INNOVATIVE APPROACHES TOWARDS DEVELOPMENT AND UTILIZATION OF DNA DIAGNOSTICS FOR *Salmonella* Typhi

by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

Symbols/ Abbreviations	Definition
°C	Degree Celsius
µg	microgram
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
$CaCl_2$	Calcium chloride
CFTR	Cyctic fibrosis transmembrane regulator
cfu	Colony forming unit
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
g	Gram
H_2O	water
HCl	Hydrogen chloride
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kb	Kilo base
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate
KIA	Kligler Iron Agar
LPS	Lipopolysaccharide
MCS	Multiple cloning site
mg	milligram
MgCl2	Magnesium chloride
ml	milliliter

mM	millimolar
NaOH	Sodium hydroxide
ND	Not done
ng	nanogram
nm	nanometer
NPV	Negative predictive Value
OEP	Outer membrane efflux protein
OM	Outer membrane
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmol	picomole
PPV	Positive predictive Value
PVP	Polyvinyl pyrrolidone
SH	Shaking
SPS	Sodium polyanethosulfonate
ST	Static
T	Time
Ta	Annealing temperature
TAE	Tris-Acetate EDTA
TE	Tris EDTA
TMP-SMZ	Trimethoprim-sulphamethoxazole
TSI	Triple Sugar Iron
U	units
UDG	Uracil DNA glycosylase
USA	United States of America
UV	ultraviolet
V	volts
WHO	World Health Organization

PENDEKATAN INOVATIF TERHADAP PEMBANGUNAN DAN PENGGUNAAN KAEDAH DIAGNOSTIK BERASASKAN DNA BAGI *Salmonella* Typhi

ABSTRAK

Demam kepialu berpunca daripada *Salmonella* Typhi kekal sebagai masalah kesihatan di negara-negara kurang membangun dan sedang membangun. Kaedah pengkulturan konvensional dan ujian-ujian biokimia untuk mengenal pasti *S.* Typhi adalah sangat remeh dan memerlukan masa 2 hingga 7 hari untuk menghasilkan keputusan. Ujian alternatif berasaskan DNA yang pantas, sensitif dan mudah digunakan, perlu dibangunkan. Ke arah pembangunan ujian tersebut, gen yang mengkodkan protein spesifik 50 kDa daripada *S.* Typhi telah digunakan dan primer telah direka untuk mengamplifikasi produk DNA yang bersaiz 1238 bp.

Ujian PCR menggunakan primer tersebut telah dinilai terhadap DNA daripada 114 isolat bakteria *S.* Typhi dan 66 isolat bakteria bukan *S.* Typhi. Ujian ini menunjukkan 100% sensitiviti, spesifisiti, nilai ramalan positif (PPV) dan nilai ramalan negatif (NPV) 100%. Penilaian semula kaedah PCR ini telah dilakukan terhadap DNA daripada 25 isolat *S.* Typhi dan 25 isolat selain daripada *S.* Typhi dengan menggunakan kontrol amplifikasi dalaman (IAC). Keputusan sekali lagi menunjukkan nilai sensitiviti, specifisiti, PPV and NPV 100% untuk mengenal pasti *S.* Typhi. Daripada keputusan ini adalah dirumuskan yang gen ST50 sesuai digunakan sebagai sasaran untuk mengenal pasti *S.* Typhi dengan menggunakan kaedah PCR.

Untuk mengatasi masalah ini, PCR biasa, satu campuran untuk S. Typhi

telah dibangunkan yang mengandungi campuran reagen PCR, primer untuk gen ST50, IAC dan larutan pewarna gel yang dibekukeringkan dalam sebuah tiub. Ini telah dilakukan dalam satu kaedah PCR reagen-formulasi-kering dua langkah untuk mengamplifikasi gen sasaran, 1238 bp dan 810 bp IAC daripada 73 sampel kultur darah BACTEC (33 sampel positif *S*. Typhi dan 40 sampel negatif *S*. Typhi). Nilai sensitiviti, spesifisiti, PPV dan nilai NPV adalah masing-masingnya 87.9%, 100%, 100% dan 90.9%. Keputusan yang diperolehi mencadangkan yang penggunaan ujian dua-langkah PCR-reagen formulasi kering boleh digunakan untuk mendiagnos demam kepialu akut.

Ujian PCR ini juga didapati teguh (robust) kerana dapat menghasilkan keputusan yang konsisten bila digunakan dengan tiga alat PCR berlainan jenama. Satu kajian telah dilakukan untuk mengkaji keupayaan/keberkesanan PCR reagen-formulasi-kering ini dalam keadaan berbeza penyimpanannya. Kit PCR ini telah didapati stabil pada suhu 4°C, 25°C dan 37°C selama enam bulan dan pada suhu 42°C selama sebulan. Kajian ini menunjukkan kebaikan dengan tidak memerlukan suhu penyimpanan -20°C seperti reagen PCR konvensional. Kelebihan seperti tahap sensitiviti yang tinggi dan spesifik, dan mudah digunakan, serta mesra pengguna membolehkan ujian PCR ini mudah diguna di negara-negara di mana kepialu adalah endemik.

Pengesanan *S*. Typhi untuk mengesan pembawa kepialu juga telah dikaji menggunakan kaedah PCR reagen-formulasi-kering ini. Tinja telah dicemarkan dengan 25 isolat S. Typhi dan 25 bukan S. Typhi dan dibiakkan dalam kaldu Selenite F selama 24 jam untuk meningkatkan bilangan bakteria ke tahap yang boleh dikesan.

DNA kemudiannya telah diasingkan degan mendidihkan spesimen bersama PVP untk mengurangkan perencat yang menganggu. PCR telah berjaya dilakukan ke atas DNA yang diasingkan mengunakan kaedah pendidihan tanpa terdapat apa-apa petanda perencat DNA. Sensitiviti, spesifisiti, PPV dan NPV yang diperolehi adalah 100%.

Keberkesanan PCR telah dibandingkan ke atas penggunaan calitan rektal dengan calitan tinja dengan menentukan samada jangkasa masa inkubasi (T0-T168 jam) kedua-dua kultur tersebut mempengaruhi keberkesanannya. Keputusan ujian PCR menggunakan DNA yang diekstrak daripada kultur calitan rektal dan kultur tinja adalah setanding untuk sampel yang diinkubasi pada kedua-dua suhu (25°C dan 37°C) dalam keadaan statik ataupun goncangan pada 200 rpm dalam inkubator penggoncang. Kedua-dua sampel boleh digunakan untuk mengesan pembawa kepialu. Keputusan ujian PCR masih kekal positif bagi inkubasi sehingga 168 jam (7 hari). Kaedah PCR ini telah juga dibandingkan dengan satu kaedah kultur yang telah diubah suai untuk mengenal pasti *S.* Typhi. Dua daripada 16 spesimen telah didapati positif (12.5%). Namun demikian, bila dua kaedah ini dibandingkan, kaedah PCR reagen-formulasi-kering didapati lebih pantas sensitif dan mudah digunakan.

Sebagai kesimpulannya PCR reagen-formulasi-kering untuk *S.* Typhi boleh digunakan untuk mengamplifikasi gen *S.* Typhi daripada kultur darah pesakit kepialu; dan kultur tinja dan juga kultur calitan rektal pembawa kepialu.

INNOVATIVE APPROACHES TOWARDS DEVELOPMENT AND UTILIZATION OF DNA DIAGNOSTICS FOR *Salmonella* Typhi

ABSTRACT

Typhoid fever caused by *Salmonella* Typhi remains a public health problem in underdeveloped and developing countries. Conventional culture method and biochemical tests to identify *S.* Typhi lacked sensitivity, are laborious and produced results within 2 to 7 days. An alternative DNA test which is rapid, sensitive and user-friendly needs to be developed. Towards the development of such test, the gene encoding for the specific 50 kDa outer membrane protein of *S.* Typhi (ST50) was used and primers were designed to amplify this DNA product with a size of 1238 bp.

A PCR assay using such primers was evaluated against DNA extracted from pure cultures of 114 *S.* Typhi and 66 non-*S.* Typhi isolates. The assay showed sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 100%. Re-evaluation of the PCR assay was performed with DNA of 25 *S.* Typhi and 25 non-*S.* Typhi isolates, with incorporation of an internal amplification control (IAC). The results again showed sensitivity, specificity, positive predictive value and negative predictive value of 100% in detecting *S.* Typhi. From the above results, it was concluded that ST50 gene can be successfully utilised as a target for the detection of *S.* Typhi by means of PCR.

To overcome limitations of conventional PCR, a ready-to-use PCR mix test for *S.* Typhi was designed comprising PCR reagents, primers of the ST50 gene of *S.* Typhi, a built-in IAC and gel loading dye mixed that was freeze-dried in a single

tube. This was used in a two-step dry-reagent-based PCR assay to amplify the 1238 bp target gene and the 810 bp IAC gene from 73 BACTEC blood culture broths (33 true positives for *S.* Typhi and 40 true negatives for non-*S.* Typhi). The sensitivity, specificity, PPV and NPV of the PCR assay were 87.9%, 100%, 100% and 90.9%, respectively. Results obtained suggested that the rapid two-step PCR test developed could be used for the rapid diagnosis of acute typhoid fever.

This PCR assay was also found to be robust and it showed consistent and reproducible results using three different thermal cyclers of different makes. A study was also performed to observe the efficiency of the dry-reagent-based PCR and the effect of storage conditions. The PCR kit was observed to be stable at 4°C, 25°C and 37°C for six months and 42°C for one month. The study revealed that this dry-reagent-based PCR kit has an advantage of obviating cold storage at -20°C of the conventional PCR reagents. The highly sensitive, specific and user friendly PCR assay can provide accessibility to countries where typhoid is highly endemic.

The detection of *S.* Typhi from stool specimens was attempted using this dry-reagent-based PCR to detect typhoid carriers. Stool was spiked with 25 *S.* Typhi and 25 non-*S.* Typhi innocula and cultured in Selenite F broth for 24 hours to increase the number of bacteria to a detectable. DNA was then extracted by boiling the specimen in the presence of PVP to reduce interfering inhibitors. The PCR successful results on the DNA extracted using the boiling method without any sign of inhibition. The sensitivity, specificity, PPV and NPV obtained were 100%.

The efficacy of rectal swab was then compared with stool swabs by determining the length of incubation time (T0-T168 hrs) of the stools and rectal swabs cultures for PCR to remain positive. PCR amplifications using the DNA extracted from rectal swab cultures were comparable to that of DNA extracted from stool cultures at both temperatures (25°C and 37°C) in either static or shaking condition at 200 rpm in an incubator shaker. Both samples can be used for the detection of typhoid carriers. The PCR results remained positive up to 168 hours (7 days) of incubation. The PCR assay was also compared with a modified culture method to detect *S.* Typhi. Two specimens from 16 specimens (12.5%) tested positive. Nevertheless, when the two methods were compared, the dry-reagent-based PCR was found to be more rapid, sensitive and easier to use.

In conclusion, the dry-reagent-based *S.* Typhi PCR test can be used to amplify *S.* Typhi gene from blood culture broths of typhoid patients and stool culture broths as well as rectal swab culture broths of typhoid carriers.

CHAPTER 1

General Introduction

1.1 Introduction to typhoid fever

Typhoid fever is a global health problem. The real challenge is the difficulty in diagnosing because the clinical feature is confused with those of many other febrile infections (WHO, 2003). It is a systemic infection caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), a Gram-negative organism, facultative anaerobic and flagellated bacilli from the family of Enterobacteriaceae (Bhan *et al*., 2005). Typhoid patients usually present with a history of prolonged fever, headache, abdominal discomfort and general lethargy (House *et al*., 2001). It is an enteric fever that starts as an infection of the gastrointestinal tract and develops into a systemic illness. The term enteric fever includes typhoid which is caused by *S.* Typhi and paratyphoid fever which is caused by *S.* Paratyphi A, B and C. The clinical symptoms of paratyphoid fever are usually less severe (Crum, 2003). Typically, typhoid fever passes through the following four phases: incubation, invasion, status period and evolution. The severity of the illness has been associated with the virulence of *S.* Typhi strain, immunocompetence, age, and nutritional status of patients (Crum, 2003). The infectious dose of *Salmonella* depends on the serovar, the bacterial strain, bacterial growth conditions and host susceptibility (Sussman, 2001). Infectious dose was determined to be between 10^3 and 10^6 organisms given orally (Bhan *et al*., 2005). Ingestion of 10^5 organisms resulted in typhoid fever for up to 55% of the study volunteers (Guzman *et al*., 2006).

1.2 Characteristics of *S*. Typhi

The genus *Salmonella* was named after the pathologist Salmon, who first isolated the organism from animal intestines. *Salmonella* is a member of family Enterobacteriaceae and contains two species, each of which contains multiples serotypes. The two species are *S. enterica* and *S. bongori*. *Salmonella enterica* subsp. *enterica* ser. Typhi is the complete name of the bacteria which causes typhoid fever and it was designated by Center of Disease Control (CDC) as *Salmonella* ser. Typhi (Brenner *et al*., 2000) after the proposal to recognize the status *S*. Typhi together with *S*. enteritidis and *S*. typhimurium as species (Ezaki *et al*., 2000) has been rejected. *S.* Typhi is rod-shaped with a length of 2-3 μm and a 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). It is serogically positive for LPS antigens O9 and O12, protein flagellar antigen Hd and polysaccharide capsular antigen Vi (Agarwal *et al*., 2004). Vi-antigen, is also found in *Citrobacter freundii, S.* Paratyphi C, and *S.* Dublin (Hashimoto *et al*., 1995a).

Most *Salmonella* serotypes cannot be distinguished by biochemical reactions except *S*. Typhi. *S*. Typhi does possess some unique biochemical characteristics that will allow it to be differentiated via producing only trace amounts of hydrogen sulfide which is usually observed as a crescent-shaped wedge of black precipitate forming at the interface of the slant and butt in KIA or TSI media (Parry, 2006). In addition, *S*. Typhi are less active biochemically than the more common serotypes and is specifically negative for the following reactions: Simmon's citrate; ornithine decarboxylase; gas from glucose; fermentation of dulcitol, arabinose and rhamnose; and mucate and acetate utilization (Winn *et al*., 2005).

1.3 Global epidemiology of typhoid fever

Typhoid is estimated to have caused 21.6 million illnesses and 216,500 deaths globally (Crump *et al*., 2004). The incidence of typhoid was high in South-Central Asia, Southeast Asia, and possibly southern Africa with more than 100 cases per 100,000 populations per year; but moderate in the rest of Asia, Africa, Latin America, and Oceania except for Australia and New Zealand with 10-1,000 cases per 100,000 populations; and low in the other parts of the world with less than 10 per 100,000 populations (Figure1.1). Despite advances in technology and public health strategies, typhoid fever remains as a major cause of morbidity in the developing countries (Bhutta, 2008). Therefore, improvements in public health and sanitation are the most effective control methods against typhoid fever, and this requires long term investment in the infrastructure of the country (Cooke and Wain, 2004). The high incidence rate reported among young children less than five years old (Figure 1.2) may reflect high rate of transmission via food and water (Sinha *et al*., 1999, Brooks *et al*., 2006, Siddiqui *et al*., 2006, Bhutta, 2008). The main reason was that antityphoid vaccine are currently being assessed only in children of school age and in older people, and the optimum age for immunization as part of public-health programs remains unclear (Sinha *et al*., 1999). As mentioned by previous study, there are no proper plans for large scale vaccination programmes among infants and children (Bhutta, 2008). The interesting points highlighted in introducing typhoid vaccine into the paediatric vaccination schedule were the efficacy of the available vaccine and route of vaccination (Griffin, 1999, Sinha *et al*., 1999). The efficacy of typhoid vaccine; Ty21a, which has been tested only in school children and adults is not suitable for large-scale use in developing countries for logistical and financial reasons (Simanjuntak *et al.*, 1991, Levine *et al.*, 1999). Therefore, a new vaccine

such as conjugates of the Vi polysaccharide which can be effective for young children should be developed (Sinha *et al*., 1999). Recent emergence of drug resistance *S.* Typhi especially to first line antibiotics and quinolones has made the situation very difficult and expensive for health services to manage the disease (Cooke and Wain, 2004, Bhutta, 2008).

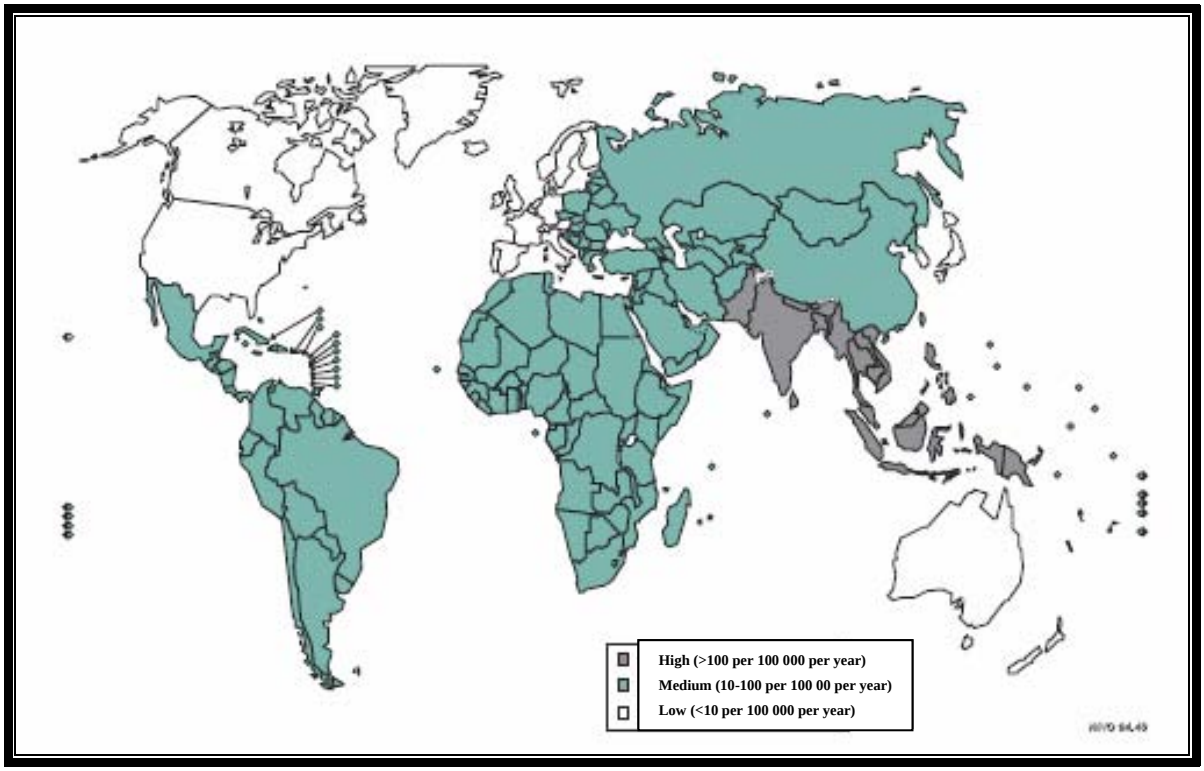

Figure 1.1: Geographical distribution of typhoid fever (Crump *et al*., 2004)

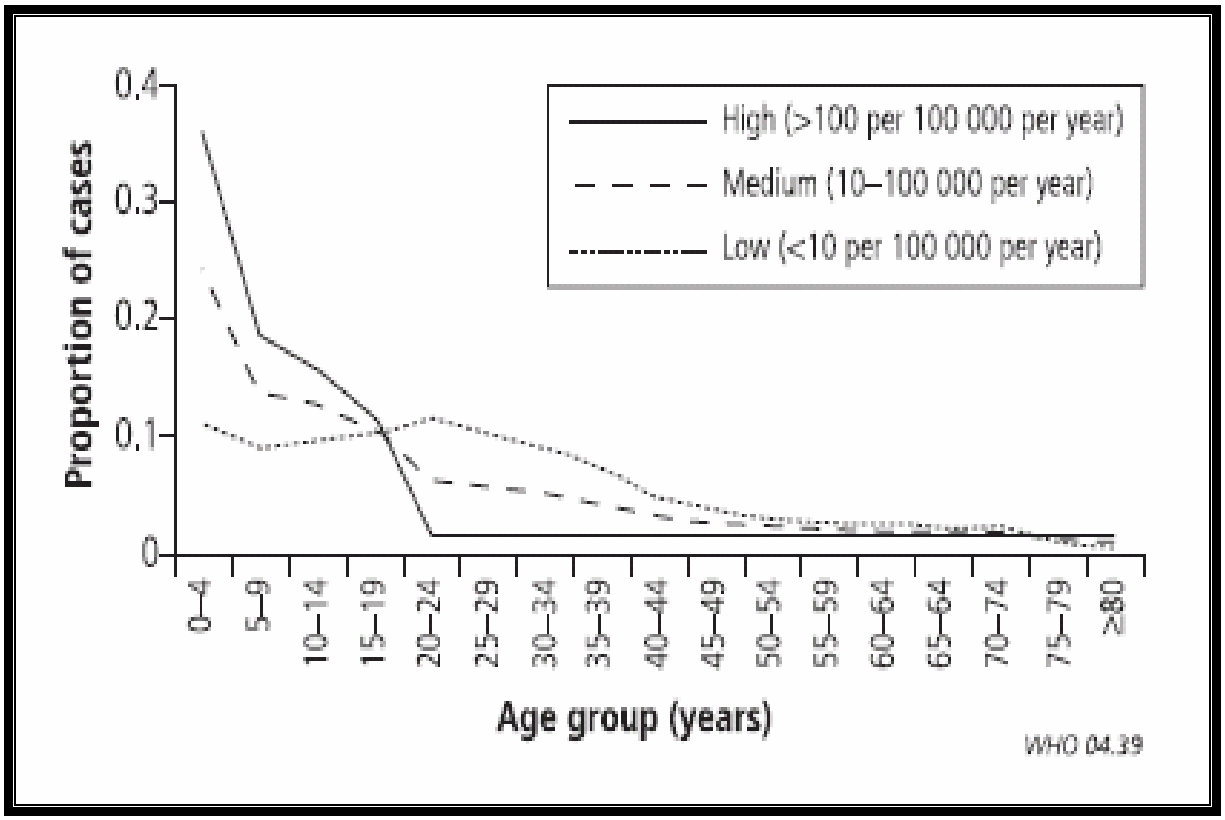

Figure 1.2: Distribution of typhoid fever, by age group, at various incidences (Crump *et al.*, 2004)

1.4 Epidemiology of typhoid in Malaysia

Malaysia has been mapped as the region with high incidence rate of typhoid by Crump *et al*. (2004) but reports from Ministry of Health showed that the incidence rate was less than 5 per 100,000 populations (Kementerian Kesihatan Malaysia, 2006). According to the report of typhoid cases in Malaysia from 2000-2005, the highest incidence rate was reported in Kelantan followed by Sabah and Terengganu (Figure 1.3). Majority of the Kelantan people especially in rural areas use water from wells for drinking and other domestic purposes and do not have access to safe water from the treated water supply system (Kementerian Kesihatan Malaysia, 2006). The high incidence of typhoid fever in 2005 (Figure 1.4) might be related to the flood at the end of 2004 whereby the contaminated water overflowed into wells. In addition to the above scenario, food handlers, especially hawkers, and those involved in selling food at food stall were found to be lacking in knowledge, attitude and practice towards food-borne diseases and food safety. Their practices were poor in hand washing, personal hygiene and safety food handling (Zain and Naing, 2002). School-children were the most affected (Choo *et al*., 1988, Yap and Puthucheary, 1998, Malik and Malik, 2001, Malik, 2002) and the contributing factors were related to unhygienic food handling practice followed by inadequate safe water supply and poor environmental sanitation (Meftahuddin, 2002). Despite being the state with the highest cases of typhoid, no data was reported for typhoid carriers. The National Antibiotic Surveillance showed that in the year 2000, 10.6% of *S*. Typhi isolates were resistant to ampicillin and 8.5% resistant to chloramphenicol (Lim, 2002). Resistance to ampicillin in Malaysia was transferred on a plasmid type IncH or IncI, and chloramphenicol was transmitted on an IncB plasmid (Ling and Chau, 1984). The ampicillin-resistant isolates harboured plasmid groups, IncA, IncC and IncC

suggest that these non-IncH plasmids were not stable in *S.* Typhi whereas IncH plasmids were stable and once acquired resulted in long term resistance (Wain and Kidgell, 2004).

1.5 Mode of transmission of typhoid

Typhoid infection is transmitted via ingestion of food or water contaminated with feces. Ice cream has been recognized as a significant risk factor for the transmission of the disease. Shellfish taken from contaminated water, and raw fruit washed with contaminated water and vegetables fertilized with sewage, have been the source of past outbreaks. The highest incidence occurred where water supplies serving large populations are contaminated with feces (WHO, 2003). There was also case of sexual transmission of typhoid fever which may be acquired by means of oral and anal sex, as well as via food and drink (Reller *et al*., 2003). The infectious dose is between 10^3 and 10^6 organisms given orally (Hornick *et al*., 1970). The pathogenesis of typhoid fever starts with adherence to and invasion of gut epithelial cells, dissemination to systemic sites, and followed by survival and replication within host cells (House *et al*., 2001). In addition, the products of certain strains of bacteria normally present in the intestinal microflora are able to trigger redistribution of cyctic fibrosis transmembrane conductance regulator (CFTR) protein in epithelial cells. CFTR is used by *S.* Typhi as a receptor on epithelial cells which mediate the translocation of this microorganism to the gastric submucosa. Therefore, Serovar Typhi-epithelial cell adhesion and CFTR-dependent invasion by serovar Typhi of epithelial cells are increased due to commensal-mediated CFTR redistribution. As a result, commensal microorganisms present in the intestinal lumen could affect the efficiency of serovar Typhi invasion of intestinal mucosa and this could be a key

factor influencing host susceptibility to typhoid fever (Lyczak and Pier, 2002, Lyczak, 2003).

1.6 Treatment of typhoid fever

S. Typhi isolates are still sensitive to the first-line drugs against typhoid such as chloramphenicol, ampicillin, amoxicillin and trimethoprin-sulfamethoxazole. Fluoroquinolones are more effective than the first-line drugs with excellent tissue penetration leading to the killing of *S.* Typhi in intracellular stationary stage as in monocyte/macrophages and hence achieve higher active drug levels in the gall bladder than other drugs (WHO, 2003). Studies have also shown that fluoroquinolones can achieve satisfactory cure rates (Van den Berg *et al.*, 1999, WHO, 2003)Table 1.1 shows the World Health Organization's recommendations for treating uncomplicated and severe cases of typhoid fever (WHO, 2003, Bhutta, 2008).

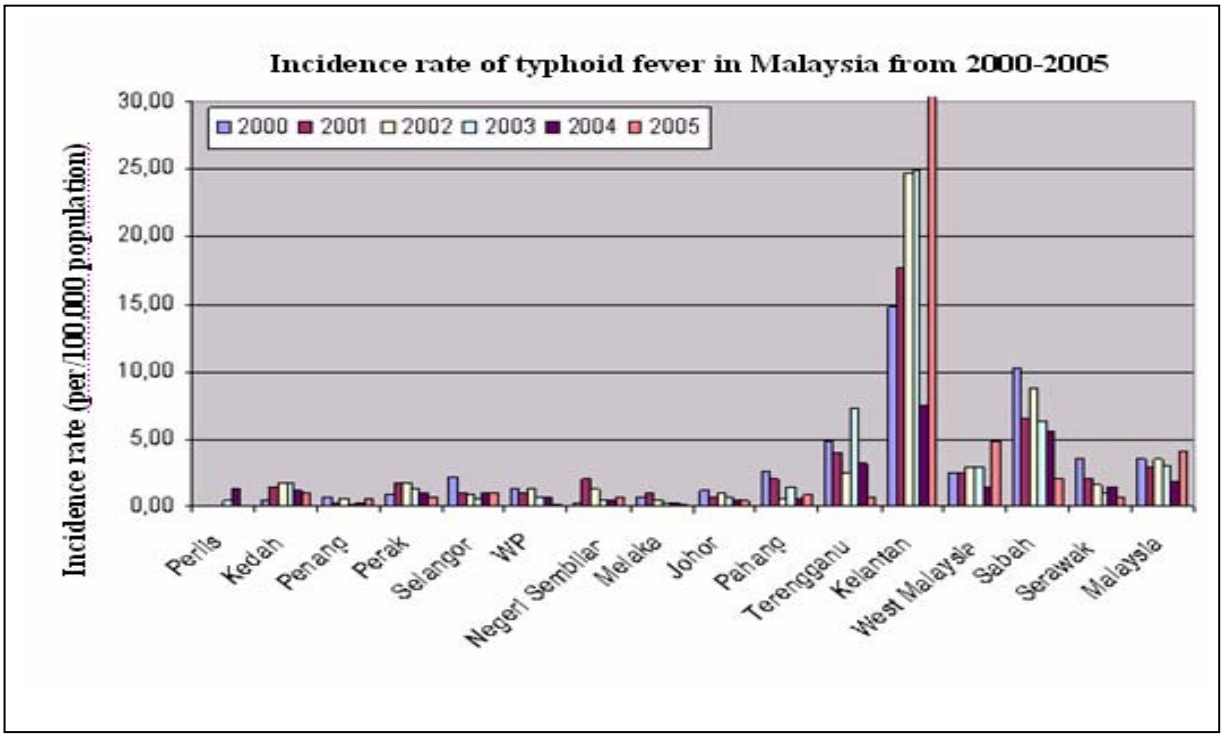

Figure 1.3: Incidence rate of typhoid fever per 100, 000 population in Malaysia (2000-2005) (Jabatan Kesihatan Negeri Kelantan, 2005)

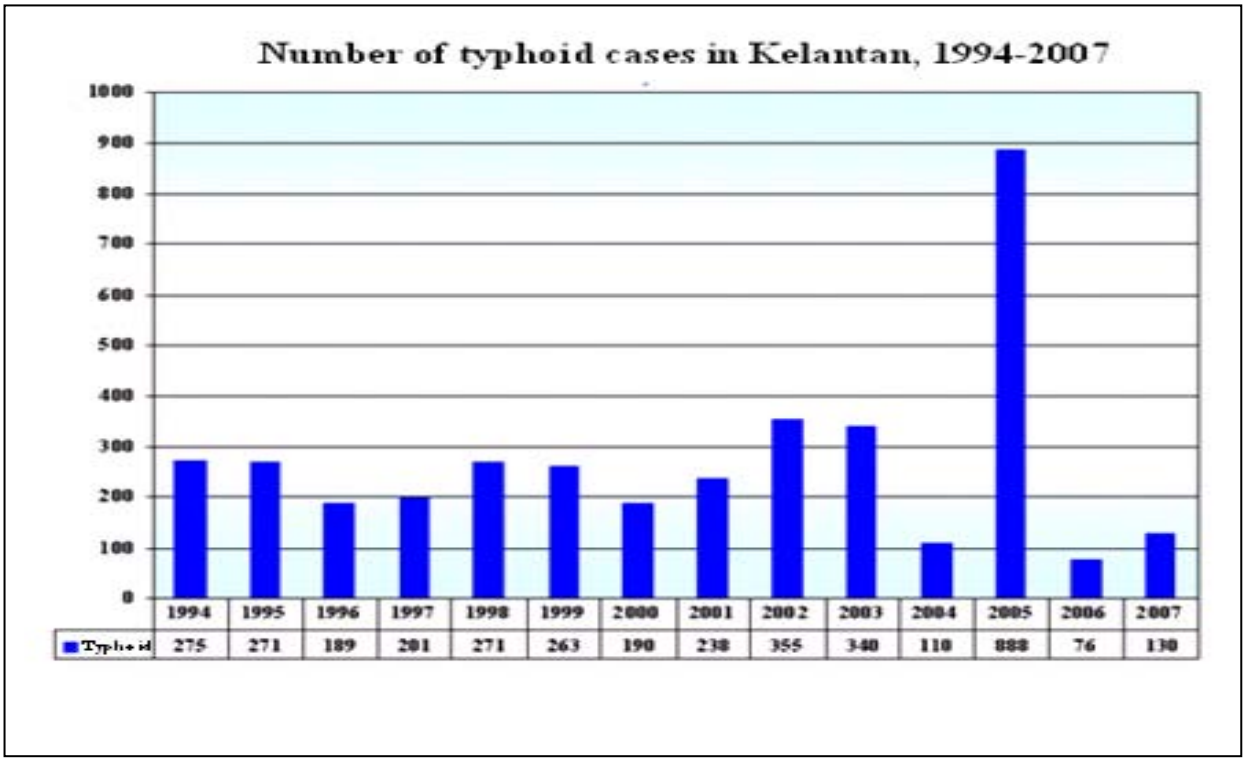

Figure 1.4: Number of typhoid cases in Kelantan, Malaysia (1994-2007) [adapted from (Mat Hussin, 2008)]

1.7 Management of typhoid carriers

According to WHO, typhoid carriers should be treated with amoxicillin or ampicillin (100 mg per kg per day) plus probenecid (Benemid®) (1 g orally or 23 mg per kg for children) or TMP-SMZ (1600 to 800 mg twice daily) for six weeks and about 60% of persons treated with either regimen are expected to have negative cultures upon follow-up (WHO, 2003). Clearence of up to 80% of chronic carriers can be achieved with the administration of 750 mg of ciprofloxacin twice daily for 28 days or 400 mg of norfloxacin (Ferreccio *et al*., 1988, Gotuzzo *et al*., 1988). However, in cases of anatomic abnormality (eg. biliary or kidney stone), eradication of carrier cannot be achieved by antibiotic therapy alone but also requires surgical correction of abnormality. Choloecystectomy in individuals with gallstones or chronic cholecystitis eliminates the carrier state by 85% (Kalra *et al*., 2003).

1.8 Definition of typhoid fever and typhoid carrier

The definition of typhoid fever is divided into two groups by the World Health Organization (WHO, 2003); confirmed case and probable case of typhoid fever. Confirmed case of typhoid is defined as a patient with fever (38°C and above) that has lasted for at least three days, with a laboratory-confirmed positive culture (blood, bone marrow, bowel fluid) of *S.* Typhi, while a probable case of typhoid is defined as a patient with fever (38°C and above) that has lasted for at least three days, with a positive serodiagnosis or antigen detection test but without *S.* Typhi isolation. A chronic carrier is an individual who excretes *S.* Typhi in stools or urine for longer than one year after the onset of acute typhoid fever.

Table 1.1: Recommended antibiotic treatment for typhoid fever [adapted from (Bhutta, 2008)]

	Optimal treatment			Alternative effective treatment		
Susceptibility	Drug	Daily dose (mg/kg)	Course (days)	Drug	Daily dose (mg/kg)	Course (days)
Uncomplicated typhoid fever						
Fully sensitive	Fluoroquinolone (such as ofloxacin or ciprofloxacin)	15	5-7*	Chloramphenicol	50-75	14-21
				Amoxicillin	75-100	14
				TMP-SMX	8-40	14
Multidrug resistance	Fluoroquinolone *or*	15	5-7	Azithromycin	8-10	7
	Cefixime	15-20	7-14	Cefixime	15-20	7-14
Quinolone resistance†	Azithromycin *or*	8-10	7	Cefixime	20	7-14
	Ceftriaxone	75	10-14			
Severe typhoid fever requiring parenteral treatment						
Fully sensitive	Fluoroquinolone (such as ofloxacin)	15	10-14	Chloramphenicol	100	14-21
				Ampicillin	100	14
				TMP-SMX	8/40	14
Multidrug resistant	Fluoroquinolone	15	10-14	Ceftriaxone *or*	60	10-14
				Cefotaxime	80	
Quinolone resistant	Ceftriaxone *or*	60	10-14	Fluoroquinolone	20	14
	Cefotaxime	80				

*Three day courses also effective, particularly so in epidemic containment.
†Optimum treatment for quinolone resistant typhoid fever has not been determined. Azithromycin, third generation cephalosporins, or a 10-14 day course of high dose fluoroquinolone is effective. Combinations of these are now being evaluated.

1.9 Diagnosis of typhoid fever

1.9.1 Culture method

Diagnosis of typhoid fever on clinical grounds is difficult because the signs and symptoms overlap with those of other common febrile illness (Crum, 2003). The standard method for diagnosing typhoid and paratyphoid fever is blood culture but the percentage of detection ranged from 40 to 70%. The detection method depends on the number of *S.* Typhi in the blood, the amount of blood used to culture, the type of culture medium used and the length of incubation period (Avendano *et al*., 1986, Hoffman *et al*., 1986). Failure to isolate the organism may be caused by several factors such as the limitations of laboratory media, the presence of antibiotics, the volume of specimen cultured or the time of collection whereby patients with a history of fever for seven to ten days would have positive blood culture less than seven days (WHO, 2003). *S.* Typhi can also be isolated from bone marrow, stool and intestinal secretion. A positive culture for *S*. Typhi is obtained in more than 90% of patients if culture is performed from all blood, bone marrow and intestinal secretions (Gilman *et al*., 1975). According to WHO (2003), bone marrow aspirate culture is the gold standard for the diagnosis of typhoid fever and is particularly valuable for patients who have been previously treated, who have a long history of illness and for those with negative blood cultures with the recommended volume of blood. Furthermore, the viable counts of organisms in bone marrow were considerably less affected by antibiotic treatment than the quantitative blood counts. Despite successful treatment with floroquinolones which have excellent cellular penetration (Wain *et al*., 2001), the bone marrow samples remained positive for up to five days or longer after starting the treatment (Gasem *et al*., 1995). Isolation of the bacteria from the blood samples is still being used since bone marrow aspiration from patient is

difficult to obtain, relatively invasive, and of little use in public health setting (Bhutta, 2008). The problem with the culture method is time consuming because it takes two to seven days to produce results and it is laborious. Isolation of non-lactose fermenting colonies from agar plates need to be identified by biochemical test and serotyping and these procedures are expensive for developing countries (Haque *et al.*, 1999, WHO, 2003). The development of a rapid and sensitive diagnostic method for typhoid, therefore, is of practical importance in endemic areas.

1.9.2 Felix-Widal test

The conventional serological method for typhoid diagnosis, Widal test; which measures agglutinating antibody, is not reliable using single titres in endemic areas (Levine *et al*., 1978). However, this test continues to be used since it is simple, cheap and requires no instrumentation. The demonstration of a four-fold rise in titre of antibodies to *S*. Typhi suggests typhoid fever but is invariably too late to help clinical decision-making (Parry, 2005). The method also showed the difficulty in determining an appropriate cut-off for positive results since it varies between areas (Clegg *et al*., 1994). The Widal test uses a crude assay method to detect antibodies by their ability to agglutinate whole bacterial cells against O and H antigens in test tubes. *S*. Typhi shares O and H antigens with other Salmonella serotypes and has cross-reacting epitopes with other Enterobacteriaceae, and this can lead to false positive results (WHO, 2003). The test also has low sensitivity since it can be negative in up to 30% of culture positive samples (WHO, 2003). New tests are currently being developed to detect acute typhoid fever.

1.9.3 Tubex test

The TUBEX test which uses a simple one step procedure is more rapid and accurate than the Widal. This test detects anti-Salmonella O9 (both IgM and IgG) antibodies in patients by inhibiting the binding between anti-O9 IgM monoclonal antibody conjugated to colored latex particles and *S.* Typhi lipoposaccharide (LPS) conjugated to magnetic latex particles (Lim *et al*., 1998, Mabey *et al*., 2004). In other words, TUBEX combines the antigen *S*. Typhi LPS as detector with a specific monoclonal antibody directed against 09 antigen as indicator. There are two disadvantages of TUBEX. This include, first, the difficulty in interpreting the results from haemolysed samples because it uses a colorimetric reaction. Secondly, the analytical sensitivity is very low and thus, early cases may be missed although both clinical sensitivity and specificity were 100% (Lim *et al*., 1998). However, these happened before the kit was commercially available. Due to the similarities in O9 antigen in both *S*. Typhi and group D (*S*. Enteritidis) which contains a sugar, α-D-tyvelose, TUBEX produced false positive results in person with recent *S*. Enteritidis infection (Oracz *et al*., 2003). Evaluation of TUBEX for detection of typhoid fever in Vietnam showed sensitivity and specificity of 78% and 89% respectively (Olsen *et al*., 2004). Previous study done in the Philiphines showed a sensitivity of 94.7%, specificity of 80.4%, PPV of 78% and NPV of 95.% (Kawano *et al*., 2007). The TUBEX need to be evaluated in larger trials in community settings (Bhutta, 2008).

1.9.4 *TYPHIDOT* and *TYPHIDOT* –M

The other test available in the market is the *TYPHIDOT* which detects the IgM and IgG antibodies against a 50 kDa protein of *S.* Typhi. The 50 kDa protein is a specific

protein on the outer membrane of *S.* Typhi (Ismail *et al*., 1991a). The dot enzyme immunoassay method developed using the 50 kDa antigen showed a sensitivity of greater than 90% and specificity of 75% (Choo *et al*., 1994). *TYPHIDOT*-M was developed to remove total IgG in order to detect for the presence of IgM especially for the convalescence or possible acute relapse cases in which IgG was boosted by secondary immune response (Ismail *et al*., 1998). The *TYPHIDOT*-M detects for the presence of IgM alone with a sensitivity of 92% and specificity of 100% among the outpatients of all ages. The test is useful in high endemic areas since it can differentiate convalescence and new cases. Studies showed that the sensitivity of *TYPHIDOT*-M was higher than *TYPHIDOT* but other evaluations suggested that the performance may not be as robust in community setting as in the hospital (Bhutta, 2008).

1.10 Diagnosis of typhoid carriers

Typhoid carriers were confirmed by isolation of *S.* Typhi from stool or rectal swab cultures and identified via biochemical tests (WHO, 2003) but these methods are costly, laborious and tedious. Three stool samples taken at different times are required and many of these cultures have to be obtained from individuals who are elderly, poorly educated, fail to understand the implications of their condition and may consequently be suspicious and uncooperative (Nolan *et al*., 1980a, Nolan *et al*., 1981). Knowing the difficulties to obtain faecal samples and perform the test, a reliable assay should be developed. An alternative serological test using crude Vi antigen from *Citrobacter freundii* was developed but this assay was not specific enough to be applied as a routine diagnostic tool for the detection of typhoid carriers. It was then revised using purified Vi antigen and specificity was found to increase

but the sensitivity remained similar to the traditional method using crude Vi antigen (Nolan *et al*., 1980a).

S. Typhi is distinguished from other salmonellae by its metabolic characteristics, its capsular polysaccharide (Vi antigen), its inability to colonize a similar disease in other animal species, and its ability to establish a chronic infection in their gall bladder in about 2-5% of individuals after an acute infection. The Vi antigen is both the virulence factor and protective antigen of *S*. Typhi. Stools of suspected typhoid carriers were cultured and the presence of the Vi antigen on these isolates were confirmed by the antiserum agar technique (Nolan *et al*., 1980b).

Using Vi antigen as a marker for detection of typhoid carriers, serological test started with agglutination assay followed by hemagglutination of erythrocytes coated with purified Vi and other method measuring the Vi antibodies (Landy and Lamb, 1951, Lanata *et al*., 1983) including counterimmunoelectrophoresis (Chau and Tsang, 1982, Sarasombath *et al*., 1983), and ELISA (Engleberg *et al*., 1983). A radioimmunoassay which is simple, rapid, and quantitative was used to identify carrier facilitated by measurement of serum Vi antibodies (Lin *et al*., 1988). No test for detection of typhoid carriers is available in the market. Since the world is moving towards the development of molecular based diagnostics, it is thus necessary to identify typhoid carriers with a more sensitive and specific molecular method to eradicate carriers.

1.11 Research background and rationale of the study

1.11.1 History of 50 kDa outer membrane protein of *Salmonella* Typhi

A 50 kDa outer membrane protein of *S.* Typhi was found to be antigenically specific (Ismail *et al*., 1991a) and a dot enzyme immunosorbent assay was developed by the group (Ismail *et al*., 1991b) which detects the presence of specific IgM and IgG antibodies in typhoid patients namely, *TYPHIDOT*. The assay was developed in order to have a rapid, easy to perform and inexpensive test for the early and accurate diagnosis of typhoid fever, as compared to the gold standard culture method and the Widal test which is difficult to interpret. The dot enzyme immunoassay (EIA) using this outer membrane protein was found to be sensitive and specific as reported by Choo *et al*. (1994, 1997). The test was then improved by the development of a new kit, *TYPHIDOT*-M which detects the presence of only IgM by removing total IgG to differentiate between new acute typhoid fever and convalescence cases (Ismail *et al*., 1998). Studies using *TYPHIDOT* and *TYPHIDOT*-M was performed on sera from febrile Malaysian children suggested that the tests was rapid and reliable in a high endemic area with 90.3% and 93.1% sensitivity respectively and combination of both tests gave a higher sensitivity of 95.2% and specificity of 87.5% (Choo *et al*., 1999). The assay was claimed and proven to be superior to Widal test shown by the higher sensitivity (85-94%) and specificity (77-89%) in a study with a consecutive group of children with suspected typhoid fever in Karachi, Pakistan (Bhutta and Mansurali, 1999). Similarly, other studies also showed that the assay was highly sensitive (78-98%) and specific (68-98.8%) (Bhutta and Mansurali, 1999, Olsen *et al*., 2004). Since 1994, *TYPHIDOT* has been used in diagnosing typhoid fever in several countries including Pakistan and Philippines. The kit received recognition by the Centres for Disease Control and Prevention (CDC), Atlanta in 2002

(www.mbdr.com.my). The gene (ST50 gene:1476 bp) which encodes the 50 kDa outer membrane protein of *S.* Typhi was submitted to Genbank with accession number BD079162 (Ismail *et al*., 2002) and was patented by Universiti Sains Malaysia (Ismail *et al.*, 2006).

1.11.2 Development of molecular methods for detection of *S*. Typhi from blood, stool and rectal swab

Molecular diagnostics are revolutionizing the clinical practice of infectious disease (Yang and Rothman, 2004). In fact, molecular testing has been heralded as the "diagnostic tool for the new millennium" whose ultimate potential could render traditional hospital laboratories obsolete (Pitt and Saunders, 2000). Previous studies reported several approaches for detection of *S.* Typhi using DNA probe and polymerase chain reaction (PCR). Different *S.* Typhi genes were used but the tests needed to be improved. The PCR technique can detect small amount of DNA by enzymatic amplification. A DNA probe against the Vi antigen was used to detect *S.* Typhi in the blood of patients with typhoid fever (Rubin *et al*., 1989). Problems with the use of a DNA probe in blood samples include low bacteraemia among typhoid patients and the sensitivity of the probe. The Vi probe detected typhoid DNA in 13 out of 32 (sensitivity of 40.6%) patients using equivalent of 2.5 ml of blood for DNA probing whereas the blood culture was positive in 17 out of 32 patients (sensitivity of 53.1%) when culturing eight ml of blood. The Vi probe also showed positive in five of 48 (10%) culture negative patients. Furthermore, the time frame for detection of the Vi DNA probe was about three days.

A PCR-based test using flagellin gene was developed to detect *S.* Typhi in the blood of typhoid patients (Song *et al*., 1993). The problem with the method was that the first PCR was not able to detect all the 12 blood samples from patients with culture-confirmed typhoid fever. Only after performing the nested PCR, amplifications were seen in 11 of 12 (sensitivity of 91.6%) specimens on the agarose gel. Southern blot hybridization with a ^{32}P-labeled internal probe showed the same pattern compared to agarose gel. The first PCR detected at least 10^6 bacteria whereas the more sensitive nested PCR detected a minimum of ten bacteria. The procedure of the first PCR and nested PCR took seven hours excluding the DNA extraction and gel electrophoresis or southern blot hybridization procedures.

The same primers from flagellin gene were used to develop PCR and nested PCR assay in Pakistan and India (Haque *et al*., 1999, Kumar *et al*., 2002). The study by Haque *et al*. (1999) showed a sensitivity of 71.9% compared to 34.1% with blood culture and 36.5% with Widal test using 25 healthy individuals and 82 typhoid patients. Although the PCR method gives the highest sensitivity rate compared to blood culture and Widal test, the percentage was unimpressive as it was less than 80% detection rate. Other study showed the sensitivity of 100% but was performed with only 20 blood culture positive cases (Kumar *et al*., 2002). Out of 20 blood culture negative cases tested, 12 (60%) were positive with PCR.

Previous prospective study showed an attempt to detect *S.* Typhi by nested PCR to amplify unique sequences in the region VI of the flagellin gene using blood, urine and stool samples from suspected typhoid patients revealed a sensitivity of 84.5%, 69.3% and 46.9% respectively (Song *et al*., 1993). In comparison, the sensitivities of

blood culture and Widal tests were 61.8% and 39.0% respectively (Hatta and Smits, 2007). This finding proved that the PCR is the best choice for diagnosis of typhoid fever but it needs to be improved. Furthermore, it is not yet available in the market. Indeed, PCR should be utilised to detect possible carriers to prevent any typhoid outbreak in the near future.

Hence, there is a need to develop a rapid and sensitive molecular method for effective management of both typhoid fever and typhoid carriers. The diagnosis of typhoid fever and typhoid carriers require accurate identification of *S.* Typhi in clinical samples by DNA based, instead of antibody detection because it shows the presence of bacteria rather than the antibody released due to past exposure to the bacteria. The aim of the present study is to develop an ideal diagnostic test with the following characteristics: (1) affordable by those at risk of infection, (2) high sensitivity and specificity, (3) user-friendly, (4) easy to perform, (5) rapid, (6) do not require refrigerated or frozen storage and, (7) contain built-in internal amplification control (IAC).

Most molecular assays rely on nucleic acid extraction, amplification and detection of an amplified product. Several approaches were performed to ensure the success of the test including:-

1. The incubation of samples in a suitable media to allow bacteria to multiply until a detectable level for single PCR to be amplified.
2. A simple boiling method for DNA extraction from BACTEC blood culture broths, rectal swab and stool culture broths that will reduce inhibitors.
3. The development of a single PCR test instead of nested PCR test which is

4. The use of built-in IAC to validate the negative PCR results whether it is truly negative or false negative due to inhibition.
5. The development of a robust *S.* Typhi dry-reagent-based PCR which is stable at room temperature or higher.

Therefore, the objectives of this study will be discussed in 1.12.

1.12 Objectives of the study

General objective:

The aim of this research project is to develop a rapid and sensitive dry-reagent-based PCR to detect typhoid fever and typhoid carriers coupled with improvements in the culture method.

Specific objectives:

The research objectives are devided into four phases as shown in the research flour chart (Figure 1.5):

Phase 1: Development of PCR assay using gene encoding for 50 kDa outer membrane protein of *Salmonella* Typhi

1. To design primers using the gene encoding for the 50 kDa outer membrane of *S.* Typhi.
2. To optimize a PCR assay using DNA extracted from pure cultures.
3. To evaluate the sensitivity and specificity of the PCR assay using DNA extracted from *S.* Typhi and other isolates.

Phase 2: Development and utilization of a dry-reagent-based PCR assay for the detection of *S*. Typhi

4. To develop a dry-reagent-based PCR assay for *S.* Typhi.
5. To evaluate the sensitivity and specificity of the PCR assay using extracted DNA from BACTEC blood cultures.

Phase 3: Stability testing and comparison of reproducibility and consistency of the dry-reagent-based PCR

6. To check the stability of the dry-reagent-based PCR in four different storage temperatures.
7. To compare the reproducibility and consistency of the dry-reagent-based PCR using different thermal cyclers.

Phase 4: Evaluation of dry-reagent-based PCR for the detection of *S*. Typhi in stool and rectal swab culture specimens

8. Optimization of DNA extraction from stool cultures and evaluation of the sensitivity and specificity of PCR from spiked stool cultures
    a. To optimize the utilization of polyvinyl pyrrolidone (PVP) procedures for DNA extraction from spiked stool cultures.
    b. To evaluate the sensitivity and specificity of the dry-reagent-based PCR using DNA extracted from stool cultures spiked with *S.* Typhi.
9. Comparative efficacies of PCR assay using stool and rectal swab cultures
    a. To compare the effectiveness of dry-reagent-based PCR assay using stool and rectal swab cultures.