IDENTIFICATION OF ANTIGENIC PROTEINS OF *Salmonella enterica* subspecies *enterica* serovar Typhi IN SERA OF PATIENTS WITH ACUTE TYPHOID FEVER

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IDENTIFICATION OF ANTIGENIC PROTEINS OF *Salmonella enterica* subspecies *enterica* serovar Typhi IN SERA OF PATIENTS WITH ACUTE TYPHOID FEVER

by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

-	Negative or minus
%	Percentage
+	Positive
<	Less than
>	More than
~	Approximately
1X	1 time
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
2D-PAGE-WB	Two-dimensional polyacrylamide gel electrophoresis-Western blot
bp	Base pair
CMI	Cell-mediated immunity
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
E. coli	*Escherichia coli*
Eff	Efficiency
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
g	Gram
HAP	High abundance protein
HRP	Horseradish peroxidase
IEF	Isoelectric focusing
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
INF	Interferon
IPG	Immobilised pH gradient
IS	Immune system
kDa	Kilo Dalton
L	Liter
LA	Luria agar
LAP	Low abundance protein
LB	Luria broth
LC	Liquid chromatography
LPS	Lipopolysaccharides
M	Molar
mA	Milliampere
MALDI	Matrix-assisted laser desorption/ionisation
MDR	Multidrug resistant

mg	Milligram
MHC	Major histocompatibility complex
mL	Milliliter
mM	Millimolar
mm	Millimeter
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
ng	Nanogram
NPV	Negative predictive value
ºC	Degree Celsius
OD	Optical density
OMPs	Outer membrane proteins
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Power of hydrogen
pI	Isoelectric points
PNHS	Pooled Normal Human Sera
PPV	Positive predictive value
PTFS	Pooled Typhoid Fever Sera
PVDF	Polyvinyl difluoride
QTRAP	Hybrid triple quadrupole/linear ion trap
SD	Standard deviation
S. Typhi	*Salmonella* Typhi
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDS-PAGE-WB	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis-Western blot
SPIs	*Salmonella* pathogenicity islands
Th	T-helper cells
™	Trademark
TOF	Time-of-flight
TTSS	Type three secretion system
V	Volt
v/v	Volume per volume
w/v	Weight per volume
xg	Relative centrifugal force
α	Alpha
β	Beta
γ	Gamma
µA	Microampere
µg	Microgram
µL	Microliter
µm	Micrometer

PENGENALPASTIAN PROTEIN ANTIGENIK *Salmonella enterica* subspecies *enterica* serovar Typhi DALAM SERUM PESAKIT DEMAM KEPIALU

ABSTRAK

Demam kepialu disebabkan oleh sejenis bakteria Gram-negatif, *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). Penyakit ini berleluasa di seluruh dunia terutamanya di negara-negara yang mundur atau sedang membangun. Oleh sebab kekurangan biopenanda yang sensitif dan spesifik di pasaran, diagnosis demam kepialu masih kekal sebagai satu masalah kepada pengamal kesihatan. Ini mungkin kerana biopenanda tersebut dikenalpasti dalam keadaan *in vitro* menggunakan kultur mikrobiologi. Infeksi adalah satu proses dinamik dan penghasilan antigen bergantung kepada cabaran persekitaran yang dihadapi oleh bakteria. Dalam kajian ini, serum pesakit demam kepialu digunakan sebagai sumber antigen *in vivo* kerana ia mengandungi produk fisiologi daripada perumah dan bakteria yang dihasilkan ketika infeksi. Namun demikian, kewujudan protein manusia dalam kuantiti yang banyak (HAPs) di dalam serum pesakit akan menghalang pengesanan protein antigenik yang wujud dalam kuantiti yang sedikit (LAPs) dan berat molekul rendah, yang mungkin mengandungi biopenanda yang berkaitan dengan penyakit tertentu. Serum daripada pesakit demam kepialu (PTFS) dan serum daripada manusia normal (PNHS) diproses menggunakan 2 kaedah, iaitu 1) afiniti kromatografi untuk memisahkan albumin dan immunoglobulin G (IgG) daripada serum, dan 2) ultraturasan membran untuk memisahkan protein berat molekul lebih daripada 100 kDa daripada serum. Hasilan pemprosesan serum telah dianalisa menggunakan 2 kaedah, iaitu 1) SDS-PAGE-Western blot, dan 2) 2D-PAGE-Western blot. Hasil kajian menunjukkan kaedah pemprosesan serum yang

berlainan memberi profil biopenanda yang berlainan. Antigen berkaitan dengan demam kepialu yang dijumpai dalam protein PTFS telah dicirikan menggunakan analisa mass spectrometri dan 11 protein *S.* Typhi dikenalpasti menggunakan LC-ESI-MS/MS. Analisa bioinformatik digunakan untuk memilih protein *S.* Typhi yang berpotensi untuk digunakan sebagai biopenanda diagnosis demam kepialu. Berdasarkan markah ramalan keantigenikan yang tinggi, dan peratusan persamaan jujukan dengan *Escherichia coli* (*E. coli*) yang rendah, 6 protein *S.* Typhi, iaitu blue copper oxidase (CueO), outer membrane protein C (OmpC), transaldolase B (TalB), cell invasion protein A (SipA), flagellar hook-associated protein 1 (FlgK), dan hemolysin E (HlyE) telah dipilih untuk kajian seterusnya. Protein-protein rekombinan (rCueO, rOmpC, rTalB, rSipA, rFlgK, dan rHlyE) telah dihasilkan menggunakan teknik pengklonan DNA untuk mendapatkan kuantiti antigen yang mencukupi bagi pembangunan Western blot dan ELISA tidak langsung yang bertujuan untuk menguji potensi diagnostik mereka. Analisa Western blot menggunakan satu panel serum yang terdiri daripada 4 demam kepialu, 2 bukan demam kepialu dan 2 manusia normal menunjukkan hanya rCueO, rOmpC, rFlgK, dan rHlyE bertindakbalas dengan antibodi hanya daripada subjek demam kepialu, dan bukannya daripada subjek bukan demam kepialu dan manusia normal. ELISA tidak langsung digunakan untuk menguji satu panel serum yang terdiri daripada 30 pesakit demam kepialu, 30 pesakit bukan demam kepialu, dan 30 manusia normal. Hasil kajian menunjukkan keempat-empat biopenanda ini adalah spesifik untuk demam kepialu dengan spesifikasi lebih daripada 90%. Namun demikian, rHlyE mempunyai sensitiviti diagnostik yang paling tinggi iaitu 90% berbanding dengan rCueO (50%), rOmpC (50%), dan rFlgK (50%). Maka, rHlyE boleh digunakan sebagai biopenanda yang tidak ternilai untuk diagnosis demam kepialu.

IDENTIFICATION OF ANTIGENIC PROTEINS OF *Salmonella enterica* subspecies *enterica* serovar Typhi IN SERA OF PATIENTS WITH ACUTE TYPHOID FEVER

ABSTRACT

Typhoid fever is caused by a Gram-negative bacteria, *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). It is a worldwide disease infecting mostly the under-developed and developing countries. Due to a lack of sensitive and specific biomarkers available in the market, diagnosis of typhoid fever remains a problem for the clinician. This may be due to the fact that these biomarkers were identified using pure microbiological cultures grown in *in vitro* conditions. Infection is a dynamic process and the expression of antigens depend on the environmental challenges faced by the bacteria. This study used serum as a source of *in vivo* antigens as it contains physiological signatures from both host and bacterial origin that are produced *in vivo* during infection. However, the presence of high abundance proteins (HAPs) in human serum masks the detection of low abundance proteins (LAPs) and low molecular weight proteins, which contain candidate protein biomarkers relevant to a particular disease state. Pooled Typhoid Fever Sera (PTFS) and Pooled Normal Human Sera (PNHS) were subjected to 2 different serum separation methods, i.e. 1) affinity chromatography to remove albumin and immunoglobulin G (IgG), and 2) membrane ultrafiltration to remove proteins with molecular weights greater than 100 kDa. These protein preparations were then subjected to 2 protein analysis methods, i.e. 1) SDS-PAGE-Western blot, and 2) 2D-PAGE-Western blot. The results showed that different separation methods gave different biomarker profiles. Typhoid-related antigens found in PTFS were

characterised using mass spectrometry analysis and 11 *S.* Typhi proteins were successfully identified using LC-ESI-MS/MS. Bioinformatic analyses were used to select *S.* Typhi proteins as potential biomarkers for diagnosis of typhoid fever. Based on high antigenicity prediction scores and low percentage sequence similarity with *Escherichia coli* (*E. coli*), 6 *S.* Typhi proteins, namely, blue copper oxidase (CueO), outer membrane protein C (OmpC), transaldolase B (TalB), cell invasion protein A (SipA), flagellar hook-associated protein 1 (FlgK), and hemolysin E (HlyE) were selected for further studies. Recombinant proteins (rCueO, rOmpC, rTalB, rSipA, rFlgK, and rHlyE) were produced using DNA cloning technique to obtain sufficient antigens for development of Western blot and indirect ELISAs to evaluate their diagnostic potential. Western blot analysis using a panel of test sera consisting of 4 typhoid fever, 2 non-typhoid fever and 2 normal human subjects showed that only antibodies from typhoid fever sera reacted with rCueO, rOmpC, rFlgK, and rHlyE, but not from non-typhoid fever and normal human subjects. Indirect ELISAs were used to test a panel of sera consisting of 30 typhoid fever, 30 non-typhoid fever, and 30 normal human subjects. The results showed that all 4 biomarkers were specific for diagnosis of typhoid fever with specificity of more than 90%. However, rHlyE had the highest diagnostic sensitivity of 90% compared to rCueO (50%), rOmpC (50%), and rFlgK (50%). Thus, rHlyE can serve as an invaluable biomarker for the diagnosis of typhoid fever.

CHAPTER 1

INTRODUCTION

1.1 Overview of Typhoid Fever

Typhoid fever, also known as enteric fever, is caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). It is a facultative anaerobic Gram-negative bacteria which belongs to the family of *Enterobacteriaceae*. It is rod-shaped in structure, non-spore forming, capsulated, and possesses peritrichous flagella for motility. Among the 2,700 serotypes in the *Salmonella* species, *S.* Typhi is the most host-restricted pathogen infecting humans, and not non-primate vertebrates (Edsall *et al.*, 1960; Raffatellu *et al.*, 2005a). This human-restricted pathogen has made studies of this bacterium and its pathogenicity very difficult.

The incubation period for typhoid fever ranges from 10 to 12 days depending on the infecting dose of the bacilli and the host immune response (Huang and DuPont, 2005). *S.* Typhi is a multi-organ pathogen that inhabits the lymphatic tissues of the small intestine, liver, spleen and bloodstream of infected humans, which leads to complications, such as ileal perforation (Chalya *et al.*, 2012), typhoid encephalopathy (Stanley *et al.*, 2008), and hepatobiliary manifestations (Jagadish *et al.*, 1994). Untreated typhoid fever cases result in mortality rates ranging from 10 to 30%, while treated cases result in 99% survival (Crump *et al.*, 2004).

Any person can get typhoid fever, but those who travel, especially to under-developed and developing countries are at increased risk due to poor sanitary systems, food hygiene, and water quality (Steinberg *et al.*, 2004; Muti *et al.*, 2014).

This is because the disease is mainly spread through fecal-oral route. The scenario is further accentuated by the present in society of 3 to 5% previously infected individuals who show no sign of the disease, but at the same time harbor the bacterium. These asymptomatic chronic carriers of the bacteria shed viable organisms in their urine or stools that can lead to outbreaks of the disease when food, prepared by the carriers, are consumed by the rest of the population (Senthilkumar and Prabakaran, 2005; Abera *et al.*, 2010).

In spite of the development of diagnostics and treatment for typhoid fever over the past 50 years, the disease remains a public health concern of global magnitude today (Buckle *et al.*, 2012). This is mainly due to the lack of sensitive and specific diagnostic biomarkers available (Keddy *et al.*, 2011), the inefficient identification of typhoid carriers (Baker *et al.*, 2010), the emergence of multidrug resistant strains (Senthilkumar and Prabakaran, 2005), and the lack of effective vaccines (Basnyat *et al.*, 2005). Moreover, its signs and symptoms are similar to that of other febrile diseases, such as malaria, dengue fever, leptospirosis and melidiosis, making clinical diagnosis of the disease to be difficult (Zhou and Pollard, 2010).

1.2 Background and History of Typhoid Fever

Typhoid fever has been known for centuries. However, clinically it is indistinguishable from and frequently confused with typhus fever due to similar clinical features, such as prolonged febrile illness. Thus, the name typhus-like fever was given to typhoid fever (Singh, 2001). In 1695, English physician, Thomas Willis documented the clinical description and symptoms of the disease. The progression of the disease was described by the French physician, Pierre-Fidèle Bretonneau in the

year 1819 through his observation of the changes of the lesions on the mucous membrane. He also reported that typhoid fever was transmitted by contact with infected sources, such as people, food, faeces, and etc. (JAMA, 1968). In 1829, the French physician, Pierre Charles Alexandre Louis proposed the name 'typhoid fever', and in 1856, William Budd hypothesised that the disease was spread through contaminated water (Moorhead, 2002). The discovery of the bacillus suspected of causing typhoid was first described by Carl Joseph Eberth in 1880. His finding was later confirmed by pathologist Georg Theodor August Gaffky in 1884 who improved the culture method for isolating the bacillus (August and Konert, 1993).

The first documented American carrier, Mary Mallon was identified in the year 1907. She was a cook in New York, and was closely associated with 47 illnesses and 3 deaths. The most deadly carrier was Tony Labella who caused 122 infected cases and 5 deaths (Emmeluth *et al.,* 2009). Both of these documented cases had no history of the carriers, having a previous clinically-detectable typhoid fever. In order to prevent the disease, Almroth Edward Wright developed an effective heat inactivated whole-cell typhoid vaccine in 1896 (Plotkin, 2011). Antibiotics were also introduced in clinical practice in 1948 (Woodward *et al.,* 1950). Together with the vaccination strategies, antibiotic treatments, and proper sanitation managements, most developed countries showed declining rates of typhoid fever in the 20th century, and some developed countries have successfully eradicated the disease. Nevertheless, typhoid fever continues to be a disease that affects population in many under-developed and developing countries today.

1.3 Epidemiology of Typhoid Fever

1.3.1 Global disease burden

In 2010, the worldwide incidence of typhoid fever was estimated to be 13.5 million but the actual incidence could be higher due to the lack of sensitivity in blood culture test for *S.* Typhi, and thus the adjusted estimation for typhoid fever incidence could be as high as 26.9 million with 269,000 deaths as proposed by Buckle *et al*. (2012). Asia was reported to have the highest incidence rate of typhoid fever, especially in South-central and South-eastern Asia, including Malaysia, India, Pakistan, and Bangladesh with more than 100 cases per 100,000 population in the year 2000 (Crump *et al.*, 2004) (Figure 1.1). However, according to the data collected by the Ministry of Health, Malaysia was classified as a low endemic region for typhoid fever with an annual incidence rate of less than 10 cases per 100,000 population from year 1995 to 2014 (Figure 1.2). Among all the states in Malaysia, Kelantan has the highest incidence rate of typhoid fever and classified as a medium endemic region (10-100 per 100,000) (Shah *et al.*, 2012). The annual typhoid incidence estimated in year 2001 for Kelantan was 37 per 100,000 (Wan Mansor *et al.*, 2005), which declined 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 year 2010 (Figure 1.3). This was attributed to introduction of proper monitoring and surveillance systems by the Kelantan State Public Health Department (Wan Mansor *et al.*, 2011). As shown in Figure 1.3, an outbreak of typhoid fever occurred in the year 2005 following a major flood in the state. It was reported that wells were contaminated with sewage overflow, and rural communities which depended on wells for water supply were infected (Shah *et al.*, 2012).

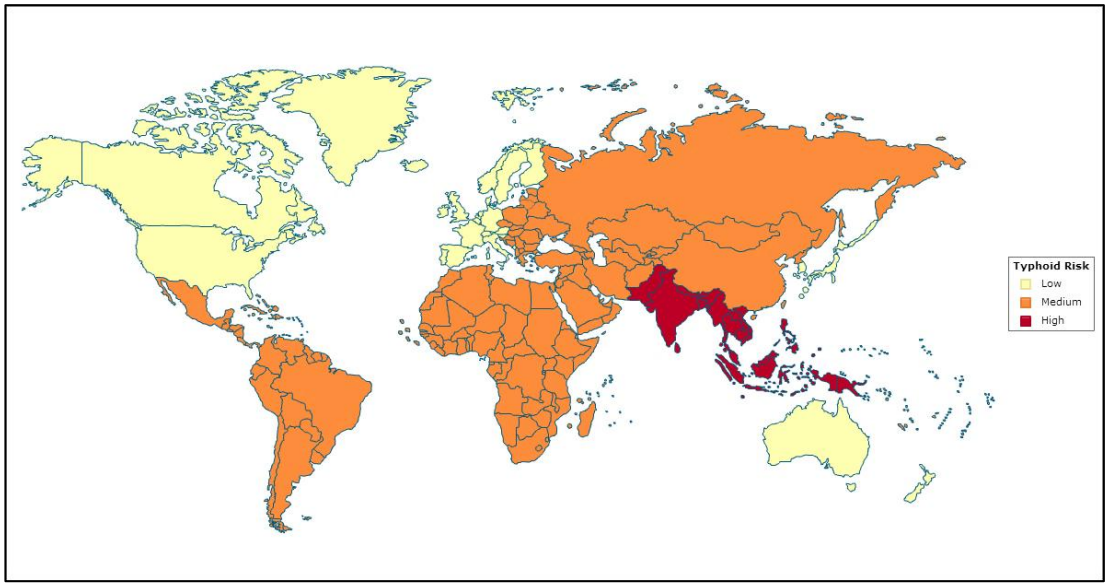

Figure 1.1: Global burden of typhoid fever (Adapted from Crump *et al*. 2004).

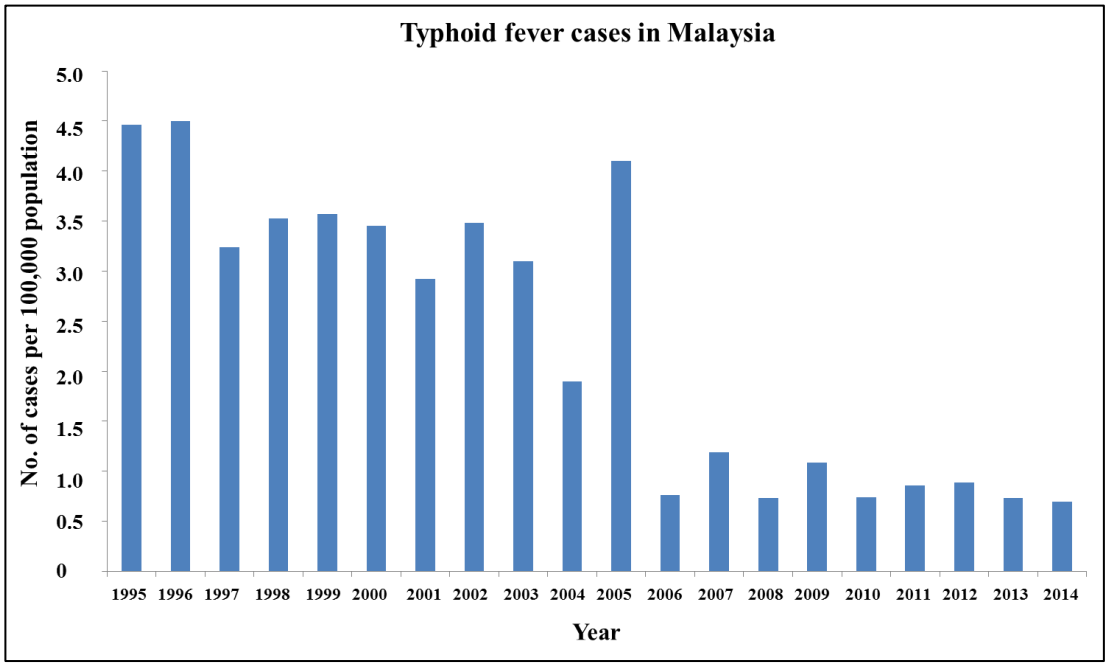

Figure 1.2: Trend of typhoid fever cases in Malaysia from year 1995 till 2014 (Data from Ministry of Health, Malaysia, 2015).

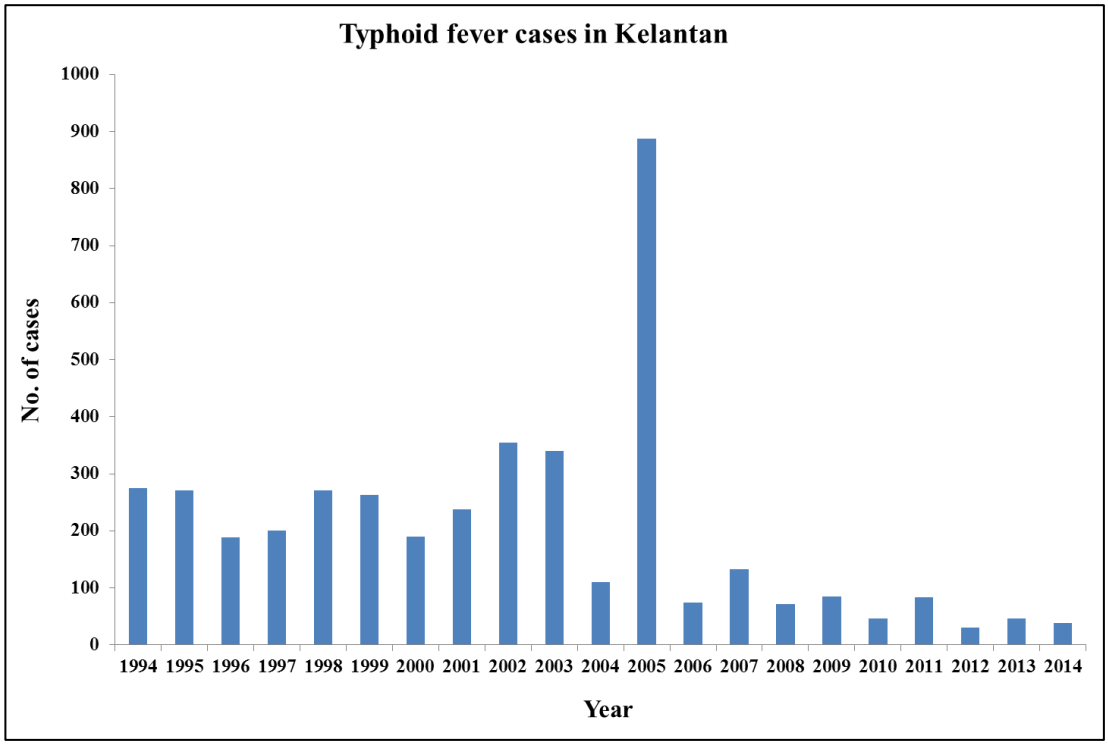

Figure 1.3: Trend of typhoid fever cases in Kelantan from year 1994 till 2014 (Data from Kelantan State Public Health Department, 2015).

Typhoid fever affects any age group but it is considered to be a disease mainly of children and young adults. Majority of typhoid fever patients in Kelantan were from the age group of 5 to 14 years old (Shah *et al.*, 2012; Ja'afar *et al.*, 2013). However, in some endemic countries, such as India, Indonesia and Pakistan, the incidence of typhoid fever amongst pre-school children, aged 2 to 5 years, were the highest (Sinha *et al.*, 1999; Ochiai *et al.*, 2008). Older people appeared to be relatively immune to the disease, presumably because of their frequently-reinforced acquired immunity through numerous sub-clinical exposures to the typhoid bacilli (Singh *et al.*, 2007).

1.3.2 Typhoid carriers

Typhoid carriers are asymptomatic persons, diagnosed through bacteria culture methods confirming the presence of *S.* Typhi in their stool or urine even a year after the acute typhoid fever episode. Approximately 3 to 5% of acute typhoid fever adults become carriers of the disease (Ismail, 2006). The carrier state of typhoid fever was observed to be higher in women in the age group 15 to 45 years (Levine *et al.*, 1982; Senthilkumar and Prabakaran, 2005). Large-scale investigations for typhoid carriers have been carried out particularly among food handlers. These studies demonstrated that food handlers harbor *S.* Typhi asymptomatically at the rate of 3.2% in Accra, Ghana (Mensah *et al.*, 2002), 2.3% in Kumasi, Ghana (Feglo *et al.*, 2004), 1.6% in Northwest Ethiopia (Abera *et al.*, 2010), and 2% in Malaysia (Pang and Puthucheary, 1983). Thus, food handlers in these developing countries may be reservoir for the bacteria because no animal carrier of the disease has been reported. The percentage of typhoid carriers could be much higher due to the lack of sensitivity in culture method. The stool culture method is the 'gold standard' for *S.* Typhi detection in typhoid carriers, and has a low sensitivity of less than 60% (Gopinath *et al.*, 2012).

The detection sensitivity for stool culture has not increased considerably in the last few decades. Amongst carriers, stool culture is more difficult due to the low numbers of viable bacteria present in the stool, and other procedural limitations, such as the need for enrichment media, and presence of other bacteria and normal flora.

During an outbreak, the source of the pathogen is usually food handlers with poor hygiene and inadequate knowledge about food safety. This is because most food handlers working in the kitchen are young workers including children, which means the majority are inexperienced or have low educational background. Also, most of them do not have medical checkup including stool examination to exclude the possibility of carrier status. Thus, health education intervention must be strengthened to ensure food safety during processing and storage in the food service industry (Abera *et al.*, 2010). Recently, in October 2015, an outbreak of typhoid fever was reported in Kuala Lumpur, and the State Public Health Department found illegal foreign workers in the local market who were without proper health screening and typhoid vaccination. They were also found to have poor personal hygiene, and their shelters were illegal construction sites which lack cleanliness and proper water supply (Low *et al.*, 2015). This incidence underscores the need to address the socio-economic problems of the people as they are linked to carriers which are human reservoirs for the disease. As a preventive measure, thousands of screening tests were conducted among food handlers in the state to identify the typhoid carriers.

1.3.3 Multidrug resistant strains of *S.* Typhi

The first multidrug resistant (MDR) strain of *S.* Typhi emerged in Southeast Asia in the late 1980s, and continued to increase and spread throughout the region (Mirza *et*

al., 1996). MDR *S.* Typhi that are resistant to all first-line antibiotics, such as chloramphenicol, ampicillin, and trimethoprim sulphamethoxazole have been associated with severe illness, higher rates of complications and deaths, especially in children under the age of 2. They were also associated with a higher probability of becoming asymptomatic carriers after recovery (Parry *et al.*, 1998). This has led to the use of second-line antibiotics such as fluoroquinolones (ciprofloxacin and ofloxacin), and cephalosporins (ceftriaxone) as alternative drugs (Capoor and Nair, 2010), but emergence of resistance even to this second-line of antibiotics has been reported in Vietnam (Parry *et al.*, 1998), Bangladesh (Ahmed *et al.*, 2006), and India (Thamizhmani *et al.*, 2012). Currently, there is no standard therapy for third-line antibiotics, raising concerns about further spread of the MDR strains to other endemic regions.

A recent study by Wong *et al.* (2015) showed that whole-genome analysis of 1,832 *S.* Typhi isolates from all over the world identified a single dominant MDR lineage, the H58 haplotype. The researchers at Sanger Institute found that the ancestral MDR strain originated from the Indian Continent and has spread to other parts of the world, including Southeast Asia, Western Asia and East Africa, where it has rapidly replaced local *S.* Typhi strains over the last 30 years, and caused high morbidity and mortality typhoid in these countries today (Wong *et al.*, 2015). In Malaysia, even though the incidence rate of the H58 haplotype is less than 1% of the isolates studied (Ja'afar *et al.*, 2013), it is possible that Malaysia might experience the same "epidemic of transmission" as her neighboring countries in the future.

1.3.4 Typhoid vaccination

There are currently 3 licensed typhoid vaccines: 1) heat-phenol inactivated whole cell parenteral vaccine, 2) a mutant *S.* Typhi Ty21a strain used as live attenuated oral vaccine, and 3) purified Vi polysaccharide parenteral vaccine. In a 3-year study of the cumulative efficacies of these vaccines, 2 doses of whole cell parenteral vaccine was found to be more effective than 3 doses of the Ty21a oral vaccine, or 1 dose of the Vi parenteral vaccine. However, whole cell vaccine is more frequently associated with adverse side effects and are highly reactogenic, causing high fever, abdominal pain, vomiting, and diarrhea (Engels *et al.*, 1998). The Ty21a oral vaccine provides protection up to 7 years, whereas the whole cell and Vi parenteral vaccine provide good protection for 3 years (WHO, 2015). Due to the limitations of these vaccines, the Vi capsular polysaccharide vaccine was modified and conjugated with recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA) (Szu *et al.*, 1987), diphtheria toxoid, or tetanus toxoid (Ali *et al.*, 2012) as carrier proteins to improve the efficacy of the vaccines for typhoid fever (Szu, 2013).

The introduction of typhoid fever vaccines in routine vaccination programs in various parts of the world has been highly beneficial in reducing the burden of the disease in terms of lost earnings for the individuals and cost of healthcare to the government. But, so far, only 2 countries, i.e. China and Vietnam, have incorporated typhoid vaccination into their routine immunisation programs, and only in a limited fashion. The reason why these efforts have not been more widely accepted lies in part to the fact that most developing countries are uncertain of the real burden of typhoid fever, due to the lack of rapid diagnostic tools, infrequency of laboratory testing, and poor national reporting system. In the developed world, vaccination

efforts face obstacles from well-organised anti-vaccination movements which claim that in societies where the prevalence of vaccine-preventable diseases are minimal, the side effects of vaccines pose a greater health threat than the diseases themselves (Bloom, 2011).

The nature of the antigen and the immune response produced are major factors in the success of a vaccination program. Current typhoid vaccines induce a predominantly humoral immune response which does not appear to eliminate the bacteria completely, especially those residing in niche areas in the body where the antibody produced has limited effect. Even when some vaccines elicit cell-mediated immune response, the memory is short-lived. These form major obstacles in the long term control and management of typhoid fever as the bacteria is not completely eliminated from the host. It could either be due to defects in the immune response of the individuals or the cleverness of the bacteria in evading the immune system of the host. Such individuals remain as healthy carriers of the disease and help the bacteria spread and establish itself in endemic areas.

1.3.5 Mode of transmission

S. Typhi is a host-restricted pathogen where humans are the only natural host and reservoir of the bacteria (Raffatellu *et al.*, 2005a). It can survive for a long time in fresh water and in cold conditions. Thus, untreated water and unhygienically prepared food are at significant risk. Shellfish taken from contaminated water, raw fruits and vegetables fertilised with sewage, contaminated milk and milk products, and foods exposed to flies are normally contaminated with *S.* Typhi (WHO, 2014). Personal hygiene especially among food handlers should also be monitored by the

Public Health Department as chronic carriers harbor *S*. Typhi in their gallbladder and continually excrete millions of the pathogenic bacteria in their faeces.

Pathogenesis of acute typhoid fever involves the enterohepatic circuit. Severe and prolonged typhoid fever infection is associated with cholecystitis and cholelithiasis. The blockage of the cystic duct with gallstones results in jaundice and accumulation of bile in the gall-bladder. This further enhances the establishment of chronic carriage as *S.* Typhi develops resistance to bile, and has the ability to form biofilms on gallstones. This helps the bacteria to settle in the biliary passages (gall-bladder) and becomes its niche habitat. The bacteria that are excreted in the bile and biofilm, can transition into planktonic (free swimming) cells, and then reinvade the intestinal wall, or are excreted with feces leading to infection of other individuals (Losonsky *et al.*, 1987; Senthilkumar and Prabakaran, 2005).

Outbreaks of typhoid fever may occur amongst institutionalised patients as within the institutionalised population there is an increased prevalence of chronic diseases that decreases host defense mechanisms against *Salmonella,* such as disorders of gastric acidity, intestinal motility, and depressed cell-mediated immunity. The frequent use of antimicrobial agents amongst inmates also decreases protective normal flora, and thus increase patient susceptibility (Rafii *et al.,* 2008). The highest mortality rates were recorded among children and the elderly due to outbreaks in nurseries and nursing homes as their immune responses are lower compared to normal healthy persons. Outbreaks at gatherings do occur due to inappropriate food handling and foods prepared in bulk off-site are likely to be contaminated during transportation.

1.4 Nomenclature of *Salmonella*

Salmonella was named after the American bacteriologist, D. E. Salmon who first isolated *Salmonella choleraesuis* from porcine intestines in 1884 (Smith, 1894). Unlike other bacterial genera, *Salmonella* organisms are differentiated by serotyping analysis. Even today, new serovars are still being discovered each year which increase the complexity of this very large bacterial population (Lin and Cheng, 2007). The latest report published in year 2014 showed that there are over 2,700 serovars in the genus *Salmonella* that are found in different hosts and environments (Ezzat *et al.*, 2014).

Seven *Salmonella* subspecies (I, II, IIIa, IIIb, IV, V, VI) were identified based on antibody reactions (Le Minor and Popoff, 1987) (Table 1.1). Six of the subspecies belonging to the *Salmonella enterica* species and were classified into groups: I, *enterica*; II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *houtenae;* and VI, *indica*. Formerly, *S. bongori* belonged to subspecies V, but later it was considered as a separate species based on differences demonstrated by DNA relatedness studies using DNA-DNA hybridisation method in year 1989. In 2005, a new species, *Salmonella subterranean* was recognised (Lin and Cheng 2007).

The classification system based on serovars in subspecies I are designated by a name usually indicative of a geographic origin or usual habitat. For the remaining subspecies, antigenic formulae including O (somatic) antigens, H (flagellar) antigens (phase 1), and H (flagellar) antigens (phase 2, if present) are determined according to the Kauffmann-White scheme for serotype naming purposes (Table 1.1). A colon is used to separate each antigen (Lin and Cheng, 2007).

Table 1.1: Current *Salmonella* nomenclature (Adapted from Lin and Cheng 2007).

Taxonomic position (writing format) and nomenclature			
Genus (capitalised, italic)	**Species (italic)**	**Subspecies (italic)**	**Serotypes (or serovars) (1st alphabet capitalised, not italic)**
Salmonella	*enterica*	*enterica* (or subspecies I)	Typhi (9,12,Vi:d:-)
		salamae (or subspecies II)	9,46:z:z39
		arizonae (or subspecies IIIa)	43:z29:-
		diarizonae (or subspecies IIIb)	6,7:l,v:1,5,7
		houtenae (or subspecies IV)	21:m,t:-
		indica (or subspecies VI)	59:z36:-
	bongori	subspecies V	13,22:z39:-
	subterranea		

For example, based on the established current nomenclature for *Salmonella* genera, *Salmonella enterica* subspecies *enterica* serovar Typhi can also be called *Salmonella* Typhi or *S.* Typhi. According to the Kauffmann-White scheme, *S.* Typhi can be named as *Salmonella* subspecies I, 9, 12, Vi:d:- as it is categorised under subspecies *enterica* with the presence of O-9 O-antigen, O-12 O-antigen, and virulence Vi polysaccharide antigen at the cell wall, with only phase 1 d-H antigen, and no phase 2 H-antigen.

1.5 Laboratory Diagnosis of Typhoid Fever

Many diagnostic tests are currently available in the market for typhoid fever. They are antigen-, antibody- or DNA-based. Antigen- and DNA-based tests are important for early diagnosis, i.e. first week of infection and their sensitivities depend on the number of bacteria presents in the circulation. When immune system recognises these foreign antigens, it activates B-cells to produce antibodies to kill the invading pathogens. Bacterial proteins and DNAs are degraded and fragmented, which may results in false negative identification if antigen- and DNA-based tests are used for detection. At this stage, antibody-based tests are more suitable for probable identification as clones of B-cells are produced, and IgM antibody peaks at 7-10 days of infection, followed by IgG antibody at 14-21 days of infection. Since typhoid fever has long incubation period (10-12 days) and the disease usually subsides after 2 months, antibody-based test might provide a greater value in diagnosis of the disease.

1.5.1 Culture methods

Bacterial culture in blood still remains the ‘gold standard’ for detection of typhoid fever as it has a specificity of 100% (Keddy *et al.*, 2011). Culture bottles containing

culture medium are incubated with 10 mL of patients' blood at 37ºC, and are checked for a period of 7 days for evidence of bacterial growth through turbidity or gas formation. Positive cultures are sub-cultured on agar plates to encourage the growth of fastidious microorganism, whereas negative cultures are discarded after 7 days. In order to identify or isolate *S.* Typhi colonies grown in the bottle, different selective or differential media are used, such as *Salmonella-Shigella* (SS) agar, deoxycholate-citrate (DCA) agar, xylose lysine deoxychoate (XLD) agar, and hektoen enteric (HE) agar. The bacterial colonies are differentiated from other *Enterobacteriaceae* by the pattern of growth and by further biochemical tests, such as triple sugar iron (TSI) medium, sulfide-indole-motility (SIM) medium, urease, citrate, and methyl-red & Vogues-Proskauer (MR-VP) tests (WHO, 2003). The results of blood culture are greatly affected by antibiotic treatments prior blood collections, and thus bone marrow cultures may provide positive identifications (Farooqui *et al.*, 1991). Also, bacteria culturing method could be done using samples from stool and urine for detection of the bacterium, *S.* Typhi. Stool culture may be positive in second week and urine culture from the third week of the infection (Abd Elseed, 2015).

1.5.2 Serotyping

Salmonella bacteria look alike under the microscope. However, there are over 2,700 serotypes of *Salmonella* that exist all around the world, which can be differentiated based on 2 antigenic structures on their cell surface, namely, the lipopolysaccharide O-antigen and the flagella H-antigen using the Kauffman-White scheme. Each of the O- and H-antigens have a unique code number. There are 46 O-antigens and 119 H-antigens that have been reported, and the distinct combination of these 2 antigens

help scientists to determine each *Salmonella* serotype (CDC, 2015), and used in current *Salmonella* nomenclature system as describe in Section 1.4.

1.5.3 Slide agglutination test – Widal test

Serological tests based on antibody detection provide a convenient laboratory method for detection of *S.* Typhi. The Widal test is an old serological assay for detecting antibodies to the O-9, O-12, and d-H antigens of *S.* Typhi based on the principal of bacterial cell agglutination (Widal, 1896). Antibody levels are considered significant at dilution of greater than or equal to 1:160, but different cut-off titers have been reported in the literature (Bakr *et al.*, 2011). Serum agglutinins rise abruptly during the second week (day 6-8) of infection for the O-antigen, and the third week (day 10-12) for H-antigen (Kundu *et al.*, 2006). If the blood is collected too early in the disease, false negative results may occur. Thus, testing a paired serum sample obtained at intervals of 7 to 10 days to read a four-fold rise in antibody level carries greater significance than single test (Sansone *et al.*, 1972). The Widal test is widely used in developing countries because of its low cost, suitability for large scale screening, simple to perform, and easy to interpret without the need for specialised or expensive equipment.

1.5.4 Enzyme immunoassay (EIA)

Another common assay used in laboratories today is the enzyme immunoassay (EIA). The detecting antigen used in typhoid EIA is usually purified extracts of *S.* Typhi cells. The antigen is derived from various subcellular structures of the organism, including lipopolysaccharide (Sippel *et al.*, 1987), outer membrane proteins (Barrett *et al.*, 1983; Verdugo-Rodriguez *et al.*, 1993a), flagella antigen and

somatic antigen (Fadeel *et al.*, 2011), and virulence Vi antigen (Barrett *et al.*, 1983). It has been found that EIA-based tests are generally superior than the Widal agglutination test in terms of sensitivity and specificity besides being suitable for large scale screening (Lim *et al.*, 1998).

1.5.5 Rapid diagnostic test kits for typhoid fever

Many rapid diagnostic test kits with higher sensitivity than the blood culture method have been developed and are available in the market (Keddy *et al.*, 2011). Typhidot™ is a commercial EIA dot-blot test kit which detects host IgM and IgG antibodies against a 50 kDa outer membrane protein, reported to be specific for *S.* Typhi. It takes approximately 2 hours using minimum laboratory resources. Detection of IgM antibody alone indicates acute typhoid fever in the early phase of infection, while presence of both IgG and IgM suggests acute typhoid in the middle phase of infection (Ismail, 2000).

TUBEX™ (IDL Biotech, Sollentuna, Sweden) is a simple, rapid, one-step test that requires 2-10 minutes to perform. It is a semi-quantitative inhibition binding assay that detects the presence of IgM antibodies to *S.* Typhi O-9 lipopolysaccharide antigen by assessing the ability of the test serum to inhibit a reaction between 2 coloured antigen/antibody-coated reagents. It is reported that this antigen contained the rare sugar residue (α-D-tyvelose) which is present not only in *S.* Typhi but also to other *Salmonella* serogroup D bacteria (Lim *et al.*, 1998), and thus can give rise to cross-reactivity and false possibility.

1.5.6 Molecular diagnostic methods

Nucleic acid amplification technology (NAAT) is a platform to amplify and detect small amount of nucleic acids from as few as 10 microorganisms (Wain and Hosoglu, 2008). It is a DNA-based method to identify the presence of foreign microorganisms in the body. It requires a sample preparation step to reduce the inhibitory substances found in serum/blood, and to increase the concentration of the target DNA. Enrichment media, such as tetrathionate broth and selenite F broth, are used to assist the growth of *Salmonella* bacteria from faeces and sewage samples because of very large numbers of competing bacteria in these samples, especially *Escherichia coli* (*E. coli*) (Pedler and Orr, 1990). After enrichment, DNA is extracted from the bacteria and tested using polymerase chain reaction (PCR) assay, which include primers or oligonucleotides that target the specific nucleic acid sequence of the organism. The PCR products are then used in various platforms, including multiplex PCR (Pui *et al.*, 2011), loop-mediated isothermal amplification (LAMP) (Abdullah *et al.*, 2014), and lateral-flow dipstick assay (Nor Amalina *et al.*, 2014), for identification of *S.* Typhi.

1.5.7 The demerits of currently available diagnostic methods

Even though many diagnostic methods for typhoid fever are currently available in the market for detection of *S.* Typhi-specific antigens and genes, and detection of host immune response (antibodies) toward target antigens, there are many drawbacks to these methods. These problems caused delay in the diagnosis of typhoid fever, and thus affect the treatment and management of the patients.

Bacterial cell culture for *S.* Typhi is the 'gold standard' for typhoid fever. However, it has low sensitivity due to failure to isolate the microorganism attributed by several factors, including patient treatment with antibiotics, variable time of sample collection, inappropriate ratio of culture medium to volume of blood used (Das, 2007), inappropriate use of bacterial enrichment media, and sampling techniques (Chua *et al.*, 2015). Bacteremia occurs in the early stage of the disease and positivity of blood cultures are as high as 90% for the first week of infection, but reduces subsequently with an overall sensitivity of less than 50%, and drops considerably with prior antibiotic therapy. Bone marrow culture has sensitivity of 80%, and bacteria may be protected from the presence of systemic antibodies and antibiotics, but it requires an invasive procedure to obtain the marrow. Stool culture is usually not positive in the acute phase of infection, and about 5% remain positive in clinically cured or convalescent cases. However, it is important to monitor the carriage of *S.* Typhi after apparent clinical cure to identify typhoid carriers who act as reservoirs for the transmission of the disease. Bacteria cell culture methods are time consuming as it requires at least 4 to 7 days for laboratory detection depending on the size of the bacterial load (Akoh, 1991; Keddy *et al.*, 2011), and require multiple equipment and reagents for bacterial identification and differentiation. Thus, it is not suitable for large scale screening. These drawbacks call for the development of more sensitive, rapid and specific diagnostic tests.

Serotyping helps to discriminate the serotype in the genus *Salmonella*, and a collection of antisera against the O- and H-antigens are required to confirm a single serotype. Thus, it is a costly, laborious and low-throughput method. Furthermore, maintaining all the regents is cumbersome and quality control is difficult when

managing a full array of antisera make from immunising rabbits (McQuiston *et al.*, 2004). Rather as a diagnostic method, serotyping is more important for epidemiological surveillance studies, outbreak detection, and investigations.

To overcome the low-throughput of the culturing method, many rapid antibody-based detection tests, such as Widal, Typhidot™, and TUBEX™, were developed. However, due to variation of immune response in each individual and cross-reactivity of the antigens, these current diagnostic tests lack sensitivity and specificity (Table 1.2).

Using Widal test, misdiagnosis may occur as the semi-quantitative slide-agglutination test is unreliable, and display inter-operator variability with poor sensitivity, poor specificity, and low positive predictive value (PPV) (Keddy *et al.*, 2011). This is because the Widal test uses crude cell extract, and the antigens are poorly standardised. It also shares many antigenic determinants with other *Salmonella* species, *E. coli*, dengue virus, and *Plasmodium* pathogen (WHO, 2003; Verma *et al.*, 2014). Hence, false positive results may be more prevalent in endemic areas due to past history of exposure to these diseases, as well as immunisation with typhoid vaccine. Also, the presence of these cross-reacting antibodies alter the base-line cut-off antibody titer of the normal population, resulting in false negative results (Pang and Puthucheary, 1983).

Table 1.2: Sensitivities and specificities of current rapid antibody-based detection tests for typhoid fever

Diagnostic tests	Sensitivity (%)	Specificity (%)	Locations	References
Widal (O- and H-antigens)	71.4	68.4	Ethiopia	(Andualem *et al.*, 2014)
	43.9	65.0	Kolkata, India	(Das *et al.*, 2013)
	72.0	87.0	Amritsar, India	(Khanna *et al.*, 2015)
Widal (O-antigen)	80.9	51.8	Kathmandu, Nepal	(Adhikari *et al.*, 2015)
	87.3	6.9	South Africa and Tanzania	(Keddy *et al.*, 2011)
Widal (H-antigen)	72.2	58.3	Kathmandu, Nepal	(Adhikari *et al.*, 2015)
	95.2	13.8	South Africa and Tanzania	(Keddy *et al.*, 2011)
Typhidot™	59.6	80.0	Bangladesh	(Islam *et al.*, 2016)
	96.0	89.5	Amritsar, India	(Khanna *et al.*, 2015)
	75.0	60.7	South Africa and Tanzania	(Keddy *et al.*, 2011)
	70.0	80.1	Papau New Guinea	(Siba *et al.*, 2012)
	26.7	61.5	Pakistan	(Mehmood *et al.*, 2015)
TUBEX™	60.2	89.9	Bangladesh	(Islam *et al.*, 2016)
	76.0	96.0	Amritsar, India	(Khanna *et al.*, 2015)
	73.0	69.0	South Africa and Tanzania	(Keddy *et al.*, 2011)
	51.1	88.3	Papau New Guinea	(Siba *et al.*, 2012)

Rapid diagnosis kits (Typhidot™ and TUBEX™) are less sensitive than the Widal test (Keddy *et al.*, 2011). They also appear to correlate poorly with blood culture results in places such as sub-Saharan Africa (Keddy *et al.*, 2011), India (Dutta *et al.*, 2006), Vietnam (Olsen *et al.*, 2004), and Egypt (Fadeel *et al.*, 2011). The sensitivity of Typhidot™ test was reported to be higher at the onset of the first week of illness as compared to TUBEX™ test (Olsen *et al.*, 2004). This is probably due to the variation in the individual's immune response, whereby the time-lapsed between the onset of symptoms and serum collection can affect the performance of antibody-based tests.

It has been found that EIA- and PCR-based tests are superior to the Widal cell agglutination test, but the former are usually tedious and require multiple steps. In EIA, enzyme conjugates require refrigeration and electronic optical density (OD) readers are expensive, and thus unsuitable for those living in low resource settings (Lim *et al.*, 1998). While for PCR, the sample preparation step is time consuming and the DNA amplification step requires sophisticated instruments which are expensive (Wain and Hosoglu, 2008).

1.6 Host Immune System

The host immune system (IS) can be divided into 2 functional categories. The Non-specific or Innate IS, and the Specific or Adaptive IS. The Innate IS is the primary immune defense against invading pathogens, while the Adaptive IS is the secondary immune defense and also confers protection against re-infection with the same pathogen. Both categories have cellular and humoral components which help them to carry out their protective functions either by themselves or in concert with each other.

1.6.1 Non-specific immune system

The Non-specific immune defense system against microorganisms entering the body is also referred to as Innate IS because it is in-born. The elements of the Innate IS include physiological barriers, secretory molecules, and cellular components. The physio-chemical barriers and the normal flora in the body are effective in preventing invading pathogens from entering and colonising the body (Murphy, 2011). *Salmonella* Typhi enters the body via oral route, and thus it must be able to survive the low pH gastric acid barrier to reach the small intestine (Giannella *et al.*, 1972). Here, it needs to compete with the normal gut flora present in the gastrointestinal tract to invade the intestinal mucosa. The resident gut flora do not cause disease because their growth is kept under control by the host's defense mechanisms and by the presence of other microorganisms. However, when the growth of the normal flora is suppressed especially during antibiotic treatment, opportunistic agents such as *Salmonella* may be able to colonise the intestine and cause disease (NIH, 2007; Rafii *et al.*, 2008).

Once the pathogen enters the body, an acute response of the Innate IS to the infection is inflammation. It is stimulated by humoral factors such as complement which could increase the vascular permeability, and enhance the recruitment of phagocytic cells (macrophages and neutrophiles), dendritic cells, and natural killer cells to the site of infection. These would activate a cascade of intracellular events which result in the production of a large array of mediator molecules that will trigger Specific IS.

The cell surface of the phagocytic cells contained receptors called Pattern Recognition Receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide-