A MULTIPLEX POLYMERASE CHAIN REACTION (mPCR) ASSAY FOR DETECTION OF *Salmonella* Typhi AND *Salmonella* Paratyphi A

By

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LIST OF ABBREVIATION

Symbols/ Abbreviations	Definition
°C	Degree Celsius
µl	Microliter
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ATCC	American Type Culture Collection
bp	Base pair
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
EDTA	Ethylene diamine tetra acetic acid
g	Gram
H_2O	Water
HCl	Hydrogen chloride
IAC	Internal amplification control
$MgCl_2$	Magnesium chloride
mA	mili ampere
ml	Milliliter
mM	Millimolar
Mins	Minutes
MR	Methyl Red test
ng	Nanogram
nm	Nanometer
PCR	Polymerase chain reaction
Pmol	Picomole
pg	Picogram
sec	Second
SIM	Sulfide indole Motility
TAE	Tris-Acetate EDTA
TSI	Triple Sugar Iron
U	Units
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Typhoid and paratyphoid fever, caused by the bacteria *Salmonella* Typhi and *Salmonella* Paratyphi A still remain major health problems worldwide. Both of these bacteria infect only human and cause systemic disease through fecal oral route. Culture and serological methods are used for diagnosis of typhoid and paratyphoid fever but the methods are laborious and produce results from 2-7 days. Thus, the study aims to develop multiplex polymerase chain reaction (mPCR) assay for specific detection of *ST50* gene and *spa4289* gene for *S.* Typhi and *S.* Paratyphi A respectively. Genomic DNA was extracted using commercial Qiagen DNA extraction kit. Gradient PCR was performed with the annealing temperature ranging from 50°C-70°C. The analytical sensitivity of mPCR was determined using DNA concentration of 50 ng – 10 pg. Seventy five bacterial isolates (25 of *S.* Typhi, 25 of *S.* Paratyphi A and 25 of *Salmonella* serovars and other enteric bacteria) were used for evaluation of this study. PCR was performed at initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec and elongation at 72°C for 1 min with a final additional elongation at 72°C for 10 min. Positive result was detected with the presence of PCR amplicon size of 1238 bp for *S.* Typhi and 549 bp for *S.* Paratyphi A when analyzed via agarose gel electrophoresis. As a result, the optimized annealing temperature of the multiplex PCR was 63°C with the detection limit of 0.39 ng when both *S.* Typhi and *S.* Paratyphi were used as DNA template. Furthermore, mPCR was capable to detect 3.13 ng for *S.* Typhi and 0.19 ng for *S.* Paratyphi A when analytical sensitivity test was performed using serially diluted DNA. The evaluation study with DNA from 75 bacterial isolates showed sensitivity and specificity of 100%. As an alternative to conventional culture method, multiplex PCR based on *ST50* and

spa4289 (hsdM) has the potential to be used as diagnostic tools for rapid detection of *S.* Typhi and *S.* Paratyphi A.

ABSTRAK

Tifiod dan paratifiod disebabkan oleh bakteria *Salmonella* Typhi dan *Salmonella* Paratyphi A masih kekal sebagai masalah kesihatan utama di seluruh dunia. Kedua-dua bakteria ini hanya menjangkiti manusia dan menyebabkan penyakit sistemik melalui mulut ke fecal. Kaedah pengkulturan konvensional dan serologi masih digunakan untuk mengesan penyakit ini tetapi kaedah ini memerlukan masa yang agak lama untuk menghasilkan keputusan ujian (2-7 hari). Oleh itu, objektif untuk kajian ini ialah untuk membangunkan tindakbalas rantai polimerase multipleks (mPCR) untuk mengesan gen *ST50* dan *spa4289 (hsdM)* bagi mengenalpasti *S.* Typhi dan *S.* Paratyphi A. Ekstraksi DNA genomik dilakukan dengan menggunakan kit ekstraksi DNA, Qiagen. Gradient PCR dilaksanakan dengan suhu sepuh lindap yang merangkumi 50°C – 70°C. Analisis sensitiviti ujian ini ditentukan dengan menggunakan kepekatan DNA dari 50 ng – 10 pg. Tujuh puluh lima bakteria isolat (25 *S.* Typhi, 25 *S.* Paratyphi and 25 dari *Salmonella* serovar dan lain-lain bakteria enterik) digunakan untuk penilaian mPCR ini. PCR dilakukan pada denaturasi awal pada 94°C selama 2 minit diikuti dengan 30 kitaran denaturasi pada 94°C selama 30 saat, suhu sepuh lindap pada 63°C selama 30 saat dan pemanjangan pada 72°C selama 1 minit dengan tambahan pemanjangan akhir pada 72°C selama 10 minit. Keputusan ujian positif ditentukan dengan kehadiran saiz amplikon PCR pada 1238 bp bagi *S.* Typhi dan 549 bp bagi *S.* Paratyphi A selepas dianalisis dengan elektroforesis gel agarose. Sebagai keputusan kajian ini, suhu optimum sepuh lindap ialah 63°C dengan tahap sensitiviti pengesanan 0.39 ng DNA apabila kedua-dua *S.* Typhi dan *S.* Paratyphi A digunakan sebagai templat sasaran. Selanjutnya, mPCR mampu mengesan 3.13 ng untuk *S.* Typhi dan 0.19 ng untuk *S.* Paratyphi A ketika ujian sensitiviti dilaksanakan secara serentak dengan menggunakan siri pencairan DNA. Keputusan mPCR daripada 75 bakteria isolat menunjukan

menunjukan sensitiviti dan spesifisiti adalah 100%. Sebagai alternatif kepada kaedah konvensional, mPCR ini mampu digunakan sebagai alternatif kepada kaedah kultur konvensional berdasarkan amplifikasi gen *ST50* dan juga *spa4289 (hsdM)*. Oleh itu, pengesanan dengan pantas, sensitif dan juga spesifik untuk mengenalpasti *S.* Typhi dan *S.* Paratyphi A ini berpotensi untuk digunakan sebagai alat diagnostik

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to Typhoid fever and Paratyphoid fever

Typhoid and paratyphoid are systemic infection caused by *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi A. They still remain as global public problems in many parts of the world mostly in developing countries due to poor sanitary conditions and improper treatment of water supplies (Parry *et al.,* 2002; Connor and Schwartz, 2005). Generally, typhoid fever is more severe compared to paratyphoid fever but recently typhoid fever is gradually being replaced by paratyphoid fever (Ochiai *et al.,* 2005). According to Crump *et al.*, (2004) these incidences of enteric fever are reported annually that account over 21.6 million cases and 216,000 associated deaths every year. Typhoid fever is defined as patients with fever (38°C and above) for at least three days, with a laboratory-confirmed positive culture (blood, bone marrow, bowel fluid) of *S.* Typhi whereas probable case of typhoid fever is defined as a patient with fever (38°C and above) for at least three days, with a positive serodiagnosis or antigen detection test but without *S.* Typhi isolation (WHO, 2003)

S. Typhi and *S.* Paratyphi A are primarily infective in human. They enter via the oral route from contaminated food or drink. During an acute infection, enteric organism (*S.* Typhi and *S.* Paratyphi A) multiplies in mononuclear phagocytic cells before being released into the bloodstream. These enteric bacilli reach the bloodstream principally by lymph drainage from mesenteric nodes, after which they enter the thoracic duct and then the general circulation. As a result of this silent primary bacteraemia the pathogen reaches the intracellular within 24 hours after ingestion throughout the organs of the reticuloendothelial system (spleen, liver, bone marrow). Typhoid fever takes about 10-14 days of incubation periods. The incubation period in a particular individual depends

on the quantity of inoculums (WHO, 2003). The clinical manifestation for typhoid fever includes malaise, headache, constipation, bradycardia and myalgia (Brooks *et al.*, 2007). Patients with paratyphoid fever are reported to develop more numerous rose-colored spots than patients with typhoid fever but it is difficult to detect in dark-skinned patients (Kudalkar *et al.*, 2004).

The severity of the disease depends on the salmonellae strains, immuno-competency, age and nutritional status of patients (Crum, 2003). Dose exposure also influences the infectiveness of the enteric fever which depends on the serovar, bacterial strains, bacterial growth condition and host susceptibility (Sussman, 2001). According to Bhan *et al.*, (2005) the infectious dose between 10^3 and 10^6 organism of enteric bacteria which accidentally ingested through oral route will cause the desired effect for development of enteric fever. Ingestion of 10^5 organisms resulted in typhoid fever for up to 55% of the study volunteers (Guzman *et al.*, 2006)

The treatment of enteric fever requires antimicrobial treatment particularly with ampicillin or trimethoprim-sulfamethoxazole. Multiple drug resistance becomes a problem in *Salmonella* infection that is transmitted genetically by plasmid among enteric bacteria. Susceptibility testing is an important adjunct to selecting a proper antibiotic (Brooks *et al.*, 2007). Fluoroquinolone and third generation of cephalosporins are the commonly used antibiotics against multi-drug resistant infections for treatment of enteric fever (Bavdeka, 1991). Furthermore, *S.* Typhi vaccine provides little cross-protection against fluoroquinolone resistant strains of *S.* Paratyphi A (Pokheral *et al.*, 2006; Tankhiwale *et al.*, 2003; Zhang *et al.*, 2004).

1.2 Characteristics of *S.* Typhi and *S.* Paratyphi A

Salmonella are facultative anaerobic Gram-negative bacilli belonging to the Family *Enterobacteriaceae* (Everest *et al.,* 2001). *Salmonella* consist of two species that are *Salmonella enterica* and *Salmonella bongori. Salmonella* Typhi and *Salmonella* Paratyphi A serovars are classified in *Salmonella enterica* species. These two bacteria can be grown on blood agar, MacConkey agar, Salmonella-shigella agar (SS), desoxycholate agar, xylose-lysine-desoxycholate agar (XLD), hektoen enteric agar (HE) and bismuth sulfite agar (WHO, 2003) as well as Selenite F broth. The colony characteristics of each media are listed in Table 1.0

Table 1.0: Colony characteristics of enteric organisms (WHO, 2003)

Media	Colony Characteristics of enteric organisms
Blood agar	Produce non-haemolytic smooth white colonies.
MacConkey agar	Produce lactose non-fermenting smooth colonies
SS agar	Produce lactose non-fermenting colonies with black centres (except *S.* paratyphi A, do not have black centres).
Desoxycholate agar	Produce lactose non-fermenting colonies with black centres (except *S.* paratyphi A, do not have black centres).
XLD agar	Produce transparent red colonies with black centres (except *S.* paratyphi A, do not have black centres).
Hektoen enteric agar	Produce transparent green colonies with black centres (except *S.* paratyphi A, do not have black centres).
Bismuth sulfite agar	Produce black colonies

Currently, culture method and biochemical test still remain as routine test in microbiology laboratories for diagnosis of typhoid and paratyphoid fever. Biochemical test includes triple sugar iron (TSI), urease, citrate, sulfide indole motility (SIM) and methyl red are performed for identification of *Salmonella* species (Appendix 1: Media preparation). The suspected colonies obtained from media agar (Table 1.0) are tested biochemically. The result obtained are listed in the Table 1.1 for diagnosis of *S.* Typhi and *S.* Paratyphi A (WHO, 2003)

Table 1.1: Biochemical test for identification of enteric organisms (WHO, 2003)

Organism	Triple Sugar Iron				Motility, Indole, Urea			Citrate
	Slant	Butt	H_2S	Gas	Motility	Indole	Urea	
S. Typhi	Alkaline	Acid	Weak	-	+	-	-	-
S. Paratyphi A	Alkaline	Acid	-	+	+	-	-	-
Other *Salmonellae*	Alkaline	Acid	V	V	+	-	-	V

V= Variable result

Kaufmann-White scheme is widely used for classification of *Salmonella* isolates based on detection of somatic (O), capsular (Vi) and flagellar (H) antigens that are present in the cell surface of *Salmonella* by slide agglutination test with specific antisera (Ochei and Kolhatkar, 2000) . The O factors determine the serogroup and H factors define the serotype of a *Salmonella* strains (Popoff, 2001; Fitzgerald *et al.,* 2003). The *S.* Typhi, *S.* Paratyphi C, some strain of *S.* Dublin and a few strains of *Citrobacter freundii* (Selander *et al.,* 1992) may possess Vi (Virulence) capsular polysaccharide antigen, a homopolymer of N-acetylgalactosamine uronic acid that forms covering layer on the outside cell wall and masks the O antigen. The presence of Vi antibody against this antigen is an indicative for identification of *S.* Typhi in an individual which may be

symptomatic or asymptomatic depends on carrier status (Ochei and Kolhatkar, 2000) and this Vi antigen is an essential factor for *S.* Typhi survival in human serum (Hashimato *et al.,* 1993). The serological characteristics of *S.* Typhi and *S.* Paratyphi A are shown in the Table 1.2.

Table 1.2: The antigenic structure of *Salmonella* species

Organism	O antigen	H antigen	Vi antigen
S. Typhi	9 or 12	D	Present
S. Paratyphi A	2	A	Absent
S. Paratyphi B	4	B	Absent
S. Paratyphi C	6 or 7	C	Present

1.3 Statistic prevalence of typhoid and paratyphoid fever in Malaysia

The highest incidence rate of typhoid fever has been reported in Kelantan (Jabatan Kesihatan Negeri Kelantan, 2005). The factors affecting the rising cases in Kelantan are poor treatment of water supplies especially in the rural area. This is because they do not have access to a treated water supply system and using untreated well (Kementerian Kesihatan Malaysia, 2006). The incidence rate of typhoid fever reported was less than 5 per 100,000 populations as reported by Kementerian Kesihatan Malaysia (2006). Unhygienic food handling, untreated water supply and poor sanitary environment may increase the typhoid cases (Meftahuddin, 2002). According to Jabatan Kesihatan Negeri Sarawak (2008), the incidence rate of typhoid and paratyphoid fever was 1 per 100, 000 of populations in Sarawak with mortality rate less than 0.05 per 100,000 populations.

The incidence of paratyphoid fever in Malaysia is low compared to other countries and isolation of *S.* Paratyphi A is poor, only 6 strains were available in Malaysia (Teh *et al.*, 2008). The increasing prevalence case of paratyphoid fever in Malaysia depends on travelers and immigrant workers that are generally implicated in the transmission of the disease. Travelers returning from endemic countries such as India and Nepal where the disease is highly prevalent and immigrant workers arriving from endemic regions especially people employed in the food and beverage sector (Connor and Schwartz, 2005). In addition, typhoid vaccine was administered to all travelers in endemic countries offer no protection against *S.* Paratyphi A infection then become predominant cause of enteric fever among vaccinated travelers (Teh *et al.*, 2008)

1.4 Diagnosis method for identification of *S.* Typhi and *S.* Paratyphi A

Different diagnosis methods such as culture of blood, stool, bone marrow, urine, bile, widal test and ELISA using Vi antigen are used for diagnosis of typhoid fever. Widal test and culture of blood and stool remain as universally practiced diagnostic procedures because other methods are either invasive, have failed to prove their utility or are expensive (Haque *et al.,* 1999). The complexity of culturing method and time involved to obtain the result usually within 4 – 7 days lead to the development of Widal test as a rapid serologic screening test which based on agglutination method required antigen-antibody reaction (Widal, 1896). But this diagnostic method has limitations such as difficulties with standardization of the antigen and the significance of specific agglutination titer is questioned and debated. Furthermore, the conventional serotyping methods based on the Kauffmann-White scheme have some limitation such as time consuming, tedious, require high quality of antisera, difficult to obtain consistency and very costly in resource limiting settings as well as subjective in interpretation (Lim and Thong, 2009)

Later, slide agglutination, IDL TUBEX (Lim *et al.*, 1998), Typhidot (Ismail *et al.,* 1991) and immunoglobulin M dipstick (Hatta *et al.,* 2002a) were developed in order to increase reliability, efficacy, integrity and quality of the test that is currently being used as diagnostic tool in the most routine microbiology laboratories. Dipstick assay for the detection of immunoglobulin M (IgM) antibodies against the lipopolysaccharide of *S.* Typhi was proposed by Hatta *et al.,* (2002) showed some limitation because these tests require several hours to develop which might be inconvenient for emergency cases (Pastoor *et al.,* 2008).

In view, DNA-based detection system has been attempted in different laboratories (Way *et al.,* 1993), for example, development of first molecular biology techniques for early detection of enteric fever is hybridization using DNA probe which said to be specific. However, this technique has poor sensitivity (Banavandi *et al.,* 2005). A polymerase chain reaction (PCR) technology has been reported as rapid diagnostic tool because this assay is effective and reproducible for the detection of *S.* Typhi and *S.* Paratyphi A (Alvarez *et al.,* 2004; Aziah *et al.,* 2007; Abubakar *et al.,* 2007). In the early development of PCR technology for diagnosis of enteric fever, PCR approach has been found to discriminate typhoid from other febrile conditions including paratyphoid and culture negative cases (Song *et al.,* 1993). However, the complexity of molecular techniques requires high costs hinder its application other than as research tool (Song *et al.,* 1993; Hatta and Smits, 2007).

The efficacy of the PCR detection method depends on the uniqueness of the sequence of the pathogen of interest as well as the specific binding of the primers and probe to the target (Chen *et al.,* 2009). These PCR approach using specific target genes have previously been reported by others such as the specific detection of *Salmonella* are associated with virulence including *invA* which defined as *Salmonella* invasion protein gene (Ferretti *et al.,* 2001; Malorny *et al.,* 2004) or *fliC* (flagellin gene) (Song *et al.,* 1993). All these previous finding showed that PCR is more sensitive and specific for diagnosis of enteric fever especially typhoid fever. However, these approaches mostly rely on the amplification of a single target gene.

Therefore, the development of multiplex PCR (mPCR) approaches that based on the simultaneous amplification of multiple sequence of multi-pathogens present in single PCR tube reaction was employed. These molecular methods are highly sensitive, very specific, fast and reproducible. One of the approach has previously been reported by Hirose *et al.,* (2002) that focused on the amplification of multiplex PCR of *tyv*, *fliC-d*, *viaB*, *fliC-a* and *prt* gene for identification of *S.* Typhi and *S.* Paratyphi A. Another approach of multiplex PCR was also developed by Ngan *et al.,* (2010); a novel serovar-specific gene for differentiation between *S.* Typhi and *S.* Paratyphi A which amplified *stgA* (fimbrial operon gene), SSPA11723a and SSPA1724 (the intergenic region genes), STY4220 (putative regulatory protein) and *ompC* (outer membrane protein C gene). Multiplex PCR is better than conventional method because its offers a significant shorter time and cost saving especially with large number of samples.

1.5 Rationale of study

S. Typhi and *S.* Paratyphi A are the causative agents of typhoid and paratyphoid. Due to an increase prevalence of these diseases, many diagnostic approaches were developed to increase the accuracy, efficacy and integrity of the test for diagnosis of typhoid and paratyphoid disease especially in molecular-based techniques. Polymerase chain reaction (PCR) is commonly used as diagnostic method since it is sensitive and specific as well as reproducible. The conventional culture method, biochemical test and serological method are still being used in most microbiology laboratories. However, all of this method requires trained personnel, time-consuming and tedious as well as need high quality of antisera and difficult to distinguish between other *Salmonella* serovars. Therefore, the aim of this study was to develop multiplex PCR assay for specific detection of *ST50* gene and *spa4289 (hsdM)* gene for *S.* Typhi and *S.* Paratyphi A respectively.

ST50 gene is a gene that encodes for 50 kDa outer membrane protein of *S.* Typhi. The 50 kDa protein was found to be antigenically specific and currently being used as antigen for Typhidot test (Ismail *et al.,* 1991). Dry-reagent based PCR using ST50 gene as a target was developed with high sensitivity and specificity (Aziah *et al.,* 2007). Based on the study, 1238 bp sequence of *ST50* gene was amplified in together with built-in internal amplification control (IAC) with amplicon size of 810 bp sequence of *hemM* gene of *Vibrio cholorae* as IAC primer. The specificity and sensitivity was reported to be 87.9% and 100% respectively.

In this study, *ST50* gene of *S.* Typhi and *spa4289 (hsdM)* gene of *S.* Paratyphi A which encode outer membrane of *S.* Typhi and the DNA methyltransferase of *S.* Paratyphi A were used to develop multiplex PCR (McClelland *et al.*, 2004) (GenBank Accession No.: AF306456.1). This multiplex PCR enabled detection of bacterial isolates that can discriminate between *S.* Typhi and *S.* Paratyphi A. Multiplex PCR has the advantages of simultaneous detection of the two genes that can reduce time, multiple pipetting techniques as well as possible contamination.

1.6 Objectives of study

The objectives of this study are

(i) To develop multiplex PCR detection method for identification of *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi A based on ST50 gene and spa4289 gene respectively.

(ii) To evaluate sensitivity and specificity of the multiplex PCR using DNA from pure culture of bacteria.

CHAPTER 2: MATERIALS AND METHODOLOGY

2.1 Materials

Chemicals, Reagents and Instruments

The chemical and reagents used in this method are listed in Table 2.1 whereas instruments used listed in Table 2.2.

Table 2.1: List of chemical, reagents and media

Chemical, reagents and media	Manufacturer
Agarose LE, Analytical Grade	Promega, USA
dNTP mix (dATP, dGTP, dTTP, dCTP)	Promega, USA
DNeasy Blood and Tissue kit	Qiagen, Hilden, Germany
EDTA	BDH Chemicals, UK
Ethidium bromide (10mg/ml)	Promega, USA
Gene Ruler 100bp DNA ladder	Promega, USA
Glacial acetic acid	Merck, Germany
M.R.V.P Medium	Oxiod Ltd, UK
Nutrient agar and broth	Oxiod Ltd, UK
S.I.M Medium	Oxiod Ltd, UK
Simmons citrate agar	Oxiod Ltd, UK
Triple Sugar Iron agar	Oxiod Ltd, UK
Taq DNA polymerase, $MgCl_2$ and PCR buffer	Promega, USA
Tris Base	Promega, USA
Urea agar base	Oxiod Ltd, UK

Table 2.2: List of Instruments

Instruments	Manufacturer
Autoclave	Kubota, Fujioka, Japan
A&D GR 300 Analytical Balance	A & D, USA
ABI PRISM® 3100 Genetic Analyzer	Applied Biosystems, USA
Electrophoresis Bio-Rad Power Pac	Bio-Rad Laboratories , USA
Electrophoresis Bio-Rad Sub Cell GT	Bio-Rad Laboratories , USA
Microcentrifuge	Eppendorf, Hamburg, Germany
Micropipette (2.5,10,20,100,200µl)	Eppendorf, Hamburg, Germany
MJ Research PTC-200 Thermal Cycler	MJ Research,USA
NanoDrop 1000 Spectrophotometer	Thermo Fisher Scientific, USA.
UV Illuminator	Syngene, UK

2.2 Methodology

The overall flow chart of the study is shown in Figure 2.0

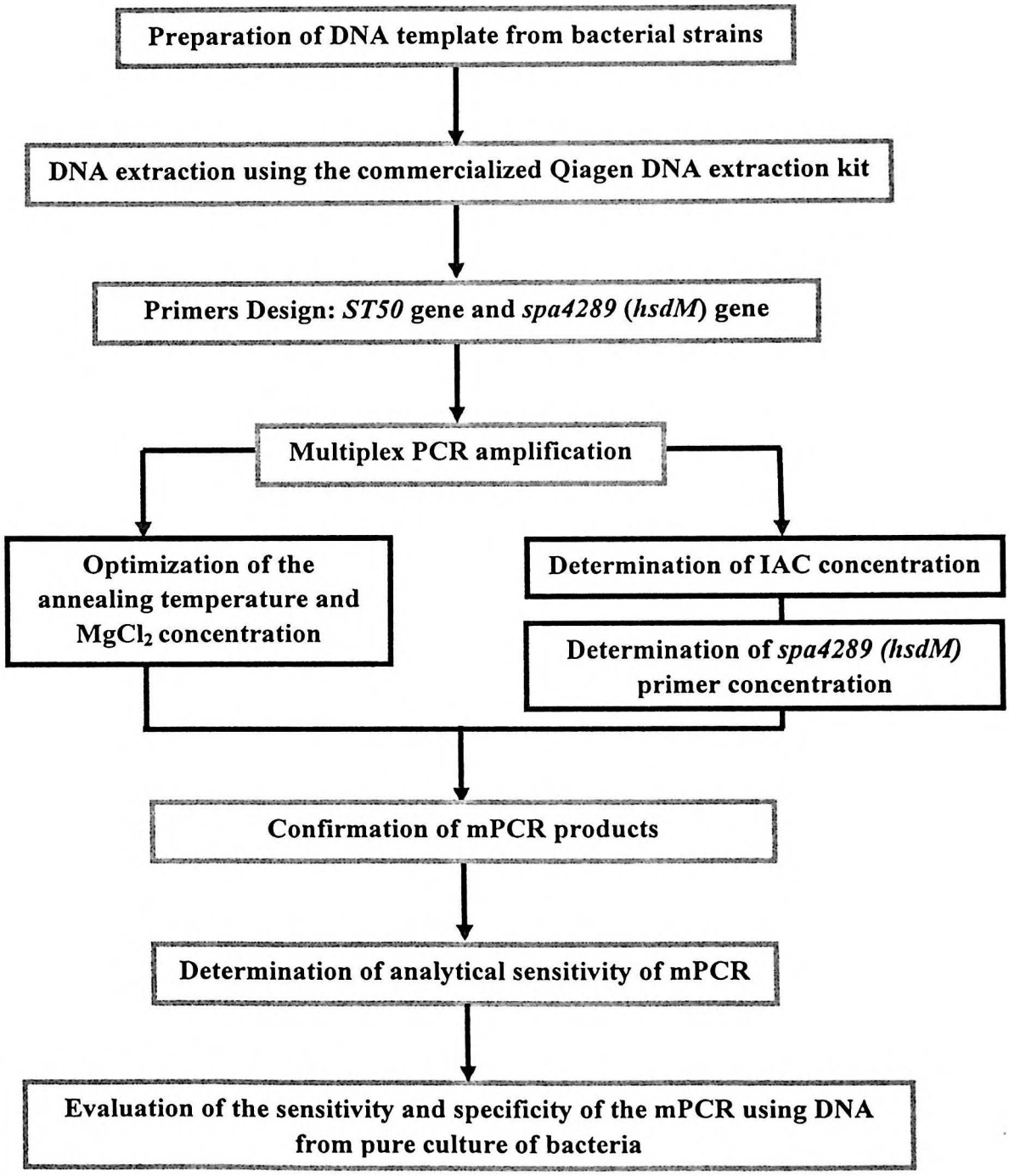

Figure 2.0 : Flow chart of overall study

2.2.1 Preparation of DNA template from bacterial strains

A total 75 bacterial isolates (25 of *S.* Typhi, 25 of *S.* Paratyphi A and 25 of *Salmonella* serovars and others enteric bacteria) were obtained from glycerol stock culture collection, Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (Table 2.3). The bacterial isolates were inoculated in nutrient broth for 18 hours at 37°C using an incubator shaker (Innova 400, USA). Then, one loop of each nutrient broth of bacterial isolates was streaked on nutrient agar and incubating at 37°C for 18 hours. After incubation for 18 hours at 37°C, single colonies were tested biochemically (Triple Sugar iron (TSI), Urease, citrate, Sulfide indole motility (SIM) and Methyl red) and serologically for confirmation of *S.* Typhi and *S.* Paratyphi A. *S.* Typhi agglutinated with poly-O, O-9 and H-d antisera as well as Vi antisera as per the standard protocols. Then, a single colony was picked and inoculated in 10 ml of nutrient broth then grown at 37°C using an incubator shaker (Innova 400, USA) for 18 hours. Three milliliters of each bacterial culture were subjected to DNA extraction.

Table 2.3: List of bacterial isolates for evaluation of multiplex PCR

Species	No. of Isolates
Salmonella Typhi	25
Salmonella Paratyphi A	25
Salmonella Paratyphi B	2
Salmonella Paratyphi C	1
Salmonella Brandii	1
Salmonella Walter	1
Salmonella Typhimurium	2
Salmonella Choleraesuis	1
Shigella boydii	1
Shigella sonnei	1
Shigella dysenteriae	1
Shigella flexneri	1
Acinetobacter baumanni	1
Klebsiella pneunomoniae	1
Pseudomonas aeruginosa	1
Escherichia coli	7
Enteroinvasive *E. coli* (EIEC)	1
Enterohaemorrhagic *E. coli* (EHEC)	1
Vibrio cholera	1
Total	**75**

2.2.2 DNA Extraction

The genomic DNA of all bacterial strains was extracted using the commercialized Qiagen DNeasy Blood and Tissue extraction kit (Qiagen, Hilden, Germany), according to the protocol supplied by manufacturer. One milliliter of each bacterial culture in ten ml nutrient broth was transferred into 1.5 ml tube and centrifuged at 8000 rpm for one minute. The supernatant was discarded. The pellet was resuspended in 180 µl buffer ATL by pipetting the solution up and down, and then the samples was first lysed using 10 µl of proteinase K and mixed by vortex. This mixture was incubated for two hours at 55°C in water bath until all the cells were completely lysed. After incubation, four µl of RNase A (100mg/ml) was added, mixed and incubated at room temperature for two minutes. Then, 200 µl of buffer AL was addedinto the tube and mixed thoroughly by vortexingfollowed by incubation at 70°C for ten minutes. Then, 200 µl of absolute ethanol was added and vortex briefly. The DNeasy Mini spin column was placed into a two ml collection tube. The sample was added to mini spin column, centrifuged at 8000 rpm for one minute and the flowthrough was discarded. Then, 500 µl of buffer AW1 was added into the DNeasy Mini spin column and centrifuged again at 8000 rpm for one minute. The flow-through was discarded. The spin column was washed again by adding 500 µl of buffer AW2 and centrifuged at 13000 rpm for one minute. The flow-through was then discarded. Lastly, the DNeasy mini spin column was placed in clean 1.5 ml microcentrifuge tube and 50µl of Tris-HCl elution (AE) buffer was pipette directly onto DNeasy membrane. The mixture was centrifuged at 8000 rpm for one minute to elute the DNA.

Finally, the concentration of DNA was determined by spectrophotometer (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE). The purified DNA obtained has A_{260}/A_{280} ratios of 1.7-1.9 and absorbance scans show a symmetric peak at 260nm confirming high purity. DNA samples were stored at -20°C prior to use.

2.2.3 Primers Design: *ST50* gene and *spa4289* (*hsdM*) gene

The multiplex PCR assay in this study contained target primers based on 1238 bp sequence of the *ST50* gene which encode the 50 kDa outer membrane protein of *S.* Typhi (GenBank Accession No. BD079162) (Aziah *et al.,* 2007). The target primer of the *spa4289 (hsdM)* gene which encodes the DNA methyltransferase of *S.* Paratyphi A was also used in this study as mentioned earlier (GenBank Accession No.: AF306456.1) (McClelland *et al,* 2004). Specific primer for *spa4289 (hsdM)* gene was selected using Primer3 program and analyzed with BLAST (Basic Local Alignment Search Tool) program to check the specificity of primer. The primer for internal amplification control (IAC) was included in the assay to validate the negative results. IAC used was based on the 810 bp sequence of the *hemM* gene of *Vibrio cholorae* (GenBank Accession No. AF22752). The IAC template was prepared in-house by cloning of *hemM* gene in pCR2.1-TOPO and the recombinant plasmid, pVcHMII was transformed in *E. coli* (TOP10) for plasmid extraction (Aziah *et al.,* 2007). The primers were described in the Table 2.4.

Table 2.4: Primers used in the multiplex PCR

Primer name	Primer sequence (5'-3')	Target gene	Amplicon size	Source
ST50 – F	TTCGAAAAAATTAACGAAGCA	*ST50* (*S.* Typhi)	1238 bp	Aziah *et al.*, (2007)
ST50 – R	CCGTCTGCGGCAGCATCCTGC			
VHMFP	GTTGAACGATTGTCGCTGATC	*hemM (IAC)*	810 bp	Aziah *et al.*, (2007)
18R-1	GTTTCTGTTCTTACCCGTTTC			
spa4289- F	GAATCGTGCTGGATGACCTTTAAC	*spa4289* (*S.* Paratyphi A)	549 bp	McClelland *et al,* (2004)
spa4289 – R	GATCAAGAACAGTACATCGATTGGTT			

2.2.4 Multiplex PCR amplification

2.2.4.1 Optimization of the annealing temperature

The gradient PCR was performed to determine the optimum annealing temperature for mPCR. This mPCR was tested with different annealing temperature ranging from 50°C – 70°C. The DNA template was prepared by mixed of 100 ng/µl *S.* Typhi ATCC 7251 and 100 ng/µl *S.* Paratyphi A ATCC 9150 in one tube to produce approximately 50 ng/µl of *S.* Typhi and *S.* Paratyphi A in addition of built in IAC template. This DNA amplification was performed in a reaction of 20 µl. The concentration and volume of PCR composition was described in the Table 2.5.

Table 2.5: PCR composition for optimization of annealing temperature

PCR reagent	Final Concentration	Volume per reaction (µl)
PCR grade water	-	4.0
5X tag polymerase buffer	1X	4.0
5mM $MgCl_2$	3 mM	2.4
10mM dNTPs mix	0.2 mM	0.4
10 pmol ST50-F	10 pmol	1.5
10 pmol ST50-R	10 pmol	1.5
10 pmol 18R1	10 pmol	1.0
10 pmol VHMFP	10 pmol	1.0
10 pmol spa4289-F	3 pmol	1.0
10 pmol spa4289-R	3 pmol	1.0
pVcHM II Template	80 pg/µl	1.0
5U Tag polymerase	1 U	0.2
50 ng/µl DNA template	50 ng/µl	1.0
TOTAL VOLUME		**20**

2.2.4.2 Optimization of $MgCl_2$ concentration

The optimization of $MgCl_2$ concentration as cofactor of PCR was performed at concentration ranging from 0 – 4 mM. The final volume of each PCR reaction was 20µl. The PCR master mix was described in Table 2.6.

Table 2.6: PCR composition for optimization of $MgCl_2$ concentration

PCR reagent	Final Concentration	Volume per reaction (µl)				
PCR grade water	-	6.4	5.6	4.8	4	3.2
5X tag polymerase buffer	1 X			4.0		
5mM $MgCl_2$	0 mM – 4 mM	0	0.8	1.6	2.4	3.2
10mM dNTPs mix	0.2 mM			0.4		
10 pmol ST50-F	10 pmol			1.5		
10 pmol ST50-R	10 pmol			1.5		
10 pmol 18R1	10 pmol			1.0		
10 pmol VHMFP	10 pmol			1.0		
10 pmol spa4289-F	3 pmol			1.0		
10 pmol spa4289-R	3 pmol			1.0		
pVcHM II Template	80 pg/µl			1.0		
5U Tag polymerase	1 U			0.2		
50 ng/µl DNA template	50 ng/µl			1.0		
TOTAL VOLUME				**20**		

2.2.4.3 Determination of IAC template concentration

The optimization of IAC concentration was performed by serially diluted the amount of IAC template (8 fold dilutions) with 10 mM Tris-HCl pH 8.0 to concentration ranging from 32.4 ng/µl to 1.4 pg/µl and the optimum intensities of the IAC bands were compared. The suitable concentration was selected when 3 bands appeared on agarose gel shows optimum band intensities. The PCR protocol and composition was described in the Table 2.7.

Table 2.7: PCR composition for determination of IAC primer

PCR reagent	Final Concentration	Volume per reaction (µl)
PCR grade water	-	4.0
5X tag polymerase buffer	1 X	4.0
5mM $MgCl_2$	3 mM	2.4
10mM dNTPs mix	0.2 mM	0.4
10 pmol ST50-F	10 pmol	1.5
10 pmol ST50-R	10 pmol	1.5
10 pmol 18R1	10 pmol	1.0
10 pmol VHMFP	10 pmol	1.0
10 pmol spa4289-F	3 pmol	1.0
10 pmol spa4289-R	3 pmol	1.0
pVcHM II Template	32.4 ng/µl – 1.4 pg/µl	1.0
5U Tag polymerase	1 U	0.2
50 ng/µl DNA template	50 ng/µl	1.0
TOTAL VOLUME		**20**

2.2.4.4 Determination of spa4289 primer concentration

The optimization of primer concentration of *spa4289 (hsdM)* gene was performed with concentration ranging from 1-5 pmol to detect optimum amplification which was observed by UV illuminator. The PCR was performed with the presence of three primer pairs in reaction of 20µl. The optimum concentration was selected as spa4289 primer for all mPCR reactions after observation of optimum band intensities on agarose gel electrophoresis under UV illuminator. The PCR composition for optimization of spa4289 primer concentration was described in Table 2.8

Table 2.8: PCR composition for optimization of spa4289 (*hsdM*) primer concentration

PCR reagent	Final Concentration	Volume per reaction (µl)
PCR grade water	-	4.0
5X tag polymerase buffer	1 X	4.0
5mM $MgCl_2$	3 mM	2.4
10mM dNTPs mix	0.2 mM	0.4
10 pmol ST50-F	10 pmol	1.5
10 pmol ST50-R	10 pmol	1.5
10 pmol 18R1	10 pmol	1.0
10 pmol VHMFP	10 pmol	1.0
10 pmol spa4289-F	1 pmol - 5 pmol	1.0
10 pmol spa4289-R	1 pmol - 5 pmol	1.0
pVcHM II Template	80 pg/µl	1.0
5U Tag polymerase	1 U	0.2
50 ng/µl DNA template	50 ng/µl	1.0
TOTAL VOLUME		**20**