Preparation of immunogen from prevalent strains of diarrheagenic *Escherichia coli* and evaluation of its protective efficacy in animal model to reduce diarrheagenic *Escherichia coli* mediated clinical health burden

A thesis submitted for the degree of
Doctor of Philosophy (Science),
of

Jadavpur University

2024

By

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"Preparation of immunogen from prevalent strains of diarrheagenic *Escherichia coli* and evaluation of its protective efficacy in animal model to reduce diarrheagenic *Escherichia coli* mediated clinical health burden"** submitted by Sri Soumalya Banerjee who got his name registered on **29th November, 2021 (Index No. 124/21/LifeSc./27, Registration Number: SLSBT1112421)** 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.

Hemanta Koley.

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I dedicate the thesis to my parents, my better half, my paternal and maternal grandparents, my family, and God

Acknowledgement

This thesis would not have been possible without the guidance and support of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study. It gives me great pleasure to express my gratitude to everyone who made it possible.

I would like to express my deep gratitude to my doctoral advisor, Dr. Hemanta Koley, Scientist-F, Division of Bacteriology, ICMR- National Institute of Cholera and Enteric Diseases (ICMR-NICED), for his patient guidance, enthusiastic encouragement, useful critiques and supporting my attendance at various conferences during my tenure. His wide knowledge and his way of thinking have been of great value for me. I could not have imagined having a better advisor and mentor for my doctoral study.

I am thankful to Dr. Shanta Dutta, The Director, ICMR- National Institute of Cholera and Enteric Diseases (ICMR-NICED), for providing me the facilities to carry out the research work.

I am grateful to Professor Jeffrey H. Withey, Department of Biochemistry, Microbiology, and Immunology, Wayne State University School of Medicine, Detroit, Michigan, USA, for helping me with useful scientific critiques and ideas during my tenure.

I am thankful to Prof. Parimal Karmakar, Head of the Department, Department of Life Science and Biotechnology, Jadavpur University, for permitting me to register as Ph.D. student.

I am thankful to PATH for providing me with full travel grant and support for attending the third Vaccines Against *Shigella* and ETEC (VASE) Conference in Washington D.C., USA, from November 29 to December 1, 2022.

I am extremely thankful to my present and past lab members Mr. Prolay Halder, Mr. Sanjib Das, Mr. Pritam Nandy, Ms. Srijani Biswas, Dr. Suhrid Maiti, Dr. Ushasi Bhaumik, Mr. Subrata Sinha, and Mr. Suhasit Ranjan Ghosh for their immense help, for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we had during my tenure. I am also thankful to other lab members and staffs of ICMR-NICED for helping me out in academic and administrative work.

I would like to thank the University Grants Commission (UGC), New Delhi, India, for providing me fellowship under CSIR-UGC-NET scheme [Student ID:191620007740].

I am eternally thankful and forever indebted to my parents, my better half, my paternal and maternal grandparents, my teachers and especially my music teacher Mrs. Malabika Dutt, and my family members for their unfailing support, continuous encouragement, and belief in me throughout my life and years of study. This accomplishment would not have been possible without their unwavering support.

My heartfelt thanks to God for your wisdom and blessings that have shaped every word and thought within this work and in all the work that I have accomplished. May your divine light continue to illuminate my path as I endeavor to acknowledge you in all aspects of my life. With deepest gratitude and reverence.

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Declaration

This Ph.D. thesis was prepared at the Division of Bacteriology, ICMR- National Institute of Cholera and Enteric Diseases, in fulfilment of the requirements for acquiring the Ph.D. degree. The research work embodied in the thesis entitled **"Preparation of immunogen from prevalent strains of diarrheagenic *Escherichia coli* and evaluation of its protective efficacy in animal model to reduce diarrheagenic *Escherichia coli* mediated clinical health burden"** was carried out at **ICMR- National Institute of Cholera and Enteric Diseases, Kolkata, India under the supervision of Dr. Hemanta Koley, Scientist- F, Division of Bacteriology**.

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

The work presented in this thesis was carried out between June 2020 to June 2024.

The Ph.D. was funded by the University Grants Commission (UGC), New Delhi, India.

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Contents

Abstract

Title of thesis:

"Preparation of immunogen from prevalent strains of diarrheagenic *Escherichia coli* and evaluation of its protective efficacy in animal model to reduce diarrheagenic *Escherichia coli* mediated clinical health burden"

Submitted by: Soumalya Banerjee

Index No.: 124/21/LifeSc./27

In developing countries, diarrhea-induced morbidity and death in children can be attributed primarily to diarrhoeagenic *Escherichia coli* (DEC) pathotypes. There are currently no licensed vaccines available that offers broad-spectrum protection against infections caused by DEC. Several immunogenic proteins are found in outer membrane vesicles (OMVs), which are microvesicles secreted by gram-negative bacteria during the growth phase. We have developed a pentavalent outer-membrane vesicles (POMVs) based immunogen that targets the five primary pathotypes of DEC that cause diarrheal diseases, taking into account the prevalence of infections. Adult BALB/c mice were administered with 10μg of the POMVs intraperitoneally after OMVs were isolated from five DEC pathotypes and combined in equal amounts to develop POMVs immunogen. In adult mice, three doses of POMVs significantly increased the cellular immune response in addition to inducing a strong humoral immune response against whole cell lysates (WCLs), outer membrane proteins (OMPs), and lipopolysaccharides (LPS) isolated from DEC pathotypes. Neonatal mice were effectively protected against DEC infections by the passive transfer of POMVs immunized sera of adult mice. Overall, this study reveals that POMVs are immunogenic, providing neonatal mice with broad-spectrum passive protection against five major DEC pathotypes. Taken together, our results indicate that POMVs could serve as a potent vaccine candidate to reduce the health burden caused by DEC.

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7/6/24

Background

Diarrhoeal diseases severely affect the public health and are a major cause of morbidity and mortality especially among infants and young children [1]. *Escherichia coli* (*E.coli*) constitutes the normal microbiota of the human intestine but certain subtypes of *E.coli* are known to cause a broad range of diseases in humans. Based on the type of infections, *E.coli* are divided into intestinal or diarrhoeagenic *E.coli* (DEC) and extraintestinal pathogenic *E.coli* (ExPEC) [2-4]. DEC is a major cause of diarrhoeal episodes in developing countries and predominantly affects the children globally [5]. DEC are divided into five main pathotypes, namely, enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC) [6]. ETEC is associated with watery diarrhoea in infants and travellers, EPEC is associated acute and chronic watery diarrhoea in the paediatric population, EIEC infection results in dysentery characterized by bloody mucoid diarrhoea, identical to that caused by *Shigella* sp., EHEC causes haemorrhagic colitis and haemorrhagic uremic syndrome (HUS) and EAEC infection contributes to acute and persistent watery diarrhoea in developing countries and in immunocompromised patients [1]. Multi-drug resistance is an increasing concern among DEC pathotypes because of inappropriate usage of antibiotics, due to which, prophylactic measures like vaccines, can effectively work to reduce DEC mediated infections [7,8]. As of now, single licensed combination vaccine against DEC is not available for public health use.

Due to ease of absorption by mammalian cells and strong immunogenic character, outer membrane vesicles (OMVs), which are nanosized proteoliposomes produced from the outer membrane of gram-negative bacteria, can be used as a potent candidate for an acellular vaccine. Natural antigens found in outer membrane membranes (OMVs) include phospholipids, peptidoglycan, lipopolysaccharides (LPSs), outer membrane proteins (OMPs), and membrane lipoprotein components that resemble the outer surface of bacteria. The European Medicine Agency (EMA) has authorized an OMV-based vaccine for active human immunization against

Neisseria meningitides. Mice immunized intraperitoneally (i.p.) with *E. coli* OMVs were shown to be protected against recurrent infections in earlier investigations. Although OMVs have been demonstrated to elicit favorable mucosal immune responses, no combination OMV-based vaccination has been developed so far against the pathotypes of DEC [9- 14]. Because OMVs contain LPS, flagellin, peptidoglycans, lipoproteins, and other outer membrane proteins (OMPs) that activate different Toll-like receptors (TLRs), they have intrinsic adjuvant qualities [15] and do not require the addition of external adjuvants to increase their immunogenicity.

According to earlier research, newborn mice are more vulnerable than adult mice to infections caused by *E. coli* [16, 17]. A model of neonatal mice is used in passive protection experiments to assess a vaccine candidate's potency and efficacy [18- 21]. It is challenging to protect neonatal mice from infection through vaccination because of their underdeveloped immune systems and short susceptibility period, which leaves them with insufficient time to mount an immunological response [22, 23]. Neonatal mice were shielded from group B streptococcal infection by the passive transfer of vaccinated adult mouse sera, according to a prior investigation [24]. Our group recently demonstrated that suckling mice were passively protected against non-typhoidal *Salmonella*-mediated gastroenteritis by the transfer of OMVs vaccinated adult mouse sera [25].

An ideal vaccine candidate should demonstrate less reactogenicity, significant immunogenicity and broad-spectrum protective efficacy. Combination-based vaccines are considered as an advantageous approach for prevention of diarrhoeal associated infections. Therefore, I have formulated a combined pentavalent outer membrane vesicles-based immunogen (POMVs) from five pathotypes of DEC (EPEC, ETEC, EAEC, EIEC and EHEC) and determined the adaptive immune response induced by the immunogen. Effect of POMVs on induction of mucosal and systemic immune responses in adult mice was assessed in this study. Passive protective efficacy conferred by POMVs immunized adult mouse sera against five prevalent

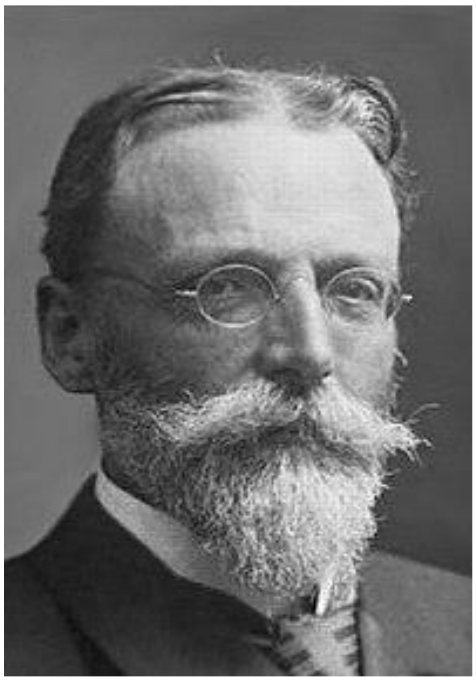
DEC pathotypes in neonatal mice was also assessed in this study. The study aims to assess the POMVs ability to act as a potent vaccine candidate for reduction of infections by five prevalent pathotypes of DEC.

Chapter 1

Review of literature

1. An overview of diarrhoeagenic Escherichia coli

In 1885, the pioneering German-Austrian pediatrician, Theodor Escherich, isolated a motile gram-negative bacterium from the stool of infants and named it *Bacterium coli commune*. A few decades later, the bacterium was officially named *Escherichia coli* after its discoverer [1].



Theodor Escherich

This microorganism has shaped bacterial genetics and the rise in modern microbiology is synonymous with the 130-year-old history of the bacteria. *E. coli* exists as a natural harmless component of the intestinal microbiota of animals including human beings but some of its subtypes can cause diseases. At present, *E.coli* is the most intensively studied and best-understood microorganism in the world [2].

The subtypes of *E. coli* that are pathogenic in nature, can cause a wide range of diseases in humans due to evolutionary pressure, because of which it developed pathogenic features, enabling its adaptation and survival in a variety of environments. These varied range of environments extend from the gastrointestinal tract where infections can occur as a result of food poisoning or contamination, to extraintestinal sites like the urinary tract, or meninges. Based on the bacterial genetics, the pathologies, and the phenotypes that specific isolates engender in their host, *E. coli* is divided into diarrheagenic *E. coli* (DEC) that mainly causes diarrheal illness and extraintestinal pathogenic *E. coli* (ExPEC) that are associated with infections in other systemic sites. At least eleven different *E. coli* pathotypes have been defined for the two categories (intestinal and extraintestinal pathogenic *E. coli*). Members of pathogenic *E. coli* are by far more prevalent than any other members of the family [3, 1].

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

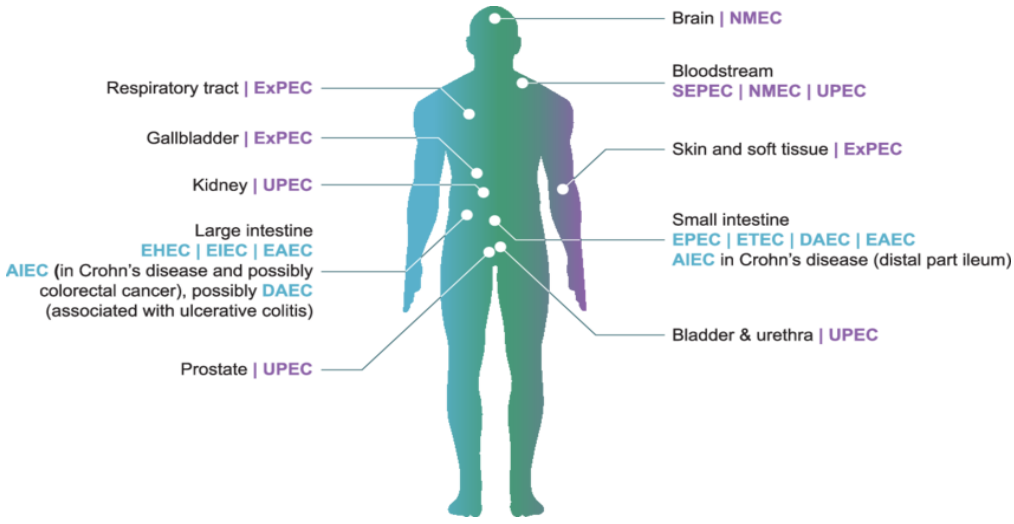
Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *Escherichia coli*

Taxonomy of *Escherichia coli*

DEC is divided into five main pathotypes. Enteropathogenic *E. coli* (EPEC) is associated acute and chronic watery diarrhoea in the paediatric population, enteroaggregative *E. coli* (EAEC) contributes to acute and persistent watery diarrhoea in developing countries and in immunocompromised patients, enterotoxigenic *E. coli* (ETEC) is associated with watery diarrhoea in infants and travellers, enterohemorrhagic *E. coli* (EHEC) causes haemorrhagic colitis and haemorrhagic uremic syndrome (HUS), and enteroinvasive *E. coli* (EIEC) results in dysentery characterized by bloody mucoid diarrhoea, identical to that caused by *Shigella* sp. [4, 5].



Jeroen Geurtsen et al FEMS Microbiology Reviews, DOI: 10.1093/femsre/fuac031

Figure 1: Site of infection of different pathogenic *E.coli* in the human host

2. Global epidemiology of DEC infection

DEC causes more than 300 million infections each year with nearly 200,000 deaths globally [6]. 30–40% of diarrhoeal episodes are caused by DEC in developing countries and in 2011, DEC caused 120,000 deaths in children worldwide [7, 8]. Infections associated with DEC are widespread globally, but infections of some pathotypes are associated with specific geographical zones. In regions of Mexico, Colombia, and Nicaragua, the majority of DEC cases are associated with ETEC infections, while in regions of Brazil, Paraguay and Peru, the majority of cases are due to EAEC infections. EPEC infections are prevalent in regions of Venezuela, Chile, Argentina and Uruguay, and infections due to EHEC are also prevalent in South American countries [9].

In Africa, 80% of diarrheal infections in humans are caused by DEC which results in 70,000 deaths each year, mostly affecting children under 5 years of age. 12-18 months children are more prone to DEC-mediated infections due to exploration and hand-to-mouth behaviour. In Kenya, 34–60% of children were reported to have DEC infections. In Egypt, DEC infections in children aged under 5 years were reported to be 20.6% in the community. Uganda was reported to have 38.2% DEC infections in children suffering from acute diarrhoea [10].

Previous studies reported that 34% of all diarrhoeal episodes were attributable to infections mediated by DEC [11]. Another study conducted in a cohort of children reported EAEC infection to be 68.8%, EPEC to be 55.9%, ETEC to be 44%, EIEC to be 19.4% and Shiga-toxin producing *E. coli* (STEC) to be 3.2% [12].

The prevalence of DEC infections reported by a study conducted in Kolkata, India was found to be 12%. In patients infected with DEC, EAEC infections were found to be most prevalent, followed by ETEC and EPEC. In children of two years of age and above, EAEC and EPEC infections were found to be significantly high compared to other DEC pathotypes [13]. Another

study conducted in Kolkata, India, from 2012 to 2019, reported that 54% of the diarrhoeal cases were due to DEC-mediated infections with ETEC being predominantly detected from the samples followed by EAEC and EPEC [14].

3. Morphological and biochemical characteristics of DEC pathotypes

With a diameter of roughly 0.6 µm and a length of 2 µm, DEC have a structural rod-like structure. DEC typically forms green sheen colonies on EMB agar that do not spread. Bacterial movement is facilitated by peritrichous flagella. *E. coli* are facultative anaerobes that convert nitrates to nitrites and generate lactate, succinate, ethanol, acetate, and carbon dioxide. When it comes to tests for oxidase, citrate, urease, and hydrogen sulphide, the majority of *E. Coli* strains test negative, yet positive for catalase. Indol production and the methyl red tests are both positive for *E. coli*. *E. coli* can be distinguished from *Shigella* and *Salmonella* based on its capacity to ferment lactose, as over 90% of *E. coli* do so. Because 98% of *E. coli* test positive for indol, we can differentiate it from other Enterobacteriaceae [15].

4. Interaction of DEC with host gut microbiome

Environmental factors and metabolites generated by the intestinal tract's gut microbiota have a significant influence on DEC pathogenicity. Typically, intestinal inflammation, diarrhoea, virulence factor synthesis and secretion, adhesion and colonization of the intestinal surface are associated with DEC infection. There is published data that supports the virulence of DEC caused by certain strains of the normal gut microbiota. The gut microbes and enteric pathogens, including DEC, may interact through a number of compounds that the microbial community in the intestinal tract produces [16]. Pathogenic strains of *E. coli* can also overcome the resistance exhibited by commensal strains by means of differential nutrition use. In contrast to commensal

E. coli, pathogenic EHEC may use sugars such as galactose, mannose, ribose, and hexuronates as sources of carbon. Moreover, commensal *E. coli* do not have the *eut* operon, that prevents them from using ethanolamine, whereas EHEC has this operon and can assimilate nutrients like ethanolamine. In ingested food, complex proteins, carbohydrates, and fats are broken down into simpler forms via host-directed pathways in eukaryote hosts, which facilitates absorption of nutrients. Members of the microbiota also aid in the production of short-chain fatty acids that the host uses throughout the catabolic process and promote the expansion of the bacterial community. In order for an external species to engraft itself in an available niche, it must outcompete fierce competition for scarce substrates because the microbiota's members have evolved to optimize the amount of nutrients available. Because pathogenic strains like *E. coli* O157 require sugars like fucose, mannose, galactose, ribose, arabinose, and N-acetyl glucosamine, resident *E. coli* employ these sugars in the mucous layer to establish colonization resistance to invasive infections. Additionally, commensal *E. coli* outcompete foreign strains by using the preferential use of resources. For instance, resistance of commensal *E. coli* to EHEC results from competing for resources including organic acids and amino acids. According to new research, dysbiosis—which is characterized as a change in the intestinal microbiota's makeup and, consequently, a modification of the host-gut microbiota relationship—may have a role in the emergence of a number of illnesses, including irritable bowel syndrome, obesity, autoimmune diseases, allergies, and inflammatory bowel disease [17, 18].

5. Interaction between DEC and intestinal epithelial cells

Majority of pathogenic strains of *E. coli* stay extracellular, except for EIEC which can invade and replicate within macrophages and epithelial cells. While some *E. Coli* strains may be

partially internalized by epithelial cells, they do not seem to multiply within cells. Pathotypes of *E. coli* interact with epithelial cells, and these interactions are contingent upon the particular mix of virulence factors that characterize their way of interaction and their capacity to induce a particular pathology. With the exception of ETEC, which generates the primary virulence factors, heat-labile (LT) and heat-stable (ST) enterotoxins, the other five pathotypes manipulate and regulate actin filament polymerization through altering cellular regulators of this process. The cytoskeleton is altered by EIEC to enable invasion and/or movement within the host cell. EIEC travel inside the infected host cell via manipulating actin-filament dynamics subsequent to invasion and escape from membrane-bound vesicles into the cytoplasm. They accomplish this by attracting actin to one of their poles via actin nucleation driven by bacterial proteins. Actin-associated cytoskeletal elements may also be hijacked when extracellular pathogens like EPEC and EHEC are infected. EAEC secretes virulence factors that lead to the proteolysis of proteins responsible for stabilizing actin filament networks [19, 20, 21, 22].

6. Virulence factors of DEC pathotypes

6.1. EPEC virulence factors

A three-dimensional microcolonies pattern known as the localized adherence (LA) pattern is capable of being formed by EPEC, at least in vitro, on cell lines and organ cultures. In addition to mediating the LA phenotype, bundle-forming pilus (BFP) is also implicated in autoaggregation, biofilm formation, and antigenicity [23]. Using type-three secretion system (T3SS), EPEC can cause lesions by delivering effector proteins directly into the host cells. The LEE pathogenicity island encodes these effectors. The outer membrane adhesin (intimin, required for adherence on the host cells, encoded by the eae gene), translocators (EspA/B/D), effector proteins (EspF/G/H, Map, EspZ), chaperones (Ces proteins), the translocated intimin

receptor (Tir), and regulatory proteins like LEE-encoded regulator (Ler), a global regulator of LEE proteins (GrlA—activator, GrlR—repressor), and chaperones (Ces proteins) are all encoded in LEE [23, 24-28].

Six pathogenicity islands are made up of different non-LEE (Nle) encode effector genes (cif, espI/nleA, nleB, nleC, nleD, nleE, nleH), which are not part of the LEE area. By obstructing or altering the host's inflammatory response, as well as by upsetting the host cells' tight junctions and cytoskeleton, the Nle proteins can make bacteria more virulent [23, 24, 29, 30]. The disintegration of the tight junctions is aided by additional non-LEE encoded effector proteins in addition to Nle A, EspF, and Map [19].

Aside from BFP, certain EPEC strains may also exhibit other characteristics such as flagella, astA gene (encoding EAST1—the enteroaggregative *E. coli* heat-stable enterotoxin 1), fimbriae or pili (type 1 fimbriae, *E. coli* common pilus), autotransporter proteins (EspC), or a hybrid adherence phenotype in HeLa cells (LA and aggregative-like pattern). However, further research is required to determine how these additional features relate to pathogenicity [23, 31].

6.2. ETEC virulence factors

Following human consumption, ETEC initiate an infection strategy that includes colonizing the gut mucosal surface and producing toxins. ETEC colonizes the part of upper jejunum to ileum [32, 33]. The intestinal epithelium is adhered to by ETEC via a number of colonization factors (CFs).

More than 25 CFs, including fimbrial and fimbrillar structures, have been found in ETEC. These CFs vary in both antigen and structural makeup. Seven of them are typically more common than the others: CS1 to CS6 (coli surface antigen) and CFA/I (colonization factor antigen) [34]. The majority of CF receptors are yet unknown, but it has been proposed that CFs bind to glycoprotein conjugates on host cell surfaces and in small intestinal mucus fraction.

The pathogens have also been linked to non-fimbrial adhesins, such as the glycosylated autotransporter TibA, the outer membrane protein Tia, and the molecular bridge EtpA that connects the receptors of host cells to the tips of ETEC flagella [35].

ETEC strains can release one or two toxins (LT and/or ST), yet research indicates that the likelihood of an illness from LT toxin is lower than that of ST or LT/ST ETEC toxins [36]. The LT toxins produced from the eltAB gene share 80% homology with the cholera toxin, making them structurally and functionally comparable. With a pentameric ring of LT_B subunits in charge of binding and internalization and a catalytically active LT_A subunit, LT exhibits an AB_5 structure. Cystic fibrosis transmembrane regulator (CFTR) phosphorylation is triggered by a rise in cAMP brought on by LT, which results in significant fluid loss and watery diarrhea. The small, cysteine-rich peptides known as ST toxins, which are expressed by the estAB gene, resemble the human hormone guanylin. They are separated into two categories based on structure and antigen identity: STa and STb. These groups reversibly bind to sulphatide and guanylyl cyclase C (GC-C), respectively, causing diarrhea and CFTR activation [37].

6.3. EAEC virulence factors

The aggregative adhesion plasmid known as pAA, a family of virulence plasmids, and pathogenicity islands dispersed across the chromosome contain the EAEC virulence components [38, 39]. The primary virulence factor regulator of EAEC is AggR. It is present on the pAA plasmid and regulates the expression of genes encoded by chromosomal and plasmid-borne virulence factors. It belongs to the AraC family of bacterial transcriptional regulators [38, 40]. The two types of EAEC strains are atypical, which lacks the AggR regulon, and typical, which carries AggR and AggR-regulated virulence components. AAF genes encode the aggregative adhesion fimbria (AAF), that is linked to the aggregative adherence of EAEC [38, 41]. There are five known varieties of AAFs, namely AAF/I–V encoded by aggA, aafA, agg3A,

agg4A, and agg5A. They are all situated on pAA. Only EAEC pathotypes have been shown to have AAF genes [38, 42]. Other, less important variables linked to EAEC adherence include different fimbrial structures, such as type-IV pili [38, 43]. Another pathogenic mode of EAEC infection is the production of biofilms, which is completely different from the process caused by non-pathogenic *E. coli*. Biofilm development in EAEC is mediated by a type-VI secretion system (T6SS), which is encoded by aaiA-Y genes, positioned on a pathogenicity island found on the chromosome, and triggered by AggR. But globally, typical EAEC strains have been discovered to carry the aaiA and aaiC genes more frequently [38, 44]. The pet, pic, sigA, sepA, sat, and astA genes encode different potential toxins from EAEC, such as a plasmid-encoded toxin, a protein implicated in intestinal colonization, a secreted autotransporter toxin, a *Shigella* extracellular enterotoxin, and an enteroaggregative heat-stable toxin [38, 42]. The other toxin genes are situated on pAA, whereas the sigA and pic genes are found on pathogenicity islands [38, 45]. *Sat* is a SPATE toxin that damages the tight junction and promotes autophagy in epithelial cells. It also has cytotoxic and enterotoxic properties. There are physical and mechanistic similarities between the enterotoxin STa released by ETEC and the enteroaggregative heat-stable toxin [38, 46].

6.4. EIEC virulence factors

Genetic factors encoded by genes on a plasmid called pINV mediate the colonization, adhesion, and invasion of intestinal epithelial cells by EIEC. A virulence plasmid called pINV is present in EIEC and encodes the type III secretion system required for attachment, host cell invasion, and intercellular dissemination. Similar in both structure and functionality to *Shigella* strains, this plasmid has the conjugation (*tra*) and replication (*rep*) regions found in IncFIIA plasmids. Though it cannot self-transfer by conjugation due to significant deletions in the *tra* region, other conjugative plasmids can mobilize pINV. *Shigella* pINV plasmids only contain faulty copies of the IS family, despite the plasmid's several functioning insertion sequences including

the IS1111 family [47]. VirB and MxiE, two global transcriptional regulators that activate and control the expression of most virulence genes, and gene clusters encoding a T3SS apparatus (Mxi and Spa), as well as its effector proteins (IpaB, IpaC, and IpaD) and their chaperons (IpgA, IpgC, IpgE, and Spa15), comprise the PAI-structure carried by pINV. Every T3SS effector is present on pINV, except for a few chromosomal effector proteins belonging to the IpaH family [47, 48]. Genes encoding OspG and OspF proteins, which aid in evading the host innate immune response, as well as IcsA, a protein that promotes bacteria to move inside the cytoplasm, VirA, a GTPase-activating protein, and RnaG, a small RNA that negatively regulates the expression of the icsA gene, are also carried on pINV. This plasmid is said to be present in all EIEC isolates since it is necessary for the pathogenic process and pathoadaptation of this pathotype. Nonetheless, some EIEC strains have been observed to have lost this genetic component [47, 49].

A novel 63-kDa enterotoxin (ShET2) is mediated via the plasmid-borne gene *sen*, which is also present in EIEC strains [50]. The enterotoxic ability of the EIEC strain was shown to be significantly reduced due to a mutation in this gene. Toxins have a known role in inducing watery diarrhea during an *E. coli* infection, even if their role in the pathophysiology of EIEC is not entirely understood. Also, EIEC strains have plasmids expressing enterotoxigenic and cytotoxigenic factors, such as *pic*, *sepA*, *sigA*, and *sat*, which are members of the SPATEs family and have been demonstrated to contribute to intestinal accumulation of fluid in an animal model. However, not all EIEC strains possess these genes. According to two distinct investigations on the virulence gene prevalence among EIEC, the *sat* gene was only detected in 15% of the isolates, while *sen*, *sigA*, and *pic* were detected in at least 70%, 64%, and 27% of the isolates, respectively [51, 52].

6.5. EHEC virulence factors

EHEC colonization is characterized by the bacterium's close adhesion to the cell surface and loss of microvilli, as well as the attachment and effacing of (A/E) lesions on the enterocytes. On a pathogenicity island called the locus for enterocyte effacement (LEE), which codes for a type III secretion system in bacteria, are the genes encoding A/E lesion development (T3SS). Although other potential adherence factors, like long polar fimbriae, or curli, have been discovered, colonization is primarily mediated by the principal adhesin, particularly intimin (encoded by the eae gene). Many other non-fimbrial EHEC adhesins have also been linked to adhesion; these include the chromosomal-encoded Iha, Cah, adhesins and OmpA, as well as the plasmid-encoded toxB [53, 54].

Shiga toxins (Stx) are created by EHEC in the intestinal lumen. They eventually pass through the epithelial barrier to reach their target organs by mechanisms that are not well understood. The bacteria create two toxin families, Stx1 and Stx2, the latter of which is linked to the most serious consequences. These toxin families are encoded in the genomes of lysogenic lambdoid phages. The two main structural subunits of Stx are A and B. The B component binds to the globotriaosylceramide-3 (Gb3) toxin cellular receptor, which is expressed on the surfaces of host microvascular endothelial cells (kidney, gut, and brain). This elucidates the potentially fatal consequences linked to EHEC infections. The A component inhibits protein synthesis and causes cell death by exhibiting an RNA N-glycosidase activity against the 28S rRNA [55-57].

7. Pathogenesis of DEC subtypes

7.1. EPEC pathogenesis

Three phases are involved in EPEC pathogenesis: (i) early host cell attachment; (ii) bacterial toxins are translocated via a type III secretion system; (iii) pedestal development and close association [20, 58, 59]. The small intestine's enterocytes are initially the site of attachment

for EPEC strains, which subsequently aggregate or form localized microcolonies. The main factor promoting this adhesion is a bundle-forming pilus (BFP) that is encoded by a plasmid. BFP mutant strains have been demonstrated to be less likely to induce diarrhea in human volunteers [60, 61]. For EPEC infections, initially adherence and microcolony formation are crucial, and BFP is a major virulence component in pathogenesis [62-64].

A type III secretion system (TTSS), translocators (EspA, EspB, and EspD), effectors (Tir, EspG, EspF, Map, and EspH), chaperones (CesAB, CesD, CesD2, CesF, and CesT), and regulators (Ler, GrlA, and GrlR) are all encoded by the locus of enterocyte effacement (LEE). These proteins are translocated from the bacterial cytoplasm to the external environment by TTSS, which creates a pore [58, 59, 65]. Esp translocators have been demonstrated to be essential for the development of A/E lesions. By creating filamentous appendages around the bacterium and through formation of a translocation tube that interfaces with host cells, EspA promotes the translocation of EspB and EspD into the host cytosol. EspB and EspD mainly serve to transfer virulence proteins to the host cells, not to operate as translocated effectors [66, 67].

Pedestal formation, enterocyte effacement, and close bacterial attachment to host cells occur during the last stage of EPEC infection. According to the pathogenesis model of EPEC, intimin interacts via the translocated intimin receptor (Tir) that is introduced into the membrane to allow EPEC to first attach itself to epithelial cells [68]. Fluid buildup in the intestinal lumen, a rise in intestinal inflammation, and a reduction of absorbent surface area are caused by the subsequent effacement of microvilli [69, 70]. Moreover, an enterotoxin called EspC, a serine protease autotransporter, is produced by a few EPEC strains. While EspC does not play a part in the development of EPEC A/E lesions, it cleaves EspA and EspD, the translocator components of the Type III secretion system (T3SS), to regulate pore formation and cytotoxicity [31]. Interestingly, EspC may aid in EPEC infection by causing necrosis and

apoptosis in epithelial cells and breaking down many biological substrates as pepsin, glycoprotein, coagulation factor V, and spectrin [58, 71].

7.2. EHEC pathogenesis

Verotoxins (VT), commonly referred to as Stx toxins, are the primary virulence factor of EHEC. The members of the Shiga toxin family include Stx1 and Stx2. Stx1 is almost identical to the *Shigella dysenteriae* Shiga toxin and has a difference only at a single amino acid. Stx2 and Stx1 share less than 60% of their amino acid sequence [72, 73, 74]. These potent cytotoxins are secreted by bacteria, which then move from the gut lumen to surrounding tissues and the circulation. When the holotoxin attaches itself to the glycolipid globotriaosylceramide (Gb3) on the surface of the target cell, it is internalized and stops the synthesis of host cell proteins by cleaving ribosomal RNA by a single A subunit. Additionally, Stx has the ability to cause local damage in the colon that can lead to hemorrhagic colitis, necrosis, and intestinal perforation. It can also trigger apoptosis in intestinal epithelial cells [75, 76]. Serine protease autotransporter EspP mediates adhesion to host cells and cytopathic effects while creating macroscopic rope-like polymers that are resistant to antibiotics, aiding in the creation of biofilms [77]. Additionally, EspP has the ability to cleave serpins and host coagulation factor V, which can prolong bleeding and worsen the pathology of EHEC [58, 78-80].

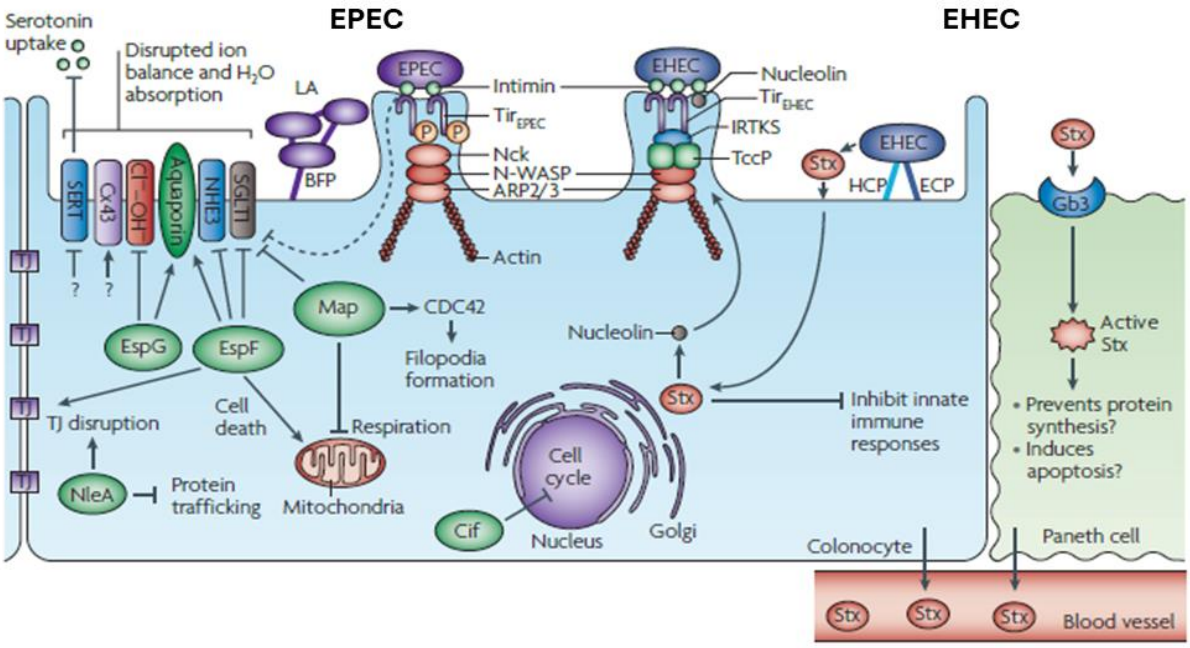


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Figure 2: Pathogenic mechanisms of EPEC and EHEC. Effectors released by the type III secretion system have the ability to block sodium-d-glucose cotransporter 1 (SGLT1), mislocalize aquaporins, and alter the activity of Cl^-–OH– and Na^+–H+ exchangers. Through the bundle-forming pilus (BFP), EPEC forms localized adhesions (LA) that are attached to the small bowel. The translocated intimin receptor (Tir) and intimin cooperate to mediate intimate connection. Host tyrosine kinases phosphorylate Tir, which in turn attracts Nck. Nck then triggers neural Wiskott-Aldrich syndrome protein (N-WASP) and the actin-related protein 2/3 (ARP2/3) complex, thereby mediating actin rearrangements and the creation of pedestals. A wide range of effector proteins are injected into the host cell, disrupting host cell pathways, using the locus of the enterocyte effacement-encoded type III secretion system. The pedestal formation mechanism employed by EPEC and EHEC differs slightly. Tir is not phosphorylated, and Nck is not required for pedestal formation. Tir cytoskeleton-coupling protein, which is connected to Tir by the host protein insulin receptor tyrosine kinase substrate, mediates the actin rearrangements required for pedestal formation. Tir cytoskeleton-coupling protein interacts with N-WASP to activate the ARP2/3 complex. Apart from this close bond, EHEC ties itself to the large intestine via the hemorrhagic coli pilus (HCP) and the E. coli common pilus (ECP). To alter host processes, EHEC introduces several effectors into the host cell, similar to those used by EPEC. Furthermore, in reaction to stress, phage-mediated lysis releases Shiga toxin (Stx),

often referred to as verocytotoxin, which exacerbates the condition. In the human intestinal mucosa, paneth cells have glycosylceramides (Gb3s) that function as Stx receptors.

7.3. ETEC pathogenesis

One or more proteinaceous pili/fimbriae, commonly known as colonization factors (CFs), assist ETEC strains adhere to the intestinal mucosa [81]. After initial adhesion and colonization, heat-labile (LT) and/or heat-stable (ST) enterotoxins encoded by plasmids are produced by ETECs, which cause diarrhea instead of entering the mucosa. The structure and function of LTs and the cholera enterotoxin generated by *Vibrio cholerae* are quite similar [82]. By activating a cAMP-dependent kinase, LTs raise the amount of intracellular cAMP in the host and open the primary chloride channel in epithelial cells, which increases the amount of chloride secreted by crypt cells. This ion imbalance modifies electrolyte homeostasis, which leads to tissue fluid loss and consequent diarrhea [83]. EatA and EtpBAC, two virulence factors encoded in plasmids, are also secreted by ETECs. EatA is an Enterobacteriaceae (SPATE) serine protease autotransporter that cleaves substrates recognized by cathepsin G [84]. EatA is exported by an extracellular adhesin system called EtpBAC, a two-partner secretion system. Another antigen, YghJ (SslE), is encoded by the *yghJ* gene, which is located on the chromosome upstream of genes encoding the type II secretion system of ETEC strains. Both EatA and YghJ break down the MUC2 mucin that is secreted by the small intestinal epithelia's goblet cells, which makes it easier for bacteria to get past the mucin barrier and release ETEC enterotoxins onto host enterocytes [58].

7.4. EAEC pathogenesis

A dense cluster of bacterial cells colonizes the intestinal mucosa during an EAEC infection, causing fluid loss and the release of EAST1 (EAEC heat-stable enterotoxin) and ShET1

(*Shigella* enterotoxin 1) [85-88]. Additionally, EAEC generates Pet, an enterotoxin that is a plasmid-encoded SPATE autotransporter [89]. Enterotoxic activity of Pet results in cytoskeletal alterations and rounding of epithelial cells because it breaks down fodrin/spectrin in host cells [90]. Furthermore, EAEC generates Pic, another mucinase-active SPATE that aids in intestine colonization. By cleaving the components of the complement cascade, C3, C4, and C2, Pic has also been demonstrated to lessen complement activation, which in turn causes PMN activation and programmed death T-cell [91- 93]. Pic activity can thus support EAEC pathogenicity and aid in immune evasion [58].

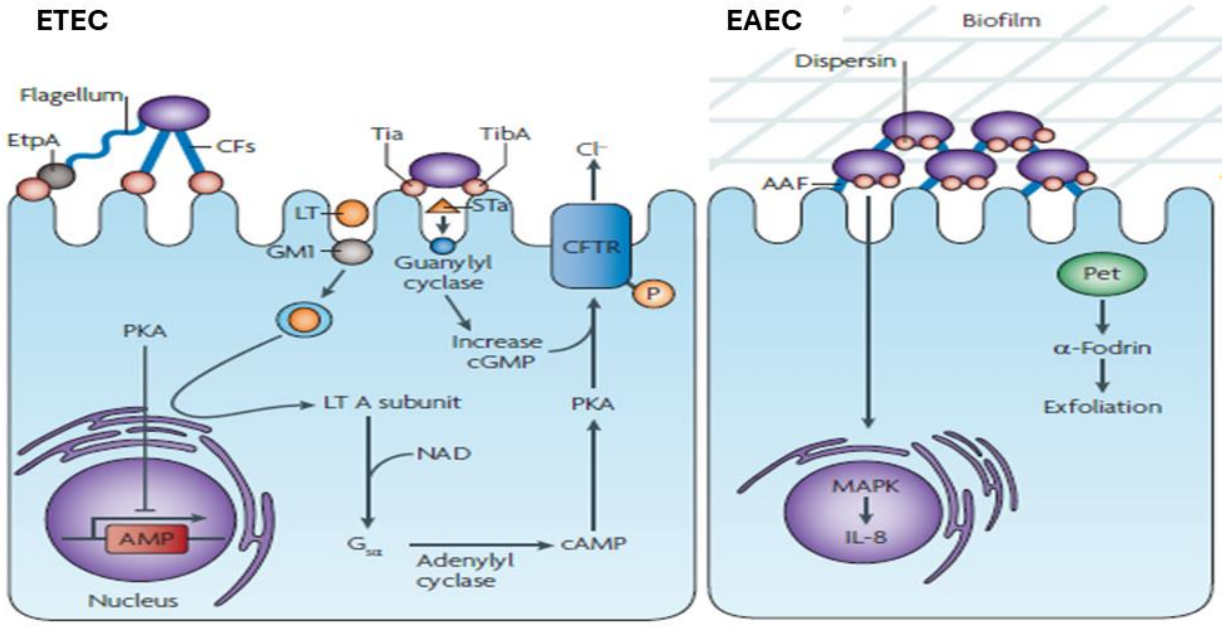

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Figure 3: Pathogenic mechanisms of ETEC and EAEC. Enterotoxigenic *Escherichia coli* (ETEC) attaches itself to small bowel enterocytes via colonization factors (CFs) and an adhesin located at the tip of the flagella (EtpA). A tighter adherence is mediated by Tia and TibA. Two toxins, heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST), are secreted and induce diarrhea by activating cystic fibrosis transmembrane conductance

regulator (CFTR). Through aggregative adherence filaments (AAF), enteroagreggative *E. coli* (EAEC) adheres to enterocytes in the small and large intestines. This triggers a potent interleukin-8 (IL 8) response, which permits the formation of biofilms on the surface of cells. α-fodrin, also referred to as SPTAN1, is the target of Plasmid-encoded toxin (Pet), a serine protease autotransporter of the Enterobacteriaceae (SPATE) that causes actin cytoskeleton disruption and exfoliation.

7.5. EIEC pathogenesis

In the course of infection, EIEC strains first lyse the endocytic vacuole, enter epithelial cells, grow within the cells, and then move across the cytoplasm to neighboring cells. T3SS penetrates host cell membranes by first inserting a pore containing IpaB and IpaC. Cell extensions are formed when IpaC stimulates the GTPases Rac and Cdc42, causing actin polymerization. IpaC induces host cell actin polymerization and lamellipodial extensions. In the same manner, IpaAs modify the cell extensions that IpaC induces by binding to vinculin and causing actin depolymerization, which results in a structure that facilitates the entry of bacteria into cells [58]. IpgD causes blebbing in the host cell membrane to aid in internalization [58, 94- 96]. The endocytic vacuole bursts in less than 10 minutes after cell entry due to a variety of factors, including host factors like Rab5 and Rab11, T3SS effectors like IpaB and IpaC, which bacteria use for phagosomal escape, and other cytosolic access factors used by intracellular bacterial pathogens [58, 96]. Following this, bacteria grow inside of cells, infiltrate adjacent epithelial cells, and cause damage. This leads to symptoms similar to Shigellosis, including diarrhea with mucus, blood and leukocytes in the stool, fever, cramping and tenesmus in the abdomen, and systemic toxicity [58, 96- 98].

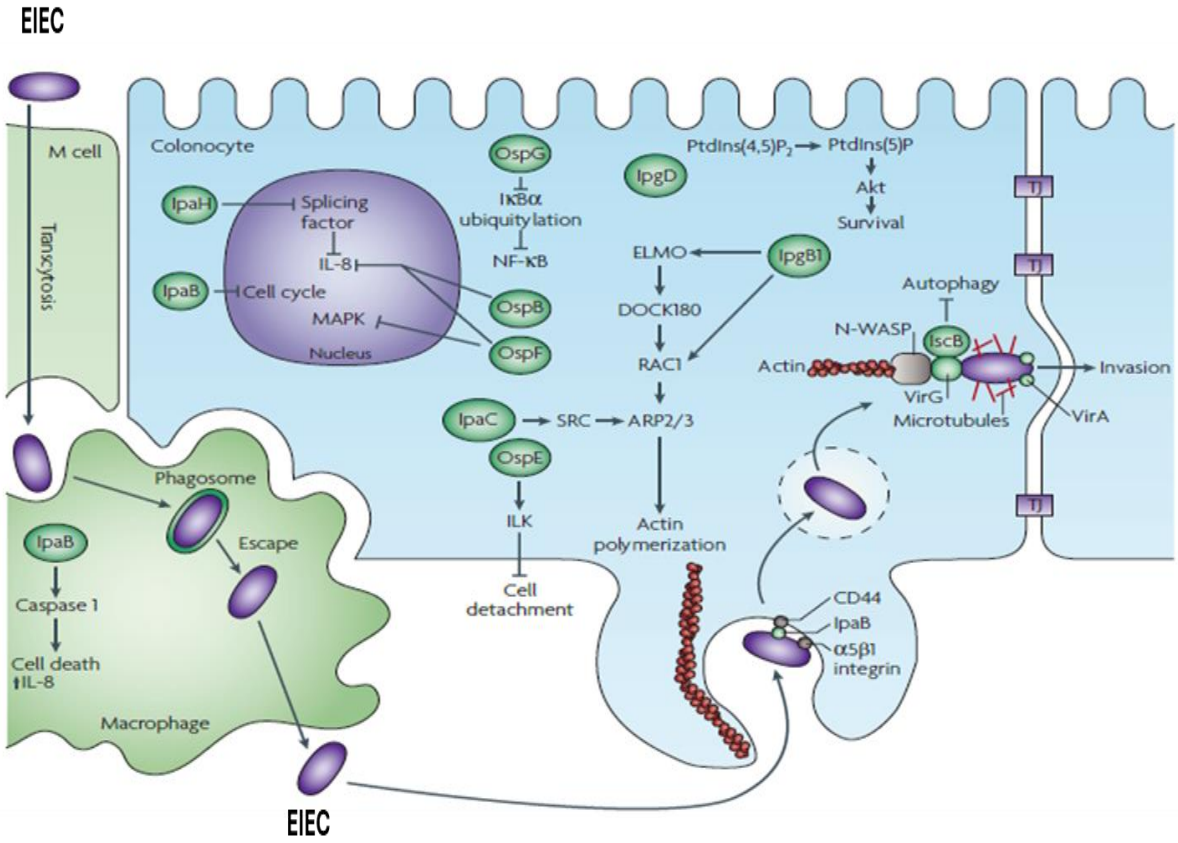

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Figure 4: Pathogenic mechanism of EIEC. EIEC infiltrate the basolateral side of colonocytes after entering the submucosa by microfold (M) cells and replicating in macrophages. Effectors produced into host cells by the type III secretion system facilitate these activities. After entering the cytoplasm of the colonocyte, more effectors are injected to take control of the host's machinery, evade the immune system, and encourage the bacteria to spread from cell to cell.

8. Treatment of DEC mediated infections

Since the primary consequence of diarrhea is dehydration, all children with diarrhea should receive appropriate fluid management, especially oral rehydration solutions (ORS). Furthermore, it is advised to continue breastfeeding in neonatal children and, if appropriate, starting solid food as soon as possible [99].

Antibiotics are not typically used for traveler's diarrhea or those with prolonged diarrhea. The lack of efficacy trials means that youngsters are not advised to use antibiotics. Adult cases of traveler's diarrhea caused by ETEC and EAEC can be treated with fluoroquinolones, azithromycin, rifaximin, amoxicillin/clavulanic acid, and nalidixic acid [100].

Antibiotic resistance in DEC strains from both symptomatic and asymptomatic patients has been documented in numerous investigations. This is a global issue that is becoming worse. Given their high morbidity and mortality rates, the rising prevalence of bla*CTX-M* genotype extended-spectrum β-lactamases (ESBL)-producing bacteria in poor nations is particularly noteworthy [101].

Antibiotic use was found to be substantially linked to an elevated risk of developing HUS in a recent systematic review [102]. Another study revealed that children who had contracted HUS before the illness developed had worse outcomes when they did not receive intravenous fluids and had high hematocrit values [103]. HUS is treated supportively; early dialysis is necessary for many youngsters.

Since they have negative side effects and are linked to antibiotic resistance, most patients with diarrheal sickness caused by *E. coli* are not advised to take antibiotics as a first-line treatment. Antibiotics might make sense for patients with severe disease (e.g., more than six stools per day, fever, hospitalization due to dehydration, diarrhea lasting more than seven days, or bloody diarrhea). The International Society of Travel Medicine (ISTM) and the Infectious Diseases

Society of America (IDSA) presently prescribe ciprofloxacin, azithromycin, and rifaximin for the treatment of *E. coli* diarrheal disease [104].

9. Antimicrobial resistance among DEC pathotypes

9.1. Antimicrobial resistance in EPEC

Research on antibiotic resistance in EPEC strains from various origins and nations has revealed that this pathogen has a high level of resistance to tetracycline, ampicillin, cefpodoxime, trimethoprim, and nalidixic acid [105]. Although it is stated that resistance to most of these antibiotics is common in typical EPEC strains, trimethoprim resistance is greater in atypical EPEC strains [106]. At least 55% of the 185 atypical EPEC isolates that were collected from healthy and diarrheal children living in seven sites in sub-Saharan Africa and South Asia in a global study demonstrated phenotypic resistance to trimethoprim, ampicillin, sulphamethoxazole, and tetracycline, while 43% of the isolates were reported to have streptomycin resistance. Surprisingly, more than half of the isolates showed resistance to three or more of the antibiotics under investigation [107].

9.2. Antimicrobial resistance in ETEC

At one point in time, a homogenous and high pattern of antibiotic susceptibility was seen for ETEC strains. However, the use of various classes of antibiotics, including rifamycin, tetracycline (doxycycline), macrolides (erythromycin and azithromycin), fluoroquinolones (norfloxacin, ofloxacin, ciprofloxacin), sulfamethoxazole-trimethoprim, and rifamycin, to treat travelers' diarrhea may have also played a role in the emergence of antimicrobial resistance in this pathotype [108]. One possible additional factor could be the widespread application of antibiotics for the treatment of viral diarrheas, which can occasionally be misdiagnosed due to similar symptoms [48].

There are several studies from different countries assessing the antibiotic resistance profile and distribution of resistance determinants in ETEC. In a study, the antimicrobial resistance profile among patients with recent travel history to ETEC endemic regions between 2001 and 2004 reported that up to 60% of the ETEC isolates were resistant to sulfamethoxazole-trimethoprim, tetracycline, and/or ampicillin. It has been observed that among patients, ciprofloxacin resistance increased significantly from 1% to 8% in a span of 10 years (1994–2004) [109]. Eight strains representing the main ETEC lineages that cause diarrheal illnesses in humans worldwide were subjected to whole genome sequence analyses in a recent study. All of the strains carried determinants of resistance for at least two of fourteen antibiotics tested, with penicillin, norfloxacin, and chloramphenicol resistance being the most prevalent [110].

9.3. Antimicrobial resistance in EAEC

Multiple-antibiotic-resistant EAEC strains have surfaced in various areas. Studies revealed more than 50% of EAEC isolates causing travelers' diarrhea from Southeast Asia, India, Africa, and Latin America were resistant to tetracycline, ampicillin, and sulphamethoxazole [111, 112]. In a related study conducted in Iran, the transposable bla_{TEM} and $bla_{CTX\text{-}M}$ genes were present in 78% and 60%, respectively, of the extended-spectrum beta-lactamase (ESBL) generating EAEC bacteria [113]. Additionally, other investigations have found plasmid-mediated quinolone resistance (PMQR) genes (qnr) that encode quinolone resistance in EAEC [114, 115].

9.4. Antimicrobial resistance in EIEC

Multidrug-resistant EIEC strains are emerging, just like *Shigella* and other *E. coli* pathotypes. A study conducted in Cameroon found that strong resistance to antibiotics, ampicillin and sulfamethoxazole-trimethoprim was present in 57.14% and 71.43%, respectively, of the EIEC isolates from adult patients with enteric infection. In 28.57% of the isolates, ampicillin

resistance determinants (*bla*$_{TEM}$ and *bla*$_{Oxa}$) were discovered. Additionally, it was observed that strains resistant to chloramphenicol had both *cat1* and *cat2* genes, but over 85 percent of the EIEC isolates had tetracycline resistant *tetA* and *tetB* genes, sulphamethoxazole-trimethoprim resistant *dfr12*, *dfr7*, *dfr1a* genes , and sulfonamide resistant *sul1* genes respectively [116]. According to an investigation conducted between 1971 and 1999, 48% of isolates of EIEC and *Shigella* isolates from eight different countries across four continents were tetracycline resistant [117]. An EIEC O164 strain that was isolated from a tourist who had diarrhea in Japan was shown to have reduced susceptibility to ciprofloxacin and was resistant to co-trimoxazole, ampicillin, streptomycin, and spectinomycin in a different investigation [118].

9.5. Antimicrobial resistance in EHEC

Beta-lactam, sulfonamide, tetracycline, and trimethoprim resistance are common in EHEC, and multidrug resistance is more common in non-O157 serotypes than in O157:H7 serotypes, according to multiple investigations on the level of antibiotic resistance in EHEC from various countries, hosts, and environments. While tetracycline and sulphamethoxazole resistances are common in strains of animal origin for EHEC O157, resistance to ampicillin and cephalothin is prevalent in strains of human origin [119, 120].

10. Vaccine strategies against DEC

10.1. Vaccine strategies for EPEC

According to one study, mice's immune responses were elicited by recombinant *Mycobacterium smegmatis* (Smeg) and *Mycobacterium bovis* BCG strains that expressed BfpA or intimin [58, 121]. Secretory IgA antibody binding to BFP is thought to potentially obstruct bacterial binding or initial attachment of EPEC, hence blocking the subsequent signal transduction pathway that leads to diarrhea [122]. TNF-α and IFN-γ were generated by spleen

cells cultured in vitro from recombinant BfpA-immunized mice [123]. Cattle have been successfully immunized against the EHEC (*E. coli* O157) strain using a combination of recombinant EspA, intimin, and Tir. Furthermore, mice inoculated with *Lactobacillus casei* strains engineered to exhibit immunological dominant epitopes of Int280 and intimin-β fragments mount humoral and cellular immune responses [124]. Together, these indicate that virulence factors can function either independently or in concert with non-infectious immunogenic vectors to provide protection against EPEC diarrhea [58].

10.2. Vaccine strategies for ETEC

A viable strategy for preventing ETEC infection may be immunization, given the high morbidity and mortality rates among children as well as the long-term effects of enteric infections on a child's growth and development. Different approaches have been developed and used in the last 20 years to produce an ETEC vaccine, with the goal of either preventing ETEC from attaching to the small intestine mucosa in the proximal region or providing immunity through the production of IgA antibodies that are directed against LT [125]. With Phase 2b clinical trials underway, ETVAX is the most advanced vaccine candidate [126]. Inactivated whole cell vaccine strains (one K-12 and three O78 positive strains of *E. coli*) that overexpress CFA/I, CS3, CS5, and CS6 antigens are used in this method together with hybrid LT/CTB (B subunit BS of cholera toxin) with and without dmLT adjuvant [127, 128]. Since ETEC strains vary in their O:H serotypes, distinct fimbrial antigens found in the most common ETEC pathotypes have been employed in this strategy to offer broad spectrum protection. In an age-descending experiment conducted in Bangladesh, this vaccine showed outstanding safety, and in both Bangladeshi newborns and in Swedish adults, the addition of the dmLT adjuvant improved the amplitude and kinetics of mucosal antibody responses [126]. With the ability to express CFA/I, CFA/II, and CFA/IV antigens, a multivalent ETEC vaccine should be able to defend against most ETEC strains globally [129- 131].

Live attenuated *E. coli* expressing ETEC fimbriae is another vaccine that is further along in the development process. The prototype strain *E. coli* strain E1392-75-2A, which has been shown in clinical trials, contains the fimbriae CS1 and CS3, but is devoid of the genes encoding LT and ST [132, 133]. The CFA/II plasmid's LT and ST genes were spontaneously removed to create this strain, which was obtained at the Central Public Health Laboratory in London, United Kingdom. After receiving 10^{10} CFU doses of strain E1392-75-2A, all volunteers showed a significant increase in sIgA antibody to CS1 and CS3 in their intestinal fluid. Additionally, the vaccinees showed a significant protective effect ($p < 0.005$, 75% vaccine effectiveness) against the ETEC challenge strain E24377A (O139:H28) [58]. After receiving this live oral vaccination, however, 15% of the participants experienced minor diarrhea. Along the same lines, ETEC fimbrial antigens and LT antigens have been expressed using live attenuated vectors containing *Salmonella* and *Shigella* [134]. In a guinea pig model, an attenuated strain of Shigella with ETEC CFA/I and CS3 fimbriae expressed those antigens and caused sIgA mucosal antibody responses [135]. A ShigETEC toxin hybrid, an LPS-free cell that expresses conserved ETEC and *Shigella* antigens, is being developed as a multivalent live oral vaccination against both *Shigella* and ETEC. It is now undergoing a Phase 1 clinical trial [136]. Similarly, a hybrid strain of *Shigella* (1208S-122), which is an attenuated strain of the vaccine strain designed to express ETEC CF and LT, is also enrolled in the Phase 1 trial [125]. Protection is seen if the attenuated *Shigella* expresses genetically detoxified LT and ETEC fimbrial colonization factors. Additionally, several preclinical stages of testing are being conducted on vaccines for ETEC virulence factors based on proteins, multiple epitope fusion antigens (MEFA), and fimbrial tip adhesin [58, 65].

10.3. Vaccine strategies for EAEC

There is currently little vaccine development for the pathotypes EAEC and DAEC. However, the best candidates are thought to be bacterial adhesins, and AAF/I and AAF/II may be the

target of EAEC. Upon immunizing Balb/c mice with three distinct vaccination modalities (DNA/DNA, DNA/Protein, or Protein/Protein of AAF/I or AAF/II of EAEC, respectively), only the Protein/Protein dosages of AAF/I and DNA/Protein vaccinations of AAF/I significantly elicited total IgG [137]. Similarly, F1845 fimbriae, Dr. hemagglutinin, and afimbrial adhesins (AFA) are significant DAEC adhesins that may be used as target antigens in vaccine development [138]. Urinary tract infections caused by homologous strains of *Escherichia coli* that carried Dr adhesin were decreased in C3H/HeJ mice that were vaccinated against the Dr fimbrial antigen. Bacterial attachment to the kidneys and bladder was prevented by immune sera with high titres of anti-Dr antibody [139]. It has not yet been determined whether Dr. Adhesin serves as an antigen in vaccinations against intestinal diseases, despite the fact that it was demonstrated to be immunogenic in this urinary tract model [58].

10.4. Vaccine strategies for EIEC

There is no animal reservoir for EIEC/*Shigella*. Usually, inadequate sanitation and hygiene allow them to spread across the human population. Since there are currently no licensed vaccinations against EIEC/*Shigella*, maintaining good sanitation and practicing basic hygiene continue to be the most effective ways to avoid infection [140, 141]. These methods are impractical in many low-income nations, though. As a result, there is a greater need for the development of vaccines. Some approaches that have been used to address this need include the use of live-attenuated strains, candidates based on glycoconjugates, novel antigens like *Shigella* outer membrane vesicles (OMV) encapsulated within nanoparticles, and protein subunit candidates [141- 146]. The potential for immunization with invasion plasmid antigens (IPAs), virG, or *Shigella* O antibodies that cross-react with EIEC O antigens has been reported in a number of research including both humans and animals [58, 147, 148].

10.5. Vaccine strategies for EHEC

Due to its severe infection, which has resulted in a rise in HUS cases, and problems that make it difficult to treat EHEC with some medications, EHEC has been the *E. coli* pathotype that has been studied the most over the past 20 years in the hopes of developing a vaccine [58]. Antibiotic use worsens EHEC sickness in contrast to other pathotypes because it can cause EHEC, which increases the generation of Shiga toxins and releases more toxins after bacterial cell lysis. Because EHEC can colonize animal intestines, studies assessing capacity of EHEC to colonize a variety of animal models have been successful. These models include neonatal calves, germ-free piglets (where they also exhibit CNS symptoms like severe infections in humans), ferrets (oral infection model) which develop HUS after O157 infection, and various murine models (intra-gastric inoculation model). A large variety of animals are readily available, which makes them useful models for both the production of vaccines and the study of host immune responses after EHEC infection [149- 156]. Various approaches have been used up to this point, such as vaccination against the Shiga toxin, which neutralizes the toxin's effects by using antibodies designed to target the A subunit of Stx2 and the B subunit of Stx1, Stx toxoids, and, attenuated bacteria, which are non-pathogenic but sufficiently immunogenic to block the toxin's pathologic effects because they lack the LEE-encoded regulator (Ler), which controls genes both inside and outside the pathogenicity island region [157- 159]. Moreover, there are additional techniques like "bacterial ghosts," in which bacteria are genetically modified to express a lysis gene under controlled conditions. This gene lyses the bacteria and creates empty bacterial cell envelopes that resemble living cells' outer envelope; these are non-infectious but trigger the mucosal immune response. It has been documented that in several animal models, alternative protein-, peptide-, plant-, DNA-, polysaccharide-, or adjuvant-enhanced vaccinations can stimulate the host immune response and lessen the lethality of EHEC [58]. EPEC and EHEC belong to the gastrointestinal bacterial diseases known as attaching and effacing (A/E) family. The LEE and O islands of EPEC and EHEC are

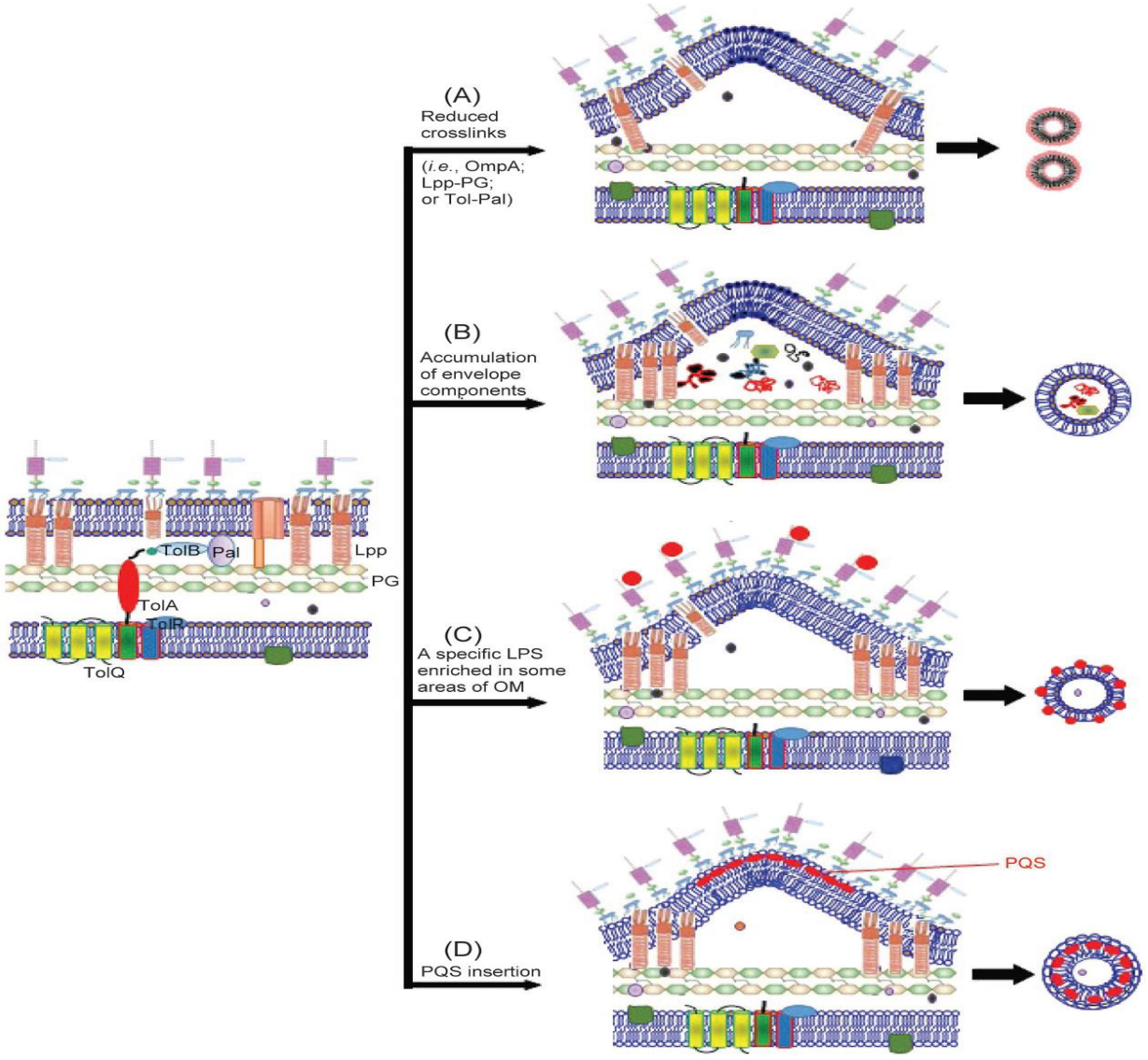
highly homologous [160]. These pathotypes' virulence is dependent on a T3SS and related secreted proteins like intimin and EspS [58]. Epidemiological studies have shown that the location wherever EPEC is endemic has much less cases of EHEC. Researchers have taken use of this technique to use the attenuated EPEC O126:H6, in which the mice that were infected with EHEC had lower mortality rates and the antibodies from the EPEC vaccination were found to be cross-reactive with those from EHEC [58, 161, 162].

11. Outer membrane vesicles (OMVs)

Different strategies are used by gram-negative bacteria to interact with human hosts. Of these systems, OMVs play a crucial role in enabling interactions between the host and the pathogen without necessitating direct contact between cells. This is especially important when bacteria invade the mucus layer covering the host epithelial cells [163- 165]. OMVs are spherical proteoliposomes with a diameter of approximately 20 to 200 nm derived from Gram-negative bacteria's outer membrane [166- 169]. These vesicles are made up of several periplasmic and cytoplasmic substances, such as metabolites, proteins, DNA, and RNA [170-172]. Lipid bilayer of OMVs shields the contents from extra-vesicular proteins like RNases and proteases [163]. Chatterjee and Das used transmission electron microscopy to characterize OMVs for the first time in 1967 using *Vibrio cholerae* [173]. Subsequent research has demonstrated that OMVs are generated by a broad spectrum of Gram-negative bacteria and were identified from both commensal and pathogenic bacteria inhabiting the human lung and gut. Outer-inner membrane vesicles (O-IMVs), which originate from the bacterial inner and outer membranes, are also released by Gram-negative bacteria; nevertheless, they make up less than 1% of all secreted vesicles [174]. Bacterial factors that alter the host's immunological response to infection are encapsulated in both OMVs and O-IMVs. Small interfering RNA

(sRNA) which binds to host mRNA transcripts and silences them is one of these factors, as are proteins that prevent epithelial chloride ion release. Although a great deal of effort has been made in defining the immunomodulatory characteristics of OMVs, much of their mechanism of action are still unclear. Researching the interaction between OMVs and the host is essential to creating new treatments for infections caused by bacteria and the resulting inflammatory response [175- 183].

It has been demonstrated that OMVs contain virulence factors including sRNA, which, like miRNA, targets genes in host immune cells to suppress the host's reaction to infection and enable the establishment of persistent infections by bacteria [163]. Little is known about the epigenetic mechanisms of action of the few bacterial factors that have been found in OMVs that cause a decreased immune response. The study of the epigenetic mechanisms underlying bacterial interactions with host immune cells has just started. In order to create persistent bacterial infections and reduce the immune system's ability to respond to subsequent infections, virulence factors that modify DNA methylation (DNAm) patterns in immune cells are involved. *Pseudomonas aeruginosa* OMVs, for example, can alter DNA sequences to suppress the immunological response of human macrophages following infection [184]. In a mouse model, OMVs generated from the human gut commensal *Bacteroides thetaiotaomicron* decrease the inflammatory response to dextran sodium sulfate, which induces colitis [185]. Even while OMVs have been shown to have immunomodulatory effects, not much study has been done on the underlying mechanisms of these interactions—more particularly, how the host immune response following infection is altered epigenetically [163].



You-Jiang Yu et al Acta Pharmacologica Sinica, DOI: 10.1038/aps.2017.82

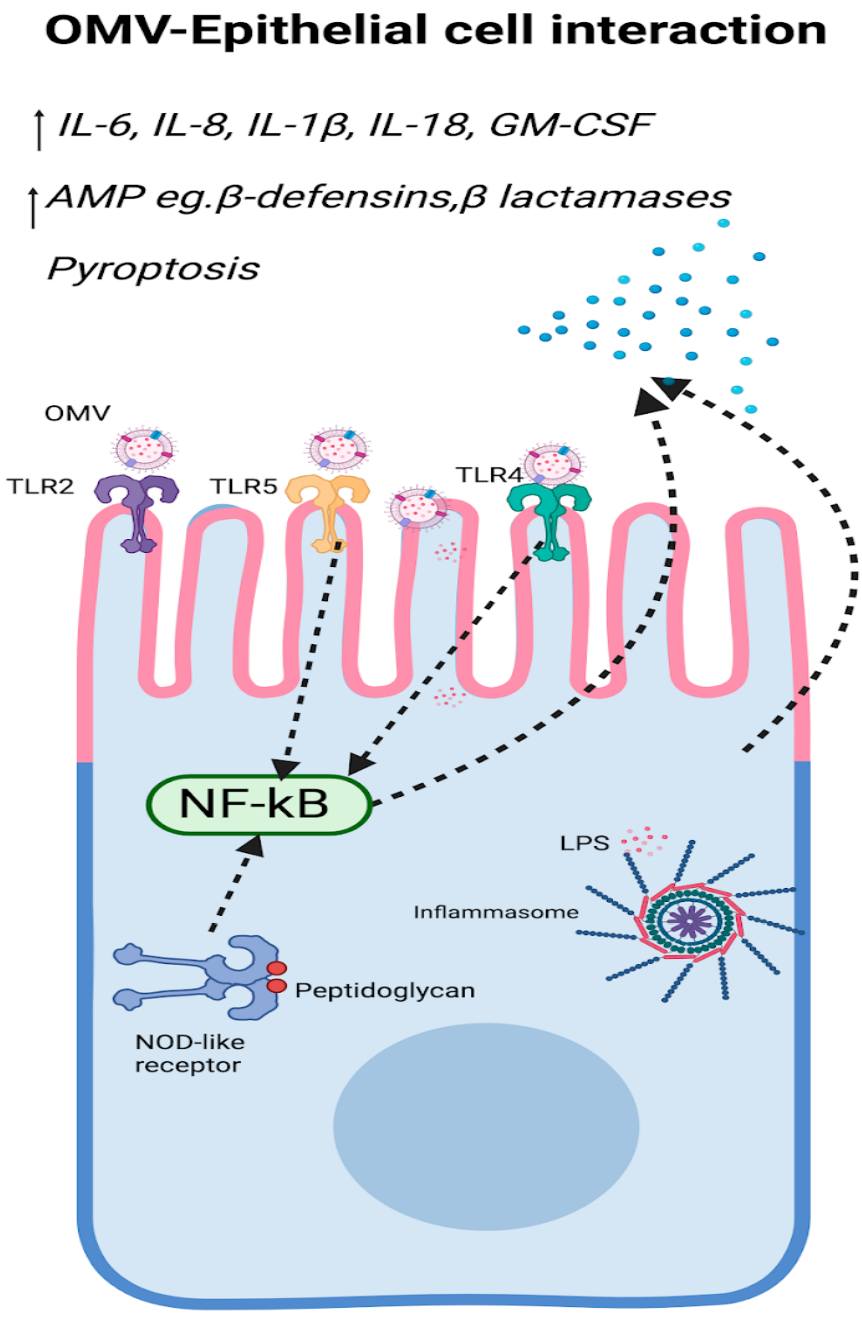
Figure 5: Models that have been proposed for the production of outer membrane vesicles (OMVs). (A) Disruption occurs in the junction between the peptidoglycan layer underlying the outer membrane. (B) The buildup of misfiled or overexpressed envelope proteins creates a physical force that pushes out the outer membrane vesicles. (C) The outer membrane curves as a result of the buildup of LPS molecules with atypical charges or configurations. (D) Extracellular signals induce localized curvature of the bacterial outer membrane.

11. Interaction of OMVs with different cell types

11.1. Interaction of OMVs with epithelial cells

OMVs diffuse across their surroundings, including mucus, to transport their contents to recipient cells after being released by microorganisms, either through explosive cell lysis or budding. OMVs can be taken up by host cells by phagocytosis or other methods such membrane fusion, clathrin-dependent endocytosis, and caveolin-mediated endocytosis. They have also been seen to fuse with lipid rafts found on the membranes of epithelial cells [163, 186- 188]. OMVs carry a variety of payloads, such as proteins, small RNAs (sRNA), and transfer RNA fragments (tRNA-fragments) that bind to genes involved in host immunity. By activating host Toll-like receptors (TLRs), certain OMV proteins, for example, have been demonstrated to upregulate the host immunological response [189- 193]. Like eukaryotic miRNAs, OMV sRNA and tRNA fragments have the ability to control target gene transcription and translation in addition to reducing the stability of host mRNA transcripts. It is favourable for the establishment and maintenance of bacterial infections to suppress the host immune response. OMVs are an important means by which microbial virulence factors are transferred to their host targets during infection, especially in conditions where mucus layers that reside above epithelia are colonized or chronic infections are present. For instance, flagellin and LPS, which are present in OMVs from infectious bacteria like *P. aeruginosa*, can stimulate TLR5 and TLR4, respectively, thereby activating the host immune system [163]. Identification of the lipid A part of LPS recognized by TLR4 triggers a signaling cascade via MyD88 and NF-κβ, raising the synthesis of cytokines that promote inflammation, like IL-8, which draws immune cells to the lungs in an effort to eradicate infection. Certain host signaling pathways can be targeted by OMV proteins to achieve their immunomodulatory effects. The effector protein BopE, for instance, is present in OMVs from *Burkholderia pseudomallei*. Its ability to bind to the host GTPase Rac1 and trigger downstream signaling cascades promotes intracellular

survival and bacterial invasion. One significant way that bacteria can control the host immune response to their benefit is through the immunomodulatory proteins that are present in bacterial OMVs [163, 194- 196].



Gisseth Magaña et al. Antibiotics, DOI: 10.3390/antibiotics13010032

Figure 6: Interaction of outer membrane vesicles with epithelial cells.

11.2. Interaction of OMVs with neutrophils

Despite being thought of as non-duplicate, inanimate nanostructures, OMVs have the ability to stimulate neutrophils and cause the host to produce inflammatory cytokines. For instance, OMVs linked to *Neisseria meningitidis* simulate the human neutrophils to generate pro-inflammatory cytokines and chemokines, such as MIP-1β, IL-8, TNF-α, interleukin-1β (IL-1β), and macrophage inflammatory protein 1α (MIP-1α). According to more research, interferon gamma (IFN-γ) can raise these cytokines' levels to sustain chronic inflammation [197, 198]. Given that these reactions resemble those of neutrophils brought on by bacterial infection, it is possible that OMVs can support infection-preventive immunity. Conversely, certain virulence factors borne by OMVs can impede neutrophil antimicrobial action, which in turn helps to reduce the amount of cytokines secreted in imitation of OMVs. For instance, cytotoxic necrotizing factor type 1 (CNF1) is present in uropathogenic *Escherichia coli* (UPEC) OMVs. Polymorphonuclear leukocytes (PMNs) are known to be toxic when their membrane fluidity is reduced by CNF1. This can lead to a reduction in PMN function and, consequently, in the cytokine and chemokine profile [199]. On the other hand, infections are the cause of neutrophil death. Neutrophils will self-defend in an attempt to thwart bacterial invasion, initiating a deadly defense mechanism against infections. Neutrophil extracellular traps (NETs) are a unique technique for killing bacteria that allows neutrophils to quickly remove pathogens. It has been observed that bacterial OMVs can also cause the creation of NETs. *Neisseria meningitidis* can, however, evade NETs through an intrinsic response, which could raise OMV levels and accelerate the infection's spread [197, 200, 201].

11.3. Interaction of OMVs with macrophages

When exposed with bacterial OMVs, macrophages, a kind of classical immune cell, can induce strong immune responses. First, OMVs compel macrophages to release cytokines that promote

inflammation. Macrophages exhibited inflammatory reactions upon pretreatment with OMVs [202- 204]. Macrophages phagocytose bacterial OMVs, and upon activation, these macrophages trigger heightened production of TNFα, IL-8, and IL-1β via NF-κβ activation [205]. *P. gingivalis* OMV-simulated macrophages secrete increased amounts of IFNγ, IL-12p70, IL-10, TNFα, IL-6, and nitric oxide [206]. The TLR2/4 pathway is necessary for the pro-inflammatory potential of isolated *Legionella pneumophila* (*L. pneumophila*) OMVs on macrophages. Moreover, OMVs promote the replication of *L. pneumophila* in macrophages. The discovery could clarify how OMVs encourage *L. pneumophila* to propagate throughout the host [197, 207]. When avirulent *Escherichia coli* OMVs are used to infect bone marrow-derived macrophages (BMDMs), guanylate binding proteins (GBP) are shown to be regulators of OMVs-mediated inflammation [208]. On the other hand, compared to *Klebsiella pneumoniae* OMVs containing porin, porin loss OMVs might cause reduced amounts of proinflammatory cytokine release [209]. Second, an adaptive immune response could be triggered by macrophages that are imitated by bacterial OMVs. The expression of molecules that support antigen presentation on the surface of macrophages is upregulated by *Neisseria meningitidis* or *Klebsiella pneumoniae* OMVs. These molecules include CD80, major histocompatibility complex-II (MHC-II), CD86, human leukocyte antigen-DR (HLA-DR), and intercellular adhesion molecules-1 (ICAM-1). Macrophages, one of the expert antigen-presenting cells, allow T cells to be stimulated by OMV antigen recognition and subsequently stimulate the adaptive immune response. *Shigella boydii* OMVs can cause naive peritoneal macrophages to polarize CD4+ T cells toward a Th1 adaptive immunological response [197, 210, 211]. Thirdly, it has been documented that bacterial OMVs can cause metabolic remodeling in macrophages and trigger apoptosis and pyroptosis. The host body may develop diseases as a result of these effects on macrophages, which may also lead to a decline in protective cell levels and malfunction [197, 212]. Bacterial OMVs can operate as anti-

inflammatory agents in infected host cells, apart from their inflammatory function in macrophages. According to earlier research, macrophages treated OMVs will release more IL-10 and other anti-inflammatory cytokines [197]. Human peripheral blood mononuclear cells (PBMCs) produce more of the immunosuppressive cytokine IL-10 as a result of *Helicobacter pylori* OMVs, and Jurkat T cells undergo apoptosis [213]. Therefore, it can be said that while bacterial OMVs effectively combat infection, they also confer advantages to the bacteria by reducing inflammation and eliminating immune cells, which in turn increases the bacteria's ability to survive in the host [197].

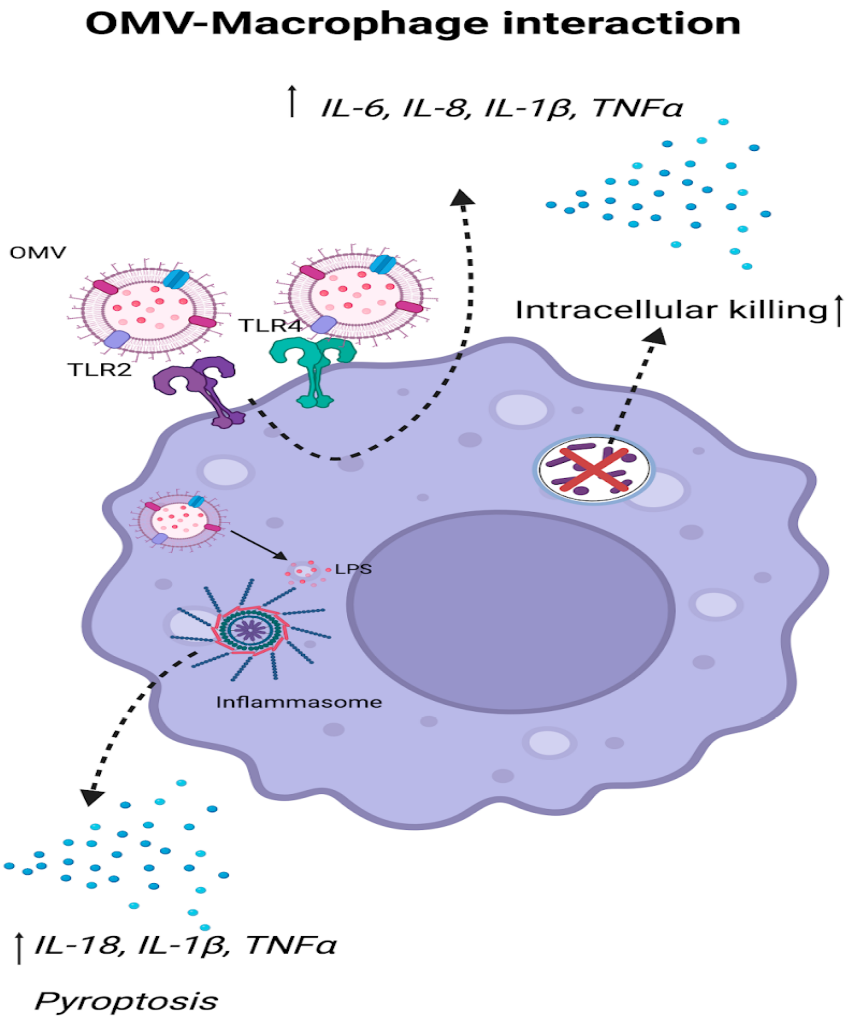


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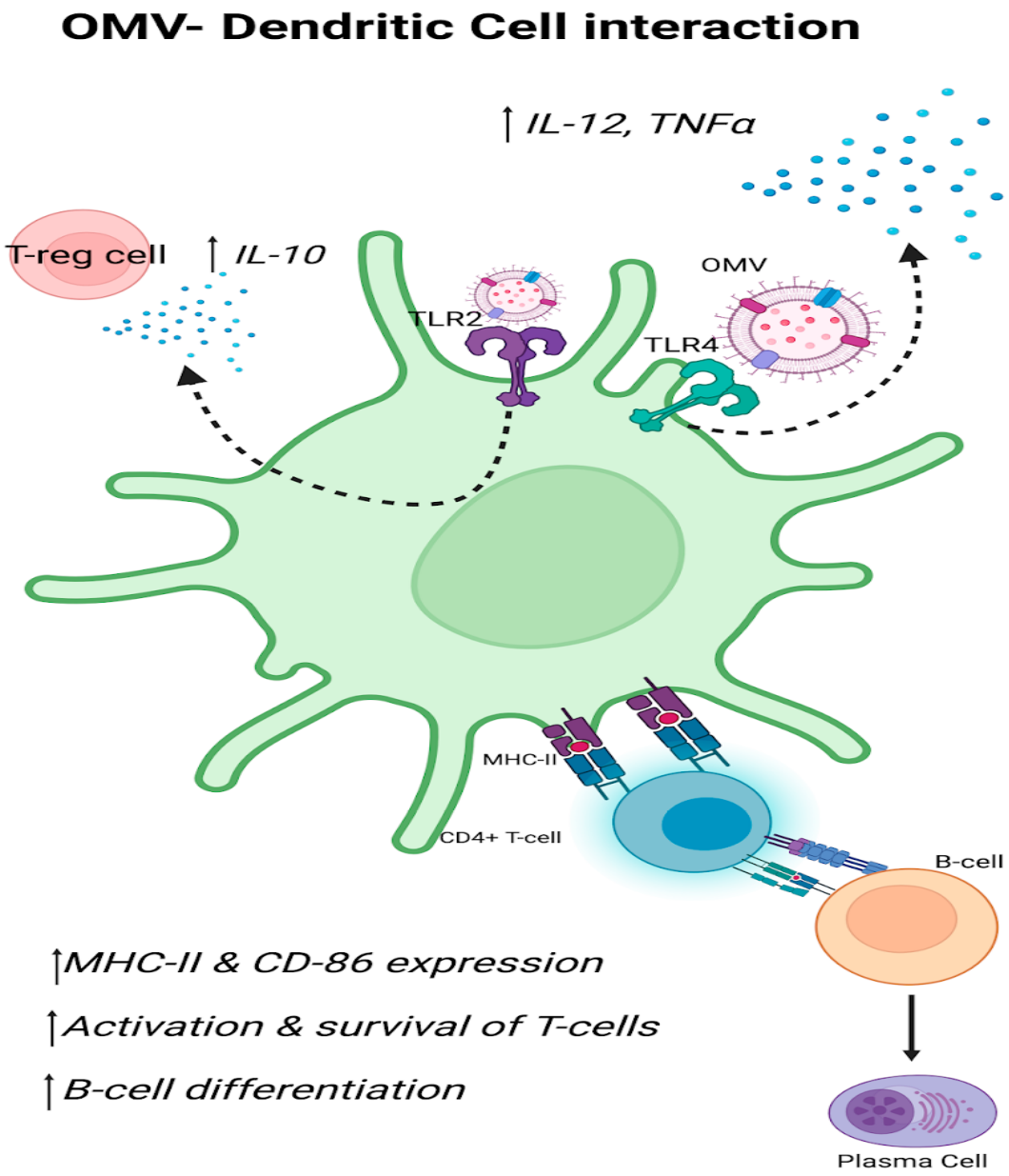
Figure 7: Interaction of outer membrane vesicles with macrophages.

11.4. Interaction of OMVs with dendritic cells

Bacterial OMVs can induce the cytokine profile and upregulate the production of co-stimulatory molecules to activate dendritic cells [214]. Dendritic cells can be stimulated by meningococcal OMVs through the upregulation of HLA-DR activation markers, CD40, CD83, CD80, CD86, and Programmed death-ligand 1 (PD-L1). Conversely, when exposed to Meningococcal OMVs, dendritic cells are activated and release higher levels of cytokines like IL-6 and IL1β than when they are not [215]. Dendritic cells express increased Heme Oxygenase-1 when *Helicobacter pylori* OMVs activate the Akt-Nrf2 and mTOR-κβ Kinase–NF-κβ pathways [216]. Dendritic cells exposed to bacterial OMVs collectively may cause an innate immune response to combat bacterial infection [217- 222]. Professional APCs play a crucial role in inducing an adaptive immune response by upregulating the production of co-stimulatory molecules [197].



Gisseth Magaña et al. Antibiotics, DOI: 10.3390/antibiotics13010032

Figure 8: Interaction of outer membrane vesicles with dendritic cells.

11.5. Interaction of OMVs with B-cells

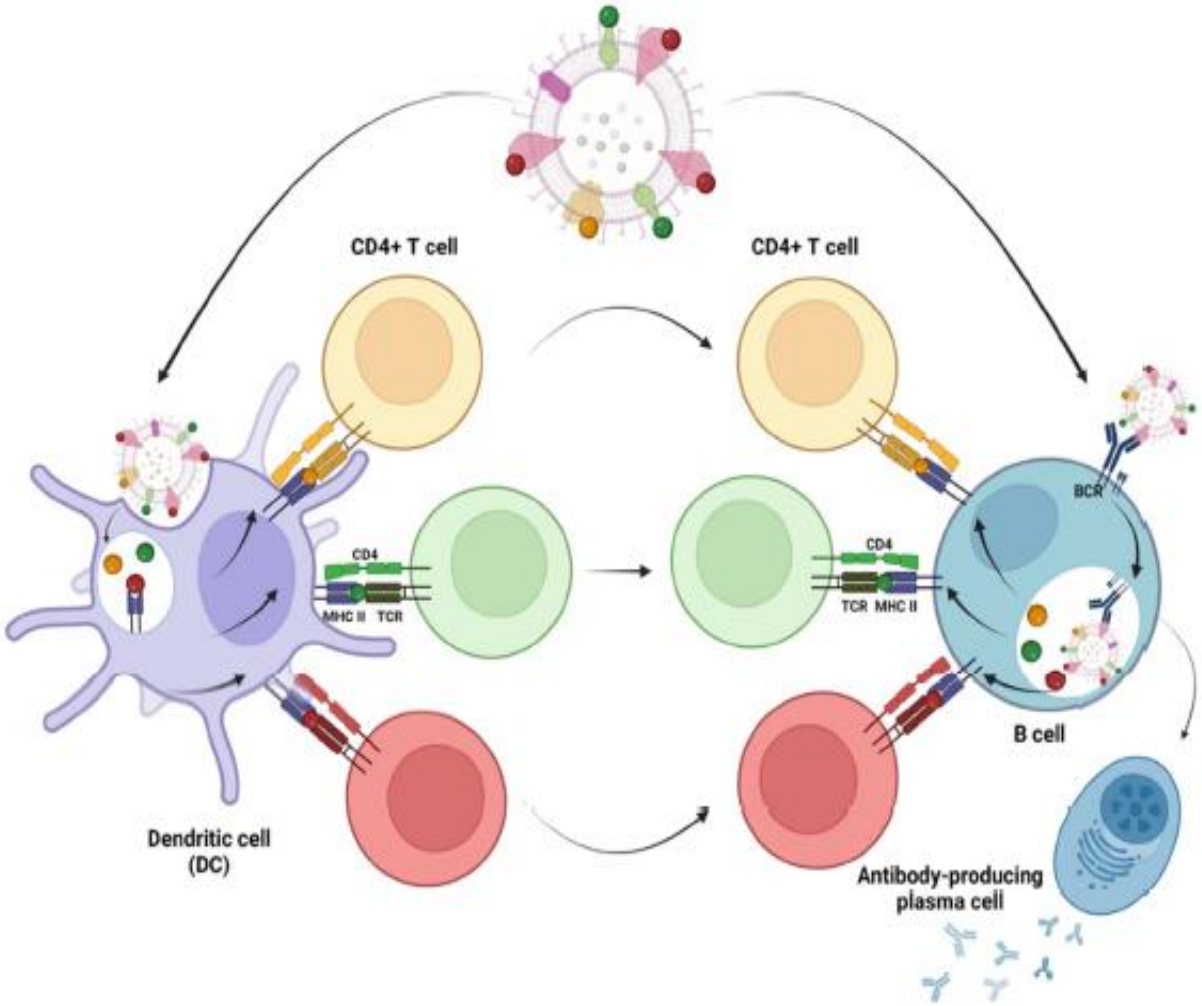
T cells aid B cells in responding to foreign antigens. OMVs of *Salmonella* Typhimurium stimulate T and B cells' responses, and mice inoculated with OMVs show the presence specific IgG. On the other hand, without the assistance of T cells, OMVs are capable of stimulating the response of B cells directly. Moreover, the simulation of OMVs will increase the proliferative activity, and this mitogenic response may require the B cell receptor (BCR). By stimulating B cell proliferation, *Neisseria lactamica* can cause the production of polyclonal IgM, which leads to colonization and immunological tolerance in the host in the absence of an adaptive immune response. In order to investigate the interaction of OMVs with B cells, a new mechanism for how bacterial OMVs activate B cells may be explored. OMVs are produced by *Moraxella catarrhalis* in order to evade the host immunological response. The author explains the process by which B cells react to OMVs. Activated B cells' membranes will first undergo BCR internalization, followed by IgD BCR clustering and Ca2+ mobilization. Then, a few patterns are essential for activation, such as unmethylated CpG-DNA and the IgD-binding super antigen MID. Finally, the activation of CD19+ IgD+ cells is followed by the generation of IL-6 and IgM as well as enhanced expression of surface markers (HLA-DR, CD45, CD64, and CD86). In summary, OMVs have a broad impact on B cells and are a crucial component of the adaptive immune response [197, 223- 225].

11.6. Interaction of OMVs with T-cells

When OMVs enter the host, APCs expose the OMV antigen to CD4+T cells, which then promotes the development of T helper cells (Th) into primarily three subtypes: Th1, Th2, and Th17. These subtypes support both the humoral and cell immunological responses.

Theoretically, OMVs may have adjuvant effects on T cells' cross-priming, which is implicated in the CD4+ and CD8+ T cell responses. To sum up, OMVs have the potential to elicit strong immune responses that are protective, which could make them a suitable choice for vaccination.

Recent research, however, indicates that OMVs may inhibit T cell proliferation and response in a number of ways. For instance, Opa proteins carried by *Neisseria meningitidis* OMVs may alter receptor binding, which in turn may impact T cell proliferation. It has been shown that *Helicobacter pylori* OMVs impede T cell proliferation by causing monocytes to produce COX-2. Furthermore, PorB proteosomes from *Neisseria gonorrhoeae* can change the immunosuppression, while PorB presented in OMVs can inhibit CD4+T cell growth. Bacterial invasion and survival may be facilitated by the detrimental effects on immunity [197, 226-229].



Lorenzo Croia et al. Membranes, DOI: 10.3390/membranes13110882

Figure 9: OMV endogenous proteins may have a function in enhancing humoral responses to heterologous antigens produced in OMVs. OMVs are taken up by DCs, which then use the MHC II molecule to deliver OMV endogenous protein epitopes, therefore stimulating a broad population of CD4+ T helper (Th) cells (three T cells, each recognizing a distinct epitope, are shown with different colors for simplicity). The identical OMV-derived epitopes that DCs display are recognized by naïve B cells and their receptors in parallel, and upon OMV internalization, they exhibit the same heterologous antigens on their MHC II molecules. Therefore, the vast number of OMV-specific effector T cells has the ability to activate B cells specific for the heterologous antigens, causing them to become memory B cells and plasma cells that produce antibodies specific for the heterologous antigens.

12. Application of OMVs as vaccines

Bacterial delivery mechanisms known as outer membrane vesicles occur naturally and are capable of delivering cargo to nearby cells, or biofilms, including lipids, proteins, and nucleic acids. Using OMVs to provide antigens for vaccination is the most obvious use for them. Originally, this technique was created to vaccinate against serogroup B of *N. meningitidis*. In order to use them to facilitate antigen delivery from homologous or heterologous strains, OMVs have since been generated from a variety of bacterial strains. Non-bacterial antigens can be expressed and then displayed on the OMV surface by fusing them with a carrier protein. VA-MENGOC-BC™ and Bexsero™, two licensed OMV vaccines, offer protection from the invasive *Neisseria meningitidis* serogroup B strain (MenB). Although there have long been vaccinations against some meningococcal serogroups of *Neisseria*, developing a vaccine against MenB has been difficult due to the bacterium's very changeable main antigen, PorA. Because PorA is highly similar to fetal brain tissue and has a poor immunogenicity, traditional vaccination strategies have not been successful. This strategy has worked well in cases of clonal outbreaks since OMVs can be produced from the precise strain that is generating a meningitis outbreak. The first licensed OMV vaccine, VA-MENGOC-BC™, was authorized for use in Cuba in 1987 as a result of years of high illness rates caused by a single serotype of MenB (B4:P1.15). The vaccine's effectiveness was assessed to be 85%, and serogroup C was also included. Subsequent MenB epidemics in Norway, New Zealand, and France were treated using OMV vaccines. Surprisingly, the vaccine used in the French and Norwegian outbreaks (MenBVac) produced broader protection against various Neisserial strains despite exhibiting a rapid drop in immune response over time. The OMV vaccine MeNZB™, which was found to be 75% effective, was used in a mass immunization program between 2004 and 2006, following the height of the MenB epidemic in New Zealand in 2001. The FDA and EMA presently only have licenses for one OMV-based vaccination, Bexsero™, which is made by

Glaxo Smith Kline in Brentford, United Kingdom. Children and young adults between the ages of 10 and 25 are administered with it intramuscularly. This vaccine is produced with three extra recombinant proteins (rMenB), which have been determined by reverse vaccinology, and it makes use of the MeNZB™ OMV vaccine that was given during the New Zealand outbreak. According to estimates, this vaccination can guard against 66–91% of the MenB strains found worldwide. Remarkably, it has been discovered that Bexsero™ exhibits cross-protection against *N. gonorrhoeae* [230].

Table 1: OMVs-based vaccines licensed and in clinical development

Status of development	Vaccine	Pathogen	Developer
Phase I	Avacc 10	COVID-19	Intravacc (Netherlands)
	iNTS-GMMA	Invasive non-typhoidal *Salmonella*	GSK (Italy)
	–	*Neisseria gonorrhoea*	GSK (Italy)
Phase II	altSonflex1-2-3	*Shigella*	GSK (Italy)
Registered	PedvaxHib (PRP-OMPC)	*Haemophilus influenzae* type b	Merck Co.
	Procomvax/Comvax (PRP-OMPC and hepatitis B)	*H. influenzae* type b and hepatitis B	Merck Co.[a]
	Vaxelis (diphtheria and tetanus toxoids, acellular pertussis, inactivated polio-virus, PRP-OMPC and hepatitis B)	Diphtheria, tetanus, pertussis, polio-myelitis, *H. influenzae* type b and hepatitis b	Merck Co. and Sanofi Pasteur
	Bexsero (4CMenB)	*N. meningitidis* B	GSK (Italy)
	MenZB (NZ dOMV)	*N. meningitidis* B	Novartis Vaccine and Diagnostics (Italy)[b] and National Institute of Public Health (Norway)
	VA-MENGOC-BC	*N. meningitidis* B	Finlay Institute (Cuba)
	Norway MenBVAC	*N. meningitidis* B	Norwegian Institute of Public Health (Norway)[a]

[a]Marketing authorization was not renewed by the market authorization holder (Procomvax)

[b]No longer licensed, NVD was taken over by GSK

Francesca Micoli et al. BioDrugs, DOI: 10.1007/s40259-023-00627-0

Chapter 2

Objectives of the study

Grounds

The public's health is greatly impacted by diarrheal infections, which are a leading cause of serious illness and death, particularly in young children and infants [1]. Enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) are the five primary pathotypes of diarrhoeagenic *E. coli* DEC [2]. ETEC is linked to traveler's diarrhea; EPEC is linked to both acute and chronic diarrhea in the pediatric population; an EIEC infection causes dysentery with bloody mucoid diarrhea, which is the same diarrhea caused by *Shigella sp*.; EHEC causes hemorrhagic colitis and hemorrhagic uremic syndrome (HUS); and an EAEC infection is linked to both acute and persistent diarrhea in patients with impaired immune systems and in developing nations [1].

Every year, DEC results in over 300 million infections and almost 200,000 fatalities worldwide. In impoverished nations, diarrheal illnesses (DECs) account for 30–40% of diarrheal episodes, and 120,000 pediatric fatalities worldwide were attributed to DECs in 2011 [3, 4, 5]. Previous research found that infection caused by DEC were responsible for 34% of all diarrheal episodes [6]. Eighty percent of human diarrheal illnesses in Africa are caused by DEC, which causes 70,000 fatalities annually, the most of which are in children under five. It has been claimed that 34–60% of children in Kenya had DEC infections. In Egypt, community-level DEC infections among children under five years old have been found to be 20.6%. It was reported that 38.2% of children in Uganda with acute diarrhea had DEC infections [7]. According to a study done in Kolkata, India, the incidence of DEC infections was 12% [8].

The misuse of antibiotics is leading to the emergence of *E. coli* strains that are resistant to many drugs. In the post-antibiotic era, preventative measures such as vaccinations can be extremely helpful in lowering the health burden caused by DECs. Presently, scientists are working to

create viable DEC vaccines, but they are encountering significant obstacles because there are numerous DEC serotypes and insufficient gut immune response. There isn't currently a single approved vaccine against DEC that can be used for public health [9- 12].

Due to their significant immunogenicity and ease of uptake by mammalian cells, outer membrane vesicles (OMVs), which are nanosized proteoliposomes produced from the outer membrane of gram-negative bacteria, have the potential to be used as acellular vaccine candidates. Natural antigens that imitate the outer surface of bacteria, including phospholipids, peptidoglycan, lipopolysaccharides (LPSs), outer membrane proteins (OMPs), and membrane lipoprotein components, are found in OMVs. The European Medicine Agency (EMA) has licensed an OMV-based vaccine against *Neisseria meningitides* for use in human active vaccination [13- 16].

According to earlier research, immunizing mice intraperitoneally (i.p.) with *E. coli* OMVs prevented them from contracting infections later on [17]. OMVs have demonstrated to elicit favorable mucosal immune responses [18]; however, no combination OMVs-based vaccines against the pathotypes of DEC has been created. In this investigation, we generated an immunogen based on combined pentavalent OMVs (POMVs) from five major DEC subgroups (EPEC, ETEC, EAEC, EIEC, and EHEC) and ascertained the adaptive immunological response that the immunogen elicited. The objective of this study is also to evaluate the passive protection that adult mouse sera vaccinated with POMVs provide to newborn mice. Our research has shown that POMVs significantly alter adult mice's mucosal and systemic immune responses. The work illustrates how adult mouse sera that have been vaccinated can passively shield neonatal mice from the five DEC pathotypes. According to our research, POMVs may be used as a vaccine candidate to lower infections caused by the five DEC pathotypes.

Objectives

- **Selection and characterization of prevalent strains of diarrhoeagenic *Escherichia coli*.**
- **Preparation of immunogen from selected diarrhoeagenic *Escherichia coli* strains.**
- **Assessment of reactogenicity and immunogenicity of immunogen in animal model.**
- **Assessment of protective efficacy offered by immunogen after subsequent immunization and challenge with currently circulating wild type virulent strains of diarrhoeagenic *Escherichia coli*.**

Chapter 3

Objective 1

Selection and characterization of prevalent strains of diarrhoeagenic *Escherichia coli*

1. Introduction

The second leading cause of death for children under five years of age worldwide is diarrheal illnesses, which are also a key factor in the need for medical attention [1, 2]. DEC mostly affects children worldwide and is a significant contributing factor to diarrheal outbreaks in underdeveloped nations [3]. Every year, DEC results in over 300 million infections and almost 200,000 fatalities worldwide [4]. In impoverished nations, diarrheal illnesses (DEC) account for 30–40% of diarrheal episodes, and 120,000 paediatric fatalities worldwide were attributed to DEC in 2011 [3]. Enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) are the five primary pathotypes of DEC [5]. Watery diarrhea is linked to ETEC in infants and travelers; EPEC is linked to both acute and chronic diarrhea in the pediatric population; an EIEC infection causes dysentery with bloody mucoid diarrhea, which is the same diarrhea caused by *Shigella* sp.; EHEC causes haemorrhagic colitis and hemorrhagic uremic syndrome (HUS); and an EAEC infection is linked to both acute and persistent diarrhea in patients with impaired immune systems and in developing nations [6]. Because of the improper use of antibiotics, multi-drug resistance is becoming a concern for DEC pathotypes. As a result, vaccinations and other preventative measures can effectively lower the incidence of DEC-mediated diseases [7, 8]. Currently, there is no licensed combination vaccination against DEC that can be used for public health purposes.

OMVs are spherical proteoliposomes that are formed from the outer membrane of Gram-negative bacteria. They have a diameter of roughly 20 to 200 nm [9- 12]. These vesicles are made up of several periplasmic and cytoplasmic substances, such as metabolites, proteins, DNA, and RNA [13- 15]. It has been demonstrated that OMVs induce significant mucosal immune responses [16]. Vaccines based on OMVs have previously demonstrated efficacy against *Neisseria*, *Shigella*, and *Salmonella* species [17- 20].

The clinical presentation and disease epidemiology impact the need for vaccinations. Selecting an antigen requires a thorough understanding of the pathogen [21]. In this work, OMVs of five pathotypes of DEC (EPEC, ETEC, EAEC, EIEC, and EHEC) were combined to create a combined pentavalent outer membrane vesicles-based immunogen (POMVs). The purpose of the study is to evaluate POMV's potential as a promising vaccine candidate for reducing infections caused by five common DEC pathotypes. Clinical isolates of DEC were collected and strains were selected for immunogen formulation based on genotypic and phenotypic characteristics.

2. Methods and Materials

2.1 Bacterial strains and culture conditions

DEC bacterial strains namely EPEC, ETEC, EAEC, EIEC and EHEC were collected from ICMR- National Institute of Cholera and Enteric Diseases (NICED), Kolkata strain repository. All the strains were preserved in 20% glycerol in Brain Heart Infusion Broth (BHIB, Difco, USA) at -80ºC. For experimentation, strains were grown in Tryptic Soy Agar (TSA, Difco, USA) plates or in Tryptic Soy Broth (TSB, Difco, USA) at 37ºC under constant shaking conditions (100 rpm). For further studies, MacConkey media and antibiotic supplemented plates were used [22].

2.2 Genotypic characterization of the strains

Strains were characterized by PCR-based method. The detailed description of the reaction cycles and the primers used in this study are described in the table below. Briefly each PCR tube contained 12.5µl of Master Mix (Takara), forward primer of 1µl, reverse primer 1µl, template 1 µl and water 9.5 µl to make final volume of 25 µl.

Table 1: Primers and detailed reaction cycles for confirmation of DEC strains used in this study

Genes	Primer (5′-3′)	Initial denaturation		Denaturation		Annealing		Extension		Final extension		Number of cycles	Amplicon size (bp)	Reference
		Temp. (°C)	Time (mins)	Temp. (°C)	Time (sec)	Temp. (°C)	Time (sec)	Temp. (°C)	Time (sec)	Temp. (°C)	Time (mins)			
***stx*$_1$**	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	95	3	95	20	58	40	72	90	72	5	35	614	[23]
***stx*$_2$**	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	95	3	95	20	58	40	72	90	72	5	35	779	[23]
AatA	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	96	4	95	20	57.5	20	72	60	72	7	35	630	[24]
bfpA	GGAAGTCAAATTCATGGGGG GGAATCAGACGCAGACTGGT	96	4	95	20	57.5	20	72	60	72	7	35	367	[24]
eae	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	96	4	95	20	57.5	20	72	60	72	7	35	881	[24]
ipaH	GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	95	5	95	60	52	60	72	60	72	10	30	619	[25]
estA	GCTAAACCAGTAGAGTC CACCCGGTACAAGCAGG	95	3	95	50	52	40	72	40	72	5	30	149	[26]
eltB	CACACGGAGCTCCTCAG CAAACTAGTTTTCCATACTG	95	3	95	50	52	40	72	40	72	5	30	324	[26]

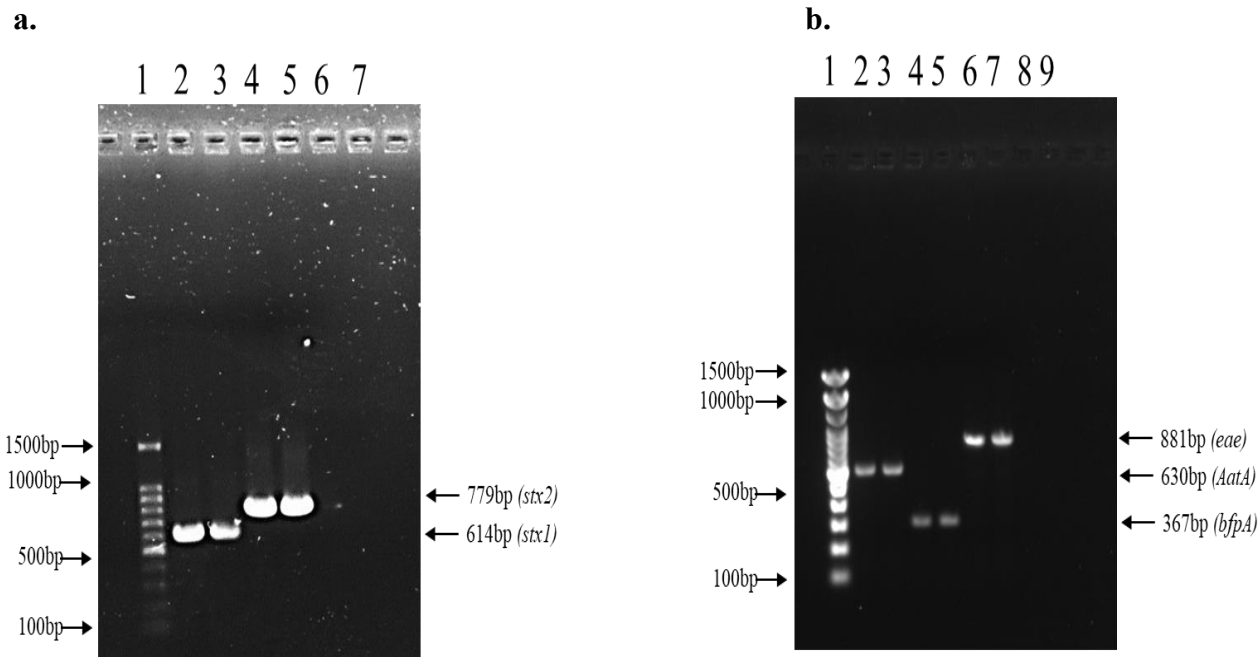
2.3 Antibiogram of DEC strains

Bacterial susceptibility to various antibiotics was determined by the standard disk diffusion method with commercial antimicrobial disks (BD, Sparks, MD). Antibiotic disks used in this study were *Cefotaxime* (CTX 30), *Ceftriaxone* (Ci 30), *Imipenem* (I10), *Ciprofloxacin* (Cf5), *Neomycin* (N30), *Cefixime* (CFX5), *Trimethoprim* (TR30), *Co-trimoxazole* (CO25), *Tetracyclin* (T30), *Streptomycin (S10), Methicillin (M30), Ampicillin* (A10), *Norfloxacin* (Nx10), *Clarithromycin* (Cw15), *Sulfamethoxazole (SXT23.75), Kanamycin (K30)*. *Escherichia coli* ATCC25922 was used as control strain for susceptibility study.

3. Results

3.1 Genotypic confirmation of DEC strains

EHEC strains were found to be positive for both *stx*$_1$ and *stx*$_2$ genes. EAEC strains were confirmed by the presence of *AatA* (CVD432) gene. All the EPEC strains used in this study were found to be typical EPEC as all of them gave positive results for both *bfpA* and *eae* genes. EIEC strains were confirmed by the presence of *ipaH* gene. Only two ETEC strains, which gave positive results for both the LT and ST toxin (*eltB* and *estA*) were subsequently chosen for immunogen preparation (Figure 1).



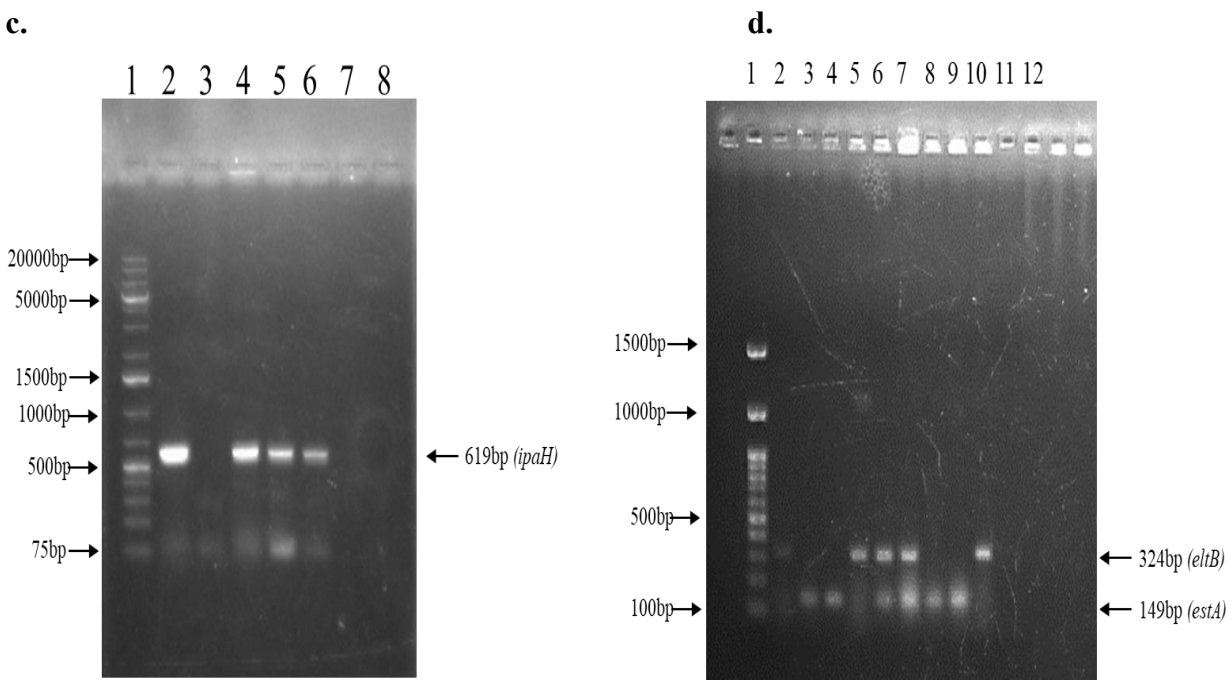


Figure 1: Genotypic Characterization of DEC by PCR. (a) EHEC *stx1 and stx2* gene positive strains- lane 1: marker, lane 2: EHEC PD124, lane 3: EHEC VT3, lane 4: EHEC PD124, lane 5: EHEC VT3, lane 6: *S*.Typhimurium SL1344 (negative control) **(b)** EAEC *AatA* gene positive and EPEC *bfpA* and *eae* gene positive strains- lane 1: marker, lane 2: EAEC DSM 411, lane 3: EAEC BCH 04060, lane 4: EPEC BCH 8865, lane 5: EPEC BCH 9327, lane 6: EPEC BCH 8865, lane 7: EPEC BCH 9327, lane 8: *S*.Typhimurium SL1344 (negative control) **(c)** EIEC *ipaH* gene positive strains- lane 1: marker, lane 2: EIEC IDH 10106, lane 3: *S*.Typhimurium SL1344 (negative control), lane 4: EIEC BCH 10790, lane 5: EIEC PT1, lane 6: EIEC IDH 5468 **(d)** ETEC *eltB* and *estA* gene positive strains- lane 1: marker, lane 2: ETEC IDH 13003, lane 3: ETEC IDH 04557, lane 4: ETEC IDH 07086, lane 5: ETEC IDH 00882, lane 6: ETEC BCH 8031, lane 7: ETEC H10407, lane 8: ETEC IDH 02316 ,lane 9: ETEC BCH 00318, lane 10: ETEC IDH 02483, lane 11: *S*.Typhimurium SL1344 (negative control).

Table 2: PCR results of DEC Strains

Strains	*stx1*	*stx2*	*AatA* (CVD432)	*bfpA*	*eae*	*ipaH*	*estA*	*eltB*
EHEC PD124	+	+	-	-	-	-	-	-
EHEC VT3	+	+	-	-	-	-	-	-
EAEC DSM411	-	-	+	-	-	-	-	-
EAEC BCH 04060	-	-	+	-		-	-	-
EPEC BCH 8865	-	-	-	+	+	-	-	-
EPEC BCH 9327	-	-	-	+	+	-	-	-
EIEC IDH 10106	-	-	-	-	-	+	-	-
EIEC BCH 10790	-	-	-	-	-	+	-	-
EIEC PT1	-	-	-	-	-	+	-	-
EIEC IDH 5468	-	-	-	-	-	+	-	-
ETEC IDH 13003	-	-	-	-	-	-	-	+
ETEC IDH 04557	-	-	-	-	-	-	+	-
ETEC IDH 07086	-	-	-	-	-	-	+	-
ETEC IDH 00882	-	-	-	-	-	-	+	-
ETEC 4266	-	-	-	-	-	-	+	+
ETEC H10407	-	-	-	-	-	-	+	+
ETEC IDH 02316	-	-	-	-	-	-	+	-
ETEC BCH 00318	-	-	-	-	-	-	+	-
ETEC IDH 02483	-	-	-	-	-	-	-	+

3.2 Antibiotic susceptibility test of DEC strains

All the strains selected for this work were tested for susceptibility against different antibiotics. All the strains were resistant to *Cefixime* and *Clarithromycin*. A large number of strains were also resistant to *Cefotaxime*. Except a few, most of the strains were sensitive to *Ceftriaxone*, and *Sulphamethoxazole* (Table 3).

Table 3: Antibiogram of DEC Strains

S Sensitive **R** Resistant **I** Intermediate

Strains / Antibiotics	E. coli ATCC 25922	EPEC BCH 8865	EPEC BCH 9327	ETEC 4266	ETEC H10407	EIEC IDH 10106	EIEC BCH 10790	EAEC BCH 04060	EAEC DSM411	EHEC PD124	EHEC VT3
Cefotaxime	S	R	R	R	R	R	R	R	R	S	R
Ceftriaxone	S	R	R	I	S	S	S	S	S	S	S
Imipenem	S	R	R	R	R	R	S	I	S	S	I
Ciprofloxacin	S	R	I	S	S	R	S	S	S	S	S
Neomycin	S	I	I	I	R	R	S	S	R	S	S
Cefixime	S	R	R	R	R	R	R	R	R	R	R
Trimethoprim	S	R	S	S	S	R	R	R	S	S	S
Lomefloxacin	S	R	S	S	S	R	S	S	S	S	S
Co trimoxazole	S	S	S	S	S	R	R	S	S	S	S
Tetracyclin	S	R	S	S	S	R	R	S	R	S	S
Streptomycin	S	R	S	S	I	R	R	I	R	S	I
Methicillin	S	I	R	I	R	R	R	R	I	I	R
Ampicillin	S	R	R	I	R	R	R	R	S	I	R
Norfloxacin	S	R	I	I	S	R	S	S	S	I	S
Clarithromycin	S	R	R	R	R	R	R	R	R	R	R
Sulfamethoxazole	S	S	S	S	S	R	R	S	S	S	S
Kanamycin	S	I	I	I	R	R	I	I	I	I	I

Based on the genotypic and phenotypic characterization, the following strains were selected for immunogenicity study and challenge study (Table 4).

Table 4: DEC strains selected for immunogenicity study and challenge study

Strain name	Purpose of use	DEC subtype	Abbreviation
H10407	Immunogen preparation	Enterotoxigenic *E. coli*	ETEC
PD124	Immunogen preparation	Enterohaemorrhagic *E. coli*	EHEC
DSM411	Immunogen preparation	Enteroaggregative *E. coli*	EAEC
BCH8865	Immunogen preparation	Enteropathogenic *E. coli*	EPEC
IDH10106	Immunogen preparation	Enteroinvasive *E. coli*	EIEC
4266	Challenge study	Enterotoxigenic *E. coli*	ETEC
VT3	Challenge study	Enterohaemorrhagic *E. coli*	EHEC
BCH04060	Challenge study	Enteroaggregative *E. coli*	EAEC
BCH9327	Challenge study	Enteropathogenic *E. coli*	EPEC
BCH10790	Challenge study	Enteroinvasive *E. coli*	EIEC

4. Discussion

In impoverished countries, diarrheal illnesses are a leading cause of childhood mortality, with children under five years old being the most vulnerable [27]. One of the key reasons behind illnesses linked to diarrhea involves the DEC pathotypes. Vaccination is a useful strategy to reduce infections caused by DEC pathotypes because of the rise of multi-drug resistant (MDR) bacteria [28, 29]. Eighty percent of human diarrheal illnesses in Africa are caused by DEC, which causes 70,000 fatalities annually, the most of which are in children under five. Because of their exploration and hand-to-mouth behavior, children aged 12 to 18 months are more vulnerable to DEC-mediated illnesses. It has been claimed that 34–60% of children in Kenya had DEC infections. In Egypt, community-level DEC infections in children under five years old were reported to be 20.6%. It was reported that 38.2% of children in Uganda with acute diarrhea had DEC infections [30]. According to a study done in Kolkata, India, the incidence of DEC infections was 12%. EAEC infections were shown to be the most common in DEC-infected patients, followed by ETEC and EPEC infections. EAEC and EPEC infections were shown to be significantly higher in children two years of age and older than other DEC pathotypes [31].

According to genotypic and phenotypic properties, strains are selected for immunogen preparation and challenge study following subsequent analysis. On the basis of both *bfpA* and *eae* gene expression, typical EPEC strains were chosen. Based on the expression of the LT and ST toxin genes, two strains of ETEC were identified: ETEC H10407 and ETEC BCH 8031. EAEC strains were chosen based on CVD432 gene expression. The selection of EIEC strains was based on *ipaH* expression. The expression of the *stx1* and *stx2* genes was used to select EHEC strains. Antibiotic susceptibility test was performed for characterizing the DEC strains phenotypically so as to select strains for OMVs-based immunogen preparation and challenge study. Antibiotic susceptibility test also indicates the suitable choice of antibiotics for treatment

of severe cases of DEC infection. Clarithromycin and Cefixime resistance was present in every strain. Additionally, a sizable portion of strains exhibited Cefotaxime resistance. With a few exceptions, the majority of the strains were susceptible to Sulphamethoxazole and Ceftriaxone.

Chapter 4

Objective 2

Preparation of immunogen from selected diarrhoeagenic *Escherichia coli* strains

1. Introduction

Although *Escherichia coli* (*E. coli*) is a typical component of the human gut microbiota, several of its subtypes have been linked to a wide spectrum of human illnesses. *E. coli* is classified as extraintestinal pathogenic *E. coli* (ExPEC) and intestinal or diarrhoeagenic *E. coli* (DEC) depending on the kind of infection [1- 3]. Globally, DEC is responsible for around 200,000 fatalities and more than 300 million infections annually [4]. In underdeveloped nations, diarrheal illnesses (DECs) account for 30–40% of diarrheal episodes, and 120,000 paediatric fatalities worldwide were attributed to DECs in 2011 [5]. Inappropriate antibiotic usage is giving rise to strains of *E. coli* that are resistant to many drugs. In the post-antibiotic time period, preventative measures such as vaccinations can be extremely helpful in lowering the health burden caused by DECs. Presently, scientists are working to create DEC vaccines, but are encountering significant obstacles because there are numerous DEC serotypes and insufficient generation of a powerful gut immune response [6- 8]. Significant immunogenicity, broad-spectrum protective effectiveness, and reduced reactogenicity are desirable qualities in a vaccine candidate. In order to avoid diseases linked to diarrhea, combination-based vaccinations are thought to be a beneficial strategy. A single licensed combination vaccination against DEC is not yet accessible for use in public health.

Bacterial transport systems called outer membrane vesicles are found in nature and are able to transport lipids, proteins, and nucleic acids to neighboring cells, or biofilms. The most obvious application for OMVs is to supply antigens for immunization. This method was originally developed as a vaccination against *N. meningitidis* serogroup B. Since then, OMVs have been produced from a range of bacterial strains to be used in the facilitation of antigen presentation from homologous or heterologous strains [9].

For producing a combined pentavalent outer membrane vesicles-based immunogen (POMVs), the OMVs of five pathotypes of DEC (EPEC, ETEC, EAEC, EIEC, and EHEC) were mixed. Examining the potential of POMVs as a viable vaccine candidate to prevent infections caused by five prevalent DEC pathotypes is the purpose of the study. Morphological characteristics of the OMVs isolated from each of the five pathotypes of DEC were determined. Following determination of the properties of OMVs from the five pathotypes of DEC, OMVs of each of the pathotypes were mixed in equal proportions for formulation of POMVs vaccine candidate.

2. Methods and Materials

2.1 Preparation of pentavalent outer-membrane vesicles (POMVs)

With some small modifications OMVs were isolated from five subgroups of DEC [10]. In summary, 10 mL of overnight cultured DEC subgroups were added to 1 liter of TSB, which was then grown to log phase (8 hours) at 37º C with continuous shaking. Centrifugation (8000 X g for 15 and 30 minutes consecutively) was used to eliminate the bacteria, and the supernatants were then collected. Subsequently, the supernatants were filtered using 0.45 µm and 0.22 µm pore size filters (Millipore, USA) in that order. In order to verify the existence of live bacteria in the culture supernatant, 100 µL of the obtained filtrate was plated on TSA plates and incubated overnight at 37º C. Protease cocktail inhibitor (Roche, Switzerland) was added to the filtrate to stop protein degradation. Subsequently, the supernatant underwent ultracentrifugation for 4 hours at 4º C using a Hitachi P27A-1004 rotor operating at 140,000 X g. The ultracentrifugation-obtained OMV pellets were resuspended in phosphate buffered saline (PBS, 7.4). Density gradient centrifugation was used to further purify OMVs. The reconstituted OMVs were carefully placed in a centrifuge tube with a gradient of 10%–50% sucrose. Following that, the tubes were centrifuged for five hours at 4º C using a swinging bucket rotor (Beckman-Coulter; SW 32.1 Ti) at 150,000 X g. Centrifuging at 150,000 X g for two hours, the OMV fractions between 20% to 30% sucrose were obtained. The resultant pellet was again filtered using 0.22 µm pore size filters and resuspended in PBS [11]. Following the extraction process, the purified OMVs of the DEC subgroups were either stored individually or combined in a 1:1:1:1:1 ratio according to the protein content to produce a final concentration of POMVs of 10 µg/100 µL PBS, which was then stored at -80º C for future experiments (Figure 1).

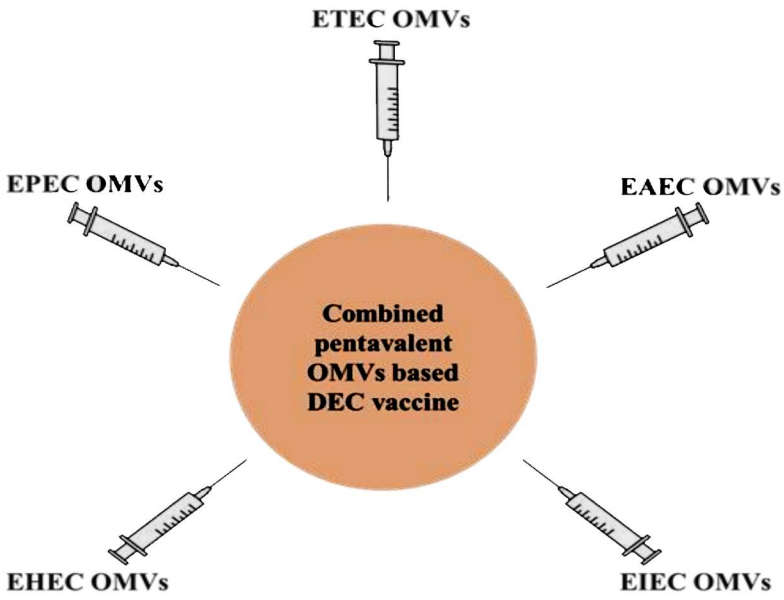


Figure 1: Schematic representation of formulation of pentavalent outer-membrane vesicles (POMVs) immunogen

2.2 Transmission electron microscopy (TEM) analysis by negative staining of OMVs

A 5 µL aliquot of OMVs was deposited on carbon-coated grids and left for one minute. It was then rinsed with two drops of Tris-HCL buffer. OMVs were negatively stained for 30 seconds using a 2% aqueous solution of uranyl acetate after the excess buffer was blotted off. After blotting away extra stain, the grids were allowed to air dry. At 100kV, a JEOL JEM-2100 transmission electron microscope was used to analyze the negatively stained OMVs [12].

2.3 Particle size analysis of OMVs by dynamic light scattering (DLS)

By using DLS, size distribution OMVs were measured. Surface charge of OMVs was analyzed by zeta potential. To put it briefly, OMVs were adjusted to a concentration of 0.1 mg/mL in milli-Q water. Next, using the Zetasizer Nano series (Malvern, Worchestershire, UK), the size distribution and zeta potential of several OMVs were analyzed [13].

3. Results

3.1 TEM analysis of OMVs secreted by DEC subtypes

TEM pictures showed that the OMVs of every DEC subgroup that were produced during the log phase had a spherical form. A distinct lipid bilayer is also seen in the OMVs of each of the DEC subgroups (Figure 2).

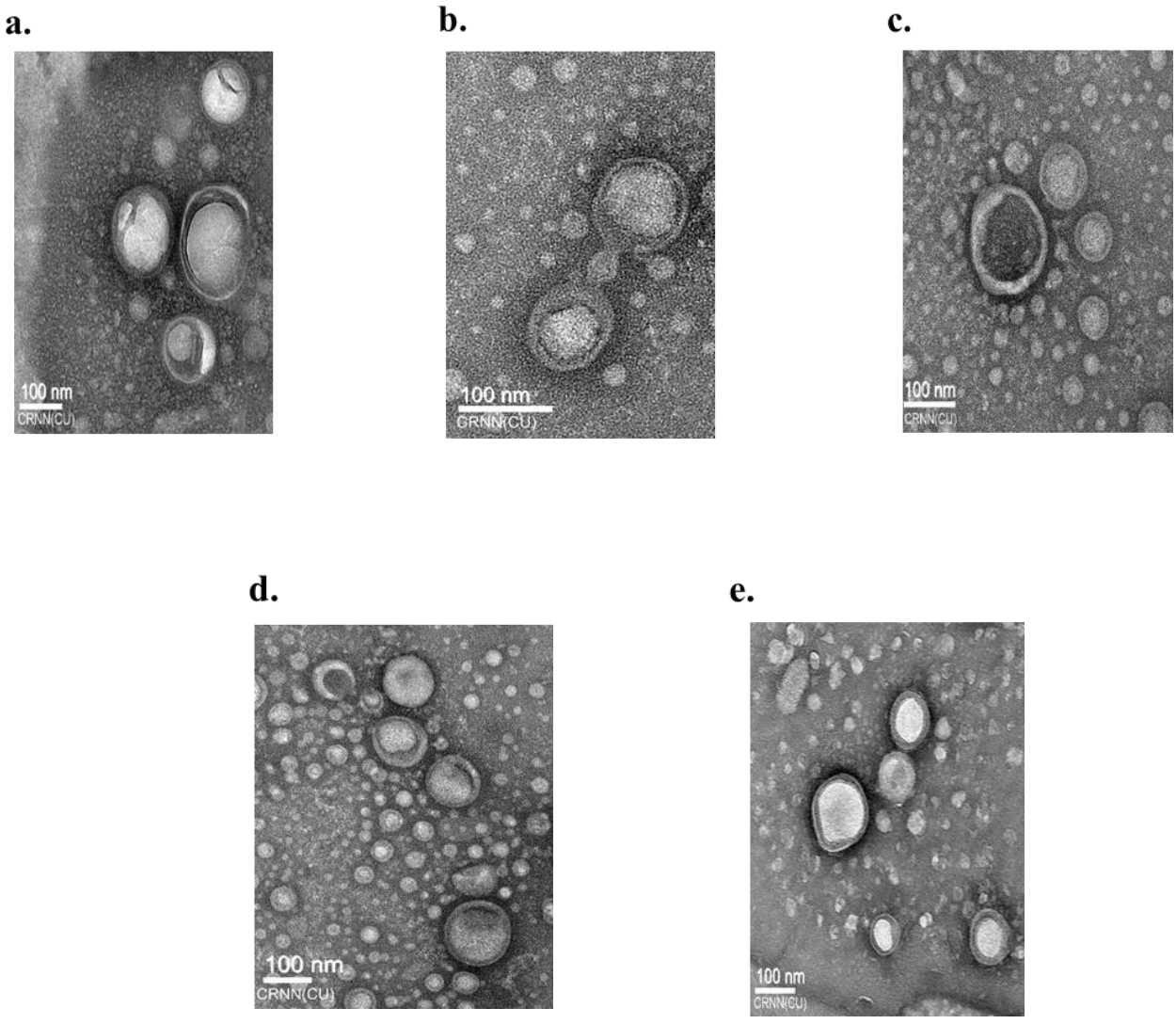


Figure 2: Transmission electron microscopic images of OMVs isolated from DEC subtypes: (a) EPEC OMVs, (b) ETEC OMVs, (c) EAEC OMVs, (d) EIEC OMVs, (e) EHEC OMVs.

3.2 DLS analysis of OMVs secreted by DEC subtypes

According to dynamic light scattering analysis, the OMV peaks of the DEC subgroups varied in size from roughly 36 nm to 101 nm. The peak of EPEC OMVs was found to be 101.10 nm, of ETEC OMVs was 50.77 nm, of EAEC OMVs was 45.30 nm, of EIEC OMVs was 36.73 nm and of EHEC OMVs was 51.39 nm (Figure 3).

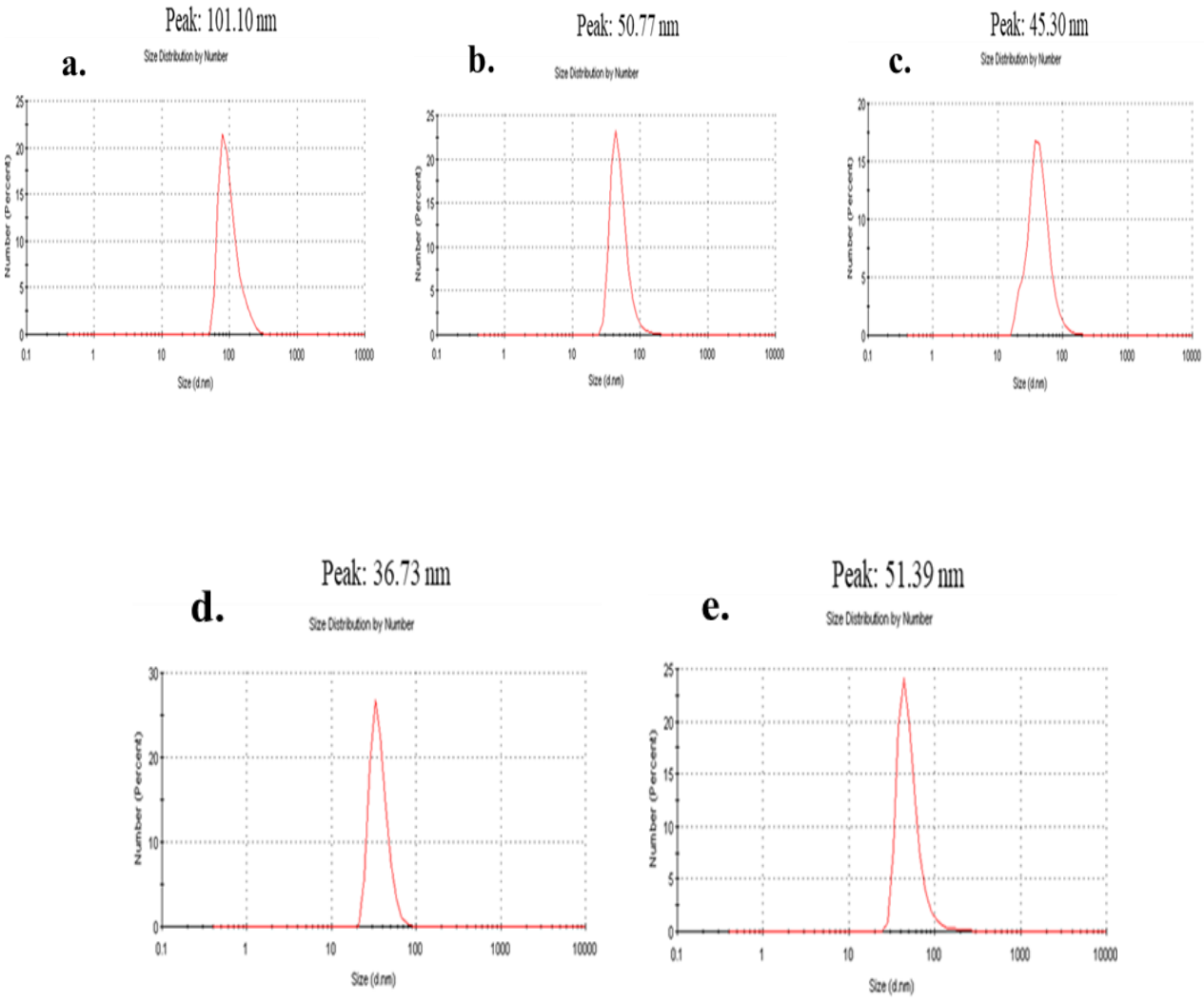


Figure 3: Dynamic light scattering analysis of OMVs from DEC subtypes with peaks ranging from 36.73 nm to 101.10 nm: (a) EPEC OMVs, (b) ETEC OMVs, (c) EAEC OMVs, (d) EIEC OMVs, (e) EHEC OMVs.

3.3 Zeta potential of OMVs secreted by DEC subtypes

All of the OMVs showed a net surface negative charge range from -0.145 mV to -5.3 mV, suggesting that these particles are stable in nature. The zeta potential of EPEC OMVs was found to be -1.6 mV, of ETEC OMVs was -5.3 mV, of EAEC OMVs was -3.69 mV, of EIEC OMVs was -0.198 mV and of EHEC OMVs was -0.145 mV. Zeta-potential measurements were also obtained for evaluation of physiochemical features of OMVs (Table 1).

Table 1: Zeta potential of OMVs isolated from DEC subtypes

Serial number	DEC subtype	Zeta potential (mV)
1.	EPEC	-1.600
2.	ETEC	-5.300
3.	EAEC	-3.690
4.	EIEC	-0.198
5.	EHEC	-0.145

4. Discussion

One of the main causes of diarrhea-related diseases is DEC pathotypes. Vaccination is an advantageous way for decreasing infections caused by DEC pathotypes associated with the rise of multi-drug resistant (MDR) bacteria [14, 15]. The effectiveness of polysaccharide conjugate vaccines against O111 *E. coli* has been previously examined. Phase 1/2 trials revealed that ETVAX, an inactivated whole-cell vaccine, was safe and immunogenic. It is made up of four recombinant *E. Coli* strains that overproduce adhesins, as well as recombinant hybrid B subunits of heat-labile toxin and cholera toxin [16, 17]. However, none of these vaccinations were successful in offering protection against several DEC subtypes and broad-spectrum immunogenicity. Vaccines based on combinations are thought to be a superior method of preventing illnesses linked to diarrhea [18]. According to a recent study, a formalin-killed whole-cell vaccine was efficient in eliciting immunogenicity and offered protection against five primary subtypes of diarrheagenic *E. coli* when it was delivered subcutaneously to BALB/c mice together with cholera toxin B subunit as an adjuvant [8].

Bacterial transport systems called outer membrane vesicles are found in nature and are able to transport lipids, proteins, and nucleic acids to neighbouring cells, or biofilms. The most obvious application for OMVs is to supply antigens for immunization. This method was originally developed as a vaccination against *N. meningitidis* serogroup B. Since then, OMVs have been produced from a range of bacterial strains to be used in the facilitation of antigen delivery from homologous or heterologous strains. Two approved OMV vaccines, VA-MENGOC-BC™ and Bexsero™, provide defence against the invasive Neisseria meningitidis serogroup B strain (MenB) [9]. Because OMVs contain LPS, peptidoglycan, flagellin, and lipoproteins, they naturally have adjuvant qualities [19, 20] that boost their immunogenicity without the requirement for additional adjuvants. Therefore, in order to offer broad spectrum protection

against DEC pathotypes, OMVs-based immunogen isolated from EPEC, ETEC, EAEC, EIEC, and EHEC have been developed in this study.

Transmission electron microscopy (TEM) analysis demonstrated the spherical nature of OMVs surrounded by a distinct lipid bilayer. The OMV peaks of the DEC subgroups ranged in size from about 36 nm to 101 nm, based on dynamic light scattering studies. The OMVs isolated from each of the DEC subtypes demonstrated a homogenous distribution of sizes as was evident from dynamic light scattering (DLS) analysis. These particles appear to be stable in nature, as was evident from zeta potential analysis which demonstrated an overall negative surface charge of OMVs, values of which ranged between -0.145 mV to -5.3 mV. Additionally, zeta-potential measurements were acquired in order to assess the physiochemical characteristics of OMVs. The estimated protein content of the OMVs was used to mix OMVs from five DEC subgroups in equal amounts (1:1:1:1:1) to formulate POMVs immunogen, which had a final concentration of 10 µg POMVs/100 µL of PBS and stored at -80º C to be used in further experiments.

Chapter 5

Objective 3

Assessment of reactogenicity and immunogenicity of immunogen in animal model

1. Introduction

One of the initial bacteria to settle in the newborn's gut after birth is *Escherichia coli* (*E. coli*), which is a component of the typical microbiota of the human intestine [1]. A number of human disorders have been linked to specific *E. coli* subgroups, which are regarded as harmful. Pathogenic *E. coli* are classified as extraintestinal *E. coli* (ExPEC) and intestinal or diarrhoeagenic *E. coli* (DEC) depending on the kind of infection [2]. Mostly affecting children worldwide, diarrheal episodes are primarily caused by DEC in developing nations. Enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) are the five primary pathotypes of DEC. EAEC infection causes acute and persistent watery diarrhea in immunocompromised patients and in developing countries; EHEC causes hemorrhagic colitis and hemorrhagic uremic syndrome (HUS); EIEC infection causes dysentery characterized by bloody mucoid diarrhea, similar to that caused by *Shigella* sp.; ETEC is linked to both acute and chronic watery diarrhea in infants and travellers; and in the paediatric population, acute and persistent watery diarrhea are linked to EPEC [3- 5].

Numerous studies have shown that DEC strains from symptomatic and asymptomatic patients exhibit antibiotic resistance. This is an increasingly widespread problem. The increasing frequency of extended-spectrum β-lactamases (ESBL)-producing bacteria with the bla*CTX-M* genotype in developing countries is especially remarkable, considering their elevated rates of sickness and death [6]. Because of the improper use of antibiotics, multi-drug resistance is becoming a concern for DEC pathotypes. As a result, vaccinations and other preventive therapies can effectively lower the incidence of DEC-mediated diseases [7, 8]. A single licensed combined vaccine for DEC is not yet accessible for use in public health.

OMVs (outer membrane vesicles) are nanosized proteoliposomes generated from the outer membrane of gram-negative bacteria. They are easily absorbed by mammalian cells and have a strong immunogenic nature, making them a promising candidate for an acellular vaccine. Phospholipids, lipopolysaccharides, peptidoglycan, outer membrane proteins (OMPs), and membrane lipoprotein components that mimic the outer surface of bacteria are examples of natural antigens present in outer membrane. An OMV-based vaccination for human immunization against *Neisseria meningitides* has been approved by the European Medicines Agency (EMA). Previous studies demonstrated protection against recurrent infections in mice inoculated intraperitoneally (i.p.) with *E. coli* OMVs. No combined OMV-based vaccination for the pathotypes of DEC has been produced to date, despite the fact that OMVs have been shown to elicit positive mucosal immune responses [9- 14].

Combination vaccinations are considered to be a beneficial strategy for preventing diseases associated with diarrhea. Thus, five pathotypes of DEC (EPEC, ETEC, EAEC, EIEC, and EHEC) were combined to create combined pentavalent outer membrane vesicles-based immunogen (POMVs) for this investigation. In this study, reactogenicity and immunogenicity induced by intraperitoneal POMVs immunization in adult BALB/c mice was evaluated. The impact of POMVs on the development of systemic and mucosal immune responses within adult mice was also evaluated in this study.

2. Methods and Materials

2.1 Bacterial strains and culture conditions

Using five circulating strains of the DEC subgroups H10407 (ETEC), PD124 (EHEC), DSM411 (EAEC), BCH8865 (EPEC), and IDH10106 (EIEC), pentavalent outer membrane vesicles-based immunogens (POMVs) were created. Five other strains of the DEC subgroups—4266 (ETEC), VT3 (EHEC), BCH04060 (EAEC), BCH9327 (EPEC), and BCH10790 (EIEC)—were employed for challenge experiments. Every strain was acquired from the strain repository located in Kolkata at the ICMR-National Institute of Cholera and Enteric Diseases (NICED). At -80ºC, all strains were maintained in Brain Heart Infusion Broth (BHIB, Difco, USA) containing 20% glycerol. Strains were cultivated for investigation at 37ºC with continuous shaking (100 rpm) in either Tryptic Soy Agar (TSA, Difco, USA) or Tryptic Soy Broth (TSB, Difco, USA) plates. MacConkey medium and plates supplemented with antibiotics were utilized for additional research.

2.2 Animals and housing

From the ICMR-NICED animal house facility, 6–8 week old BALB/c mice were obtained. For the purpose of mating, one male mouse and two female mice were kept together in a cage. Female mice were kept apart from the male during pregnancy and watched until they were born. In independent cages, ten other adult female mice were kept at a temperature of 25 ± 2 º C and a humidity of 65 ± 2%. Sterile food and water were given to the mice *ad libitum*. As directed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, all animal experiments were carried out in accordance with standard operating procedure. The Institutional Animal Ethical Committee (IAEC) of NICED authorized the animal

experimentation protocol under Registration No. 68//Rebi/S/1999/CPCSEA, which is valid until 17/7/2024. The approval number for the protocol is PRO/183/-Jan 2021-24.

2.3 Pyrogenicity and toxicity assay

Four different concentrations of combined OMVs formulation were studied here for pyrogen and toxicity test; 10µg or 20 µg or 30 µg or 40 µg of combined OMVs per 100µl of PBS. The intraperitoneal dose of 10µg of OMVs per 100µl of PBS was finalized for animal immunization after the pyrogenicity assay.

2.4 Mouse immunizations

Six female adult BALB/c mice were vaccinated intraperitoneally (i.p.) on day 0, then received booster shots on days 14 and 28 with POMVs. PBS was given to the control group on the same days. The intraperitoneal vaccination dosage for POMVs was 10 µg/mouse (Figure 1).

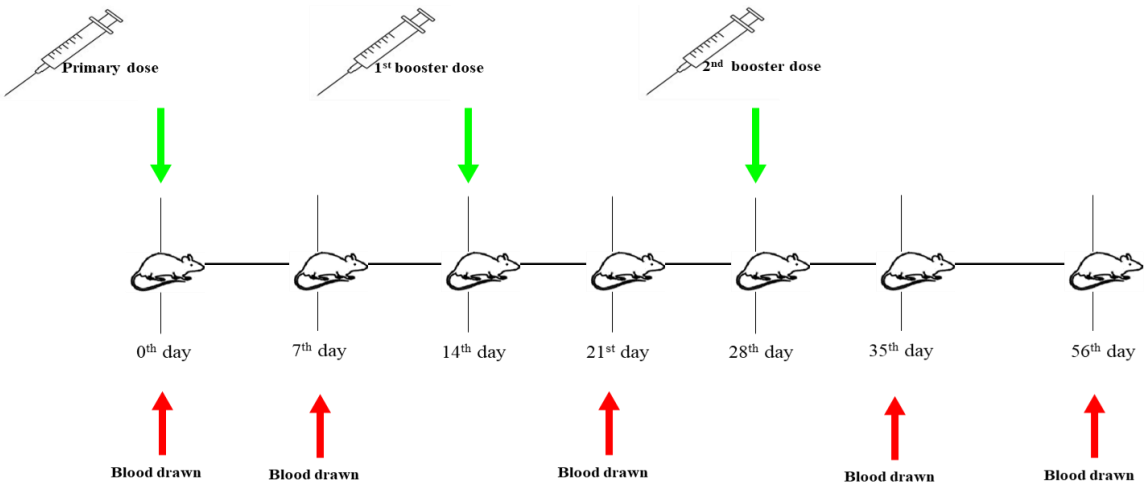


Figure 1: Immunization and sera collection schedule of adult BALB/c mice. Mice were intraperitoneally immunized with POMVs on days 0, 14, and 28 and sera were collected on days 0, 7, 21, 35 and 56.

2.5 Collection of serum and gut lavages

Blood was drawn from the lateral tail vein of mice at the following intervals: 0 days, 7 days, 21 days, 35 days, and 56 days. The obtained blood was placed in BD microtainer tubes (BD, NJ, USA) and centrifuged at 3000 rpm for 10 minutes at 4º C in order to separate the sera. Three mice from each of the POMVs immunized and non-immunized groups were sacrificed one week following the last vaccine, and the small intestines were removed. After that, PBS containing a protease inhibitor (Roche, Sigma, USA) was used to flush the intestinal lumens. Subsequently, the lavage fluid was collected and centrifuged at 1000 X g for 10 min at 4º C. The supernatant was then collected. Before being used again, the intestinal supernatant and sera were both kept at -80º C.

2.6 Preparation of whole cell lysates (WCLs) from DEC subgroups

The process described earlier was followed in order to prepare WCLs from the DEC pathotypes [15]. In short, bacteria were cultivated for a whole night before being centrifuged for ten minutes at 8,000 rpm. After that, a PBS wash and sonication (Heilcher UP100H) were performed. The cells were centrifuged at 10,000 rpm for 10 minutes after being examined for membrane lysis, and the supernatant was then removed. Until they were used again, WCLs were kept at -80º C.

2.7 Extraction of outer membrane proteins (OMPs) from DEC subgroups

With a few minor adjustments, OMPs from DEC pathotypes have been extracted using the previously described approach [16]. In short, cells were extracted after 50 mL of an overnight culture was centrifuged at 8000 rpm for 10 minutes. The separated cells were centrifuged,

resuspended in HEPES with protease inhibitor, and once again cleaned in HEPES buffer (10 mM, pH 7.5). After that, cells were sonicated with a Heilcher UP100H sonicator to induce lysis. Centrifugation at 13,000 X g for 10 minutes was used to extract the cells that were unable to lyse, and the supernatant was then collected. After that, the supernatant was ultracentrifuged for one hour at 4º C at 100,000 X g. The resulting pellets were dissolved in 10 mM HEPES with 1% sarcosyl and incubated at 37 °C for 30 minutes. They were then ultracentrifuged at 100,000 X g for 1 hour at 4 °C. The OMP-containing pellets were then re-suspended in 10 mM HEPES after being washed twice with 10 mM HEPES. A Lowry protein measurement kit (Pierce, USA) was used to determine the protein content of the OMPs. Until needed again, the OMPs were kept at -80º C.

2.8 Lipopolysaccharide (LPS) extraction from DEC subgroups.

Centrifugation at 8500 X g for 20 minutes was used to extract cells from a 50 mL overnight bacterial culture. The cells were then re-suspended in 150 mM NaCl containing 3-[N-morpholino]propen sulfonic acid (MOPS) and incubated with shaking at 65º C in a water bath for 30 minutes. The upper aqueous phase was then collected after centrifugation at 8500 X g for 30 minutes and a 10-minute incubation period on ice. The recovered aqueous phase was combined with 95% chilled ethanol (-20º C), four times the volume of aqueous phase and was kept at -20º C overnight. The next day, LPS was recovered by centrifugation at 8500 X g for 20 min at 4°C. It was then resuspended in milliQ water and kept for further use at -20º C [16].

2.9 SDS-PAGE and western blot

With an AE-6503 SDS-PAGE apparatus (ATTO corporation, Japan), WCLs, OMPs, and OMVs of the immunization and challenge strains were subjected to SDS-PAGE analysis. Samples

were denatured for 10 minutes at 100° C after their protein concentration was estimated using the Lowry method. Protein aliquots with identical quantities were separated using 12% SDS-PAGE and then stained with Coomassie blue.

The samples were electrophoresed in 12% SDS-PAGE before being transferred by semi-dry transfer (AE 6687, ATTO corporation, Japan) onto nitrocellulose membrane (Biorad, USA) for the western blot. Using 35-day POMV-immunized mouse sera obtained after the first immunization, the presence of immunogenic components in the samples was assessed. The blots were developed using BCIP/NBT (MP, USA, Cat# 980621), an alkaline phosphatase substrate.

2.10 ELISA

ELISA was used to assess immunoglobulins from intestinal lavage (sIgA) and serum (IgG and its subtypes IgG1, IgG2a, IgG2b, IgG3, and IgA), as described by Keren [17]. In short, 5 µg/well of bacterial WCLs, 5 µg/well of OMPs, and 5 µg/well of LPS of each strain from which immunogens were generated were separately coated onto disposable polystyrene micro-titre wells (Nunc, Denmark), which were then incubated for 18 hours at 4° C. After washing, wells were blocked for two hours at 37º C using 5% bovine serum albumin (BSA; Sigma Chemical, USA). Plates were treated with serially diluted serum samples and incubated for 1 hour at 37º C after rinsing the wells with PBS-T (PBS with 0.5% Tween-20, Sigma Chemicals, USA). Next, 100 µL of HRP-conjugated goat anti-mouse immunoglobulin was added, and the plates were again incubated for 1 hour at 37º C. The substrate o-phenyl-Di-amine (OPD) was added to each well after PBS wash, and the reaction was stopped after 10 minutes by addition of 100 µL of 2 N sulfuric acid. Using a microplate reader, measurements were made at OD492. With serum from individual mice that had been collected before, during, and after immunization of

both the immunized and non-immunized groups, the experiment was repeated three times for each immunoglobulin [16].

2.11 Analysis of splenic cell population by flow cytometry

On the 35th day following the three immunization doses, the spleens of both immunized and non-immunized mice were separated for flow cytometric examination. After sacrificing the mice in an aseptic manner, the spleens were separated and then disassociated using a cell strainer and a sterile plunger from a 5 mL syringe. The splenocytes were re-suspended in RPMI 1640 containing 10% FBS, 2-ME (50 mM), and antibiotics (5 U/mL penicillin G, 5 µg/mL streptomycin, and 0.1% gentamycin) (Gibco, USA) after the RBCs were lysed using RBC lysis buffer (Sigma, USA). Splenocytes were stained with CD4-phycoerythrin (PE), CD8a-PE, CD19-PE, and isotype control (PE) anti-mouse mAbs to identify the CD4+, CD8a+, and CD19+ splenic cell populations. The cells were evaluated using a BD FACS ARYA III flow cytometer, and the data was analyzed using FACS DIVA software [16].

2.12 Evaluation of the serum bactericidal characteristics of the POMV immunogen and scanning electron microscopy (SEM)

With a few minor adjustments, the previously described technique was followed while performing the serum bactericidal assay [16]. To put it briefly, on the 35th day following primary immunization, sera were taken from POMVs immunized and non-immunized mice. Heat inactivation was applied to collected sera for 30 minutes at 56º C. Every DEC challenge strain was cultivated on TSB. After all strains were cultivated for two hours (O.D.600 = 0.4), the bacterial pellet was removed and resuspended in PBS after centrifugation at 1100 X g for five minutes. To create a 50 µL reaction mixture, a 1:50 dilution of heat-inactivated sera was

added to the bacterial solution, either with or without 25% guinea pig complement. The combination was then incubated for one hour at 37º C. After adding 950 µL of TSB to halt the reaction, the reaction was finally plated on TSA plates using serial dilution to determine the CFU count. Bacteria incubated with non-immunized serum were used as control.

SEM analysis was also used to evaluate the bactericidal characteristics of the POMVs immunized serum. The strains underwent the above-mentioned culture process, were then processed for SEM analysis after being incubated with POMVs immunized and non-immunized serum for 1 hour at 37º C. To summarise, samples were fixed overnight using 3% glutaraldehyde. After dehydrating the samples in increasing alcohol grades (30, 50, 70, 90, and 100%) the following day, the samples were chemically dried using hexamethyldisilazane (HMDS) by progressively increasing the HMDS ratio (2-part ethanol: 1-part HMDS; 1-part ethanol: 1-part HMDS; 1-part ethanol: 2-part HMDS). After treating the samples with 100% HMDS and letting them evaporate in a fume hood overnight, the samples were mounted on specimen stubs, coated with gold sputter, and their pictures were examined using a Quanta 200 SEM (FEI, Netherlands).

2.13 Statistical analysis

The data in the study are expressed as mean ± S.D. Two-way analysis of variance (ANOVA) was used to compare data from three or more groups. A Student's t test was employed to examine two sets of grouped data. For the final computation, the means of three separate experiments were used. A significant level was defined as the probability of $p<0.05$ or $p<0.01$ or $p<0.001$. Statistical analyses were conducted using Microsoft Excel and GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA).

3. Results

3.1 Pyrogenicity and toxicity assessment of immunogen

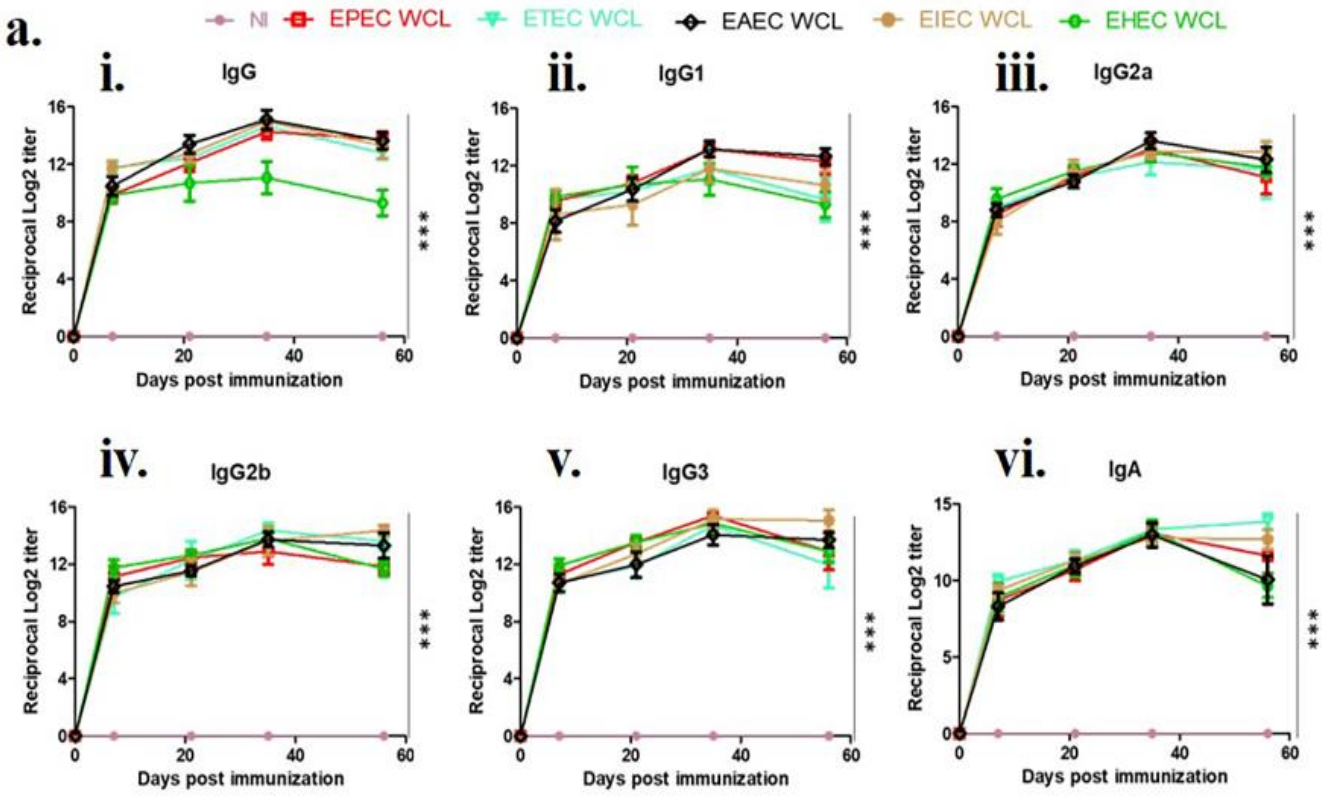
Intraperitoneal dose of 10µg of OMVs per 100µl of PBS was finalized for animal immunization after the pyrogenicity assay as mice showed no signs of lethargy, ruffled fur, and weight loss (Table 1). When 20 µg of combined OMVs /100µl of PBS were i.p. administered to the mice, 2-3 grams of weight loss were noted and on administering 30 µg of combined OMVs /100µl of PBS, 4-5 grams of weight loss, ruffled fur and lethargy were noted. On i.p. administration of 40 µg of combined OMVs /100µl of PBS all the mice died.

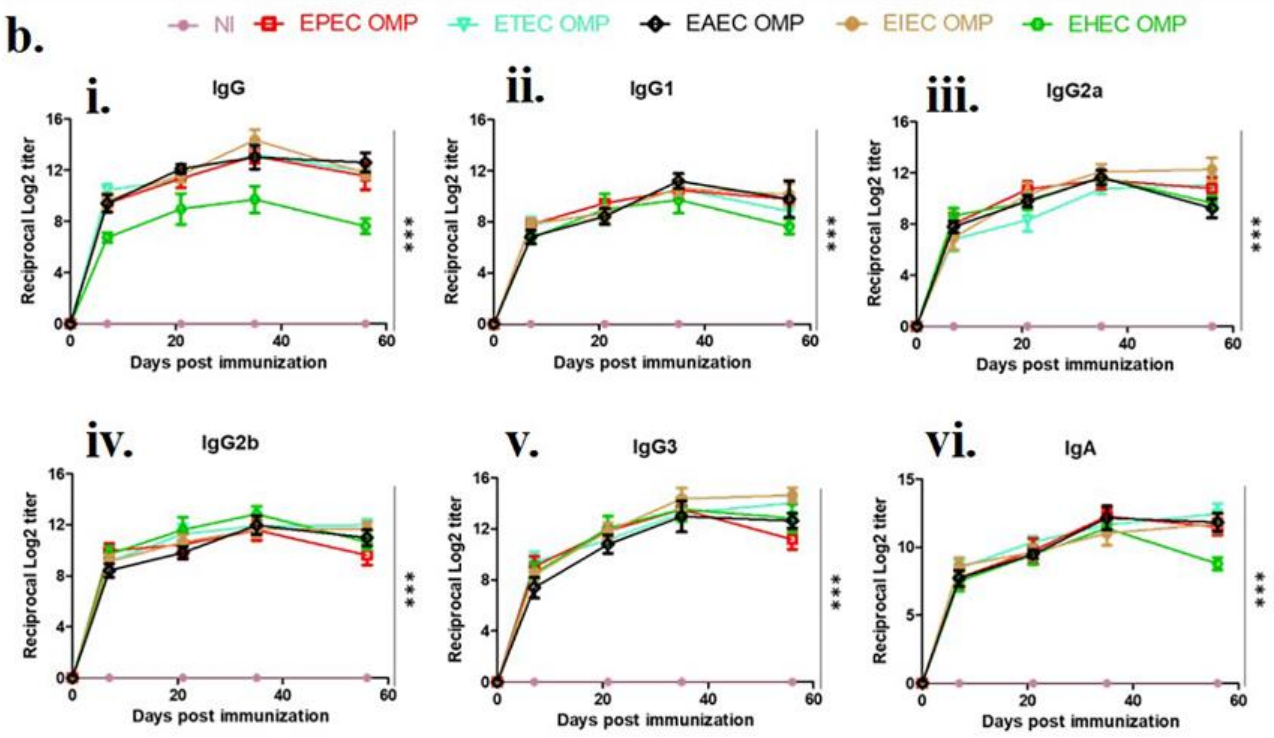
Table 1: Pyrogenicity and toxicity test to standardize immunogen dose

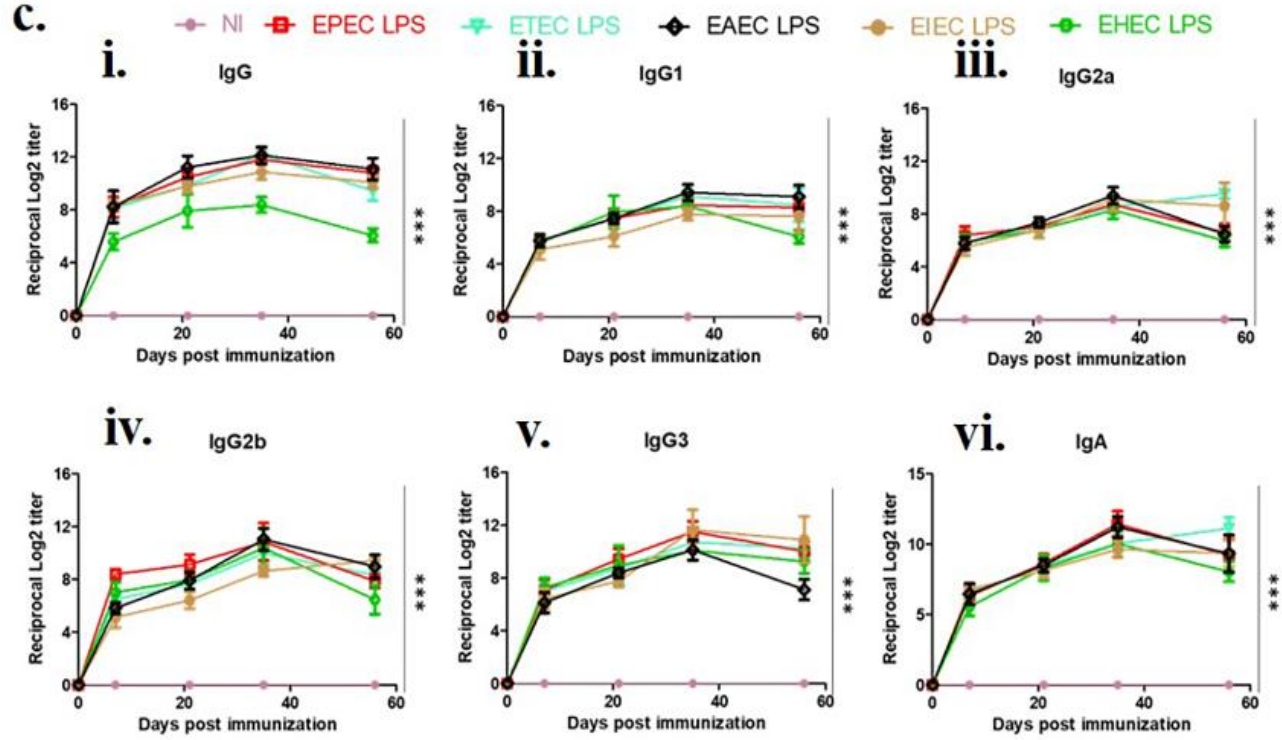
Group (6 mice in each group)	IP Dose	Parameter studied	Duration of the study	observation
1	100µl of PBS	Weight loss	3 weeks	No change
		Ruffled fur		No change
		lethargy		No change
		Death		No change
2	**10µg of combined DEC OMVs per 100µl of PBS**	Weight loss	3weeks	No change
		Ruffled fur		No change
		lethargy		No change
		Death		No change
3	**20µg of combined DEC OMVs per 100µl of PBS**	Weight loss	3 weeks	2 to 3 gram of weight loss; recovered after 2 weeks
		Ruffled fur		No change
		lethargy		lethargic,; recovered after 2 weeks
		Death		No death
4	**30µg of combined DEC OMVs per 100µl of PBS**	Weight loss	3 weeks	4 to 5 gram of weight loss; recovered after 2 weeks
		Ruffled fur		Fur get ruffled after one week; recovered after 3 weeks
		lethargy		lethargic,; recovered after 2 weeks
		Death		No death
5	**40µg of combined DEC OMVs per 100µl of PBS**	Weight loss	3 weeks	
		Ruffled fur		
		lethargy		
		Death		All are dead in 24 hrs

3.2 Immunogenicity assessment elicited by POMVs

After three intraperitoneal immunization doses (10 µg of OMVs/mice) with a 14-day interval between immunizations, the extent of immunogenicity induced by POMVs was ascertained. For comparable measurements, PBS was given intraperitoneally to the control group. Immunogenicity of serum antibodies was noted for 56 days following the initial immunization. The findings showed an increase in the generation of serum antibodies against LPS, OMPs, and WCLs isolated from homologous DEC subtypes, specifically IgG, IgG1, IgG2a, IgG2b, IgG3, and IgA. The maximum immunogenicity was seen 35 days after initial immunization and plateaued thereafter (Figure 2, a-c). When compared to non-vaccinated control groups, the intestinal lavage of immunized mice exhibited significantly greater levels of secretory IgA (sIgA) against LPS, OMPs, and WCLs of each of the five DEC subtypes, indicating a considerable activation of mucosal immune response (Figure 2d).




b.
NI
EPEC OMP
ETEC OMP
EAEC OMP
EIEC OMP
EHEC OMP
i.
IgG
ii.
IgG1
iii.
IgG2a
iv.
IgG2b
v.
IgG3
vi.
IgA
Reciprocal Log2 titer
Days post immunization
***


c.
NI
EPEC LPS
ETEC LPS
EAEC LPS
EIEC LPS
EHEC LPS
i.
IgG
ii.
IgG1
iii.
IgG2a
iv.
IgG2b
v.
IgG3
vi.
IgA
Reciprocal Log2 titer
Days post immunization
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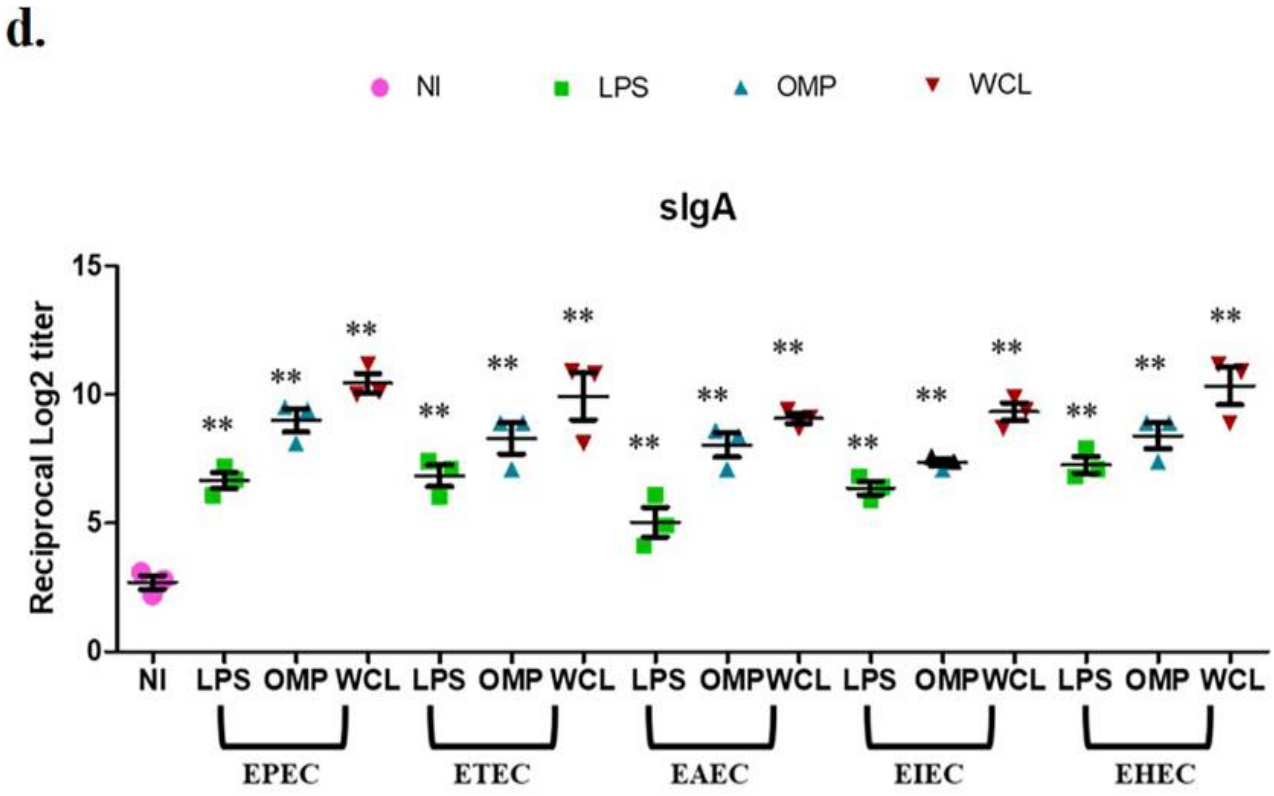


Figure 2: Reciprocal Log2 titer of serum IgG, IgG subtypes and IgA immunoglobulin from POMVs immunized and non-immunized mouse serum were measured separately after three doses if intraperitoneal immunization against WCLs (a, i-vi) OMPs (b, i-vi) and LPS (c, i-vi) of each subtype specific five DEC strains. The horizontal axis represents pre-immunization, immunization and post-immunization periods during which antibody titers were measured. Values are represented as mean ± S.D. of three independent experiments. Significant statistical difference was found between POMVs immunized and non-immunized serum (*** p value< 0.001). (d) Reciprocal Log2 titer of mucosal IgA (sIgA) from POMVs immunized mice intestinal lavage obtained at 35th day after primary immunization. Significant statistical difference was found between POMVs immunized and non-immunized groups. Results are presented as one out of three experiments using six mice per group. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Data represented as mean ± S.D., n=6. **p value<0.01. WCL- whole cell lysate, OMP- outer-membrane protein, LPS- lipopolysaccharide.

3.3 Analysis by western blot of immunogenicity elicited by POMVs

The 35th day polyvalent immunized adult mice's serum were used for Western blot analysis against elements of the immunization and challenge strains. Analysis showed that immunogenic bands against WCLs and OMPs ranged from 200 KDa to 20 KDa, with distinct bands found in the 70 KDa to 20 KDa range (Figure 4 i & ii). Unique immunogenic bands in the case of OMVs varied from 40 KDa to 20 KDa (Figure 4 iii). This shows that WCLs and OMPs have antigenic components that are present in POMVs, which is why considerable immunogenicity against a variety of proteins included in these components can be observed via western blot analysis.

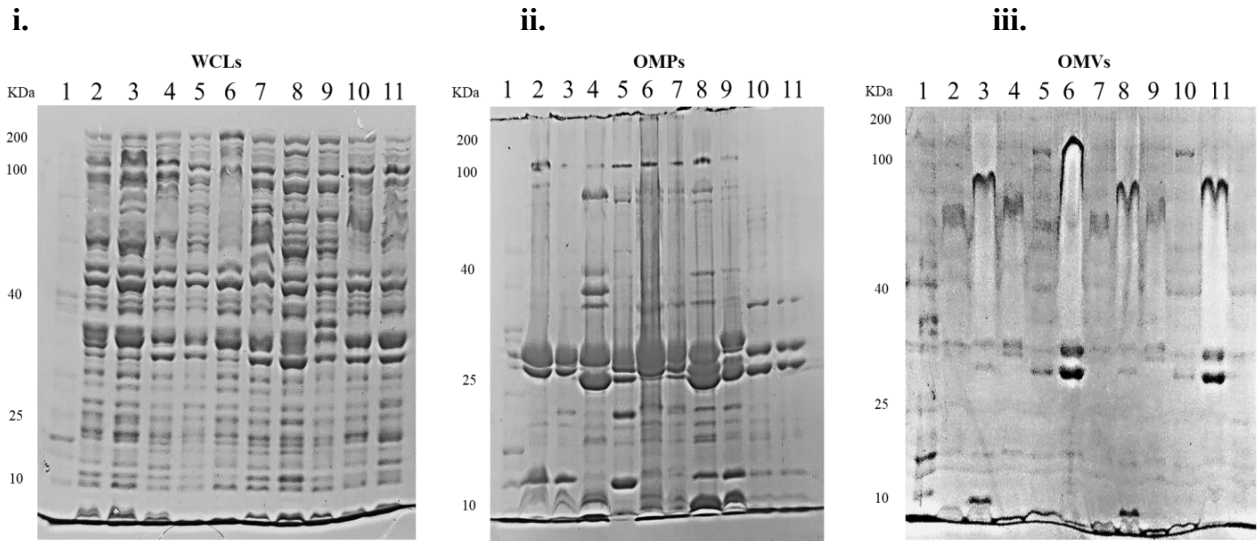


Figure 3: SDS-PAGE of WCLs (i), OMPs (ii) and OMVs (iii), isolated from both the immunization and challenge strains used in this study. For (i) WCLs and (ii) OMPs, the lanes numbered represent samples from the following: 1- Marker, 2- EPEC BCH8865, 3- EPEC BCH9327, 4- ETEC H10407, 5- ETEC 4266, 6- EAEC DSM411, 7- EAEC BCH04060, 8- EIEC IDH10106, 9- EIEC BCH10790, 10- EHEC PD124, 11- EHEC VT3. For (iii) OMVs, the following samples were loaded in the respective lanes: 1- Marker, 2- EPEC BCH8865, 3- ETEC H10407, 4- EAEC DSM411, 5- EIEC IDH10106 6- EHEC PD124, 7- EPEC BCH9327, 8- ETEC 4266, 9- EAEC BCH04060, 10- EIEC BCH10790, 11- EHEC VT3.

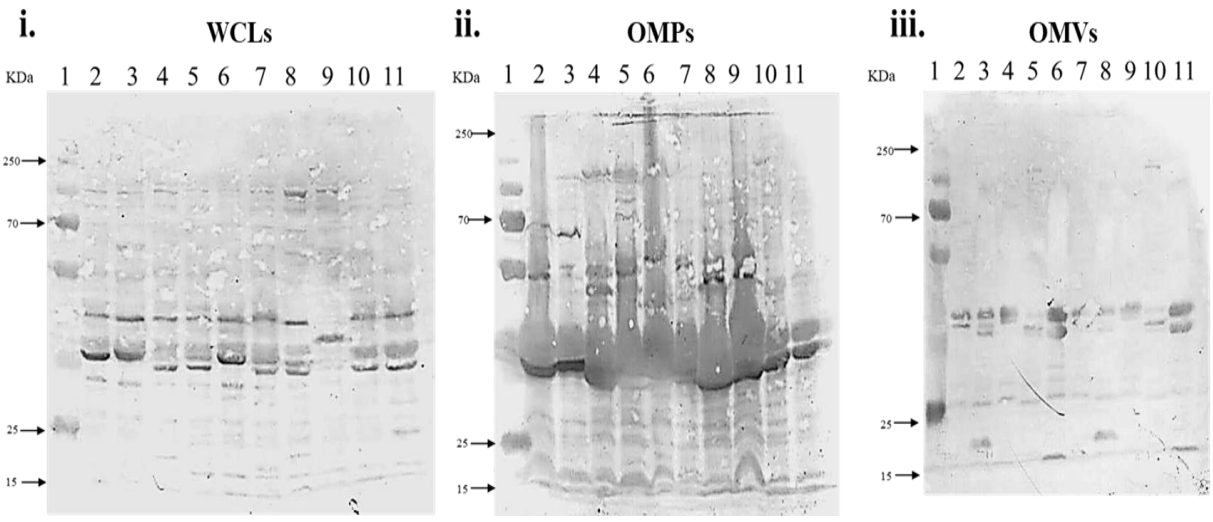


Figure 4: Immunoblot using 35th day POMVs immunized mice sera against WCLs (i), OMPs (ii) and OMVs (iii), isolated from both the immunization and challenge strains used in this study. For (i) WCLs and (ii) OMPs, the lanes numbered represent samples from the following: 1- Marker, 2- EPEC BCH8865, 3- EPEC BCH9327, 4- ETEC H10407, 5- ETEC 4266, 6- EAEC DSM411, 7- EAEC BCH04060, 8- EIEC IDH10106, 9- EIEC BCH10790, 10- EHEC PD124, 11- EHEC VT3. For (iii) OMVs, the following samples were loaded in the respective lanes: 1- Marker, 2- EPEC BCH8865, 3- ETEC H10407, 4- EAEC DSM411, 5- EIEC IDH10106 6- EHEC PD124, 7- EPEC BCH9327, 8- ETEC 4266, 9- EAEC BCH04060, 10- EIEC BCH10790, 11- EHEC VT3.

3.4 Immunization with POMVs enhanced splenic cell population

Using a flow cytometer, the splenic cell populations of mice with and without immunization were compared. Thirty-five days after the initial immunization, POMVs-immunized mice exhibited a notably larger population of CD4+, CD8a+, and CD19+ splenic cells as compared to non-immunized animals. This finding implies that OMVs antigenic components stimulate CD4+, CD8a+, and CD19+ splenic cells, which in turn helps to induce enhanced immune responses (Figure 5).

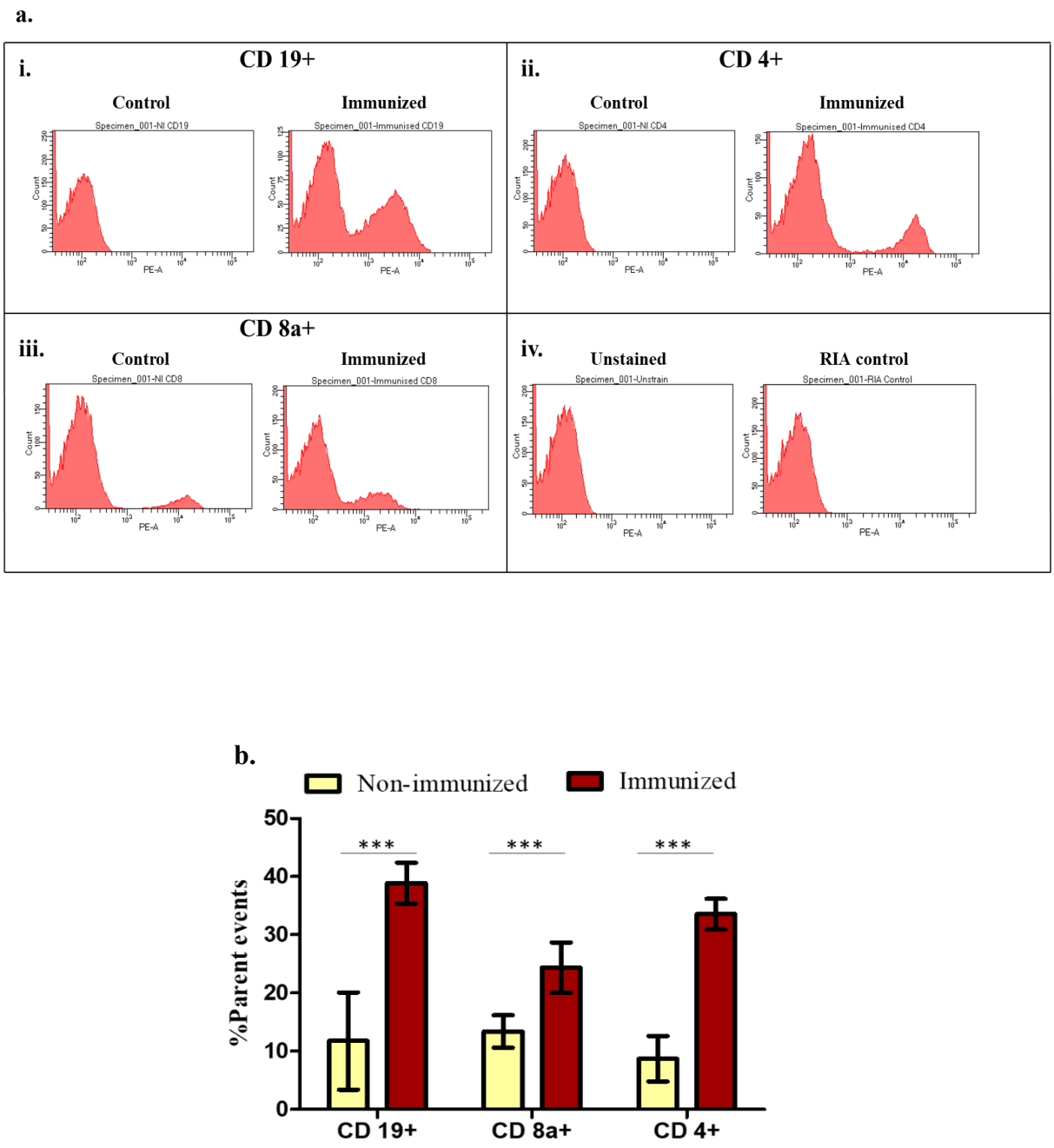
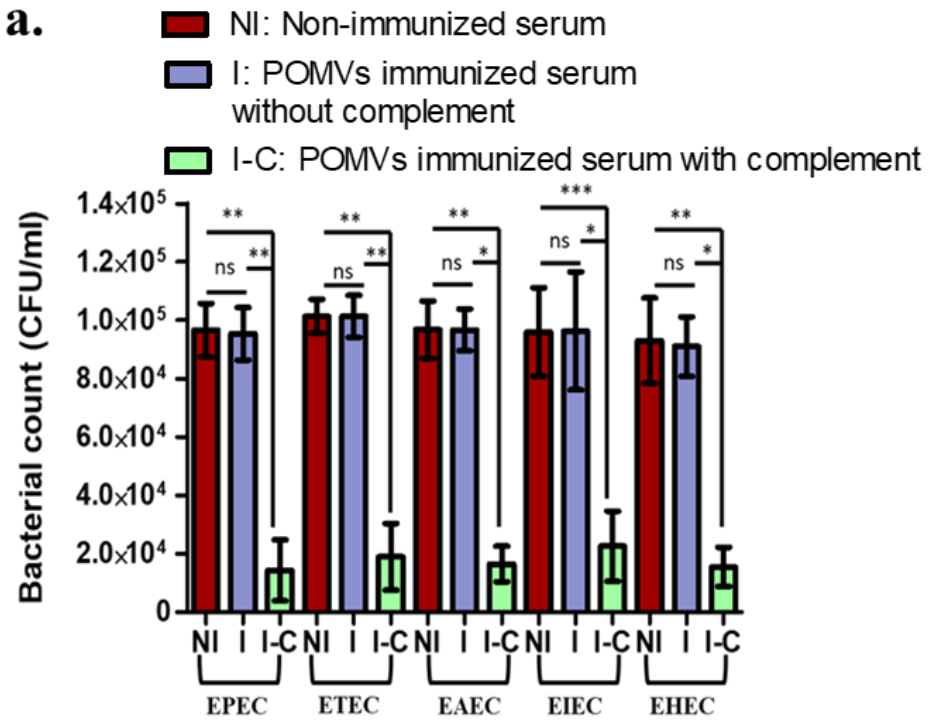


Figure 5: (a) Representative histograms of FACS analysis of POMVs immunized and non-immunized CD 19+ (i), CD 4+ (ii), CD 8a+ splenic cells (iii) stained using CD 19-PE, CD 4-PE ,CD 8a-PE and counted in FACS Aria III flow-cytometer (BD Bioscience, USA) . (iv) Represents unstained and RIA control. (b) Bar diagram comparing the percentage of CD 19+, CD 4+, CD 8a+ splenic cells from immunized and non-immunized mice. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. Significant statistical difference was found between POMVs immunized and non-immunized splenic cell population (*** p value< 0.001).

3.5 POMVs immunized adult BALB/c mice sera conferred bactericidal effect.

According to earlier findings from this study, adult BALB/c mouse sera for all five DEC subtypes exhibit significantly elevated titres of anti-WCL, anti-OMPs, and anti-LPS antibodies following POMVs immunization. The serum bactericidal assay (SBA) been utilized to analyze the bactericidal effect. Here, it was observed that, in comparison to heat-inactivated (not supplemented with guinea pig complement) and non-immunized mouse serum, immunized sera pooled from BALB/c mice, containing 25% guinea pig complement, substantially reduced the bacterial count when incubated with heterologous challenge strains of DEC subtypes. This indicates that the complement system has been activated and that the serum from BALB/c mice immunized with POMVs has large antibody titres that are adequate to destroy bacterial cells and reduce the number of bacteria (Figure 6a). SEM examination of SBA provided more evidence in support of the data. In DEC subtypes cultured with immunized mouse serum, pore formation, lysis, and agglutination of the bacterial cells were seen, but non-immunized mouse serum was unable to efficiently lyse the bacterial cells (Figure 6b).



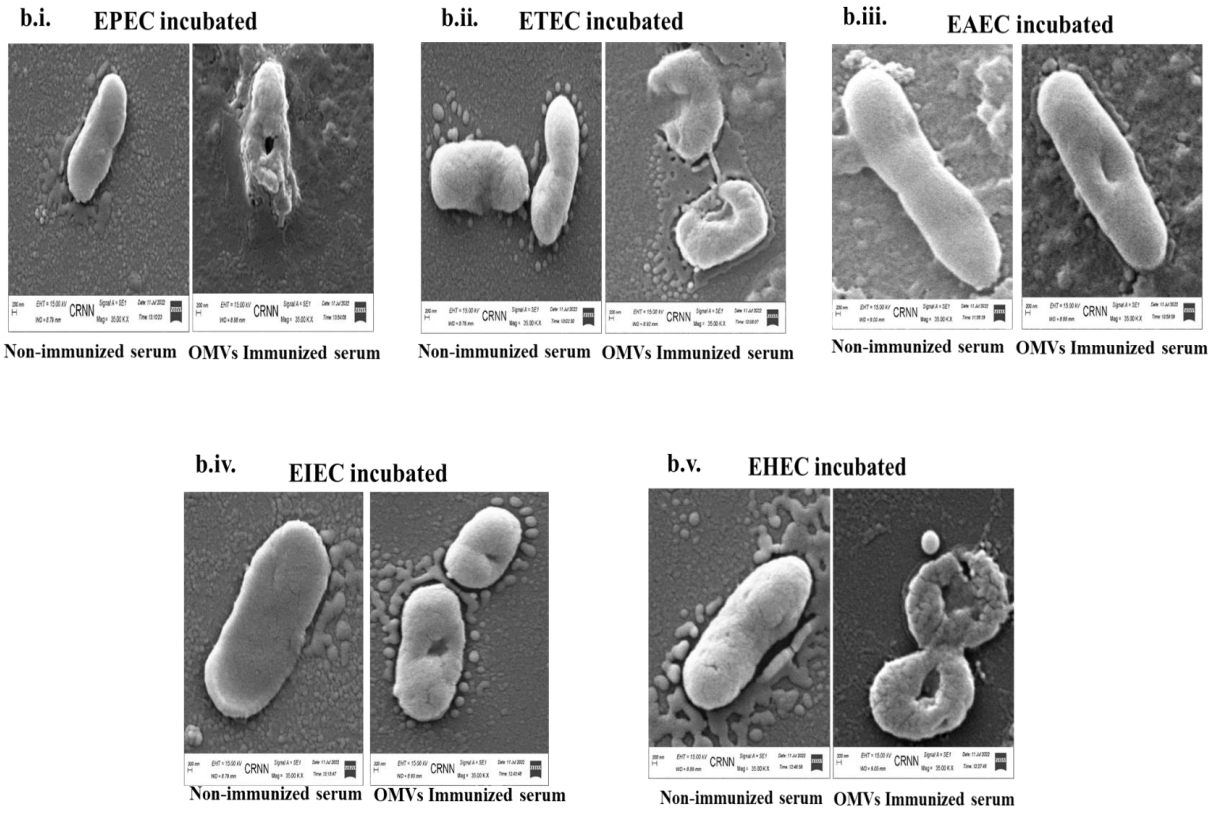


Figure 6: (a) POMVs immunized mouse serum is effective in complement mediated lysis of DEC pathotypes. 1 X 10^5CFU/mL each challenge strains of EPEC, ETEC, EAEC, EIEC, EHEC were separately incubated with the mentioned dilution of either POMVs immunized serum or non-immunized serum with or without guinea pig complement for 1 h at 37° C. Viable bacterial count were determined by plating on TSA plates. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. *** p value <0.001, ** p value<0.01, * p value<0.5. NI, non-immunized serum; I, POMVs immunized serum without complement; I-C, POMVs immunized serum with complement added. (b) Scanning electron microscopic (SEM) images of DEC challenge strains incubated with either 35th day POMVs immunized mice serum or non-immunized mice serum. (i) EPEC incubated either with or without POMVs immunized serum, (ii) ETEC incubated either with or without POMVs immunized serum, (iii) EAEC incubated either with or without POMVs immunized serum, (iv) EIEC incubated either with or without POMVs immunized serum, (v) EHEC incubated either with or without POMVs immunized serum. Scale bar 200 µm.

4. Discussion

OMVs have been found to originate from a wide variety of Gram-negative bacteria, including both pathogenic and commensal bacteria that live in the human gut and lung [18]. Natural antigens that imitate the outer surface of bacteria, such as phospholipids, peptidoglycan, lipopolysaccharides (LPSs), outer membrane proteins (OMPs), and membrane lipoprotein components, are found in OMVs. The European Medicine Agency (EMA) has licensed an OMV-based vaccine against *Neisseria meningitides* for use in human vaccination [10- 12]. OMVs do not require additional external adjuvants to boost their immunogenic qualities because they already possess intrinsic adjuvant capabilities from the presence of LPS, peptidoglycan, flagellin, and lipoproteins [19]. In my study, I have formulated a combined pentavalent OMVs-based immunogen (POMVs) to be used as a potent vaccine candidate against five prevalent pathotypes of DEC. I have assessed the activation of both the cellular and humoral arms of adaptive immune response induced by POMVs and have also assessed the bactericidal properties elicited by POMVs as a result of immunization.

It was found in this study that systemic antibody secretion can be efficiently stimulated by a minimum of 10 µg of POMVs administration per mouse intraperitoneally. 10 µg of POMVs per mouse was chosen as the ideal dose for intraperitoneal immunization as least reactogenicity was observed at this dose as compared to higher doses. It was found that following immunization, serum levels of IgA, IgG, and their subtypes increased noticeably. Secretory IgA is linked to fast bacterial removal from the intestinal lumen and resistance to host tissue invasion. Either the classical agglutination pathway or the enchained growth pathway mediates the removal of bacteria from the intestinal lumen by sIgA [20, 21]. In this study, when mice were immunized intraperitoneally with POMVs, elevated titres of anti-LPS, anti-OMPs, and anti-WCLs sIgA were also detected in the gut lumen. According to this, the POMVs

immunization may help remove DEC subtypes and ultimately offer protection from recurrent infections.

Western blot analysis used in our study shown that polyvalent POMVs immunized mouse anti-sera can identify a variety of immunogenic proteins found in WCLs, OMPs, and OMVs. In addition, immunogenic proteins from both homologous immunization strains and heterologous challenge strains can be recognized by POMVs immunized anti-sera. Immunogenic bands against WCLs and OMPs have been found to span from 200 KDa to 20 KDa, with specific bands detected in the 70 KDa to 20 KDa range, according to analysis. In the case of OMVs, distinct immunogenic bands ranged from 40 KDa to 20 KDa. This indicates that WCLs and OMPs contain antigenic components seen in POMVs, which explains why western blot analysis reveals significant immunogenicity against a range of proteins contained in these components. This suggests that POMVs might contain conserved immunogenic proteins, which may be essential for regulating host immunological responses.

Following i.p. immunization of mice with POMVs, there was an increase in the populations of CD4+ and CD8a+ splenic cells. It was additionally found that the population of CD19+ splenic cells increased following POMV immunization. Consequently, in order to provide a long-term protective immune response against DEC-mediated infections, POMVs potently stimulated both the cellular and humoral arms of the adaptive immune response.

OmpC in *E. coli* is necessary for the complement pathway to be activated by Cq1, according to a prior study. If OmpC activity is lost, serum is no longer able to efficiently kill *E. coli*, allowing it to evade the immune system [22]. By employing SBA and scanning electron microscopy analysis, I was able to evaluate the bactericidal impact of POMVs immunized serum in this work. The results showed that immunized mouse sera can effectively bind to and kill DEC subtypes by causing the bacterial cells to lyse. In preclinical research, SBA evaluates

the functionality of antibodies to determine the immunogenicity and potential protective capacity of the immunogen [23]. POMVs contain a variety of outer membrane protein components as was evident from immunoblot studies, which are important for the activation of the classical complement pathway and subsequent lysis of the bacterial cells.

Therefore, immunization with 10 µg POMVs per mouse elicits significant immunogenicity with least reactogenicity and effectively activates both the humoral and cellular arms of adaptive immune response. POMVs immunized mice sera also demonstrates bactericidal effect indicating that POMVs causes activation of the complement pathway required for effective lysis of bacterial cells.

Chapter 6

Objective 4

Assessment of protective efficacy offered by immunogen after subsequent immunization and challenge with currently circulating wild type virulent strains of diarrhoeagenic *Escherichia coli*

1. Introduction

Diarrheal illnesses have significant adverse effects on public health and are among the leading causes of morbidity and mortality, particularly in young children and newborns [1]. DEC mostly affects children worldwide and is a significant contributing factor to diarrheal outbreaks in underdeveloped nations [2]. Enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) are the five primary pathotypes of DEC [3]. Every year, DEC leads to more than three hundred million infections and approximately two hundred thousand fatalities worldwide. In impoverished nations, diarrheal illnesses (DECs) account for 30–40% of diarrheal episodes, and 120,000 paediatric deaths worldwide were attributed to DECs in 2011 [2, 4].

Due to improper antibiotic use, antibiotic resistance is becoming increasingly prevalent among DEC pathotypes. As a result, vaccines and other preventative measures can effectively lower the frequency of DEC-mediated diarrheal diseases [5, 6]. The majority of patients with *E. coli*-caused gastrointestinal infections are not recommended to use antibiotics as their first course of treatment because they have negative consequences and are associated with antibiotic resistance. Patients with severe illness (e.g., exceeding six stools per day, fever, hospitalization for dehydration, diarrhea persisting over seven days, or bloody diarrhea) may benefit from antibiotics. For the treatment of *E. coli* diarrheal disease, the International Society of Travel Medicine (ISTM) and the Infectious Diseases Society of America (IDSA) currently recommend ciprofloxacin, azithromycin, and rifaximin [7]. Currently, public health does not have access to a single licensed vaccine for DEC [8].

Gram-negative bacteria create nanosized proteoliposomes called outer membrane vesicles (OMVs). These vesicles have a strong immunogenic nature and are easily absorbed by

mammalian cells. As such, they are a promising acellular vaccine candidate. Phospholipids, lipopolysaccharides (LPSs), outer membrane proteins (OMPs), and membrane lipoprotein components are present in OMVs that mimic the outer surface of bacteria. Bacterial outer membrane vesicles are constituted with natural antigens. An OMVs-based vaccination to actively immunize humans against *Neisseria meningitides* has been approved by the European Medicines Agency (EMA). Previous studies demonstrated protection against recurrent infections in mice immunized via the intraperitoneal (i.p.) route with *E. coli* OMVs. No combined OMVs-based vaccines against the different pathotypes of DEC has been developed yet, despite the fact that OMVs have been shown to elicit positive mucosal immune responses [9- 14]. OMVs have built-in adjuvant features [15] and do not require the addition of exogenous adjuvants for enhancing their immunogenicity since they carry LPS, flagellin, peptidoglycans, lipoproteins, and other outer membrane proteins (OMPs) which cause activation of several Toll-like receptors (TLRs).

Previous studies have shown that suckling mice are more susceptible to *E. coli* infections than adult mice [16, 17]. The potency and efficacy of a vaccine candidate are assessed in passive protection tests using a model of neonatal mice [18- 21]. Due to their immature immune systems and brief susceptibility period of time, giving them a short period to generate an immune response, it is difficult to vaccinate neonatal mice against infection [22, 23]. A prior research investigation found that passive transfer of immunized adult mouse sera protected neonatal mice against group B streptococcal infection [24]. Our research group has recently shown that the transfer of OMVs vaccinated adult mouse sera to suckling mice provided passive protection against non-typhoidal *Salmonella*-mediated infections [25].

In this investigation, an immunogen was developed based on combined pentavalent OMVs (POMVs) from five major DEC pathotypes (EPEC, ETEC, EAEC, EIEC, and EHEC). The objective of this study is to evaluate the passive protection that adult mouse sera vaccinated

with POMVs provide to newborn mice. Our research has shown that POMVs significantly alter mucosal and systemic immune responses of adult mice. The work illustrates how adult mouse sera that have been vaccinated with POMVs can passively shield newborn mice from the five DEC pathotypes. According to our research, POMVs can be utilized as a vaccine candidate to mitigate infections caused by the five DEC pathotypes.

2. Methods and Materials

2.1 Bacterial strains and culture conditions

Using five circulating strains of the DEC subgroups H10407 (ETEC), PD124 (EHEC), DSM411 (EAEC), BCH8865 (EPEC), and IDH10106 (EIEC), pentavalent outer membrane vesicles-based immunogens (POMVs) were created. Five other strains of the DEC subgroups—4266 (ETEC), VT3 (EHEC), BCH04060 (EAEC), BCH9327 (EPEC), and BCH10790 (EIEC)—were employed for challenge experiments. Every strain was acquired from the strain repository located in Kolkata at the ICMR-National Institute of Cholera and Enteric Diseases (NICED). At -80ºC, all strains were maintained in Brain Heart Infusion Broth (BHIB, Difco, USA) containing 20% glycerol. Strains were cultivated for investigation at 37ºC with continuous shaking (100 rpm) in either Tryptic Soy Agar (TSA, Difco, USA) or Tryptic Soy Broth (TSB, Difco, USA) plates. MacConkey medium and plates supplemented with antibiotics were utilized for additional research.

2.2 Animals and housing

From the ICMR-NICED animal house facility, 6–8 week old BALB/c mice were obtained. For the purpose of mating, one male mouse and two female mice were kept together in a cage. Female mice were kept apart from the male during pregnancy and watched until they were born. In independent cages, ten other adult female mice were kept at a temperature of 25 ± 2 º C and a humidity of 65 ± 2%. Sterile food and water were given to the mice *ad libitum*. As directed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, all animal experiments were carried out in accordance with standard operating procedure. The Institutional Animal Ethical Committee (IAEC) of NICED authorized the animal

experimentation protocol under Registration No. 68//Rebi/S/1999/CPCSEA, which is valid until 17/7/2024. The approval number for the protocol is PRO/183/-Jan 2021-24.

2.3 Mouse immunizations

Six female adult BALB/c mice were vaccinated intraperitoneally (i.p.) on day 0, then received booster shots on days 14 and 28 with POMVs. PBS was given to the control group on the same days. The intraperitoneal vaccination dosage for POMVs was 10 µg/mouse.

2.4 Passive protection study in suckling mice

Eleven groups of one-day-old BALB/c suckling mice were created (A, B, C, D, E, F, G, H, I, J, and K, n = 6 per group). With a few small modifications, the challenge research on suckling mice was carried out as previously described [17, 18, 26]. Oral inoculation of newborn mice was performed using 1 X 10^5 CFU/neonate of challenge strains of DEC. Oral inoculations were administered to groups A and B (EPEC BCH9327), C and D (ETEC 4266), E and F (EAEC BCH04060), G and H (EIEC BCH10709), I and J (EHEC VT3), and group K (uninfected) as the control group. Groups A, C, E, G, and I received non-immunized adult mouse sera of the same dilution, while groups B, D, F, H, and J received oral administration of pooled 20 µL of 35th day POMVs immunized adult mouse sera (with a 1:10 dilution in PBS) two hours after the DEC strain challenge. After being put back with their mothers, the neonates were watched for seven days in order to analyze their survival.

In an additional set of experiments, suckling mice were once more split into 11 groups (n=6 each group) and given the same dosage of challenge DEC strains as previously mentioned. As previously mentioned, mice were passively immunized and were then sacrificed after 24 hours.

Small intestines were obtained in order to measure the bacterial load, homogenized, serially diluted, and plated on agar plates supplemented with antibiotics [20, 25, 27]. To measure the change in body weight up to three days after infection, the identical set of experiments were performed with an inoculation dose of 1 X 10^3 CFU/neonate [25].

Small intestine tissues from mice of the aforementioned groups (n = 6 per group) were homogenized in cytokine buffer 24 hours after a bacterial challenge at a dose of 1 X 105 CFU/neonate. Supernatants were then kept at -80º C for cytokine quantification, as previously described [25]. 24 hours after the bacterial challenge, small intestine samples were taken from each group and preserved in 10% buffered formalin solution for histological examination.

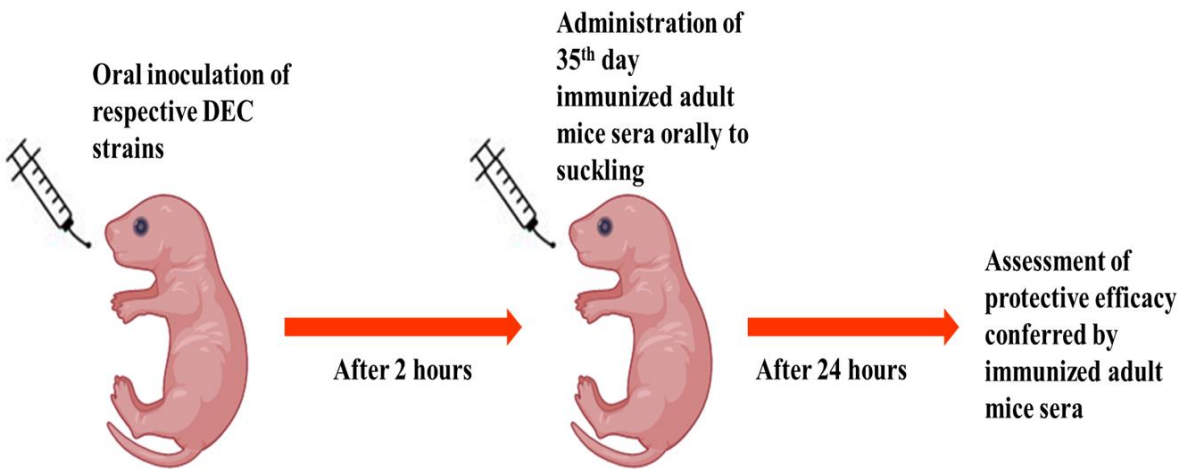


Figure 1: Protective efficacy studies in suckling mice model with immunized adult BALB/c mice sera.

2.5 Histological analysis

Small intestine samples were fixed with 10% buffered formalin, sectioned, double stained with haematoxylin and eosin (H & E), and examined using an Olympus IX51 light microscope. Samples were scored pathologically (Table 1) using the previously stated method with slight modifications [25, 28].

Table 1: Pathological score reference table for histological images.

Inflammatory cell infiltrate			Mucosal architecture			
Severity	Extent	Score 1	Severity	Extent	Mucosal and epithelial changes	Score 2
Minimal	Mucosa	1	**Minimal**	Mucosa	Minimal hyperplasia Minimal villous blunting	1 1
Mild	Mucosa and sometimes submucosa	2	**Mild**	Mucosa, sometimes submucosa	Mild hyperplasia Mild to moderate villous blunting Mild goblet cell loss	2 2 2
Moderate	Mucosa and submucosa, sometimes transmural	3	**Moderate**	Mucosa, submucosa	Moderate hyperplasia Moderate villous blunting and broadening, sometimes villous atrophy Goblet cell loss Cryptits	3 3 3 3
Marked	Transmural	4	**Marked**	Mucosa, submucosa, often transmural	Marked hyperplasia Villous atrophy Marked goblet cell loss Multiple crypt absences	4 4 4 4
						Sum of Score 1 and 2 : 0-20

2.6 Measurement of cytokines

Using cytokine ELISA kits and manufacturer's instructions (Invitrogen, USA), the following cytokines were measured from small intestine supernatants preserved in cytokine buffer: IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70, and IL-23.

2.7 Statistical analysis

The data in the study are expressed as mean ± S.D. Two-way analysis of variance (ANOVA) was used to compare data from three or more groups. A Student's t test was employed to examine two sets of grouped data. For the final computation, the means of three separate experiments were used. A significant level was defined as the probability of $p<0.05$ or $p<0.01$ or $p<0.001$. Statistical analyses were conducted using Microsoft Excel and GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA).

3. Results

3.1 Suckling mice were passively protected against infections caused by DEC pathotypes by POMVs immunized adult mouse serum

For as long as seven days, the survival of suckling mice who received adult mouse sera from both POMVs immunized and non-immunized groups was analyzed. Two hours after the challenge of suckling mice with 1 X 10^5 CFU/neonate of the corresponding heterologous challenge strains of DEC subtypes, pooled adult mouse sera from immunized and non-immunized mice (diluted in a 1:10 ratio) were given orally. Compared to groups receiving non-immunized mouse sera, those getting immunized mouse sera exhibited noticeably higher survival rates (Figure 2, a-e). Following challenge with strains of EPEC, ETEC, EAEC, EIEC, and EHEC, recipient groups of immunized sera showed survival rates of 81%, 82%, 80%, 75%, and 78%, respectively. After being administered with non-immunized mouse serum, suckling mice died within two to four days after infection. This finding implies that suckling mice can be considerably protected against DEC-mediated infections by POMVs-immunized adult mouse serum.

Following the above-mentioned DEC challenge strain administration at a similar dose, the treatment groups treated with immunized and non-immunized sera were compared for changes in small intestine colonization. Compared to neonates who were not immunized, the small intestines of those who received immunized sera treatment showed a marked reduction in the colonization of the five DEC pathotypes 24 hours after infection (Figure 2f).

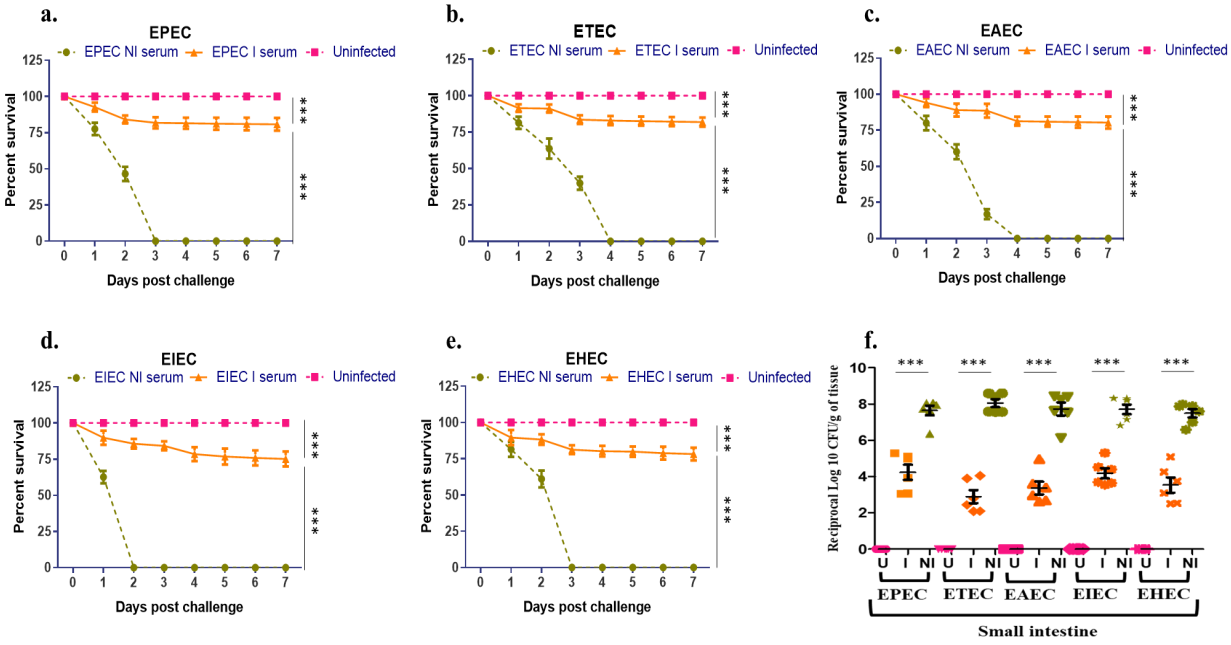


Figure 2: One day old neonatal mice were challenged with 1 X 10^5CFU of DEC heterologous challenge strains separately. After two hours of challenge, neonatal mice were administered with pooled serum from POMVs immunized and non-immunized adult mice. Neonatal mice were observed for survival up to 7 days post infection. Mortality was determined based on humane endpoints. POMVs immunized sera recipient groups after challenge represented survival rate of 81% for EPEC (a), 82% for ETEC (b), 80% for EAEC (c), 75% for EIEC (d) and 78% for EHEC (e). Data are represented as mean ± S.E. of three independent experiments (n=6). Protective efficacy was calculated using the equation {[(percent deaths of non-immunized neonates)-(percent deaths of immunized neonates)]/[percent deaths of non-immunized neonates]} X 100. (f) Comparison of protection determined from colonization data in small intestine of neonatal mice (n=6), exerted by POMVs immunized and non-immunized adult mouse serum, after challenge with 1 X 10^5CFU/ neonatal mice of DEC challenge strains. Each dot represent colonization data obtained from a single neonatal mouse. On the vertical axis, data are represented as Log10 of recovered colony forming unit per gram of small intestine of each neonatal mouse, 24 h post challenge. The difference in colonization between the immunized and non-immunized sera recipient neonatal mice were highly significant (p value <0.001). Two-way analysis of variance (ANOVA) test was used for statistical analysis. Data represented as mean value of six neonatal mice ± S.E. of three independent experiments. *** p value <0.001. UI, uninfected; I, immunized sera recipient; NI, non-immunized sera recipient.

Suckling mice were given a low dose of 1 X 10^3 CFU/neonate of challenge strains of DEC subtypes in order to measure changes in body weight. Over the course of three days, non-immunized sera treatment groups showed a considerable loss in body weight compared to immunized sera treated mice, whose body weight stayed around the same as that of uninfected groups (Figure 3).

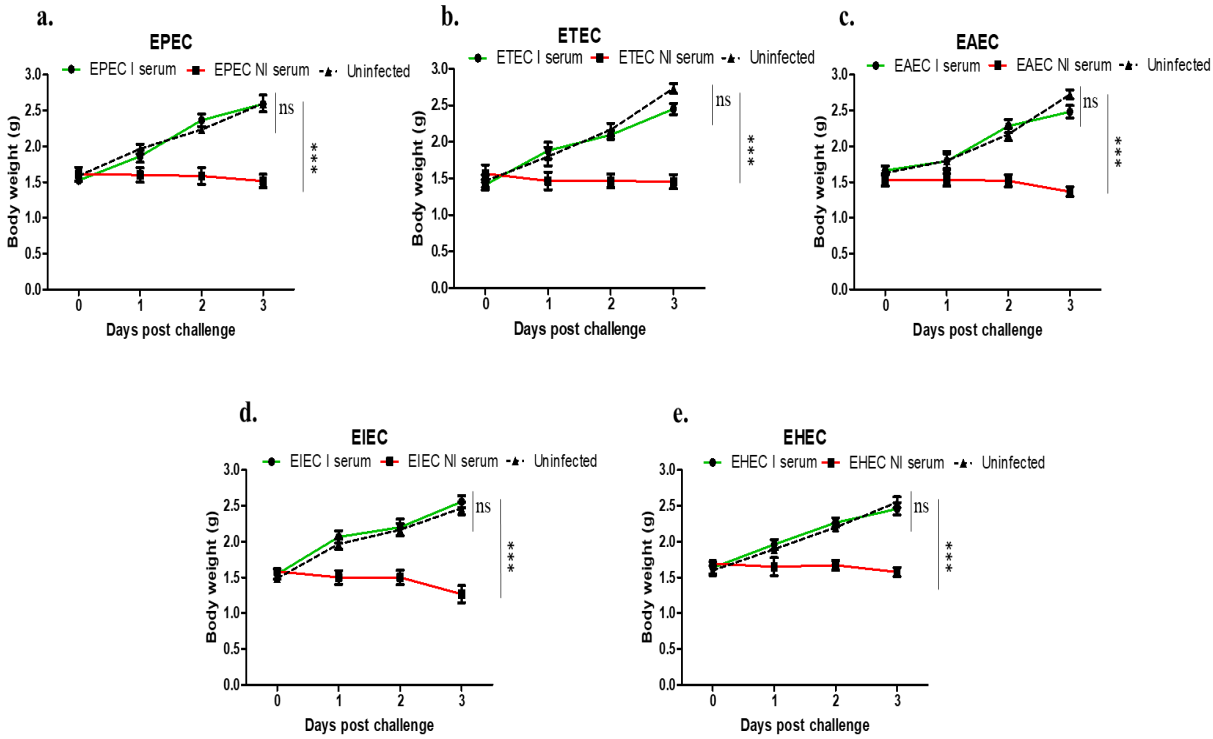
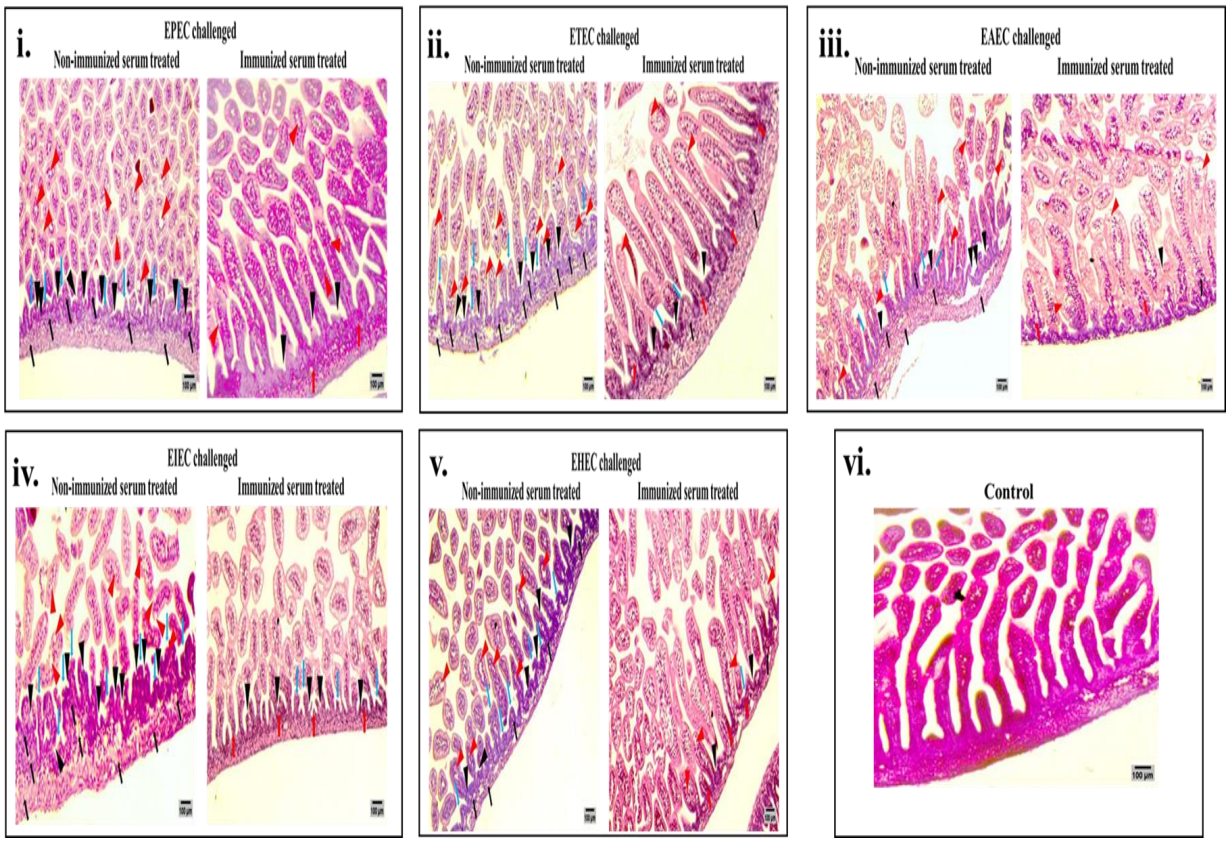


Figure 3: One day old neonatal mice were challenged with 1 X 10^3CFU of DEC heterologous challenge strains separately (n=6). 2 h post challenge, neonatal mice were administered pooled serum from POMVs immunized and non-immunized adult mice. Changes in body weight were monitored for 3 days (a-e). Every single dot represents mean and error value of six neonatal mice ± S.D. of three independent experiments. *** p value<0.001. ns, non-significant. I, immunized; NI, non-immunized.

Following infection with DEC challenge strains (1 X 10^5 CFU/neonatal mice), the histopathological alterations of the neonatal small intestine were examined 24 hours later. When compared to recipient suckling mice that did not receive immunized mice sera, recipient suckling mice that received immunized mice sera demonstrated a notable decrease in goblet cell loss, villous atrophy and hyperplasia, altered intestinal mucosa, epithelial layer damage, and PMN (polymorphic nuclear neutrophilic) infiltration (Figure 4a). In comparison to suckling mice treated with non-immunized sera, the pathological scores of mice treated with immunized sera were comparably lower (Figure 4b). Altogether, passively transferred POMVs immunized adult mouse sera demonstrated a notable degree of passive protective effectiveness against five DEC pathotypes in neonatal mice.

a.



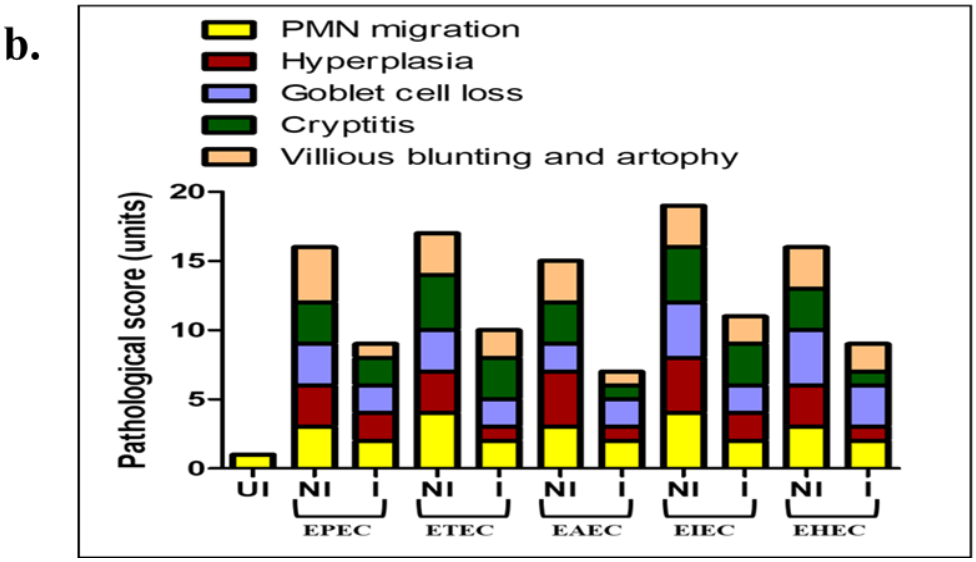


Figure 4: Pentavalent OMVs immunized mouse serum decreases small intestinal tissue damage and inflammation in neonatal mice after infection with DEC pathotypes separately (1 X 10^5CFU). (a) Histological images of small intestine of neonatal mice administered with either 35th day POMVs immunized mouse serum or non-immunized mouse serum. (i) EPEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (ii) ETEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (iii) EAEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (iv) EIEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (v) EHEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (vi) uninfected control neonatal mice small intestine. Small intestine of neonatal mice treated with POMVs immunized mouse serum showed mild epithelial layer damage, altered intestinal mucosa and polymorph nuclear neutrophilic (PMN) infiltration, whereas non-immunized sera treated neonatal mice displayed marked epithelial layer damage, altered intestinal mucosa, polymorph nuclear neutrophilic (PMN) infiltration, goblet cell loss, hyperplasia and villous atrophy. (Blue arrow– Villous blunting and atrophy; Black arrow – PMN migration marked level; Red arrow- PMN migration mild level; Black arrowhead – Crypt alteration with mucus or inflammatory cell; Red arrowhead – Goblet cell loss. Images were taken at 200X magnification, 100µm scale bar. (b) Pathological scores of immunized or non-immunized sera treated neonatal mice small intestine post challenge. Immunized sera treated neonatal mice small intestine displayed less inflammation as compared to that of non-immunized sera treated neonatal mice. UI, uninfected; I, immunized sera recipient; NI, non-immunized sera recipient.

3.2 Pro-inflammatory cytokine levels in the small intestine of neonatal mice infected with POMVs are reduced by immunized mouse serum.

After infection with DEC subtypes (1 X 10^5 CFU/neonate), there were significant differences in the amount of pro-inflammatory cytokines found in the small intestine tissue of immunized sera treated and untreated neonates. After infection, compared to non-immunized serum recipient neonatal animals, IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70, and IL-23 were significantly lower in small intestine tissues of mice who received immunized sera (Figure 5). Villous atrophy and damage to epithelial cells are caused by these pro-inflammatory cytokines. Therefore, as earlier data clearly show, treatment with POMVs immunized adult mouse sera substantially decreased intestinal inflammation in neonatal mice following infection, which in turn lowers the degree of intestinal damage.

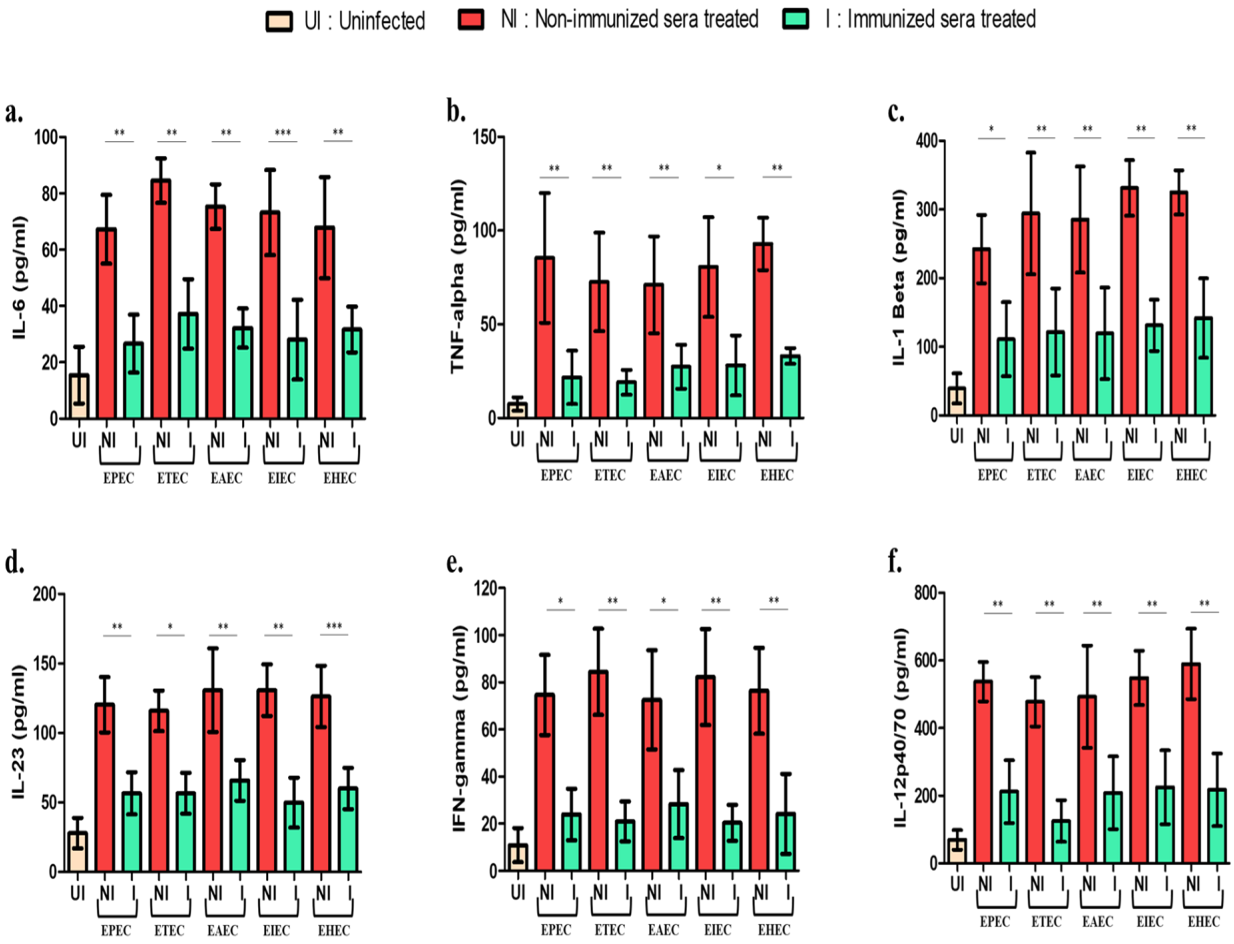


Figure 5: Passive immunization with POMVs immunized adult mouse sera significantly lowers expression of inflammatory markers in small intestinal tissues of neonatal mice. Small intestines of immunized or non-immunized sera treated neonatal mice were isolated 24 h post challenge with respective DEC pathotypes (1 X 10^5CFU) and processed as described in materials and methods. Pro-inflammatory cytokine levels were measured in small intestinal tissue extracts. Cytokine levels of (a) IL-6, (b) TNF-α, (c) IL-1β, (d) IL-23, (e) IFN-γ and (f) IL-12p40/70. (n=6). Two-way analysis of variance (ANOVA) test was used for statistical analysis. Significant statistical difference was found between uninfected, immunized sera treated and non-immunized sera treated neonatal mice small intestinal tissue inflammatory markers (*** p value <0.001, ** p value<0.01, * p value<0.5). Bars represent mean of six neonatal mice ± S.E. of three individual experiments. UI, uninfected; I, immunized sera recipient; NI, non-immunized sera recipient.

4. Discussion

As a result of improper antibiotic use, new multi-drug resistant strains of *E. coli* are evolving [29]. Prophylactic measures, including as vaccinations, can be very helpful in reducing the health burden caused by DECs in the post-antibiotic era [30]. The existence of a large variety of DEC serotypes and the inability to produce a powerful gut immune response present a significant obstacle for researchers now working to create possible vaccines against DEC [31]. Currently, public health does not have access to a single licensed vaccine against DEC [8]. Combination-based vaccinations are thought to be a more effective strategy for preventing diarrhoeal diseases [32]. According to a recent study, a formalin-killed whole-cell vaccine was efficient in eliciting immunogenicity and offered protection against five primary pathotypes of DEC when it was administered subcutaneously to BALB/c mice along with cholera toxin B subunit as an adjuvant [31].

OMVs are spherical proteoliposomes that are formed from the outer membrane of Gram-negative bacteria. They have a diameter of roughly 20 to 200 nm [33]. Proteins, metabolites, DNA, and RNA are among the periplasmic and cytoplasmic materials that make up these vesicles [34]. The most obvious application for OMVs is in the formulation of antigens for immunization. This method was initially developed as a vaccination against *N. meningitidis* serogroup B. Since then, OMVs have been produced from a range of bacterial strains to be used in the facilitation of antigen delivery from homologous or heterologous strains [35]. OMV endogenous proteins are involved in modulating humoral immune response to heterologous antigens generated within OMVs. DCs take OMVs and subsequently employ the MHC II molecule to transfer OMV endogenous protein epitopes, therefore activating CD4+ T helper (Th) cells (three T cells, each of which recognizes a different epitope). After internalizing OMV, naïve B cells and their receptors recognize the same OMV-derived epitopes that DCs display

in parallel, and their MHC II molecules express the same heterologous antigens after OMV internalization [36].

Passive transfer of adult mouse sera immunized with POMVs was found to significantly protect neonatal mice in our study from infections by DEC pathotypes. When adult mouse sera were administered orally to neonatal mice, the mice showed improved survival, minimal or no weight loss, and several times less small intestine colonization against five DEC subtypes.

Neonatal mice treated with immunized sera had small intestine histopathology scores lower than mice treated with non-immunized sera. Compared to neonatal mice treated with non-immunized sera following infection, those given immunized sera showed decreased epithelial layer damage, altered intestinal mucosa, polymorph nuclear neutrophilic (PMN) infiltration, goblet cell loss, hyperplasia, and villous atrophy.

When neonatal mice were challenged with DEC subtypes, the production of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70, and IL-23 was downregulated by administering POMVs immunized adult mouse sera. This resulted in a reduction of inflammation. This is consistent with histopathology findings showing that neonatal mice treated with immunized sera exhibited significantly lower pathological alterations than those treated with non-immunized sera.

Thus, passive transfer of POMVs immunized adult mice sera significantly lowers the colonization of DEC pathotypes, histopathological changes and inflammation in the small intestine of neonatal mice as compared to passive transfer of non-immunized adult mice sera. Passive transfer of immunized adult mice sera also demonstrated significantly improved survivability and minimal or insignificant loss of body weight in neonatal mice as compared to transfer of non-immunized adult mice sera. Therefore, from this study, it can be inferred that

POMVs can be used as a potent vaccine candidate for ameliorating infections caused by the five prevalent pathotypes of diarrhoeagenic *Escherichia coli*.

Chapter 7

General Discussion

In impoverished countries, diarrheal illnesses are a leading cause of childhood mortality, with children under five years age being the most affected [1]. One of the primary causes behind diarrhea-related diseases are DEC pathotypes. Due to development of multi-drug resistant (MDR) bacteria, vaccination is a beneficial strategy for reducing illnesses caused by DEC pathotypes [2, 3]. The effectiveness of polysaccharide conjugate vaccines against O111 *E. coli* has been previously studied [4]. Phase 1/2 trials revealed that ETVAX, an inactivated whole-cell vaccine, was safe and immunogenic. It is made up of four recombinant *E. coli* strains that overproduce adhesins, as well as recombinant hybrid B subunits of heat-labile toxin and cholera toxin [5]. However, none of these vaccines were successful in offering protection against several DEC subtypes and eliciting broad spectrum immunogenicity. Combination vaccines are thought to be a superior method of preventing diseases associated with diarrhea [6]. According to a recent study, a formalin-killed whole-cell vaccine was successful in eliciting immunogenicity and offered protection against five primary pathotypes of diarrheagenic *E. coli* when it was administered subcutaneously to BALB/c mice together with cholera toxin B subunit as an adjuvant [7]. Because OMVs comprise of LPS, peptidoglycan, flagellin, and lipoproteins, they possess intrinsic adjuvant qualities [8, 9, 10] which enhance their immunogenicity without the requirement of additional adjuvants. Therefore, in order to offer broad spectrum protection against DEC pathotypes, we have developed an OMVs-based immunogen isolated from EPEC, ETEC, EAEC, EIEC, and EHEC. It has previously been demonstrated that OMVs-based vaccinations are effective against *Shigella*, *Salmonella*, and *Neisseria* species [11- 14]. According to earlier research, the *Neisseria meningitis* OMVs-based vaccine (MenBvac and MeNZB) proved efficacious in eliciting a protective immune response when administered in three doses of 25 µg intramuscular vaccinations [15- 17]. Using an analogous approach, we found that in BALB/c mice, i.p. immunization with 3 doses of 10 µg of POMVs at an interval of 14 days between immunizations activated the humoral and cellular

arms of the immune response. Our earlier research, in which bivalent OMVs-based immunizations against *S.* Typhi and *S.* Paratyphi and, *S.* Typhimurium and *S.* Enteritidis in an adult BALB/c mouse model, revealed similar kinds of immune responses [18, 19]. It was observed in this study that systemic antibody secretion can be effectively stimulated by intraperitoneal immunization with a minimum of 10 µg of OMVs per mouse. Following immunization, it was discovered that serum levels of IgA, IgG, and their subtypes increased noticeably. The complement pathway is triggered by IgG2a and IgG2b, and this finally results in the target antigen being opsonized. IgG1 subclass facilitates efficient phagocyte uptake of bacteria [20], while bacterial capsular polysaccharides stimulate the IgG3 subclass in mice [21]. The presence of various antigens on POMVs may activate the cellular arm of the immune response necessary for the passive protection of neonatal mice, which could explain the increased levels of IgG subclass in immunized mice.

Secretory IgA is linked to quicker bacterial clearance from the intestinal lumen and resistance to host tissue invasion. Either the classical agglutination pathway or the enchained growth pathway mediates the removal of bacteria from the intestinal lumen by sIgA. By blocking interaction of bacteria with the mucosal layer, sIgA helps to eradicate bacteria from the intestinal lumen in classical agglutination pathway. When it comes to the enchained growth pathway, sIgA makes a coat that surrounds the bacterium and stops the sister cells from dividing, which eventually results in the removal of the undissociated cells [18, 22, 23]. This study also found increased titres of anti-LPS, anti-OMPs, and anti-WCLs sIgA in the intestinal lumen of mice following intraperitoneal immunization with POMVs. This suggests that the POMVs immunization can eventually defend against recurrent infections and mediate the elimination of DEC subtypes.

According to recent studies, certain *E. coli* outer membrane proteins, including OmpA, OmpC, BamA, and OmpF, have immunogenic characteristics and may therefore serve as potential

immunogens [24, 25]. Polyvalent POMVs immunized mouse anti-sera are able to recognize a variety of immunogenic proteins present in WCLs, OMPs, and OMVs, according to western blot analysis of this study. Furthermore, in addition to homologous immunization strains, polyvalent immunized anti-sera were able to identify the immunogenic proteins of heterologous challenge strains. This suggests that POMVs might contain these conserved immunogenic proteins, which may be essential for regulating host immunological responses. It has previously been reported that intraperitoneal immunization with OMVs of *Escherichia coli* boosts the number of CD4+ and CD8+ splenic T-cells [26], which are essential for a sustained humoral immune response. Increase in splenic T-cell populations have also been observed in other investigations following subsequent immunization with OMVs derived from bacteria that cause enteric diseases [18, 19, 27]. Similar to previous investigations, i.p. immunization of mice with POMVs resulted in an increase in the populations of CD4+ and CD8a+ splenic cells in the current study. Consequently, POMVs strongly stimulate the humoral and cellular components of the adaptive immune response, which are required to provide a long-lasting protection against infections mediated by DEC.

A prior study revealed that OmpC in *E. coli* is necessary to activate the complement pathway by Cq1, and that OmpC dysfunction prevents serum from efficiently killing *E. coli* and enabling it to evade the immune response [28]. By employing SBA and scanning electron microscopy analysis, we were able to evaluate the bactericidal properties of POMVs immunized serum in this work. The results showed that immunized mouse sera can efficiently bind to and kill DEC subtypes by causing the bacterial cells to lyse. In preclinical research, SBA evaluates the functionality of antibodies to determine the immunogenicity and potential protective efficacy of the immunogen [29- 31]. Based on the immunoblot results, it was shown that POMVs contain a variety of outer membrane protein components. These components can be crucial in triggering the classical complement pathway and ultimately cause lysis of the bacterial cells.

Neonates have inadequate protection against infectious diseases in their early years of life due to an underdeveloped immune system [32]. Human disease-causing pathogenic organisms have long been targeted by serum therapy or passive antibody therapy [33]. Since the immune system is still underdeveloped, neonatal mice make excellent models for research on the passive immunity provided by immunized mouse serum [18, 34]. Passive transfer of sera of POMVs immunized adult mouse was found to significantly protect neonatal mice from infections against five prevalent DEC pathotypes in our study. When immunized adult mouse sera were given orally to neonatal mice, the mice showed improved survivability, negligible or no weight loss, and several times less small intestine colonization against five DEC subtypes. Our earlier research also demonstrated the passive protection provided by OMVs isolated from *Vibrio cholerae* [27], *Shigella* sp. [35], *Salmonella* Typhimurium, and *Salmonella* Enteritidis [18]. It was found that neonatal mice treated with immunized sera showed lower small intestinal histopathological scores than those treated with non-immunized sera. Compared to neonatal mice treated with non-immunized sera following infection, those given immunized sera showed decreased epithelial layer damage, altered intestinal mucosa, polymorph nuclear neutrophilic (PMN) infiltration, goblet cell loss, hyperplasia, and villous atrophy. Anti-LPS IgG in serum has been shown in prior research to protect against *E. coli* infections [36]. In this work, we speculate that serum from immunized mice may contain anti-LPS IgG, which may trap the bacteria and protect the neonatal mice from infection.

E. coli has been shown to induce inflammation in the intestine, and IL-6 is a significant pro-inflammatory cytokine that is expressed differently depending on the severity of disease [37]. Through its effect on tight junctions, IL-1β enhances the permeability of epithelial cells. NF-κβ is upregulated by IL-1β, and this affects tight junctions, increasing permeability [38]. TNF-α is produced when LPS binds to toll-like receptors (TLRs), and excess TNF-α and IFN-γ production has been linked to the pathogenesis of inflammatory bowel disease [39, 40]. It has

been found that IFN-γ induces tight junction protein micropinocytosis into early endosomes, which increases the permeability of the barrier of intestinal epithelial cells [40]. When monocytes, macrophages, and dendritic cells are infected with bacteria, they release IL-12 and IL-23. This in turn triggers T-lymphocytes to release inflammatory cytokines, including IFN-γ. Additionally, pro-inflammatory markers including IL-1, IL-6, TNF-α, and chemokines that cause inflammation are produced by T-cells stimulated by IL-12 and IL-23 [41]. This work demonstrates that after challenge with DEC pathotypes, the administration of POMVs immunized adult mice sera decreased the production of pro-inflammatory cytokines in the small intestines of neonatal mice, particularly IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70, and IL-23. This leads to attenuation of inflammation. This is consistent with the histopathological findings that demonstrated neonatal mice treated with immunized sera exhibited fewer pathological alterations than those treated with non-immunized sera.

This study establishes that, when administered intraperitoneally to adult BALB/c mice, POMVs can be utilized as an effective immunogen to induce broad spectrum immunogenicity against five prevalent DEC pathotypes. The DEC pathotypes were successfully eliminated by the immunized mouse sera because it exhibited distinct bactericidal characteristics. Furthermore, neonatal mice received broad spectrum protection against infections caused by five prevalent pathotypes of DEC through passive transfer of POMVs immunized adult mouse sera.

Chapter 8

Pivotal findings of the thesis work in a nutshell

- ✓ **Analysis using transmission electron microscopy revealed that OMVs are spherical in shape and enclosed in a distinct lipid bilayer.**
- ✓ **Dynamic light scattering revealed a homogeneous distribution of sizes in the OMVs isolated from each of the DEC subtypes and demonstrated that the OMV peaks of the DEC subtypes varied in size from approximately 36 nm to 101 nm.**
- ✓ **The zeta potential study showed that OMVs had an overall negative surface charge, with values ranging from -0.145 mV to -5.3 mV, suggesting that OMVs are stable in nature.**
- ✓ **The optimal dose for intraperitoneal immunization was determined to be 10 µg of POMVs per mouse since, in comparison to larger doses, this dose demonstrated least amount of reactogenicity.**
- ✓ **Serum levels of IgA, IgG, and their subtypes increased significantly after immunization. Elevated titres of anti-LPS, anti-OMPs, and anti-WCLs sIgA were also seen in the intestinal lumen of mice immunized intraperitoneally with POMVs.**
- ✓ **Significant immunogenicity against a variety of proteins was found by western blot analysis. Analysis indicated that immunogenic bands against WCLs and OMPs ranged from 200 KDa to 20 KDa, with some bands observed in the 70 KDa to 20 KDa range. Unique immunogenic bands in the case of OMVs varied in size from 40 KDa to 20 KDa. This suggests that WCLs and OMPs have antigenic elements similar to those seen in POMVs.**
- ✓ **FACS analysis demonstrated that the proportions of CD19+, CD4+, and CD8a+ splenic cells increased after mice were immunized intraperitoneally with POMVs.**
- ✓ **POMVs immunized mice sera also demonstrated bactericidal effect as was evident from serum bactericidal assay and scanning electron microscopic analysis, indicating that**

POMVs causes activation of the complement pathway required for effective lysis of bacterial cells.

- ✓ **Neonatal mice were shown to be protected from infections by DEC pathotypes through the passive transfer of POMVs immunized adult mouse sera. When neonatal mice were given immunized adult mouse sera orally, they exhibited increased survival, little to no weight loss, and significantly decreased small intestinal colonization against five DEC subtypes.**
- ✓ **Neonatal mice treated with immunized sera demonstrated lower histopathological scores of small intestine than those treated with non-immunized sera. The immunized mice displayed decreased damage to epithelial layer, altered intestinal mucosa, polymorph nuclear neutrophilic (PMN) infiltration, loss of goblet cells, hyperplasia, and villous atrophy after infection as compared to the neonatal mice treated with non-immunized sera.**
- ✓ **The administration of POMVs immunized adult mouse sera downregulated the production of pro-inflammatory cytokines in the small intestine, including IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70, and IL-23, after neonatal mice were challenged with DEC subtypes. As a result, there was less inflammation in the small intestine of neonatal mice receiving immunized adult mouse sera.**
- ✓ **Thus, it can be concluded from this study that POMVs act as a potent vaccine candidate and can be utilized to mitigate diseases caused by any of the five prevalent pathotypes of diarrheagenic *Escherichia coli*.**

Chapter 9

Bibliography

Background

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Chapter 2

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Chapter 3

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Chapter 6

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Chapter 7

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Chapter 10

Patents applied

The following patents have been applied through IPR Unit of ICMR:

1. **"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.**

2. **"A bivalent typhoidal bacterial ghost (BTBG) immunogenic formulation and method for preparation thereof", the complete patent application of this invention has been filed on 15th June, 2022 and the patent application number is 202211034380.**

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 11th April, 2024 and the patent application number is 202411029436.**

Chapter 11

Original Publications

1. **Banerjee S**, Halder P, Das S, Maiti S, Bhaumik U, Dutta M, Chowdhury G, Kitahara K, Miyoshi SI, Mukhopadhyay AK, Dutta S, Koley H. **Pentavalent outer membrane vesicles immunized mice sera confers passive protection against five prevalent pathotypes of diarrhoeagenic Escherichia coli in neonatal mice.** Immunol Lett. 2023 Nov;263:33-45. doi: 10.1016/j.imlet.2023.09.009. Epub 2023 Sep 19. PMID: 37734682.

2. Das S, Halder P, **Banerjee S**, Mukhopadhyay AK, Dutta S, Koley H. **Establishment of an intragastric surgical model using C57BL/6 mice to study the vaccine efficacy of OMV-based immunogens against Helicobacter pylori.** Biol Open. 2024 Apr 11:bio.060282. doi: 10.1242/bio.060282. Epub ahead of print. PMID: 38602383.

3. 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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5. Maiti S, Halder P, **Banerjee S**, Dutta M, Kumar Mukhopadhyay A, Dutta S, Koley H. **Development of a novel trivalent invasive non-typhoidal Salmonella outer membrane vesicles based vaccine against salmonellosis and fowl typhoid in chickens.** Immunobiology. 2022 Mar;227(2):152183. doi: 10.1016/j.imbio.2022.152183. Epub 2022 Jan 31. PMID: 35121180

6. **Banerjee S**, Barry EM, Baqar S, Louis Bourgeois A, Campo JJ, Choy RKM, Chakraborty S, Clifford A, Deal C, Estrada M, Fleckenstein J, Hasso-Agopsowicz M, Hausdorff W, Khalil I, Maier N, Mubanga C, Platts-Mills JA, Porter C, Qadri F, Simuyandi M, Walker R, White JA. **The 2022 Vaccines Against Shigella and Enterotoxigenic Escherichia coli (VASE) Conference: Summary of abstract-based presentations.** Vaccine. 2023 Nov 28:S0264-410X(23)01364-6. doi: 10.1016/j.vaccine.2023.11.031. Epub ahead of print. PMID: 38030421.

Chapter 12

Published abstracts for presentation in scientific meetings

1. **Soumalya Banerjee** 1, Prolay Halder 2, Sanjib Das 3, Shanta Dutta 4, Hemanta Koley 5. 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 D.C., USA. (**International Oral Presentation with full travel grant**).

2. **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.

3. **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.

4. **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.

5. Prolay Halder*, **Soumalya Banerjee**, Sanjib Das, ShantaDutta, 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.

6. 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.

7. Sanjib Das1, Prolay Halder1, **Soumalya Banerjee**1, 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 11-13, 2022, Kolkata, India.

8. 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.

9. 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 mice model. 16th Asian Conference on Diarrhoeal Disease and Nutrition (ASCODD-2022) November 11-13, 2022, Kolkata, India.

10. 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.

11. 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.

12. 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.

13. Prolay Halder[1]*, Suhrid Maiti[1], **Soumalya Banerjee**[1], Sanjib Das[1], Moumita Dutta[2], Shanta Dutta[1], Hemanta Koley[1]**. 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.

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15. Prolay Halder[1], Suhrid Maiti[1], **Soumalya Banerjee**[1], Sanjib Das[1], Moumita Dutta[2], Shanta Dutta[1], Hemanta Koley[1]*. "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.

Immunology Letters 263 (2023) 33–45

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

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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ARTICLE INFO

Keywords:
Diarrhoeagenic *Escherichia coli*
Pentavalent outer-membrane vesicles (POMVs)
Immunogen
Adaptive immune response
Passive protection

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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https://doi.org/10.1016/j.imlet.2023.09.009
Received 23 May 2023; Received in revised form 7 September 2023; Accepted 18 September 2023
Available online 19 September 2023

diarrhoeal episodes are caused by DEC in developing countries and in 2011, DEC caused 120,000 deaths in children worldwide [6]. DEC are classified into five main pathological types, namely, enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC) [7]. ETEC is known to cause watery diarrhoea in infants and travellers, EPEC causes acute and chronic watery diarrhoea in the paediatric population, EIEC causes dysentery characterized by bloody mucoid diarrhoea, identical to that caused by *Shigella* sp., EHEC is associated with haemorrhagic colitis and haemorrhagic uremic syndrome (HUS) and EAEC causes acute and persistent watery diarrhoea in low to middle income countries and in immunocompromised patients [8].

Multi-drug resistant *E. coli* strains are emerging due to inappropriate use of antibiotics [9]. In the post-antibiotic era, prophylactic approaches like vaccines can work as a major asset for reduction of DEC mediated health burden [10]. Currently, researchers are trying to develop potential vaccines against DEC but are facing a major setback due to the presence of vast number of DEC serotypes and lack of production of an effective gut immune response [11]. As of now, no single licensed vaccine against DEC is available for public health use [12].

Outer membrane vesicles (OMVs) are nanosized proteoliposomes released from the outer membrane of gram-negative bacteria [13] and can serve as potential acellular vaccine candidates as they are readily taken up by mammalian cells and are strongly immunogenic in nature. OMVs contain natural antigens like phospholipids, peptidoglycan, lipopolysaccharides (LPSs), outer membrane proteins (OMPs) and components of membrane lipoproteins which mimic the bacterial outer surface. An OMV-based vaccine against *Neisseria meningitides* has been approved by the European Medicine Agency (EMA) for active human immunization [14–16]. Previous studies revealed that intraperitoneal (i.p.) immunization with *E. coli* OMVs protected mice against subsequent infections [17]. OMVs have been shown to elicit good mucosal immune responses [18], but currently no combination OMVs based vaccine has been developed against the pathotypes of DEC. Recently, a formalin-killed whole-cell combined vaccine candidate co-administered with either alum or Cholera toxin B (CTB) was developed and found to be effective against five main pathotypes of DEC in BALB/c mice [11]. OMVs possess intrinsic adjuvant properties due to the presence of LPS, flagellin, peptidoglycans, lipoproteins, and other outer membrane proteins (OMPs) which activate various Toll-like receptors (TLRs) [19], eliminating the need to add external adjuvants to enhance its immunogenicity.

Previous studies have reported that neonatal mice are more susceptible to *E. coli* mediated infections than adult mice [20,21]. Passive protection studies are conducted using a neonatal mice model to evaluate the potency and efficacy of a vaccine candidate [22–25]. Due to an immature immune system, protection of neonatal mice through vaccination is difficult to achieve as they lack sufficient time to generate an immune response against infection due to a short susceptibility period [26,27]. In a previous study, it was reported that passive transfer of immunized adult mice sera was able to protect neonatal mice against group B streptococcal infection [28]. Recently, our group demonstrated that transfer of OMVs immunized adult mouse sera conferred passive protection to suckling mice against non-typhoidal *Salmonella* mediated gastroenteritis [29].

In this study, we have developed a combined pentavalent OMVs-based immunogen (POMVs) from five main subgroups of DEC (EPEC, ETEC, EAEC, EIEC and EHEC) and determined the adaptive immune response induced by the immunogen. This study also aims to assess the passive protection conferred by POMVs immunized adult mouse sera in neonatal mice. From this study, we have found that POMVs induce significant mucosal and systemic immune responses in adult mice. The study depicts the role of POMVs-immunized adult mouse sera to passively protect neonatal mice against the five pathotypes of DEC. Our study indicates that POMVs can act as a vaccine candidate to reduce infections mediated by the five pathotypes of DEC.

2. Methods and materials

2.1. Bacterial strains and culture conditions

Pentavalent outer membrane vesicles-based immunogen (POMVs) were prepared from five circulating strains of DEC subgroups H10407 (ETEC), PD124 (EHEC), DSM411 (EAEC), BCH8865 (EPEC) and IDH10106 (EIEC). For challenge studies, another five strains of DEC subgroups were used 4266 (ETEC), VT3 (EHEC), BCH04060 (EAEC), BCH9327 (EPEC) and BCH10790 (EIEC). All the strains were obtained from ICMR- National Institute of Cholera and Enteric Diseases (NICED), Kolkata strain repository. All the strains were preserved in 20% glycerol in Brain Heart Infusion Broth (BHIB, Difco, USA) at −80 °C. For experimentation, strains were grown in Tryptic Soy Agar (TSA, Difco, USA) plates or in Tryptic Soy Broth (TSB, Difco, USA) at 37 °C under constant shaking conditions (100 rpm). For further studies, MacConkey media and antibiotic supplemented plates were used [30]. All the strains used in this study are listed in Table 1.

2.2. Preparation of POMVs

OMVs were isolated from five subgroups of DEC as previously described with slight modifications [29]. Briefly, 1 litre of TSB was inoculated with 10 mL of overnight culture of DEC subgroups and grown to log phase (8 h) under constant shaking at 37 °C. Bacteria were removed by centrifugation (8000 X g for 15 min and 30 min consecutively) and the supernatants were collected. This was followed by filtration of the supernatants consecutively with 0.45 μm and 0.22 μm pore size filters (Millipore, USA). To confirm the presence of viable bacteria in the supernatant, 100 μL of the collected filtrate was plated and incubated overnight at 37 °C on TSA plates. Protein degradation in the filtrate was prevented by addition of protease cocktail inhibitor (Roche, Switzerland). The supernatant was then ultracentrifuged using a Hitachi P27A-1004 rotor at 140,000 X g for 4 h at 4 °C. The OMV pellets obtained by ultracentrifugation were resuspended in phosphate buffered saline (PBS, 7.4). Further purification of OMVs were achieved using density gradient centrifugation. The resuspended OMVs were layered with care in a centrifuge tube containing a gradient of sucrose ranging from 10%−50%. Tubes were then centrifuged using a swinging bucket rotor (Beckman-Coulter; SW 32.1 Ti) at 150,000 X g for 5 h at 4 °C. The OMV fractions found between 20% and 30% of sucrose were collected and centrifuged at 150,000 X g for 2 h. The pellet obtained was resuspended in PBS and was again filtered with 0.22 μm pore size filters [31]. Purified OMVs of DEC subgroups obtained were either stored separately or mixed in a 1:1:1:1:1 ratio on the basis of protein content to make a final concentration of POMVs of 10 μg/100 μL PBS and stored at −80 °C for further experiments.

2.3. Transmission electron microscopy (TEM) analysis by negative staining of OMVs

On carbon-coated grids, a 5 μL aliquot of OMVs was placed for 1 min, after which it was washed with 2 drops of Tris-HCL buffer. After blotting of the excess buffer, OMVs were negatively stained using 2% aqueous solution of uranyl acetate for 30 s. Excess stain was blotted off and the grids were air dried. Finally, the negatively stained OMVs were analysed using a JEOL JEM-2100 transmission electron microscope operating at 100 kV [32] (Fig. 1a).

2.4. Particle size analysis of OMVs by dynamic light scattering (DLS)

Size distribution of OMVs was measured by DLS. Briefly, OMVs were diluted to a concentration of 0.1 mg/mL in milli-Q water and subsequently the sizes of different OMVs were measured using Zetasizer Nano

Table 1
List of strains used in this study.

Strain name	Purpose of use	DEC subtype	Abbreviation	Reference
H10407	Immunogen preparation	Enterotoxigenic *E. coli*	ETEC	[30] (Reference strain)
PD124	Immunogen preparation	Enterohaemorrhagic *E. coli*	EHEC	[30]
DSM411	Immunogen preparation	Enteroaggregative *E. coli*	EAEC	[30]
BCH8865	Immunogen preparation	Enteropathogenic *E. coli*	EPEC	Clinical isolate (This study)
IDH10106	Immunogen preparation	Enteroinvasive *E. coli*	EIEC	Clinical isolate (This study)
4266	Challenge study	Enterotoxigenic *E. coli*	ETEC	[30]
VT3	Challenge study	Enterohaemorrhagic *E. coli*	EHEC	[30]
BCH04060	Challenge study	Enteroaggregative *E. coli*	EAEC	[30]
BCH9327	Challenge study	Enteropathogenic *E. coli*	EPEC	Clinical isolate (This study)
BCH10790	Challenge study	Enteroinvasive *E. coli*	EIEC	Clinical isolate (This study)

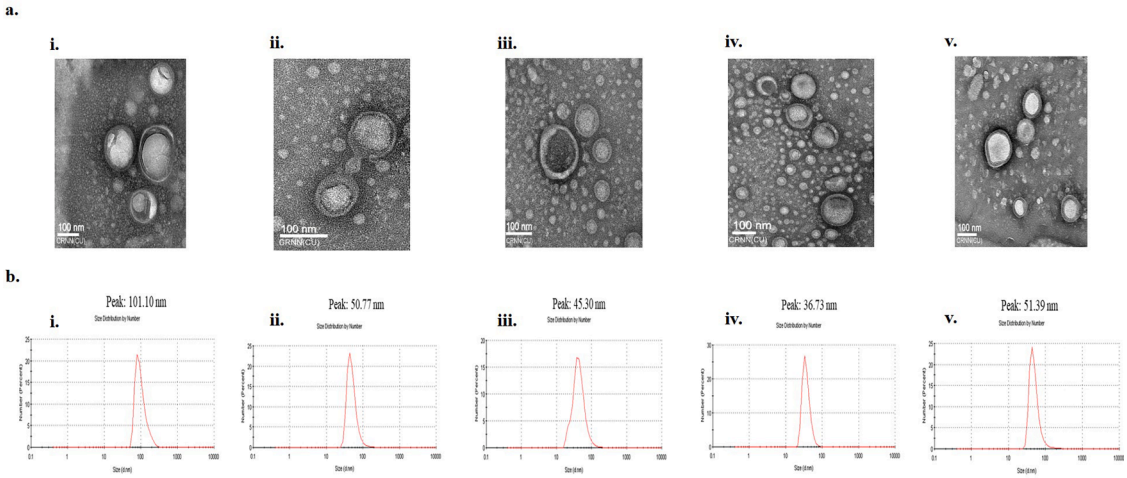

Fig. 1. (a) Transmission electron microscopic images of OMVs isolated from DEC subtypes: (i) EPEC OMVs, (ii) ETEC OMVs, (iii) EAEC OMVs, (iv) EIEC OMVs, (v) EHEC OMVs. (b) Dynamic light scattering analysis of OMVs from DEC subtypes with peaks ranging from 36.73 nm to 101.10 nm: (i) EPEC OMVs, (ii) ETEC OMVs, (iii) EAEC OMVs, (iv) EIEC OMVs, (v) EHEC OMVs.

series (Malvern, Worchestershire, UK) [33] (Fig. 1b).

2.5. Animals and housing

6–8 week old BALB/c mice were acquired from the ICMR-NICED animal house facility. Two female mice were housed with one male mouse of the same age for mating. Following conception, female mice were separated from the male and observed until birth. Other female adult mice were grouped ($n = 10$) in separate cages and maintained at 25 ± 2 °C temperature with 65 ± 2% humidity. Mice were provided with sterile food and water *ad libitum*. All the animal experiments were conducted following the standard operating procedure as outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The animal experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of NICED with Registration No. 68//Rebi/S/1999/CPCSEA valid17/7/2024, approval number PRO/183/-Jan 2021–24.

2.6. Mouse immunizations

Female adult BALB/c mice ($n = 6$) were immunized intraperitoneally (i.p.) on day 0, followed by boosters on the 14th and 28th days, with POMVs and the control group was administered with PBS on the same days. A dose of 10 μg of OMVs per mouse was used for i.p. immunizations. Immunization schedule and animal experiments are described schematically in supplementary figure 1.

2.7. Collection of serum and gut lavages

From the lateral tail vein of mice, blood was collected on the following time intervals: 0th, 7th, 21st, 35th and 56th days. The blood collected was taken in BD microtainer tubes (BD, NJ, USA) for separation of sera by centrifugation at 3000 rpm for 10 min at 4 °C. One week after the last immunization, 3 mice each from POMVs immunized and non-immunized group were sacrificed and the small intestines were dissected. The intestinal lumens were then flushed with PBS containing protease inhibitor (Roche, Sigma, USA). Then the collected lavage fluid was centrifuged at 1000 X *g* for 10 min at 4 °C, after which the supernatant was collected. Both the sera and intestinal supernatant were stored at −80 °C until further use.

2.8. Preparation of whole cell lysates (WCLs) from DEC subgroups

WCLs were prepared from the DEC pathotypes according to the previously mentioned protocol [32]. Briefly, bacteria were cultured overnight followed by centrifugation at 8000 rpm for 10 min. This was followed by a PBS wash and sonication (Heilcher UP100H). After checking the cells for membrane lysis, they were centrifuged at 10,000 rpm for 10 min and the supernatant was collected. WCLs were stored at

−80 °C until further use.

2.9. Extraction of outer membrane proteins (OMPs) from DEC subgroups

OMPs from DEC pathotypes were extracted according to the previous mentioned protocol [29] with slight modifications. Briefly, 50 mL of overnight culture was centrifuged at 8000 rpm for 10 min and cells were isolated. The isolated cells were washed with HEPES buffer (10 mM, pH 7.5) once, centrifuged and re-suspended in HEPES containing protease inhibitor. For lysis, cells were then sonicated using a Heilcher UP100H sonicator. The cells that failed to lyse were removed by centrifugation at 13,000 X *g* for 10 min and the supernatant was collected. The supernatant was then ultracentrifuged at 100,000 X *g* for 1 h at 4 °C. The pellets obtained were dissolved in 10 mM HEPES containing 1% sarcosyl and incubated for 30 min at 37 °C, followed by ultracentrifugation at 100,000 X *g* for 1 h at 4 °C. Finally, the pellets containing OMPs were washed twice with 10 mM HEPES and ultimately re-suspended in 10 mM HEPES. Protein content of the OMPs was measured using a Lowry protein estimation kit (Pierce, USA). The OMPs were stored at −80 °C until further use.

2.10. Extraction of lipopolysaccharide (LPS) from DEC subgroups

From a 50 mL overnight bacterial culture, cells were harvested by centrifugation at 8500 X *g* for 20 min, washed with normal saline and re-suspended in 150 mM NaCl containing Phenol-saturated 3-[N-*morpholino*]propen sulfonic acid (MOPS), followed by incubation with shaking at 65 °C in a water bath for 30 min. This was followed by incubation on ice for 10 min and centrifugation at 8500 X *g* for 30 min, following which the upper aqueous phase was collected. The collected aqueous phase was mixed with 95% chilled ethanol (−20 °C), 4 times the volume of aqueous phase, and kept at −20 °C overnight. LPS was ultimately isolated by centrifugation at 8500 X *g* for 20 min at 4 °C on the next day and re-suspended in milliQ water and stored at −20 °C for further use [29].

2.11. SDS-PAGE and western blot

SDS-PAGE analysis of WCLs, OMPs and OMVs of both the immunization and challenge strains was done using an AE-6503 SDS-PAGE apparatus (ATTO corporation, Japan). After estimation of protein concentration of the samples by the Lowry method, samples were denatured at 100 °C for 10 min. Aliquots of equal amount of proteins were separated by 12% SDS-PAGE and stained with Coomassie blue (Supplementary figure 2).

For western blot, the samples mentioned were first electrophoresed in 12% SDS-PAGE and transferred onto nitrocellulose membrane (Biorad, USA) by semi-dry transfer (AE 6687, ATTO corporation, Japan). Presence of immunogenic components in samples was determined using 35th day POMVs immunized mouse sera post 1st immunization. Alkaline phosphatase substrate BCIP/ NBT (MP, USA, Cat# 980,621) was used for developing the blots (Fig. 3a).

2.12. ELISA

Immunoglobulins from serum (IgG along with its subtypes IgG1, IgG2a, IgG2b, IgG3 and IgA) as well as intestinal lavage (sIgA) were measured by ELISA as described by Keren [34]. Briefly, disposable polystyrene micro-titre wells (Nunc, Denmark) were separately coated with 5 μg/well of bacterial WCLs, 5 μg/well of OMPs and 5 μg/well of LPS of each strain from which immunogens were prepared and incubated for 18 h at 4° C. Wells were washed and blocked with 5% Bovine Serum Albumin (BSA; Sigma Chemical, USA) for 2 h at 37 °C. After washing the wells with PBS-T (PBS with 0.5% Tween-20, Sigma Chemicals, USA), plates were incubated with serially diluted serum samples for 1 h at 37 °C and 100 μL of HRP-conjugated goat anti-mouse immunoglobulin was added, followed by incubation for 1 h at 37 °C. After washing with PBS, the substrate o-phenyl-Di-amine (OPD) was added to each well, followed by stopping the reaction after 10 min by addition of 100 μL of 2 N sulphuric acid. Measurement was taken at OD_{492} in a microplate reader. The experiment was repeated three times for each immunoglobulin, with the immunized and non-immunized serum, collected from individual mice, before, during and after immunization [29].

2.13. Flow cytometry analysis of splenic cells

Spleens were isolated from both non-immunized and immunized mice on the 35th day post three immunization doses for flow cytometry analysis. Mice were sacrificed under proper aseptic conditions and spleens were isolated, followed by disassociation using a sterile plunger of a 5 mL syringe and a cell strainer. RBCs were lysed using RBC lysis buffer (Sigma, USA) followed by re-suspension of the splenocytes in RPMI 1640 containing 10% FBS, 2-ME (50 mM) and antibiotics (5 U/mL penicillin G, 5 μg/mL streptomycin, and 0.1% gentamycin) (Gibco, USA). CD4+, CD8a+ and CD19+ splenic cell populations were determined by staining splenocytes with CD4-phycoerythrin (PE), CD8a-PE, CD19-PE and isotype control (PE) anti-mouse mAbs. BD FACS ARYA III flow cytometer was used for assessment of the cells and FACS DIVA software was used for analysis of data [29].

2.14. Assessment of serum bactericidal properties of POMVs immunogen and scanning electron microscopy (SEM)

Serum bactericidal assay was conducted according to the protocol mentioned previously with slight modifications [29]. Briefly, sera were collected from POMVs immunized and non-immunized mice on the 35th day post primary immunization. Collected sera was heat inactivated for 30 min at 56 °C. Each challenge strain of DEC was cultured on TSB. All the strains were grown for 2 h ($O.D._{600} = 0.4$) and bacterial pellet was collected by centrifugation at 1100 X g for 5 min and resuspended in PBS. 1:50 dilution of heat-inactivated sera with or without 25% guinea pig complement was added to the bacterial solution to make a total 50 μL reaction mixture and incubated 1 h at 37 °C. The reaction was subsequently stopped by addition of 950 μL TSB and ultimately plated on TSA plates by serial dilution for determination of CFU count. Bacteria incubated with non-immunized serum were used as control.

Bactericidal properties of POMVs immunized serum was also assessed by scanning electron microscopy (SEM) analysis. The strains were cultured as mentioned above, followed by incubation with POMVs immunized and non-immunized serum for 1 h at 37 °C and subsequently processed for SEM analysis. In short, samples were fixed using 3% glutaraldehyde and left overnight. The next day, samples were dehydrated in ascending grades of alcohol (30, 50, 70, 90, 100%), followed by chemical drying of the samples with hexamethyldisilazane (HMDS) by increasing the HMDS ratio gradually (2-part ethanol: 1-part HMDS; 1 part ethanol: 1-part HMDS; 1 part ethanol: 2-part HMDS). Finally, the samples were treated with 100% HMDS and left overnight in a fume hood for evaporation of HMDS, followed by mounting the samples on specimen stubs, sputter-coating with gold and analysis of images on Quanta 200 SEM (FEI, Netherlands).

2.15. Passive protection study in neonatal mice

One day old BALB/c neonatal mice were divided into 11 groups (A, B, C, D, E, F, G, H, I, J and K, $n = 6$ per group). Challenge study on neonatal mice was conducted as previously described with slight modifications [21,22,35]. Neonatal mice were orally inoculated with 1×10^5 CFU/neonate of challenge strains of DEC. Groups A and B were orally inoculated with EPEC BCH9327, C and D with ETEC 4266, E and F with EAEC BCH04060, G and H with EIEC BCH10790, I and J with EHEC VT3 and group K served as the control and was inoculated with PBS (uninfected). 2 h after challenge with DEC strains, groups B, D, F, H and J

were administered orally with pooled 20 μL of 35th day POMVs immunized adult mice sera (with a 1:10 dilution in PBS) while groups A, C, E, G and I received non-immunized adult mice sera of the same dilution. The neonates were returned to their dams and observed for 7 days for survival analysis.

In another set of experiments, neonatal mice were again divided into 11 groups ($n = 6$ per group) and inoculated with the same dose of challenge strains of DEC as stated above. Mice were passively immunized as described above and sacrificed after 24 h. Small intestines were collected for determination of bacterial load, homogenized, serially diluted, and plated on selective antibiotic supplemented agar plates as described previously [24,29,30]. The same set of experiments was conducted but with an inoculation dose of 1×10^3 CFU/neonate for determination of change in body weight up to 3 days post infection [29].

24 h post bacterial challenge with a dose of 1×10^5 CFU/neonate, small intestinal tissues from mice of the above mentioned groups ($n = 6$ per group), were homogenized in cytokine buffer and supernatants were stored at −80 °C as described previously for cytokine measurement [29]. For histological analysis, small intestinal samples from all the groups were collected 24 h post bacterial challenge and fixed using 10% buffered formalin solution.

2.16. Histological analysis

Following fixation using 10% buffered formalin, small intestinal samples were paraffin embedded, sectioned, double stained with haematoxylin and eosin (H & E) and visualized using an Olympus IX51 light microscope. Pathological scoring of samples (Table 2) was done as described previously with slight modifications [29,36].

2.17. Quantification of cytokines

IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70 and IL-23 were quantified from small intestinal supernatants stored in cytokine buffer using cytokine ELISA kits according to manufacturer's protocols (Invitrogen, USA).

2.18. Statistical analysis

Data are expressed as mean ± S.D. Comparison between three or more groups of data was analysed using two-way analysis of variance (ANOVA) and one tailed Student's t-test was used to analyse two grouped data sets. Mean values of triplicate experiments were taken for final calculation. A probability of $p < 0.05$ or $p < 0.01$ or $p < 0.001$ was considered as significant level. GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA) and Microsoft Excel was used for statistical analyses.

3. Results

3.1. Extraction of OMVs from DEC subgroups and preparation of POMVs immunogen

OMVs of all DEC subgroups secreted during the growth phase were found to be spherical in shape, as was revealed by TEM images (Fig. 1a i–v). The peaks of the OMVs of the DEC subgroups ranged from approximately 36 nm to 101 nm as analysed by dynamic light scattering (Fig. 1b i-v). Protein content of the OMVs was estimated and OMVs from five DEC subgroups were mixed in equal proportions (1:1:1:1:1 ratio) to formulate POMVs immunogen with a total concentration of 10 μg POMVs/100 μL of PBS.

3.2. POMVs elicited adaptive immune response in adult BALB/c mice

The magnitude of immunogenicity triggered by POMVs was determined after three doses of intraperitoneal immunization of adult BALB/c mice (10 μg of OMVs/mice) with 14 days intervening period between immunizations. The control group was administered with PBS intraperitoneally for comparative measurements. Immunogenicity of serum immunoglobulins was observed for 56 days after 1st immunization. The results demonstrated a boost in the production of serum antibodies, namely, IgG, IgG1, IgG2a, IgG2b, IgG3 and IgA against LPS, OMPs and WCLs isolated from homologous DEC subtypes with highest immunogenicity observed at 35th day post primary immunization and reached plateau afterwards (Fig. 2, a–c). Significantly higher levels of secretory IgA (sIgA) were found against LPS, OMPs and WCLs of each of the five DEC subtypes in the intestinal lavage of immunized mice as compared to non-immunized control groups, showing a significant activation of mucosal immune response (Fig 2d).

Western blot analysis was performed using 35th day polyvalent immunized adult mice sera against components of both the immunization and challenge strains. Analysis revealed immunogenic bands ranging from 200 KDa to 20 KDa against WCLs and OMPs with distinct bands in the range of 70 KDa to 20 KDa (Fig. 3a i & ii). In case of OMVs, distinct immunogenic bands ranged in the region of 40 KDa – 20 KDa (Fig. 3a iii).This signifies that POMVs contain antigenic components present in WCLs and OMPs due to which significant immunogenicity against a range of proteins present in these components can be seen in

Table 2
Pathological score reference table for histological images.

Inflammatory cell infiltrate			Mucosal architecture			
Severity	Extent	Score 1	Severity	Extent	Mucosal and epithelial changes	Score 2
Minimal	Mucosa	1	**Minimal**	Mucosa	Minimal hyperplasia Minimal villous blunting	1 1
Mild	Mucosa and sometimes submucosa	2	**Mild**	Mucosa, sometimes submucosa	Mild hyperplasia Mild to moderate villous blunting Mild goblet cell loss	2 2 2
Moderate	Mucosa and submucosa, sometimes transmural	3	**Moderate**	Mucosa, submucosa	Moderate hyperplasia Moderate villous blunting and broadening, sometimes villous atrophy Goblet cell loss Cryptits	3 3 3 3
Marked	Transmural	4	**Marked**	Mucosa, submucosa, often transmural	Marked hyperplasia Villous atrophy Marked goblet cell loss Multiple crypt absences	4 4 4 4
Sum of Score 1 and 2: 0–20						

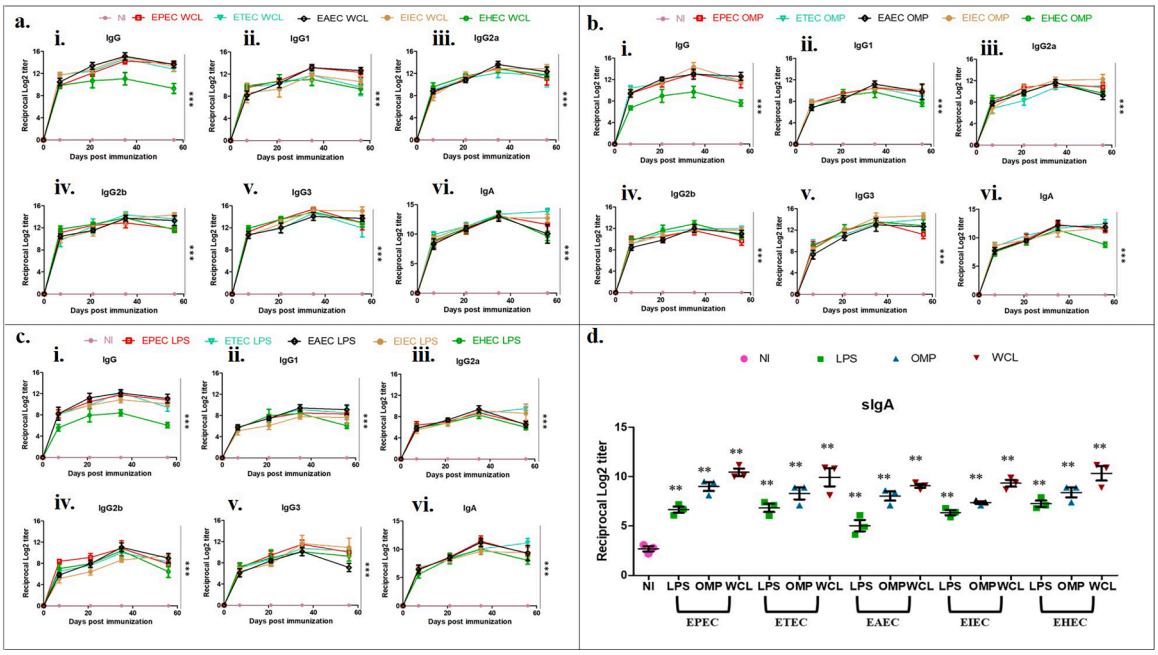

Fig. 2. Reciprocal Log2 titer of serum IgG, IgG subtypes and IgA immunoglobulin from POMVs immunized and non-immunized mouse serum were measured separately after three doses of intraperitoneal immunization against WCLs (a, i-vi) OMPs (b, i-vi) and LPS (c, i-vi) of each subtype specific five DEC strains. The horizontal axis represents pre-immunization, immunization and post-immunization periods during which antibody titers were measured. Values are represented as mean ± S.D. of three independent experiments. Significant statistical difference was found between POMVs immunized and non-immunized serum (*** p value < 0.001). (d) Reciprocal Log2 titer of mucosal IgA (sIgA) from POMVs immunized mice intestinal lavage obtained at 35th day after primary immunization. Significant statistical difference was found between POMVs immunized and non-immunized groups. Results are presented as one out of three experiments using six mice per group. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Data represented as mean ± S.D., $n = 6$. **p value < 0.01. WCL- whole cell lysate, OMP- outer-membrane protein, LPS- lipopolysaccharide.

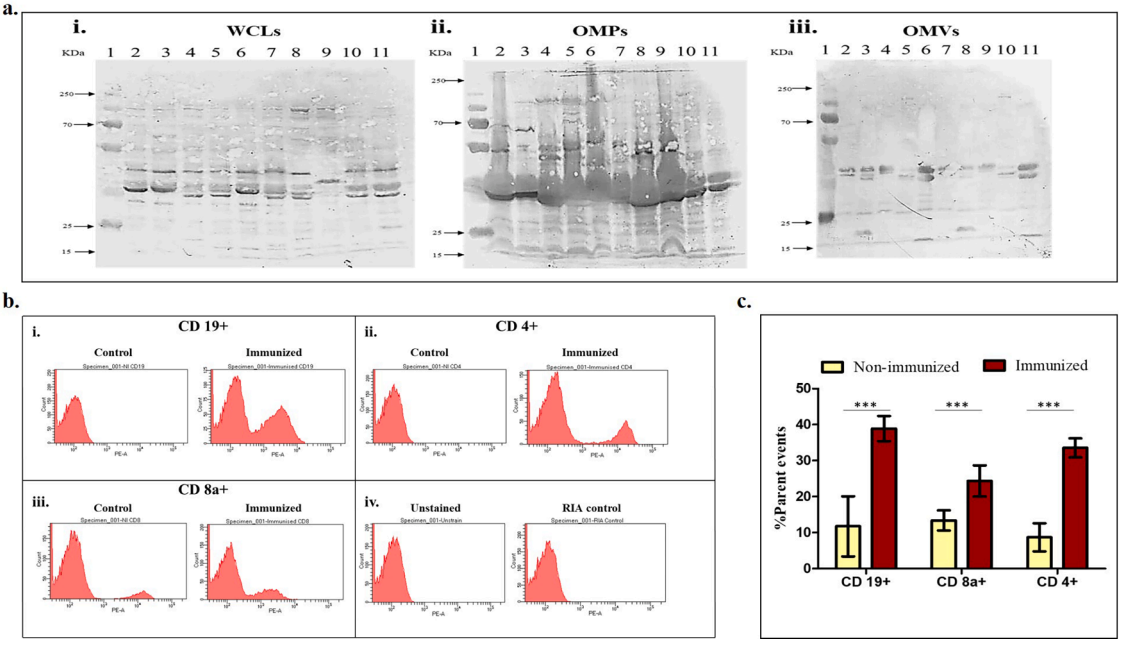

Fig. 3. (a) Immunoblot using 35th day POMVs immunized mice sera against WCLs (i), OMPs (ii) and OMVs (iii), isolated from both the immunization and challenge strains used in this study. For (i) WCLs and (ii) OMPs, the lanes numbered represent samples from the following: 1- Marker, 2- EPEC BCH8865, 3- EPEC BCH9327, 4- ETEC H10407, 5- ETEC 4266, 6- EAEC DSM411, 7- EAEC BCH04060, 8- EIEC IDH10106, 9- EIEC BCH10790, 10- EHEC PD124, 11- EHEC VT3. For (iii) OMVs, the following samples were loaded in the respective lanes: 1- Marker, 2- EPEC BCH8865, 3- ETEC H10407, 4- EAEC DSM411, 5- EIEC IDH10106 6- EHEC PD124, 7- EPEC BCH9327, 8- ETEC 4266, 9- EAEC BCH04060, 10- EIEC BCH10790, 11- EHEC VT3. (b) Representative histograms of FACS analysis of POMVs immunized and non-immunized CD 19+ (i), CD 4+ (ii), CD 8a+ splenic cells (iii) stained using CD 19-PE, CD 4-PE,CD 8a-PE and counted in FACS Aria III flow-cytometer (BD Bioscience, USA) . (iv) Represents unstained and RIA control. (c) Bar diagram comparing the percentage of CD 19+, CD 4+, CD 8a+ splenic cells from immunized and non-immunized mice. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. Significant statistical difference was found between POMVs immunized and non-immunized splenic cell population (*** p value < 0.001).

western blot.

Comparative analysis of the splenic cell populations of immunized and non-immunized mice were done using a flow cytometer. POMVs immunized mice showed a significantly greater population of CD4+, CD8a+ and CD19+ splenic cells as compared with non-immunized mice 35 days post primary immunization (Fig. 3b and c). This result suggests that antigenic components of OMVs help to elicit heightened immune responses due to activation of CD4+, CD8a+ and CD19+ splenic cells.

Altogether, i.p. immunization with POMVs generated adaptive immune responses in adult BALB/c mice and significantly activated both the cellular and humoral arms of the immune system, which could subsequently help to provide long term protective immune response against infections caused by DEC pathotypes.

3.3. POMVs immunized sera from adult BALB/c mice conferred bactericidal effect

Previous results in this study reported that POMVs immunization significantly induces high titres of anti-WCL, anti-OMPs and anti-LPS antibodies in adult BALB/c mouse sera for all the five subtypes of DEC. Bactericidal effect was analysed by serum bactericidal assay (SBA). Here, we found that immunized sera pooled from BALB/c mice, containing 25% guinea pig complement, significantly lowered the bacterial count when incubated with heterologous challenge strains of DEC subtypes, as compared to heat-inactivated (not supplemented with guinea pig complement) and non-immunized mouse serum. This signifies activation of complement pathway, along with significant antibody titres in POMVs immunized BALB/c mouse serum sufficient to effectively kill bacterial cells and subsequently lower the bacterial count (Fig. 4a). The data were further supported by SEM analysis of SBA. Pore formation, lysis and agglutination of bacterial cells were seen in DEC subtypes incubated with immunized mouse serum, whereas non-immunized mouse serum failed to effectively lyse the bacterial cells (Fig. 4b, i-v).

3.4. POMVs immunized adult mouse serum conferred passive protection to suckling mice against DEC subtypes mediated infections

Survival analysis was conducted for up to 7 days for suckling mice receiving POMVs immunized and non-immunized adult mouse sera. After challenge of suckling mice with 1×10^5 CFU/neonate of respective heterologous challenge strains of DEC subtypes, immunized and non-immunized pooled adult mouse sera (diluted in a 1:10 ratio) was administered orally 2 h post infection. Groups receiving immunized mouse sera represented significant better survival than the groups receiving non-immunized mouse sera (Fig. 5, a-e). Immunized sera recipient groups after challenge with EPEC, ETEC, EAEC, EIEC and EHEC strains represented a survival rate of 81%, 82%, 80%, 75% and 78% respectively. Suckling mice receiving non-immunized mice sera died within 2 – 4 days post infection. This result suggests that POMVs immunized adult mouse serum can significantly protect suckling mice against DEC mediated infections.

After administering a similar dose of DEC challenge strains mentioned above, assessment of changes in small intestinal colonization was analysed in immunized and non-immunized sera treated groups. 24 h post infection, a significant decrease in colonization of the five DEC pathotypes was noted in small intestine of immunized sera treated neonates as compared to non-immunized neonates (Fig. 5f).

For assessment of changes in body weight, suckling mice were administered a low dose of 1×10^3 CFU/neonate of challenge strains of DEC subtypes. Non-immunized sera treated groups displayed significant decrease in body weight observed over a 3 day period, as compared to immunized sera treated mice whose body weight remained approximately similar with that of uninfected groups (Fig. 6).

Histopathological changes of neonatal small intestine were analysed 24 h post infection with DEC challenge strains (1×10^5 CFU/neonatal mice). Recipient suckling mice administered with immunized mice sera showed a significant reduction in epithelial layer damage, altered intestinal mucosa, polymorphic nuclear neutrophilic (PMN) infiltration,

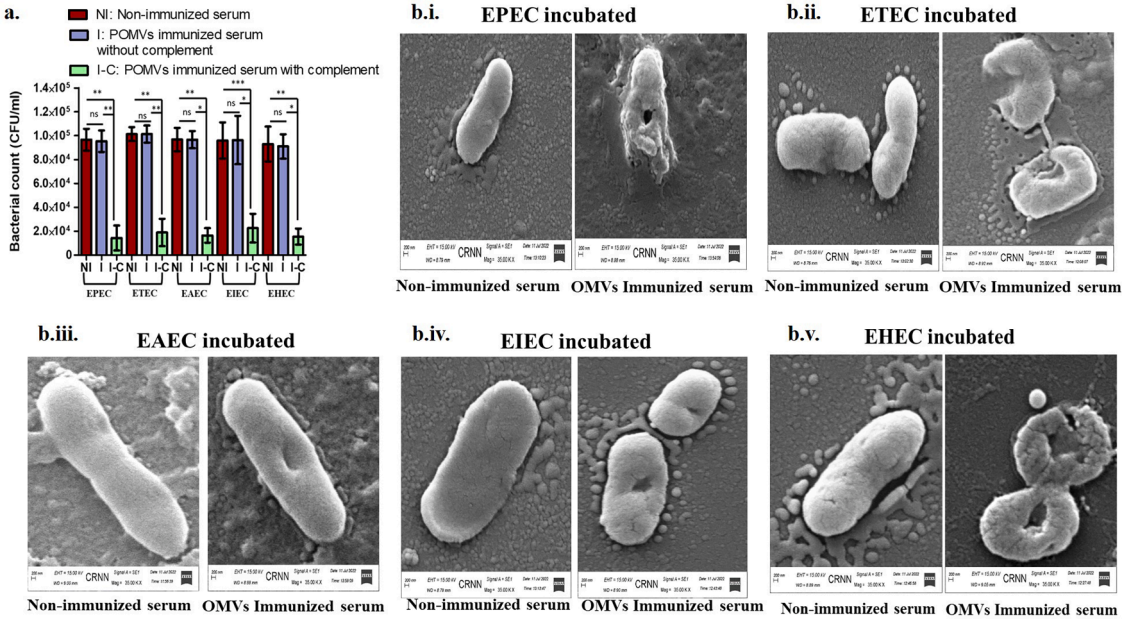

Fig. 4. (a) POMVs immunized mouse serum is effective in complement mediated lysis of DEC pathotypes. 1×10^5CFU/mL each challenge strains of EPEC, ETEC, EAEC, EIEC, EHEC were separately incubated with denoted dilution of either POMVs immunized serum or non-immunized serum with or without guinea pig complement for 1 h at 37 °C. Viable bacterial count were determined by plating on TSA plates. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. *** p value < 0.001, ** p value < 0.01, * p value < 0.5. NI, non-immunized serum; I, POMVs immunized serum without complement; I-C, POMVs immunized serum with complement added. (b) Scanning electron microscopic images of DEC challenge strains incubated with either 35th day POMVs immunized mice serum or non-immunized mice serum. (i) EPEC incubated either with or without POMVs immunized serum, (ii) ETEC incubated either with or without POMVs immunized serum, (iii) EAEC incubated either with or without POMVs immunized serum, (iv) EIEC incubated either with or without POMVs immunized serum, (v) EHEC incubated either with or without POMVs immunized serum. Scale bar 200 μm.

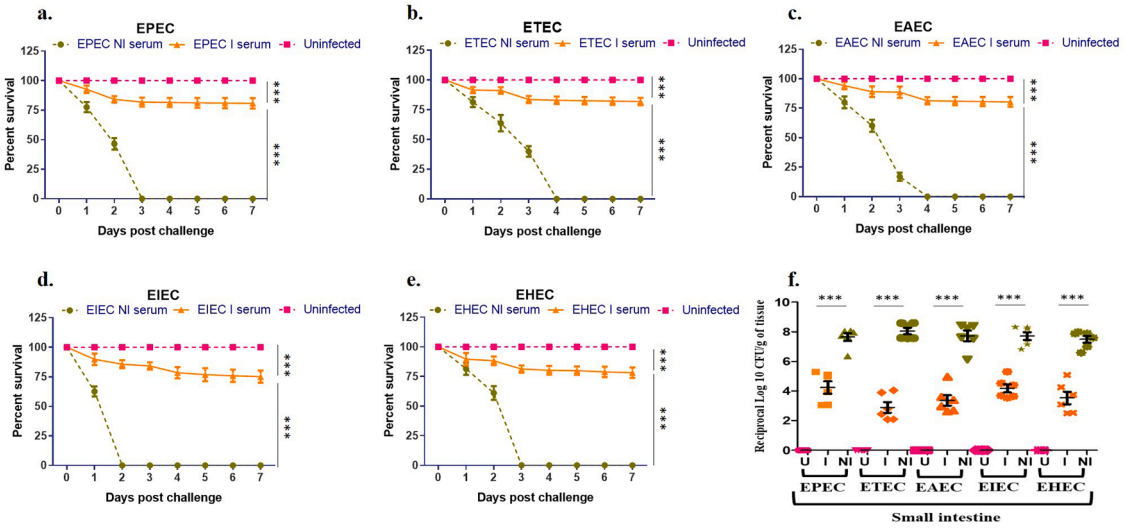

Fig. 5. One day old neonatal mice were challenged with 1×10^5CFU of DEC heterologous challenge strains separately. After two hours of challenge, neonatal mice were administered with pooled serum from POMVs immunized and non-immunized adult mice. Neonatal mice were observed for survival up to 7 days post infection. Mortality was determined based on humane endpoints. POMVs immunized sera recipient groups after challenge represented survival rate of 81% for EPEC (a), 82% for ETEC (b), 80% for EAEC (c), 75% for EIEC (d) and 78% for EHEC (e). Data are represented as mean ± S.E. of three independent experiments ($n = 6$). Protective efficacy was calculated using the equation {[(percent deaths of non-immunized neonates) - (percent deaths of immunized neonates)] / [percent deaths of non-immunized neonates]} X 100. (f) Comparison of protection determined from colonization data in small intestine of neonatal mice ($n = 6$), exerted by POMVs immunized and non-immunized adult mouse serum, after challenge with 1×10^5CFU/ neonatal mice of DEC challenge strains. Each dot represent colonization data obtained from a single neonatal mouse. On the vertical axis, data are represented as Log10 of recovered colony forming unit per gram of small intestine of each neonatal mouse, 24 h post challenge. The difference in colonization between the immunized and non-immunized sera recipient neonatal mice were highly significant (p value < 0.001). Two-way analysis of variance (ANOVA) test was used for statistical analysis. Data represented as mean value of six neonatal mice ± S.E. of three independent experiments. *** p value < 0.001. UI, uninfected; I, immunized sera recipient; NI, non-immunized sera recipient.

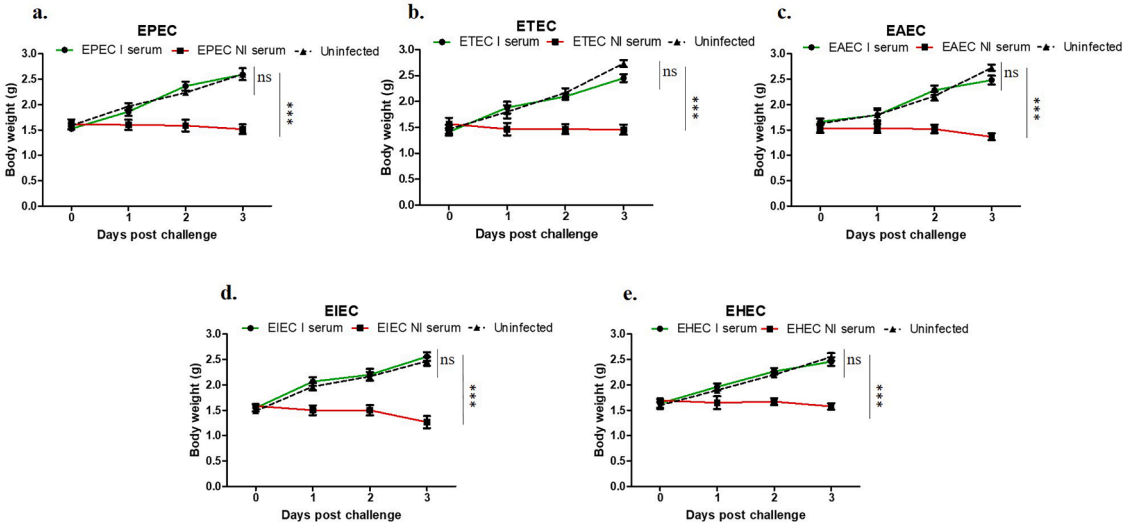

Fig. 6. One day old neonatal mice were challenged with 1×10^3CFU of DEC heterologous challenge strains separately ($n = 6$). 2 h post challenge, neonatal mice were administered pooled serum from POMVs immunized and non-immunized adult mice. Changes in body weight were monitored for 3 days (a-e). Every single dot represents mean and error value of six neonatal mice ± S.D. of three independent experiments. *** p value < 0.001. ns, non-significant. I, immunized; NI, non-immunized.

goblet cell loss, hyperplasia and villous atrophy as compared to non-immunized sera recipient suckling mice (Fig. 7a). Pathological scores of immunized sera treated suckling mice were also lower than non-immunized sera treated suckling mice (Fig. 7b). Overall, passively transferred POMVs immunized adult mouse sera, exerted a significant level of passive protective efficacy against five DEC pathotypes in neonatal mice.

3.5. POMVs immunized mouse serum lowers pro-inflammatory cytokine levels in neonatal mouse small intestine after infection

Significant differences in the amount of pro-inflammatory cytokines were noted in small intestinal tissue of immunized sera treated and non-treated neonates after infection with DEC subtypes (1×10^5 CFU/ neonate). Immunized sera recipient neonatal mice had substantially reduced levels of IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70 and IL-23 in small intestinal tissues as compared to non-immunized sera recipient neonatal mice after infection (Fig. 8). These pro-inflammatory cytokines

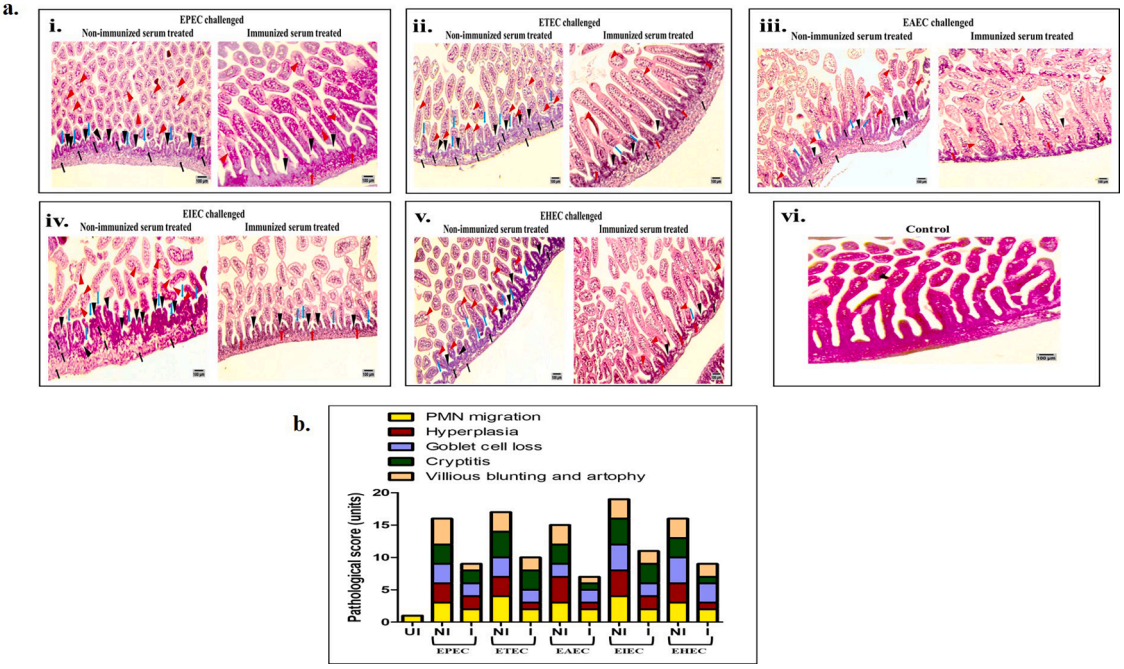

Fig. 7. Pentavalent OMVs immunized mouse serum decreases small intestinal tissue damage and inflammation in neonatal mice after infection with DEC pathotypes separately (1×10^5CFU). (a) Histological images of small intestine of neonatal mice administered with either 35th day POMVs immunized mouse serum or non-immunized mouse serum. (i) EPEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (ii) ETEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (iii) EAEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (iv) EIEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (v) EHEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (vi) uninfected control neonatal mice small intestine. Small intestine of neonatal mice treated with POMVs immunized mouse serum showed mild epithelial layer damage, altered intestinal mucosa and polymorph nuclear neutrophilic (PMN) infiltration, whereas non-immunized sera treated neonatal mice displayed marked epithelial layer damage, altered intestinal mucosa, polymorph nuclear neutrophilic (PMN) infiltration, goblet cell loss, hyperplasia and villous atrophy. (Blue arrow– Villous blunting and atrophy; Black arrow – PMN migration marked level; Red arrow- PMN migration mild level; Black arrowhead – Crypt alteration with mucus or inflammatory cell; Red arrowhead – Goblet cell loss. Images were taken at 200X magnification, 100 μm scale bar. (b) Pathological scores of immunized or non-immunized sera treated neonatal mice small intestine post challenge. Immunized sera treated neonatal mice small intestine displayed less inflammation as compared to that of non-immunized sera treated neonatal mice. UI, uninfected; I, immunized sera recipient; NI, non-immunized sera recipient.

are responsible for epithelial cell damage and villous atrophy. Therefore, administration of POMVs immunized adult mouse sera significantly lowered intestinal inflammation in neonatal mice after infection, which ultimately lowers the level of intestinal destruction as is evident from previous results.

4. Discussion

Childhood mortality due to diarrhoeal diseases are a major concern in developing nations with children less than five years of age most affected [37]. DEC pathotypes are among the main agents responsible for diarrhoea-related illnesses. Due to the emergence of multi-drug resistant (MDR) bacteria, vaccination is an advantageous approach to mitigate infections caused by DEC pathotypes [38,39]. Polysaccharide conjugate vaccine efficacy was previously studied against O111 *E. coli* [40]. ETVAX, an inactivated whole-cell vaccine, which is comprised of four recombinant *E. coli* strains overproducing adhesins, along with recombinant hybrid B subunit of heat labile toxin and cholera toxin, was found to be safe and immunogenic in phase 1/2 trials [41]. But none of these vaccines were effective in providing broad spectrum immunogenicity and protection against multiple DEC subtypes. Combination-based vaccines are considered a better approach to prevent diarrhoea associated infections [42]. Recently, a study reported formalin killed whole-cell vaccine, when administered subcutaneously in BALB/c mice along with cholera toxin B subunit as an adjuvant, was effective in eliciting immunogenicity and provided protection against five main subtypes of diarrhoeagenic *E. coli* [11]. The intrinsic adjuvant properties of OMVs, due to the presence of LPS, peptidoglycan, flagellin and lipoproteins [19,43,44], eliminates the need to supplement OMVs with external adjuvants to enhance immunogenic properties. Therefore, we have formulated OMVs-based immunogen isolated from EPEC, ETEC, EAEC, EIEC and EHEC so as to provide broad spectrum protection against DEC pathotypes. OMVs-based vaccines have already been shown to be effective against *Shigella, Salmonella* and *Neisseria* species [45–48]. Previous reports stated that three doses of 25 μg intramuscular immunizations of *Neisseria meningitis* OMVs-based vaccine (MenBvac and MeNZB) was effective in eliciting a protective immune response [16,49,50]. Using a similar approach, we found that i.p. immunization with three doses of 10 μg of POMVs at 14 day intervals activated both the cellular and humoral arms of the immune response in BALB/c mice. Similar types of immune responses were found in our previous studies, both of which were bivalent OMVs-based vaccines, one against *S.* Typhi and *S.* Paratyphi, and another against *S.* Typhimurium and *S.* Enteritidis in an adult BALB/c mouse model [29,32]. In this study, it was noticed that a minimum of 10 μg of OMVs per mice was sufficient to effectively stimulate secretion of systemic antibodies. Levels of serum IgA, IgG and its subtypes were found to significantly increase after immunization. IgG2a and IgG2b lead to activation of the complement pathway, which ultimately leads to opsonisation of the antigen of interest. Bacterial capsular polysaccharides activates IgG3 subclass in mice [51] and IgG1 subclass mediates effective uptake of bacteria by phagocytes [52]. The elevated levels of IgG subclass in immunized mice might be due to the presence of a variety of antigens on POMVs, which may cause activation of cellular arm of immune response required for passive protection of neonatal mice.

Secretory IgA is associated with prevention of host tissue invasion

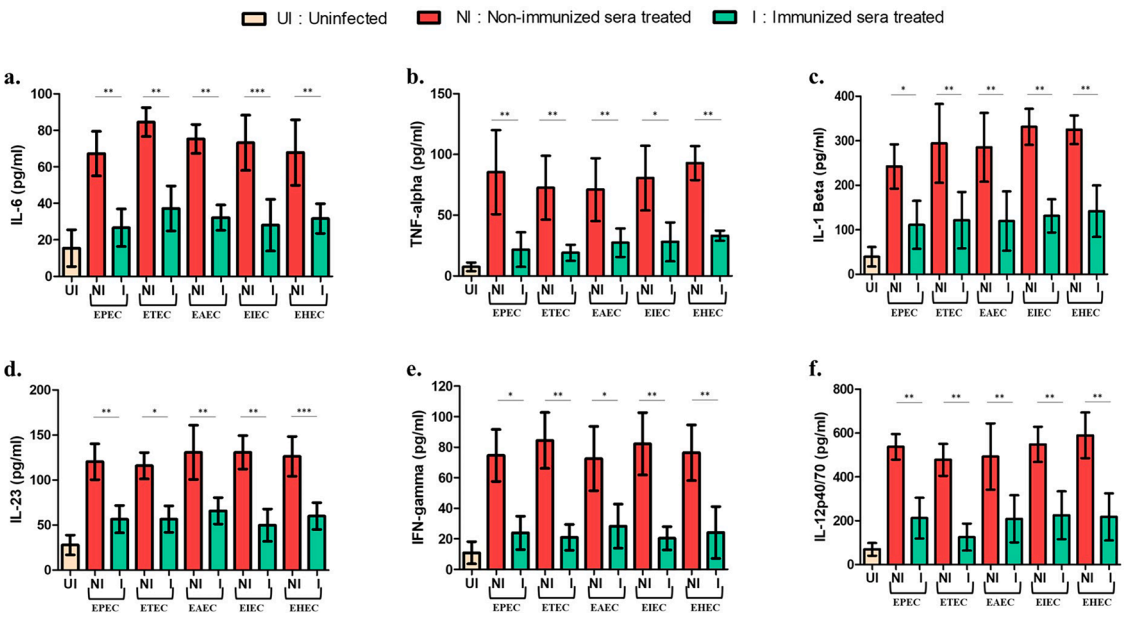

Fig. 8. Passive immunization with POMVs immunized adult mouse sera significantly lowers expression of inflammatory markers in small intestinal tissues of neonatal mice. Small intestines of immunized or non-immunized sera treated neonatal mice were isolated 24 h post challenge with respective DEC pathotypes (1×10^5CFU) and processed as described in materials and methods. Pro-inflammatory cytokine levels were measured in small intestinal tissue extracts. Cytokine levels of (a) IL-6, (b) TNF-α, (c) IL-1β, (d) IL-23, (e) IFN-γ and (f) IL-12p40/70. ($n = 6$). Two-way analysis of variance (ANOVA) test was used for statistical analysis. Significant statistical difference was found between uninfected, immunized sera treated and non-immunized sera treated neonatal mice small intestinal tissue inflammatory markers (*** p value < 0.001, ** p value < 0.01, * p value < 0.5). Bars represent mean of six neonatal mice ± S.E. of three individual experiments. UI, uninfected; I, immunized sera recipient; NI, non-immunized sera recipient.

and faster clearance of bacteria from the gut lumen. The clearance of bacteria by sIgA from the intestinal lumen is mediated either by the classical agglutination or the enchained growth pathway. In classical agglutination, sIgA prevents bacterial interaction with the mucosal layer, which results in elimination of the bacteria from the intestinal lumen. In the case of the enchained growth pathway, sIgA causes formation of a coat outside bacteria, which prevents bacterial sister cells from dividing, ultimately leading to clearance of undissociated cells [29,53,54]. Elevated titres of anti-LPS, anti-OMPs and anti-WCLs sIgA was also found in this study in the gut lumen of mice after i.p. immunization with POMVs. This indicates that POMVs immunization can mediate clearance of DEC subtypes and can eventually protect against subsequent infections.

Recently, studies have shown that different outer membrane proteins of *E.coli* like OmpA, OmpC, BamA and OmpF have immunogenic properties and can therefore act as potential immunogens [55,56]. In our study, western blot analysis revealed polyvalent POMVs immunized mouse anti-sera are able to recognize a range of immunogenic proteins present in WCLs, OMPs and OMVs. Moreover, polyvalent immunized anti-sera could recognize immunogenic proteins of heterologous challenge strains, along with those of homologous immunization strains. This indicates that these conserved immunogenic proteins may be present in POMVs and might play a pivotal role in modulating host immune responses. Previously, it was reported that i.p. immunization with *E. coli* OMVs increases the population of CD4+ and CD8+ splenic T-cells [17], necessary for a long-term humoral immune response. Other studies have also reported increases in splenic T-cell populations after subsequent immunization with OMVs isolated from enteric disease causing bacteria [25,29,32]. In the current study, a hike in the CD4+ and CD8a+ splenic cell populations was seen after i.p. immunization of mice with POMVs, which is similar to those observed previously. Therefore, POMVs potently activate both the cellular and humoral arms of the adaptive immune response, which is necessary for providing a long-term protective immune response against DEC-mediated infections.

In a previous study, it was reported OmpC in *E. coli* is required for activation of the complement pathway by Cq1 and loss of OmpC function renders serum inactive to effectively kill *E. coli*, allowing it to escape the immune response [57]. In this study, we observed bactericidal effect of POMVs immunized serum by SBA and scanning electron microscopy analysis and found that immunized mouse sera can efficiently bind and kill the DEC subtypes by causing lysis of the bacterial cells. SBA analyses the functionality of antibodies so as to assess the immunogenicity and potential protective capacity of the immunogen in preclinical studies [58]. POMVs contain various components of outer membrane proteins, as was evident from the results of immunoblot, which can play a vital role in the activation of classical complement pathway thereby leading to lysis of the bacterial cells.

Due to an immature immune system, neonates are not adequately protected in early life from infectious diseases [27]. Serum therapy or passive antibody therapy has long been used against infectious organisms responsible for causing disease in humans [59]. Neonatal mice, having an immature immune system, are a suitable animal model for studying passive protection conferred by immunized mouse sera [29,60]. In our study, passive transfer of POMVs immunized adult mouse sera significantly protected the neonatal mice from infections by DEC pathotypes. Neonatal mice administered orally with immunized adult mouse sera demonstrated better survivability, insignificant or no loss in body weight and a several fold decrease in small intestinal colonization against five DEC subtypes. Our previous studies also showed passive protection exerted by OMVs isolated from *Shigella* sp. [24], *Vibrio cholerae* [25], *Salmonella* Typhimurium and *Salmonella* Enteritidis [29]. Small intestinal histopathological scores of immunized sera treated neonatal mice were found to be lower than those treated with non-immunized sera. Neonatal mice administered with immunized sera displayed less epithelial layer damage, altered intestinal mucosa, polymorph nuclear neutrophilic (PMN) infiltration, goblet cell loss, hyperplasia and villous atrophy than those treated with non-immunized sera after infection. Previously, a study reported that anti-LPS IgG in serum protects against *E. coli* infections [61]. In this study, we hypothesize that anti-LPS IgG in immunized mice serum might trap the bacteria and

prevent infection in neonatal mice.

IL-6 was found to promote inflammation by *E. coli* in the intestine and acted as a major pro-inflammatory cytokine, expressed differentially based on disease severity [62]. IL-1β increases epithelial cell permeability by affecting tight junctions. IL-1β causes upregulation of NF-κβ, which in turn enhances permeability by affecting the tight junctions [63]. LPS binding to toll-like receptors (TLRs) leads to TNF-α production [64] and pathogenesis of inflammatory bowel disease has been found to be associated with the overproduction of TNF-α and IFN-γ [65]. IFN-γ has been found to cause micropinocytosis of tight junction proteins into early endosomes, thereby increasing permeability of the intestinal epithelial cell barrier [65]. Bacterial infection induces monocytes, macrophages and dendritic cells to secrete IL-12 and IL-23, which further stimulates T-lymphocytes to secrete inflammatory cytokines, especially IFN-γ. IL-12 and IL-23 also stimulate T-cells to produce pro-inflammatory markers such as IL-1, IL-6, TNF-*α* and chemokines causing inflammation [66]. This study suggests that administration of POMVs immunized adult mice sera downregulated the production of IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70 and IL-23 pro-inflammatory cytokines in neonatal mice small intestines after challenge with DEC subtypes, leading to attenuation of inflammation. This correlates with the histopathological observations in which immunized sera treated neonatal mice had lower pathological changes when compared to those treated with non-immunized sera.

5. Conclusion

This study demonstrates POMVs can be used as a potent immunogen to elicit broad spectrum immunogenicity against five DEC pathotypes after intraperitoneal administration in adult BALB/c mice. Immunized mouse sera displayed characteristic bactericidal properties thereby effectively killing the DEC pathotypes. Moreover, passive transfer of POMVs immunized adult mouse sera provided broad spectrum protection to neonatal mice against infections caused by five pathotypes of DEC.

Funding

This work was supported by intramural project funded by Indian Council of Medical Research-National Institute of Cholera and Enteric Diseases (ICMR-NICED, Intramural project index: Agenda [#7 (III) IM/HK)] and project funded by Program of the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) [JP23wm0125004] from the Ministry of Education, Culture, Sports, Science and Technology in Japan (MEXT), and Japan Agency for Medical Research and Development (AMED).

Declaration of Competing Interest

The authors declare no financial or personal conflicts of interest.

Acknowledgements

The authors sincerely thank Professor Jeffrey H. Withey (Department of Biochemistry, Microbiology, and Immunology, Wayne State University School of Medicine, Detroit, Michigan, USA) for his help in correcting the manuscript. The authors are highly thankful to Ms. Srijani Biswas, Mr. Benson George Chacko, Ms. Debashree Das, Ms. Ipsita Chakraborty, Ms. Sweety Bharti, Mr. Pritam Nandy, Mrs. Arpita Sarbajna, Mr. Suhasit Ranjan Ghosh and Mr. Subrata Sinha for their technical assistance. We would like to thank University Grants Commission (UGC), New Delhi, India, for providing fellowship to Soumalya Banerjee under CSIR-UGC-NET scheme [Student ID:191620007740], Indian Council of Medical Research for providing fellowship to Prolay Halder [ICMR fellowship ID No. 3/1/3/JRF-2018/HRD-066(66125)] and Sanjib Das under CSIR-UGC-NFSC scheme [SANJIB DAS/3363/(CSIR-UGC NET JUNE 2018)].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2023.09.009.

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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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Research Article

Keywords: Vaccine, Trivalent outer-membrane vesicles (TOMVs), Campylobacter jejuni, Salmonella Typhimurium, Salmonella Enteritidis.

Posted Date: March 8th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4009284/v1

Additional Declarations: No competing interests reported.

Abstract

Campylobacter and non-typhoidal *Salmonella* (NTS) are among the most common causative agents of gastroenteritis worldwide. As of now, no single combination licensed vaccine is available for public health use against both NTS and *Campylobacter*species. Outer-membrane vesicles (OMVs) are nanoscale proteoliposomes released from the surface of gram-negative bacteria during log phase and harbor a variety of immunogenic proteins. Based on epidemiology of infections, we formulated a novel trivalent outer membrane vesicles (TOMVs)-based vaccine candidate against *Campylobacter jejuni* (CJ),*Salmonella* Typhimurium (ST) and*Salmonella* Enteritidis (SE). Isolated OMVs from CJ, ST and SE were combined in equal ratios for formulation of TOMVs and 5 µg of the developed vaccine candidate was used for intraperitoneal immunization of adult BALB/c mice. Immunization with TOMVs significantly activated both the humoral and cellular arm of adaptive immune response. Robust bactericidal effect was elicited by TOMVs immunized adult mice sera. TOMVs immunization induced long-term protective efficacy against CJ, ST and SE infections in mice. The study illustrates the ability of TOMVs-based combination immunogen in eliciting broad-spectrum protective immunity against prevalent *Campylobacter* and NTS pathogens. According to the findings, TOMVs can work as a potent combination-based acellular vaccine candidate for amelioration of *Campylobacter* and NTS-mediated gastroenteritis.

Introduction

Diarrhoeal diseases continue to be a monumental cause of morbidity and mortality in developing countries especially among children less than five years of age [1, 2]. Approximately 1.6 million deaths are associated with diarrhoeal illness each year globally, with low- and middle-income countries (LMIC) having the highest-burden [3]. *Campylobacter* and non-typhoidal *Salmonella* (NTS) are foodborne zoonotic pathogens responsible for causing gastroenteritis [4–6]. 8% of global diarrhoeal cases are due to *Campylobacter* infection [7]. Common symptoms associated with campylobacteriosis are diarrhoea, abdominal pain, fever, nausea, vomiting and the main recognised consequences due to the infection are Guillain-Barré syndrome, reactive arthritis, and irritable bowel syndrome [8, 9]. Most countries around the globe consider *Campylobacter jejuni* (CJ) as the one of the major causative agents of human campylobacteriosis [10]. NTS is responsible for 93.8 million gastroenteritis cases each year, with 155 thousand deaths globally [11]. Invasive non-typhoidal *Salmonella* (iNTS) infections are characterized by abdominal pain, diarrhoea, nausea, vomiting and fever [12]. In immune-compromised individuals, iNTS is responsible for causing bacteraemia or focal infections at systemic sites [11]. *Salmonella* Typhimurium (ST) and *Salmonella* Enteritidis (SE) are the most common iNTS serotypes associated with human infection [13, 14].

Multi-drug resistant *Campylobacter* and non-typhoidal *Salmonella* are emerging due to inappropriate use of antibiotics [15]. In this generation of emerging antimicrobial resistance, prophylactic measures such as vaccines can serve as an important resource for reducing infections by foodborne pathogens [15, 16]. The primary roadblock in the development of combination-based vaccines is the genetic and antigenic

heterogeneity among various enteric pathogens. Lack of understanding of disease mechanism or pathogenesis of NTS and *Campylobacter* infections, undefined host immune correlates of protection, and short-lived protective immunity are some of the other reasons for delays in the development of combination-based vaccines against NTS and *Campylobacter* [17]. Currently, a single licensed combination vaccine against *Campylobacter* and NTS is not available in the market [18].

Combination vaccines are designed and formulated to protect the body at one time against multiple infections [19]. The combination vaccine of diphtheria, tetanus, and pertussis (DTP vaccine) was the first to be used for vaccination of infants and children in 1948 [20]. According to the World Health Organization (WHO), combination vaccines are divided into two types: (a) vaccines that contain multiple antigens in a single formulation for protection against multiple diseases and (b) those that contain antigens of different strains and serotypes of the same organism, providing protection against a single infectious disease. The advantages of combination vaccines over individual formulations include reduced frequency of clinic visits and injections, decrease in the duration of infant distress, increase in parental willingness for vaccination, lesser operational and stocking costs, and increase in the vaccine-preventable disease coverage rates [21]. Recently, our group developed a combined pentavalent outer membrane vesicles-based vaccine candidate that conferred potent protective immune response against five major pathotypes of diarrhoeagenic *Escherichia coli* in a mouse model [22].

Outer membrane vesicles (OMVs) are non-replicative membrane-derived spherical nanostructures that are released by gram-negative bacteria. These immunogenic facsimiles of secreting bacteria harbour a variety of bacteria-specific antigens and different pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS), peptidoglycan and lipoproteins, which mimic the bacterial outer surface and can therefore serve as potential acellular vaccine candidate [23–25]. Along with strong immunogenicity, OMVs have intrinsic robust adjuvanticity due to presence of PAMPs, which activate various Toll-like receptors (TLRs) [23, 26], eliminating the necessity for adding external adjuvants. A *Neisseria meningitides* OMV-based vaccine has been approved by European Medicines Agency (EMA) and Food and Drug Administration (FDA) for active human immunization [27]. Previous studies showed that intraperitoneal (i.p.) immunization with *S*. Typhimurium and *S*. Enteritidis OMVs protected mice against successive NTS infections [28, 29]. Another study also demonstrated protection mediated by chitosan-coated OMV-based antigen immunization in mice against subsequent *C. jejuni* infections [30]. OMVs are known to elicit potent mucosal immune responses [31], but at present, no single combination OMVs-based vaccine has been developed for use against both *Campylobacter* and NTS.

The aim of this study was to develop a trivalent OMVs-based vaccine candidate (TOMVs) from *C. jejuni*, *S*. Typhimurium and *S*. Enteritidis and determine the adaptive immune response the combination vaccine induces. Another aim of the study was to assess the protective efficacy mediated by TOMVs in an adult BALB/c mouse model. From this study, we have found that TOMVs significantly activate both the systemic and mucosal immune responses in BALB/c mice. The study also assessed the long-term duration of protection mediated by TOMVs immunogen. Our study elucidates that TOMVs can be used as

a combination vaccine candidate for reduction of infections mediated by *C. jejuni*, *S*. Typhimurium and *S*. Enteritidis.

Methods and materials

Bacterial strains and culture conditions

Trivalent outer membrane vesicles-based vaccine candidate (TOMVs) were prepared from three circulating strains, IDH2028 *Campylobacter jejuni* (CJ), PH94 *S*. Typhimurium (ST) and EVS111 *S*. Enteritidis (SE). Another three reference strains of *Campylobacter* and iNTS were used for challenge studies, BAA-1153 (CJ), ATCC 14028 (ST) and ATCC 13076 (SE). All the strains were obtained from the strain repository of ICMR- National Institute of Cholera and Enteric Diseases (NICED), Kolkata. *C. jejuni* strains were maintained and preserved in 20% glycerol in Mueller-Hinton Broth (MHB, Difco, USA) at -80º C. *S*. Typhimurium and *S*. Enteritidis strains were stored in 20% glycerol in Tryptic Soy Broth (TSB, Difco, USA) at -80º C. For experimentation, *C. jejuni* strains were routinely grown under microaerobic conditions (85% N2,10% CO2, 5% O2) in Mueller-Hinton Agar (MHA, Difco, USA) or Mueller-Hinton Broth (MHB, Difco, USA) with appropriate antibiotics in the following concentrations: chloramphenicol, 15 µg/ml; kanamycin, 50 µg/ml; trimethoprim, 10 µg/ml; streptomycin, 2 mg/ml [32]. *S*. Typhimurium and *S*. Enteritidis strains were grown in Tryptic Soy Agar (TSA, Difco, USA) plates or in Tryptic Soy Broth (TSB, Difco, USA) at 37º C under constant shaking conditions (100 rpm). All the strains used in this study are listed in Table 1.

Table 1
List of strains used in this study.

Strain name	Purpose of use	Organism	Abbreviation	Reference
IDH2028	Immunogen preparation	*Campylobacter jejuni*	CJ	Clinical isolate (This study)
PH94	Immunogen preparation	*Salmonella* Typhimurium	ST	Clinical isolate (This study)
EVS111	Immunogen preparation	*Salmonella* Enteritidis	SE	Clinical isolate (This study)
ATCC BAA-1153	Challenge study	*Campylobacter jejuni*	CJ	Reference strain
ATCC 14028	Challenge study	*Salmonella* Typhimurium	ST	Reference strain
ATCC 13076	Challenge study	*Salmonella* Enteritidis	SE	Reference strain

Extracting and preparing TOMVs

OMVs were extracted from CJ, ST, and SE according to the previously explained protocol with a few modifications [22]. In short, 1 L of MHB was inoculated with a stationary phase culture (10 mL) of CJ and grown until logarithmic phase (14 h) under constant shaking in microaerobic conditions at 37º C. Similarly, 1 L of TSB was inoculated with a stationary phase culture (10 mL) of either ST or SE and grown to logarithmic phase (5 h) with constant shaking at 37º C. Following centrifugation at 8000 X g for 15 mins and 30 mins subsequently, bacterial pellets were removed and the supernatants were collected. The supernatants were then consecutively filtered with pore size filters of 0.45 µm and 0.22 µm (Millipore, USA). For confirming the absence of viable bacteria, 100 µL of the collected supernatant was plated and incubated at respective conditions for CJ, ST and SE mentioned above. A cocktail of protease inhibitors (Roche, Switzerland) was used to prevent protein degradation. The supernatant was then ultracentrifuged at 140,000 X g for 4 h at 4º C, using a Hitachi P27A-1004 rotor. After ultracentrifugation, and following resuspension of the OMV pellets in phosphate buffered saline (PBS, 7.4), further purification of OMVs was achieved using density gradient centrifugation. After layering the OMVs in a centrifuge tube in a sucrose gradient ranging from 10%-50%, the tubes were ultracentrifuged using a swinging bucket rotor (Beckman-Coulter; SW 32.1 Ti) at 150,000 X g for 5 hours at 4º C. Between 20% and 30% of sucrose, the OMV fractions found were collected and again centrifuged at 150,000 X g for 2 h [22, 33]. After resuspension of the obtained pellets in PBS and filtration with 0.22 µm pore size filters, purified OMVs of CJ, ST and SE were either stored separately or mixed in a 1:1:1 ratio. Based on the protein content, a final concentration of TOMVs of 5 µg/100 µL PBS was made and stored at -80º C for further experiments.

Transmission electron microscopy (TEM) analysis by negative staining of OMVs

Negative staining of OMVs was done according to a previously mentioned protocol [22]. Briefly, 5 µL aliquot of OMVs were placed on carbon-coated grids for 1 min and after washing with Tris-HCL buffer, excess buffer was removed and OMVs were negatively stained with 2% aqueous solution of uranyl acetate for 30 s. After blotting off the excess stain, the grids were air dried. Finally, using JEOL JEM-2100 transmission electron microscope operating at 100kV, the negatively stained OMVs were analysed (Fig. 1a).

Particle size distribution and analysis of zeta potential of OMVs

The size distribution and zeta potential of OMVs were measured according to previous published protocol [22, 30]. Briefly, OMVs were adjusted to a concentration of 0.1 mg/mL in milli-Q water followed by analysing the size distribution and zeta potential of different OMVs using Zetasizer Nano series (Malvern, Worchestershire, UK) (Fig. 1b and 1c).

Proteomic analysis of OMVs

According to a previously explained protocol with minor modifications, protein in each sample was used for digestion, which was reduced with 5 mM Tris[2-carboxyethyl]phosphine (TCEP) and further alkylated

with 50 mM iodoacetamide, followed by digestion with Trypsin (1:50, Trypsin/lysate ratio) for 16 h at 37° C. Using a C18 silica cartridge, the digests were cleaned to remove the salt and dried using a speed vac, followed by resuspension of the dried pellet in buffer A (2% acetonitrile, 0.1% formic acid) [34, 35]. Experiments were conducted on an Easy-nlc-1000 system coupled with an Orbitrap Exploris mass spectrometer. On C18 column 15 cm, 3.0 µm Acclaim PepMap (Thermo Fisher Scientific, USA), 1 µg of peptide sample was loaded and separated with a 0–40% gradient of buffer B (80% acetonitrile, 0.1% formic acid, at a flow rate of 500 nl/min) and injected for MS analysis. For 60 min, LC gradients were run. In the Orbitrap, MS1 spectra were acquired (Max IT = 25ms, AGQ target = 300%; RF Lens = 70%; R = 60K, mass range = 375 − 1500; Profile data). Dynamic exclusion was applied for 30 s, excluding all charge states for a given precursor. MS2 spectra were collected (Max IT = 22ms, R = 15K, AGC target 200%) for the top 12 peptides. Following processing of all the samples, the raw files generated were analyzed using Proteome Discoverer (v2.5) against the Uniprot organism database. The precursor and fragment mass tolerances were set at 10 ppm and 0.02 Da for dual Sequest and Amanda search. Enzyme specificity was set for trypsin/P (cleavage at the C terminus of “K/R”: unless followed by “P”). Carbamidomethyl on cysteine as fixed modification and oxidation of methionine and N-terminal acetylation were taken into consideration as variable modifications for database search. 0.01 FDR was set for both peptide spectrum match and protein false discovery rate (Fig. 1d and 1e; supplementary tables S1, S2 and S3).

Animals and housing

Female adult BALB/c mice of 6–8 weeks age were acquired from the ICMR-NICED animal house facility. Mice were grouped (n = 10) in separate cages, maintained at 25 ± 2º C temperature with 65 ± 2% humidity and provided with sterile food and water *ad libitum*. All animal experiments were conducted following the standard operating procedure, as outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The approval of animal experimental protocols was given by the Institutional Animal Ethical Committee (IAEC) of NICED with Registration No. 68//Rebi/S/1999/CPCSEA valid17/7/2024, approval number PRO/174/ January 2020 - December 2022.

Immunization of Mice

I.p. immunization was done to adult female BALB/c mice (n = 6) on day 0, followed by first and second boosters on the 14th and 28th days, with TOMVs and PBS was administered to the control groups on the same time points. For i.p. immunizations, a dose of 5 µg of TOMVs per mouse was used. Immunization schedule and animal experiments are explained in supplementary figure S1.

Collection of serum and gut lavages

Blood was drawn from the lateral tail vein of mice on the following time intervals: 0th, 7th, 21st, 35th, 56th and 90th days, and taken in BD microtainer tubes (BD, NJ, USA) for separation of sera by centrifugation at 3000 rpm for 10 mins at 4º C. One week after the last immunization, 3 mice each from TOMVs immunized and non-immunized group were euthanized and the small intestines were dissected,

followed by flushing of the intestinal lumens with PBS-containing protease inhibitor (Roche, Sigma, USA). The intestinal lavage fluid collected was centrifuged at 1000 X *g* for 10 mins at 4º C, followed by collection of the supernatant. Both the collected sera and intestinal supernatants were stored at -80º C till further use.

Whole cell lysates (WCLs) extraction from CJ, ST and SE

WCLs were prepared from CJ, ST and SE according to previously described protocol [22]. Briefly, overnight bacterial culture was centrifuged at 8000 rpm for 10 mins, followed by a PBS wash and sonication (Heilcher UP100H). Bacterial cells were checked for membrane lysis and were then centrifuged at 10,000 rpm for 10 mins and the supernatant was collected. WCLs were stored at -80º C till further use.

Outer membrane proteins (OMPs) extraction from CJ, ST and SE

CJ, ST and SE OMPs were extracted according to the previous described protocol [22]. Briefly, bacterial cells were isolated from 50 mL of overnight culture, centrifuged at 8000 rpm for 10 mins. The isolated cells were washed once using HEPES buffer (10 mM, pH 7.5), centrifuged and re-suspended in HEPES buffer containing protease inhibitor. Cells were then lysed by sonication using a Heilcher UP100H sonicator. After centrifugation at 13000 X *g* for 10 mins, the cells that failed to lyse were removed and the supernatant was collected. This was followed by ultracentrifugation of the collected supernatant at 100,000 X *g* for 1 h at 4º C. Following ultracentrifugation, pellets were dissolved in 10 mM HEPES containing 1% sarcosyl and incubated for 30 mins at 37º C, which was again ultracentrifuged at 100,000 X *g* for 1 h at 4º C. Finally, the pellets were washed twice with 10 mM HEPES and OMPs were ultimately re-suspended in 10 mM HEPES. Protein content of the OMPs was estimated using Lowry protein estimation kit (Pierce, USA). The OMPs were stored at -80º C till further use.

Extraction of lipopolysaccharide (LPS) from CJ, ST and SE

Bacterial cells were harvested from 50 mL of overnight bacterial culture by centrifugation at 8500 X *g* for 20 mins and after washing with normal saline, it was re-suspended in 150 mM NaCl containing Phenol-saturated 3-[N-morpholino]propen sulfonic acid (MOPS), followed by incubation with shaking in a water bath for 30 mins at 65º C. This was followed by 10 mins incubation on ice and after subsequent centrifugation at 8500 X *g* for 30 mins, the upper aqueous phase was collected. The aqueous phase was then mixed with 95% chilled ethanol (-20º C), 4 times the collected aqueous phase volume, and kept overnight at -20º C. Finally, on the next day, LPS was isolated by centrifugation at 8500 X *g* for 20 mins at 4° C and re-suspended in milliQ water. LPS was stored at -20º C until further use [22].

Enzyme-linked immunosorbent assay

Immunoglobulins were assessed from serum (IgG with its subtypes IgG1, IgG2a, IgG2b, IgG3 and IgA) as well as intestinal lavage (sIgA) by ELISA as previously described by Keren [36]. Briefly, polystyrene disposable micro-titre wells (Nunc, Denmark) were coated separately with 5 µg/well of bacterial WCLs, 5 µg/well of OMPs and 5 µg/well of LPS of each strain from which immunogens were formulated and

incubated for 18 h at 4 C. Wells were then washed and using 5% skim milk (BD, USA) and wells were blocked for 2 h at 37º C. After washing all the wells with PBS-T (PBS plus 0.5% Tween-20, Sigma Chemicals, USA), plates were incubated for 1 h at 37º C with serially diluted serum samples, followed by addition of 100 µL of HRP-conjugated goat anti-mouse immunoglobulin to each well and again incubated for 1 h at 37º C. After washing the wells with PBS, substrate o-phenyl-Di-amine (OPD) was added, and the reaction was stopped after 10 mins by addition of 100 µL of 2 N H_2SO_4. Measurement was taken in a microplate reader at OD_{492}. Each of the experiments were repeated three times for all the immunoglobulins, using both immunized and non-immunized serum, collected before, during and after immunization from individual mice [22, 37].

TOMVs serum bactericidal properties and scanning electron microscopy (SEM)

Serum bactericidal assay was performed according to the protocol described previously with slight modifications [22]. Briefly, on the 35th day post primary immunization sera were collected from TOMVs immunized and non-immunized mice. For 30 minutes at 56º C collected sera was heat inactivated. CJ challenge strain was cultured on MHB with appropriate antibiotics as mentioned previously and challenge strains of ST and SE were cultured on TSB. The strains were grown to log phase and O.D. was adjusted to 0.4. Bacterial cell pellet was collected by centrifugation at 1100 X g for 5 min followed by resuspension in PBS. Heat-inactivated sera (1:50 dilution) with or without 25% guinea pig complement was added to the bacterial solution. The reaction mixture (50 µL) was incubated for 1 hour at 37º C. 950 µL of either MHB (for CJ) or TSB (for ST and SE) was added to stop the reaction. Finally, the reaction mixture was plated on either MHA plates containing appropriate antibiotics (for CJ) or on TSA plates (for ST and SE) by serial dilution for determination of CFU count. Non-immunized sera incubated bacteria were used as control.

By scanning electron microscopy (SEM) analysis, bactericidal properties of TOMVs immunized serum was also assessed. As mentioned above, the strains were cultured accordingly, and incubated with TOMVs immunized and non-immunized serum for 1 h at 37º C, followed by processing of the samples for SEM analysis. Briefly, using 3% glutaraldehyde samples were fixed and left overnight. On the next day, samples were dehydrated in ascending grades of alcohol and then the samples were chemically dried with hexamethyldisilazane (HMDS) by gradually increasing the HMDS ratio. The samples were finally treated with 100% HMDS and left overnight in a fume hood for HMDS evaporation. The samples were mounted on specimen stubs, sputter-coated with gold and images were analysed using Quanta 200 SEM (FEI, Netherlands).

Assessment of motility and mucin penetration

Motility assays were conducted according to a previously published method with slight modifications [37]. Either antibiotic-supplemented MHA soft agar (0.3%) plates for *Campylobacter* were used, or TSA soft agar (0.3%) plates for iNTS strains were used for the assay. Following heat inactivation, both the immunized and non-immunized serum samples were diluted in a 1:400 dilution in PBS and poured on

soft agar plates. After soaking and drying the sera mixed PBS on the plates for one hour, log-phase culture of bacteria ($O.D._{600}$ = 0.4) were pierced in the middle of the plates and incubated for 24 h at 37º C either microaerobically for *Campylobacter* or aerobically for iNTS strains. The bacterial spread across the surface of the plates were then analysed.

For mucin penetration assay, soft agar (0.3%) was prepared as stated previously and mixed with 1% w/v mucin (Sigma Aldrich, USA). The solution was allowed to gelatinize in tuberculin 1ml syringes. Heat-inactivated immunized and non-immunized sera were incubated with log phase bacterial culture ($O.D._{600}$ = 0.4) for 1 h at 37º C followed by addition of 100 µl incubated bacterial culture having the same number of bacteria (1 X 10^7 CFU/ml) on top of the gelatinized columns. The columns were then incubated vertically in static conditions at 37º C for 30 mins. After incubation, 500 µl samples were collected from the base of the columns, serially diluted, plated and bacterial numbers were enumerated either on antibiotic supplemented MHA plates for *Campylobacter* or TSA plates for iNTS strains [37].

Short-term protective efficacy evaluation of TOMVs vaccine candidate

Analysis of change in body weight

Adult BALB/c mice were divided into 7 groups (A, B, C, D, E, F and G, n = 6 per group). Groups A, C, E and G were comprised of non-immunized mice and groups B, D and F were comprised of TOMVs immunized mice. 35 days post primary immunization, groups A and B were intraperitoneally challenged with 1X 10^9 CFU per mouse of CJ BAA-1153 as in a previous study [38]. Similarly, groups C and D were intraperitoneally challenged with 1X 10^5 CFU per mice of ST ATCC 14028 and, groups E and F were intraperitoneally challenged with 1X 10^5 CFU per mouse of SE ATCC 13076 as described previously [39]. Group G was intraperitoneally administered with PBS (uninfected) and served as negative control. Changes in body weight of immunized and non-immunized groups were analysed up till 7 days post infection.

Bacterial colonization assessment

For assessment of colonization, mice were again divided into 7 groups and intraperitoneally challenged with the same dose of CJ, ST and SE challenge strains as mentioned above. After 48 hours of infection, mice from both the immunized and non-immunized groups were sacrificed, followed by collection of colon, liver, and spleen. The different organs collected from each mouse were separately homogenized, serially diluted and plated for determination of the bacterial burden. CJ load in different organs was determined by plating the serially diluted samples on *Campylobacter* selective Mueller-Hinton (MH) agar plates supplemented with 10% sheep's blood, 40 µg/mL cefoperazone, 100 µg/mL cycloheximide, 10 µg/mL trimethoprim and 100 µg/mL vancomycin. Plates were incubated at 37° C for 48 h under microaerobic conditions and the number of colonies was subsequently counted [32]. ST and SE counts were enumerated by plating the serially diluted samples on Xylose Lysine Deoxycholate (XLD) agar plates followed by incubation at 37° C for 24 h and subsequent counting of the colonies in plates.

Histological analysis

As mentioned above, after 48 h of bacterial challenge, colon tissue samples were collected from all the groups of mice and the samples were fixed using 10% buffered formalin solution. Following fixation, the colon tissue samples were embedded in paraffin, sectioned and double stained using haematoxylin and eosin (H & E). The samples were visualized using an Olympus IX51 light microscope. Pathological scoring of the respective samples (Table 2) was done as explained previously with few modifications [28, 41].

Table 2
Pathological score reference table for histological images.

Inflammatory cell infiltrate			**Mucosal architecture**			
Severity	Extent	Score 1	Severity	Extent	Mucosal and epithelial changes	Score 2
Minimal	Mucosa	1	**Minimal**	Mucosa	Minimal hyperplasia	1
					Minimal villous blunting	1
Mild	Mucosa and sometimes submucosa	1	**Mild**	Mucosa, sometimes submucosa	Mild hyperplasia	2
					Mild goblet cell loss	2
Moderate	Mucosa and submucosa, sometimes transmural	2	**Moderate**	Mucosa, submucosa	Moderate hyperplasia	3
					Goblet cell loss	3
					Cryptits	3
Marked	Transmural	3	**Marked**	Mucosa, submucosa, often transmural	Marked hyperplasia	4
					Marked goblet cell loss	4
					Multiple crypt absences	4
Sum of Score 1 and 2: 0–15						

Quantification of cytokines

Splenocyte restimulation assay

Splenic cells were isolated from TOMVs immunized BALB/c mice 1 week after last immunization and cultured in complete RPMI containing 10% FBS for 2 hours. 5 µg/ml of TOMVs was added to the isolated cells and incubated for 24 hours at 37° C in the presence of 5% CO2. IFN-γ, IL-6 and IL-17 cytokines were then quantified by cytokine ELISA kits (Invitrogen, USA) [40].

Isolation and stimulation of bone marrow-derived dendritic cells (BMDCs) with TOMVs

From naïve BALB/c mice, immature BMDCs were isolated and kept for maturation in RPMI 1640 medium supplemented with 10% FBS and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). Following maturation, 5 µg/ml of TOMVs was added to BMDCs for stimulation and kept for 24 hours at 37° C in the presence of 5% CO2. IL-6, IL-1β and IL-12p40/70 cytokines were then measured from culture supernatants using cytokine ELISA kits (Invitrogen, USA) [40].

Flow cytometry analysis of splenic cells

35 days after three immunization doses, spleens were isolated from both non-immunized and immunized mice for flow cytometry analysis. Under proper aseptic conditions mice were sacrificed for isolation of spleens. Following isolation, spleens were dissociated using a 5 mL syringe sterile plunger and a cell strainer. RBCs were lysed with RBC lysis buffer (Sigma, USA) and splenocytes were re-suspended in RPMI 1640 containing 10% FBS, 2-ME (50 mM) and appropriate antibiotics (5 U/mL penicillin G, 5 µg/mL streptomycin, and 0.1% gentamycin) (Gibco, USA). Splenocytes were stained with CD4-phycoerythrin (PE), CD8a-PE, CD19-PE and isotype control (PE) anti-mouse mAbs for determination of CD4+, CD8a + and CD19 + splenic cell populations. For assessment of the cells, BD FACS ARYA III flow cytometer was used and data were analysed using FACS DIVA software [22].

Long-term protective efficacy evaluation of TOMVs vaccine candidate

Analysis of change in body weight

Mice were divided into 7 groups and challenged with heterologous challenge strains as previously mentioned, 180 days post-boosting vaccination. Body weight of both the immunized and non-immunized groups was analysed until 7 days post infection as mentioned previously.

Bacterial colonization assessment

As stated above, mice were divided into 7 groups and challenged with heterologous challenge strains 180 days post-boosting vaccination. Bacterial load in colon, liver, and spleen of both the immunized and non-immunized groups was determined 48 hours post infection as described previously.

Histological analysis

Colon tissue samples from the above mentioned immunized and non-immunized groups were collected 48 h post infection and processed for histological analysis as described previously. The samples were visualized in Olympus IX51 light microscope and pathological scores (Table 2) were analysed accordingly.

Statistical analysis

Statistical analysis was conducted using Prism 5.00 software (GraphPad, San Diego, California, USA) and Microsoft Excel. All the data were tabulated as mean ± S.D. For three or more groups of data, analysis was performed using two-way analysis of variance (ANOVA) and for analysis of two groups of data sets, one tailed Student's t test was used. A value of $p < 0.05$ was considered statistically significant: *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

Results

Isolation, characterization of OMVs from C. jejuni, S. Typhimurium and S. Enteritidis strains, and preparation of TOMVs immunogen

OMVs secreted from log phase of growth of CJ, ST and SE strains were found to be structurally bi-layered and spherical in shape as shown in TEM images (Fig. 1a i-iii). Size distribution analysis of OMVs shows peaks ranging from 27.06 nm to 40.47 nm (Fig. 1b i-iii). Zeta-potential measurements were further acquired for characterization of physiochemical properties of OMVs. All the OMVs displayed a net surface negative charge ranging from − 2.219 mV to -22.46 mV which suggests that these particles are stable in nature (Fig. 1c i-iii).

From the proteomic profiling of the OMVs, the subcellular localization comparison of components of OMV proteins with that of CJ, ST and SE genome inferred proteins showed that log-phase derived OMVs from the three strains contained both the cytosolic and membrane bound or secretory proteins along with hypothetical unknown proteins in varied proportions (Fig. 1d i-iii). Cytosolic proteins were found to be most abundant in SE, followed by CJ and ST. Components of OMV proteins were further compared and classified based on their biological activity and molecular function with that of CJ, ST and SE genome inferred proteins. The proteins were divided and grouped into proteins associated with transport activity, proteins associated with structural activity, proteins associated with catalytic activity, proteins associated with nucleic acid binding activity and proteins associated with other molecular functions, and plotted according to their proportions (Fig. 1e i-iii). Protein identification information of all OMVs-associated CJ, ST and SE proteins are presented in supplementary tables S1, S2 and S3. Proteomic analysis portrayed that these proteins are most abundant in the OMVs of CJ, ST and SE, although other hypothetical unidentified proteins might also be present.

OMVs protein content were estimated and CJ, ST and SE OMVs were mixed in equal proportions (1:1:1 ratio) for formulation of TOMVs immunogen having a total protein concentration of 5 µg/100 µL of PBS.

Humoral and mucosal immune response generated by TOMVs in adult BALB/c mice

The degree of immunogenicity elicited by TOMVs was measured in adult BALB/c mice after three intraperitoneal immunization doses (5 µg of TOMVs/ mice), maintaining a 14 days interval gap between immunizations. Control group was intraperitoneally administered with PBS for comparative analysis. For 90 days post primary immunization, immunogenicity level of immunoglobulins was analysed. A hike in the production of serum antibodies i.e. IgG, IgG1, IgG2a, IgG2b, IgG3 and IgA was found against isolated LPS, OMPs and WCLs of CJ, ST and SE homologous strains with peak immunogenicity observed on the 35th day post first immunization (Fig. 2, a-c). Secretory IgA (sIgA) from intestinal lavage of TOMVs-immunized mice was found to be significantly higher against isolated LPS, OMPs and WCLs of CJ, ST and SE homologous strains in comparison with non-immunized control groups, which demonstrates significant activation of the mucosal immune response (Fig. 2d). Overall, TOMVs intraperitoneal immunization significantly activated the humoral arm of the adaptive immune response, which can further help in providing long-term protection against CJ, ST and SE pathotype-mediated infections.

Bactericidal effect conferred by TOMVs immunized adult mouse sera

The above results demonstrated that immunization with TOMVs induced significantly high titres of antibodies against WCLs, OMPs and LPS of CJ, ST and SE strains in sera of adult mice. Serum bactericidal assays were conducted for analysing the bactericidal effect. Pooled immunized sera from adult mice supplemented with 25% guinea pig complement lowered the viable bacterial count significantly after incubation with CJ, ST and SE heterologous challenge strains, whereas heat-inactivated sera (not containing guinea pig complement) and non-immunized sera were unable to exhibit such bactericidal effects. These results suggest complement pathway activation, along with a significant level of antibody titres in TOMVs immunized adult mice sera is necessary for effectively killing the bacteria and lowering the viable bacterial number (Fig. 3a). This result was additionally validated by SEM analysis of the bactericidal effect. Incubation with TOMVs-immunized adult mouse sera caused lysis, agglutination and pore formation in CJ, ST and SE bacterial cells whereas non-immunized adult mouse sera failed to cause lysis of incubated CJ, ST and SE cells (Fig. 3b i-iii).

Motility of CJ, ST and SE cells helps them to migrate from the intestinal lumen to the surface of intestinal epithelial cells. Effects of TOMVs on inhibition of bacterial motility was assessed by motility assays. The results revealed that TOMVs immunized adult mice sera significantly inhibited motility of CJ, ST and SE heterologous challenge strains. Bacteria in the presence of immunized mouse sera showed a significant reduction in the zone of bacterial spread as compared to bacteria in the presence of non-immunized mice sera (Fig. 3 ci, cii, ciii, d). This result demonstrates the ability of TOMVs-immunized sera to effectively agglutinate bacteria, thereby imparting protective nature to TOMVs immunogen.

Mucin penetration ability of CJ, ST and SE strains were measured in the presence of TOMVs-immunized and non-immunized sera. The number of bacteria after penetration of mucin was found to be significantly lower in the presence of TOMVs-immunized serum than in the presence of non-immunized serum (Fig. 3d). The results depict that TOMVs-immunized mouse sera can potently inhibit bacterial

motility, which in turn inhibits penetration of mucin and can therefore prevent colonization and invasion in intestinal epithelial cells.

TOMVs immunization protects adult BALB/c mice against CJ, ST and SE mediated infections

On the 35th day post primary immunization, adult BALB/c mice were challenged with 1 X 10^9 CFU/mouse of CJ and 1 X 10^5 CFU/mouse of ST and SE heterologous challenge strains separately, and changes in body weight were assessed until 7 days post infection for TOMVs- immunized and non-immunized groups. Body weight of non-immunized mice decreased significantly, with peak decrease observed at 4 days post infection. Nearly 8% peak decrease in body weight was observed for the non-immunized group challenged with CJ and 15% peak decrease was observed for ST- and SE-challenged non-immunized groups. Body weight of TOMVs-immunized mice after challenge with CJ, ST and SE, remained approximately similar when compared with uninfected control groups (Fig. 4a i-iii).

For assessment of colonization in colon and systemic organs (liver and spleen), adult mice of immunized and non-immunized groups were similarly challenged 35 days post primary immunization with the same dose of CJ, ST and SE heterologous strains as mentioned above. 48 h post-challenge, TOMVs-immunized adult mice showed a significant decrease in colonization against CJ, ST and SE challenge strains in colon, liver and spleen compared with non-immunized adult mice (Fig. 4b i-iii).

Changes in histopathology of colon of immunized and non-immunized adult BALB/c mice were measured 48 h post challenge, after 35 days of first immunization, with the same dose of CJ, ST and SE challenge strains as mentioned above. TOMVs-immunized adult mice displayed significantly decreased epithelial layer destruction, alteration in intestinal mucosa, neutrophil infiltration, loss of goblet cells, formation of cryptitis and hyperplasia as compared with non-immunized adult mice (Fig. 5a). TOMVs-immunized adult mice showed a reduction in pathological scores as compared to non-immunized mice (Fig. 5b). Altogether, immunization with TOMVs immunogen exerted a significant level of protection against CJ, ST and SE strains in adult BALB/c mice.

Induction of cell-mediated immune response and upregulation of splenic cell population by TOMVs

35 days after primary immunization, spleens were isolated from both the TOMVs-immunized and PBS-immunized (non-immunized group) mice and a single cell suspension for each of the groups were prepared separately. Both the groups were treated with TOMVs for 24 h in the presence of 5% CO_2 at 37° C. Significant upregulation of IL-17, IFN-γ and IL-6 was observed in the cell culture supernatant of splenic cells of the TOMVs-treated immunized group as compared to the non-immunized group (Fig. 6a i-iii).

The response of bone-marrow derived dendritic cells (BMDCs) in the presence of TOMVs stimulation was determined as it is responsible for controlling T-cell variation. BMDCs were isolated from adult mice and stimulated with TOMVs. IL-12p40/70, IL-1β and IL-6 were found to be significantly upregulated in the TOMVs-stimulated BMDC cell culture supernatant (Fig. 6b i-iii). Induction of these pro-inflammatory cytokines is required for controlling the differentiation of Th1/Th17 cells.

Comparative analysis of the splenic cell populations from TOMVs-immunized and non-immunized mice was performed using a flow cytometer. Immunization of adult mice with TOMVs resulted in significantly increased populations of CD4+, CD8a + and CD19 + splenic cells in the immunized group, as compared to the non-immunized group, 35 days after first immunization (Fig. 6c and d). This result suggests that the presence of a variety of antigenic components in TOMVs caused activation of CD4+, CD8a + and CD19 + splenic cell populations, which can be responsible for the heightened immune response observed previously.

Overall, i.p. immunization with TOMVs caused a significant level of activation of both the cellular and humoral arms of the adaptive immune response in adult mice, which subsequently can help in providing long-term protective immunity against CJ, ST and SE mediated infections.

Long-term protective efficacy conferred by TOMVs immunogen

For assessment of long-term protective efficacy, TOMVs-immunized and non-immunized adult BALB/c mice were challenged at 180 days post final booster immunization with 1 X 10^9 CFU/mouse of CJ and 1 X 10^5 CFU/mouse of ST and SE heterologous challenge strains separately, and changes in body weight, colonization, and histopathology of both the groups were observed as described previously. Body weight changes were assessed until 7 days post infection. Body weight of non-immunized mice decreased significantly with peak decrease observed at 4 days post infection for CJ and ST, and at 3 days post infection for SE. Approximately 10% peak decrease in body weight was observed for the non-immunized group challenged with CJ and a 14% peak decrease was observed for ST and SE challenged non-immunized group. TOMVs immunized mice also showed slight decrease in body weight, but the changes were non-significant when compared with uninfected groups. (Fig. 7a i-iii).

Colonization in colon and systemic organs (liver and spleen) of adult mice were assessed as described previously 180 days post last booster immunization. 48 h post challenge, TOMVs immunized adult mice displayed a significant decrease in colonization against CJ, ST and SE challenge strains in colon, liver and spleen compared with non-immunized adult mice (Fig. 7b i-iii).

As described previously, histopathological changes of mice were measured 48 h post challenge, 180 days after final booster immunization. TOMVs-immunized adult mice displayed significantly decreased epithelial layer destruction, alteration in intestinal mucosa, neutrophil infiltration, loss of goblet cells, formation of cryptitis and hyperplasia as compared with non-immunized adult mice (Fig. 8a). TOMVs-immunized adult mice showed reductions in pathological scores as compared to non-immunized mice (Fig. 8b). Altogether, these findings demonstrate that immunization with TOMVs immunogen exerted significant long-term protective efficacy against CJ, ST and SE infections in adult BALB/c mice.

Discussion

Diarrhoeal diseases continue to be an important public health concern globally and are a leading cause of mortality among infants and children in developing countries [3, 42]. *Campylobacter* and non-typhoidal

Salmonella are among the major causative agents of bacterial gastroenteritis in humans and impose a substantial diarrhoeal disease and economic burden in developing countries [6, 43, 44]. With increases in antimicrobial resistance among *Campylobacter* and non-typhoidal *Salmonella*, vaccination is a fruitful strategy for prevention of infections caused by these pathogens [45]. LH1120 and WT05 are two live-attenuated vaccine candidates for iNTS that have been clinically studied in humans [46]. A glycoconjugate *Campylobacter* vaccine (CJCV1) has been tested in phase 1 clinical trial for determination of safety and immunogenicity [47]. Yet, as of now, not a single vaccine candidate can provide combined protective immunity against both the pathogens. Combination vaccines are regarded as a superior approach for prevention of diarrhoeal infections [18]. Peptidoglycan, lipoproteins, LPS and flagellin are present in OMVs, which impart them with intrinsic adjuvancy [48, 49]. Due to this inherent adjuvancy, supplementation of OMVs with external adjuvants is not needed for enhancement of immunogenicity. Thus, we have developed an OMVs-based vaccine candidate against *Campylobacter jejuni*, *Salmonella* Typhimurium and *Salmonella* Enteritidis, which can provide combined broad-spectrum protective immunity against infections caused by these pathogens. *Neisseria*, *Shigella* and *Salmonella* species OMVs have already been used as potent immunogens [50–53]. Previously, 25 µg intramuscular administration of *N. meningitis* OMV vaccine (MenBvac and MeNZB) was reported to effectively elicit protective immunity [54, 55]. By utilizing a similar approach in our study, we observed profound activation of the humoral arm of the immune response after intraperitoneal immunization with 5 µg of TOMVs at 2-week intervals in adult BALB/c mice. Previously, our studies on OMVs-based immunogen against *S*. Typhi and *S.* Paratyphi, and OMVs-based immunogen against *S.* Typhimurium and *S*. Enteritidis, demonstrated similar humoral immune responses [28, 40]. This present study elucidated that minimal administration of 5 µg of TOMVs was adequate for effectively stimulating systemic antibody secretion. Significant increases in the titres of serum IgG and its subtypes, along with serum IgA were observed after immunization. Complement pathway activation is mediated by IgG2a and IgG2b, leading to antigen opsonisation. IgG3 activation is mediated by capsular polysaccharides present on bacteria [56] and phagocytosis of bacterial pathogen is effectively mediated by IgG1 [57]. Previous studies showed that OmpA, OmpC of *S*. Typhimurium, OmpA, OmpW of *S*. Enteritidis, and CjaA of *C. jejuni* OMVs-associated proteins are immunogenic in nature [58–62]. Proteomic data from our study revealed the presence of these proteins, along with a wide range of potentially other immunogenic proteins in OMVs isolated from *C. jejuni*, *S*. Typhimurium and *S*. Enteritidis. A range of these various antigens present on TOMVs might lead to the enhanced levels of IgG subtypes observed in immunized mice that may activate cell-mediated immune responses needed for imparting protective immunity.

Prevention of invasion in host tissue and faster bacterial clearance from the intestinal lumen is mediated by mucosal or secretory IgA. The classical agglutination pathway and the enchained growth pathway are the two mechanisms mediated for faster bacterial clearance by sIgA. Interaction of bacteria with the mucosal layer is prevented by sIgA in the classical agglutination pathway, which ultimately leads to bacterial clearance from the gut lumen. In the enchained growth pathway, sister cells of bacteria are prevented from dividing due to coat formation by sIgA outside bacteria, which leads to elimination of undissociated bacterial cells from the gut lumen [28, 63, 64]. Our study found significantly higher levels of

sIgA against WCLs, OMPs and LPS of CJ, ST and SE following intraperitoneal immunization with TOMVs, which suggests that clearance of these pathogens and protection against further infections can be mediated by TOMVs immunization.

The functionality of antibodies is measured by SBA to determine the desired immunogen's immunogenicity and potential protective capacity in preclinical studies [65]. This study also demonstrated the bactericidal capability elicited by TOMVs-immunized mouse sera, analysed by SBA and scanning electron microscopy. Immunized sera showed efficient binding and killing by causing lysis of CJ, ST and SE bacterial cells, as compared with non-immunized sera. As revealed by proteomic data, a range of immunogenic proteins present in TOMVs can play an important role in classical complement pathway activation, causing bacterial cells to lyse. TOMVs-immunized sera also inhibited bacterial motility and substantially decreased the ability to penetrate the mucin layer, which can considerably help in reducing bacterial attachment and invasion into intestinal epithelial cells [66]. Thus, immunization with TOMVs can block the bacterial cells from initial entry and invasion via the epithelial cells of the intestine, thereby eliciting protective immunological response against the pathogens.

Assessment of protective efficacy was carried out by challenging the TOMVs-immunized and non-immunized mice intraperitoneally with *Campylobacter* and NTS challenge strains. Intraperitoneal challenge of mice allows determination of a strong end point for assessment of vaccine efficacy, characterization of correlates of protection, reproducibility of vaccine efficacy, and prompt assessment of immunogen dosage and immunization schedules [38, 39, 67, 68]. Our study demonstrated that immunization with TOMVs significantly protected the mice against infections caused by CJ, ST and SE pathogens. TOMVs-immunized mice displayed insignificant decreases in body weight along with several fold reductions in colonization of colon, liver and spleen by CJ, ST and SE. Previously, our studies showed OMVs isolated from *Shigella* sp. [51], *Vibrio cholerae* [69] and *Salmonella* Typhi and *Salmonella* Paratyphi A [40] confer protection against these pathogens. TOMVs-immunized mice colons displayed significantly lower histopathological scores than non-immunized mice colons. TOMVs-immunized mice colons demonstrated decreased epithelial layer destruction, decreased alteration in intestinal mucosa, decreased neutrophil infiltration, decreased loss of goblet cells, and decreased formation of cryptitis and hyperplasia than non-immunized mice colons. A study previously reported anti-LPS IgG to be protective against *S.* Typhimurium infections [70]. In the present study, we hypothesized that the bacteria might be trapped by anti-LPS IgG, which ultimately prevents infection in TOMVs immunized mice.

Cell-mediated immune responses play a pivotal role in combating intracellular bacteria-mediated infections [71]. In this study, ex-vivo restimulation of TOMVs-immunized mouse splenocytes upregulated the production of IL-17, IFN-γ and IL-6 in splenic cell-culture supernatant. IL-17 functions as the most crucial biological effector of Th-17 cells. Th-17 cells play an important role in recruitment and expansion of innate immunity cells. IL-17 is known to promote dendritic cell differentiation through upregulation of expression of cell-surface co-stimulatory molecules and induces Th-1 protective immune response against intracellular pathogens [72]. IFN-γ secreted by Th-1 cells mediates CD4 + splenic cell response, necessary for infection resistance and ultimate clearance of bacteria from tissues [73]. IL-6 contributes to

bridge the gap between innate and adaptive immune responses. Recruitment and activation of neutrophils and monocytes are known to be profoundly stimulated through the secretion of IL-6 by Th-17 cells [74]. A previous study also reported increased IL-17, IFN-γ and IL-6 in re-stimulated splenic cell-culture supernatant of OMVs-immunized mice [40]. Upregulation of IL-12p40/70, IL-1β and IL-6 were observed in TOMVs-stimulated BMDCs cell-culture supernatant. NOD-2/TLR-4 signalling induces release of IL-12p40/70, which is responsible for stimulating immune cells (including dendritic cells) involved in maintaining sustained immunity and promotes naïve CD4 + cells differentiation to Th1 cell subtype, which performs a cardinal function for protection against intracellular pathogens [75]. IL-1β induces chemokines and adhesion molecules for attracting both the innate and adaptive immune cells and mediates proliferation of CD4 + and CD8 + T-cells [76, 77]. Previously, studies elucidated the role of OMVs in stimulating the increase of splenic T-cell population following immunization [22, 28, 40]. Upregulation of CD4 + and CD8a + splenic cell population was also observed in our study following i.p. immunization with TOMVs in adult BALB/c mice, which correlates to those observed in previous studies. Therefore, along with the humoral arm, TOMVs potently activates the cellular arm of immune response, essential for generating long-term protective immunity against CJ, ST and SE pathogens.

Our study also demonstrated generation of long-term protective efficacy imparted by TOMVs vaccine candidate in adult BALB/c mice. TOMVs immunized mice were significantly protected against CJ, ST and SE infections 180 days after the last booster immunization. Previous studies illustrated IFN-γ secreting Th-1 cells play a significant role in mediating long-term protection and IFN-γ $^{-/-}$ mice displayed reduced levels of protection against subsequent bacterial infections [37, 78, 79]. In our study also, significant upregulation of IFN-γ was found in splenic cell culture supernatant, which can play a pivotal role in conferring long-term protection by TOMVs immunogen. Another study demonstrated the substantial capability of IL-17 secreting T-cells to clear the bacterial infection in the long-term. IL-17 $^{-/-}$ mice harboured a significantly higher number of bacteria after challenge, indicating that IL-17 producing T-cells play a major role in clearance of bacterial infection [80]. In our study, no significant decrease in body weight was observed, along with significant reduction in colonization of colon, liver and spleen by CJ, ST and SE in TOMVs-immunized mice, 180 days after the final booster immunization. Significantly lower histopathological scores of colon were observed in TOMVs-immunized mice, which displayed decreased epithelial layer destruction, alteration in intestinal mucosa, neutrophil infiltration, loss of goblet cells, and formation of cryptitis and hyperplasia as compared to non-immunized mice, 180 days after the final booster immunization. This suggests that Th-1 and Th-17 cytokines can play an important role in conferring long-term protective efficacy after i.p. immunization with TOMVs in adult BALB/c mice.

Conclusion

This study proposes TOMVs can be used as a potent vaccine candidate effective in eliciting marked immune responses against CJ, ST and SE pathogens after intraperitoneal immunization in adult BALB/c mice. Immunization with TOMVs conferred a significant bactericidal effect, killing the CJ, ST and SE

pathogens effectively. Furthermore, intraperitoneal immunization of adult BALB/c mice with TOMVs produced long-term protection against CJ, ST and SE pathogen mediated infections.

Abbreviations

CFU, colony forming unit;CJ, *Campylobacter jejuni*; ST, *Salmonella* Typhimurium; SE, *Salmonella* Enteritidis; OMV, outer-membrane vesicle;IP, intraperitoneal; OMP, outer-membrane protein; LPS, lipopolysaccharide; WCL, whole cell lysate; MHB, Mueller-Hinton Broth; MHA, Mueller-Hinton Agar; TSB, tryptic soy broth; TSA, tryptic soy agar; PBS, phosphate buffered saline; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; sIgA, secretory IgA; IL, interleukin; IFN-γ, interferon gamma.

Declarations

Acknowledgements

The authors sincerely thank Ms. Srijani Biswas, Mr. Benson George Chacko, Ms. Debashree Das, Ms. Ipsita Chakraborty,Ms. Sweety Bharti, Mr. Pritam Nandy, Mrs. Arpita Sarbajna, Mr. Subrata Sinha and Mr. Suhasit Ranjan Ghosh for their technical assistance. We would like to thank University Grants Commission (UGC), New Delhi, India, for providing fellowship to Soumalya Banerjee under CSIR-UGC-NET scheme [Student ID:191620007740], Indian Council of Medical Research for providing fellowship to Prolay Halder [ICMR-3/1/3/JRF-2018/HRD-066(66125)] and Sanjib Das under CSIR-UGC-NFSC scheme [SANJIB DAS/3363/(CSIR-UGC NET JUNE 2018)].

Funding

This work was supported by project funded by Indian Council of Medical Research (File No. 5/3/8/66/2020-ITR; RFC No. (P-17) ITR/Ad-hoc/42/2020-21 dt.14.02.2021) and project funded by Program of the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) [JP23wm0125004] from the Ministry of Education, Culture, Sports, Science and Technology in Japan (MEXT), and Japan Agency for Medical Research and Development (AMED).

Conflict of interest The authors declare no financial or personal conflicts of interest.

Ethical approval Following the standard operating procedure as outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, all the animal experiments were conducted. The approval of animal experimental protocol was made by the Institutional Animal Ethical Committee (IAEC) of NICED with Registration No. 68//Rebi/S/1999/CPCSEA valid17/7/2024, approval number PRO/174/ January 2020 - December 2022.

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Figures

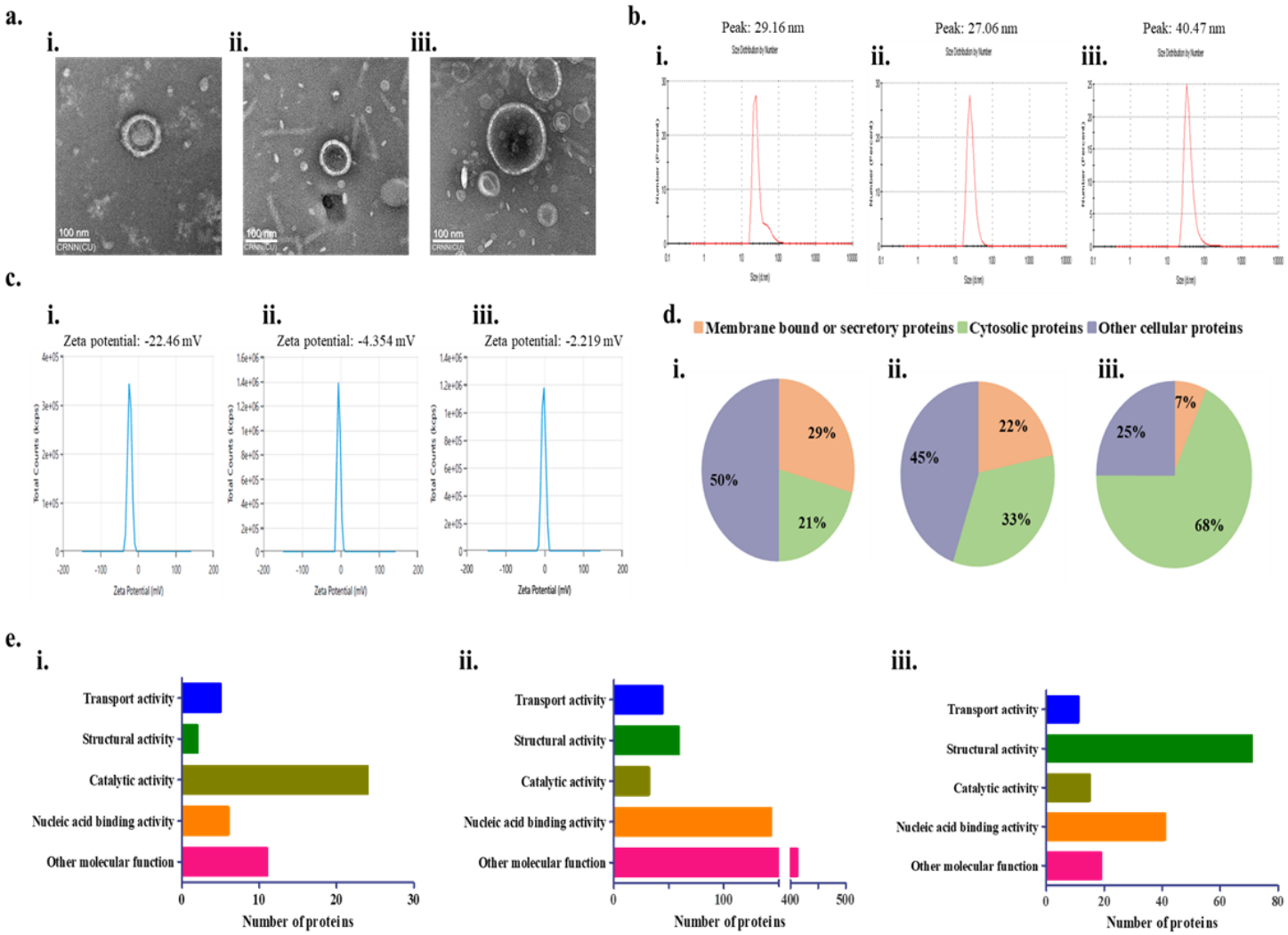

Figure 1

(a) OMVs transmission electron microscopic images isolated from (i) *C. jejuni* [CJ], (ii) *S.* Typhimurium [ST] and (iii) *S.* Enteritidis [SE]. The images show a distinct bi-layered spherical morphology of OMVs. (b) Size distribution of OMVs by dynamic light scattering (DLS) analysis: (i) CJ OMVs, (ii) ST OMVs and (iii) SE OMVs. (c) Zeta potential of OMVs: (i) CJ OMVs, (ii) ST OMVs and (iii) SE OMVs. All the OMVs have a characteristic net negative surface electrostatic charge. (d) Pie-chart showing the percentage of membrane-bound or secretory proteins, cytosolic proteins and other cellular proteins based on their localizations, analyzed from proteomic profile of OMVs: (i) CJ OMVs, (ii) ST OMVs and (iii) SE OMVs. (e) Classification of OMV-associated proteins according to their biological processes and molecular functions, analyzed from proteomic profile of OMVs. The classified proteins were grouped into proteins associated with transport activity, proteins associated with structural activity, proteins associated with catalytic activity, proteins associated with nucleic acid binding activity and proteins associated with other molecular functions, and plotted according to their proportions: (i) CJ OMVs, (ii) ST OMVs and (iii) SE OMVs.

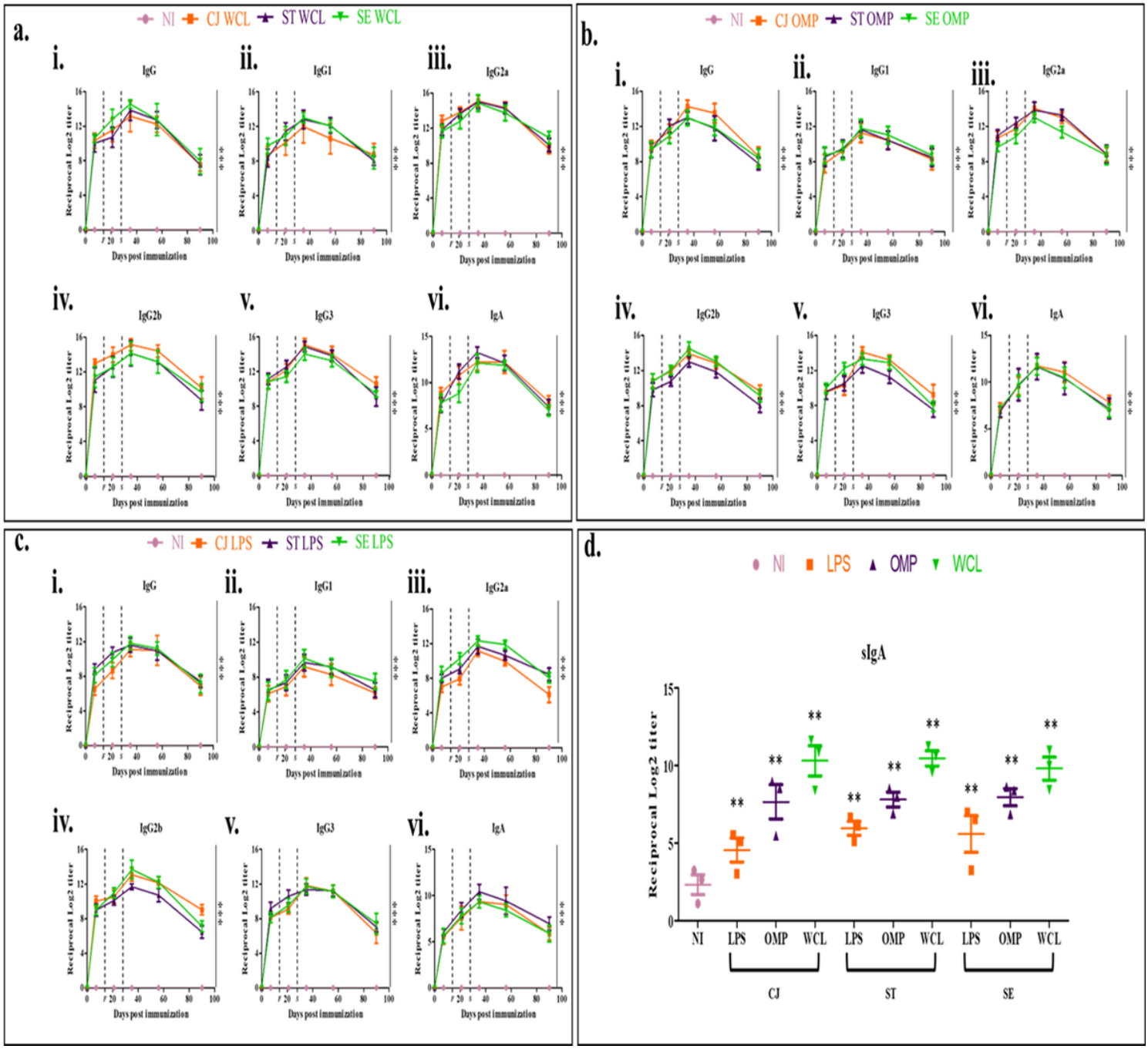

Figure 2

Reciprocal Log2 titer of IgG, IgG subtypes and IgA immunoglobulin from sera of TOMVs immunized and non-immunized mouse measured separately after three doses of intraperitoneal immunization against WCLs (a, i-vi) OMPs (b, i-vi) and LPS (c, i-vi) of CJ, ST, and SE strains. The horizontal axis represents pre-immunization, immunization and post-immunization periods during which antibody titers were measured. The dotted lines represent booster immunizations (*F* – first booster on day 14, *S* – second booster on day 28). Values are represented as mean ± S.D. of three independent experiments. Significant statistical difference was found between TOMVs immunized and non-immunized serum (*** p value< 0.001). (d) Reciprocal Log2 titer of mucosal IgA (sIgA) from TOMVs immunized mice intestinal lavage obtained at 35th day post first immunization. Significant statistical difference was found between TOMVs immunized

and non-immunized groups. Results are presented as one out of three experiments using six mice per group. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Data represented as mean ± S.D., n=6. **p value<0.01. WCL- whole cell lysate, OMP- outer-membrane protein, LPS- lipopolysaccharide, NI- non-immunized.

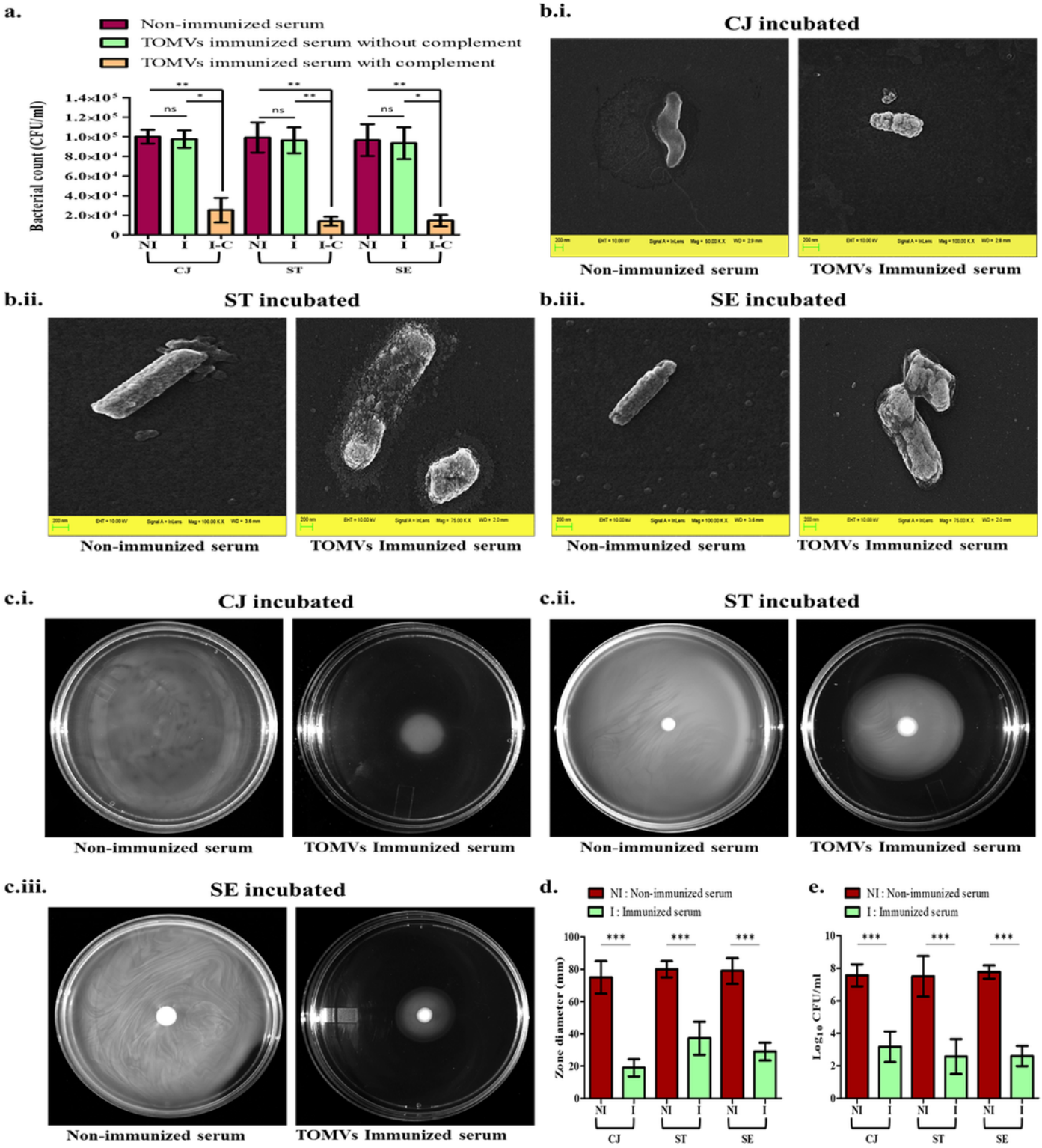

Figure 3

(a) TOMVs immunized mouse serum demonstrated effective complement mediated lysis of CJ, ST and SE challenge strains. 1 X 10^5 CFU/ml each of CJ, ST and SE challenge strains were separately incubated with indicated dilution of either TOMVs immunized serum or non-immunized serum with or without guinea pig complement for 1 hour at 37° C. Viable bacterial colonies were determined and counted by plating either on antibiotic supplemented MHA plates for CJ or on TSA plates for ST and SE. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. ** p value<0.01, * p value<0.5. NI, non-immunized serum; I, TOMVs immunized serum without complement; I-C, TOMVs immunized serum with complement added. (b) Scanning electron microscopic images of CJ, ST and SE challenge strains incubated with either 35^{th} day TOMVs immunized mice serum or non-immunized mice serum. (i) CJ incubated either with or without TOMVs immunized serum, (ii) ST incubated either with or without TOMVs immunized serum, (iii) SE incubated either with or without TOMVs immunized serum. Scale bar 200 nm. (c) Representative images of bacterial motility determined on soft agar (0.3 % agar) plates. TOMVs immunized mouse significantly reduced CJ, ST and SE motility on soft agar plates compared with non-immunized serum. (i) Spread of CJ over soft agar in presence of either non-immunized or TOMVs immunized mice serum, (ii) Spread of ST over soft agar in presence of either non-immunized or TOMVs immunized mice serum, (iii) Spread of SE over soft agar in presence of either non-immunized or TOMVs immunized mice serum. (d) Bar diagram comparing the spread of CJ, ST and SE (zone diameter- mm) in the presence of either TOMVs immunized or non-immunized mice serum. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three independent experiments. Significant statistical difference was found in zone diameter of CJ, ST and SE incubated in the presence of either TOMVs immunized or non-immunized mice serum (*** p value< 0.001). (e) Bacteria in the presence of TOMVs immunized mice serum showed reduced mucin penetration ability than in the presence of non-immunized mice serum. Bacteria were treated with either TOMVs immunized or non-immunized mice serum were loaded on the top of 1ml mucin columns and allowed to penetrate. TOMVs immunized mice serum demonstrated agglutination of bacteria resulting in reduced ability of mucin penetration. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three independent experiments. Significant statistical difference of bacterial count was noted between TOMVs immunized serum treated and non-immunized serum treated bacterial groups (*** p value< 0.001).

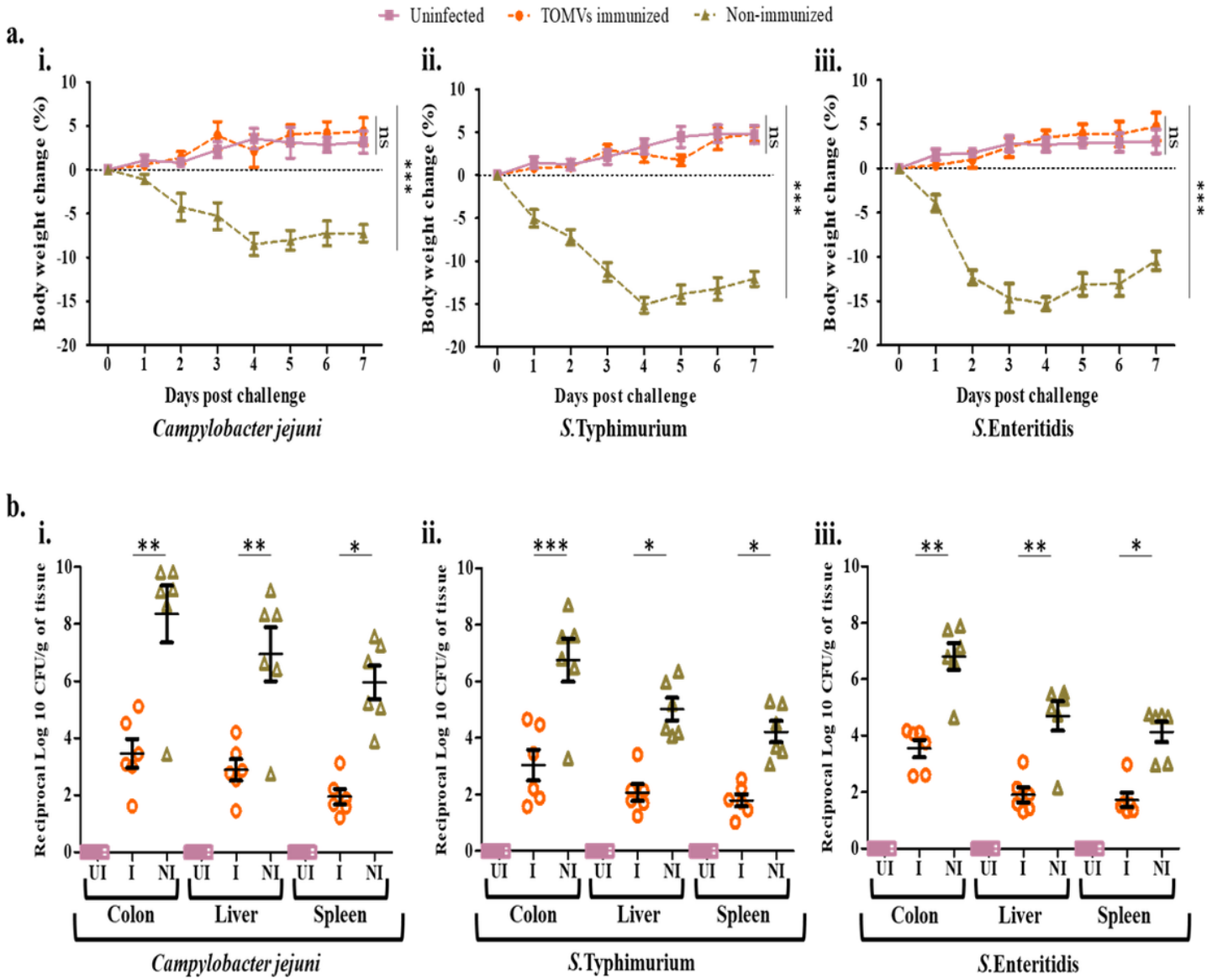

Figure 4

(a) Adult BALB/c mice of same age were challenged with either 1 X 10^9 CFU/mice of CJ heterologous challenge strain (i), or 1 X 10^5 CFU/mice of ST (ii) or SE (iii) heterologous challenge strains separately on 35^{th} day post primary immunization. Changes in body weight of TOMVs immunized, non-immunized and uninfected groups of mice were monitored for 7 days. Non-immunized group demonstrated a significant decrease in body weight when compared with TOMVs immunized and uninfected groups. No significant changes were found between TOMVs immunized and uninfected groups. Every single dot represents mean and error value of six mice ± S.D. of three independent experiments. *** p value<0.001. ns, non-significant. (b) Comparison of protection determined from colonization data in colon, liver and spleen of adult BALB/c mice (n=6), in TOMVs immunized, non-immunized and uninfected groups, after challenge with CJ, ST and SE challenge strains on 35^{th} day post primary immunization. For CJ (i), 1 X 10^9 CFU/ml was administered as the challenge dose to each mouse and immunized group displayed significantly lower colonization compared to non-immunized group. For ST (ii) and SE (iii), 1 X 10^5 CFU/ml was

administered as the challenge dose to each mouse and immunized group displayed significantly lower colonization compared to non-immunized group. Each dot represents colonization data obtained from a single mouse. On the vertical axis, data are represented as Log10 of recovered colony forming unit per gram colon or liver or spleen of each mouse, 48 h post challenge. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Data represented as mean value of six mice ± S.E. of three independent experiments. *** p value <0.001, ** p value <0.01, * p value <0.5. UI, uninfected mice; I, TOMVs immunized mice; NI, non-immunized mice.

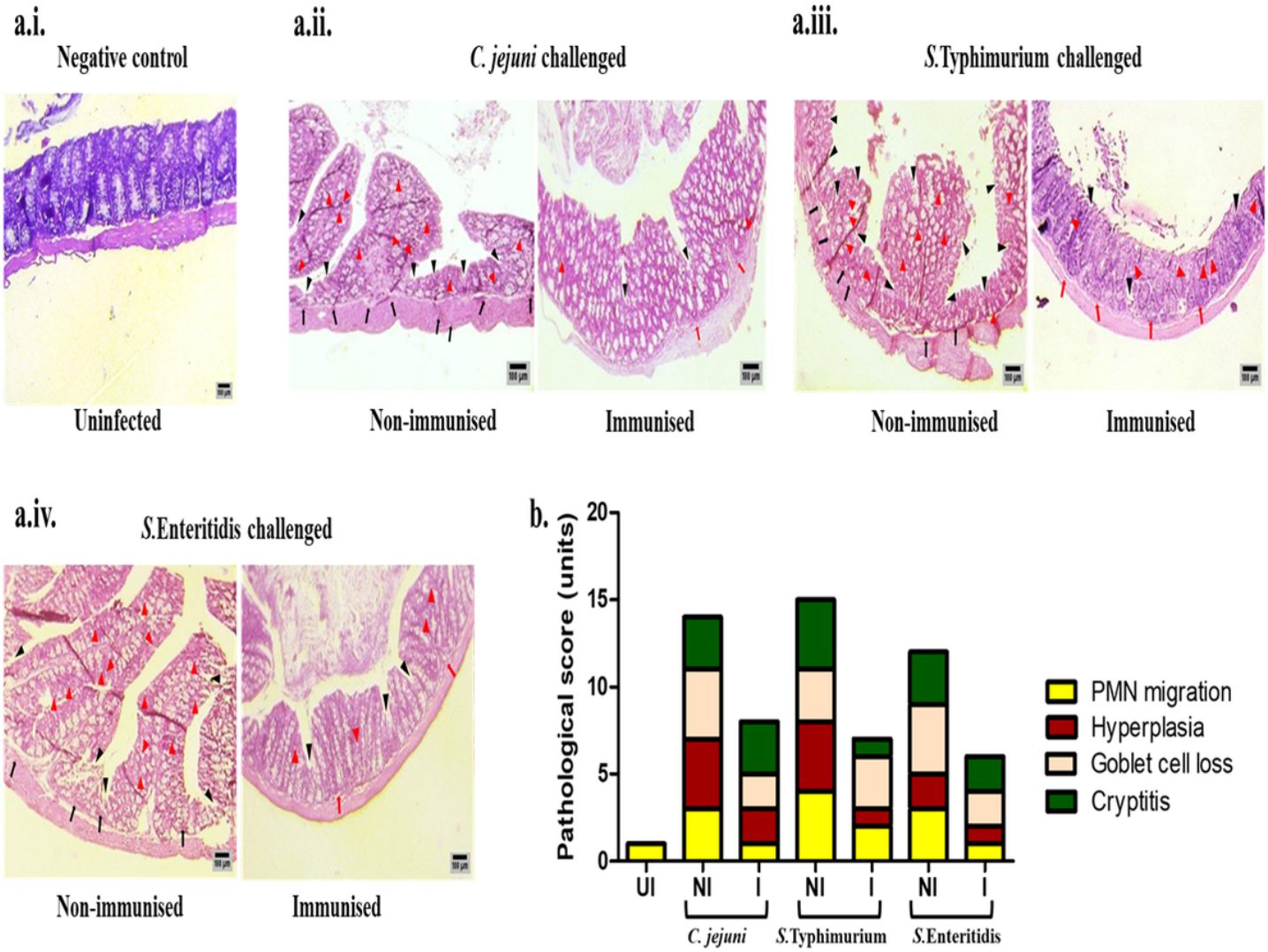

Figure 5

Trivalent OMVs immunization decreases colon tissue damage and inflammation in adult BALB/c mice after infection with CJ, ST and SE challenge pathotypes respectively. Mice of same age were challenged with either 1 X 10^9 CFU/mice of CJ heterologous challenge strain, or 1 X 10^5 CFU/mice of ST or SE heterologous challenge strains separately on 35^{th} day post primary immunization. (a) Histological images of colon tissue of adult BALB/c mice. (i) uninfected control mice colon, (ii) TOMVs immunized or non-immunized CJ challenged mice colon, (iii) TOMVs immunized or non-immunized ST challenged mice

colon, (iv) TOMVs immunized or non-immunized SE challenged mice colon. The images clearly represent marked level of polymorph nuclear neutrophilic (PMN) infiltration in submucosa and cryptic loss in the colons of non-immunized mice, whereas mild level of polymorph nuclear neutrophilic (PMN) infiltration in submucosa and cryptic loss can be seen in the colons of TOMVs immunized mice when compared with that of uninfected mice colon. Black arrow- PMN migration marked level; Red arrow- PMN migration mild level; Black Arrowhead – Crypt alteration with mucus or inflammatory cells; Red arrowhead – Goblet cell loss; All images were taken at 200X magnification, scale bar 100 μm. (b) Pathological scores of TOMVs immunized or non-immunized mice colon post challenge. TOMVs immunized mice colon displayed less inflammation as compared to that of non-immunized mice. UI, uninfected; I, TOMVs immunized mice; NI, non-immunized mice.

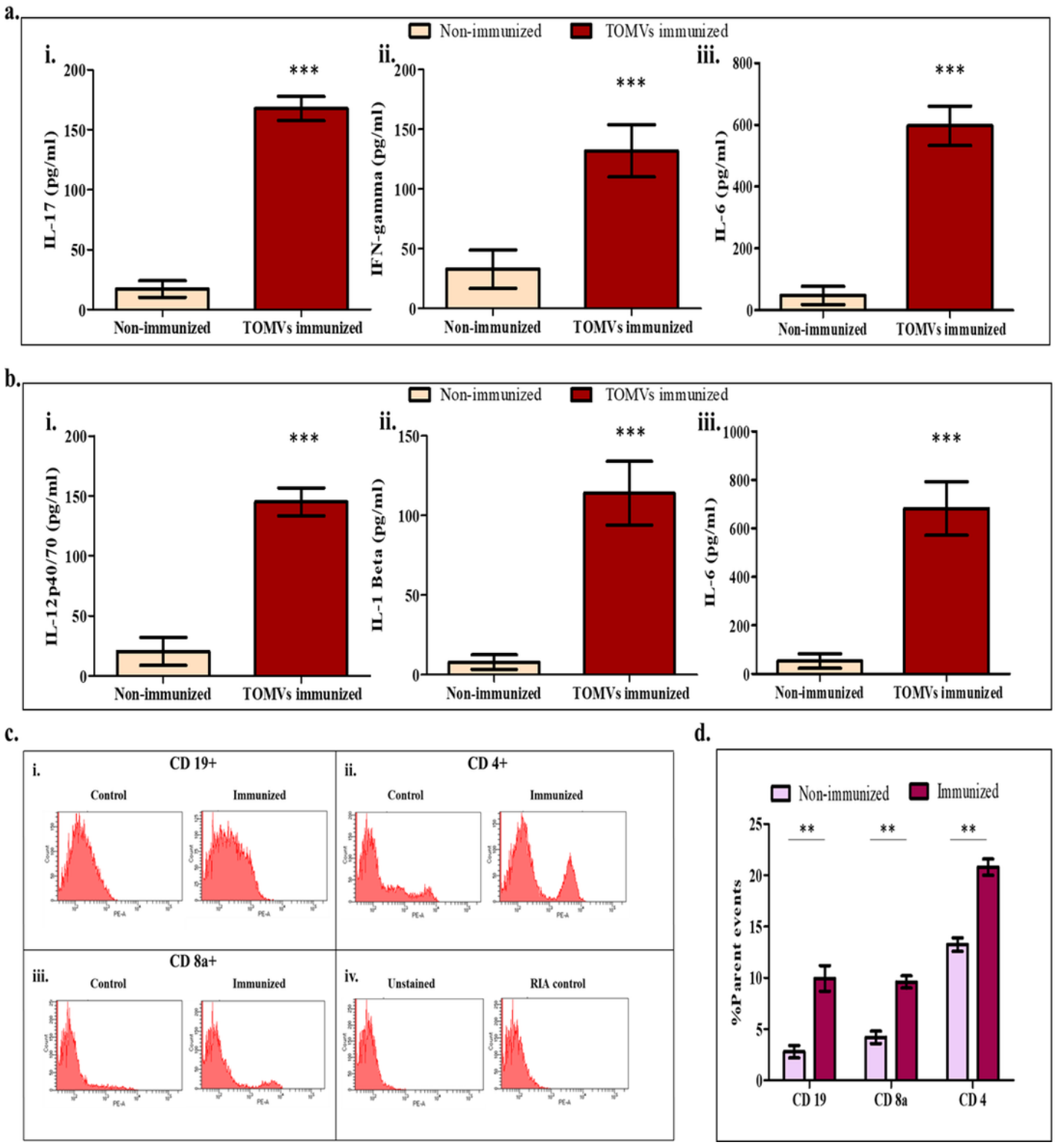

Figure 6

(a) TOMVs induces the production of T-cell mediated cytokines from splenic cells. Splenic cells collected from immunized and non-immunized mice in supplemented media were treated with 5µg/ml of TOMVs. After 24 hours, levels of IL-17 (i), IFN-γ (ii) and IL-6 (iii) from splenic cells were measured by ELISA. Statistical analyses were performed using the two-way analysis of variance (ANOVA) test. Statistical significance was found between TOMVs immunized and non-immunized groups (*** p<0.001). Each bar

represents mean and error values of six samples ± S.E. of three independent experiments. (b) TOMVs induces the production of T-cell mediated cytokines from BMDCs. BMDCs were collected from nonimmunized mice and kept in GM-CSF supplemented media until their maturation and then were treated with 5 µg/ml TOMVs. After 24 hours, levels of (i) IL-12p40/70, (ii) IL-1β and (iii) IL-6 were measured by ELISA. Statistical analyses were performed using the two-way analysis of variance (ANOVA) test. Statistical significance was found between TOMVs immunized and non-immunized groups (*** p<0.001). Each bar represents mean and error values of six samples ± S.E. of three independent experiments. (c) FACS analysis representative histograms of TOMVs immunized and non-immunized CD 19+ (i), CD 4+ (ii), CD 8a+ splenic cells (iii) stained using CD 19-PE, CD 4-PE ,CD 8a-PE and counted in FACS Aria III flow-cytometer (BD Bioscience, USA) . (iv) Unstained and RIA control are represented. (d) Bar diagram comparing the percentage of CD 19+, CD 4+, CD 8a+ splenic cells from immunized and non-immunized mice. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. Significant statistical difference was found between TOMVs immunized and non-immunized splenic cell population (*** p value< 0.001).

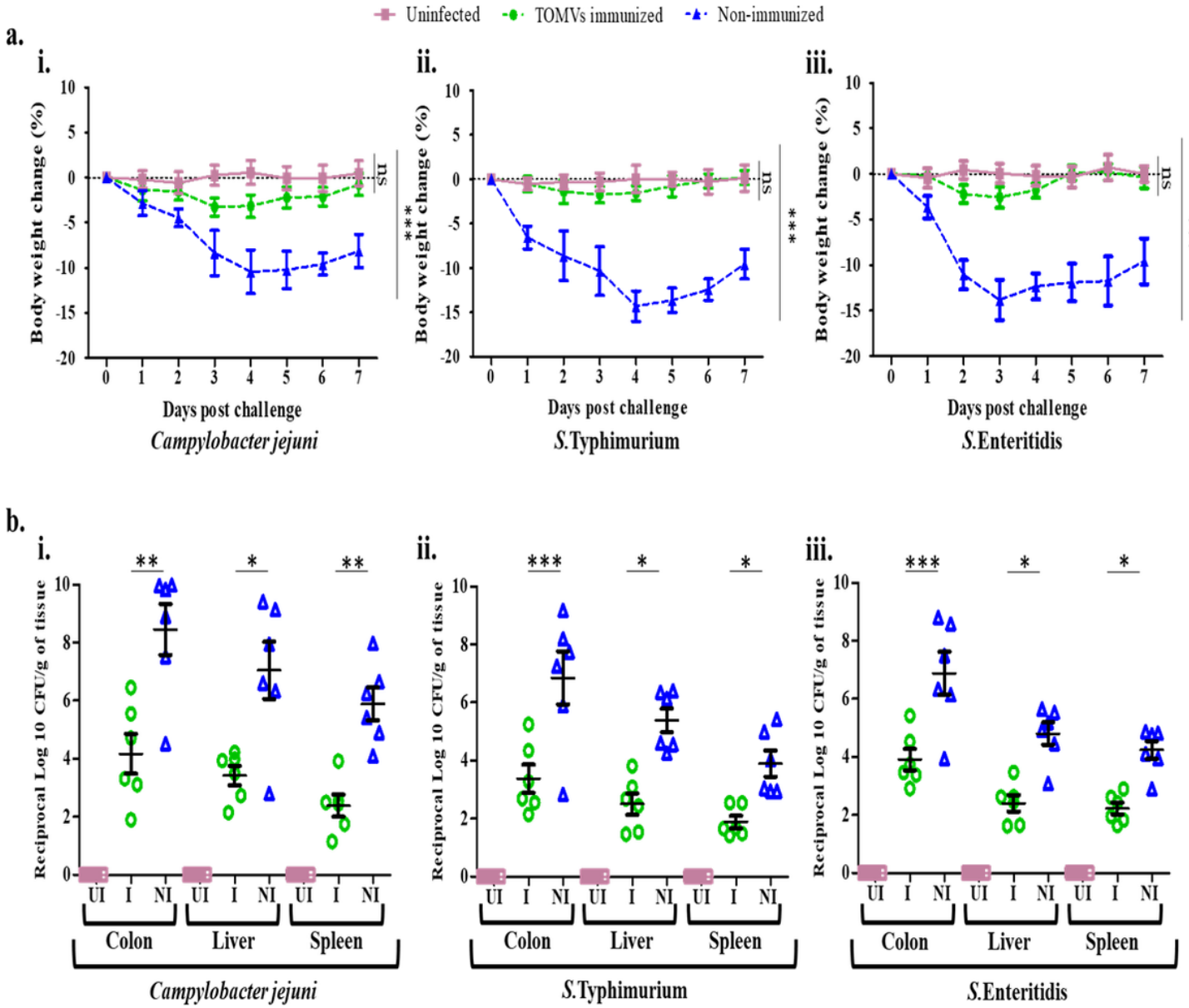

Figure 7

(a) Adult BALB/c mice of same age were challenged with either 1 X 10^9 CFU/mice of CJ heterologous challenge strain (i), or 1 X 10^5 CFU/mice of ST (ii) or SE (iii) heterologous challenge strains separately on 180^{th} day post second booster immunization. Changes in body weight of TOMVs immunized, non-immunized and uninfected groups of mice were monitored for 7 days. Non-immunized group demonstrated a significant decrease in body weight when compared with TOMVs immunized and uninfected groups. No significant changes were found between TOMVs immunized and uninfected groups. Every single dot represents mean and error value of six mice ± S.D. of three independent experiments. *** p value<0.001. ns, non-significant. (b) Comparison of protection determined from colonization data in colon, liver and spleen of adult BALB/c mice (n=6), in TOMVs immunized, non-immunized and uninfected groups, after challenge with CJ, ST and SE challenge strains on 180^{th} day post second booster immunization. For CJ (i), 1 X 10^9 CFU/ml was administered as the challenge dose to

each mouse and immunized group displayed significantly lower colonization compared to non-immunized group. For ST (ii) and SE (iii), 1 X 10^5 CFU/ml was administered as the challenge dose to each mouse and immunized group displayed significantly lower colonization compared to non-immunized group. Each dot represents colonization data obtained from a single mouse. On the vertical axis, data are represented as Log10 of recovered colony forming unit per gram colon or liver or spleen of each mouse, 48 h post challenge. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Data represented as mean value of six mice ± S.E. of three independent experiments. *** p value <0.001, ** p value <0.01, * p value <0.5. UI, uninfected mice; I, TOMVs immunized mice; NI, non-immunized mice.

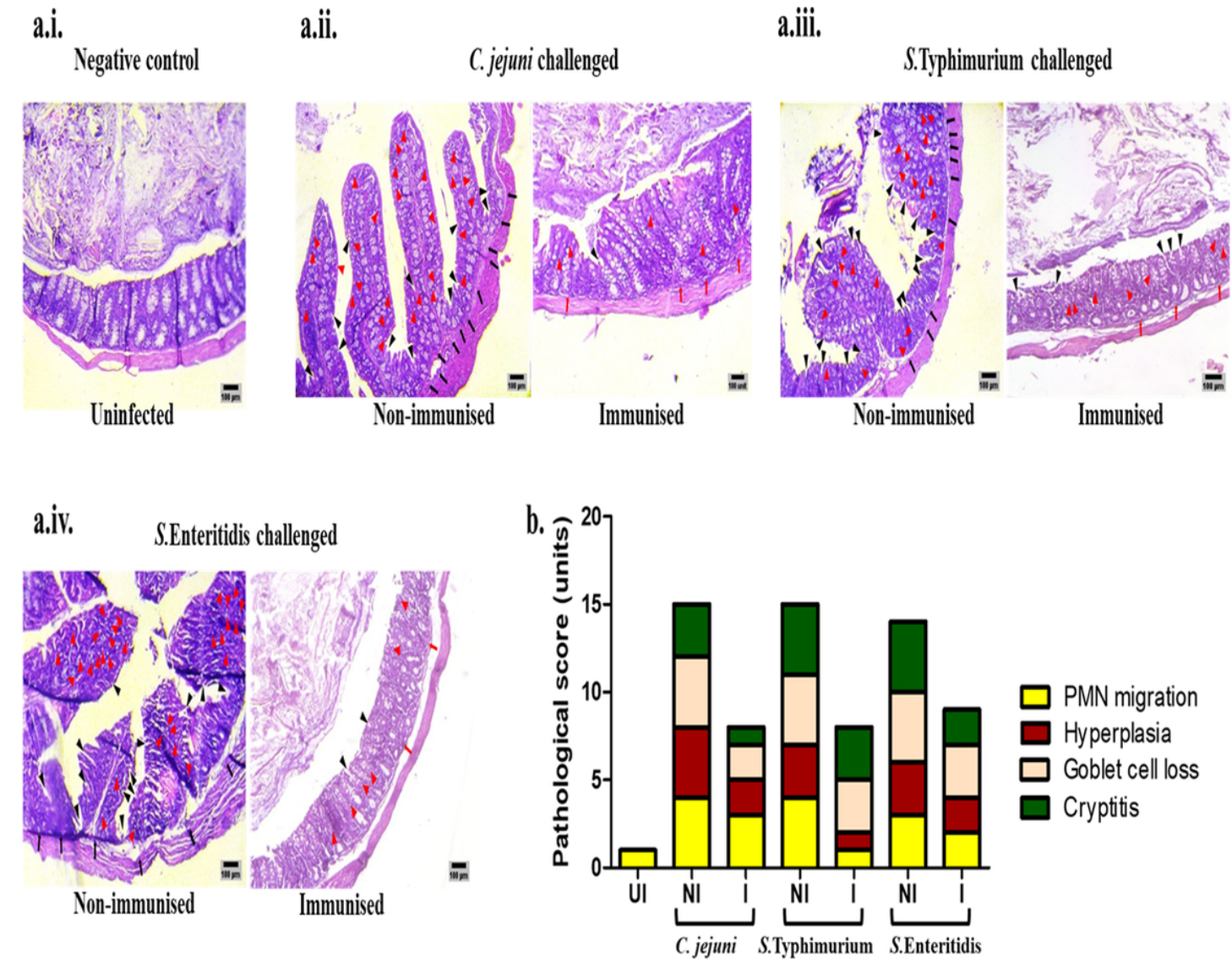

Figure 8

Trivalent OMVs immunization decreases colon tissue damage and inflammation in adult BALB/c mice after infection with CJ, ST and SE challenge pathotypes respectively. Mice of same age were challenged with either 1 X 10^9 CFU/mice of CJ heterologous challenge strain, or 1 X 10^5 CFU/mice of ST or SE heterologous challenge strains separately on 180^{th} day post second booster immunization. (a)

Histological images of colon tissue of adult BALB/c mice. (i) uninfected control mice colon, (ii) TOMVs immunized or non-immunized CJ challenged mice colon, (iii) TOMVs immunized or non-immunized ST challenged mice colon, (iv) TOMVs immunized or non-immunized SE challenged mice colon. The images clearly represent marked level of polymorph nuclear neutrophilic (PMN) infiltration in submucosa and cryptic loss in the colons of non-immunized mice, whereas mild level of polymorph nuclear neutrophilic (PMN) infiltration in submucosa and cryptic loss can be seen in the colons of TOMVs immunized mice when compared with that of uninfected mice colon. Black arrow- PMN migration marked level; Red arrow- PMN migration mild level; Black Arrowhead – Crypt alteration with mucus or inflammatory cells; Red arrowhead – Goblet cell loss; All images were taken at 200X magnification, scale bar 100 μm. (b) Pathological scores of TOMVs immunized or non-immunized mice colon post challenge. TOMVs immunized mice colon displayed less inflammation as compared to that of non-immunized mice. UI, uninfected; I, TOMVs immunized mice; NI, non-immunized mice.

Supplementary Files

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- SupplementaryfigureS1.pdf
- SupplementarytableS1.pdf
- SupplementarytableS2.pdf
- SupplementarytableS3.pdf

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

importantly, in a dose-dependent manner, indicating the usefulness of the developed model for pathophysiology, therapeutic and prophylactic studies.

Keywords: Animal model, Surgical intervention, Gastric illness, Outer membrane vesicles, Vaccine efficacy, *Helicobacter pylori*.

Background

Gastroduodenal disorders are the cumulative effect of carefully orchestrated molecular interactions between host and pathogen factors belonging to the genus *Helicobacter* [1]. With almost 50% of the population worldwide infected by the pathogen, it is one of the major health burdens in developing nations [2]. Although *H. pylori* has been recognized as a class I carcinogen by the WHO, very little has been explored thus far. This is primarily due to the asymptomatic nature of infected individuals, expensive clinical detection (e.g., endoscopy, urea breath test, etc.) and diagnosis with considerable information scarcity [3]. In addition to this, the global antimicrobial resistance (AMR) pattern of *H. pylori* is changing alarmingly, resulting in a paradigm shift in "treatment of choice" by clinical practitioner [4].

Research in *in vitro* and *in vivo* systems of *H. pylori* is continuously enriching our understanding of pathophysiology and genetic predisposition related to adaptation, survival and coevolution of the pathogen [5, 6]. For instance, *H. pylori* has the inherent ability to modulate the gastric microenvironment, such as increasing gastric pH by means of urease upregulation, employing different adhesion proteins or simply dislodging itself when the pH becomes overwhelmingly acidic [7,8]. Such responses, along with others, act as the precursor to a chronic infection that largely depends on the gastric acid neutralizing capacity unique to each strain. The pathogen is known to recruit different adhesins depending upon the stages of disease progression, such as BabA during early infection or SabA during ongoing inflammation [9]. In addition, host antigens present on the surface of host cells, mucins and other gastric cells, such as A/B-Le^b, MUC5AC, MUC1 and H type 1, play important roles in bacterial adhesion, further promoting the severity of different gastric maladies [10,11].

To date, a combination of antibiotics with a proton pump inhibitor (PPI) is the only mode of treatment available due to the lack of a potent vaccine [12]. Moreover, an efficient animal model is crucial to understanding the immunological attributes of different immunogen(s) for vaccine development, which existing models fail to satisfy. To date, considerable efforts have been made to establish a reliable murine (gerbil or mouse) model to serve this purpose, including extensive application of transgenic animals with single or double mutations, but unfortunately, no significant efforts have been made toward the route of administration to induce an infection [13]. The preexisting method relies on the oral administration of multiple doses of inoculums along with antibiotic pretreatment to induce an infection [14]. Moreover, it takes a minimum of two weeks to develop an infection using the traditional approach, which is significantly higher than any other enteric pathogens, such as *E. coli* or *Salmonella*, while using an animal model [15–17].

Therefore, in this study, we introduced an infection by surgically exposing the stomach of C57BL/6 mice and directly injecting *H. pylori* inoculums. We assessed different pathological and immunological markers for active infection and applied the same to study the vaccine efficacy of OMV-based immunogens isolated from a prevalent strain.

Methods

Bacterial strains and culture conditions

Bacterial strains were revived from glycerol stock using brain heart infusion agar (BD Difco, USA) supplemented with 7% horse blood, 0.4% IsoVitaleX with antibiotics such as amphotericin B, trimethoprim, and vancomycin (Sigma Aldrich, USA) at concentrations as described previously [18]. Inoculated plates were then kept under microaerophilic conditions (5% O2, 10% CO2, and 85% N2 at 37°C) for 48 hrs and sub-cultured before conducting any experiment.

Broth culture was prepared using Brucella Broth (BD, Difco, USA) supplemented with 10% horse serum and vancomycin (Sigma, USA). The inoculated flask was then kept in shaking conditions (100 rpm) overnight while maintaining the microaerophilic environment [19].

Characterization and selection of strains

All strains were checked for oxidase, catalase and urease as mentioned elsewhere [20]. Next, an antibiogram was performed using the agar dilution method following CLSI guidelines (**Supplementary table:1**). PCR-based detection was applied for genotypic characterization. Some major virulence factors, such as *cagA, vacA, babA* and *dupA,* were checked using either simplex or multiplex PCR [21,22]. The primers used in the present study are listed in a table **(Supplementary table: 2)**.

Animals

Six- to eight-week-old female C57BL/6 mice were received from the NICED-Animal house facility. The animals were kept in a condition maintained at 25±2°C with 65 ± 2% humidity and a 12/12-hour light/dark cycle. Animals weighing ~22 grams were selected for the study and provided with sterile food and water *ad libitum*. All experiments were performed following the standard operating procedure outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (CPCSEA Registration no. 68/GO/ReBi/S/1999/CPCSEA valid 17.07.2024) and Institutional Animal Ethics Committee (IAEC) of NICED was approved (Approval No. PRO/194/June 2022-25) and supervised experimental design and protocols from time to time.

Animal experimental design

Thirty-six C57BL/6 mice were randomly assigned into two major groups, each comprising 18 animals. To determine an infectious dose for the surgical model, the first set of mice was further divided into three subgroups and infected with a dose of either $1x10^8$ (n=6) or $2x10^8$ CFU/mL (n=6, each) along with PBS control (n=6). All groups were housed for one or two weeks under sterile conditions.

For immunological studies, the remaining 18 mice were separated into two groups as: non-immunized (NI) (n=6) and oral or i.p. immunized (IM) (n=6 in each group). An oral or

intraperitoneal immunization with 50 µg of OMVs dissolved in PBS was administered on days 0, 14, and 28. Blood was collected at different time points, and the serum was isolated and stored at -20°C for use in different immunological assays. For the protective efficacy study, both groups (IM and NI) were infected surgically on the 35th day post-first immunization and sacrificed 7 days post-infection **(Supplementary figure:1)**.

Intragastric surgical model development

Experimental animals were kept in fasting conditions overnight with sterile water. Initially, animals were sedated by an intraperitoneal injection of a mixture of ketamine (87.5 mg/kg) and xylazine (12.5 mg/kg) [23]. The stomach was exposed through a 2-3 cm midline incision without compromising any major blood supply. A disposable syringe with a 26G needle containing 200 µl (~2x10^{8} CFU) of the inoculums in PBS was directly injected into the stomach. Hydration was maintained in the exposed stomach using sterile normal saline throughout the surgery. The stomach was placed back inside the abdominal cavity, and the incision was sutured back. The incision site was monitored for any infection and occasionally washed with 5% povidone-iodine (betadine) soaked in a sterile gauge for 72 hours [24]. Sterile food and water were provided to the animals once they regained consciousness (**Fig. 1**).

Post-Surgery observation

All infected mice were observed twice a day for 7 days. Physical parameters were checked along with stool consistency and the nature of mucus or blood (if any) present in the feces. Rectal swabs were taken daily and were subjected to RUT solution and spread-plate to observe the shedding of the organism. Isolated colonies (if any) were confirmed using a PCR-based technique. *H. pylori* infection augments the modulation of both pro- and anti-inflammatory cytokines in the host [25]. Therefore, IL-1β, TNFα, IFNγ, IL-6, IL-10 and IL-17 were tested using cytokine measuring kits (Invitrogen, USA) following the manufacturer's protocol. 50µl of serum samples from 0-day, 7-day- and 14-day-infected mice were used to quantify the inflammatory response after post-operational (OP) observation due to infection.

Immunogen preparation

Outer membrane vesicles (OMVs) were isolated from the *Helicobacter* strain [A61C (1), *cagA*+, *vacA s1m1*] following the methods described previously with slight modification [26]. In brief, BB broth (BD, Difco, USA) was inoculated with log phase (OD_{600} ~0.6) pre-culture of the respective strains and kept overnight in microaerophilic conditions under constant shaking (100 rpm) at 37°C. On the next day, centrifugation was performed consecutively first at 8000xg for 15 min st 4°C, followed by 30 min with same conditions. The supernatants were then filtered twice with 0.45 µm and 0.22 µm syringe filters (Millipore, USA). To prevent protein degradation, a protease inhibitor cocktail was incorporated into the filtrate and ultracentrifuged at 140,000 x g at 4°C for 4 hrs using a P27A-1004 rotor (Hitachi). A density gradient centrifugation allowed obtaining the purified OMVs. Protein content was measured using a Lowry protein estimation kit (Pierce, USA) and stored at -20°C until further use.

Characterization of the Outer Membrane Vesicles (OMVs)

Dynamic Light Scattering

Concentrated OMVs were diluted 10-fold to reach a concentration of 0.1 mg/mL. The hydrodynamic size of OMVs was measured using a Malvern Zetasizer ZS90 (Malvern Instruments, Germany) and analyzed using ZS Xplorer version 3.1.0.64 [27].

Transmission electron microscopy

Diluted OMVs were placed on a carbon-coated grid and left for 10-20 min for absorption. The samples were then washed twice with drops of Tris buffer solution. Excess fluid was soaked using blotting paper, followed by staining with 2% uranyl acetate and air drying. Finally, the OMV-coated grids were observed under a JEOL JEM 2100 HR (JEOL, Tokyo, Japan) [28].

LC/MS of OMVs and analyses

Proteins present in OMVs were used for digestion and reduced with 5 mM TCEP and further alkylated with 50 mM iodoacetamide and again digested with trypsin (1:50, trypsin/lysate ratio) for 16 h at 37°C. Digests were cleaned using a C18 silica cartridge to remove the salt and dried using a speed vac. The dried pellet was resuspended in buffer A (2% acetonitrile, 0.1% formic acid). Experiments were performed on an Easy-nlc-1000 system coupled with an Orbitrap

Exploris mass spectrometer. One microgram of peptide sample was loaded on a C18 column (15 cm, 3.0 µm Acclaim PepMap, Thermo Fisher Scientific), separated with a 0–40% gradient of buffer B (80% acetonitrile, 0.1% formic acid at a flow rate of 500nl/min) and injected for MS analysis.LC gradients were run for 60 minutes. MS1 spectra were acquired in the Orbitrap (MaxIT = 25 ms, AGQ target=300%; RF lens = 70%; R=60 K, mass range = 375−1500; profile data). Dynamic exclusion was employed for 30s, excluding all charge states for a given precursor. MS2 spectra were collected for the top 12 peptides. MS2 (Max IT= 22 ms, R= 15 K, AGC target 200%). All samples were processed, and the generated RAW files were analyzed with Proteome Discoverer (v2.5) against the UniProt organism database. For dual Sequest and Amanda searches, the precursor and fragment mass tolerances were set at 10 ppm and 0.02 Da, respectively. The protease used to generate peptides, i.e., Enzyme specificity was set for trypsin/P (cleavage at the C-terminus of "K/R: unless followed by "P"). Carbamidomethyl on cysteine as a fixed modification and oxidation of methionine and N-terminal acetylation were considered variable modifications for the database search.

Extraction of lipopolysaccharide (LPS) and outer membrane proteins (OMPs)

LPS and OMPs were extracted following the methods described earlier [29]. LPS was then treated with proteinase-K to ensure the absence of any protein residue. The carbohydrate content of LPS was then quantified using the phenol–sulfuric acid method and measured at a wavelength of 492 nm [30]. Isolated proteins were quantified for their concentration using a Modified Lowry's Kit (Pierce, USA) and measured at 660 nm using a spectrophotometer.

ELISA

Serum immunoglobulin (IgG, IgM, IgA, IgG2c) levels were measured against OMPs or LPS following the method described previously [31]. Twofold serial dilutions were prepared from serum isolated from both immunized and non-immunized groups. HRP-conjugated secondary anti-mouse IgG, anti-IgA, anti-IgG2c and anti-IgM antibodies (Sigma Aldrich, USA) were used to detect the antibody titer. Each experiment was replicated thrice with pooled sera from different groups.

Serum Bactericidal Assay (SBA) and Scanning Electron Microscopy

The effect of immunized mouse sera on bacterial morphology was measured and visualized using scanning electron microscopy (SEM) following a previously described protocol [32]. Bacteria along with heat-inactivated mouse sera and 25% guinea pig complement (with/without) were incubated for 1 hr under microaerophilic conditions followed by plating for viable colonies or fixation with 3% glutaraldehyde overnight followed by a gradual dehydration step initially with alcohol and then substitution later with a mixture of alcohol and hexamethyldisilazane (HMDS) at ratios of 2:1, 1:1 and 1:2. Finally, the samples were mounted on specimen stubs, sputter-coated with gold and analyzed on a Quanta 200 SEM (FEI, Netherlands).

Cytokine assay

Both immunized and non-immunized mice were sacrificed, and the spleens were harvested. After isolating spleen cells; ~10^5 cells were cultured for two hours in RPMI1640 containing 10 % FBS incubated with 50 µg of OMVs and incubated overnight at 37°C (with 5 % CO2) for 24 h. IL-10, IFN-γ, IL-1β, IL-6, IL-4, TNF-α and IL-17 were measured in the culture supernatant using a cytokine measuring kit (Invitrogen, USA) [33].

Fluorescence-activated cell sorting (FACS) analysis

Spleen cells were harvested, cultured for two hours in RPMI1640 containing 10 % FBS and re-stimulated using isolated OMVs (50 µg) and incubated overnight at 37°C (with 5 % CO2) for 24 h. The next day, the cells were scraped, washed thoroughly, blocked and then incubated with mouse anti-CD4+, CD8+ or CD19+ antibodies. Splenocytes were stained with anti-Mabs: CD4-phycoerithrin (PE), CD8 PE, CD19 PE or an isotype control PE (Miltenyi Biotec, USA). Unbound antibodies were washed, and a specific epitope of the immune cell population was observed using FACS Aria II [32].

Protective efficacy Study

Seven days after the last immunization, both the immunized and non-immunized groups were challenged with the wild-type SS1 strain using a newly developed surgical procedure and housed for 7 days before being sacrificed. The antrum of stomach of both immunized and non-immunized groups was isolated and separated into two parts. Half of each part was immediately

transferred to BHI kept on ice, and the other half was transferred to neutral buffered formalin (NBF, 10%) solution to fix the tissue and left at room temperature. Harvested tissue in BHI was weighed, homogenized and serially diluted using PBS. The diluted samples were then spread onto BHIA and kept under microaerophilic conditions for 3-5 days. Any visible colonies were then counted and confirmed using RUT and PCR. Histopathological assays were performed as described elsewhere [34,35]. Briefly, samples kept in 10% formalin were washed and gradually dehydrated using the alcohol gradation method followed by preparing a paraffin block. A thin section (approximately 5 μm) was prepared using a microtome. The slides were then de-waxed, rehydrated, and stained. Hematoxylin-Eosin was used for the study because they enhance tissue or bacterial contrast. Finally, the slides were mounted and observed under a microscope. Histological scoring was assigned for each sample based on their morphological changes. The gastric tissue observed under a microscope revealed various degrees of gastritis, which was then categorized according to the Houston-updated Sydney System based on the infiltration of inflammatory cells within the lamina propria [36].

Statistical analysis

The presented data do not follow a normal distribution due to biological variations. Nonparametric tests were adopted for all data analyses. Triplicate data were expressed as the mean ± SD (standard deviation) using GraphPad Prism version 5.02. Two-way analysis of variance (ANOVA) or the Mann–Whitney test (for animal data) was performed as per the requirements, and statistical significance was determined from the *P* values mentioned in the figure legends.

Results

Characterization and selection of *H. pylori* strains used in the study

A total of 12 strains including 3 reference strains and 9 clinical strains were checked for the presence or absence of major virulence genes i.e. cytotoxin-associated gene or *cagA* representing cag pathogenicity island (cagPAI), vacuolating toxin A or *vacA*, blood group antigen binding

adhesin 2 or *babA2* and duodenal ulcer promoting gene or *dupA.* A type I or type II strain is defined by the presence of *cag* and allelic variations of *vacA* with signal region (*s1* or *s2*) and middle region (*m1* or *m2*). A *cag+s1m1* is considered to be more virulent, thereby influencing diseases development than *cag+s2m2* or any combination of *s1, s2, m1* and *m2*. Additionally, allelic variations of *babA* i.e *babA2,* plays a key role in adhesion to the Lewis B (Le^b) antigen of blood as *babA1* is known to be non-functional [21]. *dupA* belongs to a plasticity region (*jhp0917-jhp0918)* and found to be responsible in developing ulcers in *H. pylori* infected individuals [66]. Therefore, any strain positive for all these genes can be considered to be more virulent than others. A61C (1) is positive for all these virulence genes and therefore is selected for immunogen preparation. However, for model establishment and challenge study purpose, SS1 is considered to be more suitable than others as it is a mouse adapted strain. The result of genetic characterizations of all strains is listed in a table (**Supplementary table: 3)**.

Clinical response caused by surgical intervention

In the present study, $2x10^8$ CFU of bacteria were used to induce an active infection. Oral inoculation with the aforementioned dose revealed inconsistent results. Moreover, in the majority of cases, very little or no recovery of the bacterial population was observed using available detection techniques. Mice receiving WTSS1 directly to their stomachs by surgical means developed various degrees of gastric changes. Recovery of bacterial colonies from stool was insignificant and erroneous compared to gastric tissues, which were considerably higher (~2-3 times) and were confirmed to be positive upon RUT, spread-plate and PCR. The recovery rate of *H. pylori* from the 7-day infected mice was comparatively higher than that at 14 days post infection (**Fig.2**).

Intragastric surgical evoked inflammatory response Cytokine analysis of intragastrically infected mice of different time points i.e. 0-day, 7 day, and 14 days, showed drastic differences in serum cytokine levels. IFN-γ, IL-1β, TNF-α, IL-10, and IL-17 are increasing more on day 7 after infection and also reducing progressively on day 14. In case of IL-6, which is responsible

for sustaining inflammation is increasing on day 14. The majority of the pro-inflammatory cytokines were upregulated after 7 days post infection, except IL-6, which was found to be more pronounced at 14 days than at 7 days post infection (**Fig.3**) indicating active *H. pylori* infection.

Histopathological changes due to intragastric infection

Histopathological observation plays crucial role in *H. pylori* diagnosis. *H. pylori* infection causes local inflammation in gastric tissue marked by various degrees of inflammatory infiltration with substantial damage in gastric epithelium leading to the survival of the bacteria to the gastric microenvironment. Moreover, previous study has already showed a pronounced effect on gastric tissue of C57BL/6 mice upon *H. pylori* infection. Therefore, in order to establish a successful infection mediated by surgical intervention, stomach samples were taken at different time points and the topographical changes were compared. Negative control mice, receiving only PBS and Day0 had no inflammation (**Fig 4.i: a, b**) whereas inflammatory infiltration was significantly higher on day7 (**Fig 4.i:c**) compared to day 14(**Fig 4.i: d**). In addition to this, mucosal epithelium was severely damaged with exposed gastric pits in both cases. In contrast, metaplasia due to infection was more prominent on day 14 than day 7, indicating a successful infection. Histopathological scoring was assigned based on Sydney system (**Fig.4. ii**).

Isolation and characterization of OMVs from *H. pylori* strain A61C (1)

The OMVs isolated from the broth culture of A61C (1) were purified and assessed using dynamic light scattering (DLS), transmission electron microscopy (TEM) and proteomics analyses using LC/MS (**Fig. 5.A**). The data revealed uniformity in OMVs structure with a diameter of 50nm (**Fig. 5.B.i, ii**). TEM image showed the OMVs to be circular in shape with distinct bilayers. The protein components present in OMVs isolated from the immunogen strain [A61C (1)] revealed 18 major proteins including UreB, UreA, FtnA, GroEL, UbiX, Tuf, SecA, RplI, LpxK, RimO, AroB along with some other proteins with unknown localization (Fig. 5.C, **Supplementary table 4**). Presence of proteins like UreA, UreB and GroEL on OMVs indicate the potential to generate a strong immune response as these proteins are known for their

immunomodulatory activities. The sub-cellular localization of proteins indicated by the software includes cytoplasm, membrane, periplasmic space, plasma membrane of the bacteria. *H. pylori* LPS is known to have no cytotoxicity which is also evident from cytotoxicity assay (**Supplementary figure 2**).

H. pylori OMVs induce pro-inflammatory cytokine response

35th day post immunization splenic cells were harvested from both immunized and non-immunized mice and re-stimulated with 50μg of OMVs. A significant induction in IFN-γ, TNF-α, IL-1β, IL-4, IL-10, IL-17, IL-6and IL-12, IL-13 levels were observed (**Fig. 6.a-i)**. Moreover, our data revealed oral immunization to be a better route for immunization as most of pro-inflammatory cytokines. Contrary to previous studies [63], our study did not find any Th1 or Th2 biased response indicating the immune response against OMVs is independent of routes of administration, compared to the control, both oral and i.p. immunized animals revealed elevation in cytokines. Altogether, immunization invoked an array of cytokines than non-immunized implying the potential of OMVs for a vaccine candidate.

Immunization of *H. pylori* OMVs elicited higher adaptive immune response

Previous studies on bacterial extracellular vesicles revealed OMVs to be an excellent vaccine candidate against bacterial pathogens [62]. OMVs are known to induce both humoral and cellular arms of immune responses usually mediated by outer membrane proteins (OMPs) and lipopolysaccharides (LPS). We investigated serum immunoglobulin levels (**Fig 7.A.i-iv**) of oral and intraperitoneally immunized mice and found significant difference compared to the control. However, we did not find significant differences between oral and intraperitoneal immunization.

Next, we evaluated the bactericidal activity of the immunized serum. The data showed significant reduction in bacterial number when immunized serum is incubated with 25% guinea pig serum as compared to non-immunized mice serum (**Supplementary figure 3**). This confers activation of complement mediated pathway, along with sufficient antibody titer in immunized

C57BL/6 mice that effectively kill the bacteria by damaging the bacterial surface as viewed under SEM (**Fig 7. C, D.i-ii**). Comparative analyses of the splenic cell population of immunized and non-immunized mice were done using a flow cytometer. Immunization with OMVs showed significantly higher population of CD4+, CD8a+ and CD19+ (**Fig 7. B**) cells indicating a strong immune response in immunized mice.

In all, immunization with *H. pylori* OMVs generated adaptive immune responses in C57BL/6 mice and significantly activated adaptive immune responses which could in turn help to provide long term protective immune response against infections caused by *H. pylori* (**Figure 7.A.iii-iv**).

Protective efficacy study post immunization

After immunization with OMVs, the immunized and non-immunized animals were challenged with wild type SS1 with a dose mentioned before (i.e. $2x10^8$ CFU) and the colonization were analyzed 7 days post infection. A significant decrease in colonization was observed in the stomach tissue of immunized than non-immunized animals indicating a substantial reduction in bacterial load (**Fig. 8.D)**. To confirm further, DNA was extracted from the gastric tissue and subjected to *ureB* PCR for the presence of *H. pylori.* All non-immunized mice were found to be positive whereas immunized animals were found insignificant presence - of *H. pylori* DNA among OMVs immunized animals (**Fig. 8.C)**. Histopathological changes of both immunized and non-immunized mice stomachs were analyzed 7 days post infection using surgical intervention (**Fig. 8.A.i-ii)**. OMVs immunized mice showed a significant reduction in gastric epithelial damage, altered gastric mucosa, inflammatory infiltration, exposed gastric pit, and metaplasia. Pathological scores were also less in immunized mice than non-immunized mice (**Fig. 8.B)**. Overall, reductions in bacterial numbers were observed upon immunization.

Discussion:

Over the years, a number of animal models have been evaluated for pathophysiology or treatment against *H. pylori,* including gnotobiotic pigs, dogs, cats, Mongolian gerbils, guinea

pigs, rhesus monkeys, and mice [37–48]. In most cases, C57BL/6 or black mice were explored extensively because of their substantial contribution in *H. pylori*-related studies. The proper route for administering the pathogen and/or immunization also has a key impact on the development of an infection and assessment of immune response, which is another important consideration when selecting an animal model for *in vivo* studies. Therefore, cytokine alterations along with histological changes described in the present study represent a successful infection achieved through our newly developed surgical model. Conventional approaches for studying *H. pylori* infection in animals usually involve multiple oral inoculations using an oral gavage [49–51]. However, relying solely on the oral route to induce an infection and expecting the bacterium to outcompete the existing microflora and successfully colonize the stomach may not always yield a consistent result in any given experimental setting. This can cost significant time and resources being invested while still fostering uncertainty about an actual infective status in experimental animals. Therefore, it is important to consider the limitations and variability of the *in vivo* systems and look for alternative approaches that could provide a more reliable method of *H. pylori*-mediated pathogenesis in animal models. Clinical detection of *H. pylori* infection generally involves histology and PCR apart from the Rapid Urease Test (RUT) [52–54]. Serological tests are often avoided, as previously invoked antibodies fail to recognize the actual infective status of recent manifestations [55, 56]. As a consequence, this increases the chances of false positive results. In addition, histology allows visualization of pathogen-induced changes in gastric tissues, such as the intensity of inflammatory cell infiltration or aberrations in gastric topology, while PCR detects the presence of genomic DNA of *H. pylori* in gastric tissue samples [57]. However, it should be noted that neither histological observations nor negative PCR results rule out the presence of an infection [58]. Thus, a number of different techniques must be employed simultaneously to achieve a more accurate diagnosis of *H. pylori* infection [59]. Our study comprised a combination of histological observation, PCR detection and quantification of serum cytokine levels to confirm active *H. pylori* infection.

Surgical intervention initially spiked pro-inflammatory cytokines such as IFN-γ and IL-1β along with IL-17 significantly more on day7 than on day14. However, as the infection progressed, these cytokines were lowered and finally balanced, except for IL-6, which was found to be elevated more on day 14 than on day 7. A pronounced IL-6 level at later stages might indicate ongoing inflammation in the gastric lining with potentially developing chronic gastritis. Such

responses were further supported by the induction of other cytokines. IFN-γ is an early effector molecule responsible for generating a Th1-mediated response by initiating different signaling cascades. However, up-regulation of IFN-γ transiently down-regulates IL-1β production. In addition, IL-17, a cytokine regulating the Th-17-based response, plays important roles in both pathogenesis and host immunity. Studies with chronic diseases have revealed that well-balanced IL-1β and IL-17 levels are constitutively produced to sustain inflammation due to infection in the long term [67]. In the case of *H. pylori* infection, both IL-1β and IL-17 play crucial roles in pathogenesis; in particular, IL-17 influences the disease outcome upon infection. Our model showed an initial elevation in these cytokines, which decreased over time, indicating progression toward a chronic infection. However, as 7 days were not sufficient to develop a chronic infection, our model showed promising results in a time-dependent manner. Consistent with the cytokine analysis, histopathological observations also validate such changes to some extent. Intense inflammatory cell infiltration was observed on day 7 than on day 14, and the gastric lining was found to be more damaged with exposed gastric pits, indicating destruction caused by bacteria. Nevertheless, we did not find any striking structural abnormalities in gastric tissue 7 days post infection. PCR results from the same samples confirmed the presence of bacterial genomic DNA in experimental animals.

Next, we evaluated the surgical model for vaccine efficacy studies. Two different immunization routes were assessed to observe any alterations in the immune response due to changes in the route of administration. Immunization was performed both orally and intraperitoneally (i.p.) on days 0, 14 and 28. Initially, an elevation of serum IgG, IgM and IgA levels was observed against OMPs but not LPS of *H. pylori*. This can be due to the structural similarity between *H. pylori* LPS and blood antigens of the host [60-61]. Furthermore, we evaluated IgG2c (IgG subtype) and found it to be increased in immunized rather than non-immunized groups [62]. Our study found oral immunization to be better responsive than i.p. route, which can be due to the presence of different surface proteins on OMVs that are more readily absorbed and reactive to gastric epithelial cells than peritoneal immune cells. A splenic cell re-stimulation (ex vivo) assay revealed enhanced Th2-based cytokine responses, such as IL-4, IL-13, IL-10 and IL-12, coinciding with previous studies with *H.* pylori-derived OMVs used as immunogens [63]. Interestingly, our study did not find any biased immune response against OMVs, indicating that the immune response to OMVs is not general but rather unique to each strain. CD4+, CD8a+ and

CD19+ cell populations were increased due to OMV immunization independent of the route of administration. OMV immunization ultimately leads to a reduction in bacterial colonization in immunized animals but not in non-immunized animals. Serum bactericidal assay (SBA) typically denotes the functional aspect of immunogen-invoked antibody response in killing the bacterial population via complement mediated pathway. This in-vitro method involves incubation of bacteria in presence of heat-inactivated serum isolated from both OMVs immunized and PBS immunized mice. Antibodies generated in host due to immunization are sufficient enough to reduce the bacterial CFU by means of agglutination as demonstrated in OMVs induced immune response against *S. Typhi* and *Paratyphi A* [31]. However, agglutination doesn't directly imply a bacteriostatic or bactericidal activity of the antibodies. Therefore, addition of purified baby rabbit [64] or guinea-pig [65] complement externally to these antibodies ensured the lysis of the bacteria via complement-mediated pathway. In the present study, incubation of bacteria treated with OMVs immunized or PBS immunized sera in presence of guinea-pig complement lead to significant reduction in viable colony numbers in immunized compared to the PBS immunized mice groups.

Conclusion

In conclusion, the intragastric surgical model of *H. pylori* infection can be used to study the pathophysiology, immune response, and potential therapies for *H. pylori* infection. Our study indicates that a minimum of 7 days is enough to develop an infection in this model. All experimental results showed that tissue samples collected at 7days post infection can provide better results for diagnosing *H. pylori* infection than samples obtained at 14 days post infection, as histological changes and inflammatory cell infiltration are typically more pronounced at earlier time points post infection. Moreover, the cytokine response and antibody generation further support this model for vaccine efficacy studies. The immunization of mice with *H. pylori* OMVs has been shown to reduce the bacterial load with elevated antibody titers and protect gastric tissue from destruction. Therefore, the intragastric surgical model can become a valuable tool for understanding the pathophysiology of *H. pylori* infection, formulation and evaluation of potent vaccine candidates and development of potential therapeutics.

Author contributions

Sanjib Das: Conceptualization, experimental design and performance, data analysis and interpretation, manuscript preparation, **Prolay Halder:** Experiment performed, data analysis and interpretation, review and edit manuscript, **Soumalya Banerjee:** Experiment performed, data analysis and interpretation, review and edit manuscript, **Asish Kumar Mukhopadhyay:** Review and edit manuscript**, Shanta Dutta:** Review and edit manuscript, **Hemanta Koley**: Conceptualization, experimental design and supervision, data analysis and interpretation, manuscript preparation, funding acquisition.

Acknowledgments

Our heartfelt thanks go to Mr. Suhashit Ranjan Ghosh, Mr. Subrata Singha, Mr. Pritam Nandy, and Mrs. Arpita Sarbajana for their technical assistance from time to time. We extend our gratitude to the University Grants Commission (UGC), New Delhi, India, for providing the fellowships to Sanjib Das under the CSIR-UGC-NFSC scheme (Student ID: SANJIB DAS under UGC-NFSC scheme 3363/(CSIR-UGCNETJUNE2018]), the Indian Council of Medical Research for providing the fellowship to Prolay Halder [Fellowship ID: ICMR-3/1/3/JRF-2018/HRD-066(66125)] and Soumalya Banerjee under the CSIR-UGC-NET scheme [StudentID:191620007740].

Conflict of interest

The authors declare no conflicts of interest.

Funding

This work was supported by the Indian Council of Medical Research as an ICMR-extramural project (Project Index No. VIR/20/2020/ECD-I).

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Figures

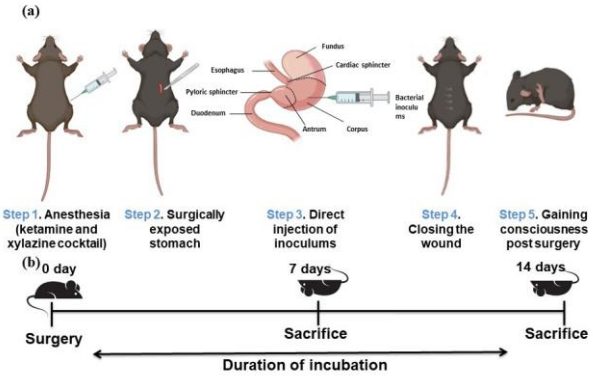

Fig. 1. Schematic diagram of the intragastrically infection model in C57BL/6 mice. (a) Graphical Representation of the "Surgical Model" using C57BL/6.Bacterial inoculation (~2x10^{8}CFU/mL) is directly injected into the stomach, **(b)** Schematic schedule from infection to sacrifice.

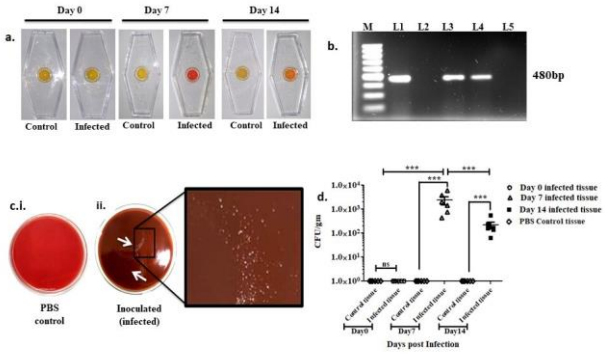

Fig. 2. Intragastric infection induced by wild type (WT) SS1 observed through urease test, *ureB* PCR, colonization in gastric tissue. (a) RUT of infected gastric tissue; Day 0, Day7 and Day 14 with respective PBS control**, (b)** Confirmatory *ureB* PCR for the presence of *H. pylori* recovered from the gastric tissue of infected mice**. M**-100bp marker, **L1**-positive control, **L2**-day 0 post infection, **L3**-day7 post infection, **L4**-14-day post infection, **L5**-PBS control. (**c**) Blood agar plates showing **(c.i.)** plate containing no *H. pylori* colonies recovered from gastric tissue of 7days post PBS inoculated mice **(c.ii)** plate containing *H. pylori* colonies recovered from gastric tissue of 7days post SS1 infected mice. **(d)** Colonies recovered from mice of 0, 7- and 14-days post infection. Data represented here are the mean values +/-Standard Deviation (SD) of three independent experiments. The differences in day wise response of each colonization assay were highly significant with respect to PBS control tissue. Statistical significance was found between 0day, 7day and 14day infected mice tissue (***p<0.001, ns-non-significant).

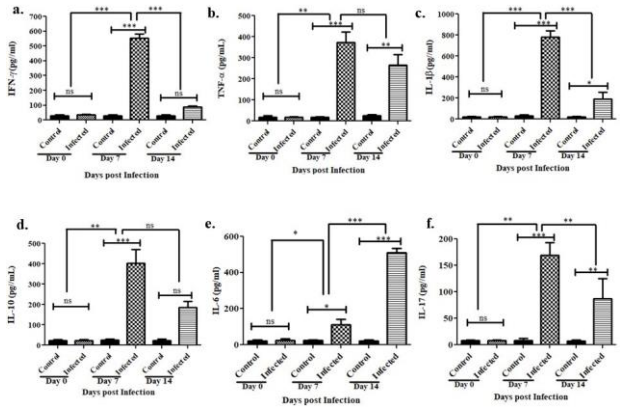

Fig. 3. *Helicobacter pylori;* wild type (WT) SS1 induces the production of cell mediated cytokines responses post-surgical intervention. (a) IFN-γ, **(b)** TNF-α, **(c)** IL-1β, **(d)** IL-10, **(e)** IL-6, **(f)** IL-17 cytokines in serum isolated on Day 0, Day 7 and Day 14 post-surgical infection with respective PBS controls. All cytokines are measured by ELISA (n = 6). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non-significant), Each bar represents median and error values of Six ± SE of three independent experiments.

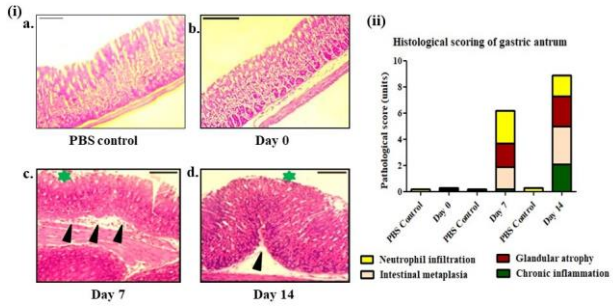

Fig. 4. Histological (H&E staining) observation and histological scoring of gastric epithelium after intragastric surgical infection with *Helicobacter pylori* (SS1). (i) a, b, c, d; all are the antrum part of the stomachs harvested from C57BL/6 mice**. (i.a)** no distinct changes observed in PBS control, mice receiving PBS only, **(i.b)** zero inflammation in Day 0 post-surgical infection of C57BL/6 mice, **(i.c)**severe inflammatory cell infiltration, glandular atrophy, intestinal metaplasia of 7 days post-surgical infection of C57BL/6 mice, **(i.d)** mild to moderate inflammatory cell infiltration, disruption in epithelial lining, glandular atrophy, chronic inflammation in 14 days post-surgical infection of C57BL/6 mice. Images were captured at 20× magnification. Scale bar represents 100 μm. Inflammatory cell infiltration indicated by (black arrowhead), gastric epithelial damage (green star); **(ii)** Histopathological scoring is done according to Huston updated Sydney classification system. Colored histogram represents the mean scores of histological scoring of experimental animals (n=6) and PBS controls (n=6). All experiments were performed in triplicate.

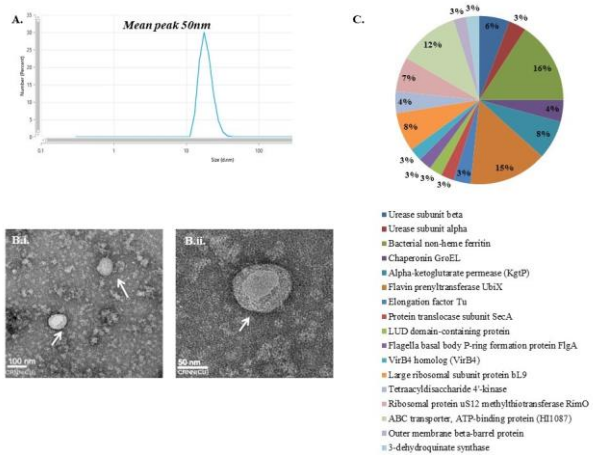

Fig. 5. Characterization of *Helicobacter pylori* OMVs isolated from strain A61C (1); (A) Dynamic Light Scattering showing a uniformity in OMVs population with mean peak at 50nm**,** (B) Transmission Electron microscopy images revealing the circular morphology of *Helicobacter pylori* OMVs of A61C (1) strain; (B.i) Image taken at 100nm scale, (B.ii) Image taken at 50nm scale. Both TEM images revealed the thick bilayer structure with hollow center of the OMVs. (C) Percentage of major proteins present on OMVs.

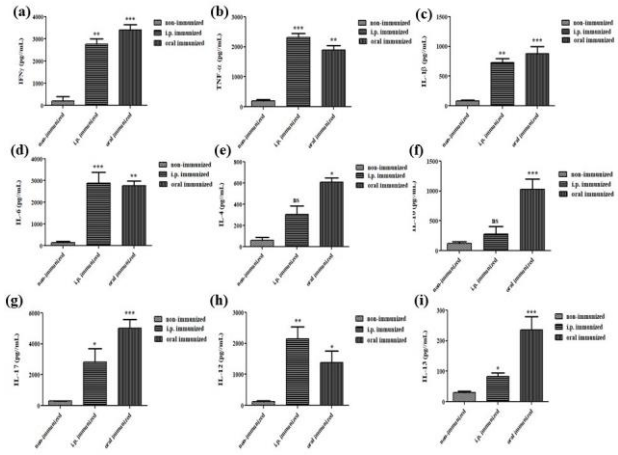

Fig. 6. Helicobacter pylori OMVs induces the production of cell mediated cytokines responses. (a) IFN-γ, **(b)** TNF-α, **(c)** IL-1β, **(d)** IL-6, **(e)** IL-4, **(f)** IL-10, **(g**) IL-17, (**h**) IL-12, (**i)** IL-13, cytokines in culture supernatant of ex-vivo cultured splenic cells of immunized and non-immunized (PBS immunized) mice after 24 h of re-stimulation with OMVs. The differences in immunized (i.p. and oral immunization) mice serum response of each of the studied cytokines were highly significant than nonimmunized. All cytokines are measured by ELISA (n = 6). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non-significant). Each bar represents median and error values of six ± SE of three independent experiments.

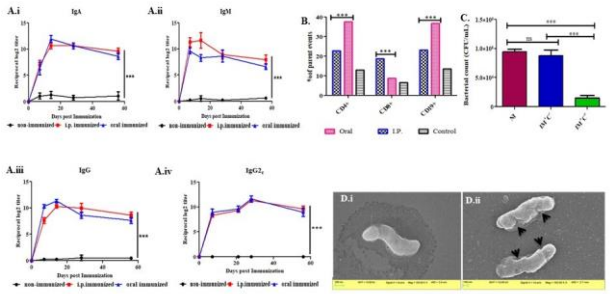

Fig. 7. Reciprocal log2 titer of serum IgA, serum IgM, serum IgG, and serum IgG2c immunoglobulins from *Helicobacter pylori* OMVs immunized and non-immunized (PBS immunized) group against OMPs. Immunization induces the population of CD4+, CD8+, and CD19+ splenic cells of immunized over non-immunized (PBS immunized) mice and the microscopic image of serum bactericidal activity of immunized and non-immunized. Mouse serum IgA (**A.i**), serum IgM (**A.ii**), serum IgG (**A.iii**), serum IgG2c (**A.iv**) was measured separately after three doses of intraperitoneal or oral immunization against Outer membrane protein (OMP) of *Helicobacter pylori*. **(B)** Bar diagram represents the percentage of CD 19+, CD 4+, and CD 8+ spleen cells from immunized and non-immunized mice using FACS analyses. Significant statistical difference was found between OMVs immunized and non-immunized spleen cell population (*** p value< 0.001). **(C)** OMVs immunized mouse serum is effective in complement mediated lysis of *H. pylori* (SS1). *H. pylori* (SS1) was separately incubated with OMVs immunized serum or non-immunized serum with or without guinea pig complement for 1 h at 37 ◦C. Viable bacterial count was determined by spread-plate method. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. (*** p value < 0.001, ns-Non-significant.). NI, non-immunized serum; IM+C-, OMVs immunized serum without complement; IM+C+, OMVs immunized serum with complement. (**D**)Scanning electron microscopic images after Serum Bactericidal Assay using non-immunized serum with complement (i) and immunized serum with complement (ii) (black arrowheads indicate immunized antibody-mediated lysis in presence of complement).

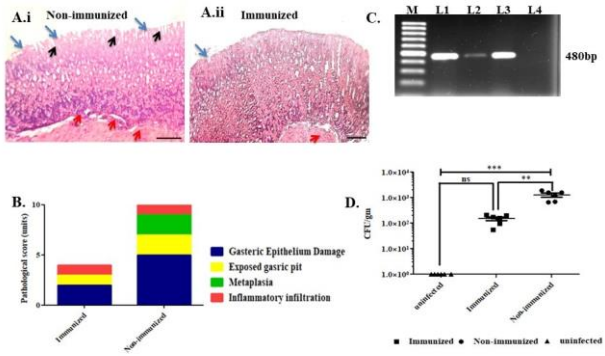

Fig. 8. OMVs immunized mice shows reduced gastric tissue damage, inflammation after infection with SS1 (2 x 10^8 CFU) and reduce bacterial colonization in gastric tissue. Histological images represent both **(A.i)** non-immunized (PBS immunized) antrum of stomach and **(A.ii)** immunized antrum of stomachs. OMVs immunized mice showed mild epithelial layer damage, less altered gastric mucosa and inflammatory infiltration, whereas non-immunized mice displayed marked epithelial damage, inflammatory infiltration, exposed gastric pit and early signs of gastric metaplasia. (Blue arrow: gastric epithelium; Black arrow: exposed gastric pit; Red arrow: Inflammatory infiltration) **(B)** Pathological scores of immunized or non-immunized mice post *Helicobacter pylori* SS1 challenge. **(C)** ***u****reB* PCR shows significant changes in bacterial DNA yield harvested from gastric tissues of both non-immunized and immunized mice post intragastric surgical challenge; **M:** 100bp ladder, **L1:** Positive control, **L2**: Immunized mice gastric tissue, **L3**: Non-immunized mice gastric tissue, **L4**: Negative control **(D)** *Helicobacter pylori* colonization in gastric tissue of immunized and non-immunized mice 7 days post challenge.

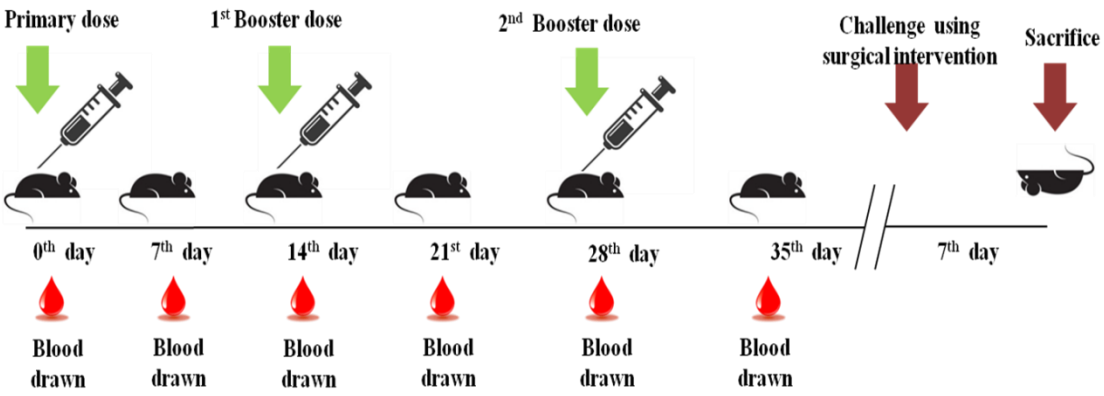

Fig. S1. Immunization and blood collection schedule Oral or Intraperitoneal (i.p.) route of immunization

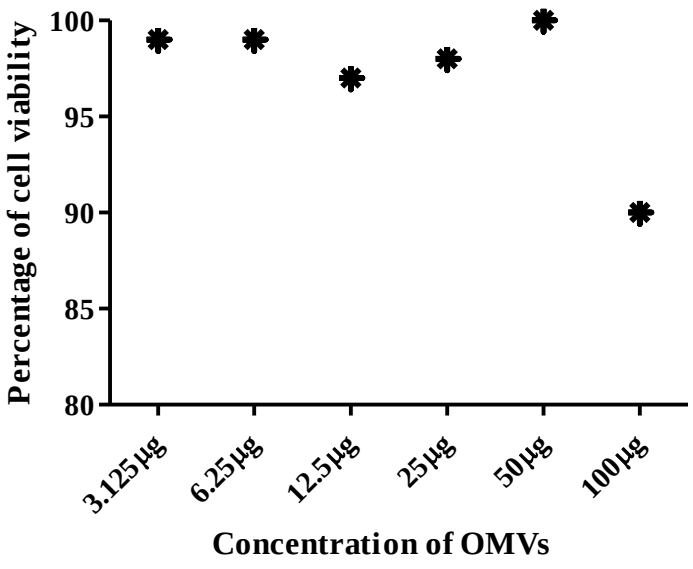

Fig. S2. *in-vitro* cytotoxicity test with different concentrations of OMVs

Table S1. The Antibiotic profile of *Helicobacter pylori*used for immunogen strain selection.

Strains			Antibiotics					
			Clarithromycin		Amoxicillin		Metronidazole	
			S	R	S	R	S	R
1	Ref. strains	26695	+	-	+	-	-	+
		J99	+	-	+	-	-	+
		SS1	+	-	+	-	-	+
2	BHU 8A		-	+	+	-	-	+
3	KO 8A		+	-	+	-	-	+
4	AS 2		+	-	+	-	-	+
5	OT-10 (A)		+	-	+	-	-	+
6	B34		+	-	+	-	-	+
7	D383		+	-	+	-	-	+
8	B6		+	-	+	-	-	+
9	M28		+	-	+	-	-	+
10	L7		+	-	+	-	-	+
11	A61C(1)		+	-	+	-	-	+
12	AM1		+	-	+	-	-	+
S - sensitive					R - resistant			

Table S2. Primers of specific genes of *Helicobacter pylori*used in this study.

Gene Assigned	Primer	Sequence	Amplicon (bp)	Reference
cagA	cag5c-F cag3c-R	5′-GTTGATAACGCTGTCGCTTCA-3′ 5′-GGGTTGTATGATATTTTCCATAA-3′	350	Chattopadhyay et al, 2004 [20]
vacA s1/s2	VA1-F VAI-R	5′-ATGGAAATACAACAAACACAC-3′ 5′-CTGCTTGAATGCGCCAAAC-3′	259/286	
vacA m1/m2	VAG-F VAG-R	5′-CAATCTGTCCAATCAAGCGAG-3′ 5′-GCGTCAAAATAATTCCAAGG-3′	567/642	
babA2	babA2R babA2F	5′-AATCCAAAAAGGAGAAAAAGTATGAAA-3′ 5′-GTTTTCTTTGAGCGCGGGTAAGC-3′	607	Ghosh et al, 2016 [21]
ureB	ureBF ureBR	5′-CGTCCGGCAATAGCTGCCATAGT-3′ 5′-GTAGGTCCTGCTACTGAAGCCTTA-3′	480	Ghosh et al, 2016 [21]
dupA	jhp0917F jhp0917R	5′-TGGTTTCTACTGACAGAGCGC-3′ 5′-AACACGCTGACAGGACAATCTCCC-3′	307	Lu et al, 2005 [66]
	jhp0918F jhp0918R	5′-CCTATATCGCTAACGCGCGCTC-3′ 5′-AAGCTGAAGCGTTTGTAACG-3′	276	
16SrRNA	16SF 16SR	5′-CTGGAGAGACTAAGCCCTCC-3′ 5′-ATTACTGACGCTGATTGCGC-3′	110	Kashyap et al., 2020 [67]

Table S3. Result of major virulence genes of *Helicobacter pylori*screened for immunogen strain selection

Genetic Features			Virulence Marker					Adhesion	Duodenal Ulcer Promoting Gene	
Strains			*cagA*	*vacA*				*babA2*	*dupA*	
				s1	*s2*	*m1*	*m2*		*jhp0917*	*jhp0918*
1	Ref. strains	26695	+	+	-	+	-	-	-	-
		J99	+	+	-	+	-	+	+	+
		SS1	+	-	+	-	+	-	-	-
2	BHU 8A		+	+	-	-	+	+	-	-
3	KO 8A		+	+	-	+	-	+	-	-
4	AS 2		+	+	-	+	-	-	-	-
5	OT-10 (A)		+	+	-	+	-	-	-	-
6	B34		+	+	-	+	-	+	-	-
7	D383		-	-	+	-	+	-	-	-
8	B6		+	+	-	-	+	+	-	-
9	M28		+	+	-	-	+	-	-	-
10	L7		+	+	-	+	-	+	-	-
11	A61C(1)		+	+	-	+	-	+	+	+
12	AM1		-	-	+	-	+	-	-	-

Table S4. Proteomic analyses of OMVs isolated from*Helicobacter pylori*strain A61C(1).

Accession	Protein assigned	Molecular mass (kDa)	No. of peptides	Sequence coverage (%)
P69996	nickel cation binding, urease activity	61.6	3	7
P14916	nickel cation binding, urease activity	26.5	1	4
P52093	ferric iron binding, ferrous iron binding, ferroxidase activity	19.3	2	19
P42383	ATP binding, ATP-dependent protein folding chaperone, isomerase activity, unfolded protein binding	58.2	3	5
O25723	transmembrane transporter activity	47.5	1	9
O26011	carboxy-lyase activity, flavin prenyltransferase activity	20.6	1	18
P56003	GTP binding, GTPase activity, guanosine tetraphosphate binding, translation elongation factor activity	43.6	2	4
O25475	ABC-type protein transporter activity, ATP binding, metal ion binding, protein-exporting ATPase	99	1	3

	activity			
O24949	Hypothetical protein	23.6	1	3
O26012	Hypothetical protein	24.3	1	3
O25206	ATP binding, DNA binding	98.4	1	3
P56035	rRNA binding, structural constituent of ribosome	16.5	1	9
O25095	ATP binding, tetraacyldisaccharide 4'-kinase activity	35.5	1	5
O25434	4 iron, 4 sulfur cluster binding, aspartic acid methylthiotransferase activity, metal ion binding,proteinmethylthiotransferase activity	49.6	1	8
O26001	ATP binding,ATP hydrolysis activity	29.2	1	14
O25992	Hypothetical protein	82.3	1	3
P56081	3-dehydroquinate synthase activity, metal ion binding, nucleotide binding	39.1	1	3

Table S5. Sydney classification of histopathological scoring used in the study

	Neutrophil infiltration	**Glandular atrophy**	**Intestinal metaplasia**	**Chronic inflammation**
Grade I	<1/3 of surface infiltrated	Mild	<1/3 of surface infiltrated	5-10 cells x 40
Grade II	1/3- 2/3 of surface infiltrated	Moderate	1/3- 2/3 of surface infiltrated	11-20 cells x 40
Grade III	>2/3 of surface infiltrated	Severe	>2/3 of surface involved	>21cells x 40

Scores assigned: mild=1, moderate=2, severe=3

Table S6. List of Antibodies used in the study

Sl no.	Antibody	Catalog no	Company
1.	Anti mouse-IgA	AB97235	Abcam
2.	Anti mouse-IgG2c	AB97255	Abcam
3.	Anti mouse-IgG	AB97023	Abcam
4.	Anti mouse-IgM	A8786	Sigma aldrich

Table S7.

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Vaccine xxx (xxxx) xxx

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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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ARTICLE INFO

Keywords:
Enteric Disease
Salmonella Typhi
Salmonella Paratyphi A
Bivalent ghost bacteria
Typhoid vaccine
Paratyphoid vaccine

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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https://doi.org/10.1016/j.vaccine.2023.08.049
Received 30 May 2023; Received in revised form 6 August 2023; Accepted 18 August 2023

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

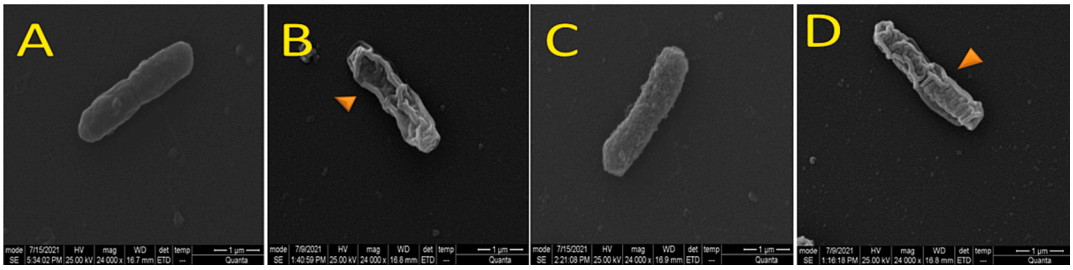

Fig. 1. Scanning electron microscopic analysis (SEM); (**A**) Untreated control S. Paratyphi A (**B**) NaOH-treated S. Paratyphi A (**C**) Untreated control S. Typhi (**D**) NaOH-treated S. Typhi. Arrows shows *trans*-membrane lysis tunnels.

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.

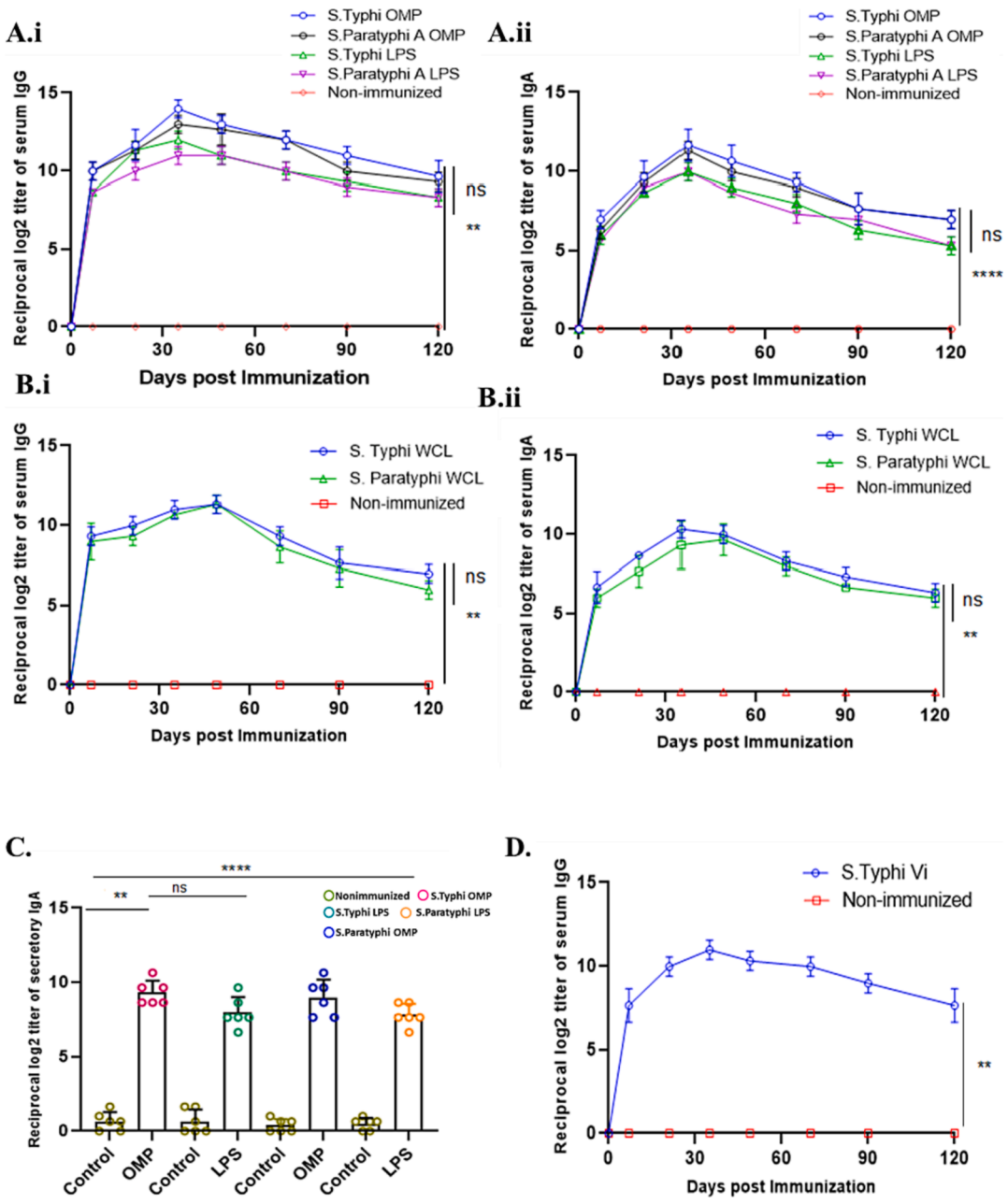

Fig. 2. Reciprocal log2 titer of serum IgG, serum IgA and secretory IgA immunoglobulin from Bivalent Typhoidal Bacterial Ghost cells immunized and non-immunized group. Mouse serum IgG (**A.i**), serum IgA (**A.ii**) was measured separately after three doses of intraperitoneal immunization against Outer 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

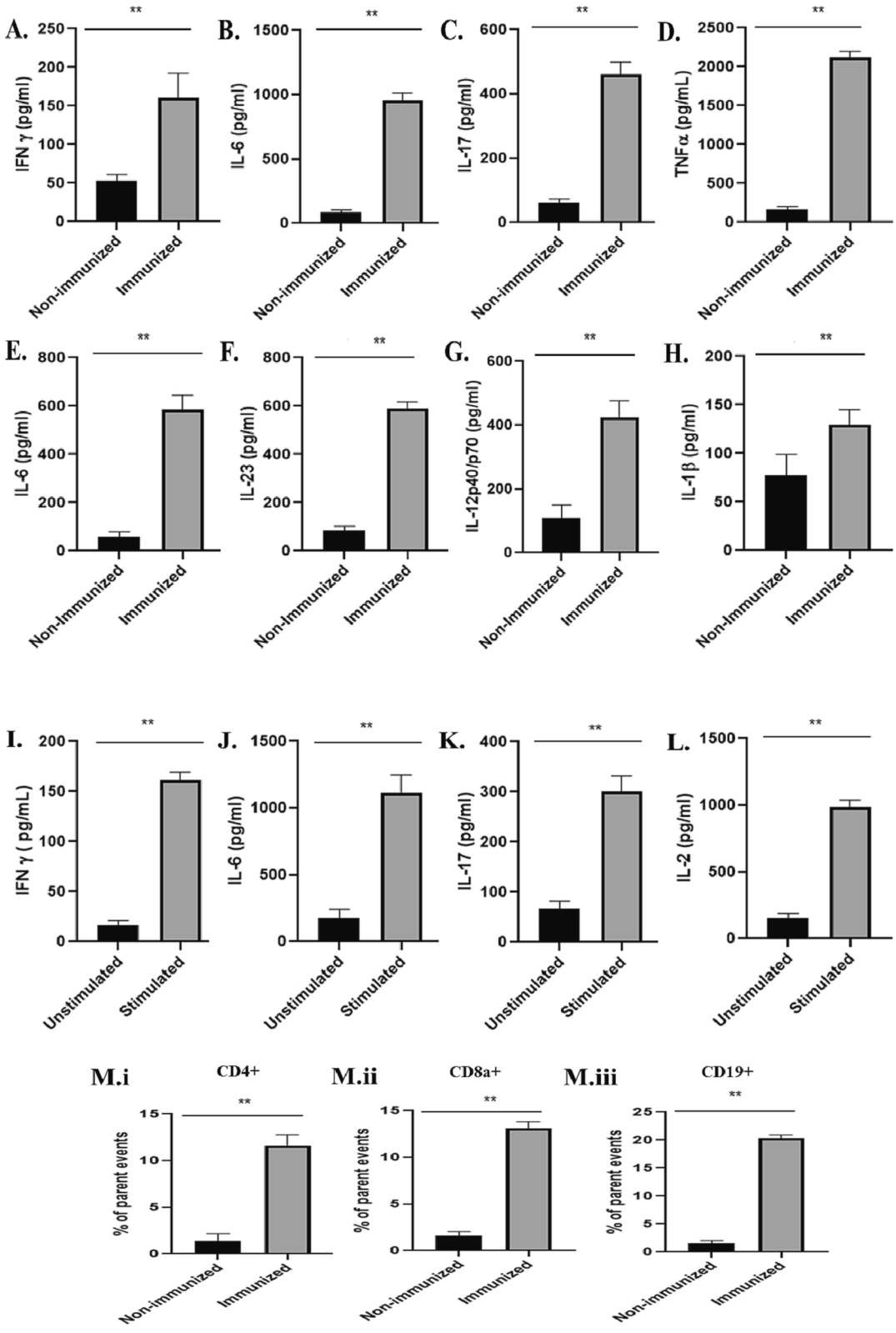

Fig. 3. Bivalent Typhoidal Bacterial Ghost cells (BTBGs) induces the production of cell mediated cytokines responses and increases the population of CD4+, CD8a+ and CD19+ splenic cells. **A.B.C.D.** IFN-γ, TNF-α, IL-6 and IL-17 cytokines in culture supernatant of ex-vivo cultured splenic cells of immunized 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.

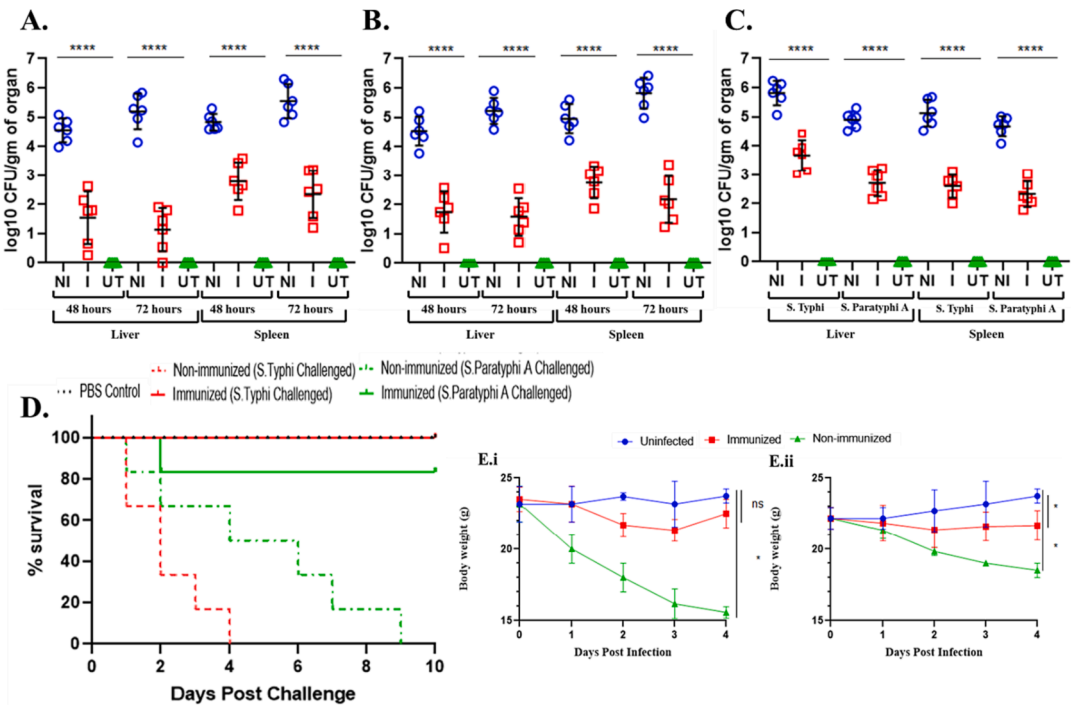

Fig. 4. Colonization and protective efficacy analysis of Ghost cells against *Salmonella* Typhi and *Salmonella* Paratyphi A infection. **A.** Colonization of *S.* Typhi **B.** Colonization of *S.* Paratyphi A, respectively after 35th day post immunization, **C.** Colonization (post 72 h post infection) 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.

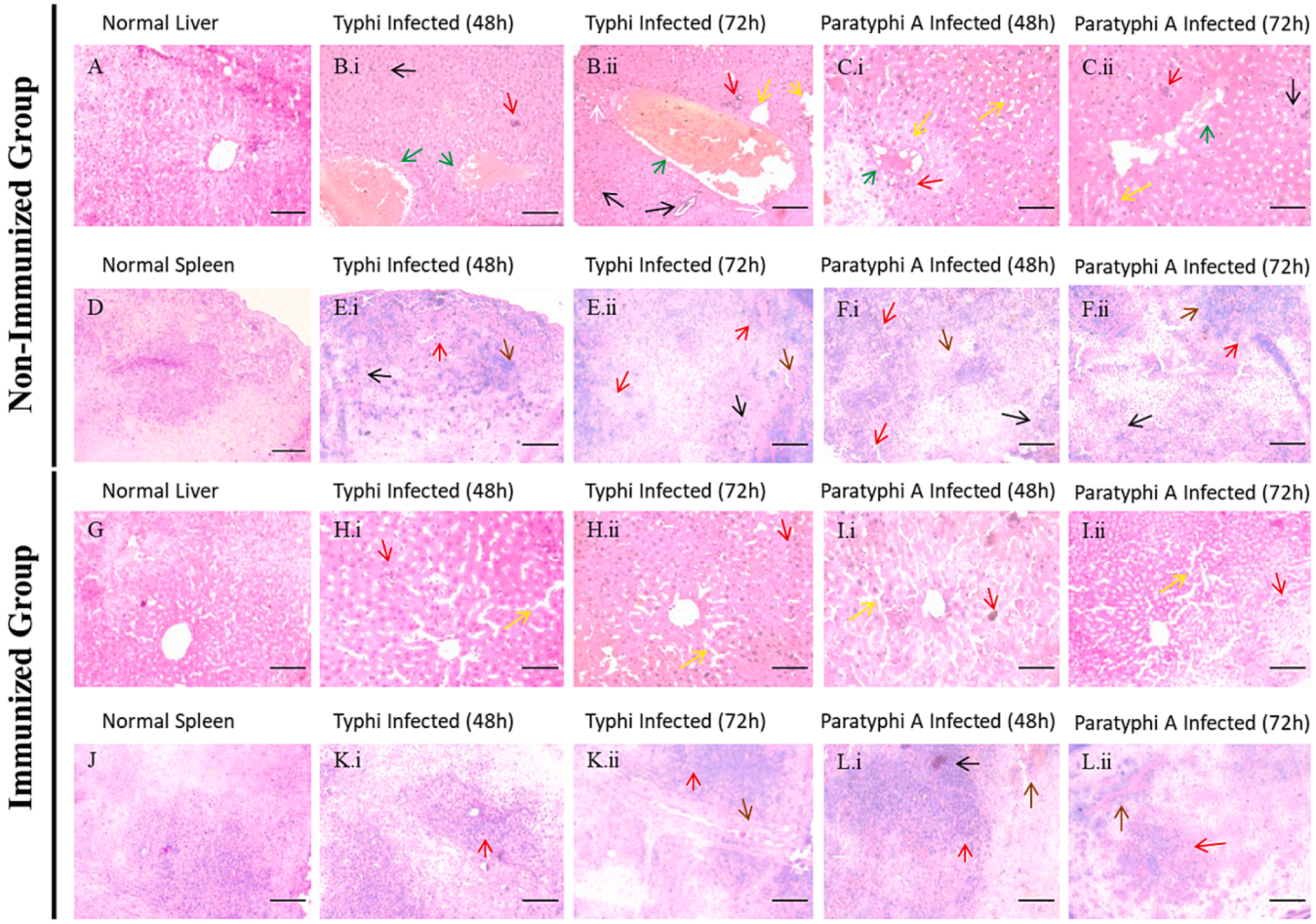

Fig. 5. Histopathology (H&E staining) of the liver and spleen of non-immunized and immunized mice after 48 h and 72 h of intra-peritoneal challenge with *Salmonella* Typhi and *Salmonella* Paratyphi A. **A.** & **D.** Liver and spleen of normal non-immunized mice. Liver and spleen of nonimmunized mice 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

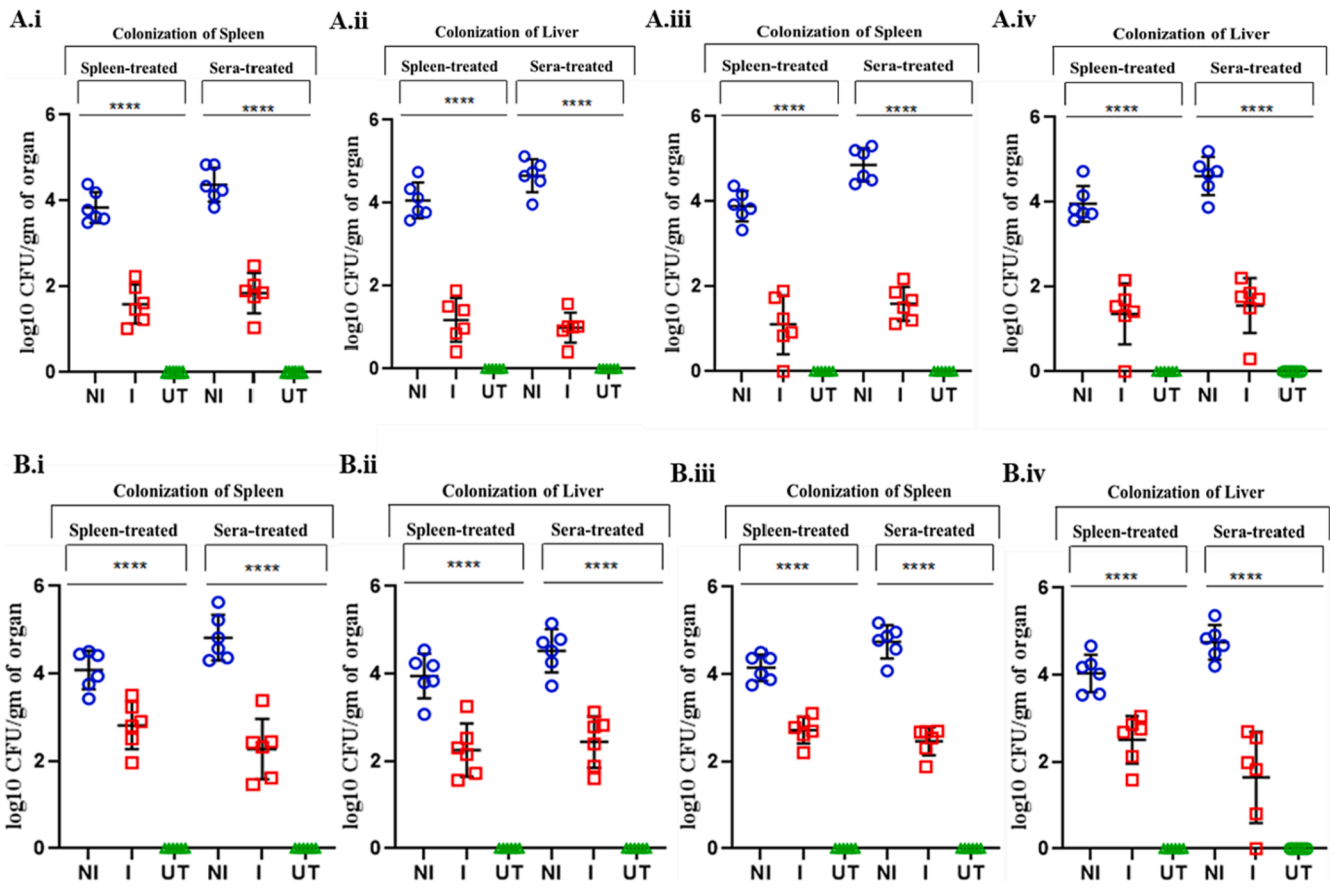

Fig. 6. Adoptive transfer (of immunized serum and splenocytes) reduces bacterial number in naïve mice after infection. **A.i, A.ii,** Bacterial number (72 h post infection) of *S.* Typhi in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 0^{th} day. **A.iii, A.iv**. Bacterial 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

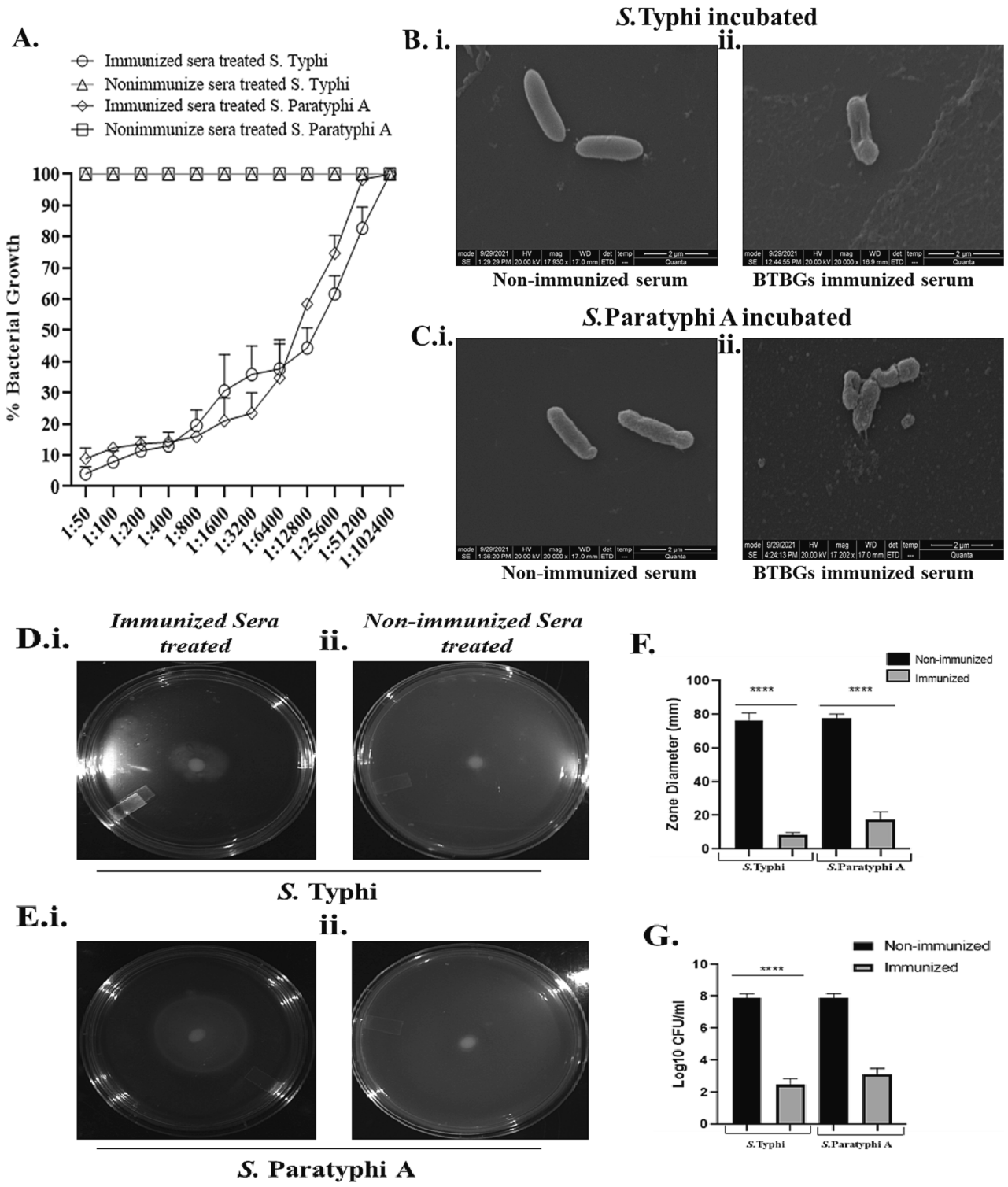

Fig. 7. Serum bactericidal activity, bacterial motility and mucin penetration assay in presence of immunized and non-immunized serum. **A.** Immunized serum is capable of complement mediated lysis of *Salmonella* Typhi (K554) and *Salmonella* Paratyphi A (K580). X-axis represents the serum dilution and 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-$\gamma^{-/-}$ 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.

Acknowledgements

The authors sincerely thank Dr. Jyl S. Matson, Associate Professor, Department of Medical Microbiology and Immunology, The University of Toledo, Health Science Campus, 3000 Arlington Avenue, MS1021, Toledo, OH 43614-2595 for her help in correcting the manuscript. Prolay Halder acknowledge Indian Council of Medical Research to get the fellowship [ICMR fellowship ID No. 3/1/3/JRF-2018/HRD-066 (66125)] for this study. Soumalya Banerjee (Student ID:191620007740), Sanjib Das (SANJIB DAS/3363/(CSIR-UGC NET JUNE 2018)) acknowledge University Grant Commission for the fellowship, and all other authors acknowledge Indian Council of Medical Research. We would also like to thank Mrs. Arpita Sarbajon for her valuable technical assistance with SEM analysis, Mr. Ananda Pal for his valuable technical assistance with FACS analysis, and Mr. Subrata Singha for his assistance with animal keeping and Mr. Suhasit Ranjon Ghosh for his help in other technical issue.

Funding

This work was supported by Indian Council of Medical Research as institutional intramural project (Project Index No. IM/HK/18-19/03).

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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Microbes and Infection 25 (2023) 105100

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

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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ARTICLE INFO

Article history:
Received 26 November 2021
Accepted 6 January 2023
Available online 22 January 2023

Keywords:
OMVs
Tetravalent immunogen
Systemic protection
Mucosal protection
Active protection
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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[2] http://www.niced.org.in/

https://doi.org/10.1016/j.micinf.2023.105100
1286-4579/

investigated as potent acellular vaccine candidates [7]. OMVs resemble the composition of bacterial outer membrane, as well as some of their periplasmic contents including membrane proteins, lipopolysaccharides etc. [8]. Presence of these innate Pathogen-Associated Molecular Patterns (PAMPs) can induce both host innate and adaptive immune response, resulting in effective protection against their respective diseases [9]. An OMVs based vaccine MeNZB is already licensed for public use in several countries against *meningitidis* serogroup B. MeNZB has proven to be a safe, cost-effective vaccine with more than 70% protective efficacy against meningococcal infection [10]. Another extracellular vesicle based *Shigella* GMMA (Generalized Modules for Membrane Antigens) vaccine candidate, which successfully entered the clinical trial phase, showed high immunogenicity and protective efficacy against shigellosis [11].

Our laboratory has reported that monovalent and hexavalent *Shigella* OMVs elicits mucosal immunity, conferring passive protection in a mouse model [12,13]. However, based on current epidemiological status provided by GEMS (Global Enteric Multicenter Study), most clinical isolates are found to be *Shigella flexneri* and *Shigella sonnei* [14]. O-antigen moieties present in *S. flexneri 2a, S. flexneri 3a* and *S. flexneri* 6 altogether cover up the entire serogroup of *S. flexneri* [14]. The negligible presence of *Shigella dysenteriae* and *Shigella boydii* clinically argue that these species do not require vaccines [15]. Consequently, a recipe with three *flexneri* and one *sonnei* strain with a broad-coverage should be adequate to provide protection against the vast majority of shigellosis.

Here, we have developed an oral tetravalent *Shigella* OMV immunogen and assessed the mucosal and systemic immune responses induced by it. Protective efficacy was then investigated in a non-surgical intra-peritoneal mouse challenge model. This study aims to provide the preliminary data pointing to the possibility of the immunogen to be a reliable and potent vaccine candidate conferring protection against major circulating serotypes of *Shigella*.

1. Materials and method

1.1. Bacterial strains and culture conditions

Shigella strains both for OMVs immunogen preparation and further challenge studies were collected from ICMR-National Institute of Cholera and Enteric Diseases (NICED), Kolkata strains repository (Table 1). All strains were preserved in 8% glycerol in Brain Heart Infusion Broth (BHIB; Difco, USA) at −80 °C. Before any experimentation, each strain was grown on Tryptic Soy Agar (TSA; Difco, USA) and in Tryptic Soy Broth (TSB; Difco, USA) at 37 °C temperature with constant shaking conditions (100 rpm).

1.2. Isolation of OMVs and formulation of the immunogen

OMVs were isolated from four wild type *Shigella* strains following the method of Balsalobre et al. (2006) with slight modification [16]. Briefly, 1 L of Luria–Bertani broth (LB; Difco) was inoculated with a 1-mL stationary phase culture of *Shigella* strains and grown for 8 h (up to the late exponential phase). Bacteria were removed by centrifugation at 8000×*g* for 25 min at 4 °C, and the supernatant was filtered through 0.45- and 0.22-μm filters (Millipore, USA). Absence of bacteria in the filtered supernatant was confirmed on TSA plates. Protease inhibitors [complete EDTA free protease inhibitor cocktail (Roche), 1 tablet per 1 L of filtrate] were added to the filtrate to prevent protein degradation. OMVs were subsequently purified from the filtrate by ultracentrifugation (140,000×*g*, 4 °C, 4 h) using a Hitachi P27A-1004 rotor and the pellet was suspended in PBS (pH 7.4). Purified OMVs were then collected and re-suspended in PBS (pH 7.4) and stored at −80 °C until further use.

Protein concentration of OMVs were measured by Bradford Assay Kit (BioRad, USA) whereas reducing sugar content in O–Ag of OMV's LPS was determined using the by Dubois et al. [17]. Finally, the OMVs were stored at-80 °C until further use.

OMVs from four *Shigella* strains were mixed in an equal 1:1:1:1 ratio, each having 5 μg protein per 200 μL of PBS. This blend of tetravalent OMVs formulation was used as the oral immunogen for adult mice.

1.3. Animals

Female albino BALB/c mice of seven-weeks age were procured from the animal resources division of ICMR-NICED, Kolkata. Animals were grouped (10 mice in each group) and maintained in separate cages at 25 ± 2 °C with 75 ± 2% humidity and fed sterile food and water. All the animal experiments were conducted following the statute given by Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Ministry of environment and forest, Government of India. Institutional Animal Ethical Committee of ICMR-NICED approved the experimental protocols (project approval no. PRO/164/-July 2022).

1.4. Oral immunization

Seven-week old BALB/c mice were immunized orally on day 0, 14 and 28. Food was withdrawn for 3–4 h prior to the immunization to reduce the stomach acid production. Stomach acidity was further neutralized by 500 μl of 5% sodium bicarbonate (SRL, India) 15 min prior to the immunization. Tetravalent OMV immunogen (total 20 μg protein content) was then mixed with 5% sodium

Table 1
Bacterial strains used in this study.

Purpose	*Shigella* serotypes	Strain	Reference
OMVs immunogen preparation	*S. flexneri* 2a	B294	[16]
	S. flexneri 3a	C519	
	S. flexneri 6	C347	
	S. sonnei phase I	IDH00968	
Heterologous strains for immunogenicity studies	*S. dysenteriae* type 1	NT4907	
	S. boydii type 4	BCH612	
Protection study	*S. dysenteriae* type 1	A1	
	S. flexneri 2a	NK3809	
	S. flexneri 3a	NK3758	
	S. flexneri 6	NK4025	
	S. boydii type 2	NK4023	
	S. sonnei phase I	NK3918	

bicarbonate and administered directly into the stomach of mice with a help of feeding needle (Harvard Apparatus). Oral immunization protocol was conducted as described by Mitra et al. (Supplementary Fig. 1) [12].

1.5. Collection of mice sera and stool

Blood was collected from the lateral tail vein of both the immunized and non-immunized mice at days 0, 14, 28, 42 and 56 days from first oral immunization. Sera was separated from blood by centrifugation at 2000 rpm for 10 min at 4 °C using BD Microtainer (BD, NJ, USA). Stools of mice were collected after one week of last immunization date from both immunized and non-immunized mice. Stools were fetched by a tweezer aseptically and then homogenized in PBS containing protease inhibitor (MP Biomedicals, USA). Supernatants from the stool samples were collected, followed by centrifugation at 10,000×*g* for 10 min. Both the sera and stool samples were stored at −20 °C for further use.

1.6. Preparation of Shigella lipopolysaccharide (LPS)

LPS from six *Shigella* strains selected for immunogenicity studies (Table 1) were purified from overnight cultures. Purification procedure was performed using the Phenol-saturated 3-[N-morpholino] propen sulfonic acid (MOPS) following *Westphal* method. The details of the protocol were described previously by Mukherjee et al. [18].

1.7. Whole cell lysate (WCL) preparation of Shigella strains

WCL of respective six *Shigella* strains used for immunogenicity studies (Table 1) were prepared from overnight bacterial culture by sonication following the protocol described elsewhere [19]. WCL of bacteria was stored at −80 °C.

1.8. Isolation of outer membrane proteins (OMPs) of Shigella strains

OMPs of six *Shigella* strains were isolated from their late exponential culture using *N*-lauryl sarcosine. Both the isolation and preservation procedures used here have been previously described [19].

1.9. Immunoblot and dot blot analysis

For immunoblot assay, OMVs, OMPs and WCL samples were first resolved by 12% SDS-PAGE and then were transferred onto a nitrocellulose membrane (Bio Rad, USA) using a semi-dry transfer apparatus from ATTO Corporation AE 6687 (Japan) [19]. Dot blot analysis was done for LPS samples following the protocol described by Mukherjee et al. [18]. Blots were developed using alkaline phosphatase substrate BCIP/NBT (MP, USA, Cat# 980,621). Serum from the 35^{th} day post 1^{st} immunization were used to recognize the immunogenic components of OMVs, OMPs and WCL from both homologous and heterologous *Shigella* strains.

1.10. ELISA

Serum immunoglobulins such as IgG, IgG2a, IgG3, IgA and secretory IgA from stool samples were determined by the ELISA method developed by Keren [20]. Specifically, disposable polystyrene (Nunc, Denmark) micro-titer wells were coated with 5 μg of either OMP, LPS or OMVs of four homologous and two heterologous *Shigella* strains and incubated for 18 h at 4 °C. Rest of the protocol was followed as described by Howlader et al. [19]. All the individual ELISA experiments were repeated thrice with both non-immunized and immunized sera collected from individual mice before, during and after the immunization.

1.11. Cytokine expression assay

Spleens were dissected from both non-immunized and immunized BALB/c mice one week after the last immunization date. Splenic monolayer was then cultured in complete RPMI 1640 media (MP Biomedicals, USA) containing 10% FBS (Gibco, Life Technologies, USA) for 2 h in a 37 °C incubator in the presence of 5% CO_2. Cells were then treated with 20 μg/ml of tetravalent OMVs and incubated under the same condition for 24 h [19]. After incubation, the supernatant was collected and assayed for TNF-α, IFN-γ, IL-6 and IL-17, using cytokine ELISA Kits (Invitrogen, USA).

1.12. Bactericidal properties of tetravalent OMVs immunized sera and scanning electron microscope (SEM)

Bactericidal activity was measured following procedure was described elsewhere [16]. Immunized (35^{th} day from first immunization) and non-immunized mice sera were heat-inactivated at 56 °C for 30 min. Log phase bacteria were then incubated with heat-inactivated sera in 1:50 dilution. Reaction was allowed to happen with or without 25% Guinea pig complement. Each of the log phase bacterial cultures of six challenge strains (Table 1) were incubated with heat-inactivated immunized and non-immunized sera (1:50 dilution) at 37 °C for 1 h and were further processed for SEM imaging as described previously [16]. Briefly, Bacteria-sera complex was fixed with 3% glutaraldehyde, followed by dehydration with ascending percentage of alcohol. Samples were then treated with increasing grades of hexamethyldisilazane (HMDS) of HMDS: ethanol composition. Completely dried samples were then imaged in Quanta 200 SEM (FEI, Netherlands) following mounting of samples in specimen stubs and sputter-coating with gold.

1.13. Protection study in adult intra-peritoneal mouse model

Mice were infected with >LD50 (1×10^9 CFU/ml) bacterial doses of six challenge *Shigella* strains i.e., *S. dysenteriae type I, S. flexneri 2a, S. flexneri 3a, S. flexneri 6, S. boydii type 2, S. sonnei I* (Table 1) intraperitoneally and observed for 10 days post challenge to observe survival rate of both immunized and non-immunized control mice [21]. A group of uninfected mice was used in each case as a negative control.

Immunized and non-immunized groups of mice were challenged with sub-lethal doses (5×10^5 CFU/ml) and both the local and systemic colonization was recorded at 24, 48 and 72 h post infection. Bacterial shedding in the stool was also examined at 24, 48 and 72 h post-infection. To study bacterial load, large intestines were collected from individual groups and washed with 1% gentamycin solution to kill extracellular bacteria. Intestines were then homogenized and plated on Hektoen Enteric Agar (HEA; Difco, NJ, USA) after serial dilution.

Changes in body weight and length of colon were studied post infection in both non-immunized and immunized mice. The mice selected for this study were of similar size and weight so that the physiological parameters, especially body weight and colon length comparison, could be done with minimum statistical error. All the variables were investigated at 24, 48 and 72 h post infection, as within this time point clinical symptoms peak in a *Shigella* infection. Fecal pathology was also observed post infection depending on the consistency like normal stool or watery loose stool [21].

1.14. Histogram of mouse colon tissues

For the histological investigation, colon tissues were collected from both the immunized and non-immunized mice post challenge. Sample tissues were fixed with 10% buffered formalin solution, followed by paraffin embedding and staining with hematoxylin & eosin (H&E) and viewed using a Olympus IX51 light microscope. Pathological scores were measured based on the following parameters: goblet cell loss (0–4), hyperplasia (0–4) and loss of barrier integrity (0–4) [21].

1.15. Statistical analysis

All the data are depicted as the mean ± SD. One-way analysis of variance (ANOVA) and two-way analysis of variance (ANOVA) tests were used to analyse two groups or more than two groups of data respectively. Data were compiled from at least three independent experiments for each study. Statistical significance was considered with a p value as ***$p < 0.001$. **$p < 0.01$, *$p < 0.05$. GraphPad Prism 5 and MS Excel were used for statistical analysis of data.

2. Result

2.1. Tetravalent Shigella OMVs-induced adaptive immune responses in mice

Immunoblot with the immunized mice sera demonstrated a large number of antigenic proteins against Whole Cell Lysate (WCL), Outer Membrane Proteins (OMPs) and Outer Membrane Vesicles (OMVs) in homologous and heterologous strains. Immune-dominant bands were seen in the cases of OMVs, OMPs and WCL. Proteins ranging in size from 100 to 20 kDa were recognized. In all cases, antisera recognized 62, 42, 38 and 34 kDa immunogenic proteins which might be IpaB, IpaC, IpaD and OmpA respectively (Fig. 1A). In depth study about the immunodominant proteins was out of scope of this work. WCL and OMPs also showed protein bands in the lower molecular weight region as well. Dot blot analysis found a very small concentration of *Shigella* LPS (0.75, 1.5 and 3 μg) to be significantly recognized by the immunized sera (Supplementary Fig. 2).

We quantified the level of elevated systemic and secretory immune responses in the mice immunized with three doses of tetravalent OMVs (Fig. 1B–E). The comparative measurement was performed with respect to non-immunized mice. Our data showed a hike in serum antibodies i.e. IgG, IgG2a, IgG3 and IgA, against *Shigella* OMVs, LPS and OMPs of all four strains used here for immunogen formulation. Comparable levels of antibodies were observed against homologous and heterologous strains. Antibodies reached their peaks after the second booster and persisted until the 42nd day, reaching a plateau stage from the 56th day onward. Peak antibodies were highest against OMPs and OMVs than the LPS of all six *Shigella* strains. . Mucosal immune responses were recorded from the stool sample collected at the 35th day after primary immunization which corresponded to the high titre values of sIgA. The difference of antibody titres between the immunized and non-immunized groups was statistically significant (Fig. 1F).

2.2. Secretion of pro-inflammatory cytokines by tetravalent Shigella OMVs immunogen ex vivo

Tetravalent OMVs primed cells showed upregulation of pro-inflammatory cytokines such as TNF-α, IFN- γ, IL-6 and IL-17 (Fig. 2). Induction of these cytokines clearly suggests that our immunogen candidate has a tremendous potency to polarize and evoke cell mediated immune responses, especially through the Th1 and Th17 pathways.

2.3. Immunized sera showed bactericidal effect

From serum bactericidal assay we have observed that heat-inactivated tetravalent *Shigella* OMVs immunized sera with 25% guinea pig complement could efficiently kill all the six *Shigella* strains. Whereas non-immunized sera and heat-inactivated immunized sera without exogenous complement were unable to show bactericidal activity (Fig. 3). This data re-confirmed the generation of bactericidal systemic antibodies in immunized sera against both homologous and heterologous *Shigella* strains. Serum bactericidal activity was supported by the SEM observation where immunized mouse sera were seen to agglutinate and lyse both homologous and heterologous *Shigella* strains. Bacterial cells were observed with disintegrated membrane and prominent pore formation. Whereas, non-immunized sera were unable to do the same and, as a result, bacterial cells remained intact (Supplementary Fig. 3).

2.4. Tetravalent Shigella OMVs conferred active protection against infection and inflammatory symptoms caused by Shigella strains in an intra-peritoneal adult mouse model

To investigate the protective efficacy of the immunogen, this standardized infection dose was used in the intra-peritoneal adult mouse model. After a challenge with > LD_{50} doses of *Shigella*, for all six strains, non-immunized mice were found to be dead 3–5 days post infection. In contrast, immunized mice survived until 10 days post infection with a significantly higher rate of survival, i.e. 66.7%–83.4% (Fig. 4A).

Later on, a sub-lethal challenge dose was used to assess the protective efficacy of the immunogen. Immunized mice had shed considerably fewer bacteria than the non-immunized ones. Shedding gradually decreased over 24h post challenge (Fig. 4B). This was comparable to the bacterial loads in large intestine. Systemic spread (in liver) was also found to be lowered at this time point. Immunized mice showed 2–4 fold fewer bacterial counts than their non-immunized counterparts. The result was similar for all six *Shigella* strains tested. Bacterial load in the tissues of large intestine and liver was the highest 24 h post infection and gradually decreased until 72 h post infection (Fig. 4C and D).

While comparing physiological parameters, it was observed that immunized mice showed a slight reduction in body weight at 24 h post infection. However, from 48 h onwards, body-weight started to stabilize and then increased, reaching their initial weights. In contrast, non-immunized mice showed drastically reduced body weight. Here, we have portrayed the changes up to 72 h post-infection as maximum inflammatory signs were seen from the time points of 24 h–72 h post infection. Colon length at 72 h post infection was also assessed, as shortening of colon length is a classical sign of sepsis. Defecation was recorded, and non-immunized mice showed classical inflammatory symptoms with mild to severe diarrhoea (Supplementary Fig. 4) [34]. Immunized mice did not show any such symptoms. Non-immunized mice were found with a prominent shortening of colon length as compared to the immunized mice. The differences of data set of two groups are highly significant (***$p < 0.001$) (Supplementary Fig. 5).

Histological analysis revealed that immunized mice showed protection against the inflammatory symptoms of *Shigella* infection. Significant epithelial cell disruption, epithelial cell shedding and hyperplasia of goblet cells in the colon tissues were observed in non-immunized mice (Fig. 5).

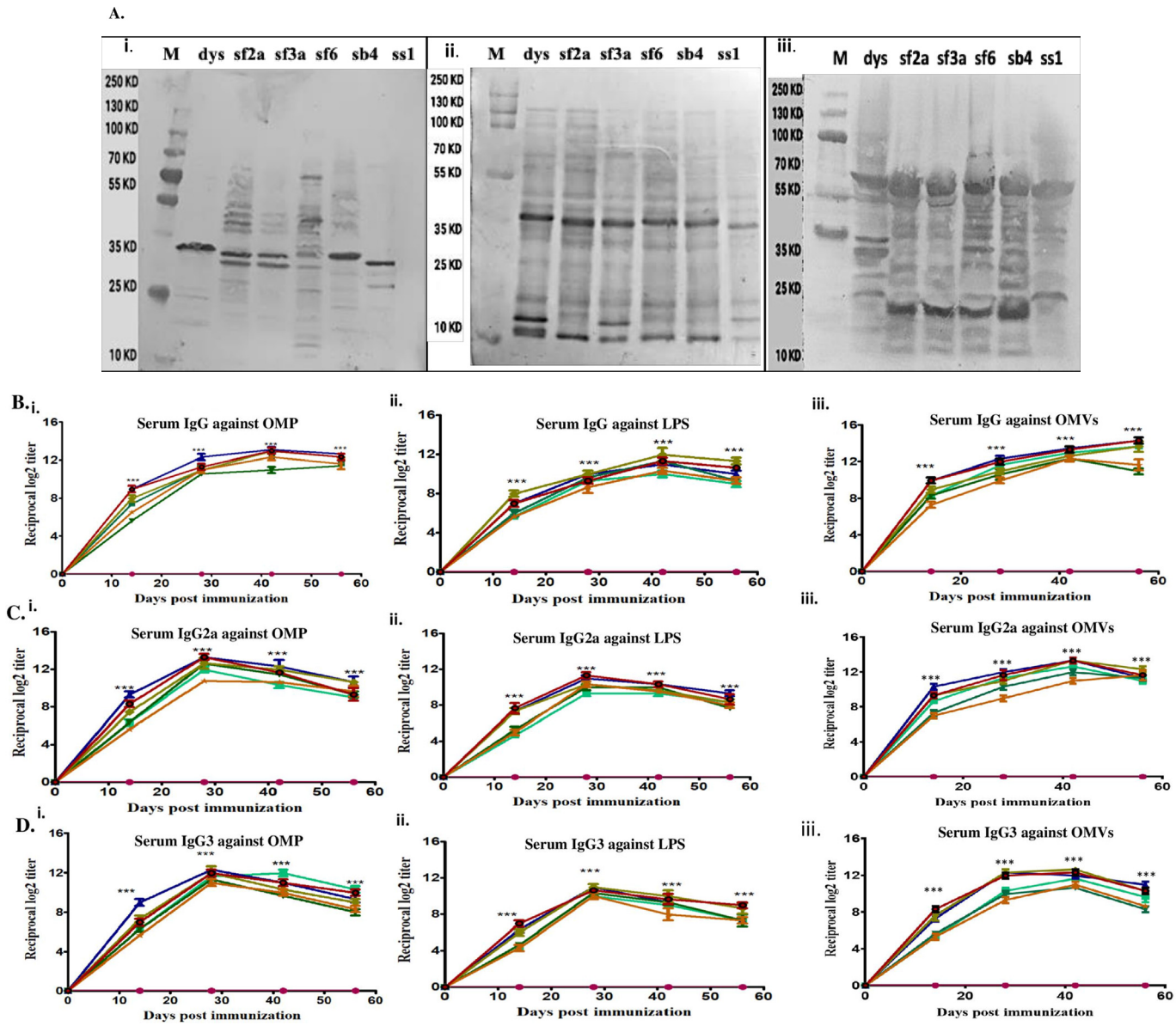
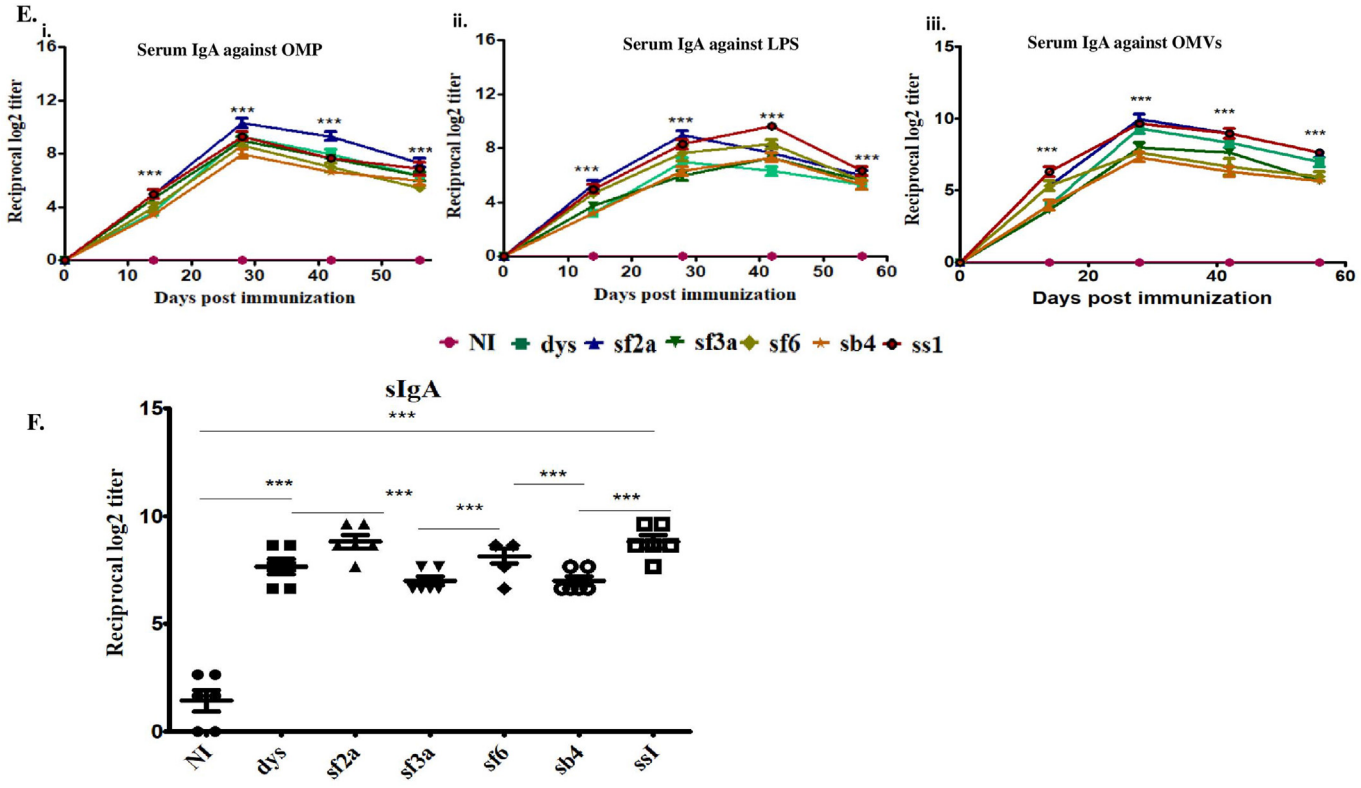


Fig. 1. A. Immunoblot analysis of tetravalent OMVs immunized mouse sera. Immunoblot against OMVs (i), OMPs (ii) and WCL (iii) of *Shigella* strains used for immunogenicity study (Table 1) with the 35th day polyclonal sera of tetravalent OMVs immunized mice. M-pre-stained molecular marker.
Antibody responses induced by tetravalent OMVs immunogen. Serum immunoglobulin IgG (**B**), IgG2a (**C**), IgG3 (**D**) titers and IgA(**E**) were separately measured against OMPs (i), LPS (ii) and OMVs (iii) of serotype specific six *Shigella* strains. Secretory IgA titer was measured against OMVs (**F**) of six *Shigella* strains in stool collected from mice at day 0 and 35th day from first immunization day. Data represented here, are the mean ± SD of three individual experiments. Differences in post immunization day wise responses in case of all antibodies were highly significant (***P value < 0.001) (dys- *S. dysenteriae type I*, sf2a- *S. flexneri 2a*, sf3a- *S. flexneri 3a*, sf6- *S. flexneri 6*, sb4- *S. boydii type 4* and ss1- *S. sonnei* designated for immunogenicity study).

3. Discussion

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

The literature suggests a number of candidate vaccines with limited success. Significant efforts are also going toward developing whole-cell bacterial vaccines, either inactivating by formalin treatment or by gathering multivalent heat-killed *Shigella* strains (HKMS) [22,23]. Live attenuated strains have been devised by mutating genes, such as *guaBA*, *virG*, and genes important for LPS and O-antigen productions [24–26]. Development of vaccines against shigellosis continues with numerous conjugate candidates having different conserved proteins of the Type Three Secretion System, such as IpaB, IpaC, IpaD, OmpA and O-antigen polysaccharides [27–29]. In the domain of new generation vaccines, Outer Membrane Vesicles (OMVs) already left their footprints under different names and forms [7]. Because of the presence of immunoreactive bacterial surface proteins and polysaccharides, OMVs have become desired molecules for vaccine formulation [9]. Generalized Modules for Membrane Antigens (GMMA) derived from different gram-negative bacteria such as *Shigella, Salmonella enterica* serovars Typhimurium, Enteritidis, and Paratyphi A, and



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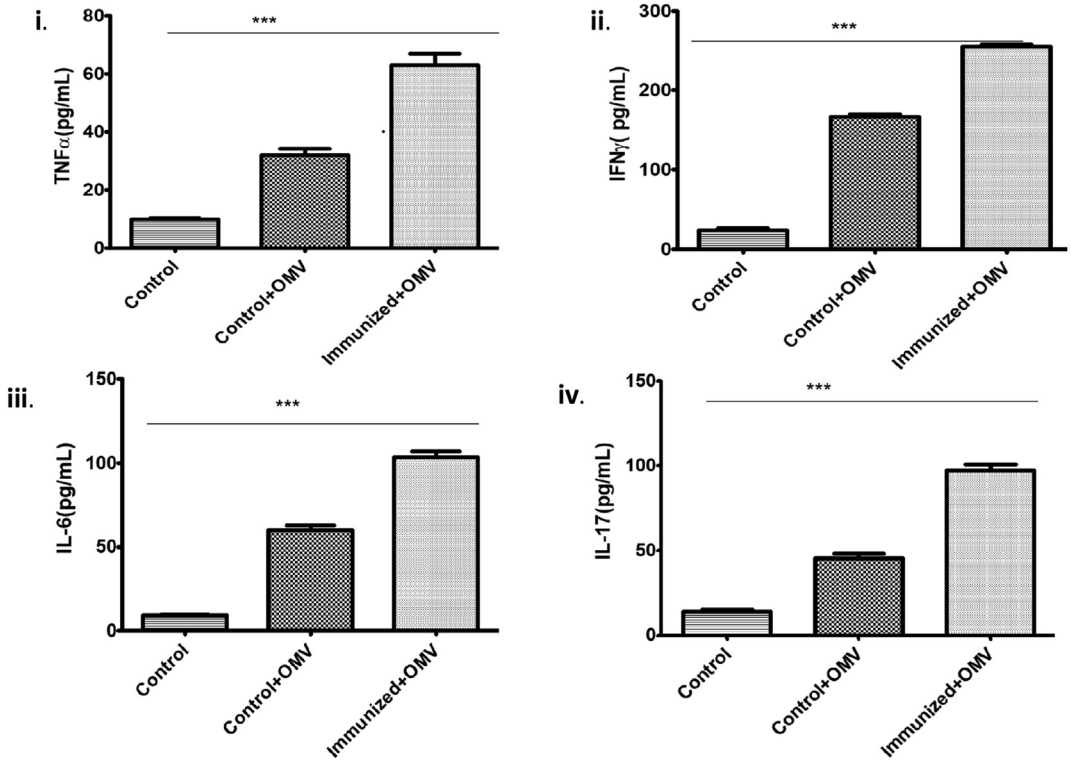


Fig. 2. Cytokine ELISA. Cytokine level of TNF-α (i), IFN- γ (ii), IL-6 (iii) and IL-17 (iv) were measured in the splenic cells isolated from both the immunized and control mice. Re-stimulation of splenic cells was done with a 24 h incubation with tetravalent *Shigella* OMVs immunogen. Differences of cytokine levels between the group of control, control primed with OMVs and immunized with OMVs was highly significant (***P < 0.001). Each bar represents the data compiled from three replica experiments with mean and error values.

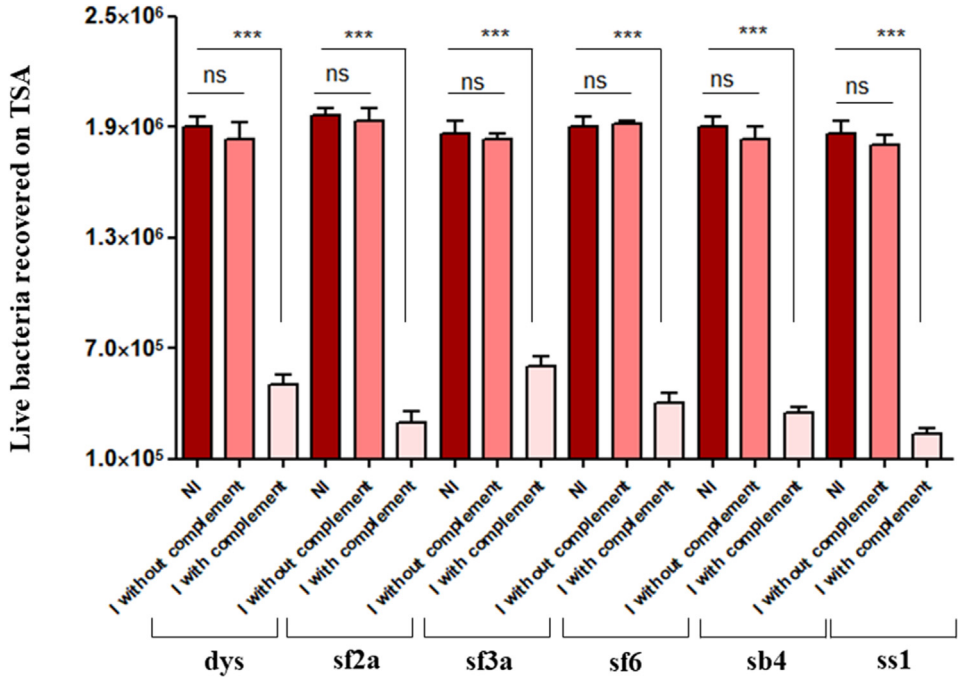


Fig. 3. Serum Bactericidal Assay. Tetravalent OMVs immunized sera are capable of complement mediated lysis of *Shigella*. All *Shigella* strains (1×10^5 CFU/ml) were incubated with indicated dilution of heat inactivated immunized serum or non-immunized serum in the presence or absence of 25% Guinea pig complement for 1 h at 37 °C. Live bacteria were counted by plating on TSA plates. Statistical analyses were performed using the two-way analysis of variance (ANOVA) test. Each bar represents mean ± SE of three independent experiments. ***$p < 0.001$. NI- non-immunized serum; I without complement-immunized serum without complement; I with complement-immunized serum with complement added.

Neisseria meningitidis, are reported as cost-effective, safe and protective candidate vaccines against respective bacterial infection [30]. GMMA derived from genetically modified *S. sonnei* entered clinical trial phase 2b and was reported to generate significant immune responses in adult human upon Intra-muscular (IM) immunization [31].

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

Here, immunoblot analysis showed that anti-OMVs sera raised in tetravalent OMVs immunized mice could recognize a number of antigenic proteins present in whole bacterial cells, bacterial membranes and OMVs. Immune-dominant protein bands representing the immunogenic factors such as Ipa B (62 kDa), Ipa C (42 kDa), Ipa D (38 kDa), Omp A (34 kDa), Vir G (120 kDa) showed more prominence [13]. These proteins present in *Shigella* are responsible for controlling T3SS machinery, invading the host cell and further inflammatory phenomena [4]. Hence, our tetravalent *Shigella* OMVs, possessing these conserved immunogenic proteins, could be a potent immunomodulator in the host. In addition, IpaB and IpaD have proved their potential for a robust induction of mucosal and systemic immune responses against *S. flexneri* and *S. sonnei* [36]. In concert, OmpA protein of *S. flexneri 2a* exerted promising passive protection in a mouse model [29]. Immunized sera not only recognized the immunogenic proteins of homologous *Shigella* strains but also of the heterologous ones, i.e. *S. dysenteriae 1* and *S. boydii 2*, indicating the evoked immunogenicity across all the serogroups of *Shigella*. Simultaneously, it was witnessed that a minimum amount of tetravalent *Shigella* OMVs immunogen (5 μg each) was remarkably successful in activating the humoral immune arm by eliciting both systemic and localized immunoglobulins against both the protein content and serotype specific LPS. Antibodies in immunized mice also recognized the OMVs, OMPs and LPS of heterologous strains, which ultimately crossed the barrier of serotype specific immunogenicity issues against *Shigella* infection. Serum IgG can effectively eliminate *Shigella* approaching mucosa, by opsonisation in the presence of complement [37]. Increased IgG3 was mainly due to the antigenic protein content of OMVs whereas IgG2a antibodies were secreted because of the carbohydrates present in OMVs [38]. Both of them play vital roles in eliminating invasive bacterial infections. Secretory IgA prevents

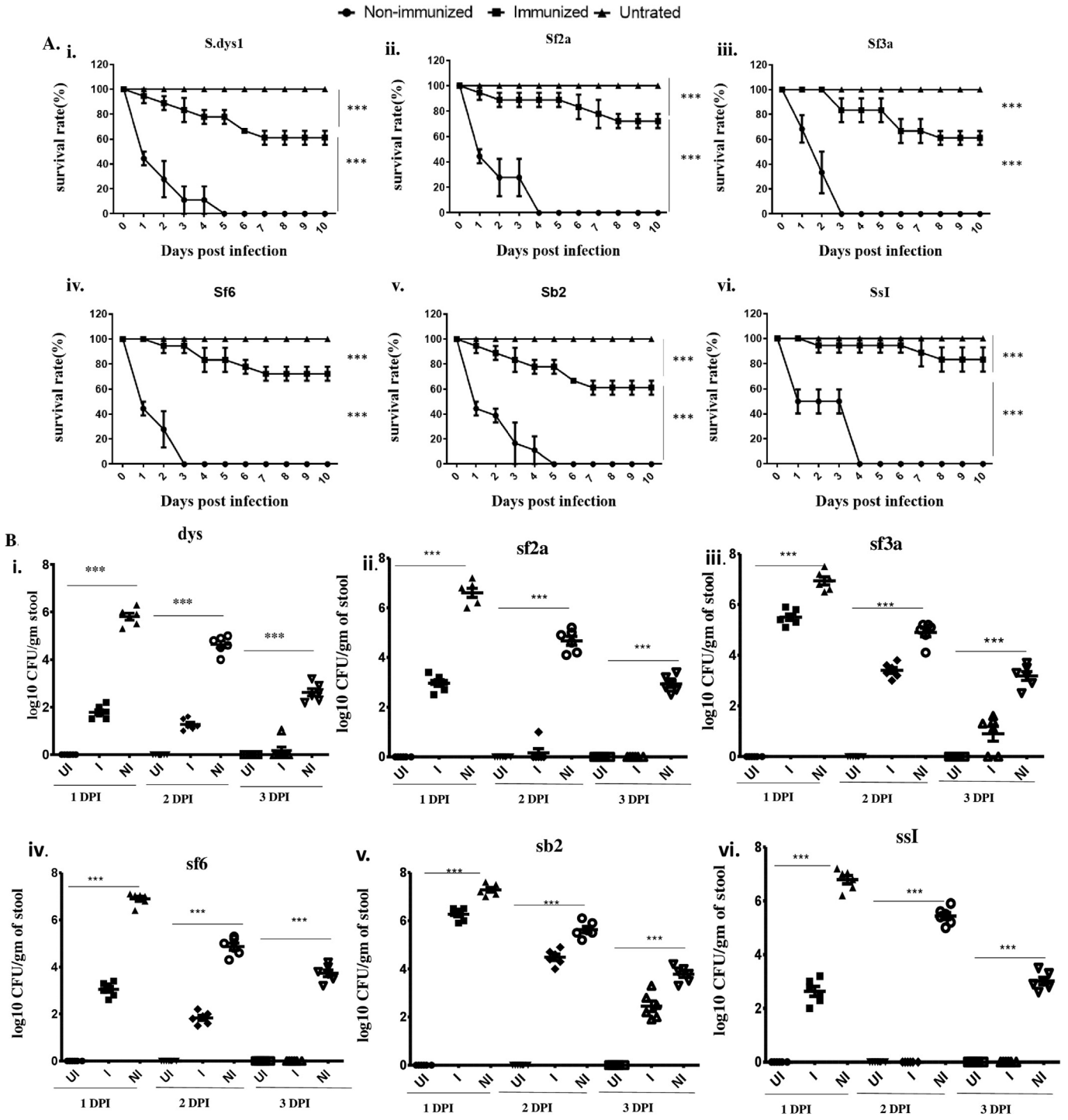
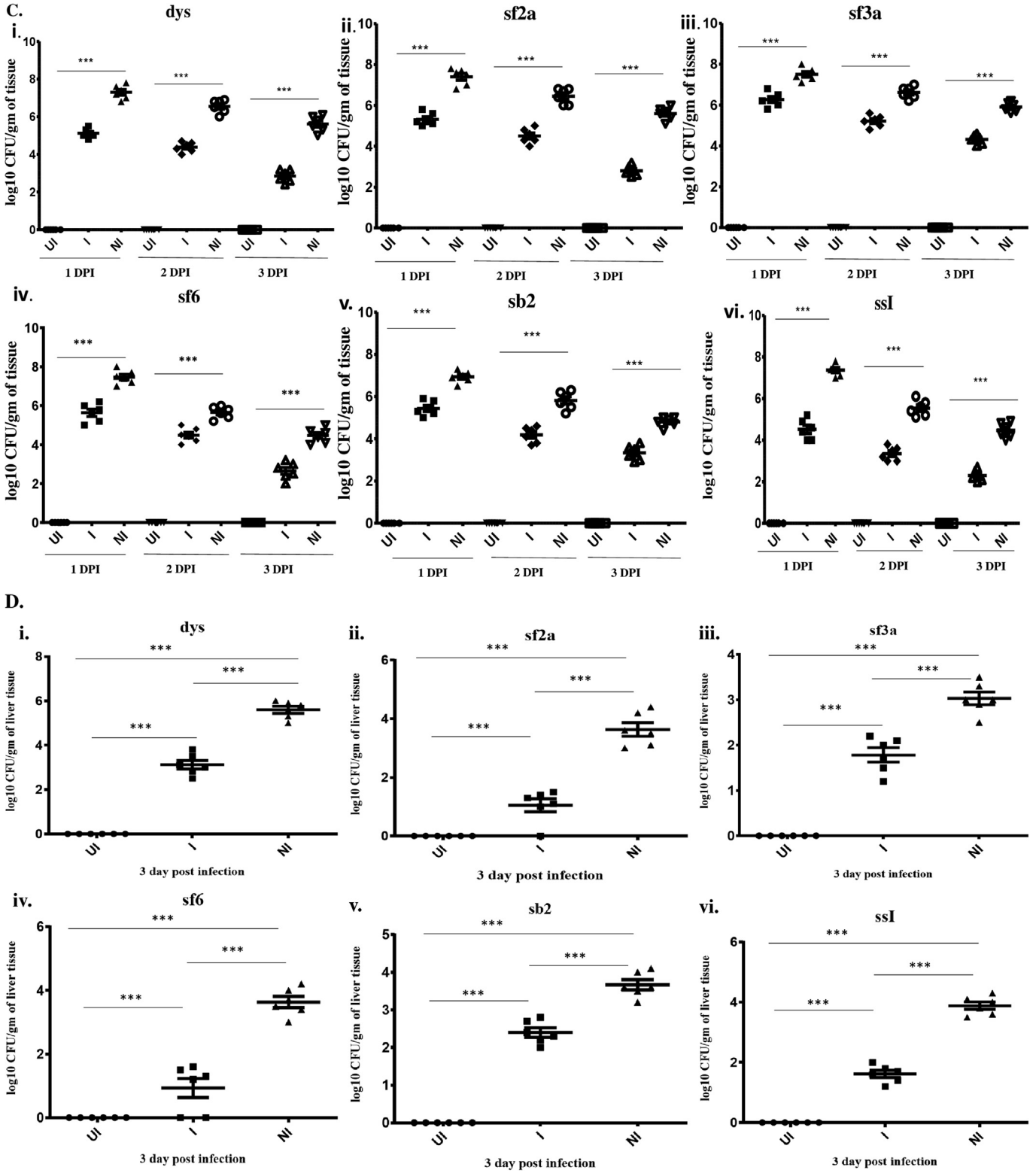


Fig. 4. Protection study in intra-peritoneal mouse model. A. Both immunized (I) and non-immunized (NI) mice were challenged with 1×10^9 CFU/ml bacterial inoculum of heterologous strains on 35th day from 1st immunization. A group of uninfected mice were also used for individual challenge strain. Survival percentages against all the heterologous strains were observed till 10 DPI (days post infection). Data compilation was done using mean ± SD of three independent experiments (n = 6) with ***p < 0.001.**B**. Bacterial shedding in the stool of I and NI mice was observed after challenging the mice with sub-lethal dose (5×10^5 CFU/ml) of challenge strains of *Shigella* till 3DPI. I mice showed remarkable lower stool bacterial count than the NI mice.**C**. Compared to group I, mice of NI group were found to have significantly higher bacterial colonization in large intestinal tissues after a challenge with sub-lethal dose. Data were collected from 1 to 3 DPI. **D**. An infection with sub-lethal dose of *Shigella* strains caused significant colonization of systemic organ i.e., liver in NI mice. Data were collected at 3 DPI as before that there was no bacterial load found in liver tissues. For bacterial shedding and colonization study (B,C,D), level of significance was ***p < 0.001, **p < 0.01. Data were expressed as Log 10 of recovered colony forming unit (CFU) per gram of stool and tissues and calculated as mean value (n = 6) of three individual experiments. Six challenge *Shigella* strains are denoted in serial number [(i) dys- *S. dysenteriae type I,* (ii) sf2a- *S. flexneri 2a,* (iii) sf3a- *S. flexneri 3a,* (iv) sf6- *S. flexneri 6,* (v) sb2- *S. boydii type 2,* (vi) ss1- *S. sonnei I*] are used for this study (Table 1), I: Immunized, NI: Non-immunized, UI: Untreated.



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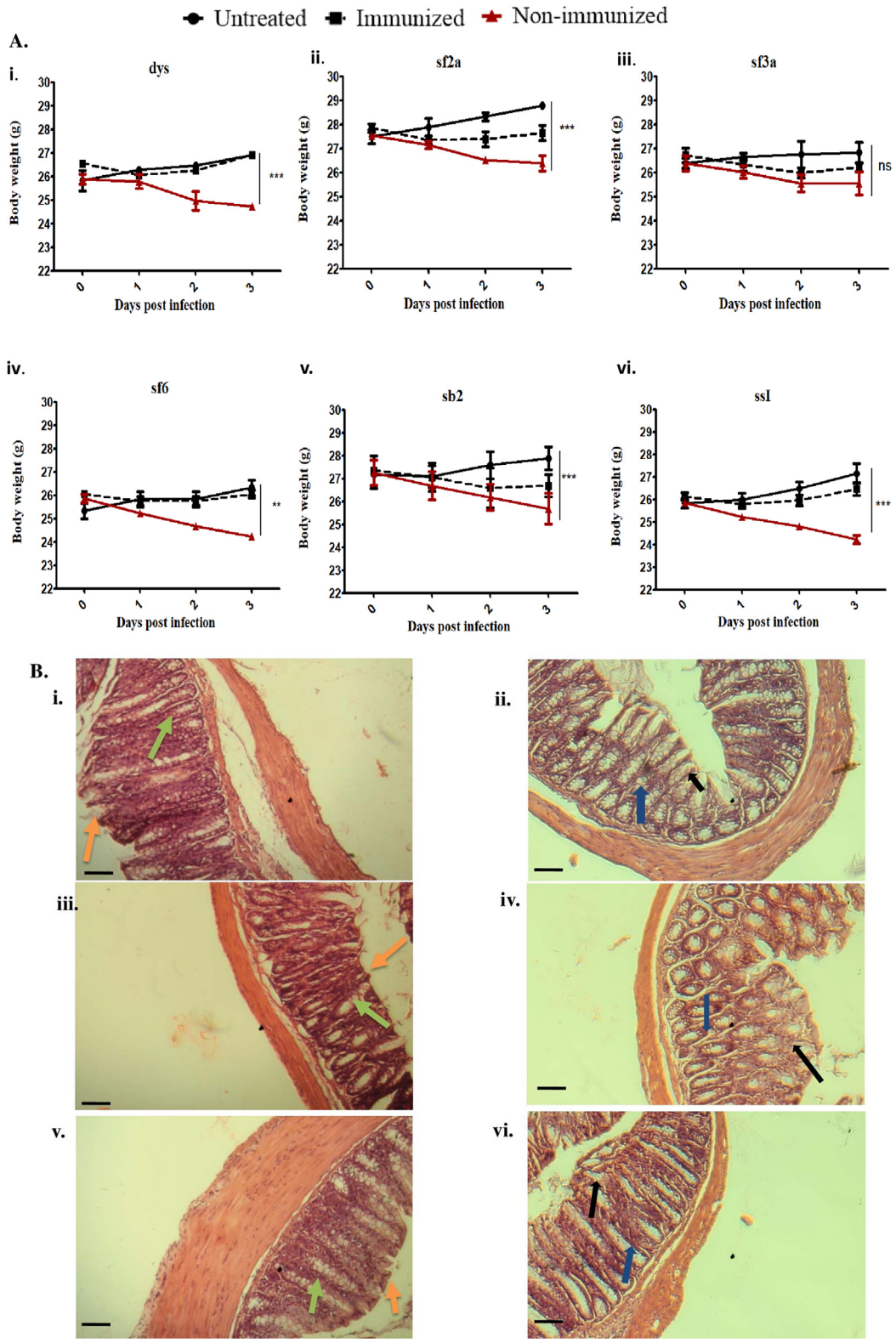
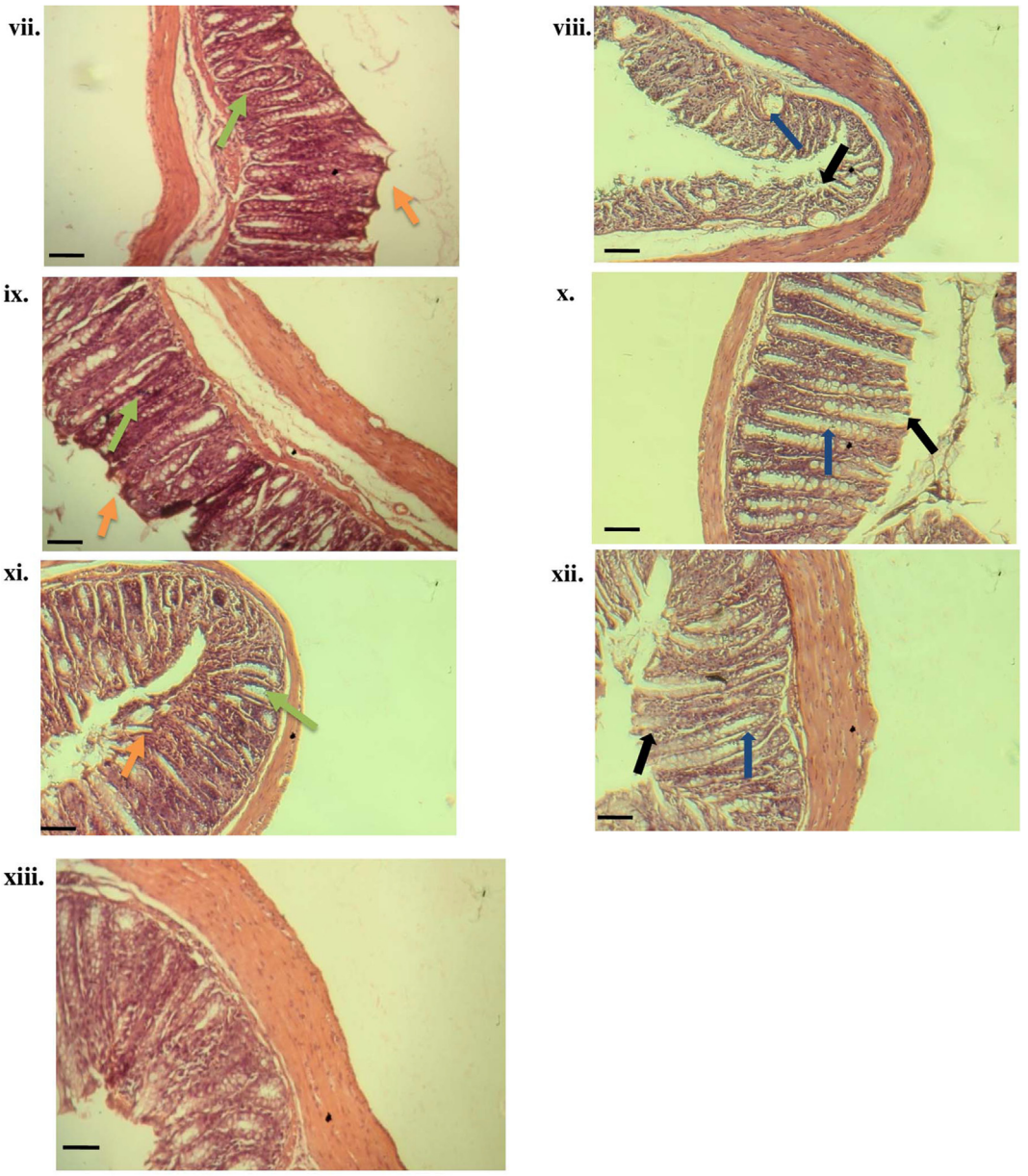
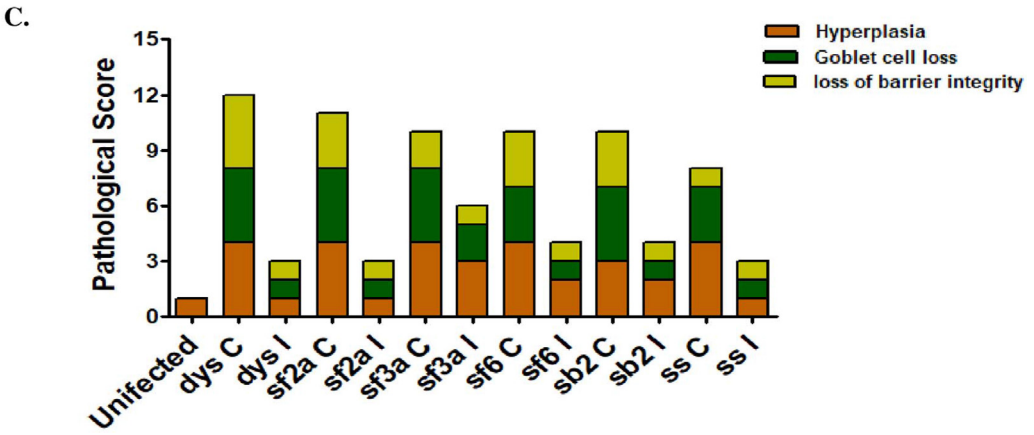


Fig. 5. A. Body weight comparison post infection with six *Shigella* strains. Non-immunized mice revealed significant reduction of body weight than immunized mice upon challenge with sublethal dose of *Shigella* strains. Each dot represented the mean and error value (n = 6) ± SD. ***p < 0.001, **p < 0.01,*p < 0.05. **B. Representative image of hematoxylin & eosin stained mice colon tissue sections.** Colon tissues of immunized mice (I) challenged with six *Shigella* strains showed no or mild hyperplasia (orange arrow) and goblet cells loss (green arrow). Whereas, tissues of non-immunized mice suffered severe hyperplasia (black arrow) and goblet cell loss (blue arrow) with the loss of membrane integrity. Scale bar-100 μm. In this study, mice challenged with *Shigella* strains are denoted as of dys- *S. dysenteriae type I* (i-immunized, ii-non-immunized), sf2a- *S. flexneri 2a* (iii-immunized, iv-non-immunized), sf3a- *S. flexneri 3a* (v-immunized, vi-non-immunized), sf6- *S. flexneri 6* (vii-immunized, viii-non-immunized), sb2- *S. boydii type 2* (ix-immunized, x-non-immunized), ss1- *S. sonnei I* (xi-immunized, xii-non-immunized) are used (Table 1). Uninfected mice tissue is image no. xiii. **C.** Pathological score based on histogram of colon tissues revealed that immunized mice (I) tissue had remarkable less inflammation than the non-immunized mice (C) upon *Shigella* challenge.





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bacteria from breaching the gut epithelial barrier either directly by neutralizing them or by the encapsulating the dividing cells of bacteria [39]. Evoked secretion of sIgA in stool samples of immunized mice confirmed the activated mucosal immunity offered by tetravalent OMVs. Anti-O antigen sera antibodies are responsible for the cross immunogenicity across heterologous serotypes of *Shigella*, as reported earlier [40]. Serum bactericidal activity proved the ability of tetravalent OMV immunogen induced complement mediated killing of *Shigella* via antibodies. The observed effect is seen due to the opsonization where complements are binding with opsonized bacteria and eventually killing them. A number of studies already reported that antibodies against LPS and other antigens of *Shigella* are involved in bactericidal activity providing clinical protection [41]. Hence, persistent antibody production following a three-dose immunization, against all the representative six serotypes of *Shigella,* strongly suggests that tetravalent *Shigella* OMVs can purvey immunogenicity covering almost all the serotypes and sub-serotypes of *Shigella*.

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

We chose an intra-peritoneal adult mouse model for the challenge study. This non-surgical model was preferred because intraperitoneal administration of bacteria induces both mucosal inflammation, producing human like diarrhoea in mice, and systemic infection, resulting in death [21]. Hence, this model is apt for vaccine efficacy studies. However, natural infection is caused through the fecal-oral route in humans with a very small amount of bacterial inoculum i.e., 10−100 microorganisms. In the protection study, when mice were challenged with virulent homologous and heterologous *Shigella* strains, immunized mice showed no or mild diarrhoea. On the other hand, non-immunized mice developed human-like diarrhoea due to severe infections in mucosal compartments. Tetravalent *Shigella* OMVs reduced intracellular invasion and bacterial dissemination leading to several fold lower bacterial colonization in tissues of large intestine and liver of immunized mice. We found non-immunized mice had prominent shortening of colon length, which is a marker of potent inflammation. On the contrary, immunized mice showed less or negligible colon shortening [21]. Our immunogen could effectively cut down the classical inflammatory signs, like reduction in goblet cells, hyperplasia and cellular destruction. Significantly higher survival rates in immunized mice than in non-immunized mice indicates protection exerted by the tetravalent OMVs immunogen against the systemic spread and sepsis caused by *Shigella* [21].

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

This study concludes that tetravalent *Shigella* OMV immunogen can, therefore, act as a cost-effective potential vaccine candidate which could be an answer to the global crisis and an ideal tool against shigellosis, addressing the barrier of cross-protection among large and diversified *Shigella* species.

Declaration of competing interest

The authors have no conflict of interest to proclaim.

Acknowledgement

We would like to thank Professor Jeffrey H. Withey, Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan, USA, for editing the manuscript. We are indebted to Indian Council of Medical Research (ICMR) for providing the fund for this work and to the Department of Science & Technology (DST) for financial contribution to the first author. Authors are whole-heartedly grateful to Late Dr. Soma Mitra, Dr. Priyadarshini Mukherjee, Mr. Suhasit Ranjan Ghosh, Mrs. Arpita Sarbajna and Mr. Subrata Sinha for their worthwhile technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2023.105100.

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Immunobiology 227 (2022) 152183

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

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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ARTICLE INFO

Keywords:
Outer membrane vesicles
Trivalent vaccine
Salmonella Typhimurium
Salmonella Enteritidis
Salmonella Gallinarum
Chicken model
Efficacy study

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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https://doi.org/10.1016/j.imbio.2022.152183
Received 10 August 2021; Received in revised form 4 January 2022; Accepted 23 January 2022
Available online 31 January 2022

et al., 1989; Ortega-Benito and Langridge, 1992). Due to this, more efforts were made to control ST infection in broiler firms but both ST and SE infection control was done in layer firms. To reduce SG infection, control measures were also taken in both type of firms. To prevent iNTS contamination and increase production in poultry items, generally antibiotics were used as the conventional strategy but long term use of antibiotics leads to increased antibiotic resistance in iNTS strains (Alvarez et al., 2020; Rabello et al., 2020; Van Boeckel et al., 2019), which poses a serious threat to human health (Michael and Schwarz, 2016; Pan et al., 2018). To minimize this threat, several other strategies were undertaken, such as increased hygiene levels in poultry farms (Davies and Wray, 1995), use of prebiotic or probiotics with normal feed to induce competitive exclusion of iNTS (Corrier et al., 1991; Fukata et al., 1999) and vaccination of flocks. Though there are several single valent or bivalent live and killed vaccines available for commercial use against chicken SG, SE and ST infection (Crouch et al., 2020a; Young J Lee et al., 2005; Okamura et al., 2007), there is no single vaccine that can deliver protection against all four prevalent serovars in India.

Outer membrane vesicles [OMVs] of Gram negative bacteria are considered as a subunit acellular vaccine candidate. OMVs are produced from the outer membrane of bacteria and possess structural similarities with the bacterial surface. Thus, OMVs are considered a potent immunomodulator against specific pathogens and contain various types of antigens including LPS and different outer membrane proteins. Previous studies have shown that OMVs of iNTS serovars induce protective immune responses in different mouse models (Liu et al., 2017, 2016; Maiti et al., 2020).

In this study, we have formulated a novel trivalent iNTS OMVs based immunogen to induce protection against the most prevalent iNTS serotypes: ST, SE, SG and SI. One day old chicks were immunized orally with trivalent iNTS-OMVs and adaptive immune response was measured. Protective efficacy of the trivalent iNTS-OMVs immunogen was measured using chicken oral and ligated intestinal loop infection models. The long term protective efficacy of the trivalent iNTS-OMVs immunogen was also determined. This study will shed light on the protective role of iNTS-OMVs immunogen against salmonellosis and fowl typhoid in chickens.

2. Material and methods:

2.1. Bacterial strains and growth conditions

Invasive non-typhoidal *Salmonella* [iNTS] outer membrane vesicles [OMVs] immunogen were prepared from three iNTS strains S.17.7 [ST], S.17.3 [SE] and PO-1 [SG]. ST IDH-3162, SE IDH-1125, SG PO-1 and SI PH-97 were used for challenge study. All strains were collected from ICMR-NICED strain repository. All strains were stored at 20% glycerol in Tryptic Soy Broth [TSB; Difco, USA] at −80 °C. All the strains were grown in Tryptic Soy Broth [TSB; Difco, USA] at 37 °C in constant shaking condition supplemented with streptomycin 100 μg/ml or in Tryptic Soy Agar [TSA; Difco, USA] plates in same condition. All the strains used in this study are mentioned on Table 1.

Table 1
Strains used in this study.

Strain Name	Serotype	Abbreviation	Reference
S.17.7	*S.* Typhimurium	ST	Poultry Isolate [This study]
S.17.3	*S.* Enteritidis	SE	Poultry Isolate [This study]
PO-1	*S.* Gallinarum	SG	Poultry Isolate [This study]
IDH-3162	*S.* Typhimurium	ST	Clinical Isolate [This study]
IDH-1125	*S.* Enteritidis	SE	Clinical Isolate [This study]
PH-97	*S.* Infantis	SI	Clinical Isolate [This study]

2.2. Preparation of OMVs

OMVs of iNTS strains were isolated using previously described protocol (Maiti et al., 2020). Purified OMVs pellets were re-suspended in PBS [pH 7.4]. Protein and LPS concentration were measured using modified Lowry protein estimation kit [pierce, USA] and Kdo method. Isolated OMVs from three strains were mixed at a ratio of 1:1:1 based on protein content and the final protein concentration of the mixture was 50 μg/200 μl PBS and stored at −80 °C.

2.3. Negative staining of OMVs

Negative staining of OMVs were done using previously described method (Maiti et al., 2020) [Fig. 1].

2.4. Animals

One day old pathogen free layer breed Road Island chicks were collected from ICMR-NICED animal house facility. Chicks were grouped [n = 10] and caged separately and maintained at 35 ± 2 °C temperature with 65 ± 2% humidity. Chicks were fed sterile food and water ad libitum. All the animal experiments were conducted following the standard operating procedure as outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA], Ministry of Environment and Forest, Government of India. The animal experimental protocol was approved by the Institutional Animal Ethical Committee [IAEC] of NICED with Registration No. 68/GO/ReBi/S/99/CPCSEA valid 17/7/2024, Approval No.: PRO/109/August2014-July2017.

2.5. Immunization of chicks

One day old chicks [n = 10] were orally immunized with trivalent iNTS-OMVs immunogen on day 0th, 14th and 28th (50 μg/chick] and negative control group received PBS. Animal experiment was schematically described for better understanding [Supplementary Figure 1]. Briefly, food were taken away 2 h before immunization. Five minutes before immunization chicks were given 1 ml of 5% sodium bicarbonate via oral gavage to neutralize stomach pH. Then 0.2 ml of iNTS-OMVs immunogen (50 μg/chick, based on total protein content) were administered via oral route mixed with 0.8 ml of 5% sodium bicarbonate solution. One hour post immunization food were supplied with water ad libitum.

2.6. Collection of serum and intestinal lavage

Blood was collected from wing vain on day 0th, 7th, 21th, 35th, 56th, 70th, 100th and 180th day from 1st immunization. Blood was collected in BD microtainer tubes [BD, NJ, USA] and centrifuged in a tabletop centrifuge [3500 rpm, 10 min, 4 °C]. One week and four weeks post final immunization 5 chicks were sacrificed from each group and small intestines were surgically separated. Intestinal lumen was flushed with PBS containing protease inhibitor [Roche, Sigma, USA] through a syringe followed by centrifugation of the intestinal fluid [1000g, 10 min, 4 °C]. Supernatants were collected and stored at −80 °C.

2.7. Isolation of outer membrane proteins [OMPs] of ST, SE and SG

Outer membrane proteins of respective iNTS strains [S.17.7, S.17.3 and PO-1] were isolated using the protocol described previously (Maiti et al., 2020). Briefly strains were inoculated in 50 ml TSB and grown overnight at 37 °C with constant shaking. Cells were isolated by centrifugation at 8000g for 10 min and cell pellets were washed once with HEPES buffer [10 mM, pH 7.5] and finally re-suspended with HEPES buffer. Protein degradation was prevented by addition of protease inhibitor cocktail [Roche, Sigma, USA] in the HEPES buffer. Cells

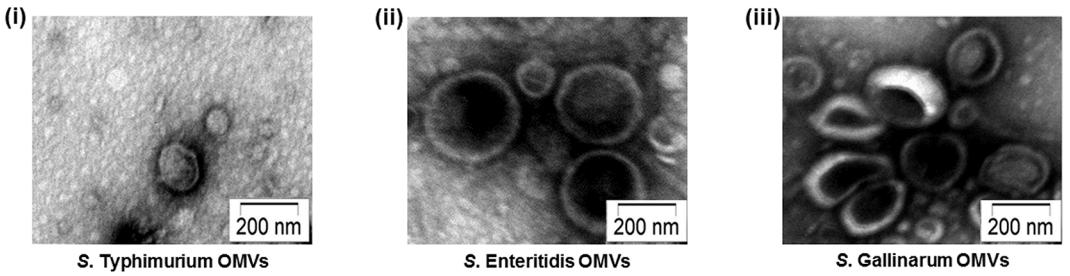


Fig. 1. Transmission electron microscopic images of iNTS OMVs. **(i)** *S.* Typhimurium OMVs, **(ii)** *S.* Enteritidis OMVs, **(iii)** *S.* Gallinarum OMVs.

were then placed in an ice bath and sonicated with Hielscher [UP100H] ultrasonic sonicator for 10 min. Unbroken cells were removed by centrifugation at 13000g for 20 min followed by collection of supernatant containing OMPs. Supernatants were transferred into an ultracentrifuge tubes and centrifuged at 100000g for 1 h at 4 °C followed by collection of pellets. Pellets were then re-suspended with HEPES buffer containing 1% sercosyl and incubated for 30 min at 37 °C followed by ultracentrifugation with same condition. Pellets were collected and washed twice with HEPES buffer and finally re-suspended with 10 mM HEPES [pH 7.5] buffer. Concentration of OMPs were determined by modified Bradford protein estimation kit [Abcam, UK] and adjusted to 5μg/μl using HEPES buffer. OMPs were aliquoted and stored at −80 °C.

2.8. Isolation of lipopolysaccharide [LPS] of ST, SE and SG

LPS of the respective iNTS strains were isolated using method stated previously (Maiti et al., 2020). Briefly cells were grown in 50 ml TSB overnight with constant shaking at 37 °C. Cells were then isolated by centrifugation at 8500g for 20 min and washed once with normal saline and finally re-suspended with 150 mM NaCl containing Phenol-saturated 3-[N-morpholino] propen sulfonic acid [MOPS], incubated at 65 °C in a water bath for 30 min with occasional shaking followed by incubation at ice for 10 min. Cells were then centrifuged at 8500g for 20 min and upper aqueous layer was collected and mixed with 4 vol chilled ethanol and kept at −20 °C overnight. Next day LPS were isolated by centrifugation at 8500g for 20 min, re-suspended with autoclaved double distilled water and stored at −20 °C.

2.9. ELISA

Different serum immunoglobulin [IgY and IgA] levels were measured using serum from iNTS OMVs immunized and non-immunized chicks against each component of OMVs like OMPs and LPS of respective strains following previously described method (Sinha et al., 2015). Briefly, disposable polystyrene microtiter 96 wells plates were coated with ST or SE or SG OMPs [5 μg/well] or LPS [5 μg/well] and incubated overnight at 4 °C. Wells were washed with PBS thrice and non-specific binding were blocked with 200 μl, 5% BSA solution and incubated at 37 °C for 2 h. Wells were then washed with PBS-T [PBS containing 0.05% Tween-20, Sigma, USA] three times and then wells were incubated with serially diluted serum/intestinal lavage samples collected from chicks for 1 h at 37 °C. Wells were then washed three times with PBS-T followed by incubation of wells with HRP conjugated Goat anti-chicken immunoglobulins [Abcam, UK] for 1 h at 37 °C. Then wells were washed with PBS for three times and substrate O-phenyl-D-amine [OPD] was added in the wells and incubated at room temperature in the dark. Reaction was stopped after 10 min by addition of 100 μl of 2-N-sulphuric acid. OD was measured at 492 nm wavelength with a microplate reader. Each experiments were repeated three times with each serum/intestinal lavage from each chicks.

2.10. Serum bactericidal assay

Serum bactericidal assay was performed following previously described protocol. Briefly, serum was collected from iNTS-OMVs immunized and non-immunized chicks on 35th day after 1st immunization. Serum from immunized chickens were divided into two followed by heat inactivation of one-part serum by incubating serum at 56 °C for 30 min in a water bath. All the cells were grown in LB medium till log phase [$OD_{600} = 0.4$] and collected by centrifugation at 1100g for 5 min and re-suspended in PBS. Heat inactivated, non-heat inactivated immunized chicken serum and non-immunized chicken serum were mixed with bacteria [1:50 dilution] and the total reaction mixture was set to 50 μl followed by incubation at 37 °C for 1 h. The reaction was fixed by addition of 950 μl Luria broth and the reaction mixtures were serially diluted and spread on Luria agar plates for CFU count. For further analysis of bactericidal activity of our trivalent iNTS-OMVs immunized chicken serum against ST, SE and SG we used SEM analysis. Invasive NTS strains were harvested as above described. Strains were then incubated with each serum [non-immunized, immunized heat-inactivated and immunized nonheat inactivated] for 1 h at 37 °C and the samples were then collected for SEM analysis. Cells were then processed using previously described protocol for scanning electron microscopic analysis and observed under previously described condition (Maiti et al., 2020).

2.11. Animal challenge experiments

Exp 1: One week post final immunization chicks were orally challenged with homologous or heterologous iNTS strains. Twenty four hour before challenge, food were withdrawn but water was given ad libitum. Fifteen minutes before challenge chicks were given 5 ml of 5% sodium bicarbonate solution to neutralize stomach pH. Then 1 ml of challenge iNTS strain containing 1×10^9 CFU bacteria was given followed by 4 ml of 5% sodium bicarbonate solutions. Chicks were then returned to their cages and food and water was given to them 4 h post challenge and monitored for 15 days for any disease symptoms. Cecal droppings were collected from each cages, serially diluted and plated on XLD agar plate [Difco, USA] for determination of iNTS bacterial load in their stools. Six- and fifteen-days post challenge, 5 chicks from each group were sacrificed and different systemic organs like liver and spleens were collected, homogenized, serially diluted and plated on XLD agar plate to determine bacterial colonization into these organs.

Exp 2: One week post 1st immunization, 5-week-old chickens were challenged with iNTS strains and ligated intestinal loop model was used to determine protective efficacy of our trivalent iNTS-OMVs immunogen. Twenty-four hour before challenge foods were withdrawn from each animals but water was supplied ad libitum. Fifteen minutes before surgery animals were anesthetized using intramuscular injection of a mixture of ketamine [35 mg/kg body weight; Nion Laboratories Pvt. Ltd, India] and xylazine [5 mg/kg body weight; Astra Zeneca Pharma India Ltd, India] solutions. Animals were restrained in a restrainer and covered with a surgical blanket. Chickens were de-feathered at abdomen portion. After the disinfection of the incision place, incision was made

and abdomen was opened and the small intestine portion was carefully removed. Two or more ligated loops were constructed with polypropylene blue monofilament surgery suture [Prolene, Ethicon, USA] without hampering normal blood supply in the intestine. Loops were 5–6 cm in length and separated by spacer loops of 1 cm to avoid any contamination. Each loop was injected with 1 ml of any of the iNTS challenge strains containing 1×10^8 CFU. PBS was injected in the loop act as negative control. After inoculation loops were reintroduced into the abdomen, and the abdominal wall was sutured and birds were returned to their cages. 16–18 h later birds were euthanized, tissue and fluids were collected from each loops and plated on XLD agar plate for determination of bacterial load. Each experiment was repeated for three times for statistical significance.

Exp 3: One-year post 1st immunization adult chickens were challenged with iNTS strains and oral challenge model was used to determine long term protective efficacy of our iNTS-OMVs immunogen following above described protocol. Each experiment was repeated for three times for statistical significance.

Exp 4: One-year post 1st immunization adult chickens were challenged with iNTS strains and ligated intestinal loop model was used to determine long term protective efficacy of our iNTS-OMVs immunogen following above described protocol. Each experiment was repeated for three times for statistical significance.

2.12. Statistical analysis:

Data shown here as the mean ± SD. Two way ANOVA tests were used to compare three or more groups of data sets. One tailed Student's *t* test was used to analyze two groups of data. All the experiments were repeated three times for statistical significance. A probability value of P either $p < 0.01$ or $p < 0.05$ were considered as level of significance. All the data were analyzed with GraphPad Prism 5 and MS Excel 2016.

3. Results:

3.1. Isolation of S. Typhimurium, S. Enteritidis and S. Gallinarum OMVs and preparation of trivalent iNTS-OMVs immunogen

Transmission electron microscopic images revealed that all three iNTS strains [*S.* Typhimurium, ST; *S.* Enteritidis, SE; *S.* Gallinarum, SG] secrete OMVs during their growth. OMVs were spherical in nature and their size ranged between 150 and 300 nm in diameter [Fig. 1]. Protein estimation results showed that S. Typhimurium OMVs protein concentration [100 μg/ml] was one-fold higher than OMVs of both SE [55 μg/ml] and SG [58 μg/ml] [Data not shown]. Post protein estimation, same amount of proteins from each OMVs were mixed together [1:1:1] to prepare trivalent iNTS-OMVs immunogen with a final protein concentration of 50 μg/200 μl.

3.2. iNTS-OMVs immunization induces adaptive immune response in one day old chicks

To determine immunogenic nature of iNTS OMVs immunization in one day old chickens, chickens were immunized orally with iNTS-OMVs [50 μg/chick] three times in two weeks intervals, and control group was immunized with PBS. Result showed following iNTS-OMVs immunization serum immunoglobulin level [IgY and IgA] induces exponentially against OMVs of ST, SE and SG upto 180 days after 1st immunization [Fig. 2]. Then, we measured the immunogenic response of iNTS-OMVs immunogen against different components of OMVs like OMPs and LPS of ST, SE and SG. In result we found that three doses of iNTS-OMVs immunization induces serum immunoglobulins like IgY and IgA against both OMPs and LPS of ST, SE and SG [Fig. 3 A, B]. Immunization also induces mucosal immune response which is important to reduce intracellular pathogens like NTS. We found that elevated levels of secretory IgA against ST, SE and SG after 1 and 4 weeks post final

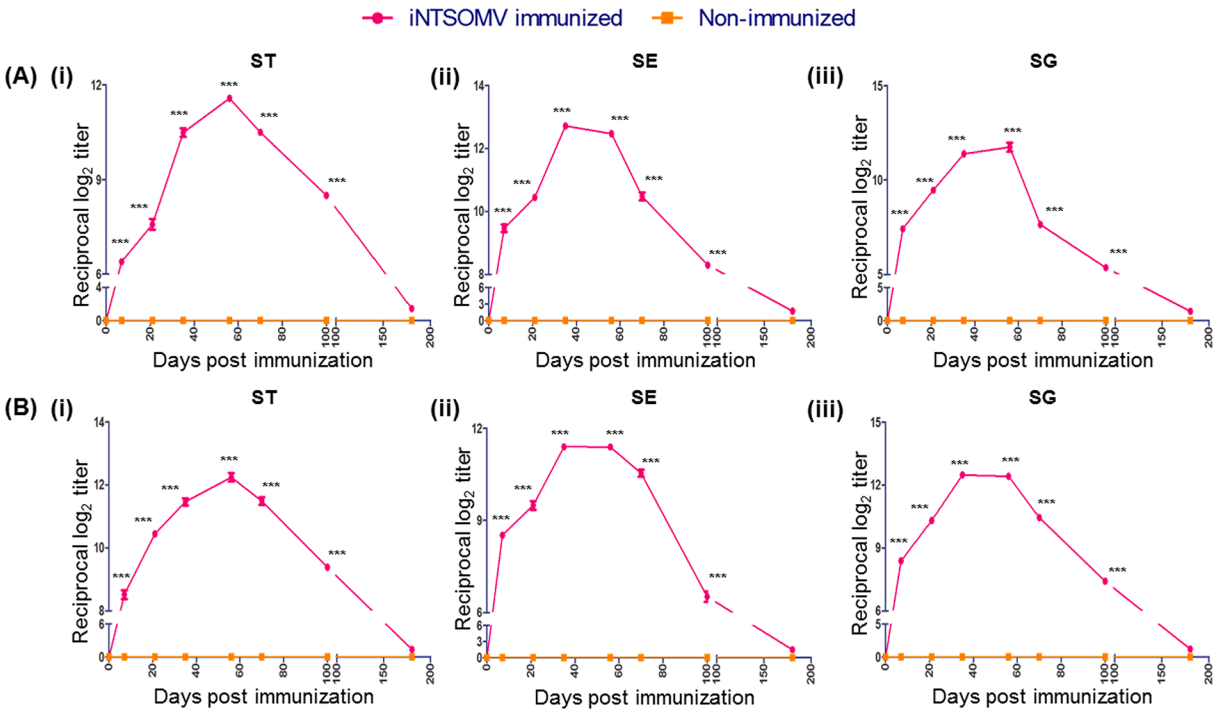


Fig. 2. Reciprocal $\log_2$ titer of serum immunoglobulin in trivalent iNTS-OMVs and non-immunized chicken serum was measured against each OMVs after three doses of oral immunization with trivalent iNTS-OMVs immunogen. Ninety six well flat bottom microtiter plates were coated with OMVs of either *S.* Typhimurium or *S.* Enteritidis or *S.* Gallinarum and trivalent iNTS-OMVs immunized or non-immunized chicken serum were used as primary antibody. (**A**) Chicken serum IgY; (**i**) *S.* Typhimurium, (**ii**) *S.* Enteritidis, (**iii**) *S.* Gallinarum. (**B**) Chicken serum IgA; (**i**) *S.* Typhimurium, (**ii**) S. Enteritidis, (**iii**) S. Gallinarum. The horizontal axis depicted the days of blood collection. Data presented here is the mean ± SD of three independent experiment. Statistical significance between immunized and non-immunized chicken serum (*** $p < 0.001$). ST, *S.* Typhimurium; SE, *S.* Enteritidis; SG, *S.* Gallinarum.

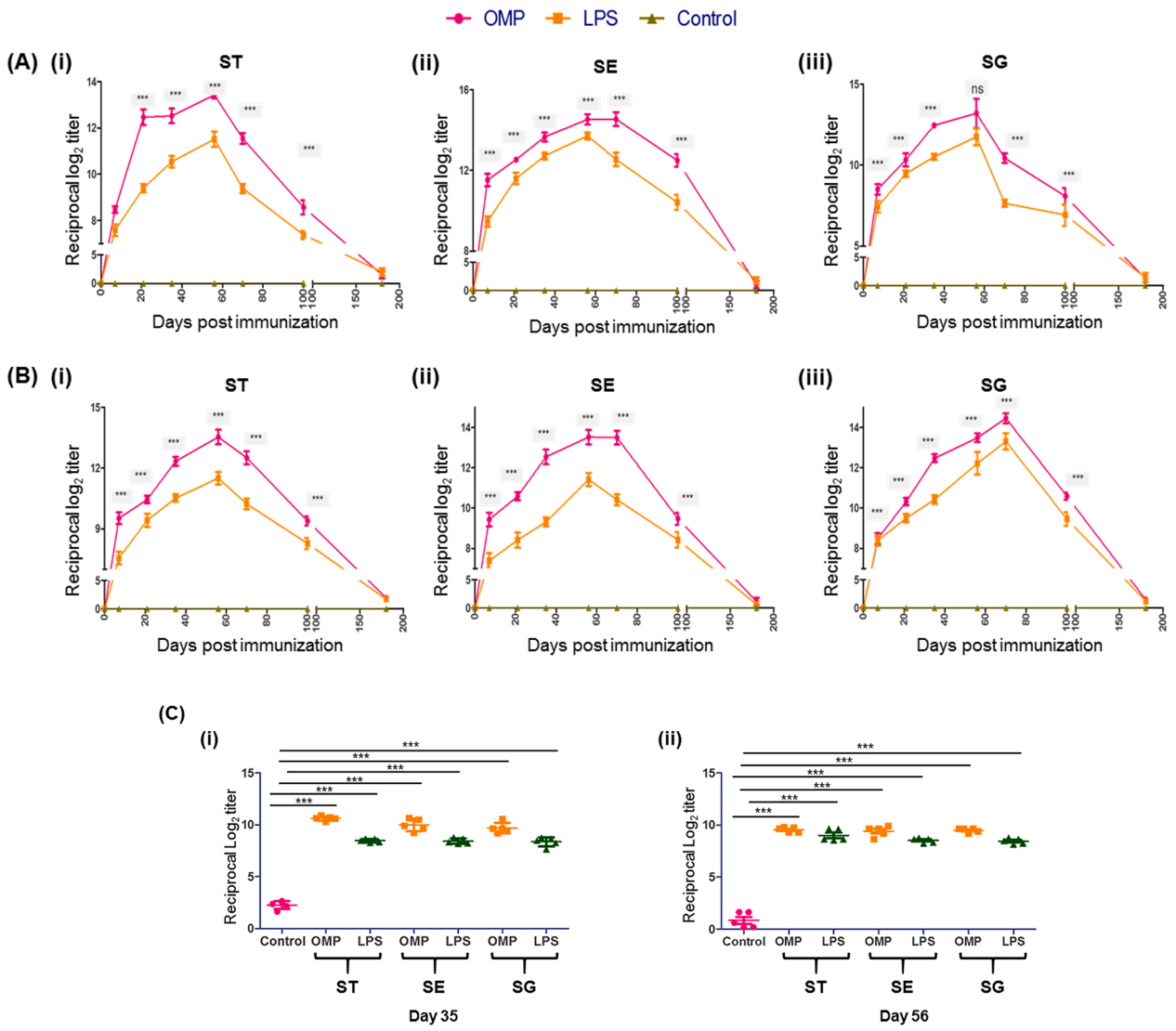


Fig. 3. Reciprocal log2 titer of serum immunoglobulin and secretory IgA in trivalent iNTS-OMVs and non-immunized chicken groups were separately measured against each serovar specific components of OMVs after three doses of oral immunization with trivalent iNTS-OMVs immunogen. Ninety six well flat bottom microtiter plates were coated with either OMPs or LPS of either *S.* Typhimurium or *S.* Enteritidis or *S.* Gallinarum and trivalent iNTS-OMVs immunized or non-immunized chicken serum were used as primary antibody at specific time point. (**A**) Serum IgY; (**i**) *S.* Typhimurium, (**ii**) *S.* Enteritidis, (**iii**) *S.* Gallinarum. (**B**) Chicken serum IgA; (**i**) *S.* Typhimurium, (**ii**) S. Enteritidis, (**iii**) S. Gallinarum. The horizontal axis depicted the days of blood collection. Data presented here is the mean ± SD of three independent experiment. Statistical significance between immunized and non-immunized chicken serum (*** $p < 0.001$). (**C**) Reciprocal log2 titer of sIgA from chicken intestinal lavage against OMPs and LPS of *S.* Typhimurium, S. Enteritidis and S. Gallinarum on (**i**) 1 week and (**ii**) 4 week from final immunization. Difference between trivalent iNTS-OMVs immunized and non-immunized group is statistically significant. Result shows one out of three independent experiments using 5 chickens in each group. Statistical analysis was performed using the two way ANNOVA test. Data were presented as mean ± S.E, each group, n = 5. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) ST, *S.* Typhimurium; SE, *S.* Enteritidis; SG, *S.* Gallinarum.

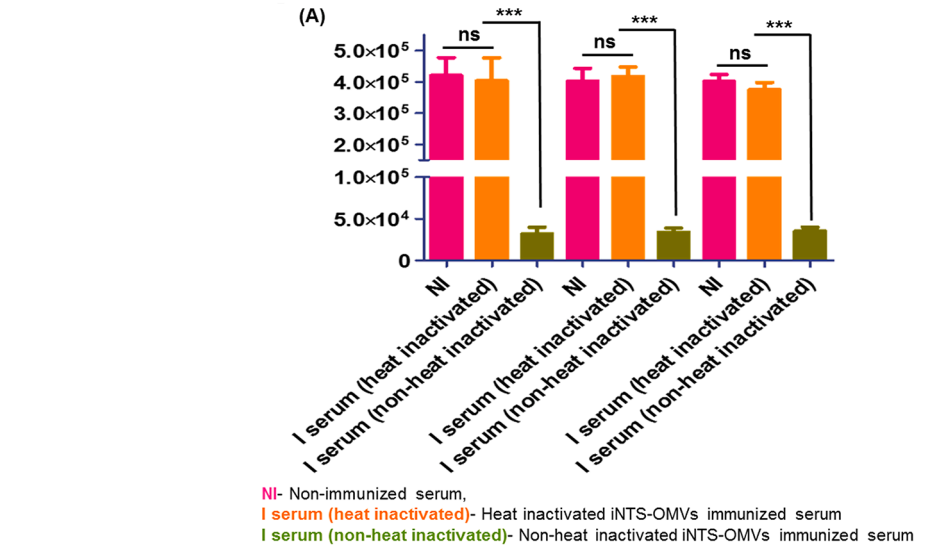
immunization in chicken's intestinal wash [Fig. 3 C].

3.3. iNTS-OMVs immunized chicken serum showed bactericidal activity

Our above result showed that immunization with iNTS-OMVs induces anti-LPS and anti-OMPs antibody against ST, SE and SG in the serum. Previous results showed OMVs immunization induces bactericidal property in mice. To measure the bactericidal activity of our iNTS-OMVs immunized chicken serum we have done serum bactericidal assay. From the result we found that trivalent iNTS-OMVs immunized chicken serum was able to kill the selected ST, SE and SG strains but non-immunized chicken serum can't [Fig. 4 A]. To inactivate the complement systems we then heat-inactivated iNTS-OMVs immunized chicken serum. When heat inactivated iNTS-OMVs immunized chicken serum was incubated with our selected iNTS strains we found that it was unable to kill the bacteria. To further support our result we have assessed the serum bactericidal activity of our trivalent iNTS-OMVs immunized chicken serum via scanning electron microscopy. SEM images showed that trivalent iNTS-OMVs immunized chicken serum can lyse ST, SE and SG strains but failed to do so when strains were incubated with non-immunized chicken serum [Fig. 4 B]. Heat-inactivated iNTS-OMVs immunized chicken serum also failed to lyse NTS strains. This phenomenon stated that iNTS-OMVs immunization induces complement pathways in chicken serum which helps bacterial killing.

3.4. iNTS-OMVs immunization confer protective efficacy in chicken

To test the protective efficacy of iNTS-OMVs immunogen chickens were orally challenged 7 days post final immunization with clinical isolates of three heterologous strains [ST IDH-3162, SE IDH-1125 and SI



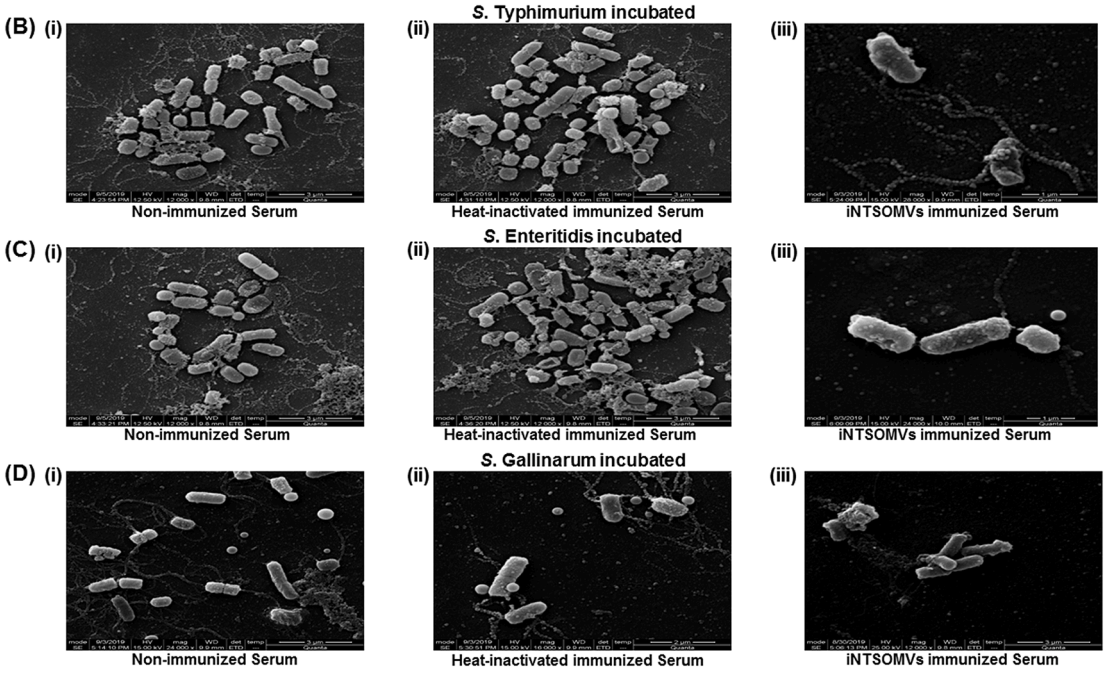


Fig. 4. Trivalent iNTS-OMVs immunized chicken serum are capable of complement killing of *S.* Typhimurium, *S.* Enteritidis and *S.* Gallinarum. ST [IDH-3162, 1×10^5] or SE [IDH-1125, 1×10^5] or SG [PO-1, 1×10^5] was incubated with indicated dilution of non-immunized serum or heat inactivated iNTS-OMVs immunized chicken serum or non-heat inactivated iNTS-OMVs immunized chicken serum for 1 h at 37 °C. A. Live bacteria were enumerated by plating on LB agar plates. Statistical analysis were performed using the two-way ANOVA test. Each bar represents mean ± SE of three independent experiments. ***$p < 0.001$. NI, non-immunized serum; I serum [heat inactivated], iNTS-OMVs immunized chicken serum heat inactivated; I serum [non-heat inactivated], iNTS-OMVs immunized chicken serum non-heat inactivated. Scanning electron microscopic images of *S.* Typhimurium, *S.* Enteritidis and *S.* Gallinarum incubated with either 35th day trivalent iNTS-OMVs or non-immunized chicken serum. (**B**) *S.* Typhimurium incubated with (**i**) non-immunized serum, (**ii**) heat-inactivated trivalent iNTS-OMVs immunized serum, (**iii**) trivalent iNTS-OMVs immunized 35th day chicken serum. (**C**) *S.* Enteritidis incubated with (**i**) non-immunized serum, (**ii**) heat-inactivated trivalent iNTS-OMVs immunized serum, (**iii**) trivalent iNTS-OMVs immunized 35th day chicken serum. (**D**) *S.* Gallinarum incubated with (**i**) non-immunized serum, (**ii**) heat-inactivated trivalent iNTS-OMVs immunized serum, (**iii**) trivalent iNTS-OMVs immunized 35th day chicken serum.

PH-97] and one homologous strain [SG; PO-1]. Chickens of both immunized and non-immunized groups were challenged orally with 1×10^9 CFU of ST or SE or SG or SI strain and their cecal droppings were collected and plated. From the result we found that bacterial shedding from immunized and non-immunized groups shows no significant difference till 6 days post challenge [Fig. 5 A]. But after that bacterial shedding greatly reduces in the immunized animals than non-immunized animals [Fig. 5 A]. Six and fifteen days post challenge five animals from each group were sacrificed and their spleens, livers were taken out to measure bacterial colonization in systemic organs. Our result showed that bacterial load in immunized animal's spleens and livers are much lower than non-immunized animal's spleen and liver [Fig. 5 B]. From this result we hypothesized that, our trivalent iNTS-OMVs immunization prevents iNTS strains to invade chicken's intestinal tissue and colonizing different systemic organs.

To, test our hypothesis we used chicken ligated intestinal loop model and challenged immunized and non-immunized chickens with iNTS strains 7 days post final immunization. After preparing loops using

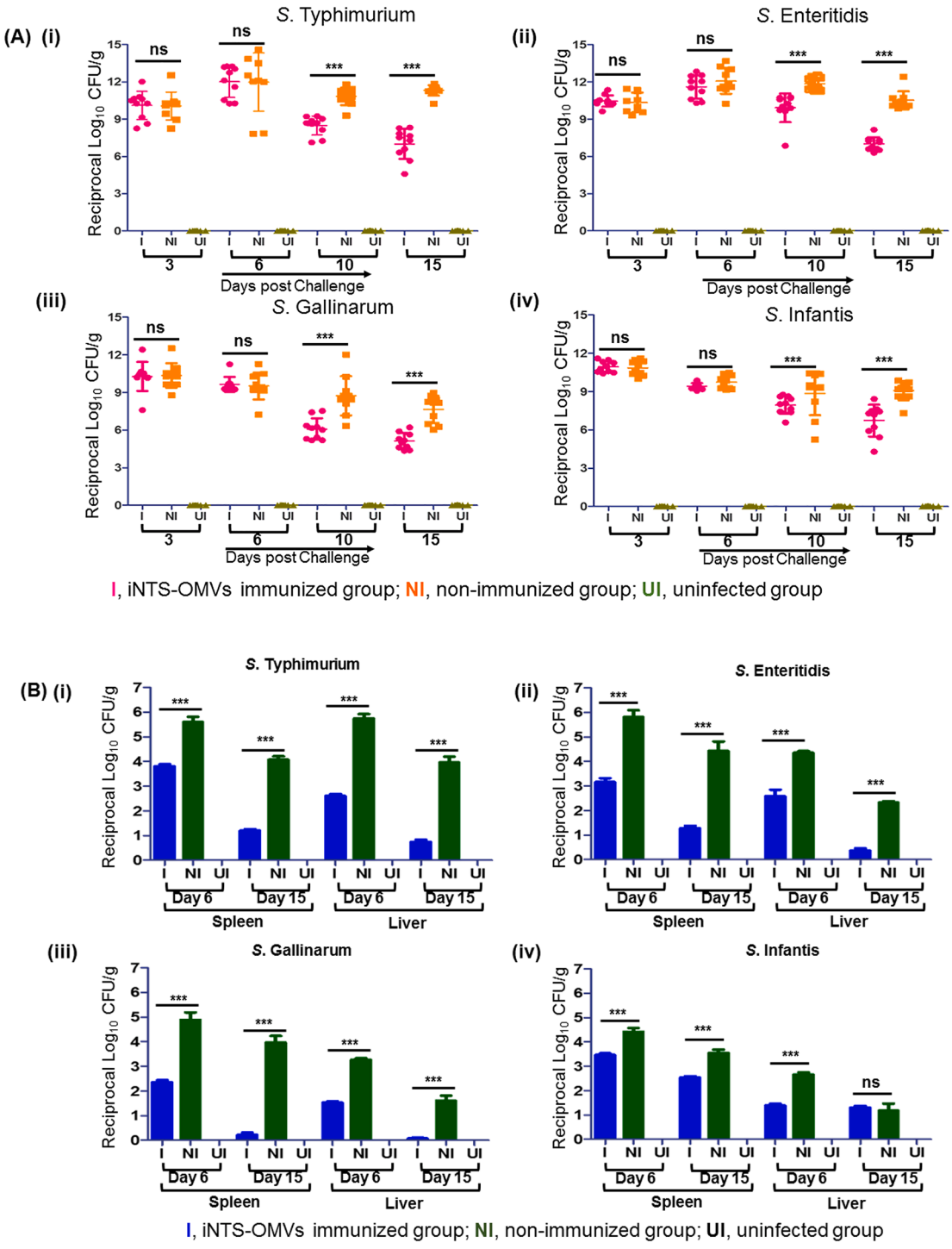


Fig. 5. Comparative protective analysis of trivalent iNTS-OMVs immunogen. One day old chicks were orally immunized with trivalent iNTS-OMVs immunogen three times with two weeks interval followed by oral challenge with homologous and heterologous iNTS strains after 1 week post immunization. (**A**) Stool was collected till 15 days post challenge. Each bar represents CFU/g of stool from ten chickens. Data was represented as $\log_{10}$ of recovered CFU/g of stool of ten chicken post 3, 6, 10 and 15 days post challenge, presented on vertical axis. Low level of *Salmonella* was detected from iNTS-OMVs immunized chicken's stool when chickens were challenged with (**i**) *S.* Typhimurium, (**ii**) *S.* Enteritidis, (**iii**) *S.* Gallinarum and (**iv**) *S.* Infantis than non-immunized groups. The differences in the stool *Salmonella* detection between the immunized and non-immunized groups were highly significant on 10 and 15 days post challenge ($p < 0.001$) but not significant on 3 and 6 days post challenge (p greater than 0.05). (**B**) Six days post oral challenge five chickens from each group were sacrificed and their systemic organs like spleen and liver, homogenized and iNTS colonization was measured by serial dilution and plating. Low level of colonization was found in iNTS-OMVs immunized group than non-immunized group on both 6 and 15 days post challenge with (**i**) *S.* Typhimurium, (**ii**) *S.* Enteritidis, (**iii**) *S.* Gallinarum and (**iv**) *S.* Infantis. Data was represented as $\log_{10}$ of recovered CFU/g of tissue of ten chicken 6 and 15 days post challenge, presented on vertical axis. The differences in the tissue colonization with *Salmonella* between the immunized and non-immunized groups were highly significant for both organs ($p < 0.001$). Statistical analysis was carried out using the two-way analysis of variance (ANNOVA) test. Data were represented as mean value of ten chicken ± S.E of three saperate experiment. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns Non-significant.

surgical method 1 ml of bacteria containing 1×10^8 CFU or 1 ml PBS were injected inside the loops. After 16–18 h of challenge chickens were sacrificed intestinal tissues and intestinal fluids were collected from each loops. We found that presence of viable bacteria in immunized chicken's tissue were lower than non-immunized chicken's [Fig. 6 A] but bacterial load in intestinal fluids of immunized group was much higher than non-immunized animals [Fig. 6 B]. We have also measured the post challenge fluid accumulation ratio.. Result showed that fluid accumulation ratio is much lower in immunized groups than non-immunized one [Fig. 6 C].

3.5. *Duration of protection conferred by trivalent iNTS-OMVs immunogen*

To test the long term protective efficacy of our trivalent iNTS-OMVs immunogen chickens were orally challenged 1 year post final immunization with clinical isolates of three heterologous strains [ST IDH-3162, SE IDH-1125 and SI PH-97] and one homologous strain [SG; PO-1]. Chickens of both immunized and non-immunized groups were challenged orally with 1×10^9 CFU of ST or SE or SG or SI strain and their cecal droppings were collected and plated. From the result we found that bacterial shedding from immunized and non-immunized groups shows little or no significant difference till 6 days post challenge [Fig. 7 A]. But then bacterial shedding were greatly reduced in the immunized animals than non-immunized animals [Fig. 7 A]. Six and fifteen days post challenge five animals from each group were sacrificed and their systemic organs like spleens and livers were taken out to measure bacterial colonization. Our result showed that bacterial load in immunized animal's spleens and livers are much lower than non-immunized animal's spleen and liver [Fig. 7 B]. From this result we hypothesized that, immunization with our trivalent iNTS-OMVs immunogen prevents iNTS strains to invade chicken's intestinal tissue and colonizing different systemic organs.

Results from our chicken oral challenge study was further evaluated with chicken ligated intestinal loop challenge study. Here both immunized and non-immunized groups were challenged after 1 year of 1st immunization. Chickens were challenged with 1×10^8CFU of bacteria injected into intestinal loops and sacrificed after 16–18 h post challenge. From the result we found that, viable bacteria in immunized animal's tissue were much lower than non-immunized groups [Fig. 8 A]. Viable CFU of NTS strains present in immunized animals were much higher in immunized animals than non-immunized animals [Fig. 8 B]. Fluid accumulation ratio was also less in case of immunized animals compared to non-immunized animals [Fig. 8 C]. From this result we can say that iNTS-OMVs immunization can protect chickens from iNTS infection till 1-year post immunization.

4. Discussion

In this study we have evaluated the safety, immunogenicity and protective efficacy of a novel trivalent outer membrane vesicles based non-typhoidal *Salmonella enterica* immunogen for chickens. Overall result showed that our immunogen appeared to be safe, with no effect on overall bird's health, food consumption and any adverse effect related to bird's mortality. Now a days vaccination programmes for poultry breeds are generally complex in nature and involves multiple vaccines being administered for vaccination against different pathogens. Thus, to reduce vaccination related stress and handling problems commercial farms usually administered multiple vaccines simultaneously (Crouch

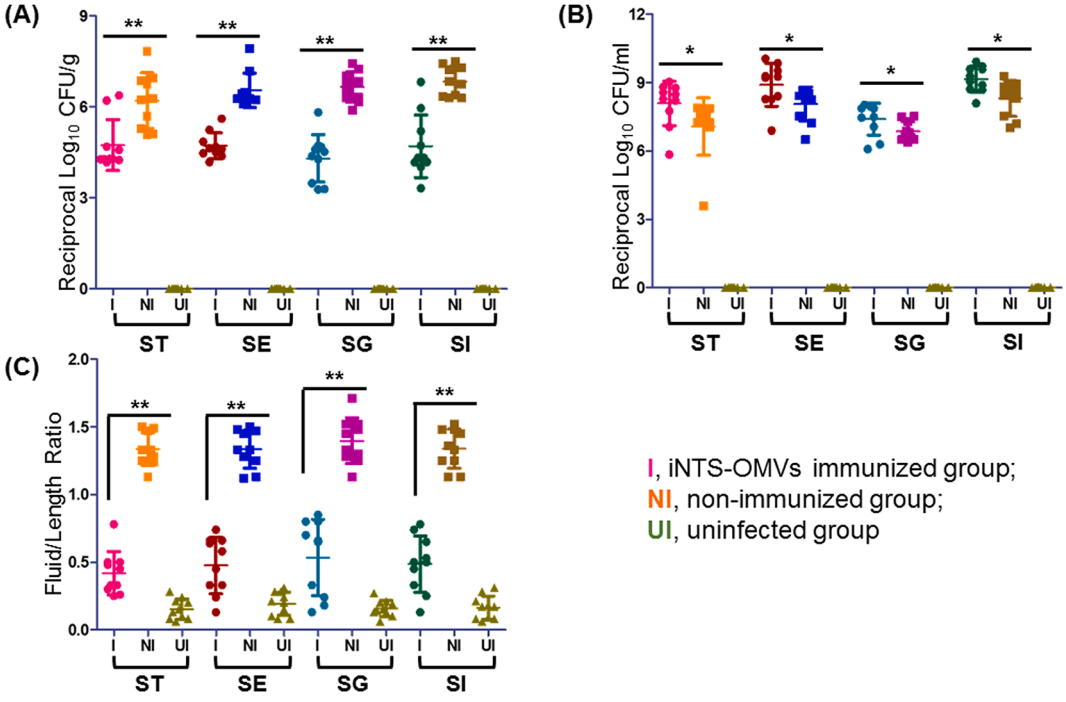
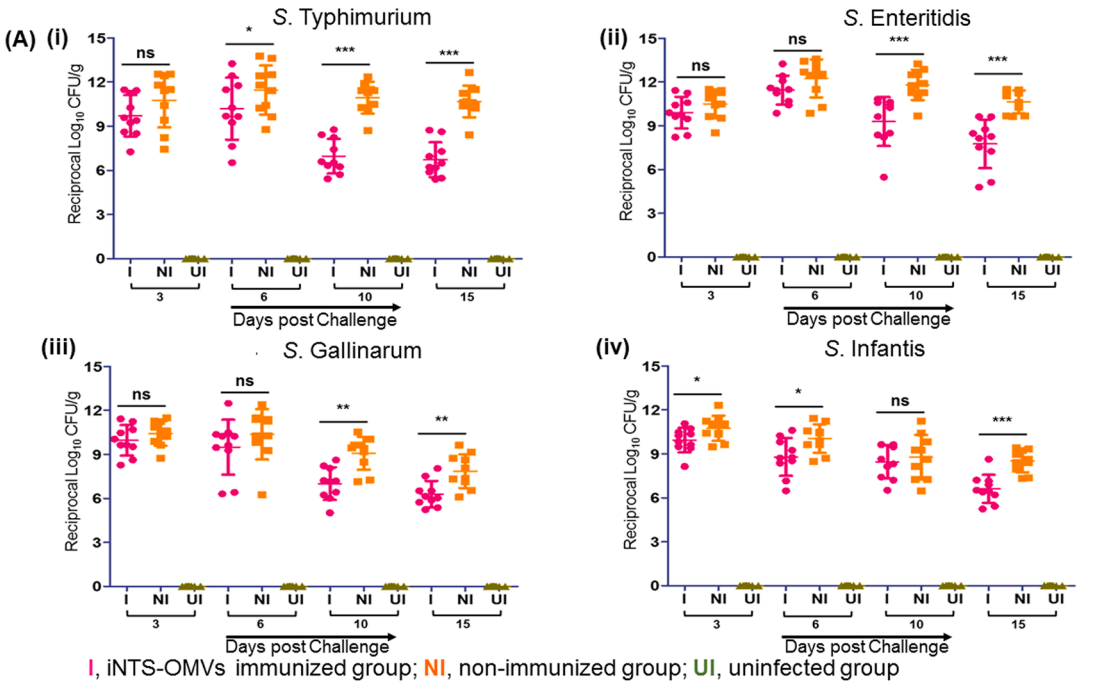


Fig. 6. One week post final immunizations chickens from each groups were challenged with homologous and heterologous iNTS strains using chicken ligated intestinal loop model. Intestines of chickens were surgically removed and loops were tied in the intestine followed by inoculation of challenge dose inside the loops then intestines were returned inside chickens. Sixteen to eighteen hours later chickens were sacrificed, tissues and fluids of loops were collected and plated for iNTS recovery. Comparative analysis of colonization of iNTS strains in (**A**) tissues, (**B**) intestinal fluids of immunized and non-immunized chickens. Less level of colonization of iNTS strains were found in immunized chickens tissues than non-immunized chickens but higher level of iNTS CFU/ml were found in immunized chickens than non-immunized chickens. (**C**) Comparative analysis of fluid volume/loop length ratio of immunized chickens and non-immunized chickens. Fluid volume/loop length ratio was less in immunized chickens than non-immunized chickens. The differences in the tissue colonization, fluid colonization with iNTS and fluid volume/loop length ratio between the immunized and non-immunized groups were highly significant. Statistical analysis was carried out using the two-way analysis of variance (ANNOVA) test. Data was represented as $\log_{10}$ of recovered CFU/g of tissue, CFU/ml of fluid and fluid volume/loop length ratio of ten chickens 1 week post challenge, presented on vertical axis. Data were represented as mean value of ten chicken ± S.E of three separate experiment. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns Non-significant. ST, *S*. Typhimurium; SE, *S*. Enteritidis; SG, *S*. Gallinarum; SI, *S*. Infantis.



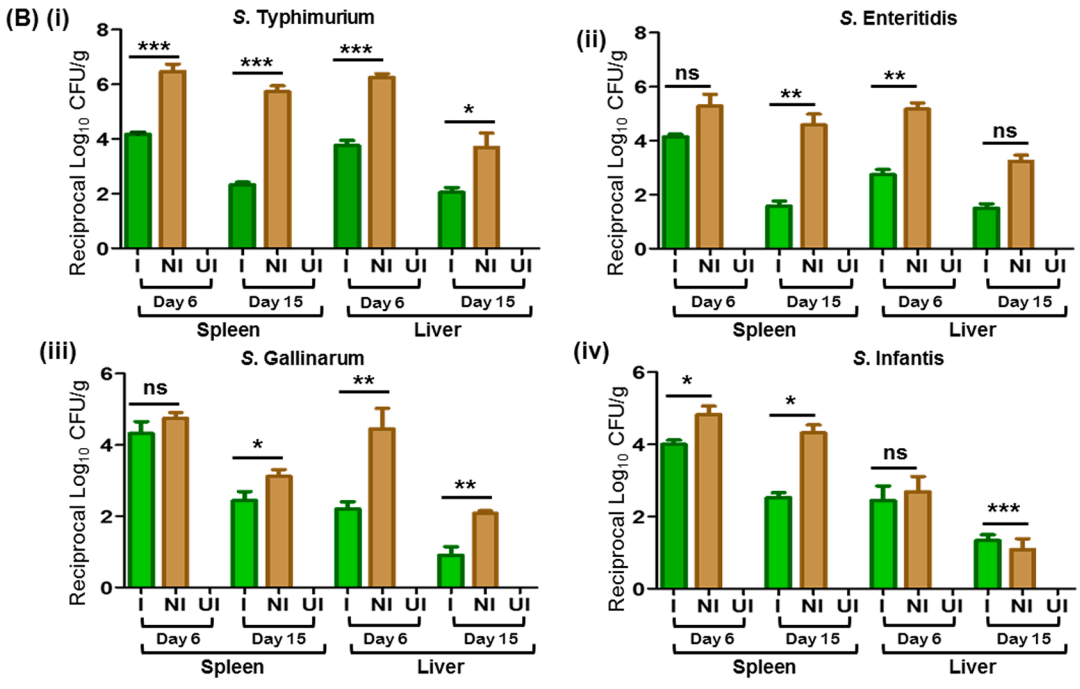


Fig. 7. Comparative protective analysis of trivalent iNTS-OMVs immunogen. One day old chicks were orally immunized with trivalent iNTS-OMVs immunogen three times with two weeks interval followed by oral challenge with homologous and heterologous iNTS strains 1 year post immunization. (**A**) Stool was collected till 15 days post challenge. Each bar represents CFU/g of stool from ten chickens. Data was represented as $\log_{10}$ of recovered CFU/g of stool of ten chicken post 3, 6, 10 and 15 days post challenge, presented on vertical axis. Low level of *Salmonella* was detected from iNTS-OMVs immunized chicken's stool when chickens were challenged with (**i**) *S.* Typhimurium, (**ii**) *S.* Enteritidis, (**iii**) *S.* Gallinarum and (**iv**) *S.* Infantis than non-immunized groups. The differences in the stool *Salmonella* detection between the immunized and non-immunized groups were highly significant on 10 and 15 days post challenge ($p < 0.001$) but not significant on 3 and 6 days post challenge (p greater than 0.05). (**B**) Six days post oral challenge five chickens from each group were sacrificed and their systemic organs like spleen and liver, homogenized and iNTS colonization was measured by serial dilution and plating. Low level of colonization was found in iNTS-OMVs immunized group than non-immunized group on both 6 and 15 days post challenge with (**i**) *S.* Typhimurium, (**ii**) *S.* Enteritidis, (**iii**) *S.* Gallinarum and (**iv**) *S.* Infantis. Data was represented as $\log_{10}$ of recovered CFU/g of tissue of ten chicken 6 and 15 days post challenge, presented on vertical axis. The differences in the tissue colonization with *Salmonella* between the immunized and non-immunized groups were highly significant for both organs ($p < 0.001$). Statistical analysis was carried out using the two-way analysis of variance (ANNOVA) test. Data were represented as mean value of ten chicken ± S.E of three separate experiment. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns Non-significant.

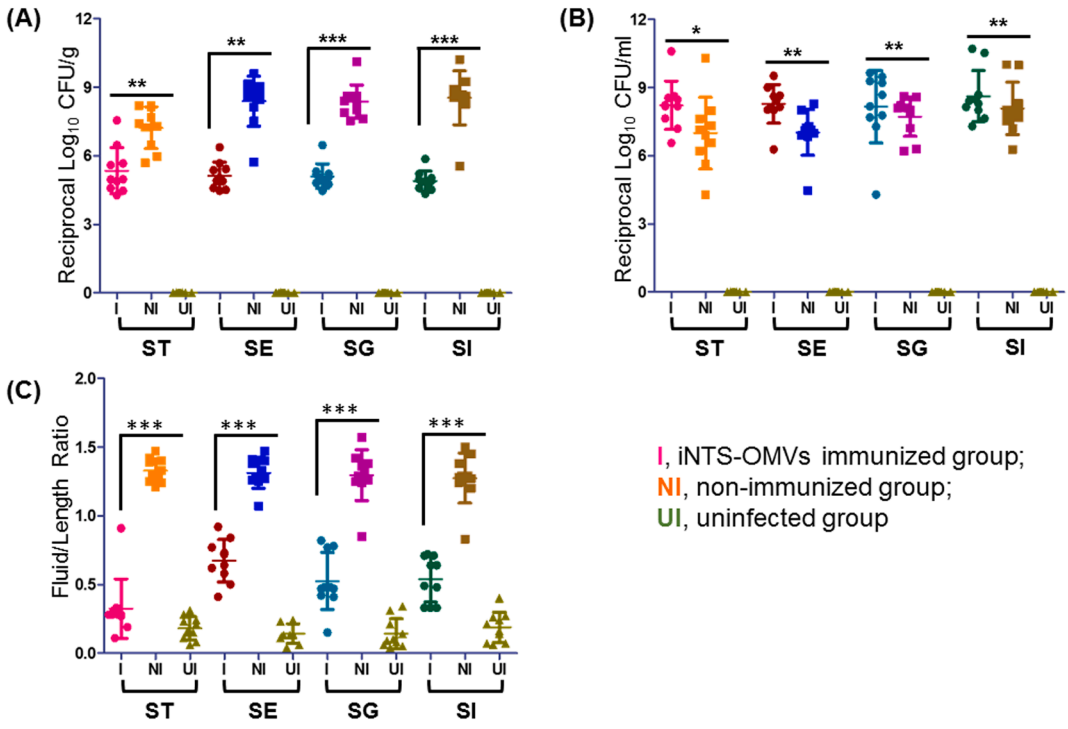


Fig. 8. One year post final immunizations chickens from each groups were challenged with homologous and heterologous iNTS strains using chicken ligated intestinal loop model. Intestines of chickens were surgically removed and loops were tied in the intestine followed by inoculation of challenge dose inside the loops then intestines were returned inside chickens. Sixteen to eighteen hours later chickens were sacrificed, tissues and fluids of loops were collected and plated for iNTS recovery. Comparative analysis of colonization of iNTS strains in (**A**) tissues, (**B**) intestinal fluids of immunized and non-immunized chickens. Less level of colonization of iNTS strains were found in immunized chickens tissues than non-immunized chickens but higher level of iNTS CFU/ml were found in immunized chickens than non-immunized chickens. (**C**) Comparative analysis of fluid volume/loop length ratio of immunized chickens and non-immunized chickens. Fluid volume/loop length ratio was less in immunized chickens than non-immunized chickens. The differences in the tissue colonization, fluid colonization with iNTS and fluid volume/loop length ratio between the immunized and non-immunized groups were highly significant. Statistical analysis was carried out using the two-way analysis of variance (ANNOVA) test. Data was represented as $\log_{10}$ of recovered CFU/g of tissue, CFU/ml of fluid and fluid volume/loop length ratio of ten chickens 1 week post challenge, presented on vertical axis. Data were represented as mean value of ten chicken ± S.E of three separate experiment. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns Non-significant. ST, *S.* Typhimurium; SE, *S.* Enteritidis; SG, *S.* Gallinarum; SI, *S.* Infantis.

et al., 2020a). There are no vaccines available in the market against *S.* Typhimurium, *S.* Enteritidis and *S.* Gallinarum combined (Young J. Lee et al., 2005). Thus to protect flocks from these widely isolated iNTS serovars, farms need to use two or more vaccines simultaneously. But, our immunogen contains all these three serovars thus it reduces vaccination related stress and being administered via oral route, our immunogen minimized immunization related stress on birds.

Chicks were infected greatly with iNTS serovars within first few days after hatching, which increases risk of systemic and horizontal spread of infection and increases death rate at young age (Wigley and Barrow, 2017). *S.* Gallinarum which causes fowl typhoid generally infect 4–5 days old chickens and most deaths were found at 1–2 weeks age there by increases economic loss in poultry industry. Though shorty after vaccination if birds infected with virulent *Salmonella enterica,* it can colonize systemic organs (He et al., 2010) but their chance of isolation declined gradually thereby reducing chance of spreading to other birds (Gast et al., 2011, 2007). Thus early vaccination is highly important to protect flocks from natural infection during or upon arrival at farms (Deguchi et al., 2009). In our study we found, the early vaccination of one day old chicks showed reduced level of bacterial invasion and colonization in intestine till 1 year of life. Similar result was found when one day old chicks were immunized with live attenuated vaccines (Barrow et al., 2000).

In this study we found oral immunization with novel trivalent iNTS-OMVs immunogen induces strong adaptive immune response against ST, SE and SG. In our study we observed, high level of anti-LPS and anti-OMPs specific antibody after 180 days post 1st immunization with trivalent iNTS-OMVs immunogen which leads to cell free or cell dependent bacterial killing which is important to prevent systemic infection. High level of serum immunoglobulin level till 180 days post immunization also indicated that our iNTS-OMVs immunogen can activate T-cell mediated immune response in chickens. Advantage of using OMVs as immunogen is, OMVs contains both OMPs and LPS in their native structure which induces serum immunoglobulin level drastically against specific pathogen (Howlader et al., 2018; Maiti et al., 2020). Previous study also shows that immunogen devoid of functional LPS or "O"-antigen in their structure failed to induce complement pathways thus unable to kill the bacteria there by clearing of pathogen from body (Rondini et al., 2013). In our study, we found tri-valent iNTS-OMVs immunized chicken serum can bind with iNTS serovars, ruptures its membrane thereby killing it.

In our oral challenge study we found our trivalent iNTS-OMVs immunogen was able to protect chickens against all three immunogen serotypes [ST, SE and SG]. Our immunogen also shown cross protective efficacy against *S.* Infantis. Previous results showed that *Salmonella* vaccine efficacy depends upon O-antigen homology of immunogen (Deguchi et al., 2009; Okamura et al., 2007) and challenge serovars but our immunogen though containing O:4, O:9 and O:12 also protected against O:7 *S.* Infantis. This indicate O-antigens of our immunogen shared some form of homology with O-antigen of *S.* Infantis. Previous results also showed that vaccine containing *S.* Typhimurium also protects chickens from S. Heidelberg infection as they share same O antigen [O:4] (Crouch et al., 2020b). So, we can predict our immunogen will also be able to protect chickens from *S.* Heidelberg infection. We found that in oral challenge study, trivalent iNTS-OMVs immunogen reduced colonization of *Salmonella* serovars in different systemic organs of

immunized chickens than non-immunized chickens. But in case of bacterial shedding we found no difference between immunized and non-immunized chicken's stool against all four serovars till 6 days post challenge. Clearance of *Salmonella* from gastrointestinal tracts of chickens hugely depends upon bird's ages, bird older than 6 week age can more effectively clear *Salmonella enterica* than younger birds (Barrow et al., 1987). Thus we found reduction of bacterial shedding in immunized chickens after 6 days post challenge when bird's age was more than 6 weeks.

We further investigated protective efficacy of trivalent iNTS-OMVs immunogen in chickens via chicken ligated intestinal loop challenge model. Here we found in case of immunized animals, fluids inside intestinal loops contains more bacteria than non-immunized chicken's loops. But tissue invasion is more in non-immunized chickens than immunized chickens. This proves, our immunogen reduces bacterial invasion in chickens. Higher bacterial load in immunized chicken's intestinal fluid may indicate the presence of serectoty IgA [sIgA] at immunized chicken's small intestine which act simultaneously in different ways; enchained growth way and classical way. In enchained growth way sIgA form a coat on bacterial membrane and prevents sister cells dissociation (Moor et al., 2017), and in classical way sIgA binds with specific receptors of *Salmonella* strains thereby blocking its interaction with intestinal cells (Mantis et al., 2011; STOKES et al., 1975). This also proves that sIgA alone can't kill *Salmonella enterica* alone which supports previous results (Endt et al., 2010).

Previous study states that in case of layer breeds, hens tend to temporarily lose T-cell response while reaching sexual maturity, this makes them susceptible for *Salmonella* infection (Johnston et al., 2012; Wigley et al., 2005), as cellular immunity is one of the major important immune response to clear *Salmonella* from chicken (Beal et al., 2006; Chappell et al., 2009; Withanage et al., 2005). All the vaccines available in the market for chickens are either inactivated vaccines or live attenuated vaccines. Though both types of vaccines have their advantages on one another but both have their negative side. Live vaccine possess risk of reversion in its wild type virulent form and spread through the environment. Generally killed vaccines possess some problem with reactogenicity. To overcome this problem different adjuvants like aluminium hydroxide or oil were added with the vaccines (Shi et al., 2001) which drives up its production cost. Live vaccines are able to induce both humoral and cell mediated immune response in chickens (Babu et al., 2003; Rana and Kulshreshtha, 2006) thus thought to be more effective to reduce *Salmonella* burdens in chickens whereas inactivated vaccine though doesn't pose any risk of environmental spreading and are mostly inducers of humoral immunity but are poor activators of T cell mediated immunity (Arnon et al., 1983; Collins, 1974, 1972). There are several advantages of our novel immunogen over other commercially available vaccines. Like our developed novel iNTS-OMVs immunogen act as subunit vaccine, thus possess no risk of environmental spread and it contains natural adjuvants thus there are no need to add external adjuvants to reduce reactogenicity like inactivated vaccines. Now a days farms adopted both live and inactivated vaccinations combined to increase protective efficacy and to reduce risk of spreading of *Salmonella* in broiler breeder hens (de Paiva et al., 2009; Dórea et al., 2010; Penha Filho et al., 2009), thus our novel immunogen could be used in future to fight against *Salmonella* infection in chickens.

5. Conclusion:

The present study shows that our novel trivalent iNTS-OMVs based immunogen is safe and substantially immunogenic in nature. This study also demonstrated that a triple vaccination schedule at very young age provide early and long lasting immune response in layer breed. It also protects chickens from invasive non-typhoidal *Salmonella* mediated fecal excretion, organ invasion when chickens were challenged with homologous and heterologous iNTS strains. Our result also demonstrated that our novel trivalent iNTS-OMVs immunogen also delivers broad spectrum protective efficacy against iNTS infections in chickens. We can say that our novel immunogen could be used in future in poultry farms to reduce iNTS burden in flocks.

Credit authorship contribution statement

Suhrid Maiti: Conceptualization, Investigation, Data curation, Writing – original draft. **Prolay Halder:** Investigation, Data curation, Writing – original draft. **Soumalya Banerjee:** Investigation, Writing – review & editing. **Moumita Dutta:** Investigation, Writing – review & editing. **Asish Kumar Mukhopadhyay:** Writing – review & editing. **Shanta Dutta:** Writing – review & editing. **Hemanta Koley:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

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.

Acknowledgement:

We like to thank Prof. Jeffrey H. Withey, (Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan, USA) for his help in correcting the manuscript. We like to thank Mr. Rudra Narayan Saha for giving us required strains. We also like to thank Mr. Subrata Singha for his help in animal keeping. We like to thank Indian Council of Medical Research for fellowship of SM [File No- 5/3/8/61/ITR-F/2019-ITR dated 26.03.2019], PH [No. 3/1/3/JRF-2018/HRD-066(66125)] and University Grant Commission for fellowship of SB.

Funding source

This work was supported by West Bengal Govt. Department of Science and Technology [Approval no- 790(Sanc.)/ST/P/S&T/9G-21/2015]. SM and PH was supported by ICMR, SB was supported by UGC.

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Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The 2022 Vaccines Against *Shigella* and Enterotoxigenic *Escherichia coli* (VASE) Conference: Summary of abstract-based presentations

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ARTICLE INFO

Keywords
Enteric diseases
Vaccine development
Shigella
Enterotoxigenic *Escherichia coli*
Campylobacter
Invasive non-typhoidal *Salmonella*

ABSTRACT

The global nonprofit organization PATH hosted the third Vaccines Against *Shigella* and Enterotoxigenic *Escherichia coli* (VASE) Conference in Washington, DC, on November 29 to December 1, 2022. With a combination of plenary sessions and posters, keynote presentations, and breakout workshops, the 2022 VASE Conference featured key updates on research related to the development of vaccines against neglected diarrheal pathogens including *Shigella*, enterotoxigenic *Escherichia coli* (ETEC), *Campylobacter*, and *Salmonella*. The presentations and discussions highlighted the significant impact of these diarrheal pathogens, particularly on the health of infants and young children in low- and middle-income countries, reflecting the urgent need for the development and licensure of new enteric vaccines. Oral and poster presentations at the VASE Conference explored a range of topics, including: the global burden and clinical presentation of disease, epidemiology, and the impact of interventions; the assessment of the value of vaccines against enteric pathogens; preclinical evaluations of vaccine candidates and models of enteric diseases; vaccine candidates in clinical trials and human challenge models; host parameters and genomics that predict responses to infection and disease; the application of new omics technologies for characterization of emerging pathogens and host responses; novel adjuvants, vaccine delivery platforms, and immunization strategies; and strategies for combination/co-administered vaccines. The conference agenda also featured ten breakout workshop sessions on topics of importance to the enteric vaccine field, which are summarized separately. This article reviews key points and highlighted research presented in each of the plenary conference sessions and poster presentations at the 2022 VASE Conference.

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https://doi.org/10.1016/j.vaccine.2023.11.031
Received 25 April 2023; Received in revised form 10 November 2023; Accepted 15 November 2023
Available online 28 November 2023

1. Introduction

From November 29 to December 1, 2022, the global nonprofit organization PATH hosted the third international Vaccines Against *Shigella* and Enterotoxigenic *Escherichia coli* (VASE) Conference in Washington, DC. With 270 attendees from more than 29 countries, it was the largest and most geographically diverse of the three VASE Conferences to date. *Shigella* and enterotoxigenic *Escherichia coli* (ETEC) were the primary focus for previous VASE Conferences, but PATH expanded the scope of VASE in 2022 to include research on other neglected enteric diseases among infants and children in low-resource settings, such as *Campylobacter*, non-typhoidal *Salmonella*, typhoid, cholera, and other diarrheagenic *E. coli*.

New findings covering a range of enteric vaccine topics were reported in 24 oral and 87 poster presentations based on submitted abstracts. In addition, the conference featured two keynote addresses. Dr. Celine Gounder of the Kaiser Family Foundation and Kaiser Health News presented on her experiences during the COVID-19 pandemic and lessons from the rapid development and roll-out of vaccines, which may be applicable to enteric vaccines. Dr. Kathleen Neuzil of the University of Maryland School of Medicine drew from her current work on typhoid conjugate vaccine introduction in low- and middle-income countries (LMICs) to highlight issues and opportunities relevant for developers of vaccines for other neglected enteric pathogens. There were also ten breakout workshop sessions (summarized separately in this issue of *Vaccine*), which directed smaller groups of conference participants to engage in deeper discussions on subjects of interest to the enteric vaccine field.

The following provides a brief summary of the research presented in each of the plenary sessions and the poster presentations at the 2022 VASE Conference in order to share the meeting content with the broader enteric vaccine field. The final conference agenda and abstracts booklet are available on the PATH website (www.path.org/resources/2022-vase-conference-abstracts-booklet/), and each presentation mentioned in this article is referenced using its assigned identifier code.

2. Global burden and clinical presentation of disease, epidemiology, and the impact of interventions

Shigella spp. continue to be a major bacterial enteric disease threat. Data from population-based surveillance in western Kenya from 2010 to 2015 demonstrated a 24% *Shigella* culture-positive rate among clinic patients with diarrhea, with an overall adjusted incidence of 7.9 episodes per 1,000 person-years and peak incidence of 15.3 episodes in children 12–23 months of age. Susceptibility to the antibiotics ceftriaxone and ciprofloxacin remained high (>98%) (GBD26). A deeper analysis of the Kenyan data further detailed the prevalence, serotype distribution, risk factors for illness, and antimicrobial susceptibility of *Shigella* (GBD16). *Shigella flexneri* remained the dominant species, with prevalence peaking in the second year of life. Similarly, data from Bangladesh from 2001 to 2020 showed that *Shigella* remained a common, if slightly declining, cause of diarrhea in urban and rural settings, with *S. flexneri* predominant. In both settings, ciprofloxacin resistance became common (>70%) and multidrug resistance significantly increased (>10%) by 2020 (GBD02). Another study that examined *Shigella* and a different class of antibiotics estimated that a 60% efficacious *Shigella* vaccine used in children could reduce fluoroquinolone and macrolide (F/M)-treated all-cause and *Shigella*-attributed diarrhea episodes by 9% and 46%, respectively, and F/M bystander exposure by 44% (AVV04).

Modeling studies added insight on the global distribution of shigellosis and the potential impact of a vaccine. A broad range of hydrometerological, environmental, household, and subject-level variables were used to perform spatiotemporal mapping of *Shigella* burden on a global scale. Based on these studies, the predicted prevalence of *Shigella* in child stool samples exceeded 25% in tropical sub-Saharan Africa, South and Southeast Asia, and limited regions of Central America and northern South America (GBD05). A study evaluating the potential impact and cost-effectiveness of *Shigella* vaccines in LMICs demonstrated a substantial impact, especially in Africa and in countries eligible for support from Gavi, the Vaccine Alliance. The inclusion of *Shigella*-attributable stunting further improved cost-effectiveness and extended the potential benefits to additional geographic regions (GBD28).

Clinical presentation and diagnosis of *Shigella* were addressed in Zambia (GBD09). While the classic shigellosis presentation is blood in stool, other clinical presentations are associated with *Shigella* infections as well, with 58% of the cases with watery diarrhea testing positive for *Shigella* and 68% presenting with mucus. Results from another study (GBD31) corroborated these results, finding that dysentery (bloody diarrhea) had a sensitivity of 8.5% in identifying *Shigella* in children by stool polymerase chain reaction (PCR). This high burden of shigellosis in Zambia also provided an opportunity for a new study to investigate its association with plasma biomarkers of environmental enteric dysfunction (EED), stunting, and the risk of subsequent *Shigella*-associated diarrhea in children (GBD12). These observations from Zambia are consistent with findings from a study in Kenya, Mali, and The Gambia, where *Shigella* was associated with a higher proportion of watery diarrhea compared to cases presenting with dysentery (GBD01).

Across pathogens, bacterial culture remains a critical tool for tracking antimicrobial susceptibility, performing serotyping, and potentiating genomic analyses. A culture optimization study identified buffered glycerol saline as a better transport media for *Shigella* than Cary Blair and highlighted the importance of rapidly transporting (<24 h) samples to the laboratory and maintaining the cold chain (4 °C) in improving recovery (GBD25). Better *Shigella* isolation also facilitates genomic analyses. A recent analysis of isolates from South Africa from 2011 to 2015 described *S. sonnei* and *S. flexneri* 2a as the dominant serotypes, but diverse sub-lineages with distinct patterns of antimicrobial resistance (AMR) and geographic distributions were also found to be circulating (GBD33). In related work from South Africa (GBD11), pathogen yields from hospital-based surveillance studies were significantly higher when molecular methods were used than when routine culture-based diagnostics were applied. The most significant difference in detection rates were noted for *Shigella* spp. (22.7% vs. 0.8%; $p < 0.001$), *Cryptosporidium* spp. (13.2% vs. 3.0%; $p < 0.001$), and *Campylobacter* spp. (11.8% vs. 0.2%; $p < 0.001$) when molecular methods and culture were compared, respectively. Separately, geospatial mapping of *Shigella* serotypes and AMR profiles was carried out using stool samples from Nigerian school children (PRE14). The data suggest continuous community spread of AMR *Shigella* strains, and the identification of focal outbreaks informs targeted interventions. Collectively, these data further highlight the value of more sensitive detection methods in assessing enteric disease burden. In comparative etiology studies from Brazil, the antigenic and virulence features of circulating strains of *Shigella* were assessed as they are important drivers of both vaccine development and uptake (GBD23). This work revealed that *S. sonnei* is the most prevalent species with *S. flexneri* still common, especially in association with shigellosis outbreaks. Moreover, the increasing prevalence of AMR among *Shigella* spp. makes effective treatment even more difficult.

ETEC was a significant pathogen in the first two years of life among children in Lima, Peru, causing one or more diarrhea episodes in 70% of children (GBD03). ETEC-attributable diarrhea (majority ST-ETEC) was high in neonates (10%) and peaked (17%) at 21–24 months of age. The most commonly occurring colonization factors (CFs) were CFA/I, CS12, CS21, CS3, and CS6. ETEC diarrhea was associated with stunting and underweight at the end of two years of follow-up. Similarly, ETEC diarrhea cases showed significantly lower mean weight-age Z-scores in children 6 months to < 3 years old in Cap-Haitien, Haiti (GBD13).

ETEC also contributed to a significant proportion of severe diarrhea cases in children and adults in Bangladesh (GBD04; GBD30). A comparison between ETEC and enteropathogenic *E. coli* (EPEC) diarrhea

showed that both were significantly more frequent in African than in Asian sites (GBD14). Both ETEC and EPEC diarrhea episodes were associated with significant growth faltering in both regions. ETEC was found year-round in two geographically distinct regions in Bangladesh, where approximately 70% of the positive samples came from the surface water, planktons, and sediments with seasonal fluctuations. ETEC isolates showed high resistance to the macrolide antibiotics like erythromycin and azithromycin (ranges of 50–90%), as well as to ampicillin (85.7%) and ceftriaxone (71.4%) (GBD24). In a study to detect ETEC in Zambia, the novel Rapid LAMP-based Diagnostic Test (RLDT) was shown to be sufficiently sensitive (85–100%) and specific (98–100%) compared to quantitative PCR (qPCR) and was easy to implement (GBD19). The detection rate of ETEC (19%) was the same by both assays, with LT-ETEC (9%) being the highest.

There was a reportedly high resistance to the combination antibiotic sulfamethoxazole-trimethoprim of between 82 and 93% from ETEC, *Shigella,* and other *E.coli* isolates in a study in Zambia with about 3.5% of these isolates being multidrug resistant (to more than three antibiotics) (GBD07; GBD08). The prevalence of *Shigella* in Zambia by culture method was 13.6% (GBD10) and incidence of 30.6/1,000 child-years (GBD08), with serotype distribution across sites being 28–56% for *S. flexneri*, 22% for *S. sonnei*, 9% for *S. boydii,* and 7.5–12% for other *Shigella* spp. The incidence of ETEC was 30/1,000 child-years with children aged one to two years having the highest burden (GDB21).

A spatiotemporal cluster identification of areas at risk for specific diarrhea pathogens revealed that Lusaka, Zambia, had a significant Relative Risk of 3.7 of a child having diarrhea compared to other sites, with cases being associated with EPEC (36.8%), ETEC (20%), *Campylobacter coli* (22%), and *C. jejuni* (12%) infections (GBD22). An investigation into other co-infections (GBD29) revealed high prevalence of diarrheagenic *E. coli* (DEC) with EPEC (45%), EAEC (38%), and ETEC (28%) as the highest. Mono infections accounted for 38% of the total cases, while double infections accounted for 61% with 10% having more than four enteric pathogens. DEC exposure was detected in children younger than 12 months of age, indicating a need for early intervention to reduce post-diarrhea sequalae.

In Ghana, the prevalence of enteric pathogens among healthcare staff was evaluated in order to curb potential outbreaks originating from health centers. Of the healthcare workers who were screened, *Salmonella* spp. (6.6%) and *Klebsiella pneumoniae* (3%) were the most common enteric pathogens recovered, highlighting the need for routine surveillance and the importance of compliance with infection prevention and control measures (GBD18).

Samples from children younger than five years of age in India who were hospitalized at three sentinel surveillance hospitals in from April 2017 to December 2020 were tested by qPCR for 16 established etiological causes of diarrhea (GBD17). Results indicated that 48% of the diarrheal hospitalizations were among children aged 0–11 months with adenovirus as the leading cause of diarrhea (28.3%) followed by rotavirus (15.3%), *Shigella* (12%), and norovirus (11%). *Shigella* (22.7%) was the most common pathogen among children older than 23 months. Based on these findings, the rotavirus burden remains significant in India despite the national introduction of rotavirus vaccines.

In more regional studies in northern India, new qPCR-based detection assays for ETEC and *Campylobacter*, along with whole genome sequencing, were used to more accurately assess burden. Overall, ETEC was detected in 4.7% of all acute diarrhea cases tested with 44% being from children younger than 15 years of age (GBD15). Many of these isolates were resistant to the antibiotics ciprofloxacin and ceftriaxone. For *Campylobacter*, whole genome Ribosomal Multilocus Sequence Typing (rMLST) revealed 13 different sequence types that clustered into two clonal complexes for which data suggested a zoonotic source of human infections in this region of India (GBD20). In AMR studies, the continued emergence of multidrug resistant *S. typhi* strains in northern India remains a concern, highlighting the importance of improving typhoid vaccine coverage in India (GBD35).

Non-typhoidal *Salmonella* (NTS) in sub-Saharan Africa are a major cause of bloodstream infections among children and a growing public health concern, particularly in children younger than five years old. Children in Kenya (GBD27) presenting with fever for more than 24 h with or without diarrhea were enrolled in a study, and household contacts of index invasive NTS (iNTS) cases were also studied. Results indicate that the presence of NTS carriers within this study site represent a risk to younger age-groups, highlighting the need for vaccine use in the prevention of NTS cases among infants and children. Similarly, the SAiNTS study in Malawi (GBD32) is a prospective community cohort study designed to measure age-stratified acquisition of lipopolysaccharide O-antigen antibody and serum bactericidal activity to the main serovars causing iNTS (*S. Typhimurium* and *Enteritidis*) in children younger than five years of age. This study is ongoing and will ultimately provide valuable information on the epidemiology of enteric NTS and subsequent acquisition of immunity in children resulting from invasive and asymptomatic NTS disease.

Antibiotic prescribing practices for moderate-to-severe diarrhea (MSD) cases were assessed in the Gambia, Mali, and Kenya (GBD06). Results indicate that antibiotic prescriptions in these countries were associated with signs and symptoms inconsistent with World Health Organization (WHO) guidelines, suggesting the need for better antibiotic stewardship and clinician awareness on diarrhea case management in these settings. Consistent with the overuse of antibiotics in many parts of Africa, studies in Ethiopia (GBD34) and Zambia (GBD07; GBD08) presented concerning AMR data. These data indicate that animal and environmental isolates play an important role in the local dissemination of antimicrobial resistant *E. coli*. In Zambia, studies are underway to determine the prevalence of AMR strains among *Shigella* isolated from children under five years of age. This work complements previous studies documenting a significant level of AMR among ETEC strains recovered from infants and young children with MSD (GBD07).

3. Assessment of the value of vaccines against enteric pathogens

Assessing the value of vaccines and optimizing their impact can help drive investment and future demand, and several studies examined different aspects of potential new *Shigella* vaccines. For example, elevated levels of maternally transmitted antibodies to *Shigella* lipopolysaccharide (LPS) in 6-week-old Zambian infants waned significantly by 14 weeks (AVV11), indicating a potential vaccination window after 14 weeks. Interviews with health experts in Asia and Africa showed that an effective vaccine at 6 to 9 months of age was only considered a moderate priority (AVV01). Another driver of increased prioritization was potential impact on growth stunting. Given the strong correlation between adult height and wage income, the potential productivity benefits of a *Shigella* vaccine that reduces child growth faltering indicated that even a moderately effective vaccine could pay for itself due to productivity gains alone (AVV02).

Typhoid fever and diarrhea caused by ETEC are each responsible for a significant disease burden in children in LMICs and are further exacerbated by emerging AMR, and vaccines could play an important role in addressing these issues. An analysis of blood culture samples from children diagnosed with typhoid fever in Pakistan found that 46.1% of *Salmonella typhi* isolates were multidrug resistant; among children 5 to 14 years old, 44.2% were extensively drug resistant (AVV13). *S. typhi* and *S. paratyphi* resistance was also high in other age groups, and the proportion of resistant isolates rose between 2017 and 2019. When children in Pakistan aged 6 months to 10 years were immunized with a single dose of a typhoid conjugate vaccine it was found that 92.8% had seroconverted (defined as fourfold increase in anti-Vi-IgG) (AVV10). An ETEC vaccine could be beneficial in reducing increasing levels of AMR (AVV12), yet the most advanced vaccine candidate, in Phase 3 clinical trials, will not be available for at least five years. An ETEC vaccine could also help prevent travelers' diarrhea (TD). To address difficulties in measuring ETEC vaccine impact on TD severity and incidence in clinical

trials, a new disease scoring system has been proposed (AVV03) and may serve as a valuable new, more sensitive metric.

Recent approaches to the development of interventions against *Shigella* include a single dose of WRSs1, a live attenuated *S. sonnei* vaccine, which elicited robust antibody responses in serum and ALS (AVV06). These studies indicate multiple doses may be necessary to provide lasting immunity. A second vaccine approach utilizes defined antigens LTA1, the ETEC mucosal adjuvant double-mutant heat-labile toxin (dmLT), and an IpaD/IpaB fusion protein to generate the self-adjuvating vaccine L-DBF (AVV09). This protected mice from lethal challenge with multiple serotypes of *S. flexneri* and *S. sonnei*, indicating potential cross-protection. An alternative strategy is to use monoclonal antibodies (Mabs) for therapy rather than antibiotics. Peripheral blood mononuclear cells from participants in several clinical trials were screened for Mabs that recognize multiple *Shigella* serotypes to identify potentially cross-reactive antibodies (AVV07). These Mabs will be evaluated for the ability to inhibit adhesion and invasion of colonic epithelium.

Evaluation of cholera vaccines in endemic areas is complicated by outbreaks. Studies of volunteers after Shanchol™ vaccination found the vaccine was immunogenic, with vibriocidal antibody titers declining quickly yet remaining elevated above baseline (AVV08). This suggests routine re-vaccination in high-risk areas may be a viable strategy since no empirical evidence supports an ideal time.

4. Preclinical evaluation of vaccine candidates and models of enteric diseases

In vivo and *in vitro* models to elucidate pathogenesis and develop interventions against enteric pathogens are being explored to facilitate vaccine development. The highly human-relevant enteroid mini gut model was used to investigate ETEC and *Shigella* pathogenesis. The role that co-expressed CFs play in ETEC was assessed. Adherence to human enteroids by clinical ETEC isolates expressing CFA/I and CS21 was reduced by deletion of CFA/I but not CS21 and was blocked by anti-CFA/I but not anti-CS21 (PRE01). Enteroids were used to quantify invasion of *S. dysenteriae*, *S. flexneri,* and *S. sonnei* using a gentamicin protection assay and intracellular bacteria were visualized by confocal microscopy (PRE08). The enteroid model was further used to identify the contribution of previously uncharacterized *S. flexneri* genes to gastrointestinal survival and virulence and to identify cellular changes that occur as a consequence of bacterial exposure to small intestine conditions (PRE02).

Antibodies from rabbits vaccinated with a quadrivalent *Shigella* bioconjugate had serum bactericidal activity (SBA) against the four component serotypes as well as cross-reactivity against *S. flexneri* 2b, 4a, and 4b (PRE011).

Residual reactogenicity has hampered advancement of some live attenuated *Shigella* vaccine candidates. Lipid A modifications introduced via ectopic expression of LpxE and/or PagL reduced the toxicity of *Shigella* LPS *in vitro* and *in vivo* (PRE05).

EtpA is a highly conserved adhesin found in diverse ETEC strains. Critical host cell binding epitopes in the C-terminal repeat region were identified by mass spectrometry and cryo-electron microscopy that inform vaccine development strategies (PRE17).

Success has been achieved in developing and refining a *Campylobacter* model in adult mice (>12 weeks of age) fed zinc-deficient diets and treated with antibiotics (PRE07; PRE21). This model exhibits the hallmarks of *C. jejuni* infection (colonization, weight loss, diarrhea associated with mucus and blood, and elevated mucosal inflammatory markers).

ETEC heat-labile (LT) and heat-stable (ST) enterotoxins were found to induce intestinal production of the epithelial alarmin IL-33 (an initiator of the inflammatory cascade) in a patent mouse assay (PRE09) and may present a novel therapeutic target and may help define correlates of ETEC morbidity and immune protection.

Preclinical evaluation of new enteric vaccine candidates is receiving much attention. The multiple epitope fusion antigen (MEFA), uses of a protein backbone fused with multiple other protective epitopes of the targeted pathogen. Given intramuscularly with the adjuvant dmLT, an ETEC MEFA vaccine, MecVax (PRE19), a *Shigella* MEFA (PRE03), and a cholera MEFA (PRE15) were each found broadly immunogenic inducing functional antibodies against vaccine antigens with reduction in intestinal colonization and protection against pathogen challenges.

The yield of *Shigella* IpaB for vaccine use has previously been unsatisfactory but may be improved using an XpressCF + TM cell-free protein synthesis platform with as much as 100-fold increase in yield of immunologically active IpaB (PRE20). Another current approach to broad coverage against *Shigella* is the use of candidates made up of synthetic oligosaccharides mimicking potentially protective determinants carried by the O-antigens of *S. flexneri* 2a and *S. sonnei* (PRE13). For ETEC, a combined formulation containing three representative Class 5 fimbrial adhesins elicited a functional immune response against all of the Class 5 ETEC strains tested (PRE12).

Efforts to identify proteins in GMMAs from non-typhoidal serovars *S. enteritidis* and *S typhimurium* revealed conserved proteins also present in *S. Typhi* with potential to elicit pan-*Salmonella* cross-reactive responses (PRE16). A live attenuated non-transmissible *S. typhimurium* vaccine was well tolerated and initiated robust immune responses against core O-polysaccharide and protected mice against a lethal challenge (PRE10).

5. Vaccine candidates in clinical trials and human challenge models

There are several enteric vaccine candidates in early- or mid-stage clinical evaluation or that are being studied in expanded immunological studies evaluating correlates of protection and duration of immunity. The safety, tolerability and immunogenicity ETVAX®, the most advanced ETEC vaccine, has been evaluated in several clinical trials in Africa. In one study, a high proportion of Zambian adults seroconverted to LTB and, in children (aged 6–9 months), the ¼ dose induced serologic responses to at least three vaccine antigens with no safety concerns (CLT08). This ¼ dose is currently being evaluated for efficacy in 6- to 18-month-old Gambian children, with the last vaccination dose administered in October 2022 (CLT01).

To assess the sustainability of immune responses, serum samples from a subset of participants were collected 200 to 400 days after vaccination with a recombinant CS6-based subunit (CssBA) prototype ETEC vaccine administered intramuscularly with dmLT (CLT03). Increased anti-LT IgG avidity was seen in participants who received 0.5 μg dmLT with no differences in avidity across CssBA doses. Anti-CS6 and anti-LT IgG remained significantly elevated in the long-term samples anti-CS6 compared to baseline samples suggesting ongoing antibody maturation.

Expanded analyses of a controlled human infection model (CHIM) were conducted to evaluate the efficacy of an *S. sonnei* GMMA-based vaccine (1790GAHB) (CLT04). Although the vaccine did not protect against shigellosis, results suggest circulating IgG levels or bactericidal activity are associated with protection against shigellosis, but the quality of 1790GAHB-induced antibodies may differ from those induced by *S. sonnei* infection. In related follow-on studies, an improved four-component formulation of the GMMA vaccine was developed and will be tested in European adults with a subsequent descending-age study planned in Africa (CLT10).

Another *Shigella* vaccine (SF2a-TT15) utilizing synthetic oligosaccharides (OS) covalently linked to a tetanus toxoid was safe and immunogenic in a Phase 1 study in Israeli adults (CLT05). A 10 μg OS dose induced long-lasting (2–3 years) functional humoral responses in the majority of subjects. The valency of a *Shigella* bioconjugate that previously demonstrated evidence of protection in a CHIM with the monovalent formulation target *S. flexneri* has been increased to include

S. sonnei, S. *flexneri* 3a, and 6. This quadrivalent vaccine is being evaluated in a descending-age, dose-escalation study in Kenya (CLT02).

Updated analyses of an *S. sonnei*-rEPA conjugate vaccine in young adults and children identified that IgG anti-*S. sonnei* LPS of at least 4.5 ELISA units corresponded to 52% protection in Israeli children aged 2 to 4 years (CLT12). This association was retained after adjusting for age and population group differences among Israeli children and adolescents. A related analysis of serum antibody results from prior efficacy trials of the *S. sonnei*-rEPA vaccine in Israel also pointed to serum IgG anti-*S. sonnei* LPS threshold antibody levels as a predictor of vaccine field efficacy (CLT14).

A live attenuated, oral vaccine, designated ShigETEC, has been manufactured to produce protection against ETEC and *Shigella* via a toxoid fusion protein to induce protective antibody responses in a serotype-independent manner due to the lack of O-antigen expression, enabling a response to minor shared and conserved antigens, respectively (CLT13). ShigETEC was evaluated in Hungarian adults and was well-tolerated up to a $5x10^{10}$ CFU/dose. CHIM studies with *Shigella* and ETEC strains are planned.

The Vi-tetanus toxoid conjugate vaccine (Vi-TT) against typhoid fever was evaluated in a cluster-randomized, double-blind trial in Bangladeshi children aged 9 months to 16 years (CLT07). Overall efficacy was 56% after 24 months with an additional 24 months of follow-up planned. These data will help guide efforts to determine the timing of a booster dose. A typhoid conjugate vaccine manufactured by EuBiologics (EuTCV) is currently being evaluated in single- and multi-dose presentations in a Phase 3 study (CLT15).

Researchers evaluated the motivating factors and experiences of CHIM participants and found the primary factors for participation were money, positive staff interactions, and food (CLT11). Additionally, most volunteers enjoy participating, discovering new things about their health status and did not feel that their participation negatively affected their well-being. Consideration of these factors is important to ensure a continued ability to successfully recruit CHIM participants.

6. Host parameters and genomics that predict responses to infection and disease

The LT enterotoxin plays an important role in intestinal inflammation. ETEC infections have been linked to stunting and malnutrition, conditions that may be driven by inflammation. Challenge of human volunteers with an LT + ETEC strain was followed by significant increases in inflammatory mediators demonstrating that infection with LT-producing strains leads to significant inflammation independent of acute presentation (GEN01). This observation is consistent with field data suggesting that even asymptomatic infection with ETEC strains producing LT toxin can cause significant intestinal inflammation and contribute to EED and stunting. In additional laboratory-based studies, LT was found to modulate many genes required for biogenesis of the brush border, the major absorptive surface of the small intestine. Infant mice challenged with toxigenic wild-type ETEC (but not nontoxigenic mutants) developed enteropathic changes in the surface of the small intestine, while maternal vaccination with LT generated antibodies that protected infant mice from enteropathy. This suggests that an effective ETEC vaccine could prevent both acute illness as well as long-term morbidity (GEN02).

Biomarkers are important for assessing enteric infection severity and impact. In continuing efforts to identify potential biomarkers of disease severity, recent ETEC CHIMs data indicate that plasma levels of proguanylin decreased significantly from baseline in volunteers experiencing severe diarrhea, potentially offering a biomarker for disease severity following infection with ST-producing ETEC (GEN06). In related field studies examining biomarkers of cholera severity, blood group O has been associated with higher risk of severe cholera disease. Individuals from a cholera-endemic area in Zambia were vaccinated with two doses of Shanchol™ and tested to determine their HBGA and secreta status in saliva. No significant differences in seroconversion status were observed among secreters compared to non-secreters or among those with blood group O verses non-O (GEN03), suggesting ABO blood group may not modulate immune responses to oral cholera vaccine. In novel psychomotor assessments, sleep and acute infectious diarrhea were examined for their impact on vigilance performance in a CHIM with ETEC. Interestingly, Illness severity, independent of sleep loss, negatively impacted performance which may have important implications for individuals infected with ETEC in military operational settings, as well as in civilian workplace and educational environments (GEN09).

Improved understanding of immune responses to ETEC infection and immunization is important for optimal vaccine development. ETEC infection or vaccination induces T follicular helper (Tfh) cell responses in peripheral blood manifesting primarily as a Th17 phenotype, consistent with strong IgA antibody promoting capacity. The possibility to monitor Tfh responses in peripheral blood provides new possibilities to study germinal center reactions in diarrheal disease patients that may enable a better assessment of immune markers for protective ETEC immunity (GEN05). Similarly, new studies in five children's hospitals in Zambia will help improve understanding of immune responses to key vaccine target antigens in children naturally infected with *Shigella*. This work should provide helpful insights into the burden of shigellosis in Zambia, as well as serum markers of immune protection and/or reduced risk of *Shigella*-associated illness to help drive vaccine development (GEN08).

Lymphostatin, a virulence factor of EPEC and non-0157 serogroup EHEC, inhibits anti-CD3 and anti-CD28 activated proliferation of CD4 + and CD8 + T cells and blocks synthesis of IL-2, IL-4, IL-5, and IFN-γ without affecting cell viability. This inhibition was not observed in T cells activated by phorbol 12-myristate 13-acetate and ionomycin, indicating that lymphostatin targets T cell receptor signaling. From analysis of CD69 expression, lymphostatin seems to suppress T-cell expression. This suppression appears to be related to a general blunting of cellular phosphorylated kinases (GEN04). These data provide insights into the mode of action of this novel bacterial virulence factor from EPEC and EHEC on host immunity.

The immunological basis for the broad protection provided in Finnish travelers to Benin by the oral ETEC vaccine candidate ETVAX needs to be better understood. To investigate this observation further, an ETEC proteomic array, developed by Antigen Discovery Inc., was used to show that volunteers without severe TD had broader IgA/IgG responses to vaccine antigens in general and more specifically to class 5 fimbriae not present in ETVAX but sharing antigen epitopes with the CFA/I vaccine component. These results suggest that broader class 5 fimbrial antibody responses may be associated with a decreased risk of ETEC diarrhea (GEN07).

7. Application of new omics technologies for characterization of emerging pathogens and host responses

Several studies have been conducted using new genomic and proteomic approaches for characterizing host responses to bacterial enteric pathogens and identifying correlates of protection and novel candidate antigens. One study employed a novel mutiplexed assay to compare *Shigella* antibodies in children from endemic regions with subjects from a CHIM study (NOT02). A similar study used a systems serology approach to define humoral correlates of protection from CHIM study subjects (NOT03). Transcriptional profiling of various ETEC strains revealed expression patterns associated with particular metabolic states as well as candidate cell surface proteins with potential to stimulate broad protection (NOT06). Finally, the well-established TaqMan Array Card platform was used to analyze samples from an ETVAX TD trial and serve as a more sensitive method than traditional culture to identify ETEC from clinical samples (NOT13).

High-throughput analysis of a large amount of sequence data

estimated the extent that CFs are shared across other *E. coli* and related species (NOT08). Hidden Markov Models have been used to determine the spread of ETEC CFs. Phylogenetic analyses were employed to describe the prevalence, distribution, and relatedness of CFs across bacterial species. Mutli-locus variable repeat analysis patterns from recent toxigenic *Vibrio cholerae* O1 Nigerian patient isolates demonstrated a *founder flush* pattern with the founder genotype giving rise to multiple other genotypes as the outbreak expands and spreads over time and space (NOT09). Large-scale BLAST score ratio and phylogenomics analysis demonstrated that CCH060 (*Sf6*) was the most genomically distant and exhibited the greatest amount of unique genomic features among three archetype *Shigella* isolates, 2457 T (*Sf*2a), J17B (*Sf*3a), and CCH060 (*Sf*6) (NOT10). Pathogenomic analysis of *Shigella* isolates from South Asia and sub-Saharan Africa revealed *S. sonnei* and protein antigen candidates (relative to other species and serotype-specific targets) to be conserved vaccine targets and identified convergent evolution of resistance against ciprofloxacin (NOT11). Moreover, serotype switching in *S. flexneri* was observed, which may lead to immune escape from O-antigen based vaccines.

Proteome microarray technology can be used to characterize the host antibody response to diarrheal pathogens. In a Zambian pediatric trial of ETVAX, pan-diarrheagenic *E. coli* proteome microarrays were used to elucidate cross-reactive IgG responses against non-vaccine class 5 fimbriae proteins that may provide protection from ETEC and other pathotypes (NOT01). Two *Campylobacter jejuni* studies utilized protein microarrays. One examined IgY responses in immunized breeder chickens and their broiler offspring (NOT05), and another analyzed IgA and IgG responses in a *C. jejuni* CHIM (NOT07). Both studies identified significant antibody reactivity against *Campylobacter* outer membrane and flagellar proteins, the former highlighting the benefit of passive antibody transfer and the latter the variation in human responses to different strains. The mucosal antibody response in understudied biological specimens such as colostrum and breast milk was also explored utilizing a multipathogen protein microarray to characterize the breadth and magnitude of secretory IgA and IgG responses in human milk (NOT04). Antibodies were associated with reduced risk of infection from rotavirus A, *Shigella,* and adenovirus 40/41 in the breastfeeding infants of mothers from Bangladesh. These early studies lay the groundwork for future vaccine antigen discovery.

8. Novel adjuvants, vaccine delivery platforms, and immunization strategies

A study was conducted on using pre-exposure prophylaxis as a way to provide protection from ETEC (ADJ04). Heavy chain nanobodies which targeted ETEC adhesins or CFs were fused with IgG to generate nanobody molecules which demonstrated cross-protective potency against eleven major pathogenic ETEC strains *in vitro* and demonstrated a significant reduction in bacterial colonization in animals.

Photochemical inactivation is being studied as a novel platform for killed whole cell vaccines (ADJ05). Unlike formalin inactivation, which is known to induce chemical modifications of proteins which can affect antigenicity, photochemical inactivation was shown to reproducibly yield inactivated bacteria which were able to generate equivalent or greater IgG levels compared to formalin-killed bacteria in mice as well as potentially expanding the antigenic repertoire presented to the immune system.

Using a Multiple Antigen Presenting System (MAPS), four *Shigella* MAPS vaccines were developed, for *S. flexneri 2a, 3a, 6*, and *S. sonnei,* using a *Shigella* surface protein as a carrier in combination with OSPs (ADJ08). *In vitro* evaluation of the quadrivalent *Shigella* MAPS vaccines demonstrated equivalent immunogenicity to each of the monovalent vaccines, generating high titers of functional antibodies against each type O antigen.

Another study examined the effect of HIV status, viral load, and CD4 count had on the immune response to oral cholera vaccine (Shanchol™) (ADJ01). Participants who were HIV-positive, had high viral load, and low CD4 counts showed reduced immunogenicity to Shanchol™, potentially affecting the protective efficacy of the vaccine. Further studies are needed to inform appropriate policy and practice for cholera vaccination.

In a study on the role that immunization route played in T-cell responses to an ETEC vaccine candidate, mice were immunized with dmLT and CssBA by intramuscular or sublingual delivery (ADJ07). Sublingual immunization was the primary source of IL-17 T-cell responses, confirming that this cytokine response was route-specific, while IL-2-secreting T cells were observed after both routes of immunization.

A reduced protein concentration formulation of the dmLT adjuvant was evaluated in order to reduce waste and the complexity of mixing with vaccines for clinical study (ADJ03). The dmLT concentration was reduced 50-fold from 1 mg/mL to 20 μg/mL, and 0.05% polysorbate 80 (PS80) was added while keeping the other excipients constant. The freeze-dried formulation was shown to have improved stability under accelerated heat stress at 40 °C for four weeks.

The comparability of *in vitro* and *in vivo* evaluations of dmLT were explored (ADJ06). Stable dmLT formulations were subjected to stress and then were evaluated in animals. Both stable and stressed dmLT samples were co-formulated with ETEC or inactivated poliovirus vaccines and administered to mice via intradermal or sublingual immunization. Vaccine efficacy was affected more by the immunization route and vaccine antigen than the predicted *in vitro* stability of dmLT. While biochemical methods are necessary for product characterization, they are not necessarily predictive of vaccine outcome. Both biochemical and animal model testing are necessary when optimizing the vaccine formulation.

9. Strategies for combination/co-administered vaccines

The future commercial success and uptake of new enteric vaccines may be improved by combining or co-administering vaccine antigens to protect against multiple pathogens at the same time. There are no licensed vaccines available for use against either NTS and *Campylobacter* species. A trivalent outer membrane vesicle (OMVs)-based immunogen was immunogenic and protective against both NTS and *Campylobacter* in a mouse model (CMB01). GMMA are outer membrane exosomes from Gram-negative bacteria that have been used as a “plug and play” technology for the development of effective multicomponent vaccines (CMB02). A combination of iNTS-GMMA and Vi-CRM197 that could result in an effective and affordable vaccine, iNTS-TCV, may be a viable option for a sustainable iNTS vaccine in sub-Saharan Africa (CMB03). Optimal batches of the iNTS conjugate and the increase in antibody titer induced was found to be dependent on the conjugation method used. This trivalent iNTS/typhoid vaccine will be tested for toxicology in other animal models (PRE06).

10. Conclusion

The plenary content and poster presentations from the 2022 VASE Conference summarized here provides a snapshot of the extensive research currently taking place around the globe in the enteric vaccine field. Topics ranged from the burden of disease and assessing the value of vaccines to preclinical and clinical evaluations of vaccine candidates, and included research on predicting responses to infection and disease and characterizing host responses, novel adjuvants, and strategies for combination vaccines.

Several themes emerged through the conference presentations, highlighting areas to prioritize for future research in the field. It is clear that enteric diseases remain a substantial public health burden in low-resource settings, and new vaccines, diagnostics, and other interventions are urgently needed. Innovative approaches to improving vaccine efficacy and delivery are likely to increase uptake of new enteric vaccines when they become available. In addition, vaccines against a

broader scope of pathogens, potentially given as combinations, will help address diarrhea and AMR more effectively. These presentations and subsequent discussions at the 2022 VASE Conference should help to accelerate the development and future introduction of vaccines against *Shigella*, ETEC, *Campylobacter*, non-typhoidal *Salmonella*, and other neglected enteric pathogens.

Funding

This work was supported by the Bill & Melinda Gates Foundation [INV-000875]. Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the Author Accepted Manuscript version that might arise from this submission.

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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

This is a conference report and does not contain original data.