IDENTIFICATION OF *Salmonella enterica* subspecies *enterica* serovar Typhi-SPECIFIC GENES FOR THE DEVELOPMENT OF DNA-BASED AND ANTIBODY-BASED DIAGNOSTICS FOR TYPHOID FEVER

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2018

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by

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Thesis submitted in fulfillment of the requirements

for the degree of

Doctor of Philosophy

February 2018

ACKNOWLEDGEMENT

Above all, it would have been impossible to write this thesis without the many wonderful people who supported me throughout this challenging period of my Ph.D. study. First and foremost, I would like to express my sincere gratitude to my supervisor, Prof. Dr. Phua Kia Kien for his constant guidance and support throughout the duration of my study. Without his knowledge, constructive comments, and effort in research and writing, this task would not have been completed. I would also like to thank my co-supervisor, Assoc. Prof. Zaidah Abdul Rahman and field supervisor Dr. Suresh Venkata Chinni for their endless support and guidance.

My sincere thanks also extend to all the kind lecturers, helpful administration and laboratory staff members, supportive friends and colleagues. Special thanks goes to Prof. Armando Acosta, Prof. Maria Elena Sarmiento, Dr. Eugene Ong Boon Beng, Assoc. Prof. Aziah Ismail, Assoc. Prof. Maizan Mohammad, Foo Phiaw Chong, Wong Weng Kin, Chin Chai Fung, Chin Kai Ling, Jason Chin, Roziana Hanafi, Aziana Ismail, Faizul Rahman, Hemaniswarri Dewi, Fadhilah Usuki, Chang Chiat Han, Kuah Vee May, Amy Amilda Anthony, Zafri Muhammad, Badrul Syam, and other members of INFORMM, who were involved directly or indirectly in this research. Thank you for all the guidance, motivation and support, as well as friendship, which have made this an experience I will cherish forever.

I am grateful for the financial support from the Ministry of Higher Education (MOHE) for giving me the National Science Fellowship (NSF) (M/0071/03/2010/S&T) in the early phase of my study, and USM for giving me the USM Fellowship [P-NFD0004/12(R)] in the later part of my study. Thanks also goes to the Division of

Research and Innovation (RCMO), USM for providing a Research University Individual (RUI) grant (reference number: 1001/CIPPM/812096) to support this study.

My family has been my strongest motivation, encouragement and support during my study. I would also like to express my deepest gratitude for their understanding and love all these years. They have helped me achieve great heights in this path of research. As a mark of appreciation, I dedicate this thesis to them and hope that I can continue to make them proud.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

ºC	Degree Celsius
%	Percent
®	Registered Trademark
<	Less Than
>	More Than
+	Positive
-	Negative
±	Both Plus and Minus Operations
×	Time
×*g*	Gravitational Force
µg	Microgram (s)
µM	Micromolar (s)
µL	Microlitre (s)
A_{260}	Absorbance at 260 nm Wavelength
A_{280}	Absorbance at 280 nm Wavelength
AFs	Amplification Facilitators
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
dATP	Deoxyadenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base Pair (s)
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
cfu	Colony Forming Unit (s)
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleoside Triphosphates
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
g	Gram (s)
H_2O	Water
HCl	Hydrogen Chloride
hr	Hour (s)
HRP	Horseradish Peroxidase
IAC	Internal Amplification Control
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IFN	Interferon
kb	Kilobase (s)
KCl	Potassium Chloride
kDa	Kilodalton (s)
L	Litre (s)

LA	Luria Agar
LAMP	Loop-Mediated Isothermal Amplification
LB	Luria Broth
LoD	Limit of Detection
M	Molar (s)
mg	Milligram (s)
mg/mL	Milligram (s) per Millilitre (s)
$MgCl_2$	Magnesium Chloride
min	Minute (s)
mL	Millilitre (s)
mM	Millimolar (s)
MRVP	Methyl Red Voges Proskauer
N	Normality
NA	Nutrient Agar
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures (NCTC)
ng	Nanogram (s)
ng/µL	Nanogram (s) per Microlitre (s)
nm	Nanometer (s)
NPV	Negative Predictive Value
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pg	Picogram (s)
pH	Power of Hydrogen
pmol	Picomole (s)
POC	Point-of-Care
poly-His-tag	Polyhistidine-tag
PPV	Positive Predictive Value
PVDF	Polyvinylidene Difluoride
PVP	Polyvinyl Pyrrolidone
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
S. Typhi	*Salmonella* Typhi
SD	Standard Deviation (s)
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium dodecyl Sulfate-polyacrylamide Gel Electrophoresis
sec	Second (s)
SIM	Sulfide Indole Motility
SPI	*Salmonella* Pathogenicity Island
TAE	Tris-Acetate-EDTA
TSI	Triple Sugar Iron
U	Unit (s)
USA	United States of America
UK	United Kingdom
UV	Ultraviolet
V	Volt (s)

v/v	Volume Per Volume
w/v	Weight Per Volume
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

PENGENALPASTIAN GEN SPESIFIK *Salmonella enterica* subspecies *enterica* serovar Typhi UNTUK PEMBANGUNAN DIAGNOSTIK BERDASARKAN DNA DAN BERDASARKAN ANTIBODI UNTUK DEMAM KEPIALU

ABSTRAK

Demam kepialu disebabkan oleh *Salmonella enterica* subspesies *enterica* serovar Typhi (*S.* Typhi). Penyakit sistemik akut ini kekal sebagai satu masalah kesihatan awam yang utama di seluruh dunia. Kekurangan penanda diagnostik yang spesifik dan sensitif untuk pengesanan *S.* Typhi pada resolusi sasaran gen tunggal menghalang usaha pengawalan penyakit yang cekap. Dalam fasa pertama kajian ini, perbandingan genomik bagi *S.* Typhi dengan patogen enteric lain dilakukan dengan menggunakan BLASTn. Enam gen iaitu STY0201, STY0307, STY0322, STY0326, STY2020 dan STY2021 didapati spesifik dan sensitif *in silico*. Enam ujian PCR sasaran gen tunggal telah dibangunkan dengan kawalan amplifikasi dalaman dan dioptimakan dengan menggunakan kaedah Taguchi. Spesifisiti analisa bagi ujian PCR yang dioptimakan telah ditentukan dengan menggunakan DNA tulen yang diperolehi daripada 39 *S.* Typhi, 62 *Salmonella* bukan-Typhi dan 10 bukan-*Salmonella* strain klinikal. Sensitiviti analisa bagi ujian PCR yang dioptimakan telah ditentukan dengan menggunakan pencairan 5-ganda genomik DNA dan pencairan 10-ganda bakteria kultur. Ujian-ujian penilaian ke atas spesifisiti diagnostik dan sensitiviti diagnostik telah diuji dengan lebih lanjut untuk salah satu daripada ujian yang paling spesifik dan sensitif. Daripada 6 calon gen, 5 gen iaitu STY0307, STY0322, STY0326, STY2020 dan STY2021 menunjukkan 100% spesifisiti analitikal (pengesanan 39/39 strain

bakteria). Gen STY0201 menunjukkan 97.2% spesifisiti analitikal (pengesanan 70/72 strain bakteria). Sensitiviti 6 ujian PCR dengan DNA genom ialah; 32 pg untuk STY0322, 6.4 pg untuk STY0201, STY0326, STY2020 dan STY2021, dan 1.28 pg untuk STY0307. Sensitiviti ujian PCR dalam kiraan bakteria ialah seperti yang berikut; 1.5×10^5 cfu/mL untuk STY0307; 1.5×10^6 cfu/mL untuk STY0201, STY0322, STY0326, STY2020 dan STY2021. Ujian PCR STY0307 menunjukkan sensitiviti yang paling tinggi. Oleh itu, ia telah dipilih untuk penilaian lanjut dengan menggunakan sampel tinja yang dicemari bakteria. Dengan pengeraman selama 18 jam, sensitiviti ujian PCR STY0307 ialah 1.5×10^4 cfu/mL. Ujian PCR STY0307 menunjukkan 100% spesifisiti dan sensitiviti diagnostik apabila dilakukan secara rawak buta terhadap 130 sampel klinikal. Fasa kedua kajian ini telah dicadangkan untuk mengkaji nilai keantigenan protein-protein yang dikodkan oleh gen-gen yang dikenal pasti dalam fasa pertama kajian ini. Analisis bioinformatik telah digunakan untuk meramal potensi calon-calon antigen. Berdasarkan rasional skor ramalan keantigenan dan spesifisiti yang tinggi, 3 gen *S.* Typhi iaitu STY0307, STY0326 dan STY2020 telah dipilih untuk produksi protein dengan menggunakan teknik DNA rekombinan. Keantigenan protein yang afiniti-ditulenkan telah diuji menggunakan ELISAs tidak langsung, terhadap 12 sera demam kepialu, 28 sera bukan demam kepialu dan 28 sera sihat. Daripada 3 calon protein yang dikaji, 2 protein, STY0307 dan STY2020 menunjukkan tindak balas dengan IgM, IgG dan IgA antibodi daripada serum demam kepialu berbanding dengan serum bukan demam kepialu dan serum sihat ($p<0.01$). Anti-STY0307 ELISA mencapai 91.7% sensitiviti (11/12) dan 92.9% spesifisiti (52/56), manakala anti-STY2020 ELISA menunjukkan 66.7% sensitiviti (8/12) dan 94.6% spesifisiti (53/56). Anti-STY0326 ELISA mencapai hanya 25.0% sensitiviti (3/12) dengan 96.4% spesifisiti (54/56). Kesimpulannya, 5 gen iaitu

STY0307, STY0322, STY0326, STY2020 dan STY2021 telah didapati 100% spesifik dan 100% sensitif sebagai penanda DNA untuk identifikasi *S.* Typhi. Dua gen, STY0307 dan STY2020 bukan sahaja sesuai sebagai penanda diagnostik DNA yang spesifik dan sensitif, tetapi protein yang diekspresikan juga berguna sebagai penanda sero-diagnostik. Hasil kajian ini menjamin pembangunan diagnostik yang dipertambahbaikan dan pembangunan vaksin untuk demam kepialu.

IDENTIFICATION OF *Salmonella enterica* subspecies *enterica* serovar Typhi-SPECIFIC GENES FOR THE DEVELOPMENT OF DNA-BASED AND ANTIBODY-BASED DIAGNOSTICS FOR TYPHOID FEVER

ABSTRACT

Typhoid fever is caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). It is an acute systemic disease which remains a major public health burden worldwide. A lack of specific and sensitive diagnostic markers for detection of *S.* Typhi at single-gene target resolution prevents effective diagnosis and therefore efficient control of the disease. In the first phase of this study, genome level comparison of *S.* Typhi with other enteric pathogens was performed using BLASTn. Six genes, i.e. STY0201, STY0307, STY0322, STY0326, STY2020 and STY2021 were found to be specific and sensitive *in silico*. Six individual single-gene target PCR assays with the incorporation of an internal amplification control (IAC) were developed and optimised using Taguchi method. The analytical specificities of the optimised PCR assays were determined using purified DNA from 39 *S.* Typhi, 62 non-Typhi *Salmonella*, and 10 non-*Salmonella* clinical strains. The analytical sensitivities of the PCR assays were assessed using 5-fold dilutions of genomic DNA and 10-fold dilutions of bacterial culture. Diagnostic specificity and diagnostic sensitivity evaluation tests were further assessed for one of these highly sensitive and specific assays. Of the 6 candidate genes, 5 genes i.e. STY0307, STY0322, STY0326, STY2020 and STY2021 demonstrated 100% analytical specificity (detection of 39/39 bacteria strains). Gene STY0201 demonstrated 97.2% analytical specificity (detection of 70/72 bacteria strains). The sensitivities of the 6 PCR assays by genomic DNA were; 32 pg for STY0322, 6.4 pg for STY0201, STY0326, STY2020 and STY2021, and 1.28 pg for STY0307. The

sensitivities of these PCR assays by bacteria counts were as follows; 1.5×10^5 cfu/mL for STY0307 and 1.5×10^6 cfu/mL for STY0201, STY0322, STY0326, STY2020 and STY2021. Since the STY0307 PCR assay demonstrated the highest analytical sensitivity, it was selected for further sensitivity evaluation using spiked stool samples. It was found that with 18 hr enrichment, the sensitivity of STY0307 PCR assay was 1.5×10^4 cfu/mL. The STY0307 PCR assay demonstrated 100% diagnostic specificity and sensitivity when 130 clinical samples were blind screened. The second phase of this study was carried out in order to study the antigenicity of the proteins encoded by the genes identified in the first phase of this study. Bioinformatics analysis was used to predict the potential antigen candidates. Based on a rationale of high antigenicity and high specificity prediction scores, 3 *S.* Typhi genes, i.e. STY0307, STY0326 and STY2020, were selected for cloning and protein expression using recombinant DNA techniques. The antigenicity of the affinity-purified proteins was evaluated using indirect ELISAs against 12 typhoid fever, 28 non-typhoid fever and 28 healthy sera. Of the 3 candidate proteins investigated, 2 proteins, STY0307 and STY2020 showed reactivity with IgM, IgG and IgA antibodies from typhoid patient sera compared to the non-typhoid and healthy control sera ($p<0.01$). The anti-STY0307 ELISA achieved 91.7% sensitivity (11/12) and 92.9% specificity (52/56), whereas the anti-STY2020 ELISA demonstrated 66.7% sensitivity (8/12) and 94.6% specificity (53/56). Anti-STY0326 ELISA achieved only 25.0% sensitivity (3/12) and 96.4% specificity (54/56). In conclusion, 5 genes namely, STY0307, STY0322, STY0326, STY2020 and STY2021, were found to be 100% specific and 100% sensitive as DNA markers for *S.* Typhi identification. Two of the genes, STY0307 and STY2020 not only could serve as sensitive and specific DNA diagnostic markers, but their corresponding proteins

were shown to be sero-diagnostically useful as well. These results hold promise for the development of improved diagnostics and vaccine for typhoid fever.

CHAPTER 1

INTRODUCTION

1.1 *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi)

Salmonella enterica subspecies *enterica* serovar Typhi (*S.* Typhi) is a Gram-negative, non-spore forming, facultative anaerobic bacteria that belongs to the family of *Enterobacteriaceae*. It is rod-shaped, 0.7-1.5 × 2.0-5.0 μm in size, and flagellated (Figure 1.1). Unlike other *Salmonella* serovars, *S.* Typhi causes typhoid fever, an acute systemic disease that is exclusive to humans. It is exquisitely adapted to the human gut before invading the bloodstream via the Peyer's patches in the small intestine (Raffatellu *et al.*, 2008), and causing gastrointestinal inflammation and high fever for extended periods of time. Worldwide, there are approximately 26.9 million typhoid cases and 269,000 deaths each year (Buckle *et al.*, 2012). Inadequate sanitation and sewage disposable systems, contaminated water supplies and poor personal hygiene, provide an opportunity for this pathogen to infect and colonise humans, leading to substantial illness, mortality and enormous financial loss (Dewan *et al.*, 2013). Correct diagnosis is essential for effective clinical management to reduce the morbidity and mortality rates. Yet, reliable diagnostic markers and tests remain tragically elusive. Without correct diagnosis and effective treatment, typhoid fever may progress to more severe illness, such as peritonitis, intestinal haemorrhage or perforation, exacerbating the case-fatality rates to 10-30% (Buckle *et al.*, 2012).

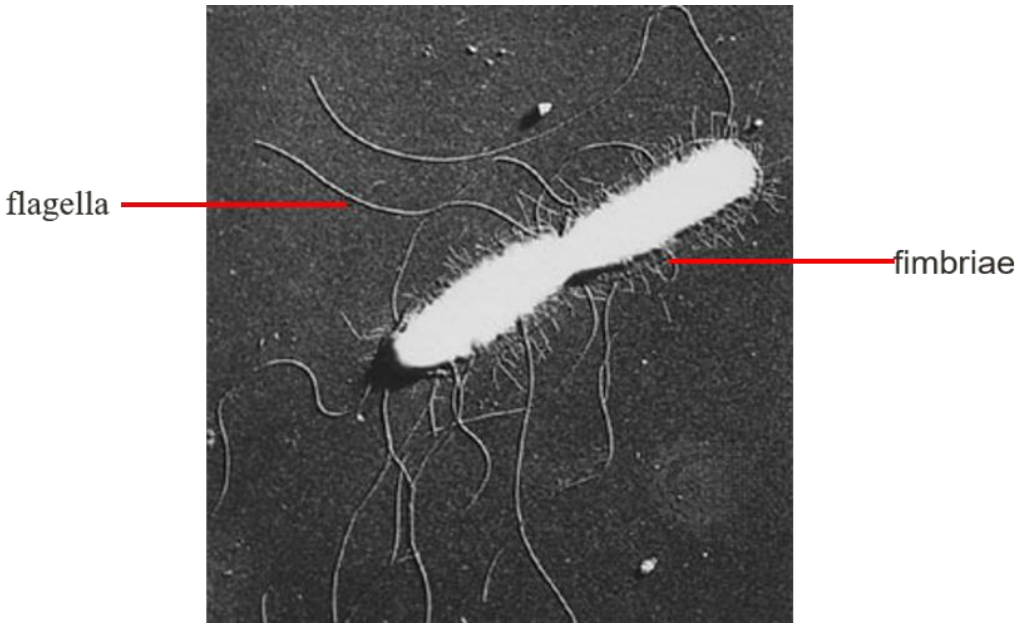

Figure 1.1 An electron microscope image of *S.* Typhi showing flagella and short fimbriae (Adapted from https://www.britannica.com/science)

1.1.1 Taxonomy

According to Le Minor and Popoff in 1987, the species *Salmonella enterica* could be classified into 7 subspecies, namely; I, *enterica*; II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *houtenae*; V, *bongori*; and VI, *indica* (Le Minor & Popoff, 1987). *S. bongor*i was later re-classified as a distinct species based on DNA-DNA hybridization studies (Agbaje *et al.*, 2011). In 2005, a new *Salmonella* species, *Salmonella subterranean* was identified (Su & Chiu, 2006). Today, it is generally accepted that the genus *Salmonella* consists of 3 species (*Salmonella enterica, Salmonella bongori* and *Salmonella subterranean*), 6 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*), and 2,659 serotypes (Issenhuth-Jeanjean *et al.*, 2014) (Table 1.1). *S.* Typhi is a serotype that belongs to *S. enterica* subspecies I (*S. enterica* subsp. *enterica*).

1.1.2 Nomenclature

Salmonella nomenclature is complex and has long been an issue of discussion. In the early 1920s, the taxonomy of *Salmonella* was in confusion until the development of the Kauffman-White scheme by Philip Bruce White in 1926. This scheme was then expanded by Fritz Kauffman from 1933-1978 (Hardy, 2004). The Kauffman-White scheme classified *Salmonella* species based on serological identification of O (somatic) and H (flagella) antigens, resulting in assignment of isolates to more than 2,500 serovars today. Many new serovars continue to be discovered each year. Currently, the *Salmonella* nomenclature system is maintained by the World Health

Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, France (Agbaje *et al.*, 2011).

When cited at the first time in a report, the genus name is italic and followed by the species (italic), subspecies (italic) and lastly the serotype name (non-italicized roman letters and starts with a capital letter) (Issenhuth-Jeanjean *et al.*, 2014), e.g. *Salmonella enterica* subspecies *enterica* serotype Typhi. This can also be stated as *Salmonella* ser. Typhi or simply *Salmonella* Typhi (*S*. Typhi). Since it is in subspecies I under serogroup D with the presence of O-9, O-12, Vi polysaccharides and phase 1 d-H antigens, *S*. Typhi can be named as *Salmonella* subspecies I, 9, 12, Vi:d. The details of *Salmonella* taxonomy and nomenclature is as shown in Table 1.1.

Table 1.1 Taxonomy and Nomenclature of *Salmonella* (Issenhuth-Jeanjean *et al.*, 2014)

Taxonomic position and nomenclature				No. of serovars in each species or subspecies
Genus (capitalized first letter, italic)	Species (italic)	Subspecies (italic)	Serovars/ serotypes (Capitalised first letter, not italic)*	
Salmonella		*enterica* (or subspecies I)	Choleraesuis, Enteritidis, Paratyphi, Typhi	1,586
	enterica	*salamae* (or subspecies II)	9, 46:z:z39	522
		arizonae (or subspecies IIIa)	43:z29:-	102
		diarizonae (or subspecies IIIb)	6,7:1, v:1,5,7	338
		houtenae (or subspecies IV)	21:m, t:-	76
		indica (or subspecies VI)	59:z36:-	13
	bongori	subspecies V	13,22:z39:-	22
	subterranea			
Total				2,659

*some selected serovars (serotypes) are listed as examples

1.2 Typhoid fever

S. Typhi infection leads to the development of enteric fever, or more commonly known as typhoid fever. It is a systemic infection of the intestinal lymphoid tissue, gallbladder and the reticulo-endothelial system. The name "typhoid" was derived from the word "typhus", owing to the indistinguishable clinical manifestations with the disease called typhus caused by Rickettsia bacteria. The burden of typhoid fever lies most critical in under-developed and developing countries, facilitated by inadequate personal hygiene, water supplies and sanitary systems. This disease is characterised by the sudden onset of a sustained high fever, constipation or diarrhea, malaise and abdominal pain (Gonzalez-escobedo *et al.*, 2011). Serious complications, such as intestinal perforation and septicaemia could occur leading to high mortality (Gonzalez-escobedo *et al.*, 2011). The disease affects all age groups, with higher incidence found in children (Darton *et al.*, 2014), immuno-compromised persons, and the elderly (Dougan, 2017). Complete control and eradication of typhoid fever remains a challenge despite improved sewage systems, socio-economic well-being and extensive efforts in public health. To further worsen the scenario, multidrug-resistant strains, which are responsible for high mortality, have risen over the past decade and appeared to be spreading worldwide (Wong *et al.*, 2015)

1.2.1 Epidemiology

According to an epidemiological study in 2000, there were 21.6 million cases and 216,000 deaths each year (Crump *et al.*, 2004). An updated study by Buckle *et al*. in 2012 indicated that typhoid fever accounted for 26.9 million cases and 269,000 deaths each year. The true incidence of the disease burden is probably higher due to a lack of

reliable data collection systems in many endemic regions (Crump, 2014), especially in Latin America and Africa (Buckle *et al.*, 2012). The paucity of information regarding the true burden of the disease makes it a truly neglected disease.

Typhoid fever is a plague of the poor. It usually occurs in low and middle-income countries (Buckle *et al.*, 2012). Regions of high incidence (>100/100,000 cases/year) include South-central Asia and Southeast Asia (Crump *et al.*, 2004). Three countries, namely, India, Bangladesh and Pakistan together account for approximately 85% of the world's typhoid cases (Maurice, 2012). Medium incidence regions (10-100/100,000 population/year) include the rest of Asia, Latin America, Africa, the Caribbean, and Oceania, except New Zealand and Australia. Regions of low incidence rates (<10/100,000 population/year) include North America and other developed countries (Crump *et al.*, 2004). The low typhoid incidence rate in the developed countries could be associated with improved patient care, water sewage system and personal hygiene. However, sporadic outbreaks do occur in developed countries with most cases amongst travellers who had returned from typhoid endemic areas. In the US, about 80% of cases in the country occur from returned travellers and immigrants (Basnyat *et al.*, 2005; Lynch *et al.*, 2009). Judged by the association between migration and introduction of disease in this interconnected world, a history of travel to typhoid-endemic countries is useful for determining people who are at risk of the infection.

Malaysia is one of the countries in Southeast Asia which is endemic for typhoid fever (Figure 1.2). Presently, typhoid fever in Malaysia is considered sporadic with occasional outbreaks confined to a few areas where safe water supply, sanitation, food-

handling and personal hygiene practices are inadequate. The highest incidence rate in the country occurred in 2005 with 4.1 cases per 100,000 population (Figure 1.3). One state hit hard was Kelantan. In 2005, several outbreaks of typhoid fever occurred in the state, resulting in a high incidence rate of 56.7 per 100,000 population in which 735 culture-confirmed cases and 2 deaths were reported (Baddam *et al.*, 2012). Several intervention programs, such as food premise grading system, law regarding food hygiene, food premise inspection, and improved water supply, have been implemented by the government and the state Public Health Department. As a consequence of these interventions and government prodding, the incidence rate of typhoid fever in Malaysia in the past 10 years has decreased to 1.42 per 100,000 population in 2015 (Figure 1.3).

S. Typhi is a bacterium that can live in water and soil for several months and then, when scuffed out, infects humans. It is common to see the rise of typhoid fever cases during natural disasters such as floods, tsunamis, and earthquakes (Sutiono *et al.*, 2010). Seasonal variation (45% of reported cases) was observed and associated with the monsoon season (July to October) in Southeast Asia.

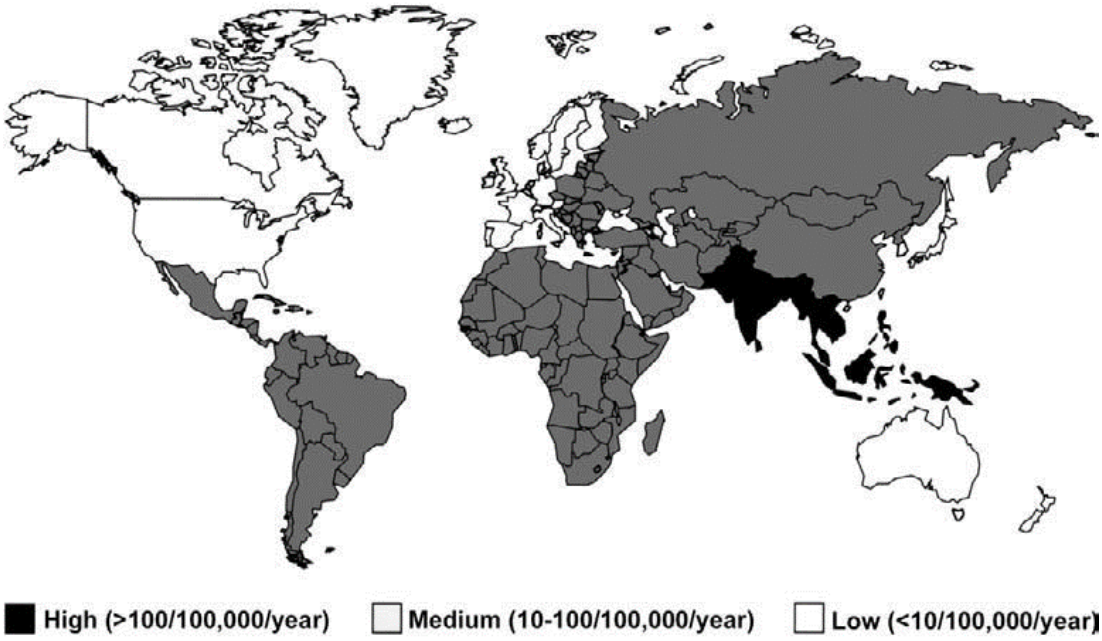

Figure 1.2 Global distribution of typhoid fever (Adapted from Crump *et al.*, 2004)

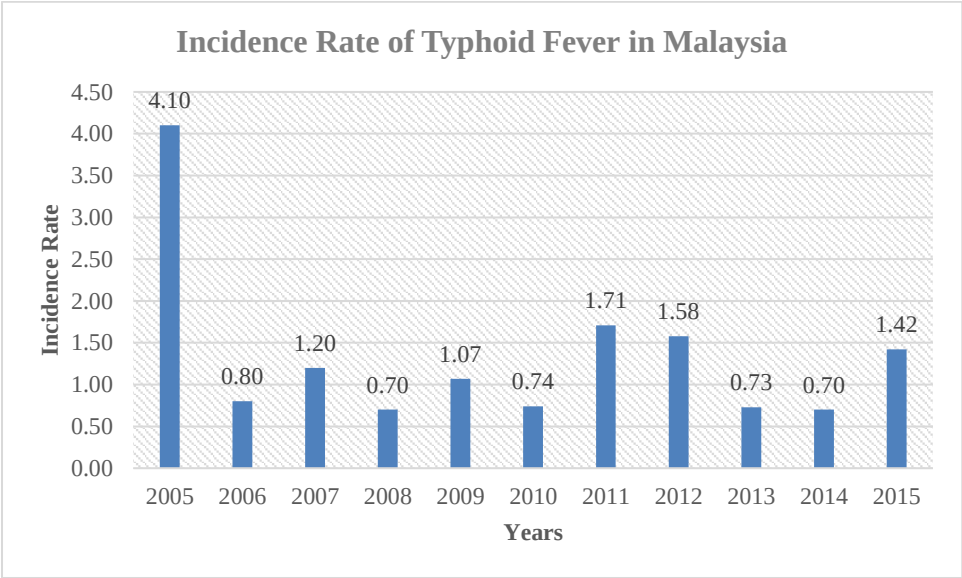

Figure 1.3 Incidence rate of typhoid fever per 100,000 population in Malaysia from 2005 to 2015 (MOH, 2016)

1.2.2 Pathogenesis

The pathogenesis of typhoid fever is difficult to study as humans are the only reservoir for the disease and there is no animal model. Current understanding of *S.* Typhi pathogenesis has been gleaned from the study of *S.* Typhimurium-infected murine model, which exhibits disseminate systemic infection with some resemble to that of human typhoid (Salazar *et al.*, 2017).

The infection starts with the ingestion of food or water contaminated with *S.* Typhi (approximately 10^3-10^6 cfu/mL) into the human alimentary canal (Ja'afar *et al.*, 2013). *S.* Typhi is able to survive the destructive effects of gastric acid and the stomach barrier, and reach the small intestine. The intestinal wall is mainly made up of epithelial cells which serve as a protective layer against harmful substances. To invade the mucosa barrier of the small intestine, *S.* Typhi employs specialised fimbriae that help it to adhere to the epithelium of the Peyer's patches, usually the M cells (Kaur & Jain, 2012). A complex attack system, the type III secretion system (T3SS) of the bacteria is employed to initiate bacterial endocytosis. *S.* Typhi injects the effectors into the intestinal cell and causes host cell membrane ruffling to engulf the bacilli in an intracellular vacuole. However, *S.* Typhi is able to escape the intracellular vacuole and reach the lymphoid follicles at the Peyer's patches. They are then transported to the mesenteric lymph nodes, formed mainly by mononuclear cells such as T lymphocytes, as well as dendritic cells (De Andrade & De Andrade, 2003), and provoke cell-mediated and humoral responses.

S. Typhi is then phagocytised by macrophages. The Vi capsular polysaccharide prevents recognition by the pattern recognition receptors (PRRs) and enables *S.* Typhi to travel within the host's circulating system undetected by the host immune system (Wilson *et al.*, 2008). Survival from the phagolysosome process of the macrophage cells and even replicating within the macrophage cell, enables *S.* Typhi to be carried through the mesenteric lymph nodes and to the reticuloendothelial system, such as bone marrow, lymph nodes, spleen and liver (Garai *et al.*, 2012). This primary bacteraemia stage is usually symptomless, and the blood culture is usually negative.

Secondary bacteraemia happens when *S.* Typhi continues to multiply and induce macrophage apoptosis, breaking out into the bloodstream. The organism re-enters the gastrointestinal tract in the bile and re-infects the Peyer's patches. They may eventually spread to the gallbladder via either vasculature or ducts from the liver. The bacteria is then excreted from the faeces, and transmission to other individuals via contamination of water and food. This secondary bacteraemia coincides with the onset of typhoid symptoms and marks the end of the incubation period (WHO, 2003).

1.2.3 Immune Response

Typhoid infection is restricted to humans. Therefore, the molecular pathogenesis of the pathogen must be unique and the host's immune response specific. The complex immune response to *S.* Typhi involves both innate and adaptive immune systems.

1.2.3 (a) Innate Immune System (Non-specific Immune System)

The innate immune system provides immediate first line host defense against the invading pathogens. Gastric acidity (pH<3.5) serves as the first line of host defence against *S.* Typhi infection as it is ingested with water and food. However, *S.* Typhi manages to survive low pH gastric acid and encounter the next barrier - intestinal mucosa layer. Mucus covers the surface of the gut epithelium lining and acts as a protective layer to prevent direct contact of harmful bacteria with the epithelium. During *S.* Typhi infection, M cells in the epithelium are employed by *S.* Typhi as a means of transportation to the lumen epithelium and reach the lamina propria. Following this epithelial invasion, the presence of *S.* Typhi is detected by monocyte-derived phagocytic cells, namely macrophages and dendritic cells. These cells express a large family of pattern recognition receptors (PRRs) on their surfaces which detect pathogen-associated-molecular-patterns (PAMPs) and danger-associated-molecular-patterns (DAMPs) on pathogens. PAMPs expressed by *S.* Typhi include flagella, fimbria, T3SS protein, lipopolysaccharide and bacterial DNA (de Jong *et al.*, 2012). Upon engagement with PAMPs and DAMPs, the PRRs trigger expression of soluble protein called cytokines, leading to the activation of the adaptive immune system (Raffatellu *et al.*, 2006).

1.2.3 (b) Adaptive Immune System (Specific Immune System)

When the innate immune response fails to prevent the entrance of *S.* Typhi, the adaptive immune system comes into play with the help of T and B cells. The adaptive immunity appears slowly, but it is more specialised and potent. Adaptive immune

system consists of cellular and humoral components which help them to carry out their protective functions.

1.2.3 (bi) Cellular Immune Response

The cellular immune response (CRI) or cell-mediated immunity (CMI) represents the cellular component of the immune response and plays a dominant role in the early response to *S.* Typhi infection since *S.* Typhi could persist intracellularly. Two major components, $CD4^+$ and $CD8^+$ cytotoxic T-cells are involved in CMI of typhoid infection (Lundin *et al.*, 2002). The recognition of $CD4^+$ and $CD8^+$ T cells against the *S.* Typhi antigens leads to secretion of pro-inflammatory cytokines, such as interferon (IFN)-γ, interleukin (IL)-6, IL-8 and Tumor Necrosis Factor (TNF)-α (Fiorentino *et al.*, 2013). The predominant cytokine, IFN-γ enhances the phagocytic ability of the macrophages (Nairz *et al.*, 2008). However, with T3SS mechanisms, *S.* Typhi can survive and use the macrophages as a vector for transport into the reticuloendothelial system of the host, such as bone marrow, lymph nodes, spleen and liver (Dougan & Baker, 2014). *S.* Typhi causes a restrained immune response in the host. Lower levels of IL-1β and TNF-α production by T- and B-cells compared to other Gram-negative bacteria infections during the acute phase of typhoid fever has been observed (Tsolis *et al.*, 2008). This result in limited neutrophil influx and explains why *S.* Typhi typically does not elicit septic shock and have prolonged incubation period (Gal-Mor *et al.*, 2012). As *S.* Typhi continues to multiply and induce macrophage apoptosis, the bacteria breaks out into the bloodstream, leading to the start of the humoral immune response.

1.2.3 (bii) Humoral Immune Response

Humoral immune response involves B cells which proliferate and differentiate into plasma cells and produce specific antibodies to neutralise and induce phagocytosis to destroy the bacteria. This immune response is important especially when *S.* Typhi is at the extracellular stage.

S. Typhi possess 3 main antigens; (1) somatic antigen O, (2) flagellar antigen H, and (3) surface antigen Vi. Antigenic structure of *S.* Typhi is shown in Figure 1.4. O, H and Vi antigens are the most studied activator of B cells to orchestrate the typhoid humoral immune response (Waddington *et al.,* 2014). O antigen is made up of polysaccharide and occurs on the surface of the outer membrane of the bacteria. It displays variable structures which are recognized by the humoral immune system leading to various *Salmonella* serovars (Liu *et al.,* 2014). H antigen constitutes the flagella, which is essential for attachment and invasion of the host intestinal epithelial cells, and also in biofilm formation (Haiko & Westerlund-Wikström, 2013). H antigen exists in 2 phases, called phase 1 and phase 2. Some *Salmonella* serotypes express one phase of H antigen (monophasic). Some others express both phases (diphasic). Some *Salmonella* serovars tend to change from one phase to another, which is termed as "phase variation". Vi antigen is a capsular polysaccharide antigen located on the surface of the bacteria, and is associated with *S.* Typhi virulence. It also plays a main role in preventing *S.* Typhi from being phagocytized by macrophages(Janis *et al.,* 2011). However, Vi antigen is not essential for *S.* Typhi infection as Vi-negative mutants of *S.* Typhi can still cause typhoid fever (Pulickal *et al.,* 2013). Reliance of Vi antigen for serological testing will result in poor prognosis.

Antibodies against these *S.* Typhi antigens could be found in serum and other secretions. However, this humoral immune response does not confer long-life protection, but, instead, relapse of typhoid fever has been observed in 15–20% of individuals who had recovered from previous typhoid fever infection (Guzman *et al.*, 2006). Anti-O, -H and -Vi antibodies have been widely used as targets in various diagnostic tests, e.g. Widal (Olopoenia & King, 2000; Andualem *et al.*, 2014), enzyme-linked immunosorbent assay (ELISA) (Fadeel *et al.*, 2004) and Tubex® (Khanna *et al.*, 2015) tests.

Humoral immune response of typhoid fever is not restricted to O, H and Vi antigens. Ty21a vaccine which is an attenuated *S.* Typhi live bacteria lack Vi antigen, gives similar protection with that of purified Vi capsular polysaccharide subunit (ViCPS) vaccine (Kantele *et al.*, 2012). These observations show that multiple adaptive immune response mechanisms are involved in eliminating the bacteria from the host. Although the humoral response in typhoid infection is mainly defined by the O, H and Vi antigens, there are other antigens such as the outer membrane proteins and heat shock proteins which have been shown to elicit specific antibody production (Sztein, 2007). However, these proteins have not been studied in detail.

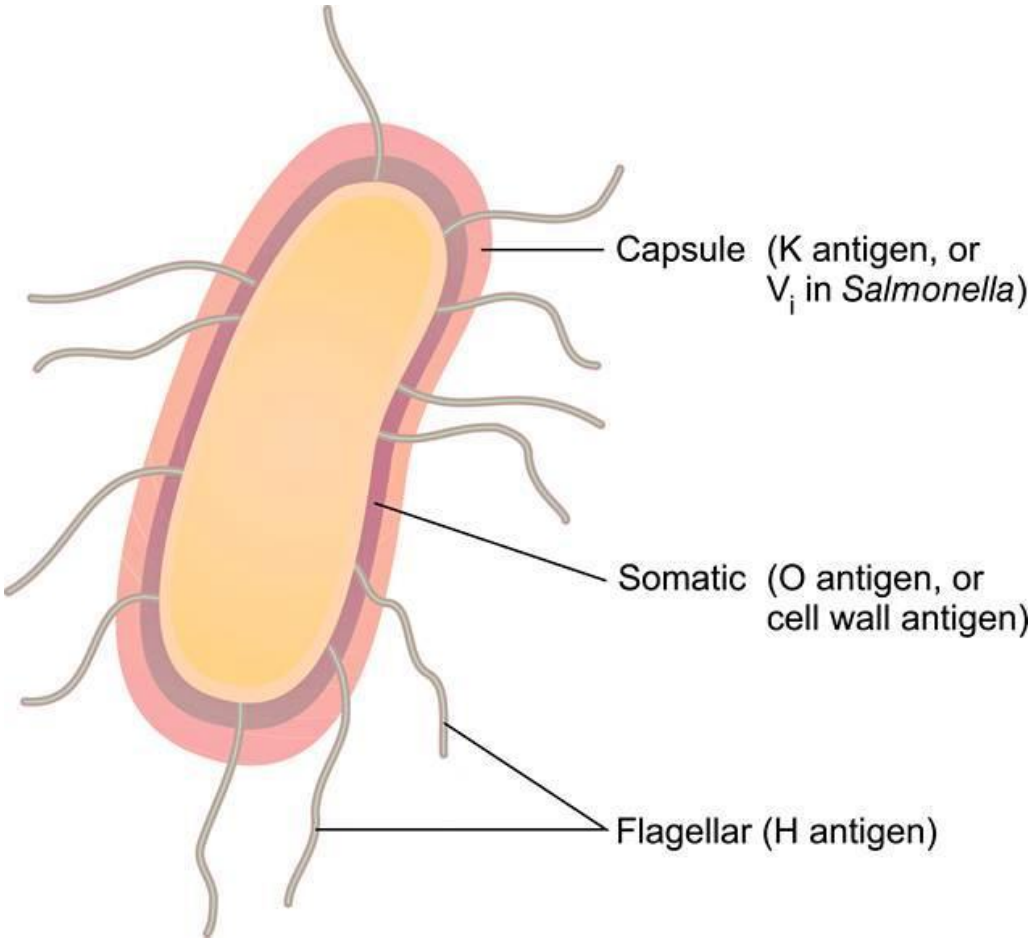

Figure 1.4 Antigenic structure of *S.* Typhi (Adapted from University of British Columbia, http://wiki.ubc.ca/Course:PATH417:2015W1/Case_2/Student_8)

1.2.3 (biii) Important Antibody Isotypes in Typhoid Serology

The initial exposure to *S.* Typhi causes the immune system to produce IgM. It activates the complement system by the Classical Pathway, promoting phagocytosis and destruction of the microbe. During *S.* Typhi infection, the level of IgM is detectable as early as 5-7 days of infection. Typhoid patients generally show significant rise in serum IgM titres during the first 10 days of illness and persists only for 45-90 days after acute illness, while serum IgG dominates the convalescent phase and can persist for more than 2 years after typhoid infection (Herath, 2003). IgM is therefore more of diagnostic significance than IgG to differentiate between acute and convalescent cases in an endemic population.

IgG functions in a variety of ways including opsonisation of antigen, complement activation, and neutralisation of pathogen. IgG production is induced by IFN-γ which responses to *S.* Typhi infection. IgG persists longer than IgM and can have a half-life of more than 2 years after typhoid infection (Ismail, 2000). Thus, it is not suitable to be used for diagnosis of typhoid fever in highly endemic areas. Approximately 80% of *S.* Typhi chronic carriers manifest high titres of serum IgG against *S.* Typhi Vi antigen (Vaishnavi *et al.*, 2005). Thus, anti-Vi IgG has been routinely utilised for screening of chronic carriers (Sztein *et al.*, 2014). It is, however, unlikely to be effective in endemic regions as false-positive results may be obtained in acute or convalescent cases (House *et al.*, 2008).

IgA is secreted in mucosal tissues and neutralises microbes including those in the lumens of the gastrointestinal and respiratory tracts, preventing adherence of the

bacteria to epithelial cells and therefore gives protection to the mucosa. Increased IgA titres in typhoid patients' saliva have been observed during the first and second weeks of illness (Herath, 2003), and therefore has diagnostic uses (Zeeba Zaka-ur-Rab *et al.*, 2012; Herath, 2003; Chin *et al.*, 2016).

1.2.4 Clinical Features

The incubation period of the infection is typically between 7-14 days (Bhutta, 2006a). Typhoid fever generally produces indistinguishable clinical features from other febrile diseases such as malaria, leptospirosis, dengue and tuberculosis. The hallmark of typhoid fever is prolonged fever, which could increase daily in a stepwise manner to as high as 38-40°C in the third or fourth day of illness (WHO, 2003). Other symptoms include mild abdominal discomfort, headache, malaise, loss of appetite, constipation or diarrhoea, hepatomegaly and splenomegaly. In certain cases, transient rash of rose-coloured spots in the abdomen can be observed. Serious complications such as intestinal perforation, intestinal haemorrhage and typhoid encephalopathy occur between 10-15% of patients, sometimes with lethal consequences (Basnyat *et al.*, 2005). Factors which influence the severity of the infection include the status of host's immune system, the infecting dose of the bacteria, history of vaccination, medication, and virulence of the bacteria strains (Bhutta, 2006a). Patients with immune-suppressed conditions are of greater risk of developing the disease with greater morbidity and mortality rates.

1.2.5 Typhoid Carrier State

Approximately 1-5% of acute typhoid patients become chronic carriers (WHO, 2003). Carriers are individuals who harbour *S*. Typhi in their gallbladder, and excrete them in stool or urine, without showing any symptoms. *S*. Typhi stays innocuous in carriers, but poses a "silent" threat to the community, sometime causing outbreaks of typhoid fever in the population.

The predisposing factor for development of the carrier state is that of the ability of certain strains of *S*. Typhi which are resistant to bile by forming biofilm in the gallbladder. The biofilm layer overcomes the action of antimicrobial agents, such as antibiotics, disinfectants and preservatives (Marathe *et al.*, 2012), allowing it to be sequestered in the gallbladder or kidneys without being detected by the host immune system. The propensity to become a carrier was reported to be higher in females especially those who are greater than 50 years old and persons with cholelithiasis or schistosomiasis (Bhan *et al.*, 2005; Ja'afar *et al.*, 2013).

Generally, carriers are divided into 3 categories based on the *S*. Typhi excretion period: i) convalescent carriers, who continue to excrete *S*. Typhi 3 weeks to 3 months after the acute illness; ii) temporary carriers, who continue to excrete *S*. Typhi between 3-12 months after the acute illness; and iii) chronic carriers who continue to excrete *S*. Typhi for more than 1 year after the acute illness. Approximately 1-5% of typhoid patients become chronic carriers after recovering from the acute illness. The occurrence of typhoid fever outbreaks is often associated with asymptomatic carriers, particularly those who work as food-handlers (Gupta *et al.*, 2006). If the carriers could

not be identified, further spreading of the disease will occur, adding more difficulties in the control of the disease. Therefore, identification of typhoid carriers and extensive antibiotic treatment for this group of people, is extremely important. However, there is currently no reliable laboratory diagnostics for identification of typhoid carriers. Stool culture, which remains the gold standard for identification of carriers, only has a recovery rate of 5%, owing to intermittent secretion of *S.* Typhi from the carriers (Ismail, 2000). As such, identification of typhoid carriers remains challenge and an obstacle for eradication of the disease.

Perhaps, the most notorious typhoid carrier was Mary Malloon or better known as "Typhoid Marry". Her career as a cook in New York city become one of the main route for spreading the disease among customers, families or friends. Eventually, 51 typhoid fever victims and 3 deaths were traced to her. She was forcibly taken into custody twice in her life by local health officials, and was detained until she die at the age of 69 (Marineli *et al.*, 2013).

1.2.6 Treatment of Typhoid Fever - Antibiotic Therapy

Typhoid fever can be controlled by antibiotic therapy. With the advent of pre-emptive antibiotic treatment, 99% of typhoid cases survival could be assured. However, without effective treatment, typhoid fever may progress to more severe illness, such as peritonitis, intestinal haemorrhage or perforation, leading to fatality in 10-30% of cases (Buckle *et al.*, 2012).

The selection of antibiotics depends on the clinical severity and antimicrobial susceptibility test. As per the WHO recommendation, the primary antibiotic treatment for *S.* Typhi infection is fluoroquinolones, followed by nalidixic acid and other antimicrobial agents. Fluoroquinolones such as ciprofloxacin, ofloxacin and pefloxacin are widely regarded as the ideal antibiotic of choice for typhoid fever (Upadhyay *et al.*, 2015; WHO, 2003). They demonstrate excellent ability to penetrate tissues and destroy the *S.* Typhi in monocytic cells such as macrophages, leading to rapid therapeutic response.

In the past decades, drug-resistant *S.* Typhi strains have emerged as a new threat. Fluoroquinolone-resistant strains have been reported in several countries, such as Nepal and India (Nobthai *et al.*, 2010; Dutta *et al.*, 2008; Afzal *et al.*, 2012). For these isolates, the use of ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole is recommended. In the mid 1980's, antimicrobial treatment has become more difficult with the emergence of pHCM1 plasmid-mediated multidrug-resistant strains, which are resistant to all 3 first-line antimicrobial agents, including chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole (Zaki & Karande, 2011). In these cases, third-generation cephalosporins, such as ceftriaxone or cefotaxime are recommended (Zaki & Karande, 2011; Bhutta, 2006a). The selection of antibiotics based on the clinical severity and drug susceptibility of the *S.* Typhi strain is shown in Table 1.2.

Table 1.2 Recommended treatment of uncomplicated typhoid fever adapted from WHO technical communities (Bhutta, 2006b; WHO, 2003)

Susceptibility	Optimal treatment			Alternative treatment		
	Antibiotics	**Daily dose (mg/kg)**	**Course (Days)**	**Antibiotics**	**Daily dose (mg/kg)**	**Course (Days)**
Uncomplicated typhoid fever						
Fully sensitive	Fluoroquinolone e.g. ofloxacine or ciprofloxacine	15	5-7	Chloramphenicol Amoxicillin TMP-SMX	50-75 75-100 8-40	14-21 14 14
Multidrug resistance	Fluoroquinolone cefixime	15 15-20	5-7 7-14	Azithromycin Cefixime	8-10 15-20	7 7-14
Quinolone resistance[b]	Azithromycin ceftriaxone	8-10 75	7 10-14	Cefixime	20	7-14
Severe typhoid fever requiring parental treatment						
Fully sensitive	Fluoroquinolone e.g. ofloxacine	15	10	Chloramphenicol Ampicilin TMP-SMX	100 100 8/40	14-21 14 14
Multidrug resistance	Fluoroquinolone	15	10-14	ceftriaxone Cefotaxime	60 80	10-14
Quinolone resistance	Azithromycin cefotaxime	60 80	10-14	Fluoroquinolone	20	14

1.2.7 Prevention of Typhoid Fever

Recognising the threat posed by typhoid fever epidemics, strategies for prevention and control of this disease are urgently needed. Some approaches to reduce typhoid fever burden include vaccination, appropriate surveillance systems, safe water supply, improved sanitation facilities, rational use of antibiotics, appropriate personal hygiene and public health education. For government to regulate resources for these purposes, accurate figures of the disease burden is essential. However, lack of adequate diagnostic tools forms the formidable obstacle to estimate the true disease burden.

1.2.7 (a) Vaccines

Immunisation is one of the keys to prevent and control of the disease. At present, there are 3 licensed typhoid vaccines available: 1) whole cell live attenuated *S.* Typhi (Ty21a) oral vaccines; 2) purified Vi capsular polysaccharide subunit (ViCPS) vaccine (Shawky Hosny *et al.*, 2015), and 3) Vi-conjugate vaccine.

Ty21a vaccine is licensed for persons aged older than 6 years and requires 3 to 4 doses oral administration on alternating days. It induces both cell-mediated and humoral immunity. It is available in both liquid and coated capsule forms (Marathe *et al.*, 2012). As a live attenuated vaccine, it should not be administered to immune-compromised persons or persons having antibiotic treatment.

ViCPS vaccine is recommended by WHO as the vaccine of choice for typhoid fever as it is free from endotoxin and requires only a single dose for effective immunity. In

addition, it is also suitable for children over 2 years old, individuals receiving antibiotic therapy and immune-compromised individuals (Shawky Hosny *et al.*, 2015). This vaccine confers 50-80% protection against typhoid fever (Anwar *et al.*, 2014).

The present available vaccines are not suitable for mass immunisation, especially to children less than 2 years old and the elderly. Continuous efforts are being made to develop vaccines which can provoke higher antibody titres and longer immunity with reduced side effects. A new Vi antigen which is conjugated to exotoxin A (rEPA) of pseudomonas aeruginosa vaccine (Vi-rEPA) has been developed (Szu, 2013). This vaccine showed enhanced immunogenicity in adults and children compared to Vi antigen alone (Jin *et al.*, 2017). Remarkably, it protects most of the recipients in trials including children below the age of 5.

Although effective vaccines are available, there is no plan for vaccination programme for infants and children. Some do not trust the vaccine, claiming the side effects of vaccine pose a greater health risk than the disease itself. Out of 16 Asia countries where typhoid is endemic, only 3 countries, i.e. India, Vietnam and Thailand have allowed mass immunisation to protect their child (Ochiai *et al.*, 2007). The main cause of this neglect is due to the inadequacies of routine diagnostics which generally make the disease burden somewhat uncertain. All these form a major obstacle in the control and management of typhoid fever.

1.3 Diagnosis of Typhoid Fever

Typhoid fever typically produces similar clinical features as with other febrile diseases, such as malaria, leptospirosis and dengue. Due to non-specific clinical manifestations, laboratory testing is therefore essential for typhoid fever diagnosis. Diagnostic methods used in diagnosis of typhoid fever include: (1) blood or stool bacterial culture method following phenotypic identification using biochemical and serotyping assays; (2) detection of antigens or antibodies by immunological assays, and (3) detection of DNA or proteins by molecular assays (Lee *et al.*, 2015). The choice of diagnostic methods depends on the laboratory facilities, budget and nature of the test samples. Presently, diagnosis of typhoid fever under the gold standard method ISO 6579:2002 (International Organization for Standardization, 2002) is through isolation of the bacteria from blood and stool samples, followed by biochemical and serological tests. However, these methods are time-consuming and laborious, delaying appropriate disease treatment and containment (Parry *et al.*, 2011).

1.3.1 Culture Systems

S. Typhi clinical strains can be isolated from blood, stool, urine, bone marrow, duodenal fluid and saliva of patients (Herath, 2003; Z. Zaka-ur-Rab *et al.*, 2012). The diagnostic efficacy of the culture method differs with the specimens tested. The highest recovery rate (detection rate) of *S.* Typhi is from bone-marrow, followed by blood, stool and urine in patients with typhoid fever (Mogasale *et al.*, 2016)

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