1.0 INTRODUCTION

Background of study

The occurrence of typhoid fever is a major threat globally with annual cases exceeds 20 million and approximately a quarter million deaths. The disease is mostly dominant in underdeveloped and developing countries where sanitation is poor, mainly in parts of South Asia (Crump and Mintz, 2010). The enteric fever is caused by the etiologic agent, *Salmonella enterica* serovar Typhi (*S.* Typhi). With early detection and medical prevalence, the severity of the diseases can be reduced and thus decrease the fatality rate. However, the diagnosis of the diseases is commonly done with conventional methods and Widal test, which specificity and sensitivity are not that high (Olopoenia and King, 2000). Furthermore, the tests are also limited by other factors of time, labour and cost which reduce its availability mainly in developing countries.

One of the rapid tests developed for detection of *S.* Typhi is through multiplex PCR, which is *Salmonella* EZplex. The system is a patented *Salmonella* detection kit developed from Laboratory of Biomedical Science and Molecular Microbiology, University Malaya (patent, PI2011005414, 2011). The method of detection amplifies a 332 bp DNA marker that is specific for *S.* Typhi. Although the DNA marker is specific for PCR detection, its specificity to serological method remains unknown.

Problem statement

The use of conventional methods (culturing and biochemical tests) for detection of *S.* Typhi is mostly applied as a confirmatory method for the presence of the pathogens. However, these methods are highly limited due to the complexity to do bacterial culture and the time taken for diagnosis. Thus, many of alternative diagnostic tools of typhoid fever are developed to utilize the antibody based methods. However, there are also limitations with this method such as the complication of having false-positive due to prior exposure to the antigen. Thus, there is a need for development of antigen based detection of the pathogens. Moreover, the amplified 332 bp DNA marker for detection of *Salmonella* Typhi is specific for its PCR based detection. However, its specificity as protein antigen is yet to be characterized.

Research questions

Does the specific DNA marker of *S.* Typhi, ST332 produce a specific protein antigen to *S.* Typhi?

If the protein is expressed, is it also specific for *S.* Typhi in serological test?

Does the protein produce a specific antigen towards *S.* Typhi?

If the ST332 marker is not antigenic, aan a specific antigen of *S.* Typhi be obtained by another approach?

Objectives

To construct an expression vector for specific DNA marker of *S.* Typhi.

To construct an epitope library of *S.* Typhi and screen for clones expressing specific epitope towards *S.* Typhi patients' sera.

Chapter 2.0 LITERATURE REVIEW

2.1 Salmonella as pathogen

Salmonella is an intracellular pathogen that causes a variety of diseases termed salmonellosis, ranging from gastroenteritis to enteric fever in humans and animals (Lan *et al.,* 2007). Its genus comprises of two distinct species which are *Salmonella bongori* and *Salmonella enterica,* the former is a commensal to cold-blooded animals while the latter comprises six subspecies; *enterica, salamae, arizonae, diarizonae, indica, and houtenae* (Levantesi *et al.,* 2011). These subspecies are further divided into 50 serogroups characterized based on O (somatic) antigen and more than 2600 serovars by H (flagellar) antigens in the genus (Sabbagh *et al.,* 2010; Salehi *et al.,* 2009). However, only 50 serovars within the subspecies *enterica* are known for common cause in infections in humans and warm-blooded animals (Levantesi *et al*., 2011). Different serovars of *Salmonella* constitute diverse geographical distribution, different host specificity and impose different syndromes during infections (Levantesi *et al.,* 2011). In clinical settings, *Salmonella* infections are distinguished into typhoidal and non-typhoidal *Salmonella* serovars (Pond, 2005 and Levantesi *et al.,* 2011).

Genetic diversity observed in *Salmonella* strains is attributed to the ability of lysogenic bacteriophages to mediate gene transfer to the bacterial chromosome (horizontal gene transfer) or by gene degradation, besides other factors (Mikasová *et al.,* 2005; Sabbagh *et al.,* 2010). However, association of *Salmonella* with human infections only involves a small fraction of its serovars, which most pertain to *Salmonella enterica* ssp. I (Sabbagh *et al.,* 2010).

In humans, *Salmonella* infection mainly routed from ingestion of contaminated food or water that causes gastroenteritis which is a localized infection, or enteric fever, a severe systemic infection (Sabbagh *et al.,* 2010). Of the prominent salmonellosis in humans are typhoid fever, an infection caused by *Salmonella enterica* serovar Typhi, and paratyphoid fever. Paratyphoid fever is another undistinguishable clinical condition caused by *Salmonella enterica* serovar Paratyphi A, B or C (Fangtham and Wilde, 2008). Infected individuals usually suffer from prolonged fevers and headache, and later coupled with diarrhea and abdominal pain. Meanwhile, several other symptoms were also observed in the patients that include a non-productive cough, constipation, meningismus, deafness, confusion, as well as weight loss. On the occurrence of death, it has mainly associated with seizures, intestinal perforation, pneumonia, delirium, and coma (Deris *et al.*, 2010).

2.2 *Salmonella* Typhi

S. Typhi is the main causative agent for typhoid fever, a systemic illness contracted mainly via ingestion of contaminated food or water (Pickard *et al.,* 1994; Abdoel *et al.*, 2007). Unlike the other serovars of *Salmonella* such as *S.* Typhimurium and *S.* Enteritidis which often associated with gastroenteritis and can infect a wider host range, *S.* Typhi infection is specific to humans (Song *et al.,* 2010).

2.3 Prevalence of typhoid fever

Incidence of typhoid fever remains a global challenge in many geographic areas, mainly in developing countries associated with poor sanitation systems and improper water treatment of water supplies, and particularly high in South-central and Southeast Asia (Crump and Mintz, 2010). The annual occurrence of the disease is estimated to reach 21 million illnesses with a death toll of approximately 216 000 (Crump *et al.*, 2004). The disease is endemic in Bangladesh with a recent record of the illness is estimated to be 390 cases per 100 000 persons (Naheed *et al.*, 2008), while in Malaysia, it periodically gives an outbreak with annual incidence of 10.2 – 17.9 cases per 100 000 persons (Deris *et al.*, 2010). Most reported cases of typhoid fever in clinical settings revealed that many of infections occur mainly in children as young as 5 years old to young adults. However, in some countries where typhoid fever is endemic; India, Indonesia, Bangladesh, Thailand and Vietnam, typhoid fever is also commonly seen in 1 to 5 years old children (Bhan *et al.*, 2005). The persistence of this fever remains a problem in these developing countries by which prolonged use of antibiotics together with misapplications and self-prescribing of the antibiotics lead to a higher health concern of drug resistance and dissemination of resistant strain in the area (Levantesi *at al.*, 2011). Meanwhile, incidence of paratyphoid fever, another form of systemic infection by another *Salmonella* serovar, *S.* Paratyphi is more inferior to typhoid fever with global cases of these diseases in 2000 are 5.4 million and 21.7 million respectively (Crump and Mintz, 2010).

2.4 Pathogenicity and clinical features

Salmonella Typhi infection is mainly caused by ingestion of food or water contaminated with the bacteria (Tischler and McKinney, 2010). In *Salmonella* species, penetration of ileal epithelial cells plays the most important step in pathogenesis (Arricau *et al.,* 1998). Similarly, *S.* Typhi also initiate infection by invading intestinal epithelial cells and transferred to lamina proria (Tischler and McKinney, 2010; Bhan *et al.,* 2005). However, unlike *S.* Typhimurium, *S.* Typhi allows the process of colonization in deeper body tissues by avoiding from triggering early inflammatory response in the human intestine (Bhan *et al.,* 2005). This stealth technique is achieved by producing factors that inhibit inflammatory response thus enabling systemic invasion and colonization of the gallbladder (Tischler and McKinney, 2010). Invasion of intestinal mucosa was done by the activation of two Type III secretion systems (T3SS-1 and T3SS-2) encoded by SPI-1 which deliver bacterial proteins into non-phagocytic epithelial cells (Bhan *et al.,* 2005; Tischler and McKinney, 2010; Kaur and Jain, 2011). These factors interfere with host cell function thus promotes bacterial uptake into the intestinal epithelial cells and allows survival and multiplication in the host macrophage (Tischler and McKinney, 2010). When infection reaches threshold level which is determined by number of bacteria, virulence and host immune response, the bacteria are disseminated systemically which starts bacteremic phase to colonize macrophages in other organs (Tischler and McKinney, 2010). The most common sites for secondary infection are liver, spleen, bone marrow, gallbladder and Peyer's patches in the terminal ileum (Kaur and Jain, 2011). It is infection in the gallbladder that would convert a person into an asymptomatic carrier of the pathogen (Tischler and McKinney, 2010).

Clinical features of typhoid fever are variable ranging from mild fever to marked toxaemia and associated complications involving many systems (Bahn *et al.,* 2005). Early onset of the fever may include headache, diarrhea or constipation and abdominal pain (Sánchez-Vargas *et al.,* 2011). In average cases of non-complicated typhoid fever, the incubation period for the fever is between 10 -14 days and may persist up to 4 weeks if left untreated (Bahn *et al.,* 2005; Sánchez-Vargas *et al.,* 2011). Other symptoms also include dull frontal headache, a dry bronchitic, anorexia, nausea, bradycardia, and rose-spots on abdomen and chest (Bahn *et al.,* 2005; Sánchez-Vargas *et al.,* 2011). Since the presentations of the disease are vary, there is a challenge to differentiate the typhoid fever symptoms to other diseases with similar onsets like tuberculosis, brucellosis, sepsis of other bacterial pathogens, and others (Bahn *et al.,* 2005). The clinical symptoms for typhoid and paratyphoid fever are very similar, therefore a better diagnosis is needed (Sánchez-Vargas *et al.,* 2011; Fangtham and Wilde, 2008). However, complications may also occur mainly in endemic regions involving between 10% - 15% of patients. Most common complications are gastrointestinal bleeding, hepatitis, pancreatitis, seizures which in some cases are fatal (Sánchez-Vargas *et al.,* 2011). Early treatment with antibiotics has shown to be highly effective in fighting infections but improper administration of the antibiotics has led to emergence of multidrug-resistance strains of the pathogen (Deng *et al.,* 2003).

2.5 Diagnosis and detection of typhoid fever

Early detection and identification of typhoid and paratyphoid fever, followed by rapid medical treatment is necessary in order to reduce the morbidity and mortality related to the diseases. Most of developing countries afflicted with these enteric fevers however still facing problem with diagnosis mainly due to high similarity of the symptoms with malaria, nonsevere dengue fever, and other febrile illnesses present in the regions, despite lacking good and simple diagnostic tests for the diseases (Rahman *et al.,* 2007). Many of the laboratories still employ Widal bacterial agglutination test, which is a serological test utilizing a suspension of killed *Salmonella* Typhi as antigen for detection of typhoid fever serum from a suspecting patient with febrile illness (Rahman *et al.,* 2007; Olopoenia and King, 2000). Although Widal test has contributed greatly in diagnosis of typhoid fever in the past, its reliability is limited to several factors. The main factor associated with this claim is the lack of significance of use with vaccinated patient or in endemic region where establishment of steady-state or baseline titre of Widal agglutination is difficult. Other associated factors include inherent variabilities of the test, cross-reactivities with other non-*Salmonella* organisms and lack of reproducibility of the results (Olopoenia and King, 2000).

Other commonly used methods for diagnosis of typhoid and paratyphoid fever are by conventional methods which involve culturing and biochemical test (Baker *et al.,* 2010). In this method, blood culture is considered to be the most reliable diagnostic test for detecting the presence of the causatic agents. However, a single blood culture is estimated to present only 50-80% in sensitivity and it is also associated with inevitable delay due to sample collection to diagnosis of up to 5 to 7 days (Naheed *et al.,* 2008). Although this method is generally used for confirmatory of diagnosis, the tests are

usually done only in laboratories with appropriate equipment and well-funded in order to afford the cost involved, other than its being tedious and not very sensitive (Rahman *et al.,* 2007). Due to these limitations, more reliable and rapid diagnostic tests are highly desired.

Over the years, several immunological methods for rapid diagnostic tests have been developed for detection of *S.* Typhi which include enzyme-linked immunosorbent assay (ELISA) (PanBio, Sinnamon Park, Qld, Australia; Mega Diagnostics, Los Angeles, CA), dot-blot ELISA (Malaysian Biodiagnostic Research, Bangi, Malaysia), immunochromatography (Standard Diagnostics, Kyonggi-do, Korea), Multi-Test Dip-S-Ticks (PANBIO INDX Inc., Baltimore, MD, USA), and a novel particle separation method used in TUBEX® (IDL Biotech, Sollentuna, Sweden) (Rahman *et al.,* 2007; Fangtham and Wilde, 2008). These tests work by the detection of immunoglobulin IgM and/or IgG of *S.* Typhi, and showed to have greater sensitivity and specificity compared to Widal test. However, as they function on the basis of antibody detection, the highest sensitivity of the tests is only achieved after a second week of illness, which poses as a delay in the diagnosis (Fangtham and Wilde, 2008). Moreover, most of the tests are not readily available in the field. In another attempt, antigen based detection gives a round of interest for detection of early onset of the disease. A Thai laboratory particularly has developed a monoclonal antibody specific to the antigen 9 of *S.* Typhi for detection of the pathogen in clinical specimens using enzyme-linked immunosorbent assay. The test showed to have promising results for detection in urine specimens with specificity of 100% and sensitivity of 65%, while having improved sensitivity in serially collected urine, 90% (Fangtham and Wilde, 2008).

Meanwhile, the development of rapid test is still improving with the lack of reliable diagnostic test for typhoid (Das *et al.,* 2013). This study utilizes a system to generate a clone library to find clones expressing antigens of *S.* Typhi while evaluating an existing ST332 gene for antigenic activity. In this way, it gives the advantage of getting more than one antigen specific to *S.* Typhi to be used in the development of a more specific and sensitive rapid test for typhoid fever.

2.6 PCR based diagnosis of typhoid fever

The use of culturing, biochemical and serological tests in early detection of typhoid fever is the most common currently. However, the methods require a long time to complete and are also costly (Schonenbrucher *et al.,* 2008), Therefore, another approach of diagnosis based on nucleic acid amplification, PCR provides a valuable tool in microbiological diagnostics as the method can provide fast and accurate results (Woodward and Kirwan, 2006). Among the PCR technologies applied in detection of *S.* Typhi are conventional PCR, nested PCR, multiplex PCR, TaqMan® real-time PCR, nested multiplex PCR and multiplex real-time PCR (Parry *et al.,* 2011). Many of PCR-based detections of *S.* Typhi target the flagella gene (*fliC*) (Baker *et al.,* 2010), but there are also several other targets utilized for *S.* Typhi PCR detection such as *stgA* fimbrial operon gene (Ngan *et al.,* 2010), v*iaB* bacterial capsule protein synthesis (Vi antigen) (Freitas *et al.,* 2010), 23S rRNA of gene specific to *S.* Typhi but unknown function (Pui *et al.,* 2011), *fliC-d* Hd Flagella gene (Song *et al.,* 1993) and *fliC-a* Ha flagella gene (Nizami *et al.,* 2006).

The use of PCR detection has shown a higher sensitivity compared to the culture method. Currently, the sensitivity of PCR in blood culture cases was reported variably

>97% with 100% specificity (Parry *et al.,* 2011). In a study done by Khan *et al.* (2012), the diagnosis of typhoid fever was done by nested PCR targeting *fliC* gene of *S.* Typhi on blood samples of 80 patients and 40 healthy individuals in Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) Hospital and Government General Hospital, Puducherry. The study showed that nested PCR for *fliC* gene gave 100% sensitivity and 76.9% specificity. They also discovered that the nested PCR was able to detect 76.2% cases compared to 61.9% of blood culture method during the initial 1-5 days of illness. The sensitivity of PCR based detection was seen higher when the illness was between 6-10 days duration with 63.1% cases compared to 45.6% cases by blood culture.

The prospective of using PCR-based detection if diagnosis of typhoid fever is paramount but developments are progressing for its utility. However, there are also limitations with this system that has to be addressed. One of them is that there is no common validated test that is being applied for detection of typhoid fever. Instead there are many in-house systems that are susceptible to varying interpretation and none have met the right quality control to make it available for worldwide application (Baker *et al.,* 2010). Moreover, there is also an issue with small number of bacteria in blood that would affect the diagnosis (Parry *et al.* 2011). With the advancement of technology, improvement could also be made in capture system to enable higher sensitivity of PCR-based detection such as the use of magnetic or nanoparticle prior to amplification (Baker *et al.,* 2010; Parry *et al.,* 2011).

Chapter 3.0 MATERIALS AND METHODS

3.1 Chemicals and biologicals

3.1.1 Bacterial strains

S. Typhi strain UJ816A was obtained from Laboratory of Biomedical Science and Molecular Microbiology, University Malaya, Kuala Lumpur.

E. coli BL21 strain was obtained from Laboratory of Clinical Chemistry, Department of Molecular Medicine, Faculty of Medicine, University Malaya.

E. coli Novablue DE3 was obtained from Novatope Cloning System kit (Novagen) and used as expression host of *S.* Typhi DNA fragment.

3.1.2 Plasmids

Plasmid pGEX-4T-1 was obtained from GE Healthcare and used as expression vector for specific DNA marker of *S.* Typhi.

pScreen-1b(+) T-vector was included in the Novatope Cloning System kit by Novagen and used for cloning of *S.* Typhi DNA fragments.

3.1.3 PCR Primers

Primers used to amplify specific *S.* Typhi DNA fragment was as used in *Salmonella* EZplex, a patented *Salmonella* detection kit developed in Laboratory of Biomedical Science and Molecular Microbiology, University Malaya (patent, PI2011005414, 2011). Restriction enzyme sites for *Eco*RI was introduced at the 5'-end of forward primer while site for *Not*1 was added at the 3'-end of the reverse primer.

PCR screening primers for Novatope cloning system clones were T7 gene *10* primer and the T7 terminator primer obtained from Novagen, patents owned by Hoffmann-La Roche.

PCR screening primers for pGEX-4T-1 clones were pGEX 5' Sequencing Primer and pGEX 3' Sequencing Primer obtained from GE Healthcare.

pGEX 5' Sequencing Primer

5'-d[GGGCTGGCAAGCCACGTTTGGTG]-3'

The pGEX 5' Sequencing Primer binds at nucleotides 869–891 on all ten pGEX vectors.

pGEX 3' Sequencing Primer

5'-d[CCGGGAGCTGCATGTGTCAGAGG]-3'

The pGEX 3' Sequencing Primer binds at the following locations on the pGEX-4T-1 vector: 1041-1019

3.1.4 Enzymes

Restriction enzymes and *Taq* polymerase were obtained from Promega, USA while T4 DNA ligase was obtained from Fermentas, USA.

3.2 Molecular Techniques

3.2.1 Genomic DNA extraction

Bacterial genomic DNA was extracted using conventional method. In this method, 5 ml of overnight bacterial culture was pelleted by centrifugation at 13,400 rpm for 3 minutes and the supernatant was discarded. The cells pellet was resuspended in 200 µl of Solution I (0.15M NaCl, 0.1M EDTA pH 8) by repeated pipetting and the tube was centrifuged again at the same speed for another 3 minutes. Then the supernatant was discarded and 100 µl of lysozyme (100 mg/ml) was added to the tube to resuspend the cells. The mixture was incubated at 37 °C with shaking for 30 minutes. Later, 100 µl of lysis buffer (1% SDS, 0.1M NaCl and 0.1M EDTA pH 8) was added into the suspension and mixed by inversion. The mixture was incubated in 60 °C waterbath for 10 minutes and cooled to room temperature. Then 50 µl of 5M sodium perchlorate ($NaClO_4$) was added into the tube and shaken on an orbital shaker at room temperature at around 200 rpm for 10 minutes. Later, 250 µl buffered phenol chloroform was added and the tubes were shaken on orbital shaker for another 20 minutes. The mixture was later centrifuged at 13,400 rpm for 15 minutes. Two hundred µl of the upper aqueous layer was transferred into another clean 1.5 ml tube by using pipette tip and added with 400 µl absolute ethanol to precipitate the genomic DNA. The

tube was inverted few times to mix the solution and placed in -20 °C overnight. The next day, the tube was centrifuged at 13,400 rpm for 15 minutes and the supernatant was discarded. Then, 500 µl of 70% (v/v) ethanol was added to the tube to wash the DNA pellet. Centrifugation was carried out again for 15 minutes. The supernatant was discarded and the ethanol was air dried. The DNA was rehydrated in 50 µl TE buffer and stored in -20 °C until further use.

3.2.2 Plasmid DNA extractions

Extraction of bacterial plasmid DNA was done by using QIAGEN miniprep kit. Here, 10 ml of bacterial overnight culture was centrifuged in a 1.5 ml microfuge tube at 13000 rpm for 2 minutes at room temperature. The supernatant was discarded and the traces of media were removed as much as possible. The pelleted cells were resuspended in 250 µl Buffer P1 added with RNase and pipetted up and down to get homogenous suspension. Then 250 µl Buffer P2 (lysis buffer) was added and the tubes were inverted a few times until the solution became viscous and clear. Then 350 µl of Buffer N3 was added into the tubes and was immediately inverted a few times to mix. The tubes were centrifuged for 10 minutes at 13000 rpm to pellet cell debris and proteins. The supernatant was aspirated out and applied to QIA PrepSpin column and centrifuged at 13000 rpm for 1 minute. The flow-through was discarded and the column was washed with 0.5 ml Buffer PB and centrifuged again. Then 0.75 ml Buffer PE was added and centrifuged. The empty column was centrifuged again to remove the residual of wash buffer. Forty µl deionized water was added to the filter membrane after that and let it stand for 2 minutes. The column was centrifuged again to collect the plasmid in a fresh 1.5 ml eppendorf tube.

3.2.3 PCR amplifications of targeted DNA sequence

PCR primers used were described in section 3.1.3. A 25 µl PCR reaction mixture was set up containing 0.5x Green GoTaq® Reaction Buffer, 3 mM Magnesium Chloride ($MgCl_2$), 0.4 mM dNTPs mix, 0.2 pmol 5' Primer, 0.2 pmol 3' Primer and 1.25 units of *Taq* DNA Polymerase (Promega). The PCR cycling profile involved predenaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 40 seconds, primer annealing at 59 °C for 20 seconds, and a primer extension at 72 °C for 20 seconds. The last cycle included a final extension at 72 °C for 7 minutes.

3.2.4 Rapid screening for inserts by colony PCR

PCR primers used were described in section 3.1.3. A 25 µl PCR reaction mixture was set up containing 1x Green GoTaq® Reaction Buffer, 1.5 mM Magnesium Chloride ($MgCl_2$), 0.2 mM dNTPs mix, 0.1 pmol 5' Primer, 0.1 pmol 3' Primer and 1.25 units of *Taq* DNA Polymerase (Promega). The PCR cycling profile involved predenaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, primer annealing at 55 °C for 1 minute, and a primer extension at 72 °C for 2 minutes. The last cycle included a final extension at 72 °C for 5 minutes.

3.2.5 Restriction digestion of DNA fragment

The reaction mixture was prepared in 1.5 ml microfuge tube with total volume of 40 µl. The reaction mixture consisted of 4 µl of 10x buffer, 20 µl of DNA, 1µl of restriction enzyme (Promega) and appropriate amount of distilled water to make up the volume to 40 µl. The reaction mixture was pulse-centrifuged for about 15 seconds and incubated at 37°C for one hour. The reaction was stopped by incubating the microfuge tube at 65 °C for 20 minutes.

3.2.6 Gel electrophoresis

Preparation and running gels used Tris-borate-EDTA (TBE) buffer which compositions were 21.6 g of tris base, 1.85 g of EDTA, and 11.0 g of Boric acid in 2 liter of distilled water. The gel composition was directly related to the amount of agarose and water. A 0.5% (w/v) gel was prepared by dissolving 0.5 g of agarose into 100 ml of 0.5x TBE, appropriate amount of agarose was used to prepare the 1% (w/v) or 1.5% (w/v) gel in 100 ml of 0.5x TBE buffer based on the similar formula. Five µl of green buffer was added into each DNA sample to be analyzed together with DNA standards of 1 kb DNA ladder and λ DNA/*Hin*dIII (3 µl DNA with 17 µl of distilled water). The samples were loaded into the gel and run in 0.5x TBE buffer at 100 V for about 25 minutes. The gel was then stained with ethidium bromide for 20 minutes and destained with distilled water for 10 minutes. Finally, the gel was photographed with Gel Doc^{TM} XR+ (Biorad) under UV light.

3.2.7 Ligation reaction of DNA fragment to vector

The ligation mix was prepared with 5 µl of digested vector plasmid, 15 µl of insert DNA, 4 µl of 10x T4 DNA ligase buffer (Fermentas), distilled water to bring to a total volume of 30 µl, and 1 µl of T4 DNA ligase. The ligation reaction was incubated overnight at 4 °C in a refrigerator or one hour at 22 °C for annealing process.

3.2.8 Preparation of *Escherichia coli* competent cells

One hundred µl of overnight *Escherichia coli* (*E. coli*) culture was inoculated to 50 ml LB broth and incubated at 37 °C with shaking at 200 rpm until the OD at 600 nm reached 0.5. One ml of the bacterial culture was then transferred into a clean 1.5 ml tube, and the cells were pelleted by centrifugation at 7,000 rpm for 10 minutes. The supernatant was discarded and the cells were resuspended in equal volume of ice-cold 50 mM $CaCl_2$. The tube was incubated on ice for 20 minutes and recentrifuged at 7000 rpm for 10 minutes. The supernatant was discarded and the cells were resuspended in 150 µl ice-cold 50mM $CaCl_2$.

3.2.9 Transformation of DNA to competent *Escherichia coli* cells

Ten µl of ligation mix of DNA was added to 200 µl competent cells in a 1.5 ml microfuge tube. The tube was tapped gently to mix the content incubated on ice for 15 minutes. The cells were incubated at 42 °C for exactly 45 seconds and transferred into ice immediately. Two hundred µl SOC broth was then added and incubated at 37 °C,

shaking 100 rpm for 1 hour to recover the cells. Then serial dilutions were made and 50 µl of each diluted culture were spread on selective LB agar.

3.2.10 Induction of protein expression with isopropyl β-D thiogalactoside (IPTG)

Fifty µl of overnight culture was inoculated into 50 ml LB broth and incubated at 37 °C with shaking at 120 rpm. When the OD_{600nm} reached 0.5-0.6, the IPTG was added at a final concentration of 1mM. The culture was further incubated for 4 hours. Then, cells were pelleted by centrifugation at 7000 rpm at 4 °C and the supernatant was discarded. The cells were washed by resuspending in ice-cold 1x PBS and centrifuged again. The supernatant was discarded and the cells were kept in -20 °C.

3.2.11 Protein extraction with NP40 buffer

One ml of ice-cold buffer was added to expressed-bacterial cell pellets in 1.5 ml microfuge tube and pipetted gently to mix. The cells were agitated gently at 4 °C for 30 minutes on a belly dancer. Then the cells were freezed-thawed by freezing in a -20 °C freezer and thawing on ice for three times. Ten µl of cold Triton X-100 was added for a final concentration of 1% to solubilize protein and further agitated gently at 4 °C for another 30 minutes. Finally the cell lysate was centrifuged at 5000 rpm for 15 minutes and the supernatant was transferred to new sterile tube. The protein lysate was stored in -20 °C until use.

3.2.12 Periplasmic protein extraction

Cells pellet was resuspended in 20 ml ice-cold extraction buffer (1x TES) for every 1 liter of culture. Then 33 ml of ice-cold 1/5 TES buffer for every liter of culture was added. The resuspended cells were incubated on ice while agitating on an orbital shaker at 50 rpm for 30 minutes. The cells debris was pelleted by centrifugation at 4000 rpm for 10 minutes at 4 °C. The supernatant was transferred into new tubes and stored in -20 °C.

3.2.13 Bradford assay for protein quantification

One hundred µl of sample was added to 100 µl of distilled water and added with 800 µl of Bradford reagent in a 1.5ml tube. The mixture was mixed and incubated at room temperature for 5 minutes. The absorbance was measured with spectrophotometer at OD_{595nm}.

3.2.14 Colony blotting of transformed *Escherichia coli* cells

Transformed *E. coli* DE3 on selective Tryptic Soy Agar (TSA) was grown overnight at 37 °C. The culture was then transferred to fresh selective TSA and incubated at 37 °C overnight. Then, a nitrocellulose membrane cut at the same size as the petri dish was blotted onto the bacterial cells and left for 15-20 minutes. The membrane was then placed onto a prewet 3M Whatman paper in perti dish containing 10% SDS for 10 minutes. Following that, the membrane was transferred into another

containers containing 3M Whatman paper prewet with Denaturing solution (0.5M NAOH, 1.5M NaCl) for 5 minutes, twice in Neutralization solution (1.5M NaCl, 0.5M Tris.Cl pH 7.4) for 5 minutes, and 2x SSC (0.3M NaCl, 0.03 M Sodium Citrate) for 5 minutes. The membrane was blocked in 3% (v/v) BSA in TBST (0.05% Tween 20) for 3 hour at room temperature. Remaining colony debris was removed by wiping with kimwipes. Then it was incubated in 1/100 pooled typhoid crude antisera in 0.5 % (v/v) BSA TBST overnight at 4 °C and washed 3 times in 0.5% (v/v) BSA in TBST 15 minutes each. The membrane was probed with 1/10000 goat-anti human IgG-AP conjugate (Abnova) in 0.5% (v/v) TBST for 1 hour at room temperature. It was washed for three times after that and developed with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) substrate (KPL).

3.2.15 Dot-blot assay

The dot-blot apparatus was assembled and connected to a pump (R300 Boeco, Germany). The pump was turned on and the pressure was adjusted to 15 cmHg. The membrane was cut at 11.3 cm x 7.8 cm and soaked in 1x *PBS.* The membrane was then sandwiched in between middle and top blocks and the apparatus was screwed. Samples were loaded to each well at 100 µl and the pump was turned on to predetermined pressure. When all wells were dried, the membrane was removed and let air-dried for 30 minutes. The membrane was blocked in 3% (v/v) BSA in PBST (0.1% Tween20) for 1 hour at room temperature with agitating on an orbital shaker at 50 rpm. Then it was incubated with 1/100 dilution of typhoid crude antisera in 3% (v/v) BSA/PBST for 1 hour at room temperature while agitating. The blot was washed five times, five minutes each with PBST while the last washing only used 1x PBS. It was probed with 1/10000

dilution of goat-anti human IgG-AP conjugate (Abnova) in 3% (v/v) BSA in PBST for 1 hour at room temperature. After washing for five times, the blot was developed using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) substrate (KPL).

3.2.16 ELISA assay

Fifty µl of antigen (20 µg/ml suspended in ice-cold 1x PBS, pH 7.4) was added into individual wells of 96-well-Greiner Elisa plate. The plate was incubated overnight at 4 °C. The wells were washed with 100 µl of 1x PBST by pipetting up and down and the solution was discarded carefully on paper towels. The washing step was repeated 3 times. The wells were coated with 50 µl blocking buffer 1% (w/v) fish gelatin in PBST and incubated for 2 hours at 37 °C. The wells were washed again with 1xPBST thrice. Then the wells were pre-hybridized with 50 µl pooled sera at 1:500 diluted in blocking buffer and incubated at 37 °C for 2 hours. After washing 3 times with 1x PBST, 50 µl of secondary antibody goat-antihuman IgG-AP (Abnova) were added and incubated for another 2 hours at 37 °C. Then the wells were washed 5 times with 1x PBST and color reaction was developed with 50 µl pNPP, incubated in dark at 37 °C for 30 minutes. The reaction was stopped by addition of 50 µl 2M NaOH and the absorbance was read at 405/415 nm (iMark Microplate Reader, Biorad).

Chapter 4.0: RESULTS

4.1 Characterization of specific DNA marker of *Salmonella* Typhi

4.1.1 Construction of an expression system for specific DNA marker of *Salmonella* Typhi

In this study, pGEX-4T-1 expression vector (GE Healthcare, Sweden) was used to clone a specific DNA fragment of *S.* Typhi, ST332. Maintaining a correct reading frame of specific DNA marker of *S.* Typhi ST332 in the pGEX-4T-1 vector is crucial to ensure correct expression of the hypothetical protein. Therefore, the reading frames and positions of the multiple cloning sites (MCS) were considered by referring to the pGEX plasmid map provided by the manufacturer (Appendix A). Introduction of gene insert was carefully done by the choice of two restrictions sites which did not overlap on the plasmid vector to facilitate double digestions and ligation, *Not*I and *Eco*RI (Figure 4.1).

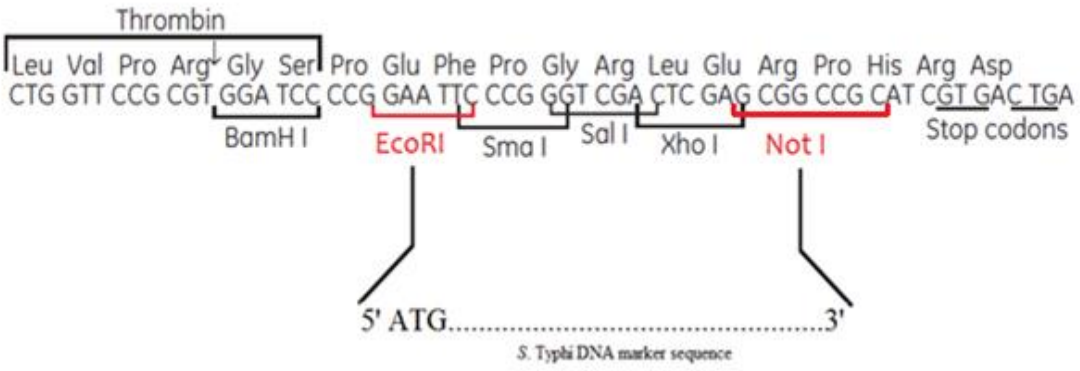

Figure 4.1 *S.* Typhi DNA marker insert and the MCS of pGEX vector. The amplified *S.* Typhi DNA fragment ST332 is situated between *Eco*RI and *Not*I sites. The gene encoding GST protein is located on the upstream of the insert site (Figure adapted from GE Healthcare GST Gene Fusion System handbook).

4.1.2 PCR amplification of the *Salmonella* Typhi DNA fragment ST332

In order to produce a DNA insert that is compatible for direct ligation to pGEX-4T-1 vector, the primer set was designed to introduce an *Eco*RI site before the ATG codon, and a *Not*I site on the terminal of the insert. The two restriction sites sequence were introduced into the ST332 primers design in which *Eco*RI site sequence was added to the 5' primer of ST332 while *Not*I restriction site sequence was added to the 3' primer of ST332. Another feature added to the primer was an addition of ATG nucleotides as a start codon in order to ensure that the insert protein has its own open reading frame for correct protein expression (Table 4.1). Consequently, this design maintained the correct reading frame of the gene while making GST-tag protein to be upstream on the N-terminal of the insert protein.

Table 4.1. Primer sequence used for the amplification of specific *S.*Typhi DNA marker

Primers	Sequences (5'-3')	Tm (°C)	Length
5' ST332	XXXGAATTCATGXXXXXXXXXXXXXXXXXXX	59 °C	31mer
3' ST332	XXXGCGGCCGCXXXXXXXXXXXXXXXXXXXX XX	59 °C	34mer

GAATTC = *Eco*RI restriction enzyme recognition site

GCGGCCGC = *Not*I restriction enzyme recognition site

ATG = start codon

XXX = patented primer sequence to amplify specific *S.* Typhi DNA marker ST332

The primers were used to PCR-amplify the gene fragment of *S.* Typhi ST332 for cloning to the pGEX-4T-1 vector. In the previous in-house study of specific *S.* Typhi DNA fragment ST332, the amplification of the gene in multiplex PCR for *Salmonella* detection produced a 332bp fragment. However, in this study, after the addition of *EcoRI* and *Not*I restriction sites and the start codon, ATG to the primers, the final size of insert produced was determined to be ~350 bp. Agarose gel electrophoresis analysis showed to be consistent with this prediction with a amplified product to be around the size of ~350 bp (Figure 4.2). Therefore, it was confirmed that the PCR was successfully carried out to amplify the target gene sequence.

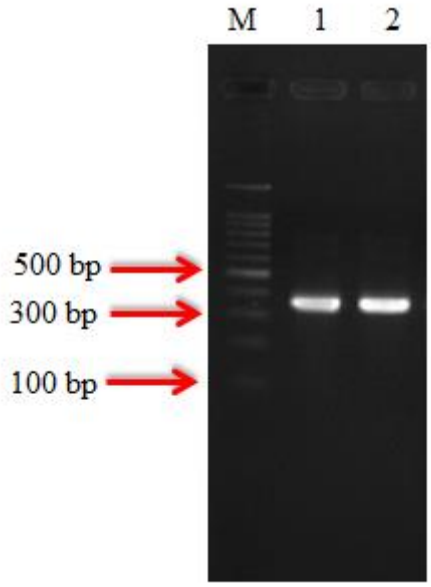

Figure 4.2. Agarose gel electrophoresis showing the size of amplified product. Lanes 1 and 2 were the replicate samples of the amplified *S.* Typhi DNA fragment ST332. The size was compared to the 100 bp ladder in the first lane (M).

Cloning of the PCR fragment into a pGEX-4T-1 vector was done at the predetermined orientation of insert by the double digestions of *Eco*RI and *Not*I on the plasmid and also the PCR product of ST332 (insert). The amplified PCR product was purified as the insert and the pGEX-4T-1 vector were digested with *Eco*RI and *Not*I restriction enzymes. The products were ligated together with T4 DNA ligase. Following ligation, the ligated vector-insert was used to transform *E. coli* BL21, and selection of clones was done by growing the transformed cells on LB agar supplemented with 100 µg/ml carbanecillin.

Screening of successful recombinants was done by PCR amplification of the inserted sequence using a pair of pGEX-sequencing primers (Table 4.2) with running conditions as section 3.4.

Table 4.2 pGEX sequencing primers

Primers	Sequences (5'-3')	Tm (°C)	Length (bp)
5' pGEX	GGGCTGGCAAGCCACGTTTGGTG	55 °C	23
3' pGEX	CCGGGAGCTGCATGTGTCAGAGG	55 °C	23

The PCR products were separated on 1.5% (w/v) agarose gel electrophoresis and visualized under UV with Gel Doc (Figure 4.3). From the plasmid vector's map (Appendix A), the flanking sequence itself would produce a 160 bp DNA fragment, thus a successful recombinant would amplify a fragment of about 500 bp in size. As seen in Figure 4.3, the agarose gel electrophoresis analysis displayed that 3 of the clones showed the predicted band in their PCR products. Thus it proved that clones bearing pGEX-4T-1-ST332 construct were successfully produced.

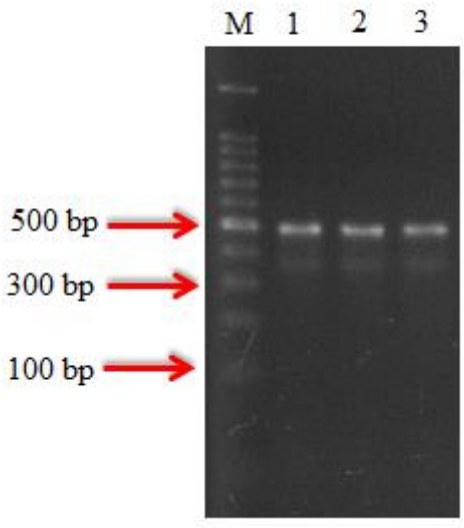

Figure 4.3. Agarose gel electrophoresis showing amplified inserts of *E. coli* BL21 clones. Lanes 1, 2 and 3 showed the amplified bands of gene insert ST332 in pGEX-4T-1 vector.

In order to verify that the DNA insert was cloned in the correct reading frame, the plasmids were purified and subjected to sequencing (Base Life Sciences Holdings). The forward and reverse sequencing results in ABI format were assembled using DNA Base v.3.5.3 to get the full length contig. Using the assembled contig of insert sequence, the open reading frame of the insert gene were searched using ORF finder (Open Reading Frame finder) from NCBI to ensure that the insert was in the correct reading frame (Figure 4.4).

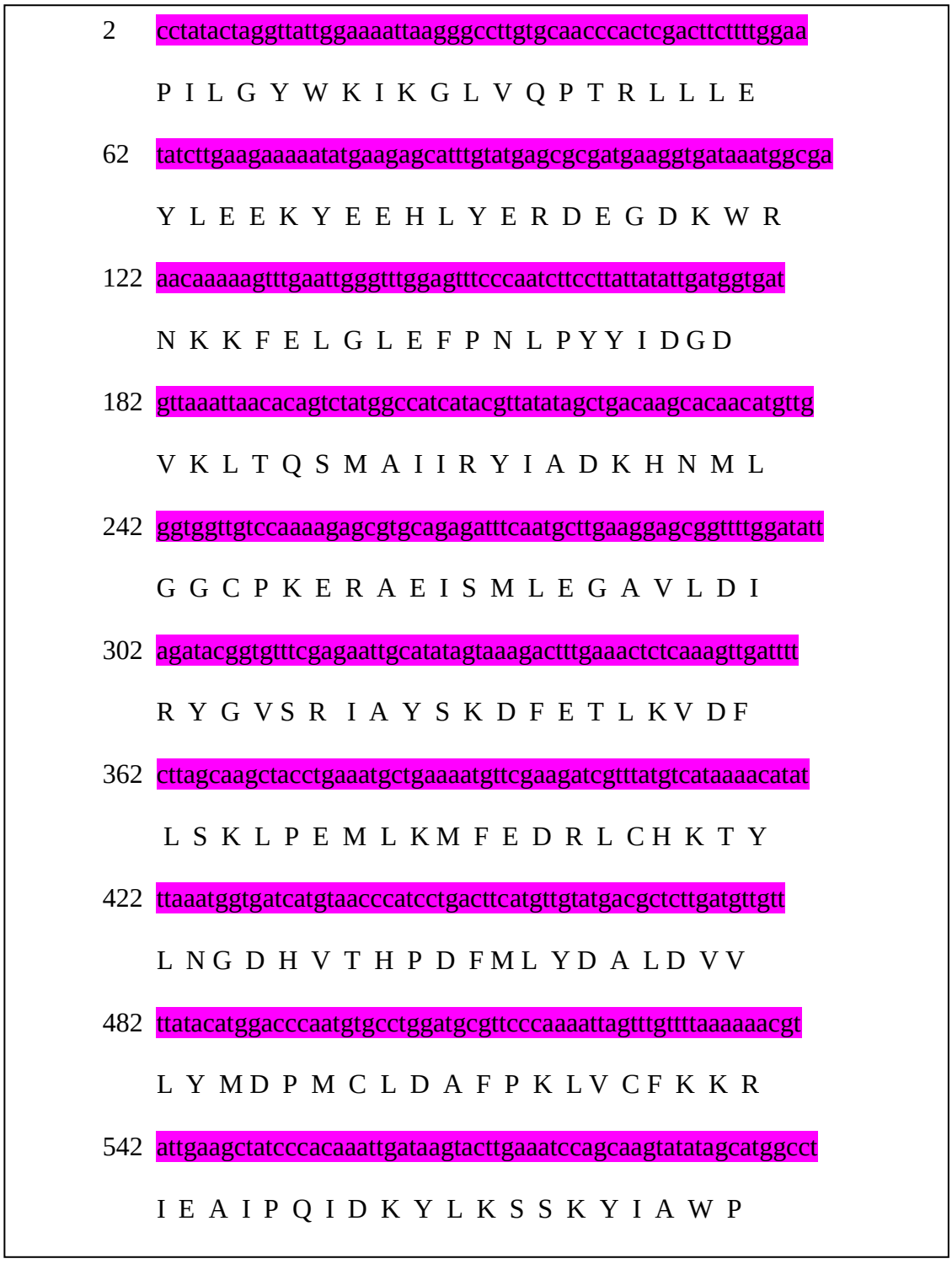

Figure 4.4: The ORF finder (NCBI) for the contiq assemblies of forward and reverse sequence of pGEX-4T-1-ST332 (figure 1 of 3). The ORF finder automatically translates DNA sequence into protein sequence at the corresponding codons; XXX = bases for

GST-tag residues before the actual *S.* Typhi DNA marker sequence; XXX = *S.* Typhi DNA marker insert sequence ST332; XXX = restriction sites flanking insert sequence.

602 ttgcagggctggcaagccacgtttggtggtggcgaccatcctccaaaatcggatctggtt

L Q G W Q A T F G G G D H P P K S D L V

662 ccgcgtggatccccggaattcatgatcctgccggaaagtgaacaacccatgctggctcaa

P R G S P E F M I L P E S E Q P M L A Q

722 cagttgaatcagcttgcacagaaatcaccagttcaggctgaacagatcgccctcagcgtg

Q L N Q L A Q K S P V Q A E Q I A L S V

782 gtgaatggctggcaccagaaacgtatcagcaatccggtgggctacttgctgactacactc

V N G W H Q K R I S N P V G Y L L T T L

842 aggcaagcgagggcaggactgtatcgtctggaacctgctgtcactcagcctgttaagagc

R Q A R A G L Y R L E P A V T Q P V K S

902 agatctgtcgtgccagctgagccatccgctgccgggaaaatcccgacatgctcagctgaa

R S V V P A E P S A A G K I P T C S A E

962 gctgatgtccctgcaggtcaggaagtcgtgaaggcgatggtggagcaaattcgttaagcg

A D V P A G Q E V V K A M V E Q I R * A

1022 gccgcatcgtgactgactgacgatctgcctcgcgcgtttcggtgatgacggtgaaaacct

A A S * L T D D L P R A F R * * R * K P

1082 ctgacacatgcagctcccggagacggtcacagcttgtctgtaagcggatgccgggagcag

L T H A A P G D G H S L S V S G C R E Q

1142 acaagcccgtcagggcgcgtcagcgggtgttggcgggtgtcggggcgcagccatgaccca

T S P S G R V S G C W R V S G R S H D P

Figure 4.4, continued: The ORF finder (NCBI) for the contiq assemblies of forward and reverse sequence of pGEX-4T-1-ST332 (figure 2 of 3). The ORF finder automatically translates DNA sequence into protein sequence at the corresponding codons; XXX = bases for GST-tag residues before the actual *S.* Typhi DNA marker sequence; XXX = *S.* Typhi DNA marker insert sequence ST332; XXX = restriction sites flanking insert sequence.

```
1202 gtcacgtagcgatagcggagtgtataattcttgaagacgaaagggcctcgtgatacgcct
      V  T  *  R  *  R  S  V  *  F  L  K  T  K  G  P  R  D  T  P

1262 atttttataggttaatgtcatgataataatggtttcttagacgtcaggtggcacttttcg
      I  F  I  G  *  C  H  D  N  N  G  F  L  D  V  R  W  H  F  S

1322 gggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatcc
      G  K  C  A  R  N  P  Y  L  F  I  F  L  N  T  F  K  Y  V  S

1442 tattcaacatttccgtgtcgcccttattccctttttgcggcattttgccttcctgtttt
      Y  S  T  F  P  C  R  P  Y  S  L  F  C  G  I  L  P  S  C  F

1382 gctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgag
      A  H  E  T  I  T  L  I  N  A  S  I  I  L  K  K  E  E  Y  E

1502 tgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagt
      C  S  P  R  N  A  G  E  S  K  R  C  *  R  S  V  G  C  T  S

1562 gggttacatcgaactggatctcaacagcggtaagatccttgagagttttcgccccgaaga
      G  L  H  R  T  G  S  Q  Q  R  *  D  P  *  E  F  S  P  R  R

1622 acgttttccaatgatgagcactttaaagttctgctatggtggcgcggtattatcccgtgt
      T  F  S  N  D  E  H  F  *  S  S  A  M  W  R  G  I  I  P  C

1682 tgacgccgggcaagagcaactcggt 1706
      *  R  R  A  R  A  T  R
```

Figure 4.4, continued: The ORF finder (NCBI) for the contiq assemblies of forward and reverse sequence of pGEX-4T-1-ST332 (figure 3 or 3). The ORF finder automatically translates DNA sequence into protein sequence at the corresponding codons; XXX = bases for GST-tag residues before the actual *S.* Typhi DNA marker sequence; XXX = *S.* Typhi DNA marker insert sequence ST332; XXX = restriction sites flanking insert sequence.

As seen in Figures 4.4, the insert ST332 was present in the correct orientation with the insert gene having its own reading frame. As the produced protein was a fusion protein of GST-tag and the insert ST332, bases of the GST-tag residues were also seen in the sequencing results. The fusion protein was estimated to be around 38 kDa which the GST-tag alone made up for a molecular weight of 26000 Dalton in size (Toye *et al.*, 1990; GST Gene Fusion Handbook) whereas the insert ST332 produced only 12284 Dalton sized protein comprised of 111 amino acids (Figure 4.4).

By using the translated amino acid sequence of the gene insert, protein identity was searched by using BLASTp in NCBI protein database and the matched results paired the sequence to hypothetical protein of *Salmonella enterica* subsp. *enterica* serovar Typhi (Table 4.3). The BLASTp results show that, the 111 amino acid sequence of the ST332 only comprises less than half of the 447 amino acid of the *S.* Typhi hypothetical protein STY 4528. Therefore, the open reading frame of the cloned ST332 gene does not represent the whole hypothetical protein.

Table 4.3. Sequence and alignment comparisons to NCBI protein database. Translated gene sequence showed a 111 a.a sequence which produced 100% similarity to hypothetical protein of *S.* Typhi.

Translated amino acid sequence:	Blastp Results
MILPESEQPMLAQQLNQLAQ KSPVQAEQIALSVVNGWHQK RISNPVGYLLTTLRQARAGLY RLEPAVTQPVKSRSVVPAEPS AAGKIPTCSAEADVPAGQEV VKAMVEQIR	Hypothetical protein SentesT_28370 [*Salmonella* enterica subsp. enterica serovar Typhi str. M223] E-value: 1e-71 Sequence Identities: 110/110 (100%) Hypothetical protein STY4528 [*Salmonella* enterica subsp. enterica serovar Typhi str. CT18] E-value: 4e-68 Sequence Identities: 110/110 (100%) Length: 447

Although the gene for the respective DNA fragment ST332 has not been characterized, it was shown to be specific for *S.* Typhi in previous research in the laboratory. As it is specific for detection by PCR, its specificity as a protein antigen for *S.* Typhi was also investigated.

4.1.3 Immunodetection of *Salmonella* Typhi specific DNA marker as a hypothetical protein

Protein expression of *S.* Typhi specific DNA marker was carried out to determine the reactivity of the protein to typhoid patients' sera. The recombinant plasmid pGEX-4T-1 carrying the DNA marker was transformed into *E.coli* BL21 competent strain.

The overnight culture of transformed *E. coli* BL21 with plasmid construct was used as an inoculum for protein expression in a 20 ml of LB broth containing 100 µg/ml carbanecillin, and induced expression with addition of 1mM IPTG (section 3.2.10). Then the protein in cell lysate was analyzed by dot-blot assay and immunoscreened with pooled crude antisera of typhoid fever patients (Figure 4.5).

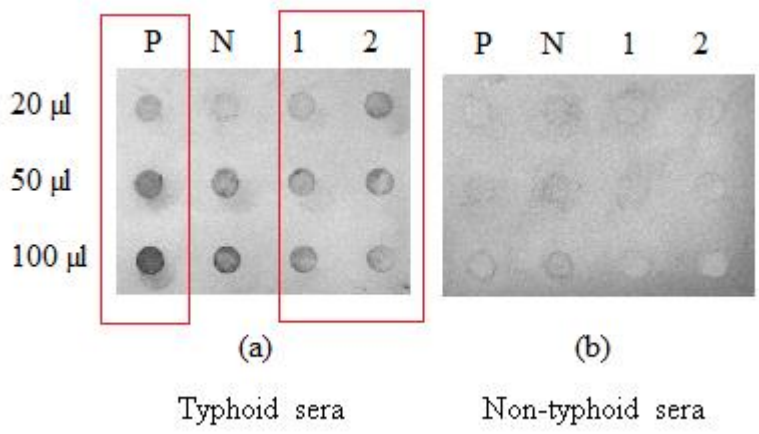

Figure 4.5 Immunoscreening of protein in cell lysate of transformed *E. coli* BL21.

P: Protein lysate of *S.* Typhi culture as positive control.

N: Protein lysate of *E. coli* BL21 protein carrying pGEX-4T-1 without insert as negative control.

1 and 2: Samples of transformed *E. coli* BL21 carrying recombinant plasmid pGEX-4T-1 with *S.* Typhi DNA marker insert as expressed protein.

Figure 4.5 (a) shows the first blot of samples with different sample volumes, hybridized with typhoid patients crude antisera (from top, 20 µl, 50 µl and 100 µl). Figure 4.5 (b) shows the same samples as (a) but the blot was hybridized with non-typhoidal patients' pooled sera.

Screening for specificity of ST332 insert protein towards *S.* Typhi antisera could not be directly done since the protein has not been characterized and no specific monoclonal antibody that binds the protein is available. Therefore, determination of its reactivity towards typhoid sera was done through hybridization of the protein against the crude serum of typhoid patients since the sera has a pool of antibodies reacting to *S.* Typhi antigens.

Dot blot results for hybridization of the insert protein against typhoid patients pooled sera is shown in Figure 4.5 (a). In the figure, it shows that the extracted protein lysate containing the fusion protein of ST332 (lane 1 and 2) showed a poor reactivity towards the sera when compared to the positive control which is the protein lysate of *S.* Typhi (P). This observation is made by comparing the intensity of color reaction of the blot marked in the red rectangles in which the color intensity of the samples blot (1 and 2) is not as high as the positive control (P). However, it is also seen in the figure that there is also a reaction between the sera and the *E. coli* proteins (negative control, N). It is suspected that the reaction occurs because the typhoid patients' pooled sera also contained antibodies reacting to *E. coli* proteins since crude sera were used in this study, not pure antibody to *S.* Typhi. However, as the intensity of the reaction is not as strong

as the positive control, it is concluded that any positive reaction should be comparable to the positive control to make it positive. Therefore, because there was weak reaction between the insert ST332 protein with the sera, the observation revealed that the protein is not highly reactive as an antigen.

A more thorough investigation is still needed for this finding to confirm the conclusion made. In this study, limitations are seen with lack of fresh typhoid serum for immunoscreening and purification system of the fusion protein. As further study is to be made by purifying the protein to make the target more specific and eliminates background reaction with *E. coli* proteins. Moreover, the use of fresh serum would be more convincing for the finding of this study.

4.2 Screening of specific *Salmonella* Typhi antigen

4.2.1 Construction of library clones of *Salmonella* Typhi genomic DNA

Another approach to look for a specific *S.* Typhi antigen was through shotgun cloning of *S.* Typhi genes and screened for protein specificity towards *S.* Typhi antisera. Novatope Cloning System was applied to construct a random peptide library of *S.* Typhi. The genomic DNA of *S.* Typhi was digested with DNaseI producing gene fragments of average sizes between 50-150 bp (Figure 4.6). Following digestion, the DNA fragments size between 50 bp to 300 bp were purified and ligated to pSCREEN-1b(+) T-vector with T4 DNA ligase. The ligated plasmids were then used to transform competent NovaBlue (*E. coli* DE3) and grown on LB agar supplemented with 50 µg/ml carbanecillin and 15 µg/ml tetracycline as selective media.

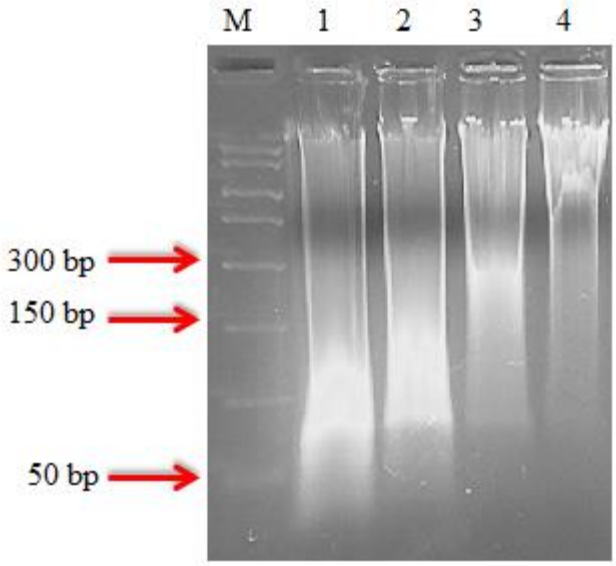

Figure 4.6. Gel electrophoresis of digested genomic DNA with different dilution of DNaseI. Lane 1 was digested with 1:133 dilution DNaseI, lane 2 with 1:200 dilution, lane 3 with 1:300 dilution and lane 4 with 1:450 dilution. M was the PCR marker as size reference.

Transformation of competent NovaBlue cells produced more than 700 clones as seen in Figure 4.7. However, because some clones grew close to each other that there was a fear of cross-contamination. Therefore, selection of clones were made where about 700 individual clones were successfully selected for further analysis and stored in glycerol stock.

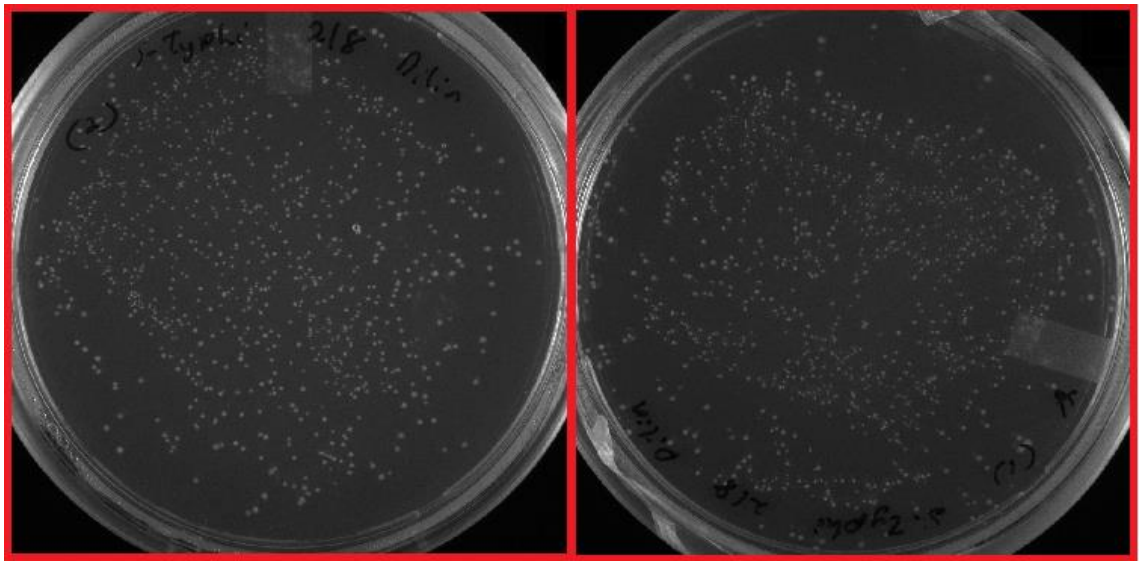

Figure 4.7 Transformed NovaBlue (*E. coli* DE3) cells with *S.* Typhi genomic DNA fragments grown on LB agar supplemented with 50 µg/ml carbanecillin and 15 µg/ml tetracycline as selective media.

Screening for successful transformation of the NovaBlue cells based on white-blue cells observation is not available on the pSCREEN-1b (+) T-vector (Novatope System Manual). Therefore, a pre-screening was done by PCR on a few selected clones in the library. The rapid screening of inserts was done by colony PCR on 59 randomly picked clones, and separated on 1.5% (w/v) agarose gel electrophoresis. The results revealed that 55 out of 59 clones tested produced a band bigger than 341 bp (Figure 4.8). As the flanking sequence of t7 gene *10* primer and T7 terminator primer on the vector amplified a 341 bp without insert, the clones with bigger bands were confirmed to have inserts. This showed that cloning of *S.* Typhi genomic DNA fragments into the pSCREEN-1b (+) T-vector was successful.

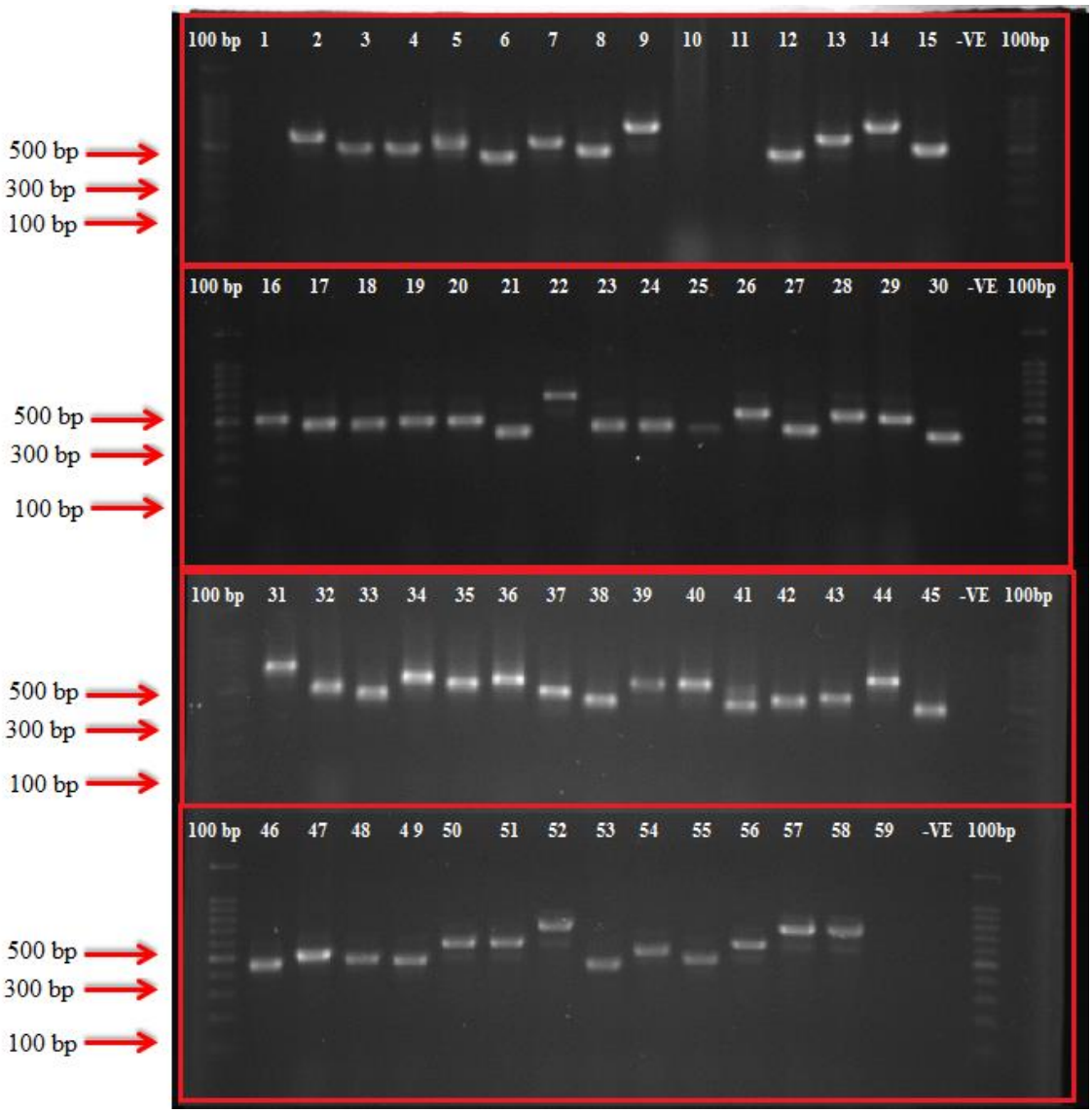

Figure 4.8 Agarose gel electrophoresis showing amplified inserts of NovaBlue transformants. Numbered lanes 1-59 showed the amplified bands of gene inserts in pSCREEN-1b(+) T-vector of different clones. 100bp: 100 bp marker as size reference, -VE: negative control.

A more thorough screening of clones carrying the *S.* Typhi gene fragments was done with colony immunoscreening, colony blot immune assay, in order to obtain a specific clone expressing protein specific to typhoid patients pooled sera.

4.2.2 Colony immunoscreening of recombinant NovaBlue (*Escherichia coli* DE3) clones

Immunoscreening of transformed NovaBlue cells carrying *S.* Typhi gene fragments were carried out in order to obtain clones expressing epitope that was specific to typhoid antisera. For this purpose, about 500 clones were selected and grown on selective TSA agar containing 50 µg/ml carbanecillin and 15 µg/ml tetracycline overnight. Upon expression, the colonies were lifted up on nitrocellulose filter and grown on fresh selective TSA agar supplemented with 250 µM of IPTG to induce the protein expression. The expressed proteins bound to the nitrocellulose filter after cell lysis were probed with the same pooled typhoid crude antisera as previous section to test their reactivity to the bound protein. Bound antibodies were detected by hybridizing with goat anti-human IgG conjugated with AP. In here, results showed that several clones exhibited significant stronger signals on the blot which indicated a higher level of reactivity towards the antisera (Figure 4.9; Membranes 1, 2 and 3). Thus those clones were selected for further immunoscreening to affirm their reactivity. As observed in previous section, screening of the expressed protein with typhoid negative antisera did not produce any signal on the membrane (Figure 4.9; Membrane 4).

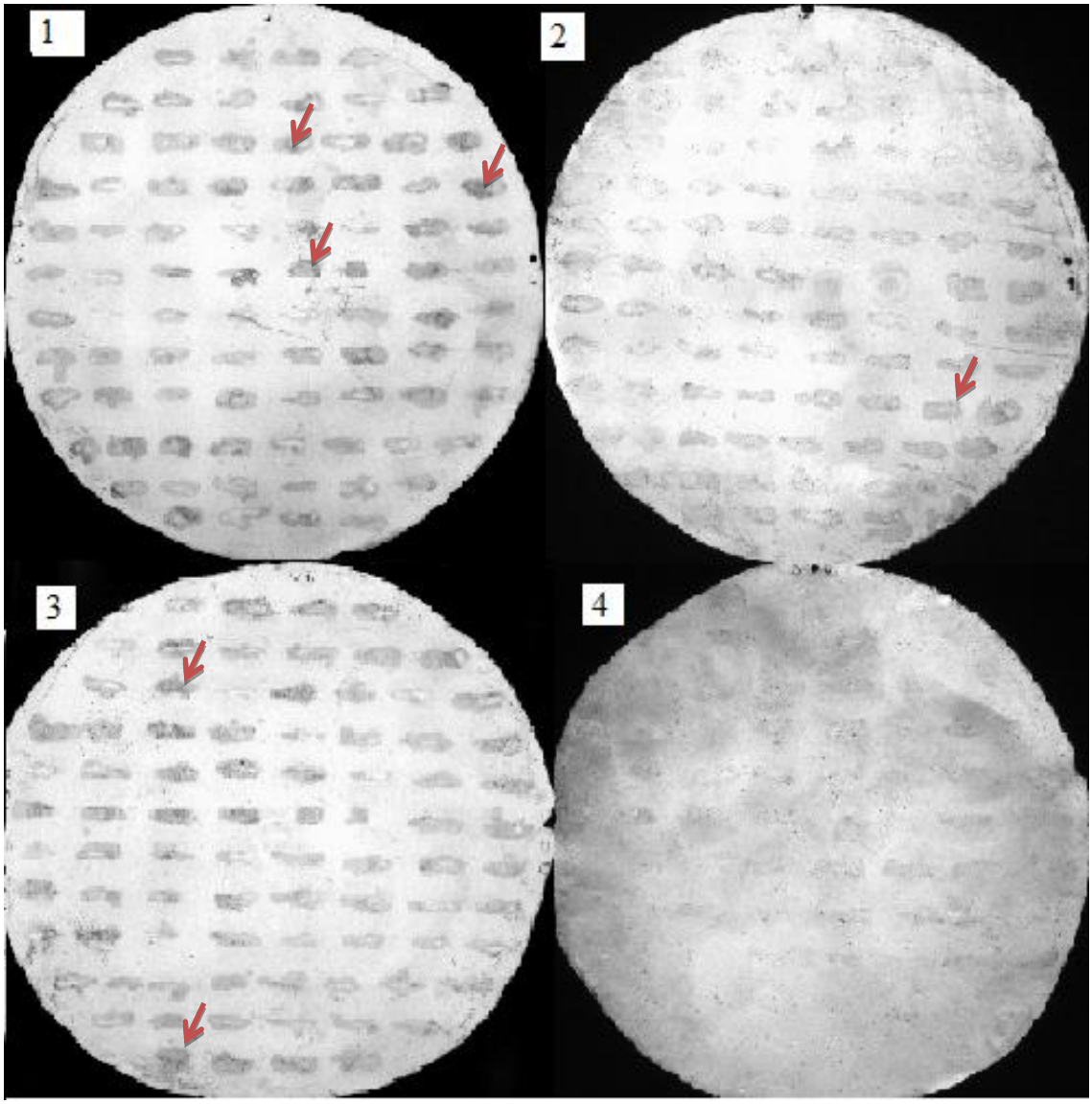

Figure 4.9 Representative results of colony immunoscreening of transformed NovaBlue (*E. coli* DE3) cells carrying pSCREEN-1b(+) vector and *S.* Typhi gene fragments with typhoid patients crude antisera. Membranes 1 – 3 show the blot of different clones probed with typhoid antisera with arrows showing several clones with stronger reactivity and selected for further screening. Membrane 4 show the clones probed with typhoid negative antisera.

4.2.3 Immunodetection of the putative reactive clones

About 30 individual clones which showed higher reactivity towards typhoid patients sera were selected and grown in LB broth supplemented with 50 µg/ml carbanecillin and 15 µg/ml tetracycline as selective agents. Then the overnight culture was used to inoculate 20 ml of selective LB broth and induced with addition of 250 µM IPTG. The cells were harvested and lysed after that, and the protein in crude cell lysate was analyzed by dot-blot assay and immunoscreened with pooled crude antisera of typhoid fever patients (Figure 4.10). It is seen in the following figure that in the reaction of the antibody to the clones protein lysate (a – g), some clones produced a higher signal compared to the negative control (N).

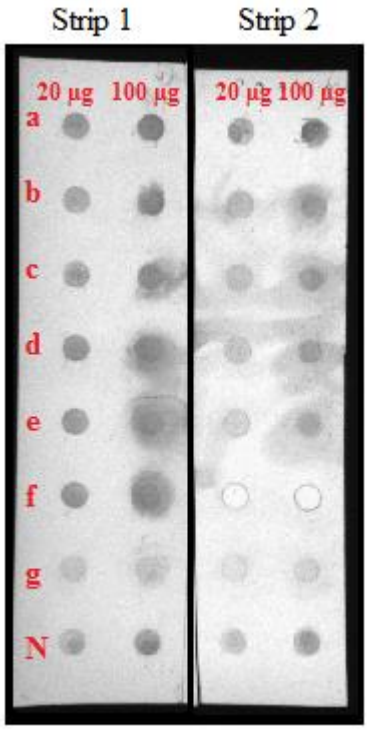

Figure 4.10 Two representative strips of immunoscreening of individual transformed NovaBlue (*E. coli* DE3) protein in cell lysate. N: *E. coli* DE3 protein in cell lysate carrying pSCREEN-1b(+) without insert as negative control. a – g were the expressed protein samples of transformed NovaBlue (*E. coli* DE3) carrying recombinant plasmid insert, with the left lane loaded at 20 µg and the right lane loaded at 100 µg on each strip (different samples on each strip).

4.2.4 ELISA immune detection of potential clones

Another immunoscreening of clones which were reactive to previous blotting was done by ELISA. About 20 clones which showed stronger reactivity in colony blot were selected and screened with ELISA by using the extracted periplasmic protein of the *E. coli* DE3 recombinant carrying *S.* Typhi DNA fragments to coat the ELISA plate wells and hybridized with pooled typhoid patients' sera and typhoid negative sera. The

bound antibody was detected with anti-human IgG conjugated with AP. The affinity of reaction was measured at 415 nm. 1% (v/v) BSA was used as a negative control to eliminate background contamination. Protein hybridization against typhoid patients pooled sera and *S.* Typhi negative sera show a distinct difference in the intensity of reactions. Most of the clones showed at least double in intensity compared to the negative sera reactions which indicate that it was highly probable that the clones have reacted towards the *S.* Typhi antibodies (Table 4.4).

Table 4.4 Affinity of periplasmic protein from recombinant *E. coli* DE3 against typhoid patients sera determined by ELISA.

Clones ID	Hybridized with positive typhoid fever sera	Hybridized with negative sera	Ratio positive/negative sera
D1	0.996	0.437	2.3
D5	1.051	0.531	2.0
D11	0.637	0.318	2.0
D13	1.102	0.555	2.0
D18	1.114	0.561	2.0
D34	1.135	0.543	2.1
D47	1.096	0.532	2.1
G11	0.919	0.439	2.1
G12	1.099	0.548	2.0
G35	1.192	0.559	2.1
I3	1.285	0.523	2.5
I11	1.430	0.653	2.2

Table 4.4 Affinity of periplasmic protein from recombinant *E. coli* DE3 against typhoid patients sera determined by ELISA (continued).

Clones ID	Hybridized with positive typhoid fever sera	Hybridized with negative sera	Ratio positive/negative sera
I23	1.170	0.514	2.3
J23	0.999	0.507	2.0
J36	0.995	0.496	2.0
K8	1.028	0.508	2.0
K35	0.038	0.483	0.1
L10	0.971	0.479	2.0
N14	0.881	0.466	1.9
N19	1.070	0.510	2.1
Negative	0.804	0.452	1.8

Table 4.4 shows affinity of periplasmic protein from recombinant E. coli DE3 against typhoid patients sera determined by ELISA. Extracted periplasmic protein of recombinant *E. coli* bearing *S.* Typhi DNA fragments were used to coat ELISA plates and incubated with pooled typhoid patients' crude sera and pooled typhoid negative sera. Bound antibody was detected with anti-human IgG conjugated to AP and developed with pNPP. Reaction color was measured with iMark microplate reader at 415nm. The absorbance values were obtained after deducting the values against background (1% (v/v) BSA). Negative: *E. coli* DE3 protein in cell lysate carrying pSCREEN-1b(+) without insert as negative control.

Of the 20 clones selected, the ones that showed a higher affinity to typhoid sera were selected for further study. From the results, clones with more than 1.5 fold affinity towards typhoid sera than typhoid negative sera were selected for confirmation of reactivity. However, since all of the clones showed high affinity of more than 1.5 ratios to the negative, clones that exhibit higher affinity were selected at range of above 2.0 ratios. The selected clones were subjected to another ELISA screening for confirmation (Table 4.5).

Table 4.5 Affinity of periplasmic protein from selected recombinant *E. coli* DE3 against typhoid patients sera determined by ELISA.

Clones ID	Hybridized with positive typhoid fever sera	Hybridized with negative sera	Ratio
D1	0.913±0.00	0.464±0.012	2.0
D34	1.01±0.001	0.521±0.011	1.9
D47	0.999±0.011	0.553±0.030	1.8
G11	0.892±0.011	0.496±0.008	1.8
G35	1.16±0.025	0.565±0.001	2.1
I3	1.033±0.005	0.511±0.002	2.0
I11	0.933±0.027	0.509±0.015	1.8
I23	0.905±0.028	0.509±0.015	1.8
N19	0.844±0.016	0.442±0.005	1.9

Extracted periplasmic protein of recombinant *E. coli* bearing *S.* Typhi DNA fragments were used to coat ELISA plates and incubated with pooled typhoid patients' crude sera and pooled typhoid negative sera. Bound antibody was detected with anti-human IgG conjugated to AP and developed with pNPP. Reaction color was measured with iMark microplate reader at 415nm. The absorbance values were obtained after deducting the values against background (1% BSA). The reaction was repeated twice. As seen in the second ELISA, all clones produced more than 1.5 fold affinity towards typhoid sera than typhoid negative sera and there were three clones (D1, G35, and I3) produced at least 2.0 fold increase in affinity towards the typhoid sera (Table 4.5).

Chapter 5.0 DISCUSSIONS

5.1 Characterization of specific DNA marker of *Salmonella* Typhi

Typhoid fever is a multi-systemic disease caused by bacterial pathogen, *S.* Typhi. The incidence of typhoid fever worldwide has been a global issue with an annual death toll of over 216 000 in 2000 (Crump *et al*., 2004). One of major problems that leads to the severity of this disease is because of the lack of reliable rapid diagnosis of the disease. Currently, the gold standard method in diagnosis of typhoid fever is through blood culture followed by microbiological identification which is tedious and time consuming (Baker *et al.,* 2010). Many studies have been conducted in finding rapid diagnosis of typhoid fever such as multiplex PCR (mPCR) assay for *Salmonella* detection (Ngan *et al.,* 2010), immunochromatographic strip serological test (Preechakasedkit *et al.,* 2011), immunochromatography as lateral flow dip stick test (IC-LFT) (Das *et al.,* 2013) and many others. One of new rapid tests targeting *S.* Typhi is called *Salmonella* EZplex that was developed in Laboratory of Biomedical Science and Molecular Microbiology, University of Malaya. This newly commercialized kit is a PCR based detection system for *S.* Typhi by the amplification of a DNA fragment, ST332. ST332 as a DNA marker shows a high specificity towards *S.* Typhi in PCR based detection. Therefore, this study intends to test for its antigenic reactivity in serological test. This is done by constructing an expression system of the gene and hybridizing the expressed protein to typhoid patients' sera in immunoscreening.

In constructing an expression system for the ST332 gene fragment, its pair of primers used in the kit were designed to incorporate appropriate restriction sites (*Not*I and *EcoRI*) and were used to generate DNA fragment for direct cloning into the plasmid

vector, pGEX-4T-1. The pGEX-4T-1 vector was employed as the expression system in this study as it allows high expression of foreign fusion proteins in *E. coli* and enables rapid purification under non-denaturing conditions (Toye *et al.,* 1990; Frangioni and Neel, 1993). Expression of the vector allows fusion of protein of interest with *Schistosoma japonicum* 26 kDa gluthathione *S*-transferase (GST) at the C-terminus of the GST (Toye *et al.,* 1990). This expression system is also preferable since the fusion protein is accumulated in the cytoplasm of the host cell thus facilitates easier purification (GST Gene Fusion Handbook). As the system has been successfully used for expression of fusion protein in various studies such as signaling mechanism of ribosomal kinase (Jensen *et al.,* 1999), vaccination against Lyme borrelis (Fikrig *et al.,* 1993), compound-protein interactions (DePalo *et al.,* 1994) and many more has made the system convincing as a good expression vector in this study.

Cloning of the *S.* Typhi DNA fragment starts with the amplification of the desired DNA fragment by PCR. As seen in Table 4.1, alterations were made to the original sequence of the primers used in the detection of *S.* Typhi in *Salmonella* EZplex by addition of *Eco*RI restriction site sequence at the 5' primer and *Not*I restriction site sequence at the 3' primer. Addition of the restriction sites were done while considering the open reading frame of the sequence to ensure correct orientation and expression of the protein. The choice of restriction sites was done according to the sequences in the multiple cloning sites of the pGEX vector (Figure 4.1). *Eco*RI and *Not*I sites were chosen since they are farther apart on the plasmid to facilitate complete digestion and their reaction conditions helped to ease the process by having similar reaction temperature at 37 °C and run on the same buffer, Buffer H (Promega). In addition, the restriction sites were also ensured to have no restriction site on the insert gene to maintain the integrity of the gene after digestion. By using these two restriction sites,

the cloning produced a predetermined orientation of the insert where the starting codon of the gene open reading frame would be at the C-terminus of the GST protein (Toye *et al.*, 1990). In the table, it is also seen that the primers sequence was not revealed since the sequence is under patent copyright PI2011005414.

In the *Salmonella* EZplex, amplification of the *S.* Typhi DNA fragment ST332 produces a DNA fragment at the size of 332 bp. However, the predicted band size for the amplified sequence using the designed primers was 350 bp. This is due to the addition of another 18 DNA bases of the restriction sites and start codon to the primers. As seen in Figure 4.2, amplified fragment was successfully produced at the predicted band size.

Cloning of ST332 DNA fragment into the pGEX-4T-1 multiple cloning site was made via *Not*I and *Eco*RI restriction sites. Confirmation of a successful construct of pGEX-4T-1-ST332 was verified by PCR amplification using PGEX sequencing primers as seen in Figure 4.3. In the figure, the band representing the construct appears at the size of 500 bp. This is because the flanking sequence of the insert from the pGEX sequencing primers recognition sites produces 160 bp without insert (Appendix B). Therefore, as the insert itself is 350 bp in size, it was verified that the band at 500 bp size represents successful vector-insert of pGEX-4T-1-ST332 construct. It is also seen in the figure that another band size at the size of 350 bp appears faintly in the figure. This suspected to be the band of the uncloned 332bp DNA insert ST332 which was taken up by the cells during transformation. This happens because competent bacteria have the ability to take free extracellular DNA (Chen and Dubnau, 2004). As transformation process uses competent *E. coli* cells made by calcium treatment (Section 3.2.8 and 3.2.9), thus the cells were able to take the ligated plasmid pGEX-4T-1-ST332

as well as non-ligated pGEX-4T-1 or ST332 fragment. Therefore, relating this to the observation in Figure 4.3, the bacterial cells must have been transformed with the vector-insert pGEX-4T-1-ST332 and also taken up free ST332 DNA fragments during transformation. Thus, as the PCR used DNA lysate of the *E. coli* transformant, it is plausible that this band of 332bp also appeared. However, as the band of pGEX-4T-1 without insert of 160 bp was absent, it shows that the transformation was successful with the transformant bearing pGEX-4T-1-ST332 construct was produced.

Another verification of successful construct was made through sequencing of the cloned plasmid. The results showed in Figures 4.4 revealed that the insert cloned into the pGEX-4T-1 vector was in frame with the start codon while having a full length amino acid sequence thus producing a whole intact protein for the gene. Identification of the 111 amino acids sequence was searched through BLASTP database search program in NCBI protein database and the matched results paired the sequence to hypothetical protein of *Salmonella enterica* subsp. *enterica* serovar Typhi (Table 4.3). This result matched with the previous study which also defined the insert sequence to be the hypothetical protein of *Salmonella* Typhi str CT18 (STY4528). In a study done by Pickard *et al.* (2003) on comparisons of SPI-7 regions among several Vi-positive *Salmonella enterica* strains, they have distinguished the *Salmonella* pathogenicity island 7 (SPI-7) to several distinct regions. The gene of interest, STY4528 was found to be in the region between truncated tRNA and STY4536. Although the specific identity and function of the STY4528 was not characterized yet, the study have predicted that the genes clustered in the region function in conjugal transfer of DNA, DNA replication or transposition of *S.* Typhi (Pickard *et al.*, 2003).

Expression of the fusion protein was induced with isopropyl β-D thiogalactoside (IPTG) as the vector expression was controlled by *tac* promoter (GST Gene Fusion Handbook). After expression with IPTG, the protein lysate was extracted from the culture and tested for reactivity by immunoscreening. High expression of fusion protein in *E. coli* normally results in the higher formation of inclusion bodies in the cell compared to proteins secreted (Marston, 1986). Besides, in most of studies, purification of GST-fusion protein was done through the extraction of the expressed cell pellets (Mercado-Pimentel *et al.*, 2002, Guan and Dixon, 1991). Therefore the GST-ST332 fusion protein in this study was extracted from cell pellets in the form of cell lysates. However, in here, the expression of the protein was not determined by SDS-PAGE since the extracted protein was in crude lysate. Due to constraints of time and unavailability of the purification system in the laboratory, the fusion protein was not purified but extracted together with the crude extract. Moreover, as this study aimed to determine the reactivity of the protein towards *S.* Typhi antisera, using the crude lysate would be sufficient to test with dot bot immunoassay. However, purification of the protein is then recommended in future works.

The reactivity of the fusion protein bearing the insert towards *S.* Typhi antisera was examined through dot blot immunoscreening, screened with typhoid patients' pooled sera. Crude sera were used because the specificity of protein in study was still unknown, which there is no specific antibody for its screening. Since typhoid patients crude sera have a broad range of antibodies reacting to *S.* Typhi proteins, specificity of ST332 could be determined if it reacts towards the sera. As seen in Figure 4.5, comparing the *E.coli* transformant crude lysates (lanes 1 and 2) to the positive control of *S.* Typhi proteins (P), the reactivity of the former was inadequate compared to the positive control.

The non-reactivity of the protein towards the pooled *S.* Typhi antisera showed that the protein was not a common antigen that involved in penetrating host immune system. Another plausible explanation on the non-reactivity of the fusion protein was about the sequence of the hypothetical protein itself, ST332. As aforementioned, the amplification of ST332 yields a 332 bp DNA fragment. However, comparing the size of the amplified sequence to the size of STY4528 revealed that the length of the studied protein was inadequate since the ST332 produced 111 amino acids while the STY4528 produced 447 amino acids (Pickard *et al.,* 2003). Therefore, it could be assumed that the translated protein was only part of the whole protein which made it non-functional.

As seen in Figure 4.5a, the negative control, expressed recombinant *E. coli* BL21 bearing empty plasmid pGEX-4T-1 showed to have reactivity towards typhoid patients sera, but not as significant as the positive control, *S.* Typhi protein. As the case of using crude serum, it is known that the composition of the serum is not restricted to *S.* Typhi antibodies only. Therefore, as the serum would also comprise of antibodies which the patients' immune system has developed towards other infecting antigens, the crude serum presents as a reservoir for other antibodies as well (GST Gene Fusion System Handbook). In this case, it is suspected that the cross reaction of the sera to the *E. coli* protein lysate in the negative control was due to the non-Typhi sera antibodies, and possibly the antibodies reacting to *E. coli* proteins.

Further analyses are needed on the fusion protein to confirm its reactivity towards typhoid sera. In this study, much of the unspecific reaction with the protein seen on the dot blot was due to the use of crude protein lysate in the analysis. Therefore, background *E. coli* proteins were suspected to have cross reaction with the sera. Many optimizations done by pre-hybridization of the sera with *E. coli* protein before the actual

hybridization with the blot did not eliminate the cross-reaction. Therefore, it is necessary to purify the fusion protein and subject it to immunoscreening again, including western blotting. In addition, immunoscreening of the fusion protein should also be done with fresh typhoid sera to obtain better results.

5.2 Epitope mapping of *Salmonella* Typhi

In another approach of obtaining specific antigen of *S.* Typhi, a clone library of *E. coli* bearing short fragments of *S.* Typhi genomic DNA was constructed by using Novatope Cloning System. The system produced more than 700 clones of *E. coli* grown on selective LB agar as seen in Figure 4.7. However, as some of the clones were grown close to each other thus has high probability of cross-contamination, thus about 700 individual clones were selected for further screening.

Transformation of Novablue with pSCREEN-1b(+) T-vector bearing inserts did not allow blue-white screening because of the features requirements for T7 expression and gene *10* sequences (Novatope System Manual). Therefore, initial quick screening could only be done with colony PCR and continued with colony immunoscreening in getting the actual clones expressing gene of interest. Colony PCR screening showed that over 93% of the tested clones bore inserts with bands higher than 341 bp (Figure 4.8). This shows that a clone library of *E. coli* expressing short DNA fragments of *S.* Typhi was successfully produced.

A more thorough screening of the clones was done by colony blot immune assay. Colony immunoblots here were used as it allows the screening of large number

of colonies with sensitive immune assay (Szakál *et al.,* 2003). Thus it served as a preliminary technique to fish out potential clones with reactivity towards *S.* Typhi antisera. Results from the colony blot screening showed that several clones on the blot were more reactive towards typhoid pooled sera compared to the others (Figure 4.9). Thus, about 30 clones which produced higher signals on the membrane were selected for further screening.

Here, instead of colony blot, the clones were expressed and the protein was extracted for analysis with protein dot blot. As seen in Figure 4.10, some of the clones produced higher signals which showed higher reactivity towards the sera compared to the other clones. Particularly for strip 1, the clones showed to have darker color developed compared to the negative controls. However, as seen in previous blotting, unspecific reactions were also observed for the negative control, *E. coli* with empty plasmid proteins. Here, the purity of the antigen itself may lead to cross-reactions in the assay as the bind protein may contain host cell proteins that compete with antibody binding (Roberts and Howes, 1992). Besides, the use of the crude antisera which contain more than just antibodies to *S.* Typhi proteins which might have reacted with the *E. coli* proteins (GST Gene Fusion Handbook). Nevertheless, it could be seen that a higher sensitivity towards the *S.* Typhi protein was observed for the clones protein lysate at 20 µg, which outstands the negative control. On the blot, it was also seen that the signals on the blot were smeared. It was suspected that the proteins might have diffused out to its surrounding during transfer and drying. However, as it was still clear on the blot for which clones have stronger signals in hybridization, thus those clones were selected for a confirmation with ELISA immunoscreening.

From the selected clones in dot blot, ELISA tests showed that the clones were also reactive towards the *S.* Typhi antisera. As shown in Table 4.4, the proteins hybridization against typhoid patients pooled sera and *S.* Typhi negative sera showed a distinct difference in the intensity of reactions. Most of the clones showed at least double in intensity compared to the negative sera reactions which indicate that it was highly probable that the clones did react towards the *S.* Typhi antibodies. Clones with more than double reactivity in this ELISA were chosen for another testing with ELISA for confirmation. A second repeat of the ELISA showed that the clones also produced a significant difference in reactivity relative to the negative sera (Table 4.5), although the ratio was rather lower than what was produced in the first ELISA (Table 4.4). However, variations in titres with same ELISA are common (Sjaak *et al.,* 2007) and it was observed in the data that although the titre values were different, the fold increase was similar. However, as seen in previous section 5.1, high background in blotting and ELISA was also observed. As the same crude serum were used against the crude protein lysate, thus it is deduced that cross-reactions of the serum with *E. coli* proteins still persist in this observation. Thus, the optimization of the method and samples need to be done in getting better results such as purification of His-tagged fusion protein.

Chapter 6.0 Conclusions and Future Works

In this study, an expression vector for specific DNA marker of *S.* Typhi (ST332) was produced by using pGEX-4T-1 plasmid vector. The expression vector bearing ST332 as insert was successfully produced by cloning at multiple cloning site of the vector through *Not*I and *Eco*RI. Expression of the fusion protein was screened against typhoid fever pooled sera in dot blot immunoassay in studying for its reactivity towards the sera. The immunoassay showed that although the DNA fragment was specific for PCR-based detection, the recombinant protein did not show strong affinity towards typhoid patients' pooled sera. Therefore, at this stage, it is yet to confirm the specific activity of the protein against typhoid sera. Future studies should include the purification of the GST-insert fusion protein and studying the hybridization using a fresh serum for typhoid fever. Moreover, a full length study of the protein could also be done in determining its function by doing protein modeling and x-ray crystallography.

In another approach of finding specific antigen towards *S.* Typhi, a NovaBlue (*E. coli* DE3) clones library was constructed producing about 700 clones bearing inserts. Screening of the clones for reactivity towards typhoid sera was carried out by colony immunoblot and followed by dot blot immunoassay and ELISA. Several clones especially D1, G35 and I3 have shown stronger reactivity towards the typhoid patients pooled. However, more studies should be done in order to reaffirm the reactivity of the potential clones expressing *S.* Typhi proteins toward typhoid sera and identify the DNA fragment of the clones by sequencing. Furthermore, another issue of eliminating cross-reacting should be tackled by studying different titres of antibodies to be used in the immunoassay.

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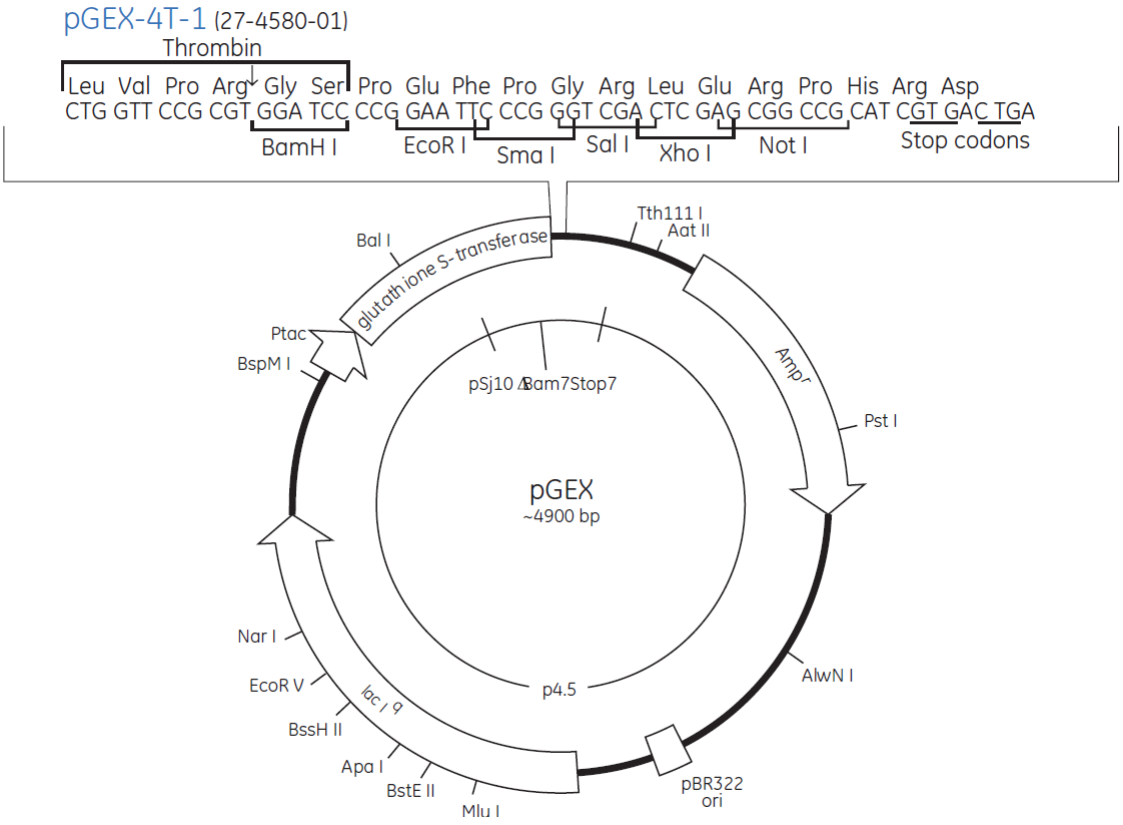
APPENDICES

Appendix A pGEX plamid map showing multiple cloning sites (GE Healthcare)



Appendix B pGEX plasmid vector sequences

ACGTTATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCC
ATCGGAAGCTGTGGTATGGCTGTGCAGGTCGTAAATCACTGCATAATTCGTG
TCGCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTTGCGCCGACATCATA
ACGGTTCTGGCAAATATTCTGAAATGAGCTGTTGACAATTAATCATCGGCTC
GTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGTA
TTCATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCA
CTCGACTTCTTTTGGAATATCTTGAAGAAAAATATGAAGAGCATTTGTATGA
GCGCGATGAAGGTGATAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGA
GTTTCCCAATCTTCCTTATTATATTGATGGTGATGTTAAATTAACACAGTCTA
TGGCCATCATACGTTATATAGCTGACAAGCACAACATGTTGGGTGGTTGTCC
AAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAG
ATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTT
GATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATCGTTTAT
GTCATAAAACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTT
GTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCG
TTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTG
ATAAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGC
AAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAATCGGATCTGGTTCCGCG
TGGATCCCCG**GAATTC**CCGGGTCGACTCG**AGCGGCCGC**ATCGTGACTGAC
TGACGATCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACAT
GCAGCTCCCGG

TTGACA = -35 tac promoter (183-188)

TATAATG = -10 tac promoter (205-211)

AATTGTGAGCGGATAACAATT = lac operator

ATG = Start codon GST protein

GGGCTGGCAAGCCACGTTTGGTG = 5’ primer (869-891)

GGATCCCCG**GAATTC**CCGGGTCGACTCG**AGCGGCCGC** = Multipe cloning site

CCTCTGACACATGCAGCTCCCGG = 3’ primer (1019-1041)

Appendix C Culture media, buffers and stock solutions

Culture media

Luria Bertani Broth

Tryptone 10.0 g

Yeast extract 5.0 g

Sodium chloride 5.0 g

10.0 g tryptone, 5.0 g yeast extract and 5.0 g sodium chloride were mixed in a schott bottle and dissolved in 1L distilled water. The broth was heat-sterilized by autoclaving at 121 °C.

Luria Bertani Agar

1.5 g bacterial agar was dissolved in 100 ml of LB broth and heat-sterilized by autoclaving.

S.O.C broth

Tryptone: 20 g

Yeast extract: 5 g

1M NaCl: 2ml

1M KCl : 2.5 ml

1M $MgCl_2$: 10 ml

1M $MgSO_4$: 10 ml

1M Glucose : 20 ml

Tryptone and yeast extract were dissolved in 500 ml distilled water and later added with the rest of the components except glucose. Then the volume was adjusted to 980 ml with distilled water and sterilized by autoclaving. When the solution has cooled to room temperature, 20 ml of sterilized 1M glucose was added to the solution and mixed. The solution was stored at room temperature.

Tryptic soy broth

30 g of tryptic soy broth powder was dissolved in 1L distilled water and heat-sterilized by autoclaving at 121 °C.

Tryptic soy agar

1.5 g of bacterial agar was dissolved in 100 ml of tryptic soy broth and heat sterilized by autoclaving at 121 °C.

Veal infusion broth

25 g of veal infusion broth powder was dissolved in 1L distilled water and heat-sterilized by autoclaving at 121 °C.

Antibiotics

Tetracycline

5mg/ml of tetracyline stock was prepared by dissolving 0.025g of tetracycline (Sigma-Aldrich) in 70% ethanol. The stock solution was stored in -20 °C.

Carbanecillin

5mg/ml of carbanecillin (Sigma-Aldrich) stock solution was prepared by dissolving 0.025g of carbanecillin in deionized water. The solution was filter-sterilized and stored in -20 °C.

Buffers

10x TBS

200 mM Tris

1500 mM NaCl

12.1 g Tris and43.8 g NaCl were dissolved in 400 ml of deionized water and adjusted to pH 7.6 with 1M HCl. The volume was adjusted to 500 ml with deionized water and then the solution was heat-sterilized by autoclaving at 121 °C.

1x TBST (0.05 % Tween 20)

0.5 ml of Tween 20 was mixed with sterile 1L of 1x TBS. The solution was stored at room temperature.

10x PBS

1.37 M NaCl

27 mM KCl

100 mM Na_2HPO_4

17.6 mM KH_2PO_4

80g of NaCl, 2g of KCl, 14.4g of Na_2HPO_4, and 2.4g of KH_2PO_4 were dissolved in 800 ml deionized water. The pH was adjusted to 7.4 with HCl and the volume was adjusted to 1L with deionized water. The solution was autoclaved and stored at room temperature.

1x PBST

0.5 ml of Tween 20 was mixed with sterile 1L of 1x PBS. The solution was stored at room temperature.

10x TBE buffer

0.89mM Tris base

0.89mM Boric acid

25 mM EDTA

108 g Tris base, 55 g Boric acid and 9.25 g EDTA were dissolved in 1L deionized water and sterilized by autoclaving at 121 °C.

NP-40 buffer

100 mM NaCl

0.5% NP50

50mM Tris

5mM EDTA

5mM EGTA

0.584g NaCl, 0.605 g Tris,0.146 g EDTA and 0.19 g EGTA was dissolved in 80 ml water. Then 50 µl of NP-40 was added to the solution and the pH was adjusted to 7.6 with HCl. The volume was adjusted to 100 ml with deionized water and then the solution was autoclaved and stored at 4 °C.

Stock Solutions

IPTG solution was prepared at 200 mM/ml by dissolving 0.025 g of IPTG powder (Fermentas) in 5 ml of distilled water. The solution was filtered through 0.2 µm membrane (Millipore) to sterilize.