Systemic and local immune responses to *Salmonella enterica* serovar Typhi by prime boost strategy with different preparations of *Salmonella* antigen(s)

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Certificate

This is to certify that the thesis entitled **"Systemic and local immune responses to *Salmonella enterica* serovar Typhi by prime boost strategy with different preparations of *Salmonella* antigen(s)"** submitted by Lovely Barai, Registration no.-137/2013-2014, Re-registration no.-166/2017-2018, carried out her research under our supervision. This is further to certify that it is an original work and the style and contents of the thesis have been approved and recommended for the fulfillment of the degree of Doctor of Philosophy (Ph.D.) in Microbiology, University of Dhaka.

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Declaration

I do hereby declare that the work submitted as a thesis entitled **"Systemic and local immune responses to *Salmonella enterica* serovar Typhi by prime boost strategy with different preparations of *Salmonella* antigen(s)"** to the Department of Microbiology, University of Dhaka for the degree of Doctor of Philosophy (Ph.D.) by the results of my own investigations and carried out under supervision of Prof. Dr. Chowdhury Rafiqul Ahsan and Prof. Dr. J. Ashraful Haq. The research work has not previously been submitted for any degree.

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Abstract

Typhoid fever, caused by *Salmonella enterica* serovar Typhi (*Salmonella* Typhi), is an important medical and public health problem in South East Asia including Bangladesh and may be prevented and controlled by vaccines. Two existing licensed vaccine namely parenteral Vi CPS and oral Ty21a for typhoid are modestly effective having 3 year cumulative efficacy of 51-55%. Though immune response to the Vi capsular polysaccharide (Vi CPS) vaccine confers protection against *Salmonella* Typhi, but it is non immunogenic in children below 2 years of age, provides protection only for 3-5 year and unable to induce booster effect. In view of the above, the present study investigated the effect of prime boost method using Vi CPS in combination with different salmonella antigens on the anti-Vi antibody response in mice model. Also, the immunogenic antigens (s) as well as humoral immune responses to Vi CPS and to other structural components of *Salmonella* Typhi were evaluated in human.

Six groups of BALB/c mice were immunized with Vi CPS in combination with Vi CPS, Vi-TT, killed whole cell (KWC) antigens through different routes. Sera from a total of 70 culture confirmed typhoid and 25 healthy/diseased cases were included in the study. Six adult human volunteers were enrolled and immunized with single dose of Vi-CPS vaccine. Anti-Vi IgM, IgG and IgA antibody secreting cell (ASC) in spleen and Peyer's patch (PP) of mice were estimated by ELISPOT assay and in serum by ELISA on 28th day of immunization. Production of functional antibodies in mice and human typhoid cases was measured by colorimetric serum salmonellacidal assay. *S.* Typhi surface, envelop and whole cell proteins were extracted by water extraction method (WEM), Tris-Sucrose-EDTA (TSE) buffer and sonication method respectively and analyzed by SDS-PAGE and Western blot (WB) methods. Antibodies to surface, envelop and whole cell extract (WCE) proteins in human sera from typhoid and control cases were measured by ELISA method.

In mice, anti-Vi IgM, IgG and IgA producing ASCs were detected in spleen following prime boosting with KWC-Vi and ViTT-ViTT antigens whereas no anti-Vi IgG ASC response was observed following prime-boosting with Vi antigen alone. Anti-Vi IgM, IgG and IgA producing ASCs were found significant ($p<0.05$) in PPs in groups primed with KWC followed by boosting with Vi in comparison to other groups.

Serum Ant-Vi IgG antibody titer response was found significantly high ($p<0.05$) in groups immunized with ViTT-ViTT and KWC-Vi antigen combinations. No significant ($p>0.05$) difference of anti-Vi ASC response was detected in spleen (except IgM), PPs and in serum when heterologous antigen (KWC-Vi) was given in similar (i.p-i.p) or alternative (oral-i.p) routes. Salmonellacidal antibody (bactericidal titer) response was observed in low titer (15±3.3) in sera of BALB/c mice immunized with KWC- Vi (i.p-i.p) and ViTT-ViTT (i.p-i.p) combinations. Similarly, very low salmonellacidal titers (2.5±1.5 and 2.3±1.5) were also detected 14 and 21 days after single dose of Vi CPS vaccine among the human volunteers. However, sera from typhoid and paratyphoid A patient showed significant ($p<0.05$) levels of salmonellacidal antibody titer (549.9±108.5 and 528.7±187.3) compared to control population (0.133±0.1). Moreover, salmonellacidal titer increased significantly ($p<0.05$) in samples collected between 7 to 10 days and between 10 to 25 days of fever (titer 535.7± 119.2 and 794.6± 235.6) compared to samples collected from cases having fever for less than 7 days (Mean titer 136.4± 52.7). The mean titer significantly ($p<0.05$) decreased to 5.5± 2.1 after 6-8 weeks onset of illness. Mean titer of anti-Vi IgG antibody of post vaccinated sera (titer 1050 ± 530.5) was 3.4 fold high from pre vaccinated sera (titer 312.5± 129.2) though it was not statistically significant ($p>0.05$). Anti-Vi IgG antibody titer response of pre and post vaccinated human sera exhibited some extent of correlation with bactericidal titer in our study (spearman's correlation coefficient test $r=0.6$, $p<0.05$). Anti-Vi IgG antibody response (>cut off OD 0.41 at 1:100 serum dilution) was positive in 67.5% (46/70) and 77.8% (7/9) of acute and convalescent typhoid cases, respectively, compared to 52% (13/25) in control cases. IgG antibody against *S*.Typhi surface, envelope and whole cell proteins was positive in all (100%) acute and convalescent typhoid cases compared to 30.8% to 55.6% in control participants. Mean IgG antibody levels against *S*.Typhi surface, envelope and whole cell proteins were significantly raised ($p<0.05$) in acute stage of typhoid fever and persisted during convalescent period (after 6-8 wks) compared to individuals in healthy and diseased groups. Water extracted surface protein of *S*.Typhi showed similar SDS-PAGE pattern to those of envelope protein extracted by Tris-sucrose-EDTA buffer. A total of ten (10) protein components, *viz*, 180, 100, 82, 66, 58, 50, 41, 37, 33, 25 kDa were common in surface and envelope proteins and three additional proteins (126 kDa, 35 kDa, 21 kDa) were also identified

in whole cell extract of *S.*Typhi. Seven (7) out of 10 surface protein of *S.* Typhi (i.e. 180, 100, 82, 66, 58, 50, 37 kDa) were immunoreactive by Western blot analysis using sera from typhoid cases. Also, the 58 kDa and 37 kDa surface proteins of *S.*Typhi showed most frequent (92% and 80%) immunoreactivity.

The results of the present study showed that prime boost strategy could be applied to get enhanced systemic and mucosal anti-Vi antibody responses using Vi CPS antigen in combination with KWC. The method was able to stimulate secondary booster effect of T independent plain Vi polysaccharide antigen. High salmonellacidal antibody detected in early period of typhoid fever could be a promising serological marker for S. Typhi acute infection. Highly immunoreactive surface or envelope protein of *S.* Typhi could be used as potential vaccine antigen or diagnostic marker.

Contents

List of Tables

List of Figures

List of Abbreviations

%	Percentage
µl	Microliter
37ºC	37 degree celsius
58kDa	58 kilo Dalton
ASC	Antibody secreting cell
ANOVA	Analysis of Variance
AP	Alkaline phosphatase
APC	Antigen presenting cell
BADAS	Diabetic Association of Bangladesh
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders.
BALB/c	albino, laboratory-bred strain of the house mouse
BSA	Bovine serum albumin
BCIP	5-bromo-4-chloro-3'-indolyphosphate
BCR	B cell receptor
BM	Bone marrow
CDC	Center for Disease Control and Prevention
CVD 908, CVD 908-htrA, CVD 909	*S*. Typhi vaccine candidates designated "CVD 908," "CVD 908-htrA," and "CVD 909,"developed at the Center for Vaccine Development, University of Maryland, based on attenuation of *S.*Typhi by deletions in the aroC, aroD, and htrA genes.
CFU	Colony forming unit
C3	Complement component 3
DEA	Diethylamine
DAB	3, 3′ diaminobenzidinetetrahydrochloride
DC	Dendritic cell
ERC	Ethical review committee

ELISA	Enzyme-linked immunosorbent assay
EIA	Enzyme immune assay
ELISPOT	Enzyme-linked immunosorbent spot
ENVP	Envelope protein
EDTA	Ethylenediaminetetraacetic acid
FAE	Follicle associated epithelium
FCS	Fetal calf serum
FDC	Follicular dendritic cell
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GALT	Gut associated lymphoid tissue
GC	Germinal center
GMT	Geometric mean titer
HRP	Horseradish peroxidase
HBSS	Hanks' Balanced Salt Solution
H_2S	Hydrogen Sulfide
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
ICOSL	Inducible T Cell Costimulator Ligand
ICPs	Immunological correlates of protection
IFN	Interferon
ILAR	Institute for Laboratory Animal Research
IL	Interleukin
i.p.	Intraperitoneal
iNOS	Inducible nitric oxide synthase
kb	Killobase
KIA	Kligler Iron agar
KWC antigen	Killed whole cell antigen
LMICs	Low and middle income countries
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MDR	Multidrug resistant

MHC	Major Histocompatibility Complex
mM	Millimole
nm	Nanometer
n.s.	Not significant
NARST	Nalidixic acid resistant *S*.Typhi
NTC	Neo tetrazolium chloride
NaCl	Sodium Chloride
NOD	Nucleotide-binding oligomerization domain
o	Oral
OD	Optical Density
OM	Outer membrane
Omp	Outer membrane protein
PAMP	Pathogen associated molecular patterns
P-B strategy	Prime boost strategy
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline solution with 0.05% Tween 20
PCR	Polymerase chain reaction
PPs	Payer's patches
PHS policy	Public Health Service Policy on Humane Care and Use of Laboratory Animals
pNPP	*para*-Nitrophenylphosphate
PRRs	Pattern recognition receptors
RPMI	Roswell Park Memorial Institute Medium
RPM	Revolutions per minute
ROS	Reactive oxygen species
RCF	Relative centrifugal force
S. enterica	*Salmonella enterica*
***S*. Typhi**	*Salmonella enterica* serovar Typhi
SBA	Serum bactericidal assay
SCV	*Salmonella*-containing vacuole
SSA	Serum salmonellacidal assay

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	Standard error
SEM	Standard error of mean
SPIs	*Salmonella* pathogenicity islands
SURP	Surface protein
TCV	Typhoid conjugate vaccine
TI type-2	T indipendent type-2
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TSB	Tryptic soy broth
TSE buffer	Tris-Sucrose-EDTA buffer
TSI	Triple sugar iron agar
TNF	Tumor necrosis factor
T1SS	Type 1 secretion system
T3SS	Type 3 secretion system
USDA	United States Department of Agriculture
Vi-PS	Vi polysaccharide
Vi CPS	Vi capsular polysaccharide
V-TT	Vi-tetanus toxoid
Vi-DT	Vi-diphtheria toxoid
Vi-rEPA	Vi-nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A
WCE	Whole cell extract
WEM	Water extraction method
WHO	World Health Organization

Chapter One

Introduction & Objectives

1.0 Introduction

Typhoid fever, caused by *Salmonella enterica* serovar Typhi (*Salmonella* Typhi), is an acute illness with a worldwide incidence rate of 154.0 cases per 100,000 population per year (Marchello et al., 2019). Global estimates of disease burden range between 11-21 million typhoid cases with approximately 128,000–161,000 deaths annually (W.H.O., 2018). Incidence of typhoid fever in low and middle-income countries (LMICs) was 17.8 million cases per year (Antillon et al., 2017). Bangladesh, Pakistan, India accounts for 85% of global typhoid cases (Maurice, 2012). It is an important communicable disease in children and is a public health problem especially in monsoon and summer seasons in Bangladesh (Naheed et al., 2010; Saha et al., 2001).

Control of typhoid fever requires an effective vaccine and public health measures. The importance of vaccine against typhoid fever has been emphasized by World health organization (W.H.O., 2017; W.H.O., 2008; W.H.O., 2000). Early killed parenteral whole-cell vaccines (T.A.B.vaccine) provided broad protection against both *S.* Typhi and Paratyphi A, B but associated with some local and systemic adverse reaction. At present, only two licensed vaccines are commercially available for typhoid fever; a subunit, Vi polysaccharide (parenteral, 25µg, single dose) and a live attenuated Ty21a (oral, 3-4 dose). Newer typhoid conjugate vaccines (Vi-rEPA, Vi-TT, Vi-DT) are at varying stages of development and use (Milligan et al., 2018).

Vi polysaccharide (Vi CPS) vaccine has certain drawbacks. It is non immunogenic in children below 2 yrs of age and vaccine-induced immunity tends to wane over time (Sahastrabuddhe and Saluja, 2019; Kossaczka et al., 1999). This vaccine is unable to induce booster effect as unconjugated Vi CPS is a T cell independent antigen (W.H.O., 2017). Subsequent re-vaccination may even cause hypo-responsiveness as is seen with meningococcal C and pneumococcal polysaccharide vaccines (Siegrist, 2018). The oral live attenuated Ty21a vaccine is recommended for children above 5-6 years of age, available in both liquid as well as enteric coated capsules. This vaccine is highly acid labile and hence stomach acidity has to be either neutralized or bypassed when Ty21a is to be fed orally (Marathe et al., 2012; Tran et al., 2010). The three year cumulative protective efficacy of two doses of killed whole cell vaccine,

three doses of Ty21a and Vi vaccine was 73%, 51% and 55% respectively (Milligan et al., 2018; Engels et al., 1998).

Many investigations are being undertaken to develop typhoid vaccine with increased immunogenicity and long lasting immunity. Major challenges are to enhance cellular as well as humoral immune responses. In last decade, several novel strategies have been explored to augment the immunity against the bacterial infection. It includes - increase dose of vaccine, use of new adjuvant or conjugate vaccine, development of new vectors, new vaccine formulation and delivery system using liposomes, proteosomes etc. (Wallis et al., 2019). One particularly promising approach is the prime boost strategy (Lu, 2009; Woodland, 2004). An effective vaccine usually requires more than one time immunization in the form of priming with first dose followed by boosting with subsequent dose. Traditionally the same vaccines are given multiple times as homologous boosts. Repeated immunization with the same vaccine results in higher levels of antibodies than single administration. Such homologous boosting is sufficient for organisms for which the protective immune response is dependent on humoral immunity (Example- DPT vaccine, Hepatitis B vaccine, oral polio vaccine etc). However, repeated homologous boosting with the same vaccine does not necessarily result in an increase in the magnitude of the cellular immune response, as pre-existing host immunity inhibits antigen presentation (Ly and McMurray, 2008). Prime boost (P-B) strategies can be used with different types of vaccines containing a common antigens (Sadlier et al., 2016; Lu, 2009; Dunachie and Hill, 2003). Alternating routes for delivery of the priming and booster doses have also examined. The strategy includes mucosal priming with vaccine antigen followed by parenteral boosting in respiratory virus infections (Gaspari, 2011). In many cases such heterologous prime-boost was found more immunogenic than homologous prime-boost. Heterologous prime boost (P-B) strategy represents a new way of immunization. The key strength of this strategy is that greater levels of immunity are established than that can be attained by a single vaccine administration or homologous boost strategies (Woodland, 2004). The prime boost (P-B) approach was applied to development of vaccine against variety of disease such as pneumococcal infection, malaria, tuberculosis and HIV infection. (Sadlier et al., 2016; Lu, 2009; Goonetilleke et al., 2003; Dunachie and Hill, 2003). This approach was employed to improve the immune response of pneumococcal polysaccharide vaccine in HIV and rheumatoid

arthritis patient (Bahuaud et al., 2018; Sadlier et al., 2016). Improved immunity was achieved by priming with pneumococcal glycoconjugate and boosted with plain polysaccharide. This strategy was also applied to improve immune response against *Salmonella* in different animal model. Priming with attenuated strain of *S.* Typhimurium (S. Typhimurium ΔznuABC) and boosted with inactivated *S.* Choleraesuis in a group of piglets was able to limit weight loss, fever and organs colonization, arising from infection with virulent *S.* Choleraesuis, more effectively, than the prime boost (P-B) vaccination with homologous *S.* Choleraesuis inactivated vaccine group (Alborali et al., 2017). Prime boost (P-B) vaccination approach was also used in *Salmonella enterica* serovar Gallinarum (SG) ghost vaccine candidate strain to optimize immunity and protection efficacy against fowl typhoid. The prime immunization with the SG ghost vaccine induced T helper type 1 (Th1) and the booster elicited both T helper type 1 (Th1) and T helper type 2 (Th2) lymphocyte mediated immune responses (Won et al., 2016). A randomized, double-blind, heterologous prime boost (P-B) clinical study conducted in human found higher and persistent anti-Vi IgG and IgA response following priming with CVD 909 and boosting with Vi compared to Vi priming recipients only (Wahid et al., 2011).

Human immune responses to *S.* Typhi involve innate and antigen specific humoral and cellular immune responses. The relative contribution of humoral arm of the immune system is still under investigation. Many large field trials have demonstrated that *S.* Typhi specific antibodies are produced in a majority of subjects following natural illness and vaccination (Lindow et al., 2011). Epidemiological, animal and human studies indicate that antibodies play an important role in eliminating extracellular *Salmonella* while specific T cells are important for clearance of intracellular bacteria (MacLennan, 2014; Siggins et al., 2014; Gondwe et al., 2010; MacLennan et al., 2008). *Salmonella* are vulnerable to antibodies at distinct point of the invasion cycle: following initial invasion, when first entering the circulation and during transiting from one phagocyte to another via the blood or extracellular fluids (MacLennan et al., 2014). Antibodies kill extracellular microorganisms through complement-mediated bacteriolysis, opsonophagocytosis or antibody dependent cellular cytotoxicity (Mitchell et al., 2014). Protection of most current vaccines is now based on induction of antibodies in serum or on mucosa that interfere infection or invasion of bloodstream by *S.* Typhi. It is important to determine that the vaccine-

induced antibodies are capable of conferring protective immunity to the host and also to establish that these antibodies can operate in such a manner *in vivo.* Impairment of antibody function cannot be ascertained by traditional serological methods that rely only on binding with an immobilizing the antigen. Functional assays are robust tool to predict vaccine-induced protection (Ndungo and Pasetti, 2020). The estimation of functional antibody responses after vaccination is also recommended by the World Health Organization Expert Committee on Biological Standardization (W.H.O., 2017). Function of antibodies mounted against bacterial pathogens rather than virus and toxin has been particularly challenging. Moreover, documentation of functional capacities of antibody in addition to their antigen recognition capacities is very essential. Opsonophagocytic antibodies have been used for detection of immunogenicity of pneumococcal polysaccharide vaccines whereas bactericidal antibody has been associated with protective immunity against meningococcal or *Vibrio cholera* infection. Capacity of serum antibodies from patients with shigellosis to promote bactericidal activity with the help of complement were also demonstrated (Ndungo and Pasetti, 2020; Plotkin and Plotkin, 2008). Studies should be done to establish the functional role of antibodies in clearance of *S.*Typhi following typhoid infection or vaccination. Enzyme-linked immunosorbent assay (ELISA) and Widal test are widely used to quantify antibodies against *S.* Typhi in serum but that does not provide information about their bactericidal capacity. Salmonellacidal antibody response can be used to assess vaccine induced bactericidal and protective capacity (functional) of antibody against *S.* Typhi (Jang et al., 2016; Boyd et al., 2014; Pulickal et al., 2009). Greater understanding of the immunogenic component of *S.* Typhi may help to facilitate the development of improved vaccine. Antibody response to Vi capsular polysaccharide (Vi CPS) can be acquired naturally during repeated exposure of *S.* Typhi in an endemic area or it can be acquired after typhoid fever or Vi based typhoid vaccination. Vi CPS antibody response is very important because it is protective and relatively well characterized in typhoid fever (House et al., 2008). Protective action of Vi is mainly mediated by serum anti-Vi IgG antibodies (Szu et al., 2014). Serum antibody responses to the LPS (O) and flagellar (H) antigens of *S.* Typhi currently used as major serological diagnostic marker, were highly variable among individuals having typhoid infection, and among healthy subjects from the community (House et al., 2001). However, human humoral immune

response to surface and envelope protein of *S.* Typhi has been less well studied. Surface proteins allow bacteria to adhere to host cells and tissues, to invade non-phagocytic epithelial and endothelial cells, to form biofilm and to evade immune responses (Scott and Barnett, 2006). Antibody responses directed against surface antigens is therefore, important for effective protection against *S.* Typhi. Studies suggested that surface associated model antigens might have intrinsically higher immunogenicity compared to internal model antigens (Barat et al., 2012). So, recognition of surface associated antigens that are involved in initial pathogenesis of *S.* Typhi is necessary to combat *S.* Typhi. A few studies have reported the immunogenicity of surface associated antigens using positive sera of the typhoid patients by Western blot assay. (Chin KL et al., 2015; El-Gayar et al., 2013).

Therefore, there is a need to explore the ways to improve immune responses of existing typhoid vaccine and to identify putative protective antigen (s) of *S.* Typhi. So, this study attempted to examine the homologous and hetelogous prime boosting approach of immunization to modulate local and systemic *Salmonella* anti-Vi humoral immune responses. Salmonellacidal (functional capacity) antibody response was examined after prime boost (P-B) vaccination and natural infection. Immunoreactive antigen (s) and antibody responses to Vi CPS, surface, envelop and cellular proteins of *S.* Typhi were also analyzed in typhoid cases.

1.1 Objectives

The objectives of the study were to:

1. determine the effect of prime boost (P-B) immunization method on the systemic and local humoral immune response to *Salmonella* Typhi Vi capsular polysaccharide (Vi CPS) antigen using Vi CPS in combination with different antigens and routes in mice model.
2. determine the salmonellacidal antibody response by prime boost immunization method using Vi CPS in combination with different antigens and routes in mice.
3. determine the salmonellacidal antibody response in typhoid cases and in human volunteers after vaccinations with Vi CPS.
4. evaluate the the humoral immune responses to Vi CPS and to other structural components of *Salmonella* Typhi in typhoid cases.
5. identify specific immunogenic antigen (s) of *Salmonella* Typhi using sera from typhoid cases by Western blot analysis.

Chapter Two

Literature Review

2.0 Review of Literatures

2.1 The *Salmonella* bacterium

2.1.1 Nomenclature

Salmonella is named after Daniel Elmer Salmon, an American veterinary surgeon, who first isolated *Salmonella choleraesuis* in 1884 together with Theobald Smith from porcine intestine (Nyirenda, 2015; Su and Chiu, 2007). The organism was originally called "Bacillus choleraesuis". Subsequently in 1900 it was changed to "*Salmonella choleraesuis*". Members of Salmonellae organisms have a high genetic similarity and are differentiated by their serotyping results. The classification of *Salmonella* serotypes (Kauffman and White) is based on antibody interactions with surface antigens of *Salmonella* organisms. All antigenic formulae are listed in the Kauffmann-White scheme (Brenner et al., 2000). The World Health Organization update this scheme regularly by Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (WHO Collaborating Centre). It is now re-designated as the White Kauffman-Le Minor Scheme (Agbaje et al., 2011). More than 2,541 *Salmonella* serovars have been identified on antibody reaction with surface antigens: O (somatic), H (flagellin) and Vi (Vi or capsular polysaccharide). The terms "serotype" and "serovar" are both frequently used where the term serovar is preferred to the term serotype according to the rules of the *Bacteriological Code* established by the Judicial Commission of the International Committee on the Systematics of Prokaryotes and used in the Kauffmann-White scheme.

According to the CDC, the genus *Salmonella* contains two species, *S. enterica*, and *S. bongori*. A third species "*Salmonella subterranea*" was recognized in 2005. *S.enterica* consists of six subspecies: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *Indica* (Jorgensen et al., 2015). The current nomenclature is summarized in the Table 2.1. All salmonellae causing human infection fall within the subspecies *enterica.* The full written designation of *Salmonella enterica* subsp. *enterica* serovar Typhi is commonly shortened to *Salmonella* Typhi (*S*. Typhi) or *S.* Paratyphi A, and this modification will be followed in this thesis.

Table 2.1: Current *Salmonella* nomenclature (Su and Chiu, 2007)

Taxonomic position (writing format) and nomenclature				No. of serotypes in each species or subspecies[22]
Genus (capitalized, italic)	Species (italic)	Subpecies (italic)	Serotypes (or serovars) (capitalized, not italic)*	
Salmonella	*enterica*	*enterica* (or subspecies I)	Choleraesuis, Enteritidis, Paratyphi, Typhi, Typhimurium	1504
		salamae (or subspecies II)	9,46:z:z39	502
		arizonae (or subspecies IIIa)	43:z29:-	95
		diarizonae (or subspecies IIIb)	6,7:l,v:1,5,7	333
		houtenae (or subspecies IV)	21:m,t:-	72
		indica (or subspecies VI)	59:z36:-	13
	bongori	subspecies V	13,22:z39:-	22
	subterranea[18]			

*: Some selected serotypes (serovars) are listed as examples.

S. Typhi, *S.* Paratyphi A and B are all restricted to human host while *S.* Paratyphi C, *S.* Typhimurium and *S.* Enteritidis have a wider host range and spread between humans and domestic animals such as chicken, goat and cattle.

2.1.2 Morphology and important properties

Salmonella are Gram-negative facultative intracellular bacilli belonging to Enterobacteriaceae family. Members of their genus are motile, facultative anaerobe, oxidase negative, urease negative, citrate-utilizing or non-utilizing and may or may not produce hydrogen sulphide (Harris and Ryan, 2015; Kim et al., 2004; Collee et al., 1996). They form non lactose fermenting colonies on MacConkey's or Eosin-Methylene Blue (EMB) agar. Media of intermediate selectivity include XLD agar, deoxycholate citrate agar, Salmonella-Shigella agar and CHROMagar Salmonella. Highly selective media include bismuth sulfite agar and brilliant green agar. In Kligler iron agar (KIA) or triple sugar iron agar (TSI) media *Salmonella* strain produce an alkaline slant, indicating that only glucose is fermented with gas and H_2S. On these media, *Salmonella* Typhi isolates characteristically produce an alkaline slant, only small amount of H_2S at the site of stab and in the stab line but do not produce gas. Phenotypic identification combination with serogrouping or serotyping by slide agglutination test with antisera O:9 are mainstay for diagnosis of S.Typhi (Jorgensen and Pfaller, 2015).

2.1.3 Antigenic structure

Various antigens of *S*. Typhi including its surface components have been reported to be most important targets of the immune response (Moreno-Eutimio et al., 2013). *Salmonella* antigens can be used in epidemiological studies, serological diagnostics and also for identifying the bacteria.

Salmonella contains mainly three major antigens (Fig 2.1): somatic O antigens (polysaccharide antigen) which is the outermost component of long chain lipopolysacharide (endotoxin); H-d antigen (protein antigen) associated with flagella; and Vi-antigen (polysaccharide antigen) only found in the capsule which lies on the exterior of the cell wall (Khan, 2004).

a. O antigen

'O' antigen projects outwards from the lipopolysaccharide (LPS) layer of outer membrane and are anchored into the cell wall by antigenically conserved lipid A and core regions. Antigen comprises a number of oligosaccharide repeats (O-units) that usually contain 2-8 sugar residues (Liu et al., 2010). This antigen exhibits extensive variation in sugar composition, types of sugar present, their arrangement within the O-unit and the linkages within and between O-units, thereby making LPS one of the most variable cell constituents. The variability of the O-antigen provides the major basis for serotyping schemes (Chart, 2007). The O-antigen appears to be a major target of the immune system and bacteriophages. They are hydrophilic and enable the bacteria to form stable, homogenous suspensions in saline solution. They are heat and alcohol stable. Over 67 different O antigens have been recognized and they are designated by arabic numerals. For determining O antigens, most common approach is to initially test the isolates by slide agglutination in antisera against O groups A to E1 because 95% of *Salmonella* isolates from human specimens belong to one of these O groups. The isolate is tested in pools containing the remaining *Salmonella* O antisera for group O:11 through O:67 (Jorgensen and Pfaller, 2015).

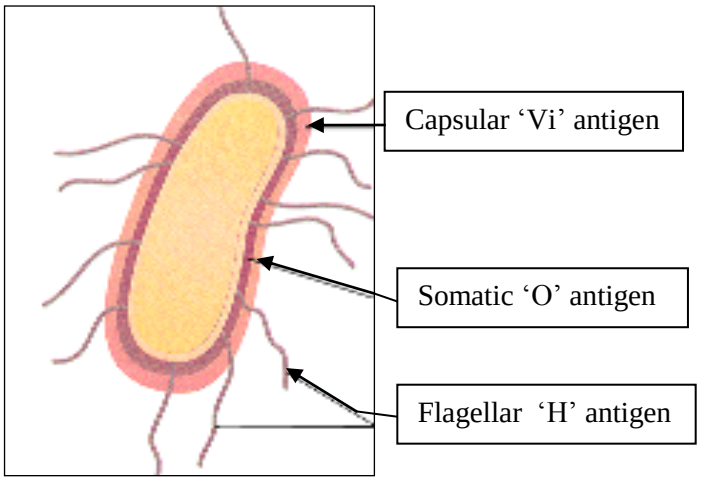

Fig 2.1: Antigenic pattern of *Salmonella* Typhi.

b. H antigen

It is a protein antigen called flagellin. Multiple flagellin subunits make up the flagellar filament. The antigenically variable portion of flagellin is the middle region, which is surface exposed. Flagellar antigens (H-d) are not species-specific to *S.*Typhi and d-antigens are present in many *Salmonella* species other than *S.*Typhi. *Salmonella* commonly express two different flagellin antigens, although specific serotypes, such as *Salmonella* Typhi (antigenic formula=9,12 [Vi]:d:-) and *Salmonella* Enteritidis (antigenic formula=1,9,12:g,m:-) possess only one flagellar antigen. The two flagellar antigens are identified as phase 1 and phase 2 antigens; Strains that expresses one and two flagellar antigens known as monophasic and diphasic strains respectively (Jorgensen and Pfaller, 2015). They are heat and alcohol labile, but are well preserved in 0.04-0.2% formaldehyde.

c. Vi-antigen

It is heat labile capsular polysaccharide, useful for the identification of *Salmonella* Typhi. Almost all recently isolated strains of Typhi form Vi antigen as a covering layer outside their cell wall. When fully developed it renders the bacteria agglutinable by Vi antibody and inagglutinable by O antibody (Old, 2006). Antigens similar to the Vi antigen have been found in some strains of Paratyphi C, Dublin and in *Citrobacter freundii* (Jorgensen and Pfaller, 2015). It interferes with the complement (C3b)-mediated opsonisation and phagocytosis of *S.* Typhi. This antigen also determines phage susceptibility.

d. Surface and envelope protein antigen

Typically, the bacterial cell envelope consists of a cell membrane and a peptidoglycan cell wall (Trefilov et al., 2015). *S*. Typhi a gram negative bacteria contain a thin peptidoglycan layer and an outer membrane. The Gram-negative outer membrane (OM) is a complex, non-uniform, asymmetric organelle, composed of phospholipids and lipoproteins in the inner leaflet and LPS in the outer leaflet. Proteins make up approximately two-thirds of the mass of the OM. (Ursell et al., 2012). Bacterial surface proteins are facing to the outside of the cell and carry out specific functions like adherence and colonization of host surfaces, invasion of cells, evasion of the host's immune response, and persistence in infected tissues. (Fischetti, 2019). Efficacy of bacterial surface antigens can also validated by usefulness oral vaccination to induce immunization (Amro et al., 2014). Surface associated antigens suggested particular promising antigens for both cellular and humoral immunity to *Salmonella* (Barat et al., 2012). Gram-negative bacteria assembled many proteins into the inner and outer membranes and export to the periplasm or extracellular medium. Periplasmic protein and outer membrane protein are first exported through the inner membrane and then inserted and spanning into the outer membrane. (Dalbey and Kuhn, 2012). Protein profile of many bacterial species have been characterized and identified by SDS-PAGE. *S*. Typhi immunogenic protein (flagellin, porin, hemolysin, outer membrane protein etc.) were reported with molecular weight less than 100 kDa smaller molecules (Chin et al., 2015). There was a common heavy protein band of 37.8 kDa found among fifty four Salmonella serovar (Begum et al., 2008). SDS-PAGE pattern of 58 kDa flagellar antigen of S. typhi were observed (Isibasi et al., 1988). 36 kDa, 26.5 kDa, 22.2 kDa and 18.6 kDa proteins were identified as four major subunits of pilli protein (Darmawati, 2005).

e. Outer membrane protein antigen

Attention has been addressed recently, to the outer membrane proteins (OMPs) as they are good immunogens in the induction of protective immunity against *Salmonella*. Major proteins among these includes porins (OmpC, OmpF, OmpD, PhoE, etc.) and the heat-modifiable protein (OmpA & OmpX) (Charles et al., 2010; Isibasi et al., 1988). Porins are the most abundant class of outer membrane proteins (OMPs) that are protective and show some degree of antigenic heterogeneity among

different strains. These proteins act as important virulence factor in the pathobiology of gram-negative bacteria and bacterial adaptation. These antigens have been reported to be targets of antibodies and T cells during the immune response to *S.* Typhi (Moreno-Eutimio et al., 2013). An apparent molecular mass of 49 kDa OMP of *Salmonella enterica* serovar Typhimurium is highly immunogenic, evokes humoral and cell-mediated immune responses. This protein is recognized by the antibodies present in serum of typhoid patients. Over the past years, several *Salmonella* OMPs have been investigated as potential vaccine candidates, virulence factors, and diagnostic antigens (Hamid and Jain, 2008). *S.* Typhi OMP have molecular sizes in the range from 17 to 80 kDa (Ortiz et al., 1989). The major OMPs with molecular masses of 15 kDa, 26 kDa, 33 kDa, 34kDa, 37 kDa, 49 kDa, 54 kDa, 60kDa were identified from SDS-PAGE gels (Aslam et al., 2012; Isibasi et al., 1988). Two major groups of proteins of the OMPs of *S.* Typhi were identified by SDS-PAGE. One was between 36 and 41 kilodaltons (kDa), which corresponds to the molecular size ranges of the known gram-negative porins and OmpA. The other group of proteins was located between 23 and 28 kDa (Isibasi et al., 1988). *S.* Typhi adhesion protein is related to OMP with a molecular weight of about 36 kDa (Muthiadin et al., 2015).

f. Whole cell protein antigen

Protein bands were detected in the all *Salmonella* serovar whole cell protein extract includes 78.1, 51.2, 41.5, 37.3, 35.1, 33.9, 30.7, 27.6, 25.4, and 24 kDa. Among them major bands 78.1, 51.2, and 41.5 kDa were observed (Aksakal, 2010).

2.1.4 Virulence factors

Several researches have been done to investigate the virulence factors of *Salmonella* Typhi bacterium. Most important of them are summarized below.

Virulence gene: Ability of *Salmonella* Typhi to invade host cells and disseminate in the body is closely related to its virulence gene. Chromosomal regions that carry majority of the virulence genes known as *Salmonella* pathogenicity islands (SPIs). It acts as a compact and distinct genetic unit known as an operon. Most of the genes in the SPI encode structures, products, and strategies that contribute to infection and play a crucial role in pathogenicity. These regions display a different composition

from the rest of the chromosome, with the characteristic presence of a greater amount of G+C (guanine and cytosine) when compared with the other parts of the DNA . To date, five SPIs have been described in *Salmonella*, with SPI-1 and SPI-2 being the most recognized and studied (Amavisit et al., 2003). SPI-1 region is necessary for the invasion of host cells and suppression of early proinflammatory cytokine expression in macrophages, including that of IL-1β, IL-8, TNF-α, IL-23α, GM-CSF, and IL-18. Gene cluster in SPI-1 consists of a 40-kb region, which includes 39 genes encoding T3SS-1 and its chaperones and effector proteins as well as some transcriptional regulators that control the expression of many virulence genes located within and outside SPI-1. Genes of SPI-2 are specifically turned on inside the cells. SPI-2 is mainly required for the survival of salmonellae within macrophages by encoding T3SS-2. In short, SPI-1 is mediating intestinal pathogenesis while SPI-2 systemic pathogenesis (Lou et al., 2019; Ibarra and Steele-Mortimer, 2009).

Type III secretion systems (T3SS): The T3SS is a complex structure made up of many bacterial proteins. The proteins that make up the T3SS apparatus are termed structural proteins. Rest of the proteins called "translocators" serve the function of translocating another set of proteins into the host cell cytoplasm. The translocated proteins are termed "effectors," as they effect the changes in the host cells, allowing the invading pathogen to colonize, multiply, and in some cases chronically persist in the host. Briefly, the T3SS-1 apparatus consists of two rings that provide a continuous path across the inner and outer membranes, including the peptidoglycan layer. By utilizing this system, bacteria are able to directly inject bacterial proteins called effectors into host cells across bacterial and host membranes, where they can manipulate host cell function (Coburn et al., 2007). The SPI-1 T3SS are expressed in response to signals sensed by bacteria in the intestine of the infected host. This system becomes active upon contact with epithelial cells, translocating effectors across the host cell plasma membrane. Several of these effectors stimulate the assembly of actin filaments, whose extension causes localized membrane ruffling and bacterial invasion. Effectors also trigger the activation of mitogen-activated protein kinase (MAPK) pathways, leading to the production of proinflammatory cytokines such as interleukin (IL)-8, stimulating the recruitment of polymorphonuclear leukocytes (PMNs) and inducing acute intestinal inflammation. The SPI-2 T3SS is a major virulence factor of *Salmonella*. It functions by

translocating effectors across the membrane of the *Salmonella*-containing vacuole (SCV) in infected host cells such as epithelial cells and macrophages (Figueira and Holden, 2012). Effectors of the SPI-2 T3SS carry out a large number of functions. These include maintaining the integrity of the SCV and its localization near the Golgi of host cells, as well as modulating the host cytoskeleton and interfering with immune signaling. Several virulent proteins (SpiC, SifA, SipA, SseJ, SseF, SspH2, PipB2, etc.) encoded by SPI-2 allow S. Typhimurium to survive and localize inside DCs. They reduce the amount of MHC molecules expressed on the surface of both mouse and human DCs and poor activation of T cells (Bueno et al., 2012).

Vi capsule: The majority of clinical *S.* Typhi isolates express a polysaccharide capsule (Vi CPS) know as Vi (Vi^+S.Typhi) which is associated virulence. Strains positive for Vi production have higher rates of infection and so continues to be the focus for improvement in current treatment and prophylaxis for this disease. Vi exhibits immune modulatory activities. Vi expression on *Salmonella* can reduce the inflammatory response in intestinal epithelial cells and throughout systemic infection by reducing levels of IL-17 and fluid influx and neutrophil recruitment. Thus it facilitates a stealth mode of pathogenesis, promoting systemic spread and limiting the clinical signatures of gastroenteritis (Tran et al., 2010). It also reduce the deposition of C3, anti-O:9 antibodies on the surface of S.Typhi and provide protection against non-specific antibody killing. This may help the establishment of initial systemic infection by *S.*Typhi and subsequent chronic carrier state. Vi capsular polysaccharide is encoded by the *via*B locus which is 14-kb DNA region containing genes required for the regulation, biosynthesis and export to surface (Wilson et al., 2011). It is expressed under low osmolarity, such as within the blood stream and surface expression being down regulated as osmolarity increases. Vi expression highest when culture at 9mM NaCl and minimal when cultured at 500mM NaCl (Kaur and Jain, 2012).

Flagella and fimbriae: Flagellar-based motility can increase the invasiveness of *Salmonella*. In the intestinal epithelium flagellin induces inflammation while inhibiting apoptosis via TLR5. In *Salmonella*-infected macrophages flagellin is translocated into the cytosol by T3SS1 resulting in activation of the inflammasome and caspase-1-mediated cell death (pyroptosis) and used for escape. Salmonella

fimbriae are required for biofilm formation, attachment and adhesion to host cells and colonization but not intracellular survival (Ibarra and Steele-Mortimer, 2009).

2.1.5 Pathogenesis

Typhoidal salmonellae are human-restricted pathogens; humans are the only natural host and reservoir of infection (Murray et al., 2016). The organism most often acquired through consumption of contaminated water or food by feces of an acutely infected or convalescent person or a chronic, asymptomatic carrier (Dougan and Baker, 2014).The infective dose to produce clinical or subclinical infection is 10^5-10^8 salmonellae. Ingested *Salmonella* Typhi and *Salmonella* Paratyphi A and B after exposure to gastric acid, efficiently traverse from the lumen across the human intestinal mucosa. After passing through the pylorus and reaching the small intestine, the bacilli rapidly penetrate the mucosa to reach the lamina propria. *Salmonella* Typhi targets M (microfold) cells overlying Peyer's patches and other gut-associated lymphoid tissue and are then ingested by dendritic cells and macrophages underlying the M cells. However, the bacilli may also invade enterocytes and enter endocytic vacuoles that transit the cells to be released into the lamina propria, without destroying the enterocyte. *Salmonella* may also pass paracellularly between enterocytes. Upon reaching the lamina propria in the nonimmune host, typhoid bacilli elicit an influx of macrophages and dendritic cells that ingest the organisms but are generally unable to kill them. Some bacilli apparently remain within macrophages of the small-intestinal lymphoid tissue. Other typhoid bacilli are drained into mesenteric lymph nodes where further multiplication and ingestion by macrophages take place. Primary bacteraemia takes place shortly after invasion of the intestinal mucosa. The main route to reach the bloodstream in this early stage is by lymph drainage from mesenteric nodes entering the thoracic duct and thence the general circulation. From the circulation it is filtered by fixed phagocytes of the reticuloendothelial system (liver, spleen, bone marrow). The pathogen resides and multiplies throughout these organs (intracellular haven) during the incubation period (usually 8–14 days) until the onset of clinical enteric fever. Clinical illness is accompanied by a secondary bacteraemia. The phase persists for several weeks if antibiotic therapy is not given. In this phase of heavy infection, the classical bowel pathology of typhoid occurs. Organisms liberated into the bile

following rupture of infected cell and causes infection of the lymphoid tissue of the small intestine paticularly in the ileum for a second time. Invasion of the mucosa causes the epithelial cells and macrophages to synthesis and release various proinflammatory cytokines including IL-1, IL-2, IL-6, IL-8, TNF- α & β, GM-CSF and other cytokines. Bleeding comes from eroded vessels in or near the Peyer's patches. Perforations of the bowel wall occur in the same sections of the gut as the hemorrhages (Harris and Ryan, 2015; Dougan and Baker, 2014; Levine et al., 2011).

During the primary bacteremia typhoid bacilli also reach the gallbladder *Salmonella* Typhi can be readily cultured from bile or from bile-stained duodenal fluid in patients with acute typhoid fever. In approximately 2–5% of patients, the gallbladder infection becomes chronic especially in female, older age and preexistant gallbladder disease. Carriers shed as many as 10^9 organisms/g feces without penetrating gastrointestinal tract or causing disease (Ismail et al., 2002).

Recent reports have revealed fascinating insights into how *Salmonella* takes advantage of the immune responses in the mucosa and turns them in its favor. Inflammation helps *Salmonella* to outcompete the microbiota by ROS (Reactive oxygen species) generating a novel respiratory electron acceptor, which can be used by *Salmonella* but not the microbiota. In addition, the detection of *Salmonella* by TLRs induces the acidification of the phagosome, which in turn provides a signal for *Salmonella*, so that it has reached its intracellular niche protected from extracellular immune responses. Finally, *Salmonella* might have evolved the ability to actively trigger NFκB and inflammasome signaling pathways through injected effector proteins. Regulation of attachment, engulfment and replication is controlled by SPI-I & II by encoding salmonella secreted invasion proteins (Ssps) and a type III secretion system (Broz et al., 2012). Invasion of salmonella depends on the SPI-I-encoded type III secretion system (T3SS) and the SPI-IV-encoded type I secretion system (T1SS). The substrate of this T1SS is the non-fimbrial giant adhesin SiiE that mediates first contact to the host cell. *Salmonella* protein SiiE also required for reduced humoral immune response by selectively reducing the number of IgG-secreting plasma cells in the BM (Barlag and Hensel, 2015).

2.2 Typhoid fever

2.2.1 Case and carrier definition (W.H.O., 2003)

Confirmed case of typhoid fever

A patient with fever (38°C and above) that has lasted for at least three days, with a laboratory confirmed positive culture (blood, bone marrow, bowel fluid) or molecular methods of *S.* Typhi or detection of *S.* Typhi DNA from a normally sterile site.

Probable case of typhoid fever

A patient with fever (38°C and above) that has lasted for at least three days, with a positive serodiagnosis or antigen detection test but without *S.* Typhi isolation.

Convalescent carrier

Evidence of shedding *Salmonella* spp. (positive stool culture or PCR) 1–12 months after finishing an appropriate course of antimicrobial treatment and the resolution of symptoms following a laboratory-confirmed episode of acute disease.

Chronic carrier

Evidence of shedding of *Salmonella* spp. (positive stool culture or PCR or repeated positive bile or duodenal string cultures) at least 12 months after finishing an appropriate course of antimicrobial treatment and the resolution of symptoms following a laboratory-confirmed episode of acute disease

Or

Two positive stool samples 12 months apart.

2.2.2 Epidemiology

a. Global burden

Burden of typhoid fever has been estimated globally 11-21 million cases and approximately 1, 28,000– 1,61,000 deaths annually (W.H.O., 2018). Systemic review and meta analysis of pooled blood culture confirmed typhoid fever incidence was overall 154 (95% CI, 115.1-198.6) cases per 100,000 per year. In Asia and Africa

pooled incidence estimate was 267.6 (95% CI, 182.8-368.2) and 112.1 (95% CI, 46.7-203.5) cases respectively per 100,000 per year (Marchello et al., 2019).

Regions with high incidence (>100/100000cases/year) of typhoid fever include south-central Asia and south-east Asia, medium incidence (10-100/100000cases/year) include rest of Asia, Africa, Latin America and the Caribbean and Oceania except for Australia and New Zealand (fig 2.2). The rest of the developed world have low incidence of typhoid fever (<10/100000cases/year) (Crump et al., 2004). Typhoid incidence data in low and middle-income countries (LMICs) was 17.8 million cases/year (95% credible interval: 6.9±48.4 million) (Antillon et al., 2017). Pakistan, India and Bangladesh-together account for about 85% of the world's cases (Maurice, 2012).

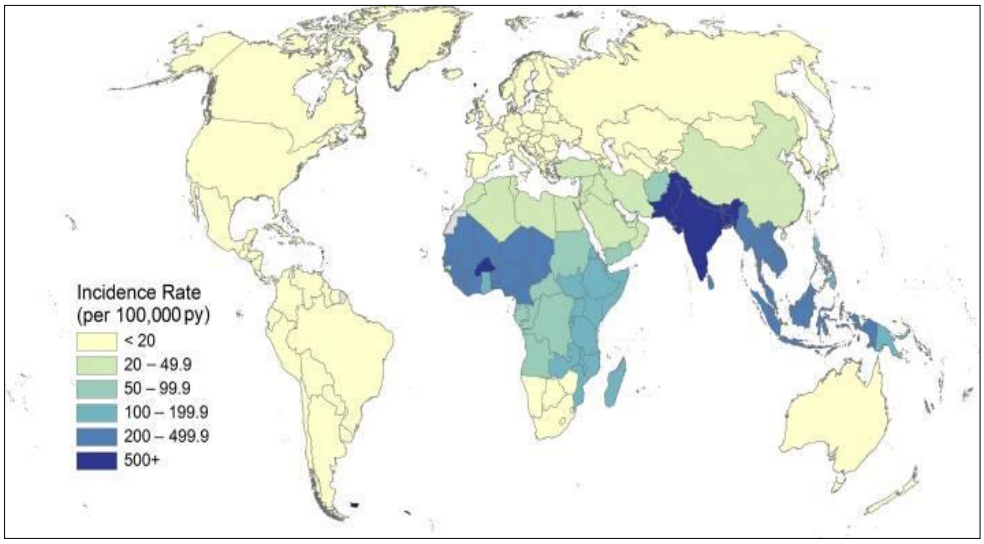

Figure 2.2: Estimated incidence of typhoid and paratyphoid fevers by country per 100,000 population; source: (Radhakrishnan et al., 2018)

b. Bangladesh scenario

Typhoid fever is an important public-health problem in Bangladesh. The overall incidence of blood culture confirmed typhoid fever was 2.0 episodes/1000 person-years, with a higher incidence in children aged <5 years (10.5/1000 person-years) than in older persons (0.9/1000 person-years) (relative risk=12, 95% confidence interval (CI) 6.3-22.6) (Naheed et al., 2010). However, absence of reliable diagnostic method and microbiology facilities in rural area of Bangladesh, hampers estimation of the true magnitude of the typhoid fever burden in our country. So, Naheed et al.

(2010) suggested that incidence of typhoid fever would have been higher at 16 episodes per 1000 person-years rather than observed 11episodes per 1000 person-years. *S.* Typhi was isolated from blood cultures every month, but isolation rate was highest in monsoon and summer seasons and lowest in winter months (Saha et al., 2001).

c. Mode of transmission

Mode of transmission is person-to-person, usually via the faecal-oral route. Faecally contaminated drinking water is a commonly identified vehicle. *S.* Typhi may also be found in urine and vomitus and, in some situations, these could contaminate food or water. Shellfish grown in sewage-contaminated water are potential vehicles, as are vegetables. Flies can mechanically transfer the organism to food, where the bacteria then multiply to achieve an infective dose. The inoculums size and the type of vehicle of organism greatly influence both the attack rate and the incubation period (W.H.O., 2011).

2.2.3 Clinical presentation

Typhoid fever is an acute, life-threatening, febrile illness. The case fatality rate of typhoid fever is 10–30% without treatment which dropping to 1–4% with appropriate therapy. Young children are at greatest risk. Common symptoms include sustained fever, chills and abdominal pain. The non-specific complaints of headache, myalgias, malaise and anorexia are common like other diseases of typhoid-endemic areas (W.H.O., 2018). Abdominal complaints may include diarrhea, constipation etc. Invasive diarrhea does not typically occur in typhoid fever. A white or yellowish coating tongue is common physical finding. Hepatosplenomegaly and rash may found in 5-10% cases.The pulse is often slower than would be expected from height of the temperature (relative bradycardia). Complication occur in untreated cases in the form of intestinal hemorrhage or perforation during third week. Urinary retention, pneumonia, thrombophlebitis, myocarditis, psychosis, cholecystitis, nephritis, osteomyelitis and meningitis are less often observed (Papadakis and McPhee, 2017; Harris and Ryan, 2015).

2.2.4 Diagnostic laboratory test

a. Culture based diagnostics

Definitive diagnosis of enteric fever is only made through the isolation of typhoidal salmonella from the blood, bone marrow, stool, urine or other clinical specimen of febrile patient. Isolation of organism also allows antimicrobial resistance testing for optimal management. In enteric fever blood, urine and stool culture results are often positive in first, second or third week of the disease respectively. Sensitivity of blood culture varies from 40% to 80% due to prior antibiotic therapy before culture and low grade bacteremia in enteric fever. Bone marrow culture has a higher sensitivity than blood culture but is a more invasive procedure. In MacConkey, Eosin methylene blue or desoxycholate differential media rapid detection of non fermenters colonies and in Bismuth sulfite medium permit rapid detection of *Salmonella* as black colonies. Salmonella-Shigella (SS) agar, Hektoen enteric agar, Xylose lysine desoxycholate (XLD) agar favor growth of salmonella and shigella over other Enterobacteriaceae. Stool specimen usually put into selenite F or tetrathionate broth to permit multiplication of salmonellae and inhibit replication of normal intestinal bacteria (enrichment culture). Suspected colonies from solid media are identified by biochemical reaction pattern and slide agglutination tests with specific anti sera (Carrol et al., 2016; Harris and Ryan, 2015).

b. Serologic methods

Currently laboratory diagnosis of typhoid fever is dependent upon either the isolation of *Salmonella* Typhi from a clinical sample or the detection of raised titers of serum antibodies against the lipopolysaccharide (LPS) (O) or flagellum (H) antigens of serotype Typhi (House et al., 2001). The antibody responses to both antigens were highly variable among infected individuals. Detection of high antibody titers from the sera of healthy subjects presumably contributes lack of specificity with serologic diagnostic tests in regions of typhoid endemicity. The serological tests could be of use for the diagnosis of typhoid fever in clinically suspected culture negative patients or in regions where bacterial culturing facilities are not available. The common serological tests for diagnosis of typhoid fever are Widal test and ELISA test (Quiroga et al., 1992). The Widal test detects agglutinating antibodies to lipopolysaccharide (LPS) (To test) and flagella (T_H test), was introduced over a century ago (Harris and Ryan, 2015). It is easy, inexpensive and relatively non-invasive test and used widely. Originally the Widal test required acute and

convalescent phase serum samples taken approximately 10 days apart. Recently acute-phase serum sample has been adapted for use (House et al., 2001). An antibody titer of 1:80 or higher were usually taken as cut off value in the manufacturer manual. H and/O agglutinin titer of 1:320 has diagnostic value for typhoid fever (Aftab and Khurshid, 2009). False negative Widal test may occur if blood collected too early in the disease processes. Elevated To and TH titers were found in sera for healthy control subject due to exposure to S.Typhi or another microorganism sharing common antigens (House et al., 2001). Enzyme-linked immunosorbent assays (ELISAs) considered as an alternative approach for the diagnosis of typhoid fever. These assays have been based on the detection of anti-LPS antibodies (IgM, IgG and IgA) or antiflagellum antibodies (IgG). Though Anti-LPS IgM antibodies reported to be more sensitive than the Widal T_O test but none of them are highly sensitive and specific. Antiflagellum IgG ELISA and Widal T_H were found highly specific but lacked sensitivity (House et al., 2001). In developing countries new commercially available typhoid rapid antibody tests (Linear Cromotest semiquantitive slide agglutination test, IDL TUBEX® TF, Typhidot etc.) can facilitate diagnosis and disease management. In semiquantitive slide agglutination test, sensitivity and specificity of the H slide agglutination test appeared to be greater, but results obtained with the O slide agglutination were inconsistent. Performance of Widal test and the newer typhoid rapid antibody tests are similar in Asia and Egypt. Some reports suggest that the Typhidot test (detect antibody against 50 kDa outer membrane protein) may be more useful (high sensitivity and specificity) than Widal test in Asia. However, new typhoid rapid antibody tests correlate poorly with blood culture in sub-Saharan Africa (Keddy et al., 2011).

c. Molecular methods

DNA-based molecular techniques detect and characterize S.Typhi at the genetic level while traditional serotyping detect phenotypic properties that may not always be expressed, regardless of the presence of the genetic material. There are several molecular techniques (eg. microarray or bead-based technology) currently available for the rapid molecular identification of *Salmonella* serotype. The use of molecular serotyping provides a valuable high-throughput alternative to traditional serotyping. It is anticipated that whole-genome sequencing will replace serotyping in public health laboratories in the future. It will not adopt in routine diagnostic laboratories

for public heath purposes until resolving the challenges including cost, data analysis, and inter laboratory comparability (Yoshida et al., 2016).

2.2.5 Treatment

General management

Supportive measures such as oral or intravenous hydration, the use of antipyretics, and appropriate nutrition and blood transfusions etc. are important in the management of typhoid fever. More than 90% of patients can be managed at home with oral antibiotics and reliable care. Rest 10% (patients with persistent vomiting, severe diarrhea and abdominal distension) may require hospitalization and parenteral antibiotic therapy (W.H.O., 2007).

Antimicrobial therapy

In late 1980s, chloramphenicol (for 2–3 weeks) was the treatment of choice for typhoid fever. Subsequently, an increased number of plasmid-mediated multidrug resistance (MDR) strains to chloramphenicol, ampicillin and cotrimoxazole are reported. Fluoroquinolone (ciprofloxacin and ofloxacin) has become the favored drug after the emergence of MDR strains (Upadhyay et al., 2015). Interestingly the spread of strains with decreased susceptibility to ciprofloxacin has limited their effectiveness particularly in Asia. In Bangladesh 80-90% of isolated S. Typhi was nalidixic acid resistant (NARST) with reduced susceptibility to ciprofloxacin (Shadia et al., 2011). Extended spectrum cephalosporins (ceftriaxone and cefixime) and azithromycin are now a day suitable alternatives for reduced fluoroquinolone susceptible *S.* Typhi. The combinations of cephalosporin and azithromycin are quite frequently used to treat the patients who failed to respond promptly. (Veeraraghavan et al., 2018). Table summery of the antibiotic option for the treatment of typhoid fever is given in Table 2.2.

Table 2.2: Antimicrobial treatment of typhoid fever (W.H.O., 2011)

Optimal Therapy				Alternative Effective Drugs		
Susteptibility	**Antibiotic**	**Daily dose mg/kg**	**Days**	**Antibiotic**	**Daily dose mg/kg**	**Days**
			Mild disease			
Fully sensitive	Ciprofloxacin or Ofloxacin	15	5-7	Chloramphenicol	50-75	14-21
					75-100	14
				Amoxycilin	8-40	14
				Cotrimoxazole		
Multi drug resistant	As above or Cefixime	15	7-14	Azyhtromycin	8-10	7
		15-20	7-14	Cefixime	15-20	7-14
Quinolone resistance	Azythromycin	8-10	7	Cefixime	20	7-14
	Ceftriaxone	75	10-14			
			Severe illness			
Fully sensitive	Ciprofloxacin or Ofloxacin	15	10-14	Chloramphenicol	100	14-21
					100	14
				Amoxycilin	8-40	14
				Cotrioxazole		
Multi drug resistant	As above or Cefixime	15	10-14	Ceftriaxone	75	10-14
		15-20	10-14	Cefotaxime	80	
Quinolone resistance	Ceftriaxone	75	10-14	Fluoroquinolone	20	7-14
	Cefotaxime	80	10-14			
	Azythromycin	8-10	10-14			

2.3 Immune responses to *Salmonella* Typhi

S. Typhi infection stimulates both local intestinal mucosal (Innate) and systemic humoral and cellular immune response (Charles et al., 2010).

2.3.1 Local intestinal mucosal immune response (Innate immunity)

Intesinal immune system provides gentle response to commensal bacteria but harsh inflammatory response with pathogens that invade via digestive tract. The cells within the lymphatic tissue of the gut are likely to be central for the orchestration of a proper and rapid response against *Salmonella*. The inflammatory response is initiated after their interaction with the intestinal epithelial cells. When pattern recognition receptor (TLR5) of dendritic cells (DCs) detect flagellin of the bacteria in lamina propria, they begin to produce IL-6. This cytokines causes naïve Th cells to become Th17. The cytokines IL-17 they produce, recruits huge numbers of neutrophils from the blood stream, strengthen intestinal tight junction, stimulate antimicrobial peptide and mucus production by goblet cells and facilitate the transcytosis of IgA antibody. These responses are important for delaying the spread of bacteria to systemic tissues.

Recruited neutrophils produce several antimicrobial and cytotoxic peptides (eg. defensins, protegrins, phospholipase A2 and cathelicidins) and important source of IFN-γ in the intestinal mucosa that play important role in host defense against intracellular pathogens (Sampayrac, 2019).

A. First line of defense

A single layer of intestinal epithelial cells of the gut provides the primary cellular barrier of Salmonella (Fig. 2.3). This gastro-intestinal barrier blocks the entry of pathogens as well as prevents the innate immune system from encountering commensal-derived antigens. Epithelial cell seals the surface of the gut by maintaining intimate association with tight junction proteins. Goblet cells in the epithelial layer help to maintain this barrier by secreting mucus or antimicrobial peptides (Paneth cells) (Fig. 2.3). Underlying this epithelial cell layer is the lamina propria, which contains gut-associated lymphoid tissue (GALT). The GALT is comprised of isolated and aggregated highly organized lymphoid follicles and is populated by a wide array of lymphocytes, such as T cells and B cells, dendritic cells, macrophages and neutrophils (Fig.2.3). These cells regulate inflammatory responses to bacteria and antigens that breach the gastrointestinal barrier, protect the mucosa against harmful pathogens, and scavenge dead cells and foreign debris. Aggregated lymphoid follicles, so-called Peyer's patches (PP), are surrounded by the follicle-associated epithelium (FAE). The FAE contains specialized cells named M (for microfold) cells, which transport luminal antigens and bacteria to the basolateral side by transcytosis. At their basal surface, the cell membrane of M cells is extensively folded around underlying lymphocytes and antigen-presenting cells. M cells are unique epithelial cells that form tight junctions with neighboring enterocytes, have an increased pinocytic activity and have the function of priming intestinal immunity by engulfing microbes from the gut and delivering them to the lymphoid cell population that resides within the follicle (Jones and Falkow, 1996). In addition, antigen-presenting dendritic cells (DCs) send processes between intestinal epithelial cells without disturbing tight junction integrity and sample antigens from commensal and pathogenic gut bacteria. In mice number of PPs has range from 5-12 and in adult human has about 200 (Cupedo et al., 2011). They contain about 20% T and 80% B lymphocytes as well as macrophages. The PPs have an important role in the immune surveillance of the intestinal lumen and facilitate the induction of

defense against pathogens as a result of the complex interplay between immune cells located in the lymphoid follicles and the FAE. B cells and memory cells get activated by antigen-presenting cells in PPs and then travel to the mesenteric lymph nodes (MLN), where the immune response is amplified. Thus the GALT plays a crucial part in the interplay between the innate and adaptive immune system (Broz et al., 2012).

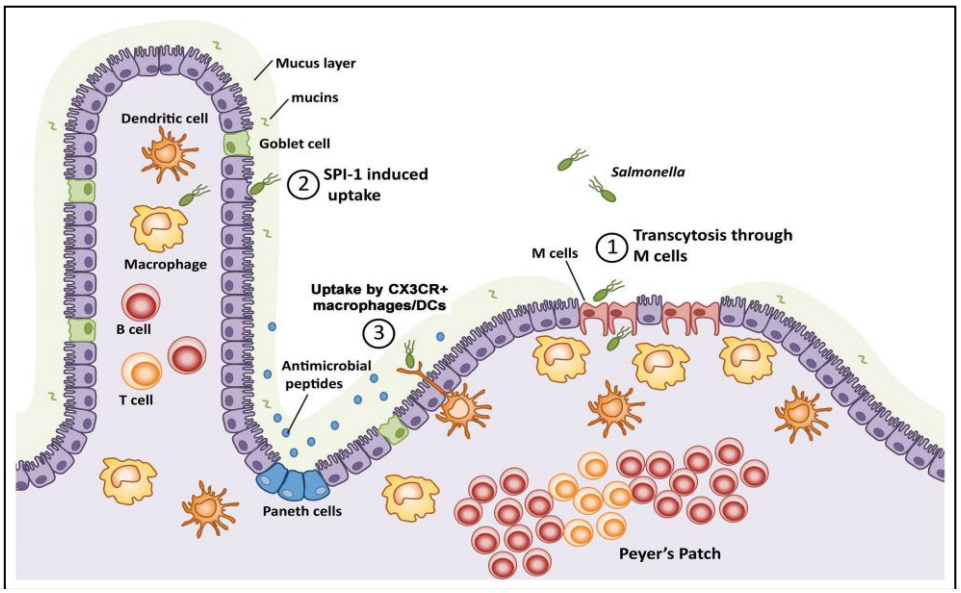

Figure 2.3: Schematic representation showing the different routes Salmonella can take in order to invade the intestinal mucosa.

(1) The major pathway of invasion is through M cell mediated transcytosis at the Peyer's patches. (2) An alternative route is via uptake by enterocytes. This route requires the injection of bacterial effector proteins by the SPI-1 Type 3 Secretion System that induce ruffling and uptake of the bacterium. (3) Uptake through intercalating CX3CR1+ macrophages/DCs might represent an additional route of invasion (Broz et al., 2012).

B. Phagocytosis and intracellular killing

Professional phagocytic cells (macrophage and neutrophil) comprising the second arm of the innate system, provides the rapid recognition and elimination of invading *Salmonella* through phagocytosis-mediated killing and the induction of inflammation. Following phagocytosis invading microbes are localized in the phagosomes (membrane-bound vesicles), which fuses with the lysosomes to form phagolysosomes. Lysosomal hydrolases play a microbicidal role at lower pH (below 5.0). Several immune mechanisms contribute to killing and elimination of intracellular pathogens. Phagocytic cells recognize and engulf pathogens through

pattern recognition receptors (PRRs) specific for microbial products, for example mannose, integrin, scavenger and toll-like receptors, and receptors for N-formyl methionine-containing peptides. Phagocytes also express receptors for complement (CR) and antibodies (FcR), which are important in complement and antibody-mediated elimination of intracellular pathogens. The uptake of IgG opsonized bacteria induces a respiratory burst, which produces reactive oxygen intermediates (ROIs), permits fusion with lysosomes, and increases production of pro-inflammatory cytokines (eg. TNF-α). Inflammatory monocytes are also recruited and produce anti-microbial factors such as iNOS, TNF-α and IL-1β etc. (Pham and McSorley, 2015)

Natural killer (NK) cells play an essential role in killing intracellular pathogens through either cytotoxic attack of infected target cells or activation of macrophages via gamma interferon (IFN-γ) production. NK cells and macrophages function cooperatively to eliminate intracellular microbes at early stages of *Salmonella* infection (Culley et al., 1996).

C. Role of cytokines and other plasma proteins

Extracellular Salmonella are detected by toll-like receptors inducing a transcriptional response leading to the expression of pro-inflammatory cytokines such as IL-23. Intracellular Salmonella activate NOD-like receptors that can induce IL-23 expression and promote the secretion of mature IL-1β and IL-18. SPI-1 mediated activation of Caspase-1 in epithelial cells might contribute to IL-18 secretion. IL-18 and IL-23 amplify the inflammatory response. IL-18 produce by macrophages, induces the release of IFNγ from Th1 cells, while IL-23 induces the release of IL-22 and IL-17. IL-18 induces TNF-α and nitric oxide (NO) production by activated macrophages. These cytokines induce the increased production of mucins and antimicrobial peptides, and promote the release of CXC chemokines leading to an influx of neutrophils into the mucosa. The combination of all of these cytokines induces a strong inflammatory environment in the intestine, which is characterized by an increased production of anti-microbial peptides (IL-22/23 axis) and a recruitment of neutrophils by the IL-17/23 axis (Broz et al., 2012). IFN-γ is the most important cytokine during the early phase of infection with intracellular pathogens. Study showed enhanced susceptibility to *Salmonella* infection by antibody-mediated neutralization of IFN- γ in vivo and also by deletion of IFN-γ gene. The second line

of cytokine involves TNF-α. IFN-γ, in combination with TNF-α activates macrophages and/or other target cells rendering the cells better able to control the growth of and/or kill several intracellular organisms. TNF-α is involved in activation of various cell types, including hepatocytes and macrophages in the liver, and recruitment and migration of phagocytes from the bone marrow to the circulation. Other pro-inflammatory cytokines are IL-1, IL-6, and IL-8 also appear to play an essential role in the innate responses and macrophage activation early in infection with other intracellular pathogens (fig 2.4). IL-1 can potentiate IL-12-mediated induction of IFN-γ from NK cells and is thus implicated in the T cell-independent resistance mechanisms to intracellular pathogens (Ismail et al., 2002).

C-reactive protein (CRP), a serum protein that is markedly elevated in response to infection and tissue injury, enhances phagocytosis of intracellular bacteria through opsonization. CRP binds to bacteria and increasing their uptake into human monocyte-derived macrophages. CRP is produced predominantly by hepatocytes. Other cells including subsets of lymphocytes, Kupfer cells, blood monocytes, and alveolar macrophages also synthesize this protein. It is also involved in mechanisms of activation of the classical complement cascade and interaction with FcRs that lead to the generation of pro-inflammatory cytokines. This recognition provides early defense and leads to activation of the adaptive immune system (Culley et al., 1996).

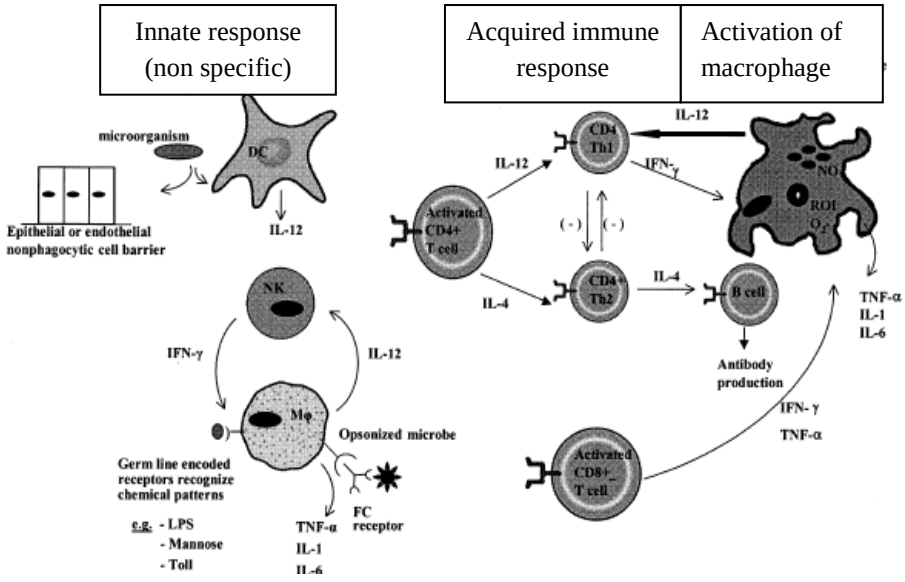

Fig. 2.4: A schematic presentation of major regulatory cytokines generated in response to infection with intracellular pathogens. Effectors Th1 CD4T cells, CD8T cells, and NK cells activate the phagocytes to kill the intracellular microbes via generation of toxic products such as NO and ROIs through IFN-γ production, (Ismail et al., 2002)

D. Complement mediated immunity

Requirement for complement in the killing of intracellular *Salmonella* is striking. There is negligible phagocytosis, oxidative burst or cellular killing of this microbe by peripheral blood cells when opsonized with antibody alone. So both antibody and complement are necessary for clearing *Salmonella* as like as other bacteria. (Gondwe et al., 2010). Complement is essential for rapid elimination of this pathogen. These proteins are present in the blood and body fluids as inactive precursors but are rapidly activated upon contact with bacterial cells. An activated complement cascade on the bacterial surface triggers a variety of responses that help to kill the bacterium. Complement-dependent bacterial killing is one of the most rapid ways to kill an invading bacterium. The swiftest response is the formation of ring-structured pores, the membrane attack complex, that directly kill *Salmonella* within minutes (Fig. 2.5). Phagocyte attraction and subsequent intracellular killing takes 30 minutes to 1 h. This potent bacteriolytic activity was recognized in 1895 by Nobel laureate Jules Bordet, who discovered complement as a system in serum that allows antibodies in vaccinated animals to kill bacteria without the help of immune cells. Nowadays, we understood that complement is not only essential for the direct killing, but that it also triggers many other innate processes such as the production of chemoattractants, and the labeling of bacteria for phagocytosis and intracellular killing by professional phagocytes (Fig. 2.5). The pathogenic bacteria have evolved mechanisms to resist various steps in the complement cascade strongly supports the crucial role of complement in human defense against bacteria (Heesterbeek et al., 2018).

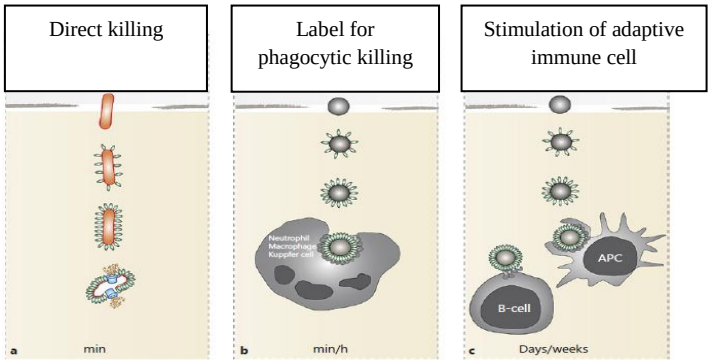

Figure 2.5: Functions of complement. a Membrane attack complex (MAC or C5b-9; blue) rapidly kills gram-negative bacteria (orange) without the help of immune cells. B. C3-derived products (C3b and C3bi; green) labels bacteria which stimulate engulfment of bacteria by phagocytes. Release of C5a is crucial for attraction of phagocytes to the site of infection. C. C3-derived products also enhances antigen presentation to B cells and triggers the development of an adaptive immune response. Source: Abbas et al., 2017.

Complement activation via antibodies depends on the large C1 complex that consists of the recognition molecule C1q and the serine proteases C1r and C1s (ratio 1:2:2). C1q has 6 globular head residues that each can bind an antibody molecule. Among the immunoglobulin subclasses, IgM is the strongest complement activator. Likely, this is due to the structure of the IgM molecule (pentamers) allow the 6 globular C1q heads to bind multiple antibody subunits at the same time. Structural analyses revealed that 6 antibody-binding headpieces of C1q could simultaneously bind to hexameric IgG, which is held together by noncovalent Fc-Fc interactions. C1r and C1s (proenzymes) are activated when the complex binds to a target surface. Activated C1s subsequently cleave C4 and C2 to deposit a C3 convertase enzyme (C4b2a) onto the target surface. The main effector functions of complement are driven by the cleavage of 2 central complement proteins: C3 and C5. Ongoing C3 cleavage by C4b2a (C3 convertase enzyme generated in classical patheway) and C3bBb (C3 convertase enzyme generated in alternate pathway) increases the density of C3b molecules on the bacterial surface. All recognition pathways (classical, alternate and lectin pathway) converge in the formation of convertase enzymes on the surface of the bacterium. Generated C3b can covalently attach to hydroxyl groups of carbohydrates on the bacterial surface. These efficiently trigger and facilitate phagocytosis by immune cells. C3b (and its breakdown product, iC3b) are recognized by complement receptors (CR) on myeloid (CR1, CR3, and CR4) and Kupffer cells (CRIg), and enhance the engulfment of opsonized particles, leading to intracellular (microbial) killing (Fig.2.5). The labeling of bacterial cells with C3-derived activation products also stimulates an adaptive immune response by directing the transport of bacteria to lymphoid organs and by enhancing antigen presentation to adaptive immune cells (Fig. 2.5). At high local C3b densities, C3 convertases (C4b2a, C3bBb) are associate with extra C3b molecules to form C5 convertases (C4b2aC3b and C3bBbC3b) and they cleave C5 into C5a and C5b. Newly formed C5b associates with components C6, C7, and C8, and 18 copies of C9 to form the lytic MAC (C5b-9) molecules. These molecules together form a heterogeneous pore and specifically kills Gram-negative bacteria. Gram-positive bacteria are protected from MAC-dependent killing, likely because their thick peptidoglycan outer layer prevents insertion of the MAC into the cell membrane (Fig. 2.6). Activation of C5 also results C5a, a strong chemoattractant that helps to recruit phagocytes towards

the site of infection and induces an oxidative burst. Additionally, C5a-mediated stimulation of basophils and mast cells triggers the production of histamine and subsequent vasodilatation (Sampayrac, 2019; Abbas et al., 2017).

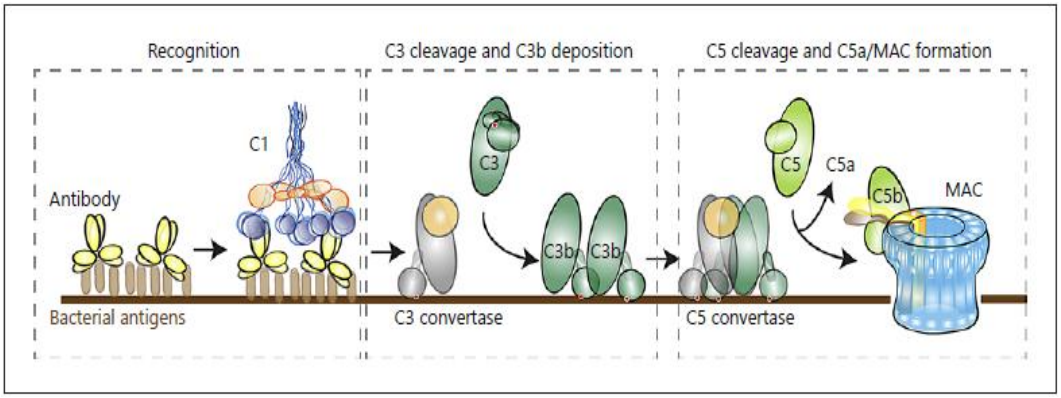

Fig. 2.6: Formation of the membrane attack complex (MAC or C5b-9; blue) in the surface of Gram-negative bacteria. Source: Abbas et al., 2017

2.3.2 Systemic immune response (Adaptive immunity)

2.3.2.1 *Salmonella* antigen processing and presentation by APC

The hallmark features of the adaptive (specific) immune response are antigen specificity as well as an ability to mount an enhanced response upon re-exposure to the same antigen. The innate immune system is linked to the specific immune system through a group of cells collectively called antigen presenting cells (APC) (Yrlid et al., 2000). During the initial stage of infection dendritic cell (DC) act as most important APC, because this cell can activate virgin T cells (Sampayrac, 2019). They have the unique ability to capture antigens and trigger the immune response by activating naive $CD4^+$ and $CD8^+$ T cells (Tobar et al., 2004). When intestinal DC detect (by PRRs) danger signal (PAMP) upon invasion of *Salmonella*, it begins to mature i.e. up regulate expression of MHC molecule and loaded with *Salmonella* antigen. After activation, DC migrate under influence of TNF-α to local lymph nodes (meseneteric LN). They uses class II MHC molecules to display fragments of the *Salmonella* proteins it has collected out in the intestines and class I MHC molecules to display peptides made by intracellular *Salmonella* infecting itself (Yrlid et al., 2001). Immunization of mice with *S.* Typhimurium phagocytosed DC generates cytotoxic T cells and IFN-γ producing CD8+ T cells specific for bacteria-encoded antigens (Yrlid et al., 2000). In the immune response to a *Salmonella* infection,

macrophages play a critical role by phagocytosing and destroying the microorganisms as well as presenting bacteria derived peptides to T cells. However activated macrophages don't travel to lymph nodes to present antigen. They stay in the local tissues and battle invaders. They are most useful for presenting antigen after the adaptive immune system has been activated. So mature DC activate virgin T cells and activated tissue macrophages mainly function to re-stimulate experienced T cells (Sampayrac, 2019). Both of these APC also produce cytokines such as IL-6 and IL-12 which influence the subsequent immune response to any gram-negative bacteria. The production of IL-12 is particular importance in mounting cell-mediated responses against *S.* Typhimurium that reside and replicate in vacuolar compartments of phagocytic cells (Yrlid et al., 2000).

B cell able to act as APC once it has been activated. Upon activation it can quickly activate helper T cells by concentrating small amounts of antigen for presentation. However B cell play major role as APC during later course of *Salmonella* infection or during subsequent infection (Sampayrac, 2019; Lopez-Medina et al., 2014). As *Salmonella* infects and resides preferentially within macrophages, the activation of these cells by IFN-γ produced by Th1 cells plays a prominent role in bacterial killing. Extensive studies in mouse and human typhoid indicates that CD4 Th1 cells play an important protective role against *Samonella* infection (Moon and McSorley, 2009). T cells are critical component of immunity to Salmonella infection and resistance to subsequent challenge. B cell required not only for antibody production but also for generation of protective immunity (Cummings et al., 2009). Greater understanding of *Salmonella*-specific T and B cell responses are necessary for the development of novel effective vaccines against both typhoidal and non typhoidal *Salmonella*.

2.3.2.2 Humoral immune response against *S.*Typhi

Human immune responses to typhoid infections involve not only innate but also antigen specific humoral and cellular immune responses. However the relative contribution of each arm of the immune system is not well understood. Protective immune responses to *Salmonella* infection is a prerequisite for new effective vaccines development. Although CD4 T cells are critical for protective immunity to Salmonella, but B cells also contributes in protection during secondary infection by Salmonella-specific antibody production, inflammatory cytokine production, and

direct antigen presentation to T cells (Nanton et al., 2012). Many large field trials have demonstrateed that S.Typhi specific antibodies are produced in a majority of subjects following natural illness and vaccination (Lindow et al., 2011). Epidemiological, animal and human studies indicate that antibodies play an important role in eliminating extracellular *Salmonella* while specific T cells are important for clearance of intracellular bacteria (MacLennan, 2014; Siggins et al., 2014; Gondwe et al., 2010; MacLennan et al., 2008). Salmonella are vulnerable to antibodies at distinct point of the invasion cycle: following initial invasion, when first entering the circulation and during transiting from one phagocyte to another via the blood or extracellular fluids (MacLennan et al., 2014). Antibodies kill extracellular microorganisms through complement-mediated bacteriolysis, opsonophagocytosis or antibody dependent cellular cytotoxicity (Mitchell et al., 2014). Antibody can also protect against *Salmonella* through opsonization of these bacteria promoting uptake and killing by phagocytic cells thereby linking humoral and cell mediated immunity against *Salmonella* (Ismail et al., 2002). However B cells also play a primary role of in adaptive immunity to *Salmonella* is via the development of protective T cell immunity. They contribute to protective immunity via antigen presentation to *Salmonella*-specific Th1 cells, or as an important source of inflammatory cytokines during infection (Nanton et al., 2012). Role of bacteria-specific IgG on the augmentation of Ag processing and presentation by DCs to T cells (Fig 2.7) against intracellular bacteria also supported by many recent data (Bueno et al., 2012; Tobar et al., 2004).

Generally IgG is the most critical antibody isotype for the clearance of bacteria and greatly contributes to the clearance of bacteria at least in the late phase of primary infection (Takaya et al., 2020). The roles of antibodies and B/plasma cells in *Salmonella* infection have recently re-evaluated. *Salmonella* inhibits the persistence of IgG-secreting plasma cells (source of serum IgG) in the BM of mice by secreting a SiiE protein (Fig. 2.8). This protein impairs the persistence of all IgG-secreting plasma cells in an antigen-specific independent manner. It may result in the loss of long-lived plasma cells secreting IgG against many kinds of bacteria and viruses generated by previous vaccination or infection (Takaya et al., 2020). Study revealed that the attenuated SiiE-deficient *Salmonella* may be a novel and efficient vaccine

against *Salmonella* as it enhances the protective high titer IgG production and the memory responses.

1. Antibody response against different structural component of *S*. Typhi

Chemical nature of the different structural component of *S*.Typhi is important factor in the determination of the character of its antibody response

a. Immune response to polysaccharide component

Carbohydrates in the form of capsular polysaccharides and/or lipopolysaccharides (LPS) are the major components on the surface of bacteria, are critical for immune recognition (Cobb and Kasper, 2005). These molecules are important virulence factors of many bacteria in infected persons (Weintraub, 2003). WHO has identified

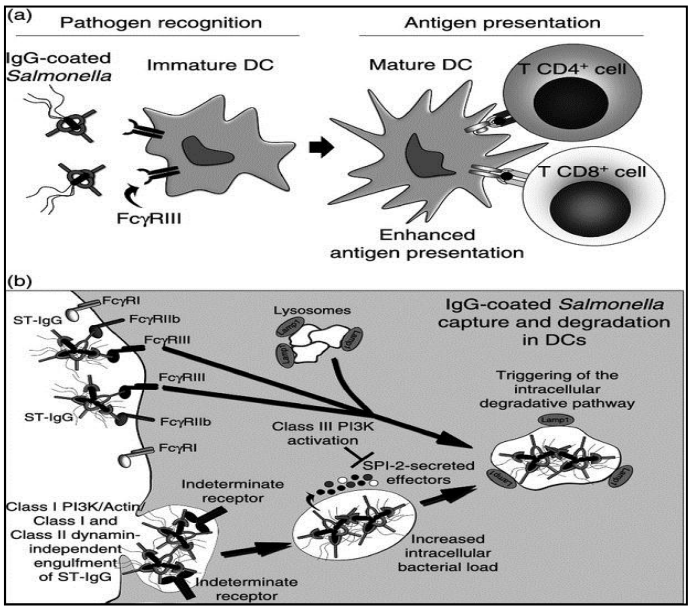

Fig 2.7: Fcγ receptor-mediated enhancement of T-cell priming by dendritic cells (DCs) after recognition and degradation of Salmonella-derived antigens.

(a) IgG opsonized *Salmonella* recognized by FcγRIII expressed on the surface of DCs and it significantly increases the DCs capacity to degrade and present bacterial antigens on MHC molecules and activate T cell. So, antigen presentation of DCs infected with *Salmonella* can be restored. (b) IgG-opsonized *Salmonella* engages FcγRIII on the surface of the DCs, triggering a PI3K dependent intracellular degradation pathway, which enhances fusion of the *Salmonella*-containing vacuoles (SCV) with lysosomes. As a result of IgG opsonization, antigen capture, degradation and presentation by DCs are significantly enhanced (Bueno et al., 2012).

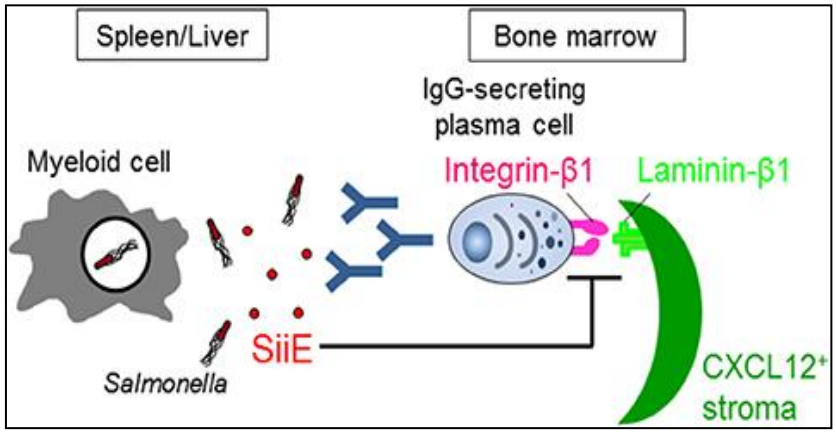

Fig 2.8: Mechanism of action of Salmonella SiiE protein to inhibit the persistence of IgG secreting plasma cell in BM. SiiE protein compete with laminin β1 of CXCL 12^{+} stromal cell of BM to interact with Integrin β1 of IgG secreting plasma cell and suppresses the retention of this plasma cells in BM survival niches. This competition induces detachment and deletion of IgG secreting plasma cell from stromal cell of BM (Takaya et al., 2020).

that most pathogenic bacteria are encapsulated which include *Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Neisseria meningitidis, Haemophilus influenza* , *Salmonella* Typhi etc. The capsule around these pathogens is composed of polysaccharides. These bacteria evade phagocytic killing as the capsule blocks complement binding and opsonization. This can be overcome by C-reactive protein (CRP) binding and the production of antibodies against the polysaccharide (Klein Klouwenberg and Bont, 2008). Fully encapsulated bacteria are poorly identified by innate immune mechanisms and defense is heavily dependent on adaptive responses producing opsonizing antibody against this type of pathogens (Vinuesa et al., 2003).

Humoral immunity act as principal defense mechanism against microbes with capsules rich in polysaccharides and lipids but T cells cannot respond to nonprotein antigens (Abbas et al., 2017). Serum antibodies against this polysaccharide antigen in concert with the complement system act by direct killing (bactericidal antibody) or by opsonophagocytic mechanism (opsonic antibody) to protect against encapsulated bacterial pathogens. Humans are capable of generating antibodies to protein antigens from birth. But an antibody to polysaccharide antigens starts to develop after 2 years of age, and reach adult levels approximately 5 years of age. The peak incidence of invasive infections with the encapsulated organisms during infancy coincides with

this period of polysaccharide unresponsiveness. Several hypotheses have been put forward for this immunological unresponsiveness which includes: immaturity of B cells, low or absent expression of CD21 (complement receptor 2) on marginal zone B cell, lack of diversity of the neonatal B cell repertoire, low level of IgG2, absence of B cells with a marginal zone phenotype, lack of a stromal component in the neonatal spleen, or deletion of polysaccharide specific B cells to prevent autoimmunity from recognition of cross reactive neuronal polysaccharide epitopes (Vinuesa et al., 2001).

However most of the bacterial polysaccharides are T-lymphocyte independent antigens (TI). In contrast to TD antigens, TI antigens do not require T-cells mediate immune response and poorly induce an immunological memory. Therefore, the antibodies produced primarily are of the IgM and in lesser quantities IgG2. Based on interaction with B cells, the TI antigens are divided into two categories: type 1 (TI-1) and type 2 (TI-2) antigens. TI-1 antigens include lipopolysaccharides of gram-negative bacteria cell wall. It induces immune responses to both adults and neonates as it can activate both immature and mature B cells. TI-2 antigens on the other hand, consist of molecule of highly repetitive structures such as bacterial capsular polysaccharides. It induces a limited immune response in children below two years of age. The older children and adults react to TI-2 antigens with the formation of sufficient antibody production by activating only mature B cells (Murphy &Weaver, 2017). So, immune responses to Gram-negative bacteria are relatively adequate in neonates and infants compared to encapsulated bacteria, but still below levels of those of adults. (Klein and Bont, 2008).

In addition, different subsets of B cells respond preferentially to polysaccharide, lipid and protein antigens. Marginal-zone B cells which are located in the peripheral region of splenic white pulp (comprise about 30% of human splenic B cell) but rare in most lymph nodes other than mesenteric lymph nodes (Vinuesa et al., 2001), respond largely to blood borne polysaccharide and lipid antigens. B-1 cells respond to non protein antigens in the mucosal tissues and peritoneum. Follicular B cells (majority of B cells) that reside in and circulate through the follicles of lymphoid organ, make the bulk of T dependent, class switched and high affinity antibody responses to protein antigens and generate long lived plasma cells. Marginal B cells and B-1 cells express antigen receptors of limited diversity and make predominantly T-independent IgM responses (Abbas et al., 2017). Among the IgG class, IgG2 are

the main subclass known to target polysaccharide antigens after any natural infection or immunisation. This can confirm by the patients with an IgG2 deficiency have an increased susceptibility for mucosal infections with encapsulated bacteria (Breukels et al., 1999).

B cell activation requires multiple cross linking of the B cell receptor by TI-2 antigens (Fig 2.9). In addition cytokines greatly enhanced the B cell responses and leads to isotype switching. Such cytokines may originate from dendritic cells. It able to bind the antigen through innate immune system receptors, present it to the B cells, and secrete a soluble TNF family cytokine called BAFF (B cell activating factor), that augment class switching of the B cell (Fig 2.9). So, dendritic cells and macrophages can provide co-stimulatory signals for activation of B cells by TI-2 antigens (Murphy and Weaver, 2017).

Bacterial capsular polysaccharides (CPSs) elicited type-specific immune responses & it conferred protection. This type of antigens exhibits a large degree of antigenic variation.

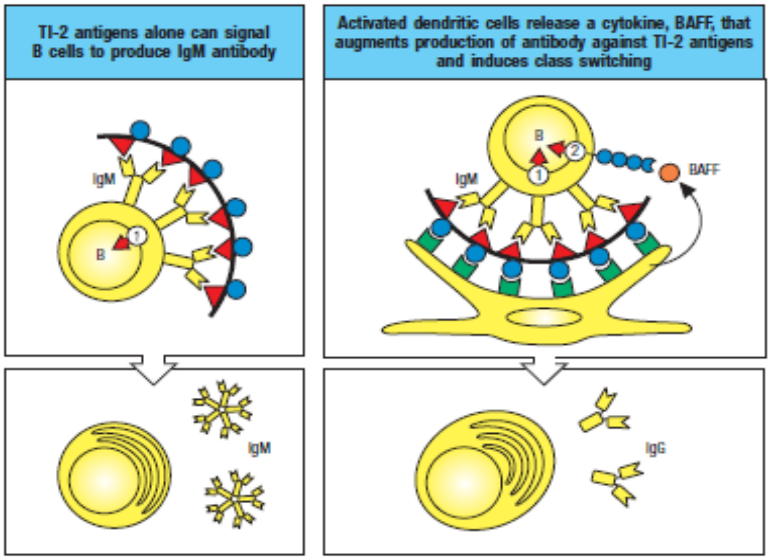

Fig 2.9: B-cell activation by TI-2 antigens (Murphy and Weaver, 2017)

The structural differences in the surface polysaccharides within the same species are the basis of serogrouping or serotyping systems. Homology between carbohydrate structures present on bacterial surface and host cell membrane could be potential virulence and evasion factor (Weintraub, 2003).

Humoral immune response to bacterial Polysaccharides (PSs) is clonally restricted compared to the response to protein antigens. That means antibody response restricted to a very small number of epitopes on any given PS. The reason is likely that PSs expresses relatively few epitopes that recognized as non self and as a result small number of B cell clones activated (Frasch, 2006). In addition, some bacterial polysaccharides are poor immunogens due to their structural similarities with glycolipids and glycoproteins present in humans. A better understanding of the immunological response to polysaccharide antigens may lead to the development of improved vaccines. This review explores the immune response on polysaccharide antigens.

i. Anti Vi response:

Antibody response to Vi capsular polysaccharide (Vi CPS) can acquired naturally during repeated exposure of *S.* Typhi in an endemic area or it can be acquired after typhoid fever or Vi based typhoid vaccination. Vi CPS antibody response is very important because it is protective and can be used for identification of S. Typhi carriers (House et al., 2008). Protective action of Vi is mainly mediated by serum anti-Vi IgG antibodies (Szu et al., 2014). Anti-Vi IgG was detected in 15% healthy control in Vietnam (House et al., 2008), 40% control in South Africa (Ferry et al., 2004), 83.3% in normal adult attending OPD in Nepal (Pulickal et al., 2009). Frequency of detectable (≥1/50 titer) serum anti-Vi IgG titers increased with age: 2% in 2-5 years, 9% in 6-10 years, 33% in those over 10 years age groups. Studies in endemic areas have reported that around 19-58% of patients are often exposed to *S.* Typhi and other related infections and therefore develop protective levels of anti-Vi antibodies in the serum prior to vaccination (Kaur and Jain, 2012). Average antibody titers in the sera from typhoid patients were higher than control. By the end of the second week of illness, anti-Vi antibodies are produced by approximately one third of acute case of typhoid fever (House et al., 2008). Whether or not these antibodies are protective for typhoid fever has yet to be determined but the evidence from vaccine studies shows that immunization with purified Vi CPS can confer protection. As data had shown a relation between risk of typhoid disease and the anti-Vi IgG titre, it may be predict that post-vaccination anti-Vi IgG titres will be a key component for assessing typhoid vaccine immunogenicity in future (Jin et al., 2017).

The attack rate of typhoid among the controls was 16.2 per 1000 and among those immunized with Vi was 4.1 per 1000 ($p<0.00001$). These data provide evidence that Vi antibodies confer protection against typhoid (Acharya et al., 1987). The data from immunization of healthy volunteers shown that geometric mean levels of anti-Vi IgG were significantly higher ($p<0.0001$) for post-vaccination subjects (39.2 AU/ml) compared to paired prevaccination (3.9 AU/ml) values. 95% (20/21) exhibited three fold increases, 67% (14/21) fourfold increases in their *S.* Typhi Vi IgG following vaccination. A threefold increase in anti-Vi IgG considered being positive vaccination response (Ferry et al., 2004). The first serological correlate of protection of anti-Vi antibody was 0.6-1.2 µg/ml at 3 years after immunization with Vi capsular polysaccharide. This value was based on the radioimmunoassay measurement of total Vi antibodies. The current estimated anti-Vi IgG protective level was 1.4 µg/ml at 46 months after immunization (Szu et al., 2014). Variation in immune responses among different individuals has shown differences in post-typhoid vaccination antibody levels in 23-40% patients. This variation in immune response is due to differences at the genetic level along with environmental factors (Gupta et al., 2008; Sur et al., 2009). An IgG titer of ≥1:200 significantly discriminated among chronic carriers (86%), patients with acute typhoid fever (12%) and a normal US population ($P< 0.001$) (Losonsky et al., 1987). IgG to the Vi antigen has been shown to have a sensitivity of 75% and specificity of >95% in outbreak investigations of S.Typhi infection (Charles et al., 2013)

ii. Anti O response:

Classical activation of antigen presenting cell by *Salmonella* LPS leads to alteration in the secretory profile of the cells and production of proinflammatory cytokines, nitric oxide (NO) etc. and thereby stimulate host's innate and adaptive immunity. During natural infection, antibodies appears first against LPS and it can be detected as S. Typhi O-antigen antibodies in sera of typhoid patient. The detection of antibody to S. Typhi O-antigen forms the diagnostic basis in Widal test, ELISA, Tubex TF, Multi-test DipSticks for typhoidal fever (Bharmoria, 2017). The antibody responses to this antigen were found highly variable among infected individuals and raised antibody titers were also detected in a high proportion in serum samples from healthy subjects from the community. The sensitivities of the anti-LPS IgM, IgG, IgA ELISAs tests and the Widal TO, TH tests were 0.75, 0.55, 0.52 and 0.47, 0.32

respectively, at a specificity of ≥0.93 (House et al., 2001). The IgM somatic "O" antibody represents the initial serologic response in acute typhoid fever in comparison to IgG flagellar "H" antibody (Kintz et al., 2017). Detection of IgM antibodies to *S.* Typhi LPS (ELISA) had a significantly higher sensitivity than the Widal TO test when used with a single acute-phase serum sample ($P \leq 0.007$) (House et al., 2001). In Bangladesh, 17.3% and 11.3% showed no response to single TO and both agglutinin (TO &TH) among the bacteriologically proven typhoid cases. TO titer >1:80 and TH titer >1:160 were considered to be significant with 88% sensitivity and 98% specificity respectively (Saha et al., 1996).

b. Immune response to protein component

Antibody response to protein antigens rely on follicular B cell subset in compare to non protein antigen which rely on mainly marginal zone B cell. Protein antigens first capture and display by follicular dendritic cells (FDCs) to B cells in the secondary lymphoid organs. Those B cells whose receptors are crosslinked by binding to their cognate antigens activate to form active or secondary lymphoid follicle. The protein antigens process in endosomal vesicles of B cell and display class II MHC-associated peptides for recognition by CD4+ helper T cells. Activated helper T cells recognize peptide antigens and express CD40 ligand, both of which bind to their receptors on the same B cells and activate the B cells by secreting cytokine (T cell dependent activation). Activated Th cell also need the assistance of activated B cells in order to mature fully. This assistance involves B7 and ICOSL on B cell to bind with CD28 and ICOS protein on the Th cell surface respectively. This fully mature Th cells are called follicular helper T (Tfh) cells.

Initial T-B interaction occurs at the edge of lymphoid follicles (extrafollicular foci) which stimulate B cell to proliferate and produce low level, low affinity antibodies and short lived plasma cells. Fully developed antibody response occur in germinal center. A few activated B cells from extrafollicular foci migrate back into the lymphoid follicle and divide rapidly (doubling time 6 hours) in response to signal from Tfh cells. These extensively proliferating B cells produce dark zone in the germinal center (Fig. 2.10).

Maturation of B cell divided into three stages: class switching (change the class of antibody), somatic hypermutation (mutation of v region of immunoglobulin gene to produce increase affinity of BCRs for their cognate antigen) and carrier decision

(decide whether to become long lived plasma cell or memory cell). So, short-lived and long-lived plasma cells make both immediate and long term antibody against the protein antigen (Sampayrac, 2019; Abbas et al., 2017). Secondary responses to a variety of protein antigens have been observed. Previous immunization with one antigen will accelerate and magnify the immune response to the second injection of related antigen. Subsequent immunization with one antigen may increase the amount of circulating antibody exclusively oriented to another antigen administered earlier, if the two antigens are related.

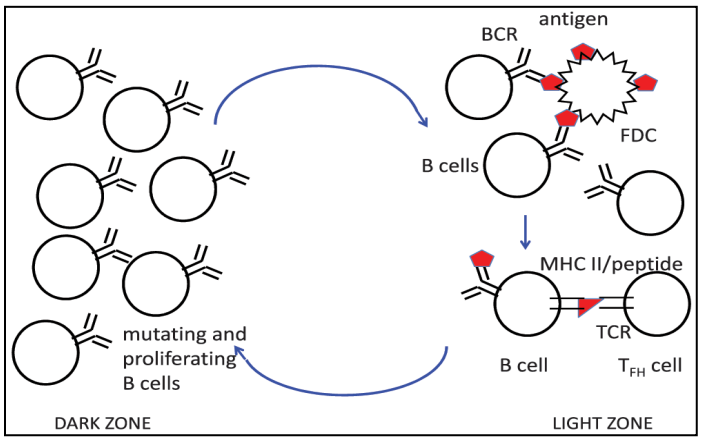

Fig 2.10: Germinal center B cell response to protein antigen in lymphoid follicle

Study found that protection in humans against invasive pneumococcal disease mediated by naturally acquired IgG is not dependent on capsular antibodies rather protection seems to require recognition of bacterial surface proteins (Wilson et al., 2017).

2. Functional antibody responses against *S*. Typhi after natural infection and vaccination.

Understanding the function of antibodies mounted in response to disease or vaccination addresses major challenges in understanding humoral immune responses to typhoid disease and aids in the evaluation of new typhoid vaccines. However, many large field trials have demonstrated *S.* Typhi specific antibodies production following vaccination or natural illness, although the mechanism of protection provided by *S*. Typhi specific antibodies has been poorly defined (Boyd et al., 2014). A passive hemagglutination assay or ELISA has been previously used to determine

serum antibodies against *S.* Typhi but it does not assess functional antibody levels. Assays are needed to measure functional antibodies (Opsonophagocytic or bactericidal or antibody dependent cellular cytotoxic activity) to assess their immunogenecities and potential protective capacities (Jang et al., 2016; Boyd et al., 2014). Opsonophagocytic antibodies bind to bacterial antigens and facilitate microbial uptake and killing by phagocytic cells. Bactericidal antibodies mediate direct bacterial killing in presence of complement. Bactericidal antibody only acts on *Salmonella* while the bacteria are in the extracellular compartment and *Salmonellae* are adapted for intracellular survival. In contrast, opsonic antibody facilitates killing of internalized Salmonella by phagocytes, although antibody still needs to bind *Salmonella* before phagocytosis. Even if Salmonella avoid opsonization by directly entering into macrophages in gut associated lymphoid tissue, the hematogenous spread of Salmonellae from one macrophage to another in the reticuloendothelial system exposes them to bactericidal and opsonic antibody (Gondwe et al., 2010). The typhoidal serovars were more susceptible to complement mediated bactericidal killing. MAC deposition in the presence of anti-Vi IgG antibodies or anti-LPS IgG antibodies correlates with killing of *S.* Typhi in the Serum Bactericidal Assay (SBA) (Jang et al., 2016; Hart et al., 2016; Boyd et al., 2014). But study by Siggins et al. (2016) suggest that MAC deposition secondary to binding of O:9 antibodies (anti-LPS) is not effective at killing in the presence of high levels of Vi antigen. Antibodies to Vi can be bactericidal and opsonic and kill Vi expressing *S.* Typhi and *S.* Typhimurium. The level of killing correlated with Vi expression. With pneumococcal conjugate vaccines, opsonophagocytic antibodies are accepted as correlates of protection (Boyd et al., 2014) and for meningococcal purified polysaccharide and polysaccharide conjugate vaccines, the correlate is serum bactericidal antibody (McIntosh et al., 2015; Frasch et al., 2009; Borrow et al., 2005). The SBA has been accepted as an in vitro surrogate assay for the evaluation of immunogenicity of cholera vaccines (Yang et al., 2007) and also bactericidal antibody response in acute and convalescent cholera patients (Son and Taylor, 2011; Qadri et al., 1995). The serum bactericidal assay measures functional S. Typhi specific antibodies capable of complement mediated bacterial killing. Serum bactericidal antibody titer also can be used as biomarker for clinical evaluation of typhoid vaccines. The SBA titer after Vi-PS typhoid vaccination showed a good

correlation with anti-Vi IgG quantity in the serum by Spearman correlation coefficient of 0.737 ($p<0.001$) (Jang et al., 2016). Future work assessing functional Vi-antibody is required to find out the differences in antibody quality in between protected and susceptible individuals.

Serum bactericidal assay can be used to detect antibodies against *Salmonella* Typhi in serum samples, might be serve as an indicator of acute infection and potential protection against typhoid similarly as vibriocidal assay in cholera (Son and Taylor, 2011). It can be utilized in research and clinical settings to test effectiveness of vaccines and also in epidemiological studies relevant to typhoid transmission and surveillance. This assay was first introduced to detect vibriocidal titer in 1962 by Finkelstein using agar plate based protocol. But this procedure was very labor intensive and time consuming as titer determination required manual colony counting method. In 1968 Benenson et al., developed microtechnique to determine titer response by adding liquid broth to detect the surviving bacteria that were not lysed by antibody (Benenson et al., 1968). Boutonnier et al in 2003 eventually refined this technique to make the protocol more user friendly, first by decreased timing, second by using small amount of broth to prevent spillover from adjacent well in the 96 microtiter plates and third by adding 1% neotetrazolium chloride to measure the growth of surviving bacterial target cells by monitoring a color change (Son and Taylor, 2011; Boutonnier et al., 2003).

The serum bactericidal (SBA) assays measure the level of antibody in serum necessary to kill bacteria by complement activation. A suspension of bacteria is mixed with dilutions of (heat-inactivated) antiserum followed by the addition of complement. The viability of the bacterial suspension is detected at each serum dilution. The bactericidal antibody titer is usually expressed as the dilution of antiserum that kills a defined proportion of bacteria The various SBA assay has been used in different studies to measure bactericidal antibody titer in cholera (Son and Taylor, 2011; Qadri et al., 1995b; Losonsky et al., 1987) or to estimate immunogenicity induced by vaccines for protection against cholera (Yang et al., 2007), typhoid (Juel et al., 2017; Jang et al., 2016; Kantele et al., 2013) or meningococcal disease (Frasch et al., 2009) or *Haemophilus influenza* type b infections (Kim et al., 2016) etc. Some factors including source and quantity of exogenous complements, test sera, bacterial strain, antigen expression in target

bacteria etc. are important for getting reliable SBA results (Jang et al., 2016). Modification of surface structure can inhibit the effective attachment of the terminal complement complex to the lethal sites on the cell surface. So to overcome the inhibitory effect, lesser amount of bacteria and higher concentrations of complement could be used (Qadri et al., 1995).

2.3.2.3 Cellular immune response against *S*. Typhi

Although several immune mechanisms contribute to killing and elimination of intracellular pathogens, persistent intracellular pathogens have found ways to thrive within a susceptible host. Role of cellular immunity in protection against *Salmonella* in man is established by the high frequency of severe *Salmonella* infections in individuals with defects in the IL12/23-IFN-γ axis. IFN-γ from $CD4^+$ lymphocytes and NK cells being important for activating macrophages to produce oxidative burst. (Gondwe et al., 2010a). CD4+ T cells are required for most antibody responses, whereas antibodies exert significant influences on T-cell responses to intracellular pathogens (Siegrist, 2018).

2.4 Current prophylaxis

Strategies for prevention and control of typhoid fever include safe water, adequate sanitation, appropriate personal and food hygiene, and vaccination. (Date et al., 2014). As the disease is spread through contaminated food and water, efforts to improve water quality, sanitation and hygiene will likely go a long way to reducing the global burden of disease. Unfortunately, improving access to clean water and sanitation is very costly and difficult to implement on a large scale. Vaccination against typhoid fever is the probable cost-effective way of reducing the global disease burden. It is the most cost effective methods of controlling typhoid fever especially protecting the population living in endemic zones.

2.4.1 *Salmonella* Typhi vaccine

2.4.1.1 History

The importance of typhoid vaccines to prevent this disease is long standing. Almroth Edward Wright, Richard Pfeiffer, and Wilhelm Kolle were first developed the typhoid vaccine in 1896. Heat killed, phenol-preserved and acetone-killed lyophilized injectable whole-cell *S. Typhi* vaccine were used that time in England and Germany (Sahastrabuddhe and Saluja, 2019). In 1904 the statistician Karl

Pearson reviewed seven studies of a heat inactivated typhoid vaccine conducted in British army units. He concluded that these vaccine studies were flawed and failed to show the efficacy of the vaccine. Though Pearson's assessment and concerns about toxicity, this vaccine was used routinely in the British army. In 1962, the results of 29 randomized controlled trials of a typhoid vaccine have been published. Whole cell vaccines, administered parenterally, were found to be effective but have a high incidence of side effects. Two vaccines developed more recently, Ty21a (an attenuated strain of *S.* Typhi administered orally) and Vi (the purified bacterial capsule, given parenterally), have seemed less toxic than the older whole cell vaccines (Engels et al., 1998). More recently, newer typhoid conjugate vaccines (TCV) have entered use, with evidence of immunogenicity in children from six months old (Milligan et al., 2018).

2.4.1.2 Vaccine Types

There have been many efforts done by different groups of scientists to develop an effective vaccine against *Salmonella* Typhi. But at present a subunit (Vi PS) and a live attenuated S. Typhi strain (Ty21a) are licensed and commercially available. Categories of different types of Typhoid vaccines are given in Table 2.3.

Table 2.3: Different types of typhoid vaccine

Types of Vaccine	Name of Vaccine	Commercial name (developer)	Age/ Volunteer age	Rout & dose	No. of dose	Schedule	Revaccination	Remarks
1. Whole organism Vaccine								
Killed	**Inactivated whole cell typhoid vaccine**	K and L vaccine	Children ≤6years of age	Intradermal (1^{st}-0.1ml, 2^{nd}-0.2ml) Or Subcutaneous (1^{st}-0.5ml, 2^{nd}-1.0ml)	2	1^{st} &2^{nd} dose separated ≥4weeks	After 3 years	Whole cell inactivated by heat,phenol (L) or acetone (K). Not in used except few countries (Thailand)
Live attenuated	**Ty21a**	Vivotif (Berna Biotech,Cruecell,Switzerland)/Zerotyph(Boryung)	Adults and Children ≥6yrs age	Oral, 1capsule (each capsule contains 2-10x10^9 viable cell)	4	Days 0,2,4,6	Every 5years	Lacks both functional galactose-epimerase gene and Vi antigen

Types of Vaccine	Name of Vaccine	Commercial name (developer)	Age/ Volunteer age	Rout & dose	No. of dose	Schedule	Revaccination	Remarks
	Ty21a			and 5-50x10^{9}non nviable S.Typhi Ty21a				
	M01ZH09 (under phase 2 clinical trial)	(Emergent Biosolution)	2 to 17	Oral, 5x10^{9}	1	-	-	New vaccine candidate, deletion in SPI gene (Ty2aroC &ssaV) required for secretion of bacterial effector protein
Live attenuated	**CVD 908** (under phase 1 clinical trial)	(CVD-centre for vaccine development University of Maryland)	18 to 35	Oral, 5x10$^{4-5}$	1	-	-	Deletion of aroC & aroD gene of virulentTy2
	CVD 908-htrA (under phase 2 clinical trial)		18 to 40	Oral, 5x10$^{6-9}$	1	-	-	Deletion of aroC, aroD& htrA genes of virulentTy2
	CVD 909 (under phase 1 clinical trial)		-	Oral, 2.5x10^{9}	1	-	-	CVD 908htrA constitutively expressing Vi
2. Subunit vaccine								
Plain polysaccharide	**Vi polysaccharide vaccine**	TyphimVi (Sanofi Pasteur)/Typherix, (GSK) /Typbar, (Bharat Biotech)/Typhivac(Sanghai institute)	Adults & children >2years of age	Intramuscular (25ug), 0.5ml	1	-	After 3years	licensed

Conjugate vaccine	**Vi-TT**	Typbar-TCV, (Bharat Biotech)/PedaTyph, (BioMed)	Children ≥6 months to 45years	Intramuscular (25ug), 0.5ml	1or 2dose	-	-	Vi conjugated to tetanus toxoid carrier protein in isotonic saline. Licensed in India
	Vi-DT (under phase 2 clinical trial)	International vaccine institute (Shanta Biotech)	-	-	-	-	-	Vi chemically conjugate with diphtheria toxin
	Vi-rEPA (under phase 3clinical trial)	National institute for health/ Lanzhou Institute, Chaina	-	-	-	-	-	Pseudomonas aeruginosa exotoxinA act as a carrier protein, Licensed in Chaina
	Vi-CRM_{197} (under phase 2 clinical trial)	NVGH	-	-	-	-	-	CRM_{197}carrier protein which is nontoxic mutant of diphtheria toxin
	O9-DT (preclinical)	International vaccine institute (IVI)	-	-	-	-	-	
Protein vaccine	**OmpC and OmpF** (phase1 in Mexico)	Institute Mexicano del Seguro social	-	-	-	-	-	
	GMMA (Generalized module for membrane antigen) (preclinical)							Deliver both surface polysaccharides and outer membrane prot to immune system

source: Theiss-Nyland et al., 2019b; Sahastrabuddhe and Saluja, 2019; Milligan et al., 2018a; Mitra et al., 2016; Mohan et al., 2015; Jackson et al., 2015; W.H.O., 2014; Puneet Garg et al., 2014; MacLennan et al., 2014; Date et al., 2014; Marathe et al., 2012; Avci et al., 2011; Sur et al., 2009; WHO, 2007; Tacket and Levine, 2007; Engels et al., 1998; Ivanoff et al., 1994; Bardhan et al., 1963; Felix, 1951

2.4.2 Immune response to *S.* Typhi vaccine

2.4.2.1 Immune response to capsular polysaccharide vaccine

It is well established that an immune response against the surface polysaccharides confers protection against capsulated pathogens. For this reason polysaccharide (PS) vaccines have been licensed for protection against several serious infections by

polysaccharide coating bacteria like *Neisseria meningitides*, *Streptococcus pneumonia*, *Haemophilus influenzae* type b (Hib) and *Salmonella enterica* serovar Typhi. (Frasch, 2006). The bacterial capsular polysaccharides (CPSs) became the target of several investigators in the 1920s and 1930s. In the mid-1940s it was established that CPS elicited type-specific protective immune responses. Vaccination with polysaccharides also reduced the carrier rate of bacteria of the same types (Weintraub, 2003). Polysaccharide antigens released from the injection site reach the marginal zone of the spleen/nodes by macrophages. This PS antigen then binds to marginal zone B cells. Repetitive structure of this antigen crosslinks the Ig receptors on the B-cell surface. This activates extrafollicular marginal zone B cells. During the week following immunization, B cells differentiate into plasma cells, undergo some degree of isotype switching from IgM to IgG/IgA. Thus, PS vaccines are usually produce moderate titers of low-affinity antibodies (mainly IgM and low extent IgG/IgA) by triggering T-independent responses (Siegrist, 2018). Subsequent revaccination with same polysaccharide does not generate secondary response. Repeated primary response follows the same kinetics in previously primed as in a naïve individual. Revaccination with some bacterial PS may even induce lower antibody responses than the first immunization, reported as hyporesponsiveness (Siegrist, 2018).

Immunization with polysaccharide vaccine in humans can generate the production of intermediate-affinity IgG antibodies. One hypothesis is that polysaccharide immunization activates “memory” B cells that have been previously formed by cross-reacting polysaccharide bacterial antigens somehow linked to protein moieties and eliciting germinal center responses. An alternative possibility is that the IgM+, IgD+, CD27+ memory B cells that appear in the blood in response to PS immunization may be recirculating splenic marginal zone B cells (Siegrist, 2018). Hib capsular polysaccharide can elicit a high IgG response from memory B cells induced by primary immunization with Hib conjugate vaccine (Granoff et al., 1993). Estimation of antibody concentrations and measurements of functional antibody are critical to polysaccharide induced immunity (Frasch, 2006).

Main limitation of plain polysaccharide vaccine is children below 2 years of age and elderly respond poorly to this type of vaccines. Immunization with polysaccharide antigens leads to limited class switching of activated B cells, no affinity maturation, and poor induction of memory cells (Jakobsen et al., 2001). The wide structural

heterogeneity among the polysaccharides within and between species is also a problem. Development of glycoconjugate vaccines have been proven to be efficient in inducing T-lymphocyte dependent immunity and to protect both infants as well as elderly from disease (Weintraub, 2003). Conjugation to a protein carrier engages T-cell help through the presentation of carrier peptides on major histocompatibility complex (MHC) molecules of antigen-presenting cells, thus overcoming the limitations of unconjugated polysaccharide (parenteral Vi CPS) vaccines. (Baliban et al., 2018).

2.4.2.2 Immune response to glycoconjugate vaccine

For vaccine-induced long-lasting protection against invasive encapsulated bacteria, a T dependent response is desirable (Beuvery et al., 1982). Glycoconjugate vaccines have played an enormous role in preventing infectious diseases caused by virulent pathogens such as *Haemophilus influenzae, Streptococcus pneumoniae, Neisseria meningitides,* Group B *streptococcus, Salmonella* Typhi etc. (Geno et al., 2015; Simon and Levine, 2012; Kelly et al., 2004; Paoletti and Kasper, 2003). The advantages of glycoconjugate vaccines over pure polysaccharide in inducing immune responses are well accepted. Covalent coupling of a CPS to a carrier protein elicits T-cell help for B cells that produce IgG antibodies to the polysaccharide (PS) component. Thus glycoconjugates induce PS-specific IgM-to-IgG switching, memory B-cell development, and long-lived T-cell memory. An understanding of the immune mechanisms involved in glycoconjugate immunization is of paramount importance in the rational design of new-generation vaccines against emerging infections (Avci et al., 2011). In glycoconjugates, the protein carrier provides the T epitopes for T-cell help recruiting, while the B epitopes for polysaccharide specific B cells engagement are provided by the saccharide moiety. The polysaccharide binds to BCR and internalized. After that B cells processed the protein moiety of glycoconjugate in peptides that are presented to carrier specific T cells in association with major histocompatibility complex (MHC) class II molecules. When B cells receive help from carrier specific Tfh (follicular T) cell, it induces formation of germinal centers [GCs] by differentiation PS specific B-cell in a highly efficient way. Recently, an additional mechanism has been anticipated in which the glycoconjugate is processed into glycan-peptides inside polysaccharide specific B cells. The resulting glycan-peptides bind MHC class II via the peptide portion allowing the glycan moiety to be exposed and recognized by the T-cell receptor of

carbohydrate specific T cell clones which then provide the cognate help to B cells (Fig. 2.11).

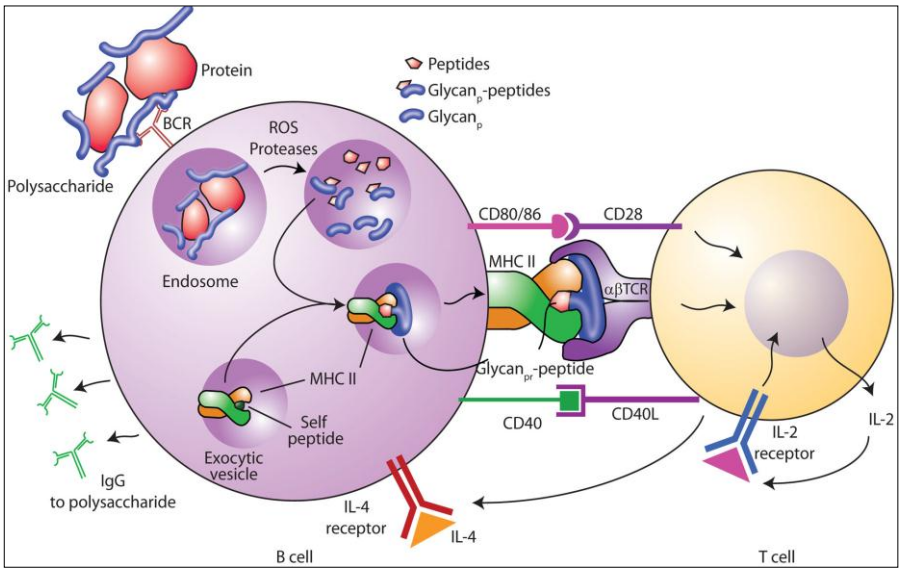

Fig 2.11: Mechanism of T-cell activation by glycoconjugate vaccines (Avci et al., 2011).

Schematic representation shows antigen processing and presentation of glycoconjugate vaccines and production of IgG antibodies to the polysaccharide. (1) The carbohydrate portion of the glycoconjugate binds to and cross-links the receptor of a B cell (BCR) (2) The glycoconjugate is internalized into an endosome of the B cell. (3) The carbohydrate is processed in the endolysosome by ROS into saccharides composed of smaller numbers of repeating units. The protein portion is processed by acidic proteases into peptides. Processing of both the protein and the carbohydrate portions generates glycanp-peptides (~10 kDa). (4) MHCII binding of the peptide portion of the glycanp-peptide allows the presentation of the more hydrophilic carbohydrate to the αβ receptor of CD4+ T cells (αβTCR). (5) The αβ receptor of CD4+ T helper cells recognizes and responds to the non-zwitterionic saccharide presented in the context of MHCII. (6) Activation of the T cell by the carbohydrate/MHCII, along with co-stimulation, results in T-cell production of cytokines such as IL-4 and IL-2, which in turn induces maturation of the cognate B cell to become a memory B cell, with consequent production of carbohydrate-specific IgG antibodies.

So, a conjugate vaccine can induce a T-cell-dependent response and be immunogenic from early infancy (Micoli and Adamo, 2018). The role of T-helper cells is obtained (i) through presentation of processed antigens in the context of class II MHC molecules on APCs to TCRs, (ii) by the interaction of the costimulatory molecules B7-1 and B7-2 on APCs with CD28 on T-helper cells, and (iii) by stimulation of CD40 on B cells through interaction with CD40L on activated T-helper cells (Guttormsen et al., 1999). Antigen-specific B cells proliferate and differentiate into higher-affinity antibody-producing cells, longer-lived plasma cells, and/or memory B cells. The latter ones, on subsequent encounter of specific antigen, can rapidly proliferate and differentiate into plasma cells, producing high antibody titers. Antibody avidity is increased through affinity maturation in germinal centers. The duration of antibody responses depends on the number and/or quality of long-lived plasma cells generated by immunization. Some germinal center (GC)-induced plasma cells are attracted toward the BM and BM stromal cells provide the signals required for their long-term survival. In BM niches, plasma cell survival and antibody production may persist for years. In the absence of subsequent antigen exposure, antibody titers persist 6 to 12 months after immunization up to the end of the short-term plasma cell response. Intensity of vaccine-induced GCs can be modulated by various determinants. The main determinants are the nature of the vaccine antigen and its intrinsic immunogenicity. For example, tetanus toxoid is found intrinsically a stronger immunogen than diphtheria toxoid. There is a risk of limiting anti-PS responses or carrier-mediated epitope suppression resulting in vaccine interference with individual conjugate vaccines (Siegrist, 2018).

2.4.3 Immunogenicity of *S.* Typhi Vaccine

To determine the immunogenicity of Vi capsular polysaccharide vaccine, Vi antibodies levels measured after 3 years in vaccine recipients had higher than the controls by radio-immunoassay (GMT 1.28 vs 0.76 microgram ml-1, P = 0.0004) and passive haemagglutination assay (GMT 10.46 vs 3.52, P = 0.0001) (Klugman et al., 1996). Seroconversion was found 92.6% (138/149), 97.9% (138/141), 84.7% (116/137), 55% (55/100) after 1, 3, 12 & 36 month respectively after single dose of Vi CPS (25ug) in adults (20-28yrs) and children (8-16 yrs) of Korea. Geometric mean titer (GMT) of anti Vi antibody was detected approximately 16, 9 & 4 times

the prevaccinated titer at 1,12 & 36 months of vaccination by passive haemagglutination method (Kim et al., 1995). Comparison of safety and immunogenicity of a Vi polysaccharide with a whole-cell killed typhoid vaccine in young adult recruits (aged 18-25 years) of the Malaysian Air Force showed a 7–9 fold rise in serum anti-Vi antibody levels with GMT values of 4.71 mg/ml and 3.64 mg/ml at 2 weeks and 6 weeks, respectively in the subjects who received the Vi vaccine in compare to whole cell killed vaccine which generated 2-fold rise in titre, with corresponding GMT values of 0.733 mg/ml and 0.796 mg/ml at 2 weeks and 6 weeks. Seroconversion rate was 75.5% vs 25% (at 2wks) and 67.0% vs 31.3% (at 6 wks) following immunization with Vi CPS & whole cell killed vaccine respectively. In Asian populations a seroconversion rate of 79% was obtained in Nepal, 68% in Indonesia, 71% in China, and 93% in the Republic of Korea (Panchanathan et al., 2001).The immune response to this vaccine is depicted by the production of IgG antibodies. So, anti Vi IgG levels are used for the assessment of protection against typhoid fever. 85 to 95% of the vaccinees develop IgG antibodies after immunization with Vi capsular polysaccharide vaccine. Protective levels of antibodies are elicited after 7 days and peak at 28 days post vaccination. The antibody levels diminished usually after 2 years. Hence revaccination is recommended every 3 years in settings where typhoid is endemic and in high-risk populations (Ochiai et al., 2014). As the Vi vaccine does not induce either protective levels of antibodies in young children (<2 years) or a booster response, Vi is added to different protein carrier (TT, DT, r-EPA, CRM197 etc) to enhanced the immunogenicity of Vi and gave it T-cell–dependent properties. The Vi conjugate vaccine elicited and maintained higher levels of anti-Vi IgG serum antibodies compared with those who received the Vi polysaccharide vaccine among children even below 6 months age in high typhoid endemic settings (Thiem et al., 2011; Lanh et al., 2003; Lin et al., 2001; Kossaczka et al., 1999). The immunogenicity of this Vi conjugate vaccine was dose dependent (Kossaczka et al., 1999). Following the administration of a single dose, detectable antibody levels were maintained for as long as 10 years in adults and 8 years in children. A threshold value of 4.3 µg/ml anti-Vi antibody (by ELISA) derived from efficacy trial of Vi-rEPA in Vietnam, associated with a high level of sustained protection lasting approximately 4 years after vaccination (Szu et al., 2013). Following a single dose of ViTT in aged 6–23 months, anti-Vi IgG antibodies proved to be long-lived and geometric mean titer still remained >5-fold above baseline at

day 720 (Mohan et al., 2015). Ty21a vaccine generate both antibodies and cell-mediated immune responses (Garmory et al., 2002; Olanratmanee T. et al., 1993; Murphy et al., 1987). The Ty2la vaccine was known to be immunogenic for older children and adults. The immunogenicity of this vaccine is age dependent. There was no evidence of a humoral or cellular immune response to S. Typhi among infants and toddlers <24 months age (Murphy et al., 1991). Continuous efforts are being undertaken to develop newer typhoid vaccine to attain higher antibody titers and increased immunogenicity (Marathe et al., 2012).

2.4.4 Efficacy of *S.* Typhi Vaccine

A meta-analysis of about 1.8 million vaccinees in efficacy trials estimated the 3-year cumulative efficacy 73% (65–80%) for two doses of whole cell vaccines, 51% (35–63%) for three doses of Ty21 live attenuated vaccine, and 55% (30–71%) for one dose of Vi vaccine (Engels et al., 1998). Efficacy of different typhoid conjugate vaccines (TCV) was evaluated in a series of studies in endemic and other areas. A placebo-controlled, randomized, double-blind study in Vietnamese children aged 2–5 years in the highly endemic area gave an estimated Vi-rEPA vaccine efficacy of 89% after nearly 4 years and anti-Vi IgG persisted for 10 years (Szu et al., 2014; Lanh et al., 2003). Seroconversion rate of Vi-TT vaccine in 6 months to 2 years aged children demonstrated 98% (Mohan et al., 2015). Recently, ViTT was prequalified by the WHO and licensed in India. Other TCVs are in various clinical trial phases globally (Lee et al., 2020). Several investigations on efficacy of typhoid vaccine are briefly summarized in the Table 2.4 .

Table 2.4: Efficacy of typhoid vaccines

Vaccine	Author, Year Journal	Country	Age range, No of subjects	F/U (yrs)	Efficacy (95%CI)
Vi CPS	Acharya et al., 1987 N Engl J Med	Nepal	5 to 44, 6438	1.6	72 (41 to 87)
	Klugman et al., 1996 Vaccine	South Africa	5 to 16, 5692	3	55 (30 to 71)
	Wang et al., 1997 Zhonghua Liu Xing Xue Za Zhi.	China (Jiansu Prov)	Adults &children, 81,506	1	71 (50-81)
	(Yang et al., 2001) Bulletin of WHO	China	3 to 50, 65, 287	1.7	69 (28-87)

Vaccine	Author, Year Journal	Country	Age range, No of subjects	F/U (yrs)	Efficacy (95%CI)
TCV (Vi-TT)	Mitra et al., 2016 Human Vaccines & Immunotherapeutics	India	6month to 12yr, 1765	1	100 (97-100)
	Jin et al., 2017 Lancet	UK	18 to 60, 41	1	55 (27-72)
	Theiss-Nyland et al., 2019a Clinical Infectious disease	Bangladesh	9 months to <16, 32,500	2	Not yet published
	Theiss-Nyland et al., 2019b Clinical Infectious disease	Nepal	9month to<16, 20,000	2	Not yet published
	Wahdan et al., 1982 J Infect Dis	Egypt	6 to 7, 32,388	3	96 (67 to 99)
Whole cell vaccine	Levine et al., 1987 Lancet	Chile	6 to 15, 10900	3	67 (46 to 79)
	Levine et al., 1990 Lancet	Chile	5 to 19, 81621	3	77 (liquid), 33 (enteric capsule)
	Black et al., 1990 Vaccine	Chile	5 to 22, 82543	2	59
	Simanjuntak et al., 1991 Lancet	Indonesia	3 to 44, 20543	2.5	53 (liquid), 42 (enteric capsule)
	YTC, 1962 Bull World Health Organ	Yugoslavia	5 to 50, 11000	6	72 (50 to 84)
	YTC, 1964 Bull World Health Organ	Yugoslavia	2 to 60, 9500	2.5	51 (heat-phenol), 79(acetone dried)
	Hejfec, 1965 Bull World Health Organ	USSR	7to ?, 22,269	0.7	37 (5-62)
	Hejfec et al., 1966 Bull World Health Organ	USSR	7 to 18, 45187	2.5	54(alcoholized), 82 (heat killed)
	PTC, 1966 Bull World Health Organ	Poland	5 to 60, 90670-116858	3	71-84
	Tapa and Cvjetanovic, 1975	Tonga	2 to 60,11128	7.5	40 (6-61)

Other sources: Sahastrabuddhe and Saluja, 2019; Milligan et al., 2018; Mohan et al., 2015; MacLennan et al., 2014; Marathe et al., 2012; Engels et al., 1998; Ivanoff et al., 1994

2.5 Prime boost vaccination strategy

Since conventional *S.*Typhi vaccination has a limited impact on the typhoid control and we are in dire need of new, safe, and more effective vaccination strategies. Key to the vaccine development is to elicit enduring protection. That means induction of strong, long-lived immunological memory to antigens that correlate with protection or ability to 'recall' previous exposures to antigen and to mount enhanced, accelerated effector responses. Several vaccine technologies and strategies have established their flexibility, expediency, robustness and potential simplicity of production and few others hold promise for the future. The list of vaccination strategies mention in the figure box. These vaccination strategies address the desired characteristics of an ideal vaccine (Table 2.5) in various ways (Levine and Sztein, 2004)

Proven and promising vaccine development strategies

- Conjugate vaccines
- Rational attenuation of known pathogens by inactivation of specific genes
- Bacterial live vector vaccines
- Viral live vector vaccines
- Subunit vaccines
- 'Reverse vaccinology' (genomics-based vaccines)
- Nonliving antigen delivery systems (such as liposomes, proteosomes, virus-like particles, virosomes and microspheres)
- DNA vaccines and replicons
- 'Heterologous' prime-boost vaccination strategies
- Powerful but well tolerated adjuvants to enhance immune responses to vaccines
- Needle-free administration of vaccines

Fig 2.12: The box showing the lists of proven and promising vaccine development strategies (Levine and Sztein, 2004)

Table 2.5: Characteristics of various vaccine development strategies (Levine and Sztein, 2004).

	Conjugate vaccines	Attenuated live vaccines	Bacterial live vector vaccines	Viral live vector vaccines	Subunit vaccines	Genomic-based vaccines	Nonliving antigen delivery systems	DNA vaccines and replicons	'Heterologous' prime-boost strategies	New adjuvants
General clinical tolerability	High	High	High	High	High	High	High	High	High	Moderate
Potential transmissibility to non-target subjects	No	Yes	Yes	Yes	No	No	No	No	Yes (if live vector vaccines used)	No
Safety concerns for immunocompromised subjects	No	Yes	Yes	Yes	No	No	No	No	Yes (if live vector vaccines used)	No
Likelihood of a single dose immunization regimen	Low	High	Moderate	Moderate	Low	Low	Moderate	Low	No	Moderate
Expected immunogenicity:										
Antibodies	High	High	High	High	Moderate	High	High	Moderate	High	High
T_H1 cytokine responses	Low	High	High	High	Low	Low/moderate	Moderate	Moderate	High	High
CTL	None	High	High	High	Low	Low	Moderate	Moderate	High	High
Potential for needle-free administration:										
Mucosal	Low	High	High	High	Low	Low	High	Moderate	High	Moderate
Transcutaneous	Medium	Low[a]	Low[a]	High	High	High	High	High	Moderate	Moderate
Needle-free injection devices	High	Moderate[a]	Low[a]	High	High	High	High	High	High	High

An effective vaccine usually requires more than one time immunization in the form of prime boost. Usually the same vaccines can be given multiple times as homologous boosts. Higher levels of antibodies can be elicited following repeat vaccination with the same vaccine than following a single vaccination. Such homologous boosting is sufficient for organisms for which the protective immune response is dependent on humoral immunity (Example- DTP vaccine, Hepatitis B vaccine, oral polio vaccine etc). Boosting with the same vaccine may inhibit antigen presentation due to pre-existing host immunity. Therefore repeated homologous boosting does not increase in the magnitude of the cellular immune response (Ly and McMurray, 2008). Recently, heterologous prime boost (P-B) immunization have been developed with the endeavor to produce stronger and longer-lasting immunity against various pathogens (Shukarev et al., 2017). Heterologous prime boost (P-B) strategy can be done with different types of vaccines containing the same antigens (Lu, 2009). Alternating routes for delivery of the priming and booster doses can also used in this strategy (Gaspari, 2011). In many situations such heterologous prime boost (P-B) are found more immunogenic than homologous prime-boost. Heterologous prime boost (P-B) strategy represents a new way of immunization. The key strength of this strategy is that greater levels of immunity are established by heterologous prime boost (P-B) than can be attained by a single vaccine administration or homologous boost strategies (Woodland, 2004).

2.5.1 History

The heterologous prime boost immunisation technique employed first in a non-human primate model was reported in landmark Scicence at 1992. In that research, Macaca fascicularis were first primed with recombinant vaccinia virus expressing SIVmne gp160 antigen and were subsequently boosted with gp160 protein produced in baculovirusinfected cells. Animals were protected from intravenous challenge with SIVmne viruses. This experiment led to one of the most promising reports of protection in the early HIV vaccine development efforts (Hu et al., 1992).

2.5.2 Goal of prime boost strategy

One of mankind's greatest achievements has been the development of vaccines against smallpox and polio. Despite these organisms certain pathogens like HIV, Mycobacterium tuberculosis, the malaria parasite, Salmonella etc. are not readily controlled by current vaccination approaches. Over the past few years, significant effort has been directed toward developing vaccines to these and related pathogens. An obvious approach for establishing strong immunity to specific pathogens is through repeated vaccination. However, homologous prime boosting approach is relatively inefficient at eliminating intracellular or polysaccharide coated pathogens. Thus a successful heterologous prime boost (P-B) strategy aimed at eliminating these pathogens by generation of large number antigen specific memory T & B cell and synergistic enhancement of immunity to target antigen (Nolz & Harty).

2.5.3. Mechanism of prime boost strategy

A homologous prime boost vaccination can induce good humoral and T cell immunity, including neutralizing antibodies. But humoral response after priming can essentially block antigen presentation to the T cells during subsequent boosting. With heterologous prime boost (P-B) immunization with different vector, the antigens seen by the T cells after boosting are packaged into a second vector which is serologically distinct from the priming vector. So abundant presentation of the target antigens to the pre-existing memory T cells occur. Following priming, naïve T cells undergo a high number of cell divisions in a extremely short period of time and become metabolically depleted. Only a few cells survive. After boosting, each

memory T cell undergoes fewer cell divisions and maintains metabolic fitness. Most of them survived. They can rapidly undergo memory differentiation upon subsequent boosting or infection. Abundant quantity of memory CD8 T cell also achieved. So both CD4+and CD8+ T cells can be strongly induced. Prime–boost is considered a form of original antigenic sin. The initial priming events elicited by a first exposure appear to be imprinted on the immune system. The synergistic enhancement of immunity to the target antigen is reflected in an increased number of antigen-specific T & B cells, selective enrichment of high avidity T& B cells. Enhanced and high magnitude memory T cell responses to the targeted antigen based on higher number of specific precursor cells. It selectively increase the numbers of memory T cells specific for the common antigen in both prime and boost vaccines. These increased numbers of specific T cells 'push' the cellular immune response over certain thresholds that are required to fight specific pathogens. T-cell responses to different antigens are highly competitive. A T-cell response to a dominant epitope will often suppress the generation of response to a subdominant epitope of pathogen. This immunodominance is controlled at two levels. First by antigen availability and processing (intrinsic mechanisms) and second by T-cell competition for antigen-presenting cells or cytokines that regulate the level of T-cell priming and expansion. So these factors enable some epitopes to dominate or suppressed. Heterologous vaccine, boost the target antigen by establishing a competition between memory cells specific for the target antigen and naı¨ve T cells of other vaccine antigen or boosting vector (Figure 2.13) (Woodland, 2004).

2.5.4 Factors influencing prime boost vaccination

Many factors can influence the outcome in a prime boost study. Most important factors are: (i) the vaccine vectors, (ii) vaccine doses, (iii) the interval between prime and boost, (iv) virus tropism for the host species, (v) non-vaccine sources of pre-existing immunity, (vi) the type of desired immune activity (e.g., B-cell versus T-cell, Th1 versus Th2), and (vii) the timing of desired immune function (acute versus long-term) (Brown et al., 2010).

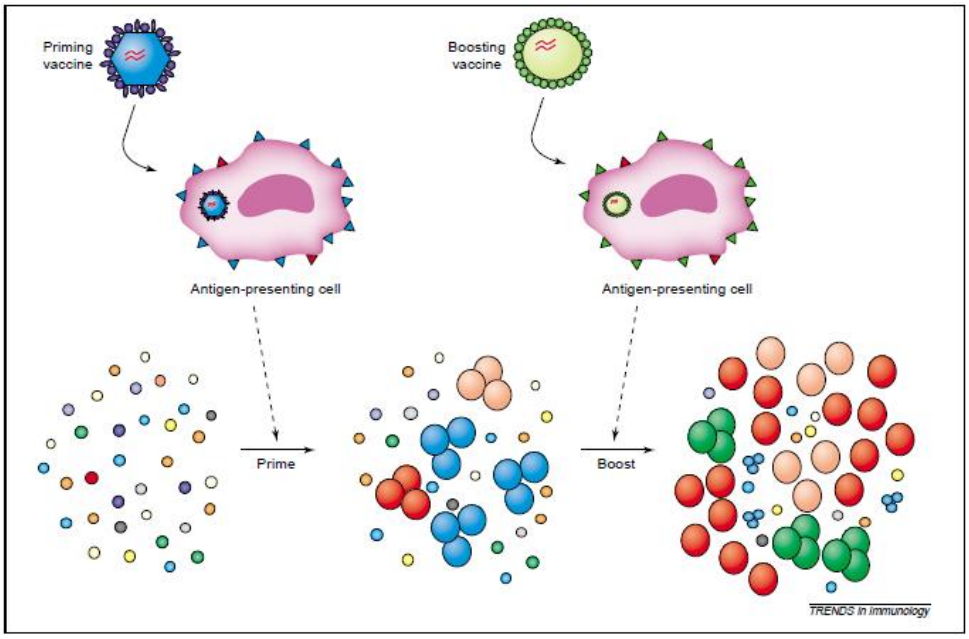

Figure 2.13: A schematic presentation of synergistically amplification of T cell immunity to specific antigens following heterologous prime-boost vaccination. Priming with the first vaccine results in the presentation of both target antigen (red triangles) and other antigens (blue triangles) on antigen-presenting cells (APCs). APCs then stimulate naïve T cells in the lymph nodes and drive expansion of both target specific T cells (red cells, high avidity cells are indicated by the darker red) and other antigen specific T cells (blue cell). Subsequent boosting with second vaccine results in the re-presentation of the target antigen (red triangles) and other antigens of second vaccine (green triangle) on APCs. These APCs then drive the expansion of target-specific memory T cells that have greater avidity for the antigen (Woodland, 2004).

There was evidence that the longer interval of 8 weeks between priming and boosting was not much better than a 3-week interval. The immunological responses of prime boost immunization with the shorter interval of 3 weeks were higher (P = 0.026) than after an 8-week interval (McConkey et al., 2003). Higher antigen content raises stronger booster responses by recruiting more memory B cells into the response at the time of boosting. This can be confirmed by observation of higher antibody responses in children primed with a glycoconjugate vaccine and boosted with a higher concentration PS (20–50 μg of PS) when compared with the glycoconjugate (1–3 μg of PS) vaccines (Siegrist, 2018).

2.5.5 Application of prime boost vaccination

A. Against pathogens:

Recently, several studies have established the efficacy of prime boost vaccination strategies in generating immunity to a variety of pathogens including M. tuberculosis, HIV, Simian immunodeficiency virus (SIV), Plasmodium, Listeria monocytogenes, Leishmania, Ebola virus, Hepatitis B & C virus, Herpes simplex virus, Human papillomavirus etc. (Woodland, 2004). Current vaccination approaches have failed against these pathogens mainly due to their inability to induce effective immune responses. Heterologous prime boost vaccines could be an avenue to tackling infectious diseases where protection has not been successfully achieved with other approaches (One health platform: Faculty club university of Leuven, 2017).

The tremendous power of prime–boosting was reported for M. tuberculosis. Mice that had been vaccinated intranasally with Bacille Calmette– Guerin and then boosted with a recombinant vaccinia virus expressing antigen complex 85A, had an 300-fold reduction in bacterial load in the lungs following aerosol challenge with M. tuberculosis (Goonetilleke et al., 2003). Alternatively BCG can also used as a boost following a DNA vaccine prime. In one study conducted in calves, DNA prime with Ag85B, MPT64, MPT83 antigens followed by a BCG boost was able to elicit higher immune responses and better protection than BCG alone against *Mycobacterium bovis* challenge (Lu, 2009). Enhanced immune response (gamma IFN, Cytoxic T cell, increased relative ratio of IgG2a to IgG1) was also elicited by a DNA vaccine encoding ESAT6 protein of Mycobacterium tuberculosis by DNA prime-protein boost protocol (Wang et al., 2004). Different routes of administration of vectored subunit vaccines based on chimpanzee-derived adenovirus serotype-3 (ChAd3) for homologous prime-boosting and modified vaccinia virus Ankara (MVA) for heterologous boosting with both vaccine vectors expressing the same antigens from Mycobacterium tuberculosis (Ag85B, ESAT6, Rv2626, Rv1733, RpfD) was also investigated (Vierboom et al., 2020).

Priming with S. Typhimurium ΔznuABC and boosted with inactivated S. Choleraesuis, applied to group of piglets was able to limit weight loss, fever and organs colonization, arising from infection with virulent S. Choleraesuis, more effectively, than the prime boost vaccination with homologous S. Choleraesuis inactivated vaccine group (Alborali et al., 2017). Prime boost (P-B)vaccination strategies was also used in Salmonella enterica serovar Gallinarum (SG) ghost vaccine candidate strain to optimize immunity and protection efficacy against fowl typhoid. Results shown that the prime immunization with the SG ghost vaccine

induced Th1 type immune response and the booster elicited both Th1- and Th2-related immune responses (Won et al., 2016). A randomized, double-blind, heterologous prime boost clinical study was conducted in human to determine whether immunity to licensed parenteral Vi vaccine could be enhanced by priming with CVD 909. Higher and persistent, though not significant, anti-Vi IgG and IgA response was obtained following priming with CVD 909 and boosting with Vi than only Vi priming recipients (Wahid et al., 2011).

Immunological response to a prime boost immunization strategy combining pneumococcal conjugate vaccine (PCV13) with polysaccharide pneumococcal vaccine (PPSV23) versus the PPSV23 alone was compared in HIV infected adults. Both PCV13 with PPSV23 elicits a greater magnitude of IgG and opsonophagocytic immune response compared to PPSV23 alone. The prime boost vaccine group achieved a ≥2fold increase in IgG level and geometric mean concentration >1 ug/ml at week 8 (odds ratio (OR) 2.00, 95% confidence interval (CI) 1.46–2.74, $p < 0.01$) and week 28 (OR 1.95, 95% CI 1.40–2.70, $p < 0.01$). This group were also shown a ≥4-fold increase in geometric mean titer at week 8 (OR 1.71, 95% CI 1.22–2.39, $p < 0.01$) and week 28 (OR 1.6, 95% CI 1.15–2.3, $p < 0.01$). This study adds strength and supporting evidence to current vaccination recommendations combining the conjugate and polysaccharide pneumococcal vaccines in the United States and Europe for HIV-infected individuals (Sadlier et al., 2016). Immunogenicity of this strategy using 13-valent/23-valent anti-pneumococcal vaccines was estimated in patients with RA. The combined pneumococcal revaccination strategy induces good protection but does not persist beyond two years in RA patients. A higher efficacy was not observed in immunization with the conjugate vaccine following polysaccharide vaccine. Possible explanation might be hypo responsiveness induced by PPV23 against the immune response elicited by the primo-injection of the PCV13 vaccine (Bahuaud et al., 2018). However prime boost vaccination strategy with PCV13 followed by PPV23 was elicited a strong antibody response compared to single-dose PCV13 in patient with inflammatory rheumatic disease (Nived et al., 2020).

Using DNA- and vaccinia based prime boost vaccination for a pre-erythrocytic malarial antigen in human were found five- to tenfold higher response than to either DNA or vaccinia virus vaccines alone (Dunachie and Hill, 2003). DNA prime and MVA boost regimens were assessed in Chimpanzees using constructs expressing the falciparum TRAP antigen. T cell response to TRAP were not detected after DNA

priming, but MVA boosting provoked high level of T cell with strong peptide specific CTLs. (Schneider et al., 1998)

DNA priming along with various forms of boost has been incorporated into almost every current major HIV-1 vaccine improvement effort. The DNA prime-protein boost approach was able to achieve "sterilizing" protection in non-human primates and proven effective in humans against HIV. In human volunteers, antigen-specific antibody responses were not detected after three DNA immunizations, but a rapid rise of anti-Env IgG responses were identified after only one protein boost in every volunteer. This result is remarkable among all other candidate HIV vaccines in human (Lu, 2009). The most advanced efficacy trials used either canarypox vector (ALVAC) boosted by adjuvanted gp120 protein or adenovirus (Ad26) vector boosted by gp140 protein against HIV-1 viruses (Excler and Kim, 2019).

The prime boost vaccine approach also improved the efficacy of existing Rabies & HBV vaccines (Gaspari, 2011). Adding a DNA prime, boosted with the licensed hepatitis B surface protein vaccine were induced stronger, more homogenous antibody responses & higher IL-12 and IFN-γ secretion in splenocytes of a mice group when compare to groups only receiving recombinant protein alone (Xiao-wen et al., 2005).

B. As therapy to cancers:

The heterologous prime boost approaches have also been studied in treatment of cancer. Using a six transmembrane epithelial antigen of the prostate (STEAP), a heterologous DNA prime and Venezuelan equine encephalitis virus-like replicon particles (VRP) boost was induced better immune responses including INF-gamma, TNF-alpha, and IL-12 against STEAP (Lu, 2009).

2.6 Measurement of vaccine induced antibody responses

One of the pivotal immunological parameter to measures immunogenicity of vaccine is assessment of the humoral immune response (antibody concentrations or antibody titres etc). Detection of the functional antibody responses by serum bactericidal antibody (SBA), toxin or virus neutralizing antibody, opsonophagocytic antibody (OPA) is most weighted measures of any vaccine immunogenicity. As this assay have low feasibility for application to large numbers of samples (e.g. very labour-intensive or requires high-level biocontainment facilities), antibody responses also determined by measuring total antibody (e.g. total IgG measured by ELISA) that binds to selected antigens (or, on occasion, to specific epitopes). Only a proportion of

the total antibody detected may be functional. All established immunological correlates of protection (ICPs) are based on measurement of functional or total IgG antibody. If ICP has already established between total and functional antibody responses to a specific microorganism or antigen, it may be sufficient to measure only the relevant functional antibody (e.g. SBA for meningococcal vaccines) or total IgG (e.g. for antibody to tetanus toxin) response. If there is no ICP, the functional antibody response needs to be measured. Functional immune responses are also important for specific age groups or target populations. Some examples of well-established ICPs include those for antibody to diphtheria and tetanus toxoids, polioviruses, hepatitis B virus and H.influenzae type b capsular polysaccharide etc. However, the data related to functional antibody response following Vi polysaccharide based typhoid vaccines and its correlation with total IgG is lacking (W.H.O., 2016).

Several methods including ELISA, Latex Agglutination test, Haemagglutination inhibition test, Chemiluminescence immunoassay, Nephelometry etc. can quantitate vaccine induced antibody response by detection of titer or concentration (Apostolopoulos and Marincola, 2010). Antibody response can also be measured by antigen specific antibody-secreting cell (ASC) or enzyme-linked immunospot (ELISPOT) assay. The ASC assay counts "spots" formed by Ig producing cells bound to a nitrocellulose plate after incubation of lymphocytes (spleen or peripheral blood) with specific antigen (Kirkpatrick et al., 2005). This immunoassay commonly used for evaluation of human clinical trials of vaccines and immunotherapy because it measures antibody quantitatively at cellular level. ELISPOT is adaptable not only to evaluation of B cells, but also T cells and various innate immune cells (Slota et al., 2011).

Chapter Three

Materials and Methods

3.0 Materials and Methods

In the present study, systemic and local (mucosal) humoral immune responses (B cell responses) were determined to *Salmonella* Typhi (*S*. Typhi) Vi CPS antigen by prime boost (P-B) immunization method using Vi CPS in combination with different antigens and routes in mice model. Specific anti-Vi IgM, IgG and IgA antibody responses were determined by homologous and heterologous prime boost (P-B) strategy using killed whole cell *S*. Typhi, conjugate vaccine (Vi-TT), and Vi capsular polysaccharide vaccine (Vi CPS) in BALB/C mice. Anti-Vi antibody secreting cell (ASC) responses in mice spleen and Peyer's patches were measured by ELISPOT assay and anti-Vi antibody titer in serum of mice by ELISA method. Immunoglobulin G (IgG) responses to Vi capsular polysaccharide, surface proteins, envelope proteins and whole cell extract of *S*. Typhi in sera of patients with typhoidal fever were analyzed by ELISA method. Functional capacity of antibody to kill *S*. Typhi was measured by colorimetric serum bactericidal assay (SBA) following infection and vaccination. Western blot test was performed to identify specific immunogenic antigens of *S*. Typhi using sera from typhoid fever patients.

3.1 Ethics statement and statistical analysis

All animals were obtained from the Animal Resources Branch of International Center for Diarrheal Diseases Research, Bangladesh (icddr,b) and all animal experiments were performed following the guidelines set by the Ethical Review Committee for Animal Experimentation of the Faculty of Biological Sciences, University of Dhaka. The work adheres to the USDA Animal Welfare Act, PHS (Public health service) Policy on Humane Care and Use of Laboratory Animals, and the "ILAR (Institute for Laboratory Animal Research) guide for the Care and Use of Laboratory Animals". The Ethical Review Committee (ERC) of the Diabetic Association of Bangladesh (BADAS) has approved the study (Ref: BADAS-ERC/EC/14/00170, date-30.08.2014). Informed written consent was obtained from all adult participants (age 18 years and above) and from the parents/guardians of all children (age up to 17 years) prior to collection of blood samples and demographic data. All data were expressed as means ± standard error (S.E.). or standard deviation (SD). The statistical significance of the differences among the three or more groups were assessed using

ANOVA (Tukey HSD Post-hoc) test and among two groups by two-tailed Student t test. Statistical significance was tested at 95% ($p<0.05$) confidence levels. SPSS software (version 22.0) was used to perform all statistical analysis.

3.2 Study population and study design

3.2.1 Study population

Animal studies included 43 BALB/c mice to measure immune response of *S.* Typhi following prime boost vaccination strategy. A total of 70 culture positive typhoidal fever patients, 11 convalescent cases, 12 healthy controls, 13 disease controls and 06 adult healthy vaccinated volunteers were examined to observe immune responses against *S.* Typhi.

3.2.2 Type of study

Experimental when animal model was involved and observational when human immune responses against *S.* Typhi was carried out.

3.2.3 Place of study

Study was conducted in the department of Microbiology of Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorder (BIRDEM) and the department of Microbiology, University of Dhaka, Bangladesh.

3.3 Measurement of anti-Vi antibody responses combining Vi CPS, Vi-TT and killed whole cell (KWC) *S.* Typhi in BALB/c mice following different prime boost immunization schedule

To investigate the efficacy of prime boost (P-B) vaccination strategies in generating systemic and local immune responses to *S.* Typhi Vi CPS antigen, mice were immunized with different homologous and heterologous combination of *S.* Typhi vaccine antigens namely killed *S.* Typhi whole cell (KWC) antigen, Vi-tetanus toxoid conjugate vaccine (Vi-TT) and Vi capsular polysaccharide (Vi CPS) vaccine in same or different route.

3.3.1. Preparation of killed *S*. Typhi whole cell (KWC) antigen

S. Typhi KWC antigen was prepared as described earlier (Diena et al., 1973; Felix, 1951). KWC antigen was prepared from wild local *S*. Typhi strain (M-5256) isolated from blood culture of a typhoid fever patient in the Department of Microbiology, BIRDEM General Hospital. The organism was identified by Gram stain, standard biochemical and serological methods (Cheesbrough, 2000; Collee et al., 1996). The strain was capsulated and the capsule of this strain was confirmed with capsular staining by India ink method (Breakwell et al., 2009). In India ink method organism was first mixed with a drop of India ink on a glass slide, spread with a loop to form a thin smear and allowed to air dry for a 5-7 minutes. Then glass slide was saturated with crystal violet for 1 minute and rinse lightly and very gently with water as water may remove the capsule from the cell. Slide was then air dried for few minutes and observed under oil immersion lens (1000x). After confirming capsule (Vi CPS), single colony of *S*. Typhi was subcultured in 100 ml TSB broth. Broth culture OD was adjusted to 0.7 in 595 nm (approximate cell concentration $2.5X10^8$CFU/ml). Five (05) ml of this suspension (cell concentration 1 $X10^9$CFU) was centrifuged at 5000 rpm for 10 minutes. Supernatant was discarded and cell pellet washed three times with PBS buffer. Cell deposit was then reconstituted with 1ml PBS buffer. Three volumes (3 ml) of 75% alcohol were then added to this bacterial suspension with shaking for one hour. Alcohol mixed *S*. Typhi suspension was left for room temperature for 3 days. Alcohol was removed by centrifugation and pellet re-suspended in 2ml PBS buffer (cell concentration- $1X10^8$CFU per 200µl). Cell death was confirmed by subculturing of this suspension.

3.3.2. Typhoid vaccines as antigen

Capsular polysaccharide vaccine (Vi CPS) was obtained commercially (Typhim®Vi, Sanofi Pasteur- France) containing 25µg Vi in 0.5ml. Vi-TT vaccine was also obtained commercially (Typbar-TCV, Bharat Biotech-India) containing 25µg Vi in 0.5ml (Mohan et al., 2015)

3.3.3 Dose of antigen and immunization schedule

Inbred, male BALB/c mice were used in this study. Mice were obtained at 6-8 weeks of age and maintained in grouped cages at animal resource center of International

Center of Diarrheal Disease Research, Bangladesh (icddr,b). Mice were fed water (10-15 ml/day) and pelleted food (4-5 gram/day). Room temperature was maintained at 20-21°C with 30-70% humidity. Animal health was routinely monitored by animal care staff. Mice showing evidence of ill health were excluded from the study.

The amount of antigen for each immunization course had been optimized per mouse by calculating Animal Effective Dose (AED) mg/kg by conversion of human dose to animal (Nair and Jacob, 2016) by using formula described below:

Animal effective dose (AED) mg/kg= Human Effective dose (HED) mg/kg x Km ratio

Km ratio= Human Km ÷ Animal Km

Human Km= Body weight of human (kg) ÷ Body surface area of human (m^2)

= 60kg ÷ 1.62 m^2 =37

Mice Km = Body weight of Mice (kg) ÷ Body surface area of Mice (m^2)

= 0.02kg ÷ 0.007 m^2

= 3

So, AED for antigen = Human dose x Km ratio

= Human dose of antigen/vaccine in mg/kg x (37÷3)

= (Human dose of antigen/vaccine x 12.3) mg/kg

Human dose of ViCPS is 25µg for adult 60kg wt man.

So, minimum AED of Vi CPS = (0.025/60) mg/kg X 12.3

= 0.00512 mg/kg=0.00512 X .025 per mice of 0.2Kg (25mg)

= 0.00128 mg= 1µg (at least)

3 µg (0.06 ml) was used here, as it was convenient to immunize mice with 0.06 ml of antigen. The dose of 2-6 µg was found to induce maximum response (Hosny Ael et al., 2015).

Mice were divided into six groups for immunized with different vaccine preparation as described in Table-3.1. Rest of the 05 mice was taken as negative control and was not immunized with any antigen/vaccine.

Table-3.1: Immunization schedule of Experimental mice

Ex. Group (Gp)	Vaccination strategy	BALB/c Mice quantity	Priming antigen (Day 0)			Boosting antigen (Day 21)			Sacrifice (Day 28)
			Type	route	Dose	type	route	Dose	
Gp.1	Priming	10	Vi*	i.p.	3µg (0.06ml)	No	-	-	Blood, Spleen, Peyer's patches were collected
Gp.2	Homo P-B	6	Vi	i.p.	3µg (0.06ml)	Vi	i.p.	3µg (0.06ml)	
Gp.3	Homo P-B	6	Vi-TT	i.p.	3µg (0.06ml)	Vi-TT	i.p.	3µg (0.06ml)	
Gp.4	Hetero P-B	6	Vi-TT	i.p.	3µg (0.06ml)	Vi	i.p.	3µg (0.06ml)	
Gp.5	Hetero P-B	5	KWC	i.p	$1X10^8$cfu (0.2ml)	Vi	i.p.	3µg (0.06ml)	
Gp.6	Hetero P-B	5	KWC	oral	$1X10^8$cfu (0.2ml)	Vi	i.p.	3µg (0.06ml)	
	No vaccination (Negative control)	5	No	-	-	No	-	-	

Note: Group 2 & 3 mice were vaccinated with Homologous prime boost (Homo P-B) immunization method and Group 4, 5 & 6 mice vaccinated with Heterologous prime boost (Hetero P-B) immunization method; Vi means Vi capular polysaccharide (Vi CPS)

3.3.4 Blood collection and preparation of serum

One week after the booster immunization, blood was collected in a sterile plain test tube after sedation of mice with chloroform followed by cardiac puncture technique. Then blood was allowed to clot and the serum was separated by centrifugation at 2000 rpm for 10 minutes and the serum was stored at -20°C for further analysis.

3.3.5 Collection of spleen and Peyer's patches

Dissection of spleen: Spleens were collected from immunized mice. Incision was made in skin of left side overlying liver and spleen. Skin was separated to reveal

muscle layer. Muscle layer was lifted away from body cavity with forceps and large incision across the body wall was made. Spleens were drawn out of the body cavities of mice being careful not to damage the organ. Scissor was inserted under the spleen (where it was attached to mesentery) and opening with the blades separated the organ from attached tissue.

Dissection of Peyer's patches (PP) from intestine: Small intestine was cut approximately 0.5 cm below the stomach, draw out of peritoneal cavity unfolding it. Intestine was cut about 1cm above the caecum. Then fat, mesenteric lymph nodes adjacent tissue was removed. Intestine was placed on an aluminium foil and kept moisten with cold Hanks' Balanced Salt Solution (HBBS) to prevent drying. Faeces was expelled by placing a curved forcep flat on the surface of the intestine and pressed gently down along the entire length to avoid breaking the intestine. PP was easily visualized when faeces were cleaned from intestine. The patches appeared as small protruding whitish nodules of PP (1-2 mm diameter) embedded in the outer intestinal wall were located and removed. About 5 to 10 PPs were obtained from a single mouse depending of age and strain. PPs were hold with curved forcep and cut PP with scissors avoiding as much as possible the surrounding intestine wall.

3.3.6 Preparation of single cell suspensions from spleen and Peyer's patches

Spleen was removed after sacrificing the mouse. Individual spleens were teased with surgical forceps in ice cold Hanks' Balanced Salt Solution (HBSS) in sterile Petri dish. Subsequently, the cell suspension was passed through a 100 µm polypropylene mesh. After passed through a mesh, the suspension was transferred to 15 ml falcon tube. Red blood cell (RBC) was lysed by adding equal volume of Tris- buffered ammonium chloride (pH 7.2) to cell suspension. The suspension was gently mixed for 5 minutes to lyse erythrocytes. Cell suspension was then centrifuged (1500 rpm) and washed two times with HBSS to remove cell debris. Pelleted cells were resuspended in 2 ml of ice cold RPMI containing 10% FCS. All cell work was performed on ice unless otherwise mentioned. Viable cell counts were made using Trypan blue exclusion method.

All PPs were transferred to a ice cold HBSS containing sterile Petri dish. PPs were teased with the surgical forceps and gently passed through polypropylene mesh. The

cells were transferred in 15 ml falcon tube. Supernatant was discarded after centrifugation at 1500 rpm at 4°C for 10 min. The cells are re-suspended in 5ml of cold HBSS and washed two times. After the last centrifugation the pellet was re-suspended in 1 ml of cold RPMI+ 10% FCS. Viable cells were counted by 0.4% trypan blue exclusion method.

3.3.7 Counting of viable lymphocytes

Cells were counted by improved Neubauer counting chamber. In a 2 ml eppendorf tube, 20 µl of 0.4% Trypan Blue was added to 20 µl cell suspension and mixed gently. Using a pipette 10 µl trypan blue treated cell suspension was applied to the Neubauer counting chamber and unstained live cells were counted in 4 corner squares (each consisting of 16 small squares) under microscope (10x). Percentage of viable cells was determined by counting live cells out of total cells (both dead and viable). Cells overlying any 2 adjacent margins of the square were included and those overlying the opposite 2 margins were excluded. Total viable cells were calculated by the following formula:

Total viable cell count = Average number of viable cells of 4 large square x 10^4 x 2 (Trypan blue dilution) x cell dilutions per ml.

3.3.8 Estimation of Vi specific antibody secreting cell (ASC) by ELISPOT assay

Salmonella Vi specific antibody secreting cells were estimated by ELISPOT method as described by (Czerkinsky et al., 1983).

I. Antigen coating

Ninety six well sterile EIA plate (Greiner bio-one, Germany, Cat no. 655161) was coated with 100µl Vi antigen (5µg/ml, Sanofi Pasteur, France) in PBS buffer (pH 7.4) ((Pulickal et al., 2009). To each well 100 µl volume of diluted Vi antigen was added and incubated overnight at 4^0C. Plate was washed 3x with PBST (Tween 0.05%) and then blocked by 200 µl of PBS-T containing 2% bovine serum albumin (BSA). The plate was incubated with blocking buffer for 2 hour at 37°C. The plates were then

washed twice with PBS-0.05% Tween followed by washing three times with PBS only to remove Tween from well.

II. Addition of cell

At first, cell number was adjusted to 1×10^7 cells/ mL. The 100 µl of cell suspension was added to each well and incubated in a vibration free incubator at 37°C in 5% CO2 and 100% humidity for 4 hours. After incubation the cells were washed off by rinsing three times with PBS-T (0.05% Tween).

III. Addition of conjugate

Isotype specific antibody secreting cells were detected by adding 100 µl of Fc specific goat anti-mouse IgG (1:20000 dilution), IgM (1:5000 dilution) or IgA (1:5000 dilutions) alkaline phosphatase conjugated antibody (Abcam, UK) to each well. The conjugate was diluted in PBST (0.05%) + 0.25% BSA. The plate was incubated overnight at 4˚C.

IV. Addition of substrate

Following incubation the plate was washed three times with PBS-0.05% Tween and the spots were developed by adding 100µl of 5-bromo-4chloro-3-indolyl phosphate (BCIP) substrate (1mg/ml) in pre-warmed (42˚C) 0.6% agarose in substrate buffer (Tris HCl, pH 9.5). The plate was incubated at room temp for 30 minutes to 1 hour and then the reaction was stopped by adding 50µl of 3M NaOH. Appearance of blue blue spot represented single antibody secreting cells.

V. Spot counting

Blue spots, each representing a single antibody secreting cell, were counted under a dissecting microscope. Antibody secreting cells were expressed as ASCs/10^7 nucleated cells.

As negative control reagent blank and spleen /PP cells from normal unimmunized mice were included in each assay.

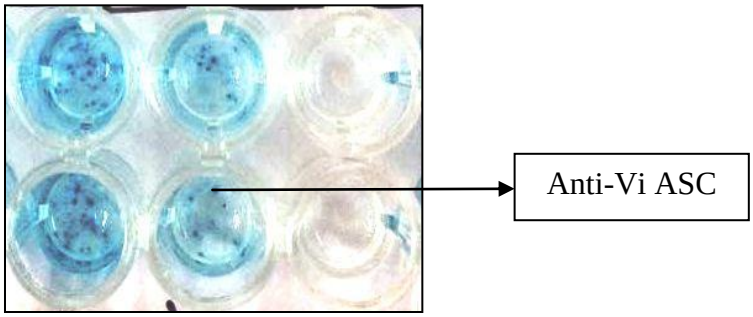

Fig 3.1 Photograph of anti -Vi antibody secreting cells (ASC) by ELISPOT assay. Each blue spot represents an antibody secreting cell developed in an antigen coated 96 well microtiter plate.

3.3.9 Measurement of anti-Vi antibody titer in mice serum by ELISA

Sera collected from mice were tested for anti-Vi IgM, IgG and IgA *S.* Typhi antibodies by ELISA method (Voller et al., 1978). Briefly,

Step 1: EIA plate was coated with Vi polysaccharide antigen and blocked with PBST+2% BSA (pH 7.4) as described in section 3.3.8

Step 2: 100µl of serum was added to each well which was diluted serially starting from 1:8 up to 1: 16384 and incubated overnight at 4 ºC.

Step 3: Then plate was washed three times 5 min with PBST. Then 100 µl of diluted alkaline phosphatase conjugated secondary antibody (Goat anti-mouse IgM mu chain 1:5000 ab 97227; Goat anti-mouse IgG Fc 1:20,000, ab97262; Goat anti-mouse IgA alpha chain 1:5000, ab97232, abcam, UK) was added in each well and incubated at 37ºC for 2 hours. The plate was washed for 5 minutes each time for 3 times with PBST washing buffer.

Step 4: To each well 100 µl of substrate buffer pNPP (p-Nitrophenyl phosphate disodium salt) 1mg/ml in diethanolamine buffer (pH 9.5) was added and incubated for 20 minutes in dark.

Step 5: The reaction was stopped by adding 100 µl of stop buffer (3N NaOH) and optical density (OD) was recorded at 405nm immediately.
A negative control was used as reagent blank and mice normal serum.

Interpretation: A cut off value of ELISA for anti-Vi IgM, IgG & IgA was determined by performing ELISA with sera from 5 normal mice (unimmunized). The mean OD+2xSD of these sera were taken as cut-off OD value to determine the antibody titer. Therefore, an OD value of 0.8 (0.4+2x0.2) was taken as the cut off value for IgM, 0.5 (0.3+2x0.1) was taken as the cut off value for IgG & IgA (Table-3.2). Highest serum dilution that had OD above this cut off value was considered anti-Vi antibody (IgM/IgG/IgA) titer of that specific mice sera.

Table-3.2: Cut off OD values for anti Vi IgM, IgG and IgA antibody in mice sera by ELISA.

Anti Vi	Mean OD value	±SD	Cut-off value (Mean+2xSD)	Baseline titer
IgM	0.4	0.2	0.8	1024
IgG	0.3	0.1	0.5	16
IgA	0.3	0.1	0.5	16

3.3.10 Determination of the functional capacity of antibody by Salmonellacidal assay in mice

Functional antibody elicited following *S.* Typhi vaccination in mice was measured by colorimetric serum bactericidal assay (SBA). Hence the SBA was termed as serum salmonellacidal assay (SSA). Salmonellacidal assay which requires fixation of complement by the antibody that is bound specifically to *Salmonella*. In SSA, target bacteria (*Salmonella* Typhi) is lysed following fixation of complement by anti-salmonella antibody.

Colorimetric serum salmonellacidal assay:

Serum salmonellacidal antibody in mice and human sera was determined by a microplate based bactericidal assay (Son and Taylor, 2011; Qadri et al., 1995). In this procedure chromogenic tetrazolium salt (Neotetrazolium chloride) in presence of substrate (glucose) is reduced by live bacterial cells resulting in the formation of a purple color. The color remains unchanged (absence of any colour) if the bacteria were lysed and dead because of presence of salmonellacidal antibody in serum in presence of exogenous source (guinea pig) of complement. The titer of the salmonellacidal antibody was determined by the highest dilution of the serum that

results in cell lysis or no change in color. Brief description of the assay is given below.

Procedure:

1. Three pure colonies of *Salmonella* Typhi were cultured overnight in 250 ml Tryptic Soy broth (TSB). Cell was counted by serial dilution and culture in MacConkey agar media and also by measuring OD. The cell count of 10^9 to 10^{10} CFU/ml and OD (OD_{595}=0.6 to 1.2) was obtained.
2. One ml culture material was then centrifuged and cell pellet re-suspended in an equal volume of cold PBS (pH 7.4). Bacteria were then adjusted to $1X10^8$ CFU/ml by adding PBS.
3. A 1:10 dilution of guinea pig complement was prepared with cold PBS.
4. A 3 ml mixture of cell and complement was prepared by adding 20 µl of cell suspension to 300 µl cold guinea pig complement and 2680 µl of cold PBS to obtain a final cell count of 2 $x10^6$ CFU.
5. Serum samples were thawed and heated at 56ºC for 30 minutes and serially diluted with cold PBS across the 12 wells (except 3 control well of last row) from neat to 1: 10240. Each well contained 25 ul of sample.
6. Then 25 µl of cell- complement mixture was added to each well and the plate was incubated for 1hour at 37 ºC for 1 hour with lid.
7. 150 µl of fresh TSB broth was added to each wells and incubated at 37 ºC for 2 hours.
8. A 10% (w/v) NTC solution was prepared by dissolving 10mg NTC (Neotetrazolium chloride, MP Biomedicals-France, Cat.# 102436) in 100µl methanol.
9. Then, 30µl of 10% NTC solution was added to 270µl of 1M glucose solution and made up to 3 ml by adding sterile distilled water to obtain a final working NTC solution (final NTC concentration 0.1%). Then, 25 µl of the NTC-glucose solution was added to each well.
10. The plate was wrapped with aluminum foils for protection from light, and placed at room temperature for overnight incubation
11. Salmonellacidal titer was determined visually as well as by measuring the OD of the solution at 630 nm.

12. In each assay, following control wells were included:

a. Only cell-complement mixture = 25 µl of cell & complement + 25 µl PBS

b. Only cell+ inactivated serum sample =25 µl of cell in PBS+ 25 µl serum sample

c. TSB+PBS=25 µl of TSB + 25 µl PBS to exclude bacterial contamination of reagent, growth medium, saline and PBS

d. For the test specificity, serum from normal mice sera (unimmunized mice), were included.

Interpretation: Salmonellacidal titer (approx-100% killing) was determined visually as the reciprocal of the highest serum dilutions at which there was no development of purple color. If the bacteria were alive because of no bactericidal activity of the antibody in the serum, NTC dye would reduce to form purple colour (Figure-3.2). Death of the bacteria was further confirmed by sub culture of the mixture from the clear well.

Also, the titer was measured by taking OD at 630 nm. Here salomonellacidal titer was defined as the reciprocal of the highest serum dilutions causing greater than 50% reduction of the OD at 630nm compared to the OD of the control wells without serum.

In case of salmonellacidal assay with human serum all the above procedures were followed except serum from healthy human, diseased and newborn babies sera were used instead of normal mice serum.

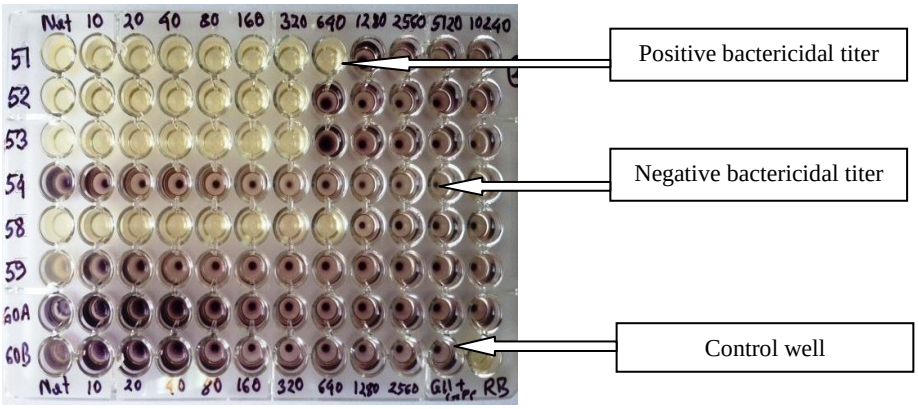

Fig 3.2: Colorimetric serum salmonellacidal (SBA) assay: Illustration of microtiter plate of colorimetric salmonellacidal assay results. Serial dilution was performed from left to right. Column 1 is neat serum, column 12 is 1:10240. Bactericidal titer was determined by reciprocal of the highest serum dilutions causing greater than 50% reduction of the OD at 630nm when compared with the OD of the control wells without serum. Bactericidal titer was also determined visually by highest dilution of serum that resulted in cell lysis with absence of purple colour. For example, sample no. 51 (row 1) last clear well is in column 8 (dilution of 1:640) and so the titer is 640.

3.4 Measurement of anti Vi IgG in Vi CPS vaccinated human volunteers and in typhoid patients by ELISA

Six healthy adult volunteers were immunized with *S.* Typhi Vi capsular polysaccharide vaccine (Typhim®Vi, Sanofi Paster- France). Each volunteer was immunized with 0.5 ml (25µg) of vaccine intramuscularly once. Anti Vi (IgG) antibody response before (0 day) and 21th day after vaccination was measured by ELISA method as described in section 3.3.9.

A total of 70 acute sera and 09 convalescent sera (after 6-8 weeks) were also collected from enteric fever cases to detect presence of *S.* Typhi anti-Vi IgG antibody. Sera from 10 widal negative healthy newborn babies (presumed to be not exposed to *Salmonella* Typhi), 12 healthy control, 13 disease control (culture negative) were included as negative control.

Here, HRP conjugated anti-human IgG (1:4000, MP Biomedicals, USA) was used instead of alkaline phosphatase conjugate. For color development 3,3`,5,5`-tetramethylbenzidine (TMB) substrate was used. The reaction was stopped by 2M sulfuric acid and the OD was measured at 450 nm. The concentration of anti-Vi IgG

was expressed in titer in vaccine volunteers and OD values (in 1:100 dilution of sera) in case of patient with typhoid fever.

Cut-off value determination: A cut off value of ELISA for *S.* Typhi anti-Vi IgG in human was determined with sera from 10 Widal negative healthy newborn babies. The mean OD+3xSD of these sera were taken as cut-off OD value. Therefore, an OD value of 0.41 (0.2+0.07x3) was taken as the cut off value at 1:100 serum dilution (Table-3.3). Any sample from study group showing OD above this cut off value (0.4 at 1:100) was considered positive for anti- Vi IgG antibody. Highest serum dilution that had OD above this cut off value was considered anti-Vi IgG titer in case of vaccinated volunteer.

Table- 3.3: Cut off OD values for anti-Vi IgG antibody in human by ELISA.

Serum dilution	Mean OD	± SD	Cut-off OD value (Mean + 3xSD)
1:100	0.2	0.07	0.4

3.5 Measurement of serum salmonellacidal antibody in vaccinated human volunteers and in typhoid patients

Serum salmonellacidal antibody titer was determined in following groups of study population:

a. culture confirmed enteric fever cases,
b. convalescent cases (6 to 8 weeks of resolution of fever),
c. culture negative fever cases,
d. apparently healthy individuals without any history of fever in preceding 3 months.
e. adult volunteers vaccinated with Vi polysaccharide typhoid vaccine (0,14th, 21st day of vaccination.

Serum salmonellacidal antibody was measured as described in section 3.3.10.

3.6 Determination of IgG antibody responses against different structural components of *S*. Typhi in enteric fever cases

Serum IgG antibody response against surface, envelope and sonicated whole cell protein of *S.* Typhi in sera of typhoid patient were determined by ELISA method.

3.6.1 Preparation of surface protein (SURP) by water extraction method (WEM)

(Ahsan and Sasaki, 1991; Ahsan and Sasaki, 1989)

Isolated *S.* Typhi was subcultured in 250 ml of Tryptic Soy Broth enrichment media and incubated overnight at 37º. The bacterial cells were harvested by centrifugation at 8000 rpm at 4 ºC for 20 min and washed 3 times with normal saline. The pelleted cells were finally suspended in 3-4 ml of sterile de-ionized distilled water and the suspension was transferred in a sterile flat bottomed (100 ml) conical flask. The flask was placed on a horizontal shaker and shacked for 6 hours at room temperature at a speed of 100 - 120 oscillations per minute. The suspension was then collected in a sterile 15 ml falcon tube and centrifuged at 16000 x g (12,500 rpm, rotor radius 9.5) for 30 minutes. The supernatant was collected in a sterile 5cc syringe and filtered through 0.2 µm Millipore filter. This fluid contains the water soluble surface proteins and designated as SURP. SURP was aliquoted and stored in 1.5 ml sterile eppendorf tubes after adding protease inhibitor.

3.6.2 Preparation of *S*. Typhi envelope protein (ENVP) by Tris-sucrose EDTA (TSE) extraction method

Envelop protein from *S.* Typhi was prepared by Tris-sucrose EDTA (TSE) extraction method as described previously (Quan et al., 2013). Bacterial cell envelope protein (ENVP) was extracted chemically by disrupting outer membrane and cell wall. Tris Sucrose EDTA (TSE) extraction method extracts bacterial envelope proteins (ENVP) which contain 78% periplasmic, 16% outer membrane and 0.4% inner membrane proteins. It also contains 5% cytosolic protein. Sucrose increases the extracellular osmolality and causes cells to shrink and release water and periplasmic contents into the surrounding medium. It also stabilizes the cell membrane and prevents bacterial cell lysis. Chelating of divalent ions by EDTA causes release of LPS and increases permeability of outer membrane. *S.* Typhi bacterial cells were harvested by centrifugation (5000 rpm, 20min at 4 ºC) of overnight 100 ml broth culture.

Supernatant was discarded and cell pellet was washed 3 times with normal saline. The pellet was re-suspended in one ml of TSE buffer in ice for 30 minutes. Cell suspension was transferred to micro centrifuge tube and centrifuged at 16000 x g (12,500 rpm, rotor radius 9.5) for 30 min at 4 ºC. Supernatant containing envelope proteins was transferred to eppendorf and preserved at -20ºC after adding protease inhibitor.

3.6.3 Preparation of Whole Cell Extract (WCE) by ultrasonication method

Whole cell antigen of *S.* Typhi was extracted by the methods described by Saranya et al. (Saranya et al., 2014). Organisms grown in 50 ml TSB by overnight incubation at 37^0C were harvested by centrifugation for 30 minutes at 8000 rpm at 4^0C for 10 minutes and supernatant was discarded. Cells were washed 3 times with PBS and re-suspended in 1 ml cold PBS in 1.5ml eppendorf tube. Then sonication (Omni Ruptor 4000, 20 kHz) was done for 4 minutes at 30 watt at 30 seconds pulse by keeping the tube in ice flakes during sonication. After sonication, centrifugation was done at 10,000 rpm for 10 minutes and the supernatant was collected as whole cell extract (WCE).

3.6.4 Estimation of protein in surface, envelope and whole cell extract of S. Typhi

Protein in the SURP, ENVP and WCE of *S.* Typhi was estimated by Bradford method (Bradford, 1976). Samples were assayed in duplicate. Diluted dye reagent (0.5 ml) was added to each tube and mixed well using vortex mixer. A mixture of dye reagent and de-ionized distilled water served as blank. All mixtures were incubated at room temperature for 30 minutes and absorbance was measured at 595 nm using a spectrophotometer (Genesys 5, Canada). The absorbance of sample was plotted on the standard curve and protein concentration was determined. Protein estimated from surface (SURP), envelope (ENVP) and whole cell extract (WCE) were 0.74 mg/ml, 0.84 mg/ml and 1.7 mg/ml respectively.

3.6.5 Measurement of antibody response against surface, envelope and whole cell extract proteins of *S*. Typhi by ELISA

The 96 well EIA plate (Greiner bio-one, Germany, Cat no. 655161) was coated with surface (SURP), envelope (ENVP) and whole cell extract (WCE) protein antigens. The antigens were diluted in coating buffer (0.5 M carbonate/bicarbonate buffer, pH 9.6). The optimal concentration of each protein (SURP, ENVP, WCE) was 10µg/ml as determined by checkerboard titration. To each well 50 µl of antigen containing coating buffer was added and incubated overnight at 4^0C. The plate was washed three times with PBS-0.05% Tween 20 (PBS-T, pH 7.4)) and blocked by incubating for 2 hours with PBS-T containing 2% bovine serum albumin (BSA) at 37^0C. The plate was then washed three times with PBS-T. A volume of 100 µl serum samples at 1:2048 dilution was added into each well and incubated overnight at 4^0C. The serum dilution of 1:2048 was determined by checkerboard titration. After washing with PBST three times, 100 µl of horseradish peroxidase conjugated anti-human IgG antibodies (1:4000, MP Biomedicals, USA) was added and incubated at 37^0C for 4 hours. After washing three times with PBST, 100 µl of 3,3`,5,5`-Tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature for 30 minutes in dark. Then 100 µl of 2(M) sulfuric acid was added in each well. The colour developed was measured at 450 nm. A cut off OD value for anti- *Salmonella* Typhi IgG was determined by performing ELISA with sera from 47 healthy newborn babies (Widal test-non reactive). The mean OD+2xSD of these sera were taken as cut-off OD value to determine the magnitude of immune response to each component of *S.* Typhi following infection. Table-3.4 shows the cut off OD values for surface, envelope and whole cell extract antigens. Any sample showing OD value above the cut-off value was referred to as exposed to *S.* Typhi.

Table- 3.4: Cut off OD values for surface (SURP), envelope (ENVP) and whole cell extract (WCE) proteins of *S*. Typhi by ELISA.

Antigen	Mean OD value	±SD	Cut-off OD value (Mean+2xSD)
SURP antigen by WEM	0.5	0.2	0.9
ENVP antigen by TSE	0.5	0.2	0.9
WCE antigen by sonication	0.9	0.4	1.7

3.7 SDS polyacrylamide gel electrophoresis and Western blot analysis of *Salmonella* Typhi protein antigens (surface, envelop and whole cell extracts)

SDS- polyacrylamide gel electrophoresis and Western blot was carried out to identify immunogenic proteins of surface (SURP), envelop (ENVP) and whole cell extracts (WCE) of *S.* Typhi. Human serum from typhoid cases were used to detect immunogenic antigens of *S.* Typhi.

3.7.1 Procedure of SDS-PAGE

S. Typhi surface protein (SURP), envelope protein (ENVP) and whole cell protein (WCE) were extracted and estimated as described in section 3.6. Briefly, 10 µg of each protein samples were mixed with 2x sample buffer (containing 0.4 ml of 0.5M Tris-HCl pH 6.8, 10% SDS, 2% β-mercaptoethanol and 20% glycerol) at a ratio of 1:1 and boiled for 3 min. Then after cooling the mixture, 5µl of tracking dye (0.1% Bromophenol blue) was added to each sample, mixed well using a vortex mixer and loaded onto the top of 1-mm slabs comprised of a 6% polyacrylamide stacking gel and 12.5% polyacrylamide separating gel. The current flow was set at 16 mA for stacking gel and but when the samples reached the separating gel, then it was set at 20-22 mA. Electrophoresis was continued until the dye reached the bottom of the gel (Mesapogu et al., 2013; Laemmli, 1970).

Gel was stained with 0.1% Coomassie brilliant blue or 0.1% Ponceau stain. After flooding the gel with staining solution and gently shaking for 1 hour, the staining solution was removed and de-staining solution (7% acetic acid solution) was added. De-staining was done overnight at room temperature in a shaking platform. The protein band was visualized in naked eye against a bright background and the molecular weight of the bands was determined by Rf value using a calibration curve prepared by standard molecular weight markers.

Preparation of all the buffers and solutions for SDS - PAGE has been described in appendix II.

3.7.2 Procedure of Western Blot analysis

For Western blotting (Towbin et al., 1979), proteins were transferred to nitrocellulose membrane (0.45 μm, Bio-Rad), and nonspecific binding sites were blocked with 2% skim milk solution prepared in PBS buffer (pH 7.4) for one hour. Sera of typhoid patient were diluted 1:100 in 2% skim milk-PBS and membranes were incubated in this solution for 1hour at room temperature. Washing was performed by shaking the membrane strips in 0.1% Tween 20 in PBS for 3 times (5 minutes each time) and in PBS once for 5 minutes. Then, Horse reddish peroxidase (anti-human IgG, Fab, HRP-conjugate, Sigma) or Alkaline phosphatase (AP) conjugate (Anti-human polyvalent α, β, γ specific immunoglobulin, Sigma) was used at a dilution of 1:10,000 in 2% skimmed milk in PBS for 1 hour. Strips were washed again as above. Substrate solution (Appendix II) was prepared and added to the washed strips in dark room. To identify immunogenic antigens, 30% H_2O_2 plus Diamino Benzedine tetrahydrochloride (DAB) were used as substrates for HRP conjugate, and were AS-MX Napthol phosphate, Fast Red TR and 50 mM Tris HCl (pH-9) were utilized as substrate for AP to perform Western blot analysis. Two negative control (E.coli culture positive patient sera and healthy control) were run on each gel to ensure test specificity.

Chapter Four

Results

4.0 Results

Six groups of BALB/c mice were immunized with *S.* Typhi Vi CPS vaccine antigen in combination with Vi CPS, Vi-TT and killed whole cell (KWC) *S.* Typhi antigens to determine the effect of homologous or heterologous P-B immunization methods on the anti-Vi antibody response in spleen, Peyer' patch and serum. In spleen and Peyer's patch, anti-Vi IgM, IgG and IgA antibody secreting cells (ASC) were determined following immunization on day 28 (7 days after the booster dose on day 21). Serum anti-Vi IgM, IgG and IgA antibodies as well as salmonellacidal antibodies were also measured in immunized mice.

Antibody (IgG) responses against Vi CPS, surface protein (SURP), envelope protein (ENVP) and whole cell extract (WCE) of *S.* Typhi were determined in 70 acute and 11 convalescent stage sera of culture positive typhoid patients and compared for corresponding antibodies in sera from 12 healthy and 13 diseased individuals. Salmonellacidal antibodies in serum were also investigated among these groups. Specific immunogenic antigen of surface, envelope and whole cell extracts of *S.* Typhi were identified by Western blot analysis using immune sera from culture confirmed typhoid cases.

4.1 Effect of prime boost method using Vi CPS in combination with Vi CPS, Vi-TT, KWC antigens on the anti-Vi ASC response in the spleen and Peyer's patch of BALB/c mice

Effect of different prime boost method using combination of Vi CPS, Vi-TT, KWC antigens on anti-Vi antibody response was measured by detecting *S.* Typhi anti-Vi IgM, IgG and IgA ASC response in spleen and Peyer's patches in different groups of BALB/c mice and compared. Anti-Vi IgM, IgG and IgA ASCs and serum antibodies were measured 7 days after booster immunization on day 28.

4.1.1 Anti-Vi ASC response in spleen of BALB/c mice

In spleen, the mean anti-Vi IgM ASC response in Group (Gr.) 2, 3, 4 and 5 was significantly ($p<0.001$) higher compared to Gr. 1 or control group (Table-4.1). Also, anti-Vi IgM ASC response in Gr. 5 was significantly ($p<0.001$) higher than that of Gr. 6 No anti-Vi IgG ASC response was detected in Gr. 1 and Gr. 2 in spleen cells. Anti-

Vi IgG ASC response was not significantly ($p>0.05$) different from each other among the other groups. In spleen, no anti-Vi IgA ASC was detected in Gr. 1 and anti-Vi IgA ASC responses in Gr. 2 to Gr. 6 were not significantly ($p>0.05$) different from each other. In spleen, predominant anti-Vi ASC response was IgM in all groups (186±26.7 ASC/10^7 to 466 ± 40 ASC/10^7 lymphocytes) except Gr. 1 and was significantly ($p<0.05$) higher compared to anti-Vi IgG and IgA ASC.

4.1.2 Anti-Vi ASC response in PP of BALB/c mice

In PPs, all three classes (IgM, IgG and IgA) of anti Vi ASC were detected in Gr. 5 and 6 while no anti-Vi IgG and IgA response was detected in Gr. 1, 2, 3 and 4 (Table-4.2). The mean anti-Vi IgM ASC response in Gr. 6 was significantly ($p<0.05$) higher than those of Gr. 2, 3 and 4 while ASC responses among the rest of the groups were not significantly different from each other. In Gr. 6, anti-Vi IgM ASC response was significantly ($p<0.05$) higher compared to anti-Vi IgG and IgA ASC response.

4.1.3 Serum Anti-Vi antibody titer response in BALB/c mice

Table-4.3 shows the detail serum anti-Vi IgM, IgG and IgA responses in BALB/c mice after immunization by P-B method using salmonella Vi CPS in combination with different antigens and routes. Mean anti-Vi IgM antibody titer was significantly higher in Gr. 5 and Gr. 6 compared to other groups. Mean serum anti-Vi IgG titer was significantly ($p<0.05$) low in Gr. 1 compared to other groups. Mean serum anti-Vi IgA response was highest in Gr. 3 and lowest in Gr. 1 compared to other groups. Serum anti-Vi IgM, IgG and IgA antibody response in different groups were not significantly different from each other except for Gr. 3 where anti-Vi IgG and IgA titers were significantly ($p<0.05$) higher than IgM.

4.1.4 Serum salmonellacidal antibody response in BALB/c mice

Salmonellacidal responses (serum bactericidal activity) in different groups of mice after immunization by P-B method using *S.* Typhi Vi CPS in combination with different antigens and routes is shown in Table 4.4. The titer was measured 7days after booster doses. Equal titer (1:15) of salmonellacidal antibody was detected only in Gr. 3 and 5 by serum salmonellacidal assay. No salmonellacidal antibody was detected in other groups.

Table 4.1: Anti-Vi IgM, IgG and IgA ASC responses in spleen of BALB/c mice after immunization by P-B method using *S*. Typhi Vi CPS in combination with different antigens and routes

Group (Gr.)	No. of Mice	Immunizing Ag and route	Mean Anti-Vi ASC /10^7 lymphocyte ± SE			P value *
			IgM[a]	IgG[b]	IgA[c]	
1	10	Vi (i.p)	35 ± 12	0	0	
2	6	Vi (i.p) -Vi (i.p)	313 ± 26.4	0	30.8 ± 5.9	a vs c <0.001
3	6	ViTT (i.p) -ViTT (i.p)	260 ± 11.7	33.3 ± 15.9	36 ± 3.2	a vs b, c <0.001
4	6	ViTT (i.p) -Vi (i.p)	296 ± 90.2	30.8 ± 13.5	17.5 ± 5	a vs b, c <0.05
5	5	KWC (i.p) -Vi (i.p)	466 ± 40	22.5 ± 3.7	18.7 ± 10.3	a vs b, c <0.001
6	5	KWC (o) -Vi (i.p)	186 ± 26.7	17 ± 5.2	13.3 ± 4.1	a vs b, c <0.001
		P value*	1vs 2,3,4,5 <0.001 5 vs 3,6 <0.05 5 vs 6 <0.001	n.s	n.s	

*P value calculated by ANOVA test. n.s., not significant; i.p., intraperitoneal; o, oral; KWC, killed whole cell; TT, tetanus toxoid, ASC, antibody secreting cell; P-B, prime -boost; SE, standard error ; a = anti-Vi IgM; b =anti-Vi IgG; c= anti-Vi IgA. No ASC was detected in unimmunized mice spleen samples.

Table 4.2: Anti-Vi IgM, IgG and IgA ASC responses in Peyer's patch of BALB/c mice after immunization by P-B method using *S*. Typhi Vi CPS in combination with different antigens and routes

Group (Gr.)	No. of Mice	Immunizing Ag and route	Mean Anti-Vi ASC /10^7 lymphocyte± SE			P value*
			IgM[a]	IgG[b]	IgA[c]	
1	10	Vi (i.p)	0	0	0	
2	6	Vi (i.p) -Vi (i.p)	15 ± 2.4	0	0	
3	6	ViTT (i.p) -ViTT (i.p)	8.3 ± 2.7	0	0	
4	6	ViTT (i.p) -Vi (i.p)	8.7 ± 2.2	0	0	
5	5	KWC (i.p) -Vi (i.p)	32 ± 13.7	25 ± 4.6	15 ± 4.7	n.s
6	5	KWC (o) -Vi (i.p)	45 ± 12.2	15 ± 5.5	8.7 ± 2.0	a vs b, c <0.05
		P value*	2,3,4 vs 6 <0.05	5 vs 6 >0.05	5 vs 6 >0.05	

*P value calculated by ANOVA test; n.s., not significant; i.p., intraperitoneal; o, oral; KWC, killed whole cell; TT, tetanus toxoid, ASC, antibody secreting cell; P-B, prime boost; PPs, Peyer's patches; SE, standard error of mean; a=anti-Vi IgM; b=anti-Vi IgG; c=anti-Vi IgA. No ASC was detected in unimmunized mice PPs samples.

Table 4.3: Serum anti-Vi IgM, IgG and IgA antibody responses after immunization by P-B method using salmonella Vi CPS in combination with different antigens and routes in BALB/c mice

Group (Gr.)	no. of mice	Immunizing Ag and route	Mean serum titer Anti-Vi ± SE			P value*
			IgM[a]	IgG[b]	IgA[c]	
1	10	Vi (i.p)	6144 ± 1448	24 ±0.7	2389±737.4	a vs b <0.001 b vs c <0.05
2	6	Vi (i.p)-Vi (i.p)	5734 ± 897.4	5120±1773.6	6553.6±897.4	n.s.
3	6	ViTT(i.p)-ViTT(i.p)	4096 ± 1003.3	13312±2660.4	14336±1773.6	a vs b,c <0.05
4	6	ViTT (i.p)-Vi (i.p)	5376 ± 1509.1	5888±3079.9	4608±1115.87	n.s.
5	5	KWC (i.p)-Vi (i.p)	10240± 1773.6	10752 ± 3018	9830 ± 1465.4	n.s.
6	5	KWC (o)-Vi (i.p)	10922±2229.5	10922 ± 2229.5	4778 ± 1474.7	n.s.
		P value*	n.s	1vs 3,5,6 <0.05	3vs1,2,4,6 <0.001 5 vs 1 <0.001	

Note: Cut off OD of anti Vi IgM, IgG and IgA were set at 0.8, 0.5, 0.5 respectively for determination of mice serum anti-Vi titer. Base line titer of unimmunized mice were 1024, 16 and 16 for anti Vi IgM, IgG and IgA respectively.

*P value calculated by ANOVA test. n.s., not significant; i.p., intraperitoneal; o, oral; KWC, killed whole cell; TT, tetanus toxoid; P-B, prime- boost; SE, standard error of mean; a =anti-Vi IgM; b =anti-Vi IgG; c= anti-Vi IgA.

Table 4.4: Serum salmonellacidal antibody titer in BALB/c mice after immunization by P-B method using salmonella Vi CPS in combination with different antigens and routes

Group	No. of mice	Immunizing Ag and route	Salmonellacidal antibody titer (Mean ± SE)
1	5	Vi (i.p)	0
2	5	Vi (i.p) -Vi (i.p)	0
3	4	ViTT(i.p) -ViTT(i.p)	15±3.3
4	4	ViTT (i.p) -Vi (i.p)	0
5	4	KWC (i.p) -Vi (i.p)	15±3.3
6	4	KWC (o) -Vi (i.p)	0

i.p., intraperitoneal; o, oral; KWC, killed whole cell; TT, tetanus toxoid; P-B, prime-boost;

4.2 Estimation of salmonellacidal antibody response in acute and convalescent stage of typhoid cases and following Vi CPS typhoid vaccination in human

Study population included 70 typhoidal cases**,** of which 62 were typhoid (mean age 30.0±2.3) and 8 were paratyphoid A fever (mean age 38.6±5.0) cases. Mean age of 11 convalescence cases were 26±3.4. Out of 25 control participants (mean age 18.1±3.2), 13 were disease control and 12 were healthy individuals. Vaccination response was measured in six (06) adult volunteers (3 male & 3 female, mean age 26.5±3.5 years). Base line characteristics of the study population are shown in Table 4.5.

4.2.1 Salmonellacidal antibody response among study population

Salmonellacidal antibody response estimated among the study population by serum salmonellacidal assay is shown in Table 4.6. Human sera from typhoid and paratyphoid A patient showed significant levels ($p<0.05$ by paired t test) of bactericidal titre (titer 549.9±108.5 and 528.7±187.3) against *S.* Typhi compared to control cases (0.133±0.1). In 11 cases paired sera were obtained and the salmonellacidal antibody titer was 363.6±142.1 and 5.5 ±2.1 in acute and convalescent period respectively. Titer decreased significantly ($p<0.05$ by paired t test) during convalescent period (363.6±142.1vs 5.5±2.1). Very low salmonellacidal titers (2.5±1.5 and 2.3±1.54) were detected 14 and 21 days after single dose of Vi CPS vaccine among the human volunteers.

4.2.2 Salmonellacidal antibody titer reponse at different interval of time during acute stage of typhoid fever

Salmonellacidal antibody titer at different interval of time after the onset of typhoid fever is depicted in Figure 4.1. The salmonellacidal titer increased significantly ($p<0.05$) in samples collected between 7 to 10 days and between 10 to 25 days of fever (titer 535.7± 119.2 and 794.6± 235.6) compared to samples collected having fever for less than 7 days (Mean titer 136.4± 52.7). The mean titer significantly ($p<0.05$) decreased to 5.5± 2.1 after 6-8 weeks of infection.

Table 4.5: Baseline characteristics of different groups of study population

Category of study population	Total No	Mean age (yrs) ± SE
1. Typhoidal cases		
Typhoid	62	30.9± 2.2
Paratyphoid	8	
2. Convalescent cases	11	26±3.4
3. Control		
Healthy	12	18.1±3.2
Disease	13	
4. Vaccine volunteer	6	26.5±3.5

Note: Convalescent samples were collected after 6-8 weeks of resolution of fever.

Table 4.6: Salmonellacidal antibody titer in sera of different category of typhoid cases and after vaccination with typhoid Vi CPS vaccine

Study population	No	Salmonellacidal titer (Mean ± SE) measured by	
		OD	visual estimation
Acute Typhoid fever[a]	62	549.9±108.5[*]	302.7±61.2
Acute Paratyphoid fever[a]	08	528.7±187.3[*]	301.4±149.5
Acute case (febrile stage)[b]	11	363.6±142.1	223.7±89.9
Convalescent case (6-8wks)[b]	11	5.5±2.1	-[c]
Control[d]	15	0.133±0.1	-
Vi vaccinated group			
At 0 day	6	0.166±0.16	-
At 14day	6	2.5±1.5[**]	-
At 21 day	6	2.3±1.54	-

a =Culture positive case; b=Paired sera taken from same individual during acute and convalescent period and antibody titer decreased significantly ($p<0.05$ by paired t test) after 6-8 weeks period; c=No antibody detected in neat serum. If antibody detected in neat serum titer considered as 1; d =Single serum samples collected from controls; * significant titer ($p<0.05$) compared with control by unpaired t test; ** Not significant ($p>0.05$) between pre and post vaccinated titer by paired t test.

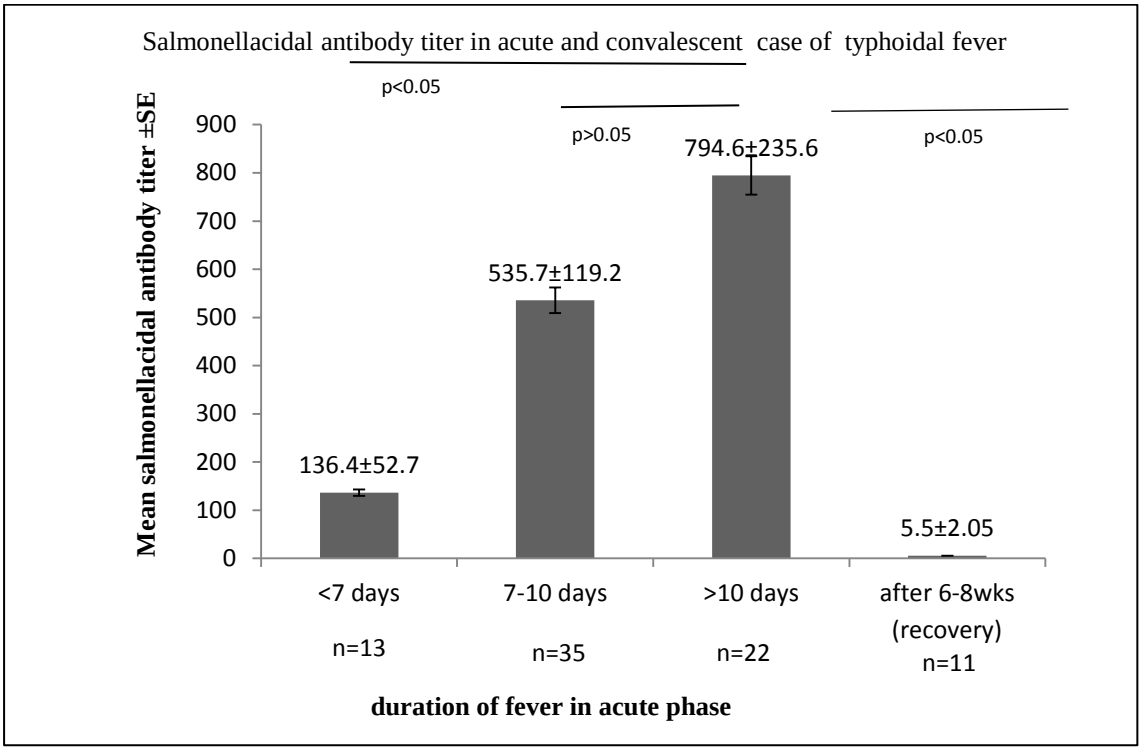

Fig 4.1. Salmonellacidal antibody titer at different interval of time following onset of typhoidal fever. *p value calculated by ANOVA (Tukey HSD Post-hoc) test.

4.3 Serum IgG antibody response to Vi capsular polysaccharide, surface, envelope and whole cell extract proteins of *S*. Typhi among the study population

Serum IgG antibody response against capsular polysaccharide, surface protein (SURP) envelope protein (ENVP) and whole cell extract (WCE) of *S*. Typhi were estimated in typhoid cases during acute and convalescent period by ELISA method. Also, Anti-Vi antibody was estimated following Vi-polysaccharide vaccination by ELISA in 6 human volunteers.

4.3.1 *Salmonella* anti-Vi IgG antibody response in typhoid cases

Out of 70 enteric fever cases, anti-Vi antibodies against *Salmonella* Typhi Vi antigen higher than the cut-off value (>0.41) were detected in 46 (65.7%) acute cases at 1:100 serum dilution (Table 4.7). Mean OD value of sera of positive cases was found two fold higher than the mean OD of negative cases (0.7 vs 0.3). The rate of positivity of anti-Vi IgG antibody in convalescent cases was 77.8% at 6-8 weeks after resolution of fever while 50% (6/12) healthy and 53.8% (7/13) diseased cases was positive.

4.3.2 *Salmonella* anti-Vi IgG antibody response in human vaccine volunteer

Response to Vi antigen was mounted in 100% (6/6) vaccine volunteer. Mean concentration of anti-Vi IgG antibody was 3.4 fold high in post vaccinated sera (1050±530.5) than that of pre vaccinated sera (312.5±129.2) but statistically titer was not significant ($p>0.05$) (Table 4.8). Salmonellacidal titer was significantly correlated with anti-Vi IgG titer (Table 4.9) in pre and post vaccinated sera (6+6= total 12 sera) shown in Table 4.9 ($r=0.6$, $p<0.05$ in spearman correlation coefficient test).

Table 4.7: Rate of positive *Salmonella* anti-Vi IgG antibody response of the study population

Study population	Total No	Anti- IgG positive n (%)	Anti- IgG negative n (%)	Mean (±SE) OD of	
				positive cases	negative cases
Acute case	70	46 (65.7)*	24 (34.3)	0.7 (±0.04)	0.3 (±0.01)
Convalescent case	9	7 (77.8)*	2 (22.2)	0.7 (±0.16)	0.3 (±0.13)
Healthy control	12	6 (50)	6 (50)	0.7 (±0.2)	0.3 (±0.02)
Disease control	13	7(53.8)	6 (46.2)	0.6 (±0.07)	0.2 (±0.04)

Cut-off OD for positivity = >0.41 at 1:100serum dilution; Convalescent case - 6 to 8weeks after resolution of fever; *p>0.05, compared acute and convalescent with control cases by Z test

Table 4.8: *Salmonella* Anti-Vi IgG antibody responses following single dose Vi CPS vaccine in human (n=6)

Vaccine volunteer	Pre-vaccine titer	Mean Pre vaccine titer (±SE)	Post-vaccine titer*	Mean Post vaccine titer (±SE)
1st	400		3200	
2nd	50		200	
3rd	800	312.5	1600	1050
4th	400	(±129.2)	800	(±530.5)*
5th	200		400	
6th	25		100	

Titer determined by cut off OD value at 0.41 at 1 in 100 dilution; * post vaccine titer calculated after 21 days of vaccination; *p value >0.05 by paired t test

Table 4.9: Spearman correlation coefficient between salmonellacidal antibody titer and anti-Vi IgG titer following vaccination with *Salmonella* Vi CPS

		Correlation	Salmonellacidal titer	anti-Vi IgG titer
Spearman's rho	**salmonellacidal titer**	Correlation Coefficient	1.000	.598*
		Sig. (2-tailed)	.	.04
		N	12	12
	anti-Vi IgG titer	Correlation Coefficient	.598*	1.000
		Sig. (2-tailed)	.04	.
		N	12	12

* Correlation is significant at the 0.05 level (2-tailed).

4.3.3 Serum IgG antibody response to surface (SURP), envelope (ENVP) and whole cell extract (WCE) proteins of *S*. Typhi in typhoidal cases

Mean serum IgG antibody to surface protein (2.4±0.06), envelope protein (2.5±0.06) and whole cell extract (2.7±0.05) of *S*. Typhi (Table 4.10) was significantly higher ($p<0.05$) during acute infection and convalescent stage compared to healthy and disease control. Serum IgG response against whole cell extract (WCE) was significantly ($p<0.05$) higher than surface (SURP) and envelope proteins (ENVP). Out of 70 acute typhoidal fever cases, IgG antibody against cell surface (SURP) and envelope protein (higher than the cut-off value >0.9) of *S.* Typhi was detected in all 70 (100%) individuals. Positive IgG antibody response was observed in 100% convalescent cases, 44-55% healthy controls and 30% disease controls against these protein antigens. IgG antibody response against whole cell extract (WCE) was almost similar to surface and envelope proteins (Table 4.11).

Table 4.10: IgG antibody response to surface, envelop and whole cell extract proteins of *S.* Typhi among the study population.

Antigen	Serum IgG antibody (mean OD±SE) in				P value by ANOVA test
	Acute case Gr-1 (n=70)	Convalescent case Gr-2 (n=9)	Diseased population Gr-3 (n=13)	Healthy population Gr-4 (n=9)	
SURP[a]	2.44 ±0.06	2.30 ±0.11	0.86 ±0.12	0.84 ±0.07	1 vs. 3,4= <0.05 2 vs 3,4= <0.05 1 vs 2=0.8, 3vs4=0.9
ENVP[b]	2.56 ±0.06	2.76 ±0.11	0.97 ±0.16	0.90 ±0.11	1 vs 3,4= <0.05 2 vs 3,4= <0.05 1 vs 2=0.6, 3 vs4=0.9
WCE[c]	2.76 ±0.05	2.86 ±0.08	1.64 ±0.18	1.82 ±0.29	1 vs 3,4= <0.05 2 vs 3,4= <0.05 1 vs 2=0.9, 3 vs 4=0.8
P value	a vs b= 0.356, b vs c= >0.5 a vs c= <0.05	a vs b=< 0.05, a vs c=< 0.05 b vs c= 0.773	a vs b= 0.875 a vs c= <0.05 b vs c= <0.05	a vs b= 0.97 a vs c=<0.05 b vs c=<0.05	

- a=Surface protein (SURP); b=Envelope protein (ENVP); c =Whole cell extract (WCE). Cut of OD for positivity to surface and envelope protein >0.9 and whole cell >1.7 at 1:2048 serum dilution

Table 4.11: Frequency of IgG antibody responses to *S*. Typhi surface (SURP), envelope (ENVP) and whole cell extract (WCE) protein antigens among the study population

Study population	Total No	Cases with positive antibody (IgG) response to		
		SURP n (%)	ENVP n (%)	WCE n (%)
Acute case	70	70 (100)	70 (100)	70 (100)
Convalescent case	9	9 (100)	9 (100)	9 (100)
Healthy control	9	5 (55.6)	4 (44.4)	5(55.6)
Disease control	13	4 (30.8)	4(30.8)	6(46.2)

*Cut off OD value of surface and envelope protein = 0.9 and whole cell=1.7;

SURP = Surface protein; ENVP = Envelope protein; WCE = Whole cell extract.

4.4 Identification of specific immunogenic antigens of *S*. Typhi by Western blot analysis

Surface, envelope and whole cell extract proteins of *S*.Typhi were analyzed for identification of immunogenic antigen by SDS-PAGE and western blot method using immune sera from culture confirmed typhoid cases.

4.4.1 SDS-PAGE analysis of surface, envelope and whole cell extract proteins of *S*. Typhi

Fig 4.2 shows the pattern of surface, envelope and whole cell extract proteins of *S*. Typhi by SDS-PAGE. *S*. Typhi was a locally isolated strain. SDS-PAGE revealed 13 proteins from whole cell extract, 10 proteins from both surface and envelope extracts of *S*. Typhi (Table 4.12). Surface and envelope extracts revealed similar pattern of bands.

4.4.2 Western blot analysis of surface, envelope and whole cell extract proteins of *S*. Typhi

Antibody response was detected against 180 kDa, 100kDa, 82kDa, 66kDa, 58kDa, 50kDa and 37 kDa molecular weight surface proteins. Immuno-reactivity was detected specifically against 58 kDa and 37 kDa protein of whole cell, surface and envelope protein extracts of *S*. Typhi with sera from typhoid patient in comparison to control by western blotting (Fig 4.3 and Fig 4.4).

Immunoreactivity of immune sera from 25 culture positive typhoid cases against suface proteins of *S*. Typhi is shown in Table 4.13. Out of 25 sera, 23 (92%) detected 58kDa protein. Antibody response (total Ig) frequency to 180 kDa, 100kDa, 82 kDa, 66kDa, 50kDa and 37kDa were 56%, 64%, 60%, 52%, 58% and 58% respectively. Frequency of IgG response was found in 80% (4/5), 60% (3/5) and 40% (2/5) cases against 37 kDa, 66 kDa and 58 kDa surface proteins respectively during acute phase of typhoid fever (Table 4.14).

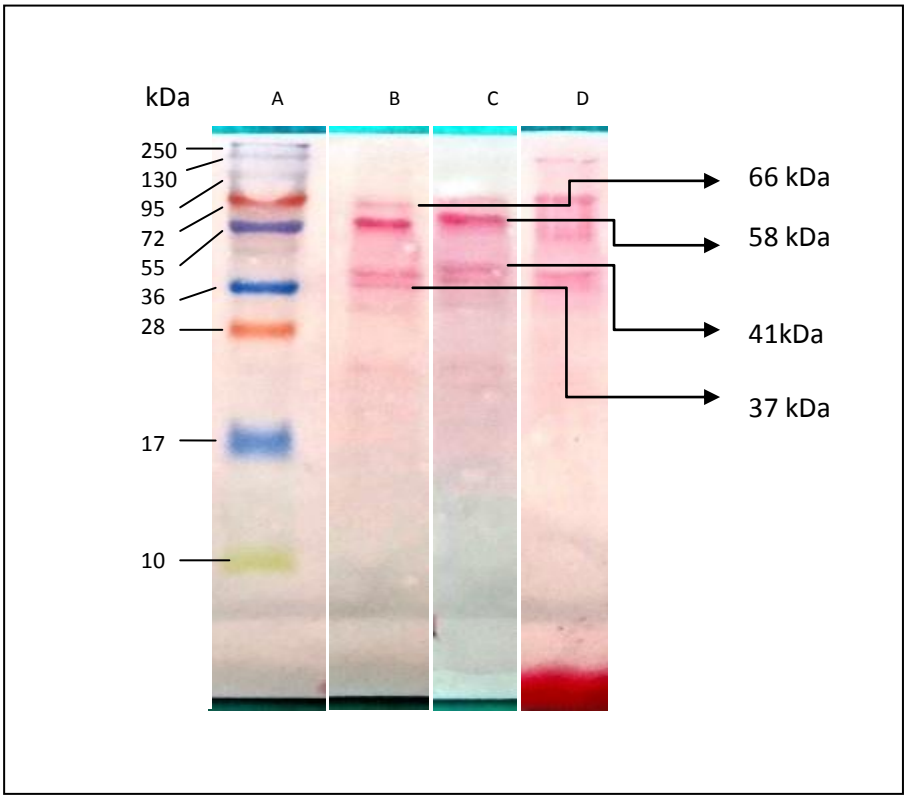

Fig 4.2: SDS-PAGE analysis of protein profile of surface, envelop and whole cell extracts of *S*. Typhi.

Patterns of protein were demonstrated by Ponceau staining. Lanes: A- protein marker; Lane B- surface protein, lane C- envelope protein, lane D- whole cell extract of *S.* Typhi.

Table 4.12: SDS-PAGE analysis of whole cell extract (WCE), surface protein (SURP) and envelope proteins (ENVP) of isolated strain of *S*. Typhi

***S*. Typhi antigen**	**SDS- PAGE analysis**	
	Size of protein (kDa)	**Total fraction of protein (N)**
Whole cell extract	180, 126, 100, 82, 66, 58, 50, 41, 37, 35,33, 25, 21	13
Surface protein	180, 100, 82, 66, 58, 50, 41, 37, 33, 25	10
Envelope protein	180,100, 82, 66, 58, 50, 41, 37, 33, 25	10

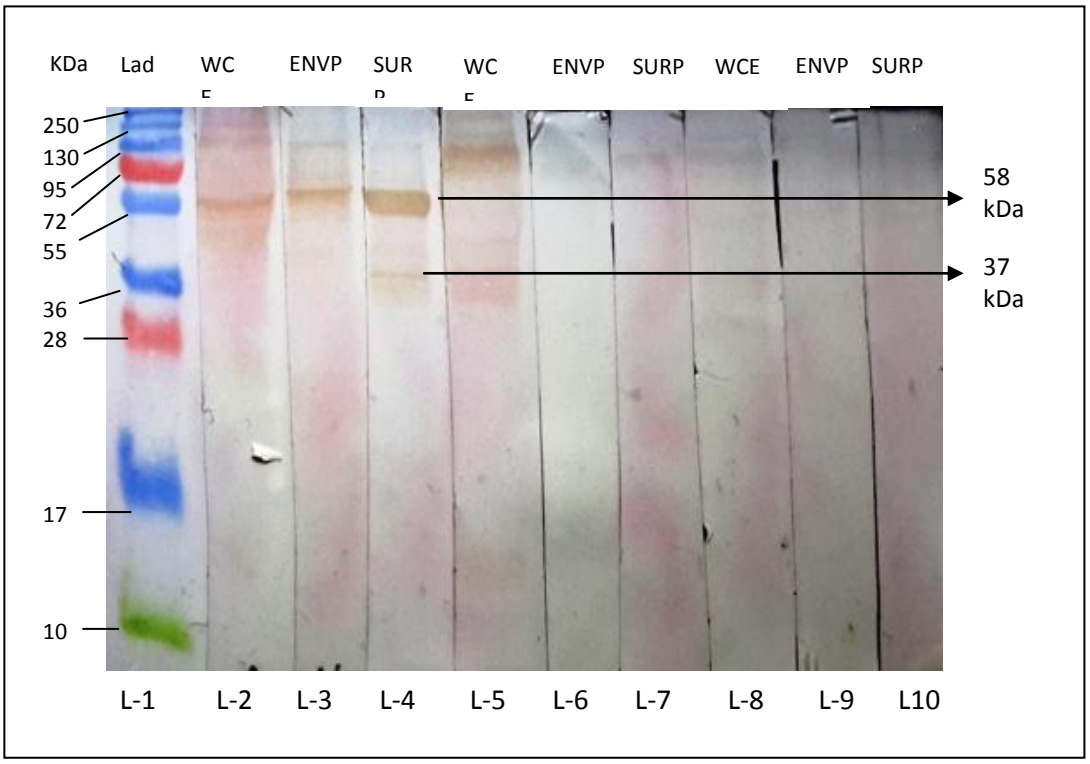

Fig. 4.3: **Immunoreactive antigens of surface (SURP), envelope (ENVP) and whole cell extract (WCE) proteins of S. Typhi by Western blot analysis with immune sera.**

Lane-1: Protein marker; Lane 2, 3, 4: using S. Typhi blood culture positive patient serum; Lane 5,6,7: using E coli culture positive septicaemic patient serum; Lane 8,9,10: using serum of healthy person; WCE: whole cell extract, ENVP: envelope protein, SURP: surface protein of S.Typhi; Anti-human IgG, Fab, HRP conjugate was used to detect immunoreactive antigen.

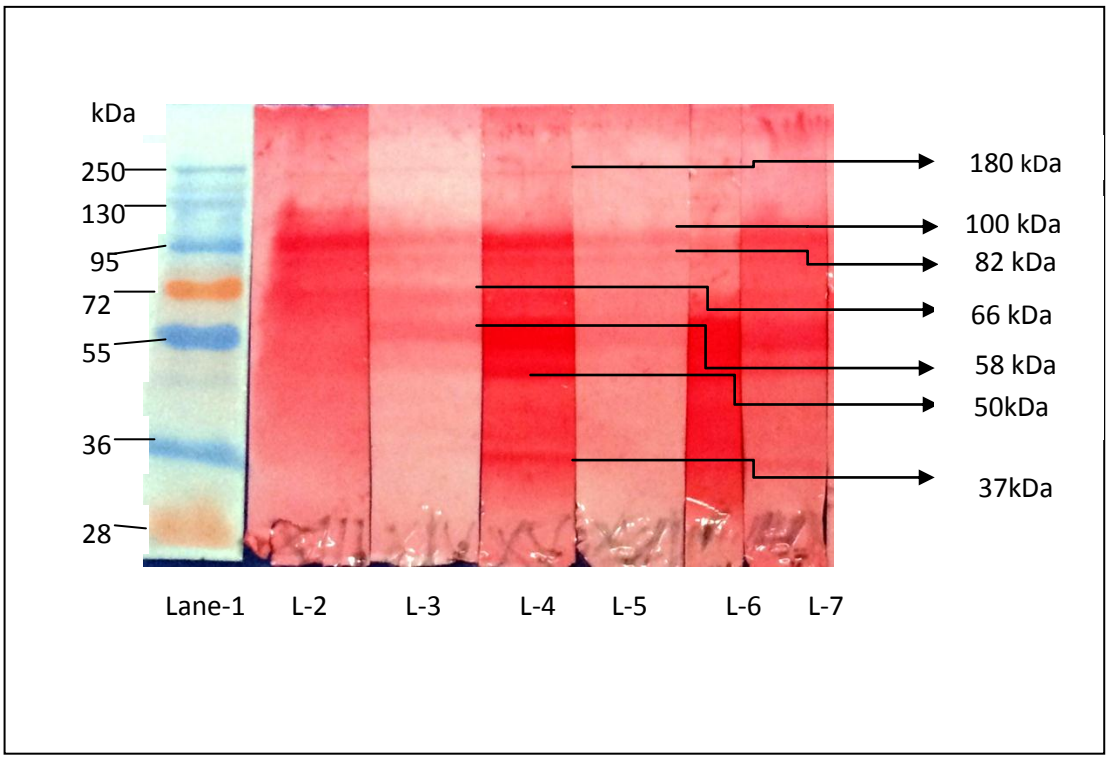

Fig 4.4: Western blot of surface proteins extracted from *S*. Typhi showing immunoreactive bands probed with sera from six culture positive typhoid cases.

Note: Lane-1: Protein marker; Lane 2, 3, 4, 5, 6, 7 surface protein probed with immune sera from typhoid cases; Total immunoglobulin (IgM, IgG and IgA) responses were detected against 180 kDa, 100kDa, 82kDa, 66kDa, 58kDa, 50kDa and 37 kDa molecular weight surface protein using anti human polyvalent α, β, γ specific immunoglobulin alkaline phosphatase conjugate.

Table 4.13: Frequency of immunoreactive sera from acute typhoid cases to different molecular weight surface proteins (n=25)

Sl no.	Surface proteins (kDa)	Number of serum with positve immunoreactive (total Ig) response	%
1	180 kDa	14	56
2	100 kDa	16	64
3	82 kDa	15	60
4	66 kDa	13	52
5	58 kDa	23	92
6	50 kDa	14	58
7	37 kDa	14	58

Note: No immunoglobulin responses were detected against 41kDa, 33kDa and 25kDa molecular weight surface protein in acute case of Typhoid fever.

Table 4.14: Frequency of IgG responses against 66kDa, 58kDa and 37kDa surface protein among typhoid fever cases (n=5)

Sl no.	Surface proteins kDa	Number of serum with positive IgG antibody response to SURP	%
1	66 kDa	2	40
2	58 kDa	3	60
3	37 kDa	4	80

Chapter Five

Discussion

5.0 Discussion

Typhoid fever caused by *Salmonella* Typhi (*S.* Typhi) which occurs exclusively in humans, is a very common systemic infection in developing countries like Bangladesh where incidence rate is >200/100,000 cases/year (Marchello et al., 2019; Radhakrishnan et al., 2018). Currently, two typhoid vaccine, parenteral Vi capsular polysaccharide (CPS) and oral live attenuated Ty21a are licensed in many countries and Vi CPS is only available in the local market of Bangladesh. It is well established that an immune response to the Vi CPS confers protection against *S.* Typhi infection (House et al., 2008; Frasch, 2006). However, three year cumulative efficacy of one dose Vi CPS vaccine is only 55% which gives protection only for 3-5 years and it is not effective for young children (<2 years of age) (Sahastrabuddhe and Saluja, 2019; Milligan et al., 2018; MacLennan et al., 2014; Marathe et al., 2012; González-Fernández et al., 2008; Lin et al., 2001). In addition, immunization with Vi CPS trigger T-independent response, thereby class switching of activated B cells is limited, no affinity maturation, and poor induction of memory cells. It produces moderate titers of low-affinity antibodies (IgM predominantly) and revaccination does not generate secondary responses rather may generate hyporesposiveness (Siegrist, 2018; Jakobsen et al., 2001). In addition, oral live attenuated Ty21a (lack of Vi antigen) is immunogenic only in children above 5-6 yrs of age and adult and highly acid labile when fed orally. Three year cumulative efficacy of three doses of this vaccine was 51% (Milligan et al., 2018; Marathe et al., 2012). Moreover, the two typhoid vaccines do not share any common antigen and the elicited immunological mechanisms are mainly targeted against different structures (Pakkanen et al., 2015). So, for the selective enhancement of immunogenicity of Vi CPS antigen, heterologous prime boost strategy was investigated in our study. Recently, this immunization strategy has shown promising results in producing stronger and longer-lasting immunity against various pathogens namely *M. tuberculosis*, Plasmodium spp and HIV (Sadlier et al., 2016; Lu, 2009; Dunachie and Hill, 2003).

In the present study, the effect of different prime boost method on the local (mucosal) and systemic anti-Vi antibody response was evaluated using combination of Vi CPS, Vi-TT, KWC *Salmonella* Typhi antigens in BALB/c mice model. The heterologous prime boost approach was conducted by priming with KWC (which also share Vi antigen, confirmed by capsular staining) or Vi-TT antigen followed by boosting with

Vi antigen and compared its effect with homologous prime boost and control groups. Anti-Vi antibody response was observed in spleen, blood (Table 4.1 and Table 4.3) and mucosal (local) response in PPs (Table 4.2) of BALB/c mice. Effect of this prime boost immunization method were measured by frequency of anti-Vi ASC with ELISPOT assay, anti-Vi antibody titer with ELISA and serum salmonellacidal antibody (functional antibody) response with colorimetric salmonellacidal assay. IgM, IgG and IgA secreting Anti-Vi ASC (all 3 classes of immunoglobulin) were detected among heterologous P-B group 4, 5, 6 and homologous P-B group 3 (ViTT-ViTT) in spleen (Table 4.1). Mice groups which received only intraperitoneal Vi CPS as single (group 1) or double dose (homologous P-B group 2), primarily produced IgM or IgM and IgA ASC but lacked IgG ASC response in spleen by inducing T independent response as plain polysaccharide was a T independent type 2 antigen (Siegrist, 2018). In PPs IgM, IgG and IgA anti-Vi ASC were found only in heterologous 5 and 6 (KWC-Vi) P-B groups. Priming with KWC antigen (i.p or oral) in group 5 and 6 and subsequent boosting with Vi antigen elicited higher anti-Vi antibody response in both spleen/ PPs and in serum compared to control group 1. Significant number of anti-Vi ASC detected in PPs when primed and boosted with KWC-Vi (group 5 & 6) could be due to the accumulation of Vi specific ASC in PPs by homing receptors. Homing receptor of ASC after KWC antigen vaccination (Oral or parenteral) possibly guided the immune effector cells to both spleen and PPs. An intestinal homing profile ($\alpha_4\beta_7$ – integrin) of majority (95%) of plasmoblast (ASC) after Ty21a vaccination and systemic homing profile (L-selectin) of majority ASC following Vi CPS vaccination (i.m.) has been documented in the different studies (Parker et al., 2018; Kantele et al., 2013; Kantele et al., 1997). Interestingly Anti-Vi IgG and IgA response in spleen (ASC) and in serum were found significantly more ($p<0.05$) among ViTT-ViTT homologous P-B group in contrast to other groups. Similar ASC response was found in greater magnitude in meningococcal glycoconjugate vaccine than plain polysaccharide vaccine following a booster dose (Mitchell et al., 2014). This suggests that T cell help may facilitate greater expansion of the plasma cell pool. Important observation in this study was the absence of booster or secondary anti Vi-IgG ASC response in Vi-Vi homologous P-B group (group 2) which was consistent with lack of induction of memory B cell response by plain polysaccharide T independent Vi antigen (Clutterbuck et al., 2012). Polysaccharide antigens predominantly activate

marginal zone B cells (MZB) of spleen in extra-follicular reactions and do not induce germinal center reaction (Murphy and Weaver, 2017). In addition, repeated immunization with plain polysaccharide vaccines/antigens might reduce the antibody levels, an effect known as 'hyporesponsiveness' and observed with meningococcal and pneumococcal vaccines (Mitchell et al., 2014; Clutterbuck et al., 2012; Lazarus et al., 2011; O'Brien et al., 2007). Presence of anti-Vi IgG ASC response observed in group 3, 4, 5, 6 might be due to the secondary T dependent response against polysaccharide Vi antigen. Such T dependent stimulation generates higher affinity antibodies, immunological memory and induces responsiveness to booster doses of vaccine (Clutterbuck et al., 2012). Class switching of IgM to IgG against Vi CPS antigen was also demonstrated in Vi based glycocojugate typhoid vaccine (Mohan et al., 2015; Szu et al., 2014; Thiem et al., 2011; Mai et al., 2003). Detection of serum anti-Vi IgG background titer (low) in mice of group 1, negative control and in group 2 (Vi-Vi) could be in response to stimulation of polysaccharide specific peritoneal B1b cell in mouse (Foote and Kearney, 2009) or induction of polysaccharide specific B cell by cross reacting antigen namely Vi of *Citrobacter freundii* (Hashimoto and Khan, 1997). Immunogenicity of heterologous P-B strategy was also studied using oral live attenuated CVD 909 typhoid candidate vaccine as priming agent (day 0) with subsequent Vi boosting (day 21) in twenty human healthy volunteer by Wahid et al. (2011). Higher and persistent, though not significant, anti-Vi IgG and IgA response were elicited following prime boost immunization in that study. In addition, Vi-specific IgA B memory (B_M) cells were significantly increased in CVD 909-primed subjects (Wahid et al., 2011). However in the present study we did not investigate the induction of memory B cell against the Vi antigen. In this study, we did not find any significant difference in anti-Vi antibody response when KWC given either through intra peritoneal (group 5) or oral route (group 6). In contrast to our study, Kinnear and Strugnell demonstrated significant difference in immune stimulation in a murine typhoid model after administering the same vaccine via oral and intravenous routes, though these differences did not impact in survival of mice challenged with a virulent strain of *S.* Typhimurium following intravenous or oral vaccination (Kinnear and Strugnell, 2015).

The immune response following any vaccination may be assessed by measuring total or isotype specific antibody or functional antibody. Most weight placed on detection

of functional antibody responses e.g. serum bactericidal antibody (SBA), toxin or virus neutralizing antibody or opsonophagocytic antibody (OPA) after any vaccination (W.H.O., 2016).

Serum bactericidal assay (SBA) is used to detect functional antibodies against specific bacteria in serum samples of patient after infection or vaccination. This assay was first introduced in 1962 by Finkelstein using agar plate based protocol (Son and Taylor, 2011). Subsequently, the technique was further refined and modified as a colorimetric assay by adding neotetrazolium dye and liquid broth to detect the end point of bacterial death (Boutonnier et al., 2003; Benenson et al., 1968). Change of colour indicated the growth of surviving bacterial target cells. The serum bactericidal assay has been used in different studies to measure bactericidal antibody titer in cholera (Son and Taylor, 2011; Losonsky et al., 1996; Qadri et al., 1995) or to estimate immunogenicity induced by vaccines for protection against cholera (Yang et al., 2007), typhoid (Juel et al., 2018; Jang et al., 2016; Kantele et al., 2013) or meningococcal disease (Frasch et al., 2009).

We estimated the salmonellacidal capacity of antibody (bactericidal antibody) generated in six groups of mice (Table 4.4) following P-B vaccination. Salmonellacidal antibody response (complement mediated killing) was observed only in one homologous (group 3) and one heterologous P-B group (group 5). The bactericidal effect could be due to generation of either complement fixing IgG1 or IgG3 antibodies following conjugate and whole cell vaccine (Igumbor and Osayande, 2009; Carson et al., 1995) in contrast to IgG2 generated predominantly by plain polysaccharide vaccine (Jakobsen et al., 2001).

In the present study, we employed colorimetric SBA to compare salmonellacidal antibody titer against *S.* Typhi during infection, convalescence period and following vaccination with Vi CPS in human volunteer (Table 4.6). Mean salmonellacidal antibody titer was found significantly high ($p<0.001$) in patient of both typhoid fever (549.9±108.5) and paratyphoid fever (528.7±187.3) when compared with control. Significant increase in salmonellacidal titer ($p<0.05$) was observed in second week (>10 days) following onset of fever in typhoid cases compared to first week (<7days) and the titer decreased significantly ($p<0.05$) in the convalescent stage (363.6±142.1 vs 5.5±2.1). It signifies that the bactericidal antibodies persists for a very short period of time during the acute phase of infection and the titer significantly decreases after

one month of infection during convalescent phase. Similar pattern of vibriocidal antibody titer response was observed in cholera (Qadri et al., 1995).

In our study, the mean salmonellacidal titer in post vaccinated sera of human (titer 2.5±1.5) and mice (titer 0 to 15± 3.3) were not significantly (P>0.05) raised from pre-vaccinated sera. It suggests that anti-Vi antibodies may not play a role in serum bactericidal activities as seen in previous report (Boyd et al., 2014). Vaccination with Vi capsular polysaccharide elicited only anti-Vi response, instead of different types of antibody as found with oral live attenuated typhoid vaccine or during natural infection to kill salmonella (Juel et al., 2018; Pulickal et al., 2009). Another reason could be the plain polysaccharide antigen induce predominantly IgG2 response (Jakobsen et al., 2001; Lottenbach et al., 1999) which is less complement fixing rather than IgG1 and IgG3 predominantly produced by conjugate based and live attenuated whole cell vaccine (Carson et al., 1995; Garred et al., 1989). High bactericidal titer observed following typhoid vaccination in a few studies could be due to the use of small concentration (10^4-10^3 CFU/ml) of salmonella in serum bactericidal antibody (SBA) assay (Boyd et al., 2014; Ahmadi et al., 2013) and use of semi automated colony counter in determination of SBA titer (Jang et al., 2016). However there are some limitations in our study. We did not investigate immunoglobulin isotypes responsible for salmonellacidal activities. But data suggests that IgM, IgG1 and IgG3 were the major subclasses to promote killing of gram negative bacteria in-vitro in the presence of complement (Igumbor and Osayande, 2009; Losonsky et al., 1996).

Infection of mice with *S.* Typhimurium results in a profound antibody response against both protein and non-protein structures of the organism (Mittrücker, 2000). However, the antibody response against different structural components (Vi, LPS, flagella, surface and envelop protein) of *S*. Typhi during natural infection is still incompletely understood despite the large number of study published (Pham and McSorley, 2015; Sztein et al., 2014; Barat et al., 2012; Mastroeni and Menager, 2003). Unlike *S. Typhimurium*, *S. Typhi* is a strict human pathogen and unable to infect mice (Mathur et al., 2012). So to measure the antibody response, we use sera from culture positive typhoid fever cases in which subjects were exposed orally to wild-type *S.* Typhi, thereby have the potential to provide a better understanding of the human immune response to infection. Antibodies (particularly IgG) perform several protective functions during the different stages of *Salmonella* infection. In the

intestinal lumen, it blocks bacterial penetration into deeper tissues, enhance bacterial engulfment via FcγR mediated phagocytosis, increasing macrophages activation with hightened bactericidal activities in gut associated lymphoid tissue, and thereby reduce the bacterial number entering the circulation and interfere in transiting of bacteria from one phagocyte to another before reaching liver and spleen (Pham and McSorley, 2015a; MacLennan, 2014; Bueno et al., 2012). Furthermore, antibodies activate complement via the classical pathway (Mitchell et al., 2014). Study confirms that pretreatment with *Salmonella*-specific antibodies accelerates the removal of bacteria from blood. Finally, antibody could block surface structures on *Salmonella* or neutralize toxic components such as LPS (Mittrücker et al., 2000). Vi capsule is a potent virulence factor of *S*.Typhi and humoral immune response against it acts as a principle host defense to combat typhoid fever (Parker et al., 2018; Abbas et al., 2017). Though, Vi CPS is T-independent type 2 antigens and produce a predominately IgM antibody response, but assessment of the IgG response is recommended following vaccination and natural infection in human (Parker et al., 2018; Szu et al., 2014; Pulickal et al., 2009; House et al., 2008). Our data have shown that positive anti-Vi IgG antibody response (cut off > 0.41 at 1:100 serum dilutions) was elicited in 65.7% (46/70), 77.8% (7/9), 52% (13/25) of acute case, convalescent and total control cases respectively (Table 4.7). Anti-Vi IgG response was found previously among 20 to 35% of acute typhoid fever cases, 15-40% non typhoid control and 83.3% of normal adult (>0.6ug/ml) in typhoid endemic area (Pulickal et al., 2009; House et al., 2008; Ferry et al., 2004). *In vivo* expression of Vi capsule is strictly regulated by multiple operons in response to environmental stresses (Kaur and Jain, 2012), though 99% of clinical isolates from typhoid fever cases possess Vi capsule *in vitro* (Wain et al., 2005). Moreover, Vi antibody response usually elicited later part of infection (>14 days) (House et al., 2008). So, prompt diagnosis and early treatment of an *S.* Typhi infection may hinder anti-Vi antibody response in acute phase typhoid fever. Anti Vi IgG antibody immune response (higher than cut off) among control in our study might be indicative of prior pathogen contact or cross reactivity with similar polysaccharide structures (Parker et al., 2018).

Immune responses directed at capsular polysaccharides vaccine are more difficult to assess in adults. This is because the majority of vaccine-naïve adults already have pre-existing immunity in the form of B cell memory and serum antibody to capsular

polysaccharides. This immunity is probably acquired following carriage of normal flora or repeated exposure to encapsulated bacteria in their life time (Mitchell et al., 2014). In our study mean titer of anti Vi IgG antibody of post vaccinated sera (titer 1050) was 3.4 fold higher from pre vaccinated sera (titer 312.5) though it was not significant ($p>0.05$) probably because of small number vaccine volunteer. Three fold increases in anti-Vi IgG usually considered to be positive vaccination response (Parker et al., 2018; Evans et al., 2018). The data from immunization of healthy volunteers from Oxford, UK have shown that geometric mean concentration of anti-S. Typhi Vi IgG were significantly higher ($p<0.0001$) in post-vaccination subjects (39.2 AU/ml vs 3.9 AU/ml) where 95% (20/21) participants exhibited fourfold increase whereas 67% (14/21) had three fold increase in contrast to prevaccination values (Ferry et al., 2004). Another recent study also estimated three fold and tenfold increase of post-vaccination IgG concentration in 100% (8/8) and 75% (6/8) Vi CPS vaccine volunteers respectively (Evans et al., 2018). A large clinical trial reported 76.9%, 79.1% and 62.5% of individuals aged 5-14 yrs., 15-44 yrs. and 45–55 years old respectively achieved sero-conversion (≥4 fold rise) in Nepal when the immunogenicity of Vi CPS vaccines were evaluated (Acharya et al., 1987). Anti-Vi IgG antibody titer response of pre and post vaccinated human sera exhibited some extent of correlation with bactericidal titer in our study (spearman's correlation coefficient test $r=0.6$, $p<0.05$) though previous data found high degree of correlation ($r=0.7$, $p<0.001$) (Jang et al., 2016). Bactericidal titer response may vary in individual vaccinee due to individual's memory or naïve B cells towards Vi capsular polysaccharides in typhoid endemic settings.

Currently, there are several serology-based diagnostic tests (Widal, Tubex, TP Test, Typhidot etc) available for typhoid fever but have poor sensitivity and/or specificity. (Andrews et al., 2018). However, detecting antibodies secreted from circulating, activated-lymphocytes requires moderately advanced laboratory capacity and requires 24–48 hours to obtain a result (Islam et al., 2016; Khanam et al., 2013). To address these issues, we have used *S*. Typhi surface (SURP), envelope (ENVP) and whole cell (WCE) protein to identify promising *Salmonella* candidate antigen (s) for possible addition in next-generation serodiagnostic assays. Immunogenicity and cross reactivity of surface, envelope and whole cell protein of *S*.Typhi were evaluated by ELISA and Western blot assay using sera of typhoid patient and control. Extraction of

surface proteins without disrupting the cells is a key simplification of conventional purification processes and isolations from whole bacteria (Trefilov et al., 2015). In this study, we used water extraction method (WEM) to obtain the surface proteins from the *S.* Typhi without significantly affecting the morphological structures of the cell. This simple low ionic strength extraction with distilled water has been used previously to release surface protein from bacteria and also from neoplastic cells (Ahsan and Sasaki, 1991; Ahsan and Sasaki, 1989). Envelope proteins extraction from any gram-negative bacteria by conventional methods such as osmotic shock can cause heavy contamination with soluble cytoplasmic protein. The cytoplasmic protein contaminants can overwhelm the genuine envelope protein components. However, Tris-Sucrose-EDTA (TSE) method from *E. coli* mostly extracted periplasmic (78%) and outer membrane protein (16%) with little contamination of cytosolic protein (5%) (Quan et al., 2013). So, we exploited this method to obtain *S.* Typhi envelope protein and subsequently used that in ELISA and immunoblot assay to detect antibody response and immunogenicity of this *S.*Typhi envelope protein. Anti-S.Typhi IgG antibody against surface, envelope and whole cell protein was detected in all (100%) acute and convalescent typhoidal fever cases (Table 4.11). Mean IgG concentration (OD) towards *S.* Typhi protein (surface, envelope and whole cell) (Table 4.10) significantly raised ($p<0.05$) during acute infection, persist also during convalescent period (after 6-8 wks) compared to control. Significantly increased IgG antibodies ($p<0.05$) was found against individual and mixed recombinant rCueO, rOmpC and rHAP1 *S.* Typhi proteins in the sera of the typhoid fever cases compared to the non-typhoid fever and the normal control cases (Chin KL et al., 2015). In our study, IgG antibody response against *S.* Typhi surface, envelop and whole cell extract proteins in 30.8% to 55.6% healthy and non-typhoid cases indicate wide or subclinical exposure of people to *S.* Typhi in this endemic region. Taken together, the data from this study indicate that the IgG antibody to a particular or specific surface, envelope or whole cell protein could serve as sensitive marker for the *S.* Typhi infection. Therefore, further study may be undertaken to identify such particular candidate protein(s).

In the present work, we have demonstrated that surface protein of *S.* Typhi obtained from water extraction method present similar SDS-PAGE pattern to those of envelope protein extracted by Tris-sucrose-EDTA buffer (Table 4.12 and Fig 4.2). Total ten (10) surface protein components were common by both extraction methods. They

were 180, 100, 82, 66, 58, 50, 41, 37, 33, 25 kDa. In addition to these proteins, three additional proteins (126 kDa, 35 kDa, 21 kDa) were identified in SDS-PAGE of whole cell extract of the same isolated strain (Table 4.12). To determine the immunogenicity of these proteins of *S.*Typhi, immunoblotting was done with sera from confirmed typhoid cases. We showed that, 7 out of 10 surface protein of *S.* Typhi (i.e. 180, 100, 82, 66, 58, 50, 37 kDa) were immunogenic (Table 4.13). Total antibody and IgG responses were detected in higher frequency against the 58 kDa (92%) and 37 kDa (80%) proteins respectively than other surface proteins among the culture confirmed typhoid fever patients (Table 4.13 and Table 4.14). The Gram-negative *Salmonella* has flagella, pili, fimbriae, outer membrane protein (OMPs) and porin proteins including other microstructures in their outer surface (Begum et al., 2008). Among these surface structures, outer membrane protein (OMP) is a major immunogenic target for typhoidal and non typhoidal serovar including *S.* Typhi (Muthiadin et al., 2015; El-Gayar et al., 2013; Aslam et al., 2012; Ortiz et al., 1989) and S. Typhimurium (Hamid and Jain, 2008; Akis et al., 2003; Foulaki et al., 1989). Several *Salmonella* OMPs have been investigated as potential vaccine candidates, virulence factors, and diagnostic candidate antigens (Dasila et al., 2017). Molecular size of *S.*Typhi OMP was detected in a range from 17 to 80 kDa. Some outer membrane proteins that are associated with the modulation of cellular permeability and antibiotic resistance are known as porin proteins. The major porins OmpC (39 kDa), OmpF (37 kDa), OmpD were recognized previously in *Salmonella* (Singh et al., 2018). Different studies have identified 60 kDa, 55kDa, 50 kDa , 36 kDa, 35 kDa, 34 kDa, 26 kDa, 25 kDa, molecular mass by SDS-PAGE of *S.*Typhi OMP (Dasila et al., 2017; Muthiadin et al., 2015; El-Gayar et al., 2013; Aslam et al., 2012; Chin KL et al., 2015). Whole cell lysates of eight different *Salmonella* serovars identified a common 37.81 kDa protein in all serovars (Begum et al., 2008). Pilli protein from *S.* Typhi isolated in Indonesia consists of 36 kDa, 26.5 kDa, 22.2 kDa and18.6 kDa major sub units and 116 kDa, 62.3 kDa, 45 kDa, 20.9 kDa minor sub units (Darmawati, 2005). The apparent molecular weight of wild-type flagellin of *Salmonella* was found between 51 to 57 kDa (Homma et al., 1987; Kondoh and Hotani, 1974). Possible explanation of various molecular sizes of *Salmonella* surface proteins in several studies may be due to use of different method of protein extraction and purification and use of different serovars and strains of *Salmonella*. However, 58 kDa and 37 kDa

surface protein of *S*.Typhi in our study showed frequent immunoreactivity and could be used in diagnostic tests for diagnosis of typhoid fever patient.

In conclusion, our results suggested that prime boost (P-B) strategy could be applied to get enhanced systemic and mucosal anti-Vi antibody responses using Vi CPS antigen in combination with KWC. The heterologous prime boost (P-B) method was able to stimulate secondary booster effect of T independent plain Vi polysaccharide antigen, and therefore might be a new mode of immunization with polysaccharide vaccine, particularly in children below two years of age. Immunization with Vi CPS induced low salmonellacidal antibody. However, high salmonellacidal antibody detected in early period of typhoid fever could be an important serological marker for the diagnosis of acute infection. As anti-*S*. Typhi IgG antibody response was observed against surface and envelop proteins in every culture confirmed typhoid case, in contrast to antibody response against Vi CPS (65.7%), identified immunogenic antigens (58kDa and 37kDa) from this surface/envelop proteins could further be refined and used as candidate for vaccine or diagnostic marker for *S*. Typhi infection.

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Appendices

Appendix I

Instruments

Important equipment used through the study is listed below:

Serial No.	Equipment	Company
1	Autoclave, Model no: HL-42AE	Hirayama corp, Japan
2	Sterilizer, Model no: NDS-600D	Japan
3	Class II Microbiology safety cabinet	Labcaire, USA
4	Room temperature horizontal shaker	Gerhardt
5	Water bath, Model: SUM	England
6	Electric balance, Scout, SC4010	USA
7	Freezer(-20^{o}C)	Liebherr,Germany
8	Refrigerator (4^{o}C)	Vest frost
9	Incubator	Japan
10	Microcentrifuge, Mikro 20	Germany
11	Micropipettes	Eppendorf, Germany
12	Microwave oven, Model: D90N30 ATP	Butterfly, China
13	Gel Documentation	Sigma
14	Power pack	Toledo, Germany
15	P^{H} meter, Model no: MP220	Eppendorf, Germany
16	Refrigerated centrifuge, Model : 5810R	Eppendorf, Germany
17	Sonicator	Omni International Inc.
18	SDS-PAGE apparatus	Bio-RAD, USA
19	Vortex	IKA
20	Binocular Microscope: CX21	Olympus
21	Dissecting or Stereo Microscope	Olympus
22	CO_2 Incubator	Sheldon Mfg. Inc.
23	ELISA Microplate Reader, ELx 800	BioTek, USA
24	Genesys5 spectrophotometer	Thermo Spectronic, ALT

Appendix II

Buffers and Reagents

1. Physiological saline (0.85%)

Ingredients	Amount
Sodium chloride	8.5 gm
Distilled water	Up to 1000 mL

2. Oxidase reagent

Ingredients	Amount
Tetramethyl-p-phenylene-diamine hydrochloride	1 gm
95% Ethanol	Up to 1000 mL

This reagent was stored at 4^{o}Cin brown bottle protecting from direct light expose.

3. Gram's staining reagents

- Crystal violet
- Gram's iodine
- Ethyl alcohol (95%)
- Safranin

4. Crystal violet Gram stain

Ingredients	Amount
Crystal violet	2 gm
95% Ethyl alcohol	20 mL
Ammonium oxalate monohydrate	0.8 gm
Distilled water	80 mL

5. Gram's iodine

Ingredients	Amount
Potassium iodine	20 gm
Iodine	10 gm
Distilled water	Up to 1000 mL

6. Ethyl alcohol (95%)

Ingredients	Amount
Absolute ethanol	475 mL
Distilled water	25 L

7. Kovac's reagents

Ingredients	Amount
Para-dimethyl aminobenzaldehyde	1.25 gm
Amylalcohol	18.75 mL
Concentrated HCl	Up to 25 mL

Prepared reagent was covered with aluminium foil and stored at $4^{o}C$.

8. Hank's balanced salt Solution

Ingredients	Amount
0.14M NaCl	8 g
0.005M KCl	400 mg
0.001M $CaCl_2$	140 mg
0.0004 M $MgSO_4$-$7H_2O$	100 mg
0.0005 M $MgCl_2$-$6H_2O$	100 mg
0.0003 M Na_2HPO_4-$2H_2O$	60 mg
0.0004 M KH_2PO_4	60 mg
0.006 M D-Glucose (Dextrose)	1 g
0.004 M $NaHCO_3$	350 mg
dH2O	1L

9. Tris-Sucrose-EDTA (TSE) buffer (pH 8.0)

Ingredients	Amount
200 mM Tris HCL	2.42 gm
500 mM Sucrose	17.11 gm
1mM EDTA	0.037 gm
Distilled water	100

10. Phosphate Buffered Saline (PBS 0.4 M) or Coating buffer for carbohydrate antigen (pH-7.2)

Ingredients	Amount
NaCl	8.0 gm
KCl	0.2gm
Na_2HPO_4	1.18 gm
KH_2PO_4	0.23 gm
Distilled water	Up to 1000 mL

The P^H was adjusted to 7.2 with HCL. PBS was stored at room temperature.

11. PBS-T buffer (0.05% Tween 20) or Wash buffer

Ingredients	Amount
PBS buffer	1L
Tween 20	500µl

12. 0.05 M Carbonate/ Bicarbonate buffer or Coating buffer for protein antigen (pH-9.6)

Ingredients	Amount
Na2CO3	0.64 gm
NaHCO3	3.7 gm
dH2O	1 L

13. Blocking buffer (1.5% BSA)

Ingredients	Amount
Bovine serum albumin	1.5 gm
PBS-T (0.05%)	100 ml

14. Tris-NH_4Cl or RBC lysis buffer

Ingredients	Amount
0.17M Tris (20.6gm/L)	10 mL
0.16 M NH_4Cl (8.3gm/L)	90 ml

15. Composition of substrate for alkaline phosphatase in ELISPOT (pH-9.5)

Ingredients	Amount
Tris-HCl	10 mL
Agarose	0.06 gm
BCIP (5-Bromo-4-chloro-3-indolyl phosphate) (1mg/ml)	10 mg (dissolve with 300 µl dH_2O)

Before adding substrate (BCIP), substrate buffer should be preheated at 42° C at water bath to dissolve the agarose and pH needs to be adjusted at 9.5.

16. 3M NaOH

Ingredients	Amount
NaOH	120 gm
dH_2O	1L

17. 30% Acrylamide

Ingredients	Amount
Acrylamide	14.5 gm
Bis-acrylamide	0.5 gm
Distilled water	Up to 50 mL

30% acrylamide was covered with aluminium foil and stored at 4^oC.

18. 0.5 M Tris HCL P^H 6.8

6.57g of Tris base was taken in 70mL distilled water. Then mixed and brought p^H to 6.8 with concentrated HCl and then the total volume of the solution was brought up to 100mL mark by adding distilled water. This solution was stored at 4^oC.

19. 1.5 M Tris HCl p^H 8.8

36.34g of Tris base was taken in 150 mL distilled water. Then mixed and brought pH to 8.8 with concentrated HCl and then the total volume of the solution was brought up to 200ml mark by adding distilled water. This solution was stored at 4^oC.

20. 10% SDS (10% Sodium Dodecyl Sulphate)

Ingredients	Amount
Sodium dodecyl sulphate	5 gm
Distilled water	Up to 50 mL

10% SDS was stored at room temperature.

21. 10% Ammonium persulphate (10% APS)

Ingredients	Amount
Ammonium persulphate	0.5 gm
Distilled water	Up to 5 mL

10% APS was aliquoted and stored at -20^oC.

22. Saturated butanol

50mL distilled water was taken in a beaker with a magnetic stirrer. Butanol was kept adding to water provided the machine was turned on. Addition of butanol was stopped when the solution got saturated and stored at 4^oC.

23. 0.1% Bromophenol blue (BPB)

At first, 2mL of 50% glycerol solution was made by mixing 1mL glycerol with 1mL distilled water. Then 2mg bromophenol blue was weighted and 50% glycerol was added up to 2mL mark. This was stored at 4^{o}C.

24. Running buffer

Ingredients	Amount
Tris base	3.0g
Glycine	14.4g
10% SDS	10mL
Distilled water	1000mL

This buffer was stored at 4^{o}C.

25. Transfer buffer

Ingredients	Amount
Methanol	200 mL
Tris base	3.03g
Glycine	14.4g
Distilled water	Up to 1000 mL

This buffer was stored at 4^{o}C.

26. 2x sample buffer (2mL)

Ingredients	Amount
0.5 M Tris HCl P^{H} 6.8	0.4mL
10% SDS	0.4mL
2-mercaptoethanol	0.04mL
Glycerol	0.4mL
Distilled water	0.76 mL

This buffer was stored at 4^{o}C.

27. 2% Skim milk

Ingredients	Amount
Skim milk	2 gm
0.4 M PBS	100 mL

This solution was prepared fresh before each use.

28. 0.1% Tween 20

Ingredients	Amount
Tween 20	0.1 mL
0.4 M PBS	100 mL

This solution was prepared fresh before each use.

29. Composition of 12.5% separating gel

Ingredients	Amount
Distilled water	1.60mL
1.5 M Tris HCl P^H 8.8	1.25mL
30 % Acrylamide	2.10mL
10% SDS	0.10mL
10% APS	35µL
TEMED (added at last)	17 L

30. Composition of stacking gel

Ingredients	Amount
Distilled water	2.137mL
0.5 M Tris HCl P^H 8.8	937µL
30 % Acrylamide	625µL
10% SDS	37µL
10% APS	25µL
TEMED (added at last)	11µL

31. Composition of 0.1% Coomassie brilliant blue

Ingredients	Amount
Acetic acid	10 mL
Methanol	10 mL
Coomassie brilliant blue	0.1 gm
Distilled water	80 mL

0.1% Coomassie brilliant blue was stored at room temperature.

32. Composition of Destaining solution

Ingredients	Amount
Acetic acid	10 ml
Methanol	10 ml
Distilled water	80 l

33. Composition of substrate for alkaline phosphatase in Western blot

Ingredients	Amount
50 mM Tris HCl (pH 9.14)	10 mL
Fast Red TR	20 mg
Napthol ASMX	10 g

34. Composition of substrate for horseradish peroxidase in Western blot

Ingredients	Amount
PBS (pH 7.4)	10 mL
30% H_2O_2	10 µL
3,3′- Diaminobenzidine	10 g

35. Composition of substrate for alkaline phosphatase in ELISA (pH-9.8)

Ingredients	Amount
1M Diethanolamine	9.7 mL
0.5 mM $MgCl_2$	10 mg
dH_2O	Up to 100 mL*
Then add pNPP (1mg/ml)	100 mg

*After preparation of Diethanolamine substrate buffer (DEA), pH was adjusted to 9.8 by adding 10 M HCl.

36. Composition of substrate for horseradish peroxidase in ELISA (pH-3.8)

Ingredients	Amount
3,3',5,5'–tetra- methylbenzidine tablet	1 Tablet
Dissolved in DMSO	1 mL
Then Add 0.05 M Phosphate-Citrate Buffer (pH-5.0)	9 mL
30% hydrogen peroxide	2 µl*

*Add fresh 30% hydrogen peroxide per 10 ml of substrate buffer solution immediately prior to use.

Appendix III

Media

Sl no	Name of Media	Company
1	MacConkey Agar (500g)	Himedia
2	Tryptone soya Broth (500g)	Himedia
3	Triple sugar iron Agar (500g)	Himedia
4	MIU Agar (500g)	Himedia
5	Simmons Citrate Agar (500g)	Himedia
6	RPMI	Sigma

1. MacConkey Agar

Ingredients	Amount
Peptic Digest of Animal Tissue	1.5 gm/L
Enzymatic digest of Casein	1.5 gm/L
Pancreatic Digest of Gelatin	17.0 gm/L
Lactose	10.0 gm/L
Bile salts	1.5 gm/L
Sodium chloride	5.0 gm/L
Crystal violet	0.001 gm/L
Neutral red	0.03 gm/L
Agar	13 gm/L

P^H 7.1 ± 0.2 at 25^{o}C.

50.0g was suspended in 1 liter of distilled water and was boiled to dissolve completely. Sterilized by autoclaving at 121^{o}C for 15 minutes.

2. Tryptone Soya Broth

Ingredients	Amount
Pancreatic digest of casein	17.0 gm/L
Enzymatic digest of soya bean	3.0 gm/L
Sodium chloride	5.0 gm/L
Di potassium hydrogen phosphate	2.5 gm/L
Glucose	2.5 gm/L

P^H 7.3 ± 0.2 at 25°C.

38.0g was suspended in 1 liter of distilled water and was boiled to dissolve completely. Sterilized by autoclaving at 121°C for 15 minutes.

3. Kligler's Iron Agar

Ingredients	Amount
'Lab-Lemco' powder	3.0 gm/L
Yeast extract	3.0 gm/L
Peptone	20.0 gm/L
Sodium chloride	5.0 gm/L
Lactose	10 gm/L
Glucose	1.0 gm/L
Ferric citrate	0.3 gm/L
Sodium thiosulphate	0.3 gm/L
Phenol red	0.05 gm/L
Agar	12.0 gm/L

P^H 7.4 ± 0.2 at 25°C.

55.0g was suspended in 1 liter of distilled water and was boiled to dissolve completely. Sterilized by autoclaving at 121°C for 15 minutes.

4. MIU Agar

Ingredients	Amount
Casein enzyme hydrolysate	10.0 gm/L
Dextrose	1.0 gm/L
Sodium chloride	5.0 gm/L
Phenol red	0.01 gm/L
Agar	2.0 gm/L

P^H 6.8 ± 0.2 at 25°C.

18.0g was suspended in 950mL of distilled water and was boiled to dissolve completely. Dispended in 95mL amounts into flasks. Sterilized by autoclaving at 121°C for 15 minutes. Cooled to 50-55°C and aseptically added 5mL sterile 40% Urea solution. Mixed well and dispensed into sterile test tube and cool in an upright position.

5. Simmons Citrate Agar

Ingredients	Amount
Magnesium sulphate	0.2 gm/L
Ammonium dihydrogen phosphate	0.2 gm/L
Sodium ammonium phosphate	0.8 gm/L
Sodium citrate, tri-basic	2.0 gm/L
Sodium chloride	5.0 gm/L
Bromothymol blue	0.08 gm/L
Agar	15.0 gm/L

P^H 7.0 ± 0.2 at 25°C.

23.0g was suspended in 1 liter of distilled water and was boiled to dissolve completely. Sterilized by autoclaving at 121°C for 15 minutes.

6. RPMI cell culture media

Ingredients	Amount
Glucose	2 gm
Phenol red	5 mg
Sodium chloride	6 gm
Sodium bicarbonate	2 gm
Disodium phosphate	1.51 gm
Potassium chloride	400 mg
Magnesium sulfate	100 mg
Calcium nitrate	100 mg
Amino acids (glutamine, arginine, cystine, lysine, serine etc.)	671 mg
Vitamins (para-aminobenzoic acid, folic acid, biotin, riboflavin etc)	39.45 mg

Appendix IV

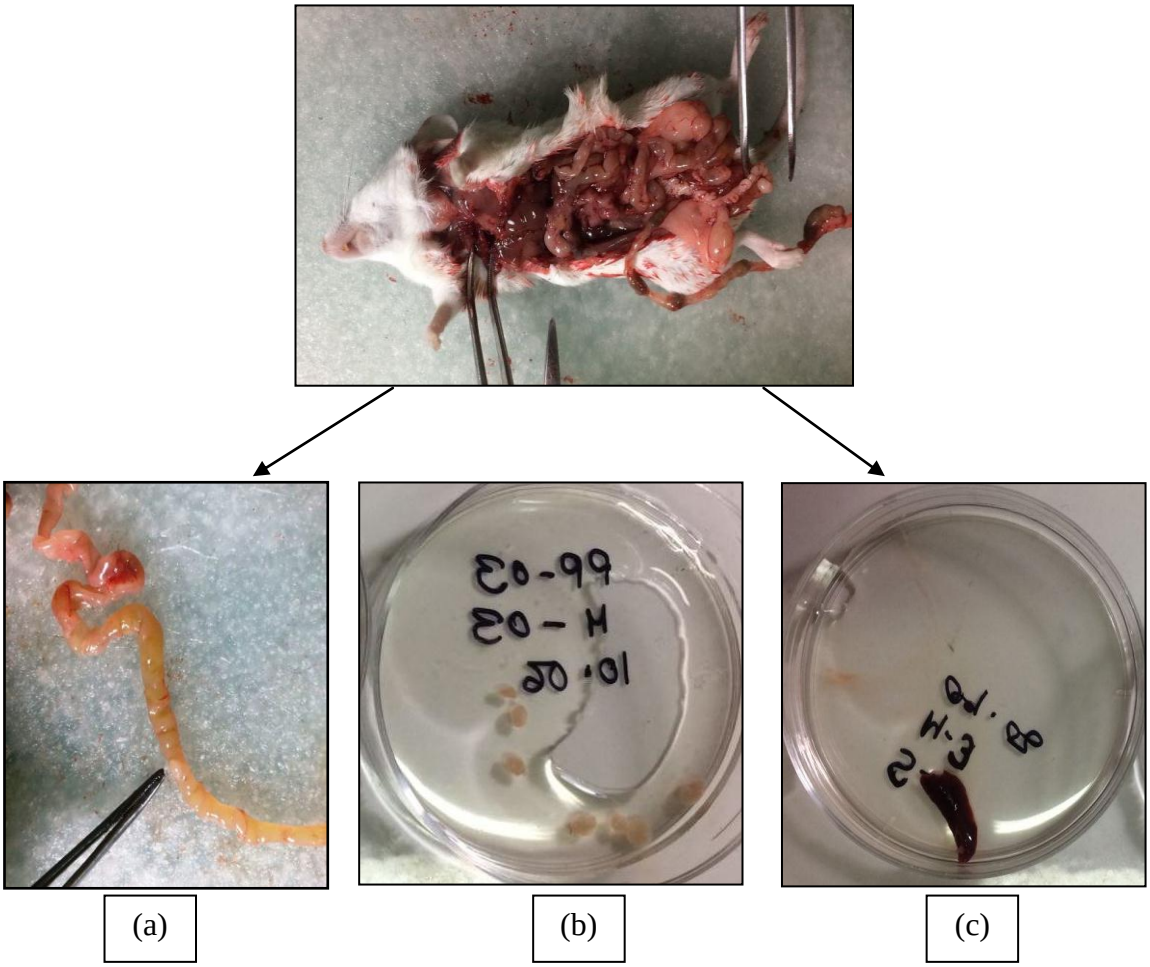

Figure A-1: Dissection of BALB/c mice. (a) Small intestine showing whitish bulging PPs (b) Petri dish containing seven (07) PPs (c) Petri dish containing spleen

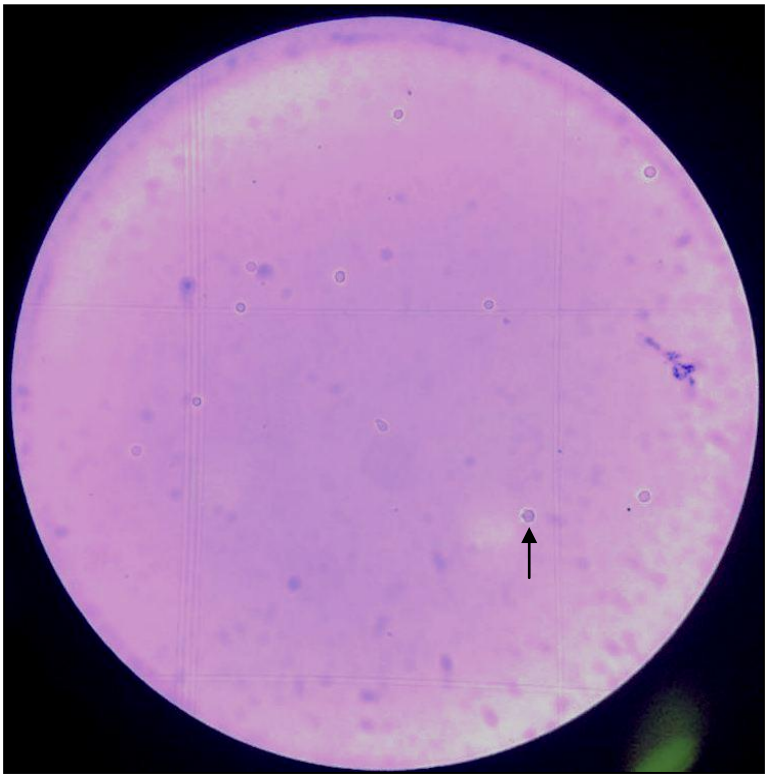

Figure A-2: Microscopic view of viable lymphocyte in Neubauer counting chamber

Appendix V

Consent Forms

Consent form for specimen (blood) and data collection for research purpose

Study title: Systemic and local immune responses to *Salmonella enterica* serovar Typhi by prime boost strategy with different preparations of Salmonella antigen (s).

Investigator: Lovely Barai, Department of Microbiology, University of Dhaka

Purpose: Typhoid fever is a public health problem in Bangladesh. It is an important communicable disease especially in school going children. Global estimates of disease burden between 11-21 million annually and Bangladesh, Pakistan and India accounts for 85% of the global typhoid cases. The two existing licensed vaccine for the typhoid fever (parenteral Vi CPS and oral Ty21a) are modestly effective (3 year cumulative efficacy 51-55%). Vaccine confers protection only for 3-5 year and non immunogenic in children below 2 yrs of age. So conventional *S.*Typhi vaccination has a limited impact on the typhoid control and we are in dire need of new vaccine or more effective strategies. So for improvement of typhoid vaccine immunogenicity we will apply prime boost strategy in mice and observe immune response against different component of S. Typhi in typhoid cases. We also find out most immunogenic antigen of S. Typhi which can help in diagnosis and vaccine development.

Risks and Discomforts: Donating 3-5 ml blood for research may have little pain. Researchers believe the chance of this risk is very small and protections are in place to lessen this risk. All personal medical information about donor and any information obtained from this study will be preserved in a secured database. Donor's name and identity will be used only for data collection and will not be disclosed to a third party. All reasonable efforts will be made to protect the confidentiality of information that can in any way be connected to donor.

Benefits: Taking part in this research will not benefit to donor directly, however what we learn may help others in the future.

Alternatives: Donor may choose not to take part in this study.

Costs: Donor will bear his/her own treatment expenditure. There will be no cost to donor for any procedures required for the research. Donor will not paid or given any other award for taking part.

Property Donation: By agreeing to take part you allow the use of your samples for the research described in the purpose section of this document. In addition, you agree that we may make any lawful use of your samples, including future research studies.

Confidentiality: Every effort will be made to keep donor's information records private. All others, including employers, insurance companies, personal physicians and relatives will be refused access to the information and to the samples, unless you provide written permission or unless we are required by law to do so. Anything that can identify will be kept in private, protected files. An ID number will be assigned to you, samples and information about your medical history. Only the investigator named on this consent form will be authorized to link the ID number to your name. The link of your ID number or any other identifying data will be stored in the established secure database. Any future research done on any of the samples must be designed in a way that protects your privacy and presents research results and data anonymously. The Ethical Review Committee must also monitor it.

Conflict of interest: The priniciple investigator and other possible research team members have no conflict of interest to declare.

Participation: Lovely Barai, cell phone: xxxxxxxxxxx has offered to answer any other questions of donors about this study. If donor has any questions regarding his/her rights as a research subject, he/she may contact us. If in the future donor decide no longer to take part in this study, we will destroy all identifying information and will not use your tissue in any future research. Donor's signature below indicates that donor has read this consent form and agrees to take part in this study.

Consent: By signing this form, I agree that:

1. You have explained this study to me. You have answered all my questions.
2. You have explained the possible harms and benefits (if any) of this study.
3. I know what I could do instead of taking part in this study. I understand that I have the right to refuse to take part in the study. My decision about taking part in the study will not affect my health care.
4. I am free now and in the future, to ask questions about the study.
5. I have been told that my medical records will be private except as described to me.
6. I understand that no information about me will be given to anyone or be published without first asking my permission.
7. I hereby knowingly and voluntarily authorized you to use my blood sample and my protected health information in the manner described in this consent form.
8. I agree or consent, that I may take part in this study.

……. …….

Date Subject's Signature

Witness-1

……. …….

Date Signature

Witness-2

……. …….

Date Signature

Appendix VI

Proforma for Collection of Data of Patient diagnosed as Typhoid fever

Date:……

1. Particulars of patient:

1.1 Name: 1.2 Age: 1.3 Sex:

1.4 Sample no.: 1.5 OPD/IPD: 1.6 Ward/Bed:

1.7 Education 1.8 Occupation: 1.9 Date of admission:

1.91 Contact No.: 1.92 Address:

2. Symptoms:

2.1 Fever : Y/ N 2.2 If Y: Duration ☐☐ days 2.3Temp : high/ low grade

2.4 Chills : Y/ N 2.5 Bowel movement: Constipation/ Loose stool

2.6 Headache: Y/ 2.7 Vomiting: Y/ N 2.8 Abdominal Pain: Y/ N

3. Signs:

3.1 Pulse: Bradycardia/Normal/Tachycardia

3.2 Hepatomegaly: Y/ N 3.3 Splenomegaly: Y/N

4. Associated conditions/Co morbidity

4.1 DM: Y/N 4.2 If Y Duration ☐☐ d/m/y 4.3 IDDM/ NIDDM

5. Treatment givem: Before admission Recent treatment

6. Previous Typhoid history:

7. Typhoid Vaccine: Y/ N

7.1 Type of Vaccine: 7.2 Total dose given: 7.3 Date of vaccination

Injectable (TyphimVi) ☐ 7.2.1 Injectable :1dose/…… 7.3.1 Injectable……

Oral (Live Ty21a) ☐ 7.2.2 Oral: 4 / 3/ 2/ 1dose 7.3.2 Oral: (1,3,5,7th day)

1st dose……

2nd dose…..……

3rd dose……..…..

4th dose……..…

8. Laboratory Investigation:

8.1 Total count WBC: /cmm DC: N- %, L- %, M- %, E- %

8.2 Platelet count: 8.3 Bilirubin level: mg/dl 8.4 ALT: IU/L

8.5 Widal test titre: TO TH AO AH BO BH

8.6 Blood Culture: Date of Sample collection for Blood culture

Culture Report: Culture positive/ negative

	Observation/ Growth of	Colony count (cfu/ml)

Antibiogram

Ampicillin	Ceftriaxone
Cotrimoxazole	Cefixime
Chloramphenical	Azythromycin
Ciprofloxacine	Cefotaxime
Nalidixic acid	

- ❖ S-Sensitive, R-Resistant

- ✓ MDR Salmonella: Y / N
- ✓ NARST: Y / N
- ✓ Date of Organism stock in -20ºC:

9.1 Date of 1st Blood collection :

9.2 Date of 2nd Blood collecton: