CHAPTER 1.0:

INTRODUCTION

CHAPTER 1.0: INTRODUCTION

1.1 Background of Study

Salmonella enterica serovar Typhi (*S.* Typhi), the etiological agent of typhoid fever, is known as exclusively adapted to infection of the human host. It is a major public health problem especially in developing countries with an estimated annual global incidence of 21.4 million cases and more than 200,000 related deaths (Crump *et al*., 2004). Infections due to *S.* Typhi result in bacteremia characterized by remittent fevers, headache, malaise, abdominal discomfort, constipation or diarrhea, and, in some cases, a characteristic "rose spot" rash (Parry *et al*., 2002). Approximately 3 to 5% of patients with acute typhoid fevers may become asymptomatic chronic carriers with persistent excretion of the organism. The majority of infections result from consuming foods or water contaminated by feces of patients or carriers (WHO, 2004).

Typhoid fever is known to be endemic in Asia, Africa, and South America where problems of hygiene and sanitation remain unresolved. The morbidity of typhoid fever is highest in Asia with 93% of the global episodes occurring in this region. Southeast Asia has an incidence of 110 cases per 100,000 populations, which is the third highest incidence rate for any region (Crump *et al*., 2004). In Malaysia, the disease is endemic, and according to the Ministry of Health Malaysia (2008), the incidence for the last ten years (1996 to 2006) is in a range of 0.71 to 4.50 cases per 100,000 populations. Hence, public health concerns and the potential for transmission have made this pathogen the subject of numerous international, national, and local surveillance programs to determine the source of infection and control the dissemination of the disease.

Several antimicrobial agents have been used for treating typhoid fever and asymptomatic carriers. However, the emergence of multidrug-resistant strains of *S.* Typhi (Gulati *et al*., 1992) and the increased incidence of typhoid fever in human immunodeficiency virus type 1-infected individuals are further causes for concern. Accordingly, controlling the presence and dissemination of *S.* Typhi strains require an effective epidemiological surveillance system based on the ability to discriminate individual isolates. A number of strain typing methods have been developed for *S.* Typhi. The classical phenotypic methods include phage tying, serotyping have their limitations with regard to practicability and discriminating capacity and therefore have been increasingly complemented by the more sensitive molecular methods such as ribotyping (Ling *et al.,* 2000; Thong *et al.,* 2000), plasmid profiling (Hampton *et al.,* 1998; Wain *et al.,* 1999), pulsed-field gel electrophoresis (PFGE) (Kubota *et al*., 2005; Thong *et al*., 1994; Thong *et al*., 1995; Thong *et al*., 1996b) and PCR-based methods including IS*200* fingerprinting (Navarro *et al.,* 1996), random amplified polymorphic DNA (RAPD) (Quintaes *et al.,* 2004), amplified fragment length polymorphism (AFLP) (Nair *et al.,* 2000; Thong *et al.,* 2003), and variable-number tandem repeat (VNTR) (Liu *et al.,* 2003). Despite the availability of these methods, there is still a lack of methods with the right combination of rapidity, ease of use, typeability, reproducibility, and discriminatory power for typing *S.* Typhi strains.

In this study, *S.* Typhi strains collected from outbreak and sporadic cases of typhoid fever during the years 1998 to 2007 in Malaysia were examined. This study has utilized a combination of three PCR-based molecular methods; restricted arbitrarily primed PCR (resAP-PCR), repetitive element PCR (rep-PCR), and variable-number tandem repeat analysis (VNTR) to explore and compare as genotypic approaches for

rapid subtyping of *S.* Typhi strains. To our knowledge, this is the first report describing the use of multiplex-PCR-based VNTR profiling for the subtyping of *S.* Typhi strains in Malaysia. The present study provides more information on molecular types that may form the bases of an epidemiological analysis of typhoid disease and contribute greatly to public health benefits in Malaysia.

1.2 Objectives of Study

The overall aim of this study was to explore and evaluate VNTR and resAP-PCR as newer molecular methods for rapid subtyping of *S.* Typhi strains and to compare the data generated with the commonly used method of rep-PCR. Specifically, the objectives are:

1. To subtype the *S.* Typhi strains by using PCR-based methods.
2. To evaluate the usefulness of VNTR for molecular subtyping of *Salmonella enterica* serovar Typhi strains in Malaysia.
3. To compare resAP-PCR, rep-PCR and VNTR on a random set of *S.* Typhi regarding their discriminatory ability, reproducibility, and typeability.

CHAPTER 2.0:

LITERATURE REVIEW

CHAPTER 2.0: LITERATURE REVIEW

2.1 The Genus *Salmonella*: Nomenclature and Classification

The genus *Salmonella* was first suggested by Lignieres in 1900 in recognition of the work carried out by the American bacteriologist, Daniel E. Salmon, who, with Theobald Smith in 1886, described the "hog cholera group of bacteria" causing "swine plaque" that they named *Bacterium suipestifer* (Dolman and Wolfe, 2003). There are many different references on the taxonomy of *Salmonella*. However, uniformity is necessary for communication between scientists, health officials, and the public (Brenner *et al*., 2000). According to the Centres for Disease Control and Prevention (CDC) system, the genus *Salmonella* contains two species, each of which contains multiple serotypes. The two species are *S. enterica*, the type species, and *S. bongori*, which was formerly subspecies V (Popoff and Le Minor, 1997; Reeves *et al*., 1989). *S. enterica* is divided into six subspecies (Brenner and McWhorter-Murlin, 1998; Popoff and Le Minor, 1997) which are differentiated biochemically and by genomic relatedness (I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *Indica*). Serotype names are further designated by antigenic formulae that are listed in a document called the Kauffmann-White scheme. Classification by this scheme is on the basis of the serologic identification of O (somatic), H (flagellar) and Vi (capsular) antigens (Popoff *et al*., 2003). The majority of the *Salmonella* serotypes belong to *S. enterica* subsp. *enterica*, in which the most common serogroups are A, B, C1, C2, D and E. These serogroups cause approximately 99% of *Salmonella* infections in humans and warm-blooded animals (Popoff, 2001) while serotypes of *S. enterica*

subspecies II, IIIa, IIIb, IV, VI, and *S. bongori* are isolated from cold-blooded animals and the environment but rarely from humans (Farmer *et al*., 1984).

2.2 Overview of *Salmonella enterica* serovar Typhi

2.2.1 Historical Overview

Typhoid fever, a systemic prolonged febrile infection caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), is an ancient disease that has been well described throughout written history (Cunha, 2004). The term "typhoid fever" was first described by Pierre Louis in 1829 as distinct clinical entity, apart from typhus and other sustained fevers. However, confusion between the diseases typhus and typhoid existed until William Jenner in 1885 clarified the differences between these two diseases based on symptomology and epidemiology. In 1873, William Budd described the contiguous nature of the disease and incriminated fecally contaminated water sources in transmission. Karl Eberth observed in 1880 rod-shaped bacterium in the spleens and mesenteric nodes of typhoid patients and is credited with discovering the serovar Typhi organism. In 1884, Georg Gaffky first successfully cultured *S.* Typhi from the spleens of infected patients. Almroth Edward Wright and, independently, Richard F.J. Pfeiffer and Wilhelm Kolle used heat-killed organisms to prepare the first vaccine against typhoid in 1896; essentially the same vaccine is still in use today. At the same time, Georges Widal reported the clumping of heat-killed serovar Typhi cells by convalescent serum. Thus was born the term "agglutinins" and the classic serological test for diagnosis of infection by *S.* Typhi (Ivanoff *et al*., 1994; Ellermeier and Slauch, 2006).

2.2.2 Morphology and Characteristics

S. Typhi is known as a member of the genus *Salmonella* in the family *Enterobacteriaceae*. It is a gram-negative, non-spore forming, facultative anaerobic, rod-shaped bacterium with a length of 2-3 µm and diameter of 0.4-0.6 µm (Le Monor, 1981). It is motile, with peritrichous flagella (H-d antigen), which is also encountered in 80 other bioserotypes of *Salmonella* (Ivanoff *et al.,* 1994). The bacterium readily grows on common laboratory media at 15-41°C, with optimal growth occurring at 37°C. It ferments glucose by the mixed acid fermentation, utilises citrate as its sole carbon source and produces hydrogen sulphite (H_2S) (except some strains). Most strains also ferment a variety of carbohydrates and decarboxylates argentine, lysine and/or ornithine (Holt, 1994).

S. Typhi possesses three antigenic structures (Levine, 1999): somatic or O antigens (O_9 and O_{12}), corresponding to bacterial endotoxin, are involved in the production of fever; H-d as a protein flagellar antigen; and Vi which is a polysaccharide capsular antigen on the exterior of the cell wall associated with virulence in human host. Flagellar antigens (H-d) are not species-specific to *S.* Typhi and d-antigens are present in many *Salmonella* species other than *S.* Typhi (Levine, 1998). The Vi capsular antigen is largely restricted to serovar Typhi, although it is shared with similar antigenic specificity in some strains of serotypes *S.* Paratyphi C, *S.* Dublin, and *Citrobacter freundii.* Unique flagella types, Hj and H_{266}, are present in some *S.* Typhi isolates from Indonesia (Parry, 2006).

2.2.3 Genomic Characteristics

The complete genome sequence of a multidrug-resistant *S.* Typhi strain (CT18), that was originally isolated from a case of typhoid in Vietnam was determined by Parkhill *et al*. (2001). The CT18 genome harbours 4,809,037 base pairs with an estimated 4,599 coding sequences revealing the presence of hundreds of insertions and deletions ranging in size from single genes to large islands (Parkhill *et al*., 2001). The genomes of *S.* Typhi CT18, *S.* Typhimurium LT2, and *Escherichia coli* are essentially collinear as there are large regions of DNA that show a high degree of conservation between species. Significantly, the genes in this core region are not only similar between the enteric bacterial species but are also in a conserved order on the chromosome (70-80% of the chromosome). This shared core region could represent a conserved gene repertoire associated with basic household functions in the *Enterobacteriaceae*, such as survival in the intestine (colonisation), survival in the environment, and transmission, and most likely originated from a successful common ancestor for the enteric bacteria. In contrast, there are regions of unique sequences that recognized to be specific to *S.* Typhi DNA. Such unique regions can be single genes or groups of genes clustered together. These unique regions are the genes that provide the distinct phenotypes of the *S. enterica* serovars such as *S.* Typhi (Wain *et al*., 2002).

A striking feature of the *S.* Typhi genome is the presence of chromosomal cluster of gene insertions, known as Salmonella Pathogenicity Islands (SPIs) that are important for invasion and survival inside host cells (including two type III secretion systems and an array of effector proteins and metal-ion transporters). SPIs are believed to be of fairly recent horizontal acquisitions and may be self-mobile. They have often been inserted adjacent to a stable RNA gene, and may carry a gene encoding an integrase or

transposase-like protein. In addition to five previously described Salmonella pathogenicity islands (SPIs 1-5), five more islands have been characterized by Parkhill *et al*. (2001). SPI-1 (40 kp) encode a type III secretion system important to invade non-phagocytic intestinal epithelial cells (Collazo and Galán, 1997) and is required for *Salmonella* induced macrophage apoptosis (Chen *et al*., 1996). SPI-2 (40 kb) encodes a second type III secretion system required for survival/replication in phagocytic cells and systemic infection (Waterman and Holden, 2003). SPI-3 (17 kb) encodes *mtgC* and *mtgB* genes, which are essential for the survival of the pathogen in macrophages (Blanc-Potard and Groisman, 1997). SPI-4 contains only 8 coding sequences (CDS) (previously predicted to encode 18 genes), three of which are predicted to encode a type I secretion system. SPI-5 (7.6 kb) encodes effectors proteins for both of the type III secretion systems encoded by SPI-1 and SPI-2. SPI-6 (59 kb) encodes the *safA-D* and *tcsA-R* chaperone-usher fimbrial operons (Townsend *et al*., 2001). SPI-7 (134 kb) encodes the Vi biosynthetic genes (Hashimoto *et al*., 1993), the SopE prophage (Mirold *et al*., 1999) and a type IVB pilus operon (Zhang *et al*., 2000). SPI-8 (6.8 kb) encodes two bacteriocin pseudogenes (STY3280 and STY3282) and a degenerate integrase; notably, genes conferring immunity to the bacteriocins remain intact. SPI-9 (16 kb), like SPI-4, encodes a type I secretory apparatus and a single, large RTX-like protein (STY2875). SPI-10 (33 kb) carries phage 46 and the *sefA-R* chaperone-usher fimbrial operon (Parkhil *et al*., 2001).

Another feature of *S.* Typhi genome is the presence of more than 200 stretches of DNA, termed as pseudogenes. These regions encode gene-like sequences but have clearly been inactivated by some sort of change, often the mutation of a single base. In *S.* Typhi many of the pseudogenes have been inactivated by the introduction of a single

frameshift or stop codon, which suggests that they are of recent origin. A substantial number are predicted to be involved in housekeeping functions or in virulence or host interactions. This apparent inactivation of genes responsible for host interactions may explain host range of *S.* Typhi, unlike other *salmonella* serotypes, is restricted to one host (i.e., humans) and suggests that *S.* Typhi may have passed through a recent evolutionary bottleneck (Parkhill *et al*., 2001).

S. Typhi CT18 harbours two plasmids. The larger conjugative plasmid, pHCM1, is 218 kb in length and shares approximately 168 kb of DNA with the plasmid R27, with more than 99 percent sequence identity (Sherburne *et al*., 2000). The plasmid R27 is an IncH1 plasmid, first isolated in the 1960s from *S. enterica* that is closely related to the chloramphenicol-resistance plasmids detected in *S.* Typhi (Taylor *et al*., 1985). The pHCM1 plasmid encodes resistance to chloramphenicol (*Cat*I), ampicillin (TEM-1, *bla)*, trimethoprim (*Dhfr*1b), sulfonamides (*Sul*II), and streptomycin (*StrAB*). The smaller plasmid, pHCM2, is 106.5 kb in length and is phenotypically cryptic, but it has striking homology with the pMT1 virulence-associated plasmid of *Yersinia pestis* (Parkhill *et al*. 2001).

2.2.4 Epidemiology of Typhoid Fever

Typhoid fever is an important cause of morbidity and mortality in many regions of the world, with an estimated of 21.4 million cases and more than 200,000 related deaths occurring annually (Crump *et al*., 2004). The vast majority of cases occur in Southeast Asia, Africa, and South America, although both the case fatality rate and the spectrum of clinical complications of typhoid fever vary considerably between different areas where the disease is endemic. In South America and parts of Southeast Asia (e.g.,

Malaysia and Thailand), typhoid fever manifests as a relatively mild illness with low fatality rates and minimal neurologic complications. In contrast, in sub-Saharan Africa and Indonesia, severe, and often, fatal disease is frequently seen with higher mortality and is often accompanied by neurologic involvement such as delirium and coma. The reasons for these differences in disease severity are not known but may be related to differences in health care facilities, host immune responses, genetic factors, and also perhaps to differences in strains of *S.* Typhi circulating in areas of endemicity (Zhu *et al.,* 1996; Everest *et al.,* 2001).

Typhoid fever is especially pronounced in developing countries due to poor sanitation, poor standards of personal hygiene, and contaminated food. However, the number of sporadic cases has remained relatively constant in the industrialized world, and with the availability of proper sanitary facilities and clean water supplies, it has been virtually eliminated in many areas (Pang *et al*., 1998). The true incidence is unknown since many hospitals lack facilities for blood culture, the clinical features is confused with many other febrile illnesses and up to 90 percent of patients with typhoid are treated as outpatients. Consequently, community based studies have consistently shown higher levels of typhoid than public health figures suggest (Parry *et al*., 2002).

Typhoid infection is transmitted through the fecal-oral route by the consumption of contaminated water and food. However, the presence of a convalescent patient or a carrier actively shedding the pathogen poses an increased risk for infection. In non-endemic areas, disease outbreaks may occur from a unique source of food or carrier (Gruner *et al*., 1997). In disease-endemic areas a recent contact with a patient or carrier has been identified as a major risk factor but other risk factors include poverty, low

education level, poor housing with inadequate food and personal hygiene and recent consumption of antimicrobials are further risk factors (Gasem *et al.*, 2001; Luxemburger *et al.*, 2001). The endemicity of typhoid fever in the developing countries of the Third World has been exacerbated by antibiotic-resistance, increased incidence among HIV-infected individuals, and the large scale movement of migrant workers from high incidence region (Thong *et al*., 2000).

Typhoid fever has also been a major health problem among all age groups with a higher incidence and more variable clinical presentation in children. In endemic regions the disease is most common in the 3-19 years age group but the peak age incidence varies regionally. Recent population based studies from South Asia suggest that the incidence is highest in children aged less than 5 years, with higher rates of complications and hospitalisation, and may indicate risk of early exposure to relatively large infecting doses of the organisms in these populations (Bhutta, 2006). In the Mekong Delta region of Vietnam, the attack rate was shown to peak among 5-9 years old, with an attack rate of 531 cases per 100,000 annually (Lin *et al*., 2000). In India typhoid fever is common among younger 1-5 years children (Sinha *et al*., 1999). High infection rates in children aged from 1-5 years have also been reported in Bangladesh, Jordan and Iran, reflecting their lack of host immunity and maternal antibodies (Ivanoff, 1998).

2.2.5 Pathogenesis and Clinical Features

Humans are the only natural host and reservoir of infection for serovar Typhi. The infectious dose in volunteers varies between 10^3-10^9 organisms (Hornick *et al.*, 1970). After ingestion of contaminated food and water, the typhoid organisms pass

through the gastric acid barrier en route to the small intestine, where they rapidly penetrate the mucosal epithelium to reach the lamina propria. After penetration, some bacilli remain within the macrophages of the small intestine lymphoid tissue while others translocate to draining mesenteric lymph nodes where there is further multiplication and ingestion by macrophages (House *et al*., 2001). Bacteria release into the general circulation via the thoracic duct disseminates (Everest *et al*., 2001). As a result of this silent primary bacteraemia, the organisms reach an intracellular haven, being taken up by macrophages lining the sinusoidal walls liver, spleen, bone marrow and replicate at these locations. After 7 to 14 day incubation period, the re-entry of bacteria into the blood stream marks the onset of a sustained secondary bacteraemia that appears in clinical disease. After a relatively sustained bacteremia (Ivanoff *et al.,* 1994), typhoid organisms are removed from blood by the liver and excreted via biliary passage to lead to re-infection of the intestinal tract.

Typhoid fever has an incubation period of about 1-2 weeks, but it may be as short as 3 days or longer than 60 days depending on the size of the infecting inoculums. The clinical presentation of typhoid fever varies from a mild illness with remittent fevers, headache, malaise and slight dry cough to a severe clinical picture with abdominal discomfort, constipation or diarrhea, and, in some cases, a characteristic “rose spot” rash. Many factors influence the severity and overall clinical outcome of the infection. They include the duration of illness before the initiation of appropriate therapy, the choice of antimicrobial treatment, age, the previous exposure or vaccination history, the virulence of the bacterial strain, the quantity of inoculums ingested, host factors (e.g. Human Leukocyte Antigens (HLA) type, Acquired Immune Deficiency Syndrome

(AIDS) or other immunosuppression) and whether the individual was taking other medications such as H_2 blockers or antacids to diminish gastric acid (WHO, 2003).

The bacteraemia of typhoid fever persists for several weeks if antibiotic therapy is not given. In this phase, the organism disseminates widely to the liver, spleen, bone marrow, gall bladder and the Peyer's patches of the terminal ileum. At these sites, the endotoxin of *S.* Typhi induces macrophages to produce an array of cytokines, including tumor necrosis factor (TNF) and interferon, and various arachidonic acid metabolites (Hoffman, 1991). Cytokines alone, when acting locally at the sites of their production or when disseminated via the blood stream, can mediate the development of fever (Newton and Krishna, 1998), intestinal necrosis (Everest *et al*., 2001), hepatic dysfunction (Tiegs, 1997), pneumonitis (Tracey *et al*., 1990), thrombosis (Naworth and Stem, 1987), vascular instability leading to shock (Tracey and Lowry, 1990), bone marrow depression (Hoffman, 1991), and altered consciousness (Newton and Krishna, 1998).

2.2.6 Diagnosis and Detection of Typhoid Fever

The diagnosis of typhoid fever on clinical grounds is difficult, as the presenting symptoms are diverse and similar to those observed with other common febrile illnesses. The definitive diagnosis of typhoid fever currently depends on the demonstration of *S.* Typhi in the blood, bone marrow, stool, or specific anatomical lesion (Punjabi *et al*., 2000). The isolation of serovar Typhi from blood remains the method of choice for the laboratory diagnosis although the limiting factor for the sensitivity of microbiological culture as a diagnostic method is the low number of bacteria in the blood (Wain *et al*., 1998). The sensitivity of blood, stool, and bone marrow aspirates cultures is 55-75%, 40-55%, and 85-95% respectively. Although

culture is currently the gold standard for diagnostics, it is difficult to see how this approach can be greatly improved (Cho and Kim, 1999).

Various biochemical and serological tests have been developed for diagnosis of *S.* Typhi. The most commonly used serological test is the Widal test, which detects agglutinating antibodies against the O (lipopolysaccharide) and H (flagella) antigens of *S.* Typhi (Wain *et al*., 2002). Although this test is widely used, it lacks sensitivity and/or specificity in typhoid-endemic regions when used with a single serum sample (House *et al*., 2001). Several alternatives to the Widal for antibody detection have been developed but none have become widely used despite the availability of commercial kits (Wain *et al*., 2002). The IDL Tubex test, developed in Sweden, detects IgM and IgG antibodies in patients by inhibiting the binding between an anti-O9 IgM monoclonal antibody conjugated to colored latex particles and *S.* Typhi lipopolysaccharide (LPS) conjugated to magnetic latex particles (Lim *et al*., 1998). Another test, Typhidot was developed in Malaysia for the detection of specific IgM and IgG antibodies against a 50 kD antigen of *S*. Typhi (Gopalakrishnan *et al*., 2002; Jesudason *et al*., 2002). A newer version of the test, Typhidot-M, was also developed to detect specific IgM antibodies only. The dipstick test, developed in the Netherlands, is based on the binding of *S.* Typhi specific IgM antibodies in samples to *S.* Typhi lipopolysaccharide (LPS) antigen and the staining of bound antibodies, by an anti-human IgM antibody, conjugated to colloidal dye particles (Ismail, 2006). Other serological tests include indirect haemagglutination, counter-immuno-electrophoresis, solid phase radioimmunoassay, fluorescent antibodies and enzyme-linked immunosorbent assay.

2.2.7 Treatment and Prevention of Typhoid Fever

In the majority of cases, infection with *S.* Typhi is not lethal if effective antimicrobial therapy is administered. In 1948, chloramphenicol was introduced and the severe, debilitating, fatal typhoid fever was transformed to a readily treatable disease. However, resistance started to develop within two years of the drug introduction and until 1972 chloramphenicol resistant *S.* Typhi became a major problem in many different countries all over the world (Mirza *et al*., 1996). Chloramphenicol resistance was associated with high-molecular-weight, self-transferable, IncHI plasmids. These strains were also found to be resistant to sulfonamide, tetracycline and streptomycin. Amoxicillin and trimethoprim-sulphamethoxazole were effective alternatives (White, 1999) till 1980s when the strains of *S.* Typhi resistant to all three of these first-line drugs were first reported. Outbreaks of infection with multidrug-resistant (MDR) *S.* Typhi strains occurred in Pakistan (Shanahan *et al*., 2000), Vietnam (Connerton *et al*., 2000; Wain *et al*., 1999), India (Shanahan *et al*., 1998; Saha *et al*., 2003), Bangladesh (Hermans *et al*., 1996), Hong Kong (Ling *et al*., 2000), Africa (Kariuki *et al*., 2000), and Japan (Hirose *et al*., 2001). The third-generation cephalosporins and the fluoroquinolones have become the drugs of choice today (Rowe *et al*., 1997). Resistant to quinolones, which has been shown to affect the outcome of treatment with fluoroquinolones is now widely reported from outbreaks (Mermin *et al*., 1999) and from endemic cases in south Asia (Threlfall and Ward, 2001). Furthermore, transferable resistance to a third generation cephalosporin, ceftriaxone, could be emerging (Saha *et al*., 1999). Azithromycin, a new macrolide antibiotic has been reported as an alternative to the fluoroquinolones for treatment of typhoid fever (Chinh *et al*., 2000).

During the recent years typhoid fever has largely disappeared from industrialized countries, although it continues to pose an important public health problem in the developing countries. Public health prevention measures include purification of water supplies, sewage control, treatment of chronic carriers, and sanitary and hygiene education especially among food handlers. However, vaccination of high-risk population is considered the most promising strategy for the control of typhoid fever. Three types of typhoid vaccines are available: a parental heat-killed whole organism vaccine, Ty21a live oral vaccine, and Vi capsular polysaccharide (ViCPS) parental vaccine. Each of these vaccines offers 55% to 85% protection for 3 to 5 years. The main differences relate to their side effects. The purified capsular Vi vaccine which is given in a single dose subcutaneous (s.c.) or intramuscularly (i.m.) has significantly fewer adverse effects than the killed whole cell parental vaccines and therefore is used as an alternative to the oral typhoid vaccine (Kalra *et al*., 2003). A novel Vi conjugate candidate vaccine bound to non-toxic recombinant *Pseudomonas aeruginosa* exotoxin A (rEPA) has enhanced immunogenicity in adults and in children aged 2 to 4 years (Kossaczka *et al*., 1999).

2.3 Methods of Bacterial Typing

Bacterial typing is a descriptive discipline based on the subdivision of bacterial species, with the aim to characterize pathogenic bacteria. A prerequisite for all existing typing schemes is the assumption that strains derived from one clone will share certain characteristics in contrast to strains derived from different clones (Busch and Nitschko, 1999). Subtyping is important epidemiologically for recognizing outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of the

infection, recognizing particularly virulent strains of organisms, and monitoring vaccination programs (Olive and Bean, 1999).

The process of subtyping has been accomplished by a number of different phenotypic and genotypic approaches. Phenotypic procedures take advantage of biochemical, physiological, and biological phenomena, whereas genetic procedures aim to detect polymorphisms at the level of nucleic acids**.** All of these methods must meet several criteria in order to be broadly useful: (a) high degree of typeability which refers to the ability of a technique to assign a definite type to each isolate; (b) discriminatory power refers to its ability to differentiate among epidemiologically unrelated strains to recognize a reasonable number of types; (c) reproducibility that yields the same results upon repeat testing of a bacterial strain over a long period of time and in different centres; (d) stability is the biological feature of clonally derived isolates to express constant markers over time and generations; (e) ease of performance reflects the cost of specialized reagents and equipments, the technical complexity of a method, and the effort required to learn and to implement the technique in the laboratory; (f) ease of interpretation refers to the effort and experience required to obtain useful, reliable typing information using a particular method (Olive and Bean, 1999; Busch and Nitschko, 1999; Tenover *et al*., 1997). Understanding the strengths and weaknesses of the chosen bacterial typing technique enhances interpretation and generalization of study results (Foxman *et al*., 2005).

In recent years, many phenotypic and genotypic methods have been utilized in the subtyping of *S.* Typhi. Epidemiological studies of this pathogen, supported by these different typing methods, are of great importance because they help determining the

source of infection to control the presence and dissemination of the causative agent. However, epidemiological investigations have been hampered by the absence of reliable and sufficiently discriminative methods of differentiating individual strains beyond the species level. Thus, there is a need for a molecular method that is cheap, discriminative, simple, and reproducible for the large-scale typing of *S.* Typhi isolates to facilitate effective surveillance and the development rational control strategies for this important human pathogen.

2.3.1 Phenotypic Methods

Conventional typing techniques based on phenotypic characteristics are those that characterize the products of gene expression in order to differentiate strains (Tenover *et al*., 1997). These methods include biotyping, serotyping, phagetyping, antibiotic susceptibility tests, polyacrylamide gel electrophoresis (PAGE), and multilocus enzyme electrophoresis (MLEE). Properties such as microbial morphology, biochemical profiles, bacteriophage types, antigens present on the cell's surface, and antimicrobial susceptibility profiles are example of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to vary, based on change in growth condition, growth phase, and spontaneous mutation (Tenover *et al*., 1997). Thus, traditional typing methods based on phenotypic characteristics are increasingly challenged by the use of genotypic DNA-based methods.

Phenotypic-based methods including antibiotic resistance patterns (Wain *et al*., 1998; Mirza *et al*., 2000), biotyping (Fica *et al*., 1996), phage typing (Hickman-Brenner *et al.* 1983; Xercavins *et al*., 1997), serotyping (Usera *et al*., 1995), lipopolysaccharide analysis (LPS) (Jimenez-Lucho and Foulds, 1990), protein profiling (Franco *et al*.,

1992), multilocus enzyme electrophoresis (MLEE) (Reeves *et al*., 1989; Selander *et al*., 1990) have been used to distinguishing different clinical *S.* Typhi isolates. However, these phenotypic methods have their limitations with regard to practicability and discriminating capacity and therefore have been complemented by the more sensitive and discriminative DNA-based, molecular techniques.

2.3.2 Genotypic Methods

Genotypic methods are those that are based on an analysis of the genetic structure of an organism. The currently available molecular methods can be classified according to their working principle in PCR-mediated typing techniques such as arbitrarily primed PCR (AP-PCR), and repetitive element PCR (rep-PCR), typing techniques combining PCR with restriction analysis, typing techniques based on chromosomal restriction fragment length polymorphisms such as ribotyping, and pulsed-field gel electrophoresis (PFGE), typing techniques combining restriction digestion with selective amplification such as amplified fragment length polymorphism (AFLP), and plasmid analysis (Heyndrickx *et al*., 2001). These molecular approaches have several advantages over conventional typing methods, including the independence of restricted numbers of organism characteristics (Busch and Nitschko, 1999), higher discriminatory power, broader application to a variety of bacterial species, and, at time, speed (Arbeit, 1995). Genotypic methods are less subject to natural variation, although they can be affected by insertions or deletions of DNA into the chromosome, the gain or loss of extrachromosomal DNA, or random mutation that may create or eliminate restriction endonuclease sites (Tenover *et al*., 1997).

In recent years, many DNA-based molecular methods have been utilized for subtyping of *S.* Typhi, including ribotyping (Ling *et al*., 2000; Thong *et al*., 2000), plasmid profiling (Hampton *et al*., 1998; Wain *et al*., 1999), pulsed-field gel electrophoresis (PFGE) (Kubota *et al*., 2005; Thong *et al*., 1994; Thong *et al*., 1995; Thong *et al*., 1996b), amplified fragment length polymorphism analysis (AFLP) (Nair *et al*., 2000; Thong *et al*., 2003), IS*200* typing (Navarro *et al*., 1996), random amplification of polymorphic DNA analysis (RAPD) (Quintaes *et al*., 2004) and variable-number tandem repeat analysis (VNTR) (Liu *et al*., 2003). However, some of these techniques such as RAPD and IS*200* typing lacked sufficient reproducibility and discriminatory power while PFGE, ribotyping and AFLP are time consuming and technically demanding. These newer techniques have been most useful in delineating epidemiological relationships between various isolates of *S.* Typhi, including investigations of outbreaks (Thong *et al*., 1994; Gruner *et al*., 1997), geographical distribution of clones (Thong *et al*., 1995), antibiotic resistance (Hermans *et al*., 1996), environmental sources (Thong *et al*., 1996a) and associations with fatal illness (Thong *et al*., 1996b).

2.3.3 Polymerase Chain Reaction (PCR)

During the past decade, polymerase chain reaction (PCR)-based methods are used for bacterial genotype identification (Persing, 1993) and discrimination of bacterial strains (van Belkum, 1994) through the use of a wide variety of techniques and primer designs. The hallmark of PCR is the ability to produce literally millions of copies of a particular DNA segment with high fidelity within 3 to 4 hours time (Tenover *et al*., 1997). The PCR technique is based on repeated cycles of high temperature for denaturation of the DNA, oligonucleotide primer annealing and an extension step which

is mediated by a heat stable polymerase. In each cycle of the PCR the number of copies of the chosen sequence is doubled so that the amount of the target DNA is exponentially increasing. The resulting PCR products represent a variety of different-sized DNA fragments that are visualized by agarose gel electrophoresis. In comparison with other genotyping methods, PCR-based techniques are generally simpler, faster, more sensitive, and require much less DNA for analysis (Williams *et al*., 1999). However, standard guidelines for interpretation of PCR-fingerprints are not yet available (Tenover *et al*., 1997).

Amplification fingerprinting which has been used to subtype diverse bacterial species (van Belkum, 1994), has also been explored to a limited extent as a molecular typing method for *Salmonella*. Although some investigators have reported discrimination of *Salmonella* at the subserotype level (Beyer *et al*., 1998; Hilton *et al*., 1997; Ling *et al*., 1998), others have found resolution limited to the serotype level, with some serotypes not differentiated (Lopez-Molina *et al*., 1998; van Lith and Aarts, 1994), or have found no serotype specificity at all (Burr *et al*., 1998). Reproducibility, although not rigorously assessed, has been noted in several studies to be somewhat or highly problematical (Burr *et al*., 1998; van Lith and Aarts, 1994). In the present study, three PCR-based molecular methods of restricted arbitrarily primed PCR (res-AP-PCR), repetitive element PCR (rep-PCR), and variable-number tandem repeat (VNTR) analysis is utilized to explore and compare their usefulness in subtyping of clinical *S.* Typhi strains collected from sporadic and outbreak cases of typhoid fevers in Malaysia.

2.3.4 Restricted Arbitrarily Primed PCR (resAP-PCR)

Arbitrarily primed PCR (AP-PCR) assay, also referred to as random amplified polymorphic DNA (RAPD) analysis, was first described by Williams *et al*. (1990) and Welsh and McClelland (1990). AP-PCR assays are based on the use of a single short random sequence primer (typically 6-10 bp) that is not targeted to amplify any specific bacterial DNA sequence. The primer will hybridize with low stringency at multiple random chromosomal locations at low annealing temperatures such that they can be used to initiate amplification of regions of the bacterial genome. If one copy of the primer binds to one strand of DNA, and another copy of the primer binds on the opposite strand of DNA but in proximity of the first primer, a DNA fragment with a molecular length corresponding to the distance between the two primers results. Since the number and location of random primer sites vary for different strains of a bacterial species, separation of the amplification products by agarose gel electrophoresis results a pattern of bands which is characteristic of the particular bacterial strain (Williams *et al*., 1990; Welsh and McClelland, 1990).

AP-PCR has remarkable general applicability and has been applied to typing eukaryotic species, as well as many bacterial species (van Belkum, 1994). Although AP-PCR has been strongly criticized for lack of reproducibility and its sensitivity to reaction conditions, a number of studies have reported success in using RAPD assays to differentiate *Salmonella* serotypes or to distinguish strains within a single serotype. Such results were obtained with *S.* Typhimurium (Carraminana *et al*., 1997), *S.* Typhi (Shangkuan and Lin, 1998; Bianca *et al*., 2002; Quintaes *et al*., 2004), *S.* Enteritidis (Radu *et al*., 2000; Betancor *et al*., 2003), and *S.* Panama (Soto *et al*., 1999).

More recently, restricted AP-PCR (resAP-PCR) is described as a modification of the AP-PCR technique employing endonuclease restriction enzymes. To perform this technique, the genomic DNA of the selected microorganism has to be digested with *Hae*III or *Alu*I restriction enzymes prior to amplification with three 9 mer oligonucleotide primes. The primers have been selected based on genome sequence of each strain, and their usage to genotype member of the same species is likely to yield comparable bands patterns by agarose gels electrophoresis. This technique is only available for organisms with appropriate C+G content (around 50% or higher) at their genome. Few data have been published on the applicability of the resAP-PCR for the analysis of bacterial strains. Bikandi *et al*. (2004) applied this technique to differentiate 27 *Salmonella* isolates belonging to 13 serotypes of *S. enterica* where they have shown that the resAP-PCR technique is discriminatory and fingerprints of the test strains were highly reproducible. In the present study, ResAP-PCR is used for subtyping of clinical *S.* Typhi strains in Malaysia.

2.3.5 Repetitive Element PCR (rep-PCR)

Repetitive element PCR (rep-PCR) is an amplification method that uses primers complementary to the short repetitive sequence elements, dispersed throughout the bacterial genome, to generate DNA fingerprints that allow discrimination between strains. These noncoding sequences appear to be conserved among many members of the *Enterobacteriaceae* and other bacterial species (Versalovic *et al*., 1991). Two main sets of repetitive elements are generally used for typing purposes. The Repetitive Extragenic Palindromic (REP) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem (Stern *et al*., 1984). The enterobacterial repetitive intergenic

consensus (ERIC) elements are 126-bp sequences which contain a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome (Hulton *et al*., 1991; Sharples and Lloyd, 1990). REP sequences have been shown to exist throughout the eubacterial kingdom, although the consensus sequences may differ among different bacteria. The palindromic nature of the REP elements and their ability to form stem-loop structures have led to multiple proposed functions including roles in transcription termination, mRNA stability, and chromosomal domain organization in vivo (Gilson *et al*., 1990; Newbury *et al*., 1987; Yang and Ames, 1988).

Rep-PCR can be performed with DNA extracted from bacterial colonies or by a modified method using unprocessed whole cells (Woods *et al*., 1993). REP or ERIC amplification can be performed with a single primer, a single set of primers, or multiple sets of primers. These consensus primers corresponding to each end of a repeated sequence are oriented such that PCR amplification of DNA sequences proceeds between adjacent repeated elements. The resulting multiple amplification products have lengths that reflect distance polymorphisms between repeated elements contained within bacterial genomes. The number and sites of these repeated sequences are variable from strain to strain; therefore simple agarose gel electrophoresis of the amplification products provides unambiguous strain-specific DNA fingerprints of limited complexity (Busch and Nitschko, 1999).

Rep-PCR has already been used for the genomic fingerprinting of various bacteria, including *Salmonella*. Burr *et al*. (1998) tested 89 *Salmonella* isolates of 22 serotypes and found that rep-PCR was able to discriminate among *Salmonella* isolates sharing similar serotypes. Gruner *et al*. (1997) used a single ERIC primer to

differentiate among *S.* Typhi isolates and reported that rep-PCR was unable to discriminate among non-related isolates. Hermans *et al*. (1996) used rep-PCR fingerprinting using ERIC-PCR to characterize 78 *S.* Typhi isolates in Dhaka, Bangladesh although the genetic diversity of strains and colonality could be deduced for all strains investigated. Johnson and Clabots (2000) modified thermal cycling condition to improve the reproducibility and resolving power of rep-PCR fingerprinting in the assay using a set of strains representing 12 serovars of *S. enterica*. The results revealed that modified thermal cycling improves the performance o rep-PCR fingerprinting for bacterial typing. Rasschaert *et al*. (2005) investigated five different Rep-PCR primers to differentiate *Salmonella* isolates at the serogroup level and reported that ERIC primer set and the $(GTG)_5$ primer were able to generate fingerprints for *Salmonella* strains. Sahilah (2000) characterised the genomic DNA of *S.* Weltevreden and *S.* Chincol by ERIC-PCR and found that the ERIC-PCR with primers ERIC1R and ERIC2 was able to discriminate the strains. Beyer *et al*. (1998) found that rep-PCR fingerprinting offered an attractive choice as a primary method to differentiate the strains within salmonella serotype *Saintpaul* since epidemic strains were adequately discriminated from cases apparently not related to the epidemic.

2.3.6 Variable-Number Tandem Repeat (VNTR)

Variable-number tandem repeats (VNTR) have emerged as valuable markers for the molecular subtyping of bacterial species. VNTR, or short sequence repeats (SSR), consist of unique DNA elements that are repeated in tandem (van Belkum, 1999). The sequence element is often maintained within a bacterial species whereas the number of repeat units at the same VNTR locus varies between individual strains. Such variability observed in VNTRs is often caused by slipped-strand nucleotide mispairing (SSM) most

commonly active during replication (Bzymek and Lovett, 2001). Since sequence homology exists between strains in the flanking region of the VNTR locus, PCR amplification with flanking-sequence-specific primers can be used to determine the variations associated with the copy numbers of repeat units at each VNTR loci that reflect the intraspecies genetic diversity. This forms the basis for using VNTR for strain typing.

The availability of complete microbial genomic sequences has greatly facilitated the identification of VNTR for strain typing. Using software programs, the genome of the bacteria can be scanned, VNTRs can be quickly located, and primers for PCR analysis can made based on the flanking sequences. VNTR analysis has been used for the strain typing of a number of bacterial species including *Bacillus anthracis* (Le Fle`che *et al*., 2001), *Legionella pneumophila* (Pourcel *et al*., 2003), *Borrelia* species (Farlow *et al*., 2002), *Pseudomonas aeruginosa* (Oteniente *et al*., 2003), *Mycobacterium tuberculosis* (Le Fle`che *et al*., 2002), *Enterococcus faecium* (Top *et al*., 2004), *Escherichia coli* O157:H7 (Keys *et al*., 2005; Lindstedt *et al*., 2004), *Yersinia pestis* (Adair *et al*., 2000; Pourcel *et al*., 2004). For genus *Salmonella*, VNTR analysis has been used for subtyping of a few serovars of *Salmonella enterica* such as *enterica* subspecies (Ramisse *et al*., 2004), serotype Typhimurium (Lindstedt *et al*., 2003; Torpdahl *et al*., 2006), serotype Typhi (Liu *et al*., 2003) and serotype Enteritidis (Boxrud *et al*., 2007; Malorny *et al*., 2008). Few data have been published on the applicability of the VNTR for the analysis of *S.* Typhi strains. Liu *et al.* (2003) described a multiplex VNTR assay based on three different VNTR loci to differentiate 59 clinical *S.* Typhi isolates from several Asian countries resulted in identifying 49 distinct VNTR profiles. Moreover, a VNTR-based analysis based on 7 VNTR loci by

Ramisse *et al*. (2004) distinguished 25 subtypes within 27 *S.* Typhi isolates. In the present study, multiplex-PCR-based VNTR profiling, previously described by Liu *et al*. (2003) is examined in order to evaluate its usefulness in subtyping of clinical *S.* Typhi strains in Malaysia.

CHAPTER 3.0:

MATERIALS AND METHODS

CAPTER 3.0: MATERIALS AND METHODS

3.1 Preparation of Common Media, Buffers and Solutions

3.1.1 Luria-Bertani (LB) Agar

Tryptone	1.0 g
Yeast extracts	0.5 g
NaCl	0.5 g
Bacteriological agar	1.5 g
dH_2O	100 ml

All the ingredients were weighted and suspended in 100 ml of dH_2O. Next, the agar medium was sterilized by autoclaving. It was cooled to 50-55 °C and poured into sterile Petri dishes.

3.1.2 Luria-Bertani (LB) Broth

Tryptone	1.0 g
Yeast extracts	0.5 g
NaCl	0.5 g
dH_2O	100 ml

All the ingredients were weighted and suspended in 100 ml of dH_2O. Next, the broth medium was sterilized by autoclaving and stored in refrigerator.

3.1.3 Bismuth Sulphite Agar (BSA)

BSA base	4.0 g
dH_2O	100 ml

BSA base was weighted and suspended in 100 ml of dH_2O. Next, the agar medium was heated gently along with frequent agitation until it starts to boil and simmer for 30 seconds to dissolve the agar completely. It was cooled to 50-55 °C, mixed well and poured into sterile Petri dishes.

3.1.4 10X Tris-borate EDTA (TBE) buffer, pH 8.3

Tris base	121.2 g
Orthoboric acid	61.8 g
EDTA	0.745 g
ddH_2O	1000 ml

All the ingredients were weighted and suspended in 500 ml of ddH_2O and then dissolved by stirring with magnetic stirrer on the hot plate. The pH of the buffer was adjusted to 8.3 by adding NaOH or HCl. Next, ddH_2O was added to top it up to 1000 ml and sterilized by autoclaving.

3.1.5 0.5X Tris-borate EDTA (TBE) buffer

10X TBE buffer	50 ml
ddH_2O	950 ml

10X TBE buffer was mixed with 950 ml of ddH_2O. It was stored at room temperature.

3.1.6 1 M Tris, pH 8.0

Tris	36.342 g
ddH_2O	250 ml

Tris base was weighted and suspended in 250 ml of ddH_2O and then dissolved by stirring with magnetic stirrer on the hot plate. The pH of the buffer was adjusted to 8.0 by adding NaOH or HCl. Next, ddH_2O was added to top it up to 300 ml and sterilized by autoclaving.

3.1.7 0.5 M EDTA, pH 8.0

EDTA	55.83 g
ddH_2O	250 ml

EDTA base was weighted and suspended in 250 ml of ddH_2O and then dissolved by stirring with magnetic stirrer on the hot plate. The pH of the buffer was adjusted to 8.0 by adding NaOH or HCl. Next, ddH_2O was added to top it up to 300 ml and sterilized by autoclaving.

3.1.8 Tris-EDTA (TE) buffer, pH 8.0

1M Tris	10 ml
0.5M EDTA	2 ml
ddH_2O	988 ml

All the ingredients were measured and mixed together thoroughly. Next, ddH_2O was added to top it up to 1000 ml. It was sterilized by autoclaving and stored at room temperature.

3.1.9 2.5% Agarose Gel for PCR

PCR agarose base	4.5 g
0.5X TBE buffer	180 ml

PCR agarose base was weighted and suspended in 180 ml of 0.5X TBE buffer. Next, it was heated to dissolve the agar completely. It was cooled to 50-55°C and poured into gel casting tray.

3.1.10 Phosphate Buffered Saline (PBS) buffer, pH 7.3

Phosphate Buffer Saline base	1 tablet
dH_2O	100 ml

Phosphate Buffer Saline tablet was dissolved in 100 ml of dH_2O. Next, it was sterilized by autoclaving and stored at room temperature.

3.1.11 80% Ethanol

100% ethanol	80 ml
ddH_2O	20 ml

100% ethanol was mixed with 20 ml of ddH_2O. It was stored at room temperature.

3.1.12 50% Glycerol

Ultra pure glycerol	25 ml
ddH_2O	25 ml

Ultra pure glycerol was mixed with 25 ml of ddH_2O. Next, it was sterilized by autoclaving and stored at room temperature.

3.1.13 Ethidium Bromide (EtBr)

Ethidum bromide base	30 µl
dH_2O	300 ml

Ethidium bromide was mixed with 300 ml of dH_2O. The solution was stored in a dark bottle at room temperature, and diluted to 0.5 µg/ml before being used.

3.2 Bacterial Strains and Purity Check

The *S.* Typhi strains used in this study are listed in **Appendix 1**. All clinical *S.* Typhi strains previously collected from sporadic and outbreak cases of typhoid fevers during the years 1998 to 2007 were obtained from the culture collections at the Biomedical Science and Molecular Typing Laboratory, Institute of Postgraduate Studies, University of Malaya. The strains were retrieved from glycerol stocks and recultured on selected media to determine viability and purity. Except for stool strains TP5/00, TP168/00, ST13/06, ST314/07, ST334/07, ST014/07, and urine strain ST3/06, all the other strains were from blood cultures.

All strains were labeled accordingly and checked for purity before analysis. One loop of the strain was inoculated into 1 ml of LB broth and incubated for overnight at 37 °C in a shaker. Next, a loopful of the culture was streaked onto LB agar plate for purification checking. The pure cultures were streaked on new LB plates for further testing while the cultures which appeared as a mixture of *S.* Typhi with other organisms were streaked onto selective media (Bismuth Sulphite Agar) for purification. After 12-18 hours incubation at 37°C, the pure black *S.* Typhi colonies were streaked onto LB agar plates. The pure cultures were kept as stocks by preparing both nutrient agar slants at room temperature and 50% glycerol stocks at -20 °C. The flowchart of the work is as illustrated in Figure 3.1.

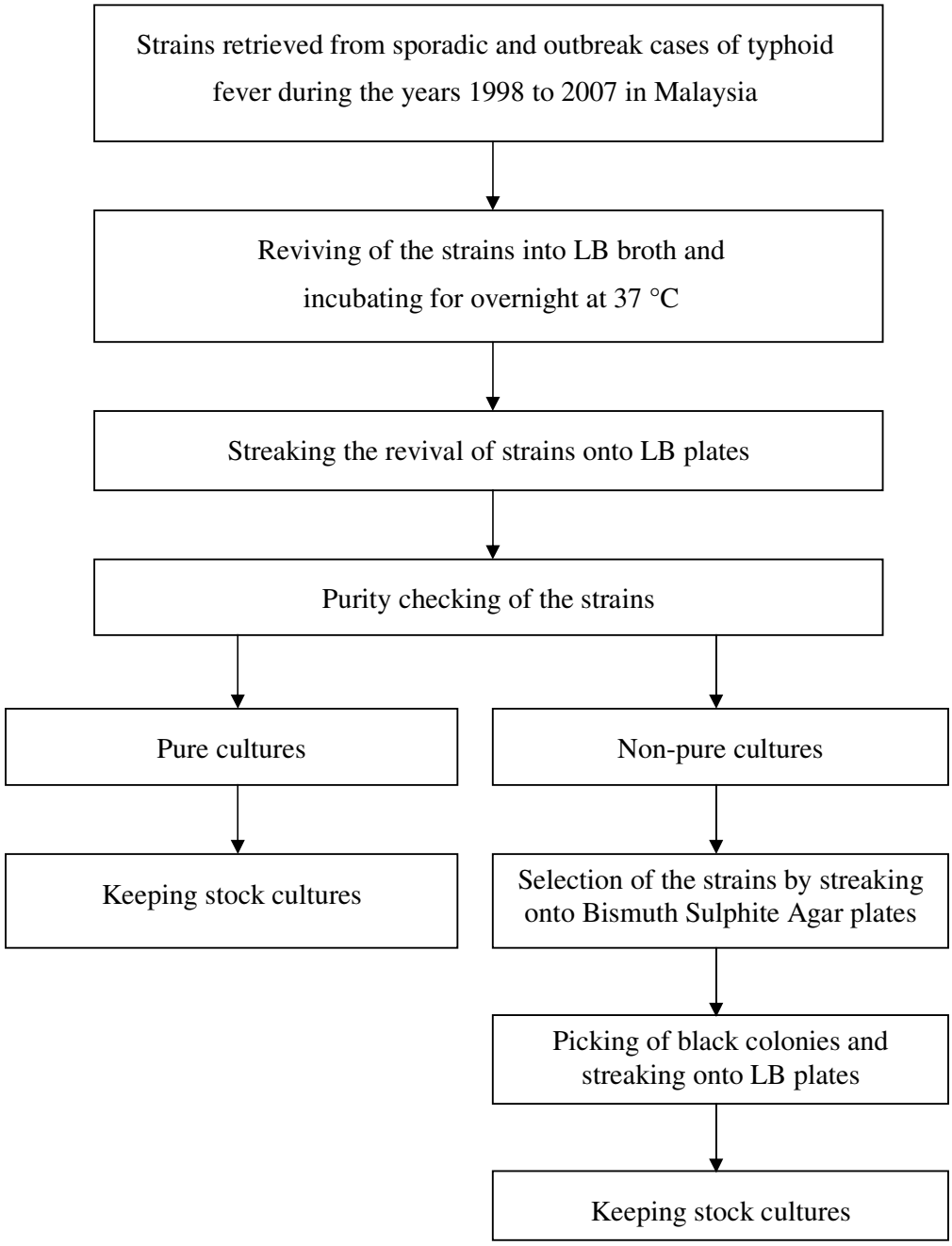

Figure 3.1: Bacterial strains and purity check.

3.3 Confirmation of *S.* Typhi Strains by PCR

The purity of *S.* Typhi strains were confirmed by application of a multiplex PCR previously developed in-house by Thong *et al*. (unpublished data). This multiplex PCR comprised of three sets of primers, Hil A (Pathmanathan *et al*., 2003), ST and SPA (proprietary information) targeting the *Salmonella* genus (789 bp), *S.* Typhi (332 bp) and *S.* Paratyphi A (496bp).

3.3.1 DNA Template Preparation

The DNA template of each strain was extracted by direct cell lysis using boiling method. A single bacterial colony was suspended in 50 µl of sterile distilled water and boiled at 99°C for 5 min and then stored at 4°C for 10 min before being used directly for PCR. The cell suspension was centrifuged at 13,400 rpm for 2 min, and an aliquot of the supernatant was used as the DNA template for PCR amplification. The balance was kept at -20°C for further analysis.

3.3.2 PCR Amplification

Multiplex PCR amplification was performed in a total reaction volume of 25 µl containing 5 µl of template DNA, 1X buffer (Promega, Madison, Wis., USA), 1.8 mM $MgCl_2$ (Promega, 25 mM), 120 µM of each dNTP (Promega, 10 mM), 1.5 U of *Taq*DNA polymerase (Promega, 5U/µl) and 0.4 µM of each Hil A, ST and SPA primers. The amplification reaction was performed in an Eppendrof thermocycler consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles at 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min and a final elongation step at 72°C for 7 min. Water was used as negative control for all PCR analysis.

3.3.3 PCR Products Analysis

The amplified products were analysed using 1.5% (wt/vol) standard agarose gel electrophoresis in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3). A 5-µl aliquot of the PCR products was loaded into the wells of the gel respectively. A 100-bp DNA marker (Promega, Madison, Wis., USA) was used as molecular size standards. After electrophoresis, the DNA fragments were stained by ethidium bromide (0.5 µg/ml) for 5 min and destained by distilled water for 1 hour. After distaining, the gel was visualized by UV transillumination and the photo of the gel was taken using BIORAD Geldoc system (USA).

3.4 Restricted Arbitrarily Primed PCR (resAP-PCR)

3.4.1 Genomic DNA Extraction

The DNA extraction was performed according to the protocol supplied in DNA Extraction Mini Kit by iNtRoN Biotechnology, Inc (Korea). In this protocol 6 different steps were conducted; Preparation step, Sample sizing step, Lysis step, DNA binding step, Washing step and Elution step. The flowchart of the work is as illustrated in Figure 3.2.

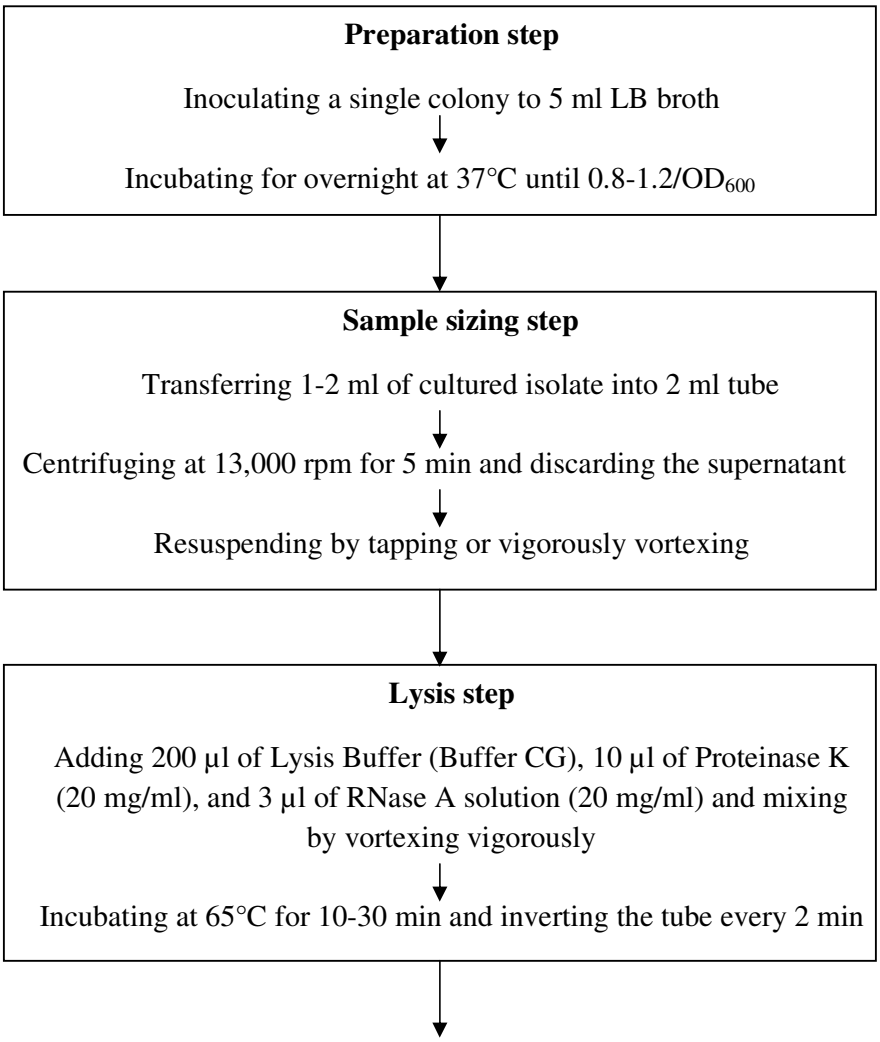

Figure 3.2: Genomic DNA extraction steps.

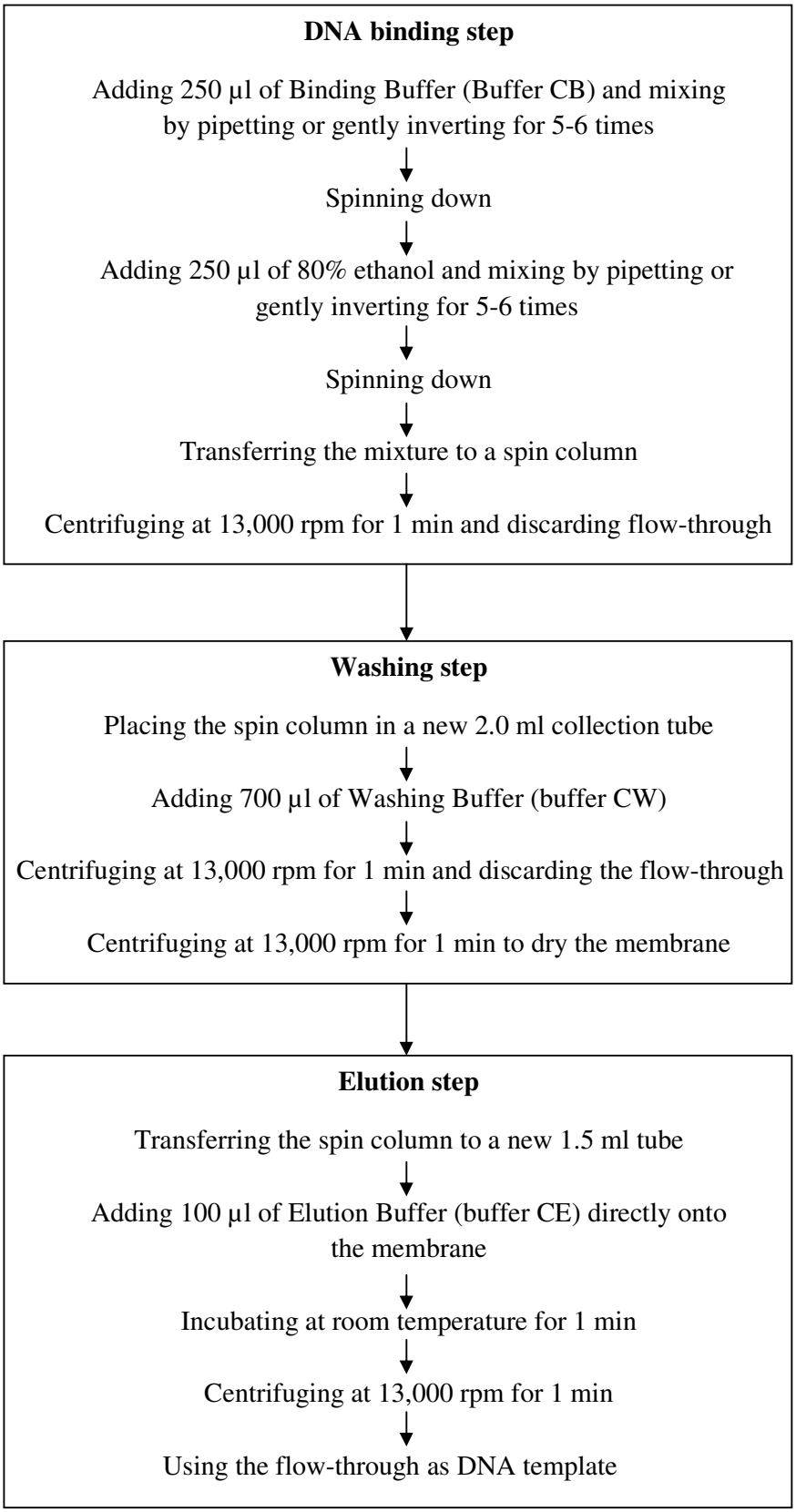

Figure 3.2, continued

3.4.2 Restriction Endonuclease Digestion

The genomic DNA of each strain was digested with two endonuclease restriction enzymes, *Hae*III (GG|CC) and *Alu*I (AG'CT) prior to amplification with three arbitrary oligonucleotide primers. The digestion reaction was performed in a final volume of 20 µl on 1 µl (1 µg/µl) of extracted genomic DNA according to manufacturer's protocol (Promega, Madison, Wis., USA). The reaction components were assembled in order (refers to Table 3.1), mixed gently by pipetting, and centrifuged for a few seconds in a microcentrifuge. The tubes incubated at 37°C for 3 hours to digest and finally heated at 65°C for 5 min to inactivate the restriction enzyme. An aliquot of the digested DNA was used as the DNA template for PCR amplification.

Table 3.1: The components of digestion reaction.

Component	Concentration	Volume (µl)
ddH_2O	-	16.3 µl
RE Buffer	10X	2.0 µl
Acetylated BSA	10 µg/µl	0.2 µl
Genomic DNA	1.0 µg/µl	1.0 µl
Restriction Enzyme	10 µ/ µl	0.5 µl

3.4.3 Restricted AP-PCR Amplification

Restricted AP-PCR amplification was performed using three various primer sets for each enzyme digested product. The 9-mer oligonucleotide primes have been selected based on genome sequence of *S.* Typhi using the *in silico* resAP-PCR program (refers to Table 3.2). PCR was initially performed on 5 strains incorporating different primer sets to evaluate their potential as molecular markers for the strain typing of *S.* Typhi. Binding patterns were only detected by using STHae8 and STAlu9 primers which were

further characterized in this study. The amplification was performed in a total reaction volume of 25 µl containing 1 µl of template DNA (digested genomic DNA, 10 ng), 1 U of *Taq*DNA polymerase (Promega, 5U/µl), 200 µM of each dNTP (Promega, 10 mM), 1 mM $MgCl_2$ (Promega, 25 mM), and 0.8 µM of each three oligonucleotide primers in 1X PCR buffer. Amplification was performed in an Eppendrof thermocycler with an amplification profile that consisted of an initial denaturation step at 95ºC for 2 min and then 30 cycles with denaturation at 95ºC for 1 min, primer annealing at 32ºC for 30 s, and extension at 72ºC for 1 min. To ensure complete strand extension, the reaction mixture was kept at 72ºC for 4 min after the final cycle. Water was used as negative control for all PCR analysis.

Table 3.2: The primers used for resAP-PCR amplification.

Primer	Primer sequence [1](1) (3'-5')	Primer sequence (2) (3'-5')	Primer sequence (3) (3'-5')	No. of Bands[2]
STHae8	GAAGCGGCG	CTGGTGGCG	CTGCTGGCG	8
STHae9	GCGTCAGCA	GGCGGCAAA	CGCCAGCCA	9
STHae10	CGCCAGCAG	CGCCACCAG	GCCGCCATT	10
STAlu8	GCGTCAGCA	CATCGCCAG	TTCCGCCAG	8
STAlu9	GCGGCGATA	GCTGGCGTT	GCTGGCGAT	9
STAlu10	CGCCGCTTT	TTCGCCAGC	ATCCGCCAG	10

[1]Primer sequences were obtained by using the *in silico* resAP-PCR program available at *http://insilico.ehu.es/resAP-PCR/* using *S.* Typhi genome as a template.

[2]The number of bands corresponds to bands amplified by different forward and reverse primers. Since forward and reverse primers are the same primer, amplification is likely to be inhibited by hairpin formation, so those bands have not been accounted in the table (but they are identified in the results).

3.4.4 PCR Products Analysis

The amplified products were analysed using 2.5% (wt/vol) standard agarose gel electrophoresis in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3). A 100-bp DNA marker (Promega, Madison, Wis., USA) was used as molecular size standards. DNA bands visualized after ethidium bromide staining and the photo of the gel was taken using BIORAD Geldoc system (USA).

3.5 Repetitive Element PCR (Rep-PCR)

3.5.1 DNA Template Preparation

The template DNA was prepared as described by Sandvang *et al.* (1998). A single bacterial colony was inoculated into 1 ml of LB broth and incubated for overnight at 37 °C in a shaker. The bacterial cells were then pelleted by centrifugation at 13,400 rpm for 5 min and washed twice with 800 µl of phosphate-buffered saline containing 85% NaCl and 800 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The cell pellets were resuspended in 100 µl of TE buffer, boiled at 95°C for 5 min, and stored at 4 for 10 min. The cell suspension was then centrifuged at 13,400 rpm for 2 min, and an aliquot of the supernatant was used as the DNA template for PCR amplification. The balance was kept at -20°C for further analysis.

3.5.2 Rep-PCR Amplification

Rep-PCR amplification was performed by using primer sequences, REP-F and REP-R (Georghiou *et al*., 1994), corresponding to the highly conserved REP repeated DNA elements (refers to Table 3.3). PCR was initially performed on 10 strains to evaluate the potential use of Rep-PCR as a molecular marker for the strain typing of *S.* Typhi. The amplification was performed in a total reaction volume of 25 µl containing 5

µl of template DNA, 1X buffer (Promega, Madison, Wis., USA), 1.5 mM $MgCl_2$ (Promega, 25 mM), 200 µM of each dNTP (Promega, 10 mM), 1 U of *Taq*DNA polymerase (Promega, 5U/µl) and 0.5 µM of each forward and reveres primers. The amplification reaction was performed in an Eppendrof thermocycler consisted of an initial denaturation step at 95°C for 7 min, followed by 30 cycles at 90°C for 30 sec, 40° C for 1 min, 65°C for 8 min and a final elongation step at 65°C for 16 min. Water was used as negative control for all PCR analysis.

Table 3.3: The primers used for rep-PCR amplification.

Primer	Primer sequence (5'-3')	Reference
REP-F	IIIICGICGICATCATCTGGG	Georghiou *et al*., 1994
REP-R	ICGICTTATCIGGCCTAC	Georghiou *et al*., 1994

3.5.3 PCR Products Analysis

The amplified products were analyzed using 1.5% (wt/vol) standard agarose gel electrophoresis in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3). 100-bp and 1-kb DNA markers (Promega, Madison, Wis., USA) were used as molecular size standards. DNA bands visualized after ethidium bromide staining and the photo of the gel was taken using BIORAD Geldoc system (USA).

3.6 Variable-Number Tandem Repeat (VNTR)

3.6.1 DNA Template Preparation

A single bacterial colony was suspended in 50 µl of sterile distilled water and boiled at 99°C for 5 min and then stored at 4°C for 10 min before being used directly

for PCR. The cell suspension was centrifuged at 13,400 rpm for 2 min, and an aliquot of the supernatant was used as the DNA template for PCR amplification.

3.6.2 VNTR Loci and Oligonucleotide Primers

The PCR primers used in this study were previously designed by Liu *et al*. (2003) through identification of five VNTR loci (labeled TR1, TR2, TR3, TR4 and TR5) in the genome of CT18 strain of *S.* Typhi (Table 3.4).

Table 3.4: The primers used for PCR amplification of selected VNTR loci from the CT18 strain of *S.* Typhi.

Primer	Locus position	Primer sequence (5'-3') [1]	No. of repeat units [2]	Predicted size (bp) [3]
TR1F1	2017115-2017136	AGAACCAGCAATGCGCCAACGA	12	261
TR1R1	2017354-2017375	CAAGAAGTGCGCATACTACACC		
TR2F1	2556810-2556831	CCCTGTTTTTCGTGCTGATACG	27	511
TR2R1	2557299-2557320	CAGAGGATATCGCAACAATCGG		
TR3F1	2926145-2926166	CGAAGGCGGAAAAAACGTCCTG	3	545
TR3R1	2926668-2926689	TGCGATTGGTGTCGTTTCTACC		
TR4F2	4396728-4396749	AAAAGCCCGTCTAGTCTTGCA	2	421
TR4R1	4397127-4397148	ATCCTTCGGTATCGGGGTATCC		
TR5F1	4624169-4624190	TGAAAACCGGCTCGTAGCAGTG	5	194
TR5R1	4624342-4624363	CATACGGTTACTGCGGGATTGG		

[1]Primer sequences from Liu *et al.* (2003).

[2,3]Number of repeat units and predicted product sizes were obtained by using the *in silico* PCR amplification program available at *http://insilico.ehu.es/PCR/* using *S.* Typhi (CT18) genome as a template.

3.6.3 PCR Amplification of VNTR Loci

PCR was initially performed on 10 strains incorporating individual primers flanking five VNTR loci (TR1, TR2, TR3, TR4 and TR5) to evaluate their potential as molecular markers for the strain typing of *S.* Typhi. Allelic variations were detected for TR1, TR2, TR3 and TR5 loci but not for TR4 locus. Therefore, the TR1, TR2, TR3 and TR5 loci were further characterized in this study. Multiplex PCR amplification was performed in a total reaction volume of 25 µl containing 5 µl of template DNA, 1X buffer (Promega, Madison, Wis., USA), 2 mM $MgCl_2$ (Promega, 25 mM), 200 µM of each dNTP (Promega, 10 mM), 2.5 U of *Taq*DNA polymerase (Promega, 5U/µl) and 0.4 µM of each forward and reverse primers. The amplification reaction consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min and a final elongation step at 72°C for 7 min. Water was used as negative control for all PCR analysis.

3.6.4 PCR Products Analysis

The amplified products were analysed using 2.5% (wt/vol) standard agarose gel electrophoresis in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3). Allele sizes were estimated by using a 50-bp DNA ladder (Invitrogen, USA) as the size marker. DNA bands visualized after ethidium bromide staining and the photo of the gel was taken using BIORAD Geldoc system (USA).

3.6.5 DNA Sequence Analysis

To confirm that length polymorphisms were the result of repeat copy number variations, the monoplex PCR products from all *S*. Typhi strains were purified using PCR quick-spin Purification Kit (iNtRoN Biotechnology, Inc., Korea), and the

nucleotide base sequence of forward strand of each product was determined using standard methods, incorporating the same primers as used in the PCRs described above, by a commercial sequencing service (Aitbiotech Pte. Ltd., Singapore).

3.6.6 Data Analysis

The VNTR alleles were defined as the copy numbers of the respective repeat units present at each locus and the VNTR profile designations were subsequently deduced. Allelic diversity of individual VNTR locus was calculated by Nei's diversity index as $D = 1 - \Sigma (\text{allele frequency})^2$ (Nei, 1978).

CHAPTER 4.0:

RESULTS

CHAPTER 4.0: RESULTS

4.1 Bacterial Strains and Purity Check

In this study, *S.* Typhi strains collected from sporadic and outbreak cases of typhoid fever during the years 1998 to 2007 were investigated. The organisms were isolated and identified by standard procedures at the hospital laboratories. However, a number of strains appeared as non-pure cultures. These strains were purified by using Bismuth Sulphite Agar (BSA) as a selective medium for isolation and preliminary identification of *Salmonella* species particularly serovar Typhi. After 12-18 hours incubation at 37°C, the black "rabbit-eye" colonies with a black zone and metallic sheen surrounding the colony were picked and streaked on LB plates for further analysis.

4.2 Confirmation of *S.* Typhi Strains by PCR

An in-house multiplex PCR assay incorporating three types of primers Hil A, ST and SPA was performed to confirm the entity of the strains as *S.* Typhi. The analysis of the amplified products by agarose gel electrophoresis revealed that all strains produced a PCR band with the Hil A primer at 784 bp indicating the identification of *Salmonella* species (Figure 4.1). However, a number of strains did not produce *S.* Typhi specific PCR band with the ST primer at 332 bp. These *Salmonella* strains were therefore excluded from this study. None of the strains produced the PCR band with SPA primer at 496 bp indicative of *Salmonella* serovar Paratyphi (Figure 4.1). Hence, PCR confirmation proved the purity of *S.* Typhi strains. Finally, 50 *S.* Typhi strains obtained from sporadic cases (36 strains) and three well-defined outbreaks (14 strains) of typhoid fever were selected for molecular subtyping by using three different PCR-based methods of restricted AP-PCR, rep-PCR and, VNTR analysis.

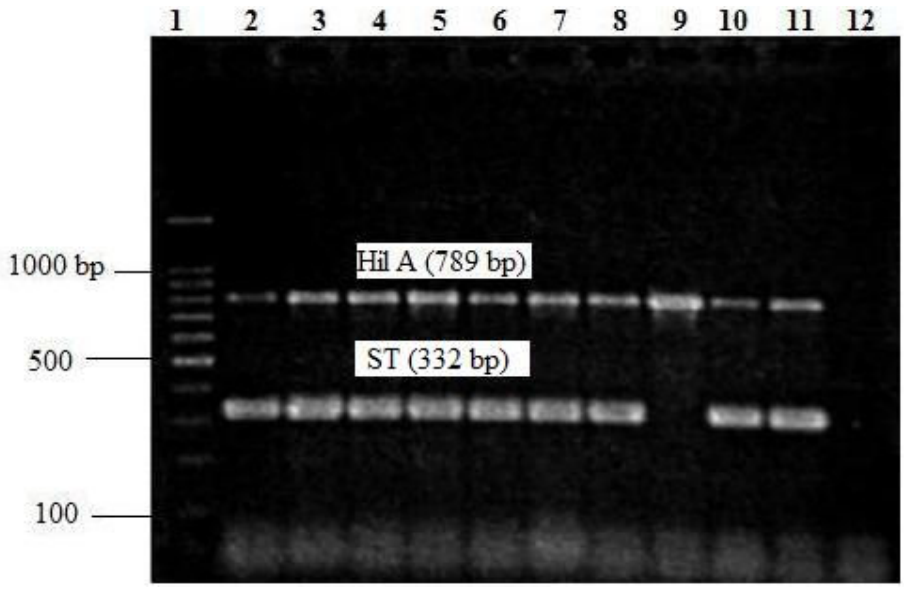

Figure 4.1: Multiplex PCR confirmation of some of *S.* Typhi strains by using three types of primers Hil A (789 bp), ST (332 bp), and SPA (496bp).

Lane 1	**100-bp Marker**	**Lane 7**	**TP41/98**
Lane 2	**TP43/98**	**Lane 8**	**TP46/98**
Lane 3	**TP189/05**	**Lane 9**	**TP132/99**
Lane 4	**TP282/03**	**Lane 10**	**TP34/98**
Lane 5	**TP3/01**	**Lane 11**	**TP130/99**
Lane 6	**TP322/03**	**Lane 12**	**Negative Control**

4.3 Restricted AP-PCR (resAP-PCR) Analysis

Restricted AP-PCR amplification was initially performed by using six different primer sets on *Hae*III and *Alu*I digested products of 5 *S.* Typhi strains to evaluate the utility of these primers. Analysis of the amplified products by agarose gel electrophoresis revealed banding patterns for STHae8 and STAlu9 primers, but not for the other primer sets which were therefore excluded from this study (results not shown). Hence, STHae8 and STAlu9 primer sets were selected for resAP-PCR assessment of other 10 *S.* Typhi strains. However, restricted AP-PCR amplification of *Hae*III digested DNA by using STHae8 primer set was not able to generate DNA polymorphism among *S.* Typhi strains tested. In addition, the DNA fingerprinting profiles were not reproducible as significant differences in the number of bands and intensities of the patterns obtained when the analysis was repeated for several times. The banding patterns consisted of 12 bands ranging from 165 bp to 1100 bp (Figure 4.2(a)). Similarly, restricted AP-PCR amplification of *Alu*I digested DNA by using STAlu9 primer set produced one pattern for all 10 *S.* Typhi strains tested (Figure 4.2(b)). The banding patterns comprised of 9 major DNA fragments ranging from 120 bp to 940 bp. However, the assay was reproducible when the analysis was repeated twice. Since these primer combinations did not enable discrimination between any of the *S.* Typhi strains tested, the assay has not been continued for remaining strains. In general, restricted AP-PCR amplification of *Hae*III and *Alu*I digested DNA was not able to discriminate *S.* Typhi strains. In addition, resAP-PCR analysis using STHae8 primer set was poor in reproducibility. Overall, resAP-PCR using these primer combinations has limited value as subtyping tool for *S.* Typhi strains due to the poor reproducibility and discriminatory power.

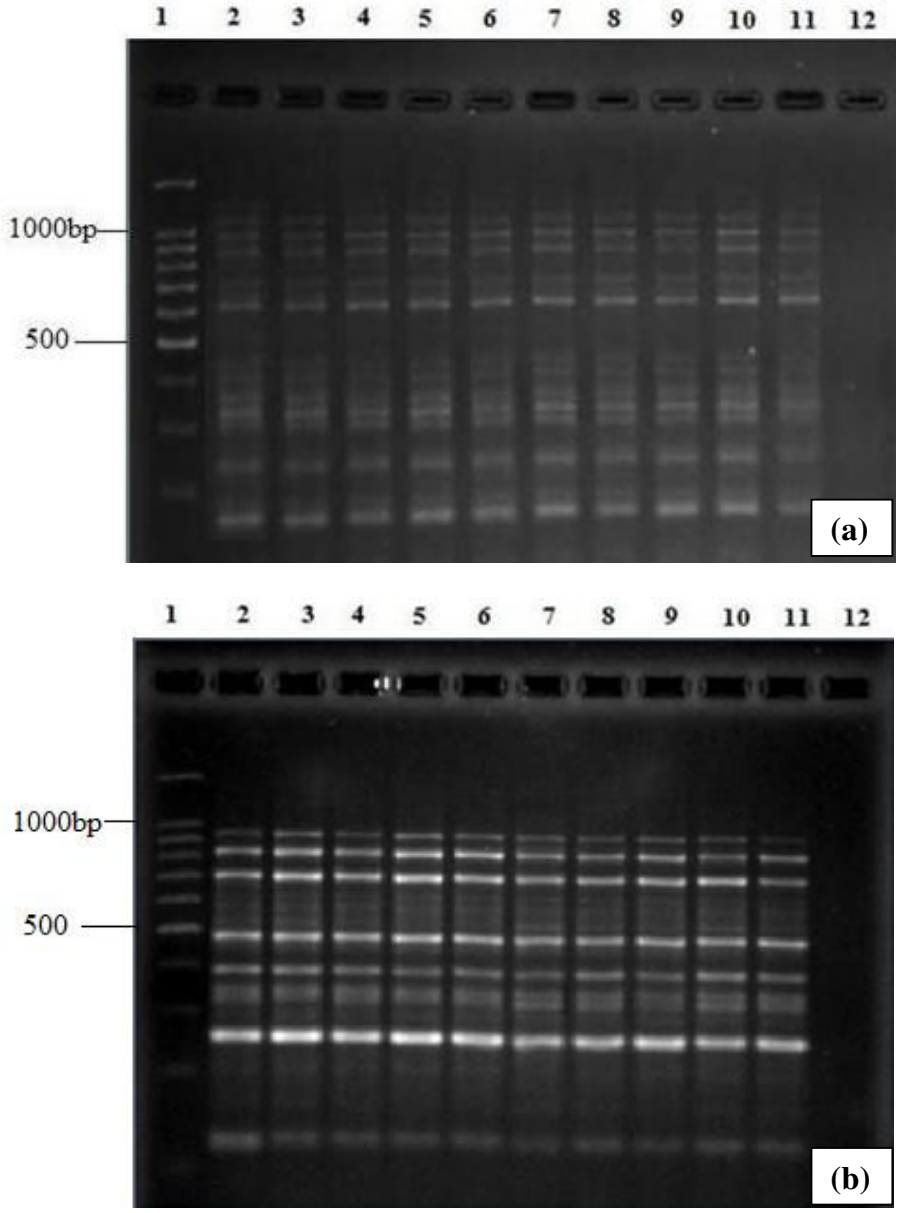

Figure 4.2: Representative resAP-PCR fingerprint patterns for some of *S.* Typhi strains; (a) *Hae*III digested DNA by using STHae8 primer set, (b) *Alu*I digested DNA by using STAlu9 primer set.

Lane 1	**100-bp Marker**	**Lane 7**	**TP323/03**
Lane 2	**TP34/98**	**Lane 8**	**TP85/04**
Lane 3	**TP247/99**	**Lane 9**	**ST156/05**
Lane 4	**TP2/00**	**Lane 10**	**ST16/06**
Lane 5	**TP38/01**	**Lane 11**	**ST014/07**
Lane 6	**TP41/02**	**Lane 12**	**Negative Control**

4.4 Repetitive Element PCR (Rep-PCR) Analysis

Rep-PCR analysis was performed on 10 strains to evaluate the potential use of REP-F and REP-R primers for the subtyping of serovar Typhi strains. Analysis of the amplified products by agarose gel electrophoresis revealed that Rep-PCR did not generate DNA polymorphism among *S.* Typhi strains tested. All the strains appeared to be homogenous showing only one REP pattern (Figure 4.3). However, DNA fingerprinting profiles were reproducible when the analysis was repeated twice. The intensity of the stained DNA varied for some strains but the same number of bands with corresponding sizes was obtained. The banding patterns comprised of 20 major DNA fragments ranging from 185 bp to 2515 bp which were identical for all strains. Bands below 150bp were not included in the analysis. Since rep-PCR did not enable discrimination between any of the *S.* Typhi strains tested, the assay has not been continued for remaining strains. In general, Rep-PCR with the primers REP-F and REP-R was not useful as a subtyping tool for *S.* Typhi. The assay was not able to discriminate *S.* Typhi strains considering that all strains shared the same predominant banding profile.

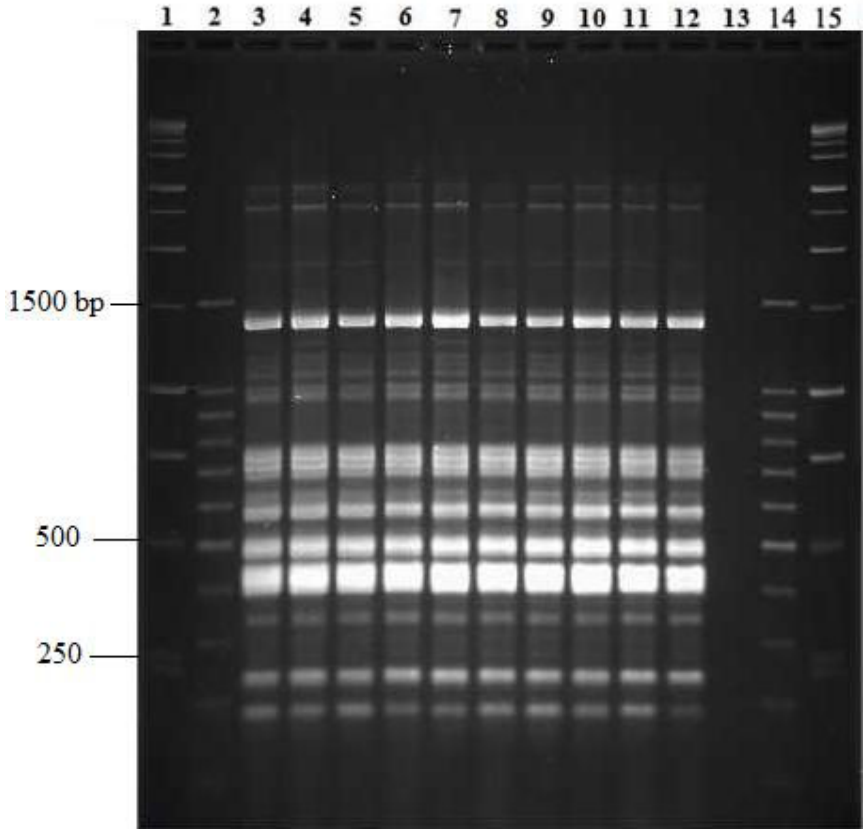

Figure 4.3: Representative Rep-PCR fingerprint patterns for some of *S.* Typhi strains.

Lane 1	**1kp Marker**	**Lane 9**	**TP85/04**
Lane 2	**100-bp Marker**	**Lane 10**	**ST156/05**
Lane 3	**TP34/98**	**Lane 11**	**ST16/06**
Lane 4	**TP247/99**	**Lane 12**	**ST014/07**
Lane 5	**TP2/00**	**Lane 13**	**Negative Control**
Lane 6	**TP38/01**	**Lane 14**	**100-bp Marker**
Lane 7	**TP41/02**	**Lane 15**	**1kb Marker**
Lane 8	**TP323/03**		

4.5 Variable-Number Tandem Repeat (VNTR) Analysis

4.5.1 PCR Analysis of VNTR Loci

Prior to performing multiplex VNTR assay, the size variation and utility of each TR locus were separately evaluated across a subset of *S.* Typhi strains. Analysis of the amplified products by agarose gel electrophoresis revealed size variations for TR1, TR2, TR3 and TR5 loci, but not for TR4 locus which was therefore excluded from this study (results not shown). Using this approach, these four loci (TR1, TR2, TR3, and TR5) were selected and taken forward for multiplex PCR assessment of 50 *S.* Typhi strains. The specificity of the primers could be seen from the single band obtained in each reaction.

Allelic variations were also observed when the strains were subjected to multiplex PCR with primer combination of TR1, TR2, TR3, and TR5. Based on visual observation, each bands produced was similar in size to its corresponding band obtained during amplification with individual primers (results not shown). Length polymorphisms were especially pronounced for TR1 and TR2. The sizes of the amplified products displayed a wide range of polymorphism, with variation of approximately 260 bp to 340 bp at TR1 locus, 320 bp to 550 bp at TR2 locus, 635 bp to 645 bp at TR3 locus, and 200 bp to 220 bp at TR5 locus, suggesting differences in tandem-repeat copy numbers in these VNTR loci (Table 4.2). All four VNTR loci yielded amplified fragments for all 50 *S.* Typhi strains studied except for 2 strains ST189/05 and ST208/05 with no amplification product at TR1 (null allele), suggesting sequence polymorphism at priming sites. The multiplex PCR amplification was robust and yielded reproducible results for all loci when the analysis was repeated twice.

VNTR banding profiles of multiplex PCR at TR1, TR2, TR3 and TR5 loci for some of *S.* Typhi strains can be seen in Figure 4.4.

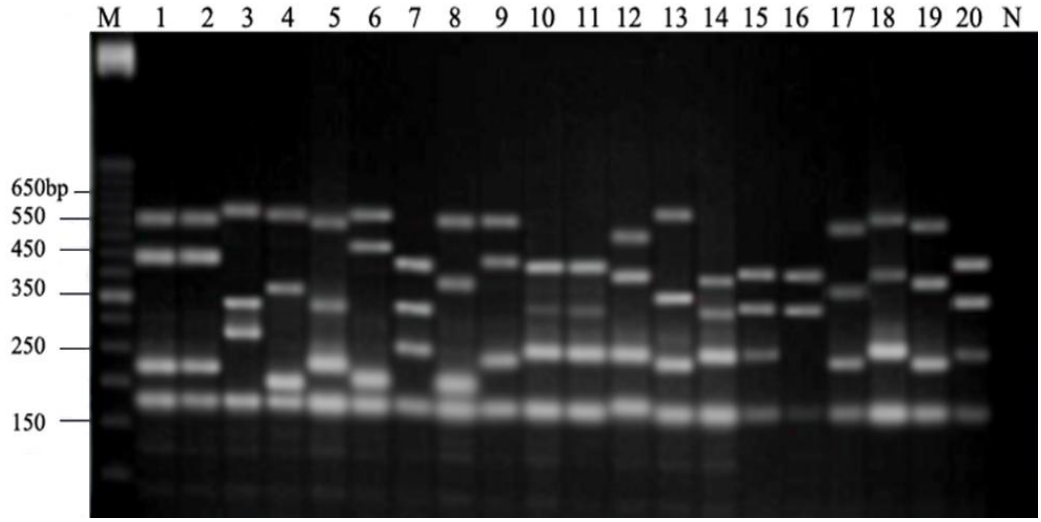

Figure 4.4: Representative VNTR banding profiles of multiplex PCR at TR1, TR2, TR3, and TR5 loci for some of *S.* Typhi strains.

Lane M	**50-bp Marker**	**Lane 11**	**TP323/03**
Lane 1	**TP40/98**	**Lane 12**	**TP329/03**
Lane 2	**TP41/98**	**Lane 13**	**TP80/04**
Lane 3	**TP128/99**	**Lane 14**	**TP85/04**
Lane 4	**TP172/99**	**Lane 15**	**ST156/05**
Lane 5	**TP5/00**	**Lane 16**	**ST189/05**
Lane 6	**TP168/00**	**Lane 17**	**ST2/06**
Lane 7	**TP5/01**	**Lane 18**	**ST16/06**
Lane 8	**TP58/01**	**Lane 19**	**ST4571/07**
Lane 9	**TP20/02**	**Lane 20**	**ST014/07**
Lane 10	**TP43/02**	**Lane N**	**Negative control**

4.5.2 Sequence Analysis of Potential VNTR Loci

Nucleotide sequencing of each of the four amplified loci (TR1, TR2, TR3 and TR5) from 50 *S.* Typhi strains was performed to verify PCR specificity and to confirm that size polymorphisms seen in the PCR products were due to variations in the VNTR copy number (Figure 4.5). Although the presence of multiples of a repeat unit was the general rule, an absence of the repeat unit and insertion and/or deletion in flanking regions were observed in a number of amplified products. The result revealed that some TR2 and TR3 amplicons with identical repeat copy number displayed different lengths of the PCR products. Sequence alignments of these amplicons with the homologous sequences in the *S.* Typhi CT18 genome revealed that these differences in amplicon sizes were due to variation in the length of the VNTR flanking regions as a result of various sequence deletion and/or insertion (results not shown). The deletions at TR2 and TR3 amplicons were found among 15 strains, while the insertion was found only in 2 strains, TP168/00 and ST2/06 (refers Table 4.1). In addition, a number of the TR2 amplicons (4 out of the 50 sequenced TR2 amplicons) contains both flanking sequences but no repeat unit (Table 4.1). Sequence analysis of TR1 amplicons, unlike the TR2 and TR3, indicated a high conservation of repeat units and flanking regions among individual strains.

TR1 locus

CGTCATGTCCCTCTTCATCCTGCATCAGAAGAAAGAAGAAAGAAGAAAGAAGAAAGAAG
AAAGAAGAAAGAAGAAAGAAGAAAGAAGAAAGAAGAATTCGTGGCATTAAAGCCCTGAC
GCCGCCAGCCTTTCCAGGCGCTGGCCGCAATTTGATCCAGTCGGTATTACCGCTTATTA
GGTGTAGGCAAATTCTGTGATCGGTGTAGTTGGCCACTTCTTGGATGCGACGGCGGAAG
CAATCCTCACGTCCCCGGGGTACTGAGAAATTCCGACTAATATGGACTCGTAGAATAAG
GA

TR2 locus

ATGTGCACAAAAAAGGCGACTTTCGTTGAGTCGCCTTTTCTTATCCCCTATGGGAGCGC
GGTGCCTTCCAGGCATTTATTTACGAAGCATGACTTCGATAAAATCTTTCCAGTTCCCC
AGTTCCCCAGTTCCCCAGTTCCCCAGTTCCCCAGTTCCCCAGTTCCCCAGTTCCCCAGT
TCCCCAGTTCCCCAGTTCACGTTCAATCATAATAGCCTCTCTTATTATTATGGGTATTC
TACGTAGTTAGCGGTATAGAGAGAAGTTCATTTAACCGATTGTTGCGATATCCTCTGAC
CGGTTGCGCAACCTCCGCGGGGATCGGTTTATCCCCGCTGGCGCGGGGATCGGTTTATC
CCCGCTGGCGCGGGGAACACTCTAAATCTACCCAATTGAATTTAAATACTTTTTTAGCG
CACAAAAAACCCACCAACTTTTCCTAATTTTTAAAGATCTCTAACTTATTGATTTTCAA
CAAGTAGAAACGCCC

TR3 locus

ATGATGCAGACAGATCGGTTTATCCCCGCTGGCGCGGGGAACACTAAAACACCGGTTGC
GCAACCTCCGCGGGGATCGGTTTATCCCCGCTGGCGCGGGGATCGGTTTATCCCCGCTG
GCGCGGGGAACACTCTAAATCTACCCAATTGAATTTAAATACTTTTTTAGCGCACAAAA
AACCCACCAACTTTTCCTAATTTTTAAAGATCTCTAACTTATTGATTTTCAACAGGTAG
AAACGCCACCAATCGCAAATTTCCGGCTTGCCCCGCTGGTGCGGGGTCCGCCTGGACCG
GTGGCGCAACCTCCGCGGGGATCGGTTTATCCCCGCTGGCGCGGGGATCGGTTTATCCC
CGCTGGCGCGGGGAACACTCTAAATCTACCCAATTGAATTTAAATACTTTTTTAGCGCA
CAAAAAACCCACCAACTTTTCCTAATTTTTAAAGATCTCTAACTTATTGATTTTCAACA
GGTAGAACGCCCT

TR5 locus

NGGCTGTTGCGTAGCTCTGTAAGTCGCCAGAGGGTTCATTTTCAACTCCGACAAGTTCC
CCCTACGCCTGCGTCGTCACGCGTCACGCGTCACGCGGCAGGCAAACGCCGATCCCGCC
AATCCCGCAGTAACCGTATGA

Figure 4.5: Nucleotide sequence of the VNTR loci. The repeat region that varies among *S.* Typhi strains is underlined.

4.5.3 Allelic Profiles and VNTR Profile Designations

Allelic profiles and VNTR profile designations were defined using the sequencing data based on the copy number of the respective repeats at each VNTR locus (Table 4.1). A total of 14 distinct VNTR alleles ranging between 0 (null allele) and 18 copies of the 7-bp repeat unit were observed at the TR1 locus, while a total of 19 distinct VNTR alleles ranging between 0 (no repeat unit) and 33 copies of the 8-bp repeat were detected at the TR2 locus. In contrast, there were only three VNTR alleles of 1, 2 and 3 copies of the 26-bp repeat at the TR3 locus and two VNTR alleles of 3 and 4 copies of the 7-bp repeat at the TR5 locus with one allele represented only once across all strains studied (see Table 4.1; Table 4.2). Ultimately, a total of 42 distinct VNTR types (MY1 to MY42) based on four VNTR banding profiles could be assigned among the 50 individual strains (Table 4.1). The results also revealed that *S.* Typhi strains investigated in this study had distinctive and different VNTR profiles compared to the 2 Malaysian strains reported by Liu *et al*. (2003).

Table 4.1: The allelic profiles and VNTR profile designations based on the copy number of the respective repeats at each VNTR locus.

Strain No./ Year of isolation	Allelic Profile (TR1/TR2/TR3/TR5)	VNTR Profile designation
TP172/99	04 / 11 / 02 / 03	MY1
TP160/99	04 / 10 / 02 / 03	MY2
TP108/04	06 / 10 / 02 / 03	MY3
TP78/04	10 / 13 / 02 / 03	MY4
ST37/06	10 / 13 / 02 / 03	MY4
TP90/04	10 / 11 / 02 / 03	MY5
TP35/02	10 / 11 / **02** / 03	MY5
TP80/04	10 / 10 / 03 / 03	MY6
TP2/00	09 / 20 / 03 / 03	MY7
TP1/00	11 / 17 / 03 / 03	MY8
ST16/06	13 / 16 / 02 / 03	MY9
TP38/01	12 / 10 / 02 / 03	MY10
ST13/06	12 / 14 / 02 / 03	MY11
ST3/06	18 / 00 / 02 / 03	MY12
TP58/01	05 / 14 / 02 / 03	MY13
TP168/00	05 / **20** / 02 / 03	MY14
TP118/00	05 / 17 / 02 / 03	MY15
TP195/03	05 / 27 / 02 / 03	MY16
TP247/99	05 / 29 / 02 / 03	MY17
TP282/03	05 / 33 / 02 / 03	MY18
TP24/02	05 / 00 / 01 / 03	MY19
TP128/99	15 / 06 / 02 / 03	MY20
TP130/99	16 / 26 / 02 / 03	MY21

Strain No./ Year of isolation	Allelic Profile (TR1/TR2/TR3/TR5)	VNTR Profile designation
TP3/01	09 / 14 / 01 / 03	MY22
ST2/06	10 / **11** / 01 / 03	MY23
ST4738/07	10 / 13 / 01 / 03	MY24
ST334/07	10 / 13 / 01 / 03	MY24
ST4571/07	10 / 13 / 01 / 03	MY24
ST314/07	10 / 13 / 01 / 03	MY24
TP2/01	10 / 15 / 01 / 03	MY25
TP5/00	08 / 06 / 01 / 03	MY26
TP40/98	07 / 19 / 01 / 03	MY27
TP34/98	06 / 19 / 01 / 03	MY28
TP46/98	06 / 19 / 01 / 03	MY28
TP43/98	07 / 21 / 01 / 03	MY29
TP41/98	07 / 19 / **02** / 03	MY30
TP20/02	10 / 19 / 01 / 03	MY31
ST208/05	Null / **18** / **02** / 03	MY32
ST189/05	Null / 00 / **02** / 03	MY33
TP41/02	12 / **17** / **02** / 03	MY34
TP43/02	12 / **22** / **02** / 03	MY35
ST117/05	11 / **18** / **02** / 03	MY36
TP323/03	11 / **18** / **02** / 03	MY36
TP322/03	12 / **25** / **02** / 03	MY37
ST156/05	12 / **19** / **02** / 03	MY38
TP5/01	12 / **18** / **02** / 03	MY39

Table 4.1, continued

Strain No./ Year of isolation	Alleic Profile (TR1/TR2/TR3/TR5)	VNTR Profile designation
ST142/05	12 / **18** / **02** / 03	MY39
ST014/07	12 / 00 / **02** / 03	MY40
TP85/04	12 / **15** / **02** / 03	MY41
TP329/03	12 / 16 / **02** / 04	MY42

Boldface type indicates groups of alleles with unexpected sizes due to the insertion and/or deletion in repeat flanking regions. Null: no amplification of the allele.

Table 4.1, continued

4.5.4 Allelic Diversity of VNTR Loci

Multiplex VNTR assay displayed diversity based on four VNTR loci studied. Diversity values at four VNTR loci was determined based on Nei's diversity index (*D*) (Nei, 1978), ranging from 0.04 to 0.93 with an overall average of 0.58 (Table 4.2). VNTR loci with higher numerical diversity value have higher discriminatory power for differentiation of the strains. The allele numbers ranged from 2 alleles for TR5 to 19 alleles for TR2 (Table 4.2).

Table 4.2: Characteristics of VNTR loci studied.

VNTR locus	Consensus sequence of repeat unit	Length of repeat unit (bp)	Size range of PCR products (bp)	Range of repeat number	No. of alleles	Allelic diversity
TR1	AGAAGAA	7	260-340	0-18	14	0.86
TR2	CCAGTTCC	8	320-550	0-33	19	0.93
TR3	CGCGGGGATCGG TTTTCCCCGCTGG	26	635-645	1-3	3	0.48
TR5	CGTCACG	7	200-220	3-4	2	0.04

CHAPTER 5.0:

DISCUSSION

CHAPTER 5.0: DISCUSSION

Typhoid fever continues to be a major public health problem in developing countries as a result of many interrelated factors, including increased urbanization, inadequate supplies of clean water, antibiotic resistance, the variable efficacies of vaccine preparations, and the increased regional movements of large numbers of migrant workers. In relation to effective surveillance and the development of rational control strategies for this important human disease, the availability of detailed and accurate data related to the molecular epidemiology of *S.* Typhi is crucial. Therefore, specific and reliable epidemiological markers for *S.* Typhi are required. A number of strain typing methods have been developed for *S.* Typhi. The classical phenotypic methods are hampered with regard to practicability and poor discrimination and therefore have been complemented by the more sensitive DNA-based, molecular techniques. However, there is still a lack of methods with the right combination of rapidity, ease of use, reproducibility, and discriminatory power for typing of *S.* Typhi.

5.1 Comparison of ResAP-PCR, Rep-PCR, and VNTR

This study explore a comparative molecular subtyping of *S.* Typhi strains using three PCR-based methods of resAP-PCR, rep-PCR and VNTR analysis. For the evaluation of each typing method based on the principles of typeability, reproducibility, discriminatory power, ease of performance, 10 selected strains from either sporadic and outbreaks typhoid cases of different years (1998 to 2007) were considered. Table 5.1 summarize the patterns produced for these strains by three typing methods used in this study.

In term of typeability, all *S.* Typhi strains were typable by resAP-PCR (*Hae*III and *Alu*I digested DNA), rep-PCR and VNTR in assigning a defined type to each strain tested. In terms of reproducibility, stable and reproducible patterns were confirmed for all typing methods, except resAP-PCR of *Hae*III digested *S.* Typhi DNA showing poor reproducibility due to differences in the number of bands and intensities of the whole patterns obtained in each repeated assay. ResAP-PCR (*Alu*I digested *S.* Typhi DNA) of 9 bands, resAp-PCR (*Hae*III digested *S.* Typhi DNA) of 12 bands, rep-PCR patterns of 20 bands, and VNTR of 4 bands were observed.

A number of problems have been reported for AP-PCR assays that contribute to a lack of reproducibility and standardization. Since the primers are not directed against any particular genetic locus, many of the priming events are the result of imperfect hybridization between the primer and the target site. Thus, the amplification process is extremely sensitive to slight changes in the annealing temperature which can lead to variability in the banding patterns. The use of empirically designed primers, each with its own optimal reaction conditions and reagents, also makes standardization of the technique difficult (Arbeit *et al*., 1994; Meunier and Grimont, 1993; Welsh and McClelland, 1990). Moreover, arbitrary amplification protocols could be affected by other factors, which include DNA extraction methods, ratio of DNA template concentration to primer concentration, batch-to-batch variation in primer synthesis, Mg^{2+} concentration, PCR conditions, model of thermocycler used, and supplier and concentration of *Taq*DNA polymerase. Most of these problems can be overcome by optimizing the reaction conditions for each organism analyzed (Penner *et al*., 1993; Berg *et al*., 1994).

Table 5.1: Comparison of 10 selected *S.* Typhi strains for the patterns produced by resAP-PCR, rep-PCR, and VNTR.

Strain Code/ Year of Isolation	ResAPs	REPs	VNTRs	Allelic profiles at VNTR loci			
				TR1	TR2	TR3	TR5
TP34/98	ResAP1	REP1	VNTR1	06	19	01	03
TP247/99	ResAP1	REP1	VNTR2	05	29	02	03
TP2/00	ResAP1	REP1	VNTR3	09	20	03	03
TP38/01	ResAP1	REP1	VNTR4	12	10	02	03
TP41/02	ResAP1	REP1	VNTR5	12	17	02	03
TP323/03	ResAP1	REP1	VNTR6	11	18	02	03
TP85/04	ResAP1	REP1	VNTR7	12	15	02	03
ST156/05	ResAP1	REP1	VNTR 8	12	19	02	03
ST16/06	ResAP1	REP1	VNTR9	13	16	02	03
ST014/07	ResAP1	REP1	VNTR10	12	00	02	03

In terms of discriminatory, rep-PCR lacked discriminatory ability showing homogenous patterns (REP1) for *S.* Typhi strains tested in this study (Table 5.1). Such substantial similarity among the rep-PCR profiles suggest that limited diversity exists at number and site of repetitive sequences among *S.* Typhi strains studied. Several findings were also noted that rep-PCR has not been useful in differentiating *Salmonella* strains. Hermans *et al*. (1996) analysed *S.* Typhi isolates by rep-PCR using primer combination ERIC1R-ERIC2, but DNA polymorphism was not obtained from strains of different geographical origins in their study. In a similar study, the use of a single ERIC primer by Gruner *et al*. (1997) gave disappointing results, being unable to discriminate among non-related *S.* Typhi isolates. In other *Salmonella* serotypes such as *S.* Dulbin, Kerouanton *et al*. (1996) reported that the REP-PCR and ERIC-PCR individually could

not differentiate the strains. Similar findings were also noted for ERIC-PCR analysis of *S.* Enteritidis giving one fingerprint (Millemann *et al.*, 1996). It is suggested that the application of both REP and ERIC-PCR to samples to be typed increases the discriminatory power over that of either technique used alone.

Similarly, resAP-PCR assays using STHae8 and STAlu9 primers were not able to generate DNA polymorphism among strains (Table 5.1), and therefore clonality could be deduced for all strains. Since the discriminatory power of these genotyping assays was poor among the nonrelated strains, we conclude that the usefulness of the individual methods for molecular typing of *S.* Typhi is questionable. In contrast, multiplex VNTR assay was highly discriminative in identifying 10 distinct VNTR patterns among selected 10 *S.* Typhi strains (Table 5.1). The discrimination was especially pronounced at TR1 and TR2 loci by assigning 6 and 9 VNTR types respectively while only 3 VNTR types at TR3 locus and 1 type at TR4 locus were observed (Table 5.1). In terms of ease of performance, all three methods were simple to perform although careful attention must be given to the preparation of template, master mixes and agarose gels to ensure reproducibility and ease of gel to gel comparison. All methods were cost-effective, except VNTR assay due to the costs for DNA sequencing.

5.2 Variable-Number Tandem Repeat (VNTR) Analysis

5.2.1 PCR and Sequence Analysis of VNTR Loci

Multiplex-PCR-based VNTR analysis using a combination of TR primers displayed a wide range of allelic variations at TR1, TR2, TR3, and TR5 loci. Length polymorphisms were especially pronounced for TR1 and TR2. However, the application of conventional gel electrophoresis met several limitations to determine the repeat copy

numbers due to the existence of alleles with unexpected size, incompatible with an exact number of repeats. This is the case, for example, for the strains TP90/04 and TP35/02 which were confirmed by sequencing to be identical in number of repeats at all four loci (see Table 4.1), were found to generate distinct amplicon sizes for TR3 locus. In addition, amplicons of the different alleles which differed by very few changes of repeat copy number could not be separated in size by agarose gel electrophoresis. The use of another approach, such as polyacrylamide gel or capillary electrophoresis, which offers higher degrees of resolution, would probably solve this issue, albeit at a higher overall cost.

The VNTR analysis based on sequencing data confirmed the presence of VNTRs between strains and provided reliable and adequate results for the repeat copy numbers at each locus. The results confirm that the sequencing of the TR1 amplicons would not be necessary for subsequent strain typing attempts. Sequencing of TR2 and TR3 might be needed, since the length polymorphism of these amplicons could be the result of various deletions and/or insertions at flanking regions. However, given the high copy number variability among strains at the TR1 locus, the sequencing of TR2 and TR3 would be quite an infrequent event, since length polymorphisms at TR1 is varied enough for strain separation.

All three VNTR loci are found in noncoding regions approximately 200 to 700 bp upstream of hypothetical open reading frames. TR1 is adjacent to an open reading frame that codes for a hypothetical protein with hydrophobic membrane-spanning regions that is highly homologous to the *Escherichia coli* YedE putative permease protein (Liu *et al.*, 2003). VNTR copy number variations in the promoter region or within the coding

regions of virulence genes have been documented to have an effect on a pathogen's ability to adapt to a hostile host environment (van Belkum, 1999).

5.2.2 Allelic Diversity of VNTR Loci

The allelic diversity, which reflects the value of a VNTR locus for the purpose of typing, was studied based on number of alleles and frequency. It was observed that the larger the repeat array in *S.* Typhi CT18, the greater the allelic diversity ($R^2 = 0.57$) and number of alleles ($R^2 = 0.87$) among strains (Figure 5.1). This correlation has also been observed previously in *F. tularensis, B. anthracis, B. burgdorferi* and *Yersinia pestis* (Farlow *et al*., 2001; Keim *et al*., 2000; Farlow *et al*., 2002; Klevytska *et al*., 2001). For example, the TR2 locus with the repeat copy number of 27 in strain CT18, exhibited 19 alleles with allelic diversity of 0.93 while the TR5 locus with a copy number of 5 in strain CT18, exhibited only two alleles with allelic diversity of 0.04 among *S.* Typhi strains studied (Table 4.2). VNTR loci with high diversity value, such as TR2 (D = 0.93), have great discriminatory power for identification of genetically highly homogenous strains. In contrast, VNTR loci with low diversity value, such as TR5 (D = 0.04), may be applied for species identification and evolutionary analysis. Predicting and understanding of diversity level of VNTR loci provides greatest power for selection in future studies.

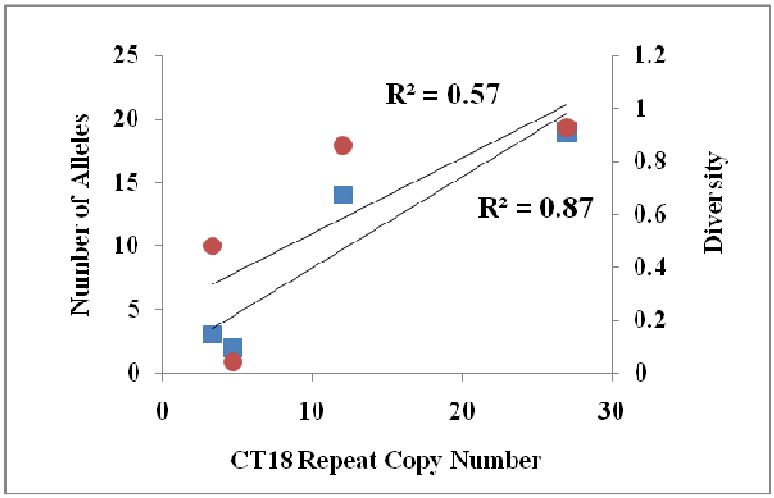

Figure 5.1: Correlation between repeat copy number and *D* values. The *S.* Typhi strain CT18 repeat copy number (Table 3.4) was compared with the *D* value and the total observed allele numbers at each VNTR locus. A square sign indicates total observed allele number versus the CT18 repeat copy number at an individual VNTR locus. A circle sign indicates the calculated allelic diversity versus the CT18 repeat copy number at an individual VNTR locus.

5.2.3 Epidemiological Analysis and Genetic Diversity

The *S.* Typhi strains used in this study were obtained from sporadic cases and three well-defined typhoid outbreaks in 1998, 2005 and 2007 in Kelantan, Malaysia. The sporadic strains mostly showed distinctly different VNTR patterns although some exceptions were also observed. For example, strains from similar geographical areas, TP78/04 and TP 37/06 have the same VNTR profile (MY4). Similarly, two individual strains TP90/04 and TP35/02 were found to share identical profile (MY5) although they were obtained from different research centres. The results suggest that multiple clones of *S.* Typhi are endemic to Malaysia and coexist simultaneously, causing sporadic cases of typhoid fever throughout the years. In contrast, the 1998 and 2007 typhoid outbreaks seem to be caused by a single clone or very closely related clones with very similar VNTR patterns in each outbreak. The 1998 outbreak strains comprised of two strains TP34/98 and TP46/98 having identical VNTR profile (MY28) and three other strains (TP43/98, TP41/98, and TP40/98) showing different profiles with trivial allelic

differences of 1-2 repeat units. The 2007 outbreak strains (ST314/07, ST334/07, ST4738/07, and ST4571/07) share identical VNTR profile (MY24) suggesting that this outbreak belong to a single clone. However, analysis of five individual strains from 2005 typhoid outbreak revealed greater allelic differences showing five different VNTR genotypes. The strain ST189/05 was found to have no repeat unit at TR2 locus while the other strains (ST208/05, ST117/05, ST156/05, and ST142/05) have 18 and 19 repeat units. In addition, two strains ST208/05 and ST189/05 have no amplification product for TR1 locus (null allele). However, all these strains were found to have deletions at TR3 flanking regions (see Table 4.1). The strain ST142/05 share identical VNTR profiles with the sporadic strain TP5/01 (MY39) as originated from identical geographic area. The strain ST117/05 is also similar in profile with the sporadic strain TP323/03 (MY36) although they were obtained from different research centres. It can be clearly concluded that 2005 outbreak relates to different *S.* Typhi subtypes.

The VNTR typing assay showed that a significant genetic heterogeneity exists at TR1, TR2 and TR3 loci among *S.* Typhi strains from Malaysia. Similarly, Liu *et al.* (2003) found substantial heterogeneity at these VNTR loci among *S.* Typhi strains within and among Asian countries. Previous studies using other genotypic markers also demonstrated that *S.* Typhi contains multiple genetic variants. Pang (1998) proposed that significant genetic diversity exists among *S.* Typhi strains globally. Nair *et al*. (1994) reported that many PFGE types are circulating in Asia. A Malaysian study by Thong *et al*. (1994) using PFGE analysis revealed that individual outbreaks were associated with closely related PFGE patterns, whereas strains from sporadic cases were very diverse. Analysis of ribosomal RNA gene restriction patterns also made similar conclusion showing genetic heterogeneity among *S.* Typhi strains in Malaysia (Thong *et*

al., 2000). Work on 120 *S.* Typhi isolates from Southeast Asia (Malaysia, Indonesia and Thailand) by PFGE reported that considerable genetic heterogeneity exists among individual strains (Thong *et al*., 1995). Franco *et al.* (1992) also found genetic heterogeneity in 69 Indonesian and Peruvian isolates by using envelope protein profiles and chromosomal restriction endonuclease digestion patterns. A study of Chilean strains isolated between 1977 and 1986 revealed multiple *S.* Typhi ribotypes, suggesting that there were multiple sources of infection due to different strains being in circulation (Fica *et al*., 1996). Work on 73 Vietnamese and 217 Hong Kong strains using plasmid profile analysis, plasmid fingerprinting, ribotyping, and total-DNA fingerprinting revealed a high level of genetic heterogeneity within each country, as well as among the countries (Ling *et al*., 2000). The VNTR findings from this study are therefore in agreement with these reports.

The VNTR analysis has been successfully used in the characterization of various pathogenic bacteria. More recently, multiple-locus VNTR analysis, a scaled up version of VNTR amplification, has been used to increase discriminative power for differentiating among isolates from genetically highly homogenous bacterial species, such as *Y. pestis* (Klevytska *et al*., 2001), *B. anthracis* (Keim *et al*., 2000) and *F. tularensis* (Farlow *et al*., 2001). To our knowledge, PCR-based VNTR profiling has not been reported for studying the relationship between the *S.* Typhi strains in Malaysia. This highly versatile method can be further improved by using primers tagged with fluorescent dyes, allowing accurate sizing of amplicons after electrophoresis on an automated DNA sequencer. New primers can also be added to increase the discriminative power.

The high degree of reproducibility and discriminative power of VNTR multiplex PCR method suggests that it could be used for both comparative typing and library typing. Comparative typing methods are used for outbreak investigations with the aim of segregating strains (Struelens *et al.*, 1998). These methods must therefore be highly typeable and discriminative. Library typing methods are used for epidemiological surveillance and hence must be highly reproducible within and between laboratories for an extended period. These techniques should also have some standard type nomenclature for easy comparison of results. The VNTR based multiplex PCR used in this study fulfils these requirements by giving simple, fast, discriminative, and reproducible results.

CHAPTER 6.0:

CONCLUSION

CHAPTER 6.0: CONCLUSION

In conclusion, Restricted AP-PCR and rep-PCR analysis were of limited value for subtyping of *S.* Typhi strains due to the poor reproducibility and discriminatory power. The results revealed that resAP-PCR analysis of *Alu*I digested *S.* Typhi DNA using STAlu9 primer demonstrated low polymorphism among *S.* Typhi strains while resAP-PCR analysis of *Hae*III digested *S.* Typhi DNA using STHae8 primer is hampered by both poor reproducibility and discriminatory power. Similarly, rep-PCR analysis incorporating REP-F and REP-R primers was not able to generate DNA polymorphisms among *S.* Typhi strains since all strains shared the same predominant banding profile. In contrast, VNTR analysis using four TR primers in this study was highly discriminative in identifying 42 distinct VNTR profiles from 50 strains. The VNTR assay was robust and gave identical results when repeated. DNA sequencing of the amplified VNTR regions substantiated the presence of VNTRs in these strains. The VNTR analysis displayed diversity based on four VNTR loci studied with total allele numbers ranging from 2 to 19 and Nei's diversity (*D*) values ranging from 0.04 to 0.93. The results revealed that the copy number of repeats in CT18 strain of *S.* Typhi was highly correlated with the *D* value (R^2 = 0.57) and number of alleles (R^2 = 0.87) observed across diverse strains. The VNTR analysis showed that a significant genetic heterogeneity exists at TR1, TR2 and TR3 loci among *S.* Typhi strains from Malaysia. Analysis of the VNTR patterns indicated that *S.* Typhi strains obtained from sporadic cases were much more heterogeneous than those obtained during outbreaks of typhoid fever. The multiplex-PCR-based VNTR profiling used in this study provided a simple, rapid, reproducible, and highly discriminative molecular tool for strain subtyping and epidemiological analysis of *S. enterica* serovar Typhi strains.

BIBLIOGRAPHY

Adair, D. M., Worsham, P. L., Hill, K. K., Klevytska, A. M., Jackson, P. J., Friedlander, A. M., & Keim, P. (2000). Diversity in a variable-number tandem repeat from *Yersinia pestis. J. Clin. Microbiol.* 38: 1516-1519.

Arbeit, R. D., Maslow, J. N., & Mulligan, M. E. (1994). Polymerase chain reaction-mediated genotyping in microbial epidemiology. *Clin. Infect. Dis.* 18: 1018-1019.

Arbeit, R. D. (1995). Laboratory procedures for the epidemiologic analysis of microorganisms, p. 190-208. In: Murray P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C., and Yolken, R. H. (ed.), Manual of Clinical Microbiology, 6th edition American Society for Microbiology, Washington, D.C.

Berg, D. E., Akopyants, N. S., & Kersulyte, D. (1994). Fingerprinting microbial genomes using the RAPD or AP-PCR method. *Methods Mol. Cell. Biol*. 5: 13-24.

Betancor, L., Felipe, S. F., Martinez, A., Pereira, M., Algorta, G., Rodríguez, M. A., Vignoli, R., & Chabalgoity, J. A. (2003). Random amplified polymorphic DNA and phenotypic analysis of *Salmonella enterica* Serovar Enteritidis isolates collected from humans and poultry in Uruguay from 1995 to 2002. *J. Clin. Microbiol.* 42(3): 1155-1162.

Beyer, W., Mukendi, F. M., Kimmig, P., & Bohm, R. (1998). Suitability of repetitive-DNA sequence-based PCR fingerprinting for characterizing epidemic isolates of *Salmonella enterica* serovar Saintpaul. *J. Clin. Microbiol.* 36: 1549-1554.

Bhutta, Z. A. (2006). Current concepts in the diagnosis and treatment of typhoid fever. *BMJ*. 333(7558): 78-82.

Bianca, R. Q., Nilma, C. L., Eliane, M. F. R., Érica, L. F., & Ernesto, H. (2002). Conventional and molecular typing of *S. typhi* strains from Brasil. *Rev. Inst. Med. Trop. Sao Paulo.* 44(6): 315-319.

Bikandi, J., Millàn, R. S., Rementeria, A., & Garaizar, J. (2004). In Silico analysis of complete bacterial genomes: PCR, AFLP-PCR and endonuclease restriction. *Bioinformatics.* 20(5): 798-799.

Blanc-Potard, A. B., & Groisman, E. A. (1997). The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. *EMBO J.* 16: 5376-5385.

Boxrud, D., Pederson-Gulrud, K., Wotton, J., Medus, C., Lyszkowicz, E., Besser, J., & Bartkus, J. M., (2007). Comparison of multiple-locus variable number tandem repeat analysis, pulsed-field gel electrophoresis, and phage typing for subtype analysis of *Salmonella enterica* serotype Enteritidis. *J. Clin. Microbiol*. 45: 536-543.

Brenner, F. W., & McWhorter-Murlin, A. C. (1998). Identification and serotyping of *Salmonella*. Centers for Disease Control and Prevention, Atlanta, Ga.

Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R., & Swaminathan, B. (2000). *Salmonella* Nomenclature. *J. Clin. Microbiol.* 38: 2465-2467.

Burr, M. K., Josephson, K. L., & Pepper, I. L. (1998). An evaluation of ERIC PCR and AP PCR fingerprinting for discriminating *Salmonella* serotypes. *Lett. Appl. Microbiol*. 27: 24-30.

Busch, U. & Nitschko, H. (1999). Methods for the differentiation of microorganisms. *J. Chromatogr. B.* 722: 263-278.

Bzymek, M., & Lovett, S. T. (2001). Instability of repetitive DNA sequences: the role of replication in multiple mechanisms. *Proc. Natl. Acad. Sci. USA*. 98: 8319-8325.

Carraminana, J. J., Humbert, F., Ermel, G., & Colin, P. (1997). Molecular epidemiological investigation of *Salmonella typhimurium* strains related to an egg-borne outbreaks. *Res. Microbiol.* 7: 633-636.

Chen, L. M., Kaniga, K., & Galán, J. E. (1996). *Salmonella* spp. are cytotoxic for cultured macrophages. *Mol. Microbiol.* 21: 1101-1115.

Chinh, N. T., Parry, C. M., Ly, N. T., Ha, H. D., Thong, M. X., Diep, T. S., Wain, J., White, N. J., & Farrar, J. J. (2000). A randomized controlled comparison of azithromycin and ofloxacin for treatment of multidrug-resistant or nalidixic acid-resistant enteric fever. *Antimicrob. Agents Chemother.* 44: 1855-1859.

Cho, J. C. & Kim, S. J. (1999). Green fluorescent protein-based direct viable count to verify a viable but nonculturable state of *Salmonella typhi* in environmental samples. *J. Microbiol. Methods.* 36: 227-235.

Connerton, P., Wain, J., Hien, T. T., Ali, T., Parry, C. M., Chinh, N. T., Vinh, H., Ho, V. A., Diep, T. S., Day, N. P. J., White, N. J., Dougan, G., & Farrar, J. J. (2000). Epidemic typhoid in Vietnam: molecular typing of multiple-antibiotic-resistant

Salmonella enterica serotype Typhi from four outbreaks. *J. Clin. Microbiol*. 38: 895-897.

Collazo, C. M., & Galán, J. E. (1997). The invasion-associated type III protein secretion in *Salmonella. Gene.* 192: 51-59.

Crump, J. A., Luby, S. P., & Mintz, E. D. (2004). The global burden of typhoid fever. *Bull. World Health Organ.* 82: 346-353.

Cunha, B. A. (2004).Osler on typhoid fever: differentiating typhoid from typhus and malaria. *Infect. Dis. Clin. N. Am.* 18: 111-125.

Dolman, C. E., & Wolfe, R. J. (2003). Suppressing the diseases of animals and man: Theobald Smith, microbiologist. Cambridge, MA: Boston Medical Library in the Francis A. Countway Library of Medcine;. Xii, 691p.

Ellermeier, C. D., & Slauch, J. M. (2006). The genus *Salmonella*, p. 123-158. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. H., and Stackebrandt, E. (ed.), The prokaryotes, 3rd ed. Springer, New York, NY.

Everest, P., Wain, J., Roberts, M., Rook, G., & Dougan, G. (2001). The molecular mechanisms of severe typhoid fever. *Trends Microbiol.* 9: 316-319.

Farlow, J., Smith, K. L., Wong, J., Abrams, M., Lytle, M., & Keim, P. (2001). *Francisella tularensis* strain typing with multiple-locus, variable-number tandem repeat analysis. *J. Clin. Microbiol.* 39: 3186-3192.

Farlow, J., Postic, D., Smith, K. L., Jay, Z., Baranton, G., & Keim, P. (2002). Strain typing of *Borrelia burgdorferi*, *Borrelia afzelii*, and *Borrelia garinii* by using multiple-locus variable-number tandem repeat analysis. *J. Clin. Microbiol*. 40: 4612-4618.

Farmer, J. J., III, McWhorter, A. C., Brenner, D. J., & Morris, G. K. (1984). The *Salmonella-Arizona* group of *Enterobacteriaceae*: nomenclature, classification, and reporting. *Clin. Microbiol. Newsl*. 6: 63-66.

Fica, A. E., Prat-Miranda, S., Fernandez-Ricci, A., D'Ottone, K., & Cabello, F. C. (1996). Epidemic typhoid in Chile: analysis by molecular and conventional methods of *Salmonella typhi* strain diversity in epidemic (1977 and 1981) and nonepidemic (1990) years. *J. Clin. Microbiol.* 34: 1701-1707.

Foxman, B., Zhang, L., Koopman, J. S., Manning, S. D., & Marrs, C. F., (2005). Choosing an appropriate bacterial typing technique for epidemiologic studies. Epidemiologic Perspectives & Innovations. 2: 10.

Franco, A., Gonzalez, C., Levine, O. S., Lagos, R., Hall, R. H., Hoffman, S. L., Moechtar, M. A., Gotuzzo, E., Levine, M. M., & Hone, D. M. (1992). Further consideration of the clonal nature of *Salmonella typhi*: evaluation of molecular and clinical characteristics of strains from Indonesia and Peru. *J. Clin. Microbiol.* 30: 2187-2190.

Gasem, M. H., Dolmans, W. M., Keuter, M. M., & Djokomoeljanto, R. R. (2001). Poor food hygiene and housing as risk factors for typhoid fever in Semarang, Indonesia. *Trop. Med. Int. Health.* 6: 484-490.

Georghiou, P. R., Doggett, A. M., Kielhofner, M. A., Stout, J. E., Watson, D. A., Lupski, J. R., & Hamill, R. J. (1994). Molecular fingerprinting of *Legionella* species by repetitive element PCR. *J. Clin. Microbiol*. 32: 2989-2994.

Gilson, E., Perrin D., & Hofnung, M. (1990). DNA polymerase I and a protein complex bind specifically to *E. coli* palindromic unit highly repetitive DNA: implications for bacterial chromosome organization. *Nucleic Acids Res.* 18: 3941-3952.

Gopalakrishnan, V., Sekhar, W. Y., Soo, E. H., Vinsent, R. A., & Devi, S. (2002). Typhoid fever in Kuala Lumpur and a comparative evaluation of two commercial diagnostic kits for the detection of antibodies to *Salmonella* typhi. *Singapore Med. J*. 43(7): 354-358.

Gruner, E., Flepp, M., Gabathuler, U., Thong, K. L., & Altwegg, M. (1997). Outbreak of typhoid fever in a non-endemic area: comparison of three molecular typing methods. *J. Microbiol. Methods.* 28: 179-185.

Gulati, S., Marwaha, R. K., Prakash, D., Ayyagari, A., Singhi, S., Kumar, L., Singhi, P., & Walia, B. N. (1992). Multi-drug-resistant *Salmonella* typhi: a need for therapeutic reappraisal. *Ann. Trop. Paediatr.* 12: 137-141.

Hampton, M. D., Ward, L. R., Rowe, B., & Threlfall, E. J. (1998). Molecular Fingerprinting of Multidrug-Resistant *Salmonella enterica* serotype Typhi. *Emerg. Infect. Dis*. 4(2): 317-320.

Hashimoto, Y., Li, N., Yokoyama, H. & Ezaki, T. (1993). Complete nucleotide sequence and molecular characterization of ViaB region encoding Vi antigen in *Salmonella typhi*. *J. Bacteriol*. 175: 4456-4465.

Hermans, P. W. M., Saha, S. K., van Leeuwen, W. J., Verbrugh, H. A., van Belkum, A., & Goessens, W. H. F. (1996). Molecular typing of *Salmonella typhi* strains from Dhaka (Bangladesh) and development of DNA probes identifying plasmid-encoded multidrug-resistant isolates. *J. Clin. Microbiol.* 34: 1373-1379.

Heyndrickx, M., Rijpens, N., & Herman, L. (2001). Molecular detection and typing of foodborne bacterial pathogens: a review, p. 193-238. In: Durieux A., and Simon, J. P. (ed.), Trends in biotechnology. Applied microbiology. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Hickman-Brenner, F. W., Farmer, J. J., III & Regional Centers for *Salmonella typhi* Bacteriophage Typing in the United States (1983). Bacteriophage types of *Salmonella typhi* in the United States from 1974 through 1981. *J. Clin. Microbiol.* 17: 172-174.

Hilton, A. C., Banks, J. G., & Penn, C. W. (1997). Optimization of RAPD for fingerprinting of *Salmonella*. *Lett. Appl. Microbiol*. 24: 243-248.

Hirose, K., Tamura, K., Sagara, H., & Watanabe, H. (2001). Antibiotic susceptibility for *Salmonella enterica* serovar Typhi and *S. enterica* serovar Paratyphi A isolated from patients in Japan. *Antimicrob. Agents Chemother*. 45: 956-958.

Hoffman, S. L. (1991). Typhoid fever, p. 344-358. In: Strickland, G. T. (ed.). Hunter's Tropical Medicine. 7th edn. Philadelphia, WB Saunders.

Holt, J. G. (1994). *Salmonella lignieres*, p. 101-119. In: Holt, J. G. (ed.): The shorter Bergey's manual of determinative bacteriology, 8th edition. Williams and Wilkins Company, Baltimore.

Hornick, R. B., Greisman, S. E., Woodward, T. E., DuPont, H. L., Dawkins, A. T., & Snyder, M. J. (1970). Typhoid fever: pathogenesis and immunological control. *N. Engl. J. Med.* 283(13): 686-691.

House, D., Wain, J., Ho., V. A., Diep, T. S., Chinh, N. T., Bay, P. V., Vinh, H., Duc, M., Parry, C. M., Dougan, G., White, N. J., Hien, T. T., & Farrar, J. J. (2001). Serology of typhoid fever in an area of endemicity and its relevance to diagnosis. *J. Clin. Microbiol*. 39: 1002-1007.

Hulton, C. S. J., Higgins, C. F., & Sharp, P. M. (1991). ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium*, and other enterobacteria. *Mol. Microbiol.* 5: 825-834.

Ismail, T. F. (2006). Rapid diagnosis of typhoid fever. *Indian J. Med. Res*. 123(4): 489-492.

Ivanoff, B., Levine, M. M., & Lambert, P. H. (1994). Vaccination against typhoid fever: Present status. *Bull. World Health Organ*. 72(6): 957-971.

Ivanoff, B. (1998). Typhoid fever: a global overview. *Med. J. Indonesia.* 7: 5-8.

Johnson, J. R., & Clabots, C. (2000). Improved repetitive-element PCR fingerprinting of *Salmonella enterica* with the use of extremely elevated annealing temperatures. *Clin. Diagn. Lab. Immun*. 7: 258-264.

Jesudason, M., Esther, E., & Mathai, E. (2002). Typhidot test to detect IgG and IgM antibodies in typhoid fever. *Indian J. Med. Res.* 116: 70-72.

Jimenez-Lucho, V., & Foulds, J. (1990). Heterogeneity of lipopolysachharide phenotypes among *Salmonella typhi*. *J. Infect. Dis.* 162: 763-764.

Kalra, S. P., Naithani, N., Mehta, S. R., & Swamy, A. J. (2003). Current trends in the management of typhoid fever. *Med. J. Armd. Forces. Ind.* 59: 130-135.

Kariuki, S., Gilks, C., Revathi, G., & Hart, C. A. (2000). Genotypic analysis of multidrug-resistant *Salmonella enterica* serovar typhi, Kenya. *Emerg. Infect. Dis.* 6:649-651.

Keim, P., Price, L. B., Klevytska, A. M., Smith, K. L., Schupp, J. M., Okinaka, R., Jackson, P. J., & Hugh-Jones, M. E. (2000). Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. *J. Bacteriol*. 182: 2928-2936.

Kerouanton, A., Brisabois, A., Grout, J., & Picard, B. (1996). Molecular epidemiological tools for *Salmonella dulbin* typing. *FEMS. Immonol. Med. Micobiol*. 14: 25-29.

Keys, C., Kemper, S., & Keim, P. (2005). Highly diverse variable number tandem repeat loci in the *E. coli* O157:H7 and O55:H7 genomes for high resolution molecular typing. *J. Appl. Microbiol.* 98: 928-940.

Klevytska, A. M., Price, L. B., Schupp, J. M., Worsham, P. L., Wong, J., & Keim, P. (2001). Identification and characterization of variable-number tandem repeats in the *Yersinia pestis* genome. *J. Clin. Microbiol.* 39: 3179-3185.

Kossaczka, Z., Lin, F. C., Ho, V. A., Thuy, N. T. T., Bay, P. V., Thanh, T. C., Khiem, H. B., Trach, D. D., Karpas, A., Hunt, S., Bryla, D. A., Schneerson, R., Robbins, J. B., & Szu, S. C. (1999). Safety and immunogenicity of Vi conjugate vaccines for typhoid fever in adults, teenagers, and 2 to 4 year-old children in Vietnam. *Infection and Immunity*. 67: 5806-5810.

Kubota, K., Barrett, T. J., Ackers, M. L., Brachman, P. S., & Mintz, E. D. (2005). Analysis of *Salmonella enterica* serotype Typhi pulsed-field gel electrophoresis patterns associated with international travel. *J. Clin. Microbiol.* 43: 1205-1209.

Le Fle`che, P., Hauck, Y., Onteniente, L., Prieur, A., Denoeud, F., Ramisse, V., Sylvestre, P., Benson, G., Ramisse, F., & Vergnaud, G. (2001). A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. *BMC Microbiol*. 1: 2.

Le Fle`che, P., Fabre, M., Denoeud, F., Koeck, J. L., & Vergnaud, G. (2002). High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. *BMC Microbiol*. 2: 37.

Le Monor, L. (1981). The genus *Salmonella*, p. 1148-1159. In: Starr, M. P., Stoup, H., Trupper, H. G., (ed.): The prokaryotes. A handbook on habitat, isolation, and identification of bacteria. Vol. 2. Berlin, Springer-Verlag.

Levine M. M. (1998). Typhoid fever, p. 839-858. In: Evans AS, Brachman PS, (ed.). Bacterial Infections of Humans. Epidemiology and Control. 3rd ed. New York, Plenum Medical Book Company.

Levine, M. M. (1999). Typhoid fever vaccines, p. 781-814. In: Plotkin S. A., Orenstein W. A. (ed.): Vaccines 3rd ed. Saunders Company.

Lim, P. L., Tam, F. C., Cheong, Y. M., & Jegathesan, M. (1998). One-step 2-minute test to detect typhoid-specific antibodies based on particle separation in tubes. *J. Clin. Microbiol*. 36(8): 2271-2278.

Lin, F. Y., Vo, A. H., Phan, V. B., Nguyen T. T., Bryla, D., Tran, C. T., Ha, B. K., Dang, D. T., & Robbins, J. B. (2000). The epidemiology of typhoid fever in the Dong Thap Province, Mekong Delta region of Vietnam. *Am. J. Trop. Med. Hyg.* 62(5): 644-648.

Lindstedt, B. A., Heir, E., Gjernes, E., & Kapperud, G. (2003). DNA fingerprinting of *Salmonella enterica* subsp. *enterica* serovar Typhimurium with emphasis on phage type DT104 based on variable number of tandem repeat loci. *J. Clin. Microbiol*. 41: 1469-1479.

Lindstedt, B. A., Vardund, T., & Kapperud, G. (2004). Multiple-locus variable-number tandem-repeats analysis of *Escherichia coli* O157 using PCR multiplexing and multi-colored capillary electrophoresis. *J. Microbiol. Methods.* 58: 213-222.

Ling, J. M., Koo, I. C., Kam, K. M., & Cheng, A. F. (1998). Antimicrobial susceptibilities and molecular epidemiology of *Salmonella enterica* serotype Enteritidis strains isolated in Hong Kong from 1986 to 1996. *J. Clin. Microbiol*. 36: 1693-1699.

Ling, J. M., Lo, N. W., Ho, Y.M., Kam, K. M., Hoa, N. T., Phi, L. T., & Cheng, A. F. (2000). Molecular methods for the epidemiological typing of *Salmonella enterica* serotype Typhi from Hong Kong and Vietnam. *J. Clin. Microbiol.* 38: 292-300.

Liu, Y., Lee, M. A., Ooi, E. E., Mavis, Y., Tan, A. L., & Quek, H. H. (2003). Molecular typing of *Salmonella enterica* serovar Typhi isolates from various countries in Asia by a multiplex PCR assay on variable-number tandem repeats. *J. Clin. Microbiol*. 41: 4388-4394.

Lopez-Molina, N., Laconcha, I., Rementeria, A., Audicana, A., Perales, I., & Garaizar, J. (1998). Typing of *Salmonella enteritidis* of different phage types of PCR fingerprinting. *J. Appl. Microbiol*. 84: 877-882.

Luxemburger, C., Chau, M. C., Mai, N. L., Wain, J., Tran, T. H., Simpson, J. A., Le, H. K., Nguyen, T. T., White, N. J., & Farrar, J. J. (2001). Risk factors for typhoid fever in the Mekong delta, southern Vietnam: a case-control study. *Trans. R. Soc. Trop. Med. Hyg*. 95: 19-23.

Malorny, B., Junker, E., & Helmuth, R. (2008). Multi-locus variable-number tandem repeat analysis for outbreak studies of *Salmonella enterica* serotype Enteritidis. *BMC Microbiology.* 8: 84.

Mermin, J. H., Villar, R., Carpenter, J., Roberts, L., Samaridden, A., Gasanova, L., Lomakina, S., Bopp, C., Hutwagner, L., Mead, P., Ross, B., & Mintz, E. D. (1999). A massive epidemic of multidrug-resistant typhoid fever in Tajikistan associated with consumption of municipal water. *J. Infect. Dis.* 179: 1416-1422.

Meunier, J. R., & Grimont, P. A. D. (1993). Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Res. Microbiol*. 144: 373-379.

Millemann, Y., Lesage-Descauses, M. C., Lafont, J. P. & Chaslus-Dancla, E. (1996). Comparison of random amplified polymorphic DNA analysis and enterobacterial repetitive intergenic consensus-PCR for epidemiological studies of *Salmonella. FEMS. Immunol. Med. Microbiol*., 14: 129-134.

Mirold, S., Rabsch, W., Rohde, M., Stender S., Tschäpe, H., Rüssmann, H., Igwe, E., & Hardt, W. (1999). Isolation of a temperate bacteriophage encoding the type III effector protein SopE from an epidemic *Salmonella typhimurium* strain. *Proc. Natl Acad. Sci. USA*. 96: 9845-9850.

Mirza, S. H., Beeching, N. J., & Hart, C. A. (1996). Multi-drug resistant typhoid: a global problem. *J. Med. Microbiol.* 44: 317-319.

Mirza, S. H., Kariuki, S., Mamun, K. Z., Beeching, N. J., & Hart, C. A. (2000). Analysis of plasmid and chromosomal DNA of multi-drug resistant *Salmonella enterica* serovar Typhi from Asia. *J. Clin. Microbiol*. 38: 1449-1452.

Nair, S., Poh, C. L., Lim, Y. S., Tay, L., & Goh, K. T. (1994.) Genome fingerprinting of *Salmonella typhi* by pulsed-field gel electrophoresis for subtyping common phage types. *Epidemiol. Infect.* 113: 391-402.

Nair, S., Schreiber, E., Thong, K. L., Pang, T., & Altwegg, M. (2000). Genotypic characterization of *Salmonella typhi* by amplified fragment length polymorphism fingerprinting provides increased discrimination as compared to pulsed-field gel electrophoresis and ribotyping. *J. Microbiol. Methods.* 41: 35-43.

Navarro, F., Llovet, T., Echeita, M. A., Coll, P., Aladuena, A., Usera, M. A., & Prats, G. (1996). Molecular typing of *Salmonella enterica* serovar Typhi. *J. Clin. Microbiol.* 34: 2831-2834.

Naworth, P. P., & Stem, D. M. (1987). Tumor necrosis factor/cachectin-induced modulation of endothelial cell haemostatic properties. *Onkologie* 10: 254-258.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small sample of individuals. *Genetics*. 89: 583-590.

Newbury, S. F., Smith, N. H., Robinson, E. C., Hiles, I. D., & Higgins, C. F. (1987). Stabilization of translationally active mRNA by prokaryotic REP sequences. *Cell.* 48: 297-310.

Newton, C. R., & Krishna, S. (1998). Severe falciparum malaria in children: current understanding of pathophysiology and supportive treatment. *Pharmacol. Ther.* 79(1): 1-53.

Olive, D. M., & Bean, P. (1999). Principles and application of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* 37: 1661-1669.

Oteniente, L., Brisse S., Tassios, P. T., & Vergnaud, G. (2003). Evaluation of the polymorphism associated with tandem repeats for *Pseudomonas aeruginosa* strain typing. *J. Clin. Microbiol*. 41: 4991-4997.

Pang, T. (1998). Genetic dynamics of *Salmonella typhi:* diversity in molecular analysis of environmental and human isolates of clonality. *Trends Microbiol.* 6: 339-342.

Pang, T., Levine, M. M., Ivanoff, B., Wain, J., & Finlay, B. B. (1998). Typhoid fever: important issues still remain. *Trends Microbiol.* 6: 131-133.

Parkhill, J., Dougan, G., James, K. D., Thomson, N. R., Pickard, D., Wain, J., Churcher, C., Mungall, K. L., Bentley, S. D., Holden, M. T., Sebaihia, M., Baker, S., Basham, D., Brooks, K., Chillingworth, T., Connerton, P., Cronin, A., Davis, P., Davies, R. M., Dowd, L., White, N., Farrar, J., Feltwell, T., Hamlin, N., Haque, A., Hien, T. T., Holroyd, S., Jagels, K., Krogh, A., Larsen, T. S., Leather, S., Moule, S., O'Gaora, P., Parry, C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., & Barrell, B. G. (2001). Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature.* 413: 848-852.

Parry, C. M., Hien, T. T., Dougan, G., White, N. J., & Farrar, J. J. (2002). Typhoid fever. *N. Engl. J. Med.* 347: 1770-1782.

Parry, C. M. (2006). Epidemiological and clinical aspects of human typhoid fever. In: Mastroeni, P., and Maskell, D. (ed.): *Salmonella* Infections: Clinical, Immunological and Molecular Aspects. Cambridge University Press, England.

Pathmanathan, S. G., Cordona-Castro, N., Sanchez-Jimenez M. M., Correa-Ochoa, Puthucheary, S. D., & Thong, K. L. (2003). Simple and rapid detection of *Salmonella* strains by direct PCR amplification of the *hilA* gene. *J. Med. Microbiol.* 52: 773-776.

Penner, G. A., Bush, A., Wise, R., Kim, W., Domier, L., Kasha, K., Laroche, A., Scoles, G., Molnar, S. J., & Fedak, G. (1993). Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. *Applications.* 2: 341-345.

Persing, D. H. (1993). *In vitro* nucleic acid amplification techniques, p. 51-87. In: Persing, D. H., Smith, T., Tenover, F. C., White T. (ed.). *Diagnostic Molecular Microbiology and Applications. Washington*, DC: American Society for Microbiology.

Popoff, M. Y., & Le Minor, L. (1997). Antigenic formulas of the *Salmonella* serovars, 7th revision. World Health Organization Collaborating Centre for Reference and Research on *Salmonella*, Pasteur Institute, Paris, France.

Popoff, M. Y. (2001). Antigenic formulas of the *Salmonella* serovars, 8th revision. W.H.O. Collaborating Centre for Reference and Research on *Salmonella*. Institute Pasteur, Paris, France.

Popoff, M. Y., Bockemuhl, J., & Gheesling, L. L. (2003). Supplement 2001 (no. 45) to the Kauffmann-White scheme. *Res. Microbiol.* 154: 173-174.

Pourcel, C., Vidgop, Y., Ramisse, F., Vergnaud, G., & Tram, C. (2003). Characterization of a tandem polymorphism in *Legionella pneumophila* and its use for genotyping. *J. Clin. Microbiol*. 41: 1819-1826.

Pourcel, C., Andre´-Mazeaud, F., Neubauer, H., Ramisse, F., & Vergnaud, G. (2004). Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. *BMC Microbiol*. 4: 22.

Punjabi, N. H. (2000). Typhoid fever, p. 161-165. In: Conn, H. F. (ed.). Current Therapy. Latest Approved Methods of Treatment for the Practising Physicians. Philadelphia, WB Saunders.

Quintaes, B. R., Leal, N. C., Reis, E. M., & Hofer, E. (2004). Optimization of randomly amplified polymorphic DNA-polymerase chain reaction for molecular typing of *Salmonella enterica* serovar Typhi. *Rev. Soc. Bras. Med. Trop.* 37: 143-147.

Radu, S., Sahilah, A. M., Rusul, G., Samuel, L., Zuraini, M. I., & Nasreldin, E. H. (2000). Comparison of arbitrarily primed PCR, antibiotic resistance and plasmid profiling for differentiating *Salmonella enteritidis* isolated from fish. *Asian Fisheries Science.* 13: 13-20.

Ramisse, V., Houssu, P., Hernandez, E., Denoeud, F., Hilaire, V., Lisanti, O., Ramisse, F., Cavallo, J., & Vergnaud, G. (2004). Variable number of tandem repeats in *Salmonella enterica* subsp. *enterica* for typing purposes. *J. Clin. Microbiol*. 42: 5722-5730.

Rasschaert, G., Houf, K., Imberechts, H., Grijspeerdt, K., De Zutter, L., & Heyndrickx, M. (2005). Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of *Salmonella enterica* isolates. *J. Clin. Microbiol.* 43(8): 3615-3623.

Reeves, M. W., Evins, G. M., Heiba, A. A., Plikaytis, B. D., & Farmer, J. J. 3rd. (1989). Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis and proposal of *Salmonella bongori* comb. nov. *J. Clin. Microbiol.* 27(2): 313-320.

Rowe, B., Ward, L. R., & Threlfall, E. J. (1997). Multidrug-resistant *Salmonella typhi*: a worldwide epidemic. *Clin. Infect. Dis.* 24: 106-109.

Saha, S. K., Talukder, S. Y., Islam, M., & Saha, S. (1999). A highly ceftriaxone-resistant *Salmonella typhi* in Bangladesh. *Pediatr. Infect. Dis. J.* 18: 387.

Saha, M. R., Dutta, P., Palit, A., Dutta, D., Bhattacharya, U. M., & Bhattacharya, S. K. (2003). Incidence of typhoid fever in diverse age groups in Kolkata, India. *Jpn. J. Infect. Dis.* 56.

Sahilah, A. M. (2000). Molecular typing of *Salmonella weltevreden* and *Salmonella chincol* by pulsed-field gel electrophoresis (PFGE) and enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). *World J. Microbiol. Biotech*. 16(7): 621-624.

Sandvang, D., Aretrup, F. M., & Jensen, L. B. (1998). Characterization on integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS. Microbiol. Lett*. 160: 37-41.

Selander, R. K., Beltran, P., Smith, N. H., Helmuth, R., Rubin, F. A., Kopecko, D. J., Ferris, K., Tall, B. D., Cravioto, A., & Musser, J. M. (1990). Evolutionary genetic

relationships of clones of *Salmonella* serovars that cause human typhoid and other enteric fevers. *Infect. Immun.* 58: 2262-2275.

Sinha, A., Sazawal, S., Kumar, R., Sood, S., Reddaiah, V. P., Singh, B., Rao, M., Naficy, A., Clemens, J. D., & Bhan, M. K. (1999). Typhoid fever in children aged less than 5 years. *Lancet.* 354: 734-737.

Shanahan, P. M., Jesudason, M. V., Thomson, C. J., & Amyes, S. G. (1998). Molecular analysis of and identification of antibiotic resistance genes in clinical isolates of *Salmonella typhi* from India. *J. Clin. Microbiol.* 36: 1595-1600.

Shanahan, P. M., Karamat, K. A., Thomson, C. J., & Amyes, S. G. B. (2000). Characterization of multi-drug resistant *Salmonella typhi* from Pakistan. *Epidemiol. Infect.* 124: 9-16.

Shangkuan, Y. K., & Lin, H. C. (1998). Application of random amplified polymorphic DNA, analysis to differentiate different strains of *Salmonella typhi* and other *Salmonella* species. *J. Appl. Microbiol.* 85: 693-702.

Sharples, G. J., & Lloyd, R. G. (1990). A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. *Nucleic Acids Res.* 18: 6503-6508.

Sherburne, C. K., Lawley, T. D., Gilmour, M. W., Blattner, F. R., Burland, V., Grotbeck, E., Rose, D. J., & Taylor, D. E. (2000). The complete DNA sequence and analysis of R27, a large *Inc*HI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. *Nucleic Acids Res.* 28: 2177-2186.

Soto, S. M., Guerra, B., Gonzalez-Hevia, M. A., & Mendoza, M. C. (1999). Potential of three-way randomly amplified polymorphic DNA analysis as a typing method for twelve *Salmonella* serotypes. *Appl. Environ. Microbiol*. 65(11): 4830-4836.

Stern, M. J., Ames, G. F. L., Smith, N. H., Robinson, E. C., & Higgins, C. F. (1984). Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell.* 37: 1015-1026.

Struelens, M. J., De Gheldre, Y., & Deplano, A. (1998). Comparative and library epidemiological typing systems: outbreak investigations versus surveillance systems. *Infect. Control Hosp. Epidemiol.* 19: 565-569.

Taylor, D. E., Chumpitaz, J. C., & Goldstein, F. (1985). Variability of *Inc*HI1 plasmids from *Salmonella typhi* with special reference to Peruvian plasmids encoding resistance to trimethoprim and other antibiotics. *Antimicrob. Agents Chemother.* 28: 452-455.

Tenover, F. C., Arbeit, R. D., & Goering, R. V. (1997). How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infectious: A review for healthcare epidemiologists. *Infect. Control Hosp. Epidemiol.* 18: 426-439.

Thong, K. L., Cheong, Y. M., Puthucheary, S. D., Koh, C. L., & Pang, T. (1994). Epidemiologic analysis of sporadic *Salmonella typhi* isolates and those from outbreaks by pulsed-field gel electrophoresis. *J. Clin. Microbiol* 32: 1135-1141.

Thong, K. L., Puthucheary, S. D., Yassin, R. M., Sudarmono, P., Padmidewi, M., Soewandojo, E., Handojo, I, Sarasombath, S., & Pang, T. (1995). Analysis of *Salmonella typhi* isolates from Southeast Asia by pulsed-field gel electrophoresis. *J. Clin. Microbiol.* 33: 1938-1941.

Thong, K. L., Cordano, A. M., Yassin, R. M., & Pang, T. (1996a). Molecular analysis of environmental and human isolates of *Salmonella typhi*. *Appl. Env. Microbiol*. 62: 271-274.

Thong, K. L., Passey, M., Clegg, A., Combs, B. G., Yassin, R. M., & Pang, T. (1996b). Molecular analysis of isolates of *Salmonella typhi* obtained from patients with fatal and nonfatal typhoid fever. *J. Clin. Microbiol.* 34: 1029-1033.

Thong, K. L., Altwegg, M., & Pang, T. (2000). Comparative analysis of *Salmonella typhi* by rRNA gene restriction and phage typing. *Pakistan J. Biological Sciences.* 3(5): 738-739.

Thong, K. L., Elgar, S., Bhutta, Z., Rohani, Y., & Schreiber, E. (2003). Fluorescent Amplified Fragment Length Polymorphism and Pulsed-Field Gel Electrophoresis Analyses of Multidrug Resistant Salmonella enterica serotype Typhi from different geographical endemic regions in Asia. *Asia Pacific Journal of Molecular Biology & Biotechnology.* 11(1): 43-50.

Threlfall, E. J., & Ward, L. R. (2001). Decreased susceptibility to ciprofloxacin in *Salmonella enterica* serotype typhi, United Kingdom. *Emerg. Infect. Dis.* 7: 448-450.

Tiegs, G. (1997). Experimental hepatitis and role of cytokines. *Acta. Gastroenterol. Belg.* 60: 176-179.

Top, J., Schouls, L. M., Bonten, M. J., & Willems, R. J. (2004). Multiple-locus variable-number tandem repeat analysis, a novel typing scheme to study the genetic relatedness and epidemiology of *Enterococcus faecium* isolates. *J. Clin. Microbiol*. 42: 4503-4511.

Townsend, S. M., Kramer, N. E., Edwards, R., Baker, S., Hamlin, N., Simmonds, M., Stevens, K., Maloy, S., Parkhill, J., Dougan, G., & Bäumler, A. J. (2001). *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences. *Infect. Immun*. 69: 2894-2901.

Tracey, K. J., & Lowry, S. F. (1990). The role of cytokine mediators in septic shock. *Adv. Surg.* 23: 21-56.

Torpdahl, M., Sorensen, G., Ethelberg, S., Sando, G., Gammelgard, K., & Porsbo, L. J. (2006). A regional outbreak of *S. typhimurium* in Denmark and identification of the source using MLVA typing. *Euro. Surveill*. 11: 134-136.

Usera, M. A., Echeita, A., Aladuena, A., Alvarez, J., Carreno, C., Orcau, A., & Planas, C. (1995). Investigation of an outbreak of water-borne typhoid fever in Catalonia in 1994. *Enferm. Infec. Microbiol. Clin.* 13(8): 450-454.

van Belkum, A. (1994). DNA fingerprinting of medically important microorganisms by use of PCR. *Clin. Microbiol. Rev.* 7: 174-184.

van Belkum, A. (1999). The role of short sequence repeats in epidemiologic typing. *Curr. Opin. Microbiol.* 2: 306-311.

van Lith, L. A., & Aarts, H. J. (1994). Polymerase chain reaction identification of *Salmonella* serotypes. *Lett. Appl. Microbiol*. 19: 273-276.

Versalovic, J., Koeuth, T., & Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* 19: 6823-6831.

Wain, J., Diep, T. S., Ho, V. A., Walsh, A. M., Hoa, N. T. T., Parry, C. M., & White, N. J. (1998). Quantitation of bacteria in blood of typhoid fever patients and relationship between counts and clinical features, transmissibility, and antibiotic resistance. *J. Clin. Microbiol.* 36: 1683-1687.

Wain, J., Hien, T. T., Connerton, P., Ali, T., Parry, C. M., Chinh, N. T. T., Vinh, H., Phuong, C. X. T., Ho, V. A., Diep, T. S., Farrar, J. J., White, N. J. & Dougan, G.

(1999). Molecular typing of multiple-antibiotic-resistant *Salmonella enterica* serovar Typhi from Vietnam: application to acute and relapse cases of typhoid fever. *J. Clin. Microbiol*. 37: 2466-2472.

Wain, J., House, D., Parkhill, J., Parry, C., & Dougan, G. (2002). Unlocking the genome of the human typhoid bacillus. *Lancet Infectious Diseases* 2: 163-70.

Waterman, S. R. & Holden, D. W. (2003). Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. *Cell Microbiol.* 5: 501-511.

Welsh, J., & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res*. 18: 7213-7218.

White, N. (1999). *Salmonella typhi and paratyphi*. In: Yu, L., Merigan, C., Barriere, S. (ed.) Antimicrobial therapy and vaccines. Baltimore: Williams and Wilkins.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalsky, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic Acids Res.* 18: 653-6535.

Williams, D. W., Wilson, M. J., & Lewis, M. A. O., (1999) Deoxyribonucleic acid typing methods for medically important microorganisms. *Br. J. Biomed. Sci.* 56: 56-65.

Woods, C. R., Versalovic, J., Koeuth, T., & Lupski, J. R. (1993). Whole-cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. *J. Clin. Microbiol.* 31: 1927-1931.

World Health Organization. (2003). Background document: the diagnosis, treatment and prevention of typhoid fever. WHO, Department of Vaccines and Biologicals, Geneva, Switzerland.

World Health Organization. (2004). Water related diseases. In: Typhoid and paratyphoid Enteric fevers. WHO, Department of Vaccines and Biologicals, Geneva, Switzerland.

Xercavins, M., Llovet, T., Navarro, F., Morera, M. A., More´, J., Bella, F., Freixas, N., Simó, M., Echeita, A., Coll, P., Garau, J., & Prats, G. (1997). Epidemiology of an unusually prolonged outbreak of typhoid fever in Terrassa, Spain. *Clin. Infect. Dis*. 24: 506-510.

Yang, Y., & Ames, G. F. L. (1988). DNA gyrase binds to the family of prokaryotic repetitive extragenic palindromic sequences. *Proc. Natl. Acad. Sci. USA* 85: 8850-8854.

Zhang, X. L., Tsui, I. S. M., Yip, C. M .C, Fung, A. W. Y., Wong, D. K. H., Dai, X., Yang, Y., Hackett, J., & Morris, C. (2000). *Salmonella enterica* serovar typhi uses type IVB pili to enter human intestinal epithelial cells. *Infect. Immun.* 68: 3067-3073.

Zhu, Q., Lim, C. K., & Chan, Y. N. (1996). Detection of *Salmonella typhi* by polymerase chain reaction. *J. Appl. Microbiol.* 80: 244-251.

APPENDIX

Appendix 1: Background data for *S.* Typhi strains used in this study.

NO.	Strain Code	Year of Isolation	Type of sample	Source
1.	**TP34/98**	1998	Blood	HKB
2.	**TP40/98**	1998	Blood	HKB
3.	**TP41/98**	1998	Blood	HKB
4.	**TP43/98**	1998	Blood	HKB
5.	**TP46/98**	1998	Blood	HKB
6.	TP128/99	1999	Blood	HKB
7.	TP160/99	1999	Blood	HKB
8.	TP130/99	1999	Blood	HKB
9.	TP172/99	1999	Blood	HKB
10.	TP247/99	1999	Blood	Penang
11.	TP1/00	2000	Blood	IMR
12.	TP2/00	2000	Blood	HTAR
13.	TP5/00	2000	Stool	HKB
14.	TP168/00	2000	Stool	Penang
15.	TP118/00	2000	Blood	HKL
16.	TP2/01	2001	Blood	HKB
17.	TP3/01	2001	Blood	HKB
18.	TP5/01	2001	Blood	HKB
19.	TP38/01	2001	Blood	Sandakan
20.	TP58/01	2001	Blood	HTAR
21.	TP20/02	2002	Blood	IMR
22.	TP24/02	2002	Blood	IMR
23.	TP35/02	2002	Blood	IMR
24.	TP41/02	2002	Blood	IMR

NO.	Strain Code	Year of Isolation	Type of sample	Source
25.	TP43/02	2002	Blood	IMR
26.	TP195/03	2003	Blood	IMR
27.	TP223/03	2003	Blood	IMR
28.	TP282/03	2003	Blood	IMR
29.	TP322/03	2003	Blood	IMR
30.	TP329/03	2003	Blood	IMR
31.	TP80/04	2004	Blood	HSA
32.	TP85/04	2004	Blood	HJ
33.	TP90/04	2004	Blood	HKB
34.	TP108/04	2004	Blood	HAS
35.	TP78/04	2004	Blood	HTM
36.	**ST117/05**	2005	Blood	HKB
37.	**ST142/05**	2005	Blood	HKB
38.	**ST156/05**	2005	Blood	HKB
39.	**ST189/05**	2005	Blood	HKB
40.	**ST208/05**	2005	Blood	HKB
41.	ST2/06	2006	Blood	HUSM
42.	ST3/06	2006	Urine	HUSM
43.	ST13/06	2006	Stool	HUSM
44.	ST16/06	2006	Blood	HUSM
45.	ST37/06	2006	Blood	HUSM
46.	ST014/07	2007	Stool	HUSM
47.	**ST314/07**	2007	Stool	HUSM
48.	**ST334/07**	2007	Stool	HUSM
49.	**ST4571/07**	2007	Blood	HUSM

NO.	Strain Code	Year of Isolation	Type of sample	Source
50.	**ST4738/07**	2007	Blood	HUSM

Boldface type indicates group of strains obtained from defined typhoid outbreaks in Malaysia. HKB, Hospital Kota Bharu; HTAR, Hospital Tengku Ampuan Rahimah; HKL, Hospital Kuala Lumpur; HTM, Hospital Tanah Merah; HSA, Hospital Sultanah Aminah; HJ, Hospital Jeli; HAS, Hospital Alor Setar; HUSM, Hospital Universiti Sains Malaysia.