2.0 REVIEW OF LITERATURE

2.1 General

*Salmonella enterica Typhi*, the causative agent of typhoid fever is an obligate pathogen. Typhoid is a widespread and potentially lethal infection. Before the nineteenth century, discriminating typhus and typhoid fever was difficult. William Jenner in 1850 settled that typhus and typhoid were different. 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. It was only in late 19th century that the disease was finally established as a distinct clinical entity.

In 1869 the term enteric fever was proposed by Wilson as an alternative to typhoid fever, given the anatomic site of infection (Singh, 2001). The typhoid bacillus was isolated by Gaffky in Germany in 1884 from the spleen. *Salmonella enterica* subspecies *enterica* subtype *Typhi* (*S. Typhi*), only colonizes human; therefore, disease can only be acquired through proximity with a person who has had typhoid fever or is a chronic carrier. Most often this involves acquisition of organisms via ingestion of food or water contaminated with human excreta.

**Scientific classification of Salmonella Typhi**

2.2 *Salmonella enterica* serovar *Typhi*

The initial classification of the genus *Salmonella Typhi* started during the first quarter of the 20th century. White (1925) discovered antigenic variation among the strains of this genus. Rahn (1937) reported that *Salmonella Typhi* belonged to genus *Salmonellae*, which belonged to family *Enterobacteriaceae*. The genus *Salmonellae* contains two species, *enterica*...
and bongori (Brenner et al., 1998). S. enterica is further divided into six subspecies (enterica, salamae, arizonae, diarizones, houtenae and indica) containing 2443 serovars. Most of the Salmonellae that cause disease, with some important exceptions, are in the subspecies Salmonella Typhi enterica subspecies enterica. The agents that cause enteric fever are therefore Salmonella enterica subspecies enterica serovar Typhi (commonly referred to as S. enterica serovar Typhi) and serovars paratyphi A, B and C (Parry, 2005).

2.3 Classification

Scientific classification

Bacteria
Kingdom : Bacteria
Phylum : Proteobacteria
Class : Gamma Proteobacteria
Order : Enterobacteriales
Family : Enterobacteriaceae
Genus : Salmonella
Species : Typhi

Salmon and Smith (1884) first cultured this bacterium on artificial media. Schutze (1921) first time introduced serological classification of genus Salmonella by using specific antisera. A simplified diagnostic scheme using a limited number of sera was suggested by Kauffmann, White and Edward (1952). Their scheme made possible the presumptive serotyping of most Salmonella Typhi. Shaw (1956) reported biochemical classification of
various species of this genus. The name *S. enterica* was suggested by Kauffmann, White and Edwards (1952). *S. Typhi* is in group D *Salmonella* according to the classification by Kauffman and White. Later Ewing, 1963, proposed the name enteritidis.

### 2.4 Cultural Characteristics and Morphology

*Salmonella enterica* serovar *Typhi* is a gram negative bacterium, facultative anaerobic and flagellated bacilli from the family of *Enterobacteriaceae* (Bhan *et al.*, 2005). *Salmonella Typhi* measures 2-4 µm long and 0.6 µm wide. *S. Typhi* is a non-acid fast and motile bacterium with peritrichous flagella. These organisms are non-spore forming, facultative anaerobic bacilli, which produce acid on glucose fermentation and reduce nitrates. *Salmonella Typhi* is a non-fastidious organism which can grow in basal media like nutrient agar. On MacConkey agar, these organisms produce colorless colonies since *S. Typhi* is a non-lactose fermentor. Low selective media such as MacConkey and deoxycholate agar and intermediate selective agar such as *Salmonella-Shigella* agar are widely used in laboratories to cultivate the organism. High selective media such as brilliant green and bismuth sulfite agar can also be used in the detection of *Salmonella Typhi*. Strontium selenite and selenite F broth were the enrichment broth for the organism (Chau *et al.*, 1972).

### 2.5 Biochemical characteristics of *Salmonella Typhi*:

After primary isolation, possible *Salmonella Typhi* isolates can be tested in commercial identification systems or conventional system such as
Triple sugar iron agar (TSI) and Lysine iron agar. The biochemical characters of *S. Typhi* given in Table 1.

### 2.6 Epidemiology of Typhoid Fever

#### 2.6.1 Typhoid Fever Global Scenario

Typhoid fever continues to be a global health problem. It is a disease of poor environmental sanitation and hence occurs in parts of the world where water supply is unsafe and sanitation is sub-standard. Each year more than 33 million people suffer from typhoid globally. Typhoid is a bacterial infection caused by *Salmonella Typhi* (Vollard, 2004) that affected 21.7 million people and caused 21, 6510 deaths worldwide in 2004 (Hornick, 1970; Crump, 2004). Complications occur in about a third of untreated cases and account for 75% of all deaths. Without effective treatment, the case fatality is 10%. Following recovery, up to 5% of patients become chronic carriers (Park, 2005). Humans are the only reservoir of infection (Black, 1985; Stroffolini, 1992; Egoz, 1998; Mermin, 1999).

Enteric fever is an increasingly common diagnosis in returning travelers in the UK. In a retrospective, descriptive study of culture-confirmed cases of enteric fever admitted to University Hospitals Leicester, UK between January 1999 and April 2009, 100 cases of enteric fever were identified in adults (n = 76) and children (n = 24). The median age of adult subjects was 38 (range 18-71) and 55% were male (Tristan, 2010).
2.6.2 Typhoid Fever Asian Scenario

Asia reports high incidences (>100/100,000 cases/year) of typhoid, accounting for almost 80% of world’s population (Puran, 2009). It is estimated that in Asia, the crude annual typhoid incidence rate was 274 per 100,000 persons in 2000 (Crump, 2004). Epidemic typhoid is a common disease in most of Africa, Asia and central South America. In the developing countries, usually the modern parts of the cities and towns are almost free of typhoid, general hospitals derive a large majority of their typhoid patients from slums, peripheral shabby towns, and rural districts where sanitation and the water supply are rudimentary. It is prevalent in developing countries where it remains a major health problem, (Trujillo et al., 1991; Arora et al., 1992) and exists both in endemic and epidemic forms (Saha, 2003).

2.6.3 Indian Scenario of Typhoid

A retrospective, hospital based study at Safdarjang hospital, India was undertaken between January 1999 and December 2003 to estimate age related epidemiological, clinical and microbiological characteristics in enteric fever cases, which showed that more than 24% of cases were in children upto 5 years of age (Walia et al., 2006). Typhoid is the 5th most common communicable disease in India. It is a major cause of absenteeism in schools and workplaces. Children constitute about 69% of hospitalized typhoid victims in India. Even sophisticated drugs are proving to be ineffective against resistant strains of typhoid bacteria.

In India, the disease is endemic in almost all parts of the country with periodic outbreaks, of water borne or food borne diseases. In 1992, about
3,52,980 cases with 735 deaths were reported. The number was 3,57,452 cases and 888 deaths in 1993, whereas in 1994, it declined to 2,78,451 cases and 304 deaths (Singh, 2001). Hospital-based studies and outbreak reports from India indicate that enteric fever is a major public health problem in this country, with *Salmonella Typhi* (*S. Typhi*) the most common etiologic agent but with an apparently increasing number of cases due to *S. paratyphi A* (Kanungo, 2008).

### 2.7 Typhoid Carriers

Convalescent and chronic carriers are the major cause of transmission of *Salmonella Typhi*. Convalescent carriers are patients who have recovered from the clinically detectable disease but continue to excrete *Salmonella Typhi* in stools or urine for a limited period to several weeks. Excretion ceases after that, but during the excretory phase in convalescence, the situation is potentially dangerous to others since the water supply or food and drink may readily be contaminated and thus spread the infection. Proper medical management of every case of typhoid therefore, includes keeping the patients in hospital or in isolation until or at least three consecutive specimens of stool and urine is negative for *Salmonella Typhi* after the full regimen of drug therapy has been completed.

The chronic carrier is quite different from the convalescent carrier. A chronic carrier state can occur following symptomatic or subclinical infections of *Salmonella Typhi*. Transmission may occur by ingestion of food or water contaminated by the urine or feces of acute cases or carriers. Among untreated cases, 10% will shed bacteria for three months after initial onset of symptoms and 2-5% will become chronic carriers. The chronic
carrier state occurs most commonly among middle-aged women (Acute Communicable Disease Control, 2009).

2.8 Symptoms of Typhoid

Following infection through ingestion, an asymptomatic period follows that usually lasts 7-14 days. The onset of bacteremia is marked by fever and malaise. Patients typically present, after the onset of fever, with influenza like symptoms with chills (although rigors are rare), a dull frontal headache, malaise, anorexia, and nausea, but with few physical signs. Hepatomegaly and splenomegaly may exist. Relative bradycardia is considered common in typhoid fever, although it is not specific for it. Rose spots, blanching erythematous maculopapular lesions usually 2-4 mm in diameter- are reported in 5-30% of cases, and usually occur on the abdomen and chest. Fever often occurs in a stepwise fashion with 5-7 days of daily increments in maximal temperature of 0.5-1°C. More serious complications e.g. gastrointestinal bleeding, intestinal perforation and typhoid encephalopathy may occur in 10-15% of typhoid patients in endemic countries (Connor, 2005; Gordon, 2008).

2.9 Pathogenicity of *Salmonella Typhi*

Typhoid fever starts as an infection of the gastrointestinal tract and develops into a systemic illness. Only 8-10 organisms/ml can cause severe disease. The bacteria find their way to food, drinks and water through house-flies and other insects. These contaminated food or drinks, when consumed, causes typhoid fever. The bacteria are disseminated by typhoid
patients and carriers in large quantities through stools and vomit. Almost all *Salmonella Typhi* are transmitted through oral ingestion of bacteria. The infective dose of bacteria really depends on the host defenses such as gastric acidity, the inhibitory effects of the normal intestinal flora and the gut peristaltic movements.

About $10^5$ to $10^6$ *S. Typhi* bacteria are required for initial infection. Following ingestion, *Salmonella Typhi* penetrates the intestinal mucous layer at the distal ileum of the small intestine and the proximal large bowel and become localized in the Peyer’s patches. M cells, which are specialized ileal epithelial cells lying at the Peyer’s patches, are thought to be the site for penetration of *S. Typhi*. Following penetration of the mucosa the organisms replicate within the macrophages of the Peyer’s patches and spread to the mesenteric lymph nodes. In the case of gastroenteritis, the bacteria do not further penetrate the gut and the gut-associated lymphoid tissue. However, it is common for *S. Typhi* to penetrate, and the bacteria may spend four to seven days spreading via thoracic duct to systemic circulation (transient primary bacteremia).

The bacteria are removed from blood by macrophages that are located in the sinusoids of the liver, spleen and bone marrow and the bacteria can replicate again and re-enter the blood (second bacteremia). The bacteria removed by the liver may infect the gall bladder, which, in turn, may lead to re-infection of the intestinal tract (second exposure of Peyer’s patches) and cause inflammation, ulceration and necrosis. However, the common causes of death in typhoid fever are peritonitis and septicemia that occur in the third week of illness, or the perforation of Peyer’s patches (Everest *et al.*, 2001).
Figure 1: Shows the schematic diagram of the first and second exposure of Peyer’s patches to *S. Typhi* in the intestine


*Salmonella Typhi* continues to cause severe disease. Its most learned complication being perforation of ulcerated peyer’s patches within the small intestine, leading to peritonitis with associated mortality. The pathogenesis of this process is not well understood (Everest *et al.*, 2001).

### 2.10 Antigenic characteristics

*Salmonellae* have 3 important antigens – 1. Somatic O antigen, 2. Flagellar H antigen and 3. Vi surface antigen (Figure : 2). The O antigen which is also called as Boivin antigen, this antigen is a phosphor lipid protein polysaccharide complex which forms integral part of the cell wall.

The antigen present in flagella is a heat labile protein. The H antigen is strongly immunogenic and induces antibody formation rapidly. In many
strains of genus *Salmonella Typhi*, the production of flagellar antigen is diphasic, each strain varying spontaneously and reversibly between two phases with different sets of H antigens. In phase 1 (the specific phase), different antigens were designated as Z1, Z2, Z3, etc. In phase 2 (the group phase), the antigens first discovered were given Arabic numerals, but later, certain phase-I antigens, especially e, n, x and z were also found to be present in the phase 2 of some strains. The Vi antigens are poorly immunogenic and only low titres of antibody produced following infection. Felix and Pitt (1934) discovered that the Vi antigen plays some role in the virulence of this bacillus (Paniker, 2005). Another antigen (D) has also been used previously for the detection of *S. Typhi* using ELISA (Rockhill *et al.*, 1980).

Savage (1956) reported that large numbers of organisms are required to cause disease in human. Clemmer *et al* (1960) claimed that the portal of entry could also affect the number of organisms required to produce the disease. When organisms were administered in aerosols to chimpanzees and chickens, substantially lower doses of the organism caused illness as compared to larger number of organisms when orally ingested. Similar findings were reported in another epidemiological survey (Crozier and Woodward, 1962).

### 2.10.1 Vi – Agglutination:

Many known carriers of typhoid bacilli possess antibody against the Vi (virulence) antigen of *S. Typhi*. This is a surface antigen easily lost during culture. (Vi titres seem tocorrelate better with the carrier state than do O or H titres). For this reason, Felix *et al.* suggested the use of Vi agglutination for detection of carriers. An elevated Vi titer may be indicative of the typhoid carrier state; the titer usually disappears within a few weeks after the termination of the carrier state.
2.11 Identification of *Salmonella Typhi*:

Most *Salmonella* serotypes cannot be distinguished by biochemical reactions except *S. Typhi*. *S. Typhi* does possess some unique biochemical characteristics that allow the differentiation. In TSI the organism produce only trace amounts of hydrogen sulfide which is usually observed as a crescent shaped wedge of black precipitate forming at the interface of the slant and butt in KIA or TSI media (Parry, 2005). In addition, *S. Typhi* is less active biochemically than the more common serotypes and is specifically negative for the following reactions: Simmon’s citrate, ornithine decarboxylase, gas from glucose, fermentation of dulcitol, rhamnose and mucate and acetate utilization.

2.12 Typing Methods

2.12.1 Phenotypic Typing Methods

2.12.1.1 Conventional methods of microbe typing

Due to growing awareness of different factors that influence the spread of human and animal pathogens in various environments, conventional typing methods for microorganisms were developed. In conventional methods, the major biological typing techniques are phage typing, serotyping, bacteriocin typing and typing with antibiotic susceptibility pattern. These methods have been used in wide range of microorganisms (Tsen, 2002).
2.12.1.2 Antibiotic Resistance

The advent of antibiotic treatment has led to a change in the presentation of typhoid, and the classic mode of presentation with a slow step ladder rise in fever and toxicity is rarely seen. However, rising antibiotic resistance has been associated with increased severity of illness and related complications.

A study was undertaken to compare the changing trends of antibiogram of *Salmonella enterica* serovar *Typhi* and *Salmonella enterica* serovar *paratyphi A* isolates. A total of 80 isolates of *Salmonella* were obtained from blood cultures during 2001-2004. Identification and antibiotic sensitivity of the isolates were performed by using mini API (Bio Merieux, France). Sixty isolates were identified as *Salmonella enterica* serovar *Typhi* and 20 were identified as *Salmonella enterica* serovar *paratyphi A*. More than 67% of S. Typhi and 80% of S. paratyphi A isolates were sensitive to chloramphenicol. Sensitivity of S. Typhi isolates to cephalosporins was found to have increased from 2001-2004 while that of S. paratyphi A showed a decline. With increasing resistance to ciprofloxacin and the possibility of re-emergence of sensitivity to chloramphenicol, the policy of empirical treatment of enteric fever needs to be rationalized (Lakshmi et al., 2006).

A total of 464 *Salmonella Typhi* were isolated from blood of patients suspected with enteric fever in Calcutta school of medicine from 1991-2003. All the 464 isolates were susceptible to Amikacin and Gentamycin. Both antibiotics showed bactericidal activity at concentrations of 2 µg/ml respectively after incubation for 6 hrs. These drugs were administrated for the treatment for typhoid fever (Mandal, 2009). Molecular characterization
of *Salmonella Typhi* with full resistance to ciprofloxacin and particularly the presence of plasmid borne integron in ciprofloxacin resistant *Salmonella Typhi*, which will lead to a situation of untreatable enteric fever was first reported by Gaind *et al.*, in 2006.

In another work, all the *Salmonella Typhi* isolates were less sensitive to ciprofloxacin but no resistance was seen, whereas 76% of the same isolates showed resistant to nalidixic acid. In this work, it was shown that nalidixic acid resistant isolates had decreased susceptibility to ciprofloxacin (Khan, 2007). Similar work was carried out by Malini *et al.*, (2007) which reveals same pattern of result as Khan *et al.* (2007).

Changing trends of antibiogram of *S. Typhi* and *S. paratyphi A* says that 67% of *S. Typhi* in the study were sensitive to chloramphenicol and the sensitivity of *S. Typhi* isolates to cephalosporin was found to have increased from 2001-2004 (Lakshmi *et al.*, 2006). Resistance to amoxicillin, chloramphenicol, ampicillin and co-trimoxazole were significantly high in a study conducted at Kasturba Medical College (Chowta, 2005). Plasmid mediated multi-drug resistance to ampicillin, chloramphenicol and cotrimoxazole has been described in various parts of Asia (Bhutta, 2006).

### 2.12.1.3 Sugar Fermentation

Phenotypic and genotypic characteristics of 30 strains of *Salmonella Typhi* isolated in different years, from some areas in Brazil were studied. Conventional typing methods were performed by biochemical tests, Vi phage-typing scheme, and antimicrobial susceptibility test. Molecular typing methods were performed by analysis of plasmid DNA and by Random Amplified Polymorphic DNA (RAPD-PCR). For the latter, an
optimization step was performed to ensure the reproducibility of the process in genetic characterization of *S. Typhi*. The predominance of 76.7% of Biotype I (xylose +, arabinose -) was noticed in all studied areas (Quintaes, 2002).

2.12.2 Molecular Typing Methods:

2.12.2.1 Random Amplified Polymorphic DNA (RAPD)

This method of DNA polymorphism analysis was developed independently by two different laboratories (Welsh and McClelland, 1990; Williams *et al.*, 1990). This procedure detects nucleotide sequence polymorphisms in a DNA amplification based assay using only a single primer of arbitrary nucleotide sequence using Polymerase Chain Reaction (PCR). In this reaction, a single primer binds to the genomic DNA at two different sites on opposite strands of the DNA template; if these primary sites are within an amplifiable distance for thermocyclic amplification a band would be obtained. The presence of each amplification product identifies complete or partial nucleotide sequence homology, between the genomic DNA and the oligonucleotide primer at each end of the amplified product. On an average, each primer will direct the amplification of several discrete loci in the genome, making the assay an efficient way to screen for nucleotide sequence polymorphism between individuals.

The major advantage of this assay over RFLP method is that there is no prior requirement for DNA sequence information of the genome to be analyzed. The protocol is also relatively quick and easy to perform and uses fluorescence *in lieu* of radioactivity (Williams *et al.*, 1992). Because the RAPD technique is amplification based assay, only nanogram quantity of
DNA are required and automation is feasible. This technique can be used to determine taxonomic identity, assess kinship relationships, detect interspecific gene flow, analyze hybrid speciation, and create specific probes. Advantages of RAPD include suitability for work on anonymous genomes, applicability to work where limited DNA is available, efficiency and low expense (Hadrys et al., 1992). It is also useful in distinguishing individual strains (Karp et al., 1996).

High throughput epidemiological typing systems that provide phylogenetic and genotypic information are beneficial for tracking bacterial pathogens in the field. The study provided insight into the evolution of serovar Typhi and demonstrates the value of a molecular epidemiological technique that is exchangeable (Baker, 2008). Detailed strain identification is essential for useful investigations of Salmonella Typhi outbreaks. Epidemiological investigations have traditionally relied on biochemical and serological methods for the primary identification of strains. Recognition of a number of phenotypic properties has been used for their subsequent differentiation. In contrast, modern typing methods are based on characterization of the genotype of the organism by analysis of plasmid and chromosomal DNA (Threlfall, 1994).

In a study RAPD, ERIC-Enterobacterial Repetitive Intergenic Consensus fingerprinting by Ribotyping PCR and SSCP were used to differentiate 57 strains of Salmonella Typhi. It showed that a combination of RAPD and ERIC was better than any other combinations (Lim, 2005). Rapid genotyping methods like RAPD and Ribotyping PCR were used to characterize Salmonella Typhi enteritidis causing food borne outbreaks. Antimicrobial resistance analysis was also done, 62.1% strains were
susceptible to all drugs tested and RAPD analysis gave 4 different bands which helped in identification (Fernanda, 2007).

To discriminate 62 strains of *Salmonella Typhi*, RAPD was used and 5 primers were used in the work, which revealed 21 RAPD types. The DNA of various strains of *Salmonella Typhi* from this simple extraction procedure could be discriminated within a few hrs using the RAPD technique (Shangkuan *et al*, 1998). Culture methods, biochemical methods, serological and molecular methods were used in characterization of 18 *Salmonella Typhi* isolates from human and food source. Dendrogram was constructed using the data obtained, which showed clear discrimination between human and non-human isolates by forming clusters except two isolates (Albufera, 2009). To elucidate the clonal relationship among *S. typhimurium*, *S.Typhi* and *enteritidis* techniques, like phage typing, antibiogram susceptibility study and RAPD were used. High genetic diversity was observed between *S. Typhi* and *S. typhimurium* (Tsen, 2002).

### 2.13 Diagnosis of Typhoid Fever:

#### 2.13.1 Culturing in plates

The diagnosis of typhoid means the detection of the etiological agent, which is possible, if the organism can be isolated by cultivation in bacteriological media. The organism, *Salmonella Typhi*, can be isolated from blood and the other important possible sources of obtaining organism are stool, urine and bone marrow. MacConkey agar, *Salmonella Shigella* agar, XLD agar and Wilson Blair bismuth sulphate agar can be used for the cultivation of the organism. These media are selective and differential in which *Salmonella Typhi* grows luxuriously and other bacterial contamination will not grow in these media.
The main disadvantage of using the culture method for the diagnosis of organism from the sample is that, this method is time consuming. Minimum of 3 days is required for the isolation and identification of the organism. Rapid diagnosis is the immediate requirement for any infection. Typhoid fever may be difficult to distinguish clinically from other causes of febrile illness like malaria as the symptoms and signs overlap. Hence febrile patients tend to be treated simultaneously for malaria and typhoid fever (Martins et al., 2003).

A total of 80 patients at the University of Uyo Teaching Hospital (UUTH) suspected of having enteric infections were screened for the presence of *Salmonella* species using blood, urine and stool samples along with Widal agglutination tests. Although 39 patients tested positive for the Widal agglutination test with titers ranging from 1:80 to 1:320, no *Salmonella* organism was encountered in some cultures. Statistical analysis revealed significant differences ($\chi^2$) at the 5% probability level between the Widal test and the cultures of the clinical samples. The results suggest that serological investigations alone may not be a reliable index for the diagnosis of *Salmonella* infections (Itah, 2004).

### 2.13.2 Serological and Biochemical Methods Used for the Diagnosis of Typhoid

#### 2.13.2.1 Widal Agglutination Test

The Widal test is the important serological test used for the diagnosis of antibodies to *Salmonella Typhi* antigens. Widal agglutination test can be performed by either tube method or by slide method. A single test result is not diagnostically significant unless the titer is unusually high. In the
unvaccinated patient a serologic diagnosis is usually made if there is a 4-fold rise in O antigen titer.

2.13.2.2 Tube Agglutination Test

Agglutination reaction performed in tubes with different antigens (O, H, Vi) and their respective antibodies raised, showed variable nature of agglutination reactions. Somatic (O) antigen showed fine agglutination whereas, H and Vi showed coarse and very fine agglutination reactions respectively. Although the mainstay of diagnosing typhoid fever is a positive blood culture, the test is positive in only 40-60% of cases, usually early in the course of the disease. Stool and urine cultures become positive after the first week of infection. In much of the developing world, widespread antibiotic availability and indiscriminate prescribing is another reason for the low sensitivity of blood cultures. Although bone marrow cultures are more sensitive, they are difficult to obtain, relatively invasive, and of little use in public health settings.

Other haematological investigations are non-specific. Blood leukocyte counts are often low in relation to the fever and toxicity, but the range is wide. In younger children leucocytosis is a common association and may reach 20000-25000/mm$^3$. Thrombocytopenia may be a marker of severe illness and accompany disseminated intravascular coagulation. Liver function test results may be imbalanced but significant hepatic dysfunction is rare. The value of the *Salmonella Typhi* agglutination tests has declined as the incidence of typhoid fever has decreased, atleast in the West, the general use of vaccines has increased, and ever increasing numbers of antigenically related serotypes of *Salmonella Typhi* have been recognized.
Widal test is often used when isolation of *Salmonella Typhi* is not feasible, but produces false negative and false positive reactions and does not provide a definitive diagnosis.

The Widal test, which detects agglutinating antibodies to lipopolysaccharide (LPS) (TO) and flagella (TH), was introduced over a century ago and is widely used for the serological diagnosis of typhoid fever. In the original format, the Widal test required acute and convalescent phase serum samples taken approximately 10 days apart. Most recently, the test has been adapted for use with a single, acute phase serum sample. ELISA has been considered an alternative approach for the diagnosis of typhoid fever. For the most part, these assays have been based on the detection of anti-LPS antibodies and have been reported to be more sensitive than the Widal test.

Newer diagnostic tests have been developed such as the typhidot or tubex, which directly detect IgM antibodies against a host of specific *S. Typhi* antigens but these have not proved to be sufficiently robust in large scale evaluations in community settings. A nested polymerase chain reaction using H1-d primers has been used to amplify specific genes of *S. Typhi* in the blood of patients and is a promising means of making a rapid diagnosis. Table 2 showed the comparison of the performance of the various tests for typhoid (Bhatta, 2006).

### 2.13.2.3 Isolation of *Salmonella Typhi* in Urine Sample:

*Salmonella Typhi* can be detected by its antigens present over the surface of the bacteria and by the flagellar H antigen and Vi antigen. A study in Jakarta showed that detection of *Salmonella Typhi* using groupD9
antigen from urine (Chaicumpa, 1992). Since typhoid fever is still a major cause of illness in many developing countries, there is a clear need for a sensitive and specific test that will permit rapid laboratory diagnosis of the disease. A study was carried out both in laboratory and in a clinical situation, for its ability to detect Vi antigen in urine. The work showed that ELISA was capable of detecting as little as 1 ng of purified Vi antigen per ml in urine, compared with 100 ng/ml detectable by co-agglutination method (Barrett, 1982).

Another study using Vi antigen in diagnosis of typhoid fever, concluded that using a cut-off value that maximally separated typhoid patients from controls, the ELISA was positive in 62.4% of 141 patients with culture proven typhoid infections and in 13.2% of 159 afebrile control subjects (Taylor, 1983).

Monovalent antisera coupled to protein A rich staphylococci used in slide coagglutination method for the detection of *Salmonella Typhi* D, Vi and d antigens in the urine. These antigens were detected in the urine of 59 out of 61 (97%) bacteriologically confirmed typhoid fever. The results suggest that the method of slide coagglutination of urine can be used to screen suspected patient with high degree of reliability (Rockhill, 1980).

In any infection or disease rapid diagnosis and cost are the main concern. Advance immunological kits are available in rapid diagnosis. While evaluating 3 immunological kits for the serological diagnosis of typhoid fever in Vietnam it was found that sensitivity and specificity findings were 89 and 53% for multi test dip stick, 79 and 89% for Typhidot, 78 and 89% for TUBEX and 64 and 76% for Widal testing in hospitals and 61% and 100% for Widal testing at the Pasteur institute. The Widal test was
insensitive and displayed inter-operator variability. Two rapid kits Typhidot and TUBEX demonstrated promising results (Olsen, 2004).

### 2.14 Avian Yolk Immunoglobulins (IgY)

The eggs of immunized chickens form an economical and abundant source of polyclonal antibody. Immunoassays are rapidly becoming important for the detection and quantification of various components. For the purpose of analysis, the common sources of antibodies are the blood of the immunized animals. Yolk antibodies are also used for detection and quantification of antigens. Yet yolk is a good potential source of specific antibodies and it contains only one known class of immunoglobulin (IgY) (Lesslie et al., 1969).

Immunoglobulins are glycoprotein, which play a vital role in protection against diseases. The protective effect is due to the specific binding of the antibodies with foreign antigens.

It is advantageous over conventional antibody production. The advantages are, capable of producing most specific antibodies against mammalian antigens in birds compared to mammals, because of the phylogenetic distance between birds and mammals (Jensenius et al., 1981); lower cost and convenience (Polson et al., 1980); compatibility with modern animal protection regulations and production and maintenance of higher levels of specific antibodies is relatively easier (Orlans, 1967; Rose et al., 1974).

So, avian egg yolk is considered as a cheaper alternative source of immunoglobulins during the past decade. IgY does not react with anti-mammalian immunoglobulins such as rheumatoid factors, anti-human IgG in
human serum and can reduce non-specific background reactions due to cross reactions. Egg yolks from immunized chickens have been recognized as an excellent source of polyclonal antibodies. Polyclonal IgY is far more superior to polyclonal IgG since, IgY does not bind to human complement thus minimizing interference in human assay systems (Yogeeswaran et al., 1999). Avian yolk antibodies are utilized as immunological supplements in infant formulae (Facon et al., 1993).

IgY is the only antibody, with low molecular weight that is present in chick’s egg yolk in the concentration of 5 to 20 mg per ml. The isoelectric point ranges from pH (5.7 to 7.6). The term IgY was proposed by Leslie et al. in 1969. IgY is also called as Chicken IgY, egg yolk IgG, 7SIgG. Structurally, avian egg yolk immunoglobulin is identical to the major immunoglobulin found in the serum of mammals but it differs from the mammalian IgG in the hinge region of aminoacids of immunoglobulin. The term IgY was originally coined to refer the large molecular mass immunoglobulin found in the avian egg yolk which is now accepted as the major antibody in avian.

In the present study, the antibodies were produced against the three antigens of *S. Typhi* and the ability of these antibodies to detect the presence of the pathogen in the urine of carriers and convalescents was being tested. The urine of carriers and convalescents carry antigens which can be detected using specific antibodies and the detection of this antigen in urine is a non-invasive method.
2.15 Counter Immuno Electrophoresis

*Salmonella Typhi* antigen and antibody were detected simultaneously in the serum samples by counter immuno electrophoresis (CIE). The conventional Widal test was performed to elicit *S. Typhi* H and O agglutination.

Counter-current immuno-electrophoresis was carried out in a study, where, test for the serodiagnosis of typhoid fever with somatic (O), flagellar (H) and capsular polysaccharide (Vi) antigens of *Salmonella Typhi* on the sera of patients who were blood culture positive (confirmed typhoid cases) or had high Widal agglutination titres, 1:320, (presumptive typhoid cases). Of the 37 sera from confirmed cases, 30% showed positivity with O antigen, 24% with H antigens and 51% with Vi antigen.

In patients with a presumptive diagnosis, 45% were positive for O antibody, 27% for flagellar antibody and 52% for Vi antibody. When all three antigens were combined the reactivity to any of the antigens was found to be 59% in confirmed typhoid cases, 79% in presumptive typhoid cases and 93% in patients who were simultaneously positive by blood culture and Widal agglutination. However, none of the sera from 45 controls gave a positive precipitation reaction with any of the antigens. It is concluded that countercurrent immuno-electrophoresis is a rapid test with low sensitivity and high specificity with Vi antigen, a panel of antigens being most effective (Sharma, 1997).

Chicken egg yolk (IgY) raised against *Salmonella Typhi*, *S. enteritidis* or *S. typhimurium* was found in highly specific activity levels by ELISA. *S. enteritidis* and *S. typhimurium* specific IgY powder, prepared by freeze drying the egg yolk water soluble fraction. *Salmonella Typhi* specific IgY
was demonstrated to inhibit *Salmonella Typhi* growth in liquid medium. The growth rate of the above said organisms were 4 fold less comparing control when incubated for 6 hours. The specific binding activity of IgY was further evaluated by using immunoflorescence and immunoelectron microscopy (Lee, 2002).

### 2.16 Dot Blot

Clinical application of a dot blot test to detect immunoglobulin G (IgG) (88% sensitivity and specificity) and IgM (12.1% sensitivity and 97% specificity) against flagellar antigen from *Salmonella Typhi* was performed in Peruvian and Colombian patients with typhoid fever. This test can be used as a ground predictor of *Salmonella Typhi* infection (Cardona, 2000).

Solid phase immunoenzymatic test, dot blot test were used for the diagnosis of typhoid, using lipopolysaccharide, crude and flagellar antigens and their respective IgG and IgM and compared with Widal. The best diagnosis efficiency was obtained by detecting IgG against a flagellar antigen by dot blot, giving a sensitivity and specificity of 88%; widal and ELISA tests showed lower diagnostic efficiencies (Cardona, 1998).

The diagnostic sensitivity and specificity of two dot-enzyme-linked immunoassays (Typhidot and Typhidot-Mt; Malaysian Bio diagnostic Research SDN BHD, Kuala Lumpur, Malaysia) was evaluated, assessing IgG and IgM antibodies against the outer membrane protein (OMP) of *Salmonella Typhi*, and the Widal test in comparison with blood culture in a consecutive group of children with suspected typhoid fever. Of 97 children with suspected typhoid fever, the disease was confirmed bacteriologically in 46 (47%), whereas 25 (26%) were considered to have typhoid fever on
clinical grounds. An alternative diagnosis was made in 26 (27%). The Typhi dot and Typhi dot-Mt were superior to the Widal test in their diagnostic sensitivity and specificity, although values (sensitivity 5 85–94% and specificity 5 77–89%) were significantly lower than in other regional reports (Bhutta, 1999).

2.17 FTIR
2.17.1 Analyzing *Salmonella Typhi* by Fourier Transform Infrared Spectroscopy

FTIR spectroscopy has been demonstrated to be a highly sensitive and reproducible method for microbial analysis and process control (Amiali, 2007)

The identification of the disease typhoid is caused by *Salmonella Typhi* and its counter parts like *Salmonella paratyphi A, B, C*, by classical methods of Widal test; ELISA is still in use widely and are considered as standard means for diagnosis. The use of other analytical instruments for this identification is less explored. The study aimed at finding a prospect for the disease identification process. This becomes novel as the classical standard methods proves undependable in identification of different strains of *Salmonella spp* and are time consuming (Goodacre et al., 1998). The analytical instrument which showed some promise was Fourier Transform Infrared Spectroscopy (FTIR).

FT-IR allows the chemically based discrimination of intact microbial cells, without their destruction, and produces complex biochemical fingerprints which are reproducible and distinct for different bacteria and fungi (Timmins et al., 1998). Earlier studies involving FTIR were used to
identify the agents in any of the chemical preparations and food spoilage identification (Whittakera et al., 2003). The ability of the instrument to identify the nanogram quantities (Mandal et al., 2011) of chemicals or contaminants in the given sample remained the reason for using it in the species identification process.

The infection process for *Salmonella Typhi* is via blood and thus the serum samples of the diseased patients were used for the study. The sodium chloride cells were smeared with the serum of different concentrations. The key markers taken for analysis were in lipid, protein, glycoprotein and nucleic acids. These are also present in the serums of normal healthy people but their increase in concentration levels will be seen only in patients with typhoid. The major contributor for the increase is the lipopolysaccharide in cell wall of the bacteria, which contains repeating units of $\alpha$-d-galactosyl-(1 → 2)$\alpha$-d-mannosyl-(1 → 4)-l-rhamnosyl-(1 → 3) and has short branches of single 3,6-dideoxyhexose residues (Kita and Nikaido, 1973). The detectable quantities of the sugar moieties were taken into consideration for their identification in the spectra.

In the literatures it has been reported that the infection caused by typhoid increases thrombomodulin (Ohnishi, 1998). Thrombomodulin is a glycoprotein that can bind to thrombin and activate protein C, thus mitigating the effects of cytokines produced by inflammatory and immunological processes. The molecule exerts a protective function on endothelial cells. Thrombomodulin is cleaved to its soluble form by neutrophil elastase and by other substances produced during acute and chronic inflammatory responses, immunologic reactions and complement activation. ELISA technique yields normal serum levels of $3.1 \pm 1.3\text{ng/ml}$; in males these levels are higher; TM levels also rise during menopause.
Other circumstances associated with an increase of serum TM levels are smoking, disseminated intravascular coagulation (DIC), cardiac surgery, atherosclerosis, ARDS, liver cirrhosis, diabetes mellitus, cerebral and myocardial infarction, and multiple sclerosis. Serum levels of TM represent a useful prognostic index, because they are associated with an increase in mortality rate, or however a progression of the underlying pathological condition (Califano et al., 2000). Thus the glycoprotein thrombomodulin levels became important in the analysis.

One other contributor in the immune modulatory processes was Lipid A of *Escherichia coli* and *Salmonella minnesota* belonging to an important class of immunomodulating molecules represented by bacterial lipopolysaccharides which are the integral constituents of the outer membrane of gram-negative bacteria also found in *Enterobacteriaceae, Neisseriacea*, and *Chlamydiaceae* (Brandenburg et al., 1998). The bulk of Lipid A in the LPS will also give an alternate perspective for the study if its identification was positive.

Fourier-transform infrared spectroscopy (FTIR) provides a biochemical absorption spectrum due to vibrational modes of functional groups in cell components (i.e., protein, nucleic acids, carbohydrates and lipids) or complex skeletal group vibrations that are reproducible and distinct for different bacteria (Helm et al., 1991; Helm and Naumann, 1995; Naumann et al., 1996). Limited information is available on the use of FTIR for the differentiation and identification of *S.enterica* serovars, and previous research has been done using only cells grown in non-selective plating media (Baldauf et al., 2006) or in broth (Kim et al., 2005; Kim et al., 2006; Baldauf, 2007).
FT-IR spectroscopy measures vibrations of functional groups and highly polar bonds such as O-H stretches. Thus, these “fingerprints” are made up of the vibrational features of all the cell components, i.e., DNA, RNA, proteins, and membrane and cell-wall components. FT-IR allows the chemically based discrimination of intact microbial cells, without their destruction, and produces complex biochemical fingerprints which are reproducible and distinct for different bacteria and fungi. Naumann and coworkers have shown that FT-IR absorbance spectroscopy (in the mid-IR range, usually defined as 4,000 to 400 cm⁻¹) provides a powerful tool with sufficient resolving power to distinguish microbes at the strain level (Timmins, 1998)

2.18 In Vitro Bioactivity of Plant Extracts on Salmonella Typhi

Swietenia mahagoni (Linn.) is a large, deciduous, and economically important timber tree which is native to the West Indies which is commonly known as “Mahogani” which has 146 genera and about 1500 species. Mahogany can reach 75 feats in height with a 50 foot spread but is more often seen at 40 to 50 feet tall and wide. The leaves are semi evergreen, alternative, pinnate, 12-25 centimeters long, with four to eight leaflets. Flowers are unisexual and trees monoecious. This tree is mainly cultivated at tropical zones, such as India, Malaysia and China (Figure. 4) (Mulholland et al., 2000).
2.18.1 Scientific Name - Swietenia mahagoni

Classification - Based upon – Bentham and Hooker (1862)
- Division: Phanerogams
- Class: Dicotyledones
- Sub class: Polypetalae
- Series: Disciflorae
- Order: Geraniales
- Family: Meliaceae
- Genus: Swietenia
- Species: mahagoni

2.18.2 The genus Swietenia

This genus is found only in the neotropics and consists of three species: Swietenia mahagoni Jacq., Swietenia macrophylla King, and Swietenia humilis Zucc.; and two natural hybrids. One of these is a product of a cross between S. macrophylla and S. humilis this is found in the areas of the distribution range in which the two species overlap; the other is a cross between S. macrophylla and S. mahagoni, named S. xaubrevilleana which has been found close to plantations of the two species.

2.18.3 Traditional Usage of Swietenia Mahagoni

Biological Activity of Swietenia

Compounds isolated from Swietenia mahagoni cotyledons swietemahonin-A, Swietemahonin-E, 3-O-acetylswietenolide and 6-O-acetylswietenolide belongs to the class of tetranortriterpenoids exhibiting antagonistic effect on platelet activating factor (PAF) (Ekimoto et al., 1991).
Methylene chloride and methanol extracts of 20 Indonesian plants with ethno medical uses have been assessed for in vitro antibacterial and antifungal properties by disk diffusion method. Extracts of the six plants: *Terminalia catappa*, *Swietenia mahagoni* Jacq., *Phyllanthus acuminatus*, *Ipomoea* spp., *Tylophora asthmatica* and *Hyptis brevipes* demonstrated high activity in this bioassay system (Goun, 2003).

**2.19 Bioautography**

Phytochemical and antimicrobial activity of *Swietenia mahagoni* crude methanolic seed extract was studied. The crude methanolic extract was used for various photochemical analysis and the antimicrobial activity against gram positive, gram negative, fungi and yeast was evaluated based on disc diffusion assay, minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC). The extract showed activity against *S. aureus*, *P. aeruginosa*, *Candida*, and *Proteus mirabilis*- MIC ranging from 25 mg/ml to 50 mg/ml (Ramanathan et al., 2009).

Different nupe plants were used to study their efficacy against *S. Typhi* in Nigeria showed good results when used in higher concentration (Ewans, 2002). Cytotoxic properties of the crude ethanolic extracts of seed, bark and leaf of *Swietenia mahagoni* and their various fractions were studied using brine shrimp lethality bioassay. The LC$_{50}$ of the leaf extract of *S. mahagoni* was found to be 13.18 µg/ml (Akbar, 2009).

*Swietenia mahagoni* extracts were used to check MIC, MBC and antibacterial activity study against Methicillin resistant *Staphylococcus aureus*. The extract showed potential activity against MRSA (Alusi, 2010). Antimicrobial efficacy of methanol and water extracts of seeds of the plant
Swietenia macrophylla was evaluated against various gram positive and gram negative bacteria and fungi. It was reported that the methanol extract showed better activity than the water extract (Maiti, 2007).

Brine shrimp is one among the vital model to check the Cytolytic activity of natural compounds and toxic substances. In a study of brine shrimp lethality activity for various plants belonging to the Meliaceae family like Azadirachta indica, Azedirachta indica var siamensis, Melia azedarach, Sandoricum indicum and Swietenia macrophylla, stem, bark and leaf extracts of the plants were used for the study. The result showed that the leaf and seed of Annona squamosa exhibited high potency than the stem, bark (Pisthutonon, 2004).
3. AIMS AND OBJECTIVES OF THE STUDY

1. To carry out typing of *Salmonella Typhi* based on biochemical reactions and antibiogram
2. To study the molecular pattern of clinical isolates of *Salmonella Typhi*.
3. To evaluate a simple method for antigen detection in urine of patients with enteric fever
4. *In vitro* bioactivity of plant extracts on *Salmonella Typhi*
4.0 MATERIALS AND METHODS

4.1 Sample Collection and Processing

A total of 194 isolates were collected during the period June 2003 to August 2009 from samples such as blood, urine and stool of patients attending the government hospital and Sri Ramachandra University, Chennai. The samples were processed in the respective microbiology laboratory and the positive *Salmonella Typhi* cultures were streaked on Brain heart infusion agar slants.

4.1.1 Laboratory Confirmation of Isolates

The *Salmonella Typhi* isolates obtained from laboratories in slants were again confirmed in SRU biotechnology by subculture onto MacConkey agar, SS agar and BHI agar and by a series of biochemical tests, as per the recommendations of Murray *et al.* (2003), and by agglutination with antisera.

4.2 DNA Isolation

Genomic DNA was isolated from all the isolates and used for RAPD. The confirmed isolates were stored in BHI agar slants at 4°C with proper packing and labeling.
4.3 Stock Maintenance

Glycerol stock of the isolates were maintained by inoculating a single colony in BHI broth and incubated till the OD reached 0.6. Then 500 µl of this culture was taken in a sterile eppendorf tube and to this 500 µl of sterile glycerol was added, mixed and stored at -20°C.

4.4 Standard Isolates

Throughout the study as a control standard ATCC cultures were used. The cultures were,

*Salmonella Typhi* ATCC 6539
*Staphylococcus aureus* ATCC 25923
*E. coli* ATCC 25922

4.5 Laboratory identification of *Salmonella Typhi*

4.5.1 Microscopic Examination with Gram’s staining

Gram’s staining was done with single colony grown in BHI agar. A drop of saline was placed over grease free slide and half colony was emulsified in the saline and thin smear was prepared. The thin film was air dried and heat fixed. The smear was flooded with crystal violet stain for 1 minute and washed, then stained with iodine and after a minute washed in running water and decolourizer was flooded and kept for half minute and finally one minute with safranin and washed with running water. Then the smear was air dried and focused under oil immersion objective to observe the morphology.