# EVALUATION OF *Salmonella* Typhi ANTIGENS HlyE AND YncE FOR THE DETECTION OF TYPHOID FEVER AND ITS CARRIERS

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**by**

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| | |
|---|---|
| µL | Microliter |
| µg | Microgram |
| µm | Micrometre |
| µM | Micromolar |
| °C | Degree Celsius |
| % | Percent |
| Amp | Ampicillin |
| Kan | Kanaymycin |
| CMP | Chloramphenicol |
| BSA | Bovine serum albumin |
| bp | Base pair |
| DNA | Deoxyribonucleic acid |
| HlyE | Hemolysin E |
| LC | Liquid chromatography |
| kDa | Kilodalton |
| LB | Luria Broth |
| M | Molar |
| min | Minutes |
| ml | Millilitre |
| mM | Millimolar |
| SEC | Size exclusion chromatography |

**PENILAIAN ANTIGEN HlyE DAN YncE DARIPADA *Salmonella* Typhi UNTUK PENGESANAN DEMAM TIFOID DAN PEMBAWANYA**

## ABSTRAK

*Salmonella* Typhi (*S.*Typhi) merupakan agen penyebab demam tifoid (DT) yang menyebabkan jutaan kes infeksi dan 1% kematian di seluruh dunia. DT sangat endemik di Asia Tenggara dan Afrika sub-Sahara. Individu tertentu yang menghidap DT mungkin tidak pulih sepenuhnya dan boleh menjadi pembawa yang tidak menonjolkan sebarang simptom DT. Pembawa ini boleh menyebarkan penyakit melalui sentuhan dengan makanan dan air. Paras antibodi terhadap protein HlyE adalah tinggi dalam kalangan pesakit DT berbanding dengan individu sihat, manakala paras antibodi terhadap protein YncE dilaporkan tinggi dalam kalangan pembawa *S.* Typhi. Kaedah yang cepat dan tepat untuk diagnosis individu dijangkiti DT atau adalah pembawa penting untuk mengawal penyakit ini. Dalam kajian ini, antigen rekombinan HlyE dan YncE didapati berguna untuk mengenal pasti individu dengan DT dan individu yang berkemungkinan adalah pembawa *S.*Typhi. Pertama sekali, antigen rekombinan HlyE dan YncE telah diekspresi dan ditulenkan. Seterusnya, ELISA rHlyE dan rYncE telah dimajukan untuk membolehkan penyaringan kuantitatif banyak sampel serentak untuk mengenal pasti sampel yang mempunyai paras antibodi IgM, IgG dan IgA yang tinggi terhadap antigen tersebut. Sejumlah 425 sampel serum (pesakit tifoid akut, pembawa tifoid, pengendali makananan, demam lain, dan individu sihat) telah disaring dengan ELISA rHlyE dan rYncE. ELISA rHlyE-IgG, -IgA dan -IgM telah menunjukkan kepekaan sebanyak 70%, 80% dan 11% dalam pengesanan pesakit tifoid akut, dan kepekaan sebanyak 83% apabila ketiga-tiganya digabungkan. ELISA rYncE mengenal pasti 16 individu yang berkemungkinan adalah pembawa

*S.*Typhi berdasarkan profil antibodi mereka. Kesimpulannya, kajian ini menunjukkan bahawa antigen HlyE boleh digunakan untuk mengenal pasti individu dengan DT manakala antigen YncE boleh digunakan untuk mengenal pasti individu yang berkemungkinan adalah pembawa tetapi pengesahan selanjutnya diperlukan.

**EVALUATION OF *Salmonella* Typhi ANTIGENS HlyE AND YncE FOR THE DETECTION OF TYPHOID FEVER AND ITS CARRIERS**

## ABSTRACT

*Salmonella* Typhi (*S*. Typhi) is the causative agent of typhoid fever (TF) that causes millions of infections and 1% of mortality worldwide. TF is highly endemic in Southeast Asia and sub-Saharan Africa. Certain individuals with TF may not recover fully and can become carriers that do not show any symptoms of the disease. The carriers can then spread the disease when they come into contact with food and water. Antibodies against *S*. Typhi HlyE are elevated among patients with TF compared to healthy individuals, while antibodies against YncE were reported to be elevated among carriers of *S*. Typhi. Rapid and accurate methods to diagnose individuals with TF or who are carriers are crucial to manage the disease. In this study, recombinant HlyE and YncE antigens were found to be useful for the identification of individuals with TF and those who are possible carriers. Firstly, recombinant HlyE and YncE were expressed and purified. Next, rHlyE and rYncE ELISAs were developed to enable quantitative screening of many samples simultaneously to identify those with elevated IgM, IgG and IgA antibodies against the antigen. A total of 425 serum samples (acute typhoid patients, carriers, food handlers, other febrile, and healthy individuals) were screened with the ELISAs.The rHlyE-IgG, -IgA and -IgM ELISAs demonstrated a sensitivity of 70%, 80% and 11% respectively to detect acute typhoid patients, and with a combined sensitivity of 83%.The rYncE ELISA identified 16 possible carriers based on their antibody profiles. Taken together, this study showed that HlyE antigen could be used to identify individuals with TF while the YncE antigen may be used to identify possible carriers, but further validation is required.

# CHAPTER ONE

# INTRODUCTION

Typhoid fever is a systemic infection caused by the human-specific pathogen *Salmonella enterica* subsp. *enterica* serovar Typhi (*S.* Typhi). One of the common symptoms of typhoid fever is prolonged fever and is estimated to be responsible for 26.9 million cases in endemic regions all over the world with 1% mortality rate in 2017 (Buckle, Walker, & Black, 2012; Radhakrishnan et al., 2018). WHO estimates the global typhoid fever disease burden at 11-20 million cases annually, resulting in about 128 000–161 000 deaths per year (Antillón et al., 2017; Mogasale et al., 2014). A small number of individuals that could not clear out the infection after one year, harbours the pathogen in the biliary tract and intermittently sheds the bacteria in the stool. Poor hygiene practice spreads the disease through contact with food and water. These individuals who do not display any clinical symptoms are called carriers.

Laboratory diagnostic method of acute typhoid fever using conventional method such as stool and blood culturing is time- consuming and laborious. In addition, as of today there is no reliable rapid detection of carriers of *S.* Typhi. The current detection method for carriers such as PCR is not sensitive whereas bile culture from patients undergoing cholecystectomy is considered invasive. Detection of acute typhoid fever and carriers is crucial for the elimination of the disease.

Levels of antibodies in patients indicate the status and stage of infection. Many immunoassays have been developed to diagnose the disease with Enzyme-Linked Immunosorbent Assay (ELISA) being one of the most common due to being robust

and able to assess multiple samples simultaneously (Lai et al., 2004; Mikulskis, Yeung, Subramanyam, & Amaravadi, 2011; Pruslin, To, Winston, & Rodman, 1991).

HlyE antigen from *S.* Typhi has been reported to be suitable for the development of ELISA in detecting individuals with acute typhoid infection (Davies et al., 2016; Felgner et al., 2017). On the other hand, a recent discovery reported that protein YncE from *S.* Typhi is a biomarker with potential aiding the detection of suspected *S.* Typhi carriers (Charles et al., 2013).

In a laboratory setting, production of recombinant antigens using molecular techniques is advantageous as protein production can be upscaled to produce higher concentration with lower cost and further modification can be done to improve the downstream processes such as purification. In general, the production of recombinant proteins involves two steps; cloning of the protein-coding gene and protein expression. Recombinant DNA cloning is a widely utilised technique that involves genetic manipulation or modification by combining DNA from different organisms. Traditionally, cloning protocols involve the usage of the enzyme DNA ligase but ligase independent cloning (LIC) such as homologous recombination cloning is preferred in the research field as it is cost-efficient, less laborious and can be easily adapted in most cloning protocols. Homologous recombination, a LIC method involves the assembly of a gene into a vector based where the gene insert contains some identical overlapping bases with cloning vector at 5' and 3'. The recombinant plasmid is then inserted into a host such as *Escherichia coli* (*E. coli*) for protein expression. Expression of recombinant proteins is induced by an inducer.

The purity of a protein is crucial for downstream applications such as ELISA and lateral flow. Protein samples without any contaminant are essential to obtain an

accurate result and to avoid cross-reaction in the case of ELISA. Affinity tags such as poly-histidine tags are usually added to increase the solubility of the recombinant protein and to facilitate the purification process. The poly-histidine tags can be placed on the C-terminal or N-terminal of the protein and the position may affect the solubility of the protein. In addition, the presence of contaminant proteins with external histidine residues is usually co-purified with the targets and requires a series of optimisation (Antaloae, Montigny, le Maire, Watson, & Sørensen, 2013).

Immunoassays such as ELISA and lateral flow assays have been the go-to for disease diagnosis. ELISA is preferred as it can analyze multiple samples at a time, and it is easy to use. ELISA also exhibits higher sensitivity compared to other available diagnostic methods. In the past, indirect ELISA has been utilized for the detection of diseases such as dengue, leptospirosis, Lyme disease, and other diseases.

In this study, IgA, IgG, and IgM ELISAs were conducted using two recombinant antigens; HlyE and YncE against serum samples representing different groups of individuals mainly from endemic Kelantan and other states in Malaysia.

## Objectives

The main objective of this study is to develop rHlyE ELISA as a diagnostic tool for the detection of individuals with acute typhoid fever. The secondary objective of this study is to evaluate the potential of rYncE ELISA for the detection of suspected carriers of *S.* Typhi. Therefore, the specific objectives of this study are:

1. To produce and purify recombinant HlyE and YncE proteins of *Salmonella* Typhi.
2. To develop rHlyE ELISA for the detection of individuals with typhoid fever.
3. To evaluate the potential of rYncE ELISA in detecting suspected typhoid carriers.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Immune Response to Infection

Pathogenic bacteria are one of the main cause of infections among humans. The number of individuals exposed to infections is higher than those succumbing to the disease. This is due to the fact that most humans except for immunocompromised individuals can clear and destroy most these of microorganisms by establishing barriers and through the activation of classes of innate resistance and adaptive immunity (Wood, 2006). Innate resistance which is also known as cell-mediated immunity (CMI) includes different types of white blood cells whereas adaptive immunity involves antibodies production.

### 2.1.1 Cell-mediated and Adaptive Humoral Immune Response

There are two types of immunity in human that protects the body from toxins, cells and foreign bodies and invading microbes. They are known as cell-mediated immunity and adaptive humoral immune response.

CMI is mediated by T lymphocytes using Th1 ($CD4^+$) and Tc-cell ($CD8^+$) and is responsible for detecting and destroying intracellular pathogens such as cells infected with viruses or bacteria. CMI involves the activation of phagocytes, macrophages and Natural Killer (NK)-cells and the production of antigen-specific cytotoxic T-lymphocytes through the release of cytokines which causes the destruction of the pathogens or infected cells (Janeway, Travers, Walport, & Shlomchik, 2005).

Bacteria cause diseases by multiplying in extracellular spaces in the body such as in the circulatory and lymphatic system. In order to prevent intracellular infection, the body as a part of an adaptive humoral response secretes antibodies through B cells (Janeway et al., 2005). These antibodies also are known as immunoglobulins (Ig) which will cause the destruction of the pathogens through a chain of events (Murphy, Travers, & Walport, 2012).

### 2.1.2 Immunoglobulins

Immunity is established through adaptive humoral response through the aid of immunoglobulins (Ig). There are five classes of immunoglobulin which are IgG, IgM, IgA, IgE, and IgD.

IgM is the first immunoglobulin expressed during B cell development and is produced during primary immune response after the exposure of a pathogen. Therefore, IgM is usually used to diagnose early acute infection to a pathogen. The half-life of IgM is reported to be lower than the half-lives of IgA and IgG which is around 3-5 to 6-5 days in the blood (Curtis & Bourne, 1973).

IgG is the predominant isotype found in the blood serum with the longest serum half-life. Earlier studies reported that the half-life of IgG is approximately 10–21 days (Mankarious et al., 1988). However, the half-life is dependent on the IgG isotype with IgG1 having slightly longer half-life compared to the other isotypes. IgG antibodies are produced by the B cells as a part of the secondary immune response and provide the longest immunity protection against invading pathogens. High levels of IgG antibody are an indication of chronic infection. Therefore, levels of IgG can be used to diagnose past infection or current acute infection.

Humans can produce two types of IgA, the monomeric serum IgA which is produced in the bone marrow and dimeric secretory IgA which is produced in the plasma cells in the mucosal surfaces. The IgA serum level is reported to be higher than IgM but lower than IgG (Mestecky, Russell, Jackson, & Brown, 1986). In contrast, secretory IgA at mucosal surfaces such as the gut, respiratory tract and urogenital tract are much higher than IgG including the saliva and breast milk (Schroeder Jr & Cavacini, 2010). The main function of secretory IgA is to prevent passage of pathogens into the circulatory system and acts the first line of defense whereas serum IgA serves during infection-inflammation conditions by strengthening the immune response by interacting with serum compartment, regulation of cytokines and non-inflammatory regulator of immunity (Leong & Ding, 2014; Russell, Sibley, Nikolova, Tomana, & Mestecky, 1997).

IgD is an antigen receptor co-expressed with IgM on naive B cells. While IgD is able to substitute for IgM loss, no other biological effector function has been identified for IgD (Lutz et al., 1998).

IgE has been associated with allergic reactions that are responsible for symptoms for hay fever, asthma, hives and anaphylactic shock (Kindt, Goldsby, Osborne, & Kuby, 2007). IgE has a high binding affinity towards FC receptors with an extremely high affinity of basophils, Langerhans cells, and eosinophil (Kawakami & Galli, 2002).

### 2.1.3 Primary and Secondary Immune Response

The first exposure of an antigen on the pathogen ignites a series of reaction termed primary immune response. Pathogens can enter the body through the

bloodstream, epithelial and by traversing mucosal surfaces igniting immune response in the spleen, nearby lymphatic nodes and submucosal lymphoid tissues respectively. After the contact of the antigen with macrophages or other classes of Antigen Presenting Cells (APC), antigens specific antibodies IgM is produced by the differentiating B cells. Levels of IgM are relatively low and overtime the antibody level declines to the point of being undetectable. A small amount of IgG is sometimes detected during the primary response. The primary response usually lasts up to 14 days depending on the nature and the route of infection of the pathogen.

During the re-exposure of the same antigen, the production of antigen-specific antibodies due to the presence of memory cells. Immunity is established in a shorter time span than the primary immune response. The amount of antibody produced during the stage is very high and long-lasting. IgG is the main antibodies produced during the secondary immune response although IgM can be sometimes detected. Other antibodies such as IgA and IgE can be produced in the case of allergy. Figure 2.1 shows the antibody production levels during the primary and secondary immune response.

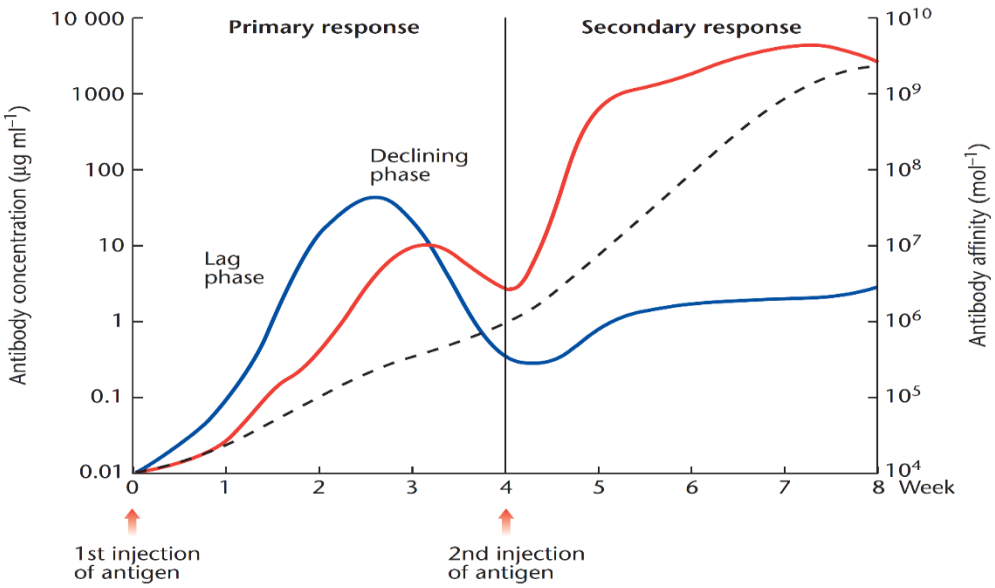


**Figure 2.1: Antibody Levels During an Infection.**

The red line indicates IgG level whereas the blue line indicates IgM levels during the primary and secondary exposure of an antigen (Ademokun & Dunn-Walters, 2001)

### 2.1.4 Immunoglobulin Level in the Detection of Diseases

Antibody levels can be used as an indicator to detect diseases and stages of infection. Measurement of antibody levels through immunoassays such as ELISA has been used to detect various infectious diseases such as leptospirosis, Lyme disease, syphilis, dengue fever, cancer-causing viruses such as Epstein Barr virus and many more. Antibody levels against specific antigens from the pathogens have been used to develop ELISA kits which help to cut down the cost of detection and save time.

Leptospirosis is caused by pathogenic spirochetes of the genus *Leptospira.* In the past, measurement of the IgM level against the heated supernatant culture of leptospires was carried out to detect individuals with leptospirosis (Terpstra, Ligthart, & Schoone, 1985). Later, IgG ELISA using recombinant proteins such as LipL32, OmpL1, LipL41, and Hsp58 recombinant proteins to detect patients with current or

recent leptospirosis (Flannery et al., 2001). The ELISA demonstrated higher sensitivity during convalescence stage but lower sensitivity detecting acute infection with LipL32 scoring the highest sensitivity. In addition, recombinant LipL32 IgG ELISA also resulted in 95% specificity among healthy sera samples and 90 to 97% among other febrile. In another study using small sample size, ELISA IgG using Hsp58 recombinant protein was successful in detecting 18 out of 22 of patients with Leptospirosis (S.-H. Park, Ahn, & Kim, 1999).

Lyme disease is a common vector-borne disease caused by *Borrelia burgdorferi***.** ELISAs against VlsE antigen from *B. burgdorferi* have been used to detect patients with Lyme disease (Lawrenz et al., 1999; F. T. Liang et al., 1999). During the 90s, IgG and IgM ELISAs against multiple recombinant outer surface protein C (OspC), OspE, OspF, p22, p35 have been successfully used to detect individuals with Lyme disease (Akin, McHugh, Flavell, Fikrig, & Steere, 1999; Engstrom, Shoop, & Johnson, 1995; Gerber, Shapiro, Bell, Sampieri, & Padula, 1995; Magnarelli, Fikrig, Padula, Anderson, & Flavell, 1996; Simpson, Schrumpf, & Schwan, 1990).

Syphilis is a sexually transmitted disease that is caused by *Treponema pallidum.* IgG ELISA against flagellum (axial filament) of *Treponema pallidum* was successful in the serodiagnosis of syphilis (Pedersen, Petersen, Vejtorp, & Axelsen, 1982) whereas recombinant antigens Tpn47, TpN17, and TpN15 were able to distinguish the patients with acute infection from healthy individuals (Sambri et al., 2001). Recently an ELISA using recombinant protein Tp0663 was developed to detect patients with syphilis (Xu et al., 2016).

Dengue fever is a tropical disease that is caused by the dengue virus which is carried by mosquitoes. IgM levels against NS1, a glycoprotein, was used to detect

patients with acute infection (Castro-Jorge et al., 2010). It was reported that rNS1-IgM was more sensitive in detecting primary dengue infection (Hang et al., 2009; Kumarasamy et al., 2007). In addition, dengue antigens (DEN-2 and DEN-3) was used in IgG ELISAs which was able to distinguish primary and secondary dengue infections (Matheus et al., 2005).

Cancer-causing Epstein Barr virus or also known as Human *gamma herpesvirus is* a member of the herpes family. It was reported that EBNA1 and VCA-p18 peptides from Epstein Barr virus have used in IgG and IgA ELISA to successfully detect patients with nasopharyngeal carcinoma (Fachiroh et al., 2006).

Antibody levels have been widely used to detect individuals with typhoid fever caused by *Salmonella* Typhi (*S.* Typhi). Antigens such as lipopolysaccharide (LPS), (O) antigen and flagellar (H) antigen, OmpA, GroEL have been reported to be immunoreactive to detect patients with typhoid fever (Charles et al., 2010) Recently, it was reported that HlyE from *S.* Typhi is the most immunoreactive antigen when compared to the other known antigen (Charles et al., 2014) (Reviewed further in Section 2.3 ).

## 2.2 Typhoid Fever

### 2.2.1 *Salmonella* Typhi

*Salmonellae* are facultative anaerobic, Gram-negative bacteria. A member of the Enterobacteriaceae family, they are rod-shaped bacteria with size approximately a 2–5 microns long by 0.5–1.5. *Salmonellae* are motile due to its flagella (Andino & Hanning, 2015). *Salmonella* can be divided into two genus, *Salmonella enterica,* and *Salmonella bongori*.

*Salmonella enterica* subsp. *enterica* serovar Typhi (*S.* Typhi) belongs to the *Salmonella enterica* subspecies. Infection of *S.* Typhi leads to the development of typhoid, or enteric fever. Upon oral entry into the small intestine, *S.* Typhi passes through the intestinal epithelial barrier and are engulfed by macrophages and spreads systemically thus producing acute infection (Vladoianu, Chang, & Pechère, 1990; Wain et al., 1998). Globally, it is estimated that typhoid fever causes 16 million illness and 600 000 deaths (Organization, 1996). *S.* Typhi is transmitted by the fecal-oral route through contaminated food and water source. Typhoid fever is endemic in third world countries due to the lack of hygiene and access to clean water. Infections of *S.* Typhi results in diarrhea, fever, headache, and malaise within 6–30 days of bacterial ingestion (Crump, Luby, & Mintz, 2004). After prolonged fever patients may also have a coated tongue (typhoid tongue) and rose spots on abdomen and chest (Bhan, Bahl, & Bhatnagar, 2005).

### 2.2.2 Pathogenesis of *Salmonella* Typhi

Infection of *S.* Typhi starts in the gut after oral consumption of infected food or water. The human-restricted pathogen reaches the intestinal mucosal cells rapidly. Penetration of the epithelial cells such as M cells and other lymphoid tissues is achieved by the *Salmonella* pathogenicity islands which encode proteins such as the multi-channel protein to release the effectors triggering their own phagocytosis (Collazo & Galán, 1997) (Kohbata, Yokoyama, & Yabuuchi, 1986). While some bacilli will remain in the macrophages, some are drained into lymph nodes where the bacilli enter the bloodstream. The capability of the bacteria to persist in the macrophages allows them to be carried in the reticuloendothelial system (RES)

(Monack, Mueller, & Falkow, 2004). The bacteria reside in the RES during the incubation period until the onset of clinical typhoid fever. As *S.* Typhi interacts with both the mucosal system and circulatory system, both mucosal and systemic host immune response is stimulated after the infection. IgA and IgM are released during the first exposure of *S.* Typhi antigens with both mucosal and circulatory system respectively whereas IgG is produced at a high amount during the second exposure to the pathogen.

### 2.2.3 Global Incidence of Typhoid Fever

Typhoid fever is not common in Western countries however it is highly endemic in regions such as Southeast Asia and sub-Saharan Africa where young children represent a subgroup with the highest disease burden (Lozano et al., 2012). It was reported that 12.4% of blood culture isolates from adolescents and children from Ghana were *S.* Typhi positive (Marks et al., 2010), whereas in Nigeria 20.9% of isolates in children less than 5 years old were *S.* Typhi positive (Obaro et al., 2011). In Asian countries such as Pakistan and India, the annual rate of typhoid fever among children are 573.2 per 100 000 and 340.1 per 100 000 person-years respectively. Whereas the annual rate of typhoid fever in countries such as China, Vietnam, and Indonesia was reported to be 24.2, 29.3 and 180.3 per 100 000 person-years. (Ochiai et al., 2008). Figure 2.2 illustrates the prevalence of typhoid fever.

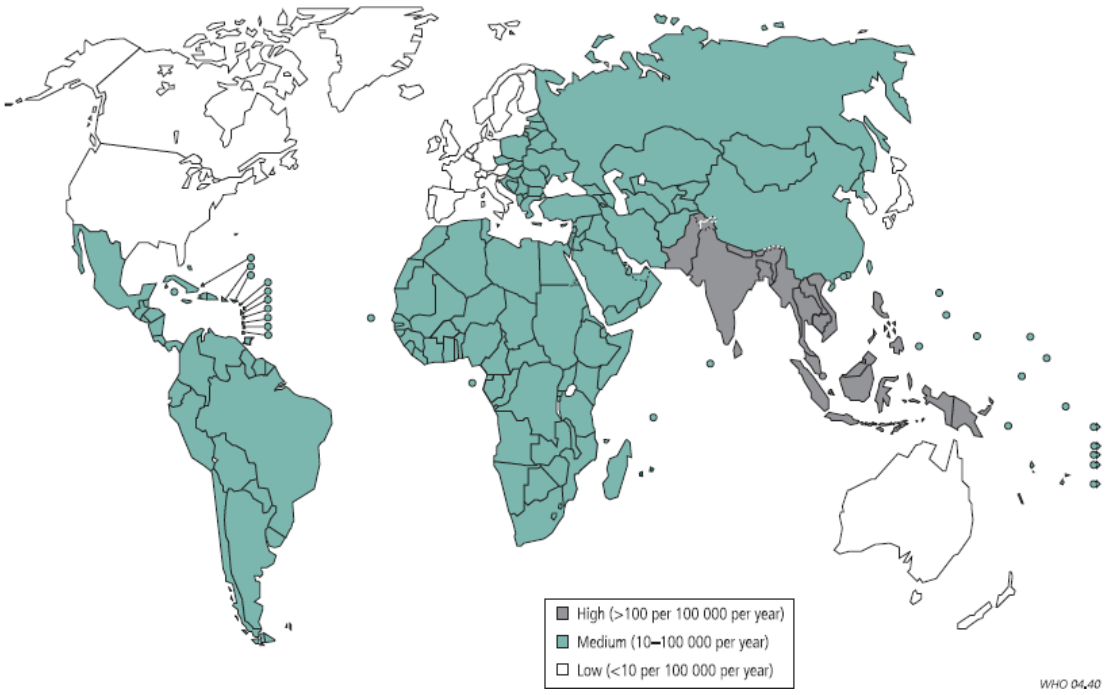


**Figure 2.2: Geographical Distribution of Typhoid Fever**

The global prevalence of typhoid fever with southeast Asia having the highest reported cases of typhoid fever (Crump et al., 2004).

### 2.2.4 Incidence of Typhoid Fever in Malaysia

The northeastern state in Malaysia, Kelantan is endemic for typhoid fever. Previous studies reported that children and adolescences are the most affected age group by typhoid fever in Kelantan (Lin et al., 1988). It was reported that majority of the typhoid cases during the year 1999 and 1998 involved children in Kelantan, Sabah and Terengganu, Selangor and Sarawak (Choo, Davis, Ismail, Ibrahim, & Ghazali, 1999; Choo, Razif, Ariffin, Sepiah, & Gururaj, 1988; Malik & Malik, 2001). The most recent typhoid outbreaks in Malaysia were reported to be in Kudat, Sabah (Joibi, 24 Feb 2019), Belaga Sarawak (Yasak, 2017) and in the capital of Malaysia, Kuala Lumpur (Bernama, 2015).

### 2.2.5 Historical Accounts of *S*. Typhi Carriers

The first documented carrier of typhoid fever in human history is Mary Mallon or widely known Typhoid Mary (Marineli, Tsoucalas, Karamanou, & Androutsos, 2013). Mary was an Irish cook in New York City during the twentieth century and allegedly responsible for infecting at least 54 people (Soper, 1939). Mary was identified as a healthy carrier as she did not exhibit any symptoms of typhoid fever which is today coined as an asymptomatic carrier. Besides Mary, another chronic carrier who as anonymously reported as 'Mr. N' the milker' is responsibly infecting approximately 200 people in the time span of 14 years (Mortimer, 1999).

### 2.2.6 Asymptomatic Carriers of *S*. Typhi

A small percentage of patients with acute typhoid fever are unable to clear the infection fully within one year of recovery. These individuals establish into a state of carriage (Levine, Black, & Lanata, 1982) and act as a reservoir for those specific pathogens harbouring it in their intestines and gallbladder (Ochiai et al., 2008). WHO also reported that some individuals are short term carriers but their epidemiological role is not as important as that of chronic carriers (WHO, 2011). In addition, some chronic carriers do not have a history of typhoid fever. Studies carried out in endemic regions demonstrated that there is a strong correlation between the establishment of the chronic carrier state and gallstones formation. It was reported that 90% of chronic carriers have gallstone (Schiøler *et al*., 1983). While most of the carriers progress into the asymptomatic carrier but 1-6% becomes chronic carriers. Asymptomatic carriers of *S*. Typhi shed large numbers *S*. Typhi in their stools and urine from time to time (Bhan et al., 2005). Chronic infections can prolong for years and these infected

individuals are very contagious and transmit disease through contact with food and water. However, they show any physical symptoms of typhoid fever, thus making the identification of carriers tough.

It was reported 147/700 of school children in Vellore, a district in Tamil Nadu to be convalescent asymptomatic carriers (Sivaji, Duraisamy, Balakrishnan, & Periasamy, 2015). Senthilkumar *et al* (2005) reported an astounding rate of 16% of typhoid carriers among food handlers in India. Whereas, in another study sampling food handlers in Karachi, Pakistan reported that around 4.3 % food handlers that do not display any symptoms of the illness were carriers of *S.* Typhi (Siddiqui, Bibi, Mustufa, Ayaz, & Khan, 2015). The carrier rate of *Salmonella enterica* serovars in China and the UK was reported to be approximately 9.5% and 12.3 % respectively where high immigration rate to the UK was cited as a reason for high carriers rate for the latter (Dryden, Keyworth, Gabb, & Stein, 1994). On the other hand, the previous study on the prevalence of non-typhoidal *Salmonella* carriers among food handlers in Ghana reported a rate of 1.1% (Feglo, Frimpong, & Essel-Ahun, 2004).

### 2.2.7 Diagnostics of Acute Typhoid fever

Traditionally, the detection of *Salmonella* is usually carried out by culturing enriched media followed by selective media, biochemical and serological testing for the confirmation of the identity of the bacteria. Xylose-lysine-deoxycholate agar (XLD) and Mac Conkey agar are usually used for the detection of *S.* Typhi. Culture from blood, urine, and stool sample (rectal swabs) are considered as the golden standard in the diagnosis of typhoid fever. However, culture sensitivity gets lower as the duration of the acute infection increases (Wain et al., 1998) as the bacterial load in blood declines during an infection. In addition, typhoid fever diagnosis by culture takes

a long time and requires serial culturing (Naravaneni & Jamil, 2005). Developed in the 1980s, the Widal Test used to be the go-to test for the diagnosis of typhoid fever. Widal test is an agglutination test where the serum from patients are reacted with the flagellum (H) and lipopolysaccharide (O) antigen to measure the degree of antibody agglutination with the antigens (Felix & Bensted, 1954). However, the Widal test demonstrated lower sensitivity due to the high background of the antibody's residual among people in endemic areas (Olopoenia & King, 2000) (Andualem et al., 2014). In addition, the Widal test does not distinguish *S*. Typhi and *S*. Paratyphi infection. The performance of the Widal test was reported to have the lowest sensitivity and specificity when compared to the other rapid tests available for typhoid fever (Keddy et al., 2011).

Typhidot assays is a qualitative antibody test that detects the presence of IgM and IgG antibodies to a 50kDa outer membrane protein(Choo, Oppenheimer, Ismail, & Ong, 1994). A positive Typhidot result (IgG and IgM) was defined as a visible reaction of an intensity equal to or greater than that of the control reaction on the commercially prepared filter paper.

TUBEX assay is a semiquantitative colorimetric test that detects anti-O9 antibody titres in patient specimens on visual examination. A positive TUBEX result was defined as a reading of $\geq 4$ but requires re-testing after 48 hours (Lim, Tam, Cheong, & Jegathesan, 1998).

Today, molecular approach to diagnose typhoid fever is preferable due to its rapid, cheap and sensitivity. Polymerase Chain reaction (PCR) and ELISA are two commonly employed diagnostic measures due to its high sensitivity and specificity compared to conventional methods.

PCR amplification of genes such as fliC-d and fliC-a has been used to diagnose *S*. Typhi and *S*. Paratyphi infection respectively. Song *et al*.,1993 utilised nested PCR on suspected typhoid patients and detected four patients positive for typhoid fever despite being a negative culture. PCR based diagnosis was reported to be more sensitive than the Widal test and six times sensitive than blood culture (Massi et al., 2003). Detection of typhoid fever through PCR using stool and urine sample also demonstrated excellent specificity but variable sensitivity (Hatta & Smits, 2007; Sánchez-Jiménez & Cardona-Castro, 2004). The pros of running PCR would be that it can be carried out within a day whereas blood culture diagnosis requires at least five days. However, PCR based diagnosis requires laboratory equipment such as the PCR thermocycler and gel image viewer which is not practical in third world countries where the disease is endemic.

Performance comparison between Widal, TUBEX-TF, Typhidot assays, and ELISA reported that ELISA depicted excellent sensitivity and specificity compared to the other (Fadeel et al., 2011). Besides being more sensitive, ELISA is cost-effective and high throughput suitable for mass screening during an outbreak.

### 2.2.8 Identification of Typhoid Carriers

Identification typhoid carriers require multiple cultures for a time span due to low and intermittent shedding patterns in the stool among carriers(Engleberg et al., 1983). Serial tests of stool and urine samples are considered as the best method to detect typhoid carriers (Howlader et al., 2017). Samples from bile, gallstones or tissues from patients undergoing cholecystectomy is another effective practice in identifying carriage but not preferred due to its invasive nature. Blood and stool samples for

molecular approaches such as nested PCR amplification for *S.* Typhi unique genes such as putative fimbrial staA or fliC (Pratap et al., 2013) have been carried out to detect carriers but displays poor sensitivity when further re-tested with culture confirmatory test (Kumar, Pratap, Mishra, Kumar, & Nath, 2012).

### 2.2.9 Treatment of Typhoid Carriers

While carriers, do not display symptoms of typhoid fever, they are able to transmit the diseases to others. Therefore, identifying and treating carriers is important. As carriers harbours *S.* Typhi in the gallbladder for years with the aid of biofilm formation on the gallstone, the removal of the gallbladder may stop the individual from shedding the bacteria(Gonzalez-Escobedo, Marshall, & Gunn, 2011). However, this does not always result in the elimination of the pathogen (Freitag, 1964). On the other hand, oral drug therapy using different drugs combination is capable of curing 90% of the carriers. Chloramphenicol and trimethoprim have been used to treat typhoid fever until the emergence of multidrug-resistant (MDR) strains (Chau et al., 2007). Current treatment involves fluoroquinolones, including nalidixic acid and ciprofloxacin, are able to cure two third of carriers cases (Kalra, Naithani, Mehta, & Swamy, 2003). However, in certain cases of anatomic abnormality such as patients with kidney stones, antibiotic therapy alone is not enough to eradicate carrier state but also requires surgical assistance.

## 2.3 Biomarkers of Typhoid Fever

Antibodies against several biomarkers have been identified for the detection of individuals with typhoid fever. Anti-lipopolysaccharide and anti-flagella antigens ELISA against IgG, IgA, and IgM have been developed for the diagnosis of typhoid

fever (Sippel et al., 1989) (House et al., 2001). The levels of the three antibodies were found to be higher in acute typhoid patients when compared to other febrile and healthy individuals.

Immunoassay using the Vi antigen after series purification has been used to identify carriers of typhoidal. Vi capsular polysaccharide is a virulence factor of *S.* Typhi and it is highly immunogenic. Antibodies against the antigen were previously used in immunoassays to detect carriers (Lin et al., 1988). Despite being more sensitive than the Widal test, the reliability and the sensitivity of the assay in highly endemic areas remains questionable as people living in high endemic areas demonstrated high background levels of antibodies against Vi despite being cleared of the disease. Gupta *et al*. (2006) reported no *S.* Typhi was isolated from 96.3% of rectal swabs from people with high titer anti-Vi antibody. Therefore, a less invasive, cheap and sensitive assay to detect typhoid carriers is essential.

### 2.3.1 Hemolysin E (HlyE)

HlyE (UniProt ID Q8Z727) from *S.* Typhi a 34-kDa protein is a virulence factor that belongs to the cytolysin family. HlyE which is also denoted as ClyA and SheA are large, pore-forming toxin proteins that are present in *Salmonella Typhi, Escherichia coli,* and *Shigella flexneri*. HlyE possesses hemolytic and cytolytic activity that is absent in other *Salmonella* serovars such as Typhimurium, Paratyphi B and Paratyphi C (Oscarsson et al., 2002)

In terms of protein topology, HlyE is a rod-shaped molecule with four major α-helices, coiled around each other with a small surface-exposed hydrophobic β-hairpin (Figure 2.3). HlyE is transported through a vesicle-mediated transport

mechanism (Wai et al., 2003) and the assembly of 13-mer oligomers of HlyE into ring-shaped toxins are required for the formation of a functional pore protein (Edwards, Olsen, & Maloy, 2002; Tzokov et al., 2006; Wallace et al., 2000). This toxin is responsible for the lysis of red blood cells and cytotoxic effects on human and murine macrophages which facilitates the virulence factor and survival of *Salmonella* (Libby et al., 1994).

HlyE protein is reported to be a known crucial antigen for the antibody response to *S.* Typhi infection (Charles et al., 2013; L. Liang et al., 2013). Its utility in serodiagnostic for typhoid fever has been reported in independent studies against anti rHlyE-IgG responses (Davies et al., 2016; Ong et al., 2015), anti rHlyE-IgM and rHlyE-IgA responses (Andrews et al., 2018; Chin, Redhuan, Balaram, Phua, & Ong, 2016; Felgner et al., 2017).

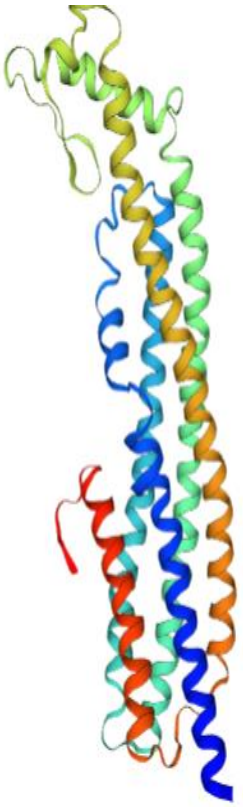

**Figure 2.3: 3D Protein Structure of HlyE**

3D model of Hemolysin E (1QOY) from SWISS model

### 2.3.2 Uncharacterized YncE Protein

YncE encodes a 353-residue protein and is conservative among Enterobacteriaceae. As of today, the function of the YncE protein is putative and unknown. X-ray crystallography of YncE protein depicted YncE with seven-bladed β-propeller structure (Baba-Dikwa, Thompson, Spencer, Andrews, & Watson, 2008; Kagawa, Sagawa, Niki, & Kurumizaka, 2011). YncE (STY1479) was identified as the dominant protein biomarker expressed among carriers of typhoid fever indicating that YncE potentially is responsible in *S.* Typhi survival in human biliary tract (Charles et al., 2013).

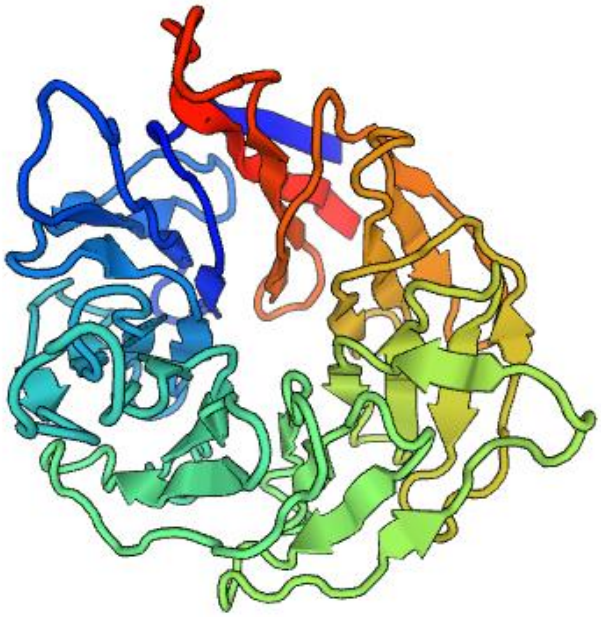

**Figure 2.4: 3D Protein Structure YncE**

3D model of uncharacterized YncE (Q8Z740) from SWISS model.

## 2.4 Recombinant Protein Production

### 2.4.1 Gene Cloning: Homologous Cloning

Generation of identical copies of a gene to produce recombinant protein is termed as gene cloning. Different types of vectors that can be used for cloning include a plasmid, phage lambda, and cosmid. Ideal vectors should be easy to be manipulated, able to amplify the genomic insert, contains multiple cloning sites and contains a selectable marker for identification. Plasmids are frequently used in the gene cloning as it is a small double-stranded DNA possessing the three functional elements of a vector; an origin of replication, multiple cloning sites, and antibiotic resistance gene. Conventionally, the cloning protocols involve DNA ligase and restriction endonucleases and many steps. However, many cloning assemblies involving ligation-

independent cloning (LIC) has been introduced where the usage of ligase was replaced with PCR.

Homologous recombination cloning, a method a LIC method (Bubeck, Winkler, & Bautsch, 1993) is dependent to the production of single-stranded DNA upon a double-stranded break with the aid of exonucleases and the annealing of the homologous stretches and repairing of overhangs by the enzyme polymerase. In terms of principle, homologous recombination is the insertion of the gene with overlapping homology sequence into a vector which was linearized using restriction enzyme the target gene insert which is amplified through PCR contains sequence on both sides matching sequence of the end of the linearized vector. Figure 2.5 is a schematic diagram to produce recombinant DNA through in vitro homologous recombination and single-strand annealing. The length of the overlapping region between the insert and the vector is vital where 20bp and more are deemed to have higher cloning efficiency (Jacobus & Gross, 2015). Homologous recombination cloning is widely used due to its efficient process, being cost effective and can be universally adapted incorporated into any laboratory protocol. After the introduction of the desired gene into the vector, the recombinant vector is then transformed into the appropriate host cell for expression.