1.0 Introduction

Typhoid fever is caused by *Salmonella typhi*, a gram negative bacterium and was a major endemic threat across Africa, Asia, Middle East and few southern and European countries during the early centuries. Its widespread distribution drew attention of the scientific community towards preventing typhoid fever. The name ‘Typhoid’ was first used by Louis Dublin in 1829. The disease was later referred to as gastric fever, nervous fever, pathogenic fever, abdominal fever and as infantile remittent fever. The bacterium *S. typhi* was first isolated by Karl J. Erberth in 1880. The word ‘*Salmonella*’ is actually named in honour of Daniel E Salmon, an American veterinarian, in 1885. This bacterium can be transmitted by the ingestion of food or water contaminated with faeces from a person already infected with *S. typhi*. Around 16 to 33 million cases of typhoid fever are reported annually, resulting in 216,000 deaths, in endemic areas around the world.

Various studies have shown that the global burden of typhoid fever in different parts of the world varies. More than 100 cases in 100,000 populations per year reported in South Central Asia and South-East Asia, Asia, Africa, Latin America and the Caribbean are estimated to have medium incidence of typhoid fever, i.e., 10 to 100 cases in 100,000 populations per year, New Zealand, Australia and Europe have low to very low incidence (Crump *et al.*, 2004).
In India 3, 52,980 cases with 735 deaths, 2, 78,451 cases with 304 deaths, and 3, 57,452 cases with 888 deaths were reported in the years 1992, 1993 and 1994 respectively. Case fatality rate due to typhoid varies between 1.1% to 2.5% in last few years (Bir Singh, 2001). Refer Figure 1.1 Depicting incidence of typhoid fever. (http://en.wikipedia.org/wiki/Typhoid_fever).

**Figure 1.1 Incidence of typhoid fever**

- Strongly endemic
- Endemic
- Sporadic cases
1.1 Classification of *Salmonella*:

*Salmonella* belongs to the family of Enterobacteraeae that includes the genera *Shigella*, *Escherichia*, and *Vibrio*. The genus of *Salmonella* contains two different species, *S. enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*) containing 2443 serovars.

**Kingdom:** Eubacteria  
**Phylum:** Proteobacteria  
**Class:** Gammaproteobacteria  
**Order:** Enterobacterales  
**Family:** Enterobacteriaceae  
**Genus:** *Salmonella*  
**Species:** *S. enterica*  
**Subspecies:**

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

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. A serovar or a serotype can be defined as a strain that has a unique surface molecule which is responsible for the production of specific antibody. Each serotype has subtle chemical differences in their antigenic region (Brenner et al., 2000).

*Salmonella enterica typhi* (referred to as *S. typhi*), is a gram negative facultative rod-shaped bacterium. It is aerobic and motile and resides in the intestinal tracts of warm and cold-blooded animals. The cell wall of bacterium contain peptidoglycans in limited amounts, while the outermost layer of cell consists of lipopolysaccharide (LPS) with proteins attached by hydrophobic interactions in the presence of cations Ca$^{2+}$ and Mg$^{2+}$.

1.2 Antigenic structure:

As with all Enterobacteriaceae, the genus *Salmonella* has three kinds of major antigens with application for diagnosis or identification, namely somatic, surface, and flagellar antigens. Somatic antigens are heat-stable and alcohol-resistant. Cross-adsorption studies have identified a large number of antigenic factors which are used for serological identification. Surface antigens are commonly observed in other genera of enteric bacteria (*Escherichia coli* and *Klebsiella*), and may be found in some *Salmonella* serovars. Surface antigens in *Salmonella* may mask ‘O’ antigen, resulting in the
bacteria being not agglutinated with ‘O’ antisera. One specific surface antigen is well known: the Vi antigen. The Vi antigen occurs in only three Salmonella serovars namely S. typhi, S. paratyphi C, and S. dublin. Strains of these three serovars may or may not have the Vi antigen. Flagellar antigens are heat-labile proteins. A few S. enterica serovars (enteritidis, typhi) produce flagella which always have the same antigenic specificity. Such an ‘H’ antigen is then called monophasic. Most Salmonella serovars, however, can alternatively produce flagella with two different ‘H’ antigenic specificities; the ‘H’ antigen is then called diphasic. However S. typhimurium cells can produce flagella with either one or two ‘H’ antigen serotype specific. If a clone is derived from a bacterial cell with ‘H’ antigen, it will consist of bacteria with flagellar antigen. However, at a frequency of 10^-3 -10^-5, bacterial cells with two ‘H’ antigen specificity flagellar antigenic patterns will appear in this clone. (Kennethtodar, http://textbookofbacteriology.net/salmonella_2.html).

1.3 Virulence factors:

S. typhi has a combination of characteristics that make it an effective pathogen. This species contains an endotoxin typical of gram negative organisms, as well as the Vi antigen which is thought to increase virulence. It also produces and excretes a protein known as “invasin” that allows non-phagocytic cells to take up the bacterium,
allowing it to live intracellularly. It is also able to inhibit the oxidative burst of leukocytes, making innate immune response ineffective.

*Salmonella typhi* is an obligate parasite that has no known natural reservoir outside of humans. The principal habitat of the *Salmonella* is the intestinal tract of humans. In humans, *Salmonella* are the cause of two diseases called salmonellosis and enteric fever (Typhoid) resulting from bacterial invasion of the bloodstream and acute gastroenteritis resulting from a food-borne infection/intoxication (Bir Singh, 2001). In the pathogenesis of typhoid, the bacteria enter the human digestive tract, penetrate the intestinal mucosa (causing no lesion) and are stopped in the mesenteric lymph nodes. The bacterial multiplication occurs and part of the bacterial population lyses. From the mesenteric lymph nodes, viable bacteria and LPS (endotoxin) may be released into the bloodstream resulting in septicemia. Release of endotoxin is responsible for cardiovascular collapse and tephosis (a stuporous state, origin of the name typhoid) due to action on the ventriculus neurovegetative centers of the brain.

Food-borne *Salmonella* toxic infections are caused by ubiquitous *Salmonella* serovars (*typhimurium*). About 12-24 hours following ingestion of contaminated food, symptoms appear (diarrhoea, vomiting, fever) and last 2-5 days. *Salmonella* contamination may be associated with all kinds of food. Contamination of meat (cattle, pigs,
goats, chicken, etc.) may originate from animal salmonellosis, but most often it results from contamination of muscles with the intestinal contents during evisceration of animals, washing and transportation of carcasses. Surface contamination of meat is usually of little consequence, as proper cooking will sterilize it. However, when contaminated meat is ground, multiplication of *Salmonella* may occur within the ground meat and if cooking is superficial, ingestion of this highly contaminated food may produce a *Salmonella* infection. Infection may follow ingestion of any food that supports multiplication of *Salmonella* such as eggs, cream, mayonnaise, creamed foods, etc., as a large number of ingested *Salmonella* are needed to produce symptoms. Vegetables and fruits may carry *Salmonella* when contaminated with fertilizers of faecal origin, or when washed with polluted water.

The incidence of food-borne *Salmonella* infection/intoxication remains relatively high in developed countries because of commercially prepared food or ingredients for food. Any contamination of commercially prepared food will result in a large-scale infection. In underdeveloped countries, food-borne *Salmonella* intoxication is less spectacular because of the smaller number of individuals simultaneously infected but also because the bacteriological diagnosis of *Salmonella* toxic infection may not be available. However, the
incidence of *Salmonella* carriage in underdeveloped countries is known to be high.

**1.4 Treatment of typhoid and antibiotic susceptibility:**

During the last decade, *Salmonella* species have been found to acquire more and more antibiotic resistance. The cause appears to be the increased and indiscriminate use of antibiotics in the treatment of Salmonellosis of humans and animals, and the addition of growth promoting antibiotics to the food of breeding animals. Plasmid-borne antibiotic resistance is very frequent among *Salmonella* strains involved in pediatric epidemics. Resistance to ampicillin, streptomycin, kanamycin, chloramphenicol, tetracycline, ceftriaxone, cefotaxine, cefoperazone and sulfonamides is commonly observed; Colistin-resistance has not yet been observed.

*Salmonella* strains should be systematically checked for antibiotic resistance to aid in the choice of an efficient drug when needed and to detect any change in antibiotic susceptibility of strains (either from animal or human source). Until 1972, *S. typhi* strains had remained susceptible to antibiotics, including chloramphenicol (the antibiotic most commonly used against typhoid), but in 1972 a widespread epidemic in Mexico was caused by a chloramphenicol resistant strain of *S. typhi*. Other chloramphenicol-resistant strains have since been isolated in India, Thailand and Vietnam.
1.5 Vaccination against typhoid fever:

Vaccination against typhoid is essential for people travelling into areas where typhoid fever is endemic. As the bacterium has the ability to acquire multi-drug resistance ability, antibiotics may not offer complete protection. Three types of typhoid vaccines are currently available for use: (1) Parenteral killed whole cell vaccine, (2) Oral live-attenuated vaccine and (3) Typhoid-Vi capsular polysaccharide vaccine for parenteral use.

1.5.1 Parenteral killed whole cell vaccine:

The parenteral heat-phenol-inactivated vaccine has been widely used for many years. In field trials involving a primary series of two doses of heat-phenol-inactivated typhoid vaccine, efficacy over the 2 to 3 year follow-up period ranged from 51% to 77%. Efficacy for the acetone-inactivated parenteral vaccine, available only to the Armed Forces, ranges from 75% to 94%. Since the inactivated vaccines contain the ‘O’ antigen (endotoxin), local and general reactions occur.

The inactivated typhoid vaccine should not be given to children younger than 2 years of age. One dose provides protection and it should be given at least 2 weeks before travel to allow the vaccine time to work. A booster dose is needed every 2 years for people who remain at risk.
1.5.2 Oral live-attenuated vaccine:

Although oral killed vaccines have poor efficacy, vaccines using live avirulent bacteria have shown promise. A mutant *S. typhi* lacking galactose-epimerase has given very good results in field trials. Mutants of *S. typhimurium* that have given good protection in animals include mutants lacking adenylate-cyclase and AMP receptor protein, and mutants auxotrophic for p-amino benzoate and adenine. *S. typhi* with the same mutations does not cause adverse reactions and is immunogenic in humans.

The live oral attenuated Ty21a typhoid vaccine should not be given to children younger than 6 years of age. It is given in four doses. The last dose is given at least one week before travel to allow the vaccine time to work. A booster dose is needed every 5 years for people who remain at risk.

1.5.3 Typhoid Vi capsular polysaccharide vaccine:

The newly licensed parenteral vaccine Vi capsular polysaccharide (ViCPS) is composed of purified Vi ("virulence") antigen; the capsular polysaccharide was isolated from *S. typhi* propagated in blood cultures. In recent studies, 25µg/0.5mL injection of purified ViCPs produced sero-conversion (i.e., at least a four-fold rise in antibody titers) in 93% of healthy U.S. adults. Two field trials in disease-endemic areas have demonstrated the efficacy of ViCPs in
preventing typhoid fever. In one trial in Nepal, in which vaccine recipients were observed for 20 months, one dose of ViCPs among persons 5-44 years of age resulted in 74% fewer cases of typhoid fever. ViCPs has not been tested among children less than 1 year of age.

1.5.4 Conjugate vaccines:

The three typhoid vaccines have limitations and draw-backs such as side effects and limited efficacy, particularly in children below 5 years of age. Considering these draw-backs, the International Vaccine Institute (IVI), Seol, South Korea, focused on a better vaccine that can combat the global issue of typhoid fever. IVI has been very particular in discovering the potentials of conjugate vaccines. Dr John Robbins, for the first time, developed a Vi conjugate vaccine in late 1990s. The efficacy of this vaccine has been studied in China and in Vietnam. The conjugate vaccine increased serum antibodies 48-fold in adults, 252-fold in 5-14 year old children and 400-fold in 2-4 year old children. The results indicated that the Vi conjugate vaccine was thus highly well tolerated, safe and immunogenic in adults and young children. The conjugation of immunogenic proteins to a poorly immunogenic polysaccharide raises high avidity antibodies. The factors that influence the coupling of polysaccharides and proteins depend upon molecular weight and activation of the functional groups. Low molecular weight can result in efficient coupling different
proteins like tetanus toxoid, diphtheria CRM 197, the B subunit of the heat-labile toxin (LT-B) of *Escherichia coli*, the recombinant exoprotein A (rEPA) of *Pseudomonas aeruginosa* and Horseshoe rab Haemocyanin (HCH) have been mostly used for conjugation. The vaccines currently used have limitations. An efficient vaccine must be capable of triggering a good immune response and must be applicable for use in children.

1.6 Objective:

In order to achieve better conjugation and test the resulting molecule the thesis work was planned with the objective ‘DEVELOPMENT OF TYPHOID Vi POLYSACCHARIDE TETANUS TOXOID CONJUGATE VACCINE’.

1.7 Work plan:

Based on the this objective, the following work was planned for the study. All experiments were performed at the facilities available in Bharat Biotech International limited (BBIL), Hyderabad, India.

1.7.1 Bacterial growth and characterization:

a. Preparation of Master seed lot

b. Preparation of Working seed lot
1.7.2 Fermentation Process:
   a. Inoculum development
   b. Batch mode fermentation
   c. Fedbatch mode fermentation

1.7.3 Downstream process:
   a. Cell separation
   b. Concentration and diafiltration
   c. Cetrimide precipitation
   d. Ethanol precipitation
   e. Lyophilization

1.7.4 Conjugation process of Vi polysaccharide with Tetanus toxoid:
   a. Modification of Vi polysaccharide
   b. Concentration and diafiltration of Tetanus Toxoid
   c. Coupling of modified Vi polysaccharide (ViPs) with-protein (TT) molecules
   d. Purification of coupled ViPs-TT molecules

1.7.5 Formulation and Filling
1.7.6 Quality Control testing of Vi polysaccharide bulk, ViPs-TT conjugate bulk, Formulated and filled final lots:

a. Assay of O-acetyl content
b. Endotoxin test
c. Serological test
d. Protein content
e. Moisture content
f. Nucleic acid content
g. Molecular size determination
h. Quantification of free ViPs
i. Detection of free protein by HPLC
j. HPLC analysis
k. ViPs/protein ratio
l. Sterility test
m. pH of test vaccine
n. Mice immunogenicity test
o. Challenge test in mice models

1.7.7 Pre-Clinical toxicity study:

a. Abnormal toxicity test
b. Acute toxicity test
c. Systemic toxicity test

1.7.8 Human clinical trials