A STUDY OF THE ANTIGENICITY OF *Salmonella enterica* subspecies *enterica* serovar Typhi 50 KDa RECOMBINANT PROTEINS

TEH BOON AUN

UNIVERSITI SAINS MALAYSIA

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A STUDY OF THE ANTIGENICITY OF *Salmonella enterica* subspecies *enterica* serovar Typhi 50 kDa RECOMBINANT PROTEINS

by

TEH BOON AUN

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

-	Negative or minus
%	Percentage
^{o}C	Degree Celsius
®	Registered Trademark
+	Positive
<	Less than
>	More than
~	Approximately
1X	1 time
2X	2 times
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
A_{260}	Absorbance at 260 nm Wavelength
A_{280}	Absorbance at 260 nm Wavelength
ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
bp	Base pair
CCR6	Chemokine receptor type 6
$CaCl_2$	Calcium chloride
CMI	Cell-mediated immunity
DC	Dendritic cells
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DotEIA	Dot enzyme immunoassay
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
g	Gram
HAP	High abundance protein
HCl	Hydrogen chloride
HRP	Horseradish peroxidase

IAA	Trans-3-indoleacrylic acid
IEF	Isoelectric focusing
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
INF	Interferon
IPG	Immobilised pH gradient
IPTG	Isopropyl β –D-1-thiogalactopyranoside
IS	Immune system
kDa	Kilo Dalton
L	Liter
LA	Luria agar
LB	Luria broth
LC	Liquid chromatography
LF	Lateral flow
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
NADPH	Nicotinamide adenice dinucleotide phophate
ng	Nanogram
NPV	Negative predictive value
℃	Degree Celsius
OD	Optical density
OMPs	Outer membrane proteins
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Power of hydrogen
pI	Isoelectric points
PMSF	Phenylmethylsulfonyl fluoride
PNHS	Pooled Normal Human Sera
POC	Point of care
PPV	Positive predictive value
PRR	Pattern Recognition Receptor
PTFS	Pooled Typhoid Fever Sera
PVDF	Polyvinyl difluoride
®	Registered Trademark
RPM	Revolutions per minute
SD	Standard deviation
S. Typhi	*Salmonella* Typhi
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPIs	Salmonella pathogenicity islands
TNF	Tumor Necrosis Factor
Th	T-helper cells
™	Trademark
TOF	Time-of-flight
TTSS	Type three secretion system
V	Volt
v/v	Volume per volume
WB	Western blot
xg	Relative centrifugal force
α	Alpha
β	Beta
γ	Gamma
µA	Microampere
µg	Microgram (s)
µL	Microliter (s)
µM	Micromolar (s)

KAJIAN TERHADAP KEANTIGENAN

***Salmonella enterica* subspecies *enterica* serovar Typhi**

50 KDa PROTEIN REKOMBINAN

ABSTRAK

Protein membran luar (OMP) 50 kDa *Salmonella* Typhi dilaporkan antigenik dan telah digunakan untuk membuat kit Typhidot® yang berjaya dikomersilkan. Walau bagaimanapun spesifikasi Typhidot® mempunyai julat variasi antara 37.5 - 98.8%. Ini mungkin disebabkan oleh pencemaran protein lain semasa penyediaan protein membran luar 50 kDa dan ianya mungkin telah menjejaskan spesifikasi OMP 50 kDa. Oleh itu, objektif kajian adalah untuk menyiasat identiti protein individu yang terkandung kompleks OMP 50 kDa untuk menentukan protein immunodominant yang bertanggungjawab terhadap keantigenannya dan meningkatkan sensitiviti dan spesifikasi ujian Typhidot®. Gel elektroforesis 2-D dan LC-MS / MS telah digunakan untuk identifikasi protein yang terkandung dalam OMP 50 kDa. Daripada protein yang telah dikenal pasti, protein TolC, GlpK dan SucB telah dipilih sebagai protein antigenik yang berpotensi dalam 50 kDa OMP dan protein rekombinan telah dihasilkan dalam *E. coli*. Oleh disebabkan oleh sifat protein yang tidak larut, proses penulenan protein telah dilakukan dengan menggunakan keadaan asli dan kenyahaslian untuk meningkatkan hasil protein yang larut. Keputusan ELISA telah menunjukkan bahawa protein rekombinan GlpK (rGlpK) dan protein rekombinan FliC (rFliC) bertindakbalas terhadap serum tifoid yang dikumpulkan bersama telah menunjukkan bacaan penyerapan ELISA yang tertinggi. Sedangkan protein rekombinan TolC (rTolC) dan protein rekombinan SucB (rSucB) menunjukkan bacaan penyerapan ELISA yang rendah. Didapati bahawa antibodi bertindak balas dengan lebih kuat dengan protein rGlpK dan rFliC yang ditulenkan di bawah keadaan asli. Ini mencadangkan kehadiran epitope linear dan berkonformasi pada protein ini. Walau bagaimanapun kedua-dua protein ini tidak sesuai untuk digunakan sebagai biopenanda kerana kedua-dua protein bergabung dengan tidak spesifik dengan antibodi yang berkandung dalam serum normal dan protein rFliC akan

bertindak balas dengan antibodi dalam sera pesakit dijangkiti oleh serovar *Salmonella* lain dan organisma yang bukan *Salmonella*. Antigen rSucB mempunyai afiniti pengikatan yang rendah dengan sera tifoid yang dikumpulkan bersama, oleh itu, ianya tidak sesuai digunakan sebagai biopenanda. Walaupun skor prediksi antigeniti yang tinggi untuk TolC, TolC didapati sebagai antigen yang lemah untuk mengesan antibodi tifoid oleh ELISA. Ini disebabkan oleh antigen rTolC yang diekspresi dalam sistem expresi *E. coli* kurang larut. Sistem ekspresi hos *S*. Typhi digunakan untuk meningkatkan kelarutan TolC. Keantigenan TolC yang diekspreskan dalam dua sistem ekspresi yang berbeza telah dinilai menggunakan ELISA. Keputusan menunjukkan bahawa keantigenan protein TolC yang diekspresi dalam *S*. Typhi (rTyTolC) jauh lebih tinggi daripada rTolC yang diekspresi dalam *E. coli*. (rEcTolC). Oleh itu, antigen rTyTolC dipilih untuk pembangunan immunoassay dalam tiga platform teknologi yang berbeza iaitu dot enzyme immunoassay (DotEIA), ELISA dan lateral flow. rTyTolC didapati mampu mengesan ketiga-tiga subkelas antibodi (IgM, IgG and IgA) yang berguna untuk membezakan pelbagai peringkat jangkitan tifoid. Keputusan sensitiviti dan spesifikasi untuk rTyTolC-DotEIA adalah seperti berikut: 67.5% dan 90.0% untuk rTyTolC-DotEIA-IgM, 94.5% dan 53.3% untuk rTyTolC-DotEIA-IgG, 97.3% dan 90.0% untuk rTyTolC-DotEIA-IgA. Keputusan sensitiviti dan spesifikasi untuk rTyTolC-ELISA adalah seperti berikut: 32.4% 96.6% untuk rTyTolC-ELISA-IgM, 64.8% dan 96.0% untuk rTyTolC-ELISA-IgG, 78.0% dan 93.3% untuk rTyTolC-ELISA-IgA. Lateral Flow didapati sebagai pilihan terbaik di antara tiga platform yang berbeza. Keputusan sensitiviti dan spesifikasi untuk rTyTolC-LF adalah seperti berikut: 100.0% untuk rTyTolC-LF-IgG dan 100.0% dan 90.0% untuk rTyTolC-LF-IgA. Ini menunjukkan kegunaan antigen rTyTolC dalam setiap ujian (DotEIA, ELISA dan aliran lateral) untuk diagnosis demam kepialu. Berbanding dengan OMP 50 kDa, protein rTyTolC adalah antigen alternatif yang lebih baik untuk diagnosis deman kepialu.

A STUDY OF THE ANTIGENICITY OF

***Salmonella enterica* subspecies *enterica* serovar**

Typhi 50 KDa RECOMBINANT PROTEINS

ABSTRACT

S. Typhi 50 kDa outer-membrane protein (OMP) was reported to be antigenic and has been used to develop the commercially successful Typhidot® kit. However, the specificity of the kit varies from 37.5 to 98.8%. This might be due to contamination with other proteins during the 50 kDa OMP preparation and it could have affected the specificity of the 50 kDa OMP. The objective of this study was to investigate the identities of the individual proteins contained in the 50 kDa protein complex to pinpoint the immunodominant protein(s) responsible for its antigenicity, and thus improve the sensitivity and specificity of the Typhidot® test. 2D-gel electrophoresis and LC-MS/MS were used to identify the proteins in the complex. From the pool of identified proteins, TolC, GlpK, FliC and SucB were identified as potential antigenic proteins in the 50 kDa OMP complex and recombinant proteins were produced in *E. coli*. Due to the insoluble nature of the proteins, purification was carried out using native and denaturing conditions to improve the yield of soluble recombinant proteins. The ELISA results showed that recombinant GlpK (rGlpK) and recombinant FliC (rFliC) against pooled typhoid sera have the highest absorbance reading, while recombinant TolC (rTolC) and recombinant SucB (rSucB) showed low absorbance reading. The ELISA results also showed that the antibodies reacted stronger with the rGlpK and rFliC proteins when there were purified under native condition. This suggested the presence of linear and conformational epitopes on these proteins. However these two proteins were found to be unsuitable as biomarkers due to non-specific binding with pooled normal serum and rFliC protein cross-reacted with antibodies in sera of subjects infected with other *Salmonella* and non-*Salmonella* serovars. The rSucB antigen had lower binding affinity with pooled typhoid sera and was not suitable to be used as a biomarker. Despite its high antigenicity prediction score, TolC was found to be a poor antigen (less

specific) for detection of typhoid antibodies by ELISA. This may be due to the rTolC antigen expressed in *E. coli* expression system lack solubility. A native *S.* Typhi host cell expression system was used to improve the solubility of the rTolC. The antigenicity of the rTolC expressed in the two different expression systems was evaluated using ELISA. The results showed that the antigenicity of the rTolC protein expressed in native *S.* Typhi (rTyTolC) was significantly higher than the rTolC expressed in *E. coli* (rEcTolC). Hence, the rTyTolC antigen was selected for immunoassay development in 3 different technological platforms: Dot Enzyme Immunoassay (DotEIA), ELISA and Lateral Flow formats. It was found that rTyTolC was able to detect different subclasses of antibody (IgM, IgG and IgA), which can be useful in differentiating the different stages of typhoid infection. The sensitivity and specificity of the rTyTolC DotEIA were as follows: 67.5% and 90.0% for rTyTolC-DotEIA-IgM, 94.5% and 53.3% for rTyTolC-DotEIA-IgG, 97.3% and 90.0% for rTyTolC-DotEIA-IgA. The sensitivity and specificity of the rTyTolC-ELISA were as follows: 32.4% and 96.6% for rTyTolC-ELISA-IgM, 64.8% and 96.0% for rTyTolC-ELISA-IgG, 78.0% and 93.3% for rTyTolC-ELISA-IgA. The Lateral Flow format was found to be the best among 3 different platforms. The sensitivity and specificity of the rTyTolC-LF were as follows: 100.0% and 75.0% for rTyTolC-LF-IgM, 100.0% and 85.0% for rTyTolC-LF-IgG, and 100.0% and 90.0% for for rTyTolC-LF-IgA. This demonstrated the usefulness of rTyTolC antigen in each of the assays (DotEIA, ELISA and lateral flow) for the diagnosis of typhoid fever. In comparison with the 50 kDa OMP, the rTyTolC protein is a better alternative antigen for diagnosis of typhoid fever.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Typhoid fever still remains a major health problem in developing countries due to limitations of current diagnostic tests for the detection of *S.* Typhi infection. Rapid and accurate diagnosis is of crucial importance to ensure effective disease management and control. The lack of case detection hinders proper disease control and surveillance. If left untreated, typhoid fever patients can have dire consequences, such as intestinal perforation, septicaemia, chronic carrier state, and even death (Huang and DuPont, 2005).

The current "gold standard" for typhoid fever diagnosis is the blood culture method. This method gives excellent specificity but has low sensitivity. In addition to that, it is time consuming, requiring 4 to 7 days to perform the test. Whereas for bone marrow culture, it has high sensitivity but is difficult to perform. Another method for diagnosis of typhoid fever is the stool culture method. This method had poor sensitivity during the acute phase of the infection but is important to monitor typhoid carriers as typhoid carriers shed the bacteria intermittently from the gall bladder into the stool (Akoh, 1991). All these methods requires technician with proper laboratory training and equipment. Many new rapid diagnostic tests have been developed but they lack sensitivity and specificity (Olsen *et al.*, 2004). One of the reasons for lack of specificity is because *S.* Typhi antigen tends to cross-react with other *Salmonella* species. On top of that, *S.* Typhi and *E. coli*, another human pathogen, have similar sequence in genomic DNA. Thus, antigen cross-reactivity tends to occur. Therefore, there is a need to develop more specific biomarkers for typhoid fever diagnosis.

Recent advancement in the field of molecular immunology has led to the identification of a new marker for typhoid fever which have good sensitivity and specificity. This allowed the development of more practical and inexpensive test kits for rapid diagnosis of typhoid fever. Example of a current rapid test for detection of typhoid fever in the market today is Typhidot®. Typhidot® utilizes a *S*. Typhi specific antigenic, 50 kDa outer membrane protein (OMP) of *S.* Typhi to detect the presence of anti-50 kDa antibodies of IgG and IgM isotypes (Ismail *et al.*, 1991b). Typhidot has a reported sensitivity greater than 90% with 75% specificity (Choo *et al.*, 1994). A derivative of the Typhidot is the Typhidot-M, which exclusively detects the presence of anti-50 kDa IgM antibody with a sensitivity and specificity of 92% and 100% respectively.

Further development based on the 50 kDa OMP lead to the development of a Dot blot that was used for screening of typhoid carriers. The screening for typhoid carriers was conducted on food handlers. The Dot blot test was designed based on the immunological response of the patient against the 50 kDa OMP complex. The test detects the presence of anti-50 kDa OMP complex antibodies of the IgA and IgG isotypes in the serum of suspected carriers (Chua *et al.*, 2015). Specific IgA antibodies serves as a marker for typhoid carriers as anti-*S* Typhi IgA antibodies are known to be elevated during the first few weeks of acute typhoid infection and resides significantly afterward.

The original work of the 50 kDa OMP complex was carried out using SDS-PAGE and Western blot analysis, and revealed the presence of a 50 kDa specific antigenic protein on the outer membrane of *S.* Typhi (Ismail *et al.*, 1991b). During the preparation of the 50 kDa OMP, the 50 kDa band was eluted out from the polyacrylamide gel by electroelution. The 50 kDa OMP was used to produce the Typhidot® kit. This kit had been marketed worldwide and considered as the test of choice for typhoid fever due to its high sensitivity and

specificity. However, recent reports have suggested that the kit lacks specificity, with specificity ranges from 37.5 to 98.8% (Narayanappa *et al.*, 2010; Mehmood *et al.*, 2015; Olsen *et al.*, 2004; Membrebe, 1999). Therefore, the suitability of the 50 kDa OMP as a diagnostic marker was questioned. Since only SDS-PAGE and Western blot were done, the possibility that the 50 kDa OMP might be a mixture of proteins that would contain other OMPs which migrated together to yield a band at the 50 kDa molecular weight. The presence of proteins co-purified together with the 50 kDa OMP could have affected the specificity of the 50 kDa OMP. Thus, it is important to investigate on the possibility of the 50 kDa OMP being a single protein or a protein complex. In order to investigate on this, the 50 kDa OMP was subjected to 2D-PAGE gel analysis and LC-MS/MS analysis to isolate and identify the proteins present in the 50 kDa band. By using 2D-PAGE, proteins with the same molecular weight but with different isoelectric points can be separated. LC-MS/MS can be used to identify all the proteins contained in the 50 kDa band. The identification of the other proteins in the 50 kDa protein complex possessed a significant question to the application of the 50 kDa OMP as the antigen of the Typhidot diagnostic kit. The antigenicity, specificity and sensitivity of these proteins individually as a diagnostic biomarker for typhoid fever, have yet to be explored. The efficacy of these individual antigenic proteins have to be elucidated to allow further improvement and development of more sensitive and specific diagnostic tests for typhoid fever.

Current production of the 50 kDa OMP from *S.* Typhi is time-consuming and tedious with a low protein yield. To overcome the current bottleneck of low yield for production of diagnostics, an *E. coli* recombinant protein expression system was used to overcome this. Producing recombinant proteins also ensure sufficient proteins for validation test. The production of recombinant antigens will be beneficial in reducing the cost of production of the antigen, which will result in lower cost of the test and making it more affordable. The production of recombinant Histidine-tagged proteins enables large-scale protein purification.

The purified proteins can then be purified easily using affinity-chromatography in sufficient quantities to study their specificity and sensitivity using ELISA. Thus, the focus of the next part of this thesis was on cloning and purifying each selected proteins in the 50 kDa complex of *S.* Typhi for the development of protein-based diagnostics.

Previous studies have shown that OMPs of *S.* Typhi were mostly expressed as inclusion bodies or insoluble form in *E. coli* recombinant expression system (Jindal *et al.*, 2012). OMPs are known to have low protein solubility when expressed as recombinant proteins due to their hydrophobicity. In addition, the proteins expressed might vary between different hosts due to different protein folding tendencies. To overcome the problem of OMP being expressed as inclusion bodies, a native host expression system was selected to produce these proteins for comparison with the recombinant *E. coli* system. The host cells used was *S.* Typhi Ty21a, a strain commonly used for vaccination. The use of Ty21a would allow the homologous expression of the proteins in the native form. The expression of recombinant proteins in its native form would allow the expression and folding of the protein to be the same as the proteins from *S.* Typhi, where the 50 kDa OMP was extracted. This will lower the possibility of the recombinant OMP expressed as inclusion bodies. The gene of each individual protein was cloned in an expression plasmid with his-tag to allow easy purification. These systems were then compared with the recombinant plasmid expressing his-tag proteins in *E. coli* and *S.* Typhi host for production of recombinant proteins.

The final part of this study involved the selection of the best recombinant protein candidate to use as protein-based rapid diagnostics on DotEIA, ELISA and lateral flow platforms. Each platform has its own benefits. The sensitivity and specificity of each of the assays were evaluated. DotEIA test offers cheap and simple test suitable for resource-limited countries. ELISA provides a diagnostic platform that has higher sensitivity to determine antibody

present in small samples. Due to automation capabilities, ELISA enables processing of large quantities of samples in a single run. Principle of both DotEIA and ELISA tests is as shown in Figure 1.1. While for lateral flow platform, an immunochromatography test, is based on detection of antibodies present in the blood of typhoid patient. Principle of lateral flow assay is as shown in Figure 1.2. For lateral flow test, low cost tests can be produced quickly and can be easily shipped without cold chain control. The lateral flow platform does not require highly trained professionals to perform the test. Lateral flow systems are easy to use without the need for any instrumentation and are ideal for POC applications. Thus, the antigenic proteins were evaluated using these technological platforms to evaluate the sensitivity and specificity of each platform for diagnosis of typhoid fever.

1.2 Objectives

1.2.1 General Objective

To identify and produce specific antigenic proteins of *S.* Typhi, for development of diagnostic tests for typhoid fever and/or carriers.

1.2.2 Specific Objectives

Identification of specific proteins

1) To characterize the composition of the 50 kDa OMP complex of *S.* Typhi using 2D-PAGE and LC-MS/MS

2) To construct and clone the identified specific protein(s) into an expression plasmid(s) for producing recombinant protein(s) for antigenicity studies.

3) To optimize the conditions for the culture and expression of individual proteins identified for expression systems in *E. coli* and of *S.* Typhi host cells.

4) To compare the antigenicity of the recombinant proteins expressed in *E. coli* and *S.* Typhi host cells.

5) To purify the recombinant proteins via affinity chromatography.

6) To evaluate the recombinant proteins' antigenicity using indirect ELISA.

7) To develop and evaluate the protein-based diagnostics using the DotEIA, ELISA and LF platforms.

CHAPTER 2

LITERATURE REVIEW

2.1 Typhoid Fever

Typhoid fever, also known as enteric fever, is a global health problem. This life-threatening illness is caused by the pathogenic bacteria name *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi). *Salmonella* is a member of the family *Enterobactericeae*. The *Salmonella* genus contains two species known as *S. enterica* and *S. bongori*. *S. enterica* can be further separated into six subspecies (*S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*). Most common serotypes of *Salmonella* that cause human infections are Enteritidis, Typhimurium, Newport and Javiana. Based on their serotype, pathogenic *Salmonella* bacteria are classified as either "typhoidal" or "non-typhoidal". Typhoid fever and paratyphoid fevers caused by typhoidal *Salmonella* include the serotypes Typhi, Paratyphi A, Paratyphi B and Paratyphi C. Non-typhoidial *Salmonella* refers to all other *Salmonella* serotypes that cause gastroenteriditis in human (Townes, 2010).

S. Typhi is a Gram-negative, facultative anaerobic, non-encapsulated, flagellated bacilli with a diameter of 0.4 - 0.6 μm and a length of 2 - 3 μm (Crum, 2003). The life cycle of this bacterium generally involves colonization of the lumen of the intestine of human and animals, and transmission via the external environment occurs between hosts (Baker and Dougan, 2007). Symptoms of typhoid fever vary from mild illness with low-grade fever, malaise, and slight dry cough to a severe abdominal discomfort and multiple complications (WHO, 2003). Untreated, typhoid fever might progress to delirium, obtundation, intestinal hemorrhage, bowel perforation, and death within 1 month of contracting the disease. A less severe form of the disease is caused by the serotype *Salmonella* Paratyphi A, and the less

common serotypes by *S.* Paratyphi B and *S.* Paratyphi C (Crum, 2003). Other bacteria which are known to cause gastroenteritis enteric fever include *Escherichia coli* and *Shigella* species.

Human is the only natural host for *S.* Typhi. The genome of *S.* Typhi comprises of 4.857-kilobase (kb) pairs encoding around 4,000 genes, of which more than 200 are functionally inactive. Serological testing is commonly done for confirmation of the clinical isolate since the *S.* Typhi bacterium share common antigenic determinants with other *Salmonella* serovars. However, two key phenotypic feature of *S.* Typhi is the ability of the cell to colonizes the gall bladder serving as a reservoir for the further spread of the disease and the ability to invade the intestinal mucosa potentially through microfold (M) cells and establish an initially clinically undetectable infection as the pathogen does not trigger a rapid inflammatory or diarrheal response (Baker and Dougan, 2007). The virulence of *S.* Typhi is dependent on its ability to invade cells, possession of a complete lipopolysaccharide coat and presence of Vi antigen. However, there were cases of Vi-negative strains of *S.* Typhi that are able to cause the disease (Jegathesan, 1983). Analysis indicated that *S.* Typhi had seven of the 12 fimbrial-like genes and several genes which are associated with the pathogenicity islands in the bacterial genome such as SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5 have become inactivated when compare with non-typhoidal *Salmonella* serotypes (Townsend *et al.*, 2001). Interestingly a few genes that are associated with intestinal attachment and persistence were inactivated in *S.* Typhi but not in *S.* Paratyphi (Baker and Dougan, 2007). Loss of function of these genes explained why *S.* Typhi invades the systemic tissues rather than confined to the luminal gut colonization.

2.2 Typhoid Fever Global Epidemiology

Typhoid fever is a global health problem. It is found primarily in developing countries where sanitary conditions are poor. A study conducted in year 2000, estimated that typhoid fever caused 21.7 million illness and 217 thousand deaths globally (Crump *et al.*, 2004). Countries endemic for typhoid fever include Asia, Africa, Latin America, the Caribbean, and Oceania. However, 80% of the cases were from Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan and Vietnam (Chau *et al.*, 2007), where low socio-economic conditions prevail. The reason for this is because low socio-economic conditions are linked to poor access to clean water and good sanitation but a lack of laboratory diagnostics and health infrastructures contribute to the persistence of the disease. Cases that occur in developed countries are usually due to travelers who were infected outside of their native country. With proper antibiotic therapy, case fatality rates of patients suffering from typhoid fever is less than 1% and patients usually recover with a median of 6 days of hospitalization. If left untreated, typhoid fever is life-threatening with a fatality rate of approximately 15%. High risk regions such as South-Central Asia, Southeast Asia and southern Africa, have more than 100 incident cases per 100,000 persons in the population each year. Medium risk regions include Asia, Africa, Latin America, and Oceania where 10 to 100 cases occur per 100,000 persons. The rest of the world including Australia and New Zealand and other developed countries are classified as low risk with less than 10 persons infected per 100,000 persons per year (Figure 2.1). *S.* Typhi infects humans of all age groups. However, higher number of cases are reported in children and young adults compared to the adults (Figure 2.2).

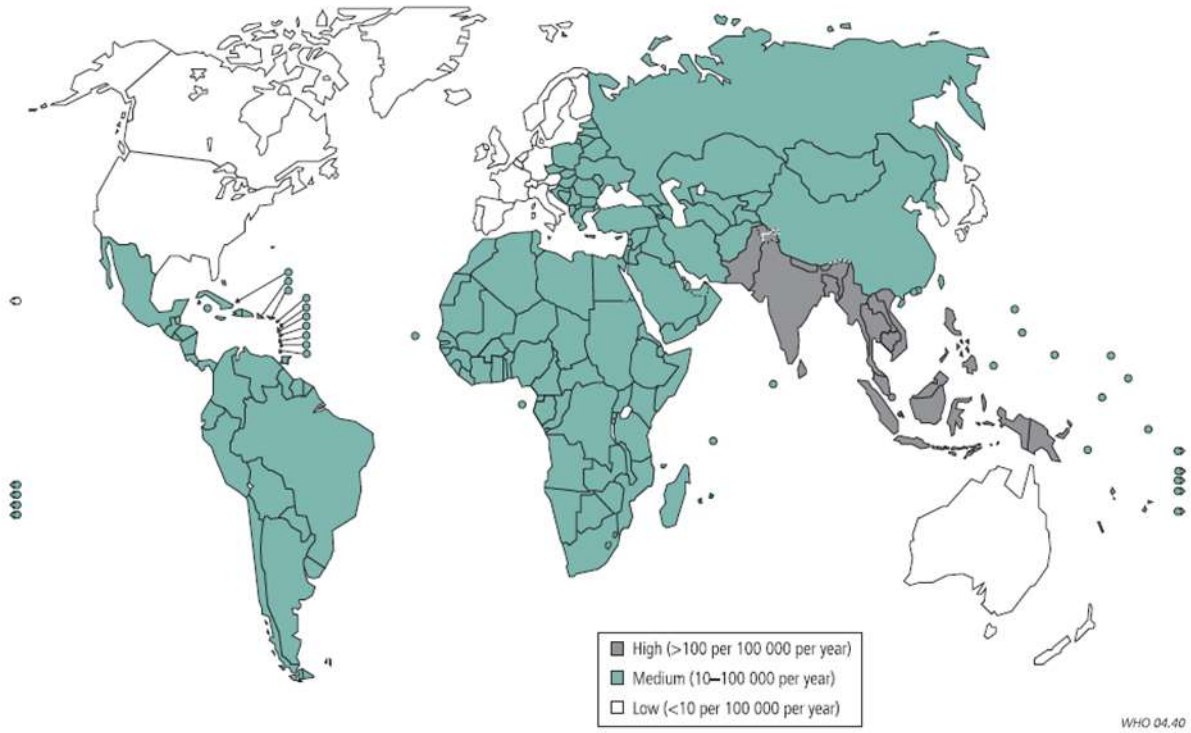

Figure 2.1: Geographical distribution of typhoid fever (Crump *et al.*, 2004)

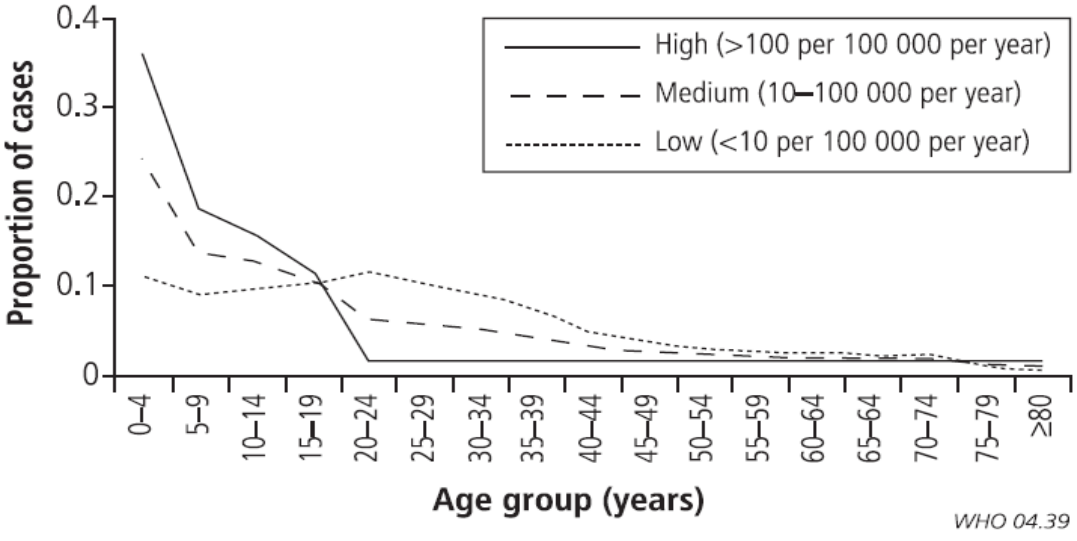

Figure 2.2: Distribution of typhoid fever by age groups at high, medium and low incidence levels (Crump *et al.*, 2004)

2.2.1 Typhoid Fever Epidemiology in Malaysia

Malaysia was mapped as a region with high typhoid incidence rate with more than 100 cases per 100,000 population (Crump *et al.*, 2004). The state of Kelantan in North Eastern Peninsular Malaysia is an endemic region for typhoid fever. Annual incidences of typhoid in Malaysia were 10.2 – 17.9 cases per 100,000 population and can reach as high as 50.3 cases per 100,000 in the state of Kelantan (Yap and Puthucheary, 1998). In Malaysia, Kelantan has the highest number of cases followed by Sabah, Terengganu, Selangor and Sarawak (Malik and Malik, 2001). A study was done to link the typhoid incidence to its distribution in Kelantan. High incidences in Kelantan are due to majority of the Kelantan people from rural areas who do not have access to safe water from treated water supplies and uses water from well for drinking and domestic purposes. In addition to that, the state of Kelantan was constantly hit by flooding. Incidence of typhoid spiked to its highest in 2005 (Figure 2.3) might be due to floods at the end of 2004 where flood waters overflowed into wells and contaminating them with *S.* Typhi. The study also concluded that there were no statistically significant association between ethnic group and typhoid fever (Safian N. *et al.*, 2008). In endemic areas, the majority of the patients were children in the age group of 1 – 19 years (Ja'afar N. *et al.*, 2013). Another study reported that the average age incidence of typhoid fever patients admitted to Hospital Universiti Sains Malaysia (HUSM) was 7.3 years (Choo *et al.*, 1988).

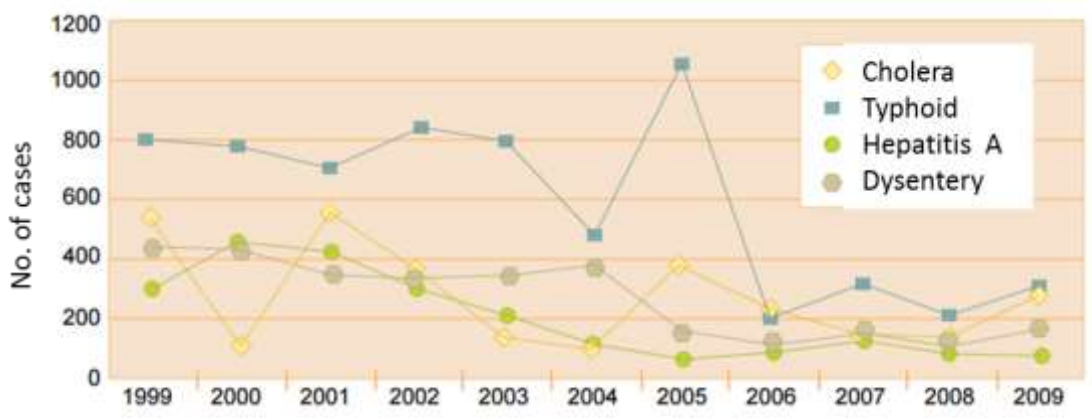

Figure 2.3: Incidence rate of food and waterborne diseases per 100,000 population in Malaysia (1999 – 2009) (Ministry of Health Malaysia, 2009)

2.3 Transmission of Typhoid Fever

Humans are the only natural host and reservoir for *S.* Typhi. This bacterium is known to survive for long periods of time in ground water, pond water, or sea water, and for months in contaminated eggs and frozen oysters (Cho and Kim, 1999; Swaddiwudhipong and Kanlayanaphotporn, 2001; Nishio *et al.*, 1981; Elsarnagawy, 1978). Shellfish from contaminated water and raw fruits and vegetable fertilized with human faeces have been the source of past outbreaks of typhoid fever. The highest incidence of typhoid outbreak happened when the water supply to large populations is contaminated with faeces. The infection dose of *Salmonella* is determined to be 1000 to 1 million bacilli when administrated orally to healthy individual (Levine *et al.*, 2001; Hornick *et al.*, 1970). The incubation period of typhoid fever is influenced by the size of the inoculum and how the organism was ingested. Primary mode of transmission is through fecal-oral route, which is through ingestion of faeces-contaminated water and food often by asymptomatic individuals or carrier, who chronically sheds the bacteria in places where typhoid fever is endemic. It also can be transmitted via hand to mouth during use of contaminated toilet and neglect of hand hygiene. There are also documented cases of typhoid fever, which are transmitted by

means of oral and anal sex (Reller *et al.*, 2003). Other person-to-person transmission of *Salmonella* has been known to occur between infected individuals and their caregivers such as nurseries and welfare homes for the elderly. With proper sanitation and good personal hygiene such as consistent hand washing, boiling water and properly cooked food, can prevent the transmission of *Salmonella*.

2.4 Diagnosis of Typhoid Fever

In endemic regions, diagnosis tests are important to detect acute cases for clinical management, to detect convalescent and chronic fecal carriages for contact tracing and the measurement of acute and convalescent cases for assessment of disease burden. Diagnosis of typhoid fever can be done by utilizing different samples, targets and methods. Diagnosis of typhoid fever is done when an isolate is serotyped as Typhi whether from blood, bone marrow, stool or other specific anatomical lesions. However, a major setback from this, is that it requires proper laboratories equipment with the right tools and technical staff which are not readily available in most developing countries. Points of care detection (POC) such as antibody- and antigen- detection kits are widely used but however it remains insensitive enough. Antigen detection kits are useful for early diagnosis such as first week of infection. Antigen detection kits have sensitivities depending on the number of bacteria presents in the test sample. While for antibody detection kits, it depends on the production of antibody against *S.* Typhi in the subject human body. Since *S.* Typhi bacteria had a long incubation period, an antibody detection test is more feasible. Antibody kit can be used to detect IgM antibodies which peak at 7-10 days of infection and IgG at 14-21 days of infection. Also there are nucleic acid detection methods but concerns and high cost for nucleic acid amplification kits makes it not feasible. Each of the diagnosis method of typhoid fever will be discussed individually.

2.4.1 Clinical Syndrome

Presence of clinical sign and symptoms of typhoid fever and a history of travel to developing countries provide a clue to doctors to diagnose the disease. The incubation period of typhoid infection is 6 to 30 days depending on the infecting dose of bacilli and host immune response. The onset of illness is insidious, with gradually increasing fatigue and a fever that increases daily from low-grade to as high as 38^{o}C to 40^{o}C by the third to fourth day of illness. Often there is a sign on hepato-splenomegaly. A transient, macular rash of rose-colored spots can occasionally be seen on the trunk. Fever is commonly lowest in the morning, reaching a peak in late afternoon or evening. This symptom is often confused with malaria. If untreated, the disease can persist for a month. Serious complication occurs 2 – 3 weeks after onset of illness, with complication like intestinal hemorrhage or perforation which can be life threatening. Healthcare workers will rely on this clinical judgment of the disease and without proper diagnostic tests cannot differentiate *S*. Typhi from *S. Paratyphi* A and other febrile illness, such as dengue, leptospirosis, rickettsia and malaria (Maskey *et al.*, 2006). Generally, healthcare workers prescribe a broad-spectrum anti-microbial agent to target the bacterium based on the observation on the infection. Due to this, inappropriate antimicrobial treatment might be administrated, which will exert selective pressure on *S*. Typhi and other gut pathogen potentially causing an increase in antimicrobial-resistance (Chau *et al.*, 2007).

2.4.2 Microbiology Culture Method

The gold standard for diagnosing typhoid fever is through isolation of *S*. Typhi from blood, bone marrow, rose spots or other sterile sites. A benefit of culture method is that it allows bacterial isolation, which confirms clinical diagnosis and allows antimicrobial-susceptibility testing so that proper therapy can be administrated. The standard method for diagnosis of typhoid fever is by blood culture method. Blood culture method had a 40 to 70% positive detection of *S*. Typhi bacteria in the blood of the patients. (Wain *et al.*, 2008). Several

reasons for this low sensitivity are that to the volume of blood drawn from the patients. Volume of blood taken from adults and school children needs to be higher compare to childrens between 12 to 36 months old. The reason for this is because children have higher levels of bacteria in their blood compared to adults (WHO, 2003). The quantity of bacteria in bloodstream is higher in the first seven days of infection compared with later weeks. Also if patients had pre-antimicrobial treatment, the quantity of bacteria viability will be lower the in bloodstream. Another factor contributing to failure of isolating viable bacteria is due to limited culture media in these laboratories. Culture media such as Oxgall media, tryptone soya broth or brain-heart infusion broth or bespoke media used for automated blood culture systems such as BACTEC® and BactAlert® are suitable to recover the bacteria. Generally 5 ml of fresh blood from the patient are inoculated with 45 ml of brain heart infusion borth or tryptic soy broth and incubated overnight at 37^{o}C. Blood agar (horse or sheep blood) is used for subculturing as *S*. Typhi produce white colonies on blood agar. MacConkey agar can be used as a substitute for blood agar as MacConkey agar allows the growth of only bile-tolerant bacteria and inhibit the growth of many Gram-positive contaminants and even *E. coli*.

The diagnosis of typhoid fever by bone marrow culture, gives a positive detection rate of up to 90% of cases even when the patients have been treated with antibiotics (Farooqui *et al.*, 1991).This is because the amount of bacteria in the bone marrow is ten times higher than in blood. However it is challenging to obtain the sample as this method is invasive, difficult to perform, requires equipment and trained laboratory personnel found primarily in developed countries. Bone marrow is important for diagnosis of patients who have previously undergone treatment, who have history of illness and who had blood culture negative.

Stool or rectal swab culture is another culture method for *S.* Typhi isolation which is less invasive. Isolation of *S.* Typhi from stool sample may indicate merely that the patient is infected with *S.* Typhi but cannot differentiate between acute or carrier status. *S.* Typhi can be isolated from stool samples after 2 weeks and after 3 weeks for urine samples after the acute infection. Stool culture method diagnostic rate is less than 50% but it can be improved to 98% when combined with blood culture, rectal swabs, bone marrow and duodenal strings (Hoffman *et al.*, 1984; Crum, 2003).

The major downside of using culture method is it requires about 2 to 7 days to obtain the results. Although culture method is specific, it lacks sensitivity and speed. As a result, diagnosis might be delayed and can influence the decision of health care workers in outpatient clinics on the management and antimicrobial selection at the time of consultation. In addition, most developing countries lack proper equipment and personnel to do the culturing method.

Characteristics of *Salmonella* Colonies Grown on Agar Plates

Blood agar

S. Typhi grows as non-haemolytic smooth white colonies on blood agar.

MacConkey agar

S. Typhi will produce lactose non-fermenting smooth colonies on MacConkey agar.

Salmonella-Shigella (SS) agar

S. Typhi produce lactose non-fermenting colonies with black centres on SS agar.

Desoxycholate agar

S. Typhi produce lactose non-fermenting colonies with black centres on desoxycholate agar.

Xylose-lysine-desoxycholate agar

S. Typhi produce transparent red colonies with black centres on xylose-desoxycholate agar.

Hektoen enteric agar

S. Typhi produce transparent green colonies with black centres on hektoen enteric agar.

Bismuth sulfite agar

S. Typhi produce black colonies on bismuth sulfite agar.

Commonly used biochemical tests used for the identification of *Salmonella* and their results are as follows (Figure 2.4);

Organism	**Klinger's iron agar**				**Motility, Indol, Urea**			**Citrate**
	Slant	**Butt**	**H_2S**	**Gas**	**Motility**	**Indol**	**Urea**	
S. Typhi	Alk	Acid	Wk+	-	+	-	-	-
S. Paratyphi A	Alk	Acid	-	+	+	-	-	-
Nontyphoidal *Salmonella* or *Salmonella* Paratyphi B or C	Alk	Acid	Wk+	+	+	-	-	V
E. coli	Acid	Acid	-	+	+	+	-	-
Klebsiella spp.	Acid	Acid	-	++	-	V	+	+
Citrobacter spp.	V	Acid	+++	+	+	V	-	+
Proteus spp.	Alk	Acid	+	+	+	V	++	V

Figure 2.4: Biochemical tests for the identification of *Salmonella*

Alk = alkaline, Wk = weak, V = variable result, + = positive, - = negative

Production of acid will turn the agar yellow. For the slant agar this means lactose fermentation and butt this means glucose fermentation.

2.4.3 Serological Diagnosis

2.4.3(a) Widal Agglutination Test

Widal test is a serological test based on the principal of bacterial cell agglutination for diagnosis of typhoid fever. It measures agglutinating antibodies in infected patients sera against the O (somatic) antigens resident within the cell wall structure and H (flagella) antigens from the bacterial flagellae and the Vi (virulence) antigens originating from the capsule surrounding the cell wall. The O and H antigens form the basis upon which the *Salmonella* genus is divided into species level. *S.* Typhi has the O-9, O-12 and d-H antigens. Patients suffering from enteric fever would possess antibodies in their sera which can react and agglutinate serial doubling dilutions of killed, *Salmonella* antigens in the agglutination test. If homologous antibody is present in a patient's serum, it will react with the antigen in the reagent and gives visible clumping on the test card. Antibody level are considered significant at dilution of greater than or equal to 1 : 160, but different cut-off titers was also reported (Bakr *et al.*, 2011). Serum agglutinins increase during the second week (day 6 – 8) of the infection for the O-antigen, and the third week (day 10 – 12) for H-antigen (Kundu *et al.*, 2006). If the blood sample is collected too early during the infection, false negative results might be obtained. The accepted criterion diagnosis of typhoid fever using Widal test is a fourfold rise in the agglutinin titer of the paired sera against the O antigens of *S.* Typhi.

Widal test is cheap and easy to use and it only requires a few minutes to complete the test. In endemic areas where proper equipment for bacteria culture is lacking or limited, the Widal test is widely used to differentiate enteric infection from other illnesses. However the test has some major limitation. It lacks standardization of reagents and inappropriate interpretations of results occur. Widal test has poor specificity and cutoff titer that differs according to endemicity of the disease. Causes of negative Widal test can be due to patient not infected by *S.* Typhi, patient in the carrier state, inadequate inoculum of the bacterial antigen in host to

induce antibody response, technical difficulty or errors in the performance of the test, patient had previous antibiotic treatment and variability in the preparation of the antigens. However the Widal test is also prone to false negative results where a defect in antibody production or infection can have an inhibitory effect on agglutinin production. While for positive result for Widal test can be interpreted as the patient being tested positive for typhoid fever, patient had previous immunization with *Salmonella* antigen, cross-reaction with non-typhoidal *Salmonella*, variability and poorly standardized commercial antigen preparation, patients infected with other enterobacteriaceae or had malaria or dengue (Olopoenia and King, 2000). With sensitivity of 64% and 76% specificity, the used of Widal test were not encouraged (Olsen *et al.*, 2004).

2.4.3(b)IDL Tubex® Test

The IDL Tubex® test is an antibody-detection kit for *S.* Typhi. This kit is a simple and rapid test which is based on the inhibition of binding between two types of reagent particles. First reagent is the colored latex particles which are coated with a monoclonal antibody specific for O9 and second reagent are the magnetic particles coated with *S.* Typhi lipopolysaccharide. The *S.* Typhi-specific antibodies from sera of suspected *S.* Typhi are detected by their ability to inhibit the binding between colored indicator particles that are coated with a monoclonal antibody specific for the *S.* Typhi O9 LPS antigen, and magnetic particles that are coated with *S.* Typhi LPS. Results can be obtained after 5 minutes by comparing the color of the supernatant with the color standards provided by the manufacturer. A Tubex® score of 4 indicate the lowest limit for a positive reaction indicating the detection of 31 micrograms of the anti-*Salmonella* antibody per milliliter (Lim *et al.*, 1998). A positive Tubex® test suggests *Salmonella* infection and the test is not known to cross react with *S.* Paratyphi A. Tubex® can detect IgM antibodies and is useful in diagnosis of acute patients. Tubex® sensitivity and specificity finding was 78% and 89%

respectively (Olsen *et al.*, 2004). Disadvantages of Tubex® are its difficulty in obtaining results from haemolysed samples as it uses a agglutination reaction and Tubex® may potentially produce a false positive in a patient with recent *S.* enterica serotype Enteritidis infection (Oracz *et al.*, 2003).

2.4.3(c) Typhidot® and Typhidot-M®

Typhidot test detects specific IgM and IgG antibodies against the outer membrane protein (OMP) of the *S.* Typhi (Ismail *et al.*, 1991b; Choo *et al.*, 1999). The OMP used in Typhidot® has a molecular weight of 50 kDa and is a specific protein on the outer membrane of *S.* Typhi (Ismail *et al.*, 1991a). The 50 kDa antigen was found to be antigenic as well as specific for *S.* Typhi as it only reacted immunologically with typhoid sera (Ismail *et al.*, 1991b). The 50 kDa demonstrated a sensitivity of greater than 90% and specificity of 75% (Choo *et al.*, 1994). Typhidot-M had been evaluated in clinical settings and demonstrated its simplicity, speed (1 to 3 hours), specificity (75%), sensitivity (95%), economy, early diagnosis and with high negative and positive predictive values (Choo *et al.*, 1994). The detection of IgM by Typhidot® indicates that the patient has acute typhoid fever in the early phase of infection. While detection of both IgG and IgM indicate that the patient has acute typhoid fever in the middle phase of infection. In endemic regions where the numbers of cases of typhoid fever are high, detection of specific IgG will increase as the IgG antibodies can persist up to 2 years after typhoid infection. As this is due to the detection of specific IgG could not differentiate between acute and convalescent patients. In addition to this, false positive results might arise due to re-infection from previously infection. Re-infection have a "boosting" effect of IgG over IgM which causes "masking" of IgM due to high concentration of IgG. Modification was done to the original Typhidot® test by inactivating total IgG in the serum sample to uncover IgM in order to increase the diagnostic accuracy of the Typhidot-M® test in such conditions. A comparison of culture, widal,

Typhidot® and Typhidot-M in clinical settings revealed that the Typhidot® and Typhidot-M® test both performed better than Widal test and culture method (Ismail *et al.*, 1998). A meta-analysis of Typhidot was performed with limited data and demonstrated that the Typhidot® kit had high variability in the sensitivity and specificity with estimates ranging from 56% to 84% and 31% to 97%, respectively (Thriemer *et al.*, 2013). Variation in the sensitivity and specificity could be due to the preparation of the 50 kDa protein.

Table 2.1: Typhidot Reference Studies

Study / published year, reference	Total no. of samples	Sensitivity (%)	Specificity (%)
Membrebe FA, Chua / 1998	185	72.0	52.0
Jesudasan MV, Sivakumar S / 2006	563	92.3	98.8
Begum Z, Hossain MA *et al.* / 2009	100	92.8	90.0
Choo KE, Oppenheimer SJ *et al.* / 1994	109	95.0	75.0
Choo KE, Davis TME *et al.* / 1999	134	93.1	80.6
Narayanappa D, Sripathi R *et al.* / 2010	105	92.6	37.5
Krishna S, Desai S *et al.* / 2011	186	100	95.5

2.4.3(d) IgM dipstick

IgM dipstick test in the market designed to diagnose typhoid fever by detection of *S.* Typhi-specific IgM antibodies in serum or whole blood samples towards *S.* Typhi LPS antigen. IgM dipstick is one of the point-of-care (POC) test which uses lateral flow assay (LFA) technology. The components of this test consist of a dipstick, a detection reagent, buffer for detection reagent, buffer to wet the test strip and tubes. One advantage of the dipstick test is that the components are stable and have a shelf life of two years if stored at temperature range 4 to 25^{o}C. Color development is based on gold nanoparticles conjugated to rabbit anti-human IgM antibody which gives the red line when bind on the test and control line on the membrane. In the dipstick test, the test line is stripped with the *S.* Typhi LPS antigen while the control line is stripped with anti-human IgM antibodies. Test is performed by incubation

of the wetted test strip with diluted serum and detection reagent for a period of 3 hour at room temperature. After incubation the strip was time and rinsed with water after the incubation. Result is interpreted by visual inspection of the test strip for staining of the antigen and control lines. The test is positive when both test and control lines are visible. Studies show that the dipstick yielded a sensitivity of 65% to 77% and specificity of 95% to 100% (WHO, 2003). Several factors influence the results of the dipstick; among them are time of sample collection and the use of antibiotics before consultation and sample collection.

LFA have been increasingly widely used in clinical analysis. The used of Lateral flow assay (LFA) as POC test had been rapidly growing for qualitative and quantitative analyses. The reasons for rapid growth of LFA is that LFA is rapid and only one step analysis, low operational cost, high specificity and sensitivity, long term stability and portability of the device are some of the advantages of LFA. LFA are simple devices to detect the presence or absence of a target analyte in the sample by using capillary technology. In addition to that, LFA does not require special equipment and trained personnel.

The lateral flow assays are built on a few components. It consists of the membrane or analytical region, the absorbent pad, sample pad and conjugate pad. The analytical region consists of the test line and control line. This region is where the target analyte binds to the antibody or antigen on test line and result in a positive detection. The membrane is the most important material used in a LFA. When a test is performed, the membrane must allow the conjugate and sample from the conjugate pad to flow consistently toward the test line and control line and allow the reaction or binding to happen, and finally allow the excess fluid to flow out without binding. Commonly used membrane matrix for LFA is nitrocellulose, which can have characteristics such as high protein-binding capacity, relative ease of handling and low cost. However, the downside of using nitrocellulose membrane is that it

may have imperfect reproducibility of performance between manufacturer lots and variable environmental conditions such as temperature and humidity. The proper performance of the LFA is based upon protein-binding capacity of the membrane, its interaction with protein and the kinetics of the protein-binding processes. Electrostatic, hydrogen and hydrophobic forces are involved in the binding of protein to the nitrocellulose membrane. One important criterion for choosing the right membrane is the capillary flow rate as there is a direct correlation between the flow rate and the sensitivity of the test. A slow rate membrane will provide higher sensitivity compared with a fast rate membrane.

Absorbent pads function as a "sink" at the end of the strip. Absorbent pad capacity to hold liquid also plays an important role in the quality of the results since with insufficient capacity will cause the test sample to flow back in the membrane and could raise the background noise or possibly cause false positives. A large capacity pad also helps to maintain a constant flow rate of the liquid over the membrane.

The sample pad could be made of cellulose and/ or glass fiber. The test sample is first applied on this pad at the beginning of the assay to transport the test sample to other components of the test strip. The sample pad act as a pretreatment before the samples moves to and reacts with the test line. This pretreatment functions such as a separation of sample components, removal of interfering components, adjustment of pH and prevent non-specific binding of the conjugate with the analyte downstream.

The function of the conjugate pad is to hold the detector particles in a dry form so that they are functionally stable until resuspended by the buffers. After the sample is applied on the sample pad, it moves up towards the conjugate pad. On the conjugate pad the detector

particles need to be released rapidly. The conjugate pad needs to have a consistent flow rate as it may cause uneven signal development at the test and control lines and affect the sensitivity of the assay. Conjugate pad are made from materials such as glass fiber, cellulose and polyesters.

Lateral flow assay can be based on two formats, the sandwich format and the competitive format. In a typical sandwich format, a label is immobilized on the conjugate pad. The label could be either secondary antibody which can be transported by capillary action of a buffer solution. The test line contains primary antibody against target analyte and control line consist of an antibody against the labeled conjugated antibody. To perform a sandwich lateral flow assay, the test sample (containing analyte) is applied on the sample pad and it will flow by capillary action to the other side of the strip. At the conjugate pad, target analyte bind with the labeled conjugated antibody, resulting in a labeled antibody conjugate/analyte complex. The complex continues to move up on the nitrocellulose membrane and when it reaches the test line, the complex and analyte binds to the primary antibody. Thus the analyte is sandwich between the two antibodies. Excess label will be captured on the control line and the absorbent pad. Formation of a colored line at the control line indicating excess reagent is used and ensures the strip works properly.

In this study LFA was chosen as one of the assay to be used with the selected diagnostic marker for the diagnosis of typhoid fever. The lateral flow assay was performed on a half-dipstick format. Half-dipstick formats are the same as the sandwich format except for the conjugate release pad which contains the gold-labelled antibody. In this format, the dipstick is first dipped into human sera and washed by dipping into washing buffer before dipped into the gold-labelled conjugates.