

Study On *Salmonella* Typhi – Isolation, Characterization, And Immunological Studies.



**A dissertation submitted to the Brac University in partial fulfillment of
the requirements for the Master degree of science in Biotechnology.**

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ABSTRACT

Typhoid fever is one of the most common febrile illnesses and its real impact is difficult to estimate because the clinical picture is confused with other febrile infections. Various diagnostic techniques are used for clinical diagnosis of typhoid patients. Unfortunately no technique so far used for can be considered as gold standard as a diagnostic tool for typhoid fever. Widal test is widely used in the developing countries for the diagnosis of the typhoid fever; however, Widal test takes a longer time for diagnosis, having high proportion of false positive results which would complicate the treatment. Blood culture technique is limited due to the indiscriminate use of antibiotics and its utility is restricted due to low numbers of bacteria in blood. Antibodies in lymphocyte supernatant (ALS) assay has recently been developed and standardized for the diagnosis of typhoid fever which is highly sensitive for detection of *Salmonella* Typhi infection at early stage. Comparison of ALS assay with blood culture and Widal test has been carried out and the method is sensitive and specific. ALS is able to confirm typhoid infection in 52% patients, whereas, blood culture test confirmed 13% and Widal test confirmed 44% patients to be positive on *Salmonella* Typhi. Using ALS assay typhoid fever was diagnosed and confirmed within 48 hrs of fever. ALS assay can be used for rapid diagnosis of typhoid fever.

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LIST OF ABBREVIATION

ALS	Antibody in lymphocytes supernatant
BSA	Bovine serum albumin
dH ₂ O	Deionized H ₂ O
ELISA	Enzyme linked immunosorbent assay
HAg	Flageller antigen
h.	Hour
ICDDR, B	International Centre for Diarrhoeal Disease Research, Bangladesh
Ig	Immunoglobulin
IL	Interleukin
INF- λ	Interferon- λ
LPS	Lipopolysaccharide
LSD	Laboratory Science Division
mAB	Milli absorbance
ml	milli Liter
MP	Membrane protein
NaCl	Sodium Chloride
O Ag	Capsular Antigen
OD	Optical Density
OPD	O-Phenylene diamine
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
RBC	Red blood cell
rpm	Rotation per minute
<i>S. Typhi</i>	<i>Salmonella</i> Typhi
TBS	Tris buffered saline
TNF- α	Tumor necrosis factor- α
TSB	Trypticase soy broth
Vi	Virulence factor
WC	Whole cell

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Chapter-1

Introduction

1.1. Introduction

Salmonella enterica serova Typhi (recently known as *Salmonella* Typhi) [1] only infects humans. *Salmonella* Typhi is a gram negative, flagellate, noncapsulated, nonsporulating, aerobic bacteria in the family Enterobacteriaceae that has more than 2501 serotypes previously described in the Kauffman-White schema. In the perspective of Bangladesh the incidence of typhoid fever is high in urban areas. The greatest incidence of infection is in children of age less than 5 years [3]. In Bangladesh about 53% of typhoid fever cases in the community are seen children aged below 5 years, with 85% of typhoid of cases occurring among the children aged 1-4 years.

1.2. History

The genus *Salmonella* was named after Daniel Elmer Salmon, an American veterinary pathologist. Salmon, along with Theobald Smith, discovered the organism that causes hog cholera, *Salmonella enterica* var. Choleraesuis [4]. At the beginning of the 19th century, typhoid was defined on the basis of clinical signs and symptoms and pathological (anatomical) changes. However, at this time, all sorts of enteric fevers were characterized as "typhoid". In 1880s, the typhoid bacillus was first observed by Eberth in spleen sections and mesenteric lymph nodes from a patient who died from typhoid. Robert Koch confirmed a related finding by Gaffky and succeeded in cultivating the bacterium in 1881. But due to the lack of differential characters, separation of the typhoid bacillus from other enteric bacteria was uncertain. In 1896, it was demonstrated that the serum from an animal immunized with the typhoid bacillus agglutinated (clumped) the typhoid bacterial cells, and it was shown that the serum of patients afflicted with typhoid

likewise agglutinated the typhoid bacillus. Serodiagnosis of typhoid was thus made possible by 1896 [5].

1.3. Transmission

The main route of transmission of *Salmonella Typhi* is water and food. The bacteria survive for a varying number of weeks in water, ice and dust, and can multiply in food. In developing countries the infection is endemic. Sometimes there may be local epidemics. The bacteria only infect humans. There is no animal reservoir, unlike the majority of the other *Salmonella* species. People can be healthy carriers and excrete *Salmonella Typhi* for prolonged periods. A classic example of a healthy carrier is the case of "Typhoid Mary", the nickname of a woman who became very famous at the beginning of the 20th century. In 1904 there was an epidemic of typhoid fever in a district of Long Island, New York. It was discovered that patients belonged to households where Mary Mallon had been cook [9].

1.4. Physiology and structural features

Around the cytoplasmic membrane lies a thin layer of peptidoglycans. This so-called murine layer consists of long chains of repetitive disaccharide links. Oligopeptide bridges connect the sugar chains. External to this second layer is a third layer, the outer membrane. It consists of a phospholipids double layer in which complex lipopolysaccharides (LPS) are anchored. These fatty sugars have the following components, seen from the inside out: a fatty part (lipid A) anchored in the membrane, a core and an external sugar part consisting of repeating oligosaccharide chains. Lipid A is very toxic (endotoxin) and causes a broad spectrum of effects such as fever and shock

during Gram-negative septicaemia. The vast majority of human isolates (>99.5%) are subspecies *S. enterica*. These organisms are rod shaped 2-3 µm long and 0.4 -0.6 µm in diameter. As with other Gram-negative bacilli, the cell envelope of salmonellae contains a complex lipopolysaccharide (LPS) structure that is liberated on lysis of the cell and, to some extent, during culture [9].

1.5. Antigenic features of *Salmonella*

There are numerous (over 2500) serovars, which are found in a disparate variety of environments and which are associated with many different diseases. *Salmonella* isolates are most commonly classified according to serology (Kauffman-White classification). The main division is first by the somatic O antigen, then by flagellar H antigens and the capsular Vi antigen.

1.5.1 O (somatic) antigens

These somatic antigens represent the side chains of repeating sugar units projecting from the outer lipopolysaccharide layer of the bacterial cell wall. They are hydrophilic and enable the bacteria to form stable, homogenous suspensions in saline solution. More than 60 different O-antigens have been identified.

1.5.2 H (flageller) antigens

These antigens represent the determinant groups in the flagellar protein. They are heat-labile as well as alcohol-labile and are well preserved in 0.04-0.2 % formaldehyde. Heating at 60°C or above causes detachment of the flagella from the bacteria and is well achieved by heating at 100°C for 30 min. The detached flagella remain immunogenic, but not the bacterium. Suspensions of such bacilli, which are freed from detached flagella by

centrifugation and washing or by heating at 100°C for 2.5 hrs, can be used for the production of O-antisera.

1.5.3. Vi-antigen

Almost all strains of *S. Typhi* produce the Vi-antigen as a covering layer outside their cell wall. It is an acidic polysaccharide and when fully developed, it renders the bacteria agglutinable by Vi-antibody.

1.6. Symptoms of typhoid fever

Primary symptoms include: loss of appetite, fever 103 or 104°F (39 or 40°C), headache, joint pain, sore throat, constipation (or, less commonly, diarrhea), abdominal pain and tenderness. As the illness progresses, fever remains high and the person may become delirious. Sustained fever is often accompanied by a slow heartbeat and extreme exhaustion. During the second week and last 2 to 5 days: 10% of infected people get clusters of small, pink spots on the chest and abdomen. Intestinal bleeding or perforation occurs in 3 to 5% of infected people. Pneumonia may develop. Infection of the gallbladder and liver may also develop. At the final stage, a blood infection (bacteremia) occasionally leads to infection of bones (osteomyelitis), heart valves (endocarditis), kidneys (glomerulitis), the genitourinary tract and tissues covering the brain and spinal cord (meningitis). Infection of muscles may lead to abscesses (collections of pus). Although the history and symptoms of illness may suggest typhoid fever, the diagnosis must be confirmed by identifying the bacteria in cultures of blood, stool, urine, or other body fluids or tissues [7].

1.7. Isolation and identification of *Salmonella*

A number of plating media have been devised for the isolation of *Salmonella*. Some media are differential and nonselective, i.e., they contain lactose with a pH indicator, but do not contain any inhibitor for non *Salmonellae* (e.g., bromocresol purple lactose agar). Other media are differential and slightly selective, i.e., in addition to lactose and a pH indicator; they contain an inhibitor for nonentities (e.g., MacConkey agar and eosin-methylene blue agar). The most commonly used media selective for *Salmonella* are SS agar, bismuth sulfite agar, Hektoen enteric medium, brilliant green agar and xylose-lisine-deoxycholate agar. All these media contain both selective and differential ingredients and are commercially available [5].

1.8. Primary host defense

Host defense mechanism is very important for resistance to *Salmonella*. Multiple bacterial and host factors determine the outcome of *Salmonella* infections. Normal gastric acidity ($\text{pH} < 3.5$) and intestinal motility is very important to reduce the infection of the bacteria. The normal intestinal microflora protects against *Salmonellae*, probably through anaerobes, which liberate short-chain fatty acids that are thought to be toxic to *Salmonellae*. Alteration of the anaerobic intestinal flora by antibiotics renders the host more susceptible to *Salmonellosis*. Secretory or mucosal antibodies also protect the intestine against *Salmonellae*. Animal strains genetically resistant to intestinal invasion by *Salmonellae* have been described. In AIDS, *Salmonella* infection is common, frequently persistent and bacteremic, and often resistant to even prolonged antibiotic treatment. Relapses are common. The role of host defenses in salmonellosis is extremely

important, and much remains to be learned. Multiantibiotics resistant strains of *Salmonella* Typhi offer a compelling argument for the development of next generation typhoid vaccines and increased the use of typhoid vaccination [8].

1.9. Epidemiology

With an estimated 16-33 million cases of annually resulting in 5×10^5 to 6×10^5 deaths in endemic areas, the World Health Organization identifies typhoid as a serious public health problem. Its incidence is highest in children between 5 and 19 years old [11]. The entry of this bacterial species into the human body is most commonly achieved by ingestion, with the importance of aerosol transmission unknown. Once ingested, the organisms multiply in the small intestine over the period of 1-3 weeks, breach the intestinal wall, and spread to other organ systems and tissues. The innate host defenses do little to prevent infection due to the inhibition of oxidative lysis and the ability to grow intracellularly after uptake. In developing countries, the incidence of typhoid fever is linked to conditions of hygiene and to the risk of oral-fecal contamination. Average incident rates are between 150 per 100,000 in South America and 1,000 per 100,000 in certain Asian countries. Typhoid fever is now rare in industrialized countries thanks to better hygiene and constant improvements in the treatment and distribution of drinking water. In France (including French overseas territories), the incidence of confirmed cases of typhoid fever is estimated to be 0.16 per 100,000. However the real incidence rate is more likely 1 per 100,000. In Guyana, it stands at 12 per 100,000. At present, 70% of all infections are contracted abroad [10].

1.10. Prevention and treatment for the typhoid fever

The most cost-effective strategy for reducing the incidence of typhoid fever is the institution of public health measures to ensure safe drinking water and sanitary disposal of excreta. The effects of these measures are long-term and reduce the incidence of other enteric infections, which are a major cause of morbidity and mortality in those areas. In the absence of such a strategy, mass immunization with typhoid vaccines at regular intervals also considerably reduces the incidence of infections. Careful food preparation and washing of hands are therefore crucial to preventing typhoid.

Health care workers caring for patients with typhoid fever should pay strict attention to adequate hand washing and safe disposal of feces and urine. Antibiotic therapy is essential and should begin empirically if the clinical evidence is strong. Patients must receive adequate fluids, electrolytes, and nutrition. Antimicrobials shorten the course, reduce the rate of complications if begun early, and drastically reduce the case-fatality rate. Chloramphenicol was introduced in 1948 and was once the mainstay of treatment. By the 1970s, widespread resistance to the drug developed. Ampicillin and cotrimoxazole became treatments of choice. However, in the late 1980s, some *S. Typhi* strains developed simultaneous plasmid-mediated resistance to all 3 drugs. Fluoroquinolones and third-generation cephalosporins have filled the breach [20].

1.11. Causes of antimicrobial resistance

Microbes, such as bacteria, viruses, fungi, and parasites, are living organisms that evolve over time. Their primary function is to reproduce, thrive, and spread, quickly and efficiently. Therefore, microbes adapt to their environment and change in ways that ensure their survival. If something stops their ability to spread, such as an antimicrobial, genetic changes can occur that enable the microbe to survive. There are several ways this happens, e.g. mutation and gene transfer which are natural causes, selective pressure, society pressure, and inadequate diagnosis, inappropriate use, hospital use and agriculture use.

1.12. Problems associated with antibiotic resistance of *Salmonella* Typhi

A drug-resistant *Salmonella* Typhimurium subtype, associated with severe human illness, has emerged in the United States, known as *S. Typhimurium* Definitive Type 104 (DT 104), characterized by multiple antimicrobial resistance, has been present in the United Kingdom since 1984. Studies in the United Kingdom showed that *S Typhimurium* is present in animals. Some variant Typhi have developed multidrug resistance as an integral part of the genetic materials of the organism. The global increase in resistance to antimicrobial drugs, including the emergence of bacterial strains that are resistant to all available antibacterial agents, has created a public health problem of potentially crisis proportion. Any time bacteria are exposed to an antibiotic, they are under selective pressure that allows only resistant forms to survive and reproduce. So the basic rule in slowing the evolution of resistance is reducing the unnecessary use of antibiotics. Resistance to ciprofloxacin is increasing up to 50% of the isolates from Finns with

typhoid fever returning from Southeast Asia had reduced susceptibility to ciprofloxacin, with a minimum inhibitory concentration (MIC) of 0.125-0.250—a 9-fold increase. For therapeutic purposes, intermediate susceptibility should be regarded as full resistance. Ciprofloxacin is no longer a good first-line treatment for *S. Typhi* infection that arises in Southeast Asia. When last evaluated, the rate of intermediate sensitivity in strains was 3.7% in the Americas [20].

1.13. Prognosis

The prognosis for recovery is good for most patients. In the era before effective antibiotics were discovered, about 12% of all typhoid fever patients died of the infection. Now, however, less than 1% of patients who receive prompt antibiotic treatment will die. The mortality rate is highest in the very young and very old, and in patients suffering from malnutrition. The most ominous signs are changes in a patient's state of consciousness, including stupor or coma [21].

1.14. Pathogenesis of salmonellosis

Most non-typhoidal *Salmonella* enter the body when contaminated food is ingested. Person-to-person spread of *Salmonella* also occurs. To be fully pathogenic, *Salmonella* must possess a variety of attributes called virulence factors. These include (1) the ability to invade cells, (2) a complete lipopolysaccharide coat, (3) the ability to replicate intracellularly, and (4) possibly the elaboration of toxin(s). After ingestion, the organisms colonize the ileum and colon, invade the intestinal epithelium, and proliferate within the epithelium and lymphoid follicles. The mechanism by which *Salmonella* invade the epithelium is partially understood and involves an initial binding to specific receptors on

the epithelial cell surface followed by invasion. Invasion occurs by the organism inducing the enterocyte membrane to undergo "ruffling" and thereby to stimulate pinocytosis of the organisms. Invasion is dependent on rearrangement of the cell cytoskeleton and probably involves increases in cellular inositol phosphate and calcium. Attachment and invasion are under distinct genetic control and involve multiple genes in both chromosomes and plasmids [8].

1.15. Invasion of intestinal mucosa by *Salmonella*

After invading the epithelium, the organisms multiply intracellular and then spread to mesenteric lymph nodes and throughout the body via the systemic circulation; the reticuloendothelial cells take them up. The reticuloendothelial system confines and controls spread of the organism. However, depending on the serotype and the effectiveness of the host defenses against that serotype, some organisms may infect the liver, spleen, gallbladder, bones, meninges, and other organs. Fortunately, most serovars are killed promptly in extraintestinal sites, and the most common human *Salmonella* infection, gastroenteritis, remains confined to the intestine. Much is now known about the mechanisms of *Salmonella* gastroenteritis and diarrhea. Summarize the pathogenesis of *Salmonella* enterocolitis and diarrhea. Only strains that penetrate the intestinal mucosa are associated with the appearance of an acute inflammatory reaction and diarrhea; the diarrhea is due to secretion of fluid and electrolytes by the small and large intestines.

Invasion of the intestinal mucosa is followed by activation of mucosal adenylate cyclase; the resultant increase in cyclic AMP induces secretion. The mechanism by which adenylate cyclase is stimulated is not understood; it may involve local production of

prostaglandins or other components of the inflammatory reaction. In addition, *Salmonella* strains elaborate one or more enterotoxin-like substances, which may stimulate intestinal secretion [8].

1.16. Intestinal epithelium responses to *Salmonella*

The first specific interactions during natural infection between pathogenic *Salmonella* and a host occur at the epithelial surface of organized patches of lymphoid tissue or follicles that are scattered throughout the small intestine. This lymphoid tissue, also called Peyer's patches, has a specialized epithelium and an underlying dome that contains mature lymphocytes as well as developing lymphoblasts. The primary function of this tissue is believed to be immune surveillance of the gut. Invasive *Salmonella* strains specifically target these Peyer's patches for the initial penetration of the host small intestine. Early infection studies with mice demonstrated that virulent *S. Typhi*, administered by an oral route, associated almost exclusively with Peyer's patch tissue of the terminal ileum as quickly as 3 hr postinoculation. M cells are specialized residents of the epithelium of lymphoid follicles and are interspersed within the epithelial layer of an intestinal lymphoid follicle at a ratio of approximately 1 per 10-20 enterocytes. M cells have several distinctive characteristics that allow them to be identified easily. These cells possess microvilli that are visibly shorter than those of enterocytes. They also lack a rigid cytoskeleton that allows migratory lymphocytes to deform and distort their cytoplasm. In addition, they have an elevated pinocytic activity that facilitates the uptake of intestinal microorganisms and particles. This suggests that a primary function of these cells is to transport and process luminal antigens to prime local intestinal immunity in the Peyer's

patches [12]. M cells also share similarities with other epithelial cells in that they form tight junctions with adjacent enterocytes and align along the basal lamina surface [8].

1.17. Phagocytic cell responses and interactions

Following passage through the intestinal epithelium of the Peyer's patch, invading organisms quickly enter the lymphatic system where interactions with professional killing cells determine the ultimate fate of the infection. These cells possess both oxygen-dependent and -independent killing mechanisms to kill internalized bacteria [19]. The production of toxic oxygen molecules such as superoxide, hydrogen peroxide, and hydroxyl radicals that are pumped into the phagolysosome are the primary oxygen-dependent killing molecules of macrophages. The oxygen-independent killing mechanisms of the macrophages include acidification of the phagolysosome as well as secretion of small bactericidal peptides into the compartment. Because the oxygen-dependent killing mechanisms of macrophages appear to have little effect on pathogenic *Salmonella*, the host is more dependent on oxygen-independent killing mechanisms. However, experimental evidence suggests that *Salmonella* has evolved mechanisms to circumvent or delay the killing activity of these mechanisms [12].

1.18. Vaccination against Typhoid Fever

Three types of typhoid vaccines are currently available for use in the United States:

- (1) An oral live-attenuated vaccine;
- (2) A parenteral heat-phenol-inactivated vaccine;
- (3) A newly licensed capsular polysaccharide vaccine for parenteral use.

1. **Live oral vaccines:** Although oral killed vaccines are without efficacy, vaccines using living avirulent bacteria have shown promise. The Live Oral Typhoid Vaccine should not be given to children younger than 6 years of age. It is given in four doses, 2 days apart, as needed for protection. The last dose should be given at least 1 week before travel to allow the vaccine time to work. A booster dose is needed every 5 years for people who remain at risk.

2. **The parenteral heat-phenol-inactivated vaccine:** It has been widely used for many years. In field trials involving a primary series of two doses of heat-phenol- inactivated typhoid vaccine, efficacy over the 2- to 3-year follow-up periods ranged from 51% to 77%. Efficacy for the acetone- inactivated parenteral vaccine, available only to the armed forces, ranges from 75% to 94%.

Since the inactivated vaccines contain the O antigen (endotoxin), local and general reactions occur. The inactivated Typhoid Vaccine should not be given to children younger than 2 years of age. One dose provides protection. It should be given at least 2 weeks before travel to allow the vaccine time to work. A booster dose is needed every 2 years for people who remain at risk.

3. **The parenteral vaccine Vi capsular polysaccharide (ViCPS):** It is composed of purified Vi ("virulence") antigen, the capsular polysaccharide elaborated by *Salmonella* Typhi isolated from blood cultures. In recent studies, one 25-ug injection of purified ViCPS produced seroconversion (i.e., at least a fourfold rise in antibody titers) in 93% of healthy U.S. adults. Two field trials in disease-endemic areas have demonstrated the efficacy of ViCPS in preventing typhoid fever. In one trial in Nepal, in which vaccine

recipients were observed for 20 months, one dose of ViCPS among persons 5-44 years of age resulted in 74% fewer cases of typhoid fever. ViCPS has not been tested among children less than 1 year of age [5].

1.19. Diagnosis

Diagnosis is made by blood, bone marrow or stool cultures and with the Widal test (demonstration of *Salmonella* antibodies against antigens O-somatic and H-flageller). The diagnosis, however, is confirmed by a blood culture. Samples of a patient's stool, urine, and bone marrow can also be used to grow *S.Typhi* in a laboratory for identification under a microscope. Cultures are the most accurate method of diagnosis. Blood cultures usually become positive in the first week of illness in 80% of patients who have not taken antibiotics. The diagnosis of the typhoid is possible by polymerase chain reaction using blood as sole source template DNA of salmonella Typhi [25]. The DNA hybridization method using DNA probe specific to the Vi polysaccharide antigen of *Salmonella* Typhi to detect the organism in the blood of patient with typhoid fever [26]. Patient of typhoid fever usually have less than 15 *Salmonella* Thyphi cells per ml of blood and probe cannot detect fewer than 500 bacteria.

1.20. Clinical specimens for the isolation of *Salmonella*

Knowledge of the natural history and pathophysiology of the infectious disease process is important in determining the optimal time for specimen collection. If carefully go through the bacteriological findings in typhoid fever, it can be seen that the onset of symptoms is associated with a bacterium. From the 1st week onwards the frequency with

which *S.Typhi* can be isolated from the blood falls. By the end of the 3rd week it can be found in about half the cases; after the 4th week its isolation is infrequent. *S.Typhi* is most easily isolated from the faeces between the 3rd and 5th week of the illness. During the 1st week only 50% of faeces cultures will yield organisms on culture. The number of organisms in the faeces increases greatly from the 1st to 3rd week of illness. The bacteria do not disappear from the intestine so quickly or as completely as from blood. Many patients excrete typhoid bacilli at a time when positive blood cultures can no longer be obtained. During the 1st week about 20% of the cases show the presence of antibodies. The curve then rises sharply, crossing the blood-culture curve just before the end of the 2nd week, and still rising attains a value of 90% or over by the 4th week. It remains at a high level for some weeks.

Various clinical specimens are:

- 1) Blood
- 2) Stool

1) Blood:

Blood samples are collected from the typhoid-suspecting patients by using a sterile needle and syringe. It is advisable to inoculate blood for culture into more than one medium, preferably three, the choice of media depending on the history of the patient. In suspected cases of enteric fever the patient's blood may be dilute 1 in 10 in 0.5 per cent

sodium taurocholate broth. Anticoagulants such as 0.2 % sodium citrate or 1% ammonium oxalate may be added to media for blood culture but these are liable to be inhibitory. Liquid (sodium polyanethol sulphate) is recommended because its inhibitory effect is only slight and it also neutralizes the natural bactericidal action of blood, reducing the need for high dilution of blood in the medium. Blood culture method advantageous because it indicates that the patient not only infected with the bacillus but also suffering from the diseases. For use, 5-10 ml patient's blood is mixed with the liquid medium and allowed to flow over the agar. The bottle is incubated in the upright position and the agar surface is examined daily for colonies, being re-seeded every 48 hr from the blood broth by allowing it to flow gently over the agar by suitably tilting the bottle.

2) Stool

Collection of Faecal Specimens

Stool specimens should be collected in clean (not necessarily sterile), wide- mouthed containers that can be covered with a tight-fitting lid. These containers should be free of preservatives, detergents, and metal ions. Contamination with urine should also be avoided. Stool specimen is inoculated on the MacConkey agar, SS agar (*Shigella Salmonella* agar). Where MacConkey agar is nonselective plate, SS agar is selective for *Shigella* and *Salmonella*. Stool sample is transferred in the enrichment media (Selenite broth media) with swabs and also incubated 24 hours at 37°C.

1.21. Widal test for *Salmonella* Typhi detection

A test involving agglutination of typhoid bacilli when they are mixed with serum containing typhoid antibodies from an individual having typhoid fever, used to detect the presence of *S. Typhi* and *S. Paratyphi*. The Widal test is one of the most utilized diagnostic tests for typhoid fever in developing countries. This test demonstrates the presence of somatic (O) and flagellar (H) agglutinins to *Salmonella* Typhi in the patient's serum using suspensions of O and H antigens. Antigens of *S. Paratyphi* A, *S. Paratyphi* B, and *S. Paratyphi* C are included in most commercial kits [24].

In rural areas where laboratory facilities are very limited, the diagnosis of typhoid fever is mainly based on clinical symptoms of the disease and the four-fold rise in the antibody titers of the Widal test [50]. To date, conventional Widal test is still widely used to support the diagnosis of typhoid fever because it can be very easily carried out and considered a very cheap test. The Widal test done on convalescent-phase serum gave more-reliable results with higher specificity and sensitivity [23]. On the other hand this test lacks proper sensitivity as well as specificity [50].

1.22. Enzyme- Linked immunosorbent assay (ELISA)

The ELISA test is used to help diagnose many different kinds of diseases in humans, animals and even plants. The test (or assay) is just a method, not a specific test for one kind of disease. In recent year this is the most acceptable and popular technique. It is very sensitive and specific technique. The principle of the enzyme-linked immunosorbent assay (ELISA) is to detect antigen purified antibody specific for antigen is linked chemically to an enzyme. The samples to be tested are coated onto the surface of plastic

wells to which they bind nonspecifically; residual sticky sites on the plastic are blocked by adding irrelevant proteins. The labeled antibody is then added to the wells under conditions where nonspecific binding is prevented, so that only binding to antigen causes the labeled antibody to be retained on the surface. Unbound labeled antibody is removed from all wells by washing, and bound antibody is detected by an enzyme-dependent color-change reaction. This assay allows arrays of wells known as microtiter plates to be read in fiberoptic multichannel spectrometers, greatly speeding the assay. The actual concentration of the specific antibody can be determined by comparison with reactivity of the standard human antibody solution.

ELISA can also be used to quantify the soluble antigen in the patient's sample. In this assay the soluble antigens in a patient sample capture and concentrated by the immobilized antibody and then detected with a different antibody labeled with enzyme [51].

1.23. Immune response determined by ELISA

ELISA technique to determine antibody responses in the serum of patients with typhoid during both the acute and convalescent phases. It can be defined as first 10 days of illness as acute and the subsequent 11–14 days as convalescent stages, in which IgG antibodies are encountered more frequently in higher titres than IgM antibodies. Both IgM and IgG antibodies were seen during the mid stage, but IgG dominated during the convalescent stage. It has showed that IgG dominated (60%) in culture confirmed typhoid with a history of one week to one month of fever, whereas IgM (20%) and IgA remained low (12%). It is reported the highest titres of IgM and IgA antibodies by two to three weeks. IgA and IgM antibodies declined to their normal values 45 to 90 days after the acute

phase. It is showed that the dynamics of salivary IgA antibodies during the acute and convalescent phases was comparable to that of serum immunoglobulin

ELISA developed for use with saliva (which can be obtained by non-invasive methods and is easy to use) is highly sensitive, specific, and efficient. The sequential study revealed that the test is most efficient during the second and third weeks of fever, the time at which patients normally present for treatment. The usefulness of the test is further enhanced by the subsequent drastic reduction of the sensitivity of the assay, enabling the diagnosis of the acute infection [2].

1.24. Aim of this study

1. To carry out microbiological isolation of *S. Typhi* from blood and stool.
2. To compare the microbiological data with the antibody in lymphocyte supernatant assay (ALS) in the same patients.

Chapter-2

Materials and methods

2.1. Isolation of *Salmonella* spp

2.1.1. Blood sample

For the culture for *S. Typhi* from the blood samples following were done:

1. In case of children 2 ml blood and in case of adults 4 ml blood was inoculated in Trypticase soy broth (TSB) in the blood culture bottle
2. The culture bottles were incubated for 48 hr at 37°C.
3. From the culture bottles blood was directly plated onto
 - A) Blood agar
 - B) Chocolate agar
 - C) MacConkey agar
4. After incubation plates were examined for non lactose fermenting colonies and identification of *S. Typhi* was carried out.

2.1.2. From Stool

Samples, stool were, were cultured onto the following plates such as

- A) MacConkey agar
- B) SS agar
- C) TTGA

2.2. Identification of *Salmonella* species

For identification of *Salmonella* species biochemical and serological tests were carried out. Suspected non-lactose fermenting colonies are subjected to the following tests.

2.3. Biochemical test

The following biochemical reactions were carried for the identification of the organism

1. Kligler's Iron Agar (KIA)
2. Motility Indole Urea (MIU)
3. Citrate agar test

1. Kligler's iron agar test (KIA)

In case of the KIA test slant i.e. upper part becomes alkaline and the lower part i.e. butts becomes acidic. *Salmonella* Typhi and other groups such as B, C, C₁, C₂, E, D and F produce hydrogen sulfite, which is visualized by black color. But the *Salmonella* paratyphi doesn't produce hydrogen sulfite resulting no visible black color All *Salmonella* groups produce gas, which is visualized by blasting of the media except *Salmonella* Typhi. Same biochemical behavior is exhibited by citrobacter and can be differentiated by serological test.

2. Motility indole urea test (MIU)

Motility Indole Urea medium was used for this test. A suspected isolated colony with a sterile straight wire and stabbed into agar very carefully down the tube without touching the bottom. An indole paper (soaked with kovac's reagent) was inserted between cotton plug and tube. The tube was incubated overnight at 37°C. The motile property of the *Salmonella* spp. was detected by presence of growth along the time of inoculation. Positive reaction on the indole paper was detected by the production of pinkish color on the paper and negative result was detected by absence of discoloration of indole paper.

3. Citrate agar test:

Citrate agar Test medium was used to perform the test. Isolated colonies were stabbed onto the soft agar. The slant then incubates overnight at 37°C.

Biochemical reaction for identification of *Salmonella* spp.

Organism	Glucose	H ₂ S	Motility	Indole	Urease	Citrate
<i>S. Typhi</i>	A	+	+	-	-	-
<i>S. Paratyphi A</i>	AG	-	+	-	-	-
<i>S. Paratyphi B</i>	AG	+	+	-	-	+

A = Acid

AG= Acid + Gas

H₂S = Hydrogen Sulfide

+ = Positive

= Negative

2.4. Serological test

The isolates were to be confirmed to be a *Salmonella* species by slide agglutination test with antiserum. A dry-clean and grease free slide was taken and marked into several parts with a wax pencil. Two drops of saline were placed on each block of the slide. An isolated colony was taken with a loop and suspended in normal saline on the slide. The slide was gently rocked for at least two minutes. The positive result was indicated by formation of clumps. Sero typing was done with specific antisera.

2.5. Widal test

The diagnostic test was carried out against six types of antigen: Flageller antigen (H antigen) and Capsular antigen (O antigen) of *Salmonella* spp.

The titers were measured using two folds dilution of serum and agglutination reactions with H and O antigen.

Procedure

1. The slide marked with the wax pencil
2. About 4 µl of serum and 2.5 µl of antigen was added in the slide
3. The slide was shaken gently and agglutination reaction observed
4. If agglutination was positive the titer was determined by following steps below
5. The serum was serially diluted to 2 to 4 dilution with normal saline or PBS in a plastic flexi plate.

Interpretation of Result	Titer
A. Undiluted Serum Agglutination	1:20
B. First diluted serum Agglutination	1:40
C. Second diluted serum Agglutination	1:80
D. Third diluted serum agglutination	1:160
E. Four diluted serum agglutination	1:320

The cut off value for detection of positive of typhoid fever was 1: 320.

2.6. Isolation of PBMC and culture for antibody in lymphocytic supernatant (ALS)

Peripheral blood mononuclear cells (PBMCs) can be isolated by density gradients centrifugation from venous blood. As the density of red blood cells (RBC) is higher than PBMC, RBC is precipitated. The plasma remains in the upper portion. Isolated PBMCs were cultured in 24-well tissue culture plate at 37°C in 5% CO₂ for 48 hrs. The PBMC primed with bacterial antigens secrete antibody during the culture period.

Procedure

1. Heparinized venous blood was diluted with equal volumes of PBS in tubes (Beckton Dickinson, BD). Diluted blood was carefully added onto Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). The tube was centrifuged at 1600 rpm (500xg) for 25 minutes at 20°C (Sorvall RT 6000B Refrigerated Centrifuge).
2. The mononuclear cells were removed from the top of the Ficoll layer carefully with a pasteur pipette.
3. The PBMCs were washed once in PBS at 2000 (750xg) rpm for 10 minute at 20°C.

4. The PBMCs were resuspended in 10 ml of PBS and counted in a hemocytometer.
5. The cells were spun down and again resuspended in RPMI medium to a concentration of 10^7 cells per ml.
6. The cells were cultured in 24 well tissue culture plates (Nunc Coaster, Denmark) at 37°C in 5% CO₂ incubator (Forma scientific) for 24hr and 48hr.
7. The cultured solution was centrifuged at 12000 rpm (11600xg (Eppendorf, centrifuge 5417R) at 20°C for 5 minute.
8. The supernatant was removed and then 10 µl of protease inhibitor cocktail solution per 1 ml of culture supernatant was added to prevent the protease mediated antibody lysis and stored at -70°C.

2.6. Serum antibody response against whole cell (WC) and Lipopolysaccharide (LPS) by ELISA.

Enzyme linked immunosorbant assay in which antibodies are detected by the binding of an enzyme coupled to either antiIg antibody or antibody specific for the antigen. This method is exquisitely sensitive for detecting antigen and antibodies and extremely economical in use of reagents. Serum antibodies to the antigens were measure by ELISA using the kinetic mode. Three isotypes of antibodies were measured: IgA, IgG, and IgM.

Procedure

1. Antigen WC, Vi and LPS were diluted with PBS at concentration of 1×10^8 CFU/ml, 1 µg/ml and 2.5 µg/ml respectively.
2. 96 well ELISA plates were coated with 100µl/well of antigen suspension.

3. The plates were kept in at room temperature for 18 hr.
4. The plates were washed 2 times with PBS and blocked with 1 % bovine serum albumin in PBS, 200 μ l/well at 37°C for 30 minutes.
5. The plates were washed three times with PBS-Tween (0.05%) and once with PBS.
6. The serum samples are diluted at 1:50 in 0.1% BSA and BS-Tween (0.05%) was added on to the plates, 100 μ l/well. Plates were incubated 37°C for 90 minutes.
7. The plates were washes three times with PBS–Tween (0.05%) and once with PBS
8. The conjugates (Rabbit anti human immunoglobulin IgG, IgA, IgM conjugated to the horse radish peroxidase (HRP) were diluted (1:1000) in a 0.1% BSA-PBS-Tween, and added to the wells (100 μ l/well).
9. The plates were incubated at 37°C for 90 minutes.
10. The plates were washed three times PBS-Tween (0.05%) and once with PBS.
11. The plates were developed by adding OPD (orthophenyldiamine, sigma), 100 μ l/ well, prepared by dissolving 10 mg of OPD in 10 ml of 0.1M sodium citrate buffer (pH 4.5) to which 4 μ l 30% H₂O₂ was added immediately before use.
12. The optical density (OD) was immediately measured kinetically at 450 nm by ELISA reader Ascent for 5 minutes.
13. The unit measured was milli absorbance unit / minute (mAB/ min).

2.7. Determination of serum/ALS against membrane protein (MP) antigen in serum

Procedure

1. The membrane protein (MP) antigen was diluted with PBS at the concentration 2 µg/ml.
2. ELISA plates were coated with 100 µl/well of antigen suspension.
3. The plates were kept in at room temperature over night.
4. The plates were washed 2 times with PBS and blocked with 1% bovine serum albumin in PBS (1%BSA-PBS), 200 µl/well at 37°C for 30 minutes.
5. 0.1% BSA-PBS Tween (0.05%), and added to the wells (100 µl/ well). Then 50 µl serum added to the first well in the column. The samples were serially diluted with 3 fold dilution to the end of the column.
6. The plates were incubated at room temperature for 90 minutes.
7. The plates were washed three times with PBS-Tween (0.05%).
8. The anti-human IgA, IgG and IgM conjugated with horse reddish peroxidase were diluted (1: 1500 dilution of IgA , 1: 4000 dilution of IgG and 1:2000 dilution of IgM.) in 0.1% BSA–PBS-Tween (0.05%) were added to the wells (100 µl/well).
9. The plates were washed three times with PBS–Tween (0.05%) and once with PBS.
10. The plates were developed by OPD (orthophenyldiamine, sigma), 100 µl/well prepared by dissolving 10 mg of OPD in 10 ml of 0.1M sodium citrate buffer (pH 4.5) to which 4µl of 30% H₂O₂ and was added immediately before use (100 µl/well).The optical density was measured after 20 minutes at 450 nm in a ELISA reader Ascent . Reaction was stopped by adding 50µl 1M H₂SO₄ to each well at 495 nm.

11. The titer was defined as the dilution giving an absorbance 0.4 above background.

2.8. Data analysis

For the analysis of the data various software such as Microsoft Excel and Wilcoxon Signed Rank Test (two tails) was used. The significance difference was considered if the P value was > 0.05 . Data was expressed as median value.

Chapter-3

Results

3.1. Results

Among the 25% of the patients enrolled in this study, blood culture test revealed only 13% positive whereas by Widal test Only 44% were found to *S. Typhi* positive and ALS assay which is a novel and new concept of this study found 52% patients to be *S. Typhi* positive (Table1).

Table1: Results of blood culture, Widal test, ALS

Patient Category	Blood Culture	Widal Test	ALS
Total patients (n=25)	13% (n=3)	44% (n=11)	52% (n=13)
Adult(n=16)	Positive(n=2)	Positive(n=7)	Positive(n=8)
Child (n= 9)	Positive(n=1)	Positive(n=4)	Positive(n=5)
Female (n=14)	Positive(n=1)	Positive(n=4)	Positive(n=5)
Male (n=11)	Positive(n=2)	Positive(n=7)	Positive(n=8)

Here "n" denotes the number of patients.

3.2. Stool Sample

Stool was be used to identify *Salmonella Typhi* and *S. Paratyphi*. In this case stool samples were inoculated on the

- MacConkey agar
- SS agar
- TTGA

Samples were also transferred to the selenite broth medium, which was used as the enrichment medium, and then it was used for the antibiogram test, where the susceptibility of the various antibiotics was determined with the zone of inhibition for the treatment treat the patient with appropriate antibiotics.

On the MacConkey agar plate the colonies, which are white in color, are treated as the *Salmonella* colonies and on the SS agar plate, black pigments were produced by *Salmonella*. These colonies were subjected to the various biochemical tests and from the following table the identification of *Salmonella* was confirmed.

Table-2: Biochemical reaction for identification of *Salmonella* spp.

Organism	Glucose	H ₂ S	Motility	Indole	Urease	Citrate
<i>S. Typhi</i>	A	+	+	-	-	-
<i>S. Paratyphoid A</i>	AG	-	+	-	-	-
<i>S. Paratyphi B</i>	AG	+	+	-	-	+

A = Acid

AG= Acid + Gas

H₂S = Hydrogen Sulfide

+ = Positive

- = Negative

3.3. Agglutination test

After reading the biochemical tests then the microorganisms were subjected to the serological test to identify their groups. In this case *Salmonella* were agglutinated with somatic antigen "O" polyvalent. After confirming with the somatic "O"

polyvalent then treated with the flagella "H" Polyvalent to specify the respective groups. In case of *Salmonella* Typhi it will show positive result with the "d" antigen. In case of *Salmonella* Paratyphi it will show positive result "a" antigen. Similarly groups B with "b and i", group C₁ with "c, c, r", group C₂ with "e, h" and group D with "d, g,"were shown positive result. After agglutination test, patients can be confirmed that they are infected with *Salmonella* Typhi or Paratyphi.

By analyzing stool as a clinical specimen only 2 to 3 percent show the positive result.

3.4. Immune response in ALS specimen against membrane protein antigen

ALS assay for the IgA response was studied at the acute stage and followed up to 21 days of onset of diseases.

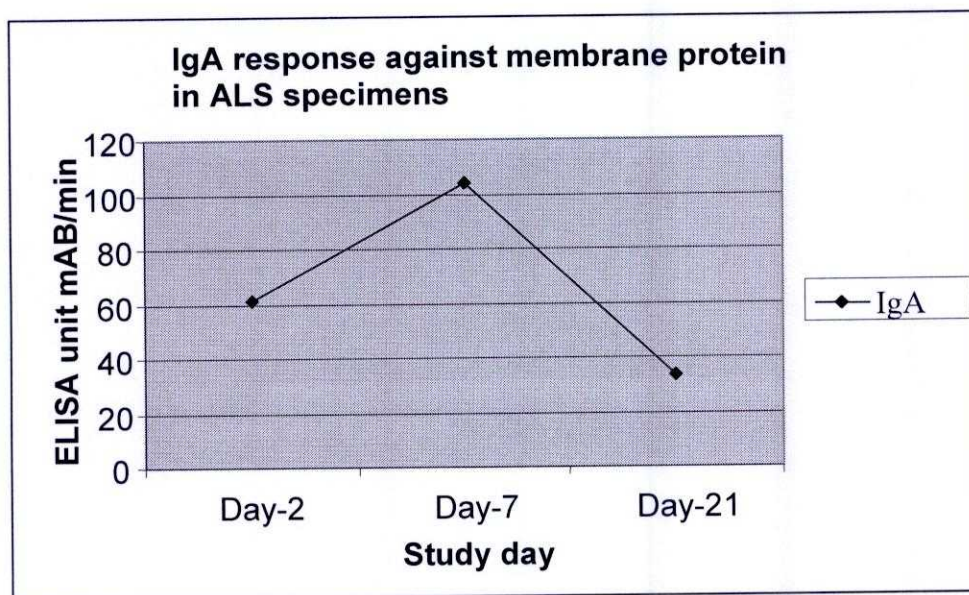


Fig-1: The line diagram of ALS response against membrane protein showed that the response at Day-7 was high where at the Day -21 was low.

3.5. Serum antibody response to the patients

Response to lipopolysaccharide antigen

Of the 13 positive patients 25 patients' serum was analyzed for immune response. Three immunoglobulin isotypes (IgA, IgM, and IgG) against lipopolysaccharide of *Salmonella* Typhi were determined. This test was done at the time interval of Day2 indicates the acute stage, Day7 indicates the early convalescence and Day21 indicates the late convalescence stage.

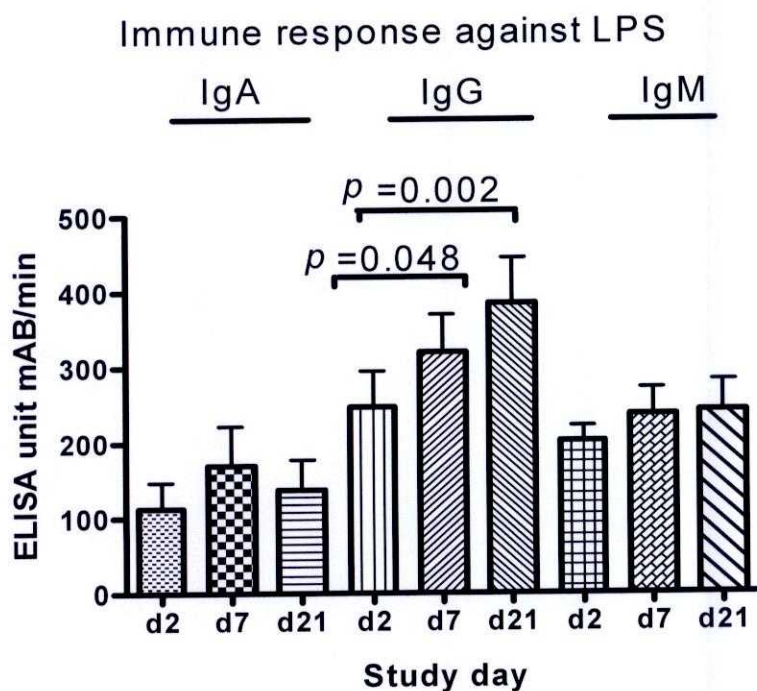


Fig-2: Here mean are shown.

3.6. Response to membrane protein antigens

Membrane protein antigen *Salmonella* Typhi was used to determine the response of antibody of all the patients. It was done in case of time interval of Day-2 indicates the acute stage, Day-7 indicates the early convalescence and Day-21 indicates the late convalescence stage. The data obtained are shown in the table;

Antibody response against membrane protein antigen

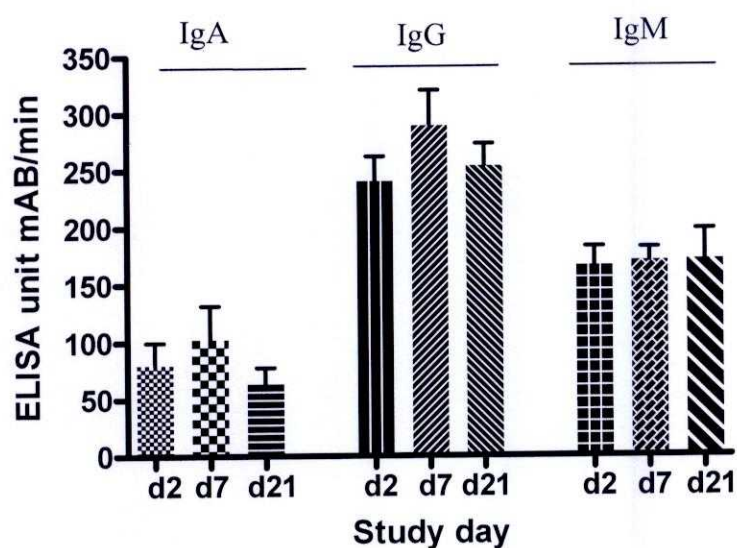


Fig-3: Here mean and Standard error mean are shown.

Chapter 4

Discussion

Discussion

Typhoid fever is a global health problem. Typhoid fever, caused by *Salmonella* Typhi, is a major cause of morbidity and mortality world wide, causing an estimated 16.6 million new infections and 600,000 deaths each year [27].

Its real impact is difficult to estimate because the clinical picture is confused with other febrile infections. Further, the disease is underestimated due to lack of bacteriology laboratories in most clinical settings of developing countries. Obviously, the disease has a very high social and economic impact [37]. Isolation of serotype Typhi from blood, urine or stool is the most reliable means of confirming an infection. However, this requires laboratory equipment and technical training that are beyond the means of most primary health care facilities in the developing world. Most serotype Typhi infections are diagnosed purely on clinical grounds and treated presumptively. As a result, the diagnosis may be delayed or missed in the case of other febrile illnesses, and patients without typhoid fever may receive unnecessary and inappropriate antimicrobial therapy [38].

Emerging drug resistance among circulating serotype Typhi strains in different parts of world [38] has complicated the treatment of typhoid fever and heightened the need for rapid accurate diagnosis and the appropriate and selective use of antimicrobial agents to which the organism has thus far remained susceptible. Researchers continue to search for the ideal rapid test to diagnose acute Typhoid fever. Several urine assays have been developed although none has proved optimal [38].

Widal test measures agglutinating antibody levels against O and H antigens. The levels are measured by using serial dilutions of sera. Usually, O antibodies appear on days 6-8 and H antibodies on days 10-12 after the onset of the disease. The test is usually

performed on acute and convalescent sera to detect the rising titres. The test has only moderate sensitivity and specificity [35]. Patients age should be considered as results of Widal test in children differ from that seen in adults [36]. In our study 44% of the patients show positive Widal test (Table 1).

Blood culture has the promise of diagnosis in the first week and is very specific, but its sensitivity is poor due to various factors. The most important factor is the very few numbers of bacteria needed to cause severe infection, which can be as low as 10/mL. Hence, positive culture yields are very low and elude definitive diagnosis. Other limiting factors, beside the bacteriostatic effect of antibiotics (already administered before the culture sample is taken), may be the nature of culture medium employed, the time of blood collection, the host's immune response system, and the intracellular characteristics of *Salmonella Typhi* [28].

In Indonesia, *S. Typhi* has been found to be the most common finding in blood cultures from febrile patients [28]. Bangladesh, in which one third (32%) of hospitalized blood culture confirmed typhoid patients were children less than 6 years of age [28]. Blood culture is positive in the first week but its utility is restricted by the very low numbers of bacteria causing severe disease (which may be less than 10/mL). As a consequence, blood culture can detect only 40%-45% of cases, and even if antibiotic treatment has not been administered, the rate of detection is not more than 70% [28]. In this study, only 13% of the patients showed the positive results by blood culture technique (Table-1).

Antibodies in lymphocyte supernatant (ALS) assays are used to assess intestinal mucosal responses to enteric infections and vaccines. The ALS assay, performed on cell supernatants, may represent a convenient alternative to the more established antibody secreting cell (ASC) assay. The specificity of the ALS assay compared to the ASC (antibody secreting cell) assay is excellent (100%), as is sensitivity (82%) [34]. In this study 52% of the patients showed the positive with ALS assay (Table1).

Chapter-5

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Chapter-6

Appendices

Appendices

Appendix: A

The composition and method of preparation of different media, chemical and reagents used in this study are given below

Media

Blood agar media **g/lit**

Trypton	14.0
Peptone neutralized	4.5
Yeast extract	4.5
Sodium chloride	5.0
Agar	12.5

MacKonkey agar (g/lit)

Peptone	20.00g
Lactose	10.00g
NaCl	5.00g
Bile salt	1.5g
Neutral red	0.05g
Crystal violet	1.00g
Agar	15.00g
pH= 7.2	

Shigella Salmonell agar (SS agar)

Dehydrated media

Trypticase soy broth (TBS)

Trypticase soy broth	30 g
Distilled water	1000ml
PABA	0.05g
Sodium polyethanol sulphonate	0.5g

Mueller hinton broth media (MHB)

Dehydrated MH (Difco)	36.5g
Distilled water	1000ml

pH= 7.4±0.2

Appendix: B

Kligler iron agar (KIA) media

Lab-lemco powder	3.0g
Yeast extracts	3.0g
Pepton	20.0g
Lactose	10.0g
Dextrose (glucose)	1.0g
Ferric citrate	0.1g
Sodium thiosulphate	0.3g
Phenol red	0.05g
Agar	12.0g

pH 7.2-7.6

Motility indole urea (MIU) media

Trypton	30.0g
Potassium dihydrogen phosphate	1.0g
Sodium chloride	5.0g
Agar	5.0g
Phenol red	2ml
Distilled water	1lit
pH	6.9-7.3

Citrate medium

Sodium ammonium sulphate	1.5 g
Potassium hydrogen phosphate	1.0g
Magnesium sulphate	0.2g
Bromothymol blue	0.016g
pH	6.6-7.0

Appendix: C

Chemical and reagents

1. FBS (Fetal bovine serum), Gibco BRL-16140-071.
2. Ficol, Pharmacia LKB Biotechnology AB Uppsala, Sweden.
3. Goat anti-human IgA-HRP, Southern Biotechnology Associates, Inc.2050-05
4. Goat anti-human IgG-AP, Southern Biotechnology Associates, Inc.2040-04
5. Goat anti-human IgM-AP, Southern Biotechnology Associates, Inc.2020-04
6. H₂O₂ (Hydrogen peroxide), Fischer Scientific, H-325.
7. MgCl₂ (Magnesium Chloride), Sigma- 7786-30-3
8. Na-acetate, Sigma – 127-09-3
9. NaHCO₃ (Sodium bicarbonate), Fischer Scientific, S233-500

Appendix-E

Preparation of 1% BSA in PBS (500ml)

Phosphate buffer saline (PBS)	500ml
Bovaine Serum Albumin (BSA)	5.0 g

Preparation of 0.1% BSA in PBS–Tween (500ml)

Phosphate buffer saline (PBS)	500ml
Bovaine Serum Albumin (BSA)	5.0 g
Tween	250µl

Buffer

Preparation of phosphate buffer saline (PBS) (pH 7.2)

NaCl	80.00 g
Na ₂ HPO ₄	11.50 g
KH ₂ PO ₄	2.00 g
KCl	2.00 g
Deionized water	1000.0 ml

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

Preparation of sodium citrate beffer (pH 4.5)

Sodium citrate buffer	14.7 g
Deionized water	400 ml

pH of the buffer was adjusted to 4.5 with 6N HCl and water was added to make the final volume 500 ml.