Comparison of Lipopolysaccharide (LPS) Specific IgA and IgG Avidity Maturation between Vivotif Vaccinees and Naturally Infected Enteric Patients in Bangladesh.

A DISSERTATION SUBMITTED TO THE BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

Submitted by
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To Whom It May Concern

This is to certify that the research work embodying the results reported in this thesis, entitled **"Comparison of Lipopolysaccharide specific IgA and IgG avidity maturation between Vivotif vaccinees and naturally infected enteric patients",** submitted by **Faisal Bin Rashed**, has been carried out by the under signed co-supervision in the Immunology Laboratory of the Centre for Vaccine Sciences at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka.

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Peace.

Author
August 31, 2014.

Abstract

Enteric fever, primarily caused by *Salmonella enterica* serovar Typhi and Paratyphi, is a potentially life-threatening systemic disease. Characterized by fever, malaise and diffused abdominal pain, it can lead to delirium, intestinal hemorrhage, bowel perforation, and even death if left untreated. High prevalence rates of enteric fever have been reported in the resource limited regions of the world, with children under 5 years of age being the largest target. Poor sensitivity and specificity of conventional diagnostic approaches result in lower or late detection of the infection. The TPTest, a novel diagnostic technique, has proved to be highly sensitive and specific for detection of the patients with enteric fever. Vaccination also is potentially important to reduce the incidence of enteric fever among young children. Vivotif, a live oral attenuated typhoid vaccine, has undergone clinical trials in different countries, including Bangladesh. However, no data on the analysis of the immune response or longevity has been carried out. The antibody avidity maturation in typhoid vaccinees or patients has also not been reported till date. Under these circumstances, this study demonstrates the lipopolysaccharide (LPS) specific immunoglobulin A (IgA) and immunoglobulin G (IgG) avidity maturation in Vivotif vaccinees and naturally infected typhoid patients aged below 5 years. Also, the age dependent variation in avidity maturation was analyzed, both in *S.* Typhi bacteremic and only TPTest positive patients. Vaccination with Vivotif generated IgA and IgG antibodies with significantly higher avidity that lasted throughout the study period of 28 days. Patients with *S.* Typhi bacteremia with an age below 5 years showed a higher IgA avidity index in the first two study day points, followed by a decrease on the second follow-up. Overall, culture positive patients had a higher IgA avidity index compared to the only TPTest positive patients, regardless of age. A similar response for IgG avidity maturation was seen among patients. Lack of significant differences in IgG avidity between different day points within a group could be attributed to the longer half-life of IgG. In short, *S.* Typhi bacteremic patients produced more highly avid IgA and IgG antibodies against LPS compared to only TPTest positive patients, indicating that higher bacterial load in the system may aid in generation of higher avidity antibodies.

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Dedicated to

My Family

CHAPTER 1

Introduction

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1.1 Typhoid fever: General Overview

Typhoid and Paratyphoid fever, collectively known as enteric fever, is a potentially fatal multi-systemic illness caused primarily by *Salmonella enterica*, subspecies *enterica* serovar Typhi (*S.* Typhi) and, to a lesser extent, related serovars Paratyphi A, B, and C.

S. Typhi has been a major human pathogen for thousands of years, thriving in conditions of poor sanitation, crowding, and social chaos. It may have been responsible for the Great Plague of Athens at the end of the Pelopennesian War (Papagrigorakis, Synodinos et al. 2007). The name *S.* Typhi is derived from the ancient Greek word "typhos", an ethereal smoke or cloud that was believed to cause disease and madness. The name echoes with the advanced stages of enteric fever, when the patient's level of consciousness is truly clouded (Christie 1974).

Although antibiotics have markedly reduced the frequency of typhoid fever in the developed world, it still eludes the understandings of the scientific community, and remains a major cause of morbidity and mortality in the developing countries (Crump and Mintz 2010). Since it follows a fecal-oral mode of transmission, the problem is more severe in regions with poor sewage system, and lack of safe water supply, food safety, maintenance of personal hygiene etc. As such, the largest burden of typhoid fever is borne by impoverished individuals in resource-limited areas (Parry, Hien et al. 2002). Typhoid is clinically difficult to differentiate from many other widespread illness associated with fever, including dengue, malaria, and leptospirosis- a feature that aids in lower or late detection of typhoid infection.

The major causative agent of typhoid fever, serovar Typhi, is a human-restricted invasive enteric pathogen- a fact that has greatly limited studies of *S.* Typhi pathogenesis (Santander and Curtiss III 2010). After ingestion, the bacterium crosses the intestinal mucosa, is taken up by gut associated lympho-reticular tissues, and then enters the systemic circulation. Consequently, it stimulates both mucosal and systemic host immune responses. Serovar Typhi is an intracellular pathogen, and antibody and cell-mediated immune responses occur after infection or immunization with live oral attenuated typhoid vaccines (Sarma, Malaviya et al. 1977, Forrest 1992, McGhee and Kiyono 1993).

1.2 Typhoid fever: Epidemiology

1.2.1 Enteric fever epidemiology: Global Perspective

The World Health Organization (WHO) identifies enteric fever (typhoid and paratyphoid fever) as a serious public health problem. It affects over 22,000,000 individuals globally each year, with an annual fatality rate of about 200,000 (Crump, Luby et al. 2004).

The south-central and south-east Asian countries have some of the highest incidence of enteric fever (>100/100,000 cases/year) whereas regions of medium incidence (10-100/100,000 cases/year) include the rest of Asia, Africa, Latin America, Caribbean and the Oceania, except for Australia and New Zealand. A noticeably lower degree of incidence is reported in Europe, North America and the rest of the developed world (with a rate of <10/100,000 cases/year) (Crump, Luby et al. 2004).

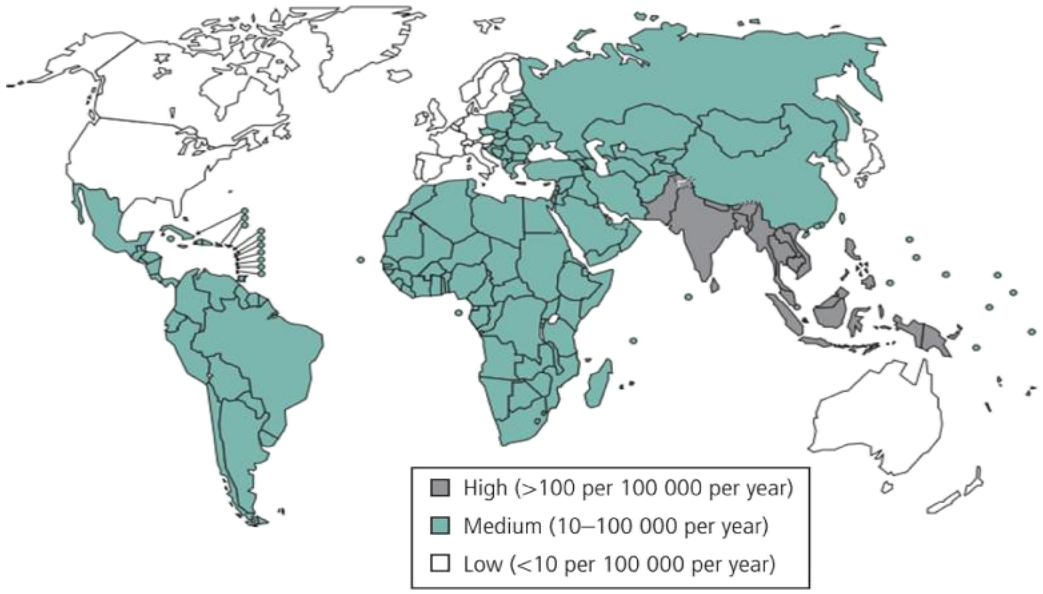

Figure 1.1: Global incidence of enteric fever (Crump, Luby et al. 2004).

Infants, children, and adolescents constitute the majority of the patients (Sinha, Sazawal et al. 1999) . Peak incidence of typhoid fever is reported in children aged between 1 to 15 years, with children below 5 years of age having the highest infection rates (Sinha,

Sazawal et al. 1999, Crump, Luby et al. 2004). Paratyphoid fever has a similar clinical feature of typhoid fever, and it is estimated that there is 0.25 paratyphoid fever illness for every typhoid fever illness (Crump, Luby et al. 2004).

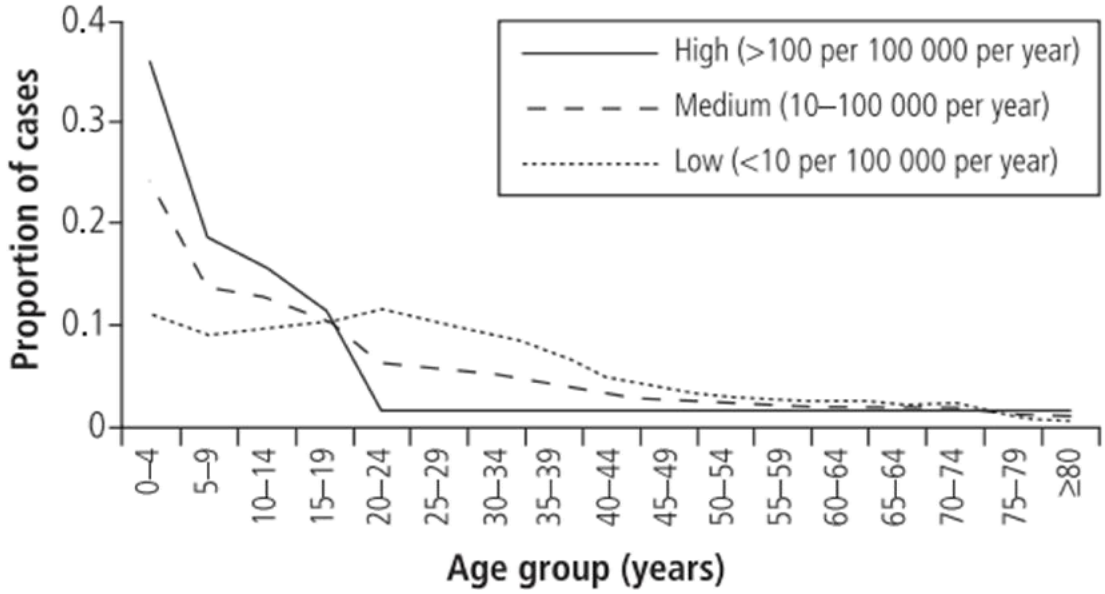

Figure 1.2: Distribution of typhoid fever by age (Crump, Luby et al. 2004).

1.2.2 Enteric fever epidemiology: Bangladesh Perspective

Bangladesh, along with China, India, Indonesia, Laos, Nepal, Pakistan and Vietnam, account for more than 80% cases of global typhoid occurrences (Chau, Campbell et al. 2007). According to a fever surveillance study in an urban area in Kamalapur in Dhaka, Bangladesh, the incidence of *S.* Typhi bacteremia for all age groups was 390 episodes/100,000 person-years (Brooks, Hossain et al. 2005). The incidence of *S.* Typhi bacteremia among individuals above 5 years of age was 210 episodes/100,000 person-years, and among children below 5 years of age, 1870 episodes/100,000 person-years. These findings indicate that children below 5 years of age had about a 9 -fold increased risk of infection when compared to other age groups (95% CI: 4.9–16.4) (Brooks, Hossain et al. 2005). Monsoon apparently acts as a trigger, and these months have the highest disease occurrences in a year (Dewan, Corner et al.).

Historical mortality rates of enteric fever are in excess of 15%, but outcomes have improved with the use of appropriate antibiotics and supportive care (Hoffman, Punjabi et al. 1984, Bitar and Tarpley 1985). Within the country, management of patients with enteric fever is confounded by the non-specific clinical presentation, inadequate current diagnostic tests, and widespread antibiotic resistance (Khanam, Sheikh et al. 2013).

1.3 Classification, Structure and Antigenic type

1.3.1 Classification

The primary causative agent of enteric fever is the bacterium *Salmonella enterica* serovar Typhi. It is a member of the genus *Salmonella* in the family of Enterobacteriaceae. From evolutionary perspective, the genus is estimated to have diverged from *Escherichia coli* approximately 100–150 million years ago (Dougan, John et al.). It contains two species: *enterica* and *bongori.* There are six subspecies of *Salmonella enterica,* namely: *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* containing 2443 serovars (Parry 2005). Among them *Salmonella enterica* subspecies *enterica* is the most pathogenic one. Within the subspecies, serovar Typhi and serovars Paratyphi A, B and C are responsible for enteric typhoid and paratyphoid respectively (Parry, Wijedoru et al.).

1.3.2 Structure

Salmonella enterica serovar Typhi is a gram-negative, flagellate, encapsulated, non-sporulating, facultative anaerobic bacterium. The organism is rod shaped (bacilli), 2-3 μm long, and 0.4-0.6 μm in diameter. The inner and outer membranes of *Salmonella* are separated by the murein cell wall layer, and flagella are attached at basal bodies. Fimbriae are thinner and shorter than flagella, and are displayed more densely around the cell (Liu 2007).

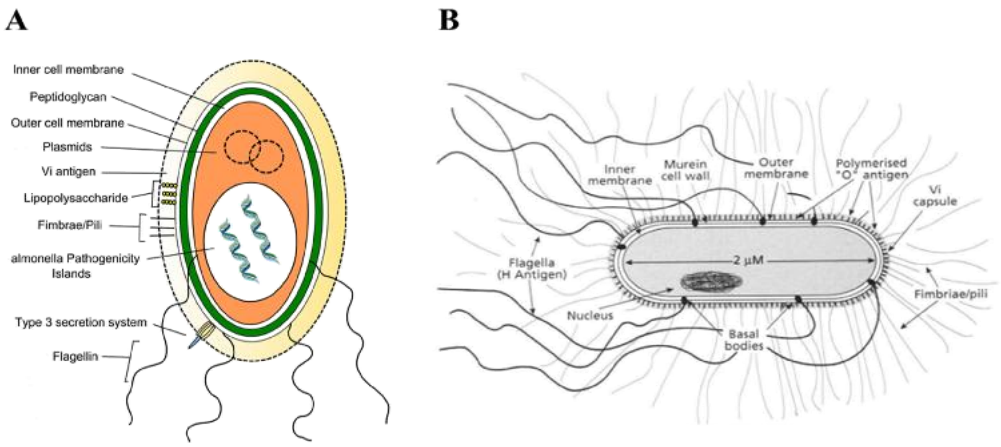

Figure 1.3: Physical structure of (A) *Salmonella enterica* serovar Typhi (de Jong, Parry et al. 2012) and (B) *Salmonella enterica* serovar Paratyphi (Kopecko 2001)

1.3.3 Genomic structure

Salmonella enterica serovar Typhi strain Ty2 is a human-specific pathogen causing typhoid fever. It is the parent of mutant strains Ty21a and CVD908 and their derivatives, used in trials of live attenuated vaccines (Deng, Liou et al. 2003). The size of the single chromosome is approximately 4,744,056 bp, with a G+C content of 53.21% and a coding percentage of 87.1. There were 4,875 protein coding sequences found, with an average length of 875 bp. The genome revealed 76 tRNA and 22 rRNA genes (Baddam, Kumar et al.) (Baker and Dougan 2007).

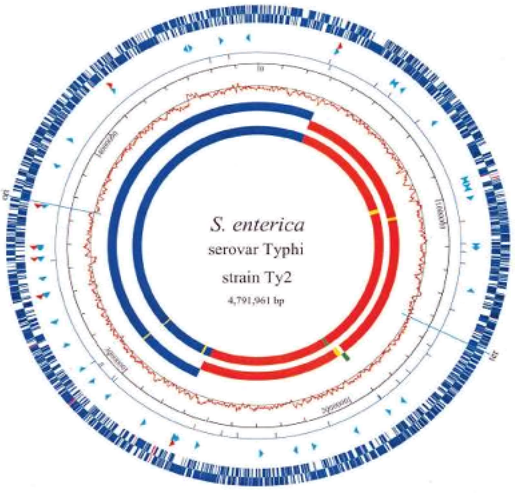

Figure 1.4: Genome of *Salmonella enterica* serovar Typhi (Deng, Liou et al. 2003)

1.3.4 Antigenic structure

1.3.4.1 O (Somatic) antigens

Somatic or cell wall antigens, also known as O antigens, are bacterial endotoxins and responsible for causing fever. In addition to 3-deoxy-D-manno-octulosonic acid and lipid A, a core contains L-glycero-D-manno-heptose, D-glucose, D-galactose, *N*-acetylglucosamine and ethanolamine pyrophosphate. From this core, a poly-O side-chain extends to the bacterial surface. The poly-O side-chain is made of repeated monomers containing D-galactose, L-rhamnose, D-mannose, and branched in position 1–3 on D-mannose (Wray 2000, Hoare, Bittner et al. 2006). It has a significant effect on the interaction between a bacterial pathogen and the host organism. Antibodies directed against O antigen are vital to the immune response to infection (Slauch, Mahan et al. 1995).

1.3.4.2 H (Flagellar) antigens

The H antigens are present in flagella and composed of protein subunits called flagellin (Collee 2006). This antigen does not have any significant effect on the virulence of *S.* Typhi. Flagella of *S.* Typhi induce different pro-inflammatory cytokines (Wyant, Tanner et al. 1999). They are heat and alcohol labile, but are well preserved in 0.2 % formaldehyde. Mixing *Salmonella* cells with flagella-specific antisera gives a characteristic pattern of agglutination (Thong 2002).

1.3.4.3 Vi (Virulence) antigen

The most significant and well known surface antigen is the Vi antigen (virulence antigen). Vi antigen is only present in outer membrane of *Salmonella enteric* serover typhi (Wain, House et al. 2005). The Vi antigen is a highly acidic polysaccharide and composed of O- and N-acetylated galactosaminouronic acid units connected through a (1-> 4) linkage. There are at least two antibody types directed against different parts of the Vi antigen molecule (Szu, Li et al. 1991).

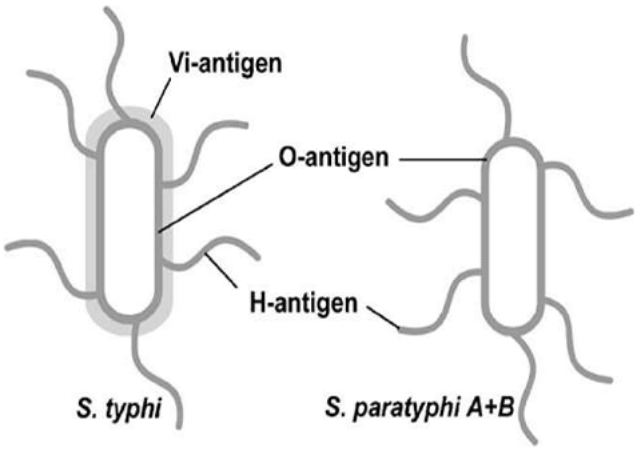

Figure 1.5: Antigenic structure of *S.* Typhi and *S.* Paratyphi (Guzman, Borsutzky et al. 2006)

1.4 Typhoid fever: Pathophysiology

Since *S.* Typhi and *S.* Paratyphi A are human-restricted pathogens, chronic carriers may act as reservoirs of infection within a community, and thus can contribute to the transmission cycle through sporadic shedding of bacteria in feces (especially in areas of low transmission) (Lynch, Blanton et al. 2009). The organisms sequester themselves either as a biofilm on gallstones or gallbladder epithelium or, perhaps, intracellularly, within the epithelium itself (Gonzalez-Escobedo and Gunn 2013). The bacteria excreted by a single carrier may have multiple genotypes, making it difficult to trace an outbreak to its origin (Chiou, Wei et al. 2013).

Typhoidal *Salmonella* have no nonhuman vectors. An inoculum as small as 100,000 organisms of Typhi causes infection in more than 50% of healthy volunteers (Levine, Tacket et al. 2001). Paratyphi requires a much higher inoculum to infect, and is less endemic in rural areas.

Following ingestion by contaminated food or water, *Salmonella enterica* serover Typhi and Paratyphi enters the small intestine via the M cells of the payer's patch. Subsequently, the pathogen migrates into the mesenteric lymph nodes where multiplication occurs, leading to the release of bacteria into the bloodstream. Afterwards,

the bacterial load is removed from blood by macrophages which are present in the sinusoids of the liver, spleen and bone marrow (Mastroeni and Sheppard 2004). If removal fails, *S.* Typhi infection of the gall bladder can lead to reinfection of the intestinal tract, causing inflammation, ulceration and necrosis (Crawford, Rosales-Reyes et al. , C Colomba 2004). Haemorrhage from ulcers can occur during the third week of illness. Perforation of the payer's patch can cause generalized peritonitis and septicaemia, the commonest cause of death in enteric fever (Ezzat, Hussein et al.)

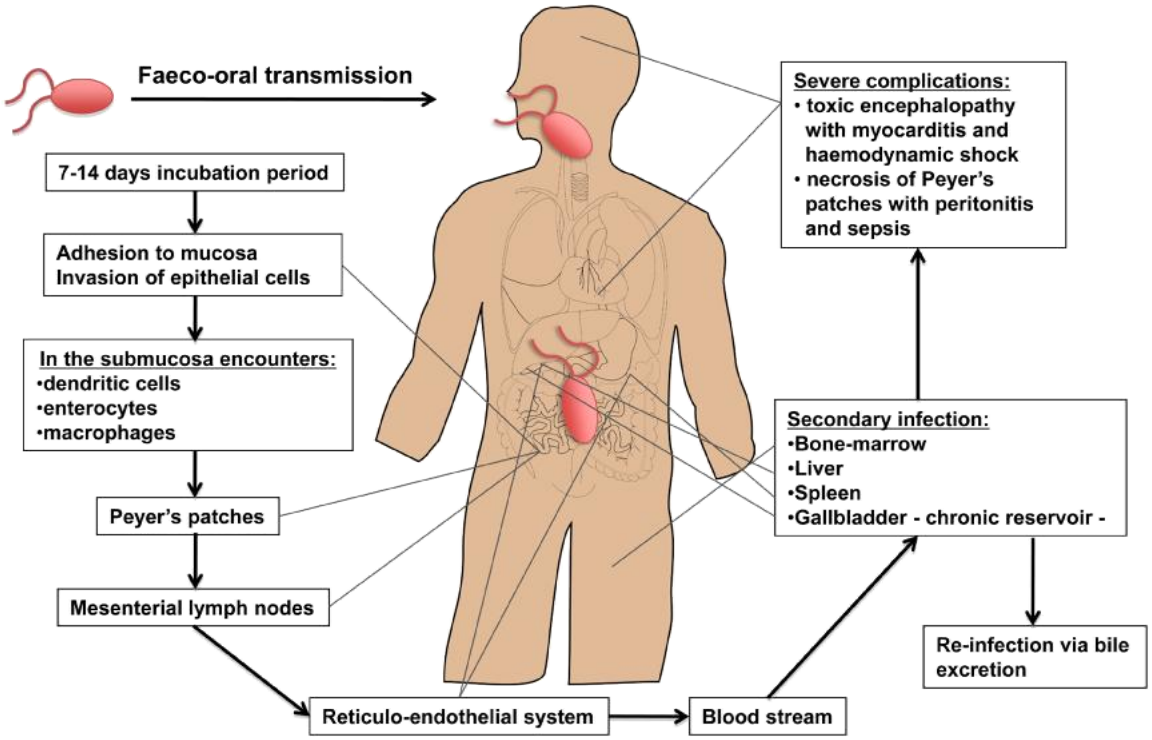

Figure 1.6: Pathophyiology of *Salmonella enterica* serovar Typhi (de Jong, Parry et al. 2012)

It has been observed that, *in vitro*, *Salmonella* are able to disrupt tight junctions, which seal the epithelial cell layer and restrict the passage of ions, water and immune cells (Jepson, Collares-Buzato et al. 1995). This, in addition to intestinal inflammatory responses, is likely to contribute in the induction of diarrhea (Haraga, Ohlson et al. 2008).

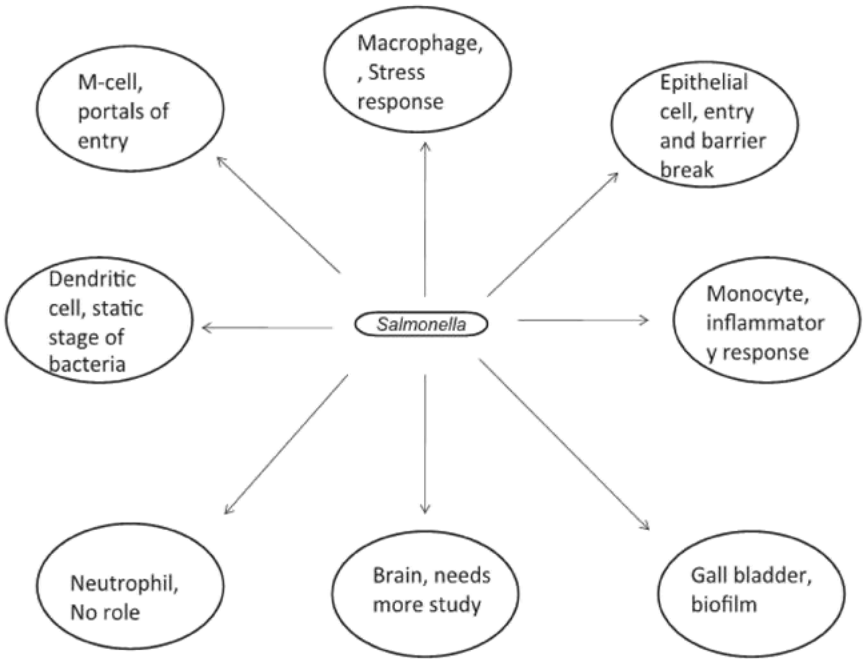

Figure 1.7: *Salmonella* infection in different cells and organs (Lahiri, Iyer et al.)

1.5 Host defense system

Salmonella enterica serovar Typhi is a facultative intracellular bacterium characterized by mucosal invasion and systemic spreading (Takaya, Tomoyasu et al. 2002) (Carter and Collins 1974). Accordingly, protection against *Salmonella enterica* serovar Typhi is mediated by mucosal and systemic immune responses, as well as cell-mediated immunity (CMI) (Capozzo, Cuberos et al. 2004) (Sheela, Babu et al. 2003).

1.5.1 Mucosal immune response to *Salmonella*

The gut microflora is able to passively restrict *Salmonella* growth by creating a nutrient-depleted environment, through releasing their metabolic by-products such as propionate or butyrate, or by production of bacteriocins (Rychlik and Barrow 2005). The epithelial cell layer also helps to maintain barrier by secreting mucus (goblet cells) or antimicrobial peptides (Paneth cells) (Nochi and Kiyono 2006). Underlying this epithelial cell layer is the lamina propria, containing a highly organized lymphoid tissue commonly referred to as gut-associated lymphoid tissue (GALT). GALT comprised of T cells and B cells,

dendritic cells, macrophages and neutrophils (Rychlik and Barrow 2005), which regulate inflammatory responses to bacteria and antigens that break the gastrointestinal barrier. Aggregated lymphoid follicles (Peyer's patches) are surrounded by a particular epithelium, the follicle-associated epithelium, which forms the interface between the GALT and the luminal microenvironment. The Peyer's patches have an important role in the immune surveillance of the intestinal lumen (Monack, Mueller et al. 2004).

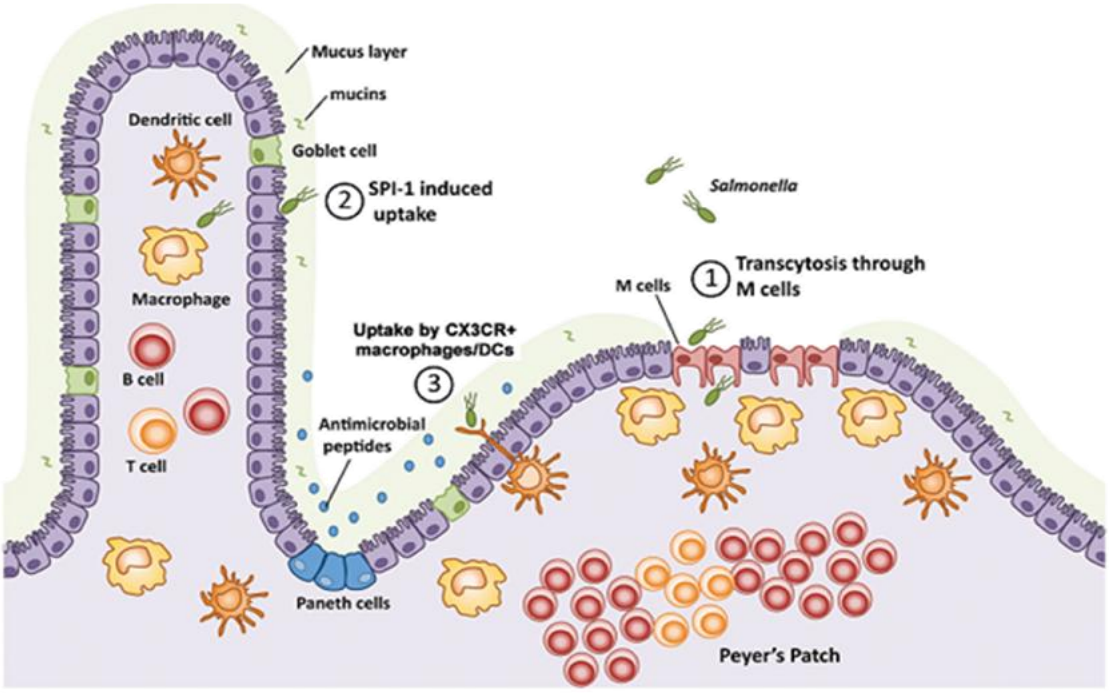

Figure 1.8: Mucosal immune response to *Salmonella enterica* serovar Typhi (Broz, Ohlson et al.)

1.5.2 Cell mediated immune response to *Salmonella*

Cell-mediated immunity plays a major role in controlling *Salmonella* infection (George, Nair et al. 1987, Hsu 1989). *Salmonella* infection induces the generation of specific CD41 and CD81 T cells, and both T cell populations are important for protection during primary and secondary responses, although the mechanisms are not yet completely understood. CD4+ and CD8+ T cells response is elicited after *Salmonella enterica*

serovar Typhi infection (Sztein 2007) (Salerno-Goncalves, Fernandez-Vina et al. 2004). During the initial period, an increased production of interleukin (IL)-2 and interferon (IFN) - γ producing cells in the spleen and Peyer's patches is observed, indicating a Th1 type response. However in the later period of infection, increased production of IL-4 producing cells is seen which suggests a Th2 type response (Sood, Rishi et al. 2005).

1.5.3 Circulating antibody response to *Salmonella*

Antibody responses to a diverse set of antigens of *Salmonella enterica* serovar Typhi and Paratyphi, including LPS, Vi polysaccharide, porins, outer membrane proteins, lipoproteins, heat shock proteins, flagella, and fimbriae, have been reported (Brown and Hormaeche 1989) (Harrison, Villarreal-Ramos et al. 1997) (Matsui and Arai 1989). IgG and IgM antibody response to H-antigen, O-antigen and Vi-antigen is significant between convalescent and acute stages of disease (Calderon, Lobos et al. 1986). During acute infection, anti-O antibodies appear first, rise progressively and then disappear within six months (Ushiba 1965). Anti-H antibodies appear later on, with a longer persistence period. Vi-antigen specific antibodies do not appear early in the disease process (House, Ho et al. 2008). Interestingly, all individuals do not mount antibody responses against all three antigens.

IgM antibody responses to anti-O: 9 antigen of *Salmonella enteric* serovar Typhi is found in plasma (Tam and Lim 2003). Specific IgA antibody response for O: 9 antigen alone can prevent initial attachment (Iankov, Petrov et al. 2004). The O9 antigen (or LPS in general) is thymus-independent type I, immunogenic in infants, and a potent B cell mitogen. It can stimulate B cells without the help of T cells (unlike protein antigens) and, consequently, anti-O9 responses are rapid (WHO 2003),

Human IgM and IgG antibodies against specific *S.* Typhi 50 kD outer membrane protein antigen in typhoid patients is observed due to either recent or current infection (Ismail, Kader et al. 1991). IgM reveals acute typhoid in the early phase of infection (Ismail 2000). IgG can persist for more than two years after typhoid infection (Choo, Davis et al. 1999).

Secretory immunoglobulin A (sIgA) represents a first line of defense against mucosal pathogens by limiting their entrance (Corthesy 2009). There is significant elevation of serum IgM and IgA levels in patients with acute typhoid fever. IgG response occur relatively later (Dham and Thompson 1982), (Kumar, Malaviya et al. 1974),(Dragomirescu, Busila et al. 1977), when it reaches in the mesenteric lymph node and the spleen. IgA antibody producing lymphocytes leak to intestinal barrier and migrate to peripheral region. These peripheral blood mononuclear cells (PBMC) produce antibody in peripheral region after 1 to 2 weeks of intestinal infection (Sheikh, Bhuiyan et al. 2009),(Charles, Sheikh et al.).

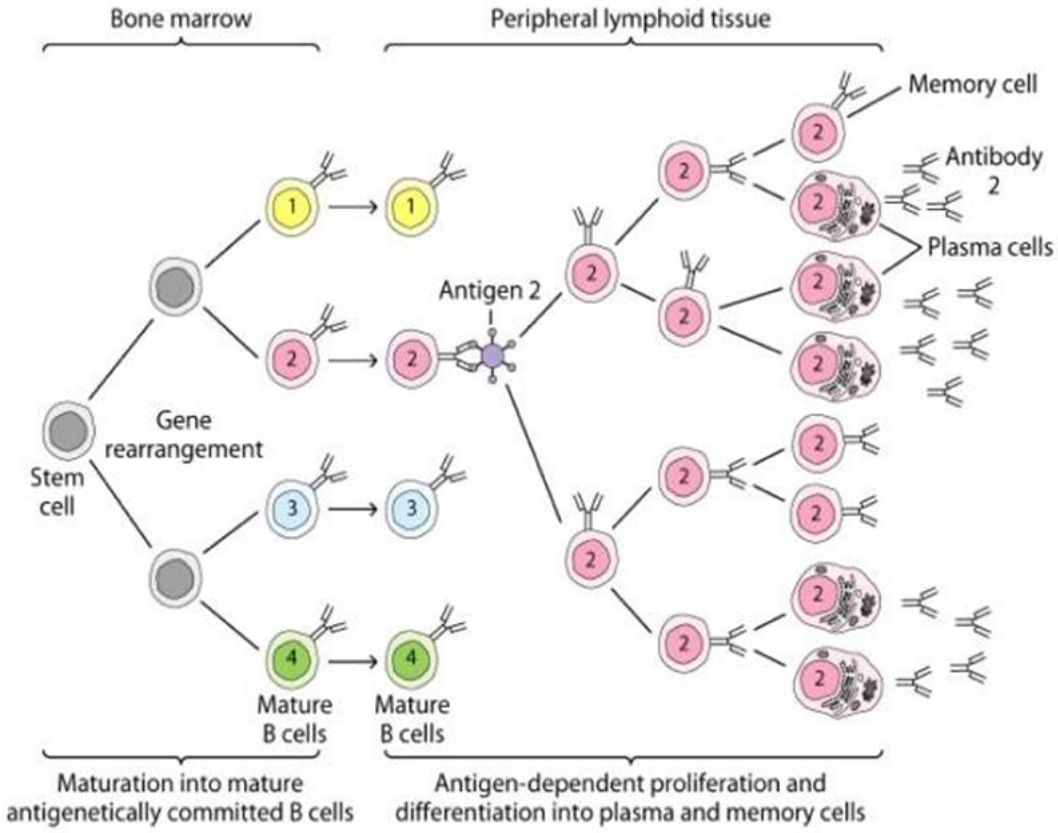

Figure 1.9: Clonal selection, expansion, proliferation and differentiation of B-cells (Kindt, Goldsby et al. 2007)

1.6 Clinical feature of Enteric fever

The clinical manifestation of typhoid fever is highly variable; ranging from fever to a severe systemic illness and associated complications involving many systems The fever might rise progressively in a stepwise manner, with 5–7 days of daily increments in maximal temperature of 0.5–1° C, to become persistent and high grade (39–41° C) by the 2nd week of illness. Continuous high-grade fever can continue for up to 4 weeks if left untreated, followed by a return to normal temperature (Leung, Bogetz et al. , Naheed, Ram et al.) . Recent study suggests the pattern of morbidity and mortality associated with typhoid fever is dependent on age and gender (Butler, Islam et al. 1991). Other clinical manifestations (Brooks, Hossain et al. 2005, Maskey, Day et al. 2006, Zimmerman, Murdoch et al. 2008) include:

- Flu-like symptoms such as- fever, headache, chills, cough, myalgia, arthralgia
- Abdominal symptoms such as- anorexia, abdominal pain, diarrhea, constipation
- Physical findings such as- coated tongue, hepatomegaly, splenomegaly, abdominal tenderness, rash, generalized adenopathy

1.7 Complications of Enteric fever

- Ileal perforation occurs if treatment is delayed (Adesunkanmi and Ajao 1997)
- *Salmonella enterica* serovar Typhi splenic abscesses are traditionally considered to be a rare complication of typhoid fever but sometimes it is observed in case of untreated patients (Calleri, Gaiottino et al. 1991, Allal, Kastler et al. 1993)
- During pregnancy, hepatic dysfunction may also be observed. (Hasbun, Osorio et al. 2006)
- Typhoid fever may pave the way for opportunistic infection.
- Gallbladder perforation is rare, but sometimes patients suffer from gallbladder perforation associated complications. (Gali, Ali et al.)
- Encephalitis
- Neuropsychiatric symptoms

- Patients may suffer from small bowel perforation in secondary infection (Dunne, Wilson et al.)

1.8 Diagnosis of Enteric fever

Diagnostic tests for typhoid fever often lack sensitivity and/or specificity, especially in the endemic regions, where clinically distinguishing typhoid fever from other febrile illnesses is difficult (Sheikh, Bhuiyan et al. 2009). Nevertheless, several diagnostic approaches with varied degree of sensitivity and specificity are available for the detection of enteric fever.

1.8.1 Diagnosis of typhoid by nested Polymerase Chain Reaction (PCR)

Diagnosis of typhoid fever is possible by Polymerase Chain Reaction (PCR) using blood and stool as sole source of *S.* Typhi template DNA (Kumar, Arora et al. 2002), since flagellin gene of *S.* Typhi can be detected by PCR. To reduce frequent contamination associated problems in conventional PCR, a nested PCR involving two sets of primers is advisable for the diagnosis. Although PCR as a diagnostic tool is promising, issues of practicality, contamination, and quality control have limited their use in many resource-poor areas of the world (Hatta and Smits 2007).

1.8.2 Bone marrow culture

Isolation of *Salmonella enterica* serovar Typhi and Paratyphi from bone marrow culture is the gold standard of diagnosis of an infection, but is undesirable due to its invasive nature (Karim, Khan et al. 1991) (Vallenas, Hernandez et al. 1985). Technical expertise and well equipped laboratory is essential in this regard. That is why blood culture is used as gold standard.

1.8.3 Blood culture

Isolation of *Salmonella enterica* serovar Typhi and Paratyphi from blood is a reliable means of confirming an infection than the previous two. However, isolation of the organism from blood requires 5 to 10 mL of blood, 2 to 7 days' time, elaborate laboratory

equipment, and a level of technical expertise, which may not be present in resource-poor laboratories (Wain, Diep et al. 1998) (Wain, Pham et al. 2001). Microbiologic culturing of blood is approximately 30% to 70% sensitive even under the best of conditions. Moreover, blood culture cannot isolate pathogen in case empirical (without knowing causative microbes) treatment of antibiotics (Akoh 1991).

1.8.4 Widal test

Widal test is used for enteric fever diagnosis due to its easy nature (Pastoor, Hatta et al. 2008). It is an immunoagglutination method that detects anti-O and anti-H antibody response in blood of enteric fever patient. Widal is a rapid and easy method, but is flawed with frequent false-positive results (Ohanu, Mbah et al. 2003) (Olopoenia and King 2000) (Parry CM 2002). False-negative reactions also may occur in confirmed typhoid cases with no detectable antibody response.

1.8.5 Tubex

Tubex is semi quantitative colorimetric test detects anti-O:9 antibody (IgM antibody) titres in patient specimens (Oracz, Feleszko et al. 2003). A positive Tubex result was defined as a reading of ≥ 4, as per manufacturer's instructions. Limitations include the potential difficulty in interpreting the results of hemolyzed samples (Olsen, Pruckler et al. 2004). Moreover, it may show a false positive in persons with recent *Salmonella enterica* serotype Enteritidis infection, resulting in inappropriate antibiotic treatment.

1.8.6 Typhidot

Typhidot is a dot-blot Enzyme Immunoassay (EIA) procedure which is based on the presence of specific IgM and IgG antibodies to a specific 50 kDa outer membrane protein (OMP) antigen on *S.* Typhi. Typhidot is a qualitative antibody detecting method (House, Wain et al. 2001). False-positive results attributable to previous infection may occur, since IgG can persist for more than 2 years after typhoid infection. False-negative results may occur in cases of reinfection, if the significant boosting of IgG masks the detection of IgM (Choo, Davis et al. 1999) (Jesudason, Esther et al. 2002).

1.8.7 TPTest

IgA is the major antibody response in mucosal immunity. Since serovar Typhi interacts with both the mucosal and the systemic immune systems, activated mucosal lymphocytes migrate from intestinal tissue and circulate within peripheral blood. This migration peaks 1 to 2 weeks after intestinal infection and may be measured by using peripheral blood mononuclear cells (PBMC) in an antibody in supernatant (ALS) assay (Sheikh, Bhuiyan et al. 2009) (Qadri, Ryan et al. 2003).

Studies conducted in Bangladesh have shown that it is possible to detect IgA antibodies targeting *Salmonella enterica* serotype Typhi in the blood of patients with typhoid fever, using a lymphocyte culture supernatant (ALS)-based system that targets serotype Typhi membrane preparation (MP) as target antigen. The assay, called TPTest, could detect all (100%) blood culture confirmed typhoid fever patients (Khanam, Sheikh et al. 2013).

1.9 Treatment of Enteric fever

Emergence of multidrug resistance *S.* Typhi is a global hindrance for treating typhoid patients (Brown, Shanahan et al. 1996). Antibiotic is the choice of drug in this regard. The most effective antibiotic is chosen for therapeutic treatment, depending on patients' antibiogram results (Rahman, Islam et al. 2001). The available antibiotics against *S.* typhi are Ampicillin, Chloramphenicol, Trimethoprim/ sulfamethoxazole, Ceftriaxone, Ciprofloxacin, Nalidixic acid, Cefixime Gentamicin.

Ironically, *S.* Typhi resistance has emerged against all of the above mention antibiotics, except for Ceftriaxone Cefixime (Hirose, Tamura et al. 2001). However, some recent reports also show an emerging resistance pattern against Cefixime and Ceftriaxone as well. At present, Azithromycin is the most commonly used antibiotic for typhoid fever treatment in Bangladesh , and has proved to be effective in treatment of multidrug-resistant (MDR) typhoid fever (Girgis, Butler et al. 1999). In case of Ciprofloxacin sensitive but Nadixic acid resistance, Ciprofloxacin is not effective to kill pathogens (Shirin Afroj 2011) (Rahman, Siddique et al. 2006). Though Fluoroquinolones were thought to be the most effective treatment option for MDR typhoid fever, emergence of

Fluoroquinolones resistant strains has aggravated the problem (Chau, Campbell et al. 2007). In Bangladesh the prevalence of Fluoroquinolones especially ciprofloxacin resistant *S.* Typhi is more pronounced (Ahmed, D'Costa et al. 2006).

1.9.1 General Prevention

1.9.1.1 Safe water and food safety

Typhoid fever is a food and waterborne disease; thus the main preventive measure is to ensure access to clean food and safe water. The water needs to be of good quality and must be sufficient to supply all the community with enough drinking water as well as for all other domestic purposes such as cooking and washing (WHO 2003). Contaminated food is another important vehicle for typhoid fever transmission. Appropriate food handling and processing is paramount and basic hygiene measures must be implemented or reinforced during epidemics.

1.9.1.2 Sanitation

The bacterium may be spread through poor hygiene habits and public sanitation conditions, and sometimes also by flying insects feeding on feces. Thus, proper sanitation greatly contributes to reducing the risk of transmission of *Salmonella* pathogens.

1.9.1.3 Health education

Health education is paramount to raise public awareness that can be an effective measure for reducing risk of transmission of *S.* Typhi.

1.10 Vaccination

Vaccine against typhoid fever appears to be the only short-term solution to protect against emerging antibiotic resistant strains of *S.* Typhi. Different approaches have been successfully pursued to develop vaccines against *S.* Typhi, which include the use of either inactivated whole cell vaccines, live attenuated vaccines or subunit vaccines.

1.10.1 Inactivated whole cell vaccines

Parenteral whole cell typhoid vaccine was formulated by heat or chemical treatments, such as acetone treatment followed by drying, which inactivated the virulent microorganisms (Hejfec, Salmin et al. 1966) (Tapa and Cvjetanovic 1975). Although these early parenteral vaccines were clearly able to confer protection against typhoid fever, they could not be used as public health tools for routine vaccination because of their high reactogenicity, like: fever, headache and severe local pain (Ashcroft, Ritchie et al. 1964), (Engels, Falagas et al. 1998). These limitations ultimately led to the development of parenteral Vi polysaccharide vaccine and live oral attenuated Ty21a vaccine (Vivotif).

1.10.2 Vi Polysaccharide Parenteral Vaccine

In the early 1980s, methods were developed to purify Vi capsular polysaccharide to obtain 99.8% purified form which was free from contaminating LPS. Purified Vi stimulates rises in serum Vi antibodies in the vast majority of vaccinated adults and school-age children. However, it is not recommended in children less than 2 years of age. Boosters do not enhance protection and memory cells, mostly due to Vi polysaccharide being a T cell independent antigen (Cui, Carbis et al. , Guzman, Borsutzky et al. 2006).

1.10.3 Ty21a Live Oral Vaccine

Ty21a is an attenuated strain of *S.* Typhi that is safe and protective as a live oral vaccine. It was developed in the early 1970s by chemical mutation of pathogenic strain Ty2. It is not recommended in child less than 5 years. Three doses of Vivotif are given on every alternative day (Simanjuntak, Paleologo et al. 1991) (Levine, Ferreccio et al. 1999).

1.10.3.1 Production of the Vivotif vaccine:

During the production of the Ty21a (Vivotif) vaccine, bacteria are grown in large-scale fermenters under controlled conditions in medium containing a digest of yeast extract, an acid digest of casein, dextrose and galactose. The bacteria are harvested by centrifugation, mixed with a stabilizer containing sucrose, ascorbic acid and amino acids,

and lyophilized. The lyophilized bacteria are mixed with lactose and magnesium stearate as excipients, and filled into gelatin capsules that are coated with an organic solution to render them resistant against stomach acid. These capsules are then packaged in capsule blisters for distribution. Each capsule contains 2 to 6×10^9 lyophilized live bacteria. The capsules are administered orally, one hour before meals, and in three doses, given in alternative days (four doses in the US and Canada) (Guzman, Borsutzky et al. 2006).

Table 1.1: Contents of one enteric coated capsule of Vivotif (Source: Berna Biotech Ltd., Switzerland)

Component	Amount
Viable *S.* Typhi Ty21a	$2\text{-}6\times10^9$ CFU (Colony Forming Unit)
Non-viable *S.* Typhi Ty21a	$5\text{-}50\times10^9$ bacterial cells
Sucrose	26-130 mg
Amino acid mixture	1.4-7 mg
Lactose	100-180 mg
Magnesium stearate	3.6-4.4 mg

1.10.3.2 Clinical trials to evaluate the efficacy of the Vivotif vaccine

Results from clinical studies indicate that adults and children older than 6 years of age can be protected from typhoid fever following administration of three doses of Vivotif. The efficacy of the vaccine has been evaluated in a series of randomized, double-blind, controlled field trials. The first trial was performed in Egypt with a study population of 32,388 children aged between 6 to 7 years. Three dose of vaccine administered in three alternative days resulted in a 95% decrease in typhoid fever over a three year surveillance (Wahdan, Serie et al. 1982). A series of field trials were subsequently performed in Santiago, Chile to evaluate efficacy of one or two doses of vaccine in 82,543 school-aged children. After 24 month of surveillance, vaccine efficacy was 29% for single dose and 59% for two doses (Black, Levine et al. 1990). A further trial was performed in Santiago, Chile involving 109,594 school-aged children. Three doses of enteric coated capsules were administered either in alternative days (short immunization schedule) or 21 days

apart (long immunization schedule). It was found that the short immunization schedule was more potent in protection against typhoid fever than long immunization schedule (Levine, Ferreccio et al. 1987). The level of protection was not diminished in four year surveillance (Levine, Ferreccio et al. 1989). A further field trial to determine the effects of dosage in school-aged children demonstrated a positively proportional relationship between efficacy and the number of doses (Ferreccio, Levine et al. 1989). Studies carried out in Plaju, Indonesia evaluating the immunogenicity of vaccine in adults showed an efficacy of about 42% (Simanjuntak, Paleologo et al. 1991). The optimum booster schedule for Vivotif vaccine has not been determined. Efficacy has been shown to persist for at least 7 years. In US, it is recommended that a re-immunization should be done every 5 years under conditions of repeated or continued exposure to typhoid fever (1990)

1.10.3.3 Immune response to Vivotif vaccine

Vivotif vaccine induces both mucosal and serum antibodies (mucosal IgA and serum IgG) as well as cell-mediated immunity (CMI) (Salerno-Goncalves, Pasetti et al. 2002) for protection against typhoid fever.

1.10.3.3.1 Antibody response to Vivotif vaccine

The induction of mucosal antibodies provides protection against both infection and disease (Dietrich, Griot-Wenk et al. 2003). Increased serum IgG antibodies and gut-derived IgA antibody secreting cells (ASC) against the O-antigen are the best surrogate markers of protection. Studies measuring antibody response against purified LPS and killed bacteria expressing O and H antigens after Ty21a vaccination in adults revealed some interesting findings. Significant rise in response was observed for IgG and IgA antibodies but not for IgM, with the highest proportional increase in IgA class in serum (Tagliabue, Villa et al. 1986), (D'Amelio, Tagliabue et al. 1988). Following vaccination with Ty21a, high levels of IgG and IgA secreting cells have been seen in adults. The response peaked at day-9 after vaccination with a gradual decrease in day-22 (Viret, Favre et al. 1999), (Kantele 1990), (Kantele, Arvilommi et al. 1986). These antibody secreting cells bear homing receptors which direct them to the gut and let them contribute

to the mucosal immunology (Kantele, Kantele et al. 1997). An increase in O-specific fecal IgA was observed 1–8 months post-immunization (Nisini, Biselli et al. 1993).

1.10.3.3.2 Cell mediated immunity to Vivotif vaccine

Ty21a also triggers cell-mediated immunity (CMI), which is crucial for protection against intracellular bacterial pathogens (Mollenkopf, Dietrich et al. 2001). Ty21a vaccination induces lympho-proliferative response on Ty21a whole-cell stimulation of PBMC, collected 21 days after vaccination. It also induces strong systemic CD4+ T-helper type 1 (Th1) responses in vaccines, characterized by the production of IFN-γ in the absence of IL-4 (Viret, Favre et al. 1999). Vaccination with Ty21a also elicites strong CD8+ cytotoxic T cells (CTL) responses, which was reported to persist for at least 2 years after immunization (Salerno-Goncalves, Pasetti et al. 2002). The *S.* Typhi-specific CD8+ T cells are phenotypically and functionally consistent with T effector memory cells (Salerno-Goncalves, Wahid et al. 2005). Vaccination induced antigen-specific $CD4^+$ and $CD8^+$ memory T cells express the gut-homing integrin $ß_7$ (Lundin, Johansson et al. 2002).

Nonetheless, very little information of immune responses after immunization with Vivotif is available in typhoid endemic countries and needs to be studied in such regions.

1.11 Hypo-responsiveness to vaccine in children of developing countries

A major problem of vaccination in developing countries is that, infants and children in the endemic regions often do not mount a consistent immune response to oral vaccines compared to children in the industrialized or developed countries. Thus, the vaccines which are effective for children in the developed countries fail to induce a similar protective immunity in children of developing countries. Comparison of data from developed and developing countries have shown that both parenteral and oral vaccines show reduced immunogenicity in developing countries. Amongst parenteral vaccines, a different immunogenicity profile has been documented for *Haemophilus influnzae* type b and *Streptococcus pneumoniae* conjugate vaccines. For oral vaccines, lower

immunogenicity has been observed for several live rotavirus vaccines (Jaimes, Rojas et al. 2004), polio vaccine (Patriarca, Wright et al. 1991, John 1993), several live oral cholera vaccines (Dukoral, Peru-15 and CVD103HgR) (Clemens, Sack et al. 1990), (Qadri, Chowdhury et al. 2007), (Simanjuntak, O'Hanley et al. 1993) and live *Shigella flexneri* 2a vaccine (Levine, Kaper et al. 1988). The reduced immunogenicity is observed regarding lower take rates, lower antibody titers among those responding, higher doses of vaccine required to achieve an acceptable response, and shorter duration of protection. Additionally, the vaccine dose required for individuals in a developed versus a developing countriy might be different. The requirement of dose of oral cholera vaccine was higher for children in Indonesia and Bangladesh compared to those in the US (Qadri, Chowdhury et al. 2007), (Kotloff, Wasserman et al. 1992), (Richie, Punjabi et al. 2000) (Bhuiyan, Lundin et al. 2009, Qadri, Bhuiyan et al. 2013)

There might be various underlying reasons for this poor immune response to vaccine in children in developing countries than that of developed countries, like-

- Differences in genetic “make-up”.
- Prior exposure or contact with antigens contained within the vaccines.
- Malnutrition, a common feature of children of developing countries, might lead to immune suppression.
- Parasitic infection in the gut, which may alter the immune response.
- The difference in gut microbial flora, which may influence mucosal immune system.

1.12 Antibody avidity

Antibody avidity is the strength with which a multivalent antibody binds with a multivalent antigen, while affinity is the strength of a single antigen-antibody binding. Avidity can also be increased when an antigen with multiple binding sites interacts with a number of different antibodies.

Low-avidity antibody is usually produced during the primary response, and the strength of the avidity of an antibody increases over time with the maturation of the IgG antibody

response. IgG avidity has been used to differentiate current from past viral infections (Chan, Sonnenberg et al. 2007). Antibody avidity increases during the secondary response when antibodies are being produced most rapidly. Ideally, increased avidity should slow down release of new antibody from an antigen template.

Antibody avidity is considered as a determinant of protective efficacy. Most likely, avidity plays a role in determining the ability of antibodies to neutralize antigen when it is encountered in the bloodstream. At low serum concentrations, antibodies of high avidity are more effective than are antibodies of lower avidity (Breukels, Jol-van der Zijde et al. 2002).

1.12.1 Avidity Index (AI) as indicators of immunological memory

During the maturation of the humoral immune response, there is an antibody selection process that results in synthesis of antibodies with increased antigen-antibody association strength. The numerical constant that represents this association strength is denoted as avidity index (AI).

Antibody avidity has been used as a marker of B cell maturation to discriminate between primary and secondary responses to a number of infections, including infections with dengue virus (de Souza, Fernandes et al. 2004), rubella virus (Pullen, Fitzgerald et al. 1986, Hedman and Rousseau 1989), cytomegalovirus (Baccard-Longere, Freymuth et al. 2001), and herpes virus (Ward, Turner et al. 2001). Furthermore, antibody avidity is an important surrogate for determining protective immunity for several vaccines, including measles vaccine (de Souza, Akico et al. 1997), Haemophilus influenza type b conjugate vaccine (Goldblatt, Vaz et al. 1998), pneumococcal capsular polysaccharide vaccine (Usinger and Lucas 1999), and mumps vaccine (Narita, Matsuzono et al. 1998).

Very recently, IgG and IgA avidity maturation has been studied in *Vibrio cholerae* and *E. coli* associated diarrhoeal patients in Bangladesh as well (Alam, Arifuzzaman et al. 2013, Alam, Aktar et al. 2014). Also, antibody avidity as humoral immune responses was analyzed in Bangladeshi children and adults following administration of an oral killed cholera vaccine (Alam, Leung et al. 2013).

In addition, the determination of antibody avidity has been gaining importance in the assessment of vaccine efficacy, where the induction of high-avidity antibodies is desired.

The AI can be measured in immune sera by enzyme-linked immunosorbent assay (ELISA) with the inclusion of one additional step: treatment of the antigen-antibody complex with denaturating reagents, such as urea or thiocyanate. Experimentally, AI is usually determined by varying the antibody concentration. Alternatively, it is also possible to vary the concentration of denaturating reagent. The latter method is used less frequently, probably because it consumes more time. AI calculation has been performed in numerous ways, all of which are based on the ratio or percentage of bound antigen in the ELISA plate with and without a denaturating agent,which would be expressed as the optical density (OD), titer or graphical distance between both titration curves (Alam, Arifuzzaman et al. 2013).

1.13 Aim of this study

Despite being an important public health problem in Bangladesh, no antibody avidity maturation study against S*almonella enterica* serovar Typhi and Paratyphi have been reported till date. Due to the disproportionately high incidences of typhoid infection among young children, avidity pattern maturation related studies could potentially provide further insight into the pathogenesis of typhoid infection. Under these circumstances, the present study aims to:

- Explore IgA and IgG avidity maturation pattern in Vivotif vaccinees aged below 5 years.
- Identify age specific IgA and IgG avidity patterns among typhoid patients in Bangladesh.
- Compare the differences between the avidity response between vaccinees and naturally infected typhoid patients of similar age groups.

CHAPTER 2

Methodology

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2.1 Place of the study

The study was carried out in the Immunology laboratory unit of International Centre for Diarrhoeal Disease and Research, Bangladesh (icddr,b).

2.2 Period of study

The duration of the laboratory works was from October 2013 to July 2014.

2.3 Study participants

The study was performed with specimens collected from vaccinees (age, below five years) immunized with Vivotif vaccine (n=24), and from patients (age, 1-59 years) with *S.* Typhi bacteremia (n= 34) and patients positive for TPTest (n= 30) [Table2.1]. For analysis, patients were categorized into three groups based on their age; group 1 (patients aged below 5 years), group 2 (patients aged between 5 to 17 years of age) and group 3 (aged 17 years onwards) [Table 2.2].

Table 2.1 Categorization of study individuals

Study participants (n=88)	
Vivotif vaccinees aged below 5 **(n=24)**	Naturally infected typhoid patients **(n=64)**

Table 2.2 Categorization of typhoid patients based on age and diagnosis method

Naturally infected typhoid patients (n=64)					
Patients aged below 5 (n=23)		**Patients aged between 5 to 17 years (n=27)**		**Patients aged above 17 years (n=14)**	
Blood culture positive **(n=16)**	Only TPTest positive **(n=7)**	Blood culture positive **(n=13)**	Only TPTest positive **(n=14)**	Blood culture positive **(n=5)**	Only TPTest positive **(n=9)**

2.3.1 Inclusion criteria

2.3.1.1 For patients

- High fever temperature (≥39^0C)
- Duration of fever 3-7 days

2.3.1.2 For vaccinees

No history of illness, fever, or diarrhea in the preceding 3 months, and were of the same socio-economic status as the patients.

2.3.1.3 Exclusion criteria

Participants who denied to providing with consent for enrollment in the study were excluded.

2.4 Ethical issue

The study was approved by the Research Review Committee (RRC) and the Ethical Review Committee (ERC) of icddr,b. Before enrollment in the study, written consent was taken from the participants and/or their parents or guardian in case of children.

2.5 Study overview

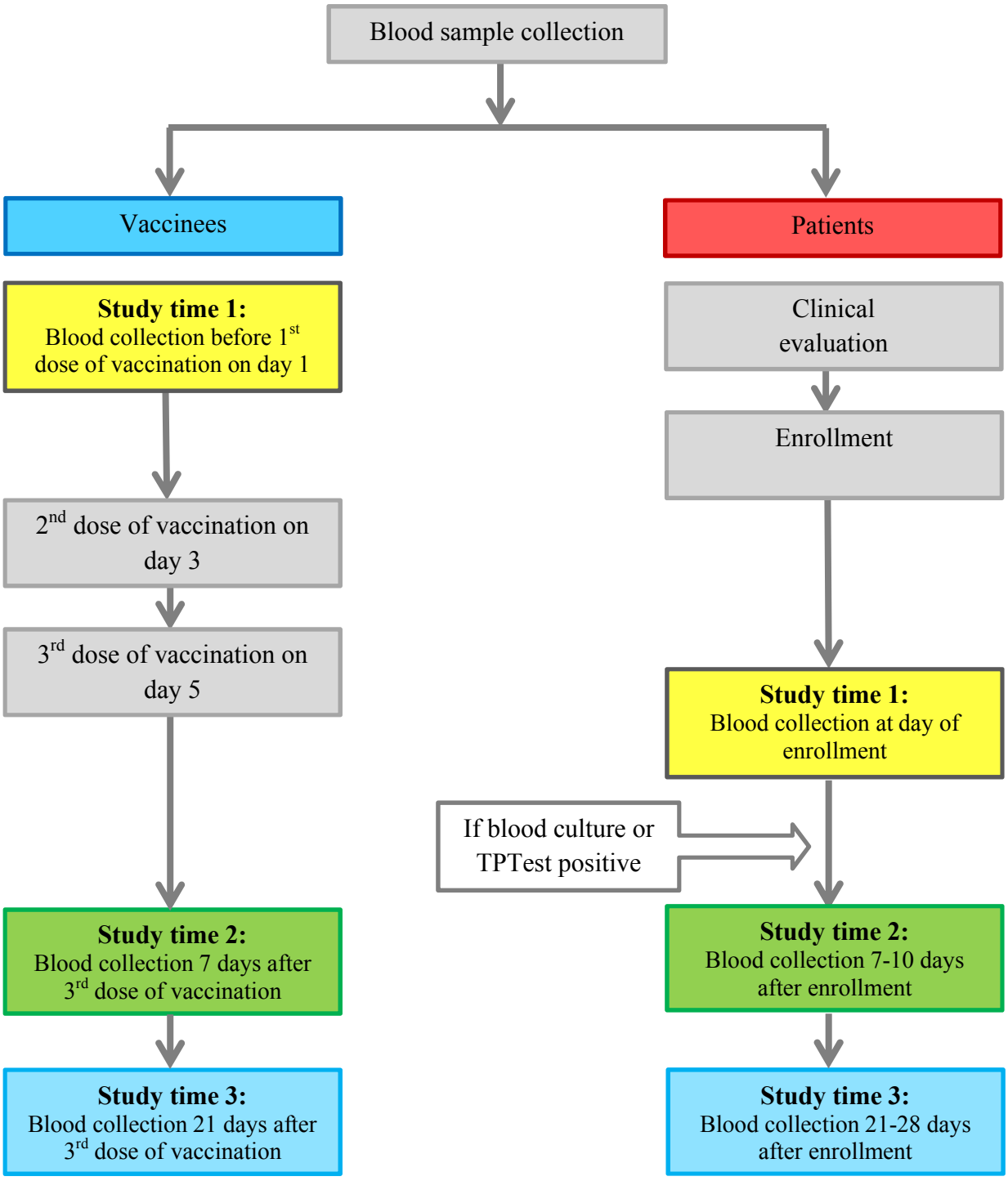

Figure 2.1: Flow-chart of the study plan

2.6 Laboratory procedures

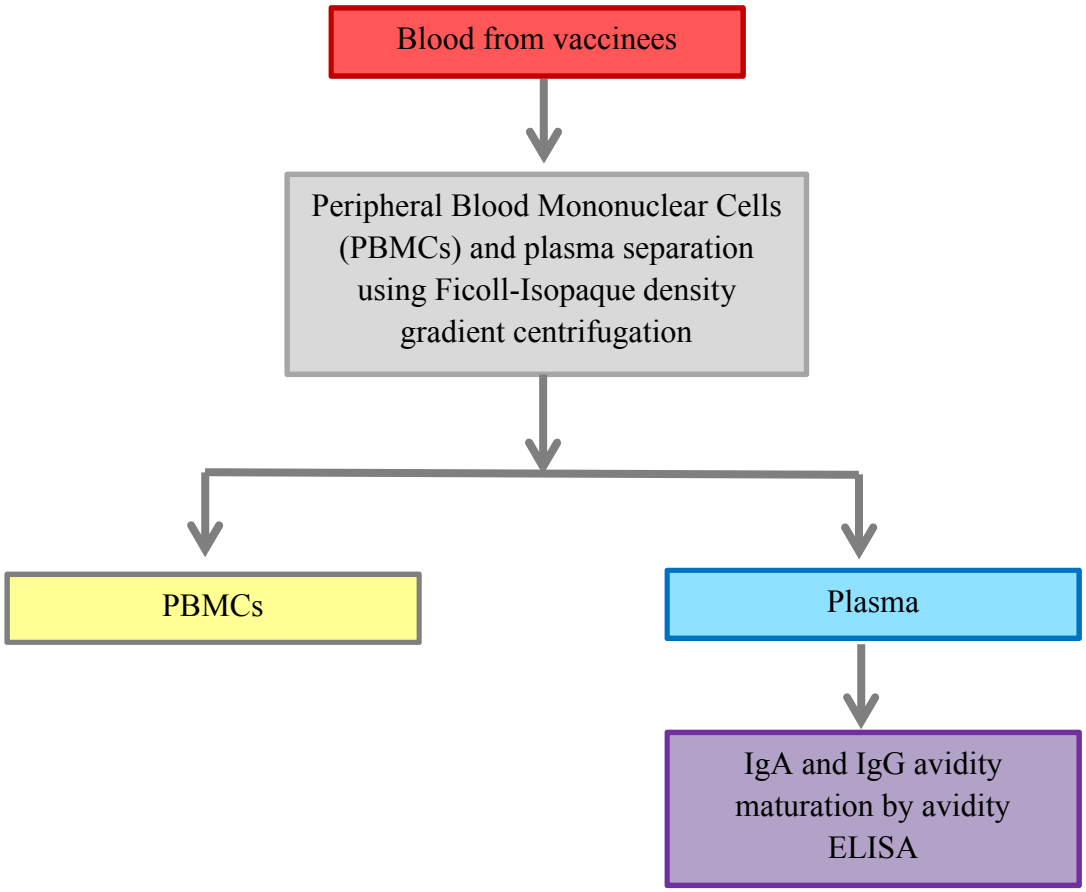

Figure 2.2: Flow chart of laboratory work for blood sample collected from vaccinees

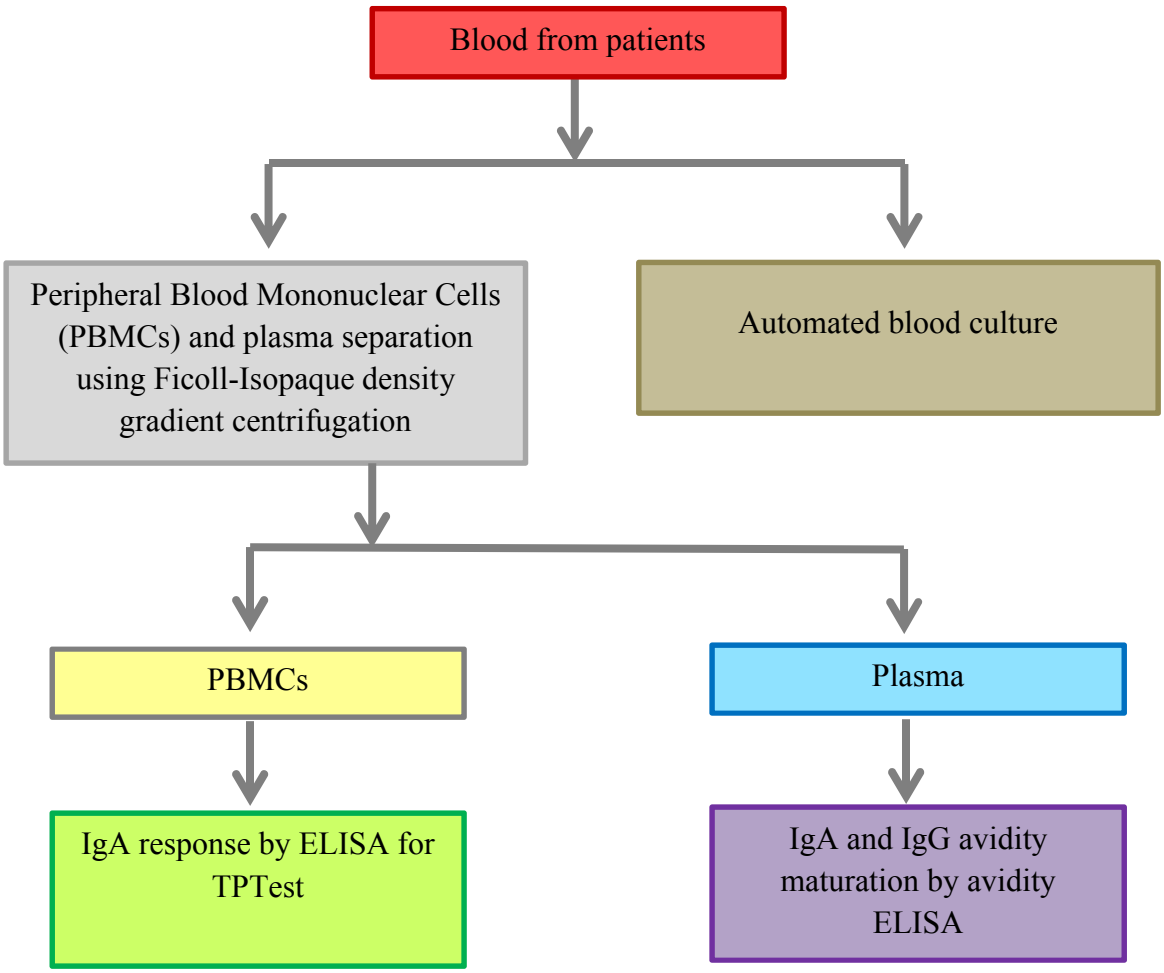

Figure 2.3: Flow chart of laboratory work for blood sample collected from patients

2.6.1 Blood culture

In case of children aged between 1 to 5 years, 3 ml of blood was collected for blood culture test. For patients aged above 5 years, 5 ml of blood was collected for the same. BacT/ALERT® 3D was used to incubate blood at 37° C. In case of positive results, growth becomes apparent by 72 hours. Nonetheless, most cultures were monitored for five to seven days. Any growth in enrichment broth is subsequently sub-cultured onto agar plates to isolate the pathogenic organism, followed by confirmation of *S.* Typhi by biochemical and with anti-sera test.

2.6.1.1 Biochemical tests

Following growth on enriched medium, *S.* Typhi organism can be identified by following biochemical reactions:

2.6.1.1.1 Kliger's Iron Agar (KIA)

Kligler Iron Agar is used for the differentiation of microorganisms on the basis of dextrose and lactose fermentation and hydrogen sulfide production.

2.6.1.1.2 Motility Indole Urea (MIU)

Motility indole urea agar (MIU) is a semisolid medium designed for detection of urease activity, motility, and indole production in Enterobacteriaceae. It was also used in combination with Kligler Iron Agar for the recognition and differentiation of *Salmonella* and *Shigella* species from colonies picked from plating media.

2.6.1.1.3 Citrate agar test

Citrate agar slants contain sodium citrate (only carbon source) and ammonium ion (the sole nitrogen source). A pH indicator (Bromothymol), included in the agar, gives a green color at pH < 7.0 and blue color at pH > 7.6. Organisms capable of utilizing citrate for energy produce alkaline compounds as by-products. Thus, a positive result for citrate utilization is the formation of a blue color. This test is among a suite of tests (Indole, Methyl-Red, Vogues-Proskauer, and Citrate) that are used to differentiate among the Gram-Negative bacilli in the family Enterobacteriaceae.

Table-2.3: The biochemical tests for identification of *S.* Typhi and Paratyphi

Organism	Glucose	Hydrogen sulphide	Motility	Indole	Urease	Citrate
S. Typhi	Acid	Positive	Positive	Negative	Negative	Negative
S. Paratyphi A	Acid + Gas	Negative	Positive	Negative	Negative	Negative
S. Paratyphi B	Acid + Gas	Positive	Positive	Negative	Negative	ositive

Figure 2.4: Biochemical test for *S.* Typhi and Paratyphi

2.6.1.2 Anti-sera test

Salmonella Grouping Antisera are used in qualitative slide agglutination tests for the serological differentiation of *Salmonella*. Using these antisera, *Salmonella* isolates may be classified into *S.* Typhi and Paratyphi A, B, C. The *Salmonella* O Polyvalent antiserum is used for screening in conjunction with biochemical tests. The *Salmonella* Vi antiserum detects the Vi antigen, a heat-sensitive envelope antigen usually found in fresh isolates of *S.* Typhi and *S.* Paratyphi.

2.6.1.3 Antimicrobial sensitivity test

Antimicrobial sensitivity was determined by the disc diffusion method of modified Kirby-Bauer (Bauer, Kirby et al. 1966) technique using Mueller-Hinton agar and commercially available antimicrobial discs (Oxoid, Hampshire, United Kingdom). Following antibiotics and their concentration per discs were used for sensitivity test:

- Ampicillin (AMP, 10µg)
- Chloramphenicol (C, 20µg)
- Trimethoprim–Sulphamethoxazol (TS, 1.25µg/ 23.75µg)
- Ciprofloaxcin (CIP, 5µg)
- Ceftriaxone (CRO, 30µg)
- Gentamicin (GM, 10µg)
- Nalidixic acid (NA, 30µg)
- Cefixime (CFM, 5µg)

2.6.1.3.1 Methods of sensitivity test

Mueller- Hinton agar plates were dried in an incubator at 37^0C for 30 minutes before use. With a sterile wire loop, half of one well isolated colony from pure culture was suspended to a sterile screw capped tube containing 2 ml of sterile Mueller- Hinton broth. The turbidity of the inoculum was standardized to the equivalent to that of 0.5 of Mac Farland standard by adding more organisms or more broth which approximately correspond to $1.5x10^8$ organisms/ ml. A sterile cotton swab was immersed into the bacterial suspension and the excess suspension was removed by rotating the swab with a firm pressure against the inner side of the tube above the fluid levels. The swab was then streaked evenly on the entire surface of a Mueller- Hinton agar plate in three different plane (by rotating the plates approximately 60° angle each time) to get a uniform distribution of the organism. The inoculum was allowed to dry for 15 minutes at room temperature with lids closed. The discs were then placed on the inoculums surface by a sterile fine forceps 15 mm away from the edge of Petri dishes with 25 mm gap in between the discs. Discs were gently pressed down to ensure contact and incubated at 37°C for 24 hours (WHO 2003) (Chart, Cheesbrough et al. 2000).

2.6.1.3.2 Measurement of inhibition zone

After overnight incubation, each plate was examined. The diameter of complete inhibition zone was measured with the help of a scale placed on the under surface of the petri dish without opening the lids. Zone of inhibition was measured in mm in two directions at right angle to each other through the center of each disc and the average of the two readings was taken.

2.6.1.3.2 Interpretation of zone

The zone of inhibition in growth produced by each antimicrobial agent on the test organisms was compared with that produced on the control organisms (ATCC). Depending on the diameter of the clear zone of inhibition around the discs the test organisms were categorized into sensitive (S), intermediate sensitive (IS) and resistant (R) to the representative antimicrobial agent (NCCLS 1988).

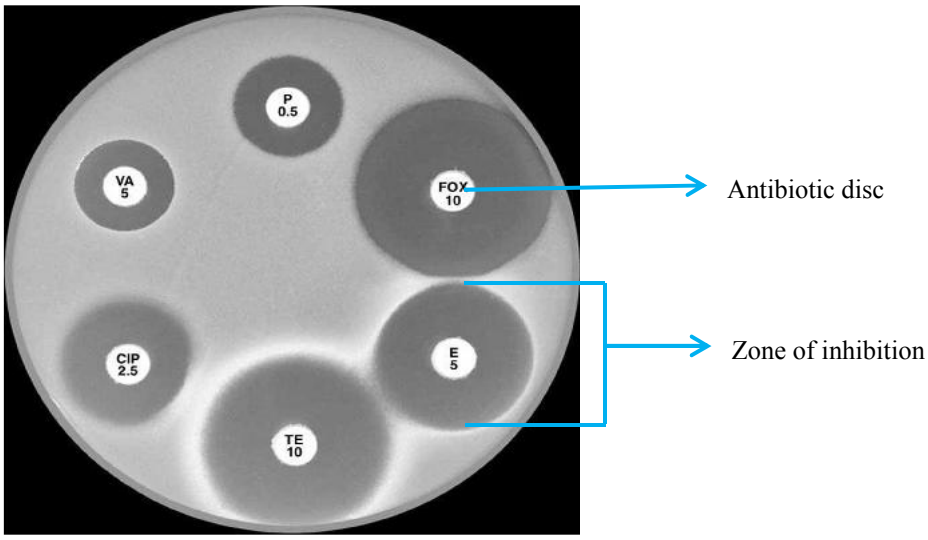

Figure 2.5: Antibiogram of *S.* Typhi and Paratyphi

2.6.2 Separation of plasma and PBMC

Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from venous blood by density gradient centrifugation on FicollIsopaque. The isolated PBMCs were cultured in RPMI complete medium for 48 hours to obtain antibody in lymphocyte supernatant (ALS).

2.6.2.1 Procedure

- Heparinized venous blood was diluted with equal volume of phosphate buffer saline (PBS) in falcon tubes.
- 4 ml of Ficoll-Isopaque was taken in falcon tube. Diluted blood was carefully layered on top of it.
- The tube was centrifuged at 1800 rpm (772xg) for 25 minute at 20°c.
- Following centrifugation, four layers were obtained- a pellet of RBCs and granulocytes at the bottom, a second of Ficoll-Isopaque on top of it, followed by a third layer of PBMCs and the final upper-most layer of plasma.

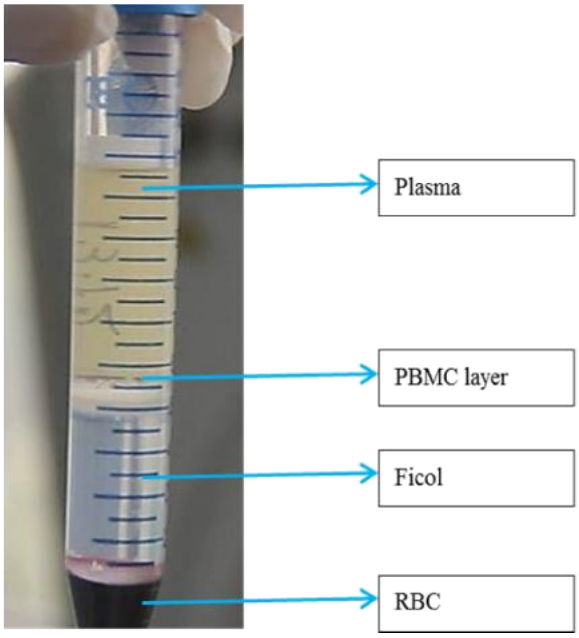

Figure 2.6: Separation of plasma and peripheral blood mononuclear cells (PBMCs)

- Plasma was carefully collected in a falcon tube with a pipette. PBMCs were isolated in a separate falcon.
- The isolated PBMCs were washed once in PBS at 2000rpm (953xg) for 10 minute at 20°C.
- Next, they were resuspended in 10 ml of PBS and counted in a hemocytometer.
- The resuspended PBMCs were washed for a second time at 2000rpm (953xg) for 10 minute at 20°C.

2.6.3 Preparation of Lymphocyte Supernatant

From the isolated PBMC, lymphocyte supernatant was prepared in the following method:

- The isolated PBMCs were taken and resuspended in RPMI- complete medium at a concentration of 1×10^7 cells per ml medium.
- The cells were cultured in tissue culture plates (Nunc Coaster) at 37°C in 5% CO_2 incubator for 48 hours.
- After 48 hours the culture supernatant was taken in an eppendorf and centrifuged at 12000 rpm (11600xg) at 4°C for 5 minute.
- The supernatant was removed and then protease inhibitor, at a concentration of 1% of culture supernatant, was added, and stored at -70°C for future analysis.

2.6.4 Antigen Preparation

For this research venture, immune response was studied against two different antigens; namely, a membrane preparation (MP) from vivotif Ty21a strain, and a Lipopolysaccharide preparation (LPS) from a laboratory control strain.

The MP antigen was used for the detection of typhoid patients using TPTest. The LPS antigen was used for avidity (IgA and IgG) measurement in patients and vaccinees.

2.6.4.1 Preparation of Membrane Preparation (MP) from Ty21a strain

- A loop of bacteria from glycerol stock was streaked on MacConkey agar plate and incubated at 37°C overnight.
- On the following day, 20-25 colonies from the plate were taken in 3 ml of LB broth for liquid culture. Growth was facilitated by shaking it in GALLENKAMP shaker incubator at 37°C with 250 rpm until an absorbance of 0.04-0.05 was achieved. The procedure took about 3 to 5 hours.
- Next, about 100 µl of bacterial suspension was spread onto Horse Blood Agar plate (5% Horse serum in Blood Agar media), and incubated at 37°C overnight.
- Bacteria grown on each plate were harvested in 3 ml of harvest buffer (5 mM $MgCl_2$-10 mMTris with p^H-8.0).
- The harvested bacterial suspension was aliquoted in 15 ml falcons, at a volume of 8 ml per falcon.
- The falcon was placed in an insulated container, containing ice with NaCl to avoid quick thawing.
- The microtip of the sonicator was immersed below enough from the surface of the suspension to avoid foaming during the sonication process.
- Each tube was sonicated 5 times at 60% amplitude for 2 min (pulse with 3s on and 3s off).
- Afterwards, the bacterial suspension was centrifuged at 1400×g for 10 min, and the supernatant was transferred into autoclaved Nalgene tubes.
- The supernatant was then centrifuged at 14900×g for 30 min.
- The supernatant was discarded, and the pellet in each tube was dissolved in 2 ml of harvest buffer.
- The membrane preparation (MP) was aliquoted in eppendorf, and kept at -70°C overnight.
- Subsequently, freeze-thawing (30 min freezing and 15 min thawing) of the prepared MP was carried out, followed by streaking of 10 µl of MP on MacConkey plate and incubation. In case if bacterial growth was found, the aliquots of MP were further kept at -70°C for 3-4 weeks.

- Finally, the protein content was estimated using Bradford method.

2.6.4.2 Preparation of Lipopolysaccharide (LPS) antigen

- The *Salmonella* strain was streaked on TSA plate, and was incubated overnight at 37°C.
- Two loops of inoculum were taken, inoculated into 50 ml of TSB broth, and was incubated at 37°C for 4 hours at 100 rpm.
- The colonies were harvested by adding 2 ml of normal saline, transferred to Nalgene tubes, and centrifuged at 10000×g for 30 min at 4°C.
- The pellet was collected in deionized water (3ml deionized water/ plate).
- Phenol (95%), preheated at 69°C, was added to the suspensions at half the volume of water.
- The mixer was then heated at 69°C for 15 min on a water bath.
- Afterwards, the mixer was cooled to 4°C on ice for 20 min, followed by another centrifugation at 10000×g for 30 min at 4°C.
- The supernatant was collected in a beaker, and 20 ml of water was added to dissolve the pellet.
- The aforementioned four steps were repeated for the second extraction.
- The two water supernatant (LPS) were combined, and dialyzed against running tap water for 48 hours to remove phenol traces.
- Next, the supernatant was dialyzed again against distilled water for another 24 hours at 4°C with stirring (cold room) and one change of autoclaved deionized water.
- The dialyzed solution was collected in a beaker and lyophilized. The white fluffy LPS attached to the wall of lyophilized vial was collected, weighed, dissolved in deionized water (3% w/v), and kept at 4°C.
- After complete dissolution, the solution was ultracentrifuged at 105000×g for 4 hours at 4°C.
- After that, the supernatant was discarded, the pellet suspended in 10 ml of deionized water, and kept at 4°C for overnight.

- The following day, the solution was vortexed to completely dissolve the pellet.
- Subsequently, 10 mg of Proteinase K was added and the volume of the suspension was made up to 10 ml. It was gently mixed by tilting the tube.
- The suspension was incubated overnight at 37°C, followed by ultracentrifugation at 105000×g for 4 hours at 4°C.
- Afterwards, the supernatant was discarded. The pellet was collected in 10 ml of distilled water, and kept at 4°C overnight.
- DNase and RNase was added at a concentration of 20µg/ ml, and incubated at 4°C for 3 to 4 hours.
- Then, the pellet was dissolved in 5 ml of deionized water in lyphilization tube to lyophilize the solution.
- Finally, the fluffy powder LAS was collected in dram vial, and stored at - 20°C.

2.6.5 TPTest

TPTest detects IgA responses in lymphocyte culture supernatant against *S.* Typhi specific MP antigen. Pooled serum is used as a positive control. The reaction rate of sample is divided by that of positive control to obtain the normalized value. A normalized value greater than 10 ELISA unit is considered as positive. The cut-off value of the TPTest method has been calculated by using the formula: > 2SD plus geometric mean of healthy Bangladeshi control.

2.6.5.1 Procedure

2.6.5.1.1 Coating of ELISA plate with antigen:

- The antigen, "Ty21a MP" was diluted with PBS at a concentration of 5.0 µg/ml.
- ELISA-plates (NuncF) were coated with 100 µl/well of antigen suspension.
- The plates were incubated at room temperature for overnight.

2.6.5.1.2 Blocking:

- The coated plates were washed thrice with PBS.

- The plates were blocked with 200 µl/well of 1.0 % bovine serum albumin in PBS (BSA-PBS) and incubated for 30 minutes at 37°C.

2.6.5.1.3 Sample loading:

- The plates were washed three times with PBS-Tween (0.05% Tween) and once with PBS.
- ALS samples were diluted at 1:2 dilutions.
- For positive control, pooled plasma of typhoid positive patients was taken and diluted with 0.1% BSA-PBS-Tween and 100µl of diluted pool solution was given in appropriate well.
- For determining antibody response in ALS, pooled plasma was diluted at 1:100 dilutions.
- For negative control, 100µl 0.1% BSA-PBS-Tween was given in appropriate wells.
- The plates were then incubated at 37°C for 90 minutes.

2.6.5.1.4 Conjugate adding:

- The plates were washed three times with PBS-Tween (0.05% Tween) and once with PBS.
- Rabbit anti human IgA, conjugated with horse reddish peroxidase were diluted in 0.1% BSA-PBS-Tween at 1:1000 dilutions and 100 µl was added in each well. The plates were incubated at 37°C for 90 minutes.

2.6.5.1.5 Plate developing:

- The plates were washed three times with PBS-Tween (0.05% Tween) and once with PBS.
- The substrate, H_2O_2-OPD, was made by dissolving 10 mg OPD (orthophenyldiamine) in 10 ml of 0.1M sodium citrate buffer (pH 4.5), to which 4 µl of 30% H_2O_2 was added immediately before use.

- The plates were developed by adding H_2O_2-OPD at 100 µl in each well.
- Then optical density (OD) was measured at 450 nm by the Ion ELISA reader immediately.

2.6.6 Avidity ELISA

Antibody avidity was assayed using avidity ELISA, a modification of end-point ELISA, with an additional step of chaotropic agent treatment to aid in the dissociation of low affinity antigen-antibody binding.

2.6.6.1 Detection of Lipopolysaccharide (LPS) specific IgG and IgA antibody avidity in plasma samples by avidity ELISA

2.6.6.1.1 Coating

- 96 well flat bottomed ELISA-plates (Nunc F) were coated with 100µL/well of LPS of *S.*Typhi at a concentration of 2.5µg/ml in PBS (pH 7.2-7.4).
- The plates were then incubated at room temperature for overnight.
- Following overnight incubation, the ELISA plates could be stored at 4^{o}C for a week.

2.6.6.1.2 Blocking

- The LPS-coated plates were washed three times with PBS and blocked with 0.1% Bovine Serum Albumin in PBS (BSA-PBS), 200µL/ well for 45 minutes at 37°C.

2.6.6.1.3 Loading of Samples

- The plates were washed three times with PBS-0.05% Tween and once with PBS.
- The plates were then incubated with two pairs of serially diluted plasma samples (1:2 to 1:3000 dillution in 0.1% BSA-PBS containing 0.05% Tween; 100 µl/well) for 90 minutes at room temperature (RT).

2.6.6.1.4 Treating with chaotropic agent: sodium thiocyanate (NaSCN)

- The plates were washed three times with PBS-0.05% Tween and once with PBS.
- One of the paired wells for each samplewas treated with 200µl/well of freshly prepared NaSCN(2M in PBS-0.3%tween) for 20 minutes (strictly) at RT whereas the other well was treated with PBS-0.3%tween alone.
- After exactly 20 minutes the plates were washed 4 times with PBS- Tween (0.05 %) and once with PBS.

2.6.6.1.5 Conjugate addition

- The horseradish peroxidase (HRP) conjugated rabbit anti-human IgA and anti-human IgG (Jackson ImmunoResearch Laboratories Inc.) were diluted in 0.1% BSA-PBS Tween and added 100µl/well.

  Anti-human IgA HRP (e.g. Jackson 309035011, 1/1000)

  Anti-human IgG HRP (e.g. Jackson 309035006, 1/1000)

- The plates were incubated for 90 min at RT.

2.6.6.1.6 Plate developing

- The plates were washed 3 times with PBS- Tween (0.05 %) and once with PBS.
- The plates were developed with the substrate orthophenylenediamine (OPD)-100 µl / well, prepared by dissolving 10 mg of OPD in 10 ml of 0.1 M sodium citrate buffer, (pH 4.5) to which was added 4 µl of 0.1 % H_2O_2 immediately before use.
- After 20 min the reaction was stopped by adding 25µl/well of 1.0 M H_2SO_4 and the reading was taken at 492 nm in the MultikanAsent ELISA Reader.
- The results are shown as the avidity index.

2.6.6.1.7 Calculation of results

The avidity index (AI) was calculated using the following equation:

$$\textbf{Avidity Index (AI)} = \frac{\textbf{OD}_{\textbf{NaSCN}}}{\textbf{OD}_{\textbf{Plain}}} \textbf{ X100}$$

Where;

- OD_{NaSCN}= Optical density of the wells treated with NaSCN
- OD_{Plain} = Optical density of the wells without NaSCN

2.6.7 Statistical analysis

For comparison of response within groups, Wilcoxon signed rank test was used. Mann-Whitney U test was used for comparing response between groups. Repeated-measures multivariate analysis of variance (MANOVA) was also used to determine differences between groups. All reported P values are two sided, with a P value of <0.05 considered a cutoff for statistical significance. Spearman's correlation analysis was used to measure bivariate associations. Graphs and figures were prepared using GraphPad Prism 5.0.

CHAPTER 3

Results

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3.1 Baseline data of the participants

3.1.1 Baseline data of vaccinees immunized with Vivotif

A total of 24 participants were enrolled in the vivotif vaccine group. 13 (54%) of them were male, and the remaining 11 (46%) of them were female. The median age was 3.6 years, with a range of 1 to 5 years (Table 3.1).

Table-3.1: Baseline data of vaccinees given the typhoid vaccine, Vivotif (n=24)

Feature	Value
Median age in year (SD)	3.6 (4.5)
No. of males (%)	13 (54%)
No. of females (%)	11 (46%)

3.1.2 Baseline data of Typhoid patients

Among 64 typhoid patients (blood culture and or TPTest positive), the number of male and female patients were 22 (34%) and 42 (66%) respectively, with a median age of 7 years and 7 months. The age range was between 1 to 44 years. The median of temperature was 39.2°C (Table 3.2).

Table 3.2: Baseline data of patients (n=64)

Feature	Value
Median age in year (SD)	7.7 (9.9)
No. of males (%)	22 (34%)
No. of females (%)	42 (66%)
Median temperature in °C (25th, 75th percentile)	39.2 (39.1, 39.5)

3.2 Clinical findings of participants

Clinical features of 64 typhoid patients included headache (78.1%), abdominal pain (57.8%), constipation (32.8%), coated tongue (53.1%), diarrhea (18.7%), vomiting (14%), rose spot (7.8%) and rash (9.3%), which are presented in (Table 3.5).

Table 3.3: Clinical findings of patients (n=64)

Clinical features	Number (%)
No. of patients (%) with:	
Headache	50 (78.1)
Abdominal pain	37 (57.8)
Constipation	21 (32.8)
Coated tongue	34 (53.1)
Diarrhea	12 (18.7)
Vomiting	9 (14)
Rose spot	5 (7.8)
Rash	6 (9.3)

3.3 Blood culture and sensitivity result

Of 35 isolated *S.* Typhi strains from the bacteremic patients, all (100%) were sensitive to cefixime, ceftriaxone and gentamicin. A total of 19 (54%) of the isolated strains were resistant to ampicillin, and trimethoprim-sulfamethoxazole. Chloramphenicol resistance was observed in 23 (66%) strains, whereas 11 (31%) and 32 (91%) strains were resistant to ciprofloxacin and nalidixic acid respectively.

Table 3.4: Antibiotic susceptibility pattern of isolated *S.* Typhi (*n*=35)

Antibiotic	Sensitive (%)	Resistant (%)
Ampicillin	16 (46%)	19 (54%)
Trimethoprim-sulfamethoxazole	16 (46%)	19 (54%)
Chloramphenicol	12 (34%)	23 (66%)
Ceftriaxone	35 (100%)	0 (0%)
Ciprofloxacin	24 (69%)	11 (31%)
Nalidixic acid	3 (9%)	32 (91%)
Cefixime	35 (100%)	0 (0%)
Gentamicin	35 (100%)	0 (0%)

3.4 IgA Antibody avidity in study participants

3.4.1 LPS-specific IgA antibody avidity indices in vaccinees

The mean avidity index (AI) for LPS-specific IgA antibody at time point 1 in Vivotif vaccinees was 43%. The avidity index significantly increased at time point 2 (AI=55%, *P*= 0.0120, SEM= 3.183) and remained elevated until time point 3 (AI= 62%, *P*= 0.0073, SEM= 3.321).

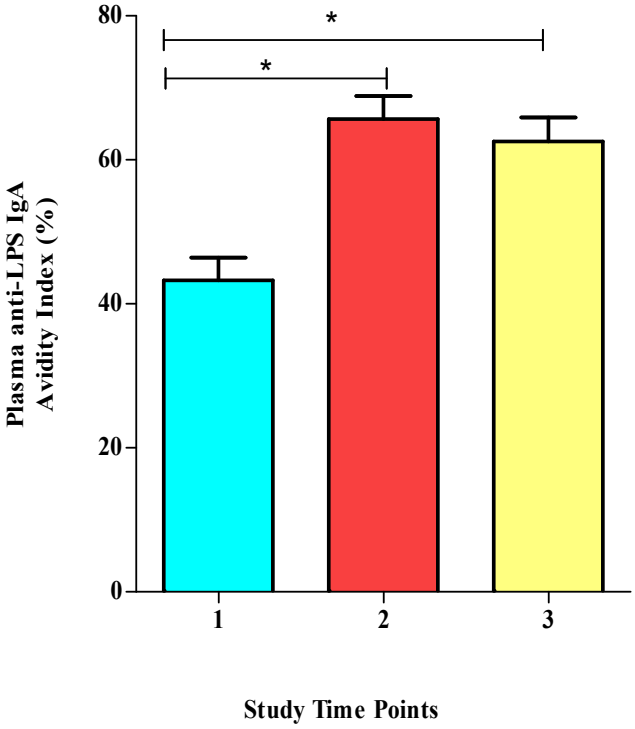

Figure 3.1: Avidity indices (AI) of LPS-specific plasma IgA antibodies in vaccinees aged below 5 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. *, statistically significant difference (P<0.05) from the baseline (study day point 1) titer. Study time points: time point 1: day of enrollment, time point 2: 7 days after 3rd dose of vaccination, time point 3: 28 days after 3rd dose of vaccination.

3.4.2 LPS-specific IgA antibody avidity indices in patients with typhoid fever

3.4.2.1 LPS-specific IgA antibody avidity indices in *S.* Typhi bacteremic patients aged below 5 years

The mean avidity indices (AI) for LPS-specific IgA antibody in patients at time point 1, 2 and 3 were 55%, 56% and 38% respectively. There was no significant difference in the avidity index between study time point 1 and 2. The avidity index was significantly decreased at time point 3 when compared to time point 1 (P= 0.0041) and time point 2 (P=0.0130). .

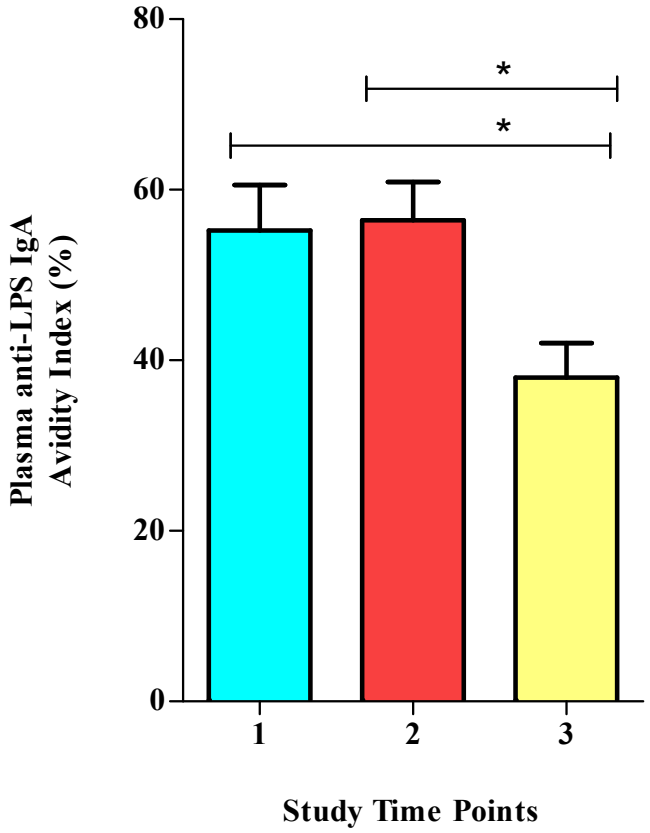

Figure 3.2: Avidity indices (AI) of LPS-specific plasma IgA antibodies in *S.* Typhi bacteremic patients aged below 5 years. Bars indicate the mean, and error bars represent standard errors of the mean (SEM) values. *, statistically significant difference (P<0.05) from the baseline (study time point 1) titer. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.4.2.2 LPS-specific IgA antibody avidity indices in only TPTest positive patients aged below 5 years

The mean avidity indices (AI) for LPS-specific IgA antibody at time point 1, 2 and 3 in this patient group was 29.81%, 31% and 33% respectively. However, no significant difference was seen in the avidity among children in the different phase of the disease. .

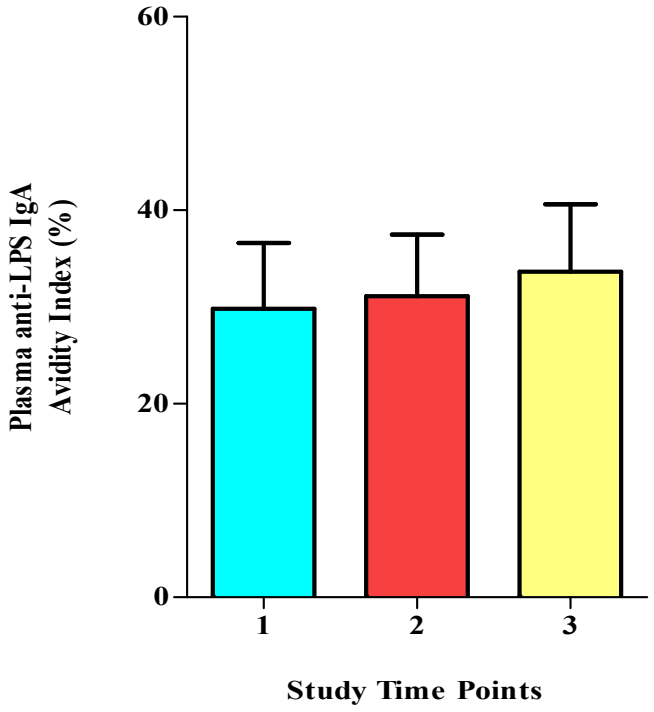

Figure 3.3: Avidity indices (AI) of LPS-specific plasma IgA antibodies in only TPTest positive children aged below 5 years of age. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. The study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.4.2.3 LPS-specific IgA antibody avidity indices in *S.* Typhi bacteremic patients aged between 5 to 17 years

The mean avidity indices (AI) for LPS-specific IgA antibody at study time point 1, 2 and 3 in this age group was 61%, 68% and 58% respectively. No statistically significant change in avidity maturation was observed during the study period.

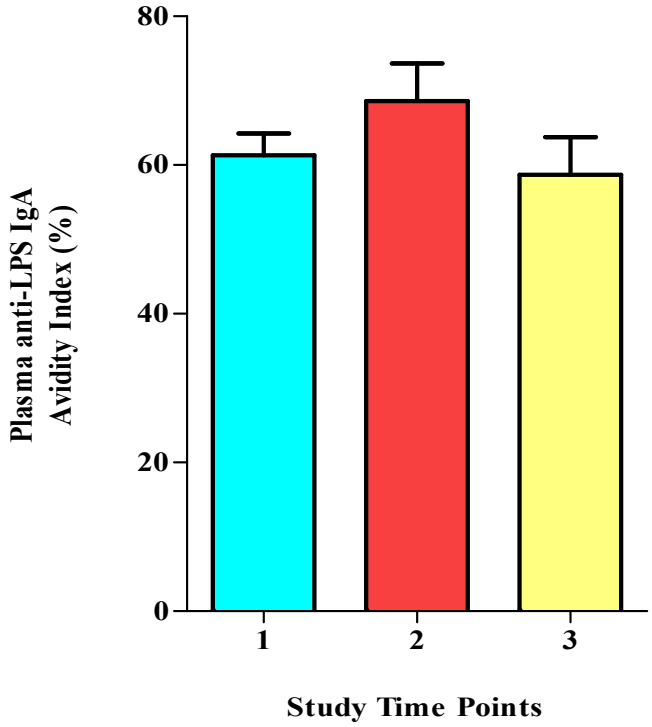

Figure 3.4: Avidity indices (AI) of LPS-specific plasma IgA antibodies in *S.* Typhi bacteremic patients aged between 5 to 17 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment

3.4.2.4 LPS-specific IgA antibody avidity indices in TPTest positive and culture negative patients aged between 5 to 17 years

The mean avidity indices (AI) for LPS-specific IgA antibody at study time point 1 and 2 in this group were 54% and 60% accordingly. Compared to the AI on study time point 2, a statistically significant decline in AI was observed on study time point 3 (AI=50%, *P*= 0.0008, SEM= 5.045).

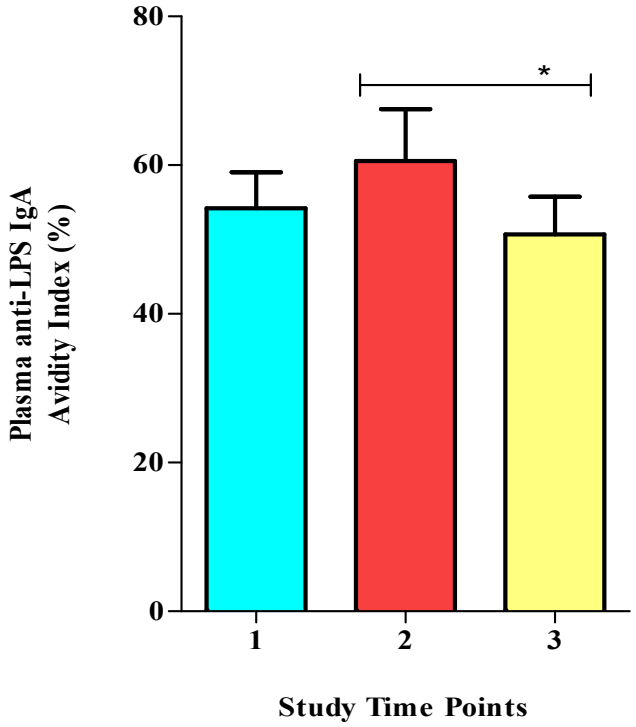

Figure 3.5: Avidity indices (AI) of LPS-specific plasma IgA antibodies in TPTest positive culture negative patients aged between 5 to 17 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. *, statistically significant difference (P <0.05) from the baseline (study time point 1) titer. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.4.2.5 LPS-specific IgA antibody avidity indices in *S.* Typhi bacteremic patients aged above 17 years

The mean avidity index (AI) for LPS-specific IgA antibody at study point 1 in patients aged above 17 years was 57%. However, no significant change in the avidity pattern was seen in subsequent follow ups. The avidity indices on study time point 2 and 3 were 60% and 47% respectively.

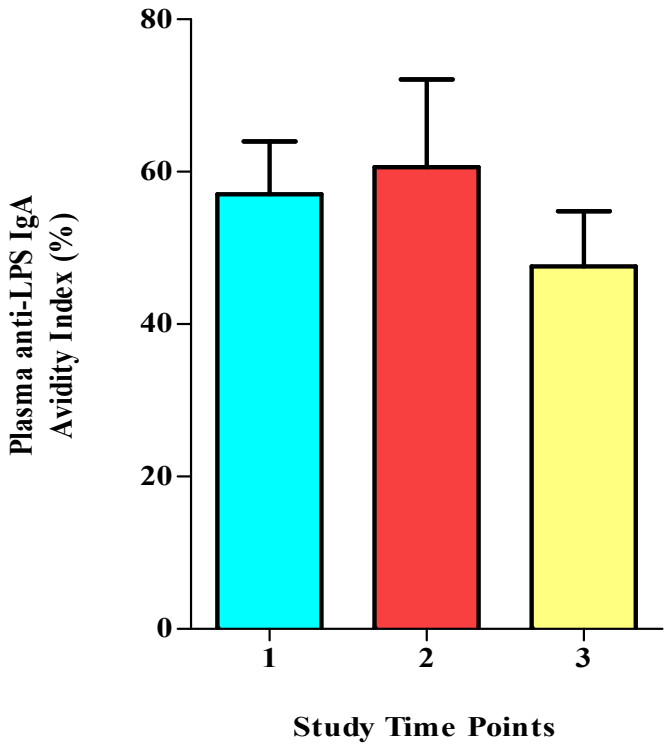

Figure 3.6: Avidity indices (AI) of LPS-specific plasma IgA antibodies in *S.* Typhi bacteremic patients aged above 17 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.4.2.6 LPS-specific IgA antibody avidity indices in only TPTest positive patients aged above 17 years

The mean avidity indices (AI) for LPS-specific IgA antibody at study time point 1 and 2 in this group were both 46%. On study time point 3, the AI decreased to 39%. No statistically significant change in avidity maturation was observed during the study period.

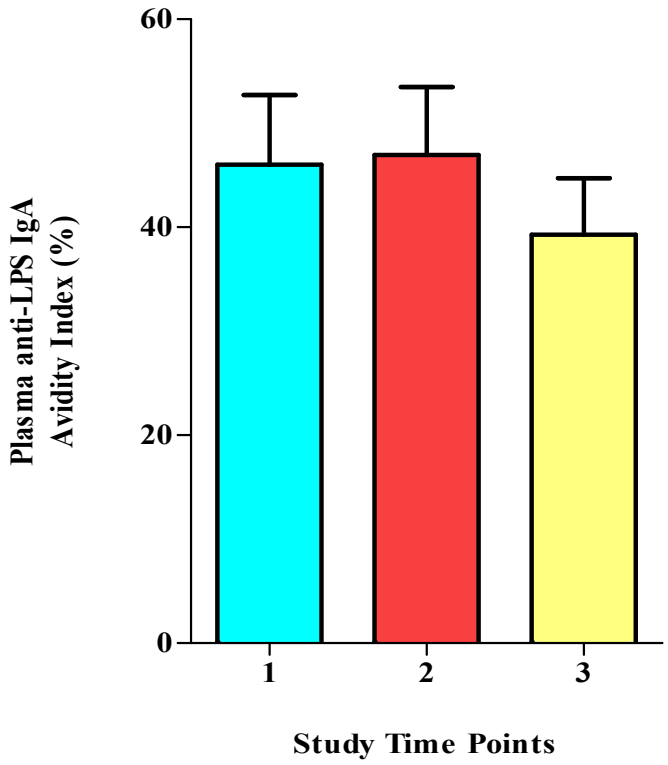

Figure 3.7: Avidity indices (AI) of LPS-specific plasma IgA antibodies in only TPTest positive patients aged above17 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.4.3 Comparison of LPS-specific IgA antibody avidity indices

3.4.3.1 Comparison of LPS-specific IgA antibody avidity indices between typhoid vaccinees and naturally infected typhoid patients aged below 5 years

Among the 24 participants vaccinated with vivotif and the 23 patients aged below 5 years of age, there was a significant difference in IgA avidity indices between study time point 1 of vaccinees and time point 1 of only TPTest positive patients (*P*= 0.0087). Also, the AI of only TPTest positive patients at time point 1 was considerably lower than that of *S.* Typhi bacteremic patients (*P*= 0.0212).

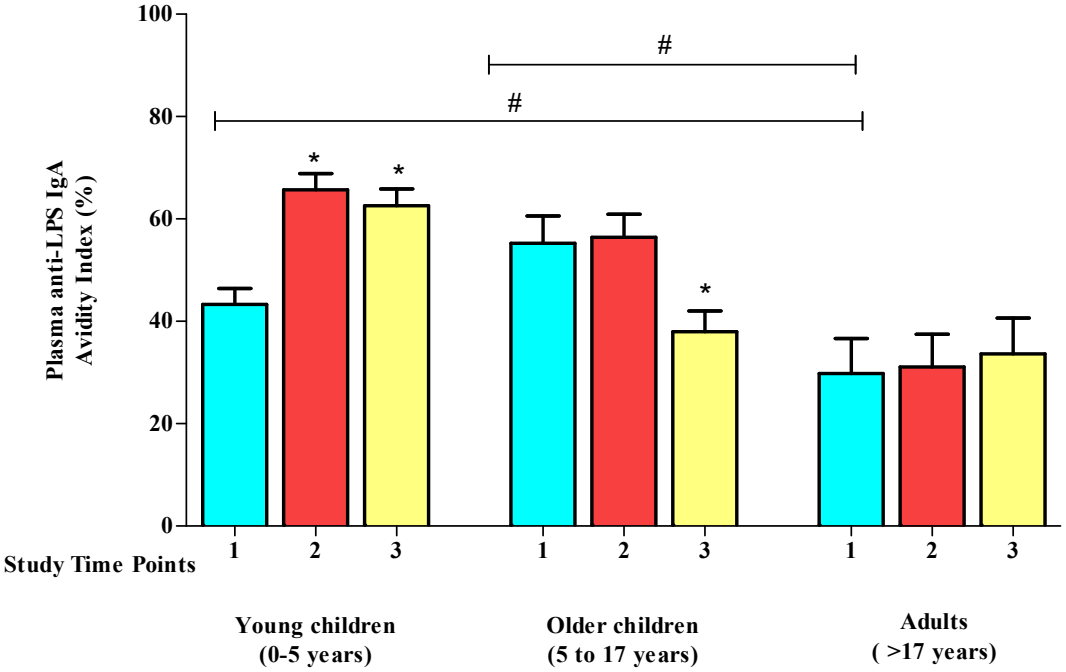

Figure 3.8: Comparison of LPS-specific plasma IgA avidity indices (AI) of between Vivotif vaccines and naturally infected typhoid patients aged below 5 (*S.* Typhi bacteremic and only TPTest positive). Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. *, statistically significant difference (P<0.05) from the baseline (day 0) titer; #, statistically significant difference between different groups (P<0.05). Study time points: time point 1: day of enrollment, time point 2: 7 days after 3rd dose of vaccination (vaccinees) or 7-10 days after enrollment (patients), time point 3: 28 days after 3rd dose of vaccination (vaccinees) or 21-28 days after enrollment (patients).

3.4.3.2 Comparison of LPS-specific IgA antibody avidity indices between different age groups of *S.* Typhi bacteremic patients

While comparing the LPS specific IgA avidity indices among *S.* Typhi bacteremic patients of three different age groups, it was seen that none of them had a significant difference among their avidity maturation profile. Nonetheless, all three age groups showed a similar pattern with study time point 3 having a lower AI than the previous two time points.

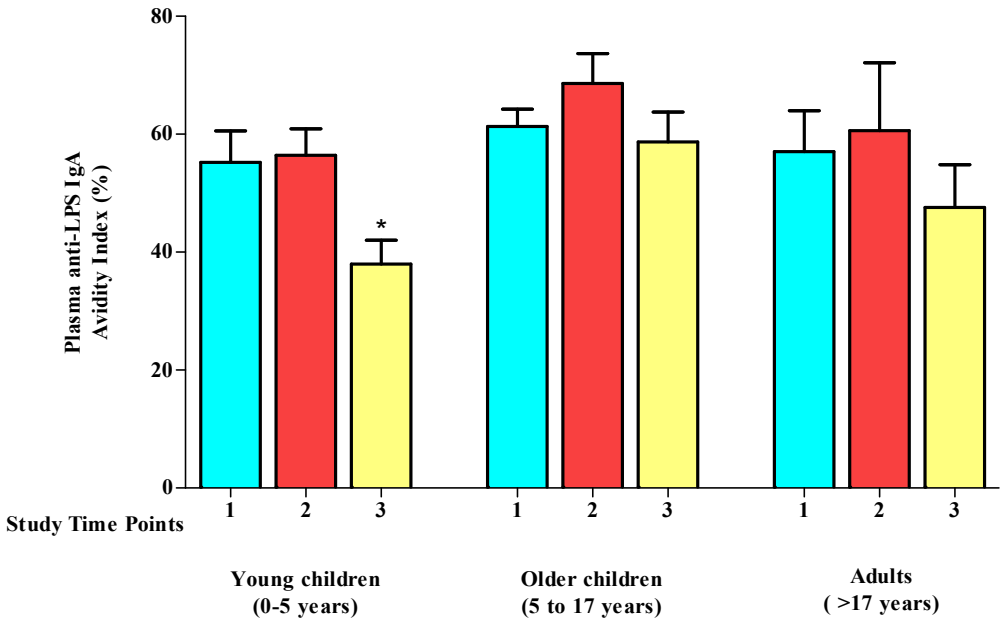

Figure 3.9: Comparison of LPS-specific plasma IgA avidity indices (AI) of among *S.* Typhi bacteremic patients of three different age groups. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. *, statistically significant difference (P<0.05) from the baseline (study time point 1) titer; #, statistically significant difference between different groups (P<0.05). Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.4.3.3 Comparison of LPS-specific IgA antibody avidity indices among different age group of patients positive by the TPTest only

During a comparison of LPS specific IgA avidity indices among TPTest positive patients of three patients in the different age groups, those in group 2 showed an overall higher avidity index (AI) profile. The AI of group 2 on study time point 1 was significantly higher than that of group 1 (AI=54%, *P*= 0.0081, SEM= 4.875).

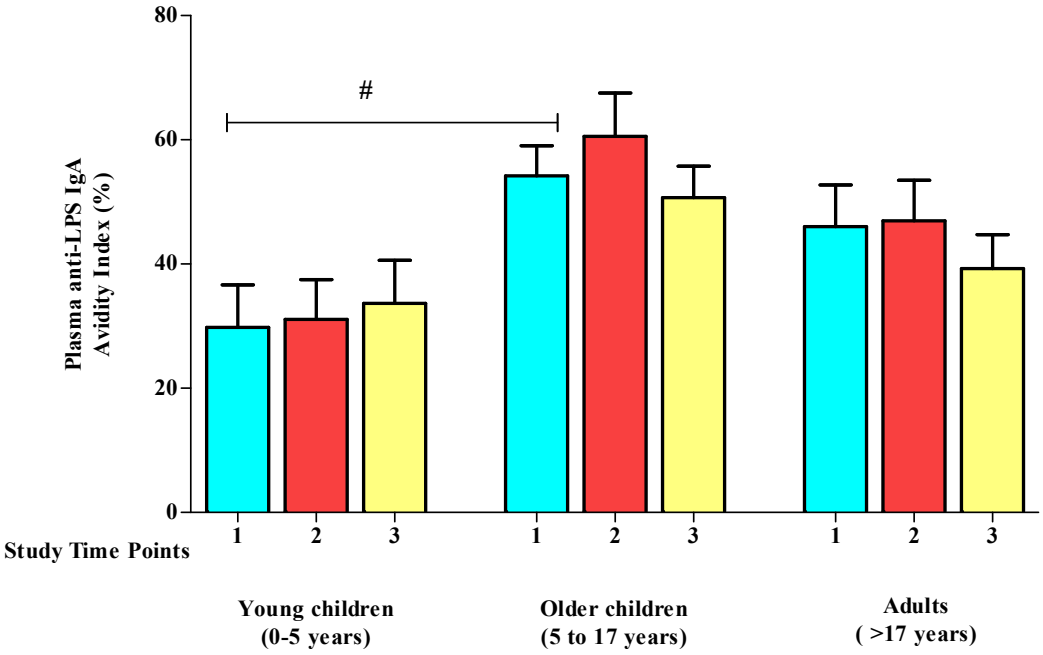

Figure 3.10: Comparison of LPS-specific plasma IgA avidity indices (AI) of among only TPTest positive typhoid patients of three different age groups. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. #, statistically significant difference between different groups (P<0.05). Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.5 IgG Antibody avidity in study participants

3.5.1 LPS-specific IgG antibody avidity indices in vaccinees

The mean avidity index (AI) for LPS-specific IgG antibody at study time point1 in Vivotif vaccinees was 36%. Avidity indices significantly increased at time point 2 (AI=51%, *P*= 0 0.0037, SEM= 2.934) and remained elevated until late convalescence (AI= 47%, *P*= 0.0385, SEM= 2.972).

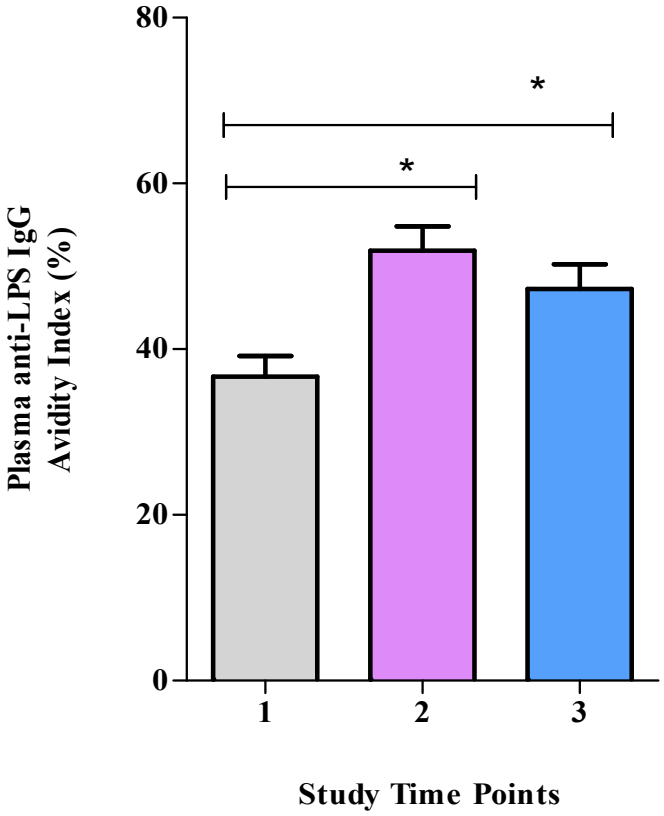

Figure 3.11: Avidity indices (AI) of LPS-specific plasma IgG antibodies in Vivotif vaccinees aged below 5 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. *, statistically significant difference (P<0.05) from the baseline (study time point 1) titer. Study time points: time point 1: day of enrollment, time point 2: 7 days after 3rd dose of vaccination, time point 3: 28 days after 3rd dose of vaccination.

3.5.2 LPS-specific IgG antibody avidity indices in typhoid patients

3.5.2.1 LPS-specific IgG antibody avidity indices in *S.* Typhi bacteremic patients aged below 5 years

The mean avidity indices (AI) for LPS-specific IgG antibody at time point 1, 2 and 3 in this patient group were 48%, 61% and 51% respectively. However, none of the changes were statistically significant.

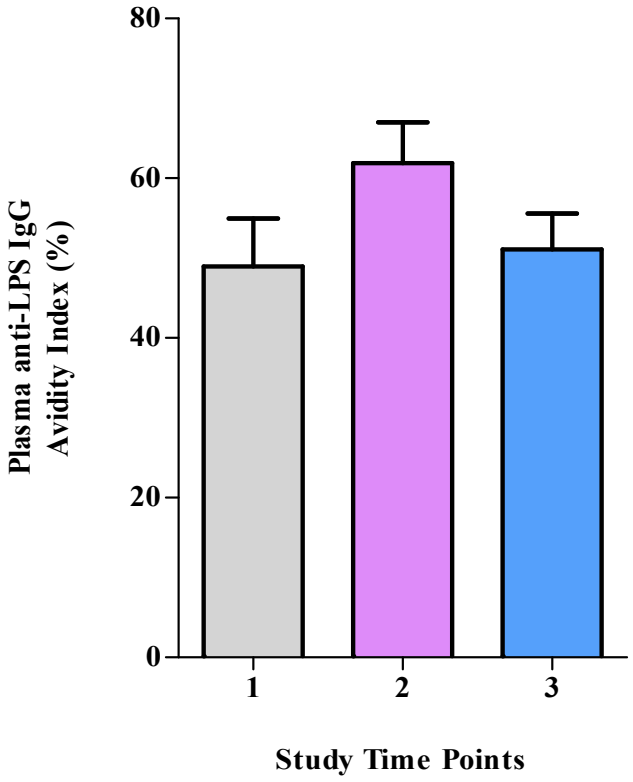

Figure 3.12: Avidity indices (AI) of LPS-specific plasma IgG antibodies in *S.* Typhi bacteremic patients aged below 5 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.5.2.2 LPS-specific IgG antibody avidity indices in TPTest positive culture negative patients aged below 5 years

The mean avidity indices (AI) for LPS-specific IgG antibody at time point 1 in only TPTest positive patients aged below 5 years was 38%. However, no significant change in avidity pattern was seen in subsequent follow ups. The avidity indices on time point 2 and 3 were 37% and 40% respectively.

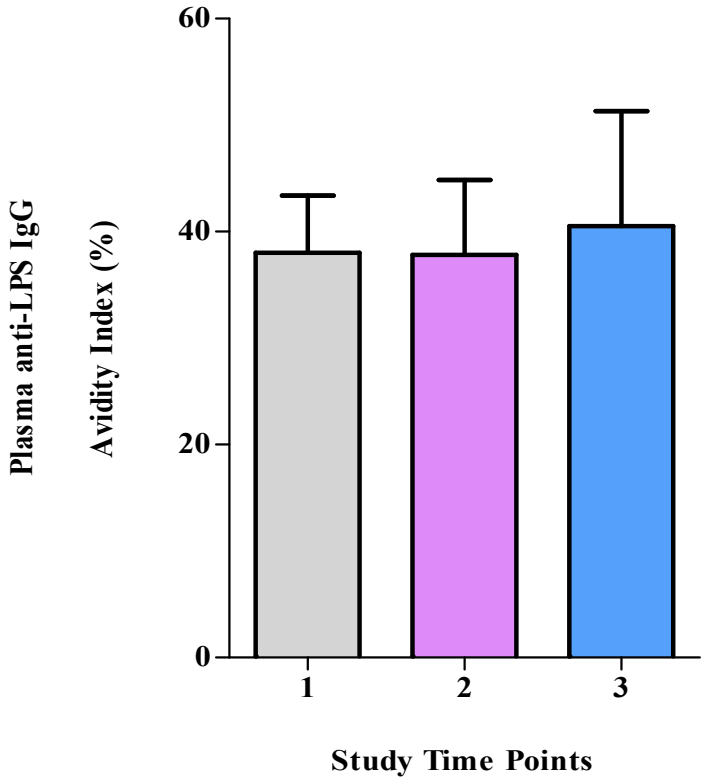

Figure 3.13: Avidity indices (AI) of LPS-specific plasma IgG antibodies in only TPTest positive aged below 5 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.5.2.3 LPS-specific IgG antibody avidity indices in *S.* Typhi bacteremic patients aged between 5 to 17 years

The mean avidity index (AI) for LPS-specific IgG antibody at study time point 1 in this group was 65%. Avidity index decreased steadily to 59% on time point 2, and to 48% on time point 3; though no statistically significant change in avidity maturation was observed during the study period.

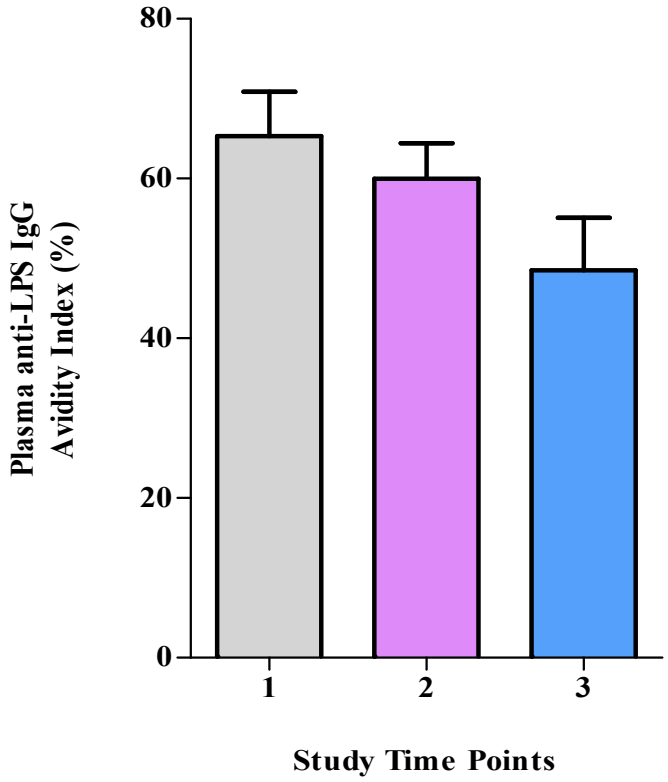

Figure 3.14: Avidity indices (AI) of LPS-specific plasma IgG antibodies in *S.* Typhi bacteremic patients aged between 5 to 17 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.5.2.4 LPS-specific IgG antibody avidity indices in TPTest positive patients aged between 5 to 17 years

The mean avidity indices (AI) for LPS-specific IgG antibody at study time point 1, 2 and 3 in only TPTest positive patients aged between 5 to 17 years were 42%, 58% and 56% respectively. However, none of the changes were statistically significant.

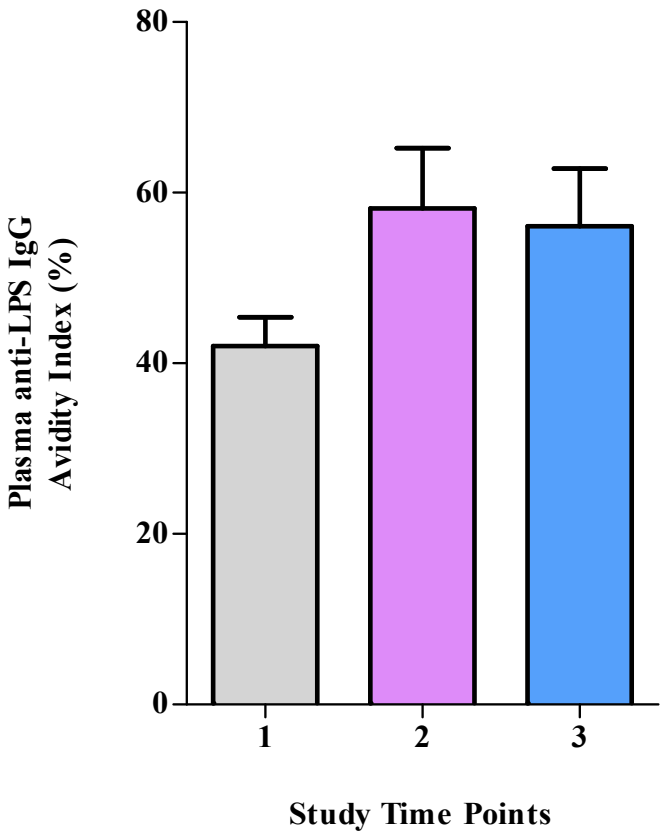

Figure 3.15: Avidity indices (AI) of LPS-specific plasma IgG antibodies in only TPTest positive aged between 5 to 17 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.5.2.5 LPS-specific IgG antibody avidity indices in *S.* Typhi bacteremic patients aged above 17 years

The mean avidity indices (AI) for LPS-specific IgG antibody at time point 1, 2 and 3 in this age group were 65%, 79% and 51% respectively. No statistically significant changes were observed during the study points.

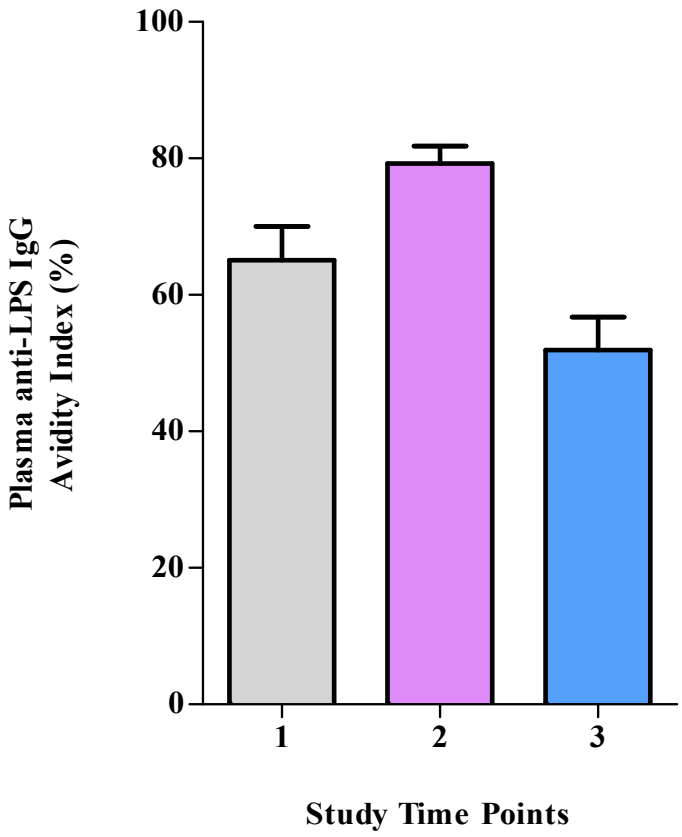

Figure 3.16: Avidity indices (AI) of LPS-specific plasma IgG antibodies in *S.* Typhi bacteremic patients aged above 17 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.5.2.6 LPS-specific IgG antibody avidity indices in TPTest positive patients above 17 years of age

The mean avidity indices (AI) for LPS-specific IgG antibody at study time point 1, 2 and 3 in this patient group were 50%, 60% and 48% respectively. None of these changes were statistically significant however.

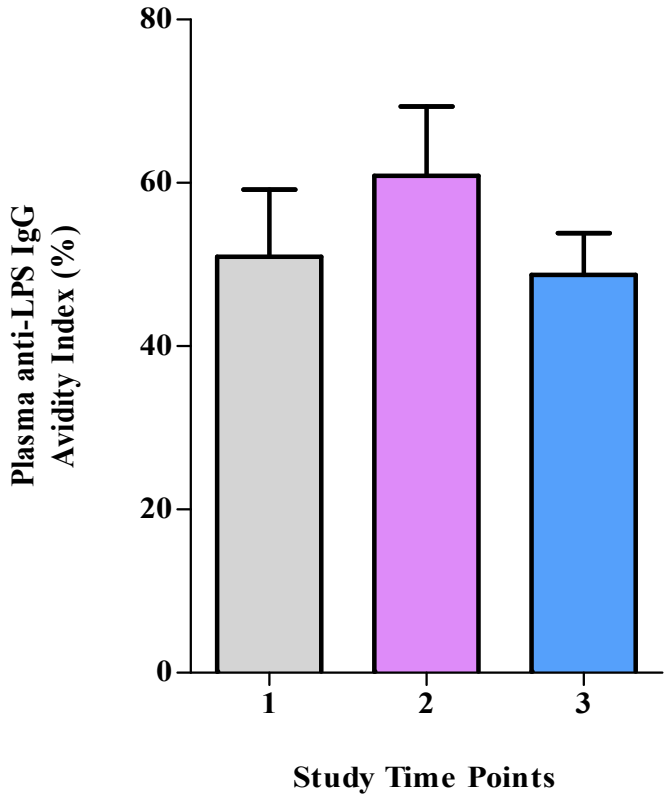

Figure 3.17: Avidity indices (AI) of LPS-specific plasma IgG antibodies in TPTest positive culture negative patients aged above 17 years. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.5.3 Comparison of LPS-specific IgG antibody avidity indices

3.5.3.1 Comparison of LPS-specific IgG antibody avidity indices between vaccinees and naturally infected typhoid patients below 5 years of age

While comparing the LPS specific IgG avidity indices among the 24 vaccinated study participants and 23 patients aged below 5 years of age, there was a significant difference between vaccinees and *S.* Typhi bacteremic patients on study time 1 (p=0.0361), and between *S.* Typhi bacteremic patients and TPTest positive patients on study time 2 (p= 0.0470).

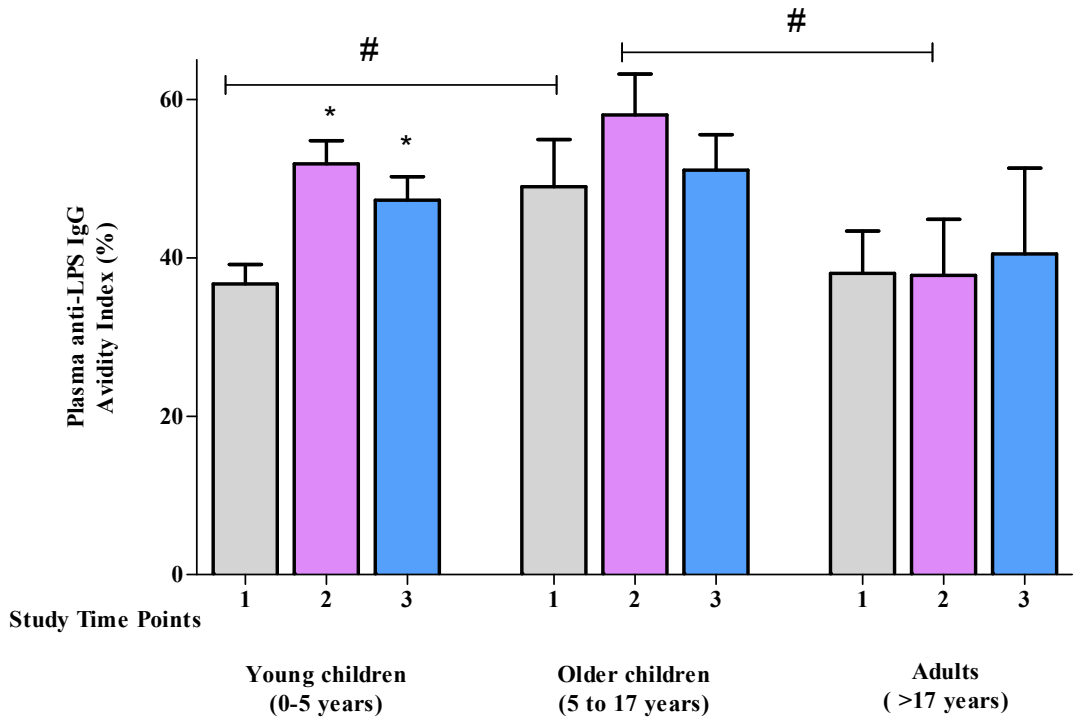

Figure 3.18: Comparison of LPS-specific plasma IgG avidity indices (AI) of between Vivotif vaccines and naturally infected typhoid patients aged below 5 (both culture positive and only TPTest positive). Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. *, statistically significant difference (P<0.05) from the baseline (study time point 1) titer; #, statistically significant difference between different groups (P<0.05). Study time points: time point 1: day of enrollment, time point 2: 7 days after 3rd dose of vaccination (vaccinees) or 7-10 days after enrollment (patients), time point 3: 28 days after 3rd dose of vaccination (vaccinees) or 21-28 days after enrollment (patients).

3.5.3.2 Comparison of LPS-specific IgG antibody avidity indices among different age groups of patients with *S.* Typhi bacteremia

Adults showed significant increase in the LPS IgG avidity indices than young children and older children (p=0.0430) at study time point 2. No difference was found between young and older children at any time point in *S.* Typhi bacteremic patients.

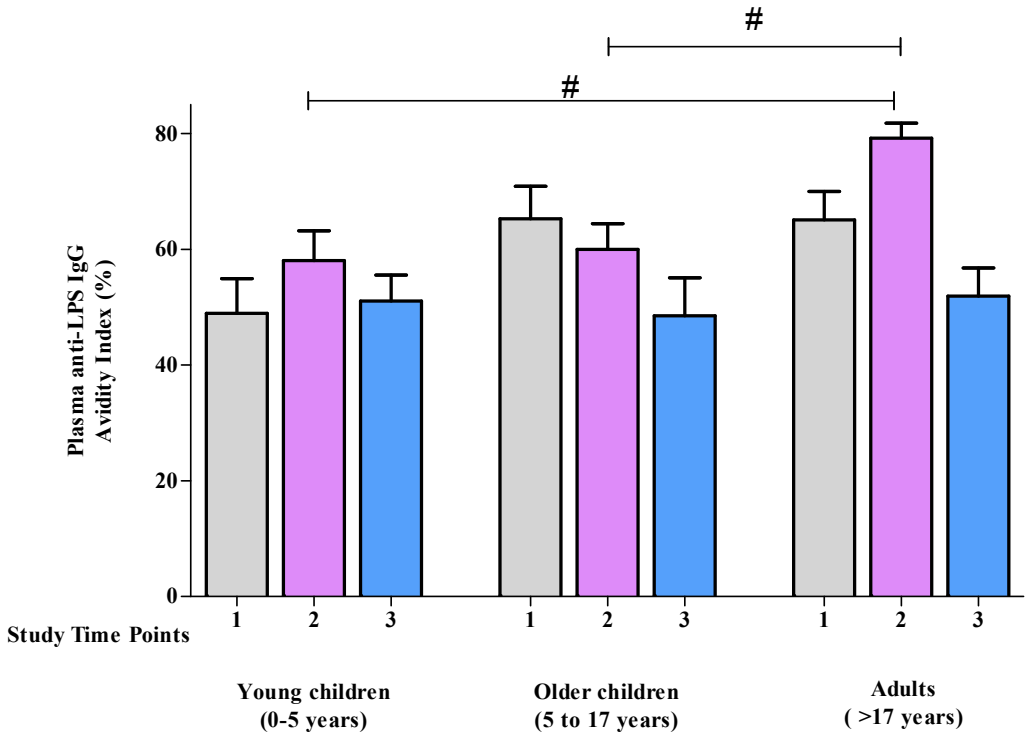

Figure 3.19: Comparison of LPS-specific plasma IgG avidity indices (AI) of among *S.* Typhi bacteremic patients of three different age groups. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. #, statistically significant difference between different groups (P<0.05). Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

3.5.3.3 Comparison of LPS-specific IgG antibody avidity indices among different age group of patients positive by the TPTest by culture negative

A significant difference was found between young children and older children at study time point 2 (p=0.0336). A significant higher response was found in adults when compared to young children at time point 2 (p= 0.0330). No difference was found between older children and adults at any time point.

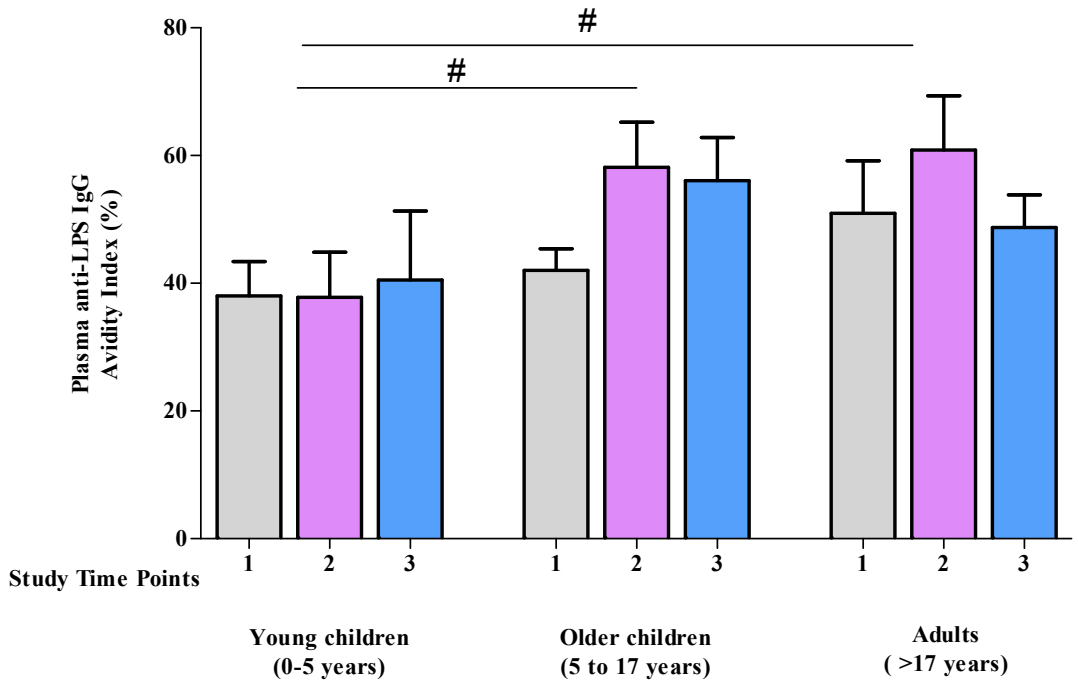

Figure 3.20: Comparison of LPS-specific plasma IgG avidity indices (AI) of among only TPTest positive typhoid patients of three different age groups. Bars indicate mean, and error bars represent standard errors of the mean (SEM) values. #, statistically significant difference between different groups (P<0.05). Study time points: time point 1: day of enrollment, time point 2: 7-10 days after enrollment, time point 3: 21-28 days after enrollment.

CHAPTER 4

Discussion

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The incidence of typhoid fever has markedly reduced in the developed world through effective health care, safe water and improved sanitation measures. Nonetheless, it still remains a public health concern in the developing nations (Crump and Mintz 2010). The south-east Asian countries, including Bangladesh, account for more than 80% of global burden of typhoid fever (Chau, Campbell et al. 2007). As such, it is of tremendous importance to study the immune response in patients with typhoid patients not only to develop effective screening and diagnostic measures, but also to uncover the differences in response in those living in endemic populations and also in different age groups. It is also important to study the differences in response of patients versus vaccinees given the oral typhoid vaccine, Vivotif.

Interestingly, typhoid fever is not only disproportionately distributed regionally, but also among different age groups (Sinha, Sazawal et al. 1999). The highest rates of typhoid fever have been documented in individuals of school going age (1 to 15 years), with children below 5 years of age having the highest incidence of infection (Sinha, Sazawal et al. 1999). Studies carried out in Bangladesh have reported about a 9-fold increased risk for *S.* Typhi infection among children below 5 years of age in comparison to other age groups (Brooks, Hossain et al. 2005). Hence, the immune response in this particular age group needs to be studied with emphasis on the avidity of immune responses generated.

Vaccination for typhoid fever is known to provide a short term protection. However, it may be useful to lower the infection rate among children. Vivotif is a live oral vaccine and contains the attenuated strain of *S.* Typhi 21a. It has been widely studied, and has undergone various clinical trials in different parts of the world; including Egypt (Wahdan, Serie et al. 1982), Chile (Black, Levine et al. 1990) (Ferreccio, Levine et al. 1989), and Indonesia (Simanjuntak, Paleologo et al. 1991). Recent studies in Bangladeshi children have reported the vaccine to be safe for administration among children aged below 5 years of age. Recipients developed IgA, IgG and IgM (32–71%) responses in plasma, and mucosal IgA responses 63–86% in lymphocyte secretions and stool specimens to a membrane preparation (MP) of the vaccine strain Ty21a. An early MP specific proliferative T cell response has also been documented (Bhuiyan, Choudhury et al. 2014).

Nonetheless, to the best of our knowledge, no data on the antibody avidity maturation pattern between Vivotif vaccines and naturally infected typhoid patients in Bangladesh have been reported till date. Therefore, the aim of this study was to characterize the immune responses in vaccinees aged below 5 years, and to compare it with that of similarly aged enteric fever patients, focusing on understanding the differences in antibody avidity responses. Also, antibody avidity pattern was studied among three different age groups of typhoid patients. Moreover, variation in avidity responses were also analyzed between blood-culture positive and TPTest positive non-bacteremic patients. As such, we believe, this is the very first study to evaluate lipopolysaccharide specific IgA and IgG avidity maturation in the Bangladeshi population.

It has already been reported that *Salmonella enterica* serovar Typhi infection stimulates both mucosal and systemic immune responses, as well as cell-mediated immunity (CMI) (Capozzo, Cuberos et al. 2004) (Sheela, Babu et al. 2003). The first line of defense against typhoid fever is mediated in the gut by secretory immunoglobulin A (Corthesy 2009). As expected, vaccinees, upon receiving oral dosage of Vivotif, generated IgA antibodies with significantly higher avidity index on their first follow-up at study time point 2 (AI=55%, *P*= 0.0120, SEM= 3.183). The higher avidity IgA antibodies sustained till the second follow-up on day 28 (AI= 62%, *P*= 0.0073, SEM= 3.321).

In contrast, blood culture positive typhoid patients aged below 5 years maintained a high avidity index (AI) for IgA on the day of enrollment (day 0) and the first follow-up (study time point 2), at 55% and 56% respectively. Compared to responses seen on study time point 1, avidity index was significantly reduced at day 28 (AI=38%, P= 0.0041). Again, in contrast to first follow-up (time point 2), avidity index was considerably lower at second follow-up (time point 3) (AI=38%, P=0.0130). On the other hand, only TPTest positive patients maintained a significantly lower AI for IgA throughout the study period of 28 days. Avidity indices on study time point 1, 2 and 3 were about 30%, 31% and 33% respectively.

This variation in the IgA avidity maturation profile may be attributed to the general practice of medication in the country and due to the infection and reinfection that goes on in the endemic population. Due to lack of awareness and proper governing bodies, people

have easy access to various antibiotics that are often misused in cases such as fever. The common scenario shows that potential enteric fever patients go to a physician only after suffering from fever for a significant length of time (3-7 days). As such, the day of enrollment for culture positive patients may very well coincide with that of the first follow-up of vaccinees. A decline in IgA AI on the second follow-up time point can be explained by the half-life of antibodies. IgA antibodies have a half-life of 6 days (Kindt, Goldsby et al. 2007), indicating that if the system does not remain activated; the IgA produced will be cleared from the system with 12 days of generation. Interestingly, though all patients enrolled in the study were positive for TPTest on study time point 1, only 53% of them generated a positive result on time point 2. Merely 18.7% remained positive until late convalescence about 3 weeks later on study time point 3. This suggests that majority of the patients had recovered during the 28 day follow-up. This is because proper regimen of antibiotic therapy was given to them (Khanam, Sheikh et al. 2013).

In general, among the three age groups, culture positive typhoid patients maintained a higher avidity index- which was comparable to the IgA- AI of time point 2 of Vivotif vaccinees. In contrary, apart from age group 2, all only TPTest positive patients maintained an avidity index which was comparable to the base line of vaccinees. This tendency could very well result from the nature of *S.* Typhi pathophysiology. An inoculum as small as 100,000 organisms of *S.* Typhi causes infection in more than 50% of healthy volunteers (Levine, Tacket et al. 2001). Although TPTest could detect such infections, blood culture requires a much higher inoculum. Hence, culture positive patients, with their higher bacterial load in the system, might have stimulated the immune system more vigorously to produce antibodies with higher affinity.

During typhoid fever, IgG response occur relatively later (Dham and Thompson 1982), (Kumar, Malaviya et al. 1974),(Dragomirescu, Busila et al. 1977), when it reaches the mesenteric lymph node and the spleen. In this study, Vivotif vaccinees produced IgG antibodies with significantly higher avidity on both first (day 14) and second (day 28) follow-up period. Culture positive patients aged below 5 years produced IgG antibodies with AI comparable to that of responses seen on day 14 and day 28 in

vaccinees. Only TPTest positive patients below 5 years of age produced much lower avidity of IgG antibodies.

All culture positive patients produced IgG antibodies with the minimum mean AI being around 49. No significant variation was observed between the different time points within and among the groups. Only TPTest positive patients, however, showed a varied degree of maturation. Group 1 produced IgG antibodies with no higher AI than around 41. Group 2 and group 3 patients, on the other hand, produced IgG with relatively higher avidity indices.

Generation of higher avidity IgG antibodies in patients aged above 5 years may be due to a result of previous exposure to the pathogen. Bangladesh, being an endemic region for typhoid, has a higher exposure rate to *S.* Typhi. The older a person is, the higher the chances are of previous exposure. Such a situation may generate higher affinity antibody production, yielding a higher avidity index. Also, a half-life of 9 days for IgG (Kindt, Goldsby et al. 2007) suggests that any IgG antibodies produced during the infection period might not have been cleared away during the follow-up, resulting in no significant variation among the follow up periods..

In summary, vaccination of children aged below 5 years with Vivotif generated LPS specific IgA and IgG antibodies with significantly high avidity. These highly avid antibodies first appeared on day 14 and were sustained until day 28. In the patient group, culture positive patients in general produced antibodies with higher affinities than those who were only TPTest positive.

In the future, a longer follow-up of both Vivotif vaccinees and typhoid patients could reveal further patterns in avidity maturation. Also, since TPTest explores the immunogenicity of a membrane preparation (MP) of *S.* Typhi strain Ty21a, it is only logical to explore the avidity pattern against MP as well. Memory induction in case of both vaccination and natural infection could further be validated using other immunological assays including the measurement of antibody secreting cells. .

CHAPTER 5

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Appendix

Laboratory Apparatus

1. Eppendorf tubes and micropipette tips were taken from Eppendorf® and Sigma, and were sterilized by autoclaving at 121°C for 20 minutes.
2. Petridishes used in the experiments were provided by either Sterilin or Gibco. Screw capped tubes and other glass wares were taken from Pyrex® Labware, USA.
3. Plastic tubes and pipettes were of Falcon®; both were the brands of Becton, Dickinson and Company. 96-well ELISA plates were obtained from Nunc™, Sweden.
4. Micropipettes were from Thermo Labsystems.
5. Mini scale centrifugations were carried out in a Sorvall® *pico* microfuge and large-scale centrifugation were carried out in a Sorvall® Legend™ *RT* super speed centrifuge. ELISA reading was taken using ASCENT Multiskan® reader.
6. Heparin-coated sterile vacutainer tubes (Becton Dickinson, Ruther-ford, NJ)
7. Multi-channel dispenser (Lab System, USA).

Chemical Reagents:

1. Ficoll, Pharmacia LKB Biotechnology AB Uppsala, Sweden.
2. Rabbit anti-human immunoglobulin horseradish peroxidase, Jackson Immuno Research, West Grove, P. A., USA.
3. H_2O_2 (Hydrogen Peroxide), Fisher Scientific, H-325.
4. FBS (Foetal Bovine Serum Albumin), Gibco BRL- 16140-071.
5. Goat Anti Human IgG $F(ab)_2$, Jackson Immuno Research 109-005-097.
6. NaCl (Sodium Chloride), Fisher Scientific, Pittsburgh, PA, USA.
7. KH_2P0_4 (Potassium Phosphate).

8. Tween 20 (polyoxietylensorbitanmonolaurat), Sigma chemical Co., St. Louis, MO, USA
9. NaCl (Sodium Chloride), Fischer Scientific, Pittsburgh, PA, USA.
10. Methanol, Merck KGA, Index No-603-001-00-X, Darmstadt, Germany.
11. Napthol, Index- A8625-25G, Sigma Chemical Co., St. Louis, MO, USA.
12. DAB, Lot-D 5637-10G, Sigma ALDRICH.Inc. USA.
13. Na-Acetate, Lot-93H-111-515, Molecular Sigma Biology.
14. Citric Acid, Lot-705455, Fischer Scientific, New Jersey, USA.
15. TMB, Lot-SLBC4166V, Sigma ALDRICH.Inc. USA.
16. Biotin, Lot-3830-4-250, Batch-3, Fischer Scientific, Pittsburgh, PA, USA.
17. Streptavidin, Lot-3420-2H (mabtech), Batch-73, Fischer Scientific, Pittsburgh, PA, USA.
18. Nitrocellulose membrane 0.45 micron, BIO-RAD.
19. Bovine Serum Albumin (BSA), Sigma A-4503.
20. Tris, Molecular Sigma Biology.

Buffers and Substrate Solutions

1. Preparation of phosphate buffer saline (PBS) (Vacutainer System; Becton Dickinson, Rutherford, NJ) (pH 7.2)

NaCl *(Fischer Scientific, Pittsburgh, PA, USA)*	80.00 g
Na_2HPO_4	11.50 g
KH_2PO_4	2.00 g
KCL (*Fischer Scientific, Pittsburgh, PA, USA*)	2.00 g
Deionized water	1000.0 ml

The concentrated solution (10xPBS) was diluted ten times and was used as working solution.

2. Preparation of 1% BSA in PBS (500 ml)

Phosphate Buffer Saline (PBS)	500 ml
Bovine Serum Albumin (BSA)	5 g

3. Preparation of 0.1% BSA in PBS-Tween (500 ml)

Phosphate Buffer Saline (PBS)	500 ml
Bovine Serum Albumin (BSA)	0.5 g
Tween	250 µl

4. Preparation of 0.1 M Sodium citrate buffer (pH 4.5) (1000ml)

Tri-natrium citrate ($Na_3C_6H_5O_4.2H_2O$)	29.4 g
H_2O (deionized)	1000.0 ml

5. Preparation of Orthophenylene diamine - H_2O_2 substrate (10 ml)

OPD	10.0 mg
0.1 M sodium citrate (pH 4.5)	10.0 ml
30% H_2O_2	4.0 m

Media Preparation:

Preparation of RPMI complete medium (200 ml)

RPMI 1640 (1X)	200 ml
Fetal Bovine Serum (FBS- 10%)	20 ml
Pen/Strp (Penicillin-Streptomycin- 1%)	2ml
Na-Pyruvate (1%)	2ml
L- Glutamine (1%)	2ml

Motility Indole Urea (MIU)

Peptone	30.0 g
KH2PO4	2.0 g
Sodium chloride	5.0 g
Phenol Red	0.005 g
Urea	20.0 g
Bacto agar	4.0 g
Distilled water pH =7.3+ 0.2	1000 mL

Citrate agar (Simmons, 1926 Modified)

NaCl	5.0 g
MgSO4	0.2 g
NH4PO4	1.0 g
K2HPO4	1.0 g
Sodium citrate	2.0 g
Bacto Agar	20.0 g

Muller Hinton Agar (1000 mL)

Beef, infusion form	300 g
Casein hydrosate	17.5 g
Starch	1.5 g
Bacto Agar	17.0 g
Distilled water pH =7.3+ 0.1	1000 mL

Reagent Preparation:

Preparation of Ammonium Chloride Lysing Solution (10X)

Ammonium Chloride Lysing Solution (200 ml):

1. Weighted the following reagents:

Ammonium Chloride	16.58gm
Potassium bicarbonate	2gm
EDTA	0.074gm

2. Dissolved in D/W to get desired volume in beaker.

3. Mixed with magnetic mixer for 15 minutes.

4. Adjust pH to 7.2 with glacial acetic acid.

5. Filtered through a 0.2 μm filter.

PBMC calculation

After PBMCs were separated and they were resuspended in 10 ml PBS

If the amount of cell counted in hemocytometer is X

Then, as the sample was 2 times diluted by adding dye,

0.1 µl of the original sample contain= 2x cells

So, 10 ml of the original sample contain= ($2x \times 10 \times 10^3$) cells=$2x \times 10^5$ cells

Preparation of Tris (1000 ml)

Tris	2.42 gm (20mM)
NaCl	29.22 gm (0.5M)

Adjust pH 7.5 with 6M HCl and made up final volume with deionized water to 1L (dissolve in approximately 600 ml deionized water).

Preparation of 4-CN (200ml)

Methanol	200 ml
Napthol	0.6 gm

Preparation of 4-CN Substrate (10 ml)

4-Chloro-1-Napthol (4-CN)	1.7 ml
Tris Buffer Saline (TBS)	8.3 ml
H_2O_2 (Hydrogen Peroxide)	10 µl

Composition of Tryphain Blue (100 ml)

Sodium Chloride (NaCl)	0.81 gm
Potassium Phosphate (K_2PO_4)	0.06 gm
Tryphan Blue	0.4 gm
Distilled Water	100 ml

Then mixed properly.Then aliquot in eppendrof tubes and seal with parafilm as Tryphan Blue is light sensitive.It is kept in room temperature.