Optimization of Immunodot Blot Assay for Diagnosis of Typhoid and Paratyphoid Fever Using the TPTest for Comparison

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SUBMITTED BY
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DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES
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DEDICATED

TO

MY BELOVED PARENTS

DECLARATION

This to declare that the research work embodying the results reported in this thesis entitled **"Optimization of Immunodot Blot Assay for Diagnosis of Typhoid and Paratyphoid Fever Using the TPTest for Comparison"** has been carried out by the under signed under joint supervision Professor Dr. Naiyyum Choudhury, Co-ordinator, Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences, BRAC University and Professor Dr. Firdausi Qadri, Senior Scientist and Head in the Immunology Laboratory of the Centre for Vaccine Science (CVS) at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). It is further declared that the research work presented here is original and submitted in the partial fulfillment for the degree of Master of Science in Biotechnology, BRAC University, Dhaka and has not been submitted anywhere else for a degree or diploma.

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Abstract

Typhoid and paratyphoid (enteric) fever are responsible for morbidity and mortality worldwide. Bangladesh is an endemic zone of enteric fever where sanitation and hygienic conditions are poor. Rapid, accurate diagnosis of enteric fever and early treatment of the patients with appropriate antimicrobials are essential for recovery and for prevention of complications and death. Bone marrow culture is the gold standard for diagnosis of enteric fever but it is an invasive procedure and blood culture method and widal test have low sensitivity and specificity respectively. Thus development of an early and reliable diagnostic method is necessary. Previous studies reported that a novel diagnostic method, TPTest is very useful for diagnosis of typhoid and paratyphoid fever and it gives a preliminary result within 24 hours and a confirmatory result after 48 hours. The sensitivity and specificity of the TPTest are 100% and 78-97% respectively. The TPTest detects *Salmonella enterica* serotype Typhi specific antibodies in peripheral blood antibodies (PBA) specimen by ELISA. The PBA specimen is prepared by isolating the lymphocytes form whole blood using ficoll density gradient centrifugation method and culturing the cells at $37^{o}C$ with 5% CO_2 supplementation. For implication of the TPTest in laboratories with less facilities and expertise immunodot blot assay was used to detect the response in PBA specimen. The specimen was prepared by isolating the blood cells using RBC lysis and culturing the cells at $37^{o}C$ with no 5% CO_2 supply. In this study, optimization of the simplified methods of the TPTest was done at different stages and the results were evaluated with the results of blood culture and with the standard TPTest method. The findings of this study suggest that the optimized immunodot blot assay and the simplified cell separation methods will be helpful for diagnosis of typhoid and paratyphoid fever.

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Chapter 1

Introduction

1.1 Background

Enteric (typhoid and paratyphoid) fever is a serious and sometimes life-threatening infection. Enteric fever is referred to as typhoid fever when it is caused by *Salmonella enterica* serovar Typhi (*S.*Typhi) and paratyphoid fever when it is caused by *Salmonella enterica* serovar Paratyphi (*S.* Paratyphi) A, B and C [1]. Enteric fever is common in resource-limited regions in the world with lack of proper sanitation and poor hygiene [2].

1.2 Epidemiology of enteric fever

1.2.1Global epidemiology

Typhoid fever is a major cause of morbidity [3]. It is estimated global incidence of 21.6 million cases and 216,510 deaths worldwide in 2000 [4] [5]. Particularly typhoid fever burden assumed to be underestimated in urban areas. It has been controlled by good sanitation and chlorination of drinking water in the developed countries. But poor sanitation, poverty and contaminated water cause 5% or more deaths due to typhoid fever in the developing countries [3]. Each year *Salmonella enterica* serotype Paratyphi A infections also causes an additional 5 million cases of enteric fever [6].

High incidence of enteric fever (>100/100,000 cases/year) is observed in south-central Asia and south-east Asia whereas regions of medium incidence (10-100/100,000 cases/year) include the rest of Asia, Africa, Latin America, and Caribbean and the Oceania, except for Australia and New Zealand. Europe, North America and the rest of the developed world have low incidence of enteric fever (<10/100,000 cases/year) [7] [8]. *Salmonella enterica* serovar Paratyphi A (*S.*Paratyphi A) is assumed to cause less severe enteric fever than *Salmonella enterica* serovar Typhi (*S.*Typhi) [9]. *S.* Paratyphi A is liable for approximately 5 million illnesses in the 2000 [1].

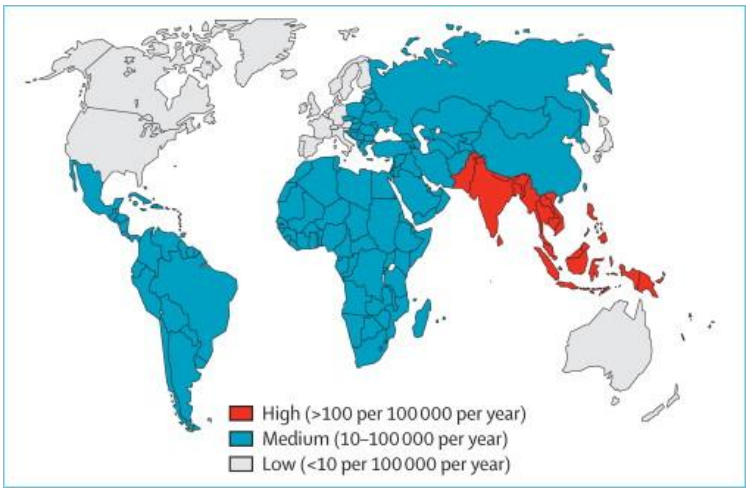


Figure 1: Global incidence of enteric fever [10]

Peak incidence of typhoid fever is reported to occur in children 1–15 years of age. Children less than 5 years of age have the highest infection rates. Paratyphoid fever has a similar clinical feature of typhoid fever [5].

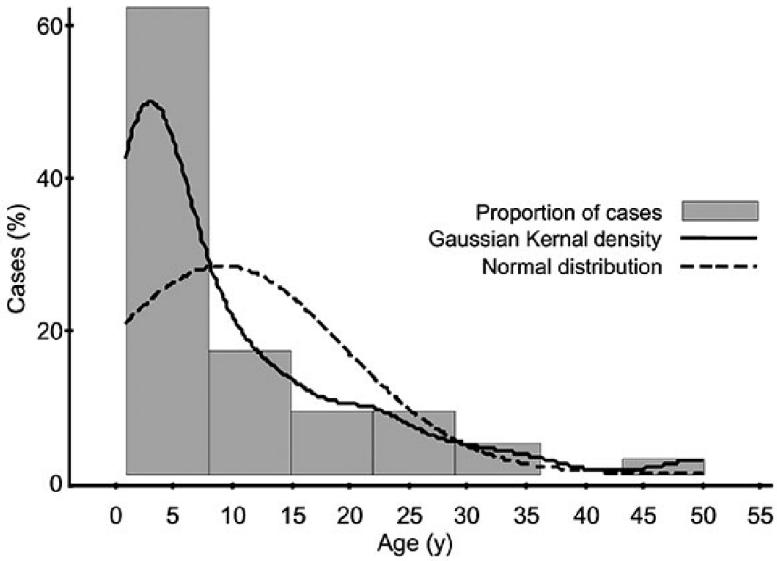


Figure 2: Distribution of enteric fever by age [5] [11]

1.2.2. Epidemiology of enteric fever in Bangladesh

In Bangladesh the incidence of typhoid fever is high in the urban population. The greatest incidence of infection is in children less than 5 years of age [12] [13] [14].53% of typhoid fever cases in the community are seen in this group. 85% of cases occurring among children aged 1-4 years of age. The overall incidence of typhoid fever is 3.9/1,000 persons per year, but in young children it is 18.7/1,000 persons per year. Typhoid fever among pre-school children varied by age, with 4% in the first year of life and 85% occurring in those 2 to 4 years of age [15] [13]. Additionally, young children appear to be clinically more ill symptoms compare to older persons. Typhoid vaccines so far developed are not effective in children. So vaccine development against typhoid fever is needed for children aged less than 2 years to protect them from the infection [16] [17] [18]. The incidence of paratyphoid fever was 0.4/1000 person per year without variation by age group [12].

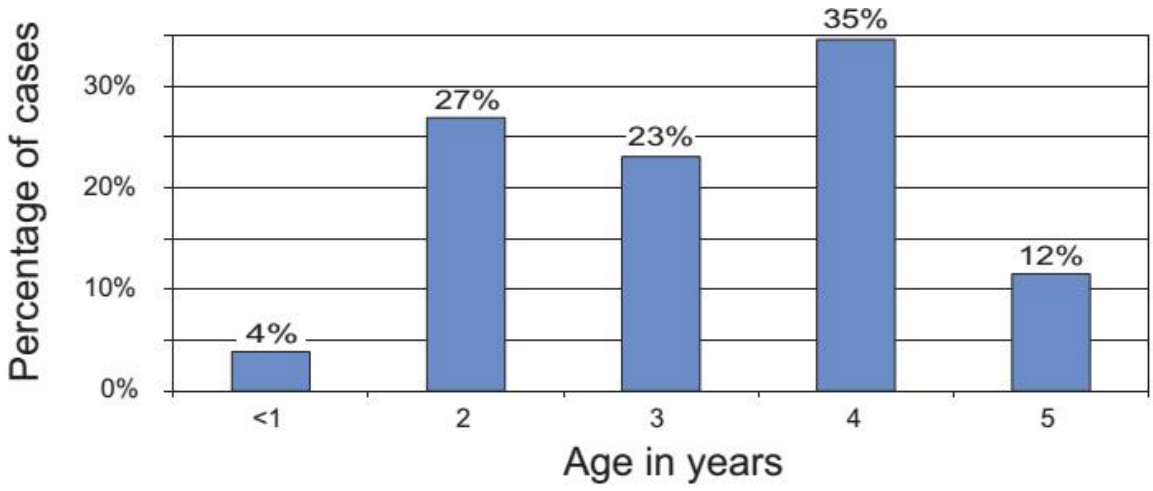


Figure 3: Age distribution of typhoid cases for patients <5 years of age, Kamalapur 2001 [14].

1.3 Classification, structure, antigenic type of *Salmonella* Typhi

1.3.1 Classification

All of the *Salmonella* serovar belong to two species: *Salmonella bongori* containing eight serovar and *Salmonella enterica* containing the remaining 2300 serovar divided among six subspecies [19].

- *Salmonella enterica subspecies enterica*
- *Salmonella enterica subspecies salamae*
- *Salmonella enterica subspecies arizonae*
- *Salmonella enterica subspecies diarizonae*
- *Salmonella enterica subspecies houtenae*
- *Salmonella enterica subspecies indica*

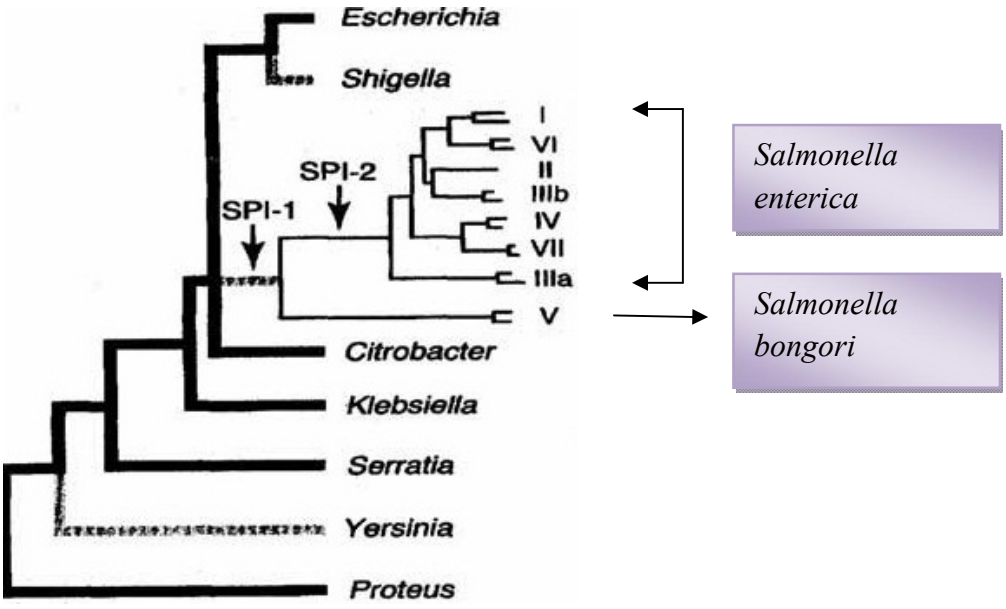


Figure 4: Phylogenetic Tree of the evolution of Salmonella species within closely related families [19].

Among them *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi are the causative agents for enteric fevers: typhoid and paratyphoid respectively.

1.3.2 Structure

Salmonella Typhi is a gram-negative, flagellate, encapsulated, nonsporulating, facultative anaerobic bacteria in the family of Enterobacteriaceae. 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 [20] [21].

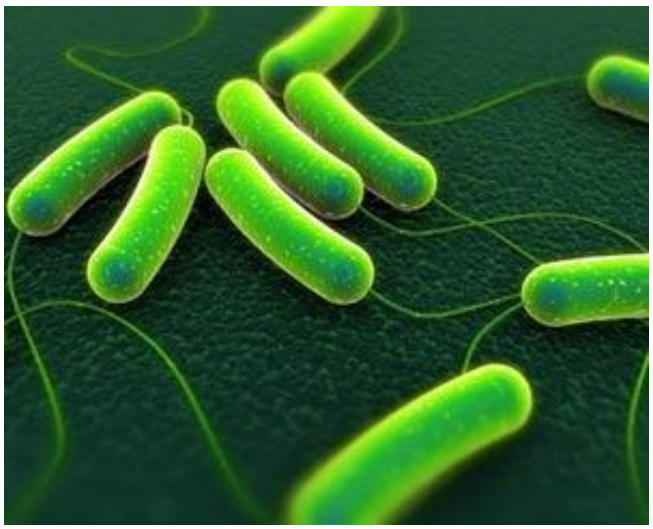


Figure 5: Physical structure *Salmonella enterica* serovar Typhi and Paratyphi [20]

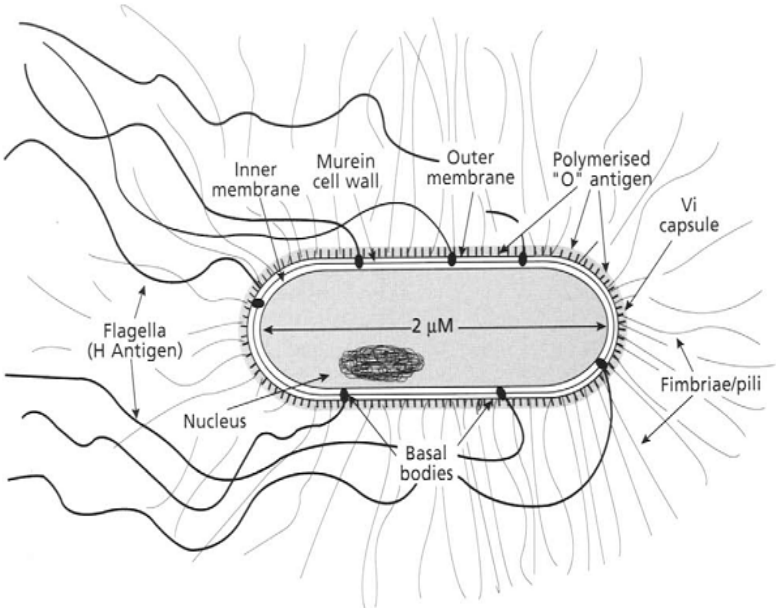


Figure 6: Physical structure *Salmonella enterica* serovar Typhi and Paratyphi [20]

1.3.3 Genome structure of *Salmonella* Typhi

The full genome of *Salmonella enterica* serovar Typhimurium strain LT2 is 4.8-Mb. The plasmids are 218 kb and 106 kb. These plasmids have different drug resistant bands [22].

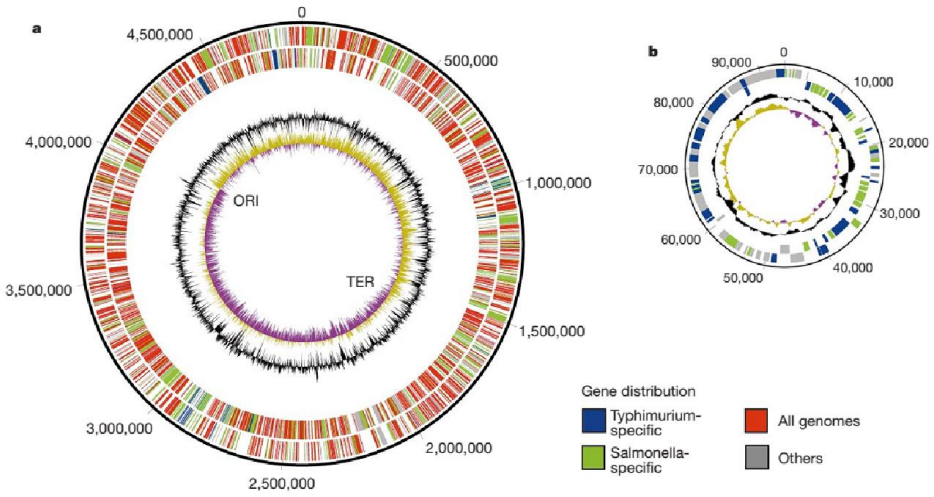


Figure 7: *Salmonella enterica* serovar Typhimurium LT2 genome. (a)The black inner circle is G+C content and the purple or yellow innermost circle is GC bias [22]

1.3.4 Antigenic types of *Salmonella* Typhi

Salmonella has three kinds of major antigens with identifying applications:

1.3.4.1 O antigens or somatic antigens

Somatic or cell wall antigens also known as O antigens. These antigens are bacterial endotoxin which causes fever. Somatic antigens are heat stable and alcohol resistant. 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 [23] [21].

1.3.4.2 H (flagellar) antigens

The H antigens are present in flagella and composed of protein subunits called flagellin. Flagellar antigens are heat-labile proteins and also alcohol labile. This antigen does not have any significant effect on the virulence of *S.* Typhi. Mixing *Salmonella* cells with flagella-specific antisera gives a characteristic pattern of agglutination. They are well preserved in 0.2 % formaldehyde. Flagella of *S.* Typhi induce different pro-inflammatory cytokines [24, 25] [26] [27].

1.3.4.3 Vi – antigen

The surface Vi (for virulance) antigen, a polysaccharide on the exterior of the cell wall. This Vi polysaccharide antigen prevents O antibodies from binding to the O antigen. Surface antigens in *Salmonella* may mask O antigens. The bacteria will not be agglutinated with O antisera [28] [29] [30] [31].

The cell envelope of Salmonellae contains a complex lipopolysaccharide (LPS) structure. The lipopolysaccharide moiety may function as an endotoxin. These may be important in determining virulence of the organisms. This macromolecular endotoxin complex consists of three components:

- An outer O-polysaccharide coat
- A middle portion (polysaccharide core)
- An inner lipid A coat

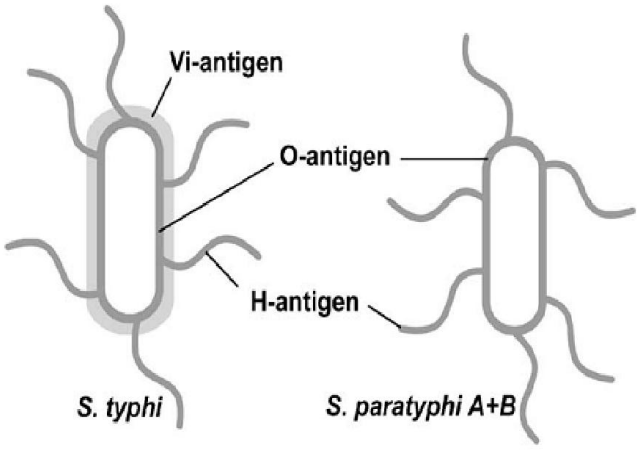


Figure 8: Antigenic structure of *S.* Typhi and *S.* Paratyphi [31]

1.3 Molecular and biologic features of *Salmonella* Typhi

A comparison of *S.* Typhi isolates from around the world indicates that they are highly related and that they emerged from a single point of origin approximately 30,000 to 50,000 years ago. Different strains may also harbor extrachromosomal DNA in the form of plasmids which usually carry virulence or antibiotic resistance genes [32].

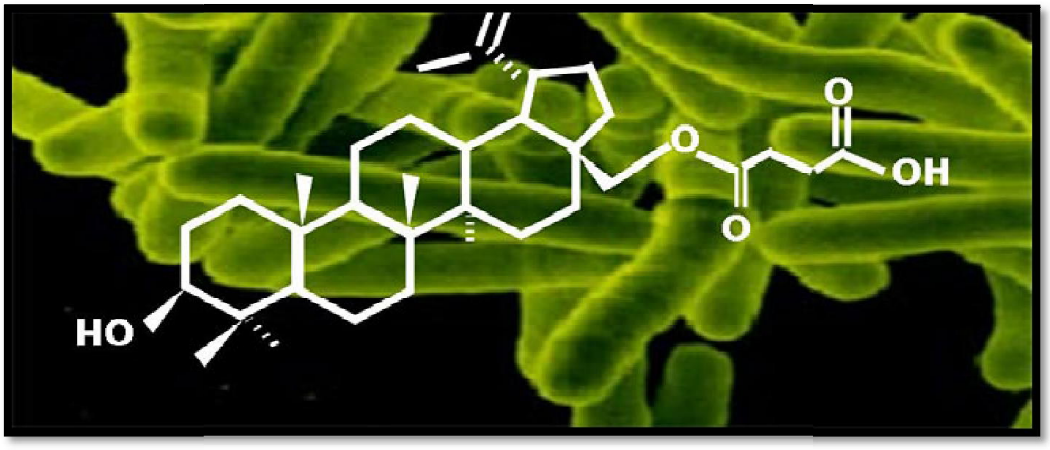


Figure 9: Molecular and Biologic Features of *Salmonella* Typhi **[33]**

1.5 Type three secretion systems (TTSS) of bacteria to translocate virulence factors to the host during infections

After a foreign genome is integrated into the bacterial chromosome the genes for virulence factors cluster in Pathogenicity islands (PI). Non-pathogenic related species of *Salmonella* do not have PIs. PIs gene expression is generally limited to specific host compartments [34].

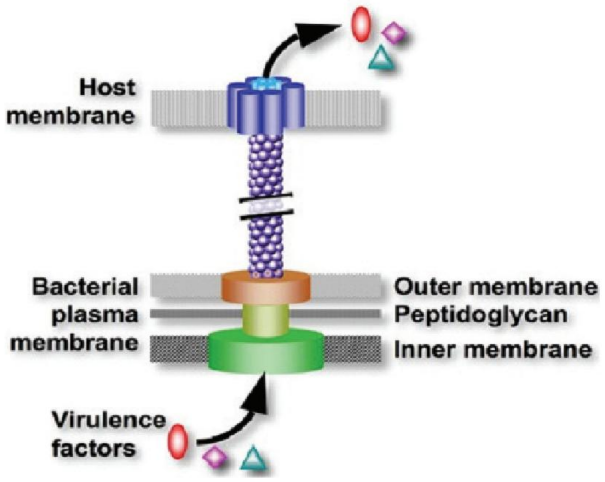


Figure 10: Type Three Secretion Systems (TTSS) of Bacteria to Translocate Virulence Factors to the Host During Infections [35]

1.6 Pathogenesis of enteric fever

The approximate number of bacteria that must be ingested to cause symptomatic disease in healthy adults is 10^6- 10^8 *Salmonella* organism. Most organisms are rapidly killed at gastric p^H ≤ 2. The gastric acidity inhibits multiplication of the Salmonellae. Achlorhydria, buffering medications, rapid gastric emptying after gastrectomy or gastroenterostomy and a large inoculum enable viable organisms to reach the small intestine. Neonates and young infants have hypochlorhydria and rapid gastric emptying, which contribute to their increased vulnerability to symptomatic Salmonellosis. In infants

who typically take fluids, the inoculum size that can produce disease is also comparatively smaller because of faster transit through the stomach [36].

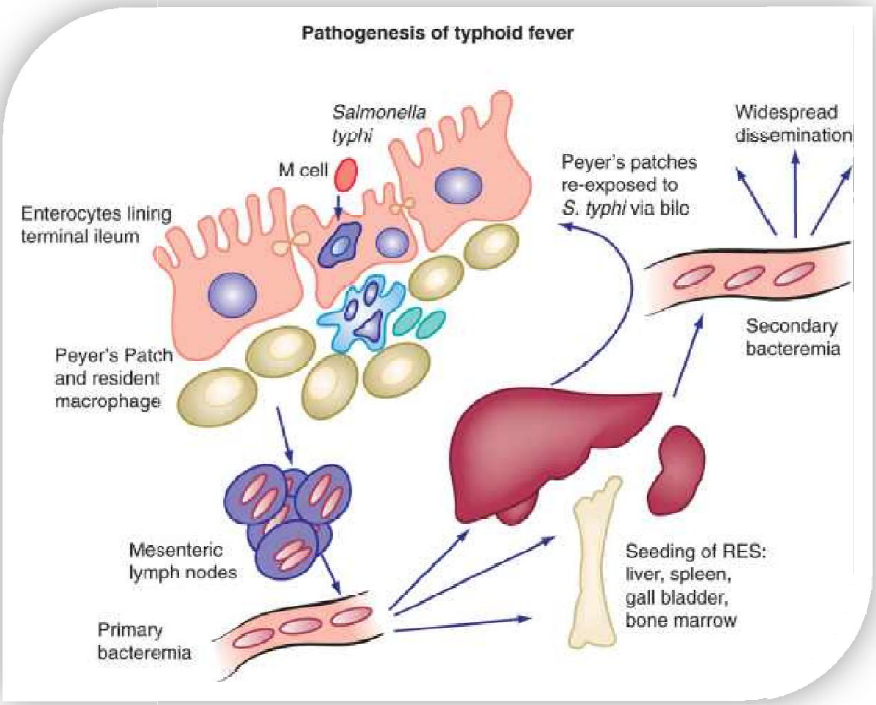


Figure 11: Pathogenesis of Typhoid Fever [36]

1.7 Transmission and risk factors of enteric fever

Risk factors for enteric fever have been identified in several epidemiologic studies, for example- waterborne [37] [38]or food borne transmission [39]. Humans are the natural host and reservoirs for *S.*Typhi *[40]*. *S.*Typhi is able to survive a stomach pH as low as 1.5. Stomach acidity is decreased by antacids, histamine-2 receptor antagonists (H2 blockers), proton pump inhibitors, gastrectomy and achlorhydria which facilitate *S.* Typhi infection [41]. The significant association has been found between the presence of serum anti-*Helicobacter pylori* IgG antibodies and typhoid fever [42]. The infectious dose varies between 10^3 to 10^6 organisms which given orally. The present study from slums in Bangladesh has been found that raw papaya is big threat for this disease. The pH of papaya is neutral. It can support the growth of various microorganisms. An environmental and behavioral risk factors that are independently associated with enteric

fever. Eating food from street vendors, living in the same household with someone who has new case of enteric fever, washing the hands inadequately, sharing food from the same plate, drinking unpurified water and living in a household that does not have a toilet are the risk factors of enteric fever [7]. Some hospitals that are situated as the middle class in South Asia seeing a large number of typhoid fever cases among relatively well-off university students who lives in group house-hold with poor hygiene [43].

1.8 Clinical manifestations of enteric fever

The clinical manifestation of enteric fever may vary widely. It largely depends on the patient population, for example-adults versus infants studied. Enteric fever is a disease of children and young adults. Most patients who present to hospital with enteric fever are in the age class of 5 to 25 years. Many cases of typhoid particular in children less than five years of age [13]. Many patients with enteric fever do not receive appropriate medical treatment in most of the developing countries [44]. The temperature increase daily (up to 40 to 41^0C) combined with headache, malaise and chills [45]. The hallmark of typhoid fever is prolonged fever. The fever may persist up to 4 to 8 weeks in untreated case [46]. The illness may be mild and brief. In rare cases an acute severe infection progresses into multiple organ failure, disseminated intravascular coagulation and central nervous system involvement and may results in early death [47].

1.9 Typhoid carrier state

Human are the sole reservoir for *Salmonella* Typhi infection. There are two types of infection-

- Asymptomatic
- Symptomatic

The percentage of people who infected with *S.*Typhi is 1 to 5%. They become asymptomatic chronic carrier and continue to excrete bacteria through their feces or urine. The frequency of chronic carriage is higher among women. These women have billiary tract infection, for example-gallstone. The tendency to become a chronic carrier

may increase by improper treatment of antibiotic and antibiotic resistance. People who handle food without proper hygiene can transmit infection. The classical story of 'Typhoid Mary' spreading typhoid infection is well known. She was responsible for an outbreak of typhoid fever in New York in 1906. Her name was Mary Million but better known as Typhoid Mary [48].

1.10 Persistent *Salmonella* infection

Bacteria enter the Peyer's patches of the intestinal tract mucosal surface by invading M cells specialized epithelial cells that take up and transcytose luminal antigens for uptake by phagocytic immune cells. This is followed by inflammation and phagocytes of bacteria by neutrophils and macrophages and recruitment of T and B cells .In systemic salmonellosis, such as typhoid fever, *S*. Typhi may target specific types of host cells, such as dendritic cells and/or macrophages that favor dissemination through the lymphatics and blood stream to the mesenteric lymph nodes (MLNs) and to deeper tissues. This then leads to transport to the spleen, bone marrow, liver and gall bladder. Bacteria can persist in the MLNs, bone marrow and gall bladder for life and periodic reseeding of the mucosal surface via the bile ducts and/or the MLNs of the small intestine occur and shedding can take place from the mucosal surface. Interferon-γ (IFN-γ) which can be secreted by T cells has a role in maintaining persistence by controlling intracellular replication. Interleukin-12 (IL-12) which can increase IFN-γ production and the *Salmonella* proinflammatory cytokine tumour-necrosis factor-α (TNF-α) also contribute to the control of persistent *Salmonella* [49].

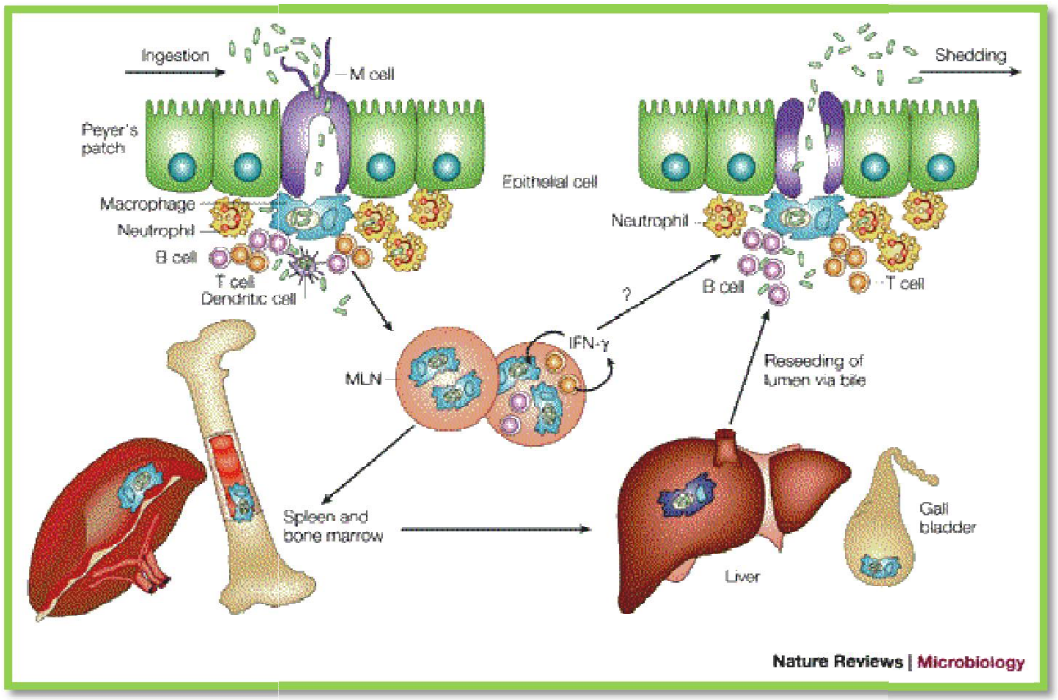


Figure 12: Schematic Representation of Persistent Infection with *Salmonella enterica* serovar Typhi in Humans [49]

1.11 Host defense system

1.11.1 Mucosal immune response to *Salmonella*

The gut microflora is able to passively restrict *Salmonella* growth by creating a nutrient depleted environment, by releasing by products of their metabolic activities such as propionate or butyrate. It can be harmful to *Salmonella*, or by production of bacteriocins. The epithelial cell layers also help to maintain by secreting mucus (goblet cells) or antimicrobial peptides (peneth cells). Underlying this epithelial cell layer is the lamina propria. It contains a highly organized lymphoid tissue commonly referred to as Gut Associated Lymphoid Tissue (GALT). The GALT is composed of T cells and B cells, dendritic cells, macrophage and neutrophils. These cells coordinate inflammatory response to bacteria and antigens that break the gastrointestinal barrier. Accumulation of lymphoid follicles which called Peyer's patches, are surrounded by a particular epithelium. The follicle associated epithelium forms the interface between the GALT and

the luminal microenvironment. It has an important role in the immune surveillance of the intestinal lumen [50] [51].

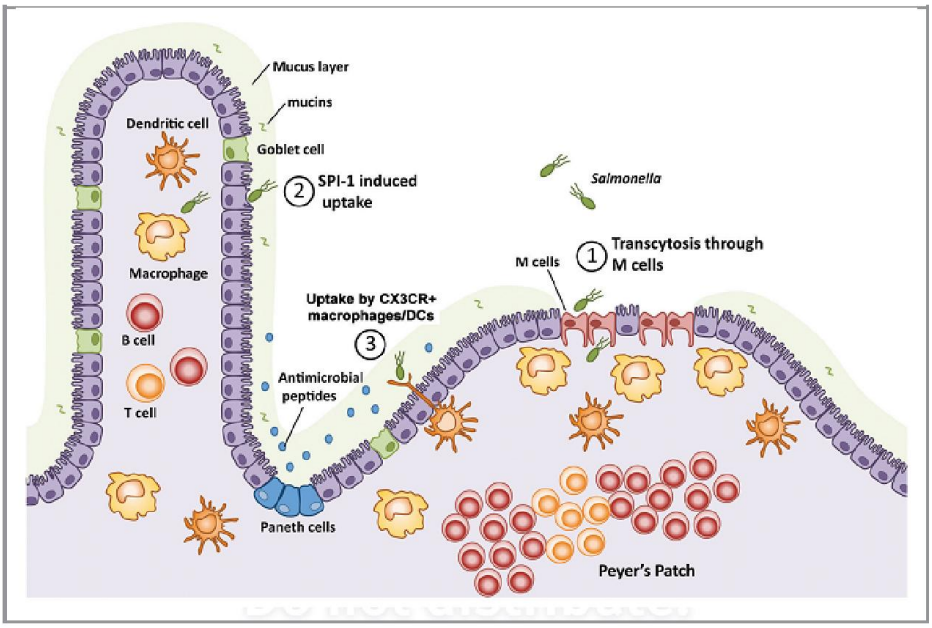


Figure 13: Schematic representation showing the different routes *Salmonella* can take in order to invade the intestinal mucosa [50]

1.11.2 Cell mediated immune response to *Salmonella*

The cell-mediated immunity plays a vital role in controlling *Salmonella* infection [52] [53]. *Salmonella* infection induces the generation of specific CD41 and CD81 T cells. CD41 and CD81 T cells populations are important for protection during primary and secondary responses, although the mechanisms underlying T cell-mediated protection are not yet completely understood [54] [55] .During the initial period there was increased production of interleukin (IL)-2 and interferon (IFN)- γ producing cells in the spleen and Peyer's patches, indicating a Th1 type response, whereas in the later period of the study, increased production of IL-4 producing cells suggested a Th2 type response [56] [54] [57] [58].

1.11.3 Circulating antibody response to *Salmonella*

Antibodies, also called Immunoglobulins or Ig's constitute the *gamma globulin* part of the blood proteins. They are soluble proteins secreted by the plasma offspring of primed B cells and react specifically to the antigen that stimulated their production [58]. Antibodies inactivate antigens by (a) complement fixation (proteins attach to antigen surface and cause holes to form, i.e., cell lysis), (b) neutralization (c) agglutination (clumping), and (d) precipitation. IgG is the predominant antibody in the secondary response and constitutes an important defense against bacteria and viruses. It is the only antibody that can cross the placental barrier to the fetus. It is responsible for the 3 to 6 month immune protection of newborns that is conferred by the mother. IgA is the main Ig in secretions such as colostrums, saliva, tears, and respiratory, intestinal and genital secretions. It prevents attachment of microbes to the mucous membranes. IgM is the main Ig produced early in the primary response. It has the highest avidity and is most efficient in agglutination, complement fixation and other antibody reactions. IgE mediates anaphylaxis and defends host against certain parasites [59].

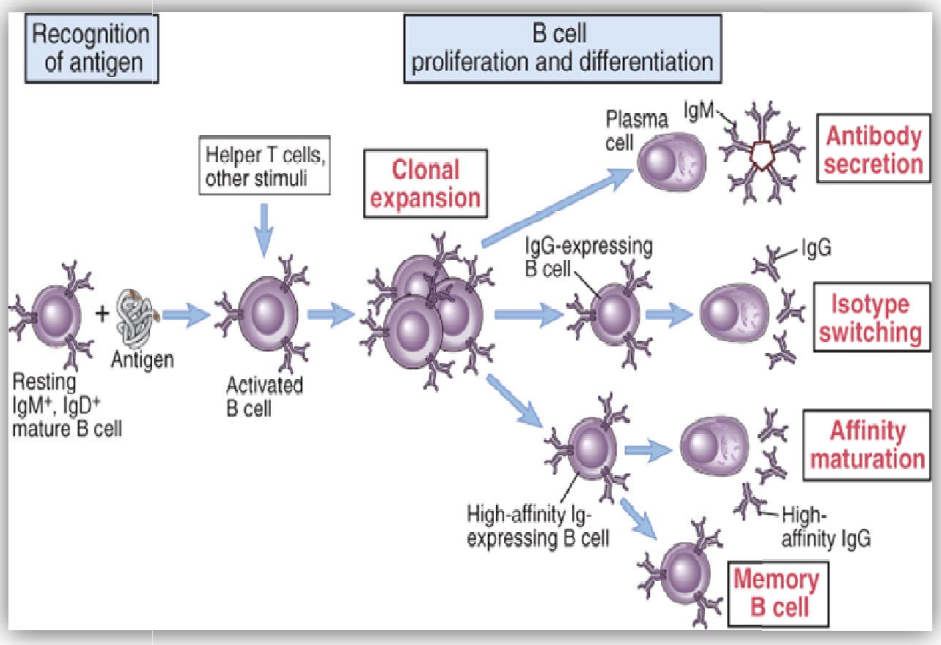


Figure 14: B-Cell Proliferation and differentiation [60] [61]

1.12 Clinical features of enteric fever

The clinical feature of typhoid fever is highly fluctuating, ranging from fever and to a severe systemic illness. It is associated complications involving many systems. The fever might rise gradually in a sidewise manner. With 5 to 7 days of daily increments in most temperature to 0.5 to 1^0C. This temperature becomes constant and high grade 39.5 to 41^0C by the second week of illness. So this high fever can continue up to 4 weeks, if left untreated then followed by return to normal temperature. Present study advises the pattern of morbidity and mortality associated with typhoid fever is reliant on age and gender [62] [63] [64]. Some people with typhoid fever develop a rash called "rose spots", which are small red spots with the abdomen and chest. Other symptoms that occur include [65] [66] -

- Abdominal tenderness
- Agitation
- Bloody stools
- Chills
- Confusion
- Difficulty paying attention (attention deficit)
- Delirium
- Fluctuating mood
- Hallucinations
- Nosebleeds
- Severe fatigue
- Slow, sluggish, lethargic feelings
- Weakness

1.13 Complications of enteric fever

- One of the most lethal complications of typhoid fever is ileal perforations. When treatment is interrupt, ileal perforation is occurred [67] [68] [67].

- *Salmonella enteric* 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 [69] [70] [71] .
- Hepatic dysfunction is clinical complications during pregnancy [72] .
- Gall bladder perforation is rare complications of typhoid fever [73] [74].
- Acute disseminated encephalomyelitis in typhoid fever [75] [75].
- Patients suffering from Small bowel perforation in secondary infection [76].

1.14 Treatment of enteric fever

The emergence of antibiotic resistant strains of *Salmonella* Typhi is major problem of treatment of typhoid fever in recent years [77]. Some variants of *Salmonella* Typhi have developed multidrug-resistance as an integral part of the genetic material of the organism. Drug-resistant *Salmonella* Typhi emerges in response to antibiotic usage, if the dose is not properly determined and time course is not followed. Selective pressure from the use of antibiotic is a major driving force behind the emergence of resistance, but other factors also need to be taken into consideration.

The available antibiotics against *S.*Typhi are Ampicillin, Chloramphenicol, Trimethoprim-sulfamethoxazole, Ceftriaxone, Ciprofloxacin, Nalidixic acid, Cefixime and Gentamicin [78]. All of antibiotics resistance *S.* Typhi is emerged except Ceftriaxone and Cefixime [79]. In some place it is reported that Cefixime and Ceftriaxone resistance *S.* Typhi are evolved. Recently Azithromycin is most commonly used in Bangladesh for treatment of typhoid fever. It is very effective in treatment of multidrug-resistant typhoid fever. In case of Ciprofloxacin sensitive but Nalidixic acid resistance, Ciprofloxacin is not effective to kill pathogens. Though Fluoroquinolones were thought to be the most effective treatment option for the multiple-drug resistance typhoid fever, emergence of Fluoroquinolones resistant strains has aggravated the problem [95, 96]. In Bangladesh the prevalence of Fluoroquinolones especially ciprofloxacin resistant *S.* Typhi is more pronounced [80] [81, 82] [83].

1.15 Prevention of enteric fever

Safe water: Enteric fever is a waterborne disease and the main preventive measure is to ensure access to 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 [84]

Food safety: Contaminated food is another important vehicle for typhoid fever transmission. Appropriate food handling and processing is paramount and the following basic hygiene measures must be implemented or reinforced during epidemics:

Sanitation: Proper sanitation contributes to reducing the risk of transmission of pathogens including *Salmonella* Typhi [84].

Health education: Health education is paramount to raise public awareness that can be an effective measure for reducing risk of transmission of S. Typhi [84].

1.16 Vaccination

1.16.1 Ty21a live oral vaccine

This is an oral vaccine containing live attenuated *Salmonella* Typhi Ty21a strains in enteric coated capsules. It consists of one enteric-coated capsule that is taken every two days, a total of 3 doses. The capsule must be refrigerated but not frozen to achieve maximum efficacy. Each capsule is taken with one glass of water. The vaccination is completed in a week and the booster dose should be given every 5 years. The vaccine recipient should not be younger than 6 years. The vaccine elicits both serum and intestinal antibodies and cell mediated immunity though, the detailed mechanism of vaccine induced immune response is unknown [85].

1.16.2 Vi capsular polysaccharide vaccine

This vaccine is composed of purified Vi ("virulence") capsular polysaccharide antigen of *Salmonella* Typhi isolated from blood cultures. This capsular polysaccharide of *S.* Typhi

is conjugated to nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A and enhances immunity. It is given by the parenteral route and only one is needed. This vaccine must be kept at 4°C temperatures. Children below two years of age are not given this vaccine. Since it is not effective or immunogenic in them a booster dose is given every 2 years to maintain protection if the exposure of *Salmonella* is expected. It is T-cell independent and, thus, cannot stimulate protective T helper cells or is used in young children [86] [17].

1.17 Diagnosis of enteric fever

The diagnosis of typhoid fever depends on the isolation of *S*.Typhi from blood, bone marrow and specific lesion. The existence of clinical symptoms characteristic of typhoid fever or the detection of a specific antibody response is symbolic of typhoid fever but not reliable. Blood culture is the mainstay of the diagnosis of this disease [84].

1.17.1 Culture methods

The diagnosis of enteric fever is ultimately based on isolation and identification of the bacterium in cultures. *Salmonella* Typhi are usually isolated from cultures of blood or bone marrow only during the first 2 weeks of illness [87].The various culture methods available are-

- Blood culture
- Stool culture
- Urine culture
- Bone marrow culture
- Bile culture

Blood culture:

This is the method of choice and has the great advantage over culture from the urine or bile showing not only that the patient is infected with the bacillus but that the infection is active and is almost certainly responsible for the disease from which he or she is

suffering [87]. Microbiologic culturing of blood is approximately 30 to 50% sensitive even under the best conditions; there may be failure to isolate the organism [6].Blood culture cannot isolate pathogen in case empirical (without knowing causative microbes) treatment of antibiotics [88].

Stool culture:

Stool culture is another way of identifying the *Salmonella* Typhi organism from infected person. Stool specimens should be examined and cultured as soon as possible after collection. As the stool specimen cools, the drop in pH soon becomes sufficient to inhibit the growth of *Salmonella* species. A small amount of faecal specimen can be added to selenite broth for enrichment [87].

Urine culture:

Except for urethral mucosa, which supports the growth of a microflora, the normal urinary tract is usually devoid of bacteria. Proper collection of sample is very important for optimum recovery of pathogens [87].

Bone marrow culture:

Bone marrow culture is more sensitive than blood culture. It is more invasive procedure. It is possible to isolate the offending organisms from the bone marrow [89] .

Bile culture:

Bile is a good culture medium for *Salmonellae*. They can be successfully recovered from culture of bile, especially from chronic carriers. Several milliliters of bile are collected in a sterile container. The sample is plated on blood agar and MacConkey agar plate and incubated at 37° C for 24 to 48 hour or until growth is obtained. It is an invasive technique [87].

1.17.2 Widal test

Widal test is used for enteric fever diagnosis due to the complexity of performing blood culture [106]. Widal is an immunoagglutination method which detects anti-O and anti-H antibody response in blood of enteric fever patient. Widal is rapid diagnostic method and easy to perform. But false-positive results are common because of antigenic cross sharing with other Salmonella serotypes, other Enterobacteriaceae, and with previous history of diseases such as malaria, dengue, and typhoid [90] [91] [41]. The Widal test also has low sensitivity, specificity and practically not being used by clinicians in this setting [41].

1.17.3 Enzyme-Linked Immune Sorbent Assay (ELISA)

ELISAs can be performed with a number of modifications to the basic procedure. The key step, immobilization of the antigen of interest can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. Antigens from the sample are attached to a surface. A further specific antibody is applied over the surface. So it can bind to the antigen. This antibody is linked to an enzyme. In the final step a substance containing the enzyme substrate is added. The subsequent reaction can produce a detectable signal, most commonly a color change in the substrate [92]

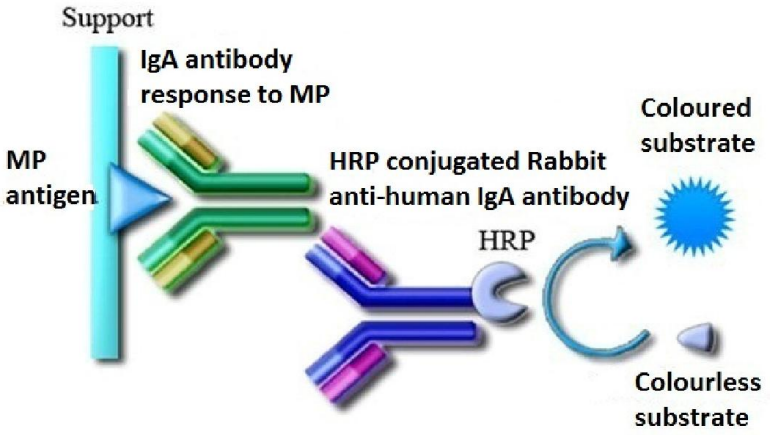


Figure 15: Assay of Antibody by ELISA[92]

1.17.4 Diagnosis of enteric fever using TPTest (Typhoid and Paratyphoid Test) and simplified methods of TPTest

The TPTest is an immunodiagnostic method that detects IgA antibody response in lymphocyte culture secretion by ELISA and used for diagnosis of enteric fever. This method gives a presumptive result within 24 hours and a confirmatory result after 48 hours and 64% positive result become positive within 24 hours. The sensitivity and specificity of the TPTest are 100% and 78-97% respectively [6, 93, 94].

In a earlier study, simplification of the TPTest was done at different stages of the method for implication of the TPTest at laboratories with less facilities and equipments [6]. Cell separation method was simplified by RBC lysis and buffy coat methods. Incubation of the isolated cells was done at 37^{o}C with 5% CO_2 and without 5% CO_2 supplementation and the antibody responses in culture supernatants were compared. Immunodot blot assay has been evaluated for identification of antibodies in lymphocyte culture supernatant and compared with the results of the standard TPTest. It has been found that immunodot blot assays could not detect positive samples of the TPTest having a value of ≤16 EU (cut-off of the TPTest: equal or less to 10 EU).

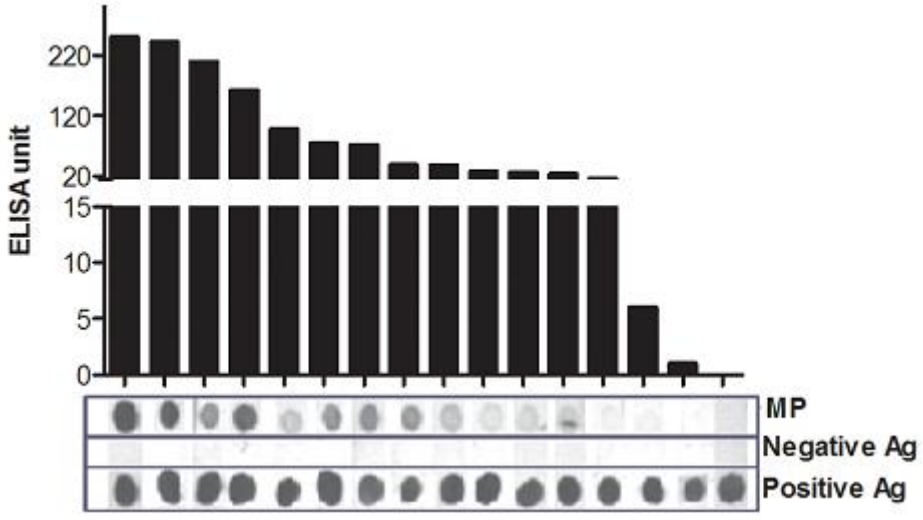


Figure 16: Immunodot blot assay for detecting antibody [6]

1.18 Hypothesis

Enteric fever can be diagnosed by measuring antibodies in lymphocyte secretion by the TPTest and its simplification is possible for use at the laboratories with less facilities and equipments.

1.19 Overall objective of the study

Previous study demonstrated that the TPTest specimens with values ≥16 ELISA unit can be detected by the immunodot blot assay. The aim of this study was to optimize the immunodot blot assay for diagnosis of the patients with positive TPTest result.

1.20 Specific objectives

a) To isolate and identify the organism using blood culture method from the patients with enteric fever.
b) To find out the antibiotic susceptibility pattern of the isolated strain.
c) To optimize the immunodot blot assay and to evaluate the assay with TPTest using ELISA.
d) To compare the performance of simple cell separation methods, such as RBC lysis with the density gradient centrifugation method of the standard TPTest.
e) To compare the effect of cell culture in an incubator without 5% CO_2 supplementation with the existing cell culture incubation requiring 5% CO_2 supply.

Chapter 2

Materials and Methods

2.1 Place of the study

This study was carried out in the Enteric Vaccine, Centre for Vaccine Science (CVS), International Centre for Diarrhoeal Disease and Research, Bangladesh (icddr,b). All the patients were enrolled from icddr,b hospital, Mirpur and Kamalapur.

2.2 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 patients and/or legal guardian of the patients in case of children. The risks and benefits had been explained in the consent form.

2.3 Study participants

Written informed consent for the study was obtained from 107 patients admitted to Mirpur, Kamalapur and icddr,b hospital with enteric fever and Positive for *Salmonella* Typhi infection. Blood samples were obtained during infection on Day 0, Day 7 and Day 21.

2.4 Inclusion criteria

- ✓ High Fever : $\geq 39.2^0$C
- ✓ Duration of Fever: 3 to 7 days

2.5 Period of the study

The study was carried out from August 2013 to May 2014.

2.6 Blood sample collection

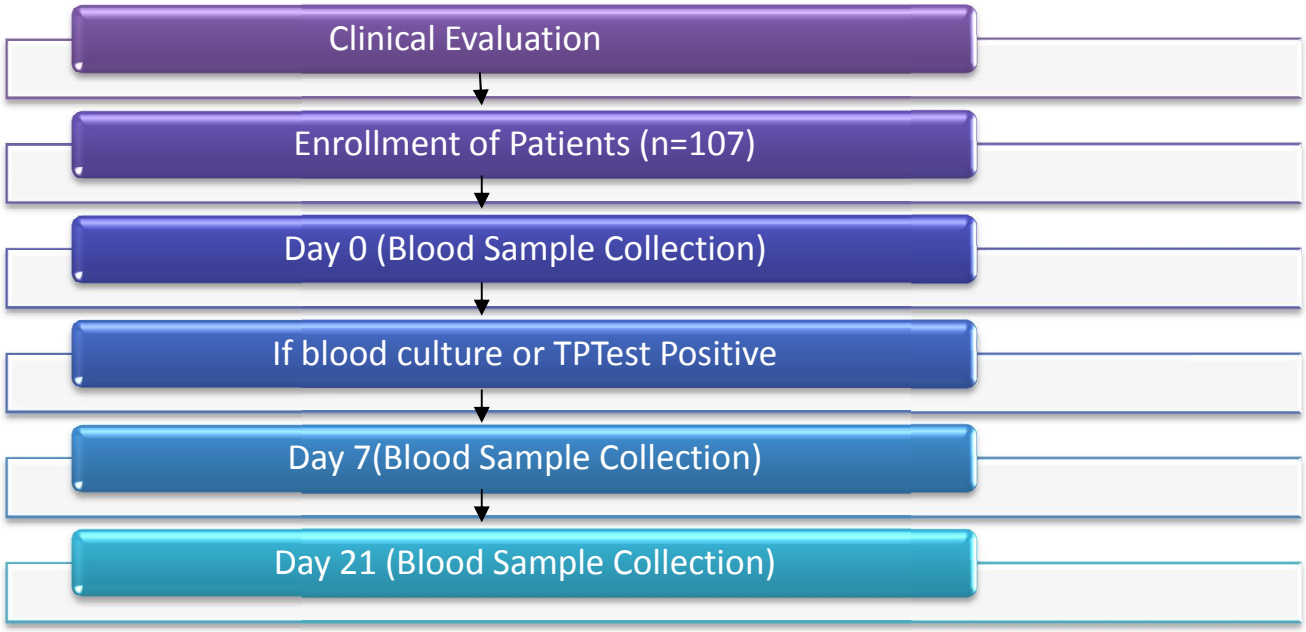


Figure 17: Blood Sample Collection Schedule

2.7 Flow chart of the study design

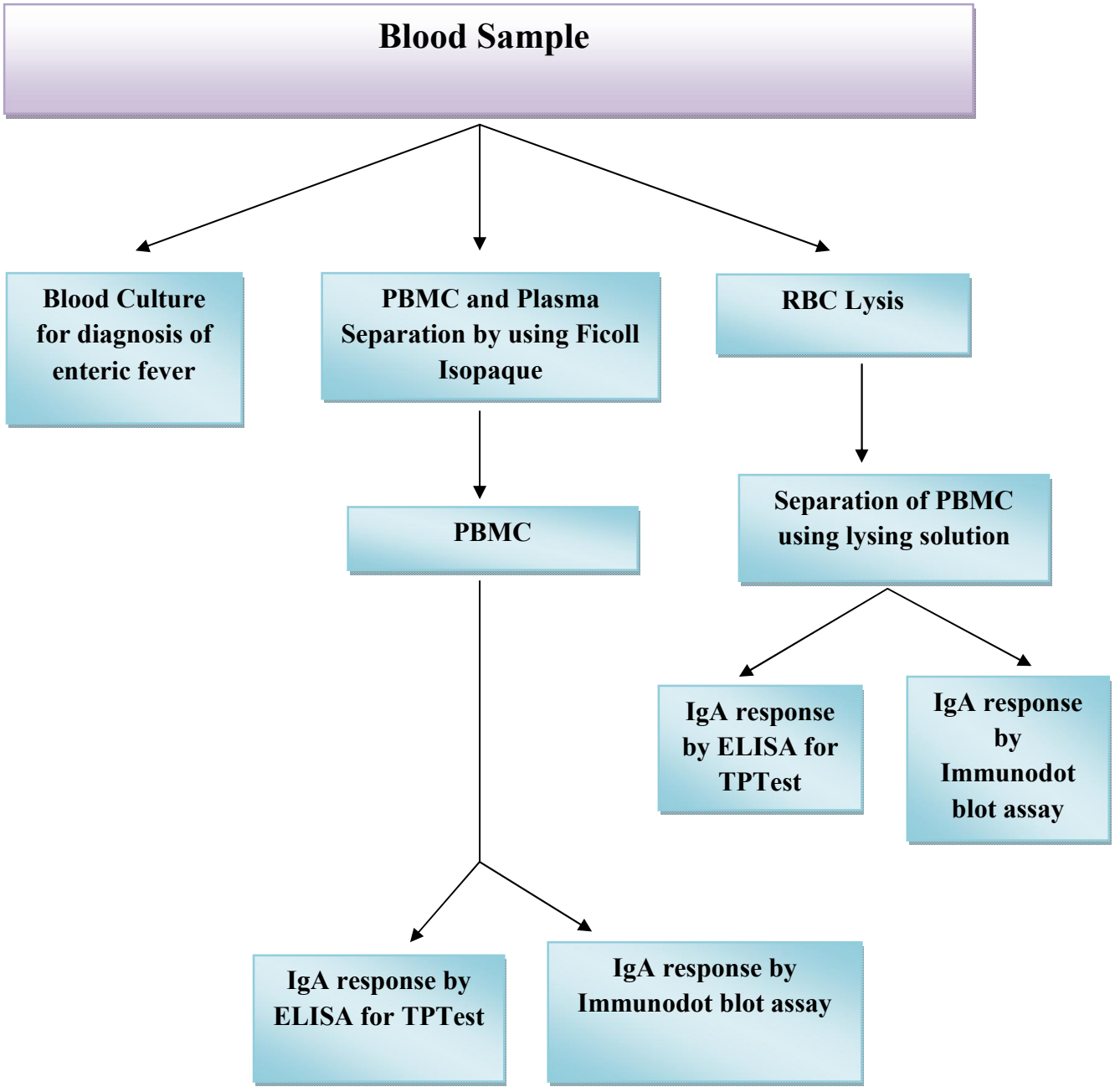


Figure 18: Flow Chart of the study design

2.7.1 Blood culture

3 ml of blood was added to enrichment broth incase of children (1-4 years) other wise 5 ml blood was collected from 5-46 year old patients. Most cultures are monitored for five to seven days. BacT/ALERT 3D was used to incubate Bacteria at 37^0C. The BacT/ALERT 3D instrument is a state-of-the-art, automated microbial detection system. The BacT/ALERT system offers advantages in every dimension of microbial detection testing. BacT/ALERT 3D is used for monitoring the presence or absence of microorganisms in broth. Any growth in enrichment broth is subsequently sub-cultured onto agar plates to isolate the pathogenic organism. Then *S.* Typhi can be confirmed by anti-sera test and biochemical tests.

2.7.1.1 Anti-Sera test

Salmonella tests are performed for determination of serological differentiation of *Salmonella* species. Anti-sera are used in qualitative slide agglutination test. Using these antisera *Salmonella* isolates in different types which are classified as *S.*Typhi and *S.*Paratyphi A and *S.*Paratyphi B.

First marked the slide into three division using glass marker. Then added 10 µl O Polyvalent antiserum into one division. After that a loop of 3 single bacterial colony using inoculating tips. Mixed the reagents by tilting the glass slide back and forth for 1 minute and the agglutination pattern were observed. Agglutination was grossly observed with light through the slide. Only strong agglutination was observed within 1 minute should be regarded as *Salmonella* species.

Then added 10 µl Vi polysaccharide antigen into another division. After that a loop of 3 single bacterial colony using inoculating tips. Mixed the reagents by tilting the glass slide back and forth for 1 minute and the agglutination pattern were observed. Agglutination was grossly observed with light through the slide. Only strong agglutination was observed within 1 minute should be regarded as *Salmonella* Typhi.

Then added 10 µl S-Poly A antigen into another division. After that a loop of 3 single bacterial colonies using inoculating tips. Mixed the reagents by tilting the glass slide back and forth for 1 minute and the agglutination pattern were observed. Agglutination was grossly observed with light through the slide. Only strong agglutination was observed wihin 1 minute should be regarded as *Salmonella* Paratyphi A.

Then added 10 µl S-Poly B antigen into another division. After that a loop of 3 single bacterial colony using inoculating tips. Mixed the reagents by tilting the glass slide back and forth for 1 minute and the agglutination pattern were observed. Agglutination was grossly observed with light through the slide. Only strong agglutination was observed within 1 minute should be regarded as *Salmonella* Paratyphi B.

Then added 10 µl of PBS into another division. After that a loop of bacterial colony using inoculating tips. Mixed the reagents by tilting the glass slide back and forth for 1 minute and the agglutination pattern were observed. Agglutination was grossly observed with light through the slide. No agglutination was found. So this method was correct for *Salmonella* Typhi and *Salmonella* Paratyphi.

2.7.1.2 Biochemical test

Biochemical tests is confirmed whether the colonies resembling *Salmonella* on XLD and BGA. These tests are described except that the Urease test is performed with urea broth instead of urea agar.

Figure 19: Biochemical Test Tube

Klinger's Iron Agar (KIA):

This is a complex medium that contains a large amount of lactose and a very small amount of glucose, a pH indicator (yellow in acid and red in base). It also contains iron which is precipitated as a black sulfide if H_2S is produced. These ingredients allow determining four biochemical properties of unknown organism.

A. Lactose (+) or (-): At the surface of the slant (aerobic conditions), only fermentation of carbohydrates present in high concentration (in this case lactose) yield more acidic products than can be oxidized to neutrality. Thus lactose (+) organisms yields a yellow slant and lactose (-) organisms yield a red slant.
B. Glucose (+) or (-): In the largely anaerobic butt of the tube, even fermentation of the trace concentrations of glucose yields enough acid to change the pH. Thus glucose (+) organisms also a yellow butt. Fermentation of lactose in the butt will obviously also change the pH. This does not confuse the interpretation since all

lactose positive organisms are also glucose-positive. If a black color from iron sulfide obscure the butt can presume it is yellow.

C. H_2S (+) or (-): if an organism forms H_2S, the lower portion of the tube will turn black, due to formation of iron sulfide.
D. Gas formation (+) or (-): If an organism's forms gas from glucose or lactose the agar in the butt will show bubbles or cracks.

Motility Urea Indole Medium: This medium is a multi-purpose medium for differentiation of Enterobacteriaceae that combines three individual tests into a single medium. Indole is tested by layering a small amount of Indole reagent onto the surface of the medium and allowed a few minutes to react. A positive result is indicated by the formation of a red line at the interface of the reagent and the medium.

Appearance: Liquid

Color: Golden

pH: 6.8 ±0.2

Recommend Incubation: Aerobically at 37±C for 18-24 hours

Shelf Life: 182 Days.

Citrate Agar: Citrate agar slants contain sodium citrate (only carbon source) and ammonium ion (the sole nitrogen source). A pH indicator, Bromothymol Blue is also included Bromothymol Blue is GREEN at pH < 7.0 and BLUE at pH > 7.6 organisms that utilize 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 1: The biochemical tests for identification of *S.* Typhi and *S.*Paratyphi

Organisms	Glucose	H_2S	Motility	Indole	Urease	Citrate
*S.*Typhi	A	+	+	-	-	-
*S.*Paratyphi A	AG	-	+	-	-	-
*S.*Paratyphi B	AG	+	+	-	-	+

Here,

A means Acid and AG means Acid plus Gas.

2.7.1.3 Antimicrobial sensitivity test

Antibiotic sensitivity is the susceptibility test of bacteria to antibiotic. This test is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection *in vivo.* The antimicrobial sensitivity test was done by "Kirby-Bauer Method". If the bacteria were sensitive to the antibiotic, a clear ring or Zone of inhibition was seen around wafer indicating poor growth.

The following antibiotics and their concentration per disc were used:

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

Figure 20: Method of Sensitivity Test

2.7.1.3.1 Measurement of inhibition zone:

Antibiotic sensitivity testing is used to determine the susceptibility of bacteria to an antibiotic. Numerous standardized tests are available to measure the effectiveness of an antibiotic against a specific organism in order to prescribe the most suitable antibiotic therapy. Kirby-Bauer Method is one of them. It is very quick an easy method for evaluating the numerous antibiotics at once. First the antibiotic disks were placed on a plate. Then the plates were incubated to allow growth of the bacteria and time for the antibiotics to diffuse into the agar. If an organism was susceptible to an antibiotic, a clear zone was appeared around the disk where the growth inhibited. The size of this zone of inhibition depends on the sensitivity of the bacteria to the specific antibiotic and the antibiotic's ability also to diffuse through the agar.

The zone of inhibition was measured by the following way:

1) Scale
2) Slide Calipers

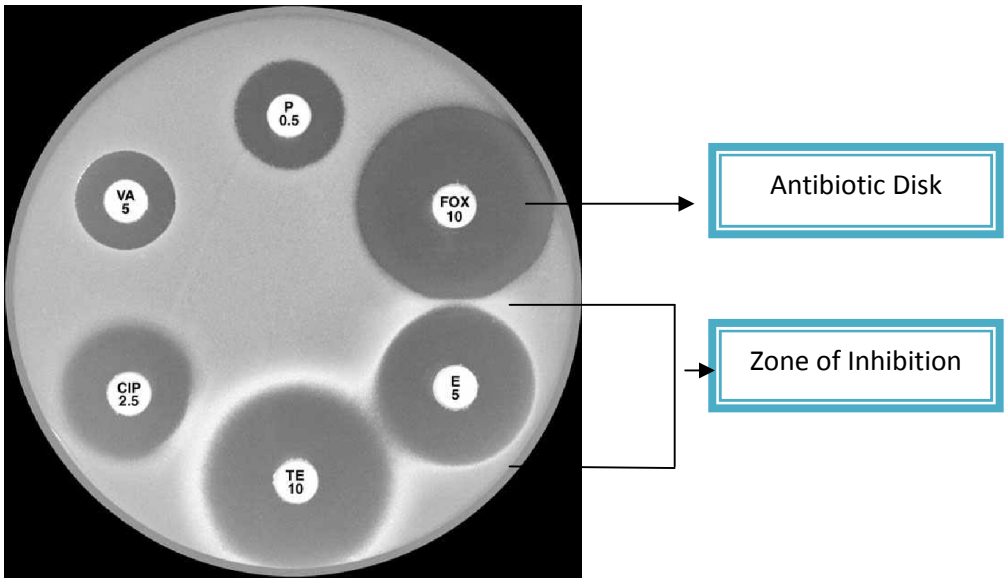


Figure 21: Zone of Inhibition

2.7.1.4 Separation of plasma and Peripheral Blood Mononuclear Cell (PBMC)

Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from venous blood by density gradient centrifugation on Ficoll Isopaque and lymphocyte culture supernatant was prepared from the isolated PBMCs.

Procedure:

- Heparinized venous blood was diluted with equal volume of phosphate buffer saline (PBS) in falcon tubes.
- Ficoll-Isopaque was taken in falcon tube and diluted blood was carefully layered on top of it.
- The tube was centrifuged at 1800 rpm (772xg) for 25 minute at 20°c.
- After centrifugation, there were following four layers- at first a pallet of RBCs and granulocytes, second layer was for the Ficoll-Isopaque. PBMCs were in the third layer and plasma was in the upper-most fourth layer.

- The plasma was carefully removed from the top with a Pasteur pipette and then PBMCs were removed from the top of the Ficoll layer similarly.
- The PBMCs were washed once in PBS at 2000rpm (953xg) for 10 minute at 20°C.
- Then they were resuspended in 10 ml of PBS and counted in a hemocytometer.
- Again it was washed at 2000rpm (953xg) for 10 minute at 20°C.

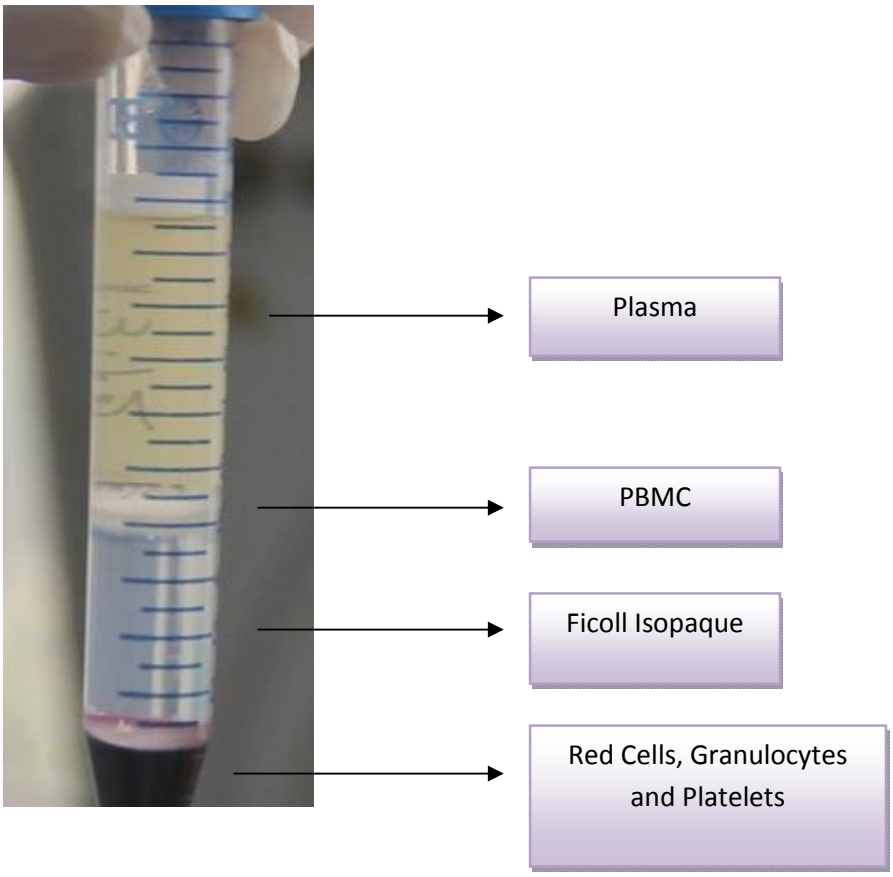


Figure 22: Separation of plasma and peripheral blood mononuclear cells (PBMC)

Preparation of lymphocyte culture supernatant:

From the isolated PBMC, lymphocyte culture supernatant was prepared in the following method used in different studied.

- The isolated PBMCs were taken and resuspended in RPMI- complete medium to 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 h.
- After 48 hrs 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 was added at a concentration of 1% of culture supernatant and stored at -70°C for future analysis.

2.7.1.5 Separation of PBMC using lysing solution

- Heparinized venous blood was diluted with 1x Lysing Solution (1:10 dilution) and mixes it well gently by inverting the falcon tube.
- Then incubated the falcon tube for 5 min in room temperature.
- The tube was centrifuged at 953g for 10 min at 20°C.
- After centrifugation the supernatant was decant quickly and then resuspended the cells in 10 ml ice cold PBS.
- Then cell was counted in a haemocytometer.
- Again the cell suspension was centrifuged at 953g for 10 min at 20°C.

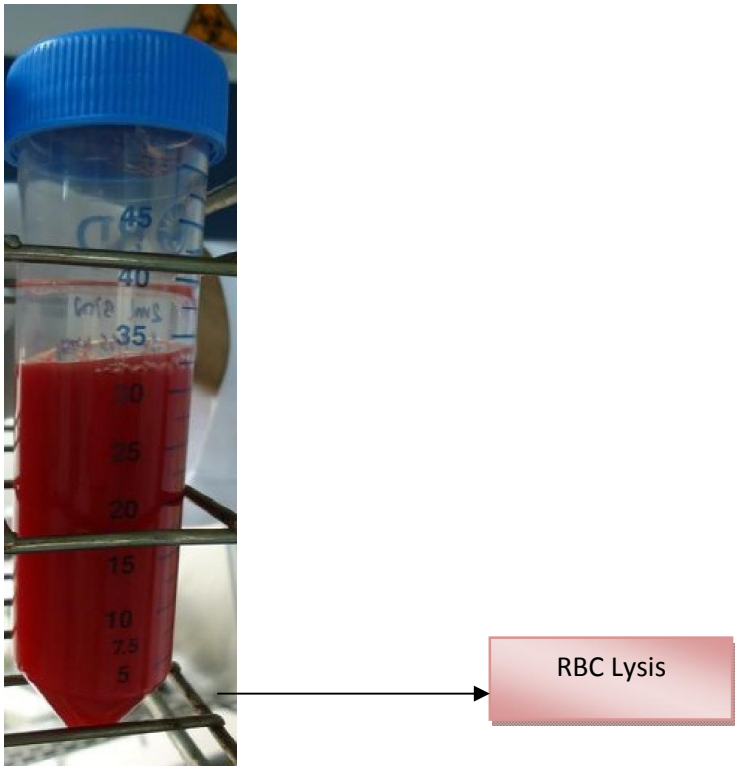


Figure 23: Separation of PBMC using Lysing Solution

Preparation of lymphocyte culture supernatant:

From the isolated PBMC, lymphocyte culture supernatant was prepared in the following method used in different studied.

- ✓ The isolated PBMCs were taken and resuspended in RPMI- complete medium to 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 h.
- ✓ After 48 hrs 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 was added at a concentration of 1% of culture supernatant and stored at -70°C for future analysis.

2.7.1.6 Assay of antibody response in lymphocyte secretion, plasma by kinetic ELISA

Enzyme linked immune-sorbant assay (ELISA) is a process of measuring the concentration of antigen or antibody. In this process, the antigen is coated in a solid surface. Upon administration of antibody containing sample, antibody binds to the antigen, which is then detected by binding to another antibody conjugated to an enzyme. When substrate is given the enzyme converts it into a colored product. The concentration of color is proportional to the concentration of sample antibody.

The IgA antibody response in lymphocyte culture supernatant against Ty21a Membrane Preparation (MP) was measured > 10 ELISA unit was considered as positive result for TPTest method. The cut-off value of the TPTest method was calculated by using the formula: > 2SD plus geometric mean of healthy Bangladeshi control.

Procedure:

Coating of ELISA plate with antigen:

- MP antigen prepared from Ty21 which was diluted with PBS at a concentration of 5.0 μg/ml for lymphocyte culture supernatant.
- ELISA-plates (NuncF) were coated with 100 μl/well of antigen suspension.
- The plates were incubated at room temperature for overnight.

Blocking:

- The coated plates were washed thrice with PBS.
- The plates were blocked with 200 μl/well of 1% bovine serum albumin in PBS (BSA-PBS) and incubated for 30 minutes at 37°C.

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 room temperature for 90 minutes.

Conjugate adding:

- The plates were washed three times with PBS-Tween (0.05% Tween) and once with PBS.
- For ALS, the 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 room temperature for 90 minutes.

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 (O.D.) was measured at 450 nm by the Multiskan Ascent ELISA reader in kinetic mode immediately.

2.7.1.7 Immunodot blot assay

Immunodot blot assay is another method for detecting the antibody response. This method is simplified in comparison to ELISA.

2.7.1.7.1 Immunodot blot assay detection of antibodies in lymphocyte secretion separated by ficoll method

2.7.1.7.1.1 Immunodot blot assay for rabbit anti-human IgA conjugate with HRP and 4-Chloro-1 –Napthol (4CN) substrate

Procedure:

- The strips were divided of 30630cm Osmonics NitroBind 0.45mm Transfer Membrane into 0.35 by 0.35 cm squares.
- Then soaked these strips in PBS and allowed the membranes to dry before coating them with MP antigen (1 and 1.5 mg/ml MP antigen) dissolved in PBS, AffinePure Goat Anti-human IgG, F $(ab^{/})_2$ Fragment (1.2 mg/ml dissolved in deionized water) as a positive control and Lipopolysaccharide (LPS) of *Vibrio cholerae* O1 X-25049 strain (Ogawa) (0.5 mg/ml dissolved in PBS) as a negative control at room temperature.
- 1 µl of antigen added each well.
- Then kept it at room temperature for 5 minutes.
- Then blocked the membranes with 1% bovine serum albumin in PBS at room temperature for 30 minutes using slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 30 minutes discarded the blocking solution and washed the membrane twice with PBS.
- To assess immuno-reactivity, added lymphocyte culture supernatants diluted 1:2 with 0.1% BSA-PBS -0.05% Tween to membrane.

- Then incubated these for 3 hours at room temperature with slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 3 hours incubation, washed the membranes 5 times with PBS-Tween (0.05%) and once with PBS.
- Then incubated the membranes with rabbit anti-human IgA conjugated to horse radish peroxidase (Jackson Immunoresearch Laboratories, Inc. West Grove. USA at a 1:500 dilution in 0.1% BSA-PBS-Tween) for 1.5 hours at room temperature with shaking.
- After that washed the membrane five times with PBS-Tween (0.05%) and once with PBS.
- Then developed the membranes by adding H_2O_2 4-Chloro-1-napthol, prepared by dissolving 1.7 ml 4-Chloro-1-Napthol (3 mg/ml in 99.9% methanol) in 8.3 ml of Tris buffered saline (TBS; 20 mM Tris; 0.5 M Nacl; pH-7.5) and 10 µl H_2O_2.
- H_2O_2 was added immediately before use.
- The reactivity was read at 5 minutes.
- Then membranes were washed with tap water and air dried.
- After that immunodots positive considered when reactivity was seen with nacked eye after membranes had dried.
- Dots were independently read with 100% concordance.

2.7.1.7.1.2 Immunodot blot assay for biotinylated IgA conjugate with streptavidin HRP and 3, 3′-Diaminobenzidine (DAB) Substrate:

Procedure:

- The strips were divided of 30630cm Osmonics NitroBind 0.45mm Transfer Membrane into 0.35 by 0.35 cm squares.
- Then soaked these strips in PBS and allowed the membranes to dry before coating them with MP antigen (1 and 1.5 mg/ml MP antigen) dissolved in PBS, AffinePure Goat Anti-human IgG, F $(ab')_2$ Fragment (1.2 mg/ml dissolved in

deionized water) as a positive control and Lipopolysaccharide (LPS) of *Vibrio cholerae* O1 X-25049 strain (Ogawa) (0.5 mg/ml dissolved in PBS) as a negative control at room temperature.

- 1 µl of antigen added each well.
- Then kept it at room temperature for 5 minutes.
- Then blocked the membranes with 1% bovine serum albumin in PBS at room temperature for 30 minutes using slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 30 minutes discarded the blocking solution and washed the membrane twice with PBS.
- To assess immuno-reactivity, added lymphocyte culture supernatants diluted 1:2 with 0.1% BSA-PBS -0.05% Tween to membrane.
- Then incubated these for 3 hours at room temperature with slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 3 hours incubation, washed the membranes 5 times with PBS-Tween (0.05%) and once with PBS.
- Then incubated the membranes with Biotinylated IgA conjugate (1:500 dilution in 0.1% BSA-PBS-Tween) for 1.15 hours at room temperature with slow shaking.
- After incubation washed the membrane one times with PBS-Tween (0.05%) and once with PBS.
- Then incubated the membrane with Streptavidin HRP (1:100 dilution in 0.1% BSA-PBS-Tween) for 45 minutes with slow shaking.
- After that washed the membrane three times with PBS-Tween (0.05%) and once with PBS.
- Then developed the membrane by adding 4 ml of PBS, 0.002 gm DAB and 6 µl H_2O_2.
- H_2O_2 was added immediately before use.
- The reactivity was read at 5 minutes.
- Then membranes were washed with tap water and air dried.

- After that immunodots positive considered when reactivity was seen with nacked eye after membranes had dried.
- Dots were independently read with 100% concordance.

2.7.1.7.1.3 Immunodot blot assay for biotinylated IgA conjugate with streptavidin HRP and 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) substrate:

Procedure:

- The strips were divided of 30630cm Osmonics NitroBind 0.45mm Transfer Membrane into 0.35 by 0.35 cm squares.
- Then soaked these strips in PBS and allowed the membranes to dry before coating them with MP antigen (1 and 1.5 mg/ml MP antigen) dissolved in PBS, AffinePure Goat Anti-human IgG, F $(ab')_2$ Fragment (1.2 mg/ml dissolved in deionized water) as a positive control and Lipopolysaccharide (LPS) of *Vibrio cholerae* O1 X-25049 strain (Ogawa) (0.5 mg/ml dissolved in PBS) as a negative control at room temperature.
- 1 µl of antigen added each well.
- Then kept it at room temperature for 5 minutes.
- Then blocked the membranes with 1% bovine serum albumin in PBS at room temperature for 30 minutes using slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 30 minutes discarded the blocking solution and washed the membrane twice with PBS.
- To assess immuno-reactivity, added lymphocyte culture supernatants diluted 1:2 with 0.1% BSA-PBS -0.05% Tween to membrane.
- Then incubated these for 3 hours at room temperature with slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 3 hours incubation, washed the membranes 5 times with PBS-Tween (0.05%) and once with PBS.

- Then incubated the membranes with Biotinylated IgA conjugate (1:500 dilutions in 0.1% BSA-PBS-Tween) for 1.15 hours at room temperature with slow shaking.
- After incubation washed the membrane one times with PBS-Tween (0.05%) and once with PBS.
- Then incubated the membrane with Streptavidin HRP (1:100 dilution in 0.1% BSA-PBS-Tween) for 45 minutes with slow shaking.
- After that washed the membrane three times with PBS-Tween (0.05%) and once with PBS.
- Then developed the membrane by adding 2.5 ml deionized water, 2.5 ml Na-acetate, 25 µl Citric acid, 50 µl TMB and 0.6 µl H_2O_2.
- TMB and H_2O_2 were added immediately before use.
- The reactivity was read at 5 minutes.
- Then membranes were washed with tap water and air dried.
- After that immunodots positive considered when reactivity was seen with nacked eye after membranes had dried.
- Dots were independently read with 100% concordance.

2.7.1.7.2 Immunodot blot assay for detection of antibodies in lymphocyte secretion separated by RBC lysis method

2.7.1.7.2.1 Immunodot blot assay for RBC lysis with 5% CO_2 :

Procedure:

- The strips were divided of 30630cm Osmonics NitroBind 0.45mm Transfer Membrane into 0.35 by 0.35 cm squares.
- Then soaked these strips in PBS and allowed the membranes to dry before coating them with MP antigen (1.5 mg/ml MP antigen) dissolved in PBS, AffinePure Goat Anti-human IgG, F $(ab^{/})_2$ Fragment (1.2 mg/ml dissolved in deionized water) as a positive control and Lipopolysaccharide (LPS) of *Vibrio cholerae* O1 X-

25049 strain (Ogawa) (0.5 mg/ml dissolved in PBS) as a negative control at room temperature.

- 1 µl of antigen added each well.
- Then kept it at room temperature for 5 minutes.
- Then blocked the membranes with 1% bovine serum albumin in PBS at room temperature for 30 minutes using slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 30 minutes discarded the blocking solution and washed the membrane twice with PBS.
- To assess immuno-reactivity, added lymphocyte culture supernatants diluted 1:2 with 0.1% BSA-PBS -0.05% Tween to membrane.
- Then incubated these for 3 hours at room temperature with slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 3 hours incubation, washed the membranes 5 times with PBS-Tween (0.05%) and once with PBS.
- Then incubated the membranes with rabbit anti human IgA conjugated to horse radish peroxidase (Jackson Immunoresearch Laboratories, Inc. West Grove. USA at a 1:500 dilution in 0.1% BSA-PBS-Tween) for 1.5 hours at room temperature with shaking.
- After that washed the membrane five times with PBS-Tween (0.05%) and once with PBS.
- Then developed the membranes by adding H_2O_2 -4-Chloro-1-napthol, prepared by dissolving 1.7 ml 4-Chloro-1-Napthol (3 mg/ml in 99.9% methanol) in 8.3 ml of Tris buffered saline (TBS; 20 mM Tris; 0.5 M Nacl; pH-7.5) and 10 µl H_2O_2.
- H_2O_2 was added immediately before use.
- The reactivity was read at 5 minutes.
- Then membranes were washed with tap water and air dried.
- After that immunodots positive considered when reactivity was seen with nacked eye after membranes had dried.
- Dots were independently read with 100% concordance.

2.7.1.7.2.2 Immunodot blot assay for RBC lysis without 5% CO_2 :

Procedure:

- The strips were divided of 30630cm Osmonics NitroBind 0.45mm Transfer Membrane into 0.35 by 0.35 cm squares.
- Then soaked these strips in PBS and allowed the membranes to dry before coating them with MP antigen (0.5 mg/ml, 1 mg/ml and 1.5 mg/ml MP antigen) dissolved in PBS, AffinePure Goat Anti-human IgG, F $(ab^{/})_2$ Fragment (1.2 mg/ml dissolved in deionized water) as a positive control and Lipopolysaccharide (LPS) of *Vibrio cholerae* O1 X-25049 strain (Ogawa) (0.5 mg/ml dissolved in PBS) as a negative control at room temperature.
- 1 µl of antigen added each well.
- Then kept it at room temperature for 5 minutes.
- Then blocked the membranes with 1% bovine serum albumin in PBS at room temperature for 30 minutes using slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 30 minutes discarded the blocking solution and washed the membrane twice with PBS.
- To assess immuno-reactivity, added lymphocyte culture supernatants diluted 1:2 with 0.1% BSA-PBS -0.05% Tween to membrane.
- Then incubated these for 3 hours at room temperature with slow shaking (230 rpm; Gyrotory Water Bath Shaker; New Brunsick Scientific).
- After 3 hours incubation, washed the membranes 5 times with PBS-Tween (0.05%) and once with PBS.
- Then incubated the membranes with rabbit anti human IgA conjugated to horse radish peroxidase (Jackson Immunoresearch Laboratories, Inc. West Grove. USA at a 1:500 dilution in 0.1% BSA-PBS-Tween) for 1.5 hours at room temperature with shaking.
- After that washed the membrane five times with PBS-Tween (0.05%) and once with PBS.

- Then developed the membranes by adding H_2O_2 -4-Chloro-1-napthol, prepared by dissolving 1.7 ml 4-Chloro-1-Napthol (3 mg/ml in 99.9% methanol) in 8.3 ml of Tris buffered saline (TBS; 20 mM Tris; 0.5 M Nacl; pH-7.5) and 10 µl H_2O_2.
- H_2O_2 was added immediately before use.
- The reactivity was read at 5 minutes.
- Then membranes were washed with tap water and air dried.
- After that immunodots positive considered when reactivity was seen with nacked eye after membranes had dried.
- Dots were independently read with 100% concordance.

Chapter 3

Results

3.1. Study participants

For optimization and evaluation of the immunodot blot assay, specimens from 107 patients were tested. Among them 46 patients were bacteremic with *S.* Typhi infection and 36 were blood culture negative but TPTest positive. . The immunodot blot assay was also tested in 23 healthy controls and 6 kala-azar and 6 tuberculosis patients. Patients with Kala-azar and tuberculosis were tested as controls with other febrile illnesses to rule out cross reactions.

The participants of the study were categorized into five groups for data analysis and for optimization of the immunodot blot assay.

Table 2: Grouping of the study participants

Groups	Number of the participants
Blood culture positive and TPTest positive	46
Blood culture negative and TPTest positive	36
Blood culture negative and TPTest negative	25
Healthy controls	23
Other febrile diseases	12
Total	142

3.2 Baseline data of the participants

Among 107 typhoid fever (blood culture positive and only TPTest positive) patients, 60 (56.07%) were male and 47 (43.92%) were female. The median age of the patients was 7 years 8 months, with a range of 1 to 44 years.

Table 3: Baseline data of the patients with febrile illness (n=107)

Features	Value
Median age in year (25th, 75th percentile)	7.8 (5.4, 18)
No. of males (%)	60 (56.07%)
No. of females (%)	47 (43.92%)
Median temperature in °C (25th, 75th percentile)	39.2 (39.1, 39.5)
Duration of fever in days at presentation (25th, 75th percentile)	4 (3, 5)
No. of patients with serovar Typhi in blood (%)	35 (40.7%)
Median pulse/min (25th, 75th percentile)	92.5 (83.5, 97.5)

3.3 Clinical findings of participants

The patients presented with the complaints of headache, abdominal pain, constipation, coated tongue, diarrhea, vomiting, rose spot and rash.

Table 4: Clinical findings of the participants (n=107)

Clinical Features	Number (%)
No. of patients (%) with:	
Headache	70 (81.4)
Abdominal pain	51 (55.4)
Constipation	28 (30.4)
Coated tongue	47(51.1)
Diarrhea	12 (13)
Vomiting	15 (17.44)
Rose spot	8 (9.3)
Rash	8 (9)

3.4 Blood culture and sensitivity result

Among 46 *S.* Typhi bacteremic patients, 25 were male (54.34%) and 21 were female (45.64%). Of 46 isolated *S.* Typhi strains, all (100%) were sensitive to cefixime, ceftriaxone and 11 (31%) were resistant to ampicillin, 15 (53.57%) were resistant to trimethoprim-sulfamethoxazole, 17 (60.71%) were resistant to chloramphenicol, 9 (32.14%) were resistant to ciprofloxacin and 27 (92.86%) were resistant to nalidixic acid.

Table 5: Antibiotic susceptibility pattern of isolated *S*. Typhi (*n*=46)

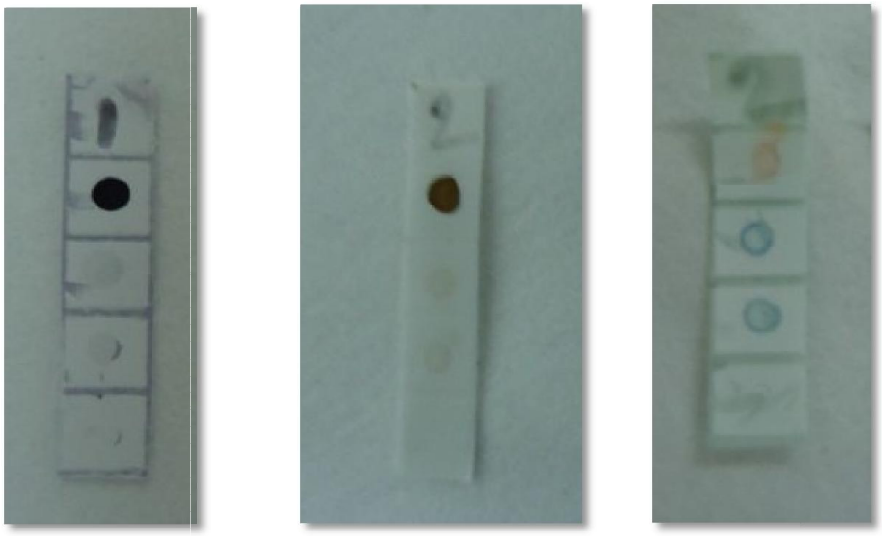
Antibiotic	Sensitive (%)	Resistant (%)
Ceftriaxone	28 (100)	0 (0.00)
Cefixim	28 (100)	0 (0.00)
Ciprofloxacin	9 (67.86)	9 (32.14)
Ampicillin	21 (60)	11 (31)
Chloramphenicol	11 (39.29)	17 (60.71)
Trimethoprim-sulphamethoxazole	26 (30)	15 (53.57)
Nalidixic acid	2 (7.14)	27 (92.86)

3.5. Comparison of the result of immunodot blot assay with the standard TPTest

Three types of substrates (4CN: 4-Chloro-1-Napthol, DAB: 3, 3$^{/}$-Diaminobenzidine, and TMB: 3, 3$^{/}$, 5, 5$^{/}$-Tetramethylbenzidine) were used for immunodot blot assay and results were compared with the TPTest. Three TPTest positive (12.32 EU, 14.08 EU and 13.6 EU) specimens were tested with three types of substrates for immunodot blot assay. Among three substrates, the intensity of the spot was higher when they tested with TMB and 4-CN was than DAB. Thereafter 4-CN and TMB were used for further analysis.

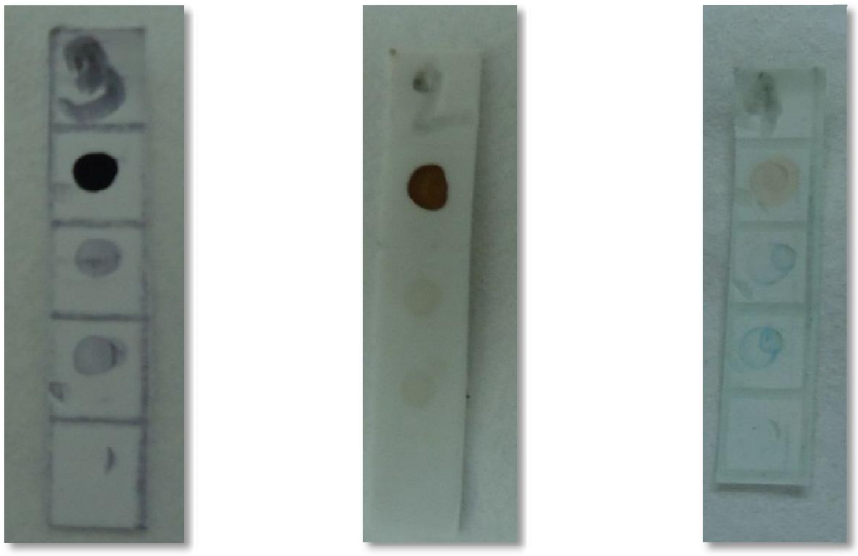
Figure 24: Comparison of the immunodot blot assay with TPTest positive specimens among three types of substrates

A. <u>TPTest result: 12.32 EU</u>



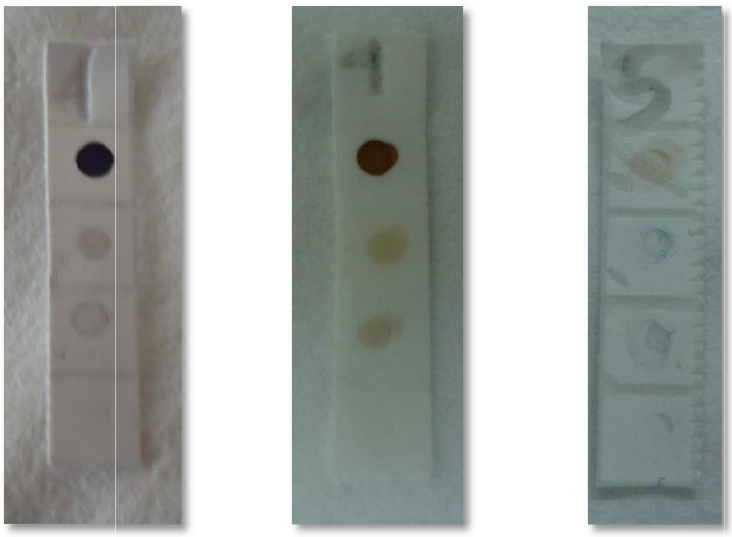
Substrate: 4-CN **Substrate: DAB** **Substrate: TMB**

<u>B.TPTest result: 14.08 EU</u>



Substrate: 4-CN **Substrate: DAB** **Substrate: TMB**

C.TPTest result: 13.6 EU



Substrate: 4-CN **Substrate: DAB** **Substrate: TMB**

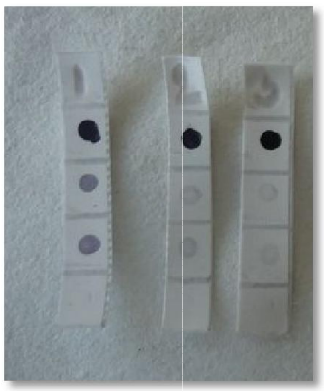
3.6 Results of the immunodot blot assay using different concentrations of antigen:

With1.5 mg/ml membrane preparation (MP) antigen:

To detect the TPTest positive (cut-off value: >10 EU) specimens by the immunodot blot technique and to increase the intensity of the spots, the concentration of the membrane preparation (MP) antigen (from 1 mg/ml to 1.5 mg/ml) and the amount of H_2O_2 were increased (from 0.5 µl/ml to 1 µl/ml). The immunodot blot assay with increased concentration of antigen and increased amount of H_2O_2 was carried out in different categories of patients:

i. Blood culture and TPTest positive:

All patients positive for blood culture positive were detected by dot blot assay.

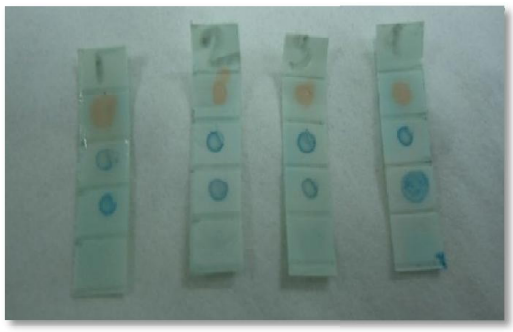


Test-Blood culture positive and TPTest positive (n=09)

01:12.06 EU; 02:11.15 EU

and 03=13.45 EU

Substrate: 4-CN



Test-Blood culture positive and TPTest positive (n=06)

01:13 EU; 02:12 EU; 03:11 EU and 04:15 EU

Substrate: TMB

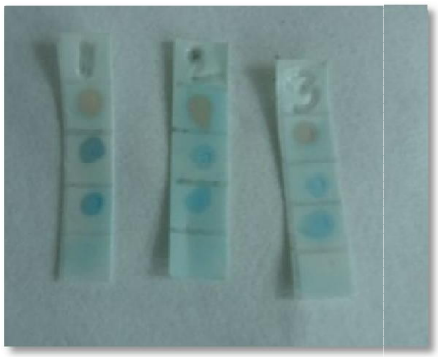
ii. Blood culture negative and TPTest positive:

All blood culture negative and TPTest positive specimens were detected by the dot blot assay.

Test-Blood culture negative and TPTest positive (n=04)

01:12.32 EU and 02:13.08 EU

Substrate: 4-CN



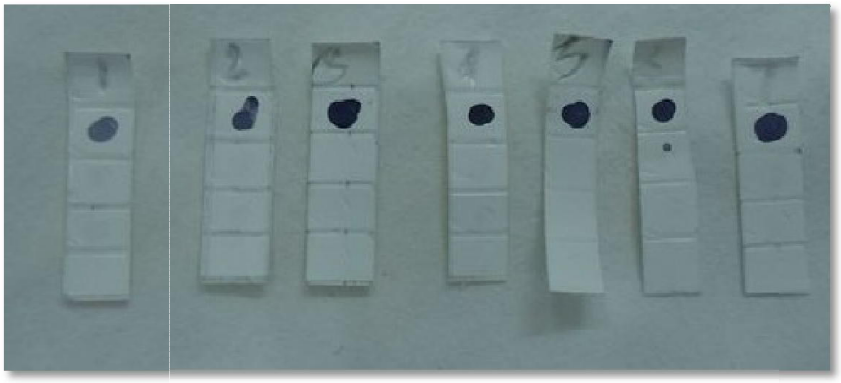
Test-Blood culture negative and TPTest positive (n=04)

01: 13 EU, 02: 12 EU and 03: 11 EU

Substrate: TMB

iii. Blood culture negative and TPTest negative:

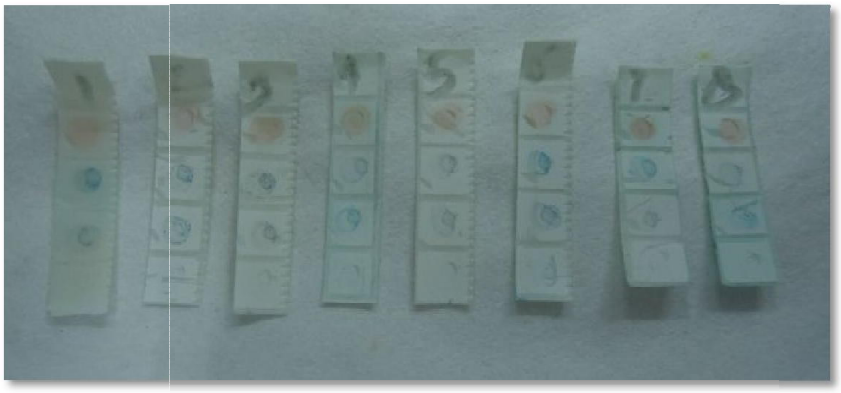
No colored dots were seen in blood culture negative and TPTest negative specimens using 4-CN. But dots were visualized in blood culture negative and TPTest negative using TMB.



Test-Blood culture negative and TPTest negative (n=14)

01: 8.0 EU; 02:1.0 EU; 03: 8.2 EU; 04:8.0 EU;05:4.0 EU; 06:9.36 EU and 07:9.57 EU

Substrate: 4-CN



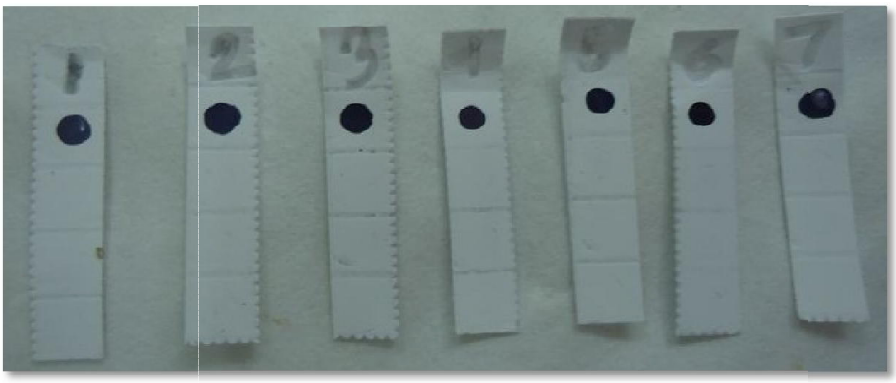
Test-Blood culture negative and TPTest negative (n=14)

01: 8.0 EU; 02: 1.0 EU; 03: 8.2 EU; 04:8.0 EU; 05: 4.0 EU, 06: 9.36 EU, 07: 9.57 EU and 08: 1.12 EU

Substrate: TMB

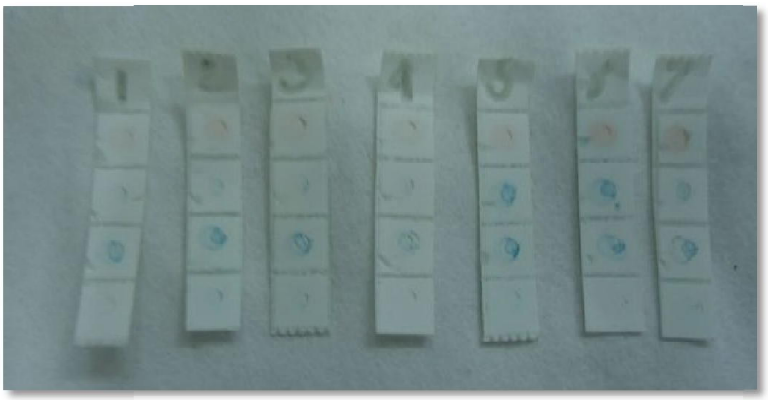
iv. Healthy control:

No dots were found in healthy control using 4-CN. But dots were found in healthy control using TMB.



Test-Healthy Control (n=12)

Substrate: 4-CN

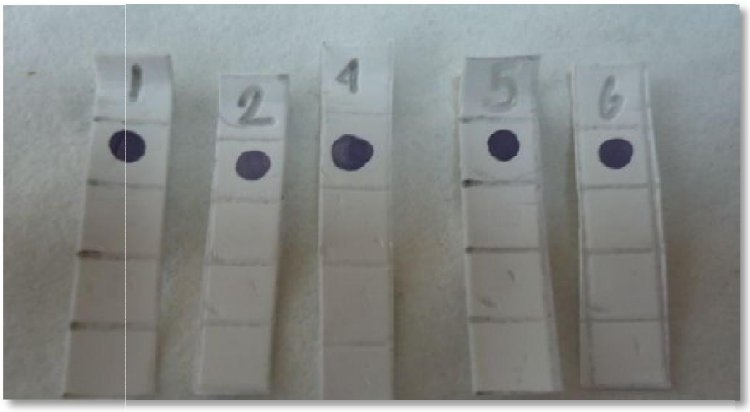


Test-Healthy Control
(n=12)

Substrate: TMB

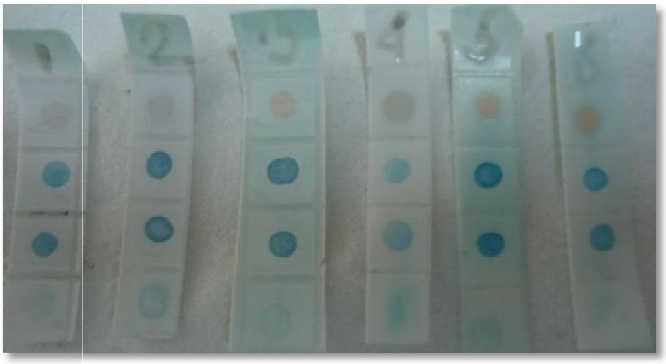
v. **Other febrile diseases:**

No dots were visualized in other febrile diseases using 4-CN. But dots were found in other febrile diseases using TMB.



Test-Other febrile illness (n=12)

Substrate: 4-CN



Test-Other febrile illness (n=12)

Substrate: TMB

Thus use TMB appeared to be more sensitive and gave positive results in TPTest positive specimens. But it had also showed positive results in negative specimens as well as healthy control and other febrile illness.

The use of 4-CN showed positive results in TPTest positive specimens and negative results in negative specimens, healthy control and other febrile illness. Hence 4-CN had taken for further analysis.

Summary of the results:

Table 6: Comparison of results between TPTest method and immunodot blot assay (Using 4-CN Substrate)

Patient Categories	TPTest Method		Immunodot Blot Assay	
	Positive	Negative	Positive	Negative
Blood culture positive and TPTest positive (n=09)	09	0	09	0
Blood culture negative and TPTest positive (n=04)	04	0	04	0
Blood culture negative and TPTest negative (n=14)	0	14	0	14
Healthy control (n=12)	0	12	0	12
Other febrile diseases (n=12)	0	12	0	12

Table 7: Comparison of results between TPTest method and immunodot blot assay (Using TMB Substrate)

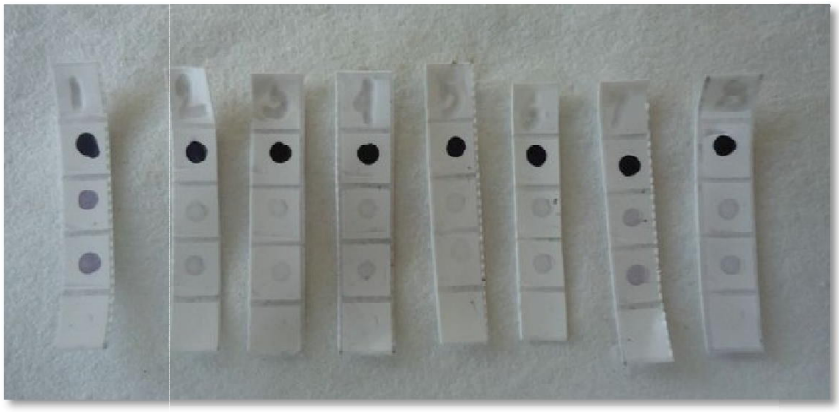
Patient Categories	TPTest Method		Immunodot Blot Assay	
	Positive	Negative	Positive	Negative
Blood culture positive and TPTest positive (n=06)	06	0	06	0
Blood culture negative and TPTest positive (n=04)	04	0	04	0
Blood culture negative and TPTest negative (n=14)	0	14	14	0
Healthy control (n=12)	0	12	12	0
Other febrile diseases (n=12)	0	12	12	0

Samples were further analyzed at three different time points- Day 0, Day 7 (early convalescence) and Day 21 (late convalescence). Samples were categorized according to their TPTest results.

- Low values (11-16 EU)
- Medium values (20- 70 EU)
- High values (70 and above)

Low values (11-16 EU):

In this category total samples were 30 in which 28 cases (93.3%) were positive by Immunodot blot assay but 30 cases (100%) positive by TPTest method. Only 2 cases (6.7%) were negative by Immunodot blot assay.



01: 15 EU; 02: 13.63 EU; 03:12.08 EU; 04:16 EU; 05:12.32 EU; 06= 15 EU, 07= 13 EU and 08= 11 EU

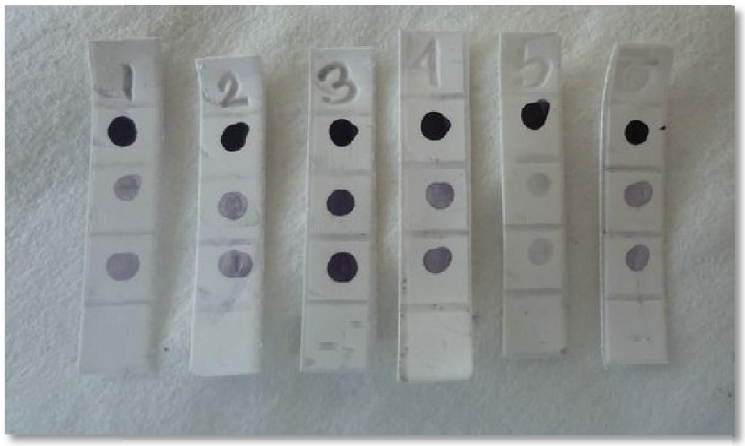
04:16 EU; 05:12.7 EU and 06:14.35 EU

Table 8: Comparison of results between TPTest method and immunodot blot assay (Low values)

Patient Categories	TPTest Method		Immunodot Blot Assay	
	Positive	Negative	Positive	Negative
Blood culture positive and TPTest positive (n=15)	15	0	15	0
Blood culture negative and TPTest positive (n=15)	15	0	13	0
Total samples (n=30)				

Medium values (20- 70 EU):

In this category total samples were 16 in which 16 cases (100%) were positive by TPTest method and Immunodot blot assay.



01=33 EU, 02=22 EU, 03=26 EU, 04=39 EU, 05=41.55 EU and 06=48.91 EU

Table 9: Comparison of results between TPTest method and immunodot blot assay (Medium values)

Patient Categories	TPTest Method		Immunodot Blot Assay	
	Positive	Negative	Positive	Negative
Blood culture positive and TPTest positive (n=09)	09	0	09	0
Blood culture negative and TPTest positive (n=07)	07	0	07	0
Total samples (n=16)				

High Values (≥70 EU):

In this category total samples were 10 in which 10 cases (100%) were positive by both the TPTest method and Immunodot blot assay.

01= 109 EU, 02=196.88 EU and 03=148.6 EU

Table 10: Comparison of results between TPTest method and immunodot blot assay (High values)

Patient Categories	TPTest Method		Immunodot Blot Assay	
	Positive	Negative	Positive	Negative
Blood culture positive and TPTest positive (n=07)	07	0	07	0
Blood culture negative and TPTest positive (n=03)	03	0	03	0
Total samples (n=10)				

Table 11: Summary of the results

Patient Categories (According to their ELISA Unit)	Positive	Negative
Low values (n=30)	28	02
Medium values (n=16)	16	0
High values (n=10)	10	0

3.7 Results of the immunodot blot assay using specimens prepared by RBC lysis:

The isolated cells from 13 patients and 11 healthy controls by the RBC lysis procedure were incubated at 37^{o}C with 5% CO_2 supply and with no CO_2 supply. After incubation, the cell culture supernatant was tested by dot blot assay.

3.7.1 Results of immunodot blot assay using specimens prepared by RBC lysis with 5% CO_2

The specimen prepared by RBC lysis with 5% CO_2 was tested by immunodot blot assay with a concentration of MP (1.5 mg/ml) antigen.

i. Blood culture and TPTest positive:

All blood culture and TPTest positive were detected by dot blot assay.

Blood culture positive and TPTest positive
(n=02)

04: EU=32.2

Substrate: 4-CN

ii. Blood culture negative and TPTest positive:

All blood culture negative and TPTest positive were detected by dot blot assay.

Blood culture negative and TPTest positive (n=01)

01: 65.57 EU

iii. Blood culture negative and TPTest negative:

All blood culture negative and TPTest negative also showed positve results by dot blot assay.

Blood culture negative and TPTest negative (n=08)

01= 10.21 EU, 02=5.95 EU, 03= 8.51 EU, 04= 1.8 EU and

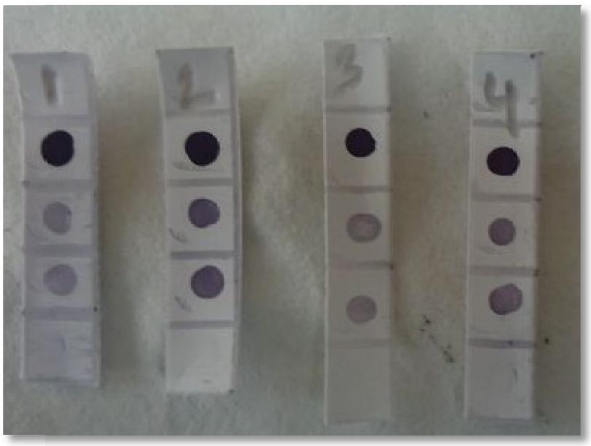
05=1.6 EU

3.7.2 Results of immunodot blot assay using specimens prepared by RBC lysis with no CO_2

The specimen prepared by RBC lysis with no CO_2 was tested by immunodot blot assay with a concentration of MP (1.5 mg/ml) antigen.

i. Blood culture and TPTest positive:

All blood culture and TPTest positive were detected by dot blot assay.



Blood culture positive and TPTest positive
(n=04)

01= 21.46 EU, 02= 103.39 EU, 03= 75.14 EU and
04=168.93 EU

ii. Blood culture negative and TPTest positive:

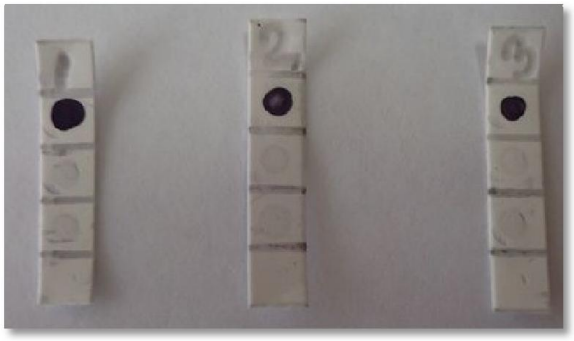
All blood culture negative and TPTest positive were detected by dot blot assay.

Blood culture negative and TPTest positive (n=01)

02: 81.04 EU

iii. Blood culture negative and TPTest negative:

All blood culture negative and TPTest negative also showed positve results by dot blot assay.

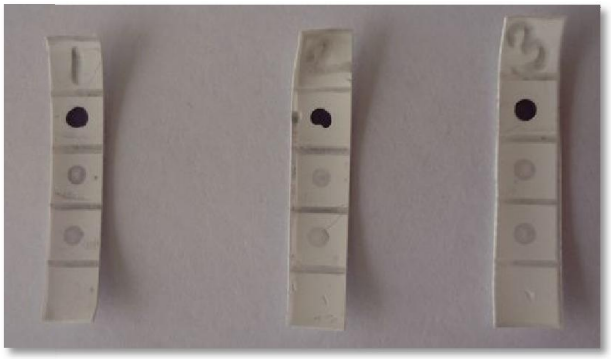


Blood culture negative and TPTest negative

01=4.30 EU, 02=3.86 EU and 03=3.02 EU

iv. Healthy control:

All healthy control also showed positve results by dot blot assay.



Healthy control (n=11)

The specimen prepared by RBC lysis with no CO_2 was tested by immunodot blot assay with a concentration of MP (0.5 mg/ml) antigen.

- **Healthy control:**

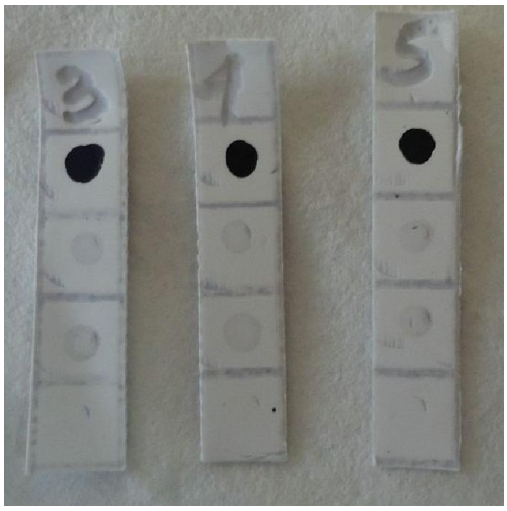
  All healthy control (n=11) also showed positve results by dot blot assay.

Healthy control (n=11)

The specimen prepared by RBC lysis with no CO_2 was tested by immunodot blot assay with a concentration of MP (1 mg/ml) antigen.

- **Healthy control:**

  All healthy controls (n=11) also showed positve results by dot blot assay.



Healthy control (n=11)

Chapter 4

Discussion

Typhoid fever is perceived as a major cause of morbidity worldwide [4] [95]. In Bangladesh, it occurs as an endemic disease [96]. Clinical diagnosis of typhoid fever is difficult because the symptoms of typhoid fever mimic other febrile diseases [97]. Isolation of *Salmonella enterica* serovar Typhi from bone marrow culture is the gold standard of diagnosis of an infection but it is an invasive method and improbable in resource limited condition. It is also clinically impractical method due to hazardous procedure and technical expert and well equipped laboratory are needed to isolate pathogen from bone marrow [89]. Blood culture has low sensitivity. The sensitivity of blood culture test also is only around 30-50% [6]. Large volume of blood is needed for culture (3 ml for children and 5 ml for adults) and it also takes at least three days to obtain a result [98] [99]. The TPTest is a diagnostic method for early diagnosis of typhoid and paratyphoid (enteric) fever. From the venous blood of the patients, the lymphocytes that have been recovered are primed and derived from the gut mucosa and are transiently circulating in the peripheral circulation and IgA antibodies represent markers of recent infection in the gut to membrane preparation (MP) antigen of *S.* Typhi [93, 100]. The sensitivity and specificity of the TPTest are 100% and 78-97% respectively [6].

In this study, automated blood culture was used for isolation of the organism and disk diffusion method was used for determination of the antibiotic susceptibility pattern of the isolated strains of *S.* Typhi. Antibiogram was carried out with commonly used antibiotics and varying susceptibility patterns were observed. Findings uncover that a higher level of resistance to first line of antibiotics which may be due to widespread and improper use of antibiotic. In this study, among 46 isolated serovar Typhi, 60% were sensitive to ampicillin, 30% were sensitive to trimethoprim-sulfamethoxazole, 39.29% sensitive to chloramphenicol and 67.86% were sensitive to ciprofloxacin. All the isolated serovar Typhi were 100% sensitive to cefixime and ceftriaxone. In this study, 7.14% nalidixicacid- resistant *Salmonella* Typhi (NARST) were isolated. The isolated serovar Typhi that are resistant to nalidixic acid (NARST) and susceptible to ciprofloxacin by current disk diffusion method, have been proposed as an indicator that such serovar Typhi strain may not respond to fluoroquinolone treatment [95].

Previous study reported that all blood culture positive typhoid fever patients could be identified by the TPTest [6]. Simplification of the TPTest was done at different stages of the method, like cell separation method, incubation of the isolated cells with CO_2 supplementation. The immunodot blot assay could not detect positive (cut-off: 10 ELISA unit) samples having ≤16 ELISA unit (EU). Hence in this study this method was optimized for the detection of positive samples with low values (≤16 EU).

Previously the immunodot blot assay was used to detect the IgA antibody response in the peripheral blood antibodies (PBA) specimens using the ficoll density gradient centrifugation and 4-Chloro-1 –Napthol (4CN) Substrate. The concentration for membrane preparation (MP) antigen was 1mg/ml. Here in addition to 4CN substrate 3, $3^{/}$-Diaminobenzidine (DAB) and 3, $3^{/}$, 5, $5^{/}$-Tetramethylbenzidine (TMB) were used to increase the intensity of the dot. The dots for positive patients were less prominent with DAB than TMB and 4-CN.

The antigen concentration and the amount of H_2O_2 were increased for TMB and 4-CN, and different categories of the participants were tested. The optimized dot blot assay using 4-CN has showed positive result for all blood culture and TPTest positive patients and the assay was negative for negative patients and healthy controls and also for the patients with other febrile illness (kala-azar and tuberculosis). The optimized dot blot assay using TMB has shown less specificity and was positive for all categories of participants including negative patients, healthy controls and other febrile illness patients. Therefore TMB was not used for further experiments.

The immunodot blot assay was also carried out with simplified RBC lysis cell separation, incubation of the isolated cells at 37^{o}C with and/or without CO_2 supply. The specimens prepared by RBC lysis procedure give nonspecific results for all categories of the participants. The experiment was also carried out with reduced concentration of the MP antigen and found the same nonspecific positive dots for negative patients, healthy controls and patients with other diseases. Further experiments need to be carried out to optimize the simplified dot blot assay using RBC lysis cell separation and incubation of cells without CO_2 supply.

At present, the TPTest positive patients with lower values can be detected by immunodot blot assay using density gradient centrifugation without CO_2 supplementation. The results of this study suggest that the optimized immunodot blot assay will be helpful for diagnosis of enteric fever at the laboratories with less facility in endemic region like Bangladesh.

The following conclusions can be drawn from the present study:

i. This study simplified and optimized the TPTest method for the detection of positive samples with low values (≤16 EU).
ii. Addition to 4-Chloro-1-Napthol (4CN) substrate, 3,3′-Diaminobenzidine (DAB) and 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) increased the intensity of the dots. The dots for positive patients were less prominent with DAB than TMB and 4-CN.
iii. The optimized dot blot assay using 4-CN showed positive results for all blood culture and TPTest positive patients and the assay was negative for negative patients and healthy controls and also for the patients with other febrile illness (kala-azar and tuberculosis).

Recommendations for future work

The different parameters of the TPTest need further modifications to make it adaptable for a rapid test that can be used in field settings and laboratories which lack facilities. These include procedures including cell separation techniques, incubation conditions, mixing of different substrates to improve the sensitivity of the test and further work in this direction will result in a simple, rapid, and effective at the same time sensitive test for diagnosis of a dreadful disease like typhoid and paratyphoid fever.

Chapter 5

References

1. Liang, L., et al., *Immune profiling with a Salmonella Typhi antigen microarray identifies new diagnostic biomarkers of human typhoid.* Sci Rep. **3**: p. 1043.
2. Waddington, C.S., T.C. Darton, and A.J. Pollard, *The challenge of enteric fever.* J Infect. **68 Suppl 1**: p. S38-50.
3. Pang, T., et al., *Typhoid fever and other salmonellosis: a continuing challenge.* Trends Microbiol, 1995. **3**(7): p. 253-5.
4. Crump, J.A., S.P. Luby, and E.D. Mintz, *The global burden of typhoid fever.* Bull World Health Organ, 2004. **82**(5): p. 346-53.
5. Brooks, W.A., et al., *Bacteremic typhoid fever in children in an urban slum, Bangladesh.* Emerg Infect Dis, 2005. **11**(2): p. 326-9.
6. Khanam, F., et al., *Evaluation of a typhoid/paratyphoid diagnostic assay (TPTest) detecting anti-Salmonella IgA in secretions of peripheral blood lymphocytes in patients in Dhaka, Bangladesh.* PLoS Negl Trop Dis. **7**(7): p. e2316.
7. Kothari, A., A. Pruthi, and T.D. Chugh, *The burden of enteric fever.* J Infect Dev Ctries, 2008. **2**(4): p. 253-9.
8. Corner, R.J., A.M. Dewan, and M. Hashizume, *Modelling typhoid risk in Dhaka metropolitan area of Bangladesh: the role of socio-economic and environmental factors.* Int J Health Geogr. **12**: p. 13.
9. Maskey, A.P., et al., *Salmonella enterica serovar Paratyphi A and S. enterica serovar Typhi cause indistinguishable clinical syndromes in Kathmandu, Nepal.* Clin Infect Dis, 2006. **42**(9): p. 1247-53.

10. Maurice, J., *A first step in bringing typhoid fever out of the closet.* Lancet. **379**(9817): p. 699-700.

11. Wahid, R., et al., *Live oral typhoid vaccine Ty21a induces cross-reactive humoral immune responses against Salmonella enterica serovar Paratyphi A and S. Paratyphi B in humans.* Clin Vaccine Immunol. **19**(6): p. 825-34.

12. Naheed, A., et al., *Burden of typhoid and paratyphoid fever in a densely populated urban community, Dhaka, Bangladesh.* Int J Infect Dis. **14 Suppl 3**: p. e93-9.

13. Sinha, A., et al., *Typhoid fever in children aged less than 5 years.* Lancet, 1999. **354**(9180): p. 734-7.

14. Agarwal, K.S., et al., *A study of current trends in enteric fever.* J Commun Dis, 1998. **30**(3): p. 171-4.

15. Walsh, A.L., et al., *Bacteremia in febrile Malawian children: clinical and microbiologic features.* Pediatr Infect Dis J, 2000. **19**(4): p. 312-8.

16. Saha, S.K., et al., *Typhoid fever in Bangladesh: implications for vaccination policy.* Pediatr Infect Dis J, 2001. **20**(5): p. 521-4.

17. Levine, M.M., D.N. Taylor, and C. Ferreccio, *Typhoid vaccines come of age.* Pediatr Infect Dis J, 1989. **8**(6): p. 374-81.

18. Engels, E.A., et al., *Typhoid fever vaccines: a meta-analysis of studies on efficacy and toxicity.* BMJ, 1998. **316**(7125): p. 110-6.

19. Fukushima, M., K. Kakinuma, and R. Kawaguchi, *Phylogenetic analysis of Salmonella, Shigella, and Escherichia coli strains on the basis of the gyrB gene sequence.* J Clin Microbiol, 2002. **40**(8): p. 2779-85.

20. Liu, S.L., *Physical mapping of Salmonella genomes.* Methods Mol Biol, 2007. **394**: p. 39-58.

21. Kopecko, L.H.a.D.J., *Salmonella Typhi and Paratyphi ,*2001. **Chapter 66**.

22. McClelland, M., et al., *Complete genome sequence of Salmonella enterica serovar Typhimurium LT2.* Nature, 2001. **413**(6858): p. 852-6.

23. Reeves, P., *Evolution of Salmonella O antigen variation by interspecific gene transfer on a large scale.* Trends Genet, 1993. **9**(1): p. 17-22.

24. Gruenewald, R., et al., *Identification of Salmonella somatic and flagellar antigens by modified serological methods.* Appl Environ Microbiol, 1990. **56**(1): p. 24-30.

25. Collee, J., Fraser, AG, Marmion, BP, Simons, *Salmonella infection, in Mackie and McCartney, in Practical Medical Microbiology.* . 2006: p. 385-402.

26. Wyant, T.L., M.K. Tanner, and M.B. Sztein, *Salmonella typhi flagella are potent inducers of proinflammatory cytokine secretion by human monocytes.* Infect Immun, 1999. **67**(7): p. 3619-24.

27. Thong, K.L., et al., *Genetic diversity of clinical and environmental strains of Salmonella enterica serotype Weltevreden isolated in Malaysia.* J Clin Microbiol, 2002. **40**(7): p. 2498-503.

28. Samuel Baron MD , R.C.P., Deborah A. James, *Medical Microbiology.* 1996.

29. Wain, J., et al., *Vi antigen expression in Salmonella enterica serovar Typhi clinical isolates from Pakistan.* J Clin Microbiol, 2005. **43**(3): p. 1158-65.

30. Szu, S.C., et al., *Relation between structure and immunologic properties of the Vi capsular polysaccharide.* Infect Immun, 1991. **59**(12): p. 4555-61.

31. Guzman, C.A., et al., *Vaccines against typhoid fever.* Vaccine, 2006. **24**(18): p. 3804-11.

32. Baker, S. and G. Dougan, *The genome of Salmonella enterica serovar Typhi.* Clin Infect Dis, 2007. **45 Suppl 1**: p. S29-33.

33. *Molecular and biologic features of Salmonella Typhi.* 2013.

34. Zhou, D. and J. Galan, *Salmonella entry into host cells: the work in concert of type III secreted effector proteins.* Microbes Infect, 2001. **3**(14-15): p. 1293-8.

35. Zhang, X.L., V.T. Jeza, and Q. Pan, *Salmonella typhi: from a human pathogen to a vaccine vector.* Cell Mol Immunol, 2008. **5**(2): p. 91-7.

36. Santos, R.L., et al., *Pathogenesis of Salmonella-induced enteritis.* Braz J Med Biol Res, 2003. **36**(1): p. 3-12.

37. Swaddiwudhipong, W. and J. Kanlayanaphotporn, *A common-source water-borne outbreak of multidrug-resistant typhoid fever in a rural Thai community.* J Med Assoc Thai, 2001. **84**(11): p. 1513-7.

38. Mermin, J.H., et al., *A massive epidemic of multidrug-resistant typhoid fever in Tajikistan associated with consumption of municipal water.* J Infect Dis, 1999. **179**(6): p. 1416-22.

39. Velema, J.P., et al., *Typhoid fever in Ujung Pandang, Indonesia--high-risk groups and high-risk behaviours.* Trop Med Int Health, 1997. **2**(11): p. 1088-94.

40. Cho, J.C. and S.J. Kim, *Viable, but non-culturable, state of a green fluorescence protein-tagged environmental isolate of Salmonella typhi in groundwater and pond water.* FEMS Microbiol Lett, 1999. **170**(1): p. 257-64.

41. Parry, C.M., et al., *Typhoid fever.* N Engl J Med, 2002. **347**(22): p. 1770-82.

42. Bhan, M.K., et al., *Association between Helicobacter pylori infection and increased risk of typhoid fever.* J Infect Dis, 2002. **186**(12): p. 1857-60.

43. Dutta, T.K., Beeresha, and L.H. Ghotekar, *Atypical manifestations of typhoid fever.* J Postgrad Med, 2001. **47**(4): p. 248-51.

44. Lin, F.Y., et al., *The epidemiology of typhoid fever in the Dong Thap Province, Mekong Delta region of Vietnam.* Am J Trop Med Hyg, 2000. **62**(5): p. 644-8.

45. Dewan, A.M., et al., *Typhoid Fever and its association with environmental factors in the Dhaka Metropolitan Area of Bangladesh: a spatial and time-series approach.* PLoS Negl Trop Dis. **7**(1): p. e1998.

46. Keystone, D., *Prolonged Fever Is Typhoid's Hallmark* 2011.

47. Bobin, A.N., N.D. Klochkov, and N.V. Bogomolova, *[Complications and the proximate causes of death in typhoid]*. Voen Med Zh, 1993(1): p. 49-52.

48. Hornick, R.B., et al., *Typhoid fever: pathogenesis and immunologic control.* N Engl J Med, 1970. **283**(13): p. 686-91.

49. Monack, D.M., A. Mueller, and S. Falkow, *Persistent bacterial infections: the interface of the pathogen and the host immune system.* Nat Rev Microbiol, 2004. **2**(9): p. 747-65.

50. Broz, P., M.B. Ohlson, and D.M. Monack, *Innate immune response to Salmonella typhimurium, a model enteric pathogen.* Gut Microbes. **3**(2): p. 62-70.

51. Hanna K. de Jong, C.M.P., Tom van der Poll and W. Joost Wiersinga, *Host–Pathogen Interaction in Invasive Salmonellosis.* PLOS Pathogens, 2012 **8**((10)).

52. George, A., et al., *Regulation of cell-mediated immunity in mice immunised with Salmonella enteritidis.* J Med Microbiol, 1987. **23**(3): p. 239-46.

53. Hsu, H.S., *Pathogenesis and immunity in murine salmonellosis.* Microbiol Rev, 1989. **53**(4): p. 390-409.

54. Sztein, M.B., *Cell-mediated immunity and antibody responses elicited by attenuated Salmonella enterica Serovar Typhi strains used as live oral vaccines in humans.* Clin Infect Dis, 2007. **45 Suppl 1**: p. S15-9.

55. Salerno-Goncalves, R., et al., *Identification of a human HLA-E-restricted CD8+ T cell subset in volunteers immunized with Salmonella enterica serovar Typhi strain Ty21a typhoid vaccine.* J Immunol, 2004. **173**(9): p. 5852-62.

56. Sood, S., et al., *Cellular immune response induced by Salmonella enterica serotype Typhi iron-regulated outer-membrane proteins at peripheral and mucosal levels.* J Med Microbiol, 2005. **54**(Pt 9): p. 815-21.

57. Wei, B., et al., *Resident enteric microbiota and CD8+ T cells shape the abundance of marginal zone B cells.* Eur J Immunol, 2008. **38**(12): p. 3411-25.

58. *Immunology.* 2001.

59. Jawetz, W.L.a.E., *Medical Microbiology & Immunology.* 2000.

60. Abul K. Abbas, S.P., Andrew H. H. Lichtman, *Cellular and Molecular Immunology.* 2011.

61. Holtmeier, W. and D. Kabelitz, *gammadelta T cells link innate and adaptive immune responses.* Chem Immunol Allergy, 2005. **86**: p. 151-83.

62. Sejvar, J., et al., *Neurologic manifestations associated with an outbreak of typhoid fever, Malawi--Mozambique, 2009: an epidemiologic investigation.* PLoS One. **7**(12): p. e46099.

63. Patel, T.A., et al., *Imported enteric fever: case series from the hospital for tropical diseases, London, United Kingdom.* Am J Trop Med Hyg. **82**(6): p. 1121-6.

64. Nakachi, S., et al., *Clinical features and early diagnosis of typhoid fever emphasizing usefulness of detecting mesenteric lymphadenopathy with ultrasound as diagnostic method.* Southeast Asian J Trop Med Public Health, 2003. **34 Suppl 2**: p. 153-7.

65. Giannella Ra, e.M., Friedman LS and Brandt LJ, *Infectious enteritis and proctocolitis and bacterial food poisoning. Sleisenger & Fordtran's Gastrointestinal and Liver Disease.* 2010.

66. Lima AAM, G.R., Mandell GL, Bennett JE and Dolin R, *Inflammatory enteritides. Principles and Practice of Infectious Diseases.* 2009.

67. Atamanalp, S.S., et al., *Typhoid intestinal perforations: twenty-six year experience.* World J Surg, 2007. **31**(9): p. 1883-8.

68. Adesunkanmi, A.R. and O.G. Ajao, *The prognostic factors in typhoid ileal perforation: a prospective study of 50 patients.* J R Coll Surg Edinb, 1997. **42**(6): p. 395-9.

69. Allal, R., et al., *Splenic abscesses in typhoid fever: US and CT studies.* J Comput Assist Tomogr, 1993. **17**(1): p. 90-3.

70. Calleri, G., et al., *[Splenic abscess in typhoid fever]*. Clin Ter, 1991. **137**(4): p. 281-3.

71. Mürvet YÜKSEL, K.U.Ö.a.S.B., *Multiple Splenic Abscesses in a Child as a Complication of Typhoid Fever.* Fırat Tıp Dergisi, 2001.

72. Hasbun, J., Jr., R. Osorio, and A. Hasbun, *Hepatic dysfunction in typhoid fever during pregnancy.* Infect Dis Obstet Gynecol, 2006. **2006**: p. 64828.

73. Anand Pandey, A.N.G.a.V.K., *Gall Bladder Perforation as a Complication of Typhoid Fever.* Saudi J Gastroenterol, 2008. **14**(4): p. 213.

74. Gali, B.M., et al., *Gallbladder perforation complicating typhoid fever: report of two cases.* Niger J Med. **20**(1): p. 181-3.

75. Ramachandran, S., H.R. Wickremesinghe, and M.V. Perera, *Acute disseminated encephalomyelitis in typhoid fever.* Br Med J, 1975. **1**(5956): p. 494-5.

76. Dunne, J.A., J. Wilson, and J. Gokhale, *Small bowel perforation secondary to enteric Salmonella paratyphi A infection.* BMJ Case Rep. **2011**.

77. Brown, J.C., et al., *Mutations responsible for reduced susceptibility to 4-quinolones in clinical isolates of multi-resistant Salmonella typhi in India.* J Antimicrob Chemother, 1996. **37**(5): p. 891-900.

78. Rahman, M., et al., *Emergence of multidrug-resistant Salmonella Gloucester and Salmonella typhimurium in Bangladesh.* J Health Popul Nutr, 2001. **19**(3): p. 191-8.

79. Hirose, K., et al., *Antibiotic susceptibilities of Salmonella enterica serovar Typhi and S. enterica serovar Paratyphi A isolated from patients in Japan.* Antimicrob Agents Chemother, 2001. **45**(3): p. 956-8.

80. Manchanda, V., et al., *Treatment of enteric fever in children on the basis of current trends of antimicrobial susceptibility of Salmonella enterica serovar typhi and paratyphi A.* Indian J Med Microbiol, 2006. **24**(2): p. 101-6.

81. Butler, T., *Treatment of typhoid fever in the 21st century: promises and shortcomings.* Clin Microbiol Infect. **17**(7): p. 959-63.

82. Basnyat, B., *The treatment of enteric fever.* Journal of The Royal Society of Medicine, 2007. **100**(4): p. 161-162.

83. Islam, A., et al., *Treatment of typhoid fever with ceftriaxone for 5 days or chloramphenicol for 14 days: a randomized clinical trial.* Antimicrob Agents Chemother, 1993. **37**(8): p. 1572-5.

84. WHO, *Background document:The diagnosis, treatment and prevention of typhoid fever.* World Health Organization, 2003.

85. Silva, B.A., et al., *Genetic characteristics of the Salmonella typhi strain Ty21a vaccine.* J Infect Dis, 1987. **155**(5): p. 1077-8.

86. Levine, M.M. and M.B. Sztein, *Vaccine development strategies for improving immunization: the role of modern immunology.* Nat Immunol, 2004. **5**(5): p. 460-4.

87. Wain, J., et al., *Quantitation of bacteria in blood of typhoid fever patients and relationship between counts and clinical features, transmissibility, and antibiotic resistance.* J Clin Microbiol, 1998. **36**(6): p. 1683-7.

88. Akoh, J.A., *Relative sensitivity of blood and bone marrow cultures in typhoid fever.* Trop Doct, 1991. **21**(4): p. 174-6.

89. Vallenas, C., et al., *Efficacy of bone marrow, blood, stool and duodenal contents cultures for bacteriologic confirmation of typhoid fever in children.* Pediatr Infect Dis, 1985. **4**(5): p. 496-8.

90. Ohanu, M.E., et al., *Interference by malaria in the diagnosis of typhoid using Widal test alone.* West Afr J Med, 2003. **22**(3): p. 250-2.

91. Olopoenia, L.A. and A.L. King, *Widal agglutination test - 100 years later: still plagued by controversy.* Postgrad Med J, 2000. **76**(892): p. 80-4.

92. *The enzyme-linked immunosorbent assay (ELISA).* Bull World Health Organ, 1976. **.54**(2): p. 129–139.

93. Qadri, F., et al., *Antigen-specific immunoglobulin A antibodies secreted from circulating B cells are an effective marker for recent local immune responses in patients with cholera: comparison to antibody-secreting cell responses and other immunological markers.* Infect Immun, 2003. **71**(8): p. 4808-14.

94. Sheikh, A., et al., *Salmonella enterica serovar Typhi-specific immunoglobulin A antibody responses in plasma and antibody in lymphocyte supernatant specimens in Bangladeshi patients with suspected typhoid fever.* Clin Vaccine Immunol, 2009. **16**(11): p. 1587-94.

95. Bhutta, Z.A., *Impact of age and drug resistance on mortality in typhoid fever.* Arch Dis Child, 1996. **75**(3): p. 214-7.

96. Ahmed, D.M.U., *Burden of Typhoid Fever in Bangladesh.*

97. Baker, S., M. Favorov, and G. Dougan, *Searching for the elusive typhoid diagnostic.* BMC Infect Dis. **10**: p. 45.

98. Bopp CA, M.P., *Manual of clinical microbiology, in Escherichia, Shigella and Salmonella.* Washington, DC: ASM Press., 2003: p. 654–671.

99. Cherian, T., et al., *Prevalence of Salmonella typhi O and H antibodies in the serum of infants and preschool children.* Indian Pediatr, 1990. **27**(3): p. 293-4.

100. Charles, R.C., et al., *Characterization of anti-Salmonella enterica serotype Typhi antibody responses in bacteremic Bangladeshi patients by an immunoaffinity proteomics-based technology*. Clin Vaccine Immunol. **17**(8): p. 1188-95.

Appendices

APPENDIX A

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.

APPENDIX B

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

APPENDIX C

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

Trypticase soy Broth (1000 mL)

Pancreatic digest of casein	15.0 g
Papaic digest of soyabean	5.0 g
Sodium chloride	5.0 g
Bacto agar	15.0 g
Distilled water	1000 mL

pH =7.3 + 0.2

Motility Indole Urea (MIU)

Peptone	30.0 g
KH_2PO_4	2.0 g
Sodium chloride	5.0 g
Phenol Red	0.005 g
Urea	20.0 g
Bacto agar	4.0 g
Distilled water	1000 mL

pH =7.3+ 0.2

Citrate agar (Simmons, 1926 Modified)

NaCl	5.0 g
$MgSO_4$	0.2 g
NH_4PO_4	1.0 g
K_2HPO_4	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	1000 mL

pH =7.3+ 0.1

APPENDIX D

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

Preparation of DAB Substrate (4ml)

Phosphate Buffer Saline	4ml
DAB	0.002gm
H_2O_2 (Hydrogen Peroxide)	6 µl

Preparation of TMB

DMSO	10 ml
TMB	0.1gm

Preparation of TMB Substrate (5ml)

Na-acetate	2.5 ml
Deionized water	2.5 ml

Citric acid	25 µl
TMB	50 µl
H_2O_2 (Hydrogen Peroxide)	0.6 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.

APPENDIX E

- MS word
- Endnote X
- Ascent software
- Microsoft excel-2007 software
- Instat2 (Graph PAD Software 10855, USA)