host immune identification, and antibodies against it work by blocking innate immune recognition and interfering with bacteria-guided neutrophil chemotaxis. Interestingly, protection coincided with sero-conversion, as determined by anti-O IgG, and the Vi deficient live-attenuated oral vaccine Ty21a demonstrated efficacy comparable to the Vi polysaccharide vaccine [79]. Conversely, with other vaccine formulations, sero-conversion of IgG O antibodies did not correlate with protection. Saliva, intestinal secretions, and faeces can all include S. Typhi-specific IgA, which is produced by natural infection or vaccination [80, 81, 82, 83]. On the other hand, there are no studies on the avidity of anti-*S.* Typhi antibodies produced by vaccination or spontaneous infection. Avidity, which indicates how strongly antibodies engage with antigens, is highest when B cells have received the best possible priming. Making educated choices on the development of vaccines will require a deeper comprehension of the avidity and correlates of protection linked to protective responses. Thus far, there have only been detection and quantification of antibodies against S. Typhi, with relatively few functional studies conducted. According to a prior

investigation, antibody dependent cellular cytotoxicity (ADCC) against S. Typhi was caused by IgA produced by Ty21a immunisation [84].

Additionally, it was noted that *S.* Typhi-specific IgG antibodies that are opsono-phagocytic bactericidal may aid in the removal of the infection [85, 86]. However, a different study carried out in Nepal, a typhoid-endemic nation, revealed that while serum bactericidal activity rose with age, there was no relationship between bactericidal titers and anti-Vi titers [87]. It is imperative that the functional role of antibodies be thoroughly studied. When compared to antibody measurement, this could result in a better knowledge of the correlates of immunogenicity and protective immunity. In conclusion, research has shown that antibodies play a function in providing protection against *S.* Typhi, however it is doubtful that these antibodies mediate protection in a dominant manner.

2.11.2. B cells immunity against *Salmonella* infection: Although previous study revealed that B cells are critical for defence against *S.* Typhimurium, it is unclear exactly what part B cells play in *S.* Typhi infection [88]. B cells can also present antigens, produce cytokines, and activate T cells, even though their main function is to produce antibodies. While the bacteria are still living in the cell, primary human B cells specific to Salmonella use their B cell receptor to engulf S. Typhimurium, display the bacterial antigen via loading onto MHC class I, and trigger a recall response by cytotoxic CD8+ T-cells [89]. These results highlight the cooperation between the immune cell compartments once again and point to a function for B cells apart from producing antibodies [90].

2.11.3. Antibody secreting cells: After coming into contact with an antigen, B cells differentiate into antibody-secreting cells (ASC) [91]. According to several studies [67, 92, 93, 94, 95, 96, 71, 72, 73 there is a brief systemic circulation of specific ASC in cases of Salmonella infection. This brief circulation of ASC peaks at about 7–10 days after exposure. The type of

antigen (killed vs. live), the quantity of vaccine doses, and the formulation all affect how much of a response a person has to a vaccination [97]. Research suggests that elevated serum antibody levels are associated with increased numbers of activated stromal cells.

2.11.4. Cell mediated immunity against *Salmonella* infection: Since S. Typhi is an intracellular pathogen, CD4+ and CD8+ T-cells play a major role in the cell mediated immune response (CMIR), which is necessary for the infection's clearance [98, 99, 100, 101]. People who have typhoid fever or have received vaccinations with Ty21a and other attenuated strains of the vaccine have been shown to have Salmonella-specific CD4+ helper T-cells and CD8+ T-cells [68, 100, 102, 103, 104, 105, 106, 107, 108, 109].

2.11.4.1. T-cell responses to *Salmonella* infection: According to several studies [100, 102, 103, 104, 105, 106, 107, 108, 109] immunisation with S. Typhi has been shown to induce the secretion of pro-inflammatory cytokines, such as IFN-γ, by pathogen-specific CD4+ and CD8+ T-cells. Immunisation against S. Typhi also results in the production of cytotoxic CD8+ T cells [105, 110]. Target apoptosis is induced by cytotoxic CD8+ T-cells via two different mechanisms [111, 112], either by killing mediated by granzymes and perforin [113] or by death mediated by the FAS ligand [114]. Nonetheless, it has been documented that granzyme and perforin are necessary for CD8+ T cell-mediated lysis of S. Typhi-infected target cells. While S. Typhi-infected targets have been demonstrated to stimulate CD8+ cells, CD4+ cells have been reported to respond more readily to soluble antigens [102, 103, 104, 105, 106]. Serum antibody titers to H and/or O antigens and CMI in recipients of live attenuated vaccinations have not been found to be correlated [73, 101, 105, 115]. These findings lend credence to the theory that host variables such as genetic makeup and gut microbiome composition have a multifaceted role in the development and dominance of humoral and/or CMI responses in individuals.

2.11.4.2. Dendritic cell cross-presentation and CD8+ T-cells: It is yet unknown how S. Typhi controls the maturation of particular T-cell responses in people. DCs have the ability to directly produce Salmonella-specific CD8+ T-cells by swallowing and digesting the bacterium, or indirectly through bystander processes [116]. Antigen-presenting cells, or DCs, can present exogenous antigens on MHC class II molecules to activate CD4+ T-cells. They can also present exogenous antigens on MHC class I molecules to activate CD8+ T-cells through a different pathway known as the cross-presentation pathway [117, 118]. Consequently, vaccination antigens must be able to influence DC cross-presentation in order to be effective. According to reports, DCs engulf infected human cells through suicidal cross-presentation, secreting pro-inflammatory cytokines such IFN-γ and IL-12p70, which causes the presentation of bacterial antigens [119].

Human DC treated with live S. Typhi released large quantities of pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α, but not much of IL-12 p70 or IFN-γ. Conversely, DCs co-cultured with S. Typhi infected cells produced high levels of TNF-α, IFN-γ, and IL-12 p70 [119]. This is consistent with reports suggesting that IFN-γ and IL-12 are necessary for resistance to Salmonella infection in humans [120, 121] and mice [122, 123, 124, 125]. Consequently, for vaccines to be effective, they must possess the desired characteristic of modulating DC cross presentation.

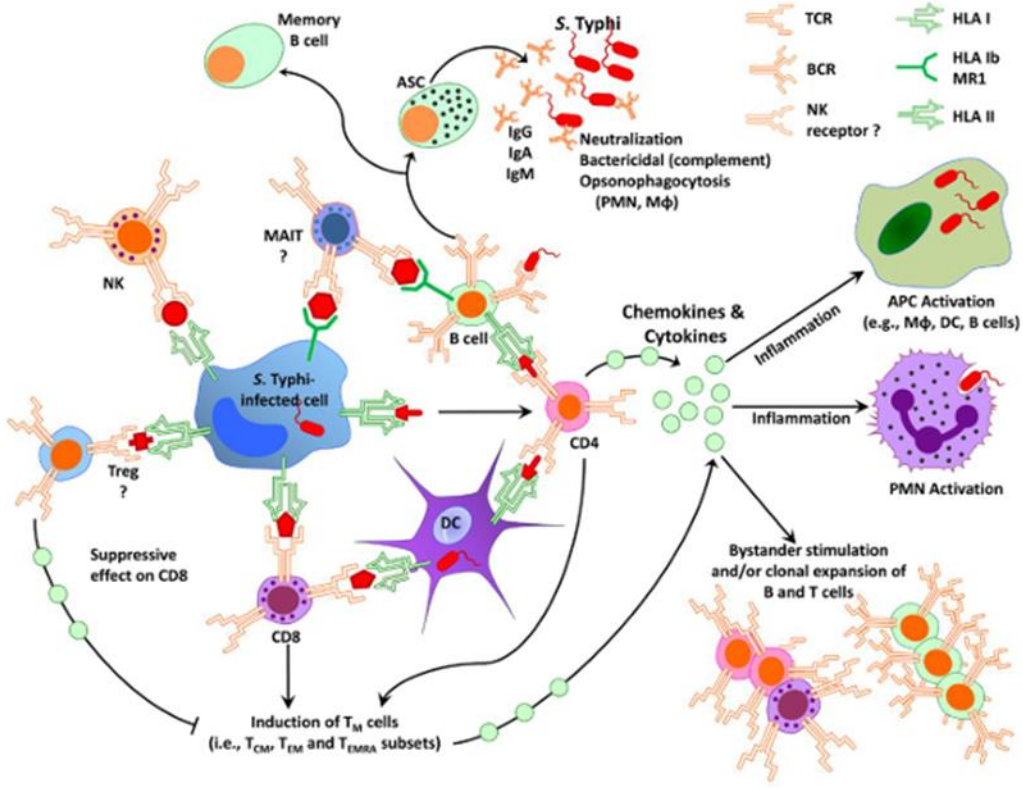


Figure 10. Mechanisms defending against infections with *Salmonella*. (Image adapted from Sztein, Marcelo B et al. "Complex adaptive immunity to enteric fevers in humans: lessons learned and the path forward." Frontiers in immunology vol. 5 516. 27 Oct. 2014, doi:10.3389/fimmu.2014.00516)

2.11.5. Memory response to *Salmonella* infection:

2.11.5.1. Memory B cells: It has been determined that immunological memory is crucial for maintaining a vaccine's long-term protective effectiveness [125, 126]. Memory B cells (phenotype CD19+ CD27+ IgD+/−) are long-lived antigen-primed cells that rapidly develop into plasmablasts and plasma cells upon re-exposure to the antigen. They play a critical role in such responses [127, 128]. According to reports, immunisation with attenuated S. Typhi vaccines produces memory B cells with the phenotype CD19+ CD27+ and a lifespan of roughly

a year that are specific to S. Typhi antigens (e.g., LPS, flagella, and Vi) and are in charge of these responses [129]. When given live attenuated vaccine strains CVD 909 orally, individuals produce strong memory B responses against both T-cell-dependent (flagella) and T-cell-independent (LPS and Vi) antigens. Thus, CVD 909 was able to mucosal prime the immune system to produce a strong and long-lasting memory B cell response to both Vi and LPS; the response to LPS was, however, less pronounced than the response to Vi; more memory B cells secreting LPS-specific IgA were seen compared to the same secreting LPS-specific IgG; additionally, all of the individuals given Ty21a developed IgA Memory B cells against LPS, but only one of them developed IgG memory B cell responses against LPS; both CVD 909 and Ty21a produced IgG and IgA memory B cell responses against S. Typhi flagella; S. Typhi porins can also induce prolonged IgG and IgM responses in humans, which are thought to be mediated by memory. It has also been documented that immunising mice with porins produces IgM memory B cells, which are in charge of providing long-term protection [130]. It has been suggested that Type-1 T follicular helpers (T_{fh}) secreting IFN-γ aid in the development of these memory B cells [130]. However, more characterization of these responses and cell subsets is necessary to ascertain.

2.11.5.2. Memory T cells: There are two primary subgroups of T-cells: memory and naïve. T cells based on the stimulation of memory and surface molecule expression One of the characteristics of a successful vaccination is T-cell responses [126, 131]. Earlier report classified T cells into two subtypes: effector memory T-cells (TEM) and central memory T-cells (TCM). The surface molecules for memory, such as CD45RO, and the chemokine receptors CCR7 and CD62L (L-selectin), which are in charge of guiding the cells to peripheral lymph nodes, are what define TCM [132, 133]. Although they can move and migrate to the spleen and non-lymphoid organs, TEM also express CD45RO. Certain CD8+ TEM in humans are referred to as TEMRA or "terminal memory" cells because they express CD45RA, which

is linked to naïve cells, rather than CD45RO [132, 133]. After Ty21a immunisation, CD8+ TCM, TEM, and TEMRA have all been linked to long-term memory (up to two years) [131]. When stimulated with targets infected with S. Typhi, these CD8+ T-cells release a variety of pro-inflammatory chemokines and cytokines, such as IFN-γ [104]. Strong long-term immunological responses were also seen in those with large numbers of CD8+ TCM subsets, which generate IL-2 and IFN-γ at early time periods [104]. This could be explained by the fact that long-lived memory T subsets are formed when CD8+ TCM secretes IL-2 and/or IFN-γ, and these subsets are responsible for quicker resolution in re-infection situations. Individuals receiving Ty21a were shown to have CD8+ cells secreting IL-17A [104]. This is particularly significant in light of the theory that the production of multifunctional CD4+ and CD8+ T-cells will determine whether a vaccine is effective [104].

2.12. Preventive measures of typhoidal *Salmonella* infection:

2.12.1. Non-vaccine measures:

A typhoidal infection was widespread in the USA and Europe in the 1800s as a result of faulty food handling practices and unsanitary water use. Enhancing the availability of food and water can take precedence over managing them as these are human-made actions. In industrialized countries, the rate of infection has decreased due to municipal water treatment, pasteurization of milk and other products, and proper handling of human waste. The same strategies could have reduced infection rates in poorer nations as well, but putting them into practice is challenging.

2.12.2. Vaccine measures: overview of vaccination.

An effective candidate vaccination ought to elicit a sustained immune response in order to provide protection against harmful pathogens. Ideally, vaccines should trigger both cell-

mediated immunity and a protective humoral immune response. According to humoral immunity might be adequate to combat invading pathogens; but, since S. Typhi and other chronic and persistent pathogens live inside specialised cellular compartments, substantial cell-mediated immunity is necessary to eradicate them [132]. When the immune system reacts to foreign antigens, dendritic cells are essential. By stimulating antigen-specific CD4+ or CD8+ T effector cells, preventive vaccinations produce immunological competence [133]. Therapeutic vaccinations, on the other hand, not only start a fresh immune response but also cause memory cells that already exist to switch from regulatory to cytotoxic immunity [133].

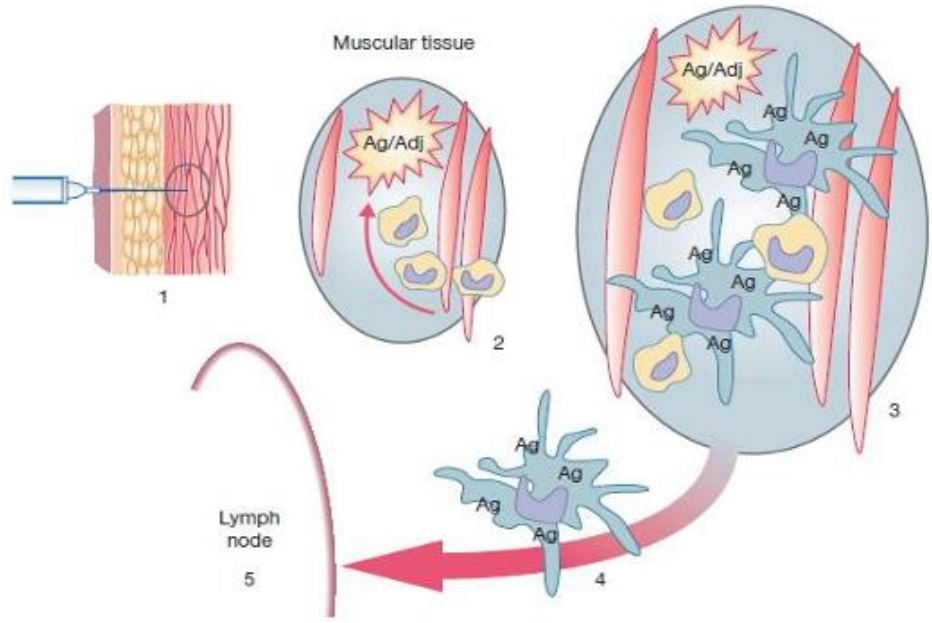


Figure 11. Commencement of a vaccination reaction. After injection (1), dendritic cells, monocytes, and neutrophils that scavenge throughout the body are drawn to vaccination antigens' pathogen-associated patterns (2). In the event that vaccine antigens or adjuvants generate enough "danger signals," monocytes and dendritic cells are activated (3), altering their surface receptors and causing them to migrate along lymphatic vessels (4) to the draining lymph nodes (5), where T and B lymphocyte activation will occur. (Image adapted from https://vaxplanations.wordpress.com/2015/02/25/239/.)

2.12.3. Immunological mechanisms of vaccination:

Immunisation programmes are the most effective means of controlling disease or eliminating infections because they can produce adaptive immunity, a long-lasting kind of defence, as opposed to strong but transient innate immune responses. The humoral immunity induced by a vaccine mainly takes the form of B lymphocyte-secreted antibodies that can attach to a particular toxin or pathogen [134]. Cytotoxic CD8+ T lymphocytes (CTL) provide cell mediated immunity by either killing or detecting contaminated cells or secreting antiviral cytokines, which can stop the spread of diseases. Growth factors secreted by CD4+ T helper lymphocytes regulate the development and maintenance of B cells as well as CD8+ T cells, which are further classified into T helper 1 (Th1) and T helper 2 (Th2) subtypes. Treg cells regulate the activity of the two subgroups and preserve immune tolerance [135]. There is a strong relationship between the humoral and cell-mediated immune responses. As an illustration, the majority of antibody responses require CD4+ T cells. T cells are significantly impacted by antibodies reactions to infections inside cells [136].

Certain antigen-presenting cells (APC), primarily dendritic cells, stimulate antigen-specific B and T lymphocytes (DC). After coming into contact with the pathogen, immature DCs that are circulated throughout the body quickly mature and go towards secondary lymph nodes, which are where T and B cell responses are induced. Mature DCs' primary function is to activate naïve T cells by presenting antigens and co-stimulating signals to T cells [133].

Using their receptors, DCs, monocytes, and neutrophils can recognise patterns of pathogens that have evolved over time. Toll-like receptors, in particular, are crucial for host defences. When infections are encountered, they alter the expression of their surface molecules and release chemokines and pro-inflammatory cytokines [137]. This causes an inflammatory milieu, which in turn causes monocytes to specialise into macrophages and immature dendritic cells to become activated. It also attracts monocytes, granulocytes, and natural killer cells

[138]. DCs with altered homing receptors go in the direction of draining lymph nodes, where they activate T and B cells.

2.12.4. Generation of immunological memory by vaccination:

One method for influencing the immune system and providing protection from human disease is vaccination [139]. Many human diseases have been successfully averted by vaccination, although there are some infections for which there are no effective vaccinations [140]. Specifically, tuberculosis (TB), AIDS, and malaria have become more difficult, most likely due to the infections' resistance to the humoral arm of the immune system [141]. Therefore, vaccinations that produce a sizable and functional memory T cell population are necessary for success.

After receiving an antigen vaccination, antigen-presenting cells, mainly known as dendritic cells (DCs), absorb the antigen. These cells then migrate to secondary lymphoid tissues where they present the processed antigens to naïve CD8+ T cells in the form of peptides loaded on major histocompatibility class I (MHC I) molecules. Following appropriate stimulation by pattern recognition receptors (PAMPs), DCs mature, increasing their capacity to release pro-inflammatory cytokines, present antigen, and trigger the development of co-stimulatory molecules [142]. APCs supply the signals (sometimes referred to as Signal 1, 2, and 3) needed to stimulate a CD8+ T cell that is naïve, leading to the cell's proliferation and the production of memory cells with a long half-life. The CD8+ T cells are influenced in vivo by minute changes in these individual signals [143]. The presentation of a peptide on MHC-I to a naïve CD8+ T cell is known as "signal 1." This encounter triggers the signalling pathways that lead to the development of an immunological synapse between the DC and T cell, which may endure for 18 to 24 hours [144, 145]. The biochemical exchange between the two immune cells during this time determines the size and functional quality of the CD8+ T cell response. "Signal 2"

describes how DCs (via CD80/86, CD70, and OX-40L, respectively) appropriately engage the TCR and co-stimulatory molecules, such as CD28, CD27, and OX-40, on CD8+ T cells. This interaction determines whether a naïve CD8 T cell becomes activated or anergic. "Signal 3" is a crucial stage that determines the strength of the T cell response and memory formation, and type I interferons (IFNα and β) and the cytokines IL-12 seem to be important regulators [146, 147]. The pathogen-dependent demand for each of these cytokines varies. According to reports, IL-2 is necessary for the development of primary memory and after naïve CD8+ T cells are first activated, it helps to generate effector cells [148, 149].

Certain features of the CD8+ T cell response have also been demonstrated to be impacted by other cytokines, including TNFα, IL-21, and IFNγ. Consequently, it is hypothesised that the entire cytokine milieu during naïve CD8+ T cell activation has a significant impact on the rate of memory formation and should be carefully considered when developing vaccination regimens.

2.12.5. Types of Vaccines

There are three types of vaccines: subunit, inactivated/killed whole cell, and live attenuated [150]. Live vaccines are made up of mutant bacteria that lack any gene, or genes responsible for one or more pathways, necessary for the growth of the bacteria. Vaccines that have been killed can be created using chemical or controlled heat treatment. The primary antigenic component(s) of the bacterium are present in subunit vaccinations. This could be covered with particles or based on vectors.

2.12.5.1. Live attenuated approach. The earliest, most well-liked, and most effective immunization methods are bacterial live vaccines [151]. It begins the infection by following the host's normal pathways and closely resembles the wild type bacterium. One can create attenuated germs through random mutation or through controlled mutagenesis. As a result, the

bacterium cannot thrive in some nutrient-derived medium, commonly referred to as auxotroph medium. These mutants are very effective in inducing both innate and adaptive immunity because they closely resemble the wild type bacterium. They are economical for use in rural settings because to their oral gavage delivery technique and cost effectiveness. The possibility of reverting back into their native wild type form makes them a challenging vaccination option, even though mucosal delivery would be successful. One of the primary methods for preparing the TS live vaccination involves attenuating one or both of the routes listed below:

Purine metabolism routes, faulty metabolic pathway(s), defects in global regulator(s), such as the phoP/phoQ system, and pathways involved in the production of aromatic amino acids [152, 153]. Furthermore, the requirement to utilize a Bio-Safety Level 3 (BSL-3) working chamber makes handling live typhoidal *Salmonella* (TS) in poor nations challenging [154]. Detail examples of live attenuated TS vaccines are following:

2.12.5.1.1. Ty21a. With a succession of N-methyl-N′-nitro-Nnitrosoguanidine (NTG) induced mutagenesis, Ty21a is a mutant strain generated from *Salmonella* strain Ty2. The galE mutant of the wild type Ty2 was chosen, and it underwent additional characterization before being mass-produced and approved as the first-ever licensed typhoidal *Salmonella* (TS) vaccine [155]. Ty21a is generally successful, however it does require a somewhat high dosage to effectively stimulate the immune system. Because it is ineffective on children younger than five, its use has been restricted. Lack of understanding about the real genetic lesions towards the alterations in the Leloir pathway enzymes and other potential physiological changes in the bacteria was another serious flaw in the procedure, compounded by the possibility of a reversal [156]. Considering all of these negative effects, some nations have already discontinued administering this vaccination, rendering it out of date.

2.12.5.1.2. 541Ty and 543Ty. Typhi CDC10-80, the wild type *Salmonella* strain, was the original source of the double mutant strain 541Ty [157]. It results from the loss of aroA, which makes purA, which is in charge of supplying adenosine, and paminobenzoic acid and 2,3-dihydroxybenzoic acid sinks. This bacterium becomes less harmful and loses its ability to produce chorismic acid. From the Vi-positive 541Ty, a Vi-negative 543Ty yields the other live attenuated vaccine, 543Ty. Even though it was discovered that the volunteers did not have the vaccination strain, a cohort analysis revealed positive faecal shedding that rises with dose . The vaccination had minimal anti-O and H-antigen activity against *Salmonella* Typhi, and the recipients did not exhibit any anti-Vi antibody reaction. When exposed to a wild-type typhoidal assault, the cell-mediated immune response was greatly elevated. Children did not demonstrate any efficacy, despite adults demonstrating strong efficacy.

2.12.5.1.3. CVD 908, CVD 908-htrA and CVD 909. A mutation in the aroC/aroD genes causes CVD 908, which results in serum anti-lipopolysaccharide (LPS) IgG response and IgA, Antigen Secreting Cells (ASCs) in vaccination recipients [153, 158]. The strain loses its ability to multiply after a considerable amount of time in volunteers with transitory bacteraemia. License is impossible due to vaccinemia, even if there is no overt clinical reactogenicity. To reduce its virulence, the heat-shock protein htrA was removed from the CVD 908 strain. Similar humoral, mucosal, and cellular immune responses were displayed by the novel strain, CVD 908-htrA, in comparison to its ancestral strain. Following vaccination, seroconversion rates with IgG anti-LPS were comparable to those with IgA ASC against O and H antigens, which occurred in 92% and 79% of cases, respectively. The volunteers experience no vaccinemia as a result.

Vi expression depends on at least two two-component systems, regulated by the PtviA promoter: rcsB-rcsC and ompR-envZ. The current CVD 908-htrA was modified to create a novel constitutively Vi expressing strain, substituting the PtviA with the Ptac. ASC and anti-

LPS responses were strong in the recently established CVD 909. The youngsters under the age of five were still not protected by it [158, 159].

2.12.5.1.4. M01ZH09. Further mutations were made to the *Salmonella* Pathogenicity Island (SPI) 2 strain's TTSS structural gene ssaV in presence of the aroC mutant, which was impaired in the chorismite synthesis pathway. In addition to losing its capacity to disseminate consistently inside its host, this strain becomes less virulent [160]. Human volunteers who received M01ZH09 vaccination show an obvious LPS-specific IgA response.

2.12.5.1.5. Ty800. A transcriptional regulator (PhoP) and a sensor kinase (PhoQ) make up the two components of the bacterial virulence regulon [161]. The PhoP gene product is in charge of the lipid A alteration that confers polymixin B resistance. It supports the bacteria's intracellular survival in endosomes and host macrophages. By using cryptidins and defensins, they also shield the bacteria from the host's anti-bacterial reaction. phoP/phoQ eliminated Ty2; that is, human volunteers with Ty800 exhibited anti-LPS IgA ASCs. There have been reports of humoral and cellular reactions to the Typhi O antigen [161].

2.12.5.1.6. χ3927 and χ4073. Another strategy for producing live vaccines was to inhibit the cAMP gene cya and its receptor, crp. CRP and cAMP are both significant transcriptional regulators. They enter cells and organelles by permease, carrying cellular metabolites. A strain known as χ4073 was created from χ3927 with a cdt mutation in order to decrease the colonization in deep tissues, as this strain was observed to have colonized the volunteer. Anti-LPS antibodies were detected in the mucosal samples, but there was no secretory immunological response [162].

2.12.5.1.7. D-Glutamate auxotrophy. Bacterial cell walls are made mostly of D-glutamate. The production of D-glutamate, which is required for the development of bacterial cell walls, is achieved by the glutamate recemase known as MurI. Stopping the gene or genes that generate

D-amino acid transaminase (Dat) and MurI can result in live attenuated strains of bacteria that are wild type [163].

2.12.5.1.8. DV-STM-07. DV-STM-07 was developed through modification and attenuation of the pmrGHIFJKLMD genes. Good protective effectiveness and humoral immune response were achieved with this single dosage formulation [164].

2.12.5.1.9. Live attenuated approach towards *Salmonella* Paratyphi A. According to published research, *Salmonella* Typhi wreaked far more havoc than *Salmonella* Paratyphi A, and it is thought to be the primary cause of enteric fever. *Salmonella* Paratyphi A infections are now the main cause of illness in many Sub-Saharan and Southeast Asian nations, yet this pattern has recently changed.

On RKS2900, ΔphoPQ was removed, and on MGN9772, MGN9773, and MGN9779, phoPQs MGN9720, MGN10028, MGN10044, and MGN10048 were produced, in that order. With the exception of MGN10028, they were all auxotrophic for fructose. Mutations in the purB gene caused MGN10044 and MGN10048 to become purine auxotroph. Once 40 generations had passed, no strain showed signs of reversion [165].

2.12.5.2. Inactivated/killed whole cell vaccine.

It is possible to make inactivated or killed vaccinations, which have naturally lost their ability to convert back into their wild type form, by employing various physical and/or chemical methods, such as formaldehyde, acetone, heat, etc [166]. The deceased bacteria nonetheless have their original structure and, like live attenuated bacteria, cause an immunological reaction in the host. This type of vaccine's reactogenicity is its primary disadvantage. Both an immunological response and reactogenicity are generated by LPS in the case of Gram-negative bacteria. Therefore, it is important to measure and maintain the endotoxin or LPS level below

the target level when creating this kind of vaccination, or any vaccine for that matter. The bacteria itself may suffer from heat and/or chemical treatment, which also has the ability to degrade surface adjuvants and lessen their ability to elicit an immune response.

2.12.5.2.1. Heat killed, phenol preserved and acetone killed. Injectable typhoid vaccinations that have been heat-killed, phenol-preserved, and acetone-killed were available in Germany and England in 1896. The acetone-killed variant outperformed the phenol- and heat-killed versions in the trials conducted in Poland and Guyana. Even though it has licenses in many nations, it has drawbacks like discomfort, fever, malaise, and localized inflammations [167].

2.12.5.2.2. Acetone killed (K66) and Heat killed vaccine (G66). Agar plate bases were used to make K66, and broth cultures were used to produce G66. While the G66 demonstrated 53% protection, the K66 had 71% protective effectiveness [168].

2.12.5.2.3. CKS362. A live attenuated strain of a typhoidal strain known as Killed but Metabolically Active (KBMA) was transformed into a killed vaccine. A viable, attenuated Typhimurium strain with double mutants phoP/phoQ and aroA was created. Together with the previously stated genes, uvrAB, which is involved in the DNA repair pathway, was also mutated in the same strain. As a result, the resulting bacteria lost their ability to produce psoralen and were vulnerable to UVA radiation. This CKS362 strain was assigned the category of KBMA based on its characteristics, even though it was a live attenuated strain [169].

2.12.5.3. Subunit vaccines.

This is a very novel approach to vaccination, in which an immunogen is derived just from the "essential" part of a pathogen [170]. They are unable to proliferate and multiply within the host because they are simply fragmenting of the pathogenic agent rather than the entire bacteria. Other non-pathogenic bacteria express a specific immunogenic portion (or parts) of the

pathogen, which are then purified. The downstream method and subsequent use determine which non-pathogenic host is best.

2.12.5.3.1. Protein subunit vaccines. This strategy is now being used to combat viral and bacterial diseases. An appropriately sized protein should be identified by the host's immune defence system, depending on the individual's immune condition. Once ingested by the host, any such protein is identified and processed by the host's immune system. The same immunogen given at the appropriate booster dose multiplies this main response many times more. However, developing a successful protein subunit vaccination requires a thorough understanding of the pathogen's whole protein repertoire as well as which and to what degree its proteins are immunogenic [171].

2.12.5.3.2. Omp28 of Typhi. Typhi is an efficient inducer of cellular and humoral immunity when it comes to Omp28. Consequently, Omp28 from Typhi MTCC 733 was expressed in E. coli M15 cells using the expression vector pQE30 and utilized in later research. When rabbits received a subcutaneous injection of 100 µg of r-Omp28, their humoral immunity was elevated and their mortality was decreased [172].

2.12.5.3.3. OmpC, OmpF and OmpA of Typhi. Under the control of ompR-envZ of *Salmonella* Typhi, three primary porin proteins—OmpC, OmpF, and OmpA—maintain osmolarity. OmpF expression was observed in low osmolarity environments, accompanied by a consistent expression of OmpC. Important cellular components are transported more easily thanks to the pores these proteins create in the bacterial cell membrane. These proteins underwent in silico study before being cloned and expressed in the BL21 system of E. coli. It was discovered that immunization with these porin proteins is dosage dependent, with an increase in protein quantity leading to an increase in immunogenicity in BALB/c mice [173].

2.12.5.3.4. OmpS1 and S2. In vitro, there is minimal expression of OmpS1 (41 KDa) and S2 (40 KDa). Toll-like receptor, TLR-4 agonists OmpS1 and S2 are also TLR2 & TLR4 agonists. To create the mutant strain expressing OmpS1, S2, STYompFC, a mutant of *Salmonella* Typhi lacking both OmpF and OmpC, was employed. After that, they were transformed using either pFM97S2 (expressing OmpS2) or pF2 (expressing OmpS1). TLR2 and 4 up-regulation as well as antibody production were regulated by IL-8 until 360 days after the first immunization. In vitro and in vivo activation of DCs and macrophages was possible for both of these porins [174].

2.12.5.3.5. Outer membrane adhesion protein T2544. T2544, a protein that binds with laminin, was selected from Ty2 and produced in an *E. coli* expression vector. Bacteria lacking T2544 showed significantly less invasion than the wild type strain due to its requirement for adhesion and ability to bind with laminin. The administration of pure T2544 subcutaneously elicited antibody responses in both serum and secretory forms, which were subsequently verified and associated with the immunogen's capacity to kill bacteria. A mouse model of iron overload showed that the immunogen provided protection [175].

2.12.5.3.6. r49 OMP of Typhi. In a separate investigation, a 1.3 KDa OMP from *Salmonella* Typhi was evaluated as a potential vaccine. The protein was produced using the pET28a expression vector and the IPTG inducible pT7/lac promoter. The T4 ligase was then used to insert and ligate the resulting 1.3 Kb fragment into E. coli DH5α. As an immunogen, this protein showed promise [176].

2.12.5.3.7. Other approaches using Typhimurium. The pathophysiology of human *Salmonella* Typhi and mouse *Salmonella* Typhimurium is comparable. Thus, a novel strategy was employed in which *Salmonella* Typhimurium was administered as a vaccine in various forms. An amalgamated protein fusion consisting of SPI 1 or 2 tip and 1st proteins was

evaluated for its immunogenic and protective properties in a mouse model. It was discovered to be efficient against *Salmonella* Enteritidis and *Salmonella* Typhimurium. This could be extended to the field of vaccination biology and infection with Typhi and Paratyphi [177]. A transmembrane β-barrel (TMBB) protein, OmpL from *Salmonella* Typhimurium is highly immunogenic and confers protection to 100% of its immunized mice against a lethal dose of *Salmonella* Typhimurium. Clearance of challenged bacteria from the mice reticuloendothelial system and further homology search in TS intrigues the hypothesis that this immunogen could also be effective against typhoidal challenge [178].

2.12.5.3.8. Polysaccharide subunit vaccines. Vi polysaccharide is the outer covering of most of the Typhi strains that aids in infection. Previous studies showed that, typhoid vaccines rich in Vi, namely K type are more potent immunogens than the low Vi-containing L types. The purest form of vaccine containing only Vi-polysaccharide from Typhi is the Typhim Vi vaccine produced by Sanofi Pasteur [179]. This is the sole licensed subunit vaccine against TS specifically for the age group of ≤ 5 years of age. This intra-mascularly administered vaccine does not contain any conjugates and works on its own based on the immunogenic properties of Vi polysaccharide [180]. Vi ensures protection of the bacteria from the harsh environment and the host's immune system by resisting phagocytosis of the bacteria [181]. Although Vi negative strains have been found in nature that causes enteric fever, but they are rare. Two formulations, a liquid and a lyophilized, were used for the assessment of efficacy of the vaccine. During 20 months of post vaccination period, 74% blood culture showed negative protection. This vaccine was intended to use for travelers and microbiological personnel as well. At 4 weeks post vaccination, greater than 96% induction of antibodies was seen in children 2 to 5 years of age. In children < 2 years ofage, the hike was greater than 94% [182]. Major drawback of this vaccine is its inability to induce T cell dependent immune response and generation of memory response thus failing to provide any additional booster responses at any age group.

2.12.5.4. Conjugate vaccines.

Conjugate subunit vaccine is another way of using a protein or polysaccharide from a pathogen in conjunction with another antigen from other organism, where the first one is unable to induce proper immune response on its own. The main drawback of the T cell independent Vi polysaccharide vaccine was its inability to protect children <5 years of age [183].

2.12.5.4.1. Vi-rEPA1 and Vi-rEPA2. One of the first approaches was to use the Pseudomonas aeroginosa recombinant exoprotein A (rEPA) as a carrier protein. Vi was conjugated with rEPA by either N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP; Vi-rEPA1) or by Adipic acid dihydrazide (ADH; Vi-rEPA2) as a linker. In a trial in Vietnam, Vi-rEPA vaccine showed more than 90% protective efficacy in the age group of 2-5 years old. Persistent efficacy was found in follow up cases in surveillance study [183, 184].

2.12.5.4.2. OAcP. In other studies, Vi polysaccharide response was compared with a structurally similar plant product, pectin [poly-a(134)-D-GalpA]. O acetylated C2 and C3 derivative (OAcP) of this product is identical to Vi. SPDP linked OAcP conjugates showed humoral response similar to the response against Vi alone [185].

2.12.5.4.3. Vi-DT. As already discussed, previously Vi was conjugated with rEPA, but due to regulatory constraints, this idea was heavily jolted. That is why, a new carrier protein, diphtheria toxin (DT) was used in conjugative form with the Vi polysaccharide. This new Vi-DT conferred significant up-regulation of the humoral arm of the immune system in mice model. A comparative analysis showed that Vi alone was16 to 42 times less effective in the generation of humoral response [186].

2.12.5.4.4. Vi-CRM197 and Vi-TT. The previous work was carried forward using Vi from a non-infectious bacterium, Citrobacter freundii and conjugated with a non-infectious DT,

CRM197. Vi was further conjugated with Tetanus Toxoid (TT). All mice showed significant upregulation of Vi-specific antibody response after immunization with either Vi-CRM or Vi-TT. On the other hand, even after three doses of immunization, the Vi, CRM or TT alone failed to provide any Vi-specific response [187].

2.12.5.5. Outer membrane vesicles (OMVs) based vaccine candidate.

The diameter of OMVs, which are closed spheroid vesicles, ranges from 10 to 300 nm. OMVs are secreted by both Gram-positive and Gram-negative cells, albeit in less quantities. The periplasmic contents and outer and inner membrane proteins make up these membrane vesicles. Cytosolic proteins are also thought to be present in these OMVs. When OMVs enter the body, the innate immune system detects them because they are inherently loaded with PAMPs (such as proteins, LPS, OMPs, DNA, etc.). This recognition triggers the humoral and cellular arms of the adaptive immune response [188].

2.13. Antibiotic resistance pattern of *Salmonella*:

Scientists, physicians and medical personal are becoming more concerned about the spread of medication resistance in bacterial species. Antibiotic resistance may arise from a variety of factors, including widespread use of the same antibiotic in a large population, widespread use of over-the-counter antibiotics (without a prescription), non-compliance with the antibiotic usage schedule, and spontaneous mutations in the bacterial genome. After acquiring the resistance gene, it is transmitted to uninitiated bacteria by horizontal gene transfer.

Chloramphenicol was the recommended medication for enteric fever from 1948 to 1970. However, because of the drug's extensive use, bacteria have developed resistance

to it, and ampicillin and trimethoprim-sulfamethoxazole have been chosen instead [189]. The same overuse of antibiotics had led to the emergence of antibiotic resistance in the 1980s. Fluoroquinolones and cephalosporins are now the preferred medications as a result. Fluoroquinolone treatment resulted in a 98% clinical cure rate, a 4-day fever clearance period, and a recurrence and fecal carriage rate of less than 2%. In Asia, the bacteria subsequently developed resistance to first-generation fluoroquinolones. Third-generation cephalosporins were so utilized in their place. Despite the high level of ciprofloxacin resistance observed in Bangladesh, cefipime, cefpodoximeproxetil, ceftriaxone, and cefizime demonstrated promising treatment outcomes. Drug resistance evolves over time, thus it's important to regularly screen for resistance patterns [189]. Patients with prolonged fever, vomiting, and diarrhea should have their antibiotic choice determined empirically. Azithromycin, tigecycline, and carbapenem have recently been the preferred medications for typhoidal *Salmonella* patients; nevertheless, prolonged usage of these medications is guaranteed to cause drug resistance.

In addition to this limited use strategy, widespread preventative actions like immunization should also be taken. Nevertheless, a relatively recent report indicates that Typhi haplotype H58 is spreading over the Indian subcontinent [190]. According to reports, this strain is both extremely drug resistant (XDR) and multi-drug resistant (MDR). It is resistant to third-generation cephalosporin, ampicillin, tri-methoprim sulfamethoxazole, fluoroquinolones, and chloramphenicol. There is also a possibility that an almost intractable strain of typhoidal *Salmonella* would arise.

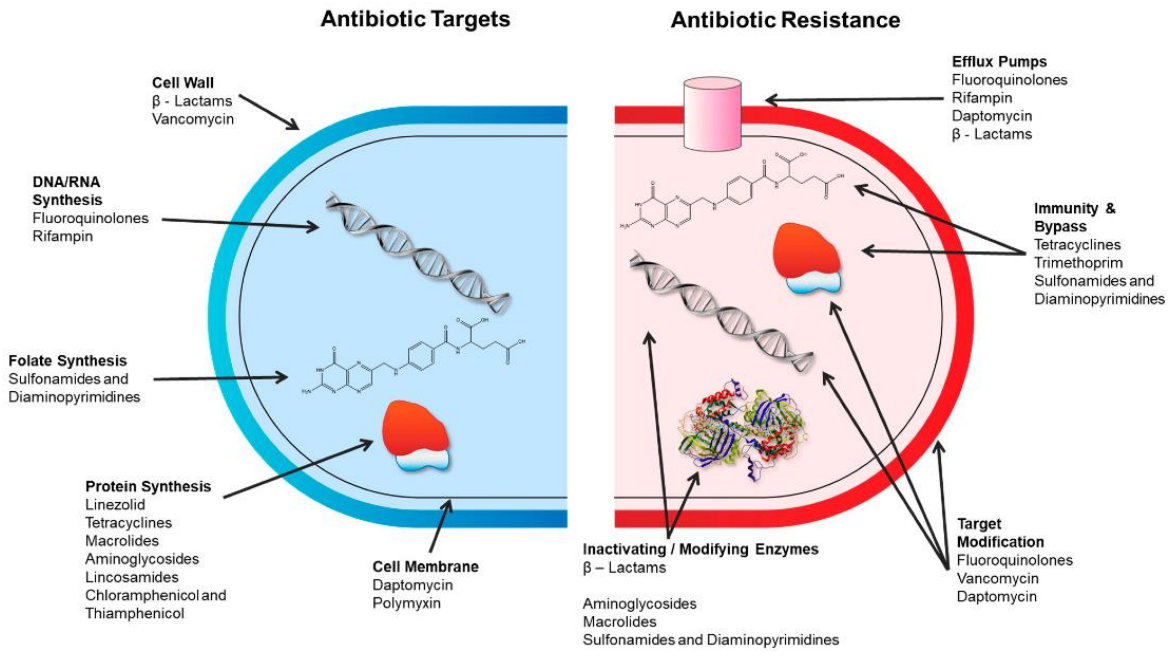


Figure 12. Antibiotic targets and mechanisms of antibiotic resistance. (image adapted from "Wright, G.D. Q&A: Antibiotic resistance: Where does it come from and what can we do about it? *BMC Biol.* 2010, *8*, 123.")

2.14. Present knowledge on experimental typhoidal vaccines:

2.14.1. Live Ty21a: **oral vaccine.** Many nations utilize the licensed galE mutant Ty2 strain as a live vaccination (Ty21a). As was previously said, there is uncertainty surrounding this vaccination strain and a possibility that it will revert to its wild type form. Despite its excellent efficacy, this vaccination has seen widespread use. Furthermore, this vaccination is recommended in locations where access to qualified medical workers is limited because of its simplicity of use (oral capsule) [191, 192].

The majority of infectious agents enter the human body through the mucosal sites and apertures, respectively. It would be ideal to have an oral vaccine against an enteric infection because the GI tract is the same path used by both the infectious agent and the vaccine. Typhoid

disease is spread by an organism that travels from person to person through the feces and mouth. Given the abundance of lymphoid cells found in the intestine, oral vaccination would be an effective way to deliver a vaccine to this site and improve both specific immunity and the protective response. The homing response in response to antigenic stimulation is controlled by the Common Mucosal Immune System (CMIS) [193, 194]. The process of homing involves making the first touch, rolling, activating the reaction, arresting, and diapedesizing. The immune cells' homing receptors interact with addressins present on the target tissues' epithelial cells, allowing the immune cells to penetrate the epithelial cell membrane. The selection of an oral vaccination for TS infection is mostly based on the expression of α4β7 integrin, a homing receptor specialized for gut mucosa that is expressed by approximately 99% of oral immunized volunteers. Despite the fact that it ought to be far more successful than it is, its effectiveness in human volunteers is only between 67% and 79% because of gastric acidity and other unidentified reasons.

2.14.2. Vi-polysaccharide: parenteral vaccine. Oral immunizations are not without limits, even though they are the best option. A parenteral strategy is used to combat them. The development of protective antibody responses against enteric illness has effectively contested the notion that parenteral vaccinations are ineffective against mucosal pathogens. Parenteral vaccination formulations with mucosal delivery components have been well received. These vaccinations stimulate homing receptors on B and T cells, which aid in mucosal defense. It has been shown that i.p. vaccination with the induction of gut associated lymphoid tissue (GALT) results in the generation of ISCs. When a typhoid infection occurs, the induction of GALT and the homing of ISCs are crucial components of the immunological generation process [195]. Since it is a polysaccharide vaccine, not every mucosal response is present. In human volunteers, it results in an immunological response that is independent of T cells and is unable to elicit a memory response. Unconjugated Vi vaccines administered in controlled pre- and

post-licensure efficacy and induction of anti-Vi IgG trials demonstrated a moderate level of protective efficacy. Therefore, Vi polysaccharide has been used to create conjugate vaccines. Here, volunteers may experience B- and T-cell-specific reactions as a result of the polysaccharide-coupled carrier proteins.

2.14.3. Typbar-TCV: intramuscular vaccine. Developed by Bharat Biotech, this is the most recent approved vaccine for typhoidal *Salmonella*. With TT and Vi polysaccharide, it is a conjugate vaccine. Participants in a randomized control experiment, ages 2 to 45, were administered a single dosage of the immunogen after two years, along with a booster. One intramuscular injection of the immunogen was administered to infants and toddlers in a different group, ranging in age from 6 to 23 months. 4.3% of the conjugate group and 2.8% of the polysaccharide group experienced fever, which was the least harmful impact. Following a single injection with a sustained antibody response, serum anti-Vi IgG was significantly greater than in the Vi alone group [196].

2.15. The current state of enteric fever: a bivalent strategy is required.

Since the Middle Ages, infectious disease effects on the general public have been documented. TS was known to cause infection and death in the same historical period (the 5th to the 15th century), and it continued into the early modern period (15th to 18th century). As early as 430 BC, some historians do think that this illness caused a massive pandemic in Athens. Three locations have been identified globally, which are low-, medium-, and high-incidence areas. Preventive measures have been implemented in these places [197]. Recent WHO figures state that there were between 11 and 21 million cases of typhoid fever worldwide, with between 75,000 and 208,000 deaths reported. 17.8 million people are predicted to get enteric fever annually out of the 5.5 billion people who live in LMIC nations [197]. While the incidence rate of typhoidal fever is substantially lower in Europe (<0.1 per 100,000 people), it can reach as

high as 724.4 per 100,000 in South Asian and Sub-Saharan African nations [198]. The basic pattern of Typhi being the primary cause of enteric fever has changed over the past few decades. According to reports from India, Paratyphi A first appeared in 2003, but in 2005 things started to alter once more [199]. South Asian nations continued to report similar trends with Paratyphi A infection findings. A few years ago, it would have been surprising to find that nearly 50% of isolates were Paratyphi A [200]. However, due to economic hardship and adequate hygienic conditions, typhoidal illnesses are declining in certain Latin American nations. The following factors contributed to the increasing trend in paratyphoid strains: widespread use causing commensal microorganisms to develop antibiotic resistance. Pathogens are acquiring these genes through a process known as horizontal gene transfer. Antibiotic resistance is consequently increasing in relatively uncommon paratyphoidal strains. Multi-Drug Resistance (MDR) is defined as resistance to the first line of treatment against TS, including ampicillin, chloramphenicol, trimethoprim, and sulfamethoxazole. Concerns were raised and a more preventive approach was required when MDR strains started to spread throughout the Indian subcontinent, Indonesia, and China [201].

Furthermore, there are potential drawbacks to having three approved vaccinations against Typhi. The true infection biology of paratyphoidal strains differs little from that of typhoidal strains, despite their pathogenetic similarity. Consequently, vaccinations against Typhi do not offer effective defense against Paratyphi. To fight against these two infections, a bivalent strategy is required.

2.16. The affordability of vaccinations against typhoidal *Salmonella* infection.

A vaccine's cost-effectiveness is dependent on a number of variables, including the vaccine's endemic status, mode of administration, immunogenicity to young patients, and duration of action [202]. The Choosing Interventions that are Cost-Effective project (WHO-CHOICE) of

the World Health Organization states that an intervention is considered “cost-effective” if it adds a Disability-Adjusted Life-Year (DALY) at a rate that is less than three times the GDP per capita [203]. While the impacts of vaccination programs are concerning in high-endemic nations, in certain sub-Saharan countries, restricted immunization of school-age children against typhoidal causes herd immunity, which reduces vaccination rates. The area in which vaccines are administered affects the concept of cost effectiveness as well. The Global Alliance for Vaccines and Immunization (GAVI) discovered that in urban slums, even Vi polysaccharide may be economically viable. A vaccine's real cost of production is determined by a number of factors, including the country's per capita income, customs fees, storage and transportation costs, and other costs [204]. The true cost of treatment after the disease starts is another crucial component of cost effectiveness. Cook et al. discovered differences in the private expenses associated with typhoidal disease in four regions of South-East Asia. Treatment for the same condition in Kolkata, India, costs no more than $12; in Jakarta, Indonesia, the cost ranges from $56 to $210. However, the expense of public treatment was different. The definition of a vaccination being cost-effective would change in such a setup. According to the same analysis from 2008, the costs associated with a "no vaccination" case are over 1.5–2 times higher in Kolkata and nearly 2.5–8 times higher in North Jakarta than the GNI criterion for cost-effectiveness. This margin even rises if a vaccination is deemed "very cost effective" based on the GNI criteria. All three groups—school-age children, younger school-aged children, and adults and children living in the community—were assessed. In Hue, Vietnam, this immunization was not found to be highly cost-effective for school-age children, although it was in Kolkata and Jakarta [205]. In this manner, before determining its cost effectiveness, a Cost Utility Analysis (CUA) must be completed. The WHO position document states that adult catch-up immunization, in addition to routine vaccination for young people, can reduce the cost of typhoidal vaccination. In order to further reduce infection, the World

Health Organization (WHO) advised the programmatic use of immunization schedule in conjunction with WASH (water, sanitation, and hygiene) [206]. Previous studies have demonstrated the cost-effectiveness of both Vi-PS and TCV vaccination, albeit their effectiveness is contingent upon specific factors. The cost-effectiveness of TCV decreases in areas with low typhoid fever incidence [207].

2.17. Vaccine pipeline for *Salmonella* Typhi and *Salmonella* Paratyphi:

The first live-attenuated S. Typhi vaccination was developed in the 1960s and provided protection for up to seven years [208]. However, these vaccinations were not included in contemporary therapy modalities due to systemic adverse outcomes that followed their usage [209]. These days, research is being done on formulations using natural and synthetic polysaccharides, protein-conjugated antigens, combination vaccinations, and alternative delivery methods like GMMA (generalized modules for membrane antigens) [210]. There are now four licensed Vi conjugate vaccines, and one of them has received WHO prequalification thus far. Vi-DT (diphtheria toxoid) and other vaccines are either in the last stages of development or are awaiting licensure. Numerous bivalent conjugate vaccinations against Typhi and Paratyphi are being developed, along with numerous Vi PS-based vaccinations (**Table. 1**).

Disease	Vaccine name/construct	Manufacturer	Status
Typhoid	Typbar-TCV / glycoconjugate Vi-TT	Bharat Biotech India Ltd, Hyderabad	Licensed in India, Nepal, Nigeria. Licensed, and prequalified by WHO in Dec 2017 for UNICEF, procurement
Typhoid	PedaTyph / glycoconjugate Vi-TT	Bio-Med Pvt. Ltd, India	Licensure in India

Typhoid	ZyVAC-TCV / glycoconjugate Vi-TT	Cadila Healthcare Limited, India	Licensed in India Launched for Typhoid (in adult volunteers) in India Adisinsight.springer.com, WHO prequalification will be sought
Typhoid	Glycoconjugate Vi-TT	Walvax	Licensed in India in 2020, Preclinical
Typhoid	TYPHIBEV/glycoconjugate Vi-CRM_{197}	Biological E Ltd, India/GVGH (GSK) Technology Transfer Agreement (TTA)	WHO prequalification obtained in Dec 2020
Typhoid	Glycoconjugate Vi-DT	SK Bioscience the International Vaccine Institute (IVITTA) PT Bio Farma (IVI TTA) Incepta (IVI TTA)	Phase II completed, enrolling into phase III, Phase-III clinical trials in typhoid (in adolescents, in children, in infants, prevention, in adults) in Indonesia (IM) ClinicalTrials.gov Identifier: NCT04051268 Preclinical
Typhoid	Glycoconjugate Vi-TT	Eubiologics, Korea	Phase 1
Typhoid	Glycoconjugate Vi-DT	DAVAC/Finlay Institute, Vietnam	Preclinical
Typhoid	Glycoconjugate Vi-PspA	IVI	Preclinical

Typhoid	Typhim Vi / Vi PS	Sanofi Pasteur SA	Prequalified by the WHO (WHO prequalified vaccines list)
Typhoid	Typherix / Vi PS	GSK	Discontinued due to better alternatives available and manufacturing struggles in 2018
Typhoid	Ty21a (Vivotif) / live attenuated	PaxVax	Prequalified by the WHO
Paratyphi A and Typhoid	Glycoconjugate O:2,12-TT + Vi-TT	NIH, Lanzhou	Phase II
Paratyphi A and Typhoid	Glycoconjugate O:2,12-CRM_{197} + Vi-CRM_{197}	GVGH, Biological E	Preclinical
Paratyphi A	Glycoconjugate CVD 1902 + CVD 909/ mutations in *guaBA* and *clpX*	University of Maryland Baltimore (UMB), Bharat Biotech	Phase I
Paratyphi A	Glycoconjugate O:2,12-DT + Vi-DT	IVI	Preclinical

Table 1. Current status of approved and clinical trial typhoid and paratyphoid vaccine

(table adapted from "Bazhenova, Aleksandra et al. "Glycoconjugate vaccines against Salmonella enterica serovars and Shigella species: existing and emerging methods for their analysis." Biophysical reviews vol. 13,2 221-246. 10 Apr. 2021, doi:10.1007/s12551-021-00791-z")

2.18. Use of novel bacterial ghost-based vaccines and its advantages.

The BG platform possesses inherent adjuvant qualities, making it a unique antigen delivery mechanism. Although the administration of vaccines via the mucosal route is a desirable concept, there are currently no adjuvants that stimulate strong immune responses at mucosal surfaces. BG can effectively trigger both systemic and mucosal immune responses in response to envelope-bound foreign antigens [211, 212]. The premise for the possibility of BG as mucosal vaccines is the presence of TLR4/TLR5 on epithelial cells, which are frequently the first and predominant cell types to meet infectious and non-infectious substances. At mucosal surfaces, immune responses can be elicited, which has the ability to eradicate or at least mitigate the negative consequences of illnesses. These investigations have also shown that BG effectively activates epithelial cells, which results in the release of defensins, chemokines, NO, and IL-6. These mediators are crucial in the vaccination or tissue injury sites' activation and recruitment of APC, which offers defense against encroaching pathogens. It was demonstrated by us and others that a single oral dose of BG generated both humoral and cell-mediated immune responses, and offered total protection against the lethal challenge [211, 213, 214]. These results suggest that BG function as strong mucosal candidate vaccines, which means they can circumvent the oral tolerance typically linked to vaccines administered orally, which is a significant obstacle to vaccinations supplied by the mucosal route [215]. In addition to being effective mucosal candidate vaccines, BG also work well as adjuvants to boost the body's defenses against foreign antigens on mucosal surfaces. According to other research, BG cause strong protective mucosal immune responses against foreign antigens supplied by ghosts and sway the immune response in favor of the Th1 subtype [216, 217]. This suggests that BG function as strong mucosal adjuvants for CD8+ T cells. Consequently, the creation of vaccines based on BG may provide human protection against a variety of intracellular organisms for which traditional vaccinations are either ineffective or nonexistent. As the immune response's

Th1 bias, a sign of CD8+ T cell activation [218], is essential for eliminating chronic infections in their natural hosts, BG may be used as adjuvants to promote sterile immunity against intracellular pathogens in susceptible animal species (**Fig. 13**).

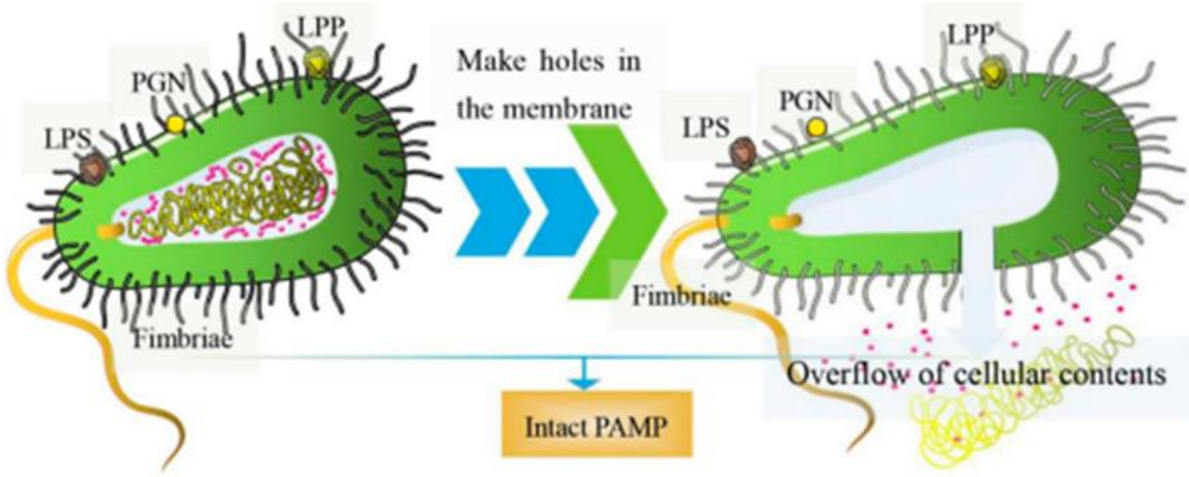


Figure 13. The Bacterial Ghost cells (BGs) diagram. Bacteria develop pores on their surface (left); biological components, such as ribosomes and nucleic acids, spill out of the cell (right). (Image adapted from "Chen, Haojie et al. "Bacterial Ghosts-Based Vaccine and Drug Delivery Systems." doi:10.3390/pharmaceutics13111892")

Potential envelope structures known as BG have effective adjuvant and delivery system qualities in addition to acting as strong candidate vaccines. The effectiveness of BG for the delivery of medications, vaccines, and biotherapeutics in animal models has been documented in a number of noteworthy studies. Nevertheless, the future of BG as drug delivery vehicles will depend on their capacity to successfully deliver biotherapeutics to their intended sites. Furthermore, the BG system implementation in humans necessitates a number of comprehensive and methodical investigations. BG's inherent adjuvant qualities and the retention of its original envelope structures would unquestionably supersede the use of live or attenuated bacteria as vaccines, which are typically linked to an unintentional infection risk. There are other topics that are pertinent to the delivery mechanism and adjuvant usage of BG

that merit further research. It would be fascinating to clarify the impact of pre-existing BG-specific immunity on the transport of heterologous foreign antigens and medications, as BG mediate active immunization against their own envelope components (**Fig. 14**).

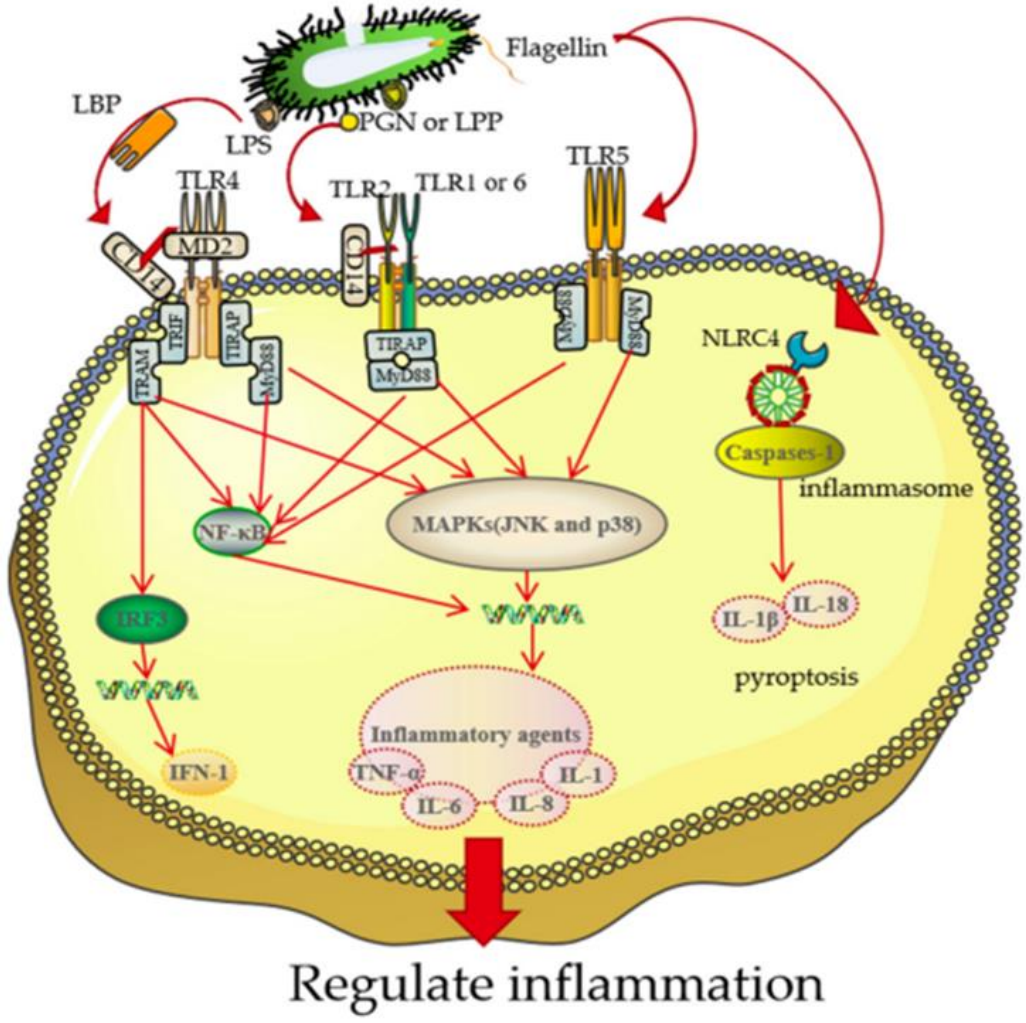


Figure 14. The mechanism by which BGs bind to TLRs on immunological or epithelial cells. (Image adapted from "Chen, Haojie et al. "Bacterial Ghosts-Based Vaccine and Drug Delivery Systems." doi:10.3390/pharmaceutics13111892")

Chapter – 3

Objectives of the study

Infectious disorders such enteric fever worry medical practitioners in developing and underdeveloped nations [1, 2]. Typhi and Paratyphi A are the deadliest of the approximately 2500 serovars of Salmonella enterica, and they have been the primary cause of enteric fever. Due to the nature of the illness, treatment with the proper antibiotic is imperative and should start as soon as a clinical diagnosis is made rather than waiting for the findings of antimicrobial susceptibility testing to be available. Since 1989, numerous developing nations, particularly Pakistan and India, have reported epidemics of Typhi caused by strains resistant to ampicillin, trimethoprim, chloramphenicol, and sulfonamides as well as increased resistance to streptomycin, tetracyclines, and sulfonamides [3]. Resistance to various antimicrobial treatments of enteric fever has been consistent for decades, as evidenced by the first reports of chloramphenicol resistance in S. Typhi in the 1970s [4]. Due to its resistance to co-trimoxazole, amoxicillin, and chloramphenicol, S. Typhi was mostly isolated in South Asia and was associated with numerous outbreaks in the late 1980s and early 1990s [5]. The treatment of enteric fever has become more difficult due to S. Typhi's antibiotic resistance. Vaccination is the only option to lessen this health burden [6].

Presently accessible typhoid vaccinations are all insufficient. Parenteral whole-cell vaccinations have been associated with few reported adverse effects. Only three of the numerous other vaccines have licenses: two are parenteral subunit vaccines based on Vi, and the third is a live-attenuated Ty21a vaccine (galE mutant) [7, 8, 9]. These two vaccinations are ineffective against other *Salmonellae* illnesses, including Paratyphi A infection, which is on the rise in South-East Asian nations, due to the lack of Vi polysaccharide in all *Salmonellae* serovars. These vaccinations do not provide long-term immunity and only offer little protection against Typhi, despite their encouraging results. All of the aforementioned live-attenuated vaccine options have the danger of reverting to the wild-type strain and posing a serious risk to human health when injected.

To fight against the diverse range of typhoidal *Salmonellae*, a new bivalent formulation is therefore required. According to studies, bacterial ghost cells (BGs) represent the antigenic content of the cells because they are just the inactivated form of a bacterial cell having cells' plasma membrane. Pathogen Associated Molecular Patterns (PAMPs) such as Vi-polysaccharides (Vi-PS), Lipopolysaccharides (LPS), peptidoglycan, outer membrane proteins (OMPs) and porins are present in the bacterial ghost cells (BGs) of Gram-negative bacteria, making them strong immunogens just like outer membrane vesicles (OMVs) of a bacteria [10,11].

Different antigenic platform including BGs, OMVs have been used in our laboratory occasionally in the quest for a novel vaccine platform for different enteric pathogens. Our previous research on mice has demonstrated the safety and efficacy of pentavalent *V. cholerae*, *Salmonella* and multi-serotype *Shigella* OMVs [10-13]. Bacterial ghost cells (BGs) would be the next generation vaccine candidates for different Gram-negative and Gram-positive bacteria

The following have been developed as the goals of my study with these considerations in mind:

i. Selection of typhoidal strains for immunogen preparation from recently circulated clinical isolates.
ii. To formulate typhoidal *Salmonellae* immunogen.
iii. To study the reactogenicity, immunogenicity and protective efficacy of the newly prepared immunogen.
iv. To study signaling pathways involved behind immunomodulation.

Chapter – 4

Methods and Materials.

4.1. Bacterial Strains: *Salmonella* Typhi (C-6.946), *Salmonella* Typhi (K554), Salmonella Paratyphi A (BCR148), and Salmonella Paratyphi A (K580) are used in this study. All Salmonella strains were kept in 20% glycerol, maintained at-80°C in Tryptic soy broth (Difco, USA). Prior to use, strains were cultured in Tryptic Soy Broth (TSB; Difco, USA) at 37°C with shaking or on Tryptic Soy Agar (TSA; Difco, USA) plates.

4.2. **Cells:** The culture medium used for RAW 264.7 cells included 10% FBS in addition to DMEM. RPMI 1640 supplemented with 10% FBS was used to cultivate ex vivo splenic cells. For five days, dendritic cells (DCs) obtained from bone marrow were cultured in RPMI 1640 supplemented with 10% FBS and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) (Sigma) in order to facilitate differentiation. For a whole day, RPMI-1640 was used in place of the previous medium, free of antibiotics. Raw 264.We bought 7 cell lines from the American Type Culture Collection. All of the materials needed for cell culture were purchased from Invitrogen. Every cell line was kept in an incubator with 5% CO2 and 37 °C humidity, along with 100 U/mL of Penicillin/Streptomycin (Invitrogen). Prior to studies, cells that were close to confluence were grown in serum-free media for a whole night.

4.3. Reagents: Molecular biology reagents were purchased as follows: Phosphate Buffer Saline (Sigma), PCR reagents (Takara), PCR DNA 100 bp ladder (Takara), PCR DNA 1 kb ladder (Takara), Bovine serum Albumin (Sigma), DH20 (Takara), Unstained Protein ladder (sigma), Prestained Protein ladder (sigma). AP- conjugated secondary antibodies were from Merck, HRP-conjugated secondary antibodies were from Merck and Fluorescent-conjugated antibodies were from Miltenyi Biotech, USA. All pharmacological inhibitors were purchased from Calbiochem. All other chemicals were purchased from Sigma-Aldrich unless otherwise mentioned.

4.4. Chemicals: All Bacterial culture media; Tryptic Soy Broth, Tryptic Soy Agar, skim milk anti-mouse CD4 Magnetic Particles were purchased from BD (Difco), USA. All kind of chemical Salts (anhydrous), Glutaraldehyde, Tris, HEPES, APS, TEMED, SDS, Acrylamide, Phenol, MOPS, Tween-20, Coomassie, silver stain, haematoxylin and eosin (H&E), baby rabbit complement, mucin were purchased from Sigma, Merck, SRL or MP biomedicals. All the acids HCL and H2So4 were purchased from Merck and MP Biomedicals. Protease inhibitor cocktail was purchased from Roche, USA. LDH assay kit, LAL assay Kit, and Gentamicin were purchased from Sigma Aldrich, USA. Murine recombinant GM-CSF and IL-2 proteins was purchased from Invitrogen and R&D systems, USA. Cytokine ELISA kits for TNF-α, IFN-γ, IL-4, IL-5, IL-1β, IL-6, IL-23, IL-12p40/70, IL-2, and IL-17 were procured from Invitrogen, USA. Vi was purchased from Bharat Biotech. RPMI, DMEM, FBS, CD4+ isolation kit was from BD Biosciences USA. NBT (nitro-blue tetrazolium chloride) /BCIP (5-bromo-4- chloro-3'-indolyphosphate p-toluidine salt) and 3-amino- 9-ethyl carbazole (AEC) were from Sigma. Total Immunoglobulin, IgA and IgG raised in goat were from Sigma-Aldrich.

4.4.1. Antibodies and Inhibitors: Protease inhibitor cocktail and BSA were purchased from Roche, Sigma, USA. All conjugated secondary antibodies are purchased from sigma, USA. CD4-PE, CD8a-PE, CD19-PE, from Miltenyi Biotech USA and CD-APC, CD62-APC, CD44-PE were from BD Biosciences USA.

4.4.2. Oligos used in this study: Oligos were custom-synthesized from IDT, USA. The table of the genes and primers as follows:

Gene and primer (oligonucleotide sequence)	**Amplified fragment size (bp)**	**Reference**
prt (rfbS) ***parat-s*** **(5'-CTT GCT ATG GAA GAC ATA ACG AAC C-3')** ***parat-as*** **(5'-CGT CTC CAT CAA AAG CTC CAT AGA-3')**	258	[1]
fliC ***fliCcom-s*** **(5'-AAT CAA CAA CAA CCT GCA GCG-3')** ***fliCd-as*** **(5'-GCA TAG CCA CCA TCA ATA ACC-3')** ***fliCa-as*** **(5'-TAG TGC TTA ATG TAG CCG AAG G-3')**	750 (*fliCcom–fliCd-as*) 329 (*fliCcom–fliCa-as*)	[1]
InvA ***InvA-F*** **(5'-CGAGCAGCCGCTTAGTATTGAG-3')** ***InvA-R*** **(5'-CCATCAAATTAGCGGAGGCTTC-3')**	881	[2]
tviA ***tviA-F*** **(5'-GTTATTTCAGCATAAGGAG-3')** ***tviA-R*** **(5'-ACTTGTCCGTGTTTTACTC-3')**	599	[3]

tviB ***tviB-F* (5'-CGAGTGAAACCGTTGGTACA-3')** ***tviB-R* (5'-CAATGATCGCATCGTAGTGG-3')**	846	[4]

Table: Details of Gene and primer used in this study.

4.5. Bacterial culture conditions:

Salmonella Typhi (C-6.946) and *Salmonella* Paratyphi A (BCR 148) were used for bacterial ghost cell preparation and Salmonella Typhi (K554) and Salmonella Paratyphi A (K580) were used for challenge studies. All strains were obtained from National Institute of Cholera and Enteric Diseases (NICED) strain culture repository. All strains were kept in 20% glycerol, maintained at-80°C in Tryptic soy broth (Difco, USA). Prior to use, strains were cultured in Tryptic Soy Broth (TSB; Difco, USA) at 37°C with shaking or on Tryptic Soy Agar (TSA; Difco, USA) plates. Table 1 lists the strains used in this study [1].

4.6. PCR Characterization of the strains used:

Although the strains were characterized once, but before starting the work, we wanted to be assured about their genotypic and phenotypic properties. Strains were characterized by both PCR-based method and serotyping. For PCR, the following genes were selected and their respective primer sequences have been shown in bracket: prtA-parat-s (5'-CTT GCT ATG GAA GAC ATA ACG AAC C-3'); parat-as (5'-CGT CTC CAT CAA AAG CTC CAT AGA-3'); fliCd-as (5'-GCA TAG CCA CCA TCA ATA ACC-3'); fliCa-as (5'-TAG TGC TTA ATG TAG CCG AAG G-3'); fliCcom-s (5'-AAT CAA CAACAA CCT GCA GCG-3') [1], invA-F (5'-CGAGCAGCCGCTTAGTATTGAG-3'), invA-R (5'-

CCATCAAATTAGCGGAGGCTTC-3') [2], tviA-F (5'-GTTATTTCAGCATAAGGAG-3'), tviA-R (5'-ACTTGTCCGTGTTTTACTC-3') [3], and tviB-F (5'-CGAGTGAAACCGTTGGTACA-3'), tviB-R (5'- CAATGATCGCATCGTAGTGG-3') [4]. The PCR was carried out with PCR mastermix (Takara), primers (prt, fliC-d, fliC-A) and boil template prepare for each strain at 100˚C for 10 min under the DNA thermal cycler conditions: 25 cycles with heat denaturation at 95°C for 30 s, primer annealing at 55°C for 60 s, and DNA extension at 72°C for 90 sec. The amplified DNA was separated by 2% agarose gel electrophoresis, stained with gel stain (sigma), and visualized by UV transillumination [5].

4.7. Serotypic and Antibiotic susceptibility patter of *Salmonella* strain:

Afterwards, anti O- and H-antibodies were used for Serotypic characterization of the strains used in the study. WHO and Clinical & Laboratory Standards Institute (CLSI) guidelines were followed for Bauer method. Antibiotic discs (AMP 10 µg, CTX 30µg, CFZ 30 µg, CFX 5µg, CRO 30 µg, CHL 30 µg, I 10 µg, NAL 30 µg, CO 25 µg, TET 30 µg, TMP 30 µg, SXT 23.75 µg, LO 30 µg, AZ 15 µg, CIP 5 µg, STR 10 µg, KAN 30 µg) were purchased used (BD Bioscience, USA). Bacterial suspensions were plated and discs of antibiotics were kept in contact with the upper surface of the plate overnight. The antibiotic sensitivity was then assessed by measuring the zone diameter of bacterial growth [5].

4.8. Preparation of Ghost immunogen:

4.8.1. Determination of MIC: Determination of the MIC of NaOH for Salmonella Typhi and Salmonella Paratyphi A will be performed by the 2-fold broth dilution method as described by with some modifications. Briefly, a virulent culture of S. Typhi and S. Paratyphi A will be grown in different broth and Solid media and adjusted to 1×108 CFU/ml. The initial concentration of NaOH will be 50 mg/ml. Two-fold dilutions of NaOH will be added to samples of the virulent bacterial culture, and they will be incubated at 37°C for 18 h. After

incubation, the turbidity of each individual tube will be assessed visually and the MIC will be determined as the lowest concentration of NaOH that completely killed the bacterial growth. Further, to determine viability, the culture that showed no visible bacterial growth will be verified by spreading 100µl of the culture onto tryptic soy agar (TSA) plates and incubating them at 37°C for 24 h. The MIC will be determined in three independent experiments [6].

4.8.2. Production of Ghost bacteria: Determination of the minimum inhibitory concentration (MIC) of NaOH for S. Typhi and S. Paratyphi A was performed by the 2-fold broth dilution method as described previously with some modifications. The biomass of overnight S. Typhi and S. Paratyphi A culture cells was centrifuged (8,000×g, 10 min, 4°C) before being resuspended in sterile phosphate buffered saline (PBS, pH-7.4). The bacterial cells were then adjusted to 1 × 108 CFU/ml. 1 ml (5×) of MIC of sodium hydroxide concentration was added to 2 ml of cell suspension with 2 ml of sterile solvent and incubated for 90 min at 37 ◦C. After centrifugation (8,000×g, 10 min, 4 ◦C) and three PBS washes, ghost cells were collected. In ice-cold PBS, the final cell pellets were resuspended. Based on bacterial cell count (1 × 108 CFU/ml), recovered bacterial ghost cells from the two strains were combined in a 1:1 ratio and the final mixture was kept at 4 ◦C until further use [6,7]. 100 µl of each strain of bacterial ghost cells were spread on agar plates to check for complete lysis of bacterial cells.

4.9. Scanning Electron Microscopy (SEM) imaging of bacterial ghost cells:

Ghost bacteria and non-treated control bacteria were fixed using buffered 2.5% glutaraldehyde (pH 7.0) for 2 h at 4°C, washed with buffer and then post fixed in 1% osmium tetroxide for 1.5 h at 4°C. Again, washed samples are dehydrated through a series of ethanol concentrations. After samples reached the critical dried point, they were mounted on SEM stubs, coated with

gold-palladium, and observed under a scanning electron microscope, Scanning Electron Microscope, FEI, Quanta 200 MK 2 (D8313) (FEI, Netherlands) [6].

4.10. Purification of whole cell lysates (WCL):

WCL were isolated according to the previously described protocol [5] as follows; Bacteria were cultured 18 h in Tryptic Soy Broth (TSB; Difco, USA), centrifuged to obtain cell pellets, washed with PBS, and sonicated. Again, samples were centrifuged and supernatant was collected and stored.

4.11. Purification of outer membrane proteins (OMPs):

Outer membrane proteins were isolated based on a previously described protocol [10] as follows; Bacteria were cultured for 18 h in Tryptic Soy Broth (TSB; Difco, USA), centrifuged, and pellets were washed with HEPES buffer (pH 7.5) and protease inhibitor cocktail (Roche, Sigma, USA). After that, they were sonicated for ten minutes using a Hielscher (UP100H) sonicator in an ice bath. After centrifugation, the supernatants were collected and centrifuged at 100000xg. The pellet was re-suspended with same buffer containing 1% N-lauryl Sarcosine and placed at 37°C for 30 minutes. Again, it was centrifuged and the final collection was re-suspended in same buffer. Then the OMP concentration was determined, which was then adjusted to 1µg/1µl with HEPES buffer and stored at -20°C.

4.12. Estimation of protein:

Pipette 40µL of unknown sample replicate into a microplate well. Add 200µL of Modified Lowry Reagent to each well at nearly the same moment using a multi-channel pipettor. Immediately mix microplate on plate mixer for 30 seconds. Cover the plate and incubate microplate at room temperature (RT) for exactly 10 minutes. Add 20µL of prepared 1X Folin-Ciocalteu Reagent to each well using a multi-channel pipettor. Immediately mix microplate on

plate mixer for 30 seconds. Cover and incubate microplate at RT for 30 minutes. Measure the absorbance at or near 750nm on a plate reader [11].

4.13. Purification of lipopolysaccharide (LPS):

LPS was isolated based on a previously described protocol [10] as follows; S. Typhi and S. Paratyphi A were cultured overnight in Tryptic Soy Broth (TSB; Difco, USA), centrifuged, and the cell pellet was re-suspended with 0.15M NaCl containing Phenol-saturated 3-[N-morpholino] propen sulfonic acid (0.02M MOPS). The mixture was incubated for 30 minutes at 65°C with random shaking, and then for 10 minutes on ice. After a second centrifugation, the upper aqueous layer was collected, mixed with four times the volume of the sample with chilled ethanol, and left overnight at -20°C. LPS was purified and collected on the following day by centrifuging at 8500xg for 20 minutes, resuspending in distilled water, and storing at -20°C.

4.14. LPS detection by silver stain:

The traditional silver staining technique of Tsai and Frasch [12] was applied to SDS-PAGE-fractionated LPS samples, with the following adjustments. Without first fixing, LPS was oxidized in the gel using 0.7% periodic acid in 40% ethanol-5% acetic acid at 22°C for 20 minutes. After that, the gel was given three 5-minute washes in distilled water. The freshly made staining solution was used to colour the gel for ten minutes. The recipe was as follows. 56 ml of 0.1 M sodium hydroxide were mixed with 4 ml of concentrated ammonium hydroxide. Approximately 200 milliliters of water were added, and then 10 milliliters of 20% (wt/vol) silver nitrate (Sigma, USA) were added in drops while stirring. 300 ml was the final volume that was adjusted with water. After that, the gel was given three 5-minute washes in distilled water. Reduction in 200 milliliters of water with 10 milligrams of citric acid and 0.1 milliliters of 37% formaldehyde produced the colour. The gel was shot right away, or it was repeatedly

washed in distilled water after being exposed to 10% acetic acid for a minute to halt the colour reaction.

4.15. Estimation of Vi-polysaccharide by Hestrin assay:

Prior to use, the alkaline hydroxylamine reagent is made fresh by combining equal amounts of Reagents 1 and 2. At room temperature, the mixture keeps for around three hours. In a test tube, 1.0 millilitre of the solution to be examined is mixed with two millilitres of the alkaline hydroxylamine reagent. One minute is required, or more if preferred, to bring the pH down to 1.2 ± 0.2. Then, 1.0 ml of acid and 1.0 ml of the iron solution are added. The density of the purple-brown colour is promptly determined at 540 nm [13].

4.16. Macrophage-mediated cytotoxicity Assay (LDH assay):

To check the cytotoxicity of NaOH treated Bacteria, we used Pierce LDH Kit (Invitrogen, USA). Murine macrophage cell line (RAW 264.7) was cultured in 96-well plates (Nunc, USA) for 24 h in a 37°C incubator (with 5% CO2). 1.0 × 105 cells/well was then incubated for 24 h with 1.0 × 108 CFU/ml of ghost cells and heat-killed cells in culture medium. LDH-positive samples are used for positive controls and PBS was used for negative control [7].

4.17. Endotoxicity measurement by LAL assay:

Equilibrate the microplate in a heating block for 10 minutes at 37ºC. With the microplate maintained at 37ºC, carefully dispense 50µL of each standard or unknown sample replicate into the appropriate microplate well, cover the plate with the lid and incubate for 5 minutes at 37ºC. Add 50µL of LAL to each well using a pipettor, cover the plate with the lid and gently shake the plate on a plate shaker for 10 seconds. Incubate the plate at 37ºC for 10 minutes. After exactly 10 minutes, add 100µL of substrate solution to each well. Pipette the substrate solution in the same manner as in step 3. Maintain a consistent pipetting speed. Cover the plate

with a lid and gently shake on a plate mixer for 10 seconds. Incubate the plate at 37ºC for 6 minutes. Add 50µL of Stop Reagent (25% acetic acid). Maintain the same pipetting order as in steps 3 and 4. Gently shake the plate on a plate mixer for 10 seconds. Measure the absorbance at 405-410 nm on a plate reader [8].

4.18. Animals:

Six weeks old female BALB/c mice were obtained from the animal house facility of NICED, Kolkata. Mice were separated into different groups with sterile food and water. The Institutional Animal Ethical Committee of NICED (CPCSEA registered, Registration No. 68//Rebi/S/1999/CPCSEA valid17/7/2024), approved the animal experimental protocol with the project approval number. PRO/167/January 2020-November 2022.

4.19. Immunization of Animals:

Mice were injected intra-peritoneally on the 0th, 14th and 28th day with 100 µl of bacterial Ghost Immunogen (1:1) containing 1×10^8 CFU/ml cell. Non-immunized control group mice received 100 µl of sterile PBS.

4.20. Collection of Serum and Intestinal lavage:

On the 0th, 7th, 21st, 35th, 49th, 70th, 90th and 120th days following the initial vaccination, at time intervals, blood was taken from tail veins. Blood was kept in microtainer tube (BD, USA) and serum isolated by centrifugation (1000 x g, 10 min, 4°C). Intestinal lavage from immunized and control mice was collected in a sterile microcentrifuge tube by PBS wash of small intestine after dissection. Lavage was centrifuged at 6000 x g, 10 min; supernatant was collected and stored at -20°C.

4.21. Haematological parameter analysis:

Animals of immunized and control groups were monitored for the signs of acute toxicity. After 7 days of every immunization, animals were bled and blood samples were collected to study haematological parameters, such as the Haemoglobin (Hb), Erythrocyte Sedimentation Rate (ESR), Total Leucocyte Count (TLC), Differential Leucocyte Count (DLC), and Packed Cell Volume (PCV) [9]. All parameters were analysed within few hours of sample collection.

4.22. Determination of antibody levels by ELISA:

96 well flat bottom ELISA plate (Tarson, India) was separately coated with whole cell lysate (WCL), outer membrane proteins (OMPs), lipopolysaccharide (LPS), and Vi-polysaccharide (Bharat biotech, India) as previously described [5, 10]. Then each plate was kept at 4ºC for 18 h. The wells were washed with PBS and blocked with skim milk (BD, USA). After that wells were washed with PBS-T (PBS having 0.5% Tween-20, Sigma, USA) and incubated for 1hr with consecutively diluted serum samples and intestinal lavage for mucosal antibody. Again, wells were washed with PBS-T and incubated after adding HRP-tagged secondary antibody (IgG, IgG1, IgG2a & IgA, Abcam & Sigma, USA). After a PBS wash, substrate was added to all wells and kept for ten mins. The reaction was stopped using sulphuric acid (2N). Finally, the OD492 nm was measured using a microplate reader.

4.23. SDS-PAGE, Immunoblot and Dot Blot.

LPS and total protein content of the OMPs from Salmonella strains were determined by SDS-PAGE. 20 μg of OMPs, were boiled in SDS-PAGE buffer and LPS samples were boiled in LPS sample buffer. The samples were then loaded onto a 12% SDS-PAGE gel separately depending on their staining reagent. 100 V was then applied for running the gel in an AE-6530 SDS-PAGE apparatus from ATTO Corporation (Japan). The gel was then stained by either

coomassie or silver stain. For immunoblot, proteins were boiled in 5x loading buffer and separated on a 12% SDS gel. Using ATTO AE-6687 (Japan) blot apparatus, proteins were transferred onto a nitrocellulose membrane for immunoblotting. Antisera from immunized mice and the ALP-conjugated goat anti-mouse secondary IgG was used for immunoblotting [10]. We measured the concentration of extracted LPS, with 5µg, 10µg and 15µg of LPS being used for a dot blot assay of either strain. First, LPS was absorbed by nitrocellulose membrane at room temperature. 5% BSA solution was used for blocking and then the membrane was washed with TBS-T following a standard protocol. Antisera from immunized mice and the ALP-conjugated anti-mouse secondary IgG was used in the dot blot [5, 10].

4.24. Serum bactericidal assay:

Serum bactericidal assay was performed according to an earlier described method [14]. Sera collected from the immunized mice on day 35 of first immunization was heat inactivated at 56°C for 20 min. Serum samples were diluted from 1:50 to 1:128000 in PBS. A master mix composed of 12.5 µl of baby rabbit complement (12.5% final concentration) with 27.5 µl of PBS, 50 µl of diluted mouse serum, and 10 µl of diluted bacteria (320 CFU, T0h) was prepared. The mixture was then incubated for 1h (T1h) with shaking at 115 rpm at 37°C. Viable bacterial colonies were counted after spreading the mixture onto the plate followed by overnight incubation at 37°C. The negative control contained bacteria and complement only. Bactericidal activity was determined as serum dilutions necessary to obtain a 50% reduction in CFU counts at T1h compared with T0h. For SEM imaging, samples (bacteria and complement with heat inactivated immunized serum, nonimmunized serum respectively) with lowest serum dilution (1:50) were fixed by adding 900 µl glutaraldehyde and incubated at 4°C overnight. Cells were then prepared using the previously described method for scanning electron microscopic analysis and observed [10].

4.25. Motility and Mucin penetration assay:

A motility assay performed based on a published method, with modifications [15]. Vaccinated and control serum were mixed with PBS in 1:400 dilutions, separately, and dispensed on soft agar (0.3%) plates. Log-phase bacteria with an OD600 value of 0.4 were punctured in the middle of the plates once the serum mixture had dried. After that, the plates were incubated for 24-hours at 37°C. Then the bacterial spreading across the surface was measured. The mucin penetration assay was performed according to a published method [16]. Briefly, a solution containing 1% (w/v) mucin (MP Biomedicals, USA) and soft agar (0.3%) media (TSB) was allowed to form a soft gel at room temperature in a 1 ml tuberculin syringe fitted with a stopcock. Log-phase bacteria were incubated for one hour at 37°C with heat-inactivated vaccinated and control serum. 100 µl aliquot (containing 1 x 107 CFU/ml organisms) of incubated bacterial suspension were placed in the top of the columns and kept at 37°C for 30 min in a vertical position. To determine the bacterial count, a 500 µl sample from the column's lower portion was collected, serially diluted, and plated.

4.26. Cytokine measurement by ELISA:

Different cytokines were measured according to the manufactures protocol. All the cytokines, TNF-α, IFN-γ, IL-6, IL-4, IL-5, IL-1β, IL-6, IL-23, IL-12p40/70, IL-2, and IL-17 (Invitrogen, USA) are stored in exact storage condition of manufacturer instruction. For quantification of cytokines, Pre-coated ELISA plate well, were washed thrice with wash buffer and then blocked with 200 µL/well of 1X ELISA diluent for 1 h. Wells were then incubated with 100 µl/well of culture supernatant, diluted serum or colonic tissue lysates (diluted in assay diluent) or culture supernatant for 2 h at room temperature. Assay diluent was used in wells of plate blank. Wells were then washed 5 times with wash buffer and incubated with 100 µl/well of diluted detection antibody at room temperature for 1 h or 30 min. Wells were washed 5 times with wash buffer,

followed by incubation with 100 µl/well of diluted Avidin-HRP at room temperature for 30 m. Wells were washed 7 times with wash buffer and then wells were incubated with 100 µl/well of 1X TMB solution for 15 m. 50 µL of stop solution was added to each well and absorbance was taken at 450 nm [5, 10].

4.27. Splenocytes re-stimulation assay.

Splenocytes from vaccinated mice were cultured for two hours in RPMI containing 10% FBS one week after the last vaccination. 1 X 10^6 CFU/ml bivalent typhoidal bacterial ghost cells were used to treat the splenocytes at 37°C (with 5% CO2) for 24 hours. From the culture supernatants, TNF-α, IFN-γ, IL-6 and IL-17 were estimated by individual ELISA kits (Invitrogen, USA) [5].

4.28. Separation of bone marrow-derived dendritic cells (BMDCs) and re-stimulation assay:

From naive BALB/c mice, bone marrow was isolated and cultured in RPMI containing 10% FBS and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). 1 X 106 CFU/ml bivalent typhoidal bacterial ghost cells were used to treat the mature BMDCs at 37°C (with 5% CO2) for 24 hours [5]. From the culture supernatants, IL-1β, IL-6, IL-23, and IL-12p40/70 were estimated by individual ELISA kits (Invitrogen, USA).

4.29. BMDC-Splenic $CD4^+$ T cell co-culture:

BMDCs (previously stimulated) and splenic CD4+ T cells were isolated (BD IMag™ anti-mouse CD4 Magnetic Particles, Cat. No. 551539, USA) and cultured at a 1:1 ratio for 24 h [17]. From the culture supernatants, IFN-γ, IL-2, IL-6 and IL-17 were estimated by individual ELISA kits (Invitrogen, USA).

4.30. Fluorescence-activated cell sorter (FACS) analysis:

The spleens of both the immunized and control mice were removed and sterilely homogenized on the 35th day after vaccination to determine the CD4+, CD8a+, and CD19+ cell populations. Using Cell Strainer (Corning, USA) and a sterile syringe, the spleen was homogenized. [10]. Splenocytes were stained with anti- Mabs: CD4-phycoerithrin (PE), CD8a PE, CD19 PE or an isotype control PE (Miltenyi Biotec, USA). Expression was measured on a BD FACS ARYA III flow cytometer and data was assessed with FACS DIVA and FlowJo (version V10.8.1).

4.31. Memory T cell assay:

As previously mentioned [18], dendritic cells were produced using myeloid precursor cells from mouse bone marrow (BM). In summary, for seven days, RPMI 1640 media supplemented with murine Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF, 20 ng/mL) was used to cultivate bone marrow cells from the femur and tibia of naïve BALB/c mice. After harvesting the cells on day 7, they were starved for 12 hours in RPMI 1640 with 1% FBS and then stimulated for 24 hours with bivalent typhoidal bacterial ghost cells. On day 120 of the initial immunization, CD4+ T cells were extracted from the inoculated mice's spleens using magnetic beads (BD IMagTM anti-mouse CD4 Magnetic Particles, USA). CD4+ T cells and antigen-pulsed BMDCs were co-cultured at a 1:1 ratio for 24 hours at 37°C with 5% CO2. The amount of IFN-g in the culture supernatants was measured using ELISA (Invitrogen, USA), and flow cytometry was used to examine the CD4+ T cells for T-effector memory cell determination markers (CD4+CD62^{Low} CD44^{high}).

4.32. Mice infection experiments:

Six weeks old female BALB/c mice were used for the mice infection study. All the animals were good in health and are caged according to our study designed. Before the experimentation

all the animals were acclimatises for a week with adequate food and water, in 12-hour day night cycle. The fresh animal with good health condition were chosen for all the immunization, bacterial infection and other animal related experimentation [5, 10].

4.32.1. Bacterial challenge study:

The immunized and non-immunized animals were infected intra-peritoneally with 5 x 10^8 CFU/mouse of heterologous strain of bacteria (S. Typhi; K-554 and S. Paratyphi A; K-580) on the 35th day after first vaccination. Total of 30 mice were divided into five groups. 6 mice per group were challenged intra-peritoneally. Two immunized group were infected with S. Typhi and S. Paratyphi A. Two nonimmunized groups were infected with S. Typhi and S. Paratyphi A. Another group received only 100 µl PBS; as the non-immunized, non-infected negative control. The infected mice were observed for 10 days to assess survival. To examine bacterial colonization in different systemic organs, animals were infected intra-peritoneally at the 35th and 180th day post first immunization with 5 x 10^5 CFU/mouse of heterologous typhoidal strains (S. Typhi; K-554 and S. Paratyphi A; K-580). Infected animals (both vaccinated and control mice) were sacrificed at different times post infection to determine the bacterial count.

4.32.2. Passive protection study:

35th day post first immunization serum and splenocytes (spleen was processed as before) were isolated from vaccinated and control group of mice then transferred to a naïve mouse via tail vein (100 µl of serum). RBCs were lysed and splenocytes were re-suspended in phosphate saline buffer and 100 µl (1 x 10^6 splenic cell) was injected via tail vein. Afterwards, animals were infected intra-peritoneally with 1 x 10^5 CFU/mouse with heterologous strains of Salmonella to observe bacterial colonization after 72 hours. One group of mice was infected on the day of the adoptive transfer (0^{th} day) and another group was infected at the 7^{th} day post adoptive transfer.

Another study was done with same protocol of immunization serum and splenocytes (spleen was processed as before) were isolated from vaccinated and control group of mice then transferred to a naïve mouse via tail. RBCs were lysed and splenocytes were re-suspended in phosphate saline buffer and 100 µl (1 x 10^6 splenic cell) was injected via tail vein. Afterwards, animals were infected intra-peritoneally with 1 x 10^8 CFU/mouse with heterologous strains of Salmonella to observe survivability of animals up to 10 days. One group of mice was infected on the day of the adoptive transfer (0^{th} day) and another group was infected at the 7^{th} day post adoptive transfer.

4.33. Histopathology:

The liver and spleen tissues that were obtained at 48 and 72 hours after infection were fixed in a solution of 10% buffered formalin, and 5 µM paraffin-embedded sections were stained with haematoxylin and eosin (H&E). In brief, deparaffinize the slide with xylene, 100% EtOH, 95% EtOH, 70% EtOH, H2O (2 min each). Then the slide with tissue section stain Mayer's Haematoxylin Solution (1 min or 2 min for Bouin's fixed sections). Then, slide rinse in running tap water for 10 min (Do not let the water run directly on the slides). Slide place in distilled H2O (30 sec), then place in 95% reagent alcohol (30 sec). After that slide counterstain with Eosin Y Solution (1 min or 2 min for Bouin's fixed sections). To complete the staining procedure, slide needs to dehydrate and clear with 2x 95% reagent alcohol, 2x absolute reagent alcohol (100% EtOH), 2x xylene (2 min each). Finally, the slide mount with resinous mounting medium [19]. The slides were viewed at 20x and 40x magnification using an Olympus I X 51 light microscope, followed by observer-blind histopathological analysis.

4.34. Statistical analysis:

All data are presented as the median± SE. A Mann-Whitney test was used to compare the experimental data of immunized group versus control group. An analysis of variance

(ANOVA) with Kruskal-Wallis for multiple comparisons was used when three or more groups were compared. Kaplan-Meier analysis was performed to compare survival curves in the challenge study. The remaining data were analysed using Student's t test. Two-tailed P values of $P < 0.05$ were considered statistically significant. All experiments were triplicated for statistical significance. All the data were evaluated using GraphPad Prism 8.0.2 (GraphPad Software, Inc.) and MS Excel software.

Chapter 5. Result

Part – 5.1.

Objective 1. Selection of typhoidal strains for immunogen preparation from recently circulated clinical isolates.

5.1.1. Genotypic selection and Characterization of S. Typhi and S. Paratyphi A strains:

Salmonella Typhi (C6.496) strains and *Salmonella* Paratyphi A (BCR148) strains were used for immunogen preparation. Another *Salmonella* Typhi (K-554) strains and *Salmonella* Paratyphi A (K-580) strains were used for live bacterial challenge study. All the strains were checked for the genus specific or *Salmonella* specific gene (*Prt A, invA*), *Salmonella* Typhi specific gene (*fliCj, tvi A and tvi B)* and *Salmonella* Paratyphi A specific gene (*fliCa)*. Presence of *fliCj* gene and *fliCa* gene is a confirmatory test of the *S*. Typhi Strains and *S*. Paratyphi A Strains respectively. Our results of these three genes show clear band of the amplified genes. Genus specific prtA was observed in all four cases, while the presence of *fliC-j* in S. Typhi strains and *fliC-a* in S. Paratyphi A strains were observed. *FliC-j* was observed via PCR-based method (**Fig. 1, Table.1**), whereas *FliC-d* was observed in serological studies (**Table. 2**). This data coincides with previously published data in other journals. All the strains were checked for the *Salmonella* "O" antigen specific gene (*prt*), *S.* Typhi "H" antigen specific gene (*fliC-d)* and *S*. Paratyphi A "H" antigen specific gene (*fliC-a*). Presence of *fliC-d* gene and *fliCA* gene is a confirmatory test of the *S*. Typhi Strains and *S*. Paratyphi A Strains respectively [1-5].

Serial Number	Name of Bacteria	PCR result					
		prtA (258 bp)	invA (881 bp)	tviA (599 bp)	tviB (846 bp)	fliC-j (489 bp)	fliC-a (329 bp)
1.	*S.* Typhi (C-6.946)	+	+	+	+	+	-
2.	*S.* Paratyphi A (BCR148)	+	+	-	-	-	+
3.	*S.* Typhi (K-554)	+	+	+	+	+	-
4.	*S.* Paratyphi A (K-580)	+	+	-	-	-	+

Table 1. Tabular representation of PCR-based Genotypic characterization of the strains used in the study.

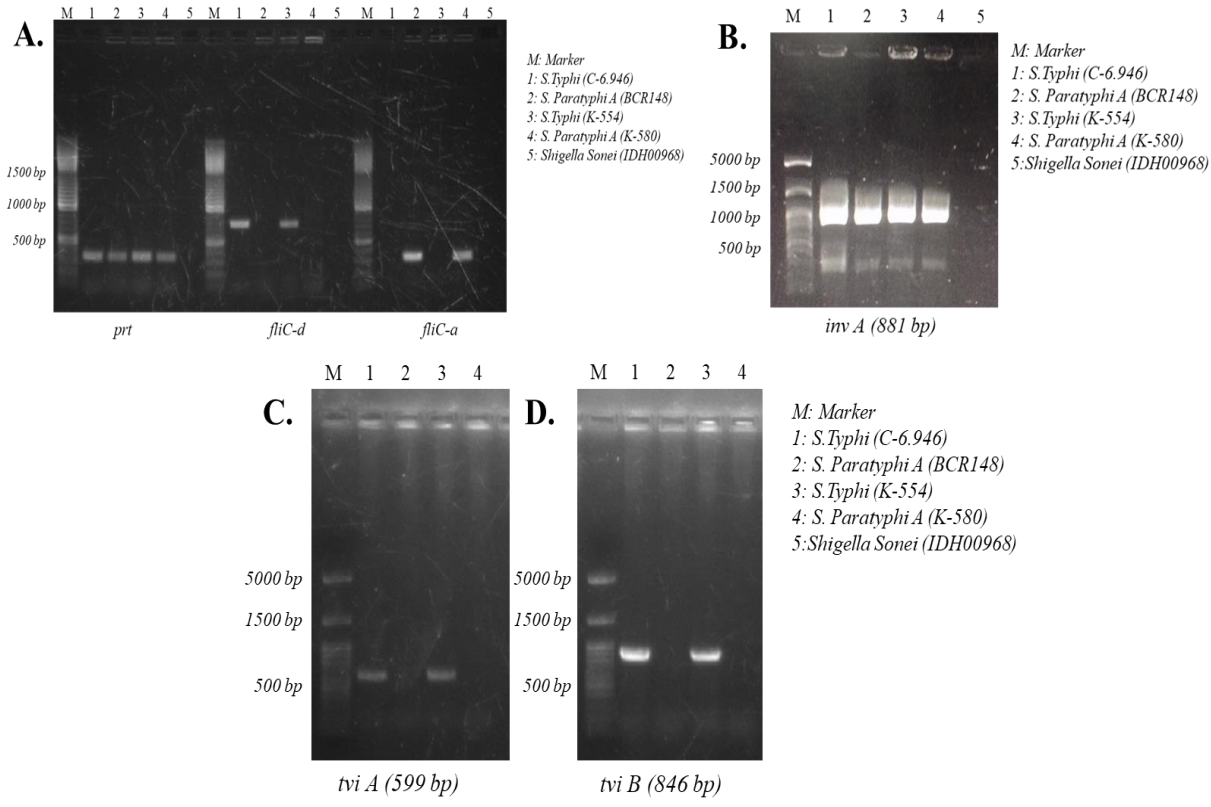


Figure 1. PCR-based Genotypic characterization of the strains used in the study. All strains were characterized by both genus, *Salmonella* specific and serotype, Typhi and Paratyphi A specific genes-based PCR method. **A.** *prt A, flic-d,* are positive in *Salmonella* Typhi whereas *prt A, flic-a* are positive in *Salmonella* Paratyphi A strains. **B.** All the *Salmonella* typhi and *Salmonella* Paratyphi A strains are positive for the *Salmonella* or genus specific *invA* gene. **C., D.** All Salmonella Typhi strains are positive in *tviA, tviB* gene but Salmonella Paratyphi A strains are negative in *tviA, tviB* gene.

5.1.2. Serotypic Characterization of S. Typhi and S. Paratyphi A strains:

Anti O- and H-antibodies were used for Serotypic characterization of the strains used in the study. White-Kauffmann-Le Minor scheme for Antigenic Formulae was followed during the

experiment [6, 7]. All the strains were checked for the *Salmonella* "O" antigen specific gene (*prt*), S. Typhi "H" antigen specific gene (*fliC-d*) and S. Paratyphi A "H" antigen specific gene (*fliC-a*) (**Table. 2**) [5].

Serial Number	Name of Bacteria	"O" Antigen	"H" antigen	
			Phase I	Phase II
1.	***S*. Typhi (C-6.946)**	9,12 [vi]	d	-
2.	***S*. Paratyphi A (BCR148)**	1,2,12	a	[1,5]
3.	***S*. Typhi (K-554)**	9,12 [vi]	d	-
4.	***S*. Paratyphi A (K-580)**	1,2,12	a	[1,5]

Table 2. Tabular representation of Serotypic characterization of the strains used in the study.

5.1.3. Antibiotic susceptibility of *Salmonella* Typhi and *Salmonella* Paratyphi A strains:

All the Strains selected for this work were tested for susceptibility against different antibiotics. Antibiotic-resistance pattern of the strains was also checked by Bauer method [8]. Selection of antibiotics was dependent on both WHO, CLSI guidelines [9, 10] and the availability of antibiotics in our facility. The *Salmonella* Typhi and *Salmonella* Paratyphi A strain were found to be resistant to six antibiotics including cefprozil, cefixime, methicillin, ampicillin, erythromycin, azithromycin but was sensitive to following antibiotics ceftriaxone, imipenem, ciprofloxacin, trimethoperim, lomefloxacin, co-trimoxaazole, nalidixic acid, tetracycline, chloramphenical, norfloxacin, sulfamethoxazole and the both strains were intermediate to cefotaxime, neomycin, streptomycin, kanamycin. Other than this *Salmonella* Paratyphi A

resistant to lomefloxacin, nalidixic acid; intermediate to cefriaxone, ciprofloxacin, tetracycline and sensitive to streptomycin (**Table. 3, Fig. 2**) [5].

SL No.	Name of Antibiotics	*S.* Typhi (C-6.946)	*S.* Paratyphi A (BCR148)	*S.* Typhi (K-554)	*S.* Paratyphi A (K-580)	*E. Coli* (ATCC 25299)
1.	**Ampicillin, AMP**	**R**	**R**	**R**	**R**	**S**
2.	**Cefotaxime, CTX**	**I**	**I**	**I**	**I**	**I**
3.	**Cefprozil, CFZ**	**R**	**R**	**R**	**R**	**S**
4.	**Cefixime, CFX**	**R**	**R**	**R**	**R**	**S**
5.	**Ceftriaxone, CRO**	**I**	**S**	**S**	**I**	**S**
6.	**Chloramphenicol, CHL**	**S**	**S**	**S**	**S**	**S**
7.	**Imipenem, I**	**S**	**S**	**S**	**S**	**S**
8.	**Nalidixic acid, NAI**	**R**	**S**	**S**	**R**	**S**
9.	**Co-trimoxaazole, CO**	**S**	**S**	**S**	**S**	**S**
10.	**Tetracycline, TET**	**S**	**I**	**S**	**I**	**S**
11.	**Trimethoperim, TMP**	**S**	**S**	**S**	**S**	**S**
12.	**Sulfamethoxazole, SXT**	**S**	**I**	**S**	**S**	**S**
13.	**Lomefloxacin, LO**	**R**	**I**	**S**	**R**	**S**
14.	**Azithromycin, AZ**	**R**	**R**	**R**	**R**	**S**
15.	**Ciprofloxacin, CIP**	**I**	**S**	**S**	**I**	**S**
16.	**Streptomycin, STR**	**R**	**I**	**I**	**S**	**S**
17.	**Kanamycin, KAN**	**I**	**I**	**I**	**I**	**S**

Table 3. Antibiotic susceptibility test. Antibiotic susceptibility pattern was tested by Kirby-Bauer disc diffusion assay.

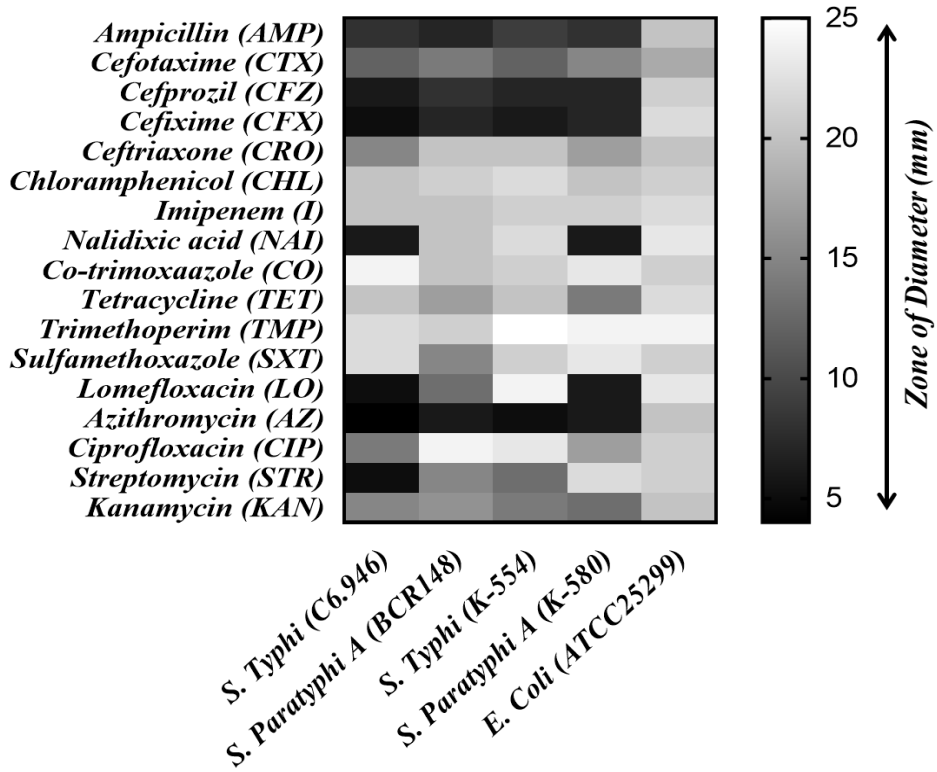


Figure 2. Heat map analysis of antibiotics profile of *S.* Typhi and *S.* Paratyphi A bacteria; A. Heat map of antibiotics profile of two different S. Typhi and S. Paratyphi A bacteria used in this study, E. coli (ATCC 25299) used as the control for the antibiotics. Resistant: <10 mm, Intermediate: 10-18 mm, Sensitive: >18 mm.

Part – 5.2.

Objective 2. To formulate typhoidal Salmonellae immunogen.

5.2.1. Production of Ghost Bacteria:

The biomass of overnight *Salmonella* Typhi and *Salmonella* Paratyphi A culture cells was harvested by centrifugation (8,000 × *g* for 10 min at 4 °C) and resuspended in sterile phosphate buffered saline (PBS, pH-7.4). The resuspended bacterial cells were adjusted to 1×10^8 CFU/ml. 1ml (5X) of above mentioned NaOH concentration (3.125 mg/ml) was added into 2 ml of bacterial suspension with 2 ml of sterile distilled water and incubated for 90 min at 37 °C. At various time points (15, 30, 45, 60, 75 and 90 min), 100 µl of suspension was spread onto TSA agar plates and incubated for 48 h at 37 °C. At the end of lysis, ghost cells were harvested by centrifugation (8,000 × *g* for 10 min at 4 °C) and washed three times with PBS. After final ghost cell production, overnight 37 °C incubated agar plate of NaOH treated ghost cell have no colony formation proves the complete lysis of bacterial cell that lose the capacity of bacterial replication in comparison with non-treated bacterial cells agar plate result (**Fig. 1**) [11, 12, 13].

The details protocol for the large or mass bacterial ghost cell preparation of *Salmonella* Typhi Ans *Salmonella* Paratyphi A is described in the flow chart below (**Fig. 2**). The multistep process would be better understandable in this flow chart. The whole procedure was completed in two days' time period with a previous overnight culture method.

5.2.2. Scanning Electron Microscopy (SEM) of *S.* Typhi and *S.* Paratyphi A bacterial ghost cells.

Morphological features of ghost bacteria cells were analysed by scanning electron microscopy (SEM) as described previously. Ghost bacteria and nontreated control bacterial cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2 h at 4°C, washed three times with the same buffer, and then post fixed in 1% osmium tetroxide for 1.5 h at 4°C. After another washing, cells were dehydrated in a graded series of ethanol concentrations (10, 30, 50, 70, and

100%). After the samples were critical point dried with liquid CO2, they were mounted on SEM stubs, subsequently sputter coated with gold-palladium, and examined with a scanning electron microscope at an accelerating voltage of 25 kV and a magnification of ×20,000 [11, 13]. Here, the treated bacterium shows a trans-membrane rupture whereas non-treated bacteria show regular structure without any rupture in the cell membrane (**Fig. 3**). As the treated bacteria form a pore through which the cytoplasmic content is released to the environment. The outer membranes of the bacteria are left as bacterial ghost after NaOH treatment.

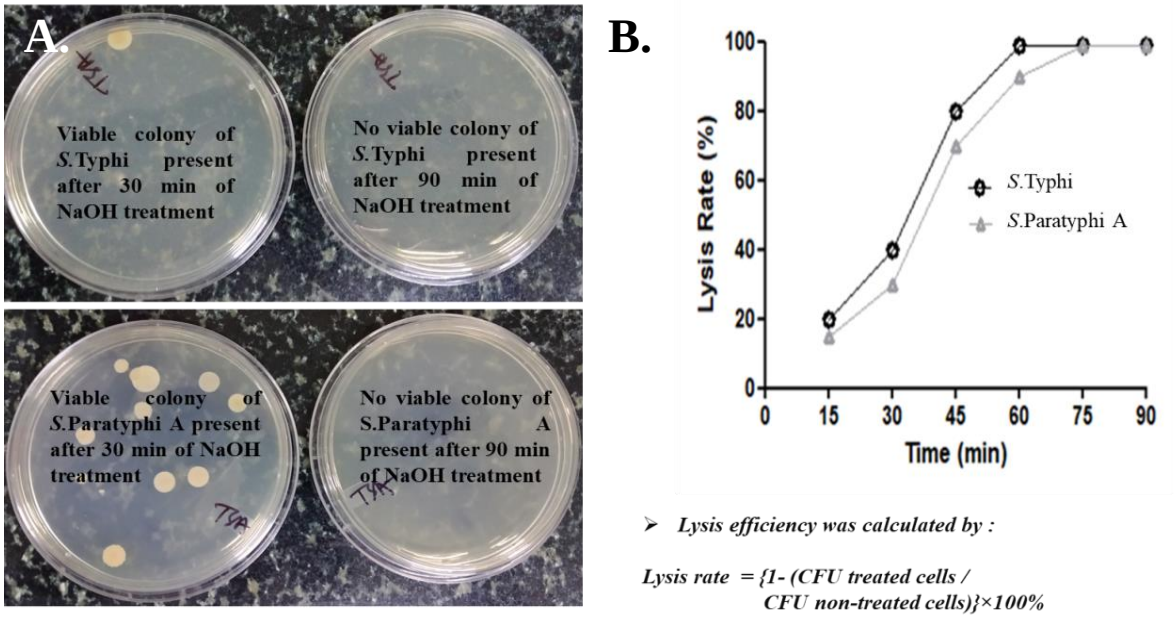


Figure 1. Determination of Minimum Inhibitory Conc. of NaOH (3.125 mg/ml) for Bacterial ghost preparation. A. Plate culture photograph of *S.* Typhi and *S.* Paratyphi A up to 90 min in the above mention MIC of NaOH with nontreated bacterial culture; **B.** Lysis curve shows the lysis rate of *S.* Typhi and *S.* Paratyphi A up to 90 min in the above mention MIC of NaOH. Lysis rate was calculated by the formula describe in the figure.

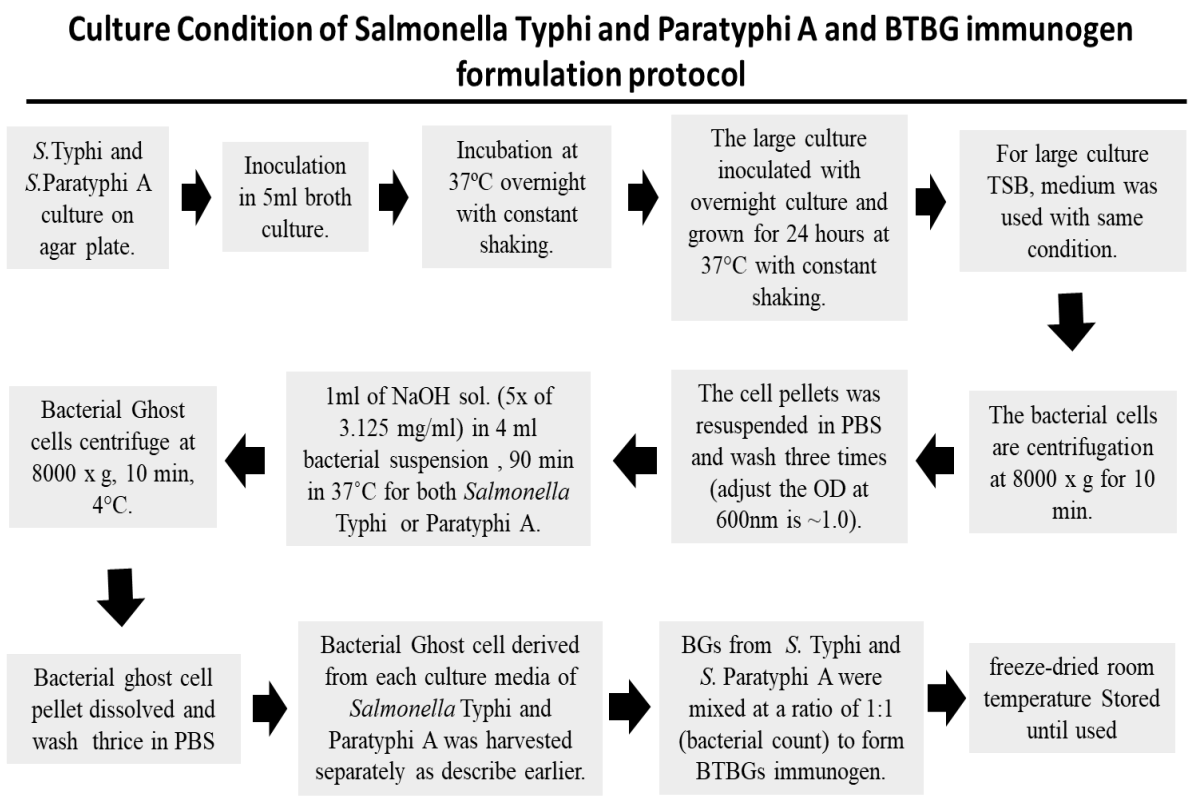


Figure 2. Schematic representation of Culture Condition of Salmonella Typhi and Salmonella Paratyphi A and BTBG immunogen formulation protocol. Detail schematic representation of culture condition of Salmonella Typhi (C6.946) and Salmonella Paratyphi A (BCR148) and bivalent typhoidal bacterial ghost cells immunogen preparation protocol.

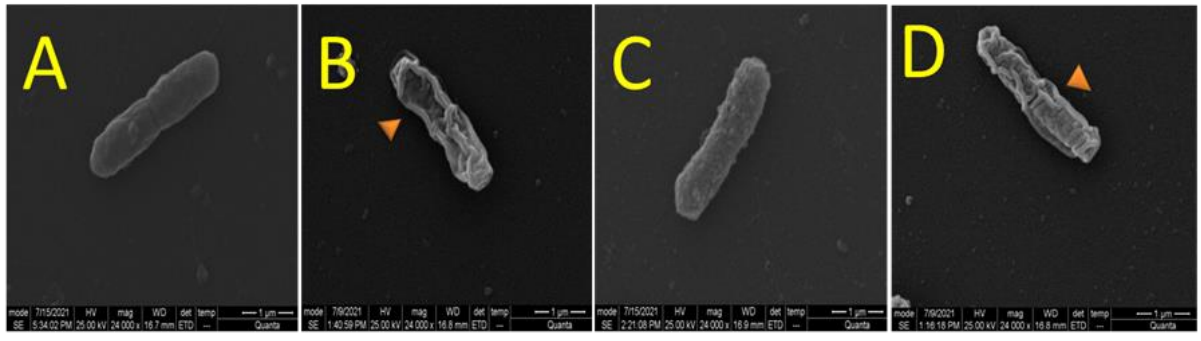


Figure 3. Scanning electron microscopic analysis (SEM) of S. Typhi and S. Paratyphi A bacterial ghost cells and normal cell; A. Untreated control *S.* Paratyphi A, **B**. NaOH-treated *S.* Paratyphi A, **C**. Untreated control *S.* Typhi, **D**. NaOH-treated *S.* Typhi. Arrows shows trans-membrane lysis tunnels.

5.2.3. SDS-PAGE analysis for the stability of Bacterial Ghost cells protein.

Bacterial Ghost cells were harvested from both Salmonella Typhi and Salmonella Paratyphi A. After the bacterial ghost cell preparation from both the strain were stored in different condition to check their stability. Different conditions like -80°C, -20°C, 4°C and freeze-dried room temperature stored sample choose to check their stability through the SDS-PAGE profile after 30 days of ghost cell preparation. SDS-PAGE profile of all the bacterial ghost sample from storage condition (-80°C, -20°C, 4°C and freeze-dried room temperature) have no such major changes in the banding pattern of the protein content. The major band was observed in the range of 35 KDa to 45 KDa. Throughout the gel we found several protein bands. In between 45KDa to 116 KDa have some faint protein band. The most important thing is that in all the storage condition have the similar kind of protein band without any alteration of banding pattern. In all storage condition (-80°C, -20°C, 4°C and freeze-dried room temperature) of bacterial ghost cells of *Salmonella* Typhi and *Salmonella* Paratyphi A have same molecular weight proteins (**Fig. 4**). From this observation of SDS-PAGE protein profile, it was clear that the bacterial ghost cells of *Salmonella* Typhi and *Salmonella* Paratyphi A have stable in different condition (-80°C, -20°C, 4°C and freeze-dried room temperature).

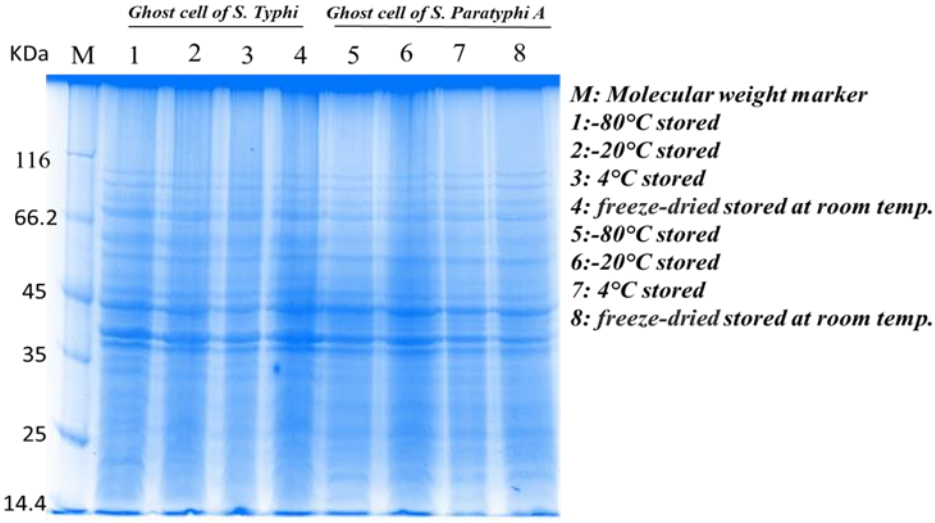


Figure 4. SDS-PAGE analysis for the stability of Bacterial Ghost cell protein. *Salmonella* Typhi and *Salmonella* Paratyphi A at -80°C, -20°C, 4°C and freeze-dried stored at room temp after 30 days of ghost cell preparation.

5.2.4. The schedule of immunization, bacterial challenge studies of animals, and blood drawn for immunological assessment.

The whole animals' experiments were followed by the described protocol. Briefly, all the immunization groups were immunized in three doses specifically, 0^{th}, 14^{th}, 28^{th} day 100 µl of bacterial Ghost Immunogen (1:1) containing 1×10^8 CFU/ml cell. Non-immunized control group mice received 100 µl of sterile PBS. After the complete immunization, at day 35^{th} the immunized and non-immunized animals were challenged with lethal and sublethal dose of live challenge bacterial strains (*Salmonella* Typhi and *Salmonella* Paratyphi A) to chcek the survivality and bacterial colonization differences in immunized compared with nonimmunized animal groups. On the 0^{th}, 7^{th}, 21^{st}, 35^{th}, 49^{th}, 70^{th}, 90^{th} and 120^{th} days following the initial vaccination, at time intervals, blood was taken from tail veins (**Fig. 5**). Blood was kept in microtainer tube (BD, USA) and serum isolated by centrifugation (1000 x g, 10 min, 4°C). Intestinal lavage from immunized and control mice was collected in a sterile microcentrifuge tube by PBS wash of small intestine after dissection. Lavage was centrifuged at 6000 x g, 10 min; supernatant was collected and stored at -20°C [5, 13, 14].

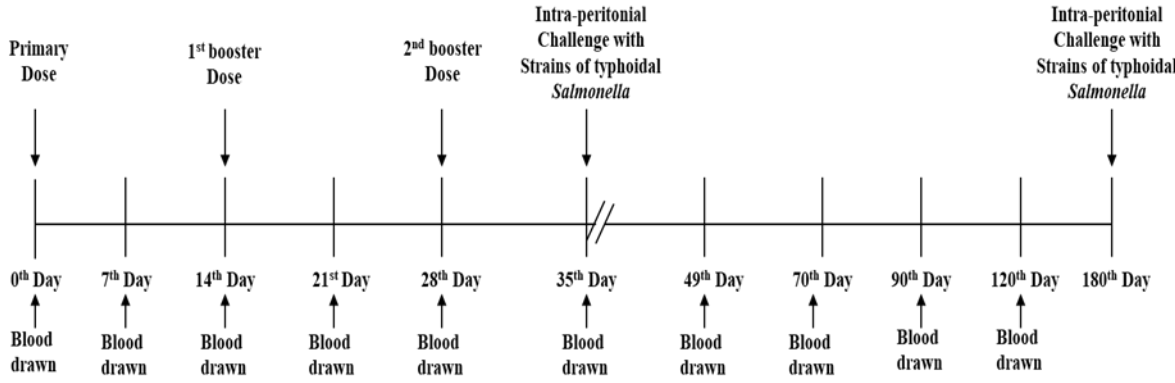


Figure 5. Scheme of immunization and challenge studies. Mice were immunized by intraperitoneally on day 0 and then two subsequent booster doses follow as stated. Mice were then challenged on day 35th and day 180th via intra-peritoneal infection model. Blood were drawn as shown by the arrows. The blood was collected from day 0 to 120th day post immunization and intestinal lavage was collected at 35th day post immunization.

Part – 5.3.

Objective 3. To study the reactogenicity, immunogenicity and protective efficacy of the newly prepared immunogen.

5.3.1. In-vitro cytotoxicity tests in murine macrophages cell line exposed to bacterial ghost cells:

To check the cytotoxicity of NaOH treated Bacteria, we were use Pierce Invitrogen Kit (USA). Murine macrophage cell line (RAW 264.7) cultured in 96-well plates (BD Falcon; BD Bioscience Discovery Labware, Bedford, MA, USA) for 24 h at 37˚C, in humidified 5% CO_2, 95% air. The cells (1.0×10^5 cells/well) were then treated with 1.0×10^4 CFU/mL of NaOH-treated Bacteria in culture medium, and incubated for a further 24 h. PBS and LDH-positive sample treated macrophages were used as negative and positive controls, respectively [12, 13]. In our experiments NaOH treated bacterial ghost cell from *Salmonella* Typhi and *Salmonella* Paratyphi A and also their combination shows much less cytotoxicity in in-vitro murine macrophage cell line than heat inactivated bacterial cells and LDH positive sample provided by the manufacturer. This suggests that the NaOH treated bacterial cells are less reactogenic in nature (**Fig. 1**). LAL assay result in comparism with positive control (LPS) also shows the lower level of endotoxin level in BTBGs.That reflects the less reacrtogenicity of BTBGs in-vitro.

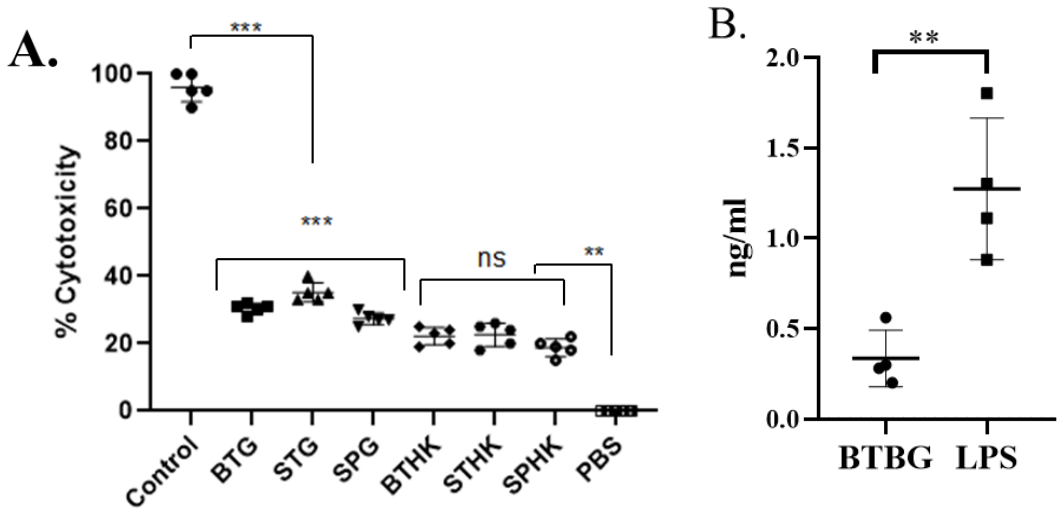


Figure 1. Cytotoxicity test (LDH assay and LAL assay) of Bivalent Typhoidal Bacterial Ghost cell. **A.** *S.* Typhi ghost cell, *S.* Paratyphi A ghost cell, Bivalent Typhoidal Bacterial heat killed and *S.* Typhi heat killed, *S.* Paratyphi A heat killed with LDH positive sample provided by manufacturer (as a control). **B.** Bivalent Typhoidal Bacterial heat killed with purified LPS (obtained from Sigma, USA) as positive sample (as a control). Statistical analyses were performed using the non-parametric one-way analysis of variance (ANOVA) test (Kruskal-Wallis test). Statistical significance was found between LDH positive control and other ghost and Heat killed sample (***p<0.0005, **p value< 0.001). Each bar represents median and error values of five sample ± SE of three independent experiments.

5.3.2. In-vivo effect of bacterial ghost cells on blood parameter:

Some haematological assessment was done to observed the haemoglobin (Hb%), Total leucocyte count (TLC), Differential leucocyte count (DLC), Red Blood cell count (RBC), Packed cell volume (PCV), Platelets count in bacterial ghost cells immunized animals in comparison with nonimmunized animals at the different time interval of complete immunization. No major alteration in any blood parameters was observed in between the immunized and PBS control mice groups [15]. All the parameters studied were within the normal range (Supplementary fig. 3.B). It's confirming us to use the NaOH treated bacterial cell in in-vivo experim ents as safe vaccine candidate with very less reactogenicity (**Table. 1**).

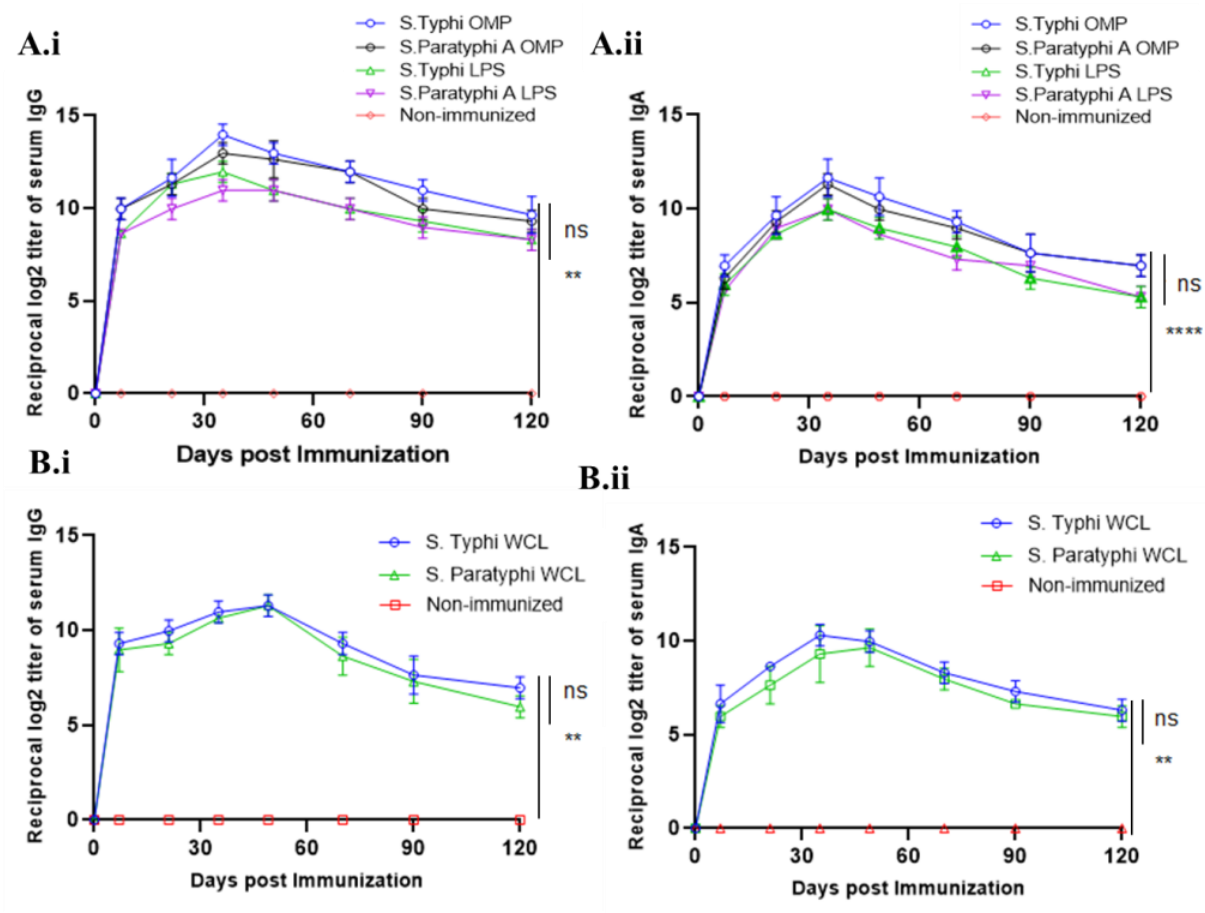
SL. No.	Haematological parameters	7 DPI		21 DPI		35 DPI	
		Control	BTBG immunized	Control	BTBG immunized	Control	BTBG immunized
1	Haemoglobin (Hb%) (g/dl)	12.866 ± 0.124	14.91 ± 0.408	12.966 ± 0.694	13.756 ± 0.524	14.566 ± 0.249	13.15 ± 0.212
2	Total leucocyte count (TLC) (10^3/µL of blood)	3.233 ± 0.679	3.676 ± 0.207	4.4 ± 0.355	4.3 ± 0.432	4.8 ± 0.374	5.166 ± 0.997
3	Differential leucocyte count (DLC) (%)	29±0.816 (N) 67±0.816 (L) 1.666±0.471 (M)	29.666± 2.054(N) 67.666± 2.054(L) 1.666± 0.471(M)	39±1.632 (N) 59±2.160 (L) 1.333±0.471 (M)	36.666± 0.942(N) 56.666± 2.494(L) 1.666± 0.471(M)	36.33±2.494 (N) 60.666±2.054 (L) 1.333±0.471 (M)	40.66±1.699 (N) 54±2.44 (L) 1.33±0.471 (M)
4	Red Blood cell count (RBC) (10^6/µL of blood)	7.336 ± 0.398	8.086 ± 0.437	7.633 ± 0.368	7.716 ± 0.487	7.86 ± 0.214	7.483 ± 0.239
5	Packed cell volume (PCV) (%)	39.066 ± 0.478	44.883 ± 0.396	41.816 ± 0.605	41.083 ± 1.637	43.266 ± 1.319	39.486 ± 0.688
6	Platelets count (10^3/µL of blood)	609.333 ± 14.704	617.333 ± 23.837	858.333 ± 34.740	786.667 ± 41.023	570.333 ± 38.577	677.666 ± 26.386

Table 1. Haematological observation of Bivalent Typhoidal Bacterial Ghost cells immunized animals. In-vivo haematological analysis after 7 days of each exposure to Bivalent Typhoidal bacterial Ghost cells vaccinated groups with one control group separately (expressed as mean ± SE). No significant changes were found in between vaccinated and control group animals.

5.3.3. Bivalent typhoidal Salmonella ghost cells immunization induces humoral and mucosal immune response in adult mice:

Up to 120 days following the first immunization, the induction of humoral immunity during and following the immunization with bivalent typhoidal bacterial ghost cells was assessed using an ELISA. In intestinal lavages of immunized mice compared to non-immunized animals, we found a considerable elevation of serum immunoglobulin IgG and IgA antibodies as well as a mucosal antibody (sIgA) response. After vaccination, the number of various antibodies rises in immunized animals from day 7 to 35 days, and they subsequently continue to have a constant level of antibody titre for up to 120 days (P value < 0.0001). After the second dose of vaccination, between days 28 and 35 after immunization, there was a notable surge in IgG and IgA titre. It takes three doses of the bivalent typhoidal bacterial ghost cells vaccination to induce a significant titre of the mucosal antibody (sIgA) in the intestinal lavage. Salmonella Typhi and Paratyphi A lipopolysaccharide (LPS), outer membrane protein (OMP), and whole cell lysate (WCL) were used to determine the total antibody titre (**Fig: 2; A.i, A.ii, B.i, B.ii**). Additionally, there was a considerable rise in mucosal antibody titres against the LPS and OMP of each bacterial ghost cell separately (**Fig. 2.C**). Additionally, a serum IgG antibody titre against Salmonella Typhi's Vi-polysaccharide (Vi-PS) was determined (**Fig. 2.D**). Our bivalent typhoidal bacterial ghost cells are immunogenic against the antigenic components of Salmonella Typhi and Paratyphi A independently as compared to non-immunized animals, as demonstrated by the ELISA results. Up to 120 days after vaccination, animals can develop strong humoral and mucosal protection from Salmonella Typhi and Paratyphi A bacterial cells. Controlling and preventing infections from Salmonella Typhi and Paratyphi A is critically dependent on this. SDS-PAGE gel separation, coomassie and silver staining, respectively, were utilized to visualize the isolated, purified OMP and LPS of S. Typhi and S. Partyphi A for ELISA, immunoblot, and dotblot, respectively (**Fig. 3.A, 3.B**). In immunoblot and dotblot

experiments, serum antibodies were able to identify the OMP, bacterial ghost cells, and WCL of both bacteria. It was determined that proteins sized between 20 and 80 KD were immunogenic (**Fig. 3.C**). Three distinct LPS concentrations (5µg, 10µg, and 15µg) from each strain were tested using the 35th day serum of mice vaccinated with bivalent typhoidal ghost cells using a dot blot assay. Against each concentration of LPS, we saw a noticeable band (**Fig. 3.D, 4.E**). This demonstrates that the lipopolysaccharide of both strains of bacteria is immunogenic to bacterial ghost cells. The entire outer membranes of bacterial ghost cells produced by NaOH have been shown to contain LPS and outer membrane proteins, which are examples of conserved epitopes [5, 13, 14].



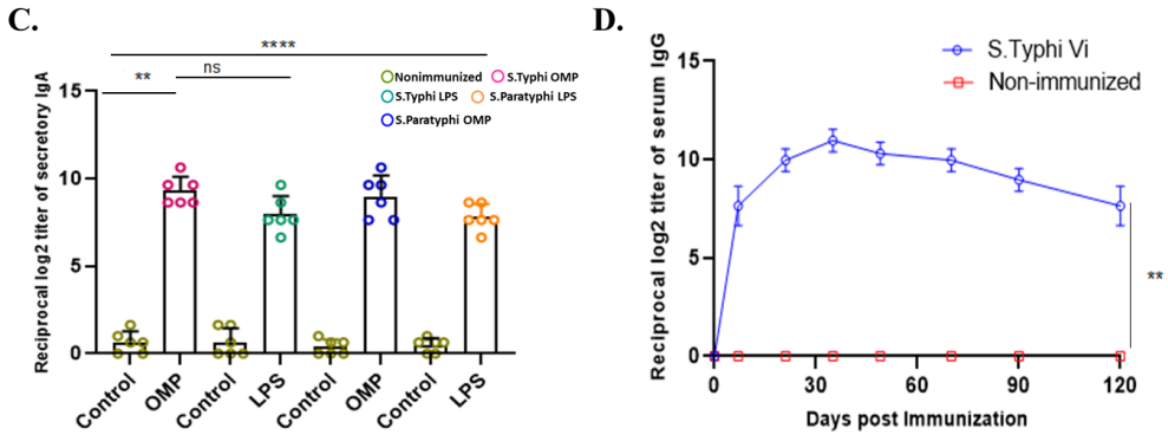


Figure 2. Reciprocal log2 titer of serum IgG, serum IgA and secretory IgA immunoglobulin from Bivalent Typhoidal Bacterial Ghost cells immunized and non-immunized group. Mouse serum IgG **(A.i),** serum IgA **(A.ii)** was measured separately after three doses of intraperitoneal immunization against Outer membrane protein (OMP) and LPS of S. Typhi and S. Paratyphi A. Mouse serum IgG **(B.i),** serum IgA **(B.ii)** were measured separately after three doses of intraperitoneal immunization against whole cell lysate (WCL) of S. Typhi and S. Paratyphi A. **(C.)** Reciprocal Log2 titter of Secretory IgA was measured after 35th day of primary immunization against OMP and LPS of S. Typhi and S. Paratyphi A. **(D.)** Mouse serum IgG was measured separately after three doses of intraperitoneal immunization against Vi-polysaccharide of S. Typhi. Difference between immunized and non-immunized group was statistically significant. Statistical analyses were performed non-parametric two tailed Student's t test (Mann-Whitney tests) and one-way analysis of variance (ANOVA) test (Kruskal-Wallis test) to evaluate data; (****P value <0.0001, **p value <0.001). Each bar represents median and error values of six sample ± SE of three independent experiments.

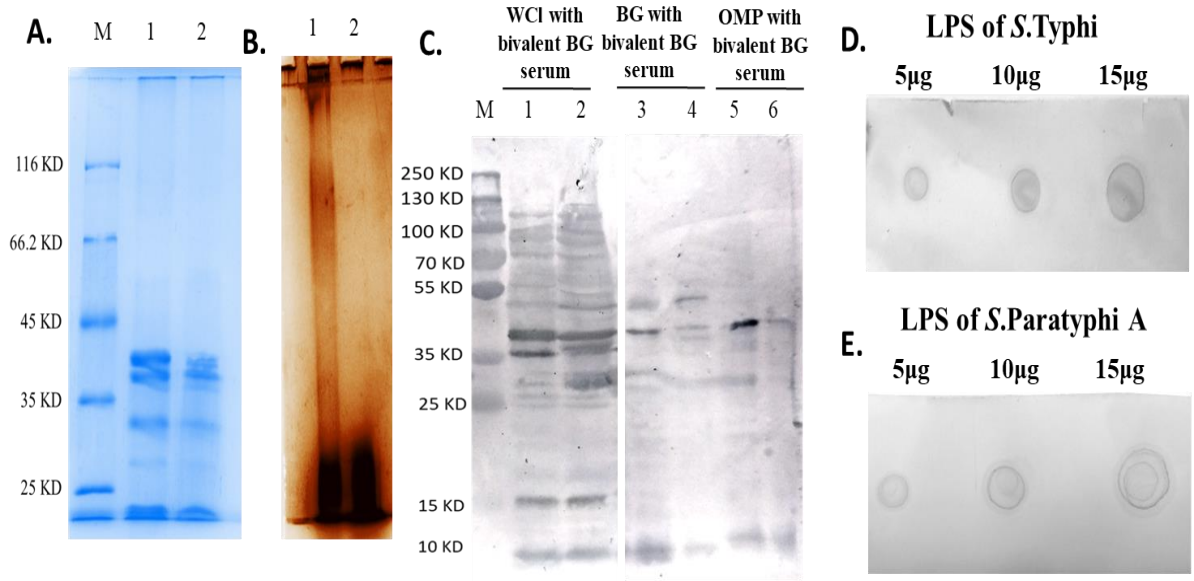


Figure 3. SDS-PAGE profile of outer membrane protein (OMP) and lipopolysaccharide (LPS) of *Salmonella* Typhi and *Salmonella* Paratyphi A followed by Coomassie and silver staing with immunoblot and Dot blot analysis. (A) SDS-PAGE profile of OMPs extracted from two strains of typhoidal *Salmonella*. Lane M: Low molecular weight marker (Sigma), Lane 1: S. Typhi OMPs, Lane 2: S. Paratyphi A OMPs. **(B)** SDS-PAGE profile of LPS extracted from two strains of typhoidal *Salmonella*, Lane 1: S. Typhi LPS, Lane 2: S. Paratyphi A LPS. **(C)** Immunoblot using Bacterial Ghosts (BG) immunized mice serum proved to contain immunogenic components in the form of antibodies against WCL, BG and OMPs of S. Typhi and S. Paratyphi A. **(D, E)** Dot Blot assay using mature LPS of S. Typhi and S. Paratyphi A (5µg, 10µg, 15µg) proved to contain antibodies against LPS of these pathogens. For both the cases, 35th day immunized sera were used as primary antibody.

5.3.4. Bacterial colonization, survivality check after heterologous bacterial challenge:

After one week of last immunization of BTBG immunized group and nonimmunized group (PBS group) were challenged with 5 x 10^5 CFU/mice of S. Typhi and S. Paratyphi A and bacterial colonization were enumerated from different systemic organs of mice. The results showed bacterial colonization was 2-3-fold less in immunized group than nonimmunized group (**Fig: 4.A, B**). At different time point post challenge, we found gradual increase of bacterial colonization in nonimmunized group but in case of immunized groups colonization was decreased. Then we checked long term protection after challenge with sublethal dose of S. Typhi and S. Paratyphi A after 180^{th} days post final immunization. We found at least 2-fold less bacterial load on organs of immunized animals against control (nonimmunized group) animals (**Fig: 4.C**). This may be due to good antibody titter present for a long period after completion of immunization. To further support our result, we challenge both group of mice with lethal dose of S. Typhi and S. Paratyphi A (5 x 10^8 CFU/mice) via intraperitoneal route and their survival was recorded. Mice were observed for 10 days post challenge and from the result we found that all mice immunized with BTBGs were survived till 10 days, but all PBS immunized mice challenged with S. Typhi died within 4 days post challenge and all PBS immunized S. Paratyphi A challenged mice died 9 days post challenge. Immunized mice showed better survival, with 100% survival for S. Typhi and ≥80% for S. Paratyphi A. All of the non-immunized control mice died during this observation period after infection (**Fig: 4.D**). The body weights of immunized mice very less decreased throughout the observation period, but dramatically decreased in non-immunized control mice (**Fig: 4.E.i, E.ii**). This result indicates that our immunogen can protect mice from *Salmonella* infection. Some other clinical signs of disease like diarrhea, lethargy and all the symptoms including weight loss were assessed to measure not only mortality, but also the morbidity signs (**Table. 2**). Non-immunized, S. Typhi and S. Paratyphi A infected mice showed major clinical signs of

morbidity i.e. severe fur ruffling was noticed over time, severe weight loss over time, severe diarrhea started overnight of infection, all animals showed severe lethargy over time, and ultimately all animals are died within 4 to 9 days of infection in nonimmunized group. Whereas the number of moralities was very less in BTBG immunized mice including the clinical sign of morbidity like very mild fur ruffling for short period and weight loss was mild but recovered shortly, in case of S. Paratyphi A challenged group. All the observed protective and good survival results in case of immunized animals were due to a Salmonella Typhi and Paratyphi A specific strong antibody mediated immunity with other cellular and humoral immune responses as was generated through BTBG immunization.

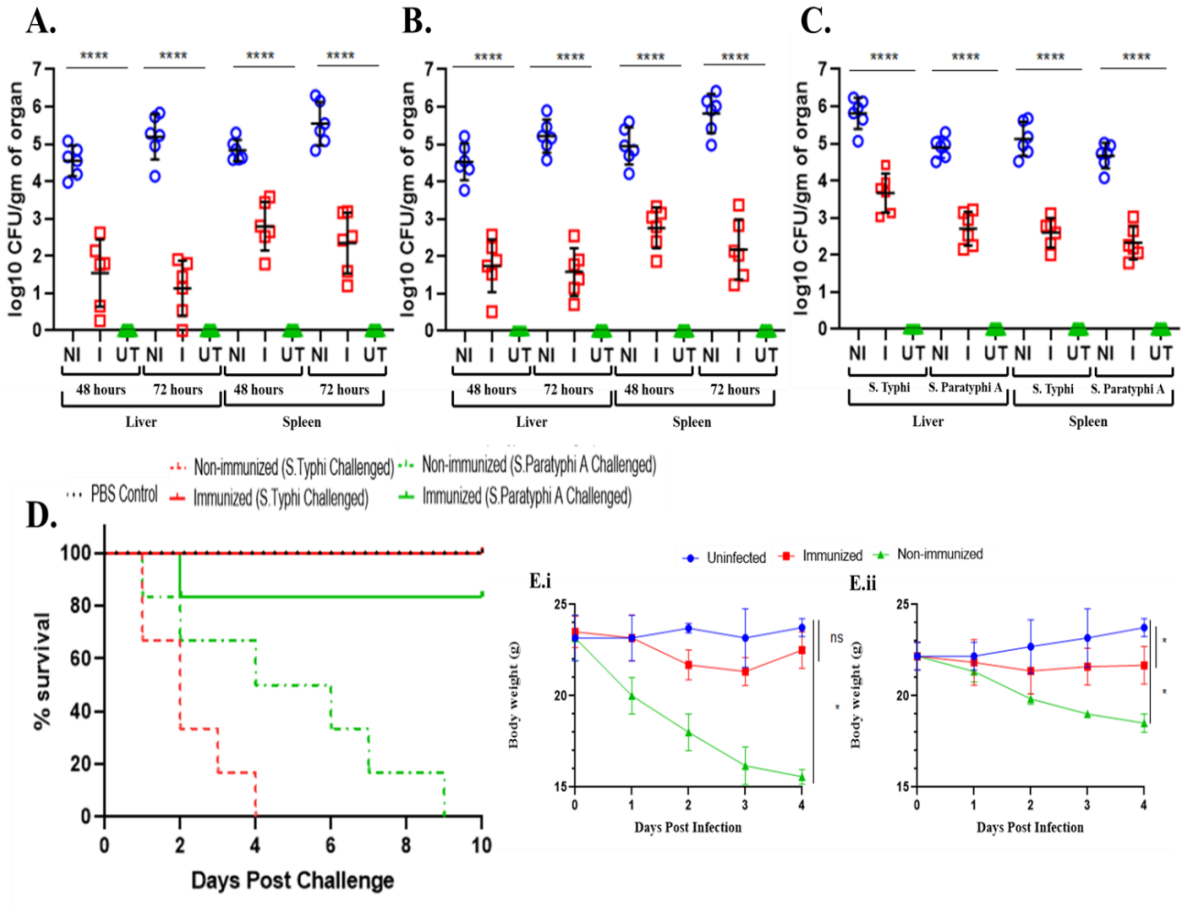


Figure 4. Colonization and protective efficacy analysis of Ghost cells against *Salmonella* Typhi and *Salmonella* Paratyphi A infection. (A.) Colonization of *Salmonella* Typhi **(B.)**

Colonization of *Salmonella* Paratyphi A, respectively after 35th day post immunization, **(C.)** Colonization (post 72 h post infection) to systemic organs after 180^{th} day post first immunization. In every case immunized and nonimmunized groups were challenged with 5 x 10^5 CFU/mice of *Salmonella* Typhi and *Salmonella* Paratyphi A. NI: non-immunized, I: immunized, UT: untreated. **(D.)** Survival graph (Kaplan-Meier Curves) depicts percentage (%) of survival of the immunized and nonimmunized BALB/c mice after Intra-peritoneal challenge with 5 x 10^8 CFU/mice of *Salmonella* Typhi and *Salmonella* Paratyphi A. **(E.i, E.ii.)** Comparative data of change in body weight between non-immunized and immunized adult mice, after challenge with *Salmonella* Typhi and *Salmonella* Paratyphi A respectively. Data were expressed as median value of six mice ± S.E of three separate experiments. Statistical analyses were performed using non-parametric two tailed Student's t test (Mann-Whitney tests) and one-way analysis of variance (ANOVA) test (Kruskal-Wallis test) (****P value <0.0001, **p value< 0.001, *p value < 0.01).

Group	Parameter studied	Duration of study	Observation
Non-immunized and Uninfected (PBS control)	Ruffled fur	4 Days with respect to challenge regimen of other groups	**No symptoms**
	Weight loss		**No symptoms**
	Diarrhea		**No symptoms**
	Lethargy		**No symptoms**
	Death		**No death**
Non-immunized and S. Typhi infected	Ruffled fur	4 Days after heterologous challenged	**Severe fur ruffling** was noticed over time
	Weight loss		**Severe weight loss over time**
	Diarrhea		**Severe diarrhea started overnight** of infection
	Lethargy		Animals shows **severe lethargy over time**

	Death		All animals are **died within 4 days of infection**
BTBG Immunized and S. Typhi infected	Ruffled fur	4 Days after heterologous challenged	**No symptoms**
	Weight loss		**No symptoms**
	Diarrhea		**No symptoms**
	Lethargy		**No symptoms**
	Death		**100% mice survived**
Non-immunized and S. Paratyphi A infected	Ruffled fur	4 Days after heterologous challenged	**Severe fur ruffling** was noticed over time
	Weight loss		**Significant weight loss over time**
	Diarrhea		**Mild to Moderate diarrhea started after next day** of infection
	Lethargy		Animals shows **severe lethargy over time**
	Death		50% animals are **died within 4 days of infection**
BTBG Immunized and S. Paratyphi A infected	Ruffled fur	4 Days after heterologous challenged	**Very mild fur ruffling** for short period
	Weight loss		**Mild**; recovered shortly
	Diarrhea		**No symptoms**
	Lethargy		**No symptoms**
	Death		**80% mice survived**

Table 2. Analysis of all clinical signs of disease (weight loss, diarrhea, lethargy and all the symptoms) to measure not only mortality, but all the morbidity signs.

5.3.5. Histopathological analysis of BTBGs from immunized and non-immunized animal tissue:

The degree of inflammation and tissue damage were determined by histopathological examination of the respective organs (**Fig: 5**). Normal uninfected immunized and non-immunized mice showed no abnormalities upon histopathological examination in the spleen or liver of both groups (**Fig: 5; A, D, G, J**). However, the infected mice displayed extensive inflammation indicative of bacterial spread. Spleens of infected non-immunized mice showed augmented states of inflammation, infiltrating lymphocytes and granulomas (**Fig: 5; Ei, E.ii, F.i, F.ii**) when compared to BTBG immunized mice (**Fig: 5; K.i, K.ii, L.i, L.ii**). Liver and spleen tissues had similar types of histopathological findings. Granulomas were detected in the livers of infected control mice, which were not present in the livers of S. Typhi and/or very reduced in S. Paratyphi A infected immunized mice. Although S. Typhi infected immunized mice presented lobular inflammation in the liver (**Fig: 5; H.i, H.ii, I.i, I.ii**), and lobular inflammation was witnessed to be higher in control mice. In addition, S. Typhi-infected PBS control mice had more portal inflammation and leukocyte infiltration in their livers (**Fig: 5; B.i, B.ii, C.i, C.ii**) than in the livers of animals that had been immunized. Immunized and unvaccinated S. Typhi-infected mice also had acidophil bodies.

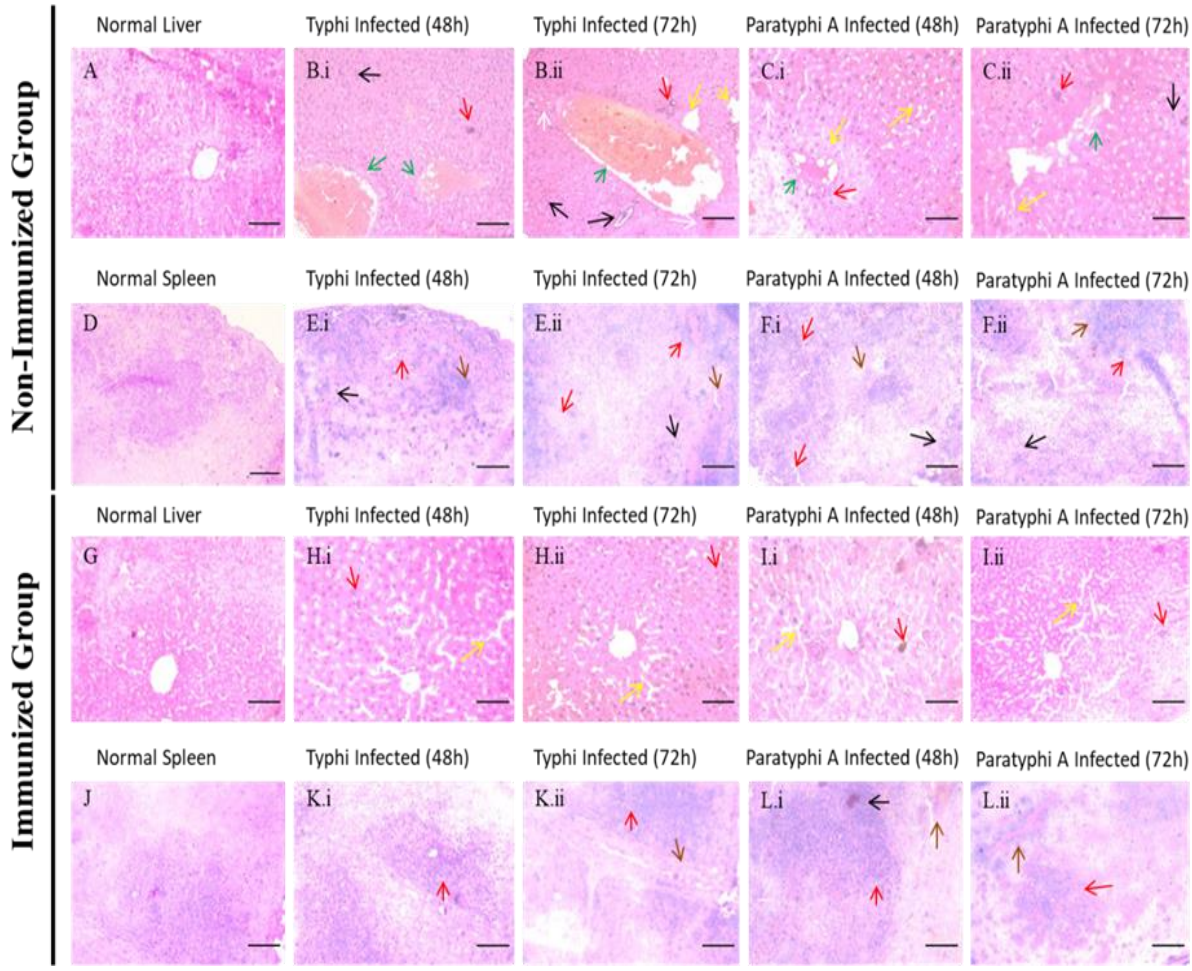


Figure 5. Histopathology (H&E staining) of the liver and spleen of non-immunized and immunized mice after 48h and 72h of intra-peritoneal challenge with Salmonella Typhi and Salmonella Paratyphi A. (**A. & D.)** Liver and spleen of normal non-immunized mice. Liver and spleen of nonimmunized mice after 48h and 72h post challenged with S. Typhi, (**B, i & ii; E, i & ii)** and S. Paratyphi A **(C, i & ii; F, i & ii).** Histological images show extensive portal inflammation, acidophilic bodies, infiltrating leukocytes, lobular inflammation and extensive granulomas. **(G. & J.)** Spleen and liver of normal immunized mice. Liver and spleen of immunized mice after 48h and 72h post challenged with S. Typhi **(H, i & ii; K, i & ii)** and S. Paratyphi A **(I, i & ii; L, i & ii).** Histological images represent mild leukocyte infiltrates, mild lobular inflammation, no portal inflammation and acidophil bodies in spleen and liver respectively. Images were captured at 20× and 40× magnification. Scale bar represents 100µm.

Inflammation (brown arrow), Extensive granulomas (black arrow), Portal inflammation (green arrow), Lobular inflammation (yellow arrow), Acidophil bodies (white arrow), Infiltrating leukocytes (red arrow).

5.3.6. Adoptive transfer of serum and splenocytes followed by bacterial challenge for colonization assay: Adoptive transfer of immunized and non-immunized mice serum and splenocytes intravenously (via tail vein) to naïve mice was done to check the humoral and cell mediated passive protection. We found that immunized blood serum and splenocytes confers protection to naïve mice similarly. We observed bacterial colonization differences in systemic organs of animals after adoptive transfer. Non-immunized sera and splenocytes transferred mice had a very high rate of colonization in the liver and spleen. However, immunized sera and splenocyte-treated mice showed considerably lower bacterial load at day 0 (**Fig: 6. A.i, ii, iii, iv**) and day 7 (**Fig: 6. B.i, ii, iii, iv**) in adoptively transferred mice.

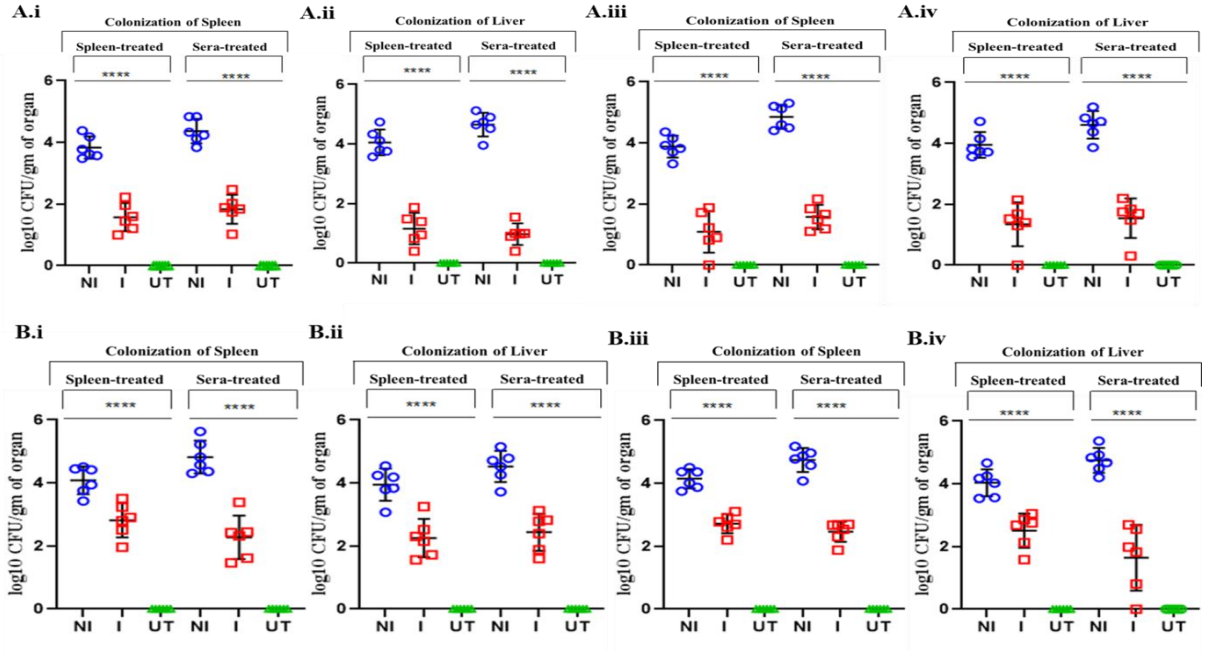


Figure 6. Adoptive transfer (of immunized serum and splenocytes) reduces bacterial number in naïve mice after infection. (A.i, A.ii) Bacterial number (72h post infection) of *S.*

Typhi in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 0^{th} day. **(A.iii, A.iv.)** Bacterial number (72h post infection) of *S.* Paratyphi A in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 0^{th} day. **(B.i, B.ii)** Bacterial number (72h post infection) of *S.* Typhi in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 7^{th} day **(B.iii, B.iv.)** Bacterial number (72h post infection) of *S.* Paratyphi A in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 7^{th} day. Data were expressed as median value of six mice ± S.E of three separate experiments. Statistical analyses were performed using non-parametric one-way analysis of variance (ANOVA) test (Kruskal-Wallis test) (****P value <0.0001, **p value< 0.001, *p value < 0.01). NI: non-immunized, I: immunized, UT: untreated.

5.3.7. Adoptive transfer of serum and splenocytes followed by bacterial challenge to check the survival:

Further the survivality of naïve animals were check after adoptive transfer of immunized mice serum and splenocytes intravenously (via tail vein) to check the humoral and cell mediated passive protection. For this assay we challenged the animals which gets immunized serum and splenocyte in comparison with normal naïve animals. Challenged with live bacterial strains (*Salmonella* Typhi and *Salmonella* Paratyphi A) at 0^{th} day of adoptive transfer and at 7^{th} day of adoptive transfer done in compare to without adoptive transfer naïve animals. Both the challenged experiment was shows that immunized sera and splenocytes have a potency to not only lower the bacterial colonization in organs but also help to survive after lethal infection. This data was supporting the humoral and cell mediated passive protection of all naïve animal. Post 7^{th} day adoptive transfer shows somewhat lower survival potentiality than 0^{th} day adoptive transfer animals might be some unknown in-vivo cellular activity still has better survivability

against without adoptive transfer animals. We found that immunized blood serum and splenocytes confers protection to naïve mice similarly. Without immunized sera and splenocytes transferred mice had a very high rate of mortality because of high bacterial colonization and systemic infection. However, immunized sera and splenocyte-treated mice showed considerably better survivality at day 0 (**Fig: 7.A.i, ii, iii, iv**) and day 7 (**Fig: 7.B.i, ii, iii, iv**).

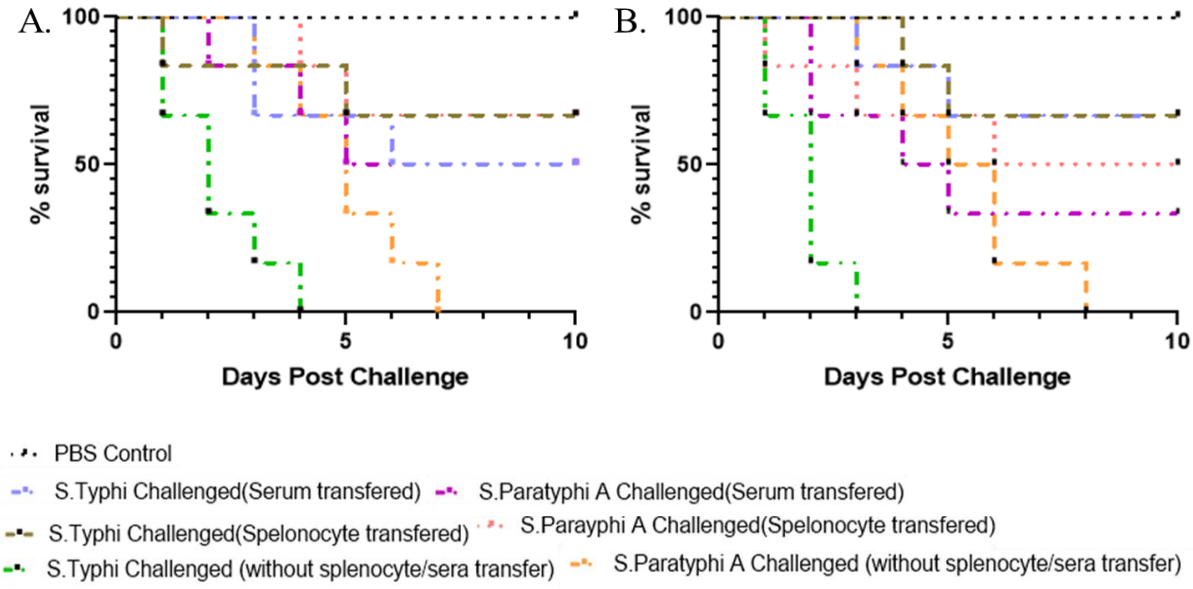


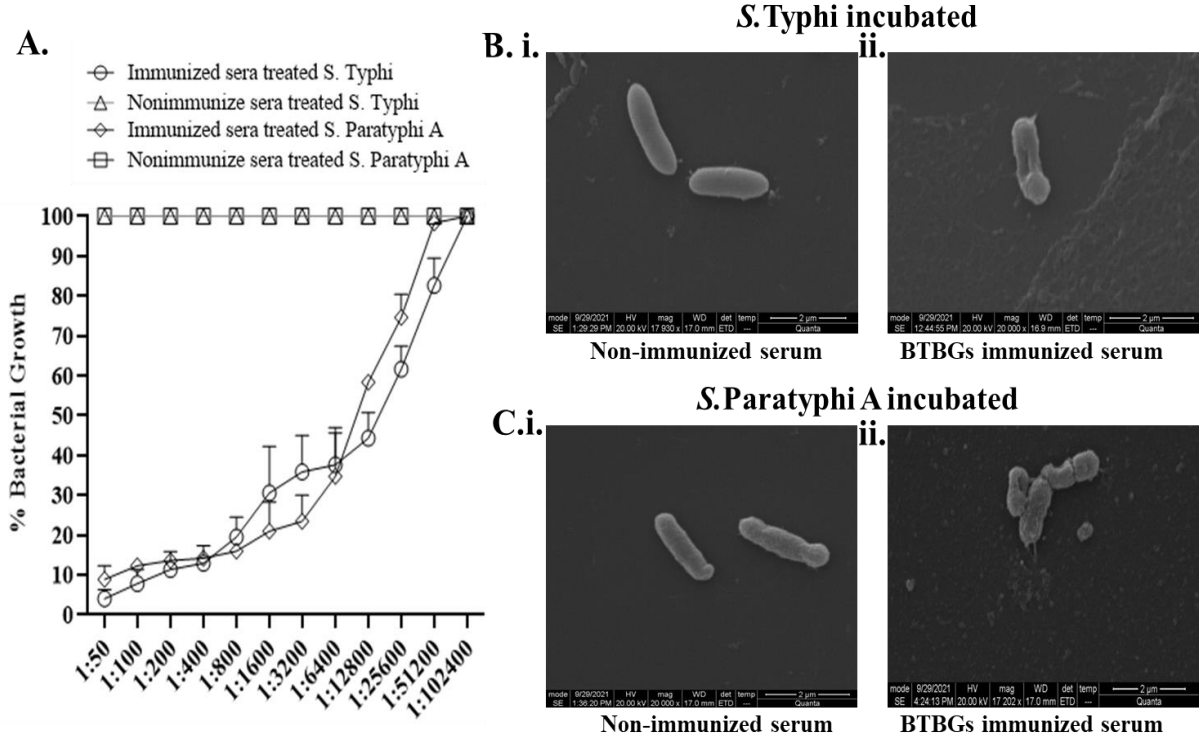
Figure 7. Survival curve of passive protective efficacies on 0th and 7th day day challenge following adoptive transfer of immunized serum and splenocytes in comparison without getting adoptive transfer of immunized serum and splenocytes animals. (A.) Both B- and T-cell mediated immune response induced by Bacterial Ghost cells is necessary for the protective immunity to bacterial infection. Splenocytes (1 x 10^6) and serum (100 µl) were injected via the tail vain. One group of mice was challenged with 5 x 10^8 CFU/ml of heterologous strains of bacteria after two hours of the adoptive transfer and kept for ten days. **(B.)** Both B- and T-cell mediated immune response induced by Bacterial Ghost cells is necessary for the protective immunity to bacterial infection. Splenocytes (1 x 10^6) and serum

(100µl) were injected via the tail vain. One group of mice was challenged with 5 x 10^8 CFU/ml of heterologous strains of bacteria after 7 days of the adoptive transfer and kept for ten days.

5.3.8. Serum bactericidal activity, bacterial motility and mucin penetration assay:

We observed that bivalent typhoidal bacterial ghost cells were able to generate protein and LPS specific serum antibodies against *S.* Typhi and *S.* Paratyphi A. Previous studies showed that both protein and LPS-specific anti *Salmonella* antibodies have complement-dependent bactericidal activity [22, 23]. So, we decided to check the bactericidal activity of our immunized mice serum. To do this, immunized mice serum was heat inactivated and then incubated in different serum dilution with bacteria and complement. Whenever we incubated the bacteria with heat inactivated immunized serum supplemented with baby rabbit complement, the bacterial cells were killed significantly. The bactericidal effect of BTBG immunized serum dilution on *Salmonella* Typhi is 1:12800 and for *Salmonella* Paratyphi A is 1:6400 (**Fig: 8. A**). Therefore, from this experiment we conclude that our typhoidal *Salmonella* ghost immunized mice serum could eliminate Typhoidal *Salmonella* by activating complement pathways. To further support our result scanning electron microscopic analysis was performed. Scanning electron microscopy (SEM) images revealed clearly visible lysis of bacterial cell when treated with immunized heat inactivated serum treated group (**Fig: 8. B.i, B.ii, C.i, C.ii**). Bacterial motility is very important for typhoidal *Salmonella's* pathogenesis as motility help them to move from epithelial lumen to epithelial surface. Thus, to find our BTBG immunogens' effect on typhoidal *Salmonella's* motility we performed bacterial motility assay. We found that immunized serum, compared to non-immunized showed a major inhibition of *Salmonella* motility. This indicates that our bivalent formulation does indeed agglutinate bacteria, which may contribute to the immunogens' protective nature (**Fig: 8. D.i, D.ii, E.i, E.ii, F**) [14].

Intestinal epithelial wall also covered with a thick layer of viscoelastic mucus gel which is formed with a dense network of entangled and cross-linked mucin. This mucin layer is always abundant with various antibodies specifically secretory IgA (sIgA) and sometimes IgG. To find the effect of the BTBGs immunization on both typhoidal *Salmonella* strains, we performed mucin penetration assay. Mucin penetration by immunized serum-treated bacteria was found to be lower whereas, that of non-agglutinated bacteria were much higher (**Fig: 8. G**). According to the findings, bacterial ghost cell-specific sera considerably hinder motility, which can prevent mucin penetration from reaching the epithelial cells and thereby prevents colonization or invasion.



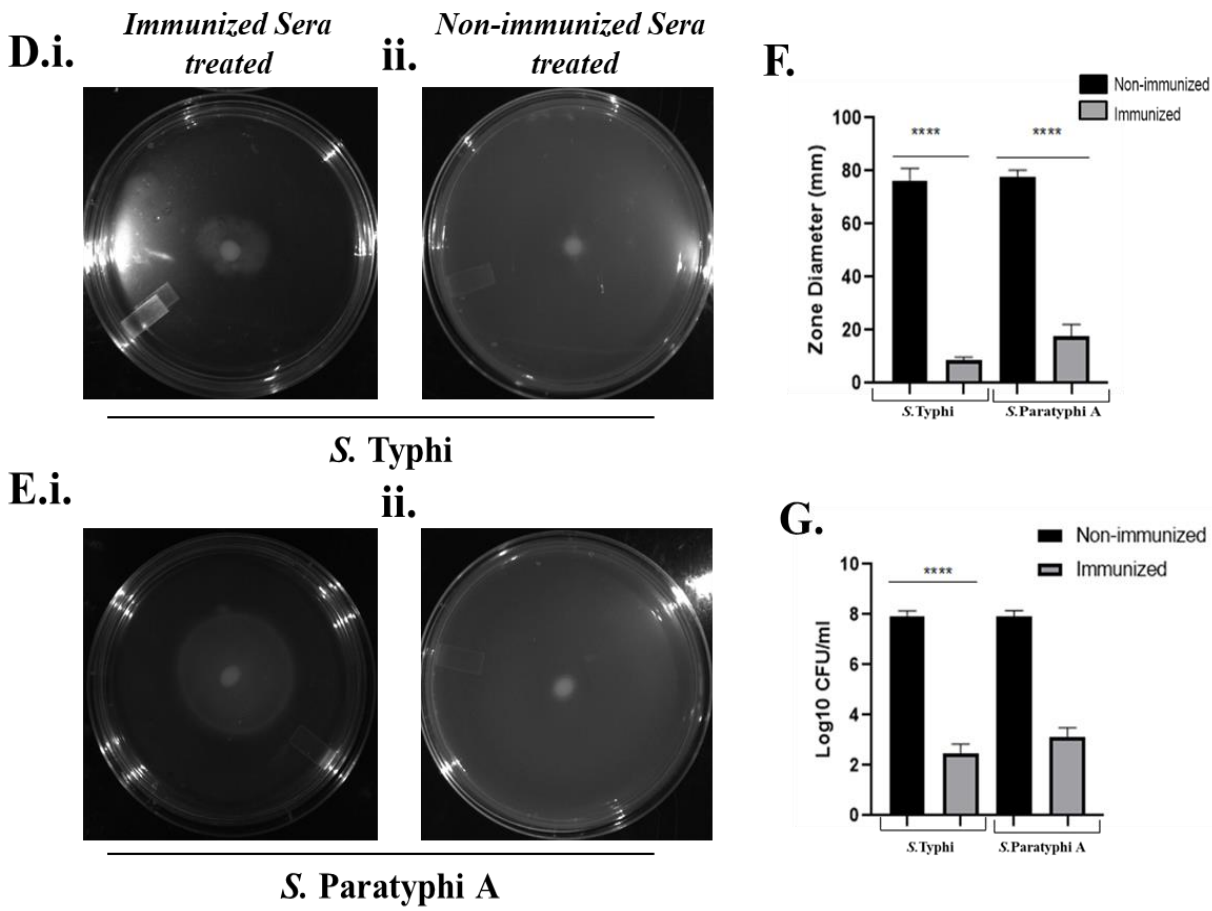
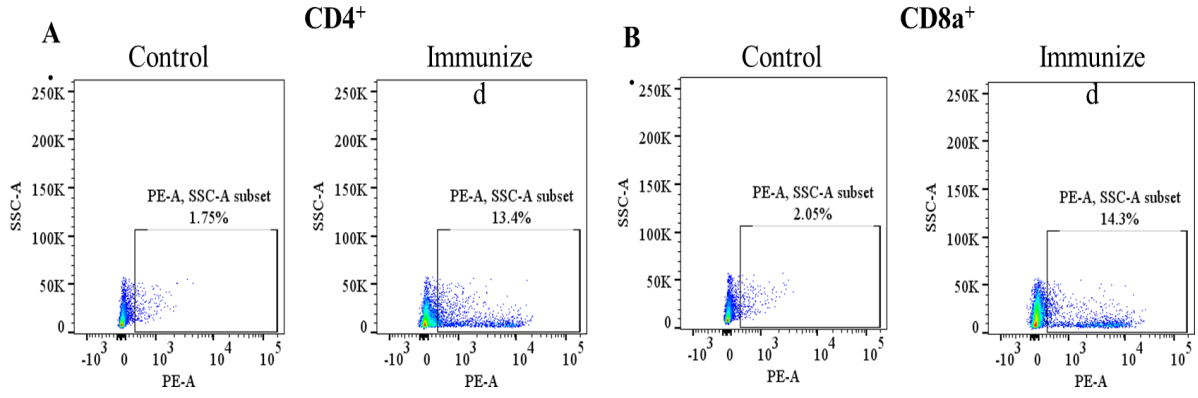


Figure 8. Serum bactericidal activity, bacterial motility and mucin penetration assay in presence of immunized and non-immunized serum. (A.) Immunized serum is capable of complement mediated lysis of *Salmonella* Typhi (K-554) and *Salmonella* Paratyphi A (K-580). X-axis represents the serum dilution and Y-axis represent the percentage of bacterial growth occur after overnight incubation. **(B, C).** Scanning electron microscopic (SEM) image of non-immunized and immunized sera treated bacteria; **(B.i.)** Nonimmunized sera treated *S.* Typhi **(B.ii.)** Immunized sera-treated *S.* Typhi **(C.i.)** Nonimmunized sera treated *S.* Paratyphi A **(C.ii.)** Immunized sera treated *S.* Paratyphi A. Scale bar represent 2μm length. **(D-F.)** Bacterial motility test on soft agar (0.3% agar) plates; **(D.i. E.i.)** The motility of *S.* Typhi and *S.* Paratyphi A was significantly reduced in case of immunized serum spreaded plate. **(D.ii. E.ii.)** The

motility of *S.* Typhi and *S.* Paratyphi A in case of nonimmunized serum spreaded plate. **(F.)** Bar diagram of zone diameter of bacterial motility in immunized and nonimmunized serum spreaded plate after overnight growth. **(G.)** Mucin penetration assay; Bacteria treated with non-immunized or immunized serum were loaded on top of the 1 ml mucin column and allowed to penetrate. Immunized serum agglutinated bacteria showed reduced ability to penetrate mucin. Statistical analyses were performed using the two tailed Student's t test (****p<0.0001).

5.3.9. Flow cytometry analysis of T-cell (CD4+, CD8+) and B-cell (CD19+) specific surface markers following bacterial ghost cell immunization in mice:

35th day post 1st immunization both BTBG and PBS immunized mice were sacrificed, and their spleens were collected, and a single cell suspension were prepared and cultured for 24 hours. To check the upregulation of adoptive immune responses post immunization with BTBGs in mice, we check the different surface markers of T-cells (CD4+, CD8a+) and B-cells (CD19+) 35th day post immunization. From the result we observed that BTBGs immunization significantly increases the both CD4+, CD8a+ and CD19+ cells populations than PBS immunized mice (**Fig: 9. A., B., C., D.i, D.ii, D.iii**).



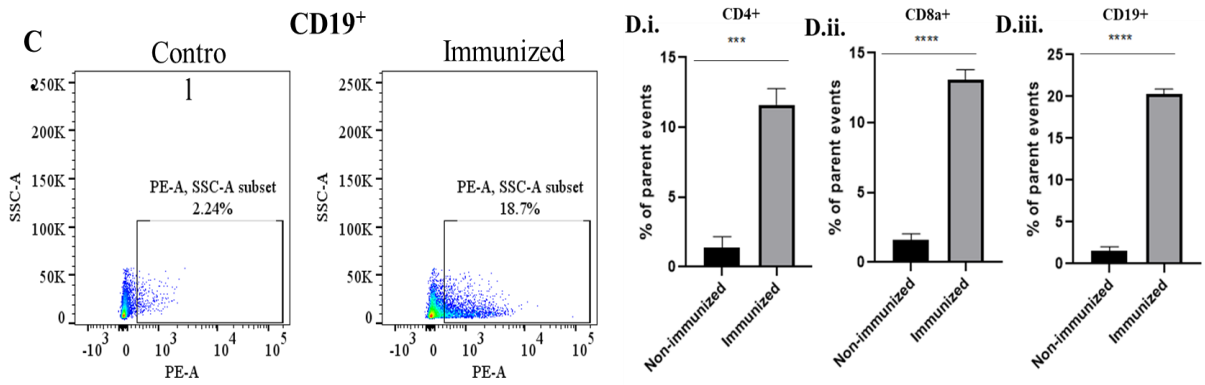


Figure 9. FACS analysis of the population of CD4+, CD8a+ and CD19+ splenic cells from immunized and control animals. 10,000 events in total population were taken in each and every case and the mean value of area under PE in P2 population was considered whilst calculating the result. **(A.)** CD4+, **(B.)** CD8a+, **(C.)** CD19+ cell population of immunized and control mice's spleen. **(D.i., D.ii., D.iii.)** graphical representation of CD4+, CD8a+ and CD19+ cell population in splenocytes of immunized and nonimmunized mice by FACS analysis. Statistical analyses were performed using the non-parametric two tailed Student's t test (Mann-Whitney tests) to evaluate data; (**p value <0.001). Each bar represents median and error values of six sample ± SE of three independent experiments.

Part – 5.4.

Objective 4. To study signaling pathways involved behind immunomodulation.

5.4.1. Bivalent Typhoidal ghost-cells (BTBGs) immunization generates IgG1 dominant immune response:

To determine the serum IgG isotypes following Bivalent Typhoidal ghost-cells (BTBGs) immunization, sera were collected on day 35 of first immunization. Both anti-protein and anti-LPS IgG subclasses were measured by ELISA. Markedly raised titers of IgG1 and IgG2a antibodies were observed in BALB/c mice immunized with BTBG while IgG2a was dominant (**Fig. 1**). This result indicates induction of both Th1 and Th2 type responses, albeit to a significantly higher level for the former.

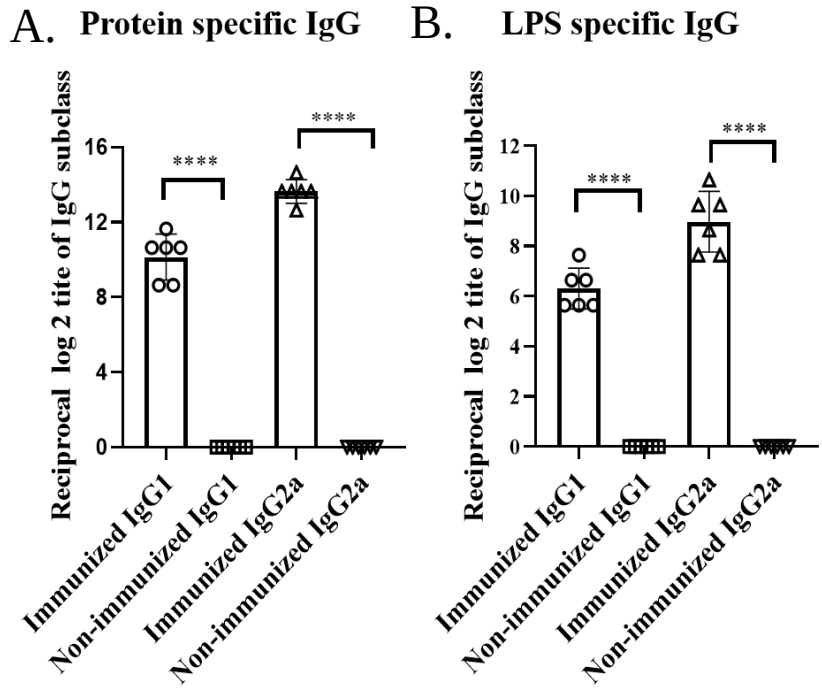
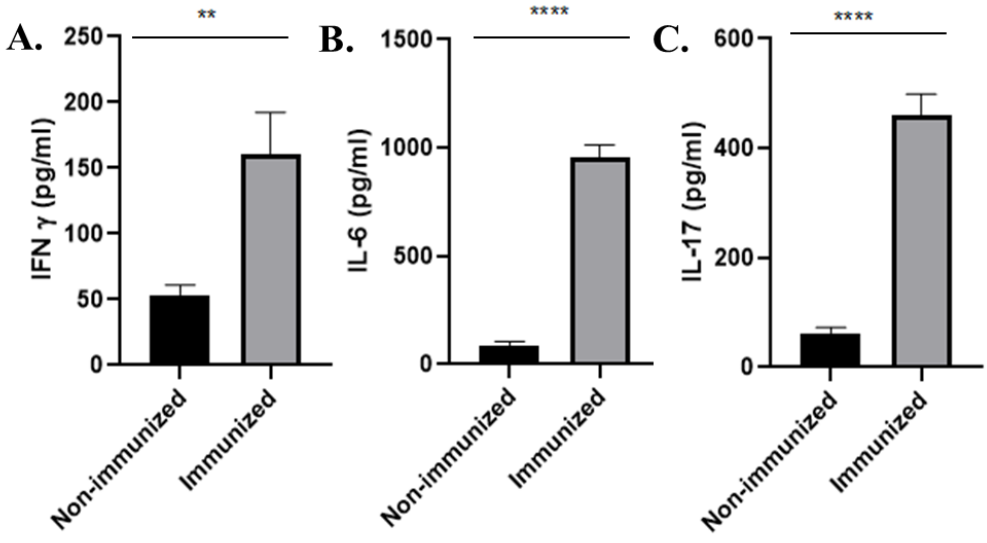


Figure 1. Reciprocal log2 titer of serum IgG1, serum IgG2a immunoglobulin from Bivalent Typhoidal Bacterial Ghost cells immunized and non-immunized group. Mouse serum IgG1 **(A),** serum IgG2a **(B)** was measured separately after 35th day of primary immunization against OMP and LPS of *S.* Typhi and *S.* Paratyphi A. Difference between immunized and non-immunized group was statistically significant. Statistical analyses were performed non-parametric two tailed Student's t test (Mann-Whitney tests) and one-way

analysis of variance (ANOVA) test (Kruskal-Wallis test) to evaluate data; (****P value <0.0001, **p value <0.001). Each bar represents median and error values of six sample ± SE of three independent experiments.

5.4.2. Ex-vivo restimulation of splenocytes with BTBG induces Th1/Th17 cytokine response:

The differentiation of T-cell subset required cytokine stimulation. To determine the types of cytokines secreted from splenic cells, CD4+ T-cells were isolated and pulsed with respective immunogen for 24h. The culture supernatants were collected and cytokines were measured by ELISA. From the culture supernatants, we found that BTBG treatment significantly upregulates levels of IFN-γ, TNF-α, IL-6 and IL-17 (**Fig. 2. A, B, C, D, E, F**) than PBS treated group.ss



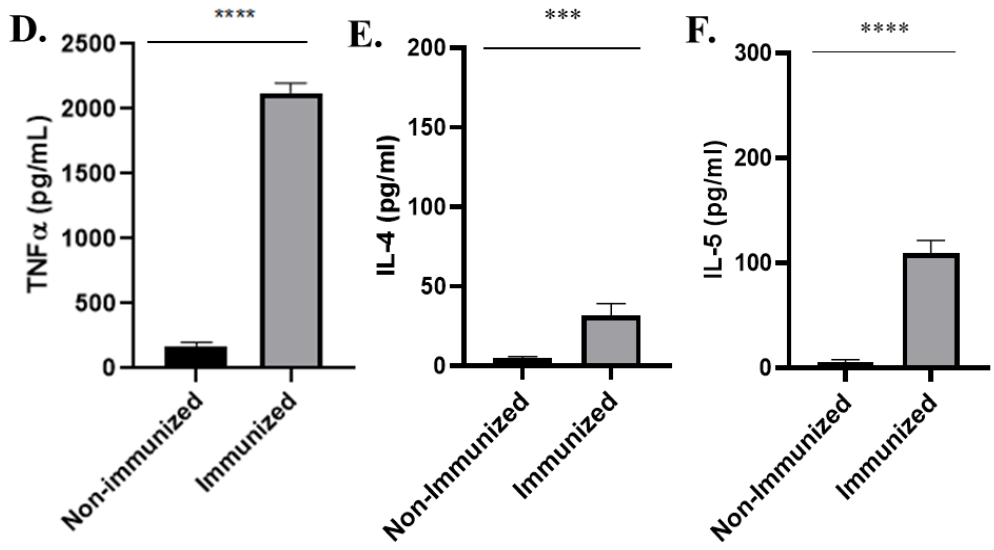


Figure 2. Bivalent Typhoidal Bacterial Ghost cells (BTBGs) mediated restimulation to splenocytes induces the cytokine responses. Cytokine ELISA assay was done to check IFN-γ **(A),** IL-6 **(B),** IL-17 **(C),** TNF-α **(D),** IL-4 **(E),** and IL-5 **(F).** Cytokines in culture supernatant of ex-vivo cultured splenic cells of immunized and nonimmunized mice after 24 hours of restimulation with BTBGs; All cytokines are measured by ELISA (n=6). Statistical analyses were performed using the non-parametric two tailed Student's t test (Mann-Whitney tests) to evaluate data; (****P value <0.0001, **p value < 0.001, *p value < 0.01). Each bar represents median and error values of six sample ± SE of three independent experiments.

5.4.3. Ex-vivo restimulation of bone marrow derived dendritic cells (BMDC) with BTBG induces Th1/Th17 cytokine response:

Signalling from antigen-presenting cells (like dendritic cells) is required to activate and differentiate T cells. To illustrate the generation of innate immune response by BTBGs; we

investigated dendritic cell (DCs) response that controls T cell variation. For this experiment, mature bone marrow-derived dendritic cells (BMDC) were isolated from naïve mice and were stimulated with BTBGs for 24h. Cytokine measurement from the culture supernatants indicated a significant induction of IL-1β, IL-6, IL-23, and IL-12p40/70 levels. This result suggested that pro-inflammatory cytokines secreted by DCs (dendritic cells) regulate Th1/Th17 cell differentiation (**Fig: 3. A, B, C, D**).

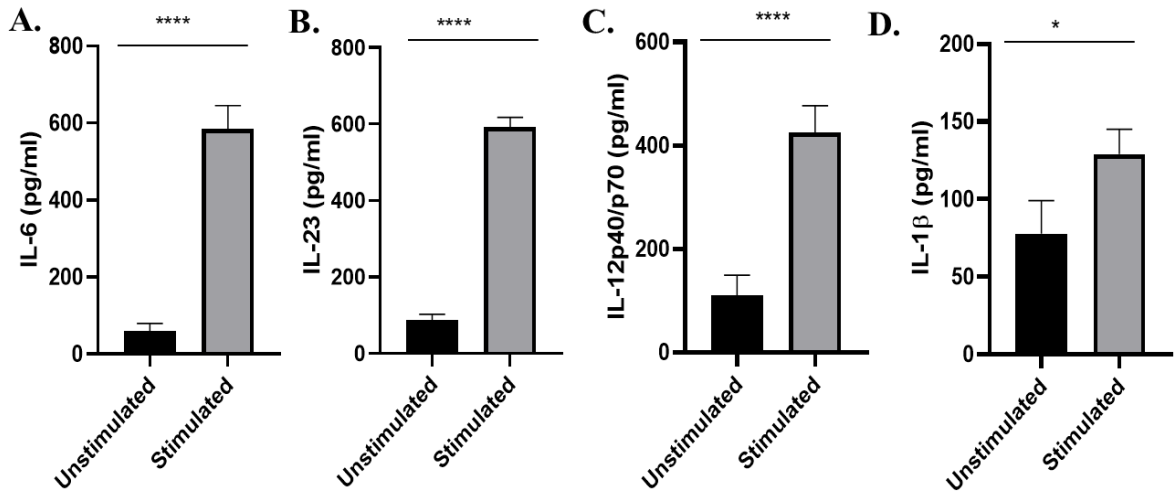


Figure 3. Bivalent Typhoidal Bacterial Ghost cells (BTBGs) mediated restimulation to bone marrow derived dendritic cells (BMDC) induces the cytokine responses. Cytokine ELISA assay was done to check IL-6 **(A),** IL-23 **(B),** IL-12p40/70 **(C),** and IL-1β **(D).** Cytokines in culture supernatant of ex-vivo bone marrow derived dendritic cells (BMDC) from naïve mice with and without BTBGs stimulation for 24 hours; All cytokines are measured by ELISA (n=6). Statistical analyses were performed using the non-parametric two tailed Student's t test (Mann-Whitney tests) to evaluate data; (****P value <0.0001, **p value < 0.001, *p value < 0.01). Each bar represents median and error values of six sample ± SE of three independent experiments.

5.4.4. Ex-vivo stimulation of bone marrow-derived dendritic cells and Splenic CD4+ T cell co-culture with BTBG induces T helper cytokine response

Bone marrow-derived dendritic cells can express both classes of MHC molecules as well as the co-stimulatory cell-surface molecules that drive the clonal expansion of naive T cells and their differentiation into armed effector T cells. To check this reaction, CD4+ T cells separated from immunized mice and PBS control mice were co-cultured with BTBGs pulsed BMDCs for 24 h. Cytokines were measured by ELISA from culture supernatants. The result showed significantly higher levels of Th1/Th17 cytokines (IFN-γ, IL-2, IL-6 and IL-17) in the culture supernatants (**Fig: 4. A, B, C, D**). All these outcomes direct a robust Th1/Th17 media immunity in BTBGs immunized mice.

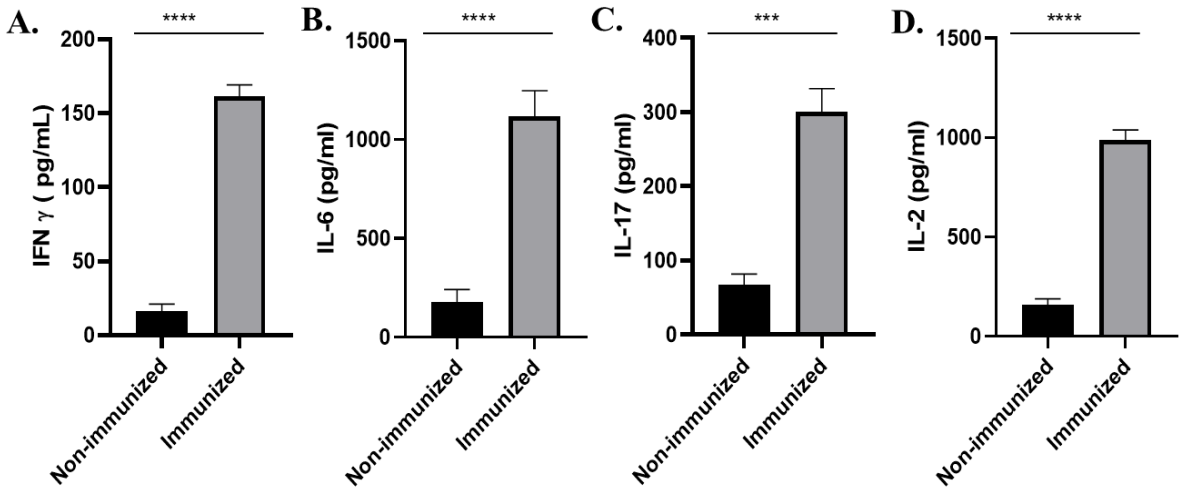


Figure 4. Bivalent Typhoidal Bacterial Ghost cells (BTBGs) mediated restimulation to bone marrow-derived dendritic cells and Splenic CD4+ T-cell co-culture induces the cytokine responses. Cytokine ELISA assay was done to check IFN-γ **(A),** IL-6 **(B),** IL-17 **(C),** and IL-2 **(D).** Cytokines in culture supernatant of ex-vivo co-culture of bone marrow derived dendritic cells (BMDC) of naïve mice and CD4+ splenic cell of immunized and nonimmunized mice after 24 hours. All cytokines are measured by ELISA (n=6). Statistical analyses were

performed using the non-parametric two tailed Student's t test (Mann-Whitney tests) to evaluate data; (****P value <0.0001, **p value < 0.001, *p value < 0.01). Each bar represents median and error values of six sample ± SE of three independent experiments.

5.4.5. Immunization with Bivalent Typhoidal Bacterial Ghost cells (BTBGs) generates memory response

To study antigen-specific memory T cells, bone marrow-derived dendritic cells (BMDCs) were isolated from the naïve BALB/c mice and pulsed in vitro with BTBG antigen for 24h. Antigen-pulsed BMDCs were then co-cultured with the experimental mice splenocytes containing CD4+ T cells. IFNγ release in the co-culture supernatants was estimated to be >10 folds higher for the splenocytes from BTBG immunized mice compared with the animals that left unimmunized, suggesting significant augmentation of T cell memory response by BTBG (**Fig. 5. A**). To determine memory T cell subsets, co-cultured CD4+ T-cells, as mentioned above were analyzed by flow cytometry after staining for the surface expression of 'Cluster of differentiation' (CD)markers (CD4+CD62LlowCD44hi). Cell subset analysis showed that augmented memory response was largely contributed by the effector memory T cells (CD62LlowCD44high) (**Fig. 5. B.i, B.ii**)

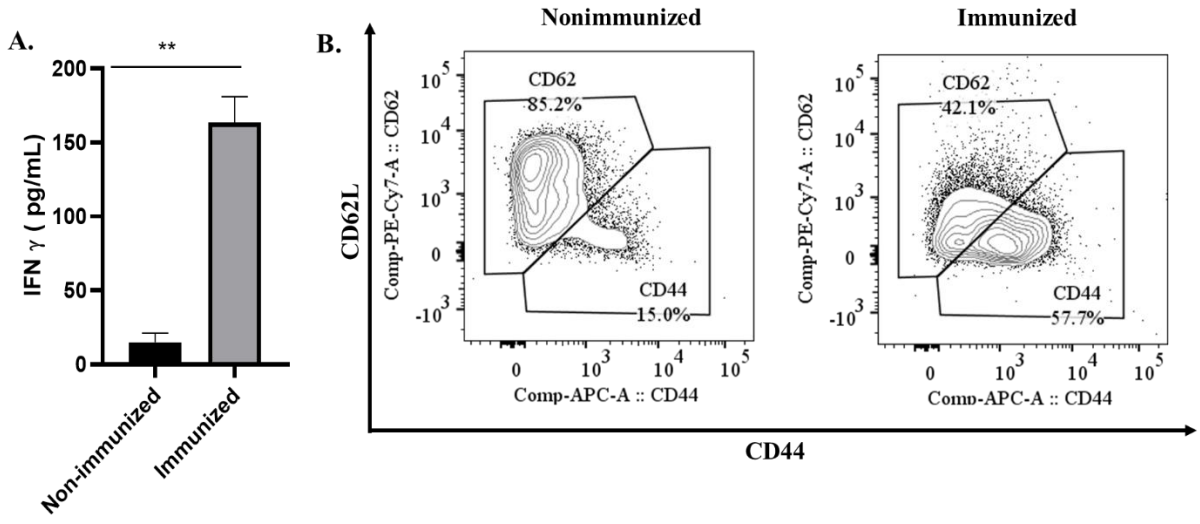


Figure 5. Induction of protective memory response. (**A**) mice were immunized as described above with the antigens indicated in the immunization scheme. Antigen-primed memory CD4+ T cells were isolated from the splenocytes of the mice 120 days of the start of the immunization. To evaluate the memory T-cells response, cells were converted to effector T-cells by presenting the respective antigens to them in association with MHC Class II. Bone marrow derived dendritic cells (BMDCs), isolated from the naïve mice were pulsed with Bivalent Typhoidal Bacterial Ghost cells (BTBGs) for 24h, followed by co-culturing of the cells with the memory T-cells. Memory response was analysed by the quantification of IFN-γ released in the co-culture supernatants using ELISA. Statistical analysis was performed using two-tailed Student's t-test (***P < 0.001; ****P < 0.0001). Data represents mean (±SEM) of three independent experiments. (B) CD4+ T cells, co-cultured with antigen-pulsed BMDCs for 24h, were analysed by flow cytometry after staining for the surface expression of 'Cluster of differentiation' (CD) markers for T-effector memory cell determination ($CD4^{+}CD62L^{low}$ $CD44^{high}$). Representative images from one out of three experiments are shown.

Chapter 6: Discussion

Salmonella Typhi and *Salmonella* Paratyphi are becoming increasingly dangerous for human health due to the global increase in multi-drug resistance. Globally, *S.* Typhi infections result in 9.9–24.2 million disease-presenting infections and 75,000–208,000 fatalities annually [1, 2, 3, 4, 5]. In contrast, *S.* Paratyphi infections cause less severe symptoms, with an annual incidence of 5.4 million, according to data from 2000 [6]. Nonetheless, a recent study [7] discovered a notable mortality rate from paratyphoid fever, which resulted in 13.7–56.3 thousand deaths in 2015. Enteric fever has a significant impact on public health and the economy, accounting for approximately 21.6 million disability-adjusted life years [8]. Patients may experience serious consequences such as meningoencephalitis, bacteraemia, hypotensive shock, intestinal perforation, multi-organ abscesses, and osteomyelitis if treatment is not received, which could result in a high fatality rate of approximately 20% [9]. Gallbladder cancer risk has been linked to persistent *S.* Typhi carriage, which is estimated to account for 3∼5% of all infections [10]. Even with the availability of potent antibiotics, treatment failures are often caused by multidrug resistance, which was first identified in the late 1980s [11] and refers to resistance to all first-line medications, including trimethoprim-sulfamethoxazole, ampicillin, and chloramphenicol [12, 13, 14]. A public health emergency has been declared due to the recent spread of the *S.* Typhi strain that is resistant to fluoroquinolones [15] and the discovery of an extensively drug-resistant (XDR) H58 subclade [16]. Multidrug resistance to *S.* Paratyphi is also becoming more common, in both endemic areas and wealthy nations [17].

Food and water contaminated by faeces can cause an outbreak of enteric fever. Based on developed world experience, it is possible to effectively control illness by improving sanitation, access to clean water, and personal and food hygiene. Shortly, though, budgetary limitations and a lack of education may prevent this from happening in low-income and lower-middle-income countries. Vaccination is still the best short-term preventive measure for these

areas, as it not only controls infections but also prevents the formation and spread of multidrug resistance [8].

Studies in the past have demonstrated that attempts have been made to create live attenuated, lethal, and subunit vaccinations [18, 19, 20], yet their effectiveness is not always at their best. A *Salmonella* vaccination that kills the entire cell has the power to induce advantageous antibody responses. Still, it is more reactogenic and only partially protects [21]. The live-attenuated vaccination can cause humoral, cellular, and mucosal immune reactions in chickens [22] although there is a high chance of reversion [23,24]. There is a pressing need to discover alternative safe and effective vaccination strategies to defend against salmonellosis to overcome these disadvantages.

Empty bacterial cell envelopes that are structurally intact and made from a variety of Gram-negative bacteria are known as bacterial ghosts (BG) [25]. As an immunogen, the BG's conserved surface features can trigger both innate and adaptive immune responses [26]. The lipopolysaccharide of gram-negative bacteria is one example of a surface-associated conserved motif (also known as a pathogen-associated molecular pattern, or PAMP) that is frequently used in vaccine formulations. These motifs ligandize host pattern recognition receptors to trigger host immune responses. Surface antigens have been shown to elicit robust humoral and cell-mediated immune responses against intracellular bacteria such as *Salmonella* [27]. Bacterial ghosts (BGs) are a reasonable, affordable, and simple platform to utilise, and their use is becoming more intense due to their expanding uses [27]. Gram-negative bacteria have been shown to use lysis gene E-based recombination to create ghost cells with regular transmembrane tunnels [28]. The BGs made using this technique is highly effective at preventing certain infections while retaining all of the cell surface antigens and their natural structure [29, 30, 31]. In particular, after lysis, the envelope components' functional and antigenic determinants remain intact. Nevertheless, the approach has many drawbacks, such as

its exclusive use for Gram-negative bacteria alone, possible dangers due to the challenge of quickly reaching a 100% lysis rate of the BGs strain [32], and an expensive, multi-step procedure that takes a long time. Conversely, BGs generated using the chemical method is not restricted to any particular type of bacterium, including Gram-positive [33] and Gram-negative [31, 34]. This technique generates BGs quickly, affordably, and with minimal effort. It is also a straightforward process that saves time. Several chemicals were used to prepare bacterial ghosts like NaOH, SDS, H_2O_2, and CaCO3 at concentrations lower than their minimum inhibitory concentrations (MIC) [35]. It was reported that Acinetobacter baumannii Ali190 ghost was created using H_2O_2, NaOH, and Na_2CO_3 solution [36]. The integrity of the cell wall is preserved using these techniques. BGs were created using a unique chemical process that involved cultivating *Salmonella* in culture media supplemented with 7% Tween 80 for a full day [37]. With lactic acid, the medium's pH was brought down to 3.6. Tween 80 dissolves hydrophobic elements in the bacterial outer membrane, causing weaker areas to develop. The abrupt drop in pH makes these places more prone to puncture development. Although NaOH may cause some surface features in BGs to change or disappear [34], NaOH-induced BGs offers effective defense against particular diseases [31, 33].

Because of their special qualities and immunogenicity, chemically induced BGs have demonstrated significant promise in the production of vaccines [38, 39, 40]. BGs are effective vehicles for delivering antigens to the immune system [39]. By inserting them into the ghost's interior or exposing them to the cell envelope's surface, they can be made to carry particular antigens [41, 42]. This enables antigens to be delivered to antigen-presenting cells precisely, resulting in efficient immune responses. BGs have inherent adjuvant qualities, which means that they strengthen the immune system's reaction to co-administered antigens [43]. When compared to live bacterial vaccinations, BGs exhibit better stability [26]. When it comes to developing vaccines, chemically induced BGs have many benefits: effective antigen delivery,

increased immunogenicity, inherent adjuvant qualities, safety, and scalability. According to research conducted on animals, it has proven to be efficient in delivering vaccines or modulating the immune system [38, 44, 45]. BGs have been effectively employed in in vivo investigations to transfer antigens from many diseases, including *Shigella*, *Salmonella*, *Escherichia coli*, and *Klebsiella pneumonia* [46, 47, 48, 49, 50]. Animals were protected against further infections by the administration of these ghost-based vaccinations, which also spurred certain immune responses. These favorable characteristics make chemically generated BGs attractive candidates for the creation of vaccines that can protect against a variety of infectious illnesses.

In several studies, effective *S.* entritidis and *S.* gallinarum ghost vaccines were produced to protect animals from *Salmonella* infections [51,52, 53]. In a rat model, it was demonstrated that mucosal immunization with *Escherichia coli* ghosts harbouring the nontypeable *Haemophilus influenzae* antigen Omp26 induced protective immunity [54]. It was found that, an oral vaccination with *Edwardsiella tarda* ghosts increases the protective efficacy in mice against infection [55]. Furthermore, the ability of BGs to target particular organs or tissues suggests that they are an effective mechanism to deliver drugs and DNA [56]. There is no ghost cell-based combination vaccine reported against *Salmonella* Typhi and *Salmonella* Paratyphi A to date. Therefore, the development of a combination vaccination against *Salmonella* Typhi and *Salmonella* Paratyphi A is a necessary step in the fight against enteric illnesses [57].

In the present study, bivalent bacterial ghost cells of *Salmonella* Typhi and *Salmonella* Paratyphi A were produced by chemical-mediated lysis using sodium hydroxide and characterized accordingly. Images captured with a scanning electron microscope reveal hollow cavities on the surface of the bacterial cells we treated. This validates the development of ghost cells in bacteria. These *Salmonella* were treated, and we found no bacterial growth, indicating

that they are dead or lysed cells. *Salmonella* Typhi and *Salmonella* Paratyphi were treated in equal proportion with sodium hydroxide and bivalent typhoidal bacterial ghost cells were created by mixing a ghost cell. We detected an anti-LPS, anti-OMP, anti-Vi-PS, and anti-WCL antibody response in mice immunized with BTBGs at two-week intervals. We analysed the blood parameters of immunized and non-immunized mice during the BTBGs immunization process to look for any anomalies. The blood profiles of animals that have received the BTBGs vaccination and those that have not shown any discernible differences. This data suggested that our BTBGs are not toxic or reactogenic in an in-vivo model [58].

Immunized mouse splenic cells demonstrated appreciable increases in the numbers of $CD4^+$, $CD8a^+$, and $CD19^+$ cells according to flow cytometry analysis. This implies that our immunogen activates B-cells and T-cells [59, 60]. The reduction of infection through humoral and cell-mediated immune responses is crucial for long-term protective immunity [61, 62, 63]. Lipopolysaccharides (LPS) and Vi-polysaccharides (Vi-PS) are crucial for producing protective immunity in the *Salmonella* Typhi vaccine [64, 65], although they both function without the involvement of T cells [66]. This explains why a very brief immunological response is produced by the typhoid vaccination based on Vi-PS [67]. Vi-PS functions in a T-cell-dependent manner, though, when it is conjugated with a protein (tetanus toxoid) [68, 69]. According to our observation, bacterial ghost cell immunization resulted in the generation of a sufficient quantity of anti-Vi-polysaccharide serum IgG. In this study, protection against *Salmonella* Typhi is mostly attributed to the anti-Vi-polysaccharide IgG antibody.

Immunization with bivalent typhoidal bacterial ghost cells has the potential to induce anti-Vi antibodies in addition to cell-mediated responses and other immunologic reactions [70]. Several surface proteins found in the outer membrane of *Salmonella* have the ability to

stimulate T-cell-dependent immunity in animals by acting as an adjuvant [71]. According to our ELISA, immunoblot, and dot blot analyses, immunized mice generate anti-LPS and anti-OMP-specific antibodies. This finding suggests that BTBGs stimulate the T-cell-facilitated immune response in immunized animals. According to earlier research, isolated porins comprising OmpC and OmpF of S. Typhi function as a potent immunogen and induce particular bactericidal antibody responses [72, 73, 74]. The immunoblot analysis conducted in our work revealed a variety of immunogenic proteins exhibiting unique immunogenic bands within the 25–55 KDa range. These findings suggest the potential existence of porin-specific antibodies in the blood of immunized individuals. In animals that have received vaccinations, BTBGs also cause an anti-LPS and anti-OMP secretory IgA response. To defend against enteric infections such as *Salmonella*, a mucosal antibody response is very necessary [60]. We observed a sustainable stable antibody titer till 120 days after immunization. This implies that BTBGs trigger a sustained immunological response.

A lethal dose of heterologous clinical isolates of *Salmonella* Typhi and Paratyphi A was administered to mice for protective efficacy study. We employed the intra-peritoneum challenge paradigm rather than the iron overload model because of its repeatability in our study on the effectiveness of vaccines against *Salmonella* Typhi [75] and Paratyphi A [59]. Mice that received the BTBGs vaccination established 100% and 80% protective efficacy or survivability against *Salmonella* Typhi and Paratyphi A, respectively. Compared to non-immunized mice, the bacterial burdens in the liver and spleen of immunized mice were noticeably reduced 48 and 72 hours after infection. After 180 days of primary immunization, the same outcomes were shown (i.e., less bacterial colonization in immune animals than in non-immunized animals after 48 hours of infection). Immunized and non-immunized mice showed clear differences in bacterial infection, spread, and inflammation based on a histological analysis of their liver and spleen tissue. The cell-mediated immune response plays a crucial role in reducing

inflammation, granulomas, portal inflammation, lobular inflammation, acidophil bodies, and infiltrating leukocytes in liver and spleen tissues of immunized mice. In nonimmunized mice, we found considerable portal inflammation, high lobular inflammation, widespread granulomas, acidophil bodies, and a high number of leukocytes infiltrating the body [76]. The bacterial count in the tissues of naïve mice is dramatically reduced by the adoptive transfer of serum and splenocytes from the immunized, confirming the protective effectiveness of the vaccine candidate. This strengthens the case for the requirement of humoral and cell-mediated immune responses in the defense against typhoidal *Salmonella* infection [77,78]. According to the results of the serum bactericidal experiment, the sera from immunized mice in our investigation showed typical bactericidal characteristics. The serum bactericidal assay is used to evaluate an antibody's functional ability to induce immunogenicity and possible protective qualities [76]. Our immune serum has the ability to attach to *Salmonella* and disrupt the cell, which helps with complement-mediated death, as demonstrated by SEM pictures from the bactericidal assay. Additionally, BTBGs immunized serum diminishes mucin layer penetration and bacterial motility, which reduces bacterial adhesion and invasion of the epithelial cell membrane [79]. The bacteria can be efficiently opsonized and killed by the antibodies present in the serum of mice immunized with BTBGs.

Th1/Th2 balance of the immune response has a major impact on the profile of immunoglobulin isotypes. This has been extensively researched, especially in mice where the Th1/Th2 predominance of immune responses has been linked to the levels of IgG1 and IgG2a [80–82]. IgG1/IgG2a ratios are higher in Th2 (IL-4-dominant responses), while IgG2a/IgG1 ratios are higher in Th1 response patterns (IL-12 and IFN- γ driven) [83]. Both IgG1 and IgG2a responses were observed in our ELISA study however, the IgG2a response was predominant. In the cytokine study (described later), it was found that IFN-γ production was highly upregulated.

IFN-γ plays an important role in humoral immunity by inducing the differentiation of Th0 cells into Th1 cells and mediating the production of IgG2a antibodies which play an important role in pathogen defense by opsonization/complement fixation [83]. This result suggested that the production of cytokines upregulated the differentiation of Th0 cells towards Th1 cells dominantly. T cells are limited to major histocompatibility complex class II (MHC-II) molecules that display peptides of extracellular or phagosomal origin. They primarily mediate surveillance through their T cell antigen receptor (TCR). Professional antigen-presenting cells (APCs), such as dendritic cells, macrophages, and B cells, constitutively express MHC-II. The three signals that APCs provide cognate antigen through the TCR, co-stimulation through co-stimulatory receptors, and cytokines—cause naive $CD4^+$ T cells to fully activate. Following this, the cells proliferate and divide into diverse effector subsets [84-87]. In our study, we have observed that IFN-γ, TNF-α, IL-6, IL-17, IL-1β, IL-12p40/70 and IL-23 cytokines are upregulated upon induction of T-helper cells through dendritic cell co-stimulation. This is necessary for differentiation between the Th1 and Th17 response in T cell receptor (TCR) stimulation [88]. IFN-γ and IL-12 cytokines may activate cytotoxic T-cell and Th1-responses. IL-6 has a standing for the gaps concerning innate as well as adoptive immune response, and helps T cells to secrete IFN-γ and IL-2 [89, 90]. IFN-γ, TNF-α also activates macrophages for antibacterial activity. IFN-γ is necessary to activate macrophages to clear the invasive bacteria [91, 92]. To prevent intracellular infection of *Salmonella* and its spread (to infect adjacent cells), cell-mediated immune responses, particularly Th1, are required [93, 94]. On the other hand, Th17 responses enhance the infiltrating leukocytes to the infection site to kill the bacteria [95] and also maintain the mucosal barrier thereby limiting the bacterial spread from the intestines [96]. In addition to this, result of splenic cytokine ELISA it was observed that the concentration of IL4 and IL-5 was much lower compared to the cytokines mentioned.

At the peak of the initial reaction, several populations of memory cells emerge, which are followed by effector CD4+ T cells devoted to a particular lineage. When foreign peptide and major histocompatibility complex class II (pMHC-II) complexes on antigen-presenting cells in secondary lymphoid organs attach to T cell antigen receptors (TCRs) on naive clones, CD4+ memory T cell formation starts. The TCR and costimulatory molecules produced from antigen-presenting cells, like CD28, send signals that cause naive cells to divide and develop into effector cell lymphoblasts [97, 98]. The ability of these effector cells to produce certain lymphokines is controlled by a differentiation process that depends on the type of cytokines produced by the innate immune system [99]. According to reports, effector cell development in the presence of interleukin 12 (IL-12) stimulates the production of interferon-γ (IFN-γ) by T helper type 1 (Th1), but not IL-4 or IL-17. On the other hand, differentiation in the presence of IL-4 stimulates the production of IL-4 by the T helper type 2 (Th2) programme, but not IFN-γ or IL-17. There is a remnant population of long-lived memory cells after the contraction phase, which lasts one to two weeks and results in the death of around 90% of the effector cells [100]. However, memory cells are diverse and are thought to belong to at least two groups. T cells with effector memory (TEM) and T cells with central memory (TCM) [101]. After TCR stimulation, effector memory T cells (TEM cells) generate a range of microbicidal cytokines, including IFN-γ, IL-4, and IL-5. TEM cells also carry homing receptors that enable migration to nonlymphoid areas of inflammation [102]. After being stimulated by the TCR, central memory T cells (TCM cells) do not immediately generate any of the classic effector cell lineage cytokines. However, they do release IL-2, multiply widely, and eventually develop the ability to make effector lymphokines. These cells express the chemokine receptor CCR7 and CD62L (L-selectin), which are important for movement across mucosal lymphoid organs and lymph nodes as well as for placement in the T cell regions of these organs. Thus, it has been hypothesised that TCM cells go through these areas and likely experience secondary reactions

there [101]. Experiments using intracellular fluorescent dye labelling have demonstrated that effector cells are more likely to lose CD62L [103] the more times they divide. These findings imply that whereas lesser stimulation promotes the development of less committed TCM cells, stronger stimulation is required for commitment to one of the TEM cell lineages. Dendritic cells present pMHC-II to excite naive T cells, which then multiply to create effector cells under the influence of innate immune system cytokines. A portion of these first effector cells, possibly those that don't engage with B cells, dedicate themselves to a specific lineage (like Th1), and a portion of these cells go on to become lineage-committed TEM cells. According to certain data, effector cells that can survive the contraction phase and develop into TEM cells include Th1, Th2, and possibly Th17.

To corroborate the antibody, recall response, we checked for $CD4^+$ effector memory cells producing IFN-γ in the culture supernatant of BTBGs immunized mice. Elevated levels of IFN-γ production were found following antigen restimulation of mouse splenocytes in the recipients of BTBGs. In addition to this, to determine memory T cell subsets, co-cultured $CD4^+$ T-cells, as mentioned above were analysed by flow cytometry after staining for the surface expression of 'Cluster of differentiation' (CD) markers ($CD4+CD62L^{low}CD44^{high}$). Cell subset analysis showed that augmented memory response was largely contributed by the effector memory T-cells ($CD62L^{low}CD44^{high}$).

All the above findings in our study indicated that our bivalent typhoidal bacterial ghost cells (BTBGs) might be employed as a vaccine candidate to combat Salmonella Typhi and Salmonella Paratyphi A near future.

Chapter 7: Conclusion

This study describes the formulation of ghost cell-based combination vaccine against *S.* Typhi and *S.* Paratyphi A. Empty bacterial cell envelopes that are structurally intact and contain structural properties that have antigenic potentiality made from a variety of gram-negative bacteria are known as bacterial ghosts (BGs) [1]. As an immunogen, the BG's conserved surface features can trigger both innate and adaptive immune responses [2]. The lipopolysaccharide of gram-negative bacteria is one example of a surface-associated conserved motif (also known as a pathogen-associated molecular pattern, or PAMP) that is frequently used in vaccine formulations. Surface antigens have been shown to elicit robust humoral and cell-mediated immune responses against intracellular bacteria such as *Salmonella* [3]. Bacterial ghosts (BGs) are a reasonable, affordable, and simple platform to utilize, and their use is becoming more intense due to their expanding uses [3]. Gram-negative bacteria have been shown to use lysis gene *E* (lysis-*E*) based recombination to create ghost cells with regular transmembrane tunnels [4]. The BGs made using this technique is highly effective at preventing certain infections while retaining all of the cell surface antigens and their natural structure [5, 6, 7]. In particular, after lysis, the envelope components' functional and antigenic determinants remain intact. Nevertheless, the approach has many drawbacks, such as its exclusive use for Gram-negative bacteria alone, possible dangers due to the challenge of quickly reaching a 100% lysis rate of the BGs strain [8], and an expensive, multi-step procedure that takes a long time. Conversely, BGs generated using the chemical method is not restricted to any particular type of bacterium, including Gram-positive [9] and Gram-negative [7, 10]. This technique generates BGs quickly, affordably, and with minimal effort. It is also a straightforward process that saves time. Several chemicals were used to prepare bacterial ghosts like NaOH, SDS, H_2O_2, and $CaCO_3$ at concentrations lower than their minimum inhibitory concentrations (MIC) [11]. It was reported that NaOH-induced BGs offers effective defense against particular diseases [7, 9]. In this study chemical-induced ghost cells were

characterized using scanning electron microscope reveal hollow cavities on the surface of the bacterial cells indicating the formation of ghost cell like structure.

BALB/c mice immunized intraperitoneally with three doses of our newly formulated bivalent immunogen (1 x 10^6 CFU/100µl) showed significant rise in the serum IgG antibody titre against outer membrane proteins (OMPs), lipopolysaccharide (LPS) and Vi-polysaccharide etc. In addition to this, IgA antibodies along with a mucosal antibody (sIgA) response in intestinal lavages of immunized mice were found higher compared to in non-immunized animals. From the ELISA results it is clear that our bivalent typhoidal bacterial ghost cells are immunogenic against the antigenic components of *Salmonella* Typhi and Paratyphi A separately compared to non-immunized animals. Bacterial cells of *Salmonella* Typhi and Paratyphi A can induce good humoral and mucosal immunity in animals up to 120^{th} day post-immunization. This is very much needed to control and prevent infections from *Salmonella* Typhi and Paratyphi A.

From T cell and B cell induction, it was found that Bacterial ghost cells (BGs) immunization induces $CD4^+$, $CD8a^+$, and $CD19^+$ populations in immunized mice spleen. The functionality of the anti-BGs antibody was determined by serum bactericidal assay and mucin penetration assay. Data from the bacterial motility inhibition assay showed that anti-BG antibody can significantly reduce the bacterial growth. Mucin penetration by immunized serum-treated bacteria was found to be lower whereas, that of non-agglutinated bacteria was much higher. These findings indicated that, bacterial ghost cell-specific sera considerably hinder motility, which can prevent mucin penetration from reaching the epithelial cells and thereby prevent colonization or invasion. Furthermore, we have evaluated the protective efficacy study in a challenged mice model. Extensive protective efficacy studies, both, in-vivo, ex-vivo and passive immunization studies proved that mice immunized with BGs significantly protect adult mice. Beside this, a passive protection study was carried out by the introduction of immunized

mice serum following an intraperitoneal (IP) challenge in naïve adult BALB/c mice. Post-challenge cytokine analysis proved that non-immunized mice were producing increased amounts of pro-inflammatory cytokines than the immunized mice. This could be the reason for the observed physiological distress in non-immunized mice due to a possible cytokine storm in-vivo.

We have evaluated the Th1/Th17 response in immunized mice as Th1/Th2 balance of the immune response has a major impact on the profile of immunoglobulin isotypes (IgG1, IgG2a). Both IgG1 and IgG2a responses were observed in our ELISA study however, the IgG2a response was predominant. Cytokine study from restimulation of immunized mice spleen, DC, or their co-culturing indicated the upregulation of Th1 cytokines (IFN-γ). IFN-γ plays an important role in humoral immunity by inducing the differentiation of Th0 cells into Th1 cells and mediating the production of IgG2a antibodies which play an important role in pathogen defense by opsonization/complement fixation. These findings suggested the induction of Th1 was dominant following BGs immunization which further correlates with the enhancement of IgG2a antibodies. We also evaluated the induction of T-effector memory responses in immunized mice. We checked for $CD4^+$ effector memory cells producing IFN-γ in the culture supernatant of BTBGs-immunized mice. Elevated levels of IFN-γ production were found following antigen restimulation of mouse splenocytes in the recipients of BTBGs. In addition to this, to determine memory T cell subsets, co-cultured $CD4^+$ Tells, as mentioned above were analyzed by flow cytometry after staining for the surface expression of 'Cluster of differentiation' (CD) markers ($CD4+CD62L^{low}CD44^{high}$). Cell subset analysis showed that augmented memory response was largely contributed by the effector memory T cells ($CD62L^{low}CD44^{high}$).

All the above findings in our study indicated that our bivalent typhoidal bacterial ghost cells (BTBGs) might be employed as a vaccine candidate to combat *Salmonella* Typhi and *Salmonella* Paratyphi A near future.

Chapter 8:

Highlights of the study – salient findings.

- Selection of strain on the basis of phenotypic and genotypic characteristics from clinical isolates for formulation of bacterial ghost cell immunogen.
- Successfully developed a *Salmonella* Typhi and *Salmonella* Paratyphi A, bivalent bacterial ghost cell vaccine using NaOH-mediated chemical treatment.
- Typhoidal and paratyphoidal bacterial ghost cells formation was confirmed by electron microscopy study.
- In-vitro cytotoxicity assay of BTBGs and In-vivo, hematopathology of adult mice during and after the BTBGs immunization period revealed that the immunogen was less reactogenic and had no negative effects on the animals' health.
- An established animal model (intraperitoneal mice model) was used to study typhoidal and paratyphoidal disease, immunogenicity and protective efficacy using BALB/c mice.
- Western blot & dot blot analysis shows the ability to induce immunogenicity against OMPs and LPS against circulating strains, after three doses of intra-peritoneal immunization in mice with BTBGs.
- ELISA results reveled significant rise in serum IgG, IgG1, IgG2a, IgA, sIgA after three doses of immunization upto 120^{th} days post immunization.
- An increased in Th1 and Th17- biased cytokines was observed in isolated splenocytes from immunized mice, indicating the induction of a strong Th1-cell mediated immunity in mice.
- Immunized mice showed significant reduction of intracellular bacterial colonization in different systemic organs, following live bacterial infection compared to non-immunized animals.
- Histopathological analysis showed, there is no significant tissue inflammation and tissue damage in immunized mice than nonimmunized mice after bacterial challenge.

- The imuunized serum have the better bacteriocidal ability than the nonimmunized control serum in presence of complement.
- Immunized sera also inhibit the bacterial motility and mucin penetration ability in in-vitro study.
- Passive transfer of immunized sera reduced the bacterial colonization ability in naïve animals.
- BTBGs immunized mice conferred 80 to100% protection against clinical isolates of Typhoidal and Paratyphoidal *Salmonellae* as a single vaccine candidate.
- Up-regulation of different surface markers induced both B-cell and T-cell mediated immune responses, which could provide long lasting immune response and protection in mice.
- Robust induction of T effector memory response indicates long term efficacy of the candidate vaccine.

Chapter 9: Bibliography

Chapter 1: Introduction.

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Chapter 7: Conclusion.

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Chapter 10:

List of original publications

1. **Halder P**, Maiti S, Banerjee S, Das S, Dutta M, Dutta S, Koley H. Bacterial ghost cell based bivalent candidate vaccine against Salmonella Typhi and Salmonella Paratyphi A: A prophylactic study in BALB/c mice. Vaccine. 2023 Sep 22;41(41):5994-6007. doi: 10.1016/j.vaccine.2023.08.049. Epub 2023 Aug 23. PMID: 37625993
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Chapter 11:

List of oral/poster presentations in national/international conferences

1. **Prolay Halder1**, Suhrid Maiti1, Soumalya Banerjee1, Sanjib Das1, Moumita Dutta2, Shanta Dutta1, Hemanta Koley1*. "Prophylactic method to reduce the enteric fever (Salmonella Typhi and Salmonella Paratyphi A) mediated health burden with bivalent typhoidal bacterial ghost immunogen in mice model." 22nd Annual Congress of Korean Society for Parenteral and Enteral Nutrition (KSPEN-2023) held in Sejong University, Gwanggaeto-gwan, Seoul, Republic of Korea, June 9-10, 2023. (International Oral Presentation)
2. **Prolay Halder1**, Suhrid Maiti1, Soumalya Banerjee1, Sanjib Das1, Moumita Dutta2, Shanta Dutta1, Hemanta Koley1*. "A bivalent Salmonella Typhi and Paratyphi A ghost-based vaccine induce protective immune response in mice model", 13th International Conference on Typhoid and Other Invasive Salmonelloses (CAT 2023), held in Kigali, Rwanda, at the Kigali Marriott, December 5 to 7, 2023. (International Poater Presentation)
3. **Prolay Halder** *, Soumalya Banerjee, Sanjib Das, Shanta Dutta, Hemanta Koley**. Bivalent typhoidal bacterial ghost acts as a protective immunogen against Salmonella Typhi and Salmonella Paratyphi A in mice model. 16th Asian Conference on Diarrhoeal Disease and Nutrition (ASCODD-2022) November 11-13, 2022, Kolkata, India.
4. **Prolay Halder** *, Soumalya Banerjee, Sanjib Das, Srijani Biswas, Shanta Dutta, Hemanta Koley**. Bivalent Typhoidal Bacterial Ghost based Immunogen of Salmonella Typhi and Salmonella Paratyphi A induces adaptive immunity and protective efficacy in mice model. 108th Indian Science Congress (ISC2023) January 3-7, 2023 at R.T.M. Nagpur University, Nagpur, Maharashtra, India.
5. **Prolay Halder** 1*, Suhrid Maiti1, Soumalya Banerjee1, Sanjib Das1, Moumita Dutta2, Shanta Dutta1, Hemanta Koley1**. Bacterial Ghost Cells mediated prophylactic study

against Salmonella Typhi and Salmonella Paratyphi A in mice model. 7th symposium of Frontiers in Modern Biology (FIMB) 2023 January 20-22, IISER Kolkata, India.

6. Debaki Ranjan Howlader, **Prolay Halder**, Suhrid Maiti, Ushasi Bhaumik, Shanta Dutta, Hemanta Koley. Cross protective nature of a newly developed bivalent typhoid vaccine in response to non-typhoidal infection. 54th US-Japan Joint Panel Conference on Cholera and Other Bacterial Enteric Infections.10th-13th December, 2019, Osaka, Japan.
7. Suhrid Maiti, **Prolay Halder**, Sounak Sarkar, Vivek Mondal, Asish Mukhopadhyay, Shanta Dutta, Hemanta Koley. "Oral Passive Immunization of NTS Outer Membrane Vesicle Derived Polyreactive Serum Protects Day Old Chicken Against Experimental Salmonellosis."54th US-Japan Joint Panel Conference on Cholera and Other Bacterial Enteric Infections. 10th-13th December, 2019 Osaka, Japan.
8. Soumalya Banerjee 1, **Prolay Halder** 2, Sanjib Das 3, Shanta Dutta 4, Hemanta Koley Development of a trivalent next generation Outer Membrane Vesicles (OMVs) based immunogen to reduce multi drug resistant non-typhoidal Salmonella and Campylobacter mediated health burden. Vaccines against Shigella and ETEC (VASE) 2022 conference, November 29- December 1, Washington, USA.
9. Soumalya Banerjee*, **Prolay Halder**, Sanjib Das, Shanta Dutta, Hemanta Koley**. Development of a pentavalent next generation Outer Membrane Vesicles based immunogen to ameliorate prevalent multi drug resistant strains of diarrhoeagenic Escherichia coli. 16th Asian Conference on Diarrhoeal Disease and Nutrition (ASCODD-2022) November 11-13, 2022, Kolkata, India.
10. Sanjib Das1, **Prolay Halder1**, Soumalya Banerjee1, Sangita Paul1, Shanta Dutta1, Asish Kumar Mukhopadhyay1, Hemanta Koley1*. Selection and characterization of Helicobacter pylori to formulate a next generation OMVs-based immunogen. 16th

Asian Conference on Diarrhoeal Disease and Nutrition (ASCODD-2022) November 11th -13th, 2022, Kolkata, India.

11. Soumalya Banerjee*, **Prolay Halder**, Sanjib Das, Srijani Biswas, Shanta Dutta, Hemanta Koley**. Studies on passive protective efficacy in suckling mice model conferred by pentavalent OMVs-based immunized mice sera against infection caused by circulating diarrhoeagenic Escherichia coli. 108th Indian Science Congress (ISC2023) January 3-7, 2023 at R.T.M. Nagpur University, Nagpur, Maharashtra, India.
12. Sanjib Das*, **Prolay Halder**, Soumalya Banerjee, Srijani Biswas, Asish Kumar Mukhopadhyay, Shanta Dutta, Hemanta Koley**. Preparation and immunogenic characterization of Helicobacter pylori derived Outer Membrane Vesicles based immunogen in C57BL/6J mice. 108th Indian Science Congress (ISC2023) January 3-7, 2023 at R.T.M. Nagpur University, Nagpur, Maharashtra, India.
13. Soumalya Banerjee*, **Prolay Halder**, Sanjib Das, Srijani Biswas, Shanta Dutta, Hemanta Koley**. A study on passive protective efficacy in suckling mice model conferred by pentavalent OMVs-based immunized mice sera against infection caused by circulating diarrhoeagenic Escherichia coli. 7th symposium of Frontiers in Modern Biology (FIMB) 2023 January 20-22, IISER Kolkata, India.
14. Sanjib Das*, **Prolay Halder**, Soumalya Banerjee, Shanta Dutta, Asish Kumar Mukhopadhyay, Hemanta Koley**. Establishment of an intra-gastric surgical model in C57BL/6 mice to study the vaccine efficacy against Helicobacter pylori. 7th symposium of Frontiers in Modern Biology (FIMB) 2023 January 20-22, IISER Kolkata, India
15. Hemanta Koley*, Suhrid Maiti , **Prolay Halder**, Soumalya Banerjee , Sanjib Das , Moumita Dutta , Asish K Mukhopadhyay & Shanta Dutta Formulation of Outer

Membrane Vesicles Based Vaccine for firm Animals : A way to reduce clinical health burden. 108th Indian Science Congress (ISC2023) January 3-7, 2023 at R.T.M. Nagpur University, Nagpur, Maharashtra, India.

16. Hemanta Koley*, Debaki Ranjan Howlader, **Prolay Halder**, Sriparna Samajpati, Shanta Dutta. Activation of humoral and cellular immune response following bivalent OMVs immunization protects mice from recently circulating typhoidal strains. US Japan Joint Panel Conference on Cholera and Other Bacterial Enteric Infections.10th-13th December, 2019, Osaka, Japan.
17. Hemanta Koley*, Suhrid Maiti, **Prolay Halder**, Soumalya Banerjee, Sanjib Das, Moumita Dutt, Asish Kumar Mukhopadhyay, Shanta Dutta. Outer Membrane Vesicles based livestock Non-typhoidal Salmonella Candidate Vaccine help to reduce Clinical Health Burden. 16th Asian Conference on Diarrhoeal Disease and Nutrition (ASCODD-2022) November 11-13, 2022, Kolkata, India.
18. Samima Sultana*, Sanjib Das, **Prolay Halder**, Anusree Giri, Ananya Roy, Hemanta Koley, Samadrita Sengupta**. "Hypocholesterolemic effect of chemically modified Mango Kernel Starch on diet induced dyslipidemia in animal model". One day National Symposium on Human Origin, Health and Diseases-New Dimension on 21st July 2023 at Sister Nivedita University, Kolkata.
19. Hemanta Koley*, Suhrid Maiti, Ushasi Bhaumik, **Prolay Halder**, Asish Mukhapadhyay, Keinosuke Okamoto, Yoshifumi Takeda, Shanta Dutta Development of heat killed EIEC immunogen protecting against circulating EIEC in mice model 54th US-Japan Joint Panel Conference on Cholera and Other Bacterial Enteric Infections.10th-13th December, 2019, Osaka, Japan.
20. Sohini Sikdar*, Debmalya Mitra, Bidisha Pal, **Prolay Halder**, Soumalya Banerjee, Shanta Dutta**, Hemanta Koley**. Studies on synbiotic therapy diminish colonization

and inflammation in ETEC induced murine diarrheal model. 16th Asian Conference on Diarrhoeal Disease and Nutrition (ASCODD-2022) November 11-13, 2022, Kolkata, India.

21. Paulami Dutta, Sohini Sikdar, Debmalya Mitra, Soumalya Banerjee, **Prolay Halder**, Hemanta Koley, Shanta Dutta*. Molecular characterisation of antimicrobial resistant non-typhoidal Salmonellae isolated from patients with acute gastroenteritis and evaluating the beneficial role of probiotic and prebiotic in NTS induced diarrhoeal Published abstracts of papers presented at different scientific meetings: mice model. 16th Asian Conference on Diarrhoeal Disease and Nutrition (ASCODD-2022) November 11-13, 2022, Kolkata, India.

Chapter 12: Patents applied

We have applied for a patent at the Indian Patent Office:

1. “A bivalent typhoidal bacterial ghost (BTBG) immunogenic formulation and method for preparation thereof”, the complete patent application of this invention was filed on 15th June, 2022 and the patent application number is 202211034380.

2. “A novel Trivalent Iron Nanoparticles Electroporated Outer Membrane vesicles-based antigen (TINEOMVs) as a vaccine candidate against Campylobacter Jejuni, Salmonella Typhimurium and Salmonella Enteritidis” the complete patent application of this invention has been filed on 30th May 2023 and the patent application number is 202311037297.

3. “A novel formulation of Sodium Alginate encapsulated green-synthesized Titanium Nanoparticle coated OMVs based vaccine (SGTiOMVs) against prevalent strains of Helicobacter pylori” The complete patent application of this invention has been filed on April 11, 2024 and application no. assigned is 202411029436.

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Bacterial ghost cell based bivalent candidate vaccine against *Salmonella* Typhi and *Salmonella* Paratyphi A: A prophylactic study in BALB/c mice

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ABSTRACT

Typhoid and emerging paratyphoid fever are a severe enteric disease worldwide with high morbidity and mortality. Licensed typhoid vaccines are in the market, but no paratyphoid vaccine is currently available. In the present study we developed a bivalent vaccine against *Salmonella* Typhi and Paratyphi A using a bacterial ghost platform. Bacterial ghost cells (BGs) are bacteria-derived cell membranes without cytoplasmic contents that retain their cellular morphology, including all cell surface features. Furthermore, BGs have inherent adjuvant properties that promote an enhanced humoral and cellular immune reaction to the target antigen. Sodium hydroxide was used to prepare ghost cells of *Salmonella* Typhi and Paratyphi A. The bacterial ghost cells were characterised using electron microscopy. Then BALB/c mice were immunized three times (0^{th}, 14^{th} and 28^{th} day) with the bivalent typhoidal bacterial ghost cells. Haematological study of adult mice throughout immunization period reflected that the immunogen was safe to administer and does not affect the animals' health. After complete immunization, we found significant serum antibody titter against whole cell lysate, outer membrane protein and lipopolysaccharide of both bacteria, and cell-mediated immunity was observed in an ex-vivo experiment. CD4+, CD8a+ and CD19+ splenic cell populations were increased in immunized animals. Bivalent Typhoidal ghost cell immunized mice showed better survival, less bacterial colonization in systemic organs, and less inflammation and/or destruction of tissue in histopathological analysis than non-immunized control mice. Serum antibodies of immunized animals can significantly inhibit bacterial motility and mucin penetration ability with better killing properties against *Salmonella* Typhi and Paratyphi A. Furthermore, significant passive protection was observed through the adoptive transfer of serum antibody and lymphocytes of immunized animals to naïve animals after bacterial infection. In summary, Bivalent Typhoidal Bacterial Ghost cells (BTBGs) enhances immunogenic properties and serves as a safe and effective prevention strategy against *Salmonella* Typhi and Paratyphi A.

1. Introduction

Enteric fever (including Typhoid fever caused by *Salmonella* Typhi and Paratyphoid fever caused by *Salmonella* Paratyphi A), is a life threating bacterial infection which poses one of the most important health problem of developing and under developed countries. *S*. Typhi is more endemic and various modelling studies have assessed the worldwide estimated risk burden was 20.6 million (17.5–24.2) cases and 223,000 (131,000–344,000) deaths [1]. A pooled assessment of local area-based reports in India proposed a paratyphoid event of 105 for every 100,000 people yearly, signifying the importance of *S*. Paratyphi (A, B and C) [2]. Enteric fever mainly effects areas with very low sanitation, poor hygiene, and congested rural areas without clean water, in addition to in travellers returning from these endemic regions. In the near future it is estimated that the major agent causing enteric fever will be *Salmonella* Paratyphi [3]. Currently, the only treatment available for typhoidal fever patients are antibiotics, but this treatment is facing a global challenge, which is increasing antibiotic resistance among enteric bacteria (both *S*. Typhi and *S*. Paratyphi A). Resistance to various antimicrobial treatments of enteric fever has been consistent for decades, as evidenced by the first reports of chloramphenicol resistance in *S*. Typhi in the 1970s [4]. *S*. Typhi's resistance to chloramphenicol, amoxicillin,

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and co-trimoxazole was linked with many outbreaks in the late 1980s and early 1990s [5], and is now majorly found in South Asia. This multi-drug resistant issue of *S.* Typhi has been complicated the therapeutic management of enteric fever. The only way to reduce this health burden is by vaccination [6]. Though there are several vaccines available against *S.* Typhi infection for global use, there is no vaccine which can protect people simultaneously from both *S.* Typhi and *S.* Paratyphi A. An important virulence factor of *S.* Typhi i.e., Vi polysaccharide (Vi-PS) has been targeted as an antigen for protective immunity against *S.* Typhi [7,8]. Vaccine efficacy report from various clinical data showed live attenuated vaccine without expressing Vi-PS has a protective efficacy of ~60 % but due to safety concerns, this is not recommended for children under the age of six [9]. The immune responses prompted by Vi-PS vaccines were short-lived and efficacy ranges-between 55 and 60 % in older children (>6years) and adults [10]. Although Subunit vaccines are safe for all ages, Vi-PS vaccines fail to make optimal immunity in children lower than 2 years of age. When Vi-PS is conjugated with a protein, it can induce anti-Vi-PS antibody in infants and young children up to 90 % efficacy [11]. In current *Salmonella* Paratyphi A vaccine research, there are a few potential subunit vaccine candidates like GMMA of *S.* Typhi A and protein capsular matrix [12]. The problem with these vaccine candidates is high production cost, and as the endemic regions of enteric fever are mainly in lower middle-income countries, the economic burden always comes before the health burden. From this point of view, it is most essential to formulate a bivalent typhoidal vaccine candidate [13] which is cost effective and also easy to make at an industrial scale.

In this study, we have developed a novel bivalent bacterial ghost based typhoidal immunogen using *S.* Typhi and *S.* Paratyphi A ghost cells. After three doses of intra-peritoneal immunization with this bivalent ghost immunogen, we checked protective efficacy in an adult mouse model. We also evaluated the humoral and cell-mediated immune response after immunization by quantifying outer membrane protein (OMP) and lipopolysaccharide (LPS) specific serum immunoglobulin and Th1/Th17 specific cytokine response from splenic cells and bone marrow derived dendritic cells. We have also aimed to induce common mucosal immunity to kill the administered bacteria through oral transmission. Low systemic circulation of bacteria after infection in fully immunized animals compared to placebo also proved the mode of protection. This preclinical study suggests that a Bivalent Typhoidal Bacterial Ghost (BTBG) cell-based vaccine might be one of the best choices for a human vaccine to reduce the typhoidal *Salmonella* mediated health burden, especially in lower middle-income countries.

2. Material and methods

2.1. Bacteria and culture method

Salmonella Typhi (C-6.946) and *Salmonella* Paratyphi A (BCR148) were used for bacterial ghost cell preparation and *Salmonella* Typhi (K554) and *Salmonella* Paratyphi A (K580) were used for challenge studies. All strains were obtained from National Institute of Cholera and Enteric Diseases (NICED) strain culture repository. All strains were kept in 20 % glycerol, maintained at-80 °C in Tryptic soy broth (Difco, USA). Prior to use, strains were cultured in Tryptic Soy Broth (TSB; Difco, USA) at 37 °C with shaking or on Tryptic Soy Agar (TSA; Difco, USA) plates. Table 1 lists the strains used in this study.

Table 1
List the strains used in this study.

Strain Name	Serotype	Abbreviation	Reference
C-6.946	*S.* Typhi	ST	Clinical Isolate [13]
BCR148	*S.* Paratyphi A	SPA	Clinical Isolate [13]
K554	*S.* Typhi	ST	Clinical Isolate [This study]
K580	*S.* Paratyphi A	SPA	Clinical Isolate [This study]

2.2. Bacterial ghost cell preparation

Determination of the minimum inhibitory concentration (MIC) of NaOH for *S.* Typhi and *S.* Paratyphi A was performed by the 2-fold broth dilution method as described previously with some modifications [14]. The biomass of overnight *S.* Typhi and *S.* Paratyphi A culture cells was centrifuged (8,000×g, 10 min, 4 °C) before being resuspended in sterile phosphate buffered saline (PBS, pH-7.4). The bacterial cells were then adjusted to 1×10^8 CFU/ml. 1 ml (5×) of MIC of sodium hydroxide concentration was added to 2 ml of cell suspension with 2 ml of sterile solvent and incubated for 90 min at 37 °C. After centrifugation (8,000×g, 10 min, 4 °C) and three PBS washes, ghost cells were collected. In ice-cold PBS, the final cell pellets were resuspended. Based on bacterial cell count (1×10^8 CFU/ml), recovered bacterial ghost cells from the two strains were combined in a 1:1 ratio and the final mixture was kept at 4 °C until further use [14,15]. 100 µl of each strain of bacterial ghost cells were spread on agar plates to check for complete lysis of bacterial cells.

2.3. Scanning electron microscopy (SEM) imaging of bacterial ghost cells

Ghost bacteria and non-treated control bacteria were fixed using buffered 2.5 % glutaraldehyde (pH 7.0) for 2 h at 4 °C, washed with buffer and then post fixed in 1 % osmium tetroxide for 1.5 h at 4 °C. Again, washed samples are dehydrated through a series of ethanol concentrations. After samples reached the critical dried point, they were mounted on SEM stubs, coated with gold–palladium, and observed under a scanning electron microscope, Tecnai12 Bio Twin Transmission Electron Microscope (FEI, Netherlands) [14].

2.4. Macrophage-mediated cytotoxicity assay (LDH assay)

To check the cytotoxicity of NaOH treated Bacteria, we used Pierce LDH Kit (Invitrogen, USA). Murine macrophage cell line (RAW 264.7) was cultured in 96-well plates (Nunc, USA) for 24 h in a 37 °C incubator (with 5 % CO2). 1.0×10^5 cells/well was then incubated for 24 h with 1.0×10^8 CFU/ml of ghost cells and heat-killed cells in culture medium. LDH-positive samples are used for positive controls and PBS was used for negative control [15].

2.5. Animals

Six weeks old female BALB/c mice were obtained from the animal house facility of NICED, Kolkata. Mice were separated into different groups with sterile food and water. The Institutional Animal Ethical Committee of NICED (CPCSEA registered, Registration No. 68//Rebi/S/1999/CPCSEA valid17/7/2024), approved the animal experimental protocol with the project approval number. PRO/167/January 2020-November 2022.

2.6. Immunization of animals

Mice were injected intra-peritoneally on the 0^{th}, 14^{th} and 28^{th} day with 100 µl of bacterial Ghost Immunogen (1:1) containing 1×10^8 CFU/ml cell. Non-immunized control group mice received 100 µl of sterile PBS. The vaccination schedule for animals is shown schematically in Supplementary Fig. 1.

2.7. Collection of serum and intestinal lavage

On the 0^{th}, 7^{th}, 21^{st}, 35^{th}, 49^{th}, 70^{th}, 90^{th} and 120^{th} days following the initial vaccination, at time intervals, blood was taken from tail veins. Blood was kept in microtainer tube (BD, USA) and serum isolated by centrifugation (1000×g, 10 min, 4 °C). Intestinal lavage from immunized and control mice was collected in a sterile microcentrifuge tube by PBS wash of small intestine after dissection. Lavage was centrifuged at

6000×g, 10 min; supernatant was collected and stored at −20 °C. The animal blood and intestinal lavage collection schedule for animals is shown in Supplementary Fig. 1.

2.8. Haematological parameter analysis

Animals of immunized and control groups were monitored for the signs of acute toxicity. After 7 days of every immunization, animals were bled and blood samples were collected to study haematological parameters, such as the Haemoglobin (Hb), Erythrocyte Sedimentation Rate (ESR), Total Leucocyte Count (TLC), Differential Leucocyte Count (DLC), and Packed Cell Volume (PCV) [16]. All parameters were analysed within few hours of sample collection.

2.9. Purification of whole cell lysates (WCL)

WCL were isolated according to the previously described protocol [13] as follows; Bacteria were cultured 18 h in Tryptic Soy Broth (TSB; Difco, USA), centrifuged to obtain cell pellets, washed with PBS, and sonicated. Again, samples were centrifuged and supernatant was collected and stored.

2.10. Purification of outer membrane proteins (OMPs)

Outer membrane proteins were isolated based on a previously described protocol [17] as follows; Bacteria were cultured for 18 h in Tryptic Soy Broth (TSB; Difco, USA), centrifuged, and pellets were washed with HEPES buffer (pH 7.5) and protease inhibitor cocktail (Roche, Sigma, USA). After that, they were sonicated for ten minutes using a Hielscher (UP100H) sonicator in an ice bath. After centrifugation, the supernatants were collected and centrifuged at 100,000×g. The pellet was re-suspended with same buffer containing 1 % N-lauryl Sarcosine and placed at 37 °C for 30 min. Again, it was centrifuged and the final collection was re-suspended in same buffer. Then the OMP concentration was determined, which was then adjusted to 1 μg/1 μl with HEPES buffer and stored at −20 °C.

2.11. Purification of lipopolysaccharide (LPS)

LPS was isolated based on a previously described protocol [17] as follows; *S.* Typhi and *S.* Paratyphi A were cultured overnight in Tryptic Soy Broth (TSB; Difco, USA), centrifuged, and the cell pellet was re-suspended with 0.15 M NaCl containing Phenol-saturated 3-[N-morpholino] propen sulfonic acid [0.02 M MOPS]. The mixture was incubated for 30 min at 65 °C with random shaking, and then for 10 min on ice. After a second centrifugation, the upper aqueous layer was collected, mixed with four times the volume of the sample with chilled ethanol, and left overnight at −20 °C. LPS was purified and collected on the following day by centrifuging at 8500×g for 20 min, resuspending in distilled water, and storing at −20 °C.

2.12. Determination of antibody levels by ELISA

96 well flat bottom ELISA plate (Tarson, India) was separately coated with whole cell lysate (WCL), outer membrane proteins (OMPs), lipopolysaccharide (LPS), and Vi-polysaccharide (Bharat biotech, India) as previously described [13,17]. Then each plate was kept at 4 °C for 18 h. The wells were washed with PBS and blocked with skim milk (BD, USA). After that wells were washed with PBS-T (PBS having 0.5 % Tween-20, Sigma, USA) and incubated for 1hr with consecutively diluted serum samples and intestinal lavage for mucosal antibody. Again, wells were washed with PBS-T and incubated after adding HRP-tagged secondary antibody (IgG & IgA, Abcam & Sigma, USA). After a PBS wash, substrate was added to all wells and kept for ten mins. The reaction was stopped using sulphuric acid (2 N). Finally, the OD_{492} nm was measured using a microplate reader. A table of antibodies used in this study are listed in Supplementary Table with RRID designations (Supplementary Table 1).

2.13. SDS-PAGE, immunoblot and dot blot

LPS and total protein content of the OMPs from *Salmonella* strains were determined by SDS-PAGE. 20 μg of OMPs, were boiled in SDS-PAGE buffer and LPS samples were boiled in LPS sample buffer. The samples were then loaded onto a 12 % SDS-PAGE gel separately depending on their staining reagent. 100 V was then applied for running the gel in an AE-6530 SDS-PAGE apparatus from ATTO Corporation (Japan). The gel was then stained by either coomassie or silver stain. For immunoblot, proteins were boiled in 5× loading buffer and separated on a 12 % SDS gel. Using ATTO AE-6687 (Japan) blot apparatus, proteins were transferred onto a nitrocellulose membrane for immunoblotting. Antisera from immunized mice and the ALP-conjugated goat anti-mouse secondary IgG was used for immunoblotting [13]. We measured the concentration of extracted LPS, with 5 μg, 10 μg and 15 μg of LPS being used for a dot blot assay of either strain. First, LPS was absorbed by nitrocellulose membrane at room temperature. 5 % BSA solution was used for blocking and then the membrane was washed with TBS-T following a standard protocol. Antisera from immunized mice and the ALP-conjugated anti-mouse secondary IgG was used in the dot blot [13]. A table of antibodies used in this study are listed in Supplementary Table with RRID designations (Supplementary Table 1).

2.14. Serum bactericidal assay

Serum bactericidal assay was performed according to an earlier described method [18]. Sera collected from the immunized mice on day 35 of first immunization was heat inactivated at 56 °C for 20 min. Serum samples were diluted from 1:50 to 1:128,000 in PBS. A master mix composed of 12.5 μl of baby rabbit complement (12.5 % final concentration) with 27.5 μl of PBS, 50 μl of diluted mouse serum, and 10 μl of diluted bacteria (320 CFU, T_{0h}) was prepared. The mixture was then incubated for 1 h (T_{1h}) with shaking at 115 rpm at 37 °C. Viable bacterial colonies were counted after spreading the mixture onto the plate followed by overnight incubation at 37 °C. The negative control contained bacteria and complement only. Bactericidal activity was determined as serum dilutions necessary to obtain a 50 % reduction in CFU counts at T_{1h} compared with T_{0h}. For SEM imaging, samples (bacteria and complement with heat inactivated immunized serum, nonimmunized serum respectively) with lowest serum dilution (1:50) were fixed by adding 900 μl glutaraldehyde and incubated at 4 °C overnight. Cells were then prepared using the previously described method for scanning electron microscopic analysis and observed [17].

2.15. Motility and mucin penetration assay

A motility assay performed based on a published method, with modifications [19]. Vaccinated and control serum were mixed with PBS in 1:400 dilutions, separately, and dispensed on soft agar (0.3 %) plates. Log-phase bacteria with an OD_{600} value of 0.4 were punctured in the middle of the plates once the serum mixture had dried. After that, the plates were incubated for 24-hours at 37 °C. Then the bacterial spreading across the surface was measured. The mucin penetration assay was performed according to a published method [20]. Briefly, a solution containing 1 % (w/v) mucin (MP Biomedicals, USA) and soft agar (0.3 %) media (TSB) was allowed to form a soft gel at room temperature in a 1 ml tuberculin syringe fitted with a stopcock. Log-phase bacteria were incubated for one hour at 37 °C with heat-inactivated vaccinated and control serum. 100 μl aliquot (containing 1×10^7 CFU/ml organisms) of incubated bacterial suspension were placed in the top of the columns and kept at 37 °C for 30 min in a vertical position. To determine the bacterial count, a 500 μl sample from the column's lower portion was collected, serially diluted, and plated.

2.16. Cytokine measurement:

2.16.1. Splenocytes re-stimulation assay

Splenocytes from vaccinated mice were cultured for two hours in RPMI containing 10 % FBS one week after the last vaccination. 1×10^6 CFU/ml bivalent typhoidal bacterial ghost cells were used to treat the splenocytes at 37 °C (with 5 % CO2) for 24 h. From the culture supernatants, TNF-α, IFN-γ, IL-6 and IL-17 were estimated by individual ELISA kits (Invitrogen, USA) [13].

2.16.2. Separation of bone marrow-derived dendritic cells (BMDCs) and re-stimulation assay

From naive BALB/c mice, bone marrow was isolated and cultured in RPMI containing 10 % FBS and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). 1×10^6 CFU/ml bivalent typhoidal bacterial ghost cells were used to treat the mature BMDCs at 37 °C (with 5 % CO2) for 24 h [13]. From the culture supernatants, IL-1β, IL-6, IL-23, and IL-12p40/70 were estimated by individual ELISA kits (Invitrogen, USA).

2.16.3. BMDC-Splenic CD4+ T cell co-culture

BMDCs (previously stimulated) and splenic CD4+ T cells were isolated (BD IMag™ anti-mouse CD4 Magnetic Particles, Cat. No. 551539, USA) and cultured at a 1:1 ratio for 24 h [21]. From the culture supernatants, IFN-γ, IL-2, IL-6 and IL-17 were estimated by individual ELISA kits (Invitrogen, USA).

2.17. Fluorescence-activated cell sorter (FACS) analysis

The spleens of both the immunized and control mice were removed and sterilely homogenized on the 35th day after vaccination to determine the CD4+, CD8a+, and CD19+ cell populations. Using Cell Strainer (Corning, USA) and a sterile syringe, the spleen was homogenized. [17]. Splenocytes were stained with anti- Mabs: CD4-phycoerithrin (PE), CD8a PE, CD19 PE or an isotype control PE (Miltenyi Biotec, USA). Expression was measured on a BD FACS ARYA III flow cytometer and data was assessed with FACS DIVA software.

2.18. Histopathology analysis

The liver and spleen tissues that were obtained at 48 h and 72 h after infection were fixed in a solution of 10 % buffered formalin, and 5 μM paraffin-embedded sections were stained with haematoxylin and eosin (H&E). The slides were viewed at 20× and 40× magnification using an Olympus IX51 light microscope, followed by observer-blind histopathological analysis.

2.19. Protective efficacy studies

2.19.1. Bacterial challenge study

The immunized and non-immunized animals were infected intra-peritoneally with 5×10^8 CFU/mouse of heterologous strain of bacteria (*S.* Typhi; K554 and *S.* Paratyphi A; K580) on the 35th day after first vaccination. Total of 30 mice were divided into five groups. 6 mice per group were challenged intra-peritoneally. Two immunized group were infected with *S.* Typhi and *S.* Paratyphi A. Two nonimmunized groups were infected with *S.* Typhi and *S.* Paratyphi A. Another group received only 100 μl PBS; as the non-immunized, non-infected negative control. The infected mice were observed for 10 days to assess survival. To examine bacterial colonization in different systemic organs, animals were infected intra-peritoneally at the 35th and 180th day post first immunization with 5×10^5 CFU/mouse of heterologous typhoidal strains (*S.* Typhi; K554 and *S.* Paratyphi A; K580). Infected animals (both vaccinated and control mice) were sacrificed at different times post infection to determine the bacterial count.

2.19.2. Passive protection study

35th day post first immunization serum and splenocytes (spleen was processed as before) were isolated from vaccinated and a control group of mouse then transferred to a naïve mouse via tail vein (100 μl of serum). RBCs were lysed and splenocytes were re-suspended in phosphate saline buffer and 100 μl (1×10^6 splenic cell) was injected via tail vein. Afterwards, animals were infected intra-peritoneally with 1×10^5 CFU/mouse with heterologous strains of *Salmonella* to observe bacterial colonization after 72 h. One group of mice was infected on the day of the adoptive transfer (0th day) and another group was infected at the 7th day post adoptive transfer.

2.20. Statistical analysis

All data are presented as the median ± SE. A Mann-Whitney test was used to compare the experimental data of immunized group versus control group. An analysis of variance (ANOVA) with Kruskal-Wallis for multiple comparisons was used when three or more groups were compared. Kaplan-Meier analysis was performed to compare survival curves in the challenge study. The remaining data were analysed using Student's *t* test. Two-tailed P values of $P < 0.05$ were considered statistically significant. All experiments were triplicated for statistical significance. All the data were evaluated using GraphPad Prism 8.0.2 (GraphPad Software, Inc.) and MS Excel software.

3. Results:

3.1. Preparation and characterization of bacterial ghost cells

For preparation of bacterial ghost cells from *Salmonella* Typhi and Paratyphi A, we used a 2-fold broth dilution method, with the MIC of NaOH found to be 3.125 mg/ml. At the end of the lysis there was no bacterial growth up to 48 h incubation at 37 °C. The determined MIC completely lysed the bacterial cells and stopped further growth (Supplementary Fig. 2). Scanning electron microscopic structure revealed that the NaOH treated *S.* Typhi and *S.* Paratyphi A had some transmembrane tunnel like structures that differentiated bacterial ghost cells from untreated live cells (Fig. 1.A, 1.B, 1.C, 1.D). Through this rupture in bacterial cell, the cytoplasmic contents leave the cell and the ability to grow was lost. The bacterial ghost cell surface structure seemed to retain normal cell morphology.

3.2. In-vitro cytotoxicity tests in murine macrophages exposed to bacterial ghost cells and in-vivo effect of bacterial ghost cells on blood parameter

In our experiments, NaOH-treated bacterial ghost cells showed much less cytotoxicity in an in-vitro murine macrophage (RAW 264.7) cell line than the LDH positive control sample provided by the manufacturer (Supplementary Fig. 3.A). This suggests that the NaOH treated bacterial cells are less reactogenic in nature. No major alteration in any blood parameters was observed in between the immunized and PBS control mice groups [16]. All the parameters studied were within the normal range (Supplementary Fig. 3.B).

3.3. Bivalent typhoidal Salmonella ghost cells immunization induces humoral and mucosal immune response in adult mice

Induction of humoral immunity during and after the immunization with bivalent typhoidal bacterial ghost cells was measured using an ELISA up to 120th days after 1st immunization. In this experiment, we observed significant induction of serum immunoglobulin IgG and IgA antibodies along with a mucosal antibody (sIgA) response in intestinal lavages of immunized mice than in non-immunized animals. The level of different antibodies in immunized animals increases from day 7 to 35 days post immunization, and then maintain a steady level of antibody titre up to 120 days (P value < 0.0001). A significant peak of IgG and IgA

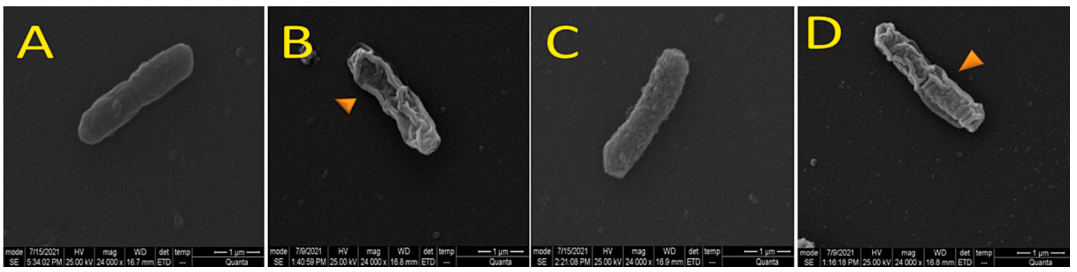

Fig. 1. Scanning electron microscopic analysis (SEM); (**A**) Untreated control S. Paratyphi A (**B**) NaOH-treated S. Paratyphi A (**C**) Untreated control S. Typhi (**D**) NaOH-treated S. Typhi. Arrows shows *trans*-membrane lysis tunnels.

titre were seen after the second dose of immunization, between day 28 and 35 post immunization. Three doses of immunization with the bivalent typhoidal bacterial ghost cells are sufficient to get a prominent mucosal antibody (sIgA) titre from the intestinal lavage. The entire antibody titre was measured against the whole cell lysate (WCL), outer membrane protein (OMP), and lipopolysaccharide (LPS) of *Salmonella* Typhi and Paratyphi A (Fig. 2; A.i,A.ii,B.i,B.ii). Mucosal antibody titres were also significant increased against these components i.e. OMP and LPS of both bacterial ghost cells individually (Fig. 2.C). As well as serum IgG antibody titre was measured against Vi-polysaccharide (Vi-PS) of *Salmonella* Typhi (Fig. 2.D). From the ELISA results it is clear that our bivalent typhoidal bacterial ghost cells are immunogenic against the antigenic components of *Salmonella* Typhi and Paratyphi A separately compared to non-immunized animals. Bacterial cells of *Salmonella* Typhi and Paratyphi A can induce good humoral and mucosal immunity in animals up to 120th day post immunization. This is very much needed to control and prevent infections from *Salmonella* Typhi and Paratyphi A.

Isolated purified OMP and LPS of *S.* Typhi and *S.* Partyphi A was used in ELISA, immunoblot and dotblot were visualised through SDS-PAGE gel separation and coomassie and silver staining respectively (Supplementary Fig. 4.A, 4.B). Serum antibodies were capable of recognizing the WCL, bacterial ghost cells, and OMP of both bacteria in immunoblot and LPS in dotblot assays. Proteins from 20 KD to 80 KD size were viewed as immunogenic (Supplementary Fig. 4.C). A dot blot assay was performed for three different concentrations of LPS (5 μg, 10 μg, and 15 μg) from either strain with 35th day sera of bivalent typhoidal ghost cell immunized mice. We observed a prominent band against every concentration of LPS (Supplementary Fig. 4.D, 4.E). That shows that bacterial ghost cells are immunogenic against lipopolysaccharide of both strains. It's confirmed that whole outer membranes of NaOH-induced bacterial ghost cells contain preserved epitopes i.e. outer membrane proteins and LPS.

3.4. *Bivalent typhoidal ghost-cells (BTBGs) induce a pro-inflammatory (Th1/Th17) cytokine response:*

35th day post 1st immunization both BTBG and PBS immunized mice were sacrificed, and their spleens were collected, and a single cell suspension were prepared and cultured for 24 h. Cells were treated with respective immunogens and checked for their cytokine response post treatment. From the culture supernatants we found BTBGs treatment significantly upregulate levels of IFN-γ, TNF-α, IL-6 and IL-17 (Fig. 3.A, B, C, D) than PBS treated group. To further illustrate the generation of innate immune response by BTBGs; we investigated dendritic cells (DCs) response that controls T cell variation. For that isolated mature bone marrow derived dendritic cells (BMDC) and were stimulated with BTBGs. We observed a significant induction of IL-1β, IL-6, IL-23, and IL-12p40/70 cytokines levels from the culture supernatants. Pro-inflammatory cytokines secreted by DCs regulate Th1/Th17 cell differentiation (Fig. 3.E, F, G, H). To check this reaction, CD4+ T cells separated or isolated from immunized mice and PBS control mice were then co-cultured for 24 h with BTBGs pulsed BMDCs. Cytokine ELISA results had significantly higher levels of Th1/Th17 cytokines (IFN-γ, IL-2, IL-6 and IL-17) in the culture supernatants (Fig. 3.I, J, K, L). All these outcomes direct a robust Th1/Th17 media immunity in BTBGs immunized mice. To check the upregulation of adoptive immune responses post immunization with BTBGs in mice, we check the different surface markers of T cells (CD4+, CD8a+) and B cells (CD19+) 35th day post immunization. From the result we observed that BTBGs immunization significantly increases the both CD4+, CD8a+ and CD19+ cells populations than PBS immunized mice (Fig. 3.M.i,M.ii,M.iii, Supplementary Fig. 5).

3.5. *Bacterial colonization, survivality check after heterologous bacterial challenge*

After one week of last immunization of BTBG immunized group and nonimmunized group (PBS group) were challenged with 5 × 10^5 CFU/mice of *S.* Typhi and *S.* Paratyphi A and bacterial colonization were enumerated from different systemic organs of mice. The results showed bacterial colonization was 2–3 fold less in immunized group than non-immunized group (Fig. 4.A, B). At different time point post challenge, we found gradual increase of bacterial colonization in nonimmunized group but in case of immunized groups colonization was decreased. Then we checked long term protection after challenge with sublethal dose of *S.* Typhi and *S.* Paratyphi A after 180 days post final immunization. We found at least 2-fold less bacterial load on organs of immunized animals against control (nonimmunized group) animals (Fig. 4.C). This may be due to good antibody titter present for a long period after completion of immunization. To further support our result, we challenge both group of mice with lethal dose of *S.* Typhi and *S.* Paratyphi A (5 × 10^8 CFU/mice) via intraperitoneal route and their survival was recorded. Mice were observed for 10 days post challenge and from the result we found that all mice immunized with BTBGs were survived till 10 days, but all PBS immunized mice challenged with S. Typhi died within 4 days post challenge and all PBS immunized *S.* Paratyphi A challenged mice died 9 days post challenge. Immunized mice showed better survival, with 100 % survival for *S.* Typhi and ≥80 % for *S.* Paratyphi A. All of the non-immunized control mice died during this observation period after infection (Fig. 4.D). The body weights of immunized mice very less decreased throughout the observation period, but dramatically decreased in non-immunized control mice (Fig. 4.E.i, E.ii). This result indicates that our immunogen can protect mice from *Salmonella* infection. Some other clinical signs of disease like diarrhea, lethargy and all the symptoms including weight loss were assessed to measure not only mortality, but also the morbidity signs. Non-immunized, *S.* Typhi and *S.* Paratyphi A infected mice showed major clinical signs of morbidity i.e. severe fur ruffling was noticed over time, severe weight loss over time, severe diarrhea started overnight of infection, all animals showed severe lethargy over time, and ultimately all animals are died within 4–9 days of infection in nonimmunized group. Whereas the number of morality was very less in BTBG immunized mice including the clinical sign of morbidity like very mild fur ruffling for short period and weight loss was mild but recovered shortly, in case of S. Paratyphi A challenged group.

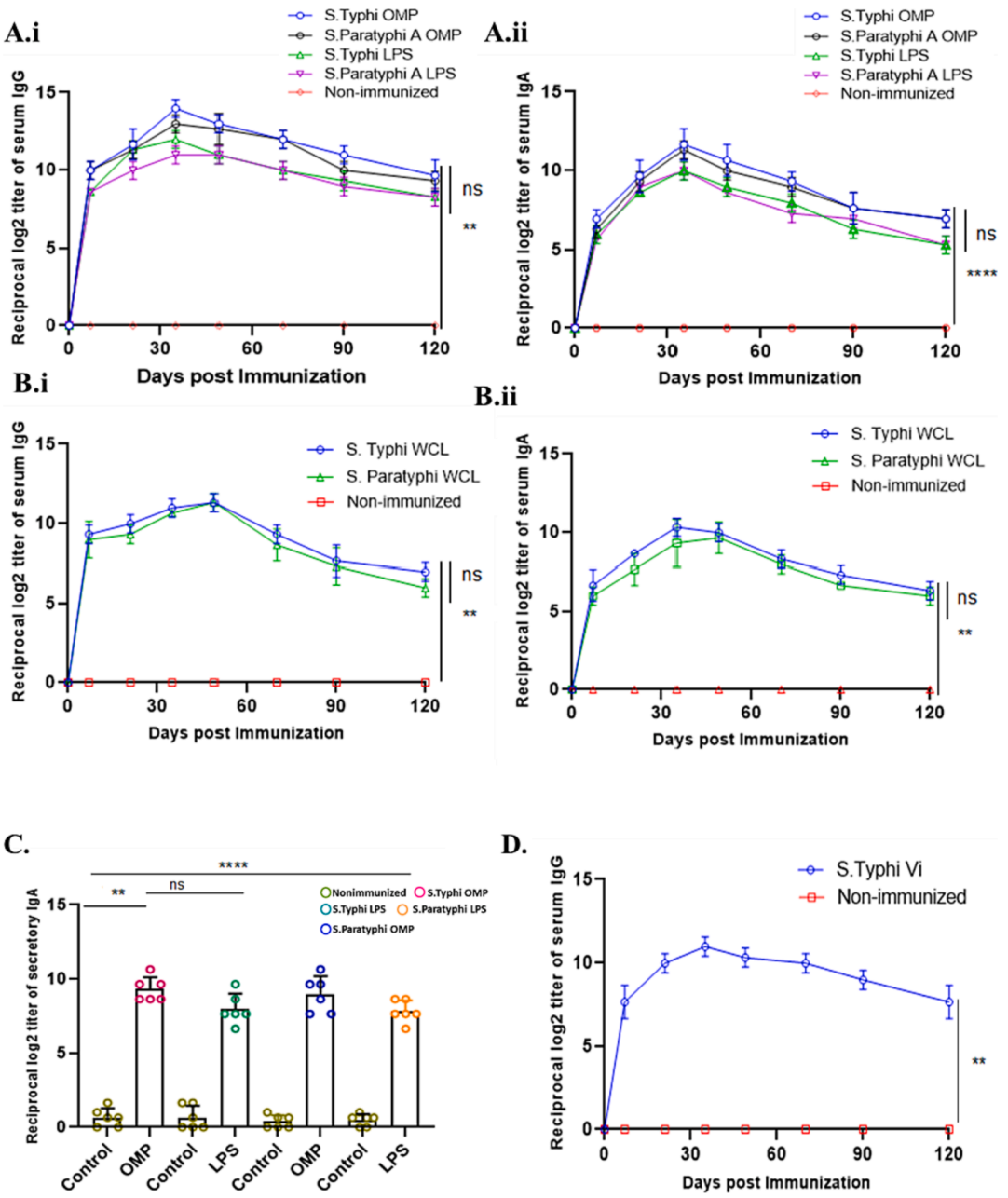

Fig. 2. Reciprocal log2 titer of serum IgG, serum IgA and secretory IgA immunoglobulin from Bivalent Typhoidal Bacterial Ghost cells immunized and non-immunized group. Mouse serum IgG (**A.i**), serum IgA (**A.ii**) was measured separately after three doses of intraperitoneal immunization against Outer membrane protein (OMP) and LPS of S. Typhi and S. Paratyphi A. Mouse serum IgG (**B.i**), serum IgA (**B.ii**) were measured separately after three doses of intraperitoneal immunization against whole cell lysate (WCL) of S. Typhi and S. Paratyphi A. **C.** Reciprocal Log2 titter of Secretory IgA was measured after 35th day of primary immunization against OMP and LPS of *S.* Typhi and *S.* Paratyphi A. **D.** Mouse serum IgG was measured separately after three doses of intraperitoneal immunization against Vi-polysaccharide of S. Typhi. Difference between immunized and non-immunized group was statistically significant. Statistical analyses were performed non-parametric two tailed Student's *t* test (Mann-Whitney tests) and one-way analysis of variance (ANOVA) test (Kruskal-Wallis test) to evaluate data; (****P value < 0.0001,**p value < 0.001). Each bar represents median and error values of six sample ± SE of three independent experiments.

All the observed protective and good survival results in case of immunized animals were due to a *Salmonella* Typhi and Paratyphi A specific strong antibody mediated immunity with other cellular and humoral immune responses as was generated through BTBG immunization.

3.6. Histopathlogical analysis of BTBGs from immunized and non-immunized animal tissue

The degree of inflammation and tissue damage were determined by histopathological examination of the respective organs (Fig. 5). Normal uninfected immunized and non-immunized mice showed no abnormalities upon histopathological examination in the spleen or liver of both groups (Fig. 5; A, D, G, J). However, the infected mice displayed extensive inflammation indicative of bacterial spread. Spleens of infected non-immunized mice showed augmented states of inflammation, infiltrating lymphocytes and granulomas (Fig. 5; Ei, E.ii, F.i, F.ii) when compared to BTBG immunized mice (Fig. 5; K.i, K.ii, L.i, L.ii). Liver and spleen tissues had similar types of histopathlogical findings. Granulomas were detected in the livers of infected control mice, which were not present in the livers of *S.* Typhi and/or very reduced in *S.* Paratyphi A

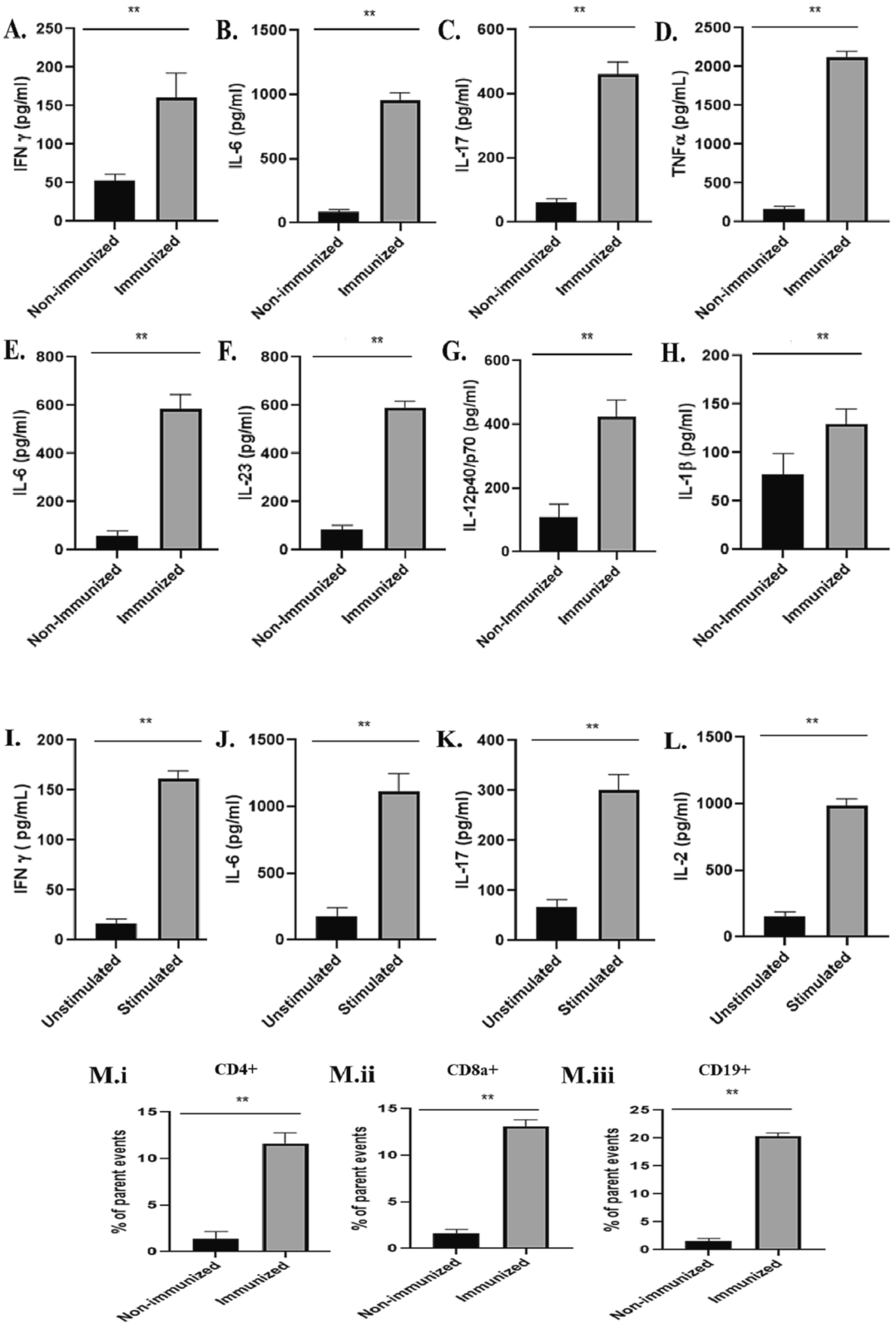

Fig. 3. Bivalent Typhoidal Bacterial Ghost cells (BTBGs) induces the production of cell mediated cytokines responses and increases the population of CD4+, CD8a+ and CD19+ splenic cells. **A.B.C.D.** IFN-γ, TNF-α, IL-6 and IL-17 cytokines in culture supernatant of ex-vivo cultured splenic cells of immunized and nonimmunized mice after 24 h of restimulation with BTBGs; **E.F.G.H.** IL-23, IL-1β, IL-6 and IL-12p40/70 cytokines in culture supernatant of ex-vivo bone marrow derived dendritic cells (BMDC) from naïve mice with and without BTBGs stimulation for 24 h, **I.J.K.L.** IFN-γ, IL-6, IL-17 and IL-2 cytokines in culture supernatant of ex-vivo co-culture of bone marrow derived dendritic cells (BMDC) of naïve mice and CD4+ splenic cell of immunized and nonimmunized mice after 24 h. All cytokines are measured by ELISA (n = 6). **M.i., M.ii., M.iii.** CD4+, CD8a+ and CD19+ cell population in splenocytes of immunized and nonimmunized mice by FACS analysis. Statistical analyses were performed using the non-parametric two tailed Student's *t* test (Mann-Whitney tests) to evaluate data; (**p value < 0.001). Each bar represents median and error values of six sample ± SE of three independent experiments.

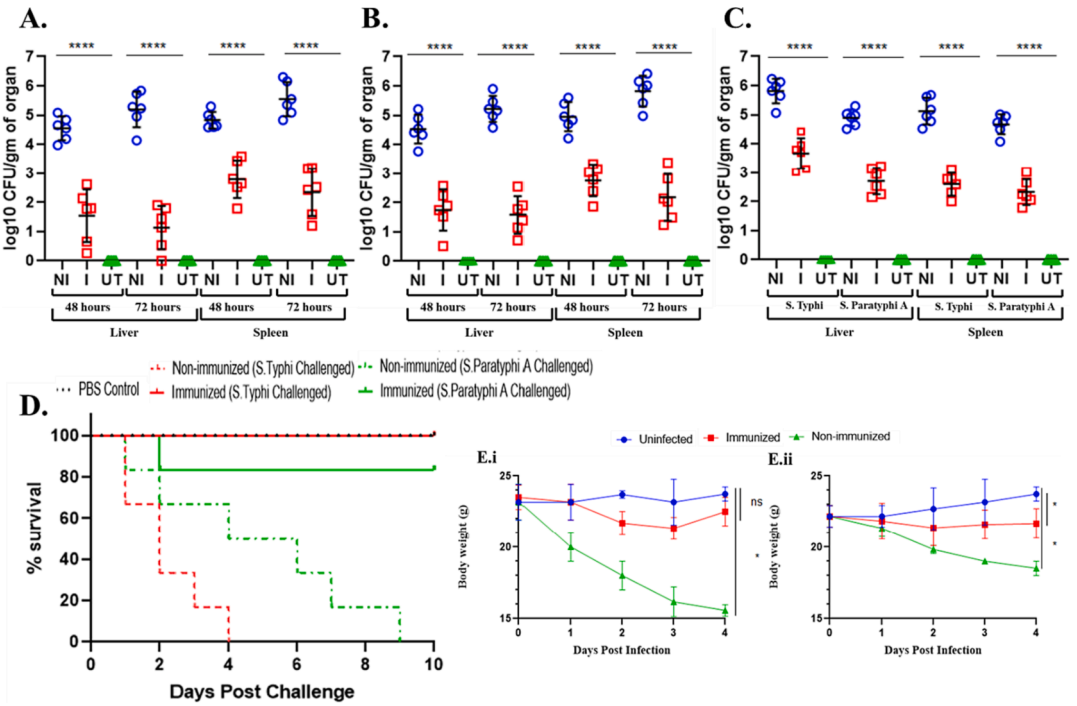

Fig. 4. Colonization and protective efficacy analysis of Ghost cells against *Salmonella* Typhi and *Salmonella* Paratyphi A infection. **A.** Colonization of *S.* Typhi **B.** Colonization of *S.* Paratyphi A, respectively after 35th day post immunization, **C.** Colonization (post 72 h post infection) to systemic organs after 180th day post first immunization. In every case immunized and nonimmunized groups were challenged with 5×10^5 CFU/mice of S. Typhi and S. Paratyphi A. NI: non-immunized, I: immunized, UT: untreated. **D.** Survival graph (Kaplan-Meier Curves) depicts percentage (%) of survival of the immunized and nonimmunized BALB/c mice after Intra-peritoneal challenge with 5×10^8 CFU/mice of S. Typhi and S. Paratyphi A. **E.i, E.ii.** Comparative data of change in body weight between non-immunized and immunized adult mice, after challenge with *S.* Typhi and *S.* Paratyphi A respectively. Data were expressed as median value of six mice ± S.E of three separate experiments. Statistical analyses were performed using non-parametric two tailed Student's *t* test (Mann-Whitney tests) and one-way analysis of variance (ANOVA) test (Kruskal-Wallis test) (****P value < 0.0001, **p value < 0.001, *p value < 0.01).

infected immunized mice. Although *S.* Typhi infected immunized mice presented lobular inflammation in the liver (Fig. 5; H.i, H.ii, I.i, I.ii), and lobular inflammation was witnessed to be higher in control mice. In addition, *S.* Typhi-infected PBS control mice had more portal inflammation and leukocyte infiltration in their livers (Fig. 5; B.i, B.ii, C.i, C.ii) than in the livers of animals that had been immunized. Immunized and unvaccinated *S.* Typhi-infected mice also had acidophil bodies.

3.7. Adoptive transfer of serum and splenocytes followed by bacterial challenge

Adoptive transfer of immunized and non-immunized mice serum and splenocytes intravenously (via tail vein) to naïve mice was done to check the humoral and cell mediated passive protection. We found that immunized blood serum and splenocytes confers protection to naïve mice similarly. We observed bacterial colonization differences in systemic organs of animals after adoptive transfer. Non-immunized sera and splenocytes transferred mice had a very high rate of colonization in the liver and spleen. However, immunized sera and splenocyte-treated mice showed considerably lower bacterial load at day 0 (Fig. 6.A.i, ii, iii, iv) and day 7 (Fig. 6.B.i, ii, iii, iv) in adoptively transferred mice.

3.8. Serum bactericidal activity, bacterial motility and mucin penetration assay

tWe observed that bivalent typhoidal bacterial ghost cells were able to generate protein and LPS specific serum antibodies against *S.* Typhi and *S.* Paratyphi A. Previous studies showed that both protein and LPS-specific anti *Salmonella* antibodies have complement-dependent bactericidal activity [22,23]. So, we decided to check the bactericidal activity of our immunized mice serum. To do this, immunized mice serum was heat inactivated and then incubated in different serum dilution with bacteria and complement. Whenever we incubated the bacteria with heat inactivated immunized serum supplemented with baby rabbit complement, the bacterial cells were killed significantly. The bactericidal effect of BTBG immunized serum dilution on *Salmonella* Typhi is 1:12,800 and for *Salmonella* Paratyphi A is 1:6400 (Fig. 7.A). Therefore, from this experiment we conclude that our typhoidal *Salmonella* ghost immunized mice serum could eliminate Typhoidal *Salmonella* by activating complement pathways. To further support our result scanning electron microscopic analysis was performed. Scanning electron microscopy (SEM) images revealed clearly visible lysis of bacterial cell when treated with immunized heat inactivated serum treated group (Fig. 7B.i, B.ii, C.i, C.ii).

Bacterial motility is very important for typhoidal *Salmonella's* pathogenesis as motility help them to move from epithelial lumen to epithelial surface. Thus, to find our BTBG immunogens' effect on typhoidal *Salmonella's* motility, we performed bacterial motility assay. We found that immunized serum, compared to non-immunized showed a major inhibition of *Salmonella* motility. This indicates that our bivalent formulation does indeed agglutinate bacteria, which may contribute to the immunogens' protective nature (Fig. 7.D.i, D.ii,E.i,E.ii,F) [14]. Intestinal epithelial wall also covered with a thick layer of viscoelastic mucus gel which is formed with a dense network of entangled and cross linked mucin. This mucin layer is always abundant with various antibodies specifically secretory IgA (sIgA) and sometimes IgG. To find the effect of the BTBGs immunization on both typhoidal *Salmonella* strains, we performed mucin penetration assay. Mucin penetration by immunized serum-treated bacteria was found to be lower whereas, that of non-agglutinated bacteria were much higher (Fig. 7.G). According to the findings, bacterial ghost cell-specific sera considerably hinder motility, which can prevent mucin penetration from reaching the epithelial cells and thereby prevents colonization or invasion.

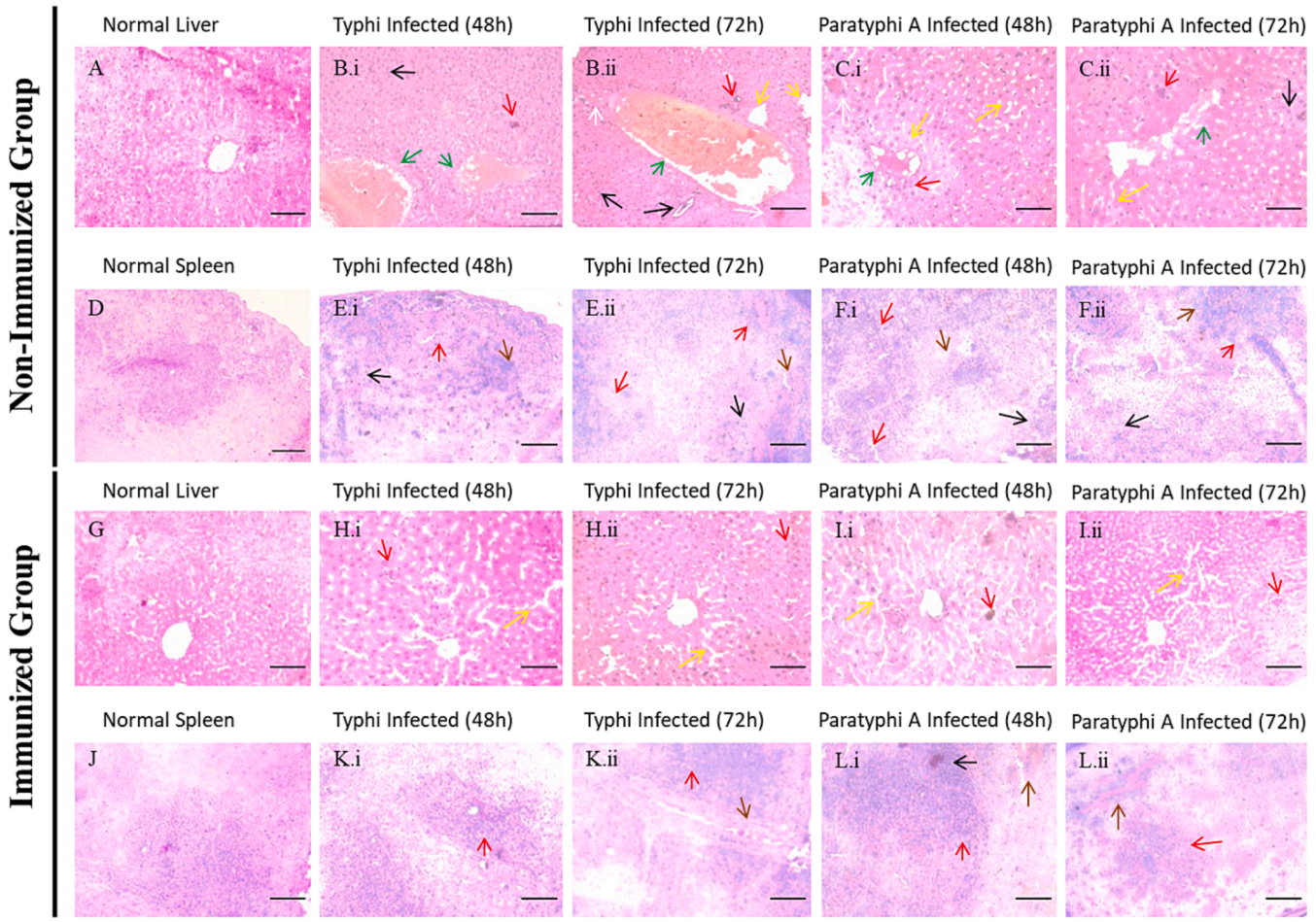

Fig. 5. Histopathology (H&E staining) of the liver and spleen of non-immunized and immunized mice after 48 h and 72 h of intra-peritoneal challenge with *Salmonella* Typhi and *Salmonella* Paratyphi A. **A.** & **D.** Liver and spleen of normal non-immunized mice. Liver and spleen of nonimmunized mice after 48 h and 72 h post challenged with *S.* Typhi (**B, i** & **ii**; **E, i** & **ii**) and *S.* Paratyphi A (**C, i** & **ii**; **F, i** & **ii**). Histological images show extensive portal inflammation, acidophilic bodies, infiltrating leukocytes, lobular inflammation and extensive granulomas. **G.** & **J.** Spleen and liver of normal immunized mice. Liver and spleen of immunized mice after 48 h and 72 h post challenged with *S.* Typhi (**H, i** & **ii**; **K, i** & **ii**) and *S.* Paratyphi A (**I, i** & **ii; L, i** & **ii**). Histological images represent mild leukocyte infiltrates, mild lobular inflammation, no portal inflammation and acidophil bodies in spleen and liver respectively. Images were captured at 20× and 40× magnification. Scale bar represents 100 μm. Inflammation (brown arrow), Extensive granulomas (black arrow), Portal inflammation (green arrow), Lobular inflammation (yellow arrow), Acidophil bodies (white arrow), Infiltrating leukocytes (red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Due to the global rise in multi-drug resistance, *Salmonella* Typhi and *Salmonella* Paratyphi are reaching an extremely concerning level with regards to human wellbeing. The advancement of vaccine development against enteric diseases should include a combination vaccine against *Salmonella* Typhi and *Salmonella* Paratyphi A [12]. Currently, no licensed bivalent vaccine or paratyphoid-only vaccines are available. In recent times, various techniques including bacterial ghost cells have been used to develop a vaccine against the cellular surface antigens of bacteria [24]. As an immunogen, both innate and adoptive immune responses can be elicited by the BGs preserved surface structures and components [25]. Vaccine formulations often include surface associated conserved motifs (i.e. pathogen associated molecular patterns (PAMP)) such as the lipopolysaccharide of gram-negative bacteria, which serve as ligands for host pattern recognition receptor to induce host immune responses. Previous work reported that surface antigens may induce strong humoral and cell-mediated immune responses against intracellular bacteria like *Salmonella* [26]. The use of bacterial ghosts (BGs) as a platform is reasonable, cost-effective, easy to produce, and at the same time intensified with expanded applications [25].

Lysis gene E-based recombination has been used in gram-negative bacteria to produce ghost cells with regular transmembrane tunnels [27]. Expense and intricacy are significant limitations for using genetic strategies to prepare bacterial ghosts. To avoid this, chemical agents are now widely used to prepare gram-positive [28], gram-negative [14,15], yeast-based fungal ghosts [29], and even viral ghosts [30]. In this study, we prepared bivalent bacterial ghost cells of *Salmonella* Typhi and *Salmonella* Paratyphi A by treatment with sodium hydroxide and characterized them. Scanning electron microscopic images show hollow structures on the surface of our treated bacterial cells. That confirms the formation of bacterial ghost cells. We observed no bacterial growth in these treated *Salmonella* suggesting that they are non-living lysed cells. An equal ratio of sodium hydroxide treated *Salmonella* Typhi ghost cells and *Salmonella* Paratyphi A ghost cells were mixed to formulate our bivalent typhoidal bacterial ghost cell mixture. After immunization of mice with BTBGs in three doses with two-week intervals, we observed an anti-LPS, anti-OMP, anti-Vi-PS and anti-WCL antibody response. Throughout BTBGs immunization, we compared the blood parameters of immunized mice with non-immunized mice to screen for any abnormalities. There are no significant changes in the blood profile between BTBG immunized and non-immunized animals. This suggests that our

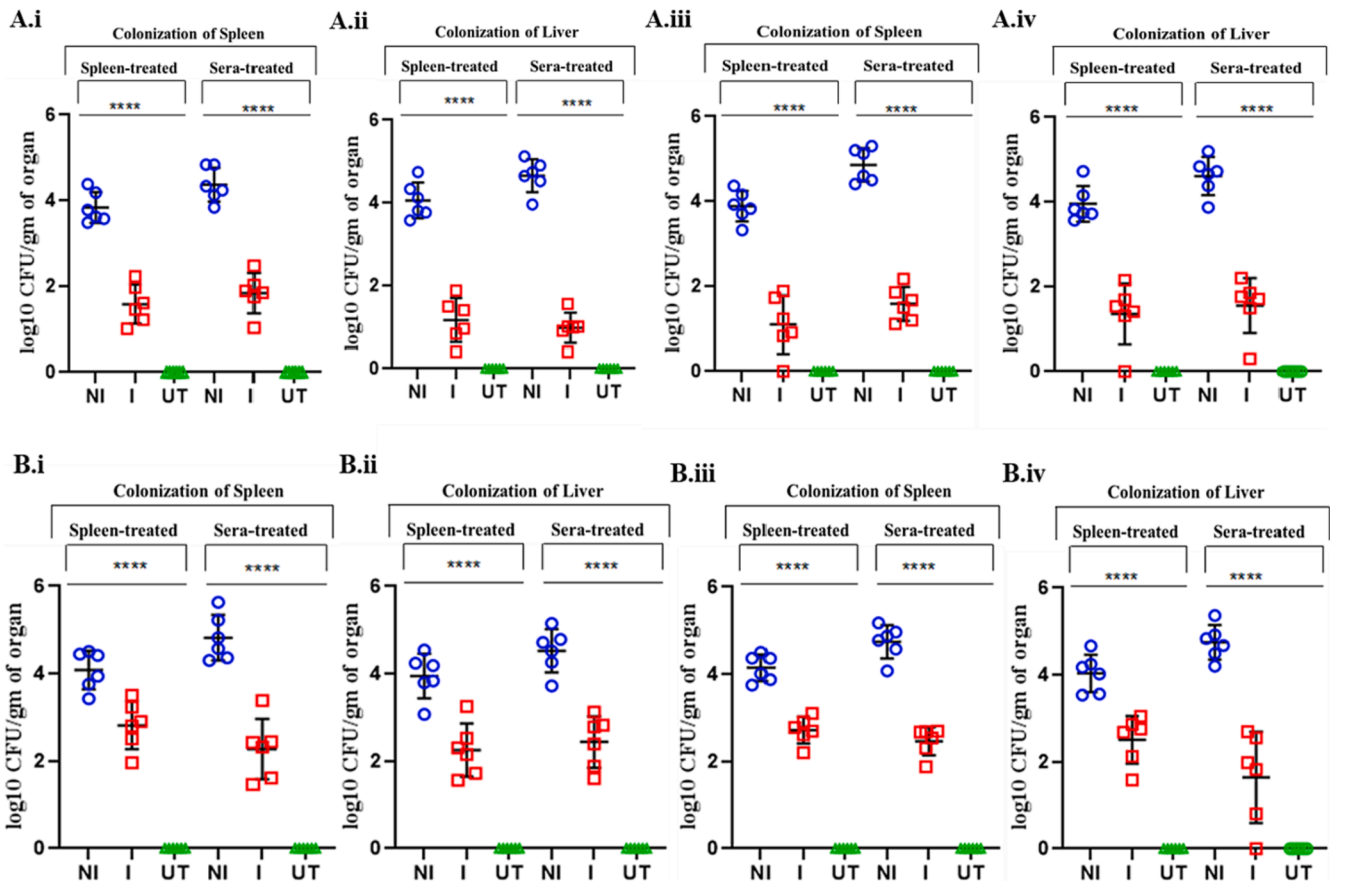

Fig. 6. Adoptive transfer (of immunized serum and splenocytes) reduces bacterial number in naïve mice after infection. **A.i, A.ii,** Bacterial number (72 h post infection) of *S.* Typhi in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 0^{th} day. **A.iii, A.iv**. Bacterial number (72 h post infection) of *S.* Paratyphi A in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 0^{th} day. **B.i, B.ii,** Bacterial number (72 h post infection) of *S.* Typhi in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 7^{th} day **B.iii, B.iv.** Bacterial number (72 h post infection) of *S.* Paratyphi A in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 7^{th} day. Data were expressed as median value of six mice ± S.E of three separate experiments. Statistical analyses were performed using non-parametric one-way analysis of variance (ANOVA) test (Kruskal-Wallis test) (****P value < 0.0001, **p value < 0.001, *p value < 0.01). NI: non-immunized, I: immunized, UT: untreated.

BTBGs are not reactogenic or toxic in an in-vivo model [16].

Flow cytometry analysis of immunized mouse splenic cells showed significant increases in CD4+, CD8a+, and CD19+ cell populations. This suggests that B-cells and T-cells are activated by our immunogen [13,17]. Long-term protective immunity relies heavily on both humoral and cell-mediated immune responses to reduce infection [31,32,33]. In the vaccine against *Salmonella* Typhi, the lipopolysaccharides (LPS) and Vi-polysaccharides (Vi-PS) very important to produce protective immunity [34,35], but both are working in a T cell-independent manner [36]. That is the reason why the Vi-PS based Typhoid vaccine produces a very short-term immune response [37]. However, when Vi-PS is conjugated with a protein (tetanus toxoid), it works in a T-cell dependent manner [38,39]. In our study, we have found that adequate amount of Vi-polysaccharide specific anti-Vi-polysaccharide serum IgG was generated after bacterial ghost cell immunization. The anti-Vi-polysaccharide IgG antibody has a significant role in the protection against *Salmonella* Typhi in this study. Protection might be achieved due to typhoid bacterial ghost cells immunization could elicit anti-Vi antibody in addition to the cell-mediated responses and other immunologic responses after bivalent typhoidal bacterial ghost cells immunization [40]. Different surface proteins present in the outer membrane of *Salmonella* can act as an adjuvant and produce T-cell dependent immunity in animals [41]. Our ELISA, immunoblot, and dotblot studies revealed that immunized mice produce both anti-LPS and anti-OMPs specific antibodies, which may be due to BTBGs inducing the T-cell facilitated immune response in immunized animals. Previous studies reported that purified porins containing OmpC and OmpF of *S.* Typhi act as strong immunogen, responsible for conferring specific bactericidal antibody responses [23,42,43]. In our study also, range of immunogenic proteins were observed in immunoblot with distinct immunogenic bands in the region of 25 KDa–55 KDa, indicating the possibility of presence of porin specific antibodies in immunized serum. BTBGs also induce an anti-LPS and anti-OMP secretory IgA response in immunized animals. A mucosal antibody response is also necessary for protection against enteric pathogens like *Salmonella* [17]. A steady antibody titre up to the 120^{th} day post immunization suggests a long-term immune response is induced by BTBGs. Ex-vivo restimulation of immunized mouse splenic cells produces a Th1 and Th17 based cytokine response. In this study, a substantial amount of IFN-γ, TNF-α, IL-6 and IL-17 were produced, which helps to activate the innate and cell-mediated responses [44,45]. As dendritic cells (DCs) are essential for bacterial-specific T-cell priming in the event of *Salmonella* infection, we examined the response of bone marrow derived mature DCs to BTBGs. This led to the induction of pro-inflammatory cytokines IL-1β, IL-6, IL-12p40/70 and IL-23. This is necessary for differentiation between the Th1 and Th17 response in T cell receptor (TCR) stimulation [46]. The BMDCs-splenic CD4 T-cell co-culture experiment suggested antigen presentation and activation of different paths for immune coordination. In this case, induction of IFN-γ and IL-12 cytokines may activate cytotoxic T-cell and Th1-responses. IL-6 has a standing for the gaps concerning innate as well as adoptive immune response, and helps T cells to secrete IFN-γ and IL-2 [47,48]. IFN-γ, TNF-α also activates macrophages for antibacterial activity. To

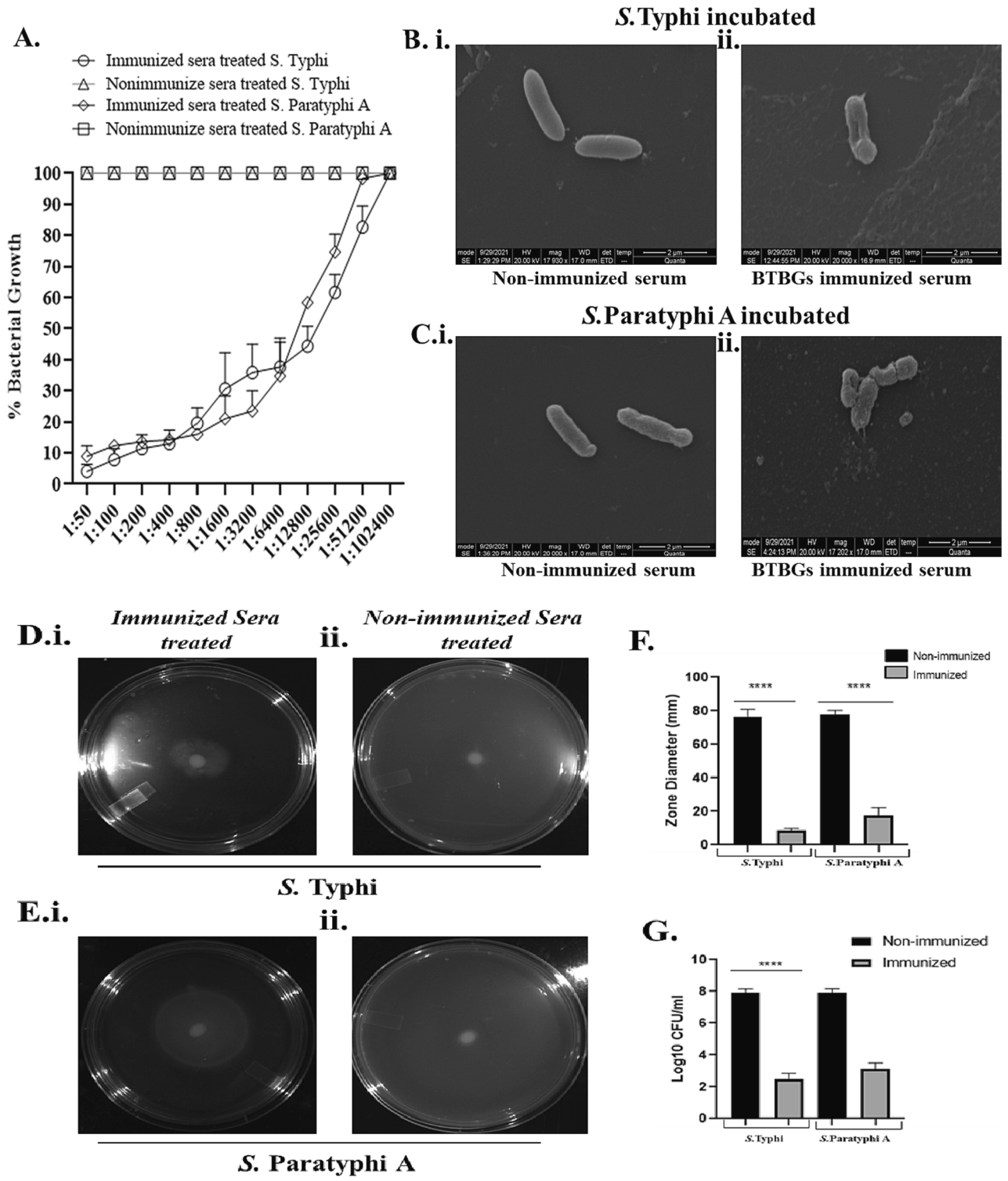

Fig. 7. Serum bactericidal activity, bacterial motility and mucin penetration assay in presence of immunized and non-immunized serum. **A.** Immunized serum is capable of complement mediated lysis of *Salmonella* Typhi (K554) and *Salmonella* Paratyphi A (K580). X-axis represents the serum dilution and Y-axis represent the percentage of bacterial growth occur after overnight incubation. **B–C**. Scanning electron microscopic (SEM) image of non-immunized and immunized sera treated bacteria; **B.i.** Nonimmunized sera treated *S.* Typhi **B.ii**. Immunized sera-treated *S.* Typhi **C.i**. Nonimmunized sera treated *S.* Paratyphi A **C.ii.** Immunized sera treated *S.* Paratyphi A. Scale bar represent 2 μm length. **D-F.** Bacterial motility test on soft agar (0.3 % agar) plates; **D.i. E.i**. The motility of *S*. Typhi and *S*. Paratyphi A was significantly reduced in case of immunized serum spreaded plate. **D.ii. E.ii.** The motility of *S*. Typhi and *S*. Paratyphi A in case of nonimmunized serum spreaded plate. **F.** Bar diagram of zone diameter of bacterial motility in immunized and nonimmunized serum spreaded plate after overnight growth. **G**. Mucin penetration assay; Bacteria treated with non-immunized or immunized serum were loaded on top of the 1 ml mucin column and allowed to penetrate. Immunized serum agglutinated bacteria showed reduced ability to penetrate mucin. Statistical analyses were performed using the two tailed Student's *t* test (****$p < 0.0001$).

prevent intracellular infection of *Salmonella* and its spread (to infect adjacent cells), cell-mediated immune responses, particularly Th1, are required [49,50]. Previous research showed that IFN-γ$^{-/-}$ mice are more prone to *Salmonella* infections than wild-type mice. It also showed that IFN-γ is necessary to activate macrophages to clear the invasive bacteria [51,52]. Th17 responses are enhancing the infiltrating leukocytes to the infection site to kill the bacteria [53] and also maintain the mucosal barrier thereby limiting the bacterial spread from the intestines [54]. All of these results indicate that BTBGs can significantly elicit both the cellular and humoral arms of the immune response that can protect against *Salmonella* infection. In our protective efficacy study, all of the animals were infected with a lethal dose of heterologous clinical isolates of *Salmonella* Typhi and Paratyphi A. Due to its reproducibility, we used the intra-peritoneum challenge model instead of the iron overload model for our vaccine efficacy study of *Salmonella* Typhi [55] and Paratyphi A [13]. BTBG immunized mice showed 100 % and 80 % protective efficacy or survivability against *Salmonella* Typhi and Paratyphi A respectively. The bacterial loads in the liver and spleen after 48 h and 72 h of infection was significantly lower in immunized mice than in non-immunized mice. The same results were obtained (i.e. lower bacterial colonization in immunized animals than nonimmunized animals after 48 h of infection) post 180th day of primary immunization. The histopathological study of spleen and liver tissue of both immunized and nonimmunized mice showed distinct differences in bacterial infection, dissemination, and inflammation. The cell mediated immune response plays a crucial role in reducing inflammation, granulomas, portal inflammation, lobular inflammation, acidophil bodies, and infiltrating leukocytes in liver and spleen tissues of immunized mice. We observed marked inflammation, extensive granulomas, significant portal inflammation, high lobular inflammation, the presence of acidophil bodies, and a large count of infiltrating leukocytes in nonimmunized mice [56]. Adoptive transfer of serum and splenocytes from immunized mice significantly lowers bacterial count in tissues of naïve mice, which also establishes the protective efficacy of our vaccine candidate. This adds to the evidence that humoral and cell-mediated immune responses are necessary to prevent typhoidal *Salmonella* infection [57,58]. In our study, immunized mice sera exhibited characteristic bactericidal properties as was evident from serum bactericidal assay. Functional capabilities of antibodies to elicit immunogenicity and potential protective capacities are measured by serum bactericidal assay [56]. SEM images from the bactericidal assay showed that our immunized serum can bind to *Salmonella* and rupture the cell, thereby aiding in complement-mediated killing. The BTBG immunized serum also inhibits bacterial motility and lowers the penetration ability of mucin layer, which reduces bacterial adherence and invasion through the epithelial cell membrane [59]. Thus, antibodies in BTBGs immunized mice serum can effectively opsonize and kill the bacteria. All of this strongly suggests that our bivalent typhoidal bacterial ghost cells (BTBGs) have the potential to be used as a vaccine candidate against *Salmonella* Typhi and *Salmonella* Paratyphi A.

5. Conclusion

A sodium hydroxide induced BTBG formulation can stimulate humoral and cellular immunity in mice. This antigen-specific long-term immune response provides protection against heterologous strains of *Salmonella* Typhi and Paratyphi A. Therefore, BTBGs can be used as vaccine candidate to prevent the typhoid and paratyphoid fever-mediated heath burden.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Authors contributions

PH: Conceptualization, design, methodology, investigation, analysis and writing; SM: methodology, analysis, review and editing. SB: methodology, review and editing; SD: methodology, review and editing. MD: methodology; SD: funding, review and editing; HK: conceptualization, design, funding, supervision and writing. All authors read and approved the final manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2023.08.049.

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Development of a novel trivalent invasive non-typhoidal *Salmonella* outer membrane vesicles based vaccine against salmonellosis and fowl typhoid in chickens

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ABSTRACT

Poultry animals act as natural reservoirs of invasive non-typhoidal *Salmonella* [iNTS] serovars and consumption of iNTS contaminated poultry meat and eggs is one of the major sources of iNTS infection in developed and developing countries. Irrational use of antibiotics in the poultry industry gives rise to the global emergence of multi drug resistant iNTS strains. Among different strategies to control iNTS infection in poultry farms, vaccination is now being widely used. There are several licensed vaccines available in the market for poultry animals to ameliorate iNTS infection but none of them have broad spectrum protective efficacy. In this study we have formulated a single novel trivalent iNTS outer membrane vesicles [OMVs] based immunogen which can confer long term broad spectrum protection against most prevalent iNTS serovars. We have isolated OMVs from *Salmonella* Typhimurium [ST], *Salmonella* Enteritidis [SE], and *Salmonella* Gallinarum [SG] and formulated the trivalent immunogen by mixing OMVs in a 1:1:1 ratio. One day old chicks were immunized thrice via oral route at two week intervals. Vaccination significantly induced serovar specific antibodies detected up to 180 days post immunization. Post challenge with both homologous and heterologous [*S*. Infantis] serovars, immunized birds showed reduced level of fecal shedding and organ invasion. A long term efficacy study also showed reduced levels of tissue invasion up to one year post immunization. These results demonstrate that our novel formulation of immunogen could be a broad spectrum potential vaccine for both layer and broiler breeds against iNTS mediated salmonellosis and fowl typhoid.

1. Introduction:

Invasive non-typhoidal *Salmonella* [iNTS] mediated foodborne diarrheal disease is a major illness globally which emerged as serious health burden over the last few decades (Kirk et al., 2015). The current estimate is 93.8 million infections and 155,000 deaths each year globally (Majowicz et al., 2010). Poultry animals act as major natural reservoirs of iNTS serovers (Dar et al., 2017). Humans generally are infected with iNTS by consumption of iNTS contaminated poultry meat, eggs and egg related food items (Antunes et al., 2016; Barua et al., 2014; Hennessy et al., 2004; Hogue et al., 1997). Invasive NTS contains more than 2500 different serovars but in India four serovars are most commonly isolated from poultry. These are *Salmonella* Gallinarum [SG, 43.7%], *Salmonella* Enteritidis [SE, 30.6%], *Salmonella* Typhimurium [ST, 21.9%] and *Salmonella* Infantis [SI, 2.7%] (Kumar et al., 2019). Most iNTS serovars are zoonotic in nature but SG only infects poultry animals causing fowl typhoid and resulting deaths at a young age and thereby causing a huge economic loss (Arora et al., 2015; Kumar et al., 2010). SE mostly causes egg contamination and a previous study in the USA showed that SE egg positivity rate was 2.75 per 10,000 eggs from naturally SE infected flocks (Okamura et al., 2007). Birds can be infected with SE naturally by the oral route and seldom produces contaminated eggs (Humphrey et al., 1989; Poppe et al., 1993) but several other routes also cause SE infection in birds, such as vaginal (Miyamoto et al., 1999) and conjunctival (Humphrey et al., 1992). Infected meat production is generally a result of ST infection(Okamura et al., 2007). Other than meat contamination, contamination of eggs with ST was also found in several previous epidemiological investigations (Chapman et al., 1988; Mitchell

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Controlling the bacterial load of *Salmonella* Typhi in an experimental mouse model by a lytic *Salmonella* phage STWB21: a phage therapy approach

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Abstract

Background *Salmonella enterica* serotype Typhi is one of the major pathogens causing typhoid fever and a public health burden worldwide. Recently, the increasing number of multidrug-resistant strains of *Salmonella spp*. has made this utmost necessary to consider bacteriophages as a potential alternative to antibiotics for *S.* Typhi infection treatment. *Salmonella* phage STWB21, isolated from environmental water, has earlier been reported to be effective as a safe biocontrol agent by our group. In this study, we evaluated the efficacy of phage STWB21 in reducing the burden of salmonellosis in a mammalian host by inhibiting *Salmonella* Typhi invasion into the liver and spleen tissue.

Results Phage treatment significantly improved the survival percentage of infected mice. This study also demonstrated that oral administration of phage treatment could be beneficial in both preventive and therapeutic treatment of salmonellosis caused by *S.* Typhi. Altogether the result showed that the phage treatment could control tissue inflammation in mice before and after *Salmonella* infection.

Conclusions To the best of our knowledge, this is the first report of phage therapy in a mouse model against a clinically isolated *Salmonella* Typhi strain that includes direct visualization of histopathology and ultrathin section microscopy images from the liver and spleen sections.

Keywords *Salmonella* Typhi, Lytic phage, In vivo, Animal model, Phage therapy

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Pentavalent outer membrane vesicles immunized mice sera confers passive protection against five prevalent pathotypes of diarrhoeagenic *Escherichia coli* in neonatal mice

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ABSTRACT

Diarrhoeagenic *Escherichia coli* (DEC) pathotypes are one of the major causative agents of diarrhoea induced childhood morbidity and mortality in developing countries. Licensed vaccines providing broad spectrum protection against DEC mediated infections are not available. Outer membrane vesicles (OMVs) are microvesicles released by gram-negative bacteria during the growth phase and contain multiple immunogenic proteins. Based on prevalence of infections, we have formulated a pentavalent outer-membrane vesicles (POMVs) based immunogen targeting five main pathotypes of DEC responsible for diarrhoeal diseases. Following isolation, OMVs from five DEC pathotypes were mixed in equal proportions to formulate POMVs and 10 μg of the immunogen was intraperitoneally administered to adult BALB/c mice. Three doses of POMVs induced significant humoral immune response against whole cell lysates (WCLs), outer membrane proteins (OMPs) and lipopolysaccharides (LPS) isolated from DEC pathotypes along with significant induction of cellular immune response in adult mice. Passive transfer of POMVs immunized adult mice sera protected neonatal mice significantly against DEC infections. Overall, this study finds POMVs to be immunogenic in conferring broad-spectrum passive protection to neonatal mice against five main DEC pathotypes. Altogether, these findings suggest that POMVs can be used as a potent vaccine candidate to ameliorate the DEC-mediated health burden.

1. Introduction

Diarrhoeal diseases are the second major cause of mortality in children less than five years of age and one of the main reasons people seek medical care globally [1,2]. *Escherichia coli* (*E. coli*) is a part of the normal microbiota of human intestine and is among the first bacteria to colonize the neonatal gut after birth [3]. Certain subgroups of *E. coli* are known to cause a broad range of diseases in humans and are considered pathogenic. On the basis of type of infections, pathogenic *E. coli* are divided into intestinal or diarrhoeagenic *E. coli* (DEC) and extra-intestinal *E. coli* (ExPEC) [4]. DEC causes more than 300 million infections each year with nearly 200,000 deaths globally [5]. 30–40% of

List of abbreviations: CFU, colony forming unit; *E.coli*, *Escherichia coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; EAEC, enteroaggregative *E. coli*; EIEC, enteroinvasive *E. coli*; EHEC, enterohemorrhagic *E. coli*; OMV, outer-membrane vesicle; IP, intraperitoneal; OMP, outer-membrane protein; LPS, lipopolysaccharide; WCL, whole cell lysate; TSB, tryptic soy broth; TSA, tryptic soy agar; PBS, phosphate buffered saline; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; sIgA, secretory IgA; TNF-α, tumor necrosis factor- alpha; IL, interleukin; IFN-γ, interferon gamma.

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Establishment of an intragastric surgical model using C57BL/6 mice to study the vaccine efficacy of OMV-based immunogens against *Helicobacter pylori*

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Abstract

Chronic gastritis is one of the major symptoms of gastro-duodenal disorders typically induced by *Helicobacter pylori* (*H. pylori).* To date, no suitable model is available to study pathophysiology and therapeutic measures accurately. Here, we have presented a successful surgical infection model of *H. pylori*-induced gastritis in C57BL/6 mice that resembles features similar to human infection. The proposed model does not require any preparatory treatment other than surgical intervention. C57BL/6 mice were injected with wild-type SS1 (Sydney strain 1, reference strain) directly into the stomach. Seven days post infection, infected animals showed alterations in cytokine responses along with inflammatory cell infiltration in the lamina propria, depicting a prominent inflammatory response due to infection. To understand the immunogenicity and protective efficacy, the mice were immunized with outer membrane vesicles (OMVs) isolated from an indigenous strain with putative virulence factors of *H. pylori* [A61C (1), *cag+/vacA s1m1*]. In contrast to the non-immunized cohort, the OMV-immunized cohort showed a gradual increase in serum immunoglobulin(s) levels on the 35th day after the first immunization. This conferred protective immunity against subsequent challenge with the reference strain (SS1). Direct inoculation of *H. pylori* into the stomach influenced infection in a short time and, more

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Original article

A tetravalent *Shigella* outer membrane vesicles based candidate vaccine offered cross-protection against all the serogroups of *Shigella* in adult mice

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Cross-protection

ABSTRACT

In today's world and mostly in low and middle income countries, *Shigella flexneri* and *Shigella sonnei* remains the major causative agent of clinical bacillary dysentery. Based on contemporary epidemiology, 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 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. This study establishes the possibilities of tetravalent OMVs immunogen to become a potent vaccine candidate against human shigellosis, overcoming the limitations of sero-specific cross-protection of *Shigella* species.

Inflammatory bacillary dysentery or bloody diarrhoea is instigated by the Gram negative entero-invasive bacteria *Shigella* sp. leading to about 164,000 deaths per annum [1]. Children under the age of five and immunosuppressed people are at high risk of infection. The situation worsens in Low- and Medium-Income Countries (LMICs) due to poor sanitary measures and unhygienic practices [2]. Additionally, shigellosis has now become prominent in developed countries as well as in travellers traveling to endemic regions [3].

After transmission by the fecal oral route, *Shigella* enters through the microfold cells of the colonic epithelial layer and is readily engulfed by the macrophages present in Gut Associated Lymphoid Tissue (GALT). *Shigella* induced macrophage pyroptosis, causing the release of inflammatory cytokines, which recruit polymorphonuclear neutrophils (PMN) in the infection zone. This ultimately leads to damage of the epithelial lining and the dispersal of the bacteria [4]. Though the primary symptom triggered by *Shigella* is stool with blood and mucus, they are also accountable for Moderate to Severe Diarrhoea (MSD) and other clinical complications such as fever, prolonged malnutrition, malaise, tenesmus etc.

Conventional antibiotic therapy is now troubled due to the global emergence of drug resistant *Shigella* strains [5]. To counter this issue, a prophylactic vaccine approach has become an ideal strategy. 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 nano particles primarily secreted from Gram-negative bacteria. OMVs are being widely

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Trivalent outer membrane vesicles-based combination vaccine candidate induces protective immunity against Campylobacter and invasive non-typhoidal Salmonella in adult mice

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Article

Stable Recombinant Invasion Plasmid Antigen C (IpaC)-Based Single Dose Nanovaccine for Shigellosis

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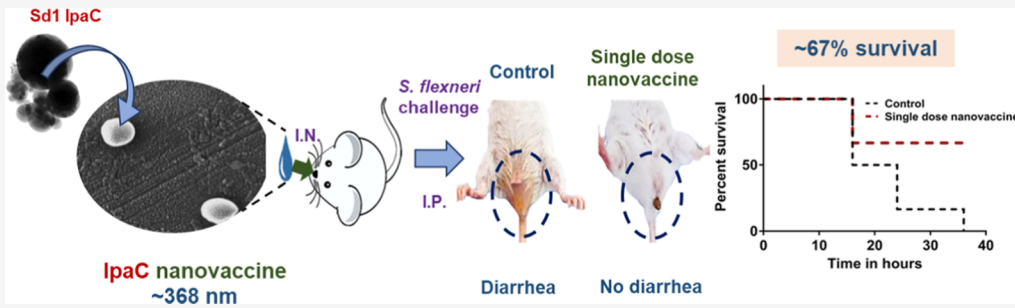

ABSTRACT: Shigellosis, caused by the bacteria *Shigella*, is the leading cause of bacterial diarrhea and the second leading cause of diarrheal death among children under the age of five. Unfortunately, *Shigella* strains have acquired resistance to antibiotics, and a commercial vaccine is yet to be available. We have previously demonstrated that *Shigella dysenteriae* serotype 1 (Sd1)-based recombinant, stabilized, "invasion plasmid antigen C" (IpaC; 42 kDa) protein can induce robust immune responses in BALB/c mice against a challenge of a high dose of heterologous *Shigella* when immunized via three intranasal doses of IpaC without an adjuvant. In this work, in order to reduce the frequency of dosing and increase possible patient compliance, based on our previous screening, the minimum protective dose of stabilized IpaC (20 μg) was encapsulated in biodegradable polymeric poly(lactide-*co*-glycolide) nanoparticles (~370 nm) and intranasally administered in BALB/c mice in a single dose. Interestingly, a single intranasal dose of the developed vaccine particles encapsulating only 20 μg of Sd1 IpaC led to a temporal increase in the antibody production with an improved cytokine response compared to free IpaC administered three times as described in our previous report. Upon intraperitoneal challenge with a high dose of heterologous *Shigella flexneri* 2a (common in circulation), the immunized animals were protected from diarrhea, lethargy, and weight loss with ~67% survival, while all the control animals died by 36 h of the challenge. Overall, the developed nanovaccine could be explored as a potential noninvasive, cross-protective, single-dose, single-antigen *Shigella* vaccine amenable for scale-up and eventual mass immunization.

KEYWORDS: *nanovaccine, Shigella vaccine, IpaC, single-antigen vaccine, minimum protective dose, single-dose vaccine*

INTRODUCTION

Shigella infection is the leading bacterial cause of diarrheal mortality causing 212,438 deaths in 2016.[1] It causes more harm in low- and middle-income countries (LMICs) resulting in a higher number of deaths in infants and the elderly.[2,3] Unfortunately, most strains of *Shigella* have been reported to develop resistance against commonly used antibiotics.[4−11] Because a licensed vaccine is still not available, the World Health Organization declared the formulation of an effective *Shigella* vaccine candidate a public health priority.[12,13]

Although there has been promising development in the field of *Shigella* vaccine production, most vaccine formulations provide low or serotype-specific protection and can be a major reason for the nonavailability of a commercial protective vaccine against *Shigella*.[12−14] Conserved antigens can effectively contribute to the formulation of cross-protective vaccines; however, a major obstacle is the low immunogenicity of such antigens.[14,15] Immunogenicity of conserved, subunit proteins can be enhanced either using adjuvants or encapsulating the antigens in nano-/microparticles.[16,17] Further, dosage and in turn immunogenicity, has also been influenced by the route of immunization with the intranasal route showing heightened immunogenicity at a lesser dose.[17,18] Nanovaccines or nanoparticle-based vaccines (1−1000 nm) have garnered more attention due to their unique size advantage.[19] Additionally, nanoparticles (NPs) less than 500 nm in size are more efficiently taken up by the antigen-presenting cells compared to NPs of higher size.[20] Nano-

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Bivalent non-typhoidal *Salmonella* outer membrane vesicles immunized mice sera confer passive protection against gastroenteritis in a suckling mice model

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ABSTRACT

Invasive non-typhoidal *Salmonella* (iNTS) serovars, especially *Salmonella* Typhimurium (ST) and *Salmonella* Enteritidis (SE), cause gastroenteritis worldwide. Due to the emergence of multi-drug resistance in iNTS, a broad-spectrum vaccine is urgently needed for the prevention of iNTS infection. Currently, there is no effective licensed vaccine against iNTS available in the market. We have formulated an outer membrane vesicles (OMVs) based bivalent immunogen as a vaccine candidate to generate broad-spectrum protective immunity against both recently circulating prevalent ST and SE. We have isolated OMVs from ST and SE and formulated the immunogen by mixing both OMVs (1:1 ratio). Three doses of bivalent immunogen significantly induced humoral immune responses against lipopolysaccharides (LPSs) and outer membrane proteins (OMPs) as well as a cell-mediated immune response in adult mice. We also observed that proteins of OMVs act as an adjuvant for generation of high levels of anti-LPS antibodies through T cell activation. We then characterized the one-day old suckling mice model for both ST and SE mediated gastroenteritis and used the model for a passive protection study. In the passive protection study, we found the passive transfer of bivalent OMVs immunized sera significantly reduced ST and SE mediated colonization and gastroenteritis symptoms in the colon of suckling mice compared to non-immunized sera recipients. The overall study demonstrated that OMVs based bivalent vaccine could generate broad-spectrum immunity against prevalent iNTS mediated gastroenteritis. This study also established the suckling mice model as a suitable animal model for vaccine study against iNTS mediated gastroenteritis.

1. Introduction

Foodborne mediated diarrheal disease is one of the major illnesses that emerge throughout the world causing millions of infections [1], and invasive non-typhoidal *Salmonella* (iNTS) is one of the major causative agents. Invasive NTS infection burden is currently estimated to be 93.8 million individuals and 155,000 death/year globally [2]. Due to the global emergence of multi-drug resistant iNTS serovars [3,4], classical antibacterial treatment therapy has become complicated for the treatment of iNTS infected patients [5]. *Salmonella* Typhimurium (ST) and *Salmonella* Enteritidis (SE) are the two most clinically predominant serovars among the iNTS family in current times. Scientists from different parts of the world are trying to develop a broad spectrum vaccine against iNTS infection to control such a global health problem and economic burden [6]. Still there is no single licensed vaccine available globally for public health use against iNTS mediated gastroenteritis.

Outer membrane vesicles (OMVs) of Gram-negative bacteria are now considered as a potential subunit acellular vaccine candidate. The structure of outer membrane vesicles is the same as the outer surface of the bacteria. Thus, OMVs considered as a good carrier of various antigens that facilitate a strong immunomodulatory role contain both lipopolysaccharides (LPSs) and different outer and inner membrane proteins. Recently, an OMV based vaccine has been licensed to use in the European market against *N. meningitides* and it can be used for individuals above 2 months of age [7,8]. Previous studies indicated that OMVs of iNTS induce a pro-

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RESEARCH

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Facile synthesis of multi-faceted, biomimetic and cross-protective nanoparticle-based vaccines for drug-resistant *Shigella*: a flexible platform technology

Namrata Baruah[1,2], Nadim Ahamad[1], Prolay Halder[3], Hemanta Koley[3] and Dhirendra S. Katti[1,2*]

Abstract

Background No commercial vaccines are available against drug-resistant *Shigella* due to serotype-specific/narrow-range of protection. Nanoparticle-based biomimetic vaccines involving stable, conserved, immunogenic proteins fabricated using facile chemistries can help formulate a translatable cross-protective *Shigella* vaccine. Such systems can also negate cold-chain transportation/storage thus overcoming challenges prevalent in various settings.

Methods We explored facile development of biomimetic poly (lactide-*co*-glycolide)/PLGA 50:50 based nanovaccines (NVs), encapsulating conserved stabilized antigen(s)/immunostimulant of *S. dysenteriae* 1 origin surface-modified using simple chemistries. All encapsulants (IpaC/IpaB/LPS) and nanoparticles (NPs)—bare and modified (NV), were thoroughly characterized. Effect of IpaC on cellular uptake of NPs was assessed in-vitro. Immunogenicity of the NVs was assessed in-vivo in BALB/c mice by intranasal immunization. Cross-protective efficacy was assessed by intraperitoneally challenging the immunized groups with a high dose of heterologous *S. flexneri* 2a and observing for visible diarrhea, weight loss and survival. Passive-protective ability of the simplest NV was assessed in the 5-day old progeny of vaccinated mice.

Results All the antigens and immunostimulant to be encapsulated were successfully purified and found to be stable both before and after encapsulation into NPs. The ~ 300 nm sized NPs with a zeta potential of ~ − 25 mV released ~ 60% antigen by 14th day suggesting an appropriate delivery kinetics. The NPs could be successfully surface-modified with IpaC and/or CpG DNA. *In vitro* experiments revealed that the presence of IpaC can significantly increase cellular uptake of NPs. All NVs were found to be cytocompatible and highly immunogenic. Antibodies in sera of NV-immunized mice could recognize heterologous *Shigella*. Immunized sera also showed high antibody and cytokine response. The immunized groups were protected from diarrhea and weight loss with ~ 70–80% survival upon heterologous *Shigella* challenge. The simplest NV showed ~ 88% survival in neonates.

Conclusions Facile formulation of biomimetic NVs can result in significant cross-protection. Further, passive protection in neonates suggest that parental immunization could protect infants, the most vulnerable group in context of *Shigella* infection. Non-invasive route of vaccination can also lead to greater patient compliance making it amenable for mass-immunization. Overall, our work contributes towards a yet to be reported platform technology for facile development of cross-protective *Shigella* vaccines.

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RESEARCH ARTICLE

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

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Debaki R. Howlader and Ushasi Bhaumik share joint first authorship. Author order was determined in order of decreasing seniority.

ABSTRACT Shigellosis has been a menace to society for ages. The absence of an effective vaccine against *Shigella*, improper sanitation, and unhygienic use of food and water allow the disease to flourish. *Shigella* can also be transmitted via natural water bodies. In the absence of a good animal model, the actual nature of pathogenesis and transmission remains unclear. Zebrafish larvae have previously been described as a model for *Shigella* pathogenesis. However, larval fish lack a mature intestinal microbiota and immune system. Here, the adult zebrafish was assessed as a potential model for *Shigella* pathogenesis. Their well-developed innate and adaptive immune responses mimic the mammalian immune system. *Shigella* showed a clear dose-, time-, and temperature-dependent colonization of the adult zebrafish gut. Efficacy of a three-dose immunization regime was tested using bath immunization with heat-killed trivalent *Shigella* immunogen. The present study demonstrates the efficacy of an adult zebrafish model for pathogenesis, transmission, and vaccine efficacy studies.

IMPORTANCE Shigellosis is a diarrheal disease that is prevalent in developing countries and especially dangerous in young children. Currently, animal models for shigellosis are unable to model some aspects of the infectious cycle. Here, we describe a new shigellosis model in adult zebrafish, an increasingly common model organism for studying bacterial pathogens. The zebrafish model can be used to study *Shigella* colonization, transmission, and immune responses, as well as test vaccine efficacy.

KEYWORDS animal models, *Shigella*, shigellosis, zebrafish

Shigellosis causes a significant economic and public health burden globally, resulting in ~164,000 mortalities annually (1). Symptoms of shigellosis range from mild watery diarrhea to inflammatory distress, including abdominal cramps along with blood- and/or mucus-containing stool. Immune-compromised individuals are at much higher risk due to the highly infective nature of the pathogen, with an infectious dose as low as 10 bacteria (2). *Shigella* transmits primarily through water; hence, countries with underdeveloped sanitary systems are at heightened risk for infection. Mice are generally resistant to oral delivery of *Shigella*. However, the disease can be modeled in mice via surgical and/or immunological manipulations (3–9). In a guinea pig luminal model, the appropriate symptoms occur, but this model is difficult to execute and needs highly trained laboratory personnel (9). Other models require prior antibiotic administration and opium or ketamine treatment, making them unsuitable for studies of natural disease biology (10). None of these models address the transmissibility of the pathogen via water.

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Suppression of classical nuclear import pathway by importazole and ivermectin inhibits rotavirus replication

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Background: Rotavirus is the foremost cause of acute gastroenteritis among infants in resource-poor countries, causing severe morbidity and mortality. The currently available rotavirus vaccines are effective in reducing severity of the disease but not the infection rates, thus antivirals as an adjunct therapy are needed to reduce the morbidity in children. Viruses rely on host cellular machinery for nearly every step of the replication cycle. Therefore, targeting host factors that are indispensable for virus replication could be a promising strategy.

Objectives: To assess the therapeutic potential of ivermectin and importazole against rotaviruses.

Methods: Antirotaviral activity of importazole and ivermectin was measured against various rotavirus strains (RV-SA11, RV-Wa, RV-A5-13, RV-EW) *in vitro* and *in vivo* by quantifying viral protein expression by western blot, analysing viroplasm formation by confocal microscopy, and measuring virus yield by plaque assay.

Results: Importin-β1 and Ran were found to be induced during rotavirus infection. Knocking down importin-β1 severely impaired rotavirus replication, suggesting a critical role for importin-β1 in the rotavirus life cycle. *In vitro* studies revealed that treatment of ivermectin and importazole resulted in reduced synthesis of viral proteins, diminished production of infectious virus particles, and decrease in viroplasm-positive cells. Mechanistic study proved that both drugs perform antirotavirus activity by inhibiting the function of importin-β1. *In vivo* investigations in mice also confirmed the antirotavirus potential of importazole and ivermectin at non-toxic doses. Treatments of rotavirus-infected mice with either drug resulted in diminished shedding of viral particles in the stool sample, reduced expression of viral protein in the small intestine and restoration of damaged intestinal villi comapared to untreated infected mice.

Conclusions: The study highlights the potential of importazole and ivermectin as antirotavirus therapeutics.

Introduction

Rotavirus is the leading cause of diarrheal mortality among children less than 5 years of age, accounting for approximately 128 500 deaths worldwide in 2016, with the majority of these deaths occurring in low- and middle-income countries of sub-Saharan Africa, Southeast Asia and South Asia.[1,2] Introduction of rotavirus vaccines in the routine immunization programme has significantly reduced the rotavirus-induced mortality in the past decade worldwide,[3–9] but the magnitude of the vaccine's efficacy has been found to be inversely influenced by the socioeconomic status of a country. The efficacy of the rotavirus vaccine is significantly lower and wanes more rapidly in high-mortality settings of low-income countries (44% effectiveness) compared with low-mortality settings of high-income countries (94% effectiveness).[10–12] The low and short-lived efficacy of oral rotavirus vaccines in resource-poor settings is associated with reduced vaccine immunogenicity.[12] Moreover, in low-income settings, the reassortment of cognate genes among co-circulating strains, genetic drift of neutralizing antigen, and zoonotic transmission of animal strains drive changes in the predominant strain, both spatially and temporarily.[13–15] This natural variability in circulating rotavirus strains may pose challenges to the prophylactic vaccination as a sole strategy of rotaviral diarrhoea management in the long run.

Rotavirus, also known as triple-layered particle (TLP), is a non-enveloped icosahedral virus of the Reoviridae family with a genome of 11 dsRNA segments surrounded by three concentric layers of proteins. During entering into the host enterocytes, rotavirus loses its outermost shell to form transcriptionally active double-layered particles (DLPs) in the cytosol. The viral polymerase complex (VP1/VP3), associated with each of the dsRNA

TITLE: Potential use of Sodium Butyrate (SB) as an anti-virulence agent against *Vibrio cholerae* targeting ToxT virulence protein.

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ARTICLE OPEN

Intranasal immunization of mice with chimera of *Salmonella* Typhi protein elicits protective intestinal immunity

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Development of safe, highly effective and affordable enteric fever vaccines is a global health priority. Live, oral typhoid vaccines induce strong mucosal immunity and long-term protection, but safety remains a concern. In contrast, efficacy wears off rapidly for injectable, polysaccharide-based vaccines, which elicit poor mucosal response. We previously reported *Salmonella* Typhi outer membrane protein, T2544 as a potential candidate for bivalent (*S.* Typhi and *S.* Paratyphi A) vaccine development. Here, we show that intranasal immunization with a subunit vaccine (chimera of T2544 and cholera toxin B subunit) induced strong systemic and intestinal mucosal immunity and protection from *S.* Typhi challenge in a mouse model. CTB-T2544 augmented gut-homing receptor expression on lymphocytes that produced Th1 and Th17 cytokines, secretory IgA in stool that inhibited bacterial motility and epithelial attachment, antibody recall response and affinity maturation with increased number of follicular helper T cells and CD4+ central and effector memory cells.

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INTRODUCTION

Salmonella enterica serovar Typhi (*S.* Typhi), a Gram-negative, intracellular pathogen and the etiological agent of typhoid fever, is a global threat to public health. The organism spreads through the faeco-oral route, causing significant morbidities and mortalities in the developing countries, especially of the south-east Asian region. As per Global Burden of Disease data, 9.24 million new typhoid fever cases occurred worldwide in 2019 (females 4.17 million and males 5.07 million), leading to 110,000 deaths and 8.05 million (3.86–13.9) disability adjusted life years (DALYs). Incidences in males and females were 7.3 (4.5–11.1) and 8.6 (5.4–12.9), respectively per 100,000 populations[1]. After crossing the intestinal barrier, *Salmonella* Typhi, being carried by the infected macrophages, spreads to the distant body sites like the liver, spleen, bone marrow and lymph nodes. As an obligate intracellular pathogen, the bacterium creates a niche for replication within the phagosomes of the infected macrophages, rendering it difficult for the host to eliminate it from the body[2]. The problem of *S.* Typhi infection is further complicated by the emergence and spread of multi-drug resistant and extensively drug-resistant (XDR) strains across continents[3]. While the mortality in untreated typhoid fever reaches nearly 20%, an incompletely cured acute infection may be followed by an asymptomatic chronic carrier state, which poses an increased risk for the development of adenocarcinoma of the gall bladder[4].

Improvement of sanitation, potable water supply and food hygiene is considered essential for elimination of *S.* Typhi. However, vaccination may play a significant role in decreasing the incidence in the endemic countries in the short run. Two vaccines, namely a live, attenuated *S.* Typhi Ty21a strain for oral administration and an injectable preparation, containing purified Vi polysaccharide (Vi-PS) are widely available commercially. Despite high seroconversion rates with good short term protection, they are at best modestly efficacious in the long run[5]. Oral typhoid vaccine induces systemic and intestinal secretory antibodies as well as good T cell response, but requires at least three to four doses for optimum protection. Further, it is not recommended for children below 6 years of age due to difficulty in swallowing large capsules of the vaccine formulation. Live typhoid vaccine has also raised safety concerns because of occasional reports of bacteremia[6]. To escalate immunogenicity with a single dose of vaccine administration, several research groups have developed alternative attenuated strains in the *S.* Typhi Ty2 background. Several mutant vaccine strains, such as Ty800 with the deletion of the global regulator phoP/phoQ, M01ZH09/ZH09 lacking aroC and ssaV genes and double mutant (aroC and aroD) strain CVD908 have passed Phase II trials and showed good mucosal and serum antibody response in the volunteers. However, CVD 908 resulted in vaccinemia with a single oral dose of 10^7 viable organisms, and was later on modified by the deletion of another stress protein, htrA that prevented vaccinemia, but retained both humoral and cellular immune responses. To ensure more consistent serum anti-Vi antibodies, Vi was constitutively expressed in CVD 908 strain, generating CVD909. A prime boost regimen with orally administered CVD 909, followed by an injection of Vi-polysaccharide vaccine failed to induce strong Vi-specific antibody response, but instead, significantly raised Vi-specific IgA^+ B memory cells. However, the need for pre-administration of buffer to neutralize stomach acid renders a potential delivery challenge for live attenuated vaccines[5]. None of the vaccine strains was shown to be effective against Paratyphoid infection.

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Quercetin, a flavonoid, combats rotavirus infection by deactivating rotavirus-induced pro-survival NF-κB pathway

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Rotavirus (RV) is the leading cause of acute gastroenteritis and watery diarrhea in children under 5 years accounting for high morbidity and mortality in countries with poor socioeconomic status. Although vaccination against RV has been implemented in more than 100 countries, the efficacy of vaccine has been challenged in low-income settings. The lack of any FDA-approved drug against RV is an additional concern regarding the treatment associated with rotavirus-induced infantile death. With the purpose for the discovery of anti-RV therapeutics, we assessed anti-rotaviral potential of quercetin, a well-characterized antioxidant flavonoid. *In vitro* study revealed that quercetin treatment resulted in diminished production of RV-SA11 (simian strain) viral particles in a concentration-dependent manner as estimated by the plaque assay. Consistent with this result, Western blot analysis also revealed reduced synthesis of viral protein in quercetin-treated RV-SA11-infected MA104 cells compared to vehicle (DMSO) treated controls. Not surprisingly, infection of other RV strains A5-13 (bovine strain) and Wa (Human strain) was also found to be abridged in the presence of quercetin compared to DMSO. The IC_{50} of quercetin against three RV strains ranges between 2.79 and 4.36 Mm, and S.I. index is greater than 45. Concurrent to the *in vitro* results, *in vivo* study in mice model also demonstrated reduced expression of viral proteins and viral titer in the small intestine of quercetin-treated infected mice compared to vehicle-treated infected mice. Furthermore, the result suggested anti-rotaviral activity of quercetin to be interferon-independent. Mechanistic study revealed that the antiviral action of quercetin is co-related with the inhibition of RV-induced early activation of NF-κB pathway. Overall, this study delineates the strong anti-RV potential of quercetin and also proposes it as future therapeutics against rotaviral diarrhea.

KEYWORDS

rotavirus, quercetin, NF-κB, antiviral therapeutics, phytochemical

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