DEVELOPMENT OF DNA APTAMERS FOR HEMOLYSIN E ANTIGEN OF *Salmonella enterica* serovar Typhi TOWARDS DIAGNOSTIC APPLICATION

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by

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LIST OF SYMBOLS

Å	Angstrom
M	Molar
%	Percentage
<	Lower than
>	Greater than
≥	Greater than or equal to
ΔG	Gibbs free energy
°C	Degree Celsius
µA	Microampere
µg	Microgram
µL	Microlitre
µM	Micromolar
Cl	Confidence intervals
g	Gram
g	Gravity force
h	Hours
Hz	Frequency
I	Currents
K	Kelvin
kcal	Kilocalorie
L	Litre
mg	Milligram
mins	Minutes
mL	Millilitre

mm	Millimeter
mol	Mole
mV	Millivolt
ng	Nanogram
nM	Nanomolar
nm	Nanometer
ns	Nanoseconds
P	p-value
pH	Potential of hydrogen
psi	Pound per square inch
R^2	R squared
s	Seconds
U	Units
V	Volt

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APS	Ammonium Persulfate
AUC	Area Under the Curve
CASP	Critical Assessment of Structure Prediction
CE	Counter Electrode
COVID-19	Coronavirus Disease 2019
CRM197	Cross-Reacting Material 197
CV	Cyclic Voltammetry
DALYs	Disability-Adjusted Life-Years
DPV	Differential Pulse Voltammetry
EIS	Electrochemical Impedance Spectroscopy
ELISA	Enzyme-Linked Immunosorbent Assay
ELONA	Enzyme-Linked Oligonucleotide Assay
FDA	Food and Drug Administration
GO	Graphene Oxide
H_2S	Hydrogen Sulfide
HlyE	Hemolysin E
HPLC	High-Performance Liquid Chromatography
HRP	Horseradish Peroxidase
ICT	Immunochromatographic Test
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IUPAC	International Union of Pure and Applied Chemistry
JEPeM	Human Ethics Committee of Universiti Sains Malaysia
Kd	Dissociation Constant
KIT	Royal Tropical Institute
LFA	Lateral Flow Assay
LMICs	Low- and Middle-Income Countries
LoD	Limit of Detection
LoQ	Limit of Quantification
LPS	Lipopolysaccharide
MCH	Mercapto-1-Hexanol

MD	Molecular Dynamics
MR	Methyl Red
MRVP	Methyl Red and Voges-Proskauer
NGS	Next-Generation Sequencing
OMP	Outer Membrane Protein
OTA	Ochratoxin A
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PLIP	Protein-Ligand Interaction Profiler
POCT	Point-of-Care Testing
qPCR	Quantitative Polymerase Chain Reaction
RDTs	Rapid Diagnostic Tests
RE	Reference Electrode
RMSD	Root Mean Square Deviation
ROC	Receiver Operating Characteristic
RT	Room Temperature
S.I.M.	Sulfide Indole Motility Test
S.O.C	Super Optimal broth with Catabolite repression
SD	Standard Deviation
SELEX	Systematic Evolution of Ligands by EXponential Enrichment
SPC	Simple Point Charge
SPE	Screen Printed Electrode
ssDNA	Single Stranded DNA
SWCNT	Single-Walled Carbon Nanotube
SWV	Square Wave Voltammetry
T1SS	Type I Secretion System
TAE	Tris-Acetate-EDTA Buffer
TCV	Typhoid Conjugate Vaccine
TEMED	N,N,N',N'-Tetramethylethylene-Diamine
TLRs	Toll-Like Receptors
TMB	3,3',5,5'-Tetramethylbenzidine
TRL	Technology Readiness Level
TSI	Triple Sugar Iron
US	United States

USA	United States of America
Vi-PS	Vi Capsular Polysaccharide Vaccine
WASH	Water, Sanitation and Hygiene
WE	Working Electrode
WHO	World Health Organization

LIST OF APPENDICES

PEMBANGUNAN APTAMER DNA UNTUK ANTIGEN HEMOLYSIN E DARIPADA *SALMONELLA ENTERICA* SEROVAR TYPHI KE ARAH APLIKASI DIAGNOSTIK

ABSTRAK

Pengesanan pantas dan tepat untuk demam kepialu menggunakan penanda biologi yang spesifik adalah penting bagi meningkatkan hasil rawatan dan mencegah penularan penyakit ini. Kajian ini bertujuan untuk membangunkan sistem pengesanan berasaskan aptamer DNA bagi *Salmonella enterica* serovar Typhi (*S*. Typhi), dengan memberi tumpuan kepada antigen hemolysin E (HlyE). Melalui proses *Systematic Evolution of Ligands by EXponential enrichment* (SELEX), aptamer DNA yang mensasarkan antigen HlyE telah diasingkan dan dinilai untuk daya ikatannya serta keperinciannya melalui ujian asai oligonukleotida terangkai enzim (ELONA). Hasil SELEX mengenal pasti 11 aptamer, dan tiga daripadanya (AptHlyE97, AptHlyE11 dan AptHlyE45) dipilih untuk pencirian lanjut. Pemalar pengurai (*Kd*) ketiga-tiga aptamer ini berada dalam julat nanomolar, dengan AptHlyE97 menunjukkan daya ikatan tertinggi pada 83.6 nM, diikuti oleh AptHlyE11 pada 102.2 nM dan AptHlyE45 pada 119.3 nM. Ujian keperincian menunjukkan aptamer ini dapat membezakan HlyE *S*. Typhi dengan berkesan daripada bakteria lain, termasuk *Salmonella* Paratyphi A ($P<0.001$), *Salmonella* Paratyphi B ($P<0.001$), *Shigella flexneri* ($P<0.001$), *Klebsiella pneumoniae* ($P<0.001$) and *Escherichia coli* ($P<0.001$). Analisis teknik pelarasan molekul menyokong penemuan ini, dengan AptHlyE97 menunjukkan tenaga ikatan tertinggi (-15.5 kcal/mol). Simulasi dinamik molekul mengesahkan kestabilan aptamer-antigen, dengan sisihan punca min kuasa dua (RMSD) sekitar ~2.0 Å sepanjang simulasi 100 ns. AptHlyE97 kemudian digunakan untuk membangunkan

aptasensor elektrokimia sebagai alat diagnostik bukti konsep. Aptasensor ini dibina dengan menggunakan AptHlyE97 pada skrin bercetak elektrod emas (Au-SPE) melalui konjugasi tiol, dengan kalium ferisianida dan ferosianida digunakan untuk pengesanan isyarat. Aptasensor ini menunjukkan prestasi diagnostik yang cemerlang, dengan mencapai 100% ketepatan dan 85.7% keperincian dalam ujian sampel serum. Selain itu, ia mempamerkan tindak balas linear yang kuat dengan had pengesanan (LoD) serendah 0.158 ng/mL. Kajian ini memperkenalkan aptamer yang baharu untuk antigen *S*. Typhi dan platform diagnostik berasaskan aptamer untuk pengesanan efisien dan selektif antigen HlyE *S*. Typhi. Kepekaan tinggi, keperincian dan had pengesanan rendah aptasensor yang dibangunkan menyerlahkan potensinya untuk memajukan diagnostik di tempat penjagaan (*point-of-care*), terutamanya di kawasan dengan sumber yang terhad. Inovasi ini menyediakan asas kukuh untuk meningkatkan pengesanan dan pengurusan demam kepialu, serta menawarkan alat yang menjanjikan untuk pemantauan dan kawalan penyakit di peringkat komuniti.

DEVELOPMENT OF DNA APTAMERS FOR HEMOLYSIN E ANTIGEN OF *SALMONELLA ENTERICA* SEROVAR TYPHI TOWARDS DIAGNOSTIC APPLICATION

ABSTRACT

The rapid and accurate diagnosis of typhoid fever using specific biomarkers is essential for enhancing treatment outcomes and preventing disease transmission. This study aimed to develop a DNA aptamer-based detection system for *Salmonella enterica* serovar Typhi (*S*. Typhi), focusing on the hemolysin E (HlyE) antigen. Using Systematic Evolution of Ligands by EXponential enrichment (SELEX), DNA aptamers targeting the HlyE antigen were isolated and evaluated for binding affinity and specificity through enzyme-linked oligonucleotide assay (ELONA). Following SELEX, 11 aptamers were identified, and three (AptHlyE97, AptHlyE11 and AptHlyE45) were selected for further characterization. Their dissociation constants (Kd) fell within the nanomolar range, with AptHlyE97 showing the highest binding affinity at 83.6 nM, followed by AptHlyE11 at 102.2 nM and AptHlyE45 at 119.3 nM. Specificity tests demonstrated that these aptamers could effectively distinguish *S*. Typhi HlyE from other bacteria, including *Salmonella* Paratyphi A ($P<0.001$), *Salmonella* Paratyphi B ($P<0.001$), *Shigella flexneri* ($P<0.001$), *Klebsiella pneumoniae* ($P<0.001$) and *Escherichia coli* ($P<0.001$). Molecular docking analysis further supported these findings, with AptHlyE97 displaying the highest binding energy (-15.5 kcal/mol). Molecular dynamics simulations confirmed the aptamer-antigen stability, with a root-mean-square deviation (RMSD) of ~2.0 Å during 100 ns simulations. AptHlyE97 was subsequently used to develop an electrochemical aptasensor as a proof-of-concept diagnostic tool. The aptasensor was constructed by

immobilizing AptHlyE97 onto a screen-printed gold electrode (Au-SPE) via thiol conjugation, with potassium ferricyanide and ferrocyanide employed for signal detection. The aptasensor demonstrated excellent diagnostic performance, achieving 100% sensitivity and 85.7% specificity in serum sample testing. Additionally, it exhibited a strong linear response with a limit of detection (LoD) of 0.158 ng/mL. This study presents a novel aptamer specific against HlyE antigen of *S.* Typhi and aptamer-based diagnostic platform for the efficient and selective detection of *S.* Typhi HlyE antigen. The high sensitivity, specificity and low detection limit of the developed aptasensor highlight its potential for advancing point-of-care diagnostics, particularly in resource-limited settings. This innovation lays a solid foundation for improving typhoid fever detection and management, offering a promising tool for community-level disease monitoring and control.

CHAPTER 1

INTRODUCTION

1.1 Research background

Typhoid fever is an infectious disease caused by the bacterium *Salmonella enterica* serovar Typhi (*S.* Typhi). The disease remains a significant public health challenge, particularly in underdeveloped and developing countries including Malaysia (Muhammad *et al*., 2020). Despite the availability of vaccines and the implementation of preventive measures, the disease continues to cause considerable morbidity and mortality worldwide. The typhoid and paratyphoid collaborators estimate that there are approximately 10.8 million cases of typhoid fever each year, resulting in around 116,800 deaths (Als *et al*., 2018). The burden of the disease is disproportionately high in areas with limited access to clean water, inadequate sanitation and poor hygiene practices.

The disease is primarily transmitted through the consumption of contaminated food, water, or beverages and is most prevalent in regions with inadequate sanitation and poor hygiene practices. Once infecting humans, the bacteria multiply and spread throughout the bloodstream, causing symptoms such as fever, headache, abdominal pain and a characteristic rash known as "rose spots" (Chowdhury *et al*., 2014). Other symptoms include fatigue, loss of appetite and constipation or diarrhoea (Crump *et al*., 2015). Typhoid fever is a serious illness that can lead to complications such as intestinal bleeding, perforation of the bowel and even death if left untreated (Contini, 2017). Treatment typically involves class of antibiotics such as chloramphenicol, amoxicillin, trimethoprim-sulfamethoxazole, ceftriaxone and ciprofloxacin, and supportive care, such as hydration and pain relief (Parry *et al*., 2023).

Rapid and accurate diagnosis of typhoid fever is crucial for effective treatment and control of the transmission of the disease. Current diagnostic methods for typhoid fever have several limitations. Culture methods are still considered the gold standard, with great performance of highly specific, but the method is time-consuming, often requiring several days to yield results, which delays treatment (Saha *et al*., 2023). Serological tests, such as the Widal test, lack sensitivity and specificity, leading to false positives and false negatives (Ashfaq *et al*., 2018). Molecular techniques like polymerase chain reaction (PCR), although highly accurate, are expensive and require sophisticated laboratory infrastructure and trained personnel, making them impractical for widespread use in resource-limited settings (Amalina *et al*., 2021). In response to the limitations of existing diagnostic methods, there is a pressing need for a new alternative diagnostic tool that is rapid and accurate that can be utilized for typhoid testing, especially in resource-limited settings.

Most of the point-of-care tests currently available on the market primarily focus on antibody detection, utilizing various proteins as biomarkers *(*Ahmad Najib *et al*., 2021). However, a significant limitation of these tests is that they only identify elevated levels of specific antibodies against unique proteins of *S*. Typhi. In contrast, ongoing research aims to detect antigen that indicates the presence of the organism itself. While studies have demonstrated that antibodies serve as the primary biomarker for antigen detection tests, they also come with several disadvantages (Deeks *et al*., 2020). To address these issues, this study explores the use of aptamers as an alternative to antibodies, potentially offering a more effective solution for detection.

Aptamers, which are short, single-stranded DNA or RNA molecules that are capable of binding to specific target molecules, such as proteins, small molecules, or even whole cells, with high affinity and specificity, offer a promising solution (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Aptamers are often referred to as "chemical antibodies" due to their ability to recognize and bind to target molecules in a manner similar to antibodies, but with several advantages over traditional antibodies, such as their smaller size, high thermal stability and easy modification (Bauer *et al*., 2019). Recent studies demonstrate that aptamers have emerged as an attractive alternative to antibodies in various fields, including biomedical research and clinical diagnosis (Zhou *et al*., 2014; Xu *et al*., 2024). Aptamers, being nucleic acid-based molecules, exhibit greater thermal stability compared to antibodies (Thiviyanathan and Gorenstein, 2012).

The absence of aptamer-based methods for detecting *S.* Typhi antigens in serum prompted our efforts to develop specific aptamers against HlyE antigen of *S.* Typhi through Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Ahmad Najib *et al*., 2021). The *S.* Typhi hemolysin E (HlyE) antigen represents a promising biomarker for typhoid diagnostics (Ong *et al*., 2015; Felgner *et al*., 2017; Andrews *et al*., 2019; Franklin *et al*., 2020; Shailendra Kumar *et al*., 2020), and could be used for fast, effective diagnostic and short-term control of typhoid fever. These newly identified aptamers aim to be utilized in the development of aptamer-based antigen detection for rapid typhoid testing. This aptamer-based sensing platform for *S.* Typhi HlyE is projected to be valuable for early detection and control of typhoid fever, especially in resource-limited settings.

1.2 Problem statements

The prevention of typhoid fever currently relies heavily on intervention strategies such as practising good hygiene and sanitation, drinking clean water and mass vaccination, especially for food handlers (Appiah *et al*., 2020). However, the implementation of these strategies is significantly hindered in underdeveloped and developing countries due to several factors such as limited access to clean water and sanitation, poor hygiene practices and inadequate vaccination coverage pose substantial barriers. As a result, these interventions are insufficient to prevent typhoid outbreaks effectively. In such contexts, the best strategy to prevent typhoid outbreaks is to rely on rapid and accurate diagnostic methods so that delayed diagnosis can be prevented. Delayed diagnosis contributes to sustained transmission within the community (Saha *et al*., 2023). Since *S.* Typhi is transmitted through the fecal-oral route, infected individuals who remain undiagnosed may unknowingly spread the disease via contaminated food or water. This is particularly concerning in areas with poor sanitation and limited access to clean water (Im *et al*., 2021).

The current gold standard for diagnosing typhoid fever is culture method which involves enrichment, isolation and biochemical identification of bacterial cultures from blood and stool samples in laboratories (Baker *et al*., 2010). However, this approach presents a significant challenge in resource-limited settings. Many areas lack essential laboratory facilities for culturing, making this method impractical. Additionally, despite the culture method is time-consuming, requiring several days to isolate and identify the causative agents, which delays diagnosis and treatment, the method is highly specific (Castonguay-Vanier *et al*., 2013).

Alternative screening techniques have been developed to provide faster sample to results turnaround time. These include the monoclonal and polyclonal antibody-based techniques such as TUBEX (Bundalian *et al*., 2019), Typhidot (Salama and Said, 2019) and Multi-Test Dip-S-Tics (Olsen *et al*., 2004). Despite their faster diagnostic capabilities, these techniques have several limitations. The high cost of antibody production, cross-reactivity with other bacteria and thermal instability pose significant challenges, especially for transportation and storage in tropical and subtropical climates where typhoid is prevalent (Bauer *et al*., 2019).

Therefore, there is a critical need for a rapid diagnostic method for typhoid fever that can overcome the limitations of current techniques. Electrochemical biosensors, particularly those utilizing aptamers as biorecognition elements, offer a promising solution. Aptamers, synthetic single-stranded DNA or RNA molecules can bind specifically to target analytes with high affinity and can be chemically synthesized at a lower cost compared to antibodies. Their stability, reusability and ease of modification make them attractive candidates for sensor development. By integrating aptamers with electrochemical transducers, an electrochemical aptasensor can be developed to detect target pathogens or biomarkers with high sensitivity and selectivity. The electrochemical aptasensor can deliver quantitative results in a short time, making them ideal for use in decentralized settings, such as rural clinics or field diagnostics. This study aims to address this need by developing DNA aptamers for the detection of *S.* Typhi, focusing on creating a robust diagnostic tool suitable for use in resource-limited settings.

1.3 Scope of study

The scope of this study was to develop DNA aptamer-based antigen detection for *S.* Typhi, specifically targeting the HlyE antigen. This research involved three distinct phases. The first phase focused on the isolation and characterization of DNA aptamers that could specifically bind to the HlyE antigen of *S.* Typhi. This was achieved using SELEX, which involved iterative rounds of selection and amplification of single-stranded DNA (ssDNA) library to enrich for aptamers with high affinity and specificity. Following the final SELEX round, the elution were cloned and 100 transformants were randomly selected, sequenced, and subjected to phylogenetic analysis to identify the aptamers sequences for further characterization.

Then, three most probable aptamers were subjected for the characterization of the binding affinity and specificity towards other *Enterobacteriaceae*. In this phase, the enzyme-linked oligonucleotide assay (ELONA) was employed to determine the binding affinity and specificity of the aptamers, ensuring the aptamers were both highly specific and strongly binding to the HlyE antigen of *S.* Typhi.

In the second phase, the present study elucidated the binding interactions between the DNA aptamers and the HlyE antigen. Structural studies using computational modelling were conducted to provide a detailed understanding of the molecular interactions and the binding interface. This phase aimed to uncover the details of how the aptamers bound to the HlyE antigen, improving our understanding of the aptamer-antigen complex structure. Data obtained from the computational binding interactions analyses also provides insight into the suitability of the aptamer to be used in developing an electrochemical aptasensor for digitalized results.

The final phase involved the development of an electrochemical aptasensor as a proof-of-concept for diagnostic applications. This phase involved integrating the best DNA aptamer into an electrochemical sensor platform. Development of the electrochemical aptasensor begins with optimization and characterization of the screen-printed gold electrode (Au-SPE) using Square wave voltammetry (SWV) responses. Finally, a feasibility study was conducted to evaluate the diagnostic performance of the newly developed electrochemical aptasensor using archived serum samples of typhoid patients, healthy individuals and patients of other bacterial diseases such as *S*. Paratyphi A, *S*. Paratyphi B, *Escherichia coli, Klebsiella pneumoniae* and *Shigella flexneri*. Finally, the limit of detection (LoD) for the developed electrochemical aptasensor was determined to evaluate its analytical sensitivity in detecting low concentrations of the *S*. Typhi HlyE antigen.

Upon successful completion of these phases, the present project has achieved early Technology Readiness Level (TRL) 3, indicating that the developed aptamer-based detection method had been validated in a laboratory environment as a proof-of-concept and was tailored to further development towards practical diagnostic applications. This comprehensive study aimed to establish a solid foundation for an innovative diagnostic tool for typhoid fever, potentially leading to improved detection and management of the disease. The flowchart of the present study design is illustrated in Figure 1.1.

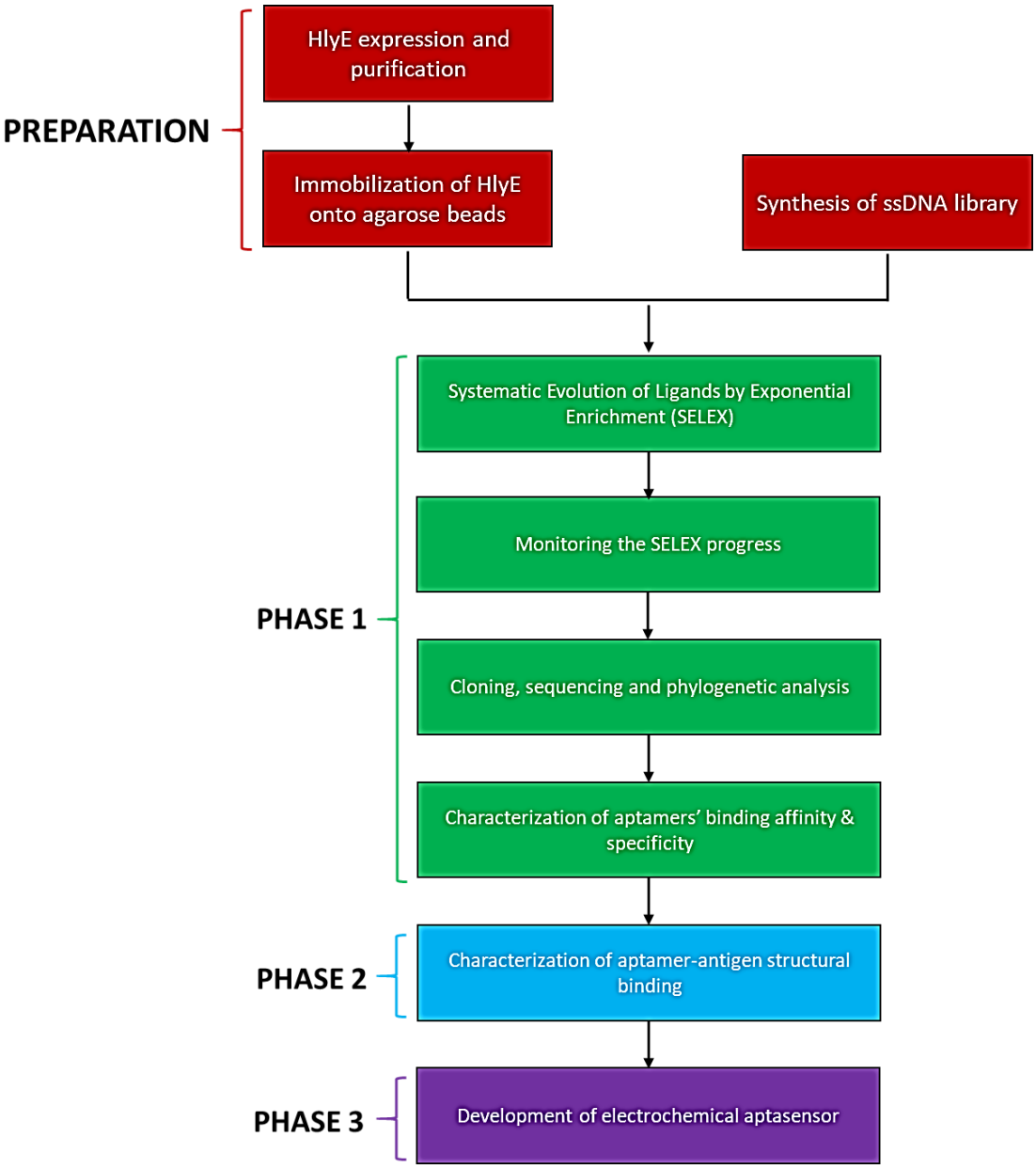

Figure 1.1 The general flow chart of study design.

1.4 Objectives of the study

1.4.1 General objective

The general objective of this study was to develop DNA aptamer-based antigen detection utilizing electrochemical sensor for *Salmonella enterica* serovar Typhi as a proof-of-concept.

1.4.2 Specific objectives

1. To isolate and characterize the ssDNA aptamers with high binding affinity and specificity against the recombinant HlyE antigen of *Salmonella* Typhi using SELEX and ELONA.

2. To validate the interaction and binding stability of selected aptamers with the HlyE antigen of *Salmonella* Typhi through molecular docking and molecular dynamics simulation.

3. To construct and evaluate an electrochemical aptasensor incorporating the optimal aptamer for the selective detection of *Salmonella* Typhi HlyE antigen in serum samples.

1.5 Research questions

1. What ssDNA aptamers can be isolated that demonstrate high affinity and specificity toward the recombinant HlyE antigen of *Salmonella* Typhi?

2. Do the selected ssDNA aptamers exhibit stable and specific molecular interactions with the HlyE antigen of *Salmonella* Typhi as demonstrated by molecular docking and molecular dynamics simulations?

3. Can an electrochemical aptasensor incorporating the optimal aptamer selectively and sensitively detect the HlyE antigen of *Salmonella* Typhi in serum samples?

1.6 Hypotheses of the study

1. H1: SELEX will yield ssDNA aptamers with strong and specific binding to the HlyE antigen, measurable through ELONA.

2. H2: Selected aptamers will form stable complexes with HlyE, characterized by favourable binding energies and sustained interactions in molecular docking and dynamics simulations.

3. H3: An electrochemical aptasensor utilizing the optimal aptamer will enable sensitive and selective detection of HlyE in serum, with minimal cross-reactivity to non-target proteins.

CHAPTER 2

LITERATURE REVIEW

2.1 *Salmonella enterica* serovar Typhi

Salmonella enterica serovar Typhi (*S.* Typhi) is a type of Gram-negative bacterium, belonging to the family *Enterobacteriaceae*. It presents a rod-shaped morphology, typically measuring 0.7-1.5 × 2.0-5.0 µm, and is equipped with flagella for motility (Figure 2.1). The *S.* Typhi is classified as an obligate human pathogen. As an obligate pathogen, *S.* Typhi requires a human host to survive and replicate. Unlike other strains of *Salmonella* which typically cause localized gastroenteritis and can infect a broader range of hosts, including various animals, *S.* Typhi is responsible for typhoid fever, a severe systemic illness unique only to humans. It demonstrates a remarkable adaptation to human intestinal environment, initially colonizing the gut before infiltrating the bloodstream via the Peyer's patches in the small intestine (Raffatellu *et al*., 2008). This invasion triggers gastrointestinal inflammation and prolonged episodes of high fever. Factors such as poor sanitation, inadequate sewage disposal, contaminated water sources and suboptimal personal hygiene create favourable conditions for *S.* Typhi transmission, resulting in significant disease burden, mortality and economic losses (Akullian *et al*., 2015). Rapid and accurate diagnosis is crucial for effective clinical management and to mitigate morbidity and mortality rates associated with typhoid fever. However, the development of dependable diagnostic markers and tests remains a challenging endeavour. Failure to promptly diagnose and treat typhoid fever can lead to severe complications such as peritonitis, intestinal hemorrhage, or perforation, substantially increasing the risk of mortality to between 10-30% (Dougan and Baker, 2014; Contini, 2017).

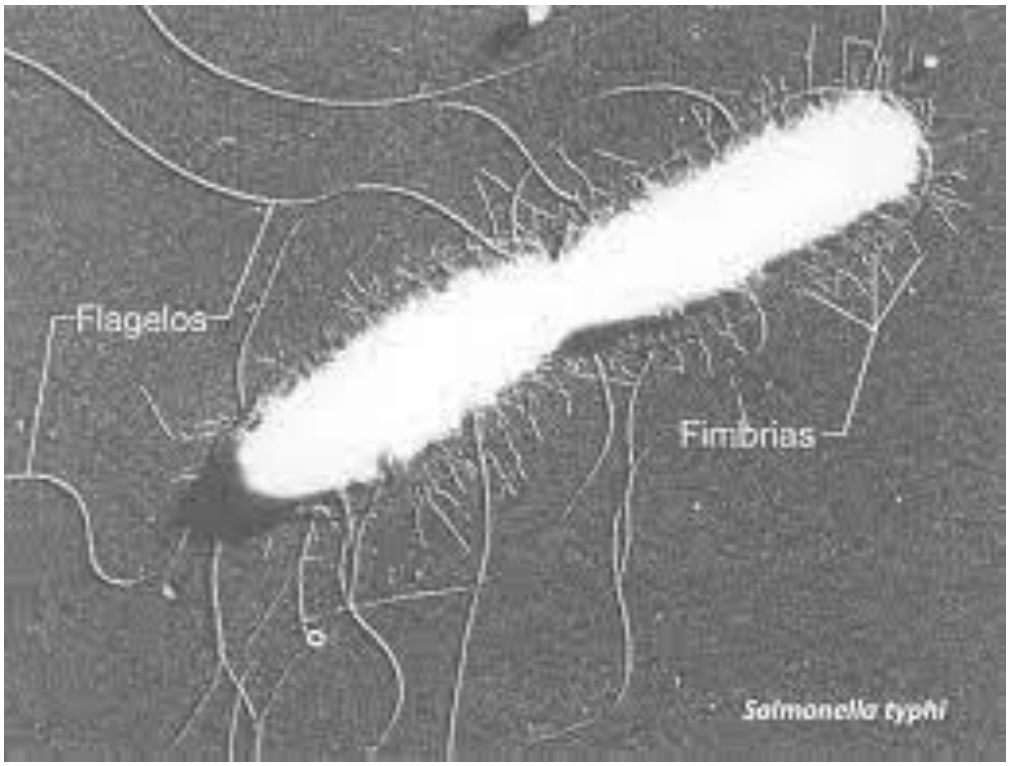

Figure 2.1 The scanning electron micrograph of *Salmonella* Typhi (image adapted from https://semicrobiologia.org).

2.1.1 Taxonomy

In 1987, Le Minor and Popoff proposed that *Salmonella enterica*, a species of considerable interest, could be categorized into seven subspecies: I, *enterica*; II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *houtenae*; V, *bongori*; and VI, *indica* (Le Minor and Popoff, 1987). However, subsequent investigations utilizing DNA-DNA hybridization techniques prompted the reclassification of *S.* bongori as an independent species (Agbaje *et al*., 2011). Currently, it is widely acknowledged that the *Salmonella* genus encompasses three primary species which are *Salmonella enterica, Salmonella bongori* and *Salmonella subterranean*, alongside six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*, aggregating a considerable number of serotypes (Issenhuth-Jeanjean *et al*., 2014). The *S.* Typhi is a serotype that belongs to *S. enterica* subspecies I (*S. enterica* subsp. enterica).

2.1.2 Nomenclature

The taxonomy and nomenclature of *Salmonella* have been subjects of longstanding complexity and debate. In the early 1920s, confusion surrounded *Salmonella* taxonomy until the introduction of the Kauffman-White scheme by Philip Bruce White in 1926. Fritz Kauffman further refined this scheme from 1933 to 1978. The Kauffman-White scheme classified *Salmonella* species based on serological identification of O (somatic) and H (flagella) antigens, leading to the recognition of over 2,500 serovars today. Despite this, new serovars continue to be identified annually. Presently, the *Salmonella* nomenclature system is overseen by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute in France (Agbaje *et al*., 2011).

2.2 Typhoid fever

Typhoid fever is an acute generalized infection of the reticuloendothelial system, intestinal lymphoid tissue and gallbladder caused by *S.* Typhi (Crump, 2019). This communicable disease is restricted to human hosts and humans (chronic carriers) serve as the reservoir of infection (Crump, 2019).

2.2.1 Epidemiology

One of the most famous outbreaks of typhoid fever is linked to Typhoid Mary, a nickname given to Mary Mallon, an asymptomatic carrier of *S.* Typhi. In the early 1900s, Mary Mallon worked as a chef in New York City, unknowingly infected 23 people, two of whom died (Marineli *et al*., 2013). Mary was the first person in the United States identified as a healthy carrier of the disease, and her case led to significant public health efforts to track and manage typhoid carriers. Despite being quarantined twice, she continued to work as a chef, leading to further infections, and her case became a landmark in understanding how asymptomatic individuals can spread infectious diseases (Marineli *et al*., 2013).

Typhoid fever is endemic in many low- and middle-income countries (LMICs), particularly in the regions of South Asia (India, Pakistan, Bangladesh and Nepal) and sub-Saharan Africa (Kenya, Ghana and Tanzania) (Crump, 2019). According to an epidemiological study in 2000, typhoid fever was estimated to cause approximately 21.7 million illnesses and 216,000 deaths worldwide (Crump *et al*., 2004). An updated study in 2010 reported a decline in the number of cases, with typhoid fever accounting for 11.9 million cases and 129,000 deaths, based on blood culture-confirmed cases from the 2010 population (Mogasale *et al*., 2014). In 2017, a new estimation for typhoid burden using epidemiological data up to 2014 estimated that 17.8 million cases of

typhoid fever occur each year in LMICs with central Africa predicted to experience the highest incidence of typhoid, followed by countries in Central Asia, South Asia and Southeast Asia (Antillón *et al*., 2017).

The 2019 Global Burden of Disease Report identified 44 countries with a high estimated burden of typhoid fever, defined as ≥100 cases per 100,000 persons (Vos *et al*., 2020). The highest estimated incidences of typhoid fever were found in Southeast Asia and the Eastern Mediterranean, with incidences of 306 and 187 cases per 100,000 persons per year, respectively, as well as in the African WHO superregions, which reported 111 cases per 100,000 persons per year (Vos *et al*., 2020). In 2019, typhoid fever was the 18th leading cause of disability-adjusted life-years (DALYs) for children aged 0 to 9 years and the 14th leading cause for those aged 10 to 24 years globally (Vos *et al*., 2020). Additionally, *S.* Typhi is the leading cause of non-hospital-acquired bloodstream infections in South and Southeast Asia (Meiring *et al*., 2023).

According to Morbidity and Mortality Weekly Report on typhoid fever incidence estimates during 2018–2022, typhoid incidence is still high in the WHO South-East Asian, Eastern Mediterranean and African regions (Hancuh *et al*., 2023). One plausible reason for this is that, since 2018, only a few countries have introduced typhoid conjugate vaccine (TCV) into their national routine immunization schedule (Hancuh *et al*., 2023).

Another recent source of epidemiological data on typhoid fever is a systematic analysis by the Global Burden of Disease Study in 2017 (Stanaway *et al*., 2019). The study reported 10.9 million cases and 116,800 deaths in 2017. Overall, the analysis showed a decline in the number of typhoid cases globally, from 20.366 million in 1990 to 10.9 million in 2017 (Table 2.1). The reported estimates are consistent with previous assessments of the typhoid fever burden, which showed a declining trend in typhoid incidence in the majority of countries where typhoid is endemic (Als *et al*., 2018). However, in some countries such as Ghana, Malawi, Fiji, China, Indonesia, Cambodia and Iraq, typhoid incidence has shown a steady increase (Stanaway *et al*., 2019). According to WHO estimates from 2019, there are 9 million cases of typhoid fever annually, resulting in approximately 110,000 deaths per year. The 2019 data is the most recent available, with no updates on typhoid epidemiology since then. This highlights a significant gap in the epidemiological understanding of typhoid fever.

Estimating the global burden of typhoid fever remains challenging due to the limited number of recent population-based studies that use blood culture confirmation from typhoid-endemic regions (Crump, 2019). Data on typhoid incidence is still limited in Asian regions. The most recent data comes from the surveillance for enteric fever in Asia project, which covered three countries: Pakistan, Bangladesh and Nepal (Garrett *et al*., 2022). The adjusted incidence of *S.* Typhi per 100,000 people per year was 913 in Bangladesh, 330 in Nepal and 176 in Pakistan (Garrett *et al*., 2022). The highest incidence rates were observed among children (Garrett *et al*., 2022). This significant disease burden emphasizes the need for effective control measures (Brockett *et al*., 2020).

Table 2.1 The global burden of typhoid fever from 1990 to 2017 (Stanaway *et al.*, 2019).

Year	Cases (Million)	Deaths (Thousands)
1990	20.366 (17.117–23.882)	202.0 (112.5–327.1)
1995	18.424 (15.562–21.584)	185.4 (104.2–302.6)
2000	16.797 (14.229–19.550)	171.6 (96.6–278.9)
2005	15.415 (13.131–17.894)	160.2 (89.8–262.9)
2010	13.769 (11.739–15.938)	145.4 (80.9–236.0)
2017	10.924 (9.343–12.597)	116.8 (65.4–187.7)

In European countries, typhoid fever is relatively rare and is mainly acquired during travel to countries where typhoid is endemic (Zuckerman *et al*., 2017). According to the European typhoid and paratyphoid fever annual epidemiological report for 2021, a total of 304 laboratory-confirmed typhoid cases were reported of which 67.9% were travel-related (European Centre for Disease Prevention and Control, 2024). The low number of cases was most likely due to the travel restrictions implemented during the COVID-19 pandemic. However, in the second half of 2021, an increase in the number of cases was observed, with the usual seasonal peak in September (European Centre for Disease Prevention and Control, 2024).

Although typhoid incidence has decreased in most developed countries, it remains prevalent in many LMICs (Als *et al*., 2018). Data on the incidence of typhoid fever are scarce in LMICs (Antillón et al., 2017). The true incidence of the disease burden is probably higher due to a lack of reliable data collection systems in many endemic regions (Als *et al*., 2018).

Even though Malaysia has been categorized as a region with high incidence rates for typhoid fever, several implementation strategies have been made to control the disease through improved sanitation, clean water access and vaccination campaigns (Jaafar *et al*., 2013). However, sporadic outbreaks still occur, especially in rural areas with limited infrastructure (Nik Mohd Hafiz, 2023). In recent years, the introduction of the vaccine has been made compulsory for food handlers to further reduce the burden of typhoid (Date *et al*., 2014). Despite these efforts, continuous surveillance, public health education and better access to healthcare are essential to fully eradicate the disease and prevent future outbreaks (Nik Mohd Hafiz, 2023).

According to a recent study on the burden of typhoid fever in Klang Valley, Malaysia, a total of 507 cumulative cases of typhoid fever were reported in the Klang Valley over 5 years from 2011 to 2015 (Muhammad *et al*., 2020). Despite rapid urbanization and development, local transmission of typhoid remains prevalent in the region. During this period, the highest number of cases was recorded in the Gombak district (34 cases, 82.9%), followed by Hulu Langat (33 cases, 66.0%), Kuala Lumpur (95 cases, 65.5%) and Petaling Jaya (79 cases, 53.4%) (Muhammad *et al*., 2020). The Klang district reported the lowest number of cases, with 27 cases (22%) (Muhammad *et al*., 2020).

Typhoid is endemic in Malaysia with an average incidence rate of 0.76 per 100,000 population reported annually in recent years (2014-2019). In 2022, the Ministry of Health (MOH) recorded 90 typhoid cases, especially from Selangor, Sabah and Kelantan. The incidence of typhoid in Kelantan is the highest compared to all other states due to the use of well water for drinking and other domestic purposes. There was an outbreak in Kelantan in 2004 whereby 888 cases were reported with 2 deaths (Jaafar *et al*., 2013). From 1994 to 2003, typhoid cases in Kelantan ranged from 189 to 361 cases. The sudden increase in typhoid cases might be related to floods at the end of year 2004. During that period, safe water supply coverage in Kelantan was only 81.8%. To date, data on the incidence of typhoid fever in Malaysia are scarce due to lack of continuous surveillance. Owing to that, the true burden of the disease is probably underestimated as there is a possibility of under-reporting of typhoid cases because of limited laboratory capacity (Muhammad *et al*., 2020).

2.2.2 Pathogenesis

The *S*. Typhi infection begins when the bacteria is ingested through contaminated food or water. The bacteria primarily invade the intestinal epithelium, particularly through specialized structures called Peyer's patches located in the terminal ileum (Baker *et al*., 2010). These patches are rich in lymphoid tissue and serve as a critical entry point for the bacteria into the host's immune system. Upon reaching the gut, *S*. Typhi utilizes fimbriae to adhere to epithelial cells, which allows for its internalization by phagocytic cells such as macrophages (Chowdhury *et al*., 2014). This process is mediated by pathogen-associated molecular patterns (PAMPs) recognized by toll-like receptors (TLRs) on macrophages (Mathur *et al*., 2012). Notably, *S*. Typhi possesses a Vi capsular antigen that helps it evade detection and destruction by neutrophils, enhancing its virulence compared to other serovars like *S*. Paratyphi A (Zhang *et al*., 2022).

Once inside macrophages, *S*. Typhi manipulates the host's cellular machinery for its replication (Chowdhury *et al*., 2014). The bacteria can survive and multiply within these immune cells, leading to their eventual apoptosis, which releases the bacteria into the bloodstream (Baker *et al*., 2010). This intracellular survival is crucial for systemic dissemination, allowing *S*. Typhi to spread to various organs such as the liver, spleen and bone marrow via the lymphatic system (Johnson *et al*., 2018).

The bacteria's ability to induce apoptosis in macrophages not only facilitates their release but also contributes to a significant inflammatory response that can lead to complications such as intestinal bleeding or perforation (Contini, 2017). The systemic spread of *S*. Typhi results in bacteremia and can lead to severe complications if untreated (Contini, 2017).

Certain genetic factors in hosts may predispose individuals to typhoid fever and affect disease severity. For example, polymorphisms in genes related to immune response can influence susceptibility to infection and severity of symptoms (Ma *et al*., 2021). Additionally, chronic carriers of *S*. Typhi, who may not exhibit symptoms but continue to shed bacteria, pose a significant risk for transmission in communities. The pathogenesis of *S*. Typhi is illustrated in Figure 2.2.

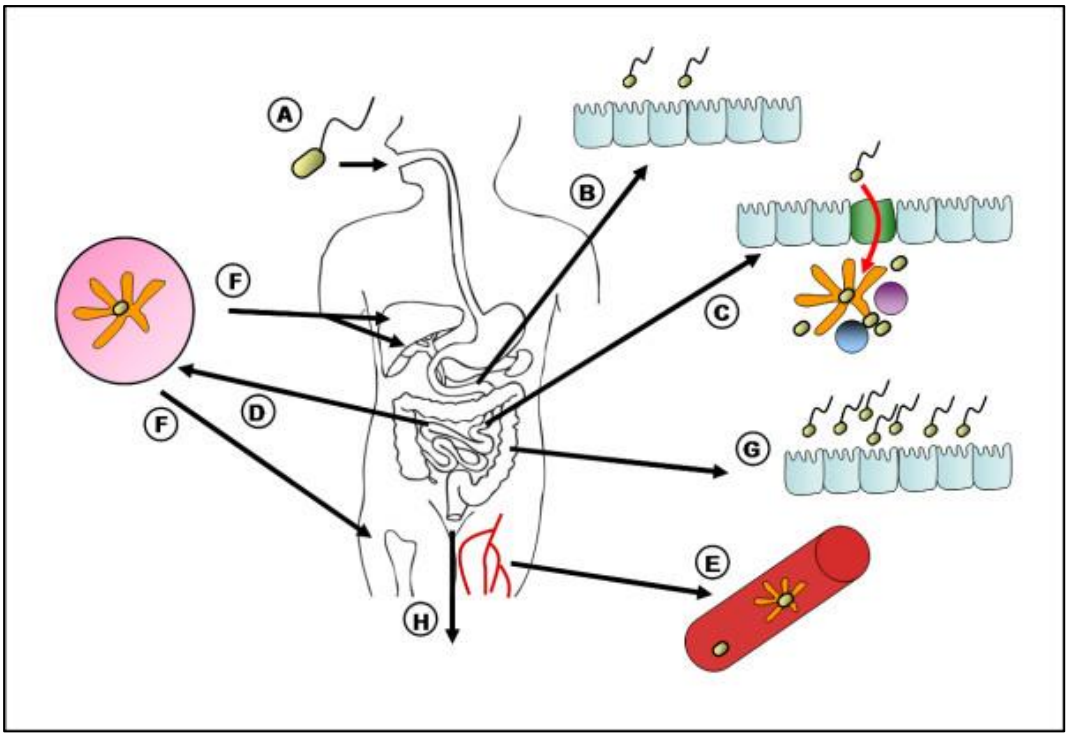

Figure 2.2 The pathogenesis of *S*. Typhi in the human host (Baker *et al.*, 2010). A; *S*. Typhi enters the human host through oral ingestion of an infectious dose. B; *S*. Typhi does not replicate in large numbers in the intestine and shedding may be sporadic and limited. C; Invasion occurs through the terminal ileum, perhaps a short time after ingestion, M cells may be the preferred portal of entry. D; *S*. Typhi is transferred to monocytic cells and is trafficked to the reticuloendothelial system, potentially in a semi-dormant state. E; *S*. Typhi re-emerges at an unknown time from the reticulo-endothelial system, possibly as the acquired immune response is activated, and re-enters the blood stream in low numbers. F; *S*. Typhi seeds into the liver, the gall bladder and the bone marrow where it can reside and may be detected for months or years. G; *S*. Typhi enter into the bile duct. H; *S*. Typhi shed sporadically into the environment via the intestine (Reproduced from Baker *et al.*, 2010 with permission from BioMed Central Ltd).

2.2.3 Clinical features

The infectious dose of *S*. Typhi in patients varies between 1000 and 1 million organisms (Chowdhury *et al*., 2014). Symptoms of typhoid typically develop after an incubation period of 7 to 14 days post-infection (Chowdhury *et al*., 2014). In some cases, infected individuals can have typhoid without developing any symptoms. These asymptomatic individuals, while not experiencing the typical symptoms of typhoid fever, can still carry and shed the bacteria. These people become chronic carriers, harbouring *S*. Typhi in their bodies and unknowingly spreading the bacteria to others through fecal-oral contamination (Gunn *et al*., 2014).

The marking onset of the clinical illness is when the bacteria is presence in the bloodstream, resulting in bacteriaemia. The major signs and symptoms of typhoid fever are relatively nonspecific. Other infections, such as malaria, influenza, COVID-19, dengue, chikungunya, scrub and murine typhus, brucellosis and leptospirosis, may cause similar symptoms (Bhargava *et al*., 2018). Distinguishing typhoid fever from these other infections is difficult due to the non-specific clinical presentation (Mukhopadhyay *et al*., 2019).

The primary symptom that emerges is fever, which gradually increases and may reach a high plateau of 39 to 40 °C (Yasin *et al*., 2018). Another main symptom is the appearance of a rash, which does not occur in all patients and is characterized by rose-coloured spots, particularly on the neck and abdomen (Yasin *et al*., 2018). Other symptoms can include fatigue, headache, nausea, abdominal pain and constipation or diarrhoea (Johnson *et al*., 2018). While adults commonly experience constipation, young children and adults with HIV infection are more prone to diarrhoea (Chowdhury