COMPARISON AMONG THE DIFFERENT DIAGNOSTIC PROCEDURES FOR EARLY AND RAPID DIAGNOSIS OF TYPHOID FEVER

DR. ZOHRA BEGUM

MBBS

DEPARTMENT OF MICROBIOLOGY
MYMENSINGH MEDICAL COLLEGE, MYMENSINGH
BANGLADESH

2008
SUMMARY

Typhoid fever still continues to be a major public health problem particularly in developing countries including Bangladesh. A simple, reliable, rapid and early diagnostic test has been a long felt need of the clinicians. To fulfill the purpose the present study was carried out in the Department of Microbiology, Mymensingh Medical College for the period from July 2006 to June 2007. This cross sectional study included 100 cases having clinical suspicion of typhoid fever. Subjects were investigated by blood culture, Widal test with rising titre and DOT EIA as a newly developed modern diagnostic method. Among them, 35 were subsequently confirmed on the basis of positive blood culture for \textit{S. typhi} and/or significant rising titre of Widal test. Another 41 cases were included as having significant titre of Widal test in first sample. Rest 24 cases neither had blood culture positive result nor significant titre on Widal test, but still were included due to the presence of strong clinical evidences in favour of typhoid fever. The control group (n = 40) comprised of age and sex matched 20 healthy persons and 20 non-typhoidal febrile patients. DOT EIA (IgM) based on detection of IgM antibodies in serum against a specific 50 KDa, outer membrane protein antigen of \textit{Salmonella typhi} by incubating nitrocellulose strips dotted with the specific antigen, was done in all groups of cases and controls using the 1st serum sample. Majority of the case (34\%) were in the age group of 1-5 years. \textit{Salmonella typhi} were isolated in 14 cases. DOT EIA test was positive for IgM in 73 cases of typhoid fever. At first week of illness, out of 14 culture positive samples, 13 were positive by DOT EIA and 06 were positive by Widal test. Out of 21 culture negative cases 19 and 9 were positive by DOT EIA and Widal test respectively. The rate of
DOT EIA positivity was significantly higher (p < 0.05) than the Widal test in both culture positive and culture negative cases. The sensitivity, specificity, positive and negative predictive value of DOT EIA (IgM) was found as 91.42%, 90%, 88.88% and 92.30% respectively. On the other hand corresponding values for Widal test were of 42.85%, 85.00%, 71.42% and 62.96% respectively. In the present study, the DOT EIA (IgM) yielded remarkable high sensitivity and specificity to diagnose typhoid fever in the first week of illness, so it was recommended to use the test in public health setting after studying a large sample at community level. It also appeared a useful for small and less equipped laboratories as a complementary test to blood culture for typhoid fever. DOT EIA needs a pretty long time for its performance but to shorten examination time, immunochromatography technique for similar detection of IgM can be studied in future.
CHAPTER 1
INTRODUCTION
INTRODUCTION

Typhoid fever is a severe multisystemic illness characterized by the classic prolonged fever, sustained bacteremia without endothelial or endocardial involvement and bacterial invasion of and multiplication within the mononuclear phagocytic cells of the liver, spleen, lymph nodes and Peyer’s patches (Brusch, 2006). Typhoid fever is potentially fatal if untreated. It is caused by *Salmonella typhi* a Gram negative bacilli (Kidgell *et al*, 2002).

Typhoid fever is a global health problem. Its real impact is difficult to estimate because the clinical picture is confused with other febrile infections. Incidence of typhoid fever has been estimated as approximately 22 million cases with at least 200,000 deaths occurring worldwide annually (Crump *et al*, 2004). The disease is endemic in the Indian subcontinent including Bangladesh, South-East and Far-East Asia, the Middle East, Africa, Central and South America (Gillespie, 2003; Saha *et al*, 1996). A recent epidemiologic study showed that South-East and South-Central Asia are the regions of highest endemicity with rates greater than 100/100,000 cases per year; the rest of Asia, Africa, Latin America, the Caribbean and Oceania (except Australia and New Zealand) are the next highest with incidence rates of 10-100/100,000 and Europe, North America and the rest of the developed world have low rates of disease (Crump *et al*, 2004). Typhoid fever represents the 4th most common cause of death in Pakistan (WHO, 2006).

The disease may occur in all ages, with the highest incidence found particularly in children (Anggraini *et al*, 2004). In Bangladesh, the overall incidence of typhoid fever
is 390 cases per 100,000 population per year. The incidence among people >5 years of age is 210 per 100,000 population per year and among children <5 years of age, the rate is 1870 per 100,000 children per year that is 8.9 times greater when compared with all other age groups (Brooks et al., 2001).

Typhoid fever emerged as an important infectious disease in the early 19th century. After an incubation period 3 to 21 days, the illness begins with mounting fever, headache, vague abdominal pain and constipation, which may be followed by appearance of rashes (Lesser & Miller, 2005; Gopalakrishnan et al., 2002). These symptoms indicate acute typhoid fever, in which the specific antibody IgM will be induced and last for several weeks, after which it is replaced by IgG (Anggraini et al., 2004). During the third week, the patient reaches a state of prolonged apathy, toxemia, delirium, disorientation and/or coma followed by diarrhea (Gopalakrishnan et al., 2002). *Salmonella typhi* lives only in humans. Persons with typhoid fever carry the bacteria in their bloodstream and intestinal tract. In addition, a small number of persons called carrier, who recover from typhoid fever but continue to excretion of the organism in their faeces or urine for more than 1 year, occurs in approximately 5% of infected persons (CDC, 2005). Case-fatality rates of 10% can be reduced to less than 1% with appropriate antibiotic therapy (WHO, 2000).

In the wake of emerging multidrug-resistant strains of bacteria causing typhoid fever, the disorder is known to be associated with significant morbidity and mortality (Rahman et al., 2006; Bhutta, 1996). It is also recognized that a delay in diagnosis and administration of appropriate therapy may significantly increase the risk of adverse outcome and mortality (Bhutta, 1996). So an accurate diagnosis of typhoid fever at an
early stage is important not only for aetiological diagnosis, but also to identify
individuals that may serve as a potential carrier, who may be responsible for acute
typhoid fever outbreaks (Parker, 1990).

The definitive diagnosis of typhoid fever depends on the isolation of *S. typhi* from
blood, bone marrow, rectal swab, urine or duodenal aspirate culture (Gasem *et al*, 1995
& Wain *et al*, 2001). Blood and bone marrow aspirate cultures are the gold standard for

Despite improved methods of bacteriologic isolation, there is a real need for rapid
serologic diagnostic tests for typhoid fever (Bhutta & Masurali, 1999). The Widal test
has been used for almost 100 years, is widely available in developing countries and is
still regarded as a useful test in endemic areas (Pang & Puthucheary, 1983).

The Widal test first described by Fernend Widal in 1896, detects agglutinating
antibodies against the O and H antigens of *Salmonella typhi* and *paratyphi* A and B
(Olopoenia & King 2000). In developed countries, the value and clinical application of
the Widal test has diminished in recent years. Unfortunately in some developing
countries, the test is still widely used though the test has many limitations. The Widal
test as a diagnostic modality has suboptimal sensitivity and specificity (Olopoenia &
negativity in early infection, prior antibiotic therapy and failure to mount an immune
response by certain individuals (Olopoenia & King 2000). Poor specificity, an even
greater problem and is a consequence of pre-existing baseline antibodies in endemic
areas, cross reactivity with other Gram-negative infections and non-typhoidal
*salmonella* and prior TAB or oral typhoid vaccination. The purity and standardization
of antigens used for the Widal test is a major problem and often results in poor specificity and poor reproducibility of test results (Olopoenia & King 2000). Widal test could correctly diagnose 74% of blood culture positive typhoid fever, however 14% results would be false positive and 10% false negative (Parry et al, 1999).

A single titre has no diagnostic significance, a paired sera is required for accurate interpretation (Olopoenia & King, 2000; Bhutta & Mansurali 1999). The test becomes positive only in the second week of illness, so its value for early diagnosis of the diseases is limited (Haque et al, 1999).

Though blood culture is gold standard, the yield of it is quite variable. In the untreated patient, blood cultures are usually positive in about 80% during the first week and declining 20%- 30% later in the course of the disease (Gillespie, 2003). A low yield is related to very low numbers of bacteria causing severe diseases which may be less than 10 per ml. of blood (Haque et al, 1999). It is not routinely requested by physicians because it is expensive and delay in the result of the test, at least 2-3 days (Hoffman et al, 1984). Although this test is highly specific but its sensitivity is affected by prior antibiotic intake and stage of illness (House et al, 2001). Bone marrow culture has a higher sensitivity despite 5 days of antibiotic therapy than blood culture but is a more invasive procedure (Farooqui et al, 1991 & Gasem et al, 1995). So, the limitation of the above traditional methods have prompted other novel tests to be developed such as Enzyme linked immunosorbent assay, latex agglutination, coagglutination and the polymerase chain reaction (Haque et al, 1999; Jesudason et al, 1994; Mukherjee et al, 1993).
The dot-enzyme immunoassay (DOT EIA) is a newer serologic test based upon the presence of specific IgM antibodies to a specific 50-KDa outer membrane protein (OMP) antigen on *S. typhi* strains and has been commercially marketed as a Typhidot. This test also can detect IgG antibodies in serum (Choo et al, 1994).

The sensitivity and specificity of the DOT EIA test has been reported to vary from 70-100% and 43-90% respectively (Khan et al, 2002, Bhutta & Mansurali 1999). The detection of IgM reveals acute typhoid fever in the early phase of infection, while the detection of both IgG and IgM suggests acute typhoid in the middle phase of infection. In areas of high endemicity where the rate of typhoid transmission is high the detection of specific IgG increase. Since IgG can persist for more than 2 years after typhoid infection (Saha et al, 1999), the detection of specific IgG can not differentiate between acute and convalescent cases. Furthermore, false positive results attributable to previous infection may occur. On the other hand IgG positivity may also occur in the event of current reinfection. In cases of reinfection there is a secondary immune response with a significant boosting of IgG over IgM, such that the later can not be detected and its effect masked. A possible strategy for solving this problem is to enable the detection of IgM by ensuring that it is unmasked (Bhatta 1996). The original Typhidot test was modified by inactivating the total IgG in the serum samples. Studies with modified test, Typhidot M, have shown that inactivation of IgG removes competitive binding and allows the access of the antigen to the specific IgM when it is present. The Typhidot M that detects only IgM antibodies of *Salmonella typhi* has been reported to be slightly more specific in a couple of studies (Hatta et al, 2002, Choo et al, 1999). This DOT EIA test offers simplicity, speed, early diagnosis and high negative and positive predictive values. The test become positive as early as in the first
week of the fever, the results can be interpreted visually and available within one hour (Ismail et al., 1991; Choo et al., 1994).

In Bangladesh, the diagnosis of typhoid fever is still based on clinical presentation and Widal test that are associated with numerous limitations. The diagnosis of typhoid fever on clinical grounds is difficult, as the presenting symptoms are diverse and similar to those observed with other febrile illnesses, especially during the first weeks of infection (Gillespie, 2003). Early and accurate diagnosis of typhoid fever by DOT EIA (IgM) would allow the clinician to decide on prompt therapy and thus prevent dreadful complications and reduce the morbidity and mortality. However, its usefulness in terms of specificity and sensitivity as compared to blood culture and Widal test has not been studied so far in our region. In this study, DOT EIA assay was applied for the detection of *S. typhi* specific IgM antibodies in serum and the results were compared with blood culture and Widal test for diagnosis of typhoid fever.
Aim and objectives

General objective

To see the usefulness of DOT Enzyme Immunoassay in the early and rapid diagnosis of typhoid fever.

Specific objectives

a) To compare among blood culture, Widal test and DOT EIA for laboratory diagnosis of typhoid fever.

b) To see the sensitivity and specificity of DOT EIA for diagnosis of typhoid fever.

c) To find out a more reliable, prompt and accurate method for diagnosis of typhoid fever.
CHAPTER 2

REVIEW OF LITERATURE
Typhoid fever is a severe multisystemic illness characterized by the classic prolonged fever, sustained bacteremia without endothelial or endocardial involvement, and bacterial invasion of and multiplication within the mononuclear phagocytic cells of the liver, spleen, lymph nodes, and Peyer’s patches (Brusch, 2006). Typhoid fever is potentially fatal if untreated. It is caused by *Salmonella typhi* (Kidgell *et al*, 2002).

### 2.1 Background and history of typhoid fever

Until the first quarter of the 19th century, typhoid fever was not recognised as a separate clinical entity and was often confused with other prolonged febrile illness such as typhus fever of rickettsial origin. “Typhos” in Greek means smoke and typhus fever got its name from smoke that was believed to cause it. Typhoid means typhus-like and thus the name given to this disease. Although typhoid fever was first discovered by Willis in 1643 (cited in Collier’s Encyclopedia 1989), it was mistakenly understood to be typhus fever for a long time. Gerhard in 1837 (cited in Collier’s Encyclopedia 1989), distinguished the two illnesses and coined the name typhoid fever which means “typhus like fever”.

The causative organism was visualized in tissue sections from Peyer’s patches and spleens of infected patients by Eberth in 1880 and named it as *Salmonella typhosum* and was grown in pure culture by Gaffky in 1884 (cited in Topley and Wilson 1990).

The significance of water contamination in the spread of the disease was first recognized by Budd in 1856 (cited in Topley and Wilson 1990). As a young practitioner in North Devon, his observations provided one of the greatest milestone in
the development of hygiene. He for the first time proved that the disease was infectious and can spread through patients faeces. He further discovered that milk and water played important role in the transmission of typhoid fever.

The most notorious carrier of typhoid fever—but by no means the most destructive—was Mary Mallon, also known as Typhoid Mary. In 1907, she became the first American carrier to be identified and traced. She was a cook in New York; some believe she was the source of infection for several hundred people. She is closely associated with forty-seven cases and three deaths (cited in Encyclopaedia). Public health authorities told Mary to give up working as a cook or have her gall bladder removed. Mary quit her job but returned later under a false name. She was detained and quarantined after another typhoid outbreak. She died of pneumonia after 26 years in quarantine.

In 1897, Almroth Edward Wright developed an effective vaccine. Most developed countries saw declining rates of typhoid fever throughout first half of 20th century due to vaccinations and advances in public sanitation and hygiene. Antibiotics were introduced in clinical practice in 1948, greatly reducing mortality (Lesser & Miller, 2005).
2.2 Epidemiology

Typhoid fever is a global health problem. A recent study estimated there to be approximately 22 million cases of typhoid fever each year with at least 200,000 deaths (Crump et al, 2004). In contrast to that seen in the rich countries, typhoid fever remains an important cause of illness in the developing world where annual incidences in Papua New Guinea and Indonesia may reach 1200/100,000 population. A recent epidemiologic study showed that South-East and South-Central Asia are the regions of highest endemicity with rates greater than 100/100,000 cases per year; the rest of Asia, Africa, Latin America, the Caribbean and Oceania (except Australia and New Zealand) are the next highest with incidence rates of 10-100/100,000 and Europe, North America and the rest of the developed world have low rates of disease (Crump et al, 2004).

Typhoid fever represents the 4th most common cause of death in Pakistan (WHO, 2006).

The majority of patients, 60%-90%, are treated as outpatients and, therefore, hospital based studies will underestimate true incidence. In Bangladesh, the overall incidence of typhoid fever is 390 cases per 100,000 population per year. The incidence among people >5 years of age is 210 per 100,000 person-year, and among children <5 years of age, the rate is 1870 per 100,000 children per year that is 8.9 times greater when compared with all others (Brooks et al, 2001).

Typhoid fever may occur at any age but it is considered to be a disease mainly of children and young adults. In endemic areas, the highest attack rate occurs in children aged 8-13 years. In a recent study from slums of Delhi, it was found that contrary to popular belief, the disease affects even children aged 1-5 years (Singh, 2001).
study from a private laboratory in Bangladesh, which found that the 57% of *S. typhi* isolates were in children less than 5 years of age and 27% less than 2 years (Saha *et al.*, 2001) Older people appear to be relatively immune, presumably because of frequently reinforced acquired immunity through numerous sub-clinical exposures to typhoid bacilli (Singh, 2001).

Humans are the only natural host and reservoir. The infection is transmitted by ingestion of food or water contaminated with faeces. Ice cream is recognized as a significant risk factor for the transmission of typhoid fever. Shellfish taken from contaminated water, and raw fruit and vegetables fertilized with sewage, have been sources of past outbreaks (WHO, 2003). Two hospital based case-control studies from Vietnam found that risk of infection was related to recent contact with an infected person, lack of education and drinking untreated water (Tran *et al.*, 2005; Luxemburger *et al.*, 2001).

The inoculum size and the type of vehicle in which the organisms are ingested greatly influence both the attack rate and the incubation period. In volunteers who ingested $10^9$ and $10^8$ pathogenic *S. typhi* in 45 ml of skimmed milk, clinical illness appeared in 98% and 89% respectively. Doses of $10^5$ caused typhoid fever in 28% to 55% of volunteers, whereas none of 14 persons who ingested $10^3$ organisms developed clinical illness (WHO, 2003).

1%-5% of patients, depending on age, become chronic carriers, harboring *S. typhi* in the gallbladder (WHO, 2003). Chronic typhoid carriers status may be responsible for the endemicity and outbreaks of the disease in the region. High prevalence of typhoid
carriers occurs in patients with biliary, gastrointestinal and other related diseases (Vaishnavi et al, 2005).

Case-fatality rates > 10% continue to be reported in developing countries despite availability of antibiotics, whereas developed countries show case fatality rates of <1% (Butler & Scheld, 2004).

*S. paratyphi* A, which normally causes about 15% - 20% of cases of typhoid fever in Asia, increasingly is becoming a pathogen in India (Tankhiwale et al, 2003) and China (Ochiai et al, 2005), possibly due to vaccination against *S. typhi*.

The risk factors for the development of enteric fever due to typhoid or paratyphoid may differ. In a study from Indonesia, paratyphoid fever was more probable with exposures outside the home (e.g. purchase of food from street vendors) compared to typhoid fever, which frequently resulted from exposure within the household e.g. sharing utensils, presence of a patient with typhoid, lack of soap or adequate toilet facilities (Vollaard et al, 2004).

*S. typhi* outbreaks in the United States are generally limited in size but can cause substantial morbidity; they are most often foodborne (Olsen et al, 2003). In 2003, a total of 356 cases of typhoid fever were reported in the United States (Hopkins et al, 2005). Approximately three-quarters of these cases occurred among persons who reported international travel during the preceding six weeks, despite the recommendation for vaccination when traveling to countries in which typhoid fever is endemic.
Recent epidemiologic studies also show the rise of multi-drug resistant (MDR) organisms (Walia et al., 2005). In a study of 1100 hospitalized children in Pakistan, the mortality rate of 1.6% was found to be related to younger age and MDR infection (Bhutta, 1996).

The importance of this illness internationally is highlighted by an outbreak of nearly 6000 cases of multi-drug resistant typhoid fever in Nepal, which was traced to municipal water supplies (Lewis et al., 2005).

### 2.3 Nomenclature and taxonomy

The nomenclature for these bacteria is confused because the criteria for designating bacteria as individual species are not clear. The members of the genus *Salmonella* are currently divided into 2,463 serovars (Popoff et al., 2000) and most of them were proved to genetically belong to a single species (Crosa et al., 1973).

In 1948, Kauffmann described 150 serotypes’ in the 6th edition of Bergey’s Manual (Kauffmann, 1948), however, in 1952, he described only three species, which could be differentiated by biochemical tests (Kauffmann & Edwards, 1952).

*S. typhi, S. enteritidis, S. choleraesuis, S. arizonae* and *S. typhimurium*, among more than 2000 serovars in the genus *Salmonella*. In 1982, based on the results of numerical analysis and DNA-DNA hybridization among 88 *Salmonella* reference strains belonging to be subgenera I-IV by Kauffman (Le Minor et al., 1982a). Le Minor et al. (Le Minor et al., 1982b) proposed to combine four *Salmonella* subgenus strains into a single species, namely *Salmonella choleraesuis*, and described six subspecies; *S. choleraesuis subsp. choleraesuis, S. choleraesuis subsp. salamae, S. choleraesuis*
subsp. arizonae, S. choleraesuis subsp. diarizonae, S. choleraesuis subsp. houtenae, S. choleraesuis subsp. bongori. Le Minor et al. intended to place all Salmonella serovars in the Salmonella choleraesuis subsp. Choleraesuis. However, an official proposal to place S. typhi, S. typhimurium and S. enteritidis as subjective synonyms of Salmonella choleraesuis subsp. choleraesuis, did not appear in a Validation List in IJSB. So, S. typhi, S. typhimurium and S. enteritidis remained as validly named species. The seventh subspecies, S. choleraesuis subsp. indica, was proposed by Le Minor et al. (Le Minor et al., 1986) and validly named in List no. 23 (Le Minor et al., 1987). Thus, at this stage, S. choleraesuis contained seven subspecies.

After the proposal of Le Minor et al. in 1982, Le Minor and Popoff requested an Opinion to designate Salmonella enterica sp. nov., nom. rev. as the type species of the genus Salmonella in 1987 (Le Minor & Popoff, 1987), i.e. they proposed S. enterica as a single species in the genus Salmonella. S. typhi, S. typhimurium and S. enteritidis were included in the S. enterica. Salmonella enterica originally was used by Edwards & Ewing (1963).

In 1989, S. choleraesuis subsp. bongori was placed as a distinct species, S. bongori (Le Minor et al., 1985; Reeves et al., 1989a; Reeves et al., 1989 b). Thus, currently, five species and six subspecies of S. choleraesuis. The species name of Salmonella typhi is still valid nomenclaturally although this species is genetically identical to S. choleraesuis subsp. choleraesuis because of their high DNA-DNA similarity value (Crosa et al., 1973; Stoleru et al., 1976). However, the name of this organism was well known and used from early in the last century because this organism causes severe epidemic disease, typhoid fever.
2.4 Kauffman – White classification

Based on their O and H antigen composition, more than 2300 *Salmonella* serotypes (species) are described in the Kauffman-White scheme. *Salmonella* are placed in groups by their O antigens (A, B, C, etc.) and subdivided by their H (phase 1 and 2) antigens. The grouping and antigenic composition of some *Salmonella* are shown in the following table (Cheesbrough, 2000).

<table>
<thead>
<tr>
<th>Group, Serogroup Serotype</th>
<th>O Antigen</th>
<th>H Antigens Phase1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A, Serogroup 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. paratyphi A</em></td>
<td>1,2,12</td>
<td>a</td>
<td>-</td>
</tr>
<tr>
<td><strong>Group B, Serogroup 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. paratyphi B</em></td>
<td>1,4,5,12</td>
<td>b</td>
<td>1,2</td>
</tr>
<tr>
<td><em>S. derby</em></td>
<td>1,4,5,12</td>
<td>f.g</td>
<td>(1,2)*</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>1,4,5,12</td>
<td>i</td>
<td>1,2</td>
</tr>
<tr>
<td><strong>Group C, Serogroup 7</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. choleraesuis</em></td>
<td>6,7</td>
<td>c</td>
<td>1,5</td>
</tr>
<tr>
<td><em>S. paratyphi C</em></td>
<td>6,7,(Vi)*</td>
<td>c</td>
<td>1,5</td>
</tr>
<tr>
<td><strong>Group D, serogroup, 9</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>9,12,(Vi)*</td>
<td>d</td>
<td>-</td>
</tr>
<tr>
<td><em>S. enteritidis</em></td>
<td>1,9,12</td>
<td>g.m</td>
<td>-</td>
</tr>
<tr>
<td><em>S. pullorum-gallinarum</em></td>
<td>1,9,12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Group E, Serogroup 3,10</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. weltevreden</em></td>
<td>3,10</td>
<td>r</td>
<td>z₆</td>
</tr>
<tr>
<td><em>S. anatum</em></td>
<td>3,10</td>
<td>e,h</td>
<td>1,6</td>
</tr>
<tr>
<td><strong>Group G, serogroup 13,22</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. Poona</em></td>
<td>13,22</td>
<td>z</td>
<td>1,6</td>
</tr>
<tr>
<td><em>S. cubana</em></td>
<td>1,13,23</td>
<td>z₂⁹</td>
<td>-</td>
</tr>
</tbody>
</table>

- Brackets indicate that the antigen may present or absent.
- Note: The O antigen in bold type is common to all members of the group.
2.5 Bacteriology

2.5.1 Definition of Genus *Salmonella*

The genus *Salmonella* belongs to family *Enterobacteriaceae*. They are fermentative, facultatively anaerobic, oxidase-negative, Gram-negative rods that generally are motile aerogenic, non-lactose fermenting, urease negative, citrate utilizing, acetyl methylcarbinol negative and KCN-negative (i.e. KCN-sensitive) (Old, 1990).

2.5.2 Habitat

*Salmonellae* are primarily intestinal parasites of man and animals, both domestic and wild. They are frequently found in sewage, river and other waters and soil. They may survive for weeks in water and for years in soil. Some serotypes are adapted to specific hosts; e.g. Abortusovis, *Gallinarum*, Typhi and Typhisuis are confined to sheep, fowl, man and swine respectively. Members of subspecies I predominate among mammals; while those from subspecies II, III and IV are found commonly in the intestinal tract of cold-blooded animals (Old, 1990).

2.5.3 Morphology

They are Gram-negative bacilli, 2-4µm × 0.6 µm, non-acid-fast, non-sporing and non-capsulated. Most serotypes are motile with peritrichous flagella, but *S. gallinarum* and *S. pullorum* are non-motile variants (OH→O variation) of other serotypes are occasionally found. Most strains of most serotypes form type-1 (mannose-sensitive, haemagglutinating) fimbriae; *S. gallinarum*, *S. pullorum* and a few strains of other serotypes form type-2 (non-haemagglutinating ) fimbriae and most *S. paratyphi A*
strains are non-fimbriate. Serovar typhi also synthesizes type IV pili and such pili are important in adherence to or invasion of human intestinal cells (Zhang et al., 2000).

2.6 Cultural Characteristics (Old, 1996)

*Salmonellae* are aerobic and facultatively anaerobic. They grow on simple laboratory media in temperature range 15º-1ºC, optimally at 37ºC. Many strains are prototrophic, i.e. capable of growing on a glucose-ammonium minimal medium but some strains are auxotrophic and require enrichment of the medium with one or more amino acids or vitamins, e.g. most *S. typhi* strains require tryptophan.

2.6.1 On nutrient agar and blood agar

The colonies of most strains are moderately large 2-3 mm in diameter after 24hrs at 37ºC. They are grey-white, moist circular discs with a smooth convex surface and entire edge. Non-virulent, rough strains (S-R variation) form opaque granular colonies with an irregular surface and indented edge.

2.6.2 Peptone water and nutrient broth

Most strains show abundant growth with uniform turbidity. A thin surface pellicle forms on prolonged incubation. Rough (R) variants, which have a hydrophobic surface and tend to autoagglutinate, produce a granular deposit and a thick surface pellicle.

2.6.3 Differential and Selective solid media

Selective culture media must be used to recover the *Salmonella* from clinical specimens that potentially harbour mixed bacteria.

The following can be used as differential and selective media for the isolation of *salmonella* from different specimens:
2.6.4 MacConkey’s agar

After 18-24 h at 37°C, the colonies are pale yellow or nearly colourless, 1-3 mm in diameter, and easily distinguished from the pink-red colonies of lactose-fermenting organisms.

2.6.5 Brilliant green MacConkey’s agar

This makes it an excellent selective as well as differential medium for *salmonellae* except *S. typhi*, which doesn’t grow well on it. *Salmonellae* produce low convex, pale-green translucent colonies, 1-3 mm in diameter. Lactose-fermenting bacteria, and some rare strains of *Salmonella* serotypes, produce blue-purple colonies.

2.6.6 Deoxycholate-citrate agar (DCA)

This medium is superior to MacConkey’s agar as far as isolation of *S. typhi* is concerned. The colonies are of the same size or slightly smaller in size than those on MacConkey’s agar. They are pale, nearly colourless, smooth, shiny and translucent. After 48 hours of incubation the colonies may show black centre, surrounded by a zone of clearance.

2.6.7 Wilson and Blair’s brilliant-green bismuth sulphite agar (BBSA)

The medium is particularly useful in the isolation of *S. typhi*. The cultures need to be examined after 24 h, and then again after 48 h. Closely packed small (about 1 mm diameter) colonies may take up the dye from the medium and appear green or pale brown. Larger discrete colonies have a black centre and a clear edge. *Salmonellae*, which produce H$_2$S, show colonies surrounded by a metallic sheen.
2.6.8 Xylose lysine deoxycholate agar (XLD)

*Salmonella* and *Shigella* grow well in this media. Both form red colonies. But salmonellae are distinguished from *Shigellae* as they produce H$_2$S which reacts with ferric ammonium citrate in the medium to produce black centres in the red colonies. The colonies of *shigellae* are red without any black centre.

2.6.9 Salmonella - Shigella agar (SS agar)

SS agar is highly selective medium formulated to inhibit the growth of most coliform organisms and permit the growth of species of *Salmonella* and *Shigella* from environmental and clinical specimens. The high bile salts concentration and sodium citrate inhibit all gram-positive bacteria and many gram-negative organisms, including coliforms. Lactose is the sole carbohydrate and neutral red is the indicator for acid detection. Sodium thiosulfate is a source of sulphur. H$_2$S production is indicated by the black precipitate formed with ferric citrate. High selectivity of SS agar permits use of heavy inoculum.

2.6.10 Enrichment media

These are liquid media used to assist the isolation of salmonellae from faeces, sewage, foodstuffs and other materials containing a mixed bacterial flora. The enriched culture is plated on selective and/or differential media, usually after 24 hours incubation.

2.6.11 Tetrathionate broth

It can be used with or without addition of brilliant green which increases the selectively of this medium to most of the *Salmonellae* but makes it inhibitory to *S. typhi* and *Shigellae*. 
2.6.12 Selenite F broth

It is the most commonly used enrichment medium for specimens that may contain either salmonellae or shigellae. It is excellent for Typhi and Dublin It is not suitable particularly for isolation of S. paratyphi A and S. choleraesuis.

This broth is found to be more efficient than other enrichment media for the isolation of salmonellae from faeces, water and foodstuffs (Old, 1996).

2.7 Biochemical reactions

Most strains of Salmonella closely resemble each other biochemically, although there are a number of exceptions; no organism should be excluded on the basis of a single test. The usual reactions include: (1) fermentation of glucose, maltose, mannitol and sorbitol with the production of acid and gas; (2) absence of fermentation of salicin, sucrose and adonitol; (3) failure to produce indole, to hydrolyse urea or to deaminate phentlalanine; and (4) a positive methyl-red reaction and a negative Voges-Proskauer reaction (Old, 1996).

Salmonella like Shigella can be presumptively identified biochemically using TSI (Triple sugar iron) agar medium and individual biochemical tests (Cheesbrough, 2000).

**TSI:** This medium is used to help identify salmonella following isolation on a selective medium. *Salmonella* produce:

- pink-red (alkaline) slope and yellow (acid) butt, indicating fermentation of glucose but not lactose.
- Cracks in the medium if serotype produce gas from glucose fermentation  
  (S. typhi does not produce gas).
- Blackening in the medium due to H$_2$S unless serotype does not produce H$_2$S,  
  e.g. S. paratyphi A. Only small amount of blackening is seen with S. typhi.

Reaction of Salmonella (most serotype) (Cheesbrough, 2000).

- Urease and indole negative
- Lactose negative
- Gas produce from glucose fermentation (S. typhi does not produce gas).
- Citrate positive (S. typhi and S. paratyphi A are citrate negative)
- Lysine decarboxylase (LDC) positive (S. paratyphi A is LDC negative)
- Beta- galactosidase (ONPG) negative.

2.8 Pathogenesis

The infectious dose of S. typhi in volunteers varies between 1000 and 1 million  
organisms (Parry et al, 2002) Vi-negative strains of S. typhi are less infectious and less  
virulent than Vi-positive strains. S. typhi must survive the gastric acid barrier to reach  
the small intestine, and a low gastric pH is an important defense mechanism. In the  
small intestine, the bacteria adhere to mucosal cells and then invade the mucosa. The M  
cells, specialized epithelial cells overlying Peyer’s patches, are probably the site of the  
internalization of S. typhi and its transport to the underlying lymphoid tissue. After  
penetration, the invading microorganisms translocate to the intestinal lymphoid follicles  
and the draining mesenteric lymph nodes, and some pass on to the reticuloendothelial  
cells of the liver and spleen (Parry et al, 2002).
### 2.8.1 Host-Cell Entry of *S. typhi*

#### Attachment to Host Cells

*Salmonella typhi* will target intestinal epithelial cells (enterocytes), causing the inflammation of other cells in the intestinal Peyer’s patches and subsequently the mesenteric lymph nodes, spleen, and bone marrow associated with typhoid fever ([Ohl and Miller, 2001](#)). The mechanism by which *S. typhi* attaches to host cell is very common in many bacteria, which utilize long, hair-like filaments known as fimbriae that are coated with receptor specific adhesins that recognize and bind to only certain types of sites on the surface of target cells ([Baulmer et al., 1996](#)). Because these receptors may only be found on certain target cells, great specificity of attachment can be achieved. For example, *Salmonella* serotypes (e.g. *S. typhimurium*), which each have their own unique antigenic factors, may bind to different cells in the intestine, such as microfold cells or different macrophages ([Ohl and Miller, 2001](#)).

#### Invasion of Host Cells

Unlike most bacteria, which rely on receptor mediated endocytosis to invade a target cell, *S. typhii* utilizes a complex process known as bacterial mediated endocytosis, where bacterial proteins enter the host cell and manipulate signaling cascades that control cytoskeletal architecture, membrane trafficking, and gene expression, all of which force the host to endocytose *S. typhi* ([Ohl and Miller, 2001](#)). After attachment, the bacteria must find a way to translocate virulence proteins across its cell membranes. This is accomplished by the formation of Type III Secretion Systems (TTSSs), which form long, hallow channels between the inner and outer bacterial membrane, known as the “needle complex” (considered an organelle). This complex is made up of three components: two inner rings which anchor the organelle to the inner membrane of the
bacteria, two rings that form the base of the structure, a central rod that links the two pairs of rings, and a needle like extension through which the proteins traverse. The exact mechanism of how this unit assembles upon activation of a signal (in this case adhesin attachment to host-cell surface receptors) is unknown (Kubori et al, 2000). The virulent proteins, now in extracellular space, are transferred across the host-cell membrane by a series of translocase proteins, which are secreted by *S. typhi* to form a protein pore on the outer membrane of the target cell. Although it is important to understand that many proteins are involved in bacterial mediated endocytosis, two virulent proteins, SopE and SptP are key players are involved in enterocyte invasion by *S. typhi* (Sah et al, 2000).

**SopE and SptP Proteins in bacterial mediated endocytosis**

Both of these proteins are part of the Rho family of G-proteins, which regulate the actin cytoskeleton and gene regulation and rearrangement via phosphorylation of lysophosphatidic acid (LPA), which then phosphorylates ezrin/radixin/moesin (ERM) proteins. The phosphorylation of ERM proteins acts as regulator for their ability to cross link plasma membrane and actin filaments. Additionally, studies have shown that LIM kinase is phosphorylated by Rho G proteins, which then phosphorylate the protein cofilin. Once phosphorylated, cofilin will suppress its normal function as an actin depolymerizing protein (Sah et al, 2000). The protein SopE will activate such a signaling cascade and cause the ruffling of the apical surface of the membrane, enveloping *S. typhi* in large vesicles. After bacterial mediated endocytosis occurs, the protein SptP has the opposite effect, reconstituting the brush border surface of the enterocyte. Currently, there are no drugs that will target these complex pathways of salmonella invasion of intestinal epithelial cells or systemic infection; however,
antibiotics are used to target the metabolic pathways of the bacteria (Ohl and Miller, 2001).

2.8.2 Persistence in Host Cells

Metabolic Fuel of *S. typhi*

*Salmonella typhi* is a facultative anaerobe, meaning it will produce ATP for a variety of functions in the presence of oxygen via glycolysis, but will resort to glucose fermentation in the absence of oxygen. This metabolic scheme is typical of *Enterobacteriaceae*, of which include *E. coli*. Most antibiotics are targeted toward one or more specific enzymes in *S. typhi* that catalyze these important reactions which ultimately kills the bacteria (Kelly *et al.*, 2004). Additionally, *S. typhi* requires various metabolites for survival, including aromatic amino acids and purines (Fang, 1997).

Host – Cell Dependent Pathways for Systemic Infection

*Salmonella typhi* will take advantage of the body’s own immune defenses in order systemically infect the body, typical of typhoid fever, while simultaneously evading these defense mechanisms. However this requires *S. typhi* to have several virulence factors to avoid destruction in a microbicidal environment. Once *S. typhi* has invaded enterocytes and M-Cells, it will inevitably trigger macrophage activity due to its antigenic factors. As a result, the bacteria will be phagocytosed in the submucousal region of the intestines, the allowing the bacteria to use the macrophage as a vector for transport to reticuloendothelial organs via blood and the lymphatic system. By doing so, *S. typhi* is able to evade the humoral immune response. This does not however, protect it from the internal environment of the macrophage itself, which contains hydrolytic enzymes, antimicrobial peptides, and other factors dangerous to *S. typhi*. To
survive, the bacteria utilize the following strategies: the lipopolysaccharide component of the bacterial lipid membrane modifies itself, altering membrane fluidity and surface charge density (Ohl and Miller, 2001). This in turn promotes resistance to antimicrobial peptide insertion, which is used by phagocytes to disrupt bacterial cell membranes. Secondly, \textit{S. typhi}, will produce several enzymes along with endogenous (produced within the bacteria) homocystein-an amino acid, that deactivates nitric oxide radicals present in the phagocyte (Fang, 1997). Thirdly, \textit{S. typhi} will begin to produce its own essential metabolites (purines, aromatic amino acids, etc.) in the nutrient deprived environment of the phagocyte. Finally, \textit{S. typhi} will produce several proteins (translocated across bacterial membrane via TTSSs) which interfere with the maturation of a phagocyte, preventing the fusion between phagosome and lysosome. These four virulence factors are all controlled by a gene regulatory system, activated when the bacteria enter the phagocyte.

**Virulence Factors Mediated by PhoP and PhoQ**

\textit{Salmonella typhi} relies on a simple “two component regulatory system”, which in general is characterized by two proteins, a sensor kinase and an effector polypeptide, that ultimately controls the expression of virulence genes that allow the bacteria to survive in the phagocyte. Upon activation due to an environmental signal, the sensor kinase phosphorylates the effector protein, which in most cases acts as a transcriptional regulator in various bacteria (Perraud, \textit{et al}, 1999). In the case of \textit{S. typhi}, the sensor kinase, PhoQ, is activated upon environmental signals unique to the internal environment of the phagocyte, including low pH and “starvation”-low nutrient levels”. After activation, phosphorylation of the cytosolic effector molecule PhoP will occur, and will subsequently act as a transcriptional regulator for over 40 virulence genes,
most important of which are called pag and prg, which mediate such virulence factors described above (Miller et al., 1989).

2.8.3 Salmonella typhi Proliferation

As mentioned before, systemic infection of the body is a typical characteristic of typhoid fever. In order for this to occur, S. typhi not only utilizes the macrophage as protection against the humoral immune response, but also as a site of proliferation. After translocation across intestinal epithelial cells and subsequent uptake by a macrophage, the bacterium will begin to multiply while being transported along blood and lymphatic system which subsequently causes the acute infection of other tissues, such as the liver (Schwan et al., 2000). Additionally, S. typhi may persist in the macrophage, undetected by immunity systems for days, weeks, or months. Interestingly, chronic infection is also facilitated by macrophage apoptosis induced by S. typhi, where vacuolization of the macrophage recruits other phagocytes to uptake the infected vacuoles of the dead macrophage. Interestingly, S. typhi controls macrophage apoptosis by same signal transduction mechanisms as membrane ruffling in bacterial mediated endocytosis, as opposed to releasing cytotoxic chemicals. During bacterial mediated endocytosis, Rho and Rac G protein cascades will trigger the increase of macrophage intracellular calcium levels, activate phospholipase A2 and protein kinase activity, as well as increase the levels of intracellular of leukotriene (a hormone). All of these factors will cause apoptosis of the macrophage, by which the exact mechanism is unknown (Monack et al., 1996)
2.9 Immune response

The nature of protective immunity in typhoid in man is not well understood. The development of the humoral immune response to O, H, and Vi antigens of *Salmonella typhi* has been regularly demonstrated during and after typhoid fever as well as after TAB vaccination (Kumer *et al*, 1974).

It is well documented in the literature that humans as well as experimental animals respond to *Salmonella* infection by activating not only humoral but also cell-mediated immune responses (CMIR) (McGhee & Kiyono, 1993).

The development of specific humoral antibody response as well as CMIR in patients with typhoid fever at various stages of their illness. These immune responses are correlated with the clinical picture and specific CMIR give protection against typhoid fever (Sarma *et al*, 1977).

2.9.1 Cell mediated immune response (CMIR)

The cell-mediated immune response in typhoid fever develops almost invariably during the second week of illness in uncomplicated cases while it was often negative in complicated cases (Sarma *et al*, 1977).

Cell-mediated immunity as assessed by the leucocyte migration inhibition test (LMI), and developed in all cases with typhoid fever. Positive LMI was evident in the first week of the illness and was maintained during the evolution of disease and in some patients was still present after a year. It also developed at the end of 3 weeks in TAB vaccinated subjects (Dham & Thompson, 1982). Positive LMT is associated with good clinical recovery (Sarma *et al*, 1977).
A studies have shown that, iron-regulated outer-membrane protein (IROMPs) expressed by *S. typhi* induce a cellular immune response against infection through Th1 and Th2 type cells (Sood *et al*, 2005). The cellular immune response induced by IROMPs resulted in an enhanced DTH (delayed-type hypersensitivity response) and exhibited a significant increase the ratio of CD4+/>CD8+ cells and increased production of interleukin (IL)-2- and interferon (IFN)-γ in early period and in the later period of the study, increased production of IL-4-producing cells. The increase in the lymphocytes in PPs (Peyer's patch) might have caused the increase in the sIgA. Therefore, it is speculated that immunization with IROMPs may evoke peripheral as well as mucosal immunity against *S. typhi* infection (Sood *et al*, 2005).

The uncomplicated cases of typhoid fever were found to have an intact CMIR as compared to the complicated cases (Rajagoplan *et al*, 1982). The ratio of T lymphocyte subpopulations was grossly imbalanced in typhoid patients, the numbers of T lymphocytes and their subpopulations were further altered in the complicated cases as compared to uncomplicated cases. CMIR become depressed in complicated patients with typhoid fever. The imbalance within the subsets of T lymphocytes may be responsible for the depressed state of CMIR in complicated cases of typhoid fever. CMIR may thus emerge as the cardinal point for recovery in typhoid fever rather than the specific antibodies (Rajagoplan *et al*, 1982; Sarma *et al*, 1977).

**2.9.2 Humoral immune response**

The humoral response to *Salmonella typhi* is important for protective immunity against typhoid fever, as indicated by the protection obtained with killed cell vaccines and
component vaccines (outer membrane proteins, Vi antigen) in animals and human beings (Aron et al, 1993).

Although antibodies to S. typhi O, H, and Vi appear to be involved in protection against S. typhi infection, it is unknown whether such antibodies mediate protection, act in conjunction with other adaptive responses, or serve as a surrogate for the presence of other, more dominant protective immune responses e.g., cell-mediated immunity (Sztein, 2007). Anti-O-polysaccharide chain antibody titers are lower at the first week and increase up to the third week of the infection. On the other hand, antilipid A antibody levels, which are already higher at the beginning of the disease, progressively augment during the following weeks (Mastroianni et al, 1988).

The antibodies and cellular reactivity developed almost simultaneously but there was no correlation between the agglutination titres and LMI positivity. Typhoid patients also showed significantly raised serum IgM and IgA levels and increased concentrations of secretory IgA in their saliva (Dham & Thompson, 1982). The antibodies appeared after the 1st week of illness and the titres gradually increased during the following days. Chloramphenicol therapy did not interfere with antibody production and antibody titres did not correlate with the severity of typhoid fever (Sarma et al, 1977).

S. typhi IROMPs have also been observed to have immunogenic potential and are able to stimulate antibody-mediated protection at systemic and mucosal levels (Sood et al, 2005). For protection against Salmonella spp., both antibody and cell-mediated immune (CMI) responses are considered to be important. The O antigen (O9, 12 serotype) is
most relevant to protection against typhoid fever; other antigens include the virulence capsule antigen and some outer membrane proteins (Viret et al, 1999).

2.10 Antigenic structure

The numerous antigenic types of *Salmonella* were organized by White in 1934 to form a logical classification scheme that was useful in epidemiological study and typing of Genus Salmonella (Joklik, 1992). They have subdivided *Salmonella* into 2,463 serovars containing different combination of antigens.

The antigens used to define serologic types of *Salmonella* include (Old, 1996)

1. The O-antigens, heat stable polysaccharide that form part of cell wall polysaccharide.
2. The H- antigens, heat labile proteins of flagella that in Salmonella have almost unique character of diphasic variation.
3. Surface polysaccharides that inhibit the agglutinability of the organism by homogenous O antisera of which the Vi-antigen of typhi is the most important example.

2.10.1 ‘O’ (Somatic) Antigens

The O-antigen is the most surface-exposed LPS component and displays enormous structural variability, resulting in a large variety of serotypes (Reeves, 1993). 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 and are designated by Arabic numeraels (Old, 1996). The O-antigens are heat-stable (unaffected by heating at
100°C for 2.5 hrs.) and alcohol-stable (withstanding treatment with 96% ethanol at 37°C for 4 hrs.) Heating destroys flagellar and fimbrial antigens, while alcohol detaches the flagella. Any of these two procedures can be used to prepare bacterial suspensions that agglutinate with O-antibodies and not with H-antibodies. O-antigens are unaffected by 0.2% formaldehyde, but in presence of flagella their fixation with formaldehyde renders the bacteria inagglutinable by O-antibodies (Old, 1996). Antibodies to O-antigens are predominantly IgM and tend to agglutinate O-antigens in granular masses. The presence and proper chain length distribution of the O-antigen polysaccharide are essential for serum resistance of Salmonella typhi but not for invasion of epithelial cells (Hoare et al, 2006).

2.10.2 H (Flagellar) Antigens (Old 1996)

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 and 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.

Many salmonellae show diphasic production of flagellar antigens and each strain can spontaneously and reversibly vary between these two phases with different sets of H-antigens. In phase-1 (the specific phase) the different antigens are designated by small letters. By now up-to 70 such antigens have been identified and the letters a – z are
insufficient. The recently discovered antigens are therefore designated Z1, Z2, Z3, etc. In phase 2 (the group phase) the antigens first discovered were given Arabic numerals.

2.10.3 Outer membrane protein (OMP) antigen

Outer membrane protein (OMPs) present on the surface of Gram negative bacteria are complex of antigens in association with lipopolysaccharide (LPS). Outer membrane protein of *S. typhi* are good immunogens and also suggests their eventual use as immunogens for the prevention or diagnosis of this disease (Fernandez-Beros *et al.*, 1989).

In addition to Vi-polysaccharide vaccines (alone or conjugated to protein), attention has also been focused on the role of outer-membrane proteins (OMPs), particularly porins of Gram-negative bacteria, in the induction of specific immunity (Isibasi *et al.*, 1988). It also act as virulence factor such as 55 kDa outer-membrane protein from short-chain fatty acids exposed *Salmonella enterica serovar Typhi* induces apoptosis in macrophages (Chander *et al.*, 2006). 50-kD outer-membrane proteins (OMPs) of *Salmonella typhi* is antigenic and produce IgM and IgG antibodies that was compared with the Widal test for the serodiagnosis of typhoid fever (Choo *et al.*, 1994). IgM has been reported to appear a few days after infection, reaching a peak within 3 to 4 weeks, and then it declines gradually over a similar period of time.

2.10.4 Other surface antigens

Although the serotype of an enterobacterium is defined mainly by its O and H antigens, there are other important antigens at the surface of the bacterial cell, which can determine agglutination with homologous antibodies.
Vi-antigen

*S. typhi* also produces a group I exopolysaccharide known as the Vi antigen, which is made of a homopolymer of high molecular mass (Virlogeux *et al*, 1996) and forms a capsular structure. The Vi antigen is found in virtually all clinical isolates from patients with acute typhoid infection. It protects *S. typhi* against complement mediate lysis as well as phagocytosis (Kossack *et al*, 1981).

M-antigen

It is an extracellular polysaccharide antigen and is produced by those strains of *S. paratyphi* B and other serotypes that form mucoid or ‘slime wall’ colonies when cultures are held for several days at room temperature after incubation for 1 day at 37ºC. This antigen also prevents the agglutination by O-antibodies.

Fimbrial antigens

The type-1 fimbriae formed by most strains of salmonellae, bear antigens that determine agglutination by sera containing anti-fimbrial antibodies. Fimbriae are not found in young (6-24 hrs old) broth cultures, but can be found in 24-48 hrs old broth cultures.

2.11 Determinants of pathogenecity (Virulence factors)

Whether an infection with *Salmonella spp.* leads to a disease largely depends on the virulence of the strain and the constitution of the host. The virulence of the strain is determined by so-called virulence factors. *Salmonella* is a complex organism that produce a variety of virulent factors. These includes- surface antigens, factors contributing to invasiveness, endotoxin, cytoxin and enterotoxin (Joklik, 1992). Whereas a number of virulence factors of *Salmonella* have been identified only
recently, others have been studied for decades. These latter virulence factors i.e., virulence-plasmids, toxins, fimbriae and flagella are therefore referred to as "classic" virulence factors (Asten & Dijk, 2005).

2.11.1 Surface antigens

a) O- antigen: The presence and proper chain length distribution of the O-antigen polysaccharide are essential for serum resistance of Salmonella typhi but not for invasion of epithelial cells (Hoare et al, 2006).

b) Vi antigen: The capsular polysaccharide Vi antigen (ViCPS) is an essential virulence factor and also a protective antigen of Salmonella typhi (Tang et al, 2003). The Vi antigen is found in virtually all clinical isolates from patients with acute typhoid infection. It protects S. typhi against complement mediate lysis as well as phagocytosis (Kossack et al, 1981).

c) Fimbrial antigen: S. typhi synthesizes type IV pili and such pili are important in adherence to or invasion of human intestinal cells (Zhang et al, 2000).

d) Outer membrane protein (OMP): Act as virulence factor such as 55 kDa outer-membrane protein from short-chain fatty acids exposed Salmonella typhi induces apoptosis in macrophages (Chander et al, 2006).

2.11.2 Invasiveness

Unlike most bacteria, which rely on receptor mediated endocytosis to invade a target cell, S. typhi utilizes a complex process known as bacterial mediated endocytosis, where bacterial proteins enter the host cell and manipulate signaling cascades that
control cytoskeletal architecture, membrane trafficking, and gene expression, all of which force the host to endocytose *S. typhi* (Ohl, et al., 2001).

2.11.3 Endotoxine

There is controversy regarding possible role of endotoxine in production of clinical manifestation presumably it is responsible for fever (Joklik, 1992). Lipopolysaccharide (LPS) has also been implicated in the adherence and/or invasion of epithelial cells (Hoare et al., 2006).

2.11.4 Virulence plasmid

Certain *Salmonella* serovars belonging to subspecies I carry a large, low copy-number plasmid that contains virulence genes. Virulence plasmids are required to trigger systemic disease; their involvement in the enteric stage of the infection is unclear. *Salmonella* virulence plasmids are heterogeneous in size (50–90 kb), but all share a 7.8 kb region, spv, required for bacterial multiplication in the reticuloendothelial system (Rotger & Casadesus, 1999).

2.12 Clinical feature

The incubation period for *salmonella typhi* ranges from 3 to 21 days, variability is most likely related to the size of the initial inoculums, the health and immune status of the host (Lesser & Miller, 2005).

Although in most cases a transient and mild episode of diarrhoea develops shortly after ingesting *S. typhi* bacteria, most cases are asymptomatic during an incubation period (Maskalyk, 2003). In classic form without treatment typhoid fever lasts about 4 weeks (Gillespie, 2003). In the first week the features are non-specific with remittent fever,
temperature variation of 103°F to 104°F, which usually is associated with chills, headache and malaise. Early intestinal manifestations may be constipation and diarrhoea, the later being more common in children and is associated with abdominal tenderness (Joshi, 2001).

**Table I: Frequency of symptoms in typhoid fever (Pearson & Guerrant, 1995).**

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Frequency in typhoid fever (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>89-100</td>
</tr>
<tr>
<td>Headache</td>
<td>43-90</td>
</tr>
<tr>
<td>Nausea</td>
<td>23-36</td>
</tr>
<tr>
<td>Vomiting</td>
<td>24-35</td>
</tr>
<tr>
<td>Abdominal cramps</td>
<td>8-52</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>30-57</td>
</tr>
<tr>
<td>Constipation</td>
<td>10-79</td>
</tr>
<tr>
<td>Cough</td>
<td>11-36</td>
</tr>
</tbody>
</table>

During the second weeks the patient looks toxic and apathetic with sustained high temperature. The abdomen is slightly distended and splenomegally is common (Gillespie, 2003). Relative bradycardia is not a consistent feature. Rose spots are reported in 5–30% of cases but are easily missed in dark-skinned patients. These rose spots are small blanching erythematous maculopapular lesions typically on the abdomen and chest. Melanesian typhoid patients may develop purpuric macules that do not blanch (Parry et al, 2002).
With the onset of third week the patient become more toxic and fever persist and a delirious confusional state sets in (Typhoid states). Abdominal distention becomes pronounced, with scanty bowel sounds. Diarrhoea is common, with liquid, foul green–yellow stools. The patient is weak with feeble pulse and rapid breathing; crackle may developed over the lung bases.

Death may occur at this stage from overwhelming toxaemia, myocarditis, intestinal haemorrhage or perforation. Considerable weight loss is common.

In patient who survive into the fourth week, the fever, mental state and abdominal distention slowly improve over a few days but intestinal complication may still occur. Convalescence is usually a slow process (Gillespie, 2003).

The tongue is covered with a thick furry white to brown coating that spares the bright red tip and edges. The classic description of “doughy” feeling on palpation on abdomen is due to the bowl filled with air and fluid especially in the ileo-caecal region commonly designated as “Caecal gurgling”.

However, the classical presentation of typhoid fever has changed over the years. Atypical presentation seen more often than not now a days may delay the clinical suspicion of the disease. Atypical manifestations observed were burning micturition with normal urine examination, diarrhoea in first week, encephalopathy in first week, isolated hepatomegaly, pneumonitis and bone marrow depression (Dutta et al, 2001).
Table II: Physical findings in patients with typhoid fever (Pearson & Guerrant, 1995)

<table>
<thead>
<tr>
<th>Physical findings</th>
<th>Typhoid fever (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>98-100</td>
</tr>
<tr>
<td>Abdominal tenderness</td>
<td>33-84</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>23-65</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>15-52</td>
</tr>
<tr>
<td>Relative bradycardia</td>
<td>17-50</td>
</tr>
<tr>
<td>Rose spots</td>
<td>2-46</td>
</tr>
<tr>
<td>Rales or rhonchi</td>
<td>8-84</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>1-21</td>
</tr>
<tr>
<td>Meningism</td>
<td>1-12</td>
</tr>
</tbody>
</table>

If untreated, the fever persists for two weeks or more and defervescence occurs slowly over the following 2–3 weeks. Convalescence may last for 3–4 months. If an appropriate antibiotic is given the fever gradually falls over 3–4 days. The duration of untreated illness prior to the initiation of therapy influences the severity of the disease. Those individuals infected with multi-drug-resistant (MDR) isolates of *S. typhi* may also suffer more severe disease (Parry *et al*, 2002).

2.12.1 Typhoid fever in children

Typhoid fever, affects all age groups and it has been stated that classical features described in textbooks were absent in children. Fever was the most common presenting symptom and diarrhoea was more common than constipation. Unproductive cough,
hepatomegaly and splenomegaly are present. Rose spots and relative bradycardia were rarely observed (Yap & Puthuchery, 1991).

2.13 Complications

Typhoid fever is a multi-system disease and if untreated or inadequately treated may involve all the organs, leading to prolonged illness, systemic complications and high mortality. Some of the complications associated with high morbidity and mortality in patients are as follows.

2.13.1 Intestinal perforation and bleeding

The most lethal complications of typhoid fever are intestinal bleeding and ileal perforations, both arising from necrosis of Peyer’s patches in the terminal ileum. The majority of typhoid fever patients who develop perforation do so within the first 2 weeks of the illness. The risk factors for enteric perforation in patients with typhoid fever are -a short duration of symptoms, inadequate antimicrobial therapy prior to admission, male sex, and leukopenia (Hosoglu et al, 2004).

2.13.2 Typhoid encephalopathy

Typhoid encephalopathy, often accompanied by shock, is associated with a high mortality. Patients may display the “typhoid” facies, a thin, flushed face with a staring, apathetic expression. Mental apathy may progress to an agitated delirium, frequently accompanied by tremor of the hands, tremulous speech and gait ataxia, and then muttering delirium, twitchings of the fingers and wrists (subsultus tendinum), agitated plucking at the bedclothes (carphology), and a staring, unrousable stupor (coma vigil) (Parry et al, 2002).
2.13.3 Hepatobiliary manifestation

Acute cholecystitis is also a fairly common complication; however, classical symptom of acute cholecystitis may be absent. *Salmonella* infection has been reported to be associated with increased incidence of gallstones. Jaundice has been reported in about 1% of patients with typhoid fever, which usually occurs as a result of typhoid hepatitis (hepatomegaly with raised transaminases), septicaemia, or liver abscesses (Joshi, 2001).

2.13.4 Typhoid fever in pregnancy:

Typhoid fever in pregnancy may be complicated by miscarriage, although antimicrobial treatment has made this less common. Vertical intra-uterine transmission from a typhoid-infected mother may lead to neonatal typhoid, a rare but severe and life-threatening complication (Joshi, 2001).

2.13.5 Complications of typhoid fever in children

Complications of typhoid fever in children includes- anicteric hepatitis, bone marrow suppression, paralytic ileus, myocarditis, psychosis, cholecystitis, osteomyelitis, peritonitis, pneumonia, haemolysis, and syndrome of inappropriate release of antidiuretic hormone (SIADH) (Malik, 2002).

2.14 Relapse

Relapse occurs in 5%–10% of patients, usually 2 to 3 weeks after defervescence. There is usually an afebrile period between the first and second episode of fever which may be a few days to a few weeks (Joshi, 2001). The illness is usually but not invariably, milder than the original attack and the relapse *S. typhi* isolate has the same susceptibility pattern as in the original episode (Parry, 2002).
2.15 Carrier

It is reported that 1%–5% of those infected become chronic carriers (WHO, 2003) and carrier status persists throughout the life of a person. Carriers of *S. typhi* are either convalescent carriers who excrete the organism for a limited period of time after apparent clinical cure, or chronic carriers in whom persistent excretion of *S. typhi* in stool or urine can be detected a year after clinical illness. Chronic faecal carriers occur more commonly than do chronic urinary ones (Singh, 2001).

Chronic typhoid carriers status may be responsible for the endemicity and outbreaks of the disease in the region. High prevalence of typhoid carriers occurs in patients with biliary, gastrointestinal and other related diseases (Vaishnavi et al., 2005). Apart from this it may be responsible for deaths due to hepatobiliary cancer.

Chronic carriers give no prior history of typhoid fever in up to 25% of cases. Faecal carriage is more frequent in individuals with gallbladder disease and is most common in women over 40; in the Far East there is an association with opisthorchiasis (Vaishnavi et al., 2005). Chronic carriage carries an increased risk of carcinoma of the gallbladder, pancreas and large bowel (Parry et al., 2002). Urinary carriage is associated with schistosomiasis and nephrolithiasis.

2.16 Review of diagnostic methods

Laboratory diagnosis of typhoid fever is based on isolation identification of *S. typhi* from a suitable clinical specimen e.g. blood, stool, urine, bone marrow, duodenal
aspirate by culture, detection of *Salmonella typhi* specific antibodies and antigen by serological test and identify DNA by PCR (Pearson & Guerrant, 1995).

Recent advances in immunochemistry has provided different new approaches. Among them Dot enzyme immunoassay (EIA) is one of major serologic importance.

### 2.16.1 Isolation of the organism

*S. typhi* can be isolated from more than 90% of patients of typhoid fever if blood, stool, rose spot, and bone marrow aspirates are cultured (Gilman *et al*, 1975). *Salmonella typhi* maximally isolated from blood in the first week of disease; from feces in the second and following weeks and urine in the third and forth weeks.

The various culture methods available are:


### 2.16.2 Blood culture

This is the method of choice and has the great advantage over culture from the faeces, urine or bile of showing not only that the patient is infected with the bacillus but that the infection is active and is almost certainly responsible or the disease from which he or she is suffering (Parker, 1990). Though it is gold standard, the yield of blood culture is quite variable. In the untreated patient, blood cultures are usually positive in about 80% during the first week and declining 20%- 30% later in the course of the disease (Gillespie, 2003). A low yield is related to very low numbers of bacteria causing severe diseases which may be less than 10 per ml. of blood (Haque *et al*, 1999).
Bacterial detection by blood culture may be influenced by the culture medium employed, the number of circulating bacteria, the time of blood collection, the volume of blood employed for the culture, the host’s immune response system, the intracellular character of these bacteria, etc (Gavirla-Ruizi and Cardona-Castro, 1995).

Adequate volumes of medium should be used in blood culture systems if negative results are to be avoided. A study findings suggested that 50 ml of medium was adequate for 8 ml of blood, presumably because of very low degrees of bacteremia in some patients (Watson, 1978).

If whole blood is to be cultured, it is essential to prevent bactericidal effects of serum either by adequate dilution of the sample in an adequate medium volume or by inhibition of serum bactericidal factors. Sodium polyanethol sulfonate (SPS) (Liquoid) and bile salt inhibit this bactericidal effect (Parker, 1990). SPS in concentration of 0.025% to 0.03% is the best anticoagulant for blood. In addition to its anticoagulants properties, SPS is also anticomplementary and antiphagocytic, and interfere with the activity of some antimicrobial agents, notable amino glycosides (Forbes et al, 2002). A study was indicated that SPS aids in early recovery of S. typhi and S. paratyphi A from blood culture (Escamilla et al, 1985).

Taking culture on several occasions may improve the yield (Le & Hoffman, 1999). Trypticase soya broth, bile broth or glucose broth, brain heart infusion broth are usually used for conventional methods of blood culture. The media is incubated aerobically at 37°C. Subculture should be done on MacConkey’s agar, blood agar media daily for 1 week (Watson, 1978).
Modern (automated) blood culture techniques permit the bacteriological confirmation of typhoid fever in a higher proportion of cases. These systems employ equipment that automatically detects an early sign of bacterial growth in a special blood culture bottle (Collee & Marr, 1996). An isolation rate of 92% of blood culture with the Bectec 460 radiometric system using a blood : broth ratio of 1:6 was found in a study (Duthie & French, 1990).

2.16.3 Clot culture

Blood clot from which serum has been removed often gives a positive result when a similar volume of whole blood yields no growth (Parker, 1990). A method of clot culture with streptokinase has been recommended (Watson, 1956). Blood is taken from the vein in the usual way and 8-ml quantities are allowed to clot in sterile screw-capped Universal containers. The separated serum is removed. The medium used consists of a Wilson & Blair agar slope in a four-ounce bottle to which is added 15 ml of streptokinase bile salt broth. The streptokinase causes rapid clot lysis with release of bacteria trapped in the clot. The cultures are then incubated. Positive results may be obtained in less than 24 hours (Watson, 1956). Clot culture is more sensitive than blood culture with an isolation rate of 92% and the clot technique has many advantages over conventional whole blood culture, both in reliability and in cost (Watson, 1978).

2.16.4 Culture of the Mononuclear Cell-Platelet Fraction of Blood

The low concentration of S. typhi cells in the blood of patients with typhoid fever, <10 bacteria per ml, undoubtedly contributes to the moderate sensitivity of blood culture. By this method blood from typhoid patients is subjected to density gradient centrifugation, virtually all S. typhi cells are in the fraction containing only
mononuclear cells (MNC) and platelets. Colonies of *S. typhi* were present in all mononuclear cell-platelet layer-positive cultures within 18 hours of plating and were identified within an additional 10 min by a coagglutination technique. In contrast, identification of all positive cultures by conventional blood culture required 3 days (Rubin *et al*, 1990).

### 2.16.5 Bone marrow culture

Bone marrow aspirates are known to yield a higher rate of positive cultures in typhoid than peripheral blood (Gilman *et al*, 1975; Farooqui *et al*, 1991). Bone marrow culture may give a positive result when blood culture fails, particularly in patients admitted to hospital while on antibiotic treatment. Unlike blood culture bone marrow culture is highly (90%) sensitive (Lesser & Miller, 2005) despite 5 days antibiotic treatment (Gasem *et al*, 1995). A study report show that the concentrations of *S. typhi* in the bone marrow are considerably higher than in peripheral blood (Wain *et al*, 2001). In the bone marrow there were over 10 times more bacteria than in peripheral blood. It seems likely therefore that a large-volume blood culture (>10 ml) would be needed to match the positivity rate of a 1-ml bone marrow culture, and the necessity of taking a bone marrow aspirate may be reduced if blood cultures of greater than 10 ml are collected (Wain *et al*, 2001). This should be true particularly for patients who have been previously treated or who present late in the illness.

### 2.16.6 Duodenal string-capsule culture (DSCC)

The string capsule device provides a useful and simple method for culturing duodenal contents for the presence of *S. typhi*. This capsule device should be useful as a simple out patient method for defining typhoid carriers (Gilman & Hornick, 1976). The string
capsule device consisted of a length of nylon yarn coiled into a weighted gelatin capsule. A thread attached to the yarn protrudes from the capsule and allows for the yarn to uncoil as the capsule is swallowed and for retrieval of the yarn from the intestine. The final 12 inches of bile-stained string was cut off and dropped into selenite F broth. After 24 h of incubation at 37 °C the broth was streaked on MacConkey’s (Gilman & Hornick, 1976).

The sensitivity of duodenal string-capsule culture (DSCC) was compared to that of bone-marrow-aspirate culture (BMAC), single 3-ml blood culture (BC), and rectal-swab culture (RSC) for isolating Salmonella typhi and Salmonella paratyphi type A from patients with typhoid and paratyphoid fever, DSCC was positive in 57.6%, RSC in 35.6%, BC in 54.2%, and BMAC in 85.6%. The sensitivity of DSCC was improved by an additional 4.7% if subcultured done daily for seven days. The DSCC has no advantage over the combination of RSC and BC and is inferior in sensitivity to the BMAC. However, when a BMAC cannot be obtained, the addition of the DSCC to BC and RSC can be expected to improve the isolation rate by greater than 17%, to at least 85% (Hoffman et al, 1984).

2.16.7 Stool Culture

Stool specimen should be collected in a sterile wide mouthed container. Specimens should preferably be processed within 2 hours after collection. If there is a delay the specimen should be stored in a refrigerator at 4°C or in a cool box with freezer packs. The sensitivity of stool culture depends on the amount of feces cultured, and the positivity rate increases with the duration of the illness. Rectal swabs should be avoided as these are less successful. Stool cultures are positive in 30% of patients with acute
enteric fever (Parry et al, 2002). For the detection of carriers, several samples should be examined because of irregular shedding of salmonella.

2.16.8 Urine culture

Urine cultures are not recommended for diagnosis in view of poor sensitivity (Parry et al, 2002; Gilman et al, 1975). Bacteria are not excreted continuously and therefore several specimens may need to be cultured before organisms can be isolated (Chessbrough, 2000).

2.16.9 Serodiagnosis of typhoid fever

Widal test

This test first described by Fernend Widal in 1896, detects agglutinating antibodies against the O and H antigens of Salmonella typhi and H antigens of paratyphi A and B (Olopoenia & King, 2000). The "O" antigen is the somatic antigen of Salmonella typhi and is shared by Salmonella paratyphi A, paratyphi B, other Salmonella species and other members of the Enterobacteriaceae family (Rodrigues, 2003). Antibodies against the O antigen are predominantly IgM, rise early in the illness and disappear early (Rodrigues, 2003). The H antigens are flagellar antigens of Salmonella typhi, paratyphi A and paratyphi B. Antibodies to H antigens are both IgM and IgG, rise late in the illness and persist for a longer time (Olopoenia & King 2000, Rodrigues, 2003). Usually, O antibodies appear on day 6-8 and H antibodies on days 10-12 after the onset of disease. The test is usually performed on an acute serum (at first contact with the patient). A convalescent serum should preferably also be collected so that paired titration’s can be performed.
Conventionally, a positive Widal test result implies demonstration of rising titers in paired blood samples 7-10 days apart (Olopoenia & King, 2000). Unfortunately, this criterion is purely of academic interest. Decisions about antibiotic therapy cannot wait for results from two samples. Moreover, antibiotics may dampen the immune response and prevent a rise in titers even in truly infected individuals. Therapeutic decisions have to be generally based on results of a single acute sample. In endemic areas, baseline anti O and anti H antibodies are present in the population owing to repeated subclinical infections with *Salmonella typhi/para typhi*, infections with other *Enterobacteriaceae* and other tropical diseases such as dengue and malaria (Olopoenia & King, 2000; Parry *et al*, 1999). These antibody titers vary with age, socio economic strata, urban or rural areas and prior immunization with the TAB vaccine.

While interpreting the results of the Widal test, both H and O antibodies have to be taken into account. There is controversy about the predictive value of O and H antibodies for diagnosis of enteric fever. Certain authorities claim that O antibodies have superior specificity and positive predictive value (PPV) because these antibodies decline early after an acute infection (Schroeder, 1968). Other studies report a poorer positive predictive value of O antibodies probably due to rise of these antibodies in other salmonella species, gram-negative infections, in unrelated infection and following TAB vaccination (Parry *et al*, 1999). For practical purpose and for optimal result this test should be done after 5-7 days of fever by tube method and level of both H and O antibodies of 1 in 160 dilution (four fold rise) should be taken as cut off value for diagnosis.
The Widal test as a diagnostic modality has suboptimal sensitivity and specificity (Olopoenia & King, 2000; Parry et al, 1999; Rodrigues, 2003). It can be negative in up to 30% of culture proven cases of typhoid fever. Suboptimal sensitivity results from negativity in early infection, prior antibiotic therapy and failure to mount an immune response by certain individuals (Olopoenia & King, 2000). Poor specificity, an even greater problem and is a consequence of pre-existing baseline antibodies in endemic areas, cross reactivity with other Gram-negative infections and non-typhoidal salmonella, anamnestic reactions in unrelated infections and prior TAB or oral typhoid vaccination. The purity and standardization of antigens used for the Widal test is a major problem and often results in poor specificity and poor reproducibility of test results (Olopoenia & King, 2000).

Notwithstanding these problems, the Widal test may be the only test available in certain resource poor set ups for diagnosis of enteric. In Vietnam, using a cutoff of >1/200 for the O agglutinin or >1/100 for H agglutinin test performed on acute-phase serum the Widal test could correctly diagnose 74% of blood culture positive typhoid fever, however 14% results would be false positive and 10% false negative (Parry et al, 1999). Hence, it is important to realize the limitations of the Widal test and interpret the results carefully in light of endemic titers so that both over diagnosis and under diagnosis of typhoid fever and the resulting consequences are avoided.

**Other serological tests**

In view of the limitations of the Widal test and need for a cheap and rapid diagnostic method, several attempts to develop alternative serologic tests have been made. These
include rapid dipstick assays, dot enzyme immuno-assays and agglutination inhibition tests (Olsen *et al.*, 2004).

2.16.10 Antibody detection:

**DOT Enzyme Immunoassay (EIA) test**

A dot enzyme immunoassay that detects IgG and IgM antibodies against a 50 KD outer membrane protein distinct from the somatic (O), flagellar (H) or capsular (Vi) antigen of *Salmonella typhi* is commercially available as Typhidot (Gasem *et al.*, 2002). The sensitivity and specificity of this test has been reported to vary from 70-100% and 43-90% respectively (Khan *et al.*, 2002, Bhutta & Mansurali 1999). This dot EIA test offers simplicity, speed, early diagnosis and high negative and positive predictive values. The detection of IgM reveals acute typhoid in the early phase of infection, while the detection of both IgG and IgM suggests acute typhoid in the middle phase of infection. In areas of high endemicity where the rate of typhoid transmission is high the detection of specific IgG increase. Since IgG can persist for more that 2 years after typhoid infection (Saha *et al.*, 1999). The detection of specific IgG can not differentiate between acute and convalescent cases. Furthermore, false positive results attributable to previous infection may occur. On the other hand IgG positivity may also occur in the event of current reinfection. In cases of reinfection there is a secondary immune response with a significant boosting of IgG over IgM, such that the later can not be detected and its effect masked. A possible strategy for solving this problem is to enable the detection of IgM by ensuring that it is unmasked (Bhutta, 1996). The original Typhidot test was modified by inactivating the total IgG in the serum samples. Studies with modified test, Typhidot M, have shown that inactivation of IgG removes
competitive binding and allows the access of the antigen to the specific IgM when it is present.

The Typhidot M that detects only IgM antibodies of *Salmonella typhi* has been reported to be slightly more specific in a couple of studies (Hatta *et al*, 2002; Choo *et al*, 1999).

**IDL Tubex test**

The Tubex test is easy to perform and takes approximately 2 minutes time (Lim *et al*, 1998). The test is based on detecting antibodies to a single antigen in *S. typhi* only. The O9 antigen used in this test is very specific found in only sero group D *salmonellae*. A positive result always suggest a *salmonellae* infection but not which group D *salmonella* is responsible. Infection by other serotypes like *S. paratyphi* A give negative result. This test detects IgM antibodies but not IgG which is further helpful in the diagnosis of current infections.

**IgM dipstick test**

The test is based on the binding of *S. typhi* specific IgM antibodies to *S. typhi* lipopolysaccharide (LPS) antigen and the staining of the bound antibodies by an antihuman IgM antibody conjugated to colloidal dye particles. This test will be useful in places where culture facilities are not available as it can be performed without formal training and in the absence of specialized equipments. One should keep in mind that specific antibodies appear a week after the onset of symptoms so the sensitivity of this test increases with time (Hatta *et al*, 2002).

**2.16.11 Antigen detection tests**

Enzyme immuno-assay’s, counterimmune electrophoresis and co-agglutination tests to detect serum or urinary somatic/flagellar/Vi antigens of *Salmonella typhi* have been
evaluated (Fadeel et al, 2004; Kalhan et al, 1999). Sensitivity of Vi antigen has been found to be superior to somatic and flagellar antigen and has been reported as ranging from 50-100% in different studies (Rao et al, 1999; Fadeel et al, 2004; Kalhan et al, 1999). Similarly, specificity estimates have been reported to vary from 25% -90%. The suboptimal and variable sensitivity and specificity estimates, inability to detect *Salmonella paratyphi* infection and Vi antigen negative strains of *S typhi* are serious limitations of the Vi antigen detection tests.

### 2.16.12 Molecular methods

The limitations of cultures and serologic tests advocate for development of alternative diagnostic strategies. PCR as a diagnostic modality for typhoid fever was first evaluated in 1993 when Song, *et al*. successfully amplified the flagellin gene of *S. typhi* in all cases of culture proven typhoid fever and from none of the healthy controls (Song *et al*, 1993). Moreover, some patients with culture negative typhoid fever were PCR positive suggesting that PCR diagnosis of typhoid may have superior sensitivity than cultures. Over the next 10 years a handful of studies have reported PCR methods targeting the flagellin gene, somatic gene, Vi antigen gene, 5S-23S spacer region of the ribosomal RNA gene, invA gene and hilA gene of *Salmonella typhi* for diagnosis of typhoid fever (Haque *et al*, 1999). These studies have reported excellent sensitivity and specificity when compared to positive (blood culture proven) and healthy controls. The turn- around time for diagnosis has been less than 24 hours.

These reports should be viewed within the context of certain limitations. Clinical utility of PCR tests has been inadequately evaluated. Performance of the test in individuals with febrile illnesses other than typhoid, in those with past history of typhoid, carriers
of *S typhi*, and those vaccinated with typhoid vaccine is not known. Patients with a clinical diagnosis of typhoid fever who are culture negative but PCR positive may in fact be false positives. Comparison of PCR to bone marrow cultures as a gold standard may be a superior way of evaluating the sensitivity and specificity of these tests, but has not been done. The tests claim to detect as few as 10 organisms, but it should be remembered that in typhoid fever the median bacteremia is 0.3 CFU/ml of blood (Wain *et al*, 2001). Using small volumes of blood for DNA extraction may significantly lower the sensitivity of these tests. The cost and requirement for sophisticated instruments is also a potential drawback of molecular methods.

### 2.17 Treatment of typhoid fever

Typhoid fever is a severe systemic infectious disease. Treatment with appropriate antibiotics is essential for recovery. Since 1990s *Salmonella typhi* has developed resistance simultaneously to all the drugs used in first line treatment (chloramphenicol, cotrimoxazole and ampicillin) and are known as Multi Drug Resistant typhoid fever (MDRTF). There are some reports of re-emergence of fully susceptible strain to first line drugs (Bhatia *et al*, 2006). But these reports are few and unless antibiotic sensitivity testing shows the organisms to be fully susceptible to first line drugs they are not advocated for empirical therapy in typhoid.

#### 2.17.1 Chloramphenicol

Chloramphenicol, which other antibiotics must be compared with, has been the "gold standard" therapy since its introduction in 1948. Treatment with chloramphenical reduces typhoid fever mortality from approximately 20% to 1% and duration of fever from 14-28 days to 3-5 days (Lesser & Miller, 2005). Reemergence of sensitivity of
*Salmonella enterica serovar typhi* to chloramphenicol and a study results in India show that a high sensitivity of *Salmonella enterica serovar typhi* to chloramphenicol (96%) (Bhatia *et al.*, 2006).

### 2.17.2 Cotrimoxazole

Cotrimoxazole is a first line drug for typhoid fever but resistance is an increasing problem. In adults TMP-SMX appears to be effective when the dose is 800 mg of sulfa plus 160 mg of trimetoprim every 12 hours for 15 days.

### 2.17.3 Fluroquinolones

Fluoroquinolones are widely regarded as the most effective drug for the treatment of typhoid fever (Bhutta *et al.*, 1999). But unfortunately, some strains of *S. typhi* have shown reduced susceptibility to fluoroquinolones (Parry *et al.*, 2007).

Ciprofloxacin, ofloxacin, perfloxacin and fleroxacin are common fluoroquinolones proved to be effective and used in adults. Ciprofloxacin is usually given orally 500 mg twice daily for 14 days, but there are report that course of seven days may be adequate (Gillespie, 2003). Fluoroquinolones have the advantage of lower rates of stool carriage than the first line drugs. However, fluoroquinolones are not approved by Drug Controller General of India to be used under 18 years of age unless the child is resistant to all other recommended antibiotics and is suffering from life threatening infection. There is now considerable amount of evidence from the long term use of fluoroquinolones in children that neither they cause bone or joint toxicity nor impairment of growth (Kaundo *et al.*, 2006).
The 4-quinolone drugs are highly effective against multi-resistant strains and trials have shown this in comparison with parenteral and oral ceftriaxone and cefixime (Gillespie, 2003).

### 2.17.4 Third generation cephalosporins

With the development of fluoroquinolones resistance third generation cephalosporins were used in treatment but sporadic reports of resistance to these antibiotics also followed. Of the third generation cephalosporins oral cefixime has been widely used in children (Matsumoto et al., 2001). Amongst the third generation cephalosporins in injectable form ceftriaxone, cefotaxime and cefoperazone are used of which ceftriaxone is most convenient.

Of the oral third generation cephalosporins, oral cefixime is used in a dose of 15-20 mg per kg per day in two divided doses. Parenteral third generation cephalosprins include ceftriaxone 50-75 mg per kg per day in one or two doses; cefotaxime 40-80 mg per kg per day in two or three doses, and cefoperazone 50-100 mg per kg per day in two doses. A short, 5-day course of ceftriaxone is a useful alternative to conventional 14-day chloramphenicol therapy in the treatment of typhoid fever in both children and adults (Islam et al., 1993).

### 2.17.5 Other Antibiotics

In case of uncomplicated typhoid oral third generation cephalosporin e.g., cefixime should be the drug of choice as empiric therapy. If by 5 days there is no clinical improvement and the culture report is inconclusive add a second line drug, e.g., azithromycin or any other drug effective against *S. typhi* depending upon the sensitivity pattern of the area (Kaundu et al., 2006)
2.17.6 Role of Steroids

The role of glucocorticoids in the management of infectious diseases in man remains controversial, although experimental data obtained both in vitro and in experimental infections in animals provide evidence of a beneficial effect of such treatment. Their use in the treatment of severe typhoid fever has been shown to be beneficial (Lesser & Miller, 2005).

Based on a study from Jakarta which showed a significant reduction in mortality in patients with severe typhoid fever (i.e. CNS symptoms, shock, disseminated intravascular coagulation), Dexamethasone (3mg/kg as a loading dose over 30 min, followed by 1mg/kg every 6h for 24h to 48h) used along with parenteral antimicrobials seems to reduce mortality. Steroid treatment over 48 hours may increase the relapse rate (Lesser & Miller, 2005).

2.17.7 Treatment of complications

For complicated typhoid the choice of drug is parenteral third generation cephalosporin e.g. ceftriaxone. In severe life threatening infection fluoroquinolones may be used as a last resort. Aztreonam and imepenem may also be used (Kaundu et al, 2006).

2.18 Drug resistance in typhoid fever (ICDDR’B, 2007)

Antibiotic resistance remains an important problem in Asia and in Africa because of the possibility to buy antibiotics across the counter in the open market. Susceptibility patterns of S. typhi strains showed high rates of multidrug resistance (MDR) in Asia and in Africa, with rates of 40% of isolates from India, 70% from Pakistan, 40% from Bangladesh, and 77% from Viet Nam. It was reported that more than 80% of isolates in Nigeria and 60% in Cameroon had resistance against ciprofloxacin and ceftriaxone. A
three-year study in Iran showed a frequency of MDR of 50%, 67%, and 33% respectively in different Iranian locations. A non-plasmid-mediated fluoroquinolone-resistant S. Paratyphi A was reported from India. In addition, resistance of S. typhi to nalidix acid varied between none in Laos to low levels in isolates from China (5%), higher rates on the India subcontinent (38%–Pakistan, 40%–Bangladesh, 61%–India), and higher levels in Viet Nam. Resistance of S. Paratyphi A to nalidixic acid was also reported in South China. Usually, the resistance to nalidixic acid is considered a marker for reduced susceptibility to fluoroquinolones (Kadhiravan et al., 2005). It is still not known if the Nalidixic acid resistance in S. Paratyphi A has a clinical profile similar to resistance of S. typhi to nalidixic acid.

CHAPTER 3
MATERIALS AND METHODS
MATERIALS AND METHODS

3.1 Type of the study

The study was designed as cross-sectional comparative study.

3.2 Place of the study

The study was carried out in the Department of Microbiology, Mymensingh Medical College. The cases were selected from outpatient / inpatient department of Mymensingh Medical College Hospital (MMCH).

3.3 Period of study

The period was from July 2006 to June 2007.
3.4 Data collection and analysis

Relevant history, clinical findings, and laboratory records of every case was collected and recorded in a pre-designed data sheet (Appendix 1). Subsequently, data were analyzed by computer programme SPSS version 12.0.

3.5 Study population

100 cases and 40 age sex matched controls were studied.

3.5.1 Cases

One hundred clinically suspected typhoid fever cases (irrespective of age and sex) were selected on the basis of following inclusion criteria -

**Inclusion criteria (Butler & Scheld, 2004)**

i) Fever for ≥ 3 days, with no obvious focus of infection,

ii) Abdominal discomfort- constipation or loose motions,

iii) Coated tongue, toxic look,

iv) Hepatomegally, splenomegally,

v) Relative bradycardia, rose spot etc.

**Exclusion criteria**

i) Persons who are immunized with typhoid vaccines.
ii) Patient suffering from fever other than typhoid.

3.5.2 Controls

A total of 40 age and sex matched controls were selected from the same place as well as same locality of which 20 were sick (non-typhoidal febrile illness) & 20 healthy controls.

3.6 Socioeconomic condition

Economic condition of each of the cases was ascertained by interviewing the patients or their guardians.

The cases were classified into three groups on the basis of their monthly income from all possible sources as describe by Islam (1992).

1. Low income group- < 3000 Tk./month
2. Middle income group 3001-20,000 Tk./month
3. Upper income group -> 20,000 Tk./month.

3.7 Study groups

Group I (Culture positive typhoid fever cases)

Culture positive typhoid fever cases (n = 14), serum samples of patients in whom the diagnosis was confirmed by the isolation of *Salmonella typhi* from blood.

Group II (Culture negative typhoid fever cases)

Culture negative typhoid fever cases (n = 21), serum samples of patients in whom the diagnosis of typhoid fever was based on clinical suspicion and significant rising
antibody titre in paired sera by Widal agglutination test but without confirmation by isolation of *S. typhi* from blood.

**Group III (Non-typhoidal febrile controls)**

Febrile control (n = 20), serum from patients with non-typhoidal febrile illness.

**Group IV (Healthy controls)**

Healthy control (n = 20), sera from healthy individuals who did not have any history of fever within last 6 months.

### 3.8 Laboratory methods

#### 3.8.1 Specimen:

Blood was taken for both culture & serological tests.

#### 3.8.2 Procedure of collection of blood and separation of serum

Written consents were taken from patients or from their attendants before collection of samples. One blood sample was collected from each patient in the first time for culture and serological test and a second sample was collected from the patient 7-10 days after collection of the first blood sample to see rising titer of Widal test.

Single sample of venous blood (preferable antecubital) was collected from each patient with sterile disposable syringe and needle after disinfection of the selected venipuncture site with 70% alcohol in a expanding circular scrubs from the centre to the periphery of the the needle insertion site followed by 2% tincture of iodine which as allowed to dry for one minute (Forbes *et al*, 2002; Chessbrough, 2000).
At least 5 ml of blood from each paediatric patient and 7 ml of blood from each adult patient were collected from single venupuncture. After removing the syringe and needle from the venipuncture site the sampling needle was discarded and replaced by a sterile needle. The top of the rubber stoppers of the blood culture bottle were disinfected with 70% alcohol and collected blood were injected immediately into the culture bottle, 3 ml in paediatric bottle and 5 ml in adult. Rest of 2 ml of blood from each paediatric and adult samples were taken in a clean dry test tube for separation of serum.

Tubes containing 2 ml of blood was kept at room temperature for one hour to allow clotting of blood and then it was centrifuged at 1500 rpm for 15 minute. Serum was separated and kept in a sterile eppendorfs tube at -20°C until further use.
3.8.3 Procedure of conventional blood culture method

Blood culture was done by conventional or traditional method using trypticase soya broth (TSB) with sodium polyanethol sulfonate (SPS) (Appendix-II).

5 ml of collected blood for adult and 3 ml of collected blood for paediatric patients were inoculated immediately into 50 ml and 30 ml TSB broth (which was brought to room temperature 30 minutes before inoculation) respectively. The inoculated bottle was inverted 3-5 times to mix blood with broth. Inoculated culture bottle was incubated at 37°C aerobically.

Bottles were examined visually daily. Growth was usually indicated by haemolysis of red blood cells, gas bubbles in the medium or turbidity in the broth. When macroscopic evidence of growth was apparent, a Gram-stained smear of an air-dried drop of medium was done. In addition to daily visual examination, blind subculture from conventional bottle after the first 24 hours of incubation was performed by aseptically removing a few drops of the well-mixed medium and spreading this inoculum onto MacConkey’s and blood agar plates. The plate was incubated at 37°C aerobically for 24 - 48 hours. Culture negative bottles were then reincubated for 5 to 7 days (Forbes et al., 2002; Chessbrough, 2000).

Suspected colonies were identified as *Salmonella typhi* by Gram’s stain, motility test (Appendix- IV) and by following biochemical tests including - Oxidase test, triple-sugar-iron agar (TSI), Citrate, Indole and Urease tests (Appendix- V).
3.8.4 Serological tests

3.8.4.1 Antibody detection by Widal agglutination test

Widal agglutination test was done by rapid slide titration method, using murex reagents (Murex Biotech limited, UK) containing ‘O’ (somatic) and ‘H’ (flagellar) antigens of *Salmonella typhi* and ‘O’ and ‘H’ antigen of *Salmonella paratyphi* A and B, with serial dilutions of sera beginning at 1:80. In case of patients paired samples were tested when second sample were available and in all cases 1st serum samples were tested. In controls single sample were tested. Widal results were expressed as the inverse of the highest dilution expressing agglutination.

**Procedure of Widal test**

**Rapid slide titration methods (murex)**

1. Using a 0.2 ml pipette, 0.08, 0.04, 0.02, 0.01 and 0.005 ml of undiluted serum was delivered into a row of 3 cm diameter circles on white tile.
2. Using a dropper one drop of appropriate well-shaken antigen suspension (TO, TH, AO, BO, AH, BH) was added to each serum aliquot.
3. Mixed by stirring for a few seconds with a wooden applicator stick, proceeding from the mixture containing 0.005 ml serum to that containing 0.08 ml serum, spreading the contents to fill the circles.
4. The tile was rotated slowly and agglutination was read at one minute.

The reactions obtained are equivalent to those which would occur in a tube agglutination test with the serum dilutions of 1/20, 1/40, 1/80, 1/160 and 1/320 respectively.
3.8.4.2 DOT Enzyme Immunoassay

Dot enzyme immunoassay for detection of IgM antibody in patient’s serum was done as per manufacturer’s (MBDR, Malaysian) instruction using the 1st serum samples from all groups of cases and controls.

Principle

It is a qualitative antibody detection test designed for rapid & early diagnosis of typhoid fever (Ismail et al, 1991). The presence of IgM antibodies in serum against a specific 50 KDa, outer membrane protein antigen of Salmonella typhi are detecting by incubating nitrocellulose strips dotted with the specific antigen. To visualize the antigen-antibody complex, the strips are simultaneously incubated with peroxidase conjugated anti human IgM. Upon addition of the chromogenic substrate; the result can be read visually. Positive result is indicated by the duplicate blue colour dot.

Requirements (Appendix - VI)

Preparation of reagents: Colour development reagents were prepared 30 minutes before use as per manufacturers instructions (Appendix - VI)

Procedure for antibody ( IgM ) detection by DOT EIA as per kit manual

i) First the reagents and serum samples to be tested were taken out of the refrigerator and were allowed to warm to room temperature.

ii) A filter paper was placed on the table. Then using a forcep, the predotted antigen strips were removed from the container and was placed in a line on the filter paper
with the marked side up. Then the code number for each patient were labeled accordingly. Similar procedure were applied for positive and negative controls.

iii) 250 µl of sample diluent was added into the reaction wells. Then 2.5 µl either control or test serum was added to the reaction wells to achieve a final serum dilution of 1:100. The solution was gently aspirated for proper mixing. For each specimen a new sterile disposable pipette tip was used to avoid cross contamination.

iv) The reaction tray was then shaken gently to allow the strips to be thoroughly wet and remain fully immersed in first antibody solutions.

v) It was then incubated on a rocker platform for 20 minutes at room temperature.

vi) Then the first antibody solution was aspirated into a jar containing disinfectant (Clorox).

vii) Then 250 µl washing buffer was added into each well and washed three times for 5 minutes.

viii) Using sterile micropipette tips, 250 µl of prediluted Anti-human IgM was added into the wells.

ix) Then the reaction tray was covered with aluminium foil and incubated for 15 minutes at room temperature on a rocker platform.

x) The second antibody solution was then aspirated into discard jar and washed three times for 5 minutes as describe before.

xi) Then 250 µl of colour development solution was added into each well. The tray was covered with aluminium foil and incubated on the rocker platform for 15 minutes for colour development.
xii) Then the reaction was stopped by aspirating the solution and briefly rinsing the strips three times in distilled water.

xiii) The strips for each patient and controls were dried by placing onto a filter paper.

xiv) Then interpretation of results.

Results

A. Positive result: The test was positive when both dots on the test strips was as dark as or was darker than their corresponding dots on the positive control strips.

B. Negative result: When the dots on the test strips was lighter compared to the corresponding dot on the positive control strip.
The present study was conducted on a total of 140 subjects. Among them, 100 were cases of typhoid fever and 40 were age and sex matched healthy and sick controls. Out of 100 cases, 14 were culture positive for *Salmonella typhi* and 62 were Widal test positive and rest of 24 were clinically diagnosed typhoid fever but blood culture and Widal test negative.

Age and sex distribution of cases and controls is shown in the Table 1 & 2 respectively. Majority of the cases (34%) and controls (15%) were in the age group of 1 to 5 years (Figure-1). Number of cases and controls gradually declined with increase in age. Total male were 60% and female 40% in the cases group (Figure-2) and corresponding values for controls was 22 (55%) and 18(45%) respectively. Male to female ratio of cases were 1.5:1 (Table-1).

Out of 100 cases highest number 82% of cases were from low income group and lowest number 04% in high income group (Table 3).
Table 1: Age and sex distribution of cases

<table>
<thead>
<tr>
<th>Age in years</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 5 = 34</td>
<td>23(67.65)</td>
<td>11(32.35)</td>
</tr>
<tr>
<td>6 - 10 = 27</td>
<td>15(55.55)</td>
<td>12(44.44)</td>
</tr>
<tr>
<td>11 - 15 = 24</td>
<td>14(58.33)</td>
<td>10(41.66)</td>
</tr>
<tr>
<td>&gt;15 = 15</td>
<td>08(53.33)</td>
<td>07(46.66)</td>
</tr>
<tr>
<td>Total = 100</td>
<td>60(60.00)</td>
<td>40(40.00)</td>
</tr>
</tbody>
</table>

**Ratio**

| Male:Female = 1.5:1 |

Figures in parenthesis indicate percentage.
<table>
<thead>
<tr>
<th>Age in years</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5 = 15</td>
<td>08(53.33)</td>
<td>07(46.66)</td>
</tr>
<tr>
<td>6 - 10 = 07</td>
<td>04(57.14)</td>
<td>03(42.85)</td>
</tr>
<tr>
<td>11-15 = 05</td>
<td>03(60.00)</td>
<td>02(40.00)</td>
</tr>
<tr>
<td>&gt;15 = 13</td>
<td>07(53.85)</td>
<td>06(46.15)</td>
</tr>
<tr>
<td>Total = 40</td>
<td>22(55.00)</td>
<td>18(45.00)</td>
</tr>
</tbody>
</table>
Table 3: Socioeconomic condition of subjects

<table>
<thead>
<tr>
<th>Income group</th>
<th>No. of cases (n=100)</th>
<th>No. of controls (n = 40)</th>
<th>Total (100+40=140)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower class</td>
<td>82 (82.00)</td>
<td>31 (77.5)</td>
<td>113 (80.71)</td>
</tr>
<tr>
<td>Middle class</td>
<td>14 (14.00)</td>
<td>06 (15.0)</td>
<td>20 (14.28)</td>
</tr>
<tr>
<td>Upper class</td>
<td>04 (4.00)</td>
<td>03 (7.50)</td>
<td>07 (5.00)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage.
Out of 100 cases, 14 were positive for *Salmonella typhi*. The highest rate of blood culture positivity for *S. typhi* 04(14.81%) was found among 6 to 10 years of age group. (Table 4). Out of 100 clinically diagnosed typhoid fever cases 14(14%) were blood culture positive for *S. typhi*, 41(41%) were Widal test positive on first instance and 73 (73%) were DOT EIA positive for IgM (Table 6). The DOT EIA (IgM) is highly significant than Widal test among clinically diagnosed typhoid fever (χ² value 17.254, p < 0.001).

Out of 100 cases 41 were Widal test positive on first instance and out of 50 culture negative cases 21 showed rise in titre in paired sera taken at interval of 7 to 10 days. Among them 9 showed 4 fold rise and 12 showed 2 fold rise (Table 5). Among 100 clinically suspected typhoid fever 24 cases were both blood culture and Widal test negative (Table 7) of which 04 were DOT EIA (IgM) positive (Table 8).

DOT EIA (IgM) was positive in 13(92.85%) out of 14 culture positive typhoid cases, 37(90.24%) out of 41 Widal positive on first sample and 19 (90.47%) out of 21 rising titer positive typhoid cases. The test was also positive in 04(20%) out of 20 febrile controls. None of the healthy controls were positive by DOT EIA (IgM) (Table 8).
Table 4: Rate of isolation of *Salmonella typhi* among study cases

<table>
<thead>
<tr>
<th>Age in years</th>
<th>Blood culture Positive (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5 = 34</td>
<td>05(14.70)</td>
</tr>
<tr>
<td>6-10 = 27</td>
<td>04(14.81)</td>
</tr>
<tr>
<td>11-15 = 24</td>
<td>03(12.50)</td>
</tr>
<tr>
<td>&gt;15 = 15</td>
<td>02(13.33)</td>
</tr>
<tr>
<td>Total = 100</td>
<td>14(14.00)</td>
</tr>
</tbody>
</table>
Figures in parenthesis indicate percentage.
Table 5: Result of Widal test in paired sera of blood culture negative typhoid cases showed rise of antibody titre (n= 21)*

<table>
<thead>
<tr>
<th>Duration of illness (days)</th>
<th>Widal titre</th>
<th>Fold of rise</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st sample</td>
<td>2nd sample</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>80/80</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>160/320</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>160/80</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>80/80</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>80/80</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td>320/320</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>320/160</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>80/80</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>80/80</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>160/160</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>80/80</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>320/320</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>80/80</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>80/80</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>160/160</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>80/80</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>80/80</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>160/160</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>80/80</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>160/160</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>80/80</td>
</tr>
</tbody>
</table>

*These 21 cases were blood culture negative but showed rising titre in Widal test considered as Group II.
Table 6: Comparison of Blood culture, Widal test and DOT EIA on first sample among clinically suspected typhoid cases

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture (n = 100)</td>
<td>14 (14.00)</td>
<td>86 (86.00)</td>
</tr>
<tr>
<td>Widal test (n = 100)</td>
<td>41 (41.00)</td>
<td>59 (59.00)</td>
</tr>
<tr>
<td>DOT EIA (n = 100)</td>
<td>73 (73.00)</td>
<td>27 (27.00)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage.

( p < 0.001 by Chi-square test )
Table 7: Category of cases

<table>
<thead>
<tr>
<th>Category</th>
<th>No. of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture positive</td>
<td>14</td>
</tr>
<tr>
<td>Positive Widal test on first sample</td>
<td>41</td>
</tr>
<tr>
<td>*Significant Widal test as per rising titre among culture negative cases</td>
<td>21</td>
</tr>
<tr>
<td>Clinically suspected typhoid but both blood culture and Widal test negative</td>
<td>24</td>
</tr>
</tbody>
</table>

* Significant rising titer of TO & TH $\geq 1:160$
Table 8. Category wise results of DOT EIA

<table>
<thead>
<tr>
<th>Category of cases and controls</th>
<th>No. of individual DOT EIA positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture positive 14(14.00)</td>
<td>13 (92.85)</td>
</tr>
<tr>
<td>Positive Widal test on first instance 41(41.00)</td>
<td>37 (90.24)</td>
</tr>
<tr>
<td>Significant Widal test as per rising titre among culture negative cases 21(42.00)</td>
<td>19(90.47)</td>
</tr>
<tr>
<td>Clinically suspected typhoid fever but both blood culture &amp; Widal test negative 24(24.00)</td>
<td>04 (16.66)</td>
</tr>
<tr>
<td>Non-typhoidal febrile illness 20</td>
<td>04 (20.00)</td>
</tr>
<tr>
<td>Healthy controls 20</td>
<td>00 (00)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage.
The Widal test was positive in 06(42.85%) out of 14 blood culture positive cases and in 09(42.85%) out of 21 culture negative typhoid cases in the first week of illness (Table 9). Out of 14 culture positive cases 12(85.71) showed significant rising titre (Table 10).

In Group I out of 14 blood culture positive typhoid cases, 13(92.85%) were DOT EIA (IgM) positive and only 06(42.85%) were Widal positive on first sample and in Group II out of 21 culture negative typhoid cases 19(90.47%) were DOT EIA positive for IgM and 09(42.85) were Widal positive on first instance. In Group III 04(20%) were DOT EIA positive of which 02 were paratyphoid A patient and other 02 were one RTI and one UTI. Group IV none were positive for DOT EIA but 02 (10%) were positive for Widal test (Table 9). The DOT EIA (IgM) is more significant in both culture positive and culture negative typhoid fever cases than Widal test ($\chi^2$ value 4.072, p<.05).

Sensitivity and specificity of DOT EIA in confirmed typhoid cases are shown in (Table 11 ). Accordingly sensitivity and specificity were calculated as 91.47% and 90.00% respectively.

Sensitivity and specificity of Widal test in confirmed typhoid cases are shown in (Table 12). Widal test was positive in 15(42.85%) out of 35 cases. In 40 controls 06(15%) were positive and 34(85%) were negative. Accordingly sensitivity and specificity were calculated as 42.85% and 85.00% respectively.

The sensitivity of DOT EIA( IgM) was found to be much higher than Widal test ( 91.47% vs 42.85%) in the early serodiagnosis of typhoid fever while the specificity
of DOT EIA and Widal was found to be 90.00% and 85.00% respectively (Table 13, Figure 3 & 4).
Table 9: Results of Widal test on 1st sample and DOT EIA in different study groups

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No. of individual</th>
<th>Widal test 1st sample positive</th>
<th>DOT EIA positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>14</td>
<td>06 (42.85)</td>
<td>13 (92.85)</td>
</tr>
<tr>
<td>Group II</td>
<td>21</td>
<td>09 (42.85)</td>
<td>19 (90.47)</td>
</tr>
<tr>
<td>Group III</td>
<td>20</td>
<td>04 (20.00)</td>
<td>04 (20.00)</td>
</tr>
<tr>
<td>Group IV</td>
<td>20</td>
<td>02 (10.00)</td>
<td>00 (00)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage.

( p <0.05 by Chi-square test )

Group I - Culture positive typhoid fever

Group II - Culture negative typhoid fever (Significant Widal test as per rising titre).

Group III - Febrile control (Non-typhoidal febrile illness).

Group IV - Healthy control.
Table 10: Result of DOT EIA and Widal test in first and second sample among confirmed typhoid cases (Group I & Group II)

<table>
<thead>
<tr>
<th>Test</th>
<th>Group I (n=14)</th>
<th>Group II (n=21)</th>
<th>Group I &amp; II (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Widal test</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; sample</td>
<td>06(42.85)</td>
<td>08(57.14)</td>
<td>09(42.85)</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; sample</td>
<td>12(85.71)</td>
<td>02(14.28)</td>
<td>21(100.00)</td>
</tr>
<tr>
<td>DOT EIA</td>
<td>13(92.85)</td>
<td>01(7.14)</td>
<td>19(90.47)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage.

**Group I** - Culture positive typhoid fever

**Group II** - Culture negative typhoid fever (Significant Widal test as per rising titre)
Table 11: Sensitivity and specificity of DOT EIA (IgM) in confirmed typhoid cases

<table>
<thead>
<tr>
<th>Test result</th>
<th>Confirmed typhoid fever cases (n= 35)</th>
<th>Controls (n= 40)</th>
<th>Total</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOT EIA positive</td>
<td>(a) 32 (91.43)</td>
<td>(b) 04 (10.00)</td>
<td>(a+b) 36</td>
<td>91.42%</td>
<td>90.00%</td>
</tr>
<tr>
<td>DOT EIA negative</td>
<td>(c) 03 (8.57)</td>
<td>(d) 36 (90.00)</td>
<td>(c+d) 39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage.

N.B: Sensitivity and specificity was calculated by the following formula

Sensitivity = 100 x a/(a+c), Specificity = 100 x d/(b+d).

a = True positive, b = False positive, c = False negative and d = True negative.
Table 12: Sensitivity and specificity of Widal test on single sample

<table>
<thead>
<tr>
<th>Test result</th>
<th>Confirmed typhoid fever cases (n= 35)</th>
<th>Controls (n= 40)</th>
<th>Total</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Widal positive</td>
<td>(a) 15(42.85)</td>
<td>(b) 06(15.00)</td>
<td>(a+b)21</td>
<td>42.85%</td>
<td>85.00%</td>
</tr>
<tr>
<td>Widal negative</td>
<td>(c) 20(57.14)</td>
<td>(d) 34(85.00)</td>
<td>(c+d)54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage.

N.B: Sensitivity and specificity was calculated by the following formula

Sensitivity = True positive x 100 /True positive + False negative =100 x a/a+c,

Specificity = True negative x 100 / True negative + False positive =100 x d/b+d.

a = True positive;  b = False positive ;  c = False negative and  d = True negative.
Table 13: Comparative results among DOT EIA, single Widal test and paired Widal test

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOT EIA</td>
<td>91.42%</td>
<td>90.00%</td>
<td>88.88%</td>
<td>92.30%</td>
</tr>
<tr>
<td>Single Widal test</td>
<td>42.85%</td>
<td>85.00%</td>
<td>71.42%</td>
<td>62.96%</td>
</tr>
<tr>
<td>Paired Widal test</td>
<td>85.71%</td>
<td>Not done</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PPV = Positive predictive value ; NPV = Negative predictive value

Positive predictive value = $a \times 100 / (a+b)$

Negative predictive value = $d \times 100 / (c+d)$

*a = True positive,  b = False positive, c = False negative and  d = True negative.
DISCUSSION

Isolation of the causative agent by culture has remained the gold standard for diagnosis of enteric fever. Blood culture has got its limited diagnostic utility due to low sensitivity. Although the Widal test has been used for more than a century in many developing countries but it is non-specific, poorly standardized, often confusing and difficult to interpret (Schroeder, 1968). Moreover, sharing of O and H antigens by other Salmonella serotypes and other members of Enterobacteriaceae makes the role of Widal test even more controversial in diagnosing typhoid fever (Parry et al, 2002). On the other hand DOT EIA is a new, inexpensive and reliable serodiagnostic test recently available commercially. It has been studied in many countries & they found significantly higher sensitivity and specificity. Keeping the described in mind we studied DOT EIA (IgM) test for its usefulness in diagnosis of typhoid fever in our country.

The disease affected all ages, however most of the cases 34% of the study were in the age group of 1–5 years (Table 1). This findings correlates with the observation made by Saha et al (2003) who found that children between 2-3 years of age are most susceptible age group (35.6%). Another study from Bangladesh done by Saha et al (2001) showed that 16.2% patients in the age group of 2-3 years are more susceptible to infection. Almost similar study done by Sinha et al (1999) showed 44% children were aged under 5 years. It is interesting that although the child aged group < 5 years were more prone to infection, which may be due to a lack of immunity transferred by mother’s milk or the no consumption of potable drinking water as is the common practice in rural areas (Saha et al, 2003). In our study, among 100 clinically
suspected typhoid cases 60% were male and 40% were female (Table 1). This finding were similar with Roxas & Mendoza (1989) who reported 56% male and 44% female. Another study done by Butler et al (1991) also showed that infection rate is slightly higher in male, perhaps reflecting greater exposure of male to contaminated food and water out side the home.

In our study, 100 febrile patients clinically suggestive of typhoid fever were screened for isolation of *S. typhi* by blood culture. Among them 14 (14%) were positive for *S. typhi* and constituted the bacteriologically proven typhoid fever (Group I) (Table 9). The remaining 86 patients were culture negative but clinically suggestive typhoid fever. Among them 21 cases selected as culture negative typhoid fever on the basis of significant rising titre of Widal test (Group II), because a single Widal test is only be suggestive but not definitive for diagnosis of typhoid fever (Roxas & Mendoza, 1989; Hoffman et al, 1986). Another study also illustrates that a single widal test may increase the chance of misdiagnosis and four fold rise titre after an interval 7-10 days is more diagnostic for typhoid fever (Conanan et al, 1989). Although in our study, maximum titre rise 2 fold rather than 4 fold. This findings correlate with Tupasi et al (1991) who stated that clinically four-fold rising titre is rarely demonstrated and two to three-fold rises are more common. This may be due to effect of antimicrobial therapy which inhibit further antibody rise. In our study, among clinically suspected 100 typhoid fever cases 24 cases were both blood culture and Widal test negative (Table 7). But this out of 24 cases 4 cases were DOT EIA positive (Table 8).

In the present study, out of 100 clinically diagnosed typhoid fever 14(14%) showed positive blood culture for *S. typhi* (Table 7). Similar finding were also reported by Saha
et al (2003) from Kolkata they found an isolated rate of 21.1%, Hossain (2001) from Bangladesh reported an isolation rate of $S. typhi$ was 16.67%. In contrast Saha et al (2001) from Bangladesh and Jesudasson & Sivakumer from India reported an isolation rate only 8.40% and 6.92% respectively. The relative low sensitivity of the blood culture in diagnosing typhoid fever due to the widespread and irrational use of antibiotics and the difficulties in obtaining large enough volume of blood for cultures from children (Bhutta & Mansurali, 1999; Mohanty & Ramana, 2007).

The most widely used serological test in typhoid fever is to detect antibody against O and H antigen of $S. typhi$ by Widal test. In the present study Widal test was carried out in all the four groups of patients and controls (Table 9). The cut off value of Widal test was considered as 1:80 for both TO and TH (Saha et al, 1996). Although Widal test usually become positive from second week, in this study out of 14 culture positive typhoid patients (Group I) 06 (42.85%) had an initial TO and TH titre $\geq$ 160 in the first week of illness. Closely similar findings were also reported by Hatta et al (2002) they found 33(47.8%) out of 69 culture positive cases in the first week of illness. Another study done by Shukla et al. (1997) who found that 44.2% had TO titre of $\geq$160 in single sample collected in the early phase of illness from patients suspected to have typhoid in an endemic area of central India. This findings were most probably attributable to a hyper immune or immunologically sensitized population which is continually exposed to $S. typhi$ and other salmonellae (Senewiratne & Senewiratne, 1977). This observation is also of practical importance as second specimens are often not sent to the laboratory. The results obtained are also of relevance to the concept that specimens which are taken in the first week of illness are of little use in the serodiagnosis of typhoid.
In this present study when paired sample were collected at 7 to 10 days interval among 14 bacteriologically proven typhoid fever cases 12( 85.71%) had a significant Widal test (Table 10). This result agree closely with those of Pang & Puthuchery (1983) who found that 93.1% and Hatta et al ( 2002 ) found 90.4% on paired sera of typhoid cases, gave a significant Widal reaction. The rate of positivity of Widal reaction on 2nd sample higher than 1st sample because antibody to ‘O’ and ‘H’ antigen usually begin to apper towards the end of the 1st week of fever and increase to maximum during the 3rd week (Parker, 1990).

The incidence of false negative Widal test among the bacteriologically proven cases of this study was 02(14.28%) (Table 10). This findings were similar to when compared with findings in Bangladesh 11.3% (Saha et al, 1996), in Iran 24%( Noorbakhsh et al, 2003) and 6.9% in Malaysian populations (Malik, 2001). Possible hypotheses put forward to explain this phenomenon are prior use of antibiotics, the existence of less immunogenic strains of S .typhi, reduced immunity from severe nutritional hypo proteinaemia ( Noorbakhsh et al, 2003).

Out of 20 non-typhoidal febrile cases 4(20%) (Table 9) showed high titre in the Widal test. This findings were closely similar with the findings of Duthie & French (1990) they found 23% false positive results of Widal test. Handojo et al (2003) in Indonesia also found 7% non typhoid fever showing a false positive Widal test. In contrast Pang & Puthuchery (1983) found that 3% of non-typhoidal fever gave a significant Widal reaction. These raised Widal titre in non- typhoidal febrile patients was perhaps due to the fact that these persons had been infected by S. typhi in the past as Salmonella
agglutinating antibodies may show a non-specific rise as a result of non-typhoidal fever (Pang & Puthucheary, 1989).

Out of 20 healthy controls 2 (10%) case was positive for TH (titre ≥ 160) (Table 9). Our findings were almost similar to those of Saha et al. (1996) they reported 4.3% out of 300 healthy Bangladeshi children had TH titre ≥160. These raised TH titre among our healthy controls was probably due to previous exposure S. typhi as typhoid is endemic in our reason (Saha et al., 1996).

In the current study, the DOT EIA for IgM was done on 35 confirmed typhoid cases (Group I & Group II) attended in the first week of illness. They include both blood culture positive cases and blood culture negative but paired sera showed rising titre. Positive DOT EIA results were read in comparison with the results of control sera and represented a titre > 1:100 (Bhatta & Mansurali, 1999).

In the present study, 13 (92.85%) out of 14 culture positive cases were positive for DOT EIA (Table 10). Our results are consistent with the finding of Bhatta & Mansurali (1999) in Pakistan who found 43 (93.47%) out of 46 culture positive typhoid fever cases and Sherwal et al. (2004) in India they found 35 (92.10%) out of 38 culture positive cases were DOT EIA positive. Closely similar findings were also reported by Choo et al. (1994) who found that 40 (95.23%) out of 42 culture positive typhoid fever cases were DOT EIA positive. The only 01 (7.14%) cases out of 14 culture positive cases was negative in DOT EIA (Table 10), was a 3 years old child attended in the 3rd day of fever having insignificant titre (TO / TH < 80/80) in the initial Widal test. The false negative DOT EIA in this case was probably due to the failure of DOT
EIA to detect the antibodies or perhaps the antibodies did not yet reach the detectable level in this patient.

19 (90.47%) out of 21 culture negative typhoid cases (Group II) were DOT EIA positive in this study (Table 10). Similar results were also reported by Choo et al. (1994) who found that 17 (94.44%) out of 18 culture negative typhoid cases were DOT EIA positive. A study done by Anggraini et al. (2004) showed that DOT EIA IgM positive 66% among clinically diagnosed typhoid fever. In contrast, two studies one from Pakistan (Bhatta & Masurali, 1999) showed that only 7 (28%) out of 25 and one from Malyasia Gopalakrishnan et al. (2002) showed that 18 (36%) out of 50 culture negative clinical typhoid fever cases were DOT EIA positive. This was probably due to the fact that they have used the clinical feature rather than rise in Widal titre for the diagnosis of typhoid fever cases which is very much variable and unreliable.

Only 2(9.52%) cases out of 21 culture negative typhoid cases were DOT EIA negative (Table 10). One was a child attended in the 3rd day of fever with initial insignificant titre (TO/TH= 80/80) in the Widal test and other was an adult presented with 4 days fever and was Widal significant (TO/TH = 160/80). The negative DOT EIA in this case was probably due to the failure of DOT EIA to detect the IgM antibodies or perhaps the antibodies did not yet reach the detectable level in this patient. DOT EIA negativity in the Widal positive case was probably due to the fact that the OMP antigen used in the DOT EIA is highly specific for *S. typhi* and a different class from the Widal antigen (O9, dH) (Choo *et al*, 1994).
Among 20 non-typhoidal febrile cases, DOT EIA was positive in 4(20%) cases (Table 9). Among 4 false positive cases 02 cases were with of *S. paratyphi* A infection (confirmed by blood culture) and other 2 cases of UTI and RTI respectively. The false positive result in paratyphoid A fever might be due to cross-reaction between the OMP antigen of *S. typhi* and *S. paratyphi* A (Olsen *et al*., 2004). The false positive result in other 2 non-typhoidal febrile cases due to high level of typhoid fever endemicity in our region indicating persistence of preexisting antibody from previous exposure (Olsen *et al*., 2004). Inconsistent with our finding, Bhutta & Mansurali (1999) from Pakistan found DOT EIA positive in 6 (23.07%) out of 26 and Sherwal *et al* (2004) from India also found 3 (12.5%) out of 24 non-typhoidal febrile cases. None of the 20 healthy controls in our study were positive for DOT EIA.

In our study, a total of 32(91.43%) out of 35 typhoid cases were DOT EIA positive while only 15(42.85%) were Widal positive in the first week of illness (Table 10). The rate of DOT EIA positivity was significantly higher than Widal test positivity among typhoid cases in the first week of illness. In agreement with our finding Bhutta & Mansurali (1999) from Pakistan has reported that among 46 culture positive typhoid cases, 43(93%) patients were DOT EIA positive while only 29(63%) had significant titre on Widal test. Furthermore, as is well known, it is not possible to come into a definite conclusion about typhoid fever by doing the Widal test in a single blood sample whereas by doing DOT EIA in a single blood sample almost certain diagnosis of typhoid cases can be done (Ismail *et al*., 1991; Choo *et al*., 1994).

In our study, it was found that DOT EIA becomes positive in first sample 73 (73%) out of 100 clinically suspected typhoid cases, whereas Widal test was positive in only 41
(41%) (Table 6). This findings were almost similar with Bhutta & Mansurali (1999) and Sherwal et al (2004) they reported DOT EIA positive 70% and 79%, Widal positive 54% and 57% respectively among clinically suspected typhoid fever.

In our study it was found that DOT EIA has a sensitivity of 91.42% (Table 11). Our result are remarkably consistent with the findings of Sherwal et al (2004) from India who reported a sensitivity of 92.0%. Almost similar sensitivity were also reported by Choo et al (1994) who found sensitivity of 90.3% of DOT EIA. Several studies have reported much higher sensitivity of DOT EIA in diagnosing typhoid fever cases. Jesudasson et al (2002) and Gopalakrishan et al (2002) reported that DOT EIA was 100% and 98% sensitivity respectively in detecting typhoid fever. This difference was probably due to the fact that in those studies unlike us have included all typhoid patients irrespective of duration of fever and result of repeat test was also included in their study.

In our study DOT EIA was found to have high specificity 90% (Table 11). In agreement with our finding Bhutta & Mansurali (1999) found that 89% specificity, Sherwal et al (2004) 87.5% and Anggraini et al (2004) found 100% specificity of DOT EIA in the diagnosis of typhoid fever. In contrast, a study from Pakistan (Bhutta & Mansurali, 1999) reported a much lower specificity (77%) of the test. This was due to a high rate of (23%) of DOT EIA positivity among non-typhoidal febrile patients. In our study a positive predictive value of 88.88% and a high negative predictive value 92.30% was found.
After analyzing the findings of the present study it was concluded that although blood culture is gold standard for diagnosis of typhoid fever and rising titre of Widal test also helpful for diagnosis but DOT EIA (IgM) might be a practical alternative test for diagnosis of typhoid fever.


Asten AJAM and Dijk JE. Distribution of "classic" virulence factors among Salmonella spp. FEMS Immunology & Medical Microbiology 2005; 44: 251-259.


CDC. Typhoid fever. Coordinating Center for Infectious Diseases / Division of Bacterial and Mycotic Diseases 2005.


Choo KE, Davis TM, Ismail A, Tuan Ibrahim TA and Ghazali WN. Rapid and reliable serological diagnosis of enteric fever: comparative sensitivity and specificity of


Crump JA, Fouad G, Youssef, Luby SP, Wasfy MO, Rangel JM et al. Estimating the Incidence of Typhoid Fever and Other Febrile Illnesses in Developing Countries. CDC 2003; 9:


Handojo I, Edijanto SP, Retnowati E and Salim SY. The widal slide agglutination test (sat) using antigen from locally prevalent *Salmonella typhi* as a diagnostic tool for typhoid fever Folia Medica Indonesiana 2003; 39: 29-35.


Hossain MS. Comparative study of Widal test and DOT enzyme immunoassay for early sero diagnosis typhoid fever. (Thesis) 2001;Bangabandhu Sheikh Mujib Medical University Dhaka, Bangladesh.


ICDDR’B. 6th International Conference on Typhoid Fever and Other Salmonelloses, Guilin, China 2005; 122 – 124.


Jesudason MV and Sivakumar S. Prospective evaluation of a rapid diagnostic test Typhidot® for typhoid fever. Indian J Med Res 2006; 123: 513-516


Kelly A, Martin D. Goldberg MD, Carroll RK, Danino V, Hinton JCD and Dorman C J. A global role for Fis in the transcriptional control of metabolism and type


Le Minor and Popoff MY. Salmonella cholerae-suis subsp. arizonae comb. nov., Salmonella choleraesuis subsp. bongori subsp. nov., Salmonella cholerae-suis subsp 1985;


**Reeves MW, Evins GM, Heiba AA, Plikaytis BD and Farmer JJ.** III *Salmonella bongori* comb. nov. In Validation of the Publication of New Names and New


**Rodrigues C.** The Widal test more than 100 years old: abused but still used. J Assoc Physicians India 2003; 51: 7-8.


**Rubin FA, Mcwhirter PD, Burr D, Punjabi NH, Lane E, Kumala SY et al.** Rapid Diagnosis of Typhoid Fever through Identification of Salmonella typhi within 18 Hours of Specimen Acquisition by Culture of the Mononuclear Cell-Platelet Fraction of Blood. Journal of Clinical Microbiology 1990; 28:825-827.


Sherwal BL, Dhamija RK, Randhawa VS, Jais M, Kaintura A, Kumar M. A Comparative Study of Typhidot and Widal Test in Patients of Typhoid Fever. JIACM 2004; 5: 244-6.


ACKNOWLEDGEMENTS

All praises to Almighty Allah, the merciful and kind enough for giving me the opportunity and courage to carry out and complete the thesis work.

I would like to express my deepest regards and sincerest gratitude to my honorable teacher and guide professor Dr. Md. Akram Hossain, Head of the Department of Microbiology, Mymensingh Medical College, Mymensingh. I shall remain evergreatful to him for his continuous and untiring guidance, active co-operation, valuable suggestions, constructive criticism and constant inspiration to carry out the work successfully.

I express my deep regards and gratitude to my respected teachers Dr. A. K. M. Musa, Associate professor of Microbiology, Dr. A.K.M Shamsuzzaman, Assistant professor of Microbiology and Dr. Md. Chand Mahmud, Assistant professor of Microbiology, Mymensingh Medical College, for their constructive criticism in correcting this thesis with their active co-operation and valuable suggestions.

I also express my thanks to all other teachers and M.phil students in the Department of Microbiology, Mymensingh Medical College, Mymensingh for their co-operation. I feel to express my sincere thanks to all laboratory technicians and other staffs of Microbiology Department, Mymensingh Medical College, Mymensingh for their active help and co-operation during my thesis work.

I express my deep regards and gratitude to Professor Md. Monwar Hossain, Principal, Mymensingh Medical College, Mymensingh for his administrative support during my study period. I am thankful to the honorable members of the Ethical review committee for giving kind approval of my thesis protocol.

I express my gratitude to professor Khandker Golam Sabbir Ahmed, Head of the Department of Microbiology, Community Based Medical College, Bangladesh for his co-operation. At the same time I would not miss the chance to thank Dr. Md. Murshed
APPENDICES
APPENDICES

Appendix-I

Data sheet

Typhoid fever study

Date: Case no Regd. No

Clinic/Hospital:

Name of patient: Age: Sex:

Occupation: Income:

Address:

**Housing Area**: City/village/ slum area.

**Latrine**: Sanitary / Non sanitary ( kacha / paka /open )

**Source of drinking water**: Supply / Tube well/ pond / River.

**Presenting Complaints:**

a) Fever for: days . b) Onset: sudden /stepladder.

c) Maximum rise of temperature: d) Type of fever:

e) Chill / Rigor / Sweating / Headache /Body ache / Malaise.

  f) Nausea / Abdominal discomfort / Constipation /Diarrhoea / Anorexia / Vomiting / Coated tongue.
On examination:

- Pulse /min.
- Anaemia:
- Coated tongue / Toxic state / Rose spot / Abdominal tenderness / Caecal gurgling
- Liver:
- Spleen:
- Temp.

Past history:

- History of antibiotic intake:
  - Name of antibiotic:
  - Dose & duration:

- History of contact: Yes / No

- History of vaccination (TAB): Yes / No

Complications:

Blood culture:

- Organism isolated:
- Time of isolation:

Antibiogram:

- Azithromycin, Amoxycilline, Cotrimoxasole, Ciprofloxacine, Cefixime, Ceforoxime, Ceftriaxone.

Widal test:

- 1st sample:
- 2nd sample:

Typhidot – IgM:

Signature:
Appendix-II

**Tryptone soya broth (TSB):**

Ingradients:

- Pancreatic digest of casein 17.00 gm.
- Papaic digest of soyabean meat 03.00 gm
- Sodium chloride 05.00 gm
- Dibasic potassium phosphate 02.50 gm
- Dextrose 02.50 gm

$\text{pH}$ after sterilization 7.3 ± 0.2

**Directions:**

Suspend 30.0 grams in 1000 ml purified / distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired. Sterilize by autoclaving at 15 lbs pressure 121°C for 15 minutes.

**Sodium polyanethol sulfonate (SPS) 5gm (HIMEDIA):**

(S, P.S Polyanethol sulphonic acid sodium salt).

SPS in concentration of 0.025% to 0.03% is the best anticoagulant for blood. In addition to its anticoagulants properties, SPS is also anticomplementary and antiphagocytic, and interfere with the activity of some antimicrobial agents, notable amino glycosides.
Appendix - III

The composition and methods of preparation of different media used in this study are given below:

**Blood agar media:**

Dehydrated blood agar base : 40 grams.
Distilled water : 1000.0ml
pH : 6.8

Autoclave at 121°C for 15 minutes under 15 lbs. Pressure, cooled down at 50°C, 7% sheep blood (defibrinated, aseptically collected) was added, mixed well and poured into sterile Petri dishes.

**MacCokey’s agar media:**

Dehydrated MacConkey’s agar base : 51.5 gm
Distilled water : 1000.0ml

Media was dissolved and pH was adjusted to 7.2 to 7.6 at 25°C and autoclave at 121°C for 15 minutes. When it was cooled to 55°C, poured into sterile Petri dish.

**Nutrient agar media:**

Dehydrated blood agar base : 40 gm
Distilled water : 1000.0ml
pH : 7.3 ± 0.2
Autoclave at 121\(^o\)C for 15 minutes. When it was cooled to 55\(^o\)C, poured into sterile Petri dish.

**Triple sugar iron (TSI) agar media:**

- Dehydrated TSI agar base : 65.0 gm.
- Distilled water : 1000.0 ml
- \(pH\) : 7.2-7.6

The ingredients were dissolved by boiling and the \(pH\) was checked, distributed into test tube in 5 ml amounts and sterilized by autoclaving at 121\(^o\)C for 15 minutes under 15 Ibs. Pressure. Allow the medium to solidify in a sloped position to give a butt 25-30 mm and a slope 20-25 mm long. Store in a cool dark place or at 2-8\(^o\)C.

**Simmon’s citrate media:**

- Dehydrated media : 24.2 gm.
- Distilled water : 1000.0 ml
- \(pH\) : 6.8

Ingredient dissolved, dispensed into test tubes and autoclave at 121\(^o\)C for 15 minutes and allowed to set as slopes.
Appendix-IV

Motility test:

Semisolid agar medium was for motility test. The test organism were inoculated into semisolid agar medium with a sterile straight making a single stab down the centre of the tube about upper one third of the depth of the media. The tube were then incubated at 37° C aerobically and examined at interval e.g 6 hours, 1,2,and 6 days. The motility of the bacteria were evident by the appearance of typical diffuse, hazy growth that spreads outwards from the stab line in the medium rendering it slightly opaque. The absence of motility of bacteria was indicated by the confinement of the growth to the stab line, having sharply defined margins with the surrounding clear transparent medium. Positive control with salmonella and negative control with shigella were tested in the same way and the result were compared with the test organism.

Gram staining technique (Cheesbrough, 2000)

Required

Crystal violet stain, Lugol’s iodine, Acetone-alcohol decolourizer, Neutral red, 1 g/l (0.1% w/v).

Method :

1. Fix the dried smear by gentle heat.
2. Cover the fixed smear with crystal violet stain for 30-60 seconds.
3. Rapidly wash off the stain with clean water.
4. Tip of all the water, and cover the smear with Lugol’s iodine for 30-60 seconds.
5. Wash of the iodine with clean water.
6. Decolourize rapidly (few seconds) with acetone-alcohol. Wash immediately with clean water.

7. Cover the smear with neutral red stain for 2 minutes.

8. Wash off the stain with clean water.

9. Wipe the back of the slide, clean, and place it in a draining rack for the smear to air dry.

10. Examine the smear microscopically, first with the 40X objectives to check the staining and to see the distribution of material, and then with the oil immersion objectives to report the bacteria and cells.
Biochemical test

Oxidase test (Cheesbrough, 2000)

A piece of filter was placed in a clean petridish and 2 to 3 drops of 1% freshly prepared oxidase reagent (tetramethyl-p-phenylene diamine dihydrochloride) was added. With a sterile glass rod 1 or 2 colonies of the test organism was taken and rubbed on the filter paper. Development of blue-purple colours within 10 seconds indicates positive test. *Pseudomonas aeruginosa* was used as positive control and *Escherichia coli* as negative control.

Indole test (Cheesbrough, 2000)

Medium:

Peptone (containing sufficient tryptphan) : 20.0gm.
Sodium chloride (Nacl) : 5.0gm.
Distilled water : 1000.0ml.

\( \text{PH} \) adjusted to 7.4, dispensed in test tubes and sterilized by autoclaving at 121\(^{0}\) C for 5 minutes.

Kovac’s reagent:

Amyl or isoamy alcohol : 150.0ml
P-Dimethyl-aminobenzaldehyde : 10.0gm
Concentrated hydrochloric acid : 50.0ml

Aldehyde was dissolved in alcohol and acid is added slowly.
Method:

Medium was inoculated with test organisms and incubated for 48 hours at 37°C. 0.5ml of Kovac’s reagent was added and shaken gently. A red colour in the alcohol layer indicates a positive reaction.

Urease test (Cheesbrough, 2000)

Medium (Christensen’s medium)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 gm.</td>
</tr>
<tr>
<td>Sodium chloride (Nacl)</td>
<td>5 gm.</td>
</tr>
<tr>
<td>Dipotassium hydrogenphosphate</td>
<td>2 gm.</td>
</tr>
<tr>
<td>Phenol red (1 in 500 aqueous solution)</td>
<td>6 ml.</td>
</tr>
<tr>
<td>Agar</td>
<td>20 gm.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Glucose 10% solution (filter sterilized)</td>
<td>10 ml</td>
</tr>
<tr>
<td>Urea 20% solution (filter sterilized)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Basal medium was prepared without glucose and urea, pH adjusted to 6.8 and sterilized by autoclaving in a flask at 121°C for 30 min. Cooled to about 50°C, sterile glucose and urea solutions were added, dispensed in test tubes and allowed to solidify as deep slopes.

Method:

Entire slope surface was inoculated heavily and incubated at 37°C. Examined after 4 hours and after overnight incubation. Urease positive cultures change the colour to purple-pink.
Triple sugar iron agar (TSI)

**Contents:** Peptone, yeast extract, sodium chloride, lactose, glucose, sucrose. Ferrous sulfate, sodium thiosulphate, phenol red and agar.

This medium is used to help identify *Salmonella typhi* following isolation on a selective medium. *Salmonella* produce:

- pink-red (alkaline) slope and yellow (acid) butt, indicating fermentation of glucose but not lactose.
- Blackening in the medium due to H$_2$S produce by *S. typhi*. 
Appendix-VI

DOT EIA (Typhidot®)

Requirements:

(a) Reagents

i) Predotted antigen strips (0.45 µm pore size), 0.5 cm by 1 cm, 1 µl containing 0.03 µg of 50 KDa OMP dotted on the strip.

ii) Sample diluent.

iii) Washing buffer.

iv) Prediluted anti-Human IgM conjugated with horse-raddish peroxidase (HRP).

v) Colour reagents-(0.06% 4 chloronaphthol, 0.015% H₂O₂ and 20% methanol).

vi) Positive control sera.

vii) Negative control sera.

Reagents are kept at 4°C.

They are allowed to warm at room temperature before doing the test.

(b) Equipments

Measuring cylinder (100 ml) ii) Micropipettes (2-20 µl; 1000 µl) and sterile micropipette tips. iii) Small conical flask (100 ml) iv) Forceps v) Wash bottles. vi) Filter paper vii) Distilled water. viii) Rocker platform. ix) Gloves. x) Aspirator.

xi) Aluminium foil xii) A dark reagent bottle.
Preparation of reagents:

1. Washing buffer (10X):

Washing buffer was diluted into 90 ml of distilled water to a final concentration of 1X. Stored at 2-8°C and used as needed.

<table>
<thead>
<tr>
<th>10X</th>
<th>Distilled water</th>
<th>1X</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ml</td>
<td>90 ml</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

2. Colour development reagent:

Substrate A and substrate B was brought to room temperature before mixing, avoiding exposure to strong light during incubation or storage. Based on the number of test (including controls) the recommended volume of substrate B was added into a bottle covered with aluminium foil. Then the recommended volume of substrate A was added and mixed well. This is prepared 30 minutes before use.

Interpretation of DOT EIA:

Basic principles:

To interpret the DOT EIA for typhoid the colour intensity of the dots produced by the test sera must be equivalent to or greater than those of the positive control. Reading DOT EIA results depends on entirely on the observation of the intensity of each dotted antigen after colour development.
There are some important principles to remember:

1. While comparing the colour intensity, the dot on the left of the test strip should be compared with the dot on the left of the positive control. Similarly, the colour intensity on the right of the test strip (next to the line marked on the strip) should be compared with the colour intensity of the dot on the right of the positive control strip. Only when both dots on the test strip are as dark as or are darker than their corresponding dots on the positive control strip the result can be interpreted as positive.

2. If one of the dots on the test strip is lighter compared to the corresponding dots on the positive control strip, the results should be reported as negative.

3. If interpretation cannot be done immediately the strip can be submerged in distilled water for up to 1 day.
Figure-5: Photograph of blood culture. Left: bottle containing trypticase soya broth with growth of *S. typhi*. Right: Inoculated broth without growth
Figure-6. Photograph of growth of *Salmonella typhi* on MacCokey’s agar media
Figure-7. Photograph of reaction of *Salmonella typhi* on TSI medium
Figure- 8. Photograph of DOT EIA reaction tray containing antigen dotted strips
Figure-9. Photograph of DOT EIA test strip showing positive and negative result
APPENDIX- XII

STATISTICAL FORMULA

Formula for mean

\[ \bar{x} = \frac{\sum x}{n} \]

- \( \bar{x} \) = mean of observations
- \( x \) = individual observations
- \( n \) = number of observation

Formula for Standard Deviation

\[ SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} \]

- \( SD \) = Standard deviation
- \( x \) = Individual observation of a series
- \( \bar{x} \) = mean of observations
- \( n \) = number of observation
- \( N \) = Number of observation
- \( n-1 \) = applicable for Sample
- \( N \) = for population
**Formula for Chi-square test ($\chi^2$):**

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Here,

- **O** = Observed frequencies
- **E** = Expected frequencies
- **df** = Degrees of freedom = (c-1) (r-1)
- **$\sum$** = Summation