FUNCTIONAL CHARACTERIZATION OF THE OUTER MEMBRANE PROTEIN TolC of *Salmonella enterica* subspecies *enterica* serovar Typhi AND ITS ASSOCIATION WITH VIRULENCE

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by

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LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

-	Negative or minus
%	Percentage
+	Positive
<	Less than
>	More than
~	Approximately
ABC	ATP-binding cassette
Amp	Ampicillin
AMP	Antimicrobial peptide
aph	Aminoglycoside phosphotransferase
bp	Base pair
BSAC	British Society for Antimicrobial Chemotherapy
cDNA	Complimentary DNA
CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EtBr	Ethidium bromide
g	Gram
IL	Interleukin
Kan^r	Kanamycin
kDa	Kilo Dalton
L	Liter
LA	Luria agar
LB	Luria broth
LPS	Lipopolysaccharides
M	Molar
mA	Millie ampere
MATE	Multidrug and toxic compound extrusion family
MDR	Multidrug-resistant
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
mg	Milligram
MIC	Minimum inhibitory Concentration
min	Minute
mL	Milli liter
mM	Milli molar
ng	Nano gram
NTS	Non-Typhoidal *Salmonella*
^oC	Degree Celsius
OD	Optical density
Omp	Outer membrane protein
OMPs	Outer membrane proteins
PAP	Periplasmic adaptor

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RND	Resistance nodulation division
rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase PCR
S	Seconds
S. Typhi	*Salmonella Typhi*
SCV	*Salmonella*-containing vacuole
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDW	Sterile distilled water
SMR	Small multidrug resistance family
SPIs	*Salmonella* pathogenicity islands
TE	Tris-EDTA buffer
TLR	Toll-like receptor
TTSS	Type three secretion system
UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
WT	Wild-type
µg	Microgram
µL	Microliter

PENCIRIAN FUNGSI PROTEIN MEMBRAN LUAR TolC *Salmonella enterica* subspecies *enterica* serovar Typhi DAN PERKAITANNYA DENGAN VIRULEN

ABSTRAK

Walaupun banyak kajian telah dijalankan ke atas *Salmonella enterica* subspesies *enterica* serovar Typhi (*S.* Typhi), agen penyebab demam kepialu manusia, hanya beberapa protein membran luarnya (OMPs) telah dicirikan secara berfungsi. Dalam kajian ini, mutan pelesapan *tolC* dihasilkan untuk mengkaji fungsi biologi TolC dalam strain *S.* Typhi yang telah diasingkan daripada pesakit demam kepialu akut. Pelesapan TolC menyebabkan peningkatan kerentanan kepada pelbagai cabaran antimikrobial, integriti membran dikompromi, dan pengurangan aktiviti pengeluaran efluks *S.* Typhi. Di samping itu, mutan Δ*tolC* mempunyai lekatan dan serangan yang lebih rendah dalam model *in vitro* jangkitan kultur sel manusia. Untuk pengesahan lanjut pelemahan ini, analisis PCR transkripsi berbalik (RT-PCR) menunjukkan pengurangan transkripsi gen bakteria yang berkaitan dengan serangan *Salmonella*. Susulan daripada itu, mutan *tolC* kurang dapat menyerang sel-sel HT-29 dan THP-1 berbanding strain asal dalam ujian serangan. Tambahan pula, analisis *in vitro* mendedahkan bahawa kehadiran mutant *tolC* intrasel adalah hipersitotoksik terhadap makrofaj manusia berbanding dengan strain liar, dan ia menyebabkan peningkatan transkripsi gen kemokin prokeradangan dalam makrofaj manusia. Secara keseluruhannya, data ini mencadangkan bahawa fungsi TolC diperlukan oleh *S.* Typhi untuk menghalang kematian sel

perumah dan melemahkan gerak balas imun perumah. Pelesapan *tolC* memberikan fenotip yang berbeza daripada strain asal liar. Ini menunjukkan bahawa fungsi TolC adalah berbeza daripada rakan proteinnya dalam efluks substrat dan virulens. Kajian ini mencadangkan kemungkinan fungsi TolC dalam virulens dan kepatogenan *S.* Typhi.

FUNCTIONAL CHARACTERIZATION OF THE OUTER MEMBRANE PROTEIN TolC OF *Salmonella enterica* subspecies *enterica* serovar Typhi AND ITS ASSOCIATION WITH VIRULENCE

ABSTRACT

Although *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi) has been well studied, only a few of its outer membrane proteins (OMPs) have been functionally characterized. In this study, a *tolC* deletion mutant was generated to study the biological functions of TolC in *S.* Typhi strain that was isolated from an acute typhoid patient. Deletion of TolC caused increase susceptibility to a range of antimicrobials challenge, compromised membrane integrity, and reduced efflux activity of *S.* Typhi. In addition, the Δ*tolC* mutant was shown to have lower adhesion and invasion capability in an *in vitro* human cell culture infection model. Further confirmation of this attenuation was investigated by the reverse transcription (RT)-PCR analysis which showed reduced transcription of bacterial genes related to the invasion of the *Salmonella*. Consequently, when invasion tests were performed with the *tolC* mutant, the *tolC* mutant was significantly less able to invade HT-29 and THP-1 cells than its parental strain. Furthermore, the *in vitro* analysis revealed that the intracellular presence of the *tolC* mutant was hyper cytotoxic to human macrophages as compared to the wild-type strain, and it elicited the increased transcription of proinflammatory chemokine genes in human macrophages. Collectively, these data suggest that TolC function is required for *S.* Typhi to inhibit host cell death and dampen host immune

responses. Deletion of the *tolC* conferred a distinct phenotype from the wild-type parent strain. This indicates that the function of TolC is distinct from its protein partners in both effluxes of substrates and in virulence. This study suggests the possible functions of TolC in the virulence and pathogenicity of *S.* Typhi.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Typhoid fever continues to be a major cause of morbidity and mortality worldwide. *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi) causes typhoid fever in the human host. Typhoid fever can be described as a normalized acute infection of an organ like intestinal lymphoid tissue, reticuloendothelial system, and gallbladder. Buckle *et al.* (2012) had proposed that typhoid fever prevalence could be as high as 26.9 million with 269,000 deaths. Most of cases were reported in young children (2008, Wain *et al.*, 2015). The disease is limited to humans, but human chronic carriers act as reservoirs for the *S.* Typhi for further spread of the infection (Gunn *et al.*, 2014). These claims may be due to *S.* Typhi ability to transmit its virulence from human-to-human. Their persistence alone makes the strains responsible for many cases worldwide. Several strategies are used by this pathogen to influence its virulence efficiency in its human host. These strategies include tolerating antimicrobial factors of the host and secreting a toxin that causes damage to the host cells. However, virulence and persistency are difficult to explain because these pathogenic bacteria have evolved various escape mechanisms and strategies for surviving in the human host even though one of the important mechanisms involve its efflux pump system. In Gram-negative bacterial pathogens, TolC is an outer membrane efflux pump protein that facilitates efflux

function and contributes to virulence and pathogenesis. Previous reports have reported that TolC-facilitated efflux functions have links to virulence and pathogenesis in several Gram-negative pathogens (Piddock, 2006b). The expression of efflux pumps appears to go along with the infection process of Gram-negative pathogens (Fernando and Kumar, 2013). According to these reports, question is rise that there is a link among the function of outer membrane efflux pump protein (TolC), and its involvement in the virulence of *S*. Typhi and various infection processes (such as colonization, and resistance from host defense). Thus, TolC potentially plays an important role in virulence; therefore, this study pays attention to the involvement of the TolC, outer membrane channel protein, in the virulence of *S*. Typhi in the human host.

1.2 Problem statement and rationale of the study

Outer membrane efflux pump proteins (OMPs) have essential functions in the physiology of bacteria, for example, adhesion and invasion of the host cell, resistance to host serum, maintenance of the membrane integrity, and passive and active transfer of substances (Tokuda, 2009). The most common OMPs, like TolC has been examined as a multifunctional protein because of its contributions to maintenance of cell membrane integrity, tolerance to acidic condition, elimination of metabolites, exportation of siderophores which are crucial in acquiring iron from surrounding environments, toxins exportation which encoded by plasmid and chromosomally, for example, hemolysin, colicin V, microcins, and virulence in host, as evident from studies in many Gram-

negative pathogens, such as *Enterobacter*, *Borrelia*, *Salmonella*, *Vibrio*, *Legionella*, *Francisella*, and *Escherichia coli* (Wandersman and Delepelaire, 1990, Hwang *et al*., 1997, Delgado *et al*., 1999, Gil *et al*., 2006, Bunikis *et al*., 2008, Ferhat *et al*., 2009a, Horiyama *et al*., 2010, Lee *et al*., 2014, Matsuo *et al*., 2013). TolC in *E. coli*, is promiscuous mainly because it facilitates the use of multidrug resistance efflux pumps of different families (Lomovskaya and Lewis, 1992, Fralick, 1996, Kobayashi et al., 2001, Nishino and Yamaguchi, 2001, Nishino and Yamaguchi, 2002, Kobayashi et al., 2003b, Nishino et al., 2003). The biological functions of the TolC homologs in several Gram-negative bacteria have already been widely explained in several published reports as mention, but the function of TolC in *S*. Typhi is currently not well understood. Hence, this study aims to investigate the function of TolC in the virulence of *S*. Typhi.

1.3 Objectives

General objective:

To study the role of TolC in the early steps of host cell invasion and TolC-dependent hypercytotoxic and proinflammatory responses from host cells by using an *in vitro* system.

Specific objectives

- To construct a *tolC* deletion mutant and its complementation strain.

- To establish phenotypic characterization of *S*. Typhi *tolC* mutant.
- To investigate the effect of *tolC* deletion on early steps of *S*. Typhi invasion in a host cell.
- To observe the effect of *tolC* deletion on hypercytotoxic and proinflammatory responses from host cells.
- To determine whether a virulence-related factor, exported by TolC of *S*. Typhi, can restore the invasiveness of the *tolC* mutant.

1.4 Overview of the study

The hypothesis is that the gene locus *tolC* is involved in the virulence of *S*. Typhi to infect human epithelial and macrophage cells to cause systemic infection, and it also has functions in the physiology of *S*. Typhi. To test this hypothesis, several goals have been set. First, a *tolC* deletion mutant of *S*. Typhi was constructed. After, the effects of the *tolC* deletion were observed on the physiology of *S*. Typhi, such as antibiotic and detergent resistance ability, efflux function, growth curve, and maintenance of bacterial membrane integrity. Thereafter, the effects of *tolC* deletion were verified in *S*. Typhi with respect to its adhesion and invasion ability to host cell, and expression of its invasion-related genes. Subsequently, the effects of *tolC* deletion were observed on the proinflamantry response of host during infection of *S*. Typhi. Finally, it was determined whether a virulence-related factor, exported by TolC of *S*. Typhi, can restore the

invasiveness of the *tolC* mutant. The overview of the study is shown in Figure 1.1.

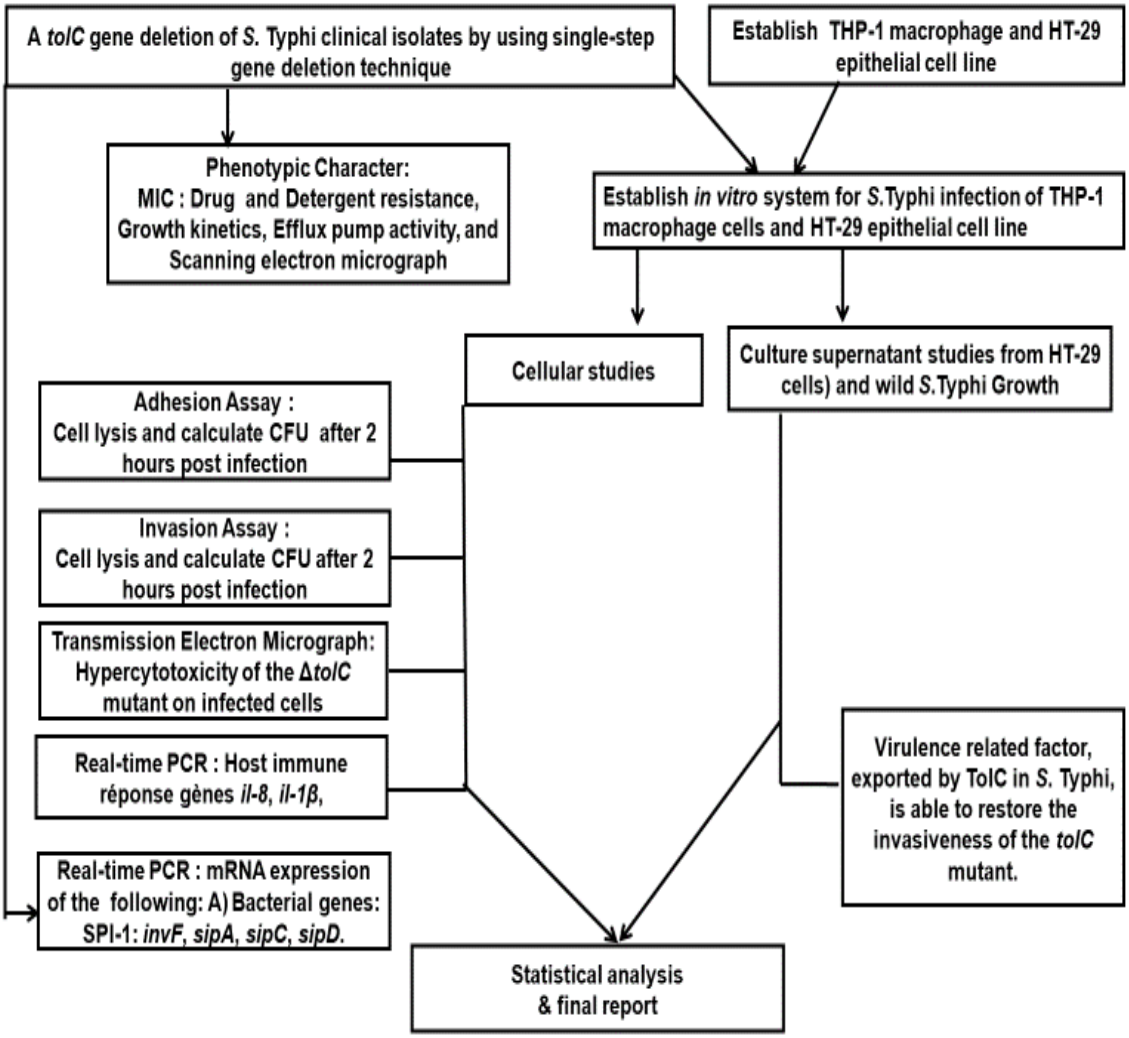

Figure 1.1: Overview of Study

CHAPTER 2

LITERATURE REVIEW

2.1 Typhoid fever

Typhoid fever is a severe systemic illness that affects the gallbladder and reticuloendothelial system of the human body, and it causes prolonged fever, hepatomegaly, splenomegaly, stomach pain, anxiety, headache, and constipation. According to Hornick *et al*. (1970), this illness arises due to consumption of meals or drinking water which is infected with 10^3-10^6 CFU/mL of *S*. Typhi. With regards to persistence within the host, *S*. Typhi has extraordinary features that have not been completely demonstrated yet (Merrell and Falkow, 2004). After intake, *S*. Typhi pass through the gastric acid-abundant stomach and arrives at the intestine, where it colonizes in the intestine. The pathogen adheres and invades the epithelial cells of the small intestinal tract; following which, they are phagocytosed by macrophages. This course of action requires a couple of type III secretion systems (T3SS), T3SS-1 and T3SS-2, which usually stimulated when *S*. Typhi get across epithelial cells of the intestine and survive within macrophages of the host respectively (Tischler and McKinney, 2010). Within the macrophages, the bacteria have the ability to survive in the phago-lysosome system and take benefit of free passage to the lymphatic and reticuloendothelial systems in the small intestinal tract, liver as well as spleen, and remain there for few days prior to being transferred back

towards the bloodstream (Everest *et al*., 2001, House *et al*., 2001, Parry *et al*., 2002), as shown in Figure 2.1.

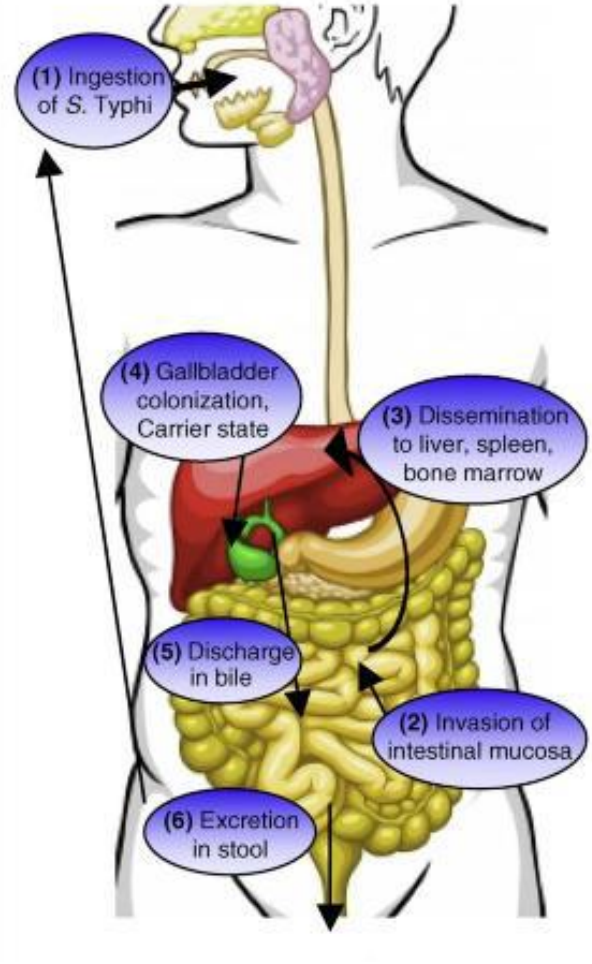

Figure 2.1: Typhoid fever pathogenesis explaining the developmental phases of the illness. Each phase is labeled in numbers. Figure adapted from Tischler and McKinney, (2010).

In recent years, fatalities of the disease have decreased, but the numbers of cases have increased. This observation is mainly due to the use of antibiotics particularly fluoroquinolones (ciprofloxacin, nalidixic acid), cephalosporins (Ceftriaxone, Cefalexin), and macrolides (azithromycin) that are being used in the treatment of Typhoid fever (Zaki and Karande,

2011). These antibiotics help to reduce the number of deaths during typhoid fever, but also lead to the development of strains which are resistant to multiple drugs (Zaki and Karande, 2011). The increasing number of *S.* Typhi strains that are resistant to antibiotics remain a serious problem in endemic regions because these strains contribute to increase numbers of typhoid fever cases and complications in the treatment of the disease (Zaki and Karande, 2011). Currently, two vaccines are available to prevent *S.* Typhi infection: the first type is the polysaccharide capsule and Vi antigens that are administered through parenteral route, and the second type is a live attenuated Ty21a strain that is given via oral route (Paterson and Maskell, 2010, Martin, 2012). Their effectiveness is about 70 %, and both types do not provide long-term protection (Paterson and Maskell, 2010, Martin, 2012). In view of this, new generation vaccines are being developed to improve efficiency and a long-term protection (Paterson and Maskell, 2010, Martin, 2012, Tennant and Levine, 2015).

2.2 Epidemiology of typhoid fever

2.2.1 History of typhoid fever

A French physician, Pierre Charles Alexandre Louis (1787-1872) initially used the title — typhoid fever in the year 1829. William Budd (1811-1880) concluded in 1873 the fact that fecal–oral path is responsible for spreading of typhoid fever. In the year 1880, Karl Eberth (1835-1926) noticed rod-shaped microorganisms within the lymph nodes and spleens of individuals suffering from typhoid fever, and he found the causative agent *Salmonella*

Typhi (*S*. Typhi). In the same way, Lignieres credited the term *Salmonella;* successively in 1885, Daniel Elmer Salmon recognized *Salmonella Choleraesuis* from pigs. In the year 1884, Georg Gaffky (1850-1918) cultured *S*. Typhi from affected individuals. In the 1940s, Fritz Kauffmann (1899-1978) stretches the research of Phillip Bruce White (White, 1926), and he set up a serological distinction of *Salmonella*. By using chloromycetin (chloramphenicol), Theodore E. Woodward (1914-2005) and colleagues effectively cured typhoid affected individuals in 1948 (Woodward *et al*., 1948, Woodward *et al*., 1950).

2.2.2 Carriers of *S*. Typhi

The colonization of *S*. Typhi in humans mainly causes serious symptoms, but sometimes the infection is not associated with any symptoms (Parry *et al*., 2002). Typically, 1-5 % typhoid fever patients become chronic carriers of *S*. Typhi (Parry *et al*., 2002). In asymptomatic carriers, bacteria usually persist in the gallbladder in the form of biofilms that protects them against the antibiotics and effectors of the host's immune system (Sinnott and Teall, 1987, Tabak *et al*., 2009, Crawford *et al*., 2010, Hoiby *et al*., 2010, Gonzalez-Escobedo *et al*., 2011, Basnyat and Baker, 2015). These asymptomatic typhoid carriers excrete bacteria in their stool, which increases the risk of infection in the population that is a major threat to public health (Parry *et al*., 2002, Bhan *et al*., 2005).

2.2.3 Distribution and infectivity of *S.* Typhi

The spread of typhoid fever is worldwide, however, it much more widespread in Oceania, The African continent, Latin America as well as Asia with an occurrence rate of cases 15.4, 49.8, 53.1, and 274.3 for each 100,000-human population respectively (Crump *et al*., 2004).

In the year 2000, the prevalence rate of typhoid fever was highest in Asia, and with more than 100 cases per 100,000 populations, particularly in Southeastern and South-central part of Asia, including Bangladesh, India, Malaysia, and Pakistan (Crump *et al*., 2004) because this serovar is mainly found in developing countries (Rhen *et al*., 2007), as shown in Figure 2.2. The World Health Organization (WHO, 2008) reports that typhoid fever approximates 16.6 million infections with an average of 600,000 deaths worldwide each year. A report in 2010 indicated that typhoid fever cause approximately 21.7 million infections and 217,000 deaths annually (Crump and Mintz, 2010). A separate report in 2012 indicated that typhoid fever incidence could be as high as 26.9 million infections with 269,000 deaths as proposed by Buckle *et al*. (2012). The complications of the disease that are related to the human-adapted bacterial pathogen in endemic regions of the world have been reported by Wain *et al*. (2015). Studies claim that typhoid incidents are greater than 70 % in developed nations tend to come from individuals who have experienced travel to a typhoid endemic region (Mead *et al*., 1999, Ackers *et al*., 2000, Reller *et al*., 2003, Connor and Schwartz, 2005, Ekdahl *et al*., 2005, Lynch *et al*., 2009). Since the source was mainly from travelers who returned from their journey, in 100,000

passengers, there are 3 to 30 cases of typhoid fever (Steinberg *et al*., 2004). However, outbreaks can happen, through imported fruits contaminated with the pathogens (Katz *et al*., 2002), or via meal services employees who are asymptomatic carriers of the *S*. Typhi (Greig *et al*., 2007). The dropped numbers of typhoid fever cases observed in the developed world in the 1940s were result of improved sanitation, disposal of waste materials, food handling, personalized cleanliness, unpolluted drinking water and the use of antibiotics to treat the disease (Mølbak *et al*., 2006, WHO, 2008). However, in 2013, in the United States, 400 cases are reported, and the disease is estimated to occur in about 5,700 people per year (CDC, 2014, Jackson *et al*., 2015).

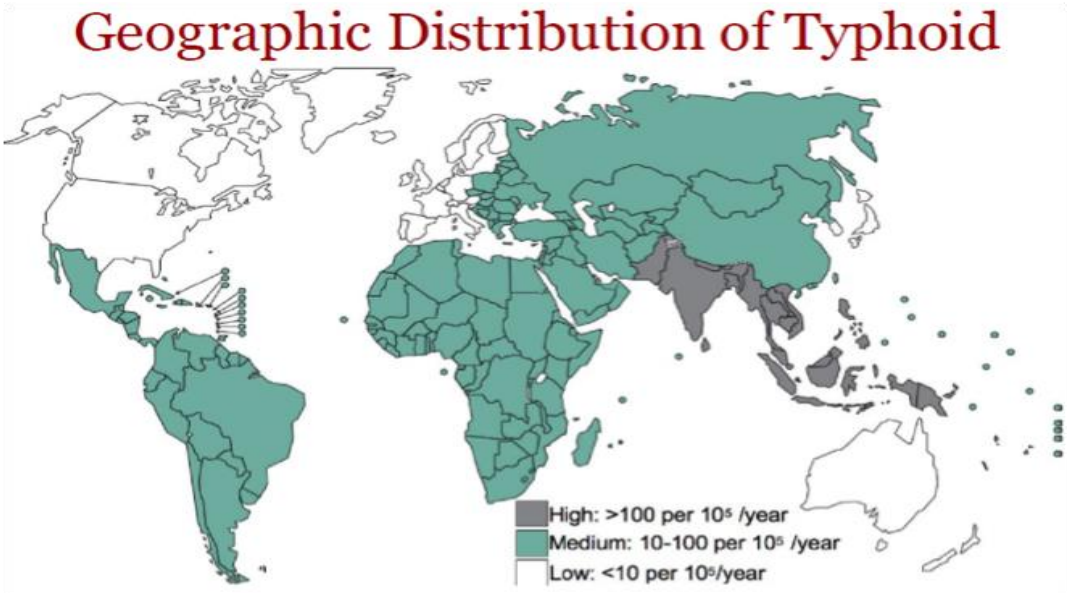

Figure 2.2: The global burden of typhoid fever. Figure adapted from Crump *et al* (2004).

As typhoid fever is associated with poor hygiene and lack of health facilities; therefore, the disease mostly occurs in underdeveloped countries

(Rhen *et al*., 2007). In endemic areas, the bacterium spread through contaminated water and food (Nguyen *et al*., 2009). The fecal-oral spread of the bacteria is a major problem because contamination is produced by asymptomatic carriers and patients who release pathogen in their feces (Basnyat, 2007).

The disease evidently occurs mostly in 1 - 19 year-aged (Lin *et al*., 2000, Merrell and Falkow, 2004). On the other hand, more recent information revealed that typhoid infection rate is 44 – 54 % in children under 5 years old (Graham, 2002, Siddiqui *et al*., 2006, Ja'afar *et al*., 2013). Fluoroquinolones work well (Parry *et al*., 2002), but resistance to these types of drugs are increasing and results in increasing numbers of resistance strains (Threlfall, 2002). *S*. Typhi that resistance to chloramphenicol is mediated by plasmid gene while nalidixic acid resistance is mediated by chromosomal gene (Ray *et al*., 2006). The risk of death may be as high as 25 % without treatment, while with treatment it is between 1- 4 % (Wain *et al*., 2015, 2008).

Generally, disease spread is an important characteristic for the complication of the disease (Rhen *et al*., 2007). This serovar infects only humans and does not colonize in animals, so it's only involved in human to human transmission (Rhen *et al*., 2007). This lack of zoonosis is a great benefit to humans. This strict adaptation to the human host; consequently, strictly limits the study of this bacteria because there is no animal model that can reproduce systemic illness caused by *S*. Typhi. Existing knowledge

about the virulence of this serovar comes largely from studies with *S.* Typhimurium in mice (Haraga *et al.*, 2008). The genomes of the two serovars *S.* Typhi and *S.* Typhimurium have more than 90 % homology (Vidal *et al.*, 1995, Alemán *et al.*, 2009). Thereby *S.* Typhimurium has long been used to reproduce a systemic infection in a mouse model having a mutation in the Nramp1 protein, making it very susceptible to mice (Vidal *et al.*, 1995), so the pathogenesis of *S* Typhi has been elucidated in large part through this mouse model. However, the interaction between the pathogen and its own host is critical to understanding pathogenesis by use of mouse model. Therefore, research is still dedicated to finding an appropriate model to conduct *in vivo* infections (Daigle *et al.*, 2001a). However, sequencing of the complete genome of *S.* Typhi CT18 was completed in 2011, and it is available on the website (http://www.sanger.ac.uk/Projetcs/S_Typhi/) since this comprehension has been useful to the advancement of research on this pathogen.

2.2.3 (a) Typhoid fever in Malaysia

In Malaysia, all classes of the community are affected by typhoid fever (Malik and Malik, 2001). Additionally, some other risk factors have been identified, for example, interaction with the infected individual, inadequate domestic cleaning, cleaning hands without utilizing soap, and earlier infection with *Helicobacter pylori* (Bhan *et al.*, 2005). In endemic areas, the youngest age group of 1-19 years have the highest prevalence of typhoid fever (Lin *et al.*, 2000, Ja'afar *et al.*, 2013). Choo *et al.* (1988) have described that 7.3 years is the average age of typhoid fever patients

who admitted to Hospital Universiti Sains Malaysia. Typhoid cases in Malaysia between 1978 to 1990 occurred yearly with an occurrence rate of 10.2-17.9 incidents per 100,000 human population with a higher as 50.3 incidents per 100,000 population in the state of Kelantan (Yap and Puthucheary, 1998). However, a significant progress was accomplished in decreasing the incidence of typhoid fever in Kelantan from 14.7 incidents per 100,000 human population in 2000 to 2.8 incidents per 100,000 human population in 2010. According to a recent report by the Ministry of Health, Malaysia was categorized as a low endemic area for typhoid fever with a yearly prevalence rate less than 10 cases per 100,000 populations from the year 1995 to 2014 (Figure 2.3). Kelantan state of Malaysia has the maximum prevalence rate (10-100 case/ 100,000) of typhoid fever and categorized as an average endemic region (Shah SA *et al.*, 2012). In the year 2001, typhoid cases in Kelantan were 37 per 100,000 (Wan Mansor *et al.*, 2011), which dropped to 24.4 per 100,000 in 2003, 10 per 100,000 in 2008, 3.29 per 100,000 in 2009, and 2.8 per 100,000 in the year 2010 (Figure 2.4). This was credited to the establishment of appropriate observing and surveillance systems by the Public Health Department of Kelantan State (Wan Mansor *et al.*, 2011), as presented in Figure 2.4. An outbreak of typhoid fever happened in the year 2005 with a major flood in the state because the wells were polluted with sewage overflow, and water sources were contaminated in rural areas (Shah SA *et al.*, 2012).

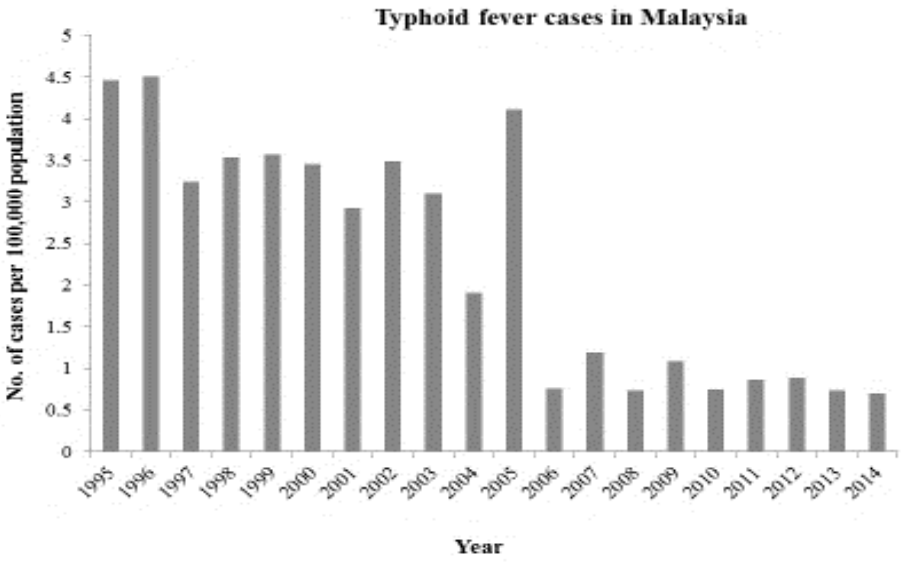

Figure 2.3: Occurrence of typhoid fever in Malaysia from the year 1995 till 2014. (Data from Ministry of Health, Malaysia, 2015).

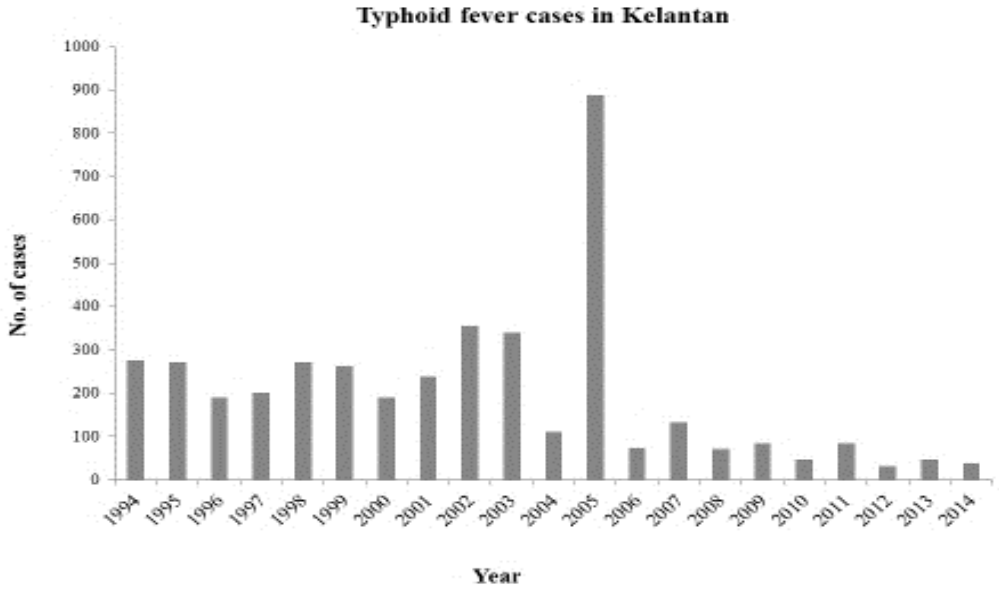

Figure 2.4: Pattern of typhoid fever in Kelantan state from the year 1994 till 2014. (Data from Kelantan State Public Health Department, 2015).

2.3 Molecular basis of systemic infection

2.3.1 Adhesion and invasion

The *S*. Typhi bacterium adhere to the epithelial cells of the host intestine, and this adhesion is the primary mechanism of action towards the establishment of the infection. Different events may happen during this period like the tissue invasion at the time of colonization (Rhen *et al*., 2007). *S*. Typhi has interactions with the M cells of Peyer's patches of the intestinal tract. This entrance makes possible for bacteria to get across the epithelial hindrance through pinocytosis which is the same as phagocytosis (Kaufmann SH *et al*., 2001). However, in this interaction, M cells also make it possible for bacteria to get transported towards the lymphoid T cells (Haraga *et al*., 2008). A few studies support the idea that the bacteria could possibly be phagocytosed by CD-18 positive cells (Wilson *et al*., 2008), such as monocytes, macrophages, dendritic cells, and neutrophils. These immune cells engulf *Salmonella* and transport them to various systemic sites through the blood and lymph (Haraga *et al*., 2008). Unlike entry through M cells, this transportation pathway permits *S*. Typhi to evade the immune system. Thus, the bacteria are able to spread without inducing a significant inflammatory response in the host.

S. Typhi can also invade epithelial cells of the intestine by injecting effectors into the host cell through utilization of its specific T3SS-1-type secretion system and interact with actin cytoskeleton of the epithelial cell (Haraga *et al*., 2008). The effector proteins that are essential to manipulate

the cytoskeleton of the host cell for invasion, include, SopE, SopE2, SopB, SipB, SipA, and SipC; thus, the membrane of the epithelial cell can be corrugated and allows the bacterium to internalize into the host cell. These entry strategies permit *S*. Typhi to adhere and invade epithelial cells of the gut and to arrive in the lamina propria. This passage via the hindrance of the epithelium is an important stage to infection because *S*. Typhi needs to pass within the body of the host to produce a systemic disease. However, *S*. Typhi can evade the immune system that produce inflammatory response that might be able to eliminate it.

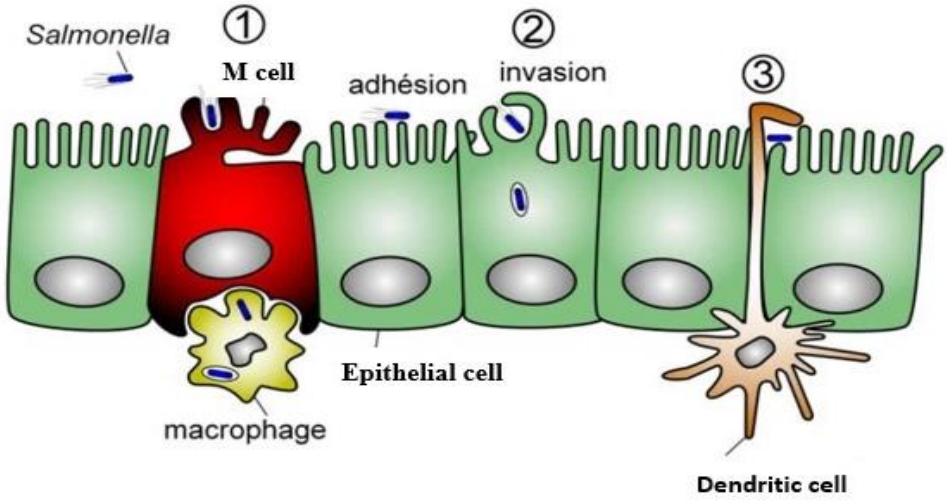

Figure 2.5: Mechanisms used by *Salmonella* to cross the cell barrier bowel.

1) *Salmonella* is internalized into cells by pinocytosis and is taken by macrophages to the underlying epithelium. 2) *Salmonella* adheres to epithelial cells and using its *T3SS-1* to secrete effector into the cytoplasm of cells and causes remodeling of actin. 3) Dendritic cells located on the basolateral side can capture the bacteria present on the apical side by extending pseudopodia. Figure adapted from Sansonetti (2004).

2.3.2 Evasion of the immune system

S. Typhi has the potential to infect epithelial cells without initiating any noticeable inflammatory reaction. When invasion happens through M cells of Peyer's patches or by endocytosis, this requires the LPS and flagellin that are very antigenic (Wilson *et al*., 2008). The capsule upregulation during access into epithelial cells which hides LPS and flagellin of *S*. Typhi, and allows it to evade being detection by the Toll-like receptor (TLR) - 4 and 5 (Wilson *et al*., 2008). In this condition, the infected epithelial cell will not produce IL-8 which is responsible for activation of a localized inflammatory reaction at this stage of the infection, so the patient infected with *S*. Typhi does not suffer from diarrhea at this stage; subsequently, this allows *S*. Typhi to cause a systemic infection (Wilson *et al*., 2008). However, *S*. Typhimurium infection in humans is associated with a strong inflammatory response in the gut (Santos *et al*., 2009) because this response is due to the recognition of several components that are found on the surface of the bacteria by the immune system of the host (Wangdi *et al*., 2012). Among others, flagellin, the major component of flagella is recognized by TLR-5 and IPAF (IL-1β converting enzyme protease activating factor), which causes the release of interleukin-8 (IL-8) by the enterocytes, and IL-18 and IL-1β by macrophages (Santos *et al*., 2009, Wangdi *et al*., 2012). LPS is recognized by the TLR-4 while T3SS-1 by NLRC4 (nucleotide-binding oligomerization domain-like receptor family caspase-associated recruitment domain-containing protein 4) (Santos *et al*., 2009, Wangdi *et al*., 2012). These interactions trigger characterized

inflammatory responses that recruit neutrophils at the infection site because the action of neutrophils causes damage to the intestinal epithelium and extravascular leakage of fluids in the intestine, thus contributing to diarrhea (Santos *et al*., 2009).

2.3.3 Survival and systemic infection

Bacteria grow and increase its number inside the macrophages. As stated previously, *S.* Typhi is a facultative intracellular bacterium. Which means, this is their benefit of getting engulfed by macrophages and resides in macrophages. To avoid digestion through phagocytic cells, intracellular bacteria can multiply in these cells. The absence of phagolysosome formation is an essential function of the virulence of *Salmonella,* which prevents digestion by macrophages (Haraga *et al*., 2008). The degree of interaction among *S.* Typhi and the phagocytic cell is essential for the progression of the disease, in which the bacteria transform the vacuole by utilizing effectors, such as SipA, SopB, SopD, and SopE2, which modify macrophage cell signaling and turns into a niche regarding survival and replication of intracellular bacteria (Haraga *et al*., 2008). *S.* Typhi has the ability to modify most crucial components of the host protection mechanism against intracellular pathogens (Daigle *et al*., 2001b).

Survival in macrophages has been demonstrated for *S.* Typhimurium in murine models of typhoid fever. In fact, *S.* Typhimurium mutant's defective in SPI-2 is severely attenuated in the mouse infection, and being incapable of proliferating in various organs (Hensel, 2000). The SPI-2 is

also essential for the survival and intracellular proliferation of *S.* Typhimurium in murine macrophages during *in vitro* infection (Hensel *et al.*, 1998). However, the intracellular survival of *S.* Typhi in human macrophages appears to be independent of the SPI-2, suggesting that the SST3-2 is not obligatory for survival and intracellular growth of *S.* Typhi (Forest *et al.*, 2010). *Salmonella* also has an important defense system to protect from antimicrobial factors in macrophage, for example, efflux pump (Fernando and Kumar, 2013). Survival in macrophages is essential for the establishment of systemic infection, and this allows passage of *S.* Typhi to the lymph nodes, liver, and spleen of the host (Fields *et al.*, 1986, Dragunsky *et al.*, 1990, Vazquez-Torres *et al.*, 1999, House *et al.*, 2001).

One to two weeks after intake of the bacterium, many bacteria are released into the bloodstream and infect the liver, gall bladder spleen, bone marrow, and Peyer's patches of the host (Parry *et al.*, 2002). At this stage of infection, the symptoms of typhoid fever appear include, high fever, lethargy, headaches, and stomach pain (House *et al.*, 2001). The bacteria associated with the gallbladder are excreted in the feces and can reinfect the intestine, which can lead to severe infections; at this point, there is a perforation, intestinal bleeding, encephalopathy, and possibly death (House *et al.*, 2001). During its cycle of infection, *Salmonella* encounters various environmental changes, and its ability to adapt quickly to changes in environmental conditions is essential for survival and virulence of *Salmonella*. In response to various environmental signals, *Salmonella* expresses several genes that are related to stress tolerance and virulence

factor that are required to meet these changing conditions. Like most bacteria, *Salmonella* has multiple control systems to encounter the different stress conditions. Among the main regulatory systems are *marA*, *soxS* or *rob* that are usually increase resistance to several antibiotics, and PhoPQ, BaeSR, and EvgAS regulons that are responsible for resistance against superoxides, endurance under acidic milieus in host, and tolerance of extracytoplasmic stress during infections (Aono *et al*., 1998, Eguchi *et al*., 2003, Nishino *et al*., 2005, Zhang *et al*., 2008).

2.4 Taxonomy and nomenclature of *S*. Typhi

The genus *Salmonella* consists of two species, *S. enterica* and *S. bongori*, and *S. bongori* is also referred to as subspecies V. These species are further divided into six subspecies (Figure 2.6), which are biochemically distinguished into serovars based on their carbohydrate, flagellar, and lipopolysaccharide (LPS) structures. An antigenic formula that depends on somatic (O) and flagellar (H) antigens, in addition to capsular (Vi) antigens, are used to describe all *Salmonella* serotypes (Fierer and Guiney, 2001).

S. Typhi is a highly conserved serovar within subspecies I of the *S*. *enterica* (Tang *et al*., 2013). The Kaufmann-White scheme classifies *S*. Typhi as Group D with O-antigen type O9-12, phase1 flagellin type H: d, and Vi capsule positive. Therefore, *S*. Typhi is normally monophasic. Most *S*. Typhi falls within the Kaufmann-White classification, but rare isolates are Vi-negative (Dougan and Baker, 2014).

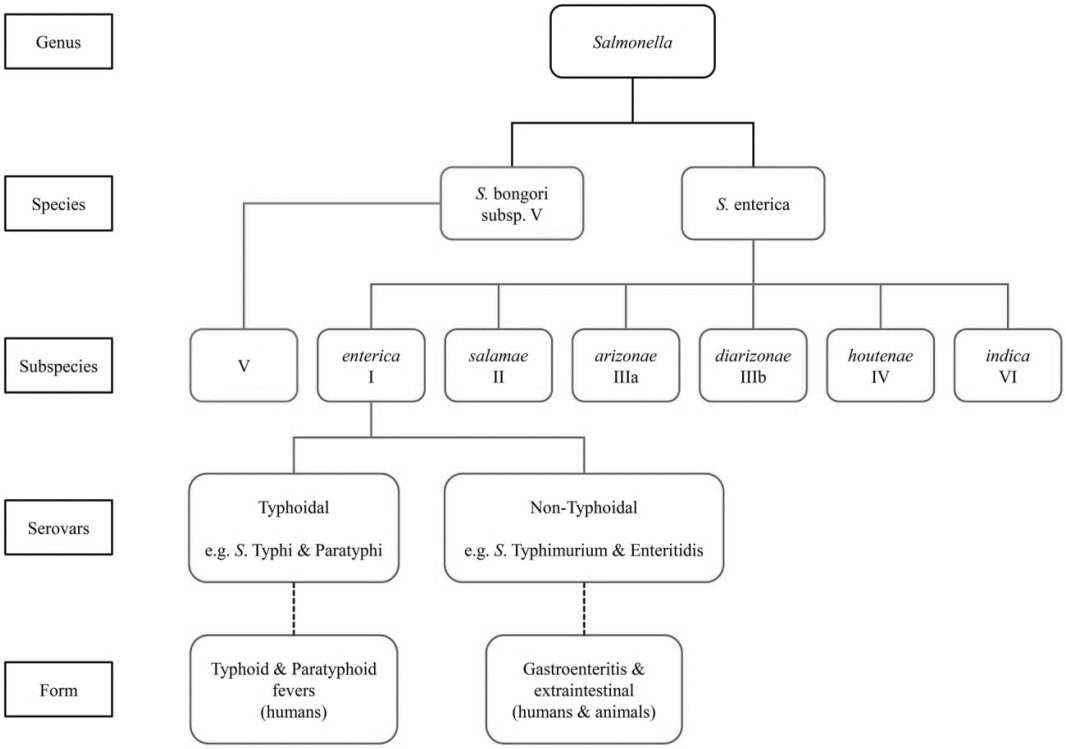

Figure 2.6: Classification of *Salmonella* species and subspecies. Figure adapted from Hurley *et al.* (2014).

2.5 Characteristics of *S.* Typhi

Salmonella is categorized as Gram-negative, straight rod-shaped, nonencapsulated, non-spore forming, facultative, and usually motile with peritrichous flagella (Gray and Fedorka-Cray, 2002, Mølbak *et al.*, 2006) (Figure 2.7). The bacterium has a length of 2.0 to 5.0 µm, and a thickness of 0.7 to 1.5 µm (Holt *et al.*, 1994). The ideal temperature range within 35 to 40°C is appropriate for growth of *Salmonella* (Dickson, 2000) although it can survive through a broad range of temperatures from 8 to 45°C (Hanes, 2003). A pH range of 4.5 to 9.0 has determined to be optional for the growth of *Salmonella* (D'Aoust, 1989). On the other hand, pH range within 6.5 to 7.5 is most favorable for growth (Garcia-Del Portillo, 1999). In addition, the pathogen *S.* Typhi can survive more than a few months in water and

soil (Tran *et al*., 2005). *Salmonella* is often aerogenic - generating gas from glucose, and can make use of citrate as an only carbon source. It is unable to ferment lactose, but it is competence to generate hydrogen sulfide (H_2S) gas from Sulphur-containing amino acids, so these features are utilize to recognize colonies on culture media (Ryan and Falkow, 1994).

The outer membrane of *Salmonella* accounts for around 60 % of the protein that is present in the whole cell envelope as well as around 90 % of the entire lipoprotein (Ohl and Miller, 2001). The outer membrane lipoproteins are usually attached to the membrane in a similar way as those located on the inner membrane, but these are situated on the inner instead of the outer leaflet of the membrane layer (Ohl and Miller, 2001). The integral proteins of the outer membrane are completely different from their inner membrane counterparts because they fold to create β-barrel conformations which span the membrane and work as channels that allow the influx of nutrients, exportation of waste materials, and efflux pump for amphipathic molecules which are unable to export from the membrane by any other means (Nikaido, 2003, Doerrler, 2006, Ruiz *et al*., 2006, Rigel and Silhavy, 2012). Although *S.* Typhi has developed various mechanisms for survival in the host and resistance against antibacterial agents, efflux function is an important mechanism that causes multiple drug resistant phenotype and complication in disease. The efflux pumps are present in the cell membrane of the bacteria because they are involved in the mechanism that pumps out any toxic substance from bacterial cells.

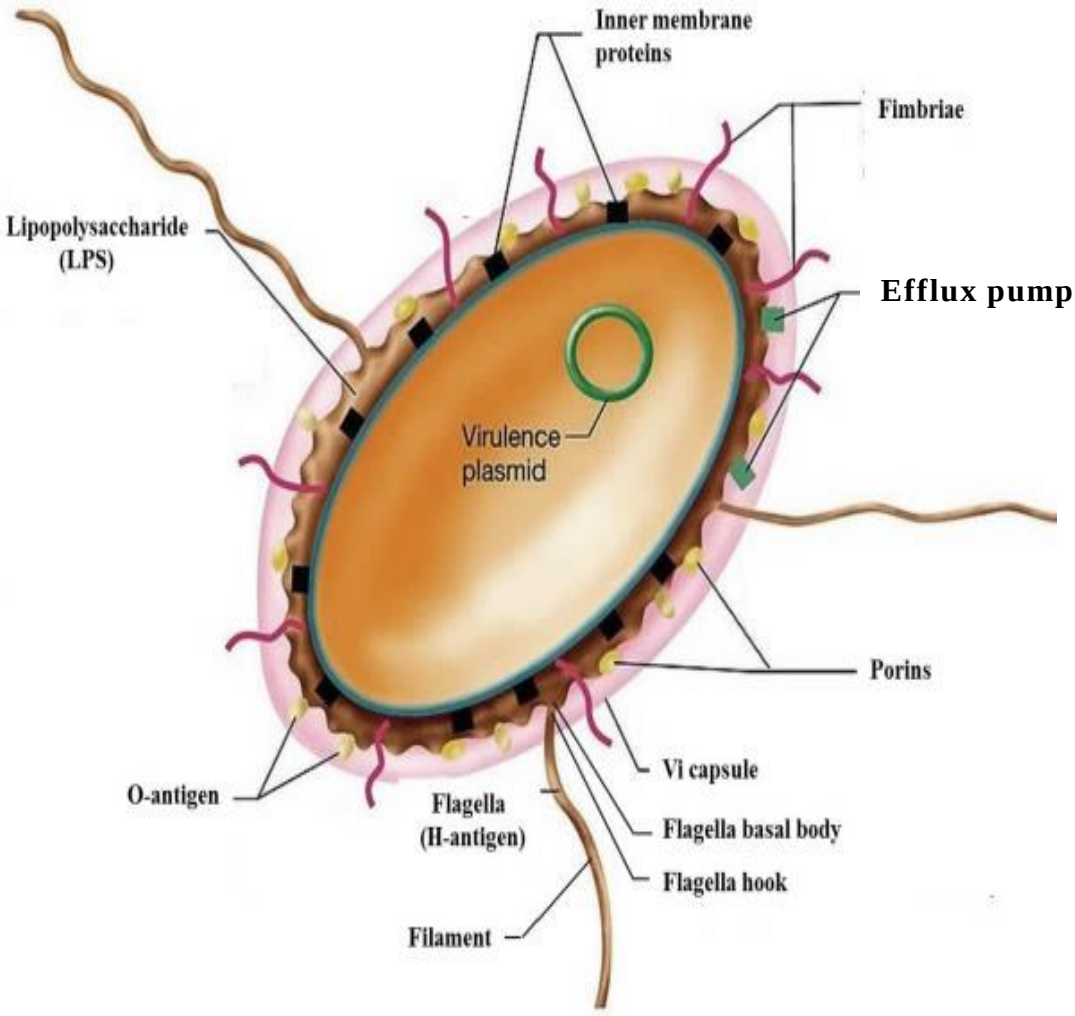

Figure 2.7: Structure of *Salmonella* Typhi.

(Figure adapted and modified from from University of British.Columbia.http://wiki.ubc.ca/File:*Salmonella_virulence_factors 1.j*).

Multidrug efflux pumps of *Salmonella* cause an obstruction to the treatment of disease because the efflux pumps facilitate the removal of structurally different substrates from the bacterial cell and giving an effective protection against antimicrobials (Nishino *et al.*, 2009). The efflux pumps