DEVELOPMENT AND OPTIMIZATION OF A MULTIPLEX PCR ASSAY FOR SIMULTANEOUS DETECTION OF SALMONELLA ENTERITIDIS, SALMONELLA TYPHIMURIUM, SALMONELLA WELTEVREDEN, SALMONELLA AGONA, AND SALMONELLA HEIDELBERG

By

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LIST OF SYMBOLS AND ABBREVIATIONS

-	Negative
+	Positive
%	Percentage
<	less than
>	more than
^{o}	degree
^{o}C	degree Celcius
A	Absorbance
AC	Amplification Control
ATCC	American Type Culture Collection
BLASTN	Basic Local Alignment Search Tool for Nucleotide
Bp	base pair
BSA	Bovine Serum Albumin
C	Cytosine
CDC	Centers for Disease Controls
CFU/ml	Colony Forming Unit per millilitre
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytosine-5'-triphosphate
ddH_2O	double distilled water
dGTP	deoxyguanosine-5'-triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine-5'-triphosphate
DTU	Denmark Technology University
E. coli	*Escherichia coli*
EHEC	Enterohaemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
F	Forward
fg/ml	femtogram per microlitre
G	Guanosine
G	gram
H_2S	Hydrogen sulphide
HUSM	Hospital Universiti Sains Malaysia
IC	Internal Control
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMR	Institute for Medical Research
INFORMM	Institute for Research in Molecular Medicine
Kb	kilobase
mg/ml	milligram per millilitre
$MgCl_2$	Magnesium chloride
Ml	millilitre
µl	microlitre

µm	micrometre
mM	millimolar
µM	micromolar
mPCR	multiplex PCR
MRVP	Methyl Red Vogas-Proskauer
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	nanogram
ng/ml	nanogram per microlitre
NS	Normal Saline
NTS	Non-Typhoidal *Salmonella*
OD	Optical density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
pg/ml	picogram per microlitre
PVP	Polyvinylpyrrolidone
R	Reverse
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SGI	*Salmonella* Genomic Island
SIM	Sulphate Indole Motility
SPI	*Salmonella* Pathogenicity Island
spp.	species
SSH	Suppression Subtractive Hybridization
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TS	Typhoidal *Salmonella*
TSI	Triple Sugar Iron
U	Unit
U/µl	Unit per microlitre
UCI	University of California, Irvine
V	Volt
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organization
X	times
x *g*	gravity
XLD	Xylose Lysine Deoxycholate

APPENDICES

PEMBANGUNAN DAN PENGOPTIMUNAN TINDAKBALAS RANTAIAN POLIMERASI (PCR) MULTIPLEKS UNTUK PENGESANAN SERENTAK SALMONELLA ENTERITIDIS, SALMONELLA TYPHIMURIUM, SALMONELLA WELTEVREDEN, SALMONELLA AGONA DAN SALMONELLA HEIDELBERG

ABSTRAK

Salmonella enterica adalah spesis pathogen bawaan makanan penting yang menyebabkan gastroenteritis dan jangkitan sistemik dalam manusia. Di Malaysia, kejadian Salmonellosis bawaan makanan yang disebabkan oleh serotip *Salmonella* bukan tifoid (NTS) semakin meningkat secara berkala, manakala kejadian yang disebabkan oleh *Salmonella* tifoid (TS) semakin menurun. NTS telah diklasifikasikan sebagai punca utama wabak penyakit bawaan makanan di negara yang maju manakaladi negara yang sedang membangun, ia merupakan unsur ancaman kesihatan yang membimbangkan. Penserotipan adalah kaedah yang lazim digunakan untuk pembezaan pathogen ini dari 2,600 jenis serotip yang wujud. Walau bagaimanapun, kaedah ini mempunyai beberapa kelemahan seperti spesifisiti yang rendah, kaedah yang menyusahkan, mahal dan kadang-kala memberi keputusan yang mengelirukan. Sehingga kini, negara yang maju dan negara yang membangun di seluruh dunia masih tidak mempunyai asai untuk pengenalpastian and pembezaan secara serentak untuk lima jenis serotip NTS yang pra-dominan dan sering dipencilkan daripada pesakit gastroenteritis di hospital, iaitu *S.* Enteritidis, *S.* Typhimurium, *S.* Weltevreden, *S.* Agona dan *S.* Heidelberg. Oleh itu, kajian ini bertujuan untuk menghasilkan asai tindakbalas berantai polymerasi multiplek (mPCR) berasaskan amplifikasi jujukan asid nukleik yang unik untuk pengesanan dan pengesahan lima jenis serotip NTS ini secara serentak. Asai mPCR ini mengandungi lima pasangan primer spesifik untuk lima jenis serotip NTS dalam satu reaksi yang menyasarkan jujukan asid nukleik yang unik seperti yang ditentukan dengan perisian bioinformatik. Analisis penjujukan DNA menunjukkan bahawa hasil teramplifikasi PCR bagi setiap serotip adalah seperti yang dijangka. Asai mPCR ini juga mengandungi 2 jenis kawalan supaya kualiti yang bermutu tinggi dapat dicapai untuk asai diagnostik, iaitu

kawalan dalaman, gen *inv*A untuk "Pan-*Salmonella*" dan kawalan amplifikasi, gen 16S rRNA untuk "Pan-bakteria". Pengoptimunan yang melibatkan pengoptimunan kepekatan primer, kepekatan $MgCl_2$, kepekatan gliserol, suhu sepuh lindap dan masa pemanjangan primer telah dijalankan untuk asai mPCR ini. Analisis kespesifikan untuk asai mPCR yang telah dioptimumkan adalah 100%. Kesemua 29 bakteria bukan sasaran yang mengandungi 10 bakteria bukan *Salmonella* dan 19 spesis *Salmonella* bakteria telah diuji negatif manakala bakteria sasaran NTS diuji positif dalam sampel spika tunggul hidup. Analisis kepekaan untuk asai mPCR bagi kesemua 5 jenis serotip NTS adalah >1000 CFU/ml atau 10 pg/µl untuk DNA genom bakteria. Asai ini juga disahkan 100% sensitif kepada 116 isolat klinikal dari sumber tempatan dan luar negara. Kesimpulannya, satu mPCR yang sensitif, spesifik dan murah telah dihasilkan untuk pengesanan secara serentak bagi 5 jenis serotip NTS yang pradominan dan sering dipencilkan di serata dunia. Teknik ini boleh digunakan secara langsung dan mudah untuk ditubuhkan dalam makmal rutin yang lengkap dengan penyediaan DNA dan kemudahan asai PCR. Asai ini adalah satu penemuan yang unggul untuk diagnosis makmal bagi *Salmonella* bukan tifoid dan ia juga boleh digunakan untuk pengawasan rapi dan kajian epidemiologi bagi penyakit yang disebabkan oleh NTS.

DEVELOPMENT AND OPTIMIZATION OF A MULTIPLEX PCR ASSAY FOR SIMULTANEOUS IDENTIFICATION OF SALMONELLA ENTERITIDIS, SALMONELLA TYPHIMURIUM, SALMONELLA WELTEVREDEN, SALMONELLA AGONA, AND SALMONELLA HEIDELBERG

ABSTRACT

Salmonella enterica are a species of important food-borne pathogens that cause gastroenteritis and systemic infections in humans. The incidence of food-borne Salmonellosis due to non-typhoidal *Salmonellae* (NTS) serotypes is increasing periodically while typhoidal Salmonellosis (TS) decreases in Malaysia. NTS have been classified as the leading cause of food-borne disease outbreaks in developed countries and are also a public health concern in developing countries. Serotyping is the conventional method used for differentiating these pathogens from the 2,600 serotypes known to exist. However, this method has a number of disadvantages including low specificity, costly, laborious and sometimes the results are ambiguous. Until today, there is still no assay available for simultaneous identification and differentiation of the five predominant NTS serotypes, i.e., *S.* Enteritidis, *S.* Typhimurium, *S.* Weltevreden, *S.* Agona, and *S.* Heidelberg, which are increasingly being isolated in hospitalized-gastroenteritis patients in both developed and developing countries worldwide. Thus, the aim of this study was to develop a multiplex PCR (mPCR) assay to enable the simultaneous detection and confirmation of these five NTS serotypes based on amplification of their unique nucleic acid sequences. The mPCR assay consists of 5 pairs of specific primers in a single reaction that target the unique nucleic acid sequences of the 5 NTS serotypes, which were determined by using bioinformatic tools. Sequencing data showed that the amplified PCR products of the serotypes were as expected of their target genes. The mPCR assay also contained two controls, i.e., an internal control (IC) Pan-*Salmonella* gene (*inv*A) and an amplification control (AC) Pan-bacteria gene (16s rRNA), to ensure high quality required for diagnostic assays. Optimization of the mPCR assay was carried out by adjusting primers' concentrations, $MgCl_2$ concentrations, glycerol

concentrations, annealing temperatures and primers' extension times. The analytical specificity of the optimized mPCR assay was 100%; all 29 non-target bacteria consisting of 10 non-*Salmonella* bacteria and 19 other known *Salmonella* species tested negative while all 5 reference NTS bacteria tested positive in spiked stool samples. The analytical sensitivity of the mPCR assay for all 5 NTS serotypes was <1000 CFU/ml or <10 pg/µl of bacteria genomic DNA.The assay was also validated using 116 clinical isolates from local and foreign sources which show 100% sensitivity. In conclusion, a sensitive, specific and cost-effective mPCR has been developed for simultaneous detection of the 5 predominant NTS serotpes isolated worldwide. The technique employed is straightforward and easily established in standard laboratories equipped with DNA preparation and PCR facilities. This assay is a breakthrough for laboratory diagnosis of non-typhoidal Salmonellosis, and is useful for surveillance and epidemiology studies of *Salmonella* infections.

CHAPTER ONE

INTRODUCTION

1.1 Non-Typhoidal *Salmonellae*

Non-typhoidal *Salmonellae* (NTS) are the main causes of bacterial food-borne disease outbreaks in developing countries and are also a public health concern in developed countries (Andrysiak *et al.*, 2008; Learn-Han *et al.*, 2008). NTS infections are not only restricted to humans but they are capable of infecting a broad range of animals, including mammals, reptiles, birds and insects (Hohmann, 2001). However, there are some NTS that have restricted ability to infect only one type of animal. For example *Salmonella* Arbortusovis is restricted to sheep and *Salmonella* Gallinarium to poultry (Bhowmick *et al*., 2011). Generally, *Salmonellae* are facultative anaerobic, gram-negative, rod-shaped bacilli and belong to the family of *Enterobacteriaceae* (Lim & Thong, 2009). The size of *Salmonellae* are approximately 0.7 to 1.5 μm wide and 2.0 to 5.0 μm in length (Giannella, 1996). NTS usually ferment glucose with production of gas and they are grouped as prototrophic because of their ability to grow in a minimal amount of medium containing glucose as a source of carbon and ammonia as a source of nitrogen. They can be phenotypically identified by urea hydrolysis, absence of tryptophan deaminase, non-lactose fermentation, production of hydrogen sulphide (H_2S) gas, and growth in Simmons citrate agar (Grimont *et al*., 2000).

1.1.1 Nomenclature of *Salmonellae*

The genus *Salmonella* has a confusing background in aspect of classification and nomenclature because scientists use different systems to refer to and communicate about this genus. There are more than 2,600 serotypes and the number is still growing with newly identified serotypes every year (Bopp *et al.*, 2003; Brenner *et al.*, 2000). *Salmonella* was discovered in the year 1886 by Theobald Smith, who worked on swine fever (hog cholera)

and later he named the genus after his supervisor, Daniel Elmer Salmon (Grimont *et al*., 2000). Classification of *Salmonella* started when Bruce White developed a method for analysis of somatic and flagella antigens of the bacteria in 1926. Later in 1961, Fritz Kauffmann expanded the method to identify more than 2,000 serotypes. In 1980, the Kauffmann-White scheme was proposed as a systematic *Salmonella* nomenclature, and is currently maintained by The World Health Organization (WHO) (Brenner *et al*., 2000; Grimont *et al*., 2000; Hohmann, 2001).

Currently, molecular methods have shown that the genus *Salmonellae* consist of only two species, *Salmonella enterica* and *Salmonella bongori* (Le Minor & Popoff, 1987; Reeves *et al.*, 1989; Threlfall & Frost, 1990). The species *Salmonella enterica* is further divided into six subspecies as follows: (1) *Salmonella enterica* subspecies *enterica*, (2) *Salmonella enterica* subspecies *salamae*, (3) *Salmonella enterica* subspecies *arizonae*, (4) *Salmonella enterica* subspecies *diarizonae*, (5) *Salmonella enterica* subspecies *houtenae*, and (6) *Salmonella enterica* subspecies *indica*. *Salmonella enterica* subspecies *enterica* is the most common clinically identified pathogen and is usually named after the geographical location in which there were first discovered in order to avoid confusion (Table 1.1) (Grimont *et al*., 2000; Su & Chiu, 2007).

1.1.2 Laboratory Identification of Non-Typhoidal *Salmonellae*

Traditionally, phenotypic identification methods like bacteriophage typing or phage typing, biotyping, colicin typing, resistance typing and serotyping have been employed for *Salmonellae* identification during an outbreak investigation (Threlfall & Frost, 1990). Currently, the conventional bacteria culture method, such as low-selective media (MacConkey agar) or intermediate-selective media (*Shigella-Salmonella* xylose-lysine deoxycholate, XLD agar), is widely used. Although this method requires less skill, it is time consuming. All NTS will grow as red colonies with black centers on XLD medium.

Salmonella bacteria are further tested on a panel of biochemical tests in order to identify the species (Table 1.2) (ISO-6579-4, 2002; Mikoleit, 2009).

Another method for *Salmonella enterica* identification is using conventional serotyping method. This method utilizes specific antisera, which are IgM and IgG antibodies against the somatic (O) and flagella (H) antigens, respectively, in order to determine the serotype (Table 1.3) (Grimont & Weill, 2007; Guibourdenche *et al.*, 2010; Hiriart *et al.*, 2013; Pokhrel *et al.*, 2009). Although this method is considered as the gold standard of *Salmonella* identification and differentiation, it has poor discriminatory power, costly and limited in the availability of the antisera (Cardona-Castro *et al.*, 2009; Zou *et al.*, 2010). Such limitations have encouraged the development of molecular "serotyping" methods based on amplification of specific DNA sequences for identification of *Salmonella* serotypes (Cardona-Castro *et al.*, 2009; Herrera-Leon, 2004; Lim & Thong, 2009; Nori, 2010; Zou *et al.*, 2010).

1.2 Salmonellosis

Salmonellosis or commonly known as gastroenteritis is an infection caused by non-typhoidal *Salmonellae* (NTS) which is clinically indistinguishable from that due to many other enteric bacteria (Hohmann, 2001). More than 95% of the transmission of *Salmonellae* are via the oral-fecal route commonly caused by consumption of contaminated food and water (Biologics, 2005). *Salmonellae* infections have a short episode of disease lasting less than 10 days, which consists of an incubation period of 12 to 72 hours post-infections, and then symptoms such as nausea, vomiting and self-limited diarrhea. Sometimes, asymptomatic infections may also occur and the illness usually lasted from 4 to 7 days (Zou *et al.*, 2010).

Table 1.1: Nomenclature of *Salmonellae* (Adapted from Su & Chiu, 2007)

Taxonomic position (writing format) and nomenclature				No. of serotypes in each species or subspecies
Genus (capitalized, italic)	**Species (italic)**	**Subspecies (italic)**	**Serotypes (serovars) (Capitalized, not italic)***	
Salmonella	*enterica*	*enterica* (subspecies I)	Typhi, Enteritidis, Typhimurium, Weltevreden, Agona, Heidelberg	1531
		salamae (subspecies II)	9, 46:z:z39	505
		arizonae (subspecies IIIa)	43:z29:-	99
		diarizonae (subspecies IIIb)	6,7:1, v:1,5,7	336
		houtenae (subspecies IV)	21 :m, t:-	73
		indica (subspecies VI)	59:z36:-	13
	bongori	subspecies V	13,22:z39:-	22
			Total	2579

*some selected serotypes (serovars) are listed as examples

Table 1.2: Biochemical Tests for Identification of NTS (Adapted from ISO 6579)

Organism	**Triple Sugar Iron Test**				**Motility, Indole, Urea Tests**			**Citrate Test**
	Slant	**Butt**	**H_2S**	**Gas**	**Motility**	**Indole**	**Urea**	
Non-Typhoidal *Salmonella* spp.	Alkaline	Acid	V	V	+	-	-	V
E. coli	Alkaline	Acid	-	+	+	+	-	-
Klebsiella spp.	Acid	Acid	-	+	-	V	+	+
Citrobacter spp.	V	Acid	+	+	+	V	-	+
Proteus spp.	Alkaline	Acid	+	+	+		+	V

'+' = Positive
'-' = Negative
Wk+ = Weak positive
V = Variable result
H_2S = Hydrogen sulphide

Table 1.3: Serological Identification of NTS (Adapted from Grimont, 2007)

Serotype	O antigen	H1 antigen	H2 antigen	Serogroup Phase 1:2
S. Enteritidis	1, 9, 12	g,m	-	Group D1
S. Typhimurium	1, 4, (5), 12	i	1,2	Group B
S. Weltevreden	3, (10), (15)	r	z_6	Group E
S. Agona	1, 4, (5), 12	f,g,s	(1,2)	Group B
S. Heidelberg	1, 4, (5), 12	r	1,2	Group B

1.2.1 Manifestation of Non-Typhoidal Salmonellosis

Non-typhoidal *Salmonellae* (NTS) infections in most cases result in self-limited gastroenteritis. During the course of infection, *Salmonella* colonizes the intestines and messentric lymph nodes (Raffatellu *et al.*, 2006; Zhang *et al.*, 2002). The symptoms, such as nausea, vomiting and diarrhea are frequently reported in the event of gastrointestinal manifestation. At this stage, stools are loose and in moderate volume, without the presence of blood. (Manatsathit *et al.*, 2002; Saphra & Winter, 1957). In most cases, fever usually resolve within 48 to 72 hours post-infection, whereas diarrhea typically lasts from 3 to 7 days (Coburn *et al.*, 2007; Saphra & Winter, 1957). Sometimes, patients require hospitalization for dehydration treatment and deaths are not common. However, deaths do occur among elderly and immunocompetent patients (Buchwald & Blaser, 1984). The infected but recovered individuals may continue to secrete stools containing NTS after resolution of the infection for 4 to 5 weeks depending on the *Salmonella* serotype (Celum *et al*., 1987). It is uncommon for patients with gastrointestinal infection caused by NTS to develop bacteremia. However, there are approximately 5% of infected individuals who develop bacteremia, which is a serious and fatal problem (Celum *et al.*, 1987; Han *et al.*, 1967; Hohmann, 2001).

1.2.2 Chronic Carrier Stage and Localized Infections

The frequency of NTS to persist in the stool and urine after 1 year course of infection is relatively low, from 0.2 to 0.6% (Cruickshank & Humphrey, 1987; Musher & Rubenstein, 1973). It is more common to have other complications like focal infections. Focal infections occur when the *Salmonella* causes localized infections in other organs rather than in the gut (Lee *et al*., 2005). The phenomena of extraintestinal infections have been classified into four groups; (1) primary bacteremia, (2) secondary bacteremia, (3) digestive focal infections, and; (4) non-digestive focal infections (Ramos *et al*., 1996). Between 8 to 17% of patients affected with NTS bacteremia develop focal complications (Galofré *et al.*, 1994; Grisaru-Soen *et al.*, 2004; Zaidi *et al.*, 1999). Commonly, urinary isolates of *Salmonella* may

erroneously be attributed to fecal contamination or urinary tract colonization (Abbott *et al*., 1999). There are other complications contributed by focal infections, such as osteomyelitis and joint infections (Workman *et al*., 1996), severe and prolonged polyarticular reactive joint disease (Mattila *et al*., 1994; Mattila *et al*., 1998), and central nervous system (CNS) infections (Kinsella *et al*., 1987).

1.2.3 Epidemiology of Non-Typhoidal Salmonellosis

Non-typhoidal *Salmonellae* (NTS) are commonly isolated bacterial pathogens in the laboratory upon the diagnosis of diarrhea (Hohmann, 2001). These microorganisms can reside in the gastrointestinal tracts of animals and humans and cause various stages of gastrointestinal disease (Winokur, 2003). The World Health Organization (WHO) has estimated that NTS cause 1.3 billion cases of gastroenteritis or diarrhea, with 3 million deaths annually (Nair *et al*., 2002). The global NTS infections surveillance data suggests that the incidence of non-typhoidal Salmonellosis has increased during the last couple of decades (Molbak, 2006). Several countries including Malaysia have experienced decrease in the incidence of *Salmonella* Typhi while NTS serotypes have lately increased proportionally and in absolute numbers (Yasin *et al*., 1995).

In Malaysia, non-typhoidal Salmonellosis is endemic, and its incidence appears to be increasing with periodic outbreaks (Thong *et al*., 1994). Similar situation is observed in the state of Kelantan, where large numbers of NTS isolated from Hospital Universiti Sains Malaysia (HUSM) usually occur during the fasting month of Ramadhan (unpublished data). In particular, onset of the outbreak during the festive season is due to lack of hygienic practices among food-handlers throughout the food-handling process. Commonly, NTS are transmitted to humans from contaminated raw vegetables and meat (Thong *et al*., 2002).

Amongst the NTS serotypes, *S.* Typhimurium and *S.*Enteritidis are the two predominant agents associated with food-borne non-typhoidal Salmonellosis (Jegathesan, 1984; Nori, 2010; Yasin *et al.*, 1995). *S.* Weltevreden is the third most common serotype isolated with hospitalized-gastroenteritis in developing countries, such as countries in Southeast Asia and India (Bangtrakulnonth *et al.*, 2004; Boonmar *et al.*, 1998; Jegathesan, 1984; Sood & Basu, 1979; Yasin *et al.*, 1995). This serotype also caused a few major food-borne outbreaks in Malaysia as well as in developed countries, such as Europe and the United States (Aarestrup *et al.*, 2003; D'Ortenzio *et al.*, 2008; Emberland *et al.*, 2007; Learn-Han *et al.*, 2008; Ponce *et al.*, 2008). *S.* Agona and *S.* Heidelberg are not the most commonly isolated serotypes in Malaysia. However, they are amongst the most predominant serotypes causing human illness in developed countries (Andrysiak *et al.*, 2008; Boyd *et al.*, 2002; Bronowski & Winstanley, 2009)

1.3 Multiplex Polymerase Chain Reaction

Multiplex Polymerase Chain Reaction (mPCR) is a subset of the Polymerase Chain Reaction (PCR). It is defined as simultaneous amplification of multiple sequences of target DNA in a single reaction. It is a technique developed to save time and effort (Markoulatos *et al*., 2002). This technique requires a combination of several primer pairs with single or multiple DNA template(s) in a single reaction (Edwards & Gibbs, 1994). The availability of the genetic information of the target DNA is important to ensure that the mPCR works. This technique has been broadly used as screening tests for microbial agents due to its versatility in simultaneously detecting multiple agents that cause similar or identical clinical syndromes, as well as those that might share similar epidemiological features (Lim, 2009). Hence, this technique is widely used in research laboratories (Markoulatos *et al*., 2002).

Although PCR is a powerful tool for amplification of nucleic acid sequences, it requires a thorough optimization in order to successfully yield an amplification of a particular DNA segment. The use of the same protocol on another gene segment may require minor

optimization on the PCR components and thermal cycling parameters (Stephenson, 2012). Since the development of an efficient mPCR assay is a greater challenge than a monoplex PCR assay, strategic planning is required to optimize the reaction conditions (Lim, 2009). The challenge of mPCR is designing the primer sets which have compatible annealing conditions (Schoske *et al*., 2003).

Ideally, there are a few parameters that need to be taken into account for primer selection such as; (1) primers length from 18 to 24 bp is long enough to confer high specificity of complementary to the desired nucleic acid sequences (Baumforth *et al.*, 1999; Wu *et al.*, 1991); (2) GC content of the primers must be within 50 to 60% to avoid formation of secondary structures (Kramer & Coen, 2001; Wu *et al.*, 1991); (3) all the primers must have similar melting temperatures ($60 \pm 1^{o}C$), and; (4) the primers' sequences homology towards the target nucleic acid sequences (Henegariu *et al.*, 1997; Nicodème & Steyaert, 1997; Robertson & Walsh-Weller, 1998). Also, primers should not exhibit significant homology to each other in order to avoid primer-dimer formation, which is a common artifact that has been observed when the primers themselves become the template and are taken through many amplification cycles (Cha & Thilly, 1993; Kramer & Coen, 2001). This mispriming may be due to high primer concentration coupled with low template concentration at the beginning of the amplification reaction.

The relative concentrations of the primers must be adjusted and optimized to achieve the same or similar amplification efficiencies for each DNA target in a single tube of reaction (Schoske *et al.*, 2003). Ideally, the primers' concentrations should be ascertained using a titration method and should not be greater than 1.0 μM because high primers' concentrations will promote mispriming as described above (Baumforth *et al.*, 1999; Brownie *et al.*, 1997; Elnifro *et al.*, 2000).

Magnesium chloride ($MgCl_2$) is one of the most critical components in any PCR assay because its concentration can affect the specificity and efficiency of the reaction (Raj, 2014). This compound is a salt which can dissociate into two ions, Mg^{2+} and Cl^-. The Mg^{2+} is divalent and it can affect the PCR differently at high and low concentrations. The primary function of Mg^{2+} is to destabilize the double helix structure of DNA, in order to allow the primer to anneal with the complementary target site, at the same time, enable the polymerase enzyme to unwound the DNA template. Excess Mg^{2+}, however, stabilizes the double-stranded DNA structure and prevent complete denaturation of the target during thermocycling process, resulting in a reduction of the PCR yield (Kidd, 1995). This could also be due to spurious annealing of primers to incorrect template sites, therefore resulted in larger amounts of undesired PCR products. On the other hand, insufficient Mg^{2+} concentration impair the primer extension process as Mg^{2+} is the co-factor for the enzymatic activity for most DNA polymerases (Baumforth *et al.*, 1999).

Optimization of the mPCR cycling parameters such as annealing temperatures is also critically to ensure that one reaction is not dominant over the other (Henegariu *et al.*, 1997). The annealing temperature chosen for a PCR usually depends on the duration of exposure to the target and composition of the primers. The ideal annealing temperature of a primer should be 5^oC below its melting temperature, as it can affect the PCR differently at high or low temperatures (Kidd, 1995). Too low annealing temperatures will result in non-specific binding of primers to DNA sequences other than the true target; thereby reducing the yield of the desired product and specificity of the test. Conversely, too high annealing temperatures may yield little product as primer annealing is reduced (Stephenson, 2012). The suggested annealing time is 30 seconds for small volume (20 μl) PCR as the temperature equilibrate quickly (Kidd, 1995).

The utilization of PCR adjuvants, such as dimethyl sulfoxide (DMSO), glycerol and bovine serum albumin (BSA) have been shown to improve PCR amplification efficiency and

specificity, and at the same time, overcome difficulties encountered with high GC content or long DNA templates (Hengen, 1997; Kramer & Coen, 2001; McElhinney, 1995). The optimum concentration of PCR adjuvants, particularly glycerol need to be optimized in order to favor mPCR assays (Henegariu *et al.*, 1997). Glycerol is used to enhance the efficiency and specificity of DNA polymerases and primers by preventing the formation of secondary structures caused by GC-rich regions during DNA amplification, and improve the association between enzyme and template by lowering the strand separation temperatures of DNA thereby facilitating DNA amplification (Lee, 1997; Rees *et al.*, 1993; Saikar *et al.*, 2013).

1.4 Significance of Study

Human Salmonellosis outbreaks and occurrences are highly related to the environment. Its increasing prevalence and high frequency in the global food chain and its virulence and adaptability has impacted the medical, public health and economy of many countries worldwide (Dunbar *et al.*, 2003; Freitas Neto *et al.*, 2010; Hornick, 1994). Globally, the incidences of *Salmonella* infections have increased markedly as good surveillance systems are lacking in many countries, including Malaysia. Although there are many established methods for detection and characterization of NTS, which includes a series of microbiological and biochemical tests (Dunbar *et al.*, 2003) followed by antisera serotyping (Hohmann, 2001; Nori, 2010; Threlfall & Frost, 1990), the whole process is laborious, time consuming, require skilled personnel, and the results obtained can be confusing due to the large numbers of antisera needed, as well as the cross-reactivity between certain serotypes or with other enteric bacteria (Bäumler *et al.*, 2000; Luderitz *et al.*, 1966; Ng *et al.*, 1976).

There has been many studies done in the past decades to differentiate *Salmonella* serotypes by using whole genome sequencing, comparative genomic techniques, molecular typing and *Salmonella* DNA fingerprinting. These studies have shown that bacteria in the genus *Salmonella* have about 90% similarities in their genome (Crosa *et al.*, 1973; Kang *et al.*,

2006; Leekitcharoenphon *et al.*, 2012). The remaining 10 to 12% of their chromosome DNA were shown to be unique for each serotype. These unique gene fragments are hypothesized to be due to horizontal gene transfer (Bäumler, 1997), lateral gene transfer (Ochman *et al.*, 2000), and gene rearrangement processes such as insertions, deletions, inversions, and translocations within its genome relative to each serotypes (Doublet *et al*., 2008). The unique regions are distributed in many sites on the chromosomes and they are likely to encode gene products which account for the different abilities of the serotypes, such as pathogenesis (Kang *et al.*, 2006; Leekitcharoenphon *et al.*, 2012; Ochman *et al.*, 2000).

The mPCR is a powerful technique because it has the ability to detect minute quantities of DNA through amplification of a particular DNA segment, as well as its ability to discriminate between different organisms in one reaction even though they are closely related. This rapid, robust and cost effective technique has been widely employed in the detection, identification, and differentiation of *Salmonella* serotypes (Edwards *et al.*, 2002; Greisen *et al.*, 1994; Jitrapakdee *et al.*, 1995; Tennant *et al*., 2010). In a recent study, a 2-sequential mPCR assay was developed to serotype somatic (O), capsular (Vi) and flagellar (H) genes present on *Salmonella* bacteria (Lim, 2009; Lim & Thong, 2009). Although this approach was aimed at *Salmonella* grouping and differentiation based on their genetic information, it has a number of drawbacks such as, the method is laborious, time consuming and the mPCR showed low specificity and sensitivity.

Therefore, this study was focused on the development and optimization of a mPCR assay based on single specific-gene detection for each serotypes. Currently, there is no single assay that allows simultaneous detection and identification on the five most commonly isolated NTS serotypes, i.e., *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Weltevreden, *Salmonella* Agona, and *Salmonella* Heidelberg based on their genomes. Since the identification of *Salmonella* serotypes using conventional methods is tedious, expensive and time consuming, this study was aimed at developing an alternative method using multi-

target genes amplification mPCR which enabled rapid and specific detection of the five NTS serotypes causing disease in humans. In addition, to providing a better diagnostic tool for early detection of *Salmonella* infections this mPCR assay can improve the *Salmonella* surveillance system in Malaysia.

1.5 Objectives of Study

The main objective of this research was to develop an optimized multiplex PCR (mPCR) assay using specific gene targets to simultaneously identify *S.* Enteritidis, *S.* Typhimurium, *S.* Weltevreden, *S.* Agona, and *S.* Heidelberg in clinical isolates. The sub-objectives of this study were:-

1. To identify unique DNA sequences of *S.* Weltevreden, *S.* Agona, and *S.* Heidelberg using bioinformatic approaches to elucidate specific gene targets for identification of these pathogens,

2. To develop and optimize five monoplex PCR assays using the gene targets delineated above,

3. To develop and optimize a mPCR assay from the above monoplex PCR assays for simultaneous detection of *S.* Enteritidis, *S.* Typhimurium, *S.* Weltevreden, *S.* Agona and *S.* Heidelberg, and

4. To determine the analytical sensitivities and specificities of the mPCR assay using confirmed bacterial isolates and spiked stool samples.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals, Media and Reagents

All chemicals, media and reagents listed in Table 2.1 were used for isolation and identification of *Salmonella* in clinical samples. These chemicals and media were imported either directly from the manufacturers or procured through their Malaysian agents.

Table 2.1: List of Chemicals, Media and Reagents

No.	Name	Source
1.	Absolute Ethanol	HmbG chemical, Germany
2.	Glacial Acetic acid, 100%	Merck, Germany
3.	Agarose powder, analytical grade	Promega, USA
4.	Dimethyl sulfoxide (DMSO)	New England Biolabs, USA
5.	Ethidium bromide (10 mg/ml)	Promega, USA
6.	Ethylenediamine tetracetic acid (EDTA)	Fluka Analytical, USA
7.	Glycerol	Merck, Germany
8.	Indole reagent (KOVACS)	Remel, UK
9.	Methyl red solution	Remel, UK
10.	Methyl red Vogas-Proskauer (MRVP) medium	Oxoid Ltd, UK
11.	Nutrient agar powder	Oxoid Ltd, UK
12.	Nutrient broth powder	Oxoid Ltd, UK
13.	Polyvinylpyrrolidone (PVP)	Calbiochem,
14.	Selenite cystine broth	Fluka Analytical, USA
15.	Simmons citrate agar	Oxoid Ltd, UK
16.	Sodium chloride (NaCl)	Merck, Germany
17.	Sulfide indole motility (SIM) medium	Oxoid Ltd, UK
18.	Triple sugar iron (TSI) medium	Oxoid Ltd, UK
19.	Tris (hydroxymethyl) aminomethane	Bio-Rad, USA
20.	Urea	Amresco, USA
21.	Urea agar base	Oxoid Ltd, UK
22.	Xylose Lysine Deoxycholate (XLD) agar	Oxoid Ltd, UK

2.1.2 Research Kits and Consumables

All research kits listed in Table 2.2 were used for extraction of *Salmonella* DNA from clinical samples. QIAGEN brand research kits were mainly used in this study.

All laboratory comsumables listed in Table 2.2 were regularly used and replacements were required throughout the research work. These laboratory comsumables were procured through their Malaysian agents. Greiner Bio-One (Germany) and Axygen (USA) were mainly used.

Table 2.2: List of Research Kits and Consumables

No.	Name	Source
1.	Cryotube with screw cap 2.0 ml	Axygen, USA
2.	DNEasy Blood & Tissue Kit (250)	QIAGEN, Germany
3.	Micro-centrifuge tubes 1.5 ml, 0.2 ml	Axygen, USA
4.	Pipette tips 5.0 ml, 1.0 ml, 200 µl, 10 µl	Axygen, USA
5.	Petri dish	Greiner Bio-One, Germany
6.	Polypropylene tubes 15 ml, 50 ml	Greiner Bio-One, Germany
7.	QIAquick Gel Extraction Kit (250)	QIAGEN, Germany

2.1.3 Software

All software listed in Table 2.3 were used in the analysis and interpretation of results obtained in this study.

Table 2.3: List of Software

No.	Name	Purpose	Source
1.	SynGene Genesnap Version 7.09	Gel documentation	SynGene, UK
2.	ND-1000 Version 3.5.2	DNA quantification	ThermoScientific, USA
3.	BioEdit Sequence Alignment Editor	Sequence Analysis	Tom Hall, USA

2.1.4 Instruments

All the instruments listed in Table 2.4were used for bacterial storage, culture and other molecular biology experiements in this study.

Table 2.4: List of Instruments

No.	Name	Source
1.	Analytical balance	A&D Company Ltd, Japan
2.	Autoclave machine	Hirayama, Japan
3.	Chiller (4^oC)	Hitachi, Japan
4.	Deep Freezer (-80^oC)	ThermoScientific, USA
5.	Dry Bath incubator	Labnet, USA
6.	Dry Oven	Memmert, Germany
7.	Freezer (-20^oC)	ThermoFisher, USA
8.	Gel electrophoresis Sub-Cell GT	Bio-Rad, USA
9.	Gel eletctrophoresis MiniSub-Cell GT	Bio-Rad, USA
10.	Gel documentation chamber	SynGene, UK
11.	Incubator	Memmert, Germany
12.	Incubator shaker	ThermoScientific, USA
13.	ND-1000 Spectrophotometer	ThermoScientific, USA
14.	PCR thermal cycler	MJ Research, Canada
15.	pH meter	Villerbanne Cedey, France
16.	Power pack	Bio-Rad, USA
17.	Steamer	KSI KOSH, Germany
18.	Table top centrifuge (1.5 ml)	FisherScientific, USA
19.	Table top centrifuge (0.2 ml)	Tomy Digital Biology, Japan
20.	Vortex	Sigma-Aldrich, USA

2.1.5 PCR Reagents

All *Taq* polymerase and dNTPs were used in the monoplex PCR and Multiplex PCR assays whilst DNA molecular weight marker was used as reference standard to determine the size of PCR amplicons were listed in Table 2.5. All the enzymes, DNTPs and molecular weight marker were stored ar -20^oC freezer (Thermo Fisher, USA).

Table 2.5: List of PCR Reagents

No.	Name	Source
1.	GoTaq® Flexi DNA Polymerase (500 U)	Promega, USA
2.	Phusion® High-Fidelity DNA Polymerase	New England Biolabs, USA
3.	DNA Ladder (100 bp)	Promega, USA
4.	Deoxyribonucleotide triphosphate (dNTP) mix	Promega, USA
5.	Green GoTaq® Flexi Buffer (5X)	Promega, USA
6.	Magnesium chloride solution, 25 mM	Promega, USA
7.	Phusion® High Fidelity Buffer	New England Biolabs, USA

2.1.6 Bacteria Isolates

Thirty-nine isolates were obtained from three different sources, i.e., Biobank, Institute for Research in Molecular Medicine (INFORMM), Hospital Universiti Sains Malaysia (HUSM) and American Type Cell Culture (ATCC) which consisted of 29 known *Salmonella* spp. and 10 non-*Salmonella* bacteria used for analytical specificity of the monoplex and multiplex PCR assays as tabulated in Table 2.6.

A total of 116 *Salmonella* isolates were obtained from four different sources, i.e., the Department of Clinical Microbiology and Parasitology, Hospital Universiti Sains Malaysia (HUSM), Denmark Technology University (DTU), University of Liverpool, and University of California, Irvine (UCI) were used for analytical sensitivity of the monoplex PCR assays as tabulated in Table 2.7.

Table 2.6: *Salmonella*and Non-*Salmonella* Strains used in Analytical Specificity Tests

No.	Strain	Strain ID No.	Source
1.	*Salmonella* Typhi	7251	ATCC
2.	*Salmonella* Paratyphi A	9150	ATCC
3.	*Salmonella* Paratyphi B	BAA 1250	ATCC
4.	*Salmonella* Paratyphi C	9068	ATCC
5.	*Salmonella* Enteritidis	13076	ATCC
6.	*Salmonella* Typhimurium	14028	ATCC
7.	*Salmonella* Poona	04840	ATCC
8.	*Salmonella* Hadar	51956	ATCC
9.	*Salmonella* Weltevreden	6534	ATCC
10.	*Salmonella* Agona	51957	ATCC
11.	*Salmonella* Heidelberg	8326	ATCC
12.	*Salmonella* Braenderup	MOB316/06	INFORMM Biobank
13.	*Salmonella* Regent	MOB491/06	INFORMM Biobank
14.	*Salmonella* Kibi	3124K/07	INFORMM Biobank
15.	*Salmonella* Tsevie	D1746K/07	INFORMM Biobank
16.	*Salmonella* Farsta	D1361K/07	INFORMM Biobank
17.	*Salmonella* Uppsala	D1354/07	INFORMM Biobank
18.	*Salmonella* Brooklyn	D1726K/07	INFORMM Biobank
19.	*Salmonella* Assinie	D1767/07	INFORMM Biobank
20.	*Salmonella* Richmond	D1832K/07	INFORMM Biobank
21.	*Salmonella* Bardo	D1874K/07	INFORMM Biobank
22.	*Salmonella* Tshiongwe	MOB1228/06	INFORMM Biobank
23.	*Salmonella* Emek	3264/07	INFORMM Biobank
24.	*Salmonella* Kissi	D1135K/07	INFORMM Biobank

Table 2.6 - *Continued*

25.	*Salmonella* Mbandaka	B08680/10	HUSM
26.	*Salmonella* Javiana	S00617/10	HUSM
27.	*Salmonella* Albany	S00306/11	HUSM
28.	*Salmonella* Standley	S00353/11	HUSM
29.	*Salmonella* Oslo	S00751/10	HUSM
30.	*Salmonella* Newport	B08147/10	HUSM
31.	*Escherichia coli*	ESCO	ATCC
32.	*Shigella dysentriae*	SHDY/150/09	INFORMM Biobank
33.	*Shigella boydii*	SHBO/150/199/A	INFORMM Biobank
34.	*Shigella flexneri*	S00001/11	HUSM
35.	*Shigella sonnei*	S00014/11	HUSM
36.	Enteropathogenic *Escherichia coli*	S00024/11	HUSM
37.	Enterohemorrhagic *Escherichia coli*	S00022/11	HUSM
38.	*Yersinia enterocolitica*	B06102/08	HUSM
39.	*Klebsiella pneumonia*	B02148/09	HUSM

Table 2.7: *Salmonella* Clinical Isolates used in Sensitivity Tests

No.	Strain	Strain ID No.	Year	Source
1.	*Salmonella* Enteritidis	B04106/10	2010	HUSM
2.	*Salmonella* Enteritidis	B05708/10	2010	HUSM
3.	*Salmonella* Enteritidis	B08268/10	2010	HUSM
4.	*Salmonella* Enteritidis	B08347/10	2010	HUSM
5.	*Salmonella* Enteritidis	B08572/10	2010	HUSM
6.	*Salmonella* Enteritidis	B10162/10	2010	HUSM
7.	*Salmonella* Enteritidis	S00194/10	2010	HUSM
8.	*Salmonella* Enteritidis	S00272/10	2010	HUSM
9.	*Salmonella* Enteritidis	S00275/10	2010	HUSM
10.	*Salmonella* Enteritidis	S00294/10	2010	HUSM
11.	*Salmonella* Enteritidis	S00457/10	2010	HUSM
12.	*Salmonella* Enteritidis	S00738/10	2010	HUSM
13.	*Salmonella* Enteritidis	S00754/10	2010	HUSM
14.	*Salmonella* Enteritidis	S00755/10	2010	HUSM
15.	*Salmonella* Enteritidis	S00764/10	2010	HUSM
16.	*Salmonella* Enteritidis	S00780/10	2010	HUSM
17.	*Salmonella* Enteritidis	S00923/10	2010	HUSM
18.	*Salmonella* Enteritidis	S01034/10	2010	HUSM
19.	*Salmonella* Enteritidis	U02775/10	2010	HUSM
20.	*Salmonella* Enteritidis	B01779/11	2011	HUSM
21.	*Salmonella* Enteritidis	B02184/11	2011	HUSM
22.	*Salmonella* Enteritidis	B03102/11	2011	HUSM
23.	*Salmonella* Enteritidis	S00054/11	2011	HUSM
24.	*Salmonella* Enteritidis	S00059/11	2011	HUSM
25.	*Salmonella* Enteritidis	S00081/11	2011	HUSM
26.	*Salmonella* Enteritidis	S00186/11	2011	HUSM
27.	*Salmonella* Enteritidis	S00230/11	2011	HUSM
28.	*Salmonella* Enteritidis	S00270/11	2011	HUSM
29.	*Salmonella* Enteritidis	S00450/11	2011	HUSM
30.	*Salmonella* Enteritidis	S00556/11	2011	HUSM
31.	*Salmonella* Typhimurium	S00481/10	2010	HUSM
32.	*Salmonella* Typhimurium	S00485/10	2010	HUSM

Table 2.7 - *Continued*

33.	*Salmonella* Typhimurium	S00497/10	2010	HUSM
34.	*Salmonella* Typhimurium	S00512/10	2010	HUSM
35.	*Salmonella* Typhimurium	S00681/10	2010	HUSM
36.	*Salmonella* Typhimurium	S00773/10	2010	HUSM
37.	*Salmonella* Typhimurium	S00938/10	2010	HUSM
38.	*Salmonella* Typhimurium	B03358/11	2011	HUSM
39.	*Salmonella* Typhimurium	S00261/11	2011	HUSM
40.	*Salmonella* Typhimurium	S00301/11	2011	HUSM
41.	*Salmonella* Typhimurium	S00372/11	2011	HUSM
42.	*Salmonella* Typhimurium	S00656/11	2011	HUSM
43.	*Salmonella* Weltevreden	2007-60-3289-1	2007	DTU
44.	*Salmonella* Weltevreden	P01485/10	2010	HUSM
45.	*Salmonella* Weltevreden	S00045/10	2010	HUSM
46.	*Salmonella* Weltevreden	S00336/10	2010	HUSM
47.	*Salmonella* Weltevreden	S00376/10	2010	HUSM
48.	*Salmonella* Weltevreden	S00409/10	2010	HUSM
49.	*Salmonella* Weltevreden	S00412/10	2010	HUSM
50.	*Salmonella* Weltevreden	S00490/10	2010	HUSM
51.	*Salmonella* Weltevreden	S00492/10	2010	HUSM
52.	*Salmonella* Weltevreden	S00516/10	2010	HUSM
53.	*Salmonella* Weltevreden	S00563/10	2010	HUSM
54.	*Salmonella* Weltevreden	S00620/10	2010	HUSM
55.	*Salmonella* Weltevreden	S00859/10	2010	HUSM
56.	*Salmonella* Weltevreden	S00930/10	2010	HUSM
57.	*Salmonella* Weltevreden	S00981/10	2010	HUSM
58.	*Salmonella* Weltevreden	S00989/10	2010	HUSM
59.	*Salmonella* Weltevreden	S00995/10	2010	HUSM
60.	*Salmonella* Weltevreden	S00996/10	2010	HUSM
61.	*Salmonella* Weltevreden	S01064/10	2010	HUSM
62.	*Salmonella* Weltevreden	B01292/11	2011	HUSM
63.	*Salmonella* Weltevreden	S00015/11	2011	HUSM
64.	*Salmonella* Weltevreden	S00057/11	2011	HUSM
65.	*Salmonella* Weltevreden	S00145/11	2011	HUSM
66.	*Salmonella* Weltevreden	S00202/11	2011	HUSM
67.	*Salmonella* Weltevreden	S00228/11	2011	HUSM
68.	*Salmonella* Weltevreden	S00382/11	2011	HUSM
69.	*Salmonella* Weltevreden	S00576/11	2011	HUSM
70.	*Salmonella* Weltevreden	S00585/11	2011	HUSM
71.	*Salmonella* Weltevreden	S00970/11	2011	HUSM
72.	*Salmonella* Weltevreden	S00974/11	2011	HUSM
73.	*Salmonella* Weltevreden	S00988/11	2011	HUSM
74.	*Salmonella* Weltevreden	S01004/11	2011	HUSM
75.	*Salmonella* Agona	MZ0008	-	UCI
76.	*Salmonella* Agona	MZ0693	-	UCI
77.	*Salmonella* Agona	MZ1431	-	UCI
78.	*Salmonella* Agona	MZ1608	-	UCI
79.	*Salmonella* Agona	MZ1609	-	UCI
80	*Salmonella* Agona	MZ1610	-	UCI
81.	*Salmonella* Agona	2009-60-2063	2009	DTU
82.	*Salmonella* Agona	2009-60-2107	2009	DTU
83.	*Salmonella* Agona	2010-60-0568	2010	DTU
84.	*Salmonella* Agona	2011-60-0239	2011	DTU

Table 2.7 - *Continued*

85.	*Salmonella* Agona	2011-60-0523	2011	DTU
86.	*Salmonella* Agona	2011-60-1289	2011	DTU
87.	*Salmonella* Agona	2011-60-1475	2011	DTU
88.	*Salmonella* Agona	2011-60-1697	2011	DTU
89.	*Salmonella* Agona	2011-60-3540	2011	DTU
90.	*Salmonella* Agona	2012-60-1061	2012	DTU
91.	*Salmonella* Agona	2012-60-1662	2012	DTU
92.	*Salmonella* Agona	2013-60-0214	2013	DTU
93.	*Salmonella* Agona	2013-60-1041	2013	DTU
94.	*Salmonella* Agona	2013-60-1082	2013	DTU
95.	*Salmonella* Agona	2013-60-1360	2013	DTU
96.	*Salmonella* Heidelberg	B00050A/10	2010	HUSM
97.	*Salmonella* Heidelberg	B00050B/10	2010	HUSM
98.	*Salmonella* Heidelberg	S00491/10	2010	HUSM
99.	*Salmonella* Heidelberg	S00840/10	2010	HUSM
100.	*Salmonella* Heidelberg	S00992/10	2010	HUSM
101.	*Salmonella* Heidelberg	17705	-	University of Liverpool
102.	*Salmonella* Heidelberg	KMS1977	-	University of Liverpool
103.	*Salmonella* Heidelberg	55/336	-	University of Liverpool
104.	*Salmonella* Heidelberg	2005-60-0545	2005	DTU
105.	*Salmonella* Heidelberg	2006-60-1066	2006	DTU
106.	*Salmonella* Heidelberg	2006-60-1251	2006	DTU
107.	*Salmonella* Heidelberg	2006-60-1833	2006	DTU
108.	*Salmonella* Heidelberg	2007-60-2447	2007	DTU
109.	*Salmonella* Heidelberg	2008-60-0842	2008	DTU
110.	*Salmonella* Heidelberg	2010-60-0508	2010	DTU
111.	*Salmonella* Heidelberg	2010-60-6148	2010	DTU
112.	*Salmonella* Heidelberg	2011-60-0380	2011	DTU
113.	*Salmonella* Heidelberg	2011-60-1487	2011	DTU
114.	*Salmonella* Heidelberg	2012-60-1704	2012	DTU
115.	*Salmonella* Heidelberg	2012-60-2239	2012	DTU
116.	*Salmonella* Heidelberg	2013-60-1631	2013	DTU

2.1.7 Target Specific Oligonucleotides

The following target specific oligonucleotides were used to develop a single multiplex PCR assay (Table 2.8). Primers for *S.* Enteritidis and *S.* Typhimurium were obtained from my colleague, Ms. Yeoh Chiann Ying (Yeoh, 2014) with kind permission. Primers for *S.* Weltevreden, *S.* Agona and *S.* Heidelberg were designed in this study. An internal control (IC) primer pair targeting Pan-*Salmonella*, invasion gene (*inv*A) which produces a 284 bp PCR product was used in this study. The primer sequences were adopted from Rahn *et al.* (1992). Based on their study, it has 95% sensitivity across *Salmonella* spp. An amplification control (AC) used in this study was the housekeeping gene of the bacteria kingdom, 16S rRNA gene. The primer sequences were designed by Marchesi *et al*. (1998) and were chosen in this study because they targeted the conserved region of the 16S rRNA gene sequence that has 97% sequence homology across all bacteria.

Table 2.8: List of Oligonucleotides Used in mPCR Assay

Target	Primers	Sequence (5'→ 3')	Product Size (bp)	Reference
Salmonella Enteritidis	SEN	F: GCTGCAGATGTACTGTGCTTTC R: CCAGGCGACTCTACTTATCCAG	692	(Yeoh, 2014)
Salmonella Typhimurium	STM	F: GCAGCCGATGATGTAGCTTA R: TTACGCTGCGGGATTAATGT	495	
Salmonella Weltevreden	SWEL	F: CACCACCTGACACAACCAGT R: GTGGTGCCTGTGACGAATAA	349	Current study
Salmonella Agona	SAGO	F: TCGCTCTTTCTGCCATACCT R: CTCCTGAATCTGCTGGTGGT	438	
Salmonella Heidelberg	SHEI-1	F: GGCGTGGTATTTGTCGAAGT R: ATAAACGCCCATCGTTCTCA	626	
Pan-*Salmonella* (IC)	*inv*A	F: GTGAAATTATCGCCACGTTCGGGCAA R: TCATCGCACCGTCAAAGGAACC	284	(Rahn *et al.*, 1992)
All bacteria species (AC)	16S rRNA	F: CAGGCCTAACACATGCAAGTC R: GGGCGGTGTGTACAAGGC	1362	(Marchesi *et al.*, 1998)

2.1.8 Media Preparation for Bacteria Culturing Purposes

2.1.8 (a) Nutrient Agar

Nutrient agar was prepared by adding 28 g of nutrient agar powder to 1000 ml of distilled water by heating and stirring. The solution was autoclaved at 121^{o}C for 15 minutes, and then the sterilized molten nutrient agar solution was placed in a water bath at 50^{o}C. Twenty five millilitre aliquots of the molten nutrient agar solution were poured into sterile petri dishes and allowed to solidify at room temperature. The nutrient agar plates were stored at 4^{o}C until ready for use.

2.1.8 (b) Nutrient Broth

Nutrient broth was prepared by adding 13 g of nutrient broth powder to 1000 ml of distilled water by heating and stirring. Ten millilitre aliquots of the solution were distributed into universal bottles and sterilized by autoclaving at 121^{o}C for 15 minutes. The universal bottles were stored at 4^{o}C until ready for use.

2.1.8 (c) Xylose Lysine Deoxycholate (XLD) Agar

Xylose Lysine Deoxycholate (XLD) agar was prepared by dissolving 53 g of XLD powder in 1000 ml of distilled water by heating and stirring. The solution was then placed in a water bath at 50^{o}C. Twenty five millilitre aliquots of the molten XLD solution were poured into sterile petri dishes and allowed to solidify at room temperature. The agar plates were stored at 4^{o}C until ready for use.

2.1.8 (d) Triple Sugar Iron (TSI) Agar

Triple sugar iron (TSI) agar was prepared by dissolving 65 g of TSI powder in 1000 ml of distilled water by heating and stirring. Four millilitre aliquots of the solution were pipetted into capped test tubes and sterilized by autoclaving at 121^{o}C for 15 minutes. The molten TSI agar was allowed to solidify at room temperature with the test tubes inclined at 45^{o}. The test tubes were stored at 4^{o}C until ready for use.

2.1.8 (e) Simmons Citrate Agar

Simmons citrate agar was prepared by dissolving 23 g of Simmons citrate agar powder in 1000 ml of distilled water by heating and stirring. Two millilitre aliquots of the solution were pipetted into capped test tubes and sterilized by autoclaving at 121^{o}C for 15 minutes. The molten agar was allowed to solidify at room temperature with the test tubes inclined at 45^{o}. The test tubes were stored at 4^{o}C until ready for use.

2.1.8 (f) Urea Solution (40%)

Urea solution (40% w/v) was prepared by dissolving 40 g of urea powder in 100 ml of autoclaved distilled water by stirring. The solution was stored at 4^{o}C until ready for use.

2.1.8 (g) Urea Base Agar

Urea agar base was prepared by dissolving 2.4 g of urea agar base powder in 95 ml of distilled water by heating and stirring. The solution was placed in a water bath at 50^{o}C and 5 ml of 40% (w/v) urea solution [*Section* 2.1.8 (f)] was added and mixed well. One thousand and five hundreds microlitre aliquots were pipetted into capped test tubes and allowed to solidify at room temperature with the test tubes inclined at 45^{o}. The test tubes were stored at 4^{o}C until ready for use.

2.1.8 (h) Sulphate Indole Motility (SIM) Medium

Sulphate indole motility (SIM) medium was prepared by dissolving 30 g of SIM powder in 1000 ml of distilled water by heating and stirring. Two millilitre aliquots of the solution were pipetted into capped test tubes and sterilized by autoclaving at 121^{o}C for 15 minutes. The solution was allowed to solidify at room temperature and stored at 4^{o}C until ready for use. One drop of KOVACS reagent was added into each inoculated test tube after overnight incubation and the colour changes were recorded immediately.

2.1.8 (i) Methyl Red Vogas-Proskauer (MRVP) Medium

Methyl red Vogas-Proskauer (MRVP) medium was prepared by dissolving 15 g of MRVP powder in 1000 ml of distilled water by heating and stirring. Two millilitre aliquots of the solution were pipetted into capped test tubes and sterilized by autoclaving at 121^{o}C for 15 minutes. The solution was allowed to solidify at room temperature and stored at 4^{o}C until ready for use. One drop of methyl red solution was added into each inoculated test tube after overnight incubation and the changes were recorded immediately.

2.1.8 (j) Selenite Cystine Broth

Selenite cystine broth was prepared by dissolving 23 g of Selenite cystine broth powder in 1000 ml of autoclaved distilled water by steaming for 7 minutes. The solution was allowed to cool at room temperature before storage at 4^{o}C.

2.1.8 (k) Sodium Chloride (NaCl) Solution

Sodium chloride (NaCl) (0.9% w/v) was prepared by dissolving 9 g of NaCl in 1000 ml of distilled water. The solution was sterilized by autoclaving at 121^{o}C for 15 minutes. The solution was stored at room temperature.

2.1.8 (l) Polyvinylpyrrolidone (PVP) Solution

Polyvinylpyrrolidone (PVP) solution (5% w/v) was prepared by dissolving 5 g of PVP in 100 ml of distilled water by vigorous mixing. The solution was sterilized by autoclaving at 121^{o}C for 15 minutes. The solution was stored at 4^{o}C until ready to use.