CHAPTER 1

1.1 INTRODUCTION

Salmonella is a group included in the family *Enterobacteriaceae,* Gram negative facultative anaerobic bacilli (Yan *et al*., 2003). *Salmonella* bacteria were considered as an important issue in medical field, therefore the detection and identification become an essential role (Hoorfara *et al*., 1999). Members of the genus *Salmonella* is one of the major contributory agents that lead to food, water borne diseases and outbreak (Molarny *et al.*, 2003). The disease caused by *Salmonella* spp. generally is moderate gastroenteritis (Dunne *et al*., 2000), but can develop to complications such as bacteremia, septicemia and meningitis (Giradin *et al.,* 2006).

Salmonella genus has two species, namely *Salmonella enterica* and *Salmonella bongori*. There are six subspecies of *S. enterica*, the most important of which is *S. enterica* subsp. *enterica* (subspecies I) (Popoff & le Minor, 1997). Members of the other five subspecies (II–VI), found in the natural environment, are primarily parasites of cold-blooded animals (Kim *et al*., 2006). At present the most common approach of identification and typing *S. enterica* serovars relys on the Kauffmann-White scheme which is designed for subspecies classification by targeting somatic, capsular, and flagella antigens that are present in *Salmonella* (Lindberg *et al.,* 1984). The somatic (O) antigens typing indicate the serogroup, and the flagella (H) antigens indicate the serotype. The characterization of about 2,523 serotypes identified and described in the Kaufmann-White scheme use 46 (O) serogroups, 11 additional O antigens, and 119 H antigens (Kim *et al*., 2006). The serogroups of *Salmonella* are designated by alphabets and the major serogroups are A to E.

These include more than 95% of the infectious *Salmonella* serotypes to man and animals (Luk & Lindberg, 1991). Identification of *Salmonella* based on somatic (O) and flagella (H) antigens is vital and useful for epidemiological surveillance and outbreak investigations for *Salmonella* serotypes, and extremely value to understand the distribution of *Salmonella* pathogen and the host range (Fritzgerald *et al*., 2006). Classical serotyping is performed using several serologic tests such as bacterial agglutination, latex agglutination, co-agglutination, fluorescent and enzyme labeling assays (Chart *et al*., 2007). The conventional serotyping method needs well trained technicians to be able to investigate and interpret the results (Herrera-loan *et al.,* 2004; Lim and Thong, 2009). The traditional method of serotyping and identification of the *Salmonella* is reliable, even though it has several limitations such as difficulty in maintaining its inherent characteristics. Different 250 sera of typing with 350 different antigens were needed for producing the antisera, which is time consuming and need minimum of 3 days to determine the serotype of an isolate. Frequently using the commercial antisera for less common antigens are unavailable, and some of untypeable *Salmonella* are autoagglutinable (rough) strains (Fritzgerald *et al*., 2006; McQuiston *et al*., 2004). Serotyping has poor discriminatory power due to large number of serotypes, cross reaction of antigens and also for presence of similar antigens in the lipopolysaccharides (Luderitz *et al*., 1966).

Such drawbacks and problems associated with conventional serotyping support the need to develop a new system for the molecular identification of serotype based on the genes and DNA responsible for expression of serotype antigens. DNA based tests are usually specific, sensitive, quick, easy for interpretation and reproducible (McQuiston *et al*., 2004).

Delineation of epidemiological relationships between various isolates have been utilized by a variety of DNA-based genotyping. Quick methods of typing are required to distinguish *Salmonella* serotypes within the sources of contamination in food-processing, and also during epidemiological investigation and controlling the food borne outbreaks (Lim *et al.*, 2005). The ability to detect the pathogens by using small quantity of DNA can be achieved by applying polymerase chain reaction (PCR), which is amplification of known DNA target, it make discrimination in a reaction among diverse organisms even for closed related strains (Hatta & Smit, 2007). PCR approach was proven to be valuable technique for the identification of pathogens in food. In some cases such as several pathogens can be detected in a single reaction by using multiplex PCR (Way *et al.,* 1993; Brasher *et al.,* 1998; Soumet *et al.,* 1999; Vantarakis *et al*., 2000).

Detection and serotyping of *Salmonella* by molecular methods is mainly dependent on the same antigens that described by Kauffmann-White scheme which are O, H, and Vi antigens (McQuiston *et al.,* 2004; Kim *et al.,* 2006; Herrera- Leon *et al.*, 2007).

1.2 Scope of study

The duration of research work was for 6 months from July –Dec 2009. Due to the short duration, the work involved application and optimization of published primers that target the somatic (O), flagella (H) and capsular (Vi) antigens of *Salmonella.*

1.3 Objective of study

The objective of the study was to apply and to develop a DNA-based typing scheme to differentiate and serotype the main *Salmonella enterica* strains based on the O, H and Vi antigens.

CHAPTER 2

LITERATURE REVIEW

2.1 Characterization of *Salmonella* species

Salmonella species are Gram-negative rod, anaerobic bacteria, classified under the *Enterobacteriaceae* family. *Salmonella* grow optimally at 35º C to 37º C, and catabolize a variety of carbohydrates into acids and gas. These organisms have oxidative and fermentative metabolic pathways. There are oxidative negative, catalaze positive, utilize citrate medium as only source of energy, usually produce H_2S and do not hydrolyze urea. Members of genus *Salmonella* is characterized as non-lactose and non-sucrose fermenters (Yousef & Calstrom, 2003). Most of *Salmonella* spp. is motile by peritrichous flagellation except for *Salmonella enterica* subsp. *enterica* serotype Gallinarium, and *Salmonella enterica* subsp. *enterica* serotype Pullorum, which lack flagella (D'Aoust, 1997). According to the Central for Disease Control and Prevention (CDC) and the World Health Organization Collaborating Center (WHOCC), the genus *Salmonella* contains two species*: S. enterica* and *S. bongori*. *S. enterica* is divided into six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae* and *indica.* All these subspecies are differentiated biochemically and are divided into different serotypes based on genomic relatedness (Brenner *et al*., 2000).

2.2 Routes of transmission

The main sources for transmitting the infection of *Salmonella* are represented in foods, which have been implicated as vehicles for transmitting infection to people because foods are recognized as reservoir of *Salmonella*. *Salmonella* spp are found mainly in the intestinal tracts of a diversity group of animals. Farm animals such as pigs, poultry, cattle, and sheep are carriers of extreme concern to the food industry, because generally they are asymptomatic carriers (Oosterom, 1991). During the slaughtering of animals, the intestinal material usually contain *Salmonella* bacteria, which in later stages can lead to extensive contamination of meat products (Kariuki *et al*., 2006). Moreover the disease can transmit through contact with farm animals, and natural pet treats (Wall *et al*., 1996). In addition, the raw wastewater is considered as a source of contamination to vegetables and fruits (Ait Melloul *et al.*, 2001).

2.3 Detection of *Salmonella*

Detection of *Salmonella* is an essential factor in microbiological analysis to control food safety. Different methods have been developed for the detection of the pathogen, such as the standard culture method (ISO 6579) which requires up to 5 days (Holbrook *et al.,* 1989). Even though the analytical parameters of this method are very good, microbiological examinations for *Salmonella* using ordinary culture approaches can be time-consuming and laborious especially with samples able to contaminate with other enteric microorganisms (Trkov *et al.,* 1999). To avoid the limitation of mentioned approaches, a number of fast, less technically and less arduous involved screening methods for *Salmonella* have been described such as immunological methods which are used in different formats. Enzyme-

linked immunosorbent assay (ELISA) is the one of frequently used immunochemical technique to identify pathogens (Bokken *et al*., 2003). The theory of immunological assays is antibody antigen reactions which the detection and serotyping of *Salmonella* targeting the immunologic antigens such as O-polysaccharide (O antigen) and flagellin protein (H antigen) (McQuiston *et al*., 2004).

2.4 Salmonellosis

Salmonellosis is a term used in medical field to describe the sickness caused by *Salmonella* spp. The clinical presentation of human Salmonellosis distinguish three clinically forms of Salmonellosis. These include enteric fever, gastroenteritis, and extraintestinal infection which include bacteremia and septicaemia (Lim & Thong, 2009). *Salmonella* serotypes which are adapted to man, such as *S*. Typhi and *S*. Paratyphi, usually cause severe diseases in humans as typhoid and paratyphoid fever. *S*. Typhi cause typhoid fever which is more prolonged and has a higher mortality rate than paratyphoid fever. The symptoms of typhoid fever may take 7 to 28 days to appear after exposure to *S*. Typhi, which are fever, headache, abdominal pain, prostration, watery diarrhea, nausea and a rash of rose spots on the shoulders and thorax (D'Aoust, 2002). Typhoid fever considered as systemic disease, *S*. Typhi may colonize the intestine, liver, spleen, and may reach to the bone marrow (Raffatellu *et al.*, 2006). Complications may include intestinal bleeding from ulcers or intestinal perforation (D'Aoust, 2002). Furthermore the complications may involve peritonitis, encephalopathy and hemorrhage (Kumar *et al.*, 2002). *S.* Paratyphi A, B and C are the cause agents of paratyphoid fever, which the incidence of occurrence less than typhoid fever (Vollaard *et al*., 2004).

Serotypes that are highly adapted to animal hosts which are usually produce mild symptoms in man such as *S*. Gallinarum (poultry) or *S.* Abortus- bovis (sheep), *S.* Choleraesuis (pig), *S.* Dublin (bovines). Ubiquitous serotypes, such as *S.* Enteritidis or *S.* Typhimurium, which affect both man and animals, which are the main causative agents cause gastrointestinal infections usually less severe than typhoid and paratyphoid fever. These commonly named as zoonotic non typhoid *Salmonella* (NTS), and it is the most common form Salmonellosis in human (Raffatellu *et al.*, 2006). However, they also have the capacity to produce typhoid-like infections in mice and in humans or asymptomatic intestinal colonization in chickens (Cowden *et al.*, 1989). Gastroenteritis is an infection of the colon which usually occurs 18–48 hours after ingestion of *Salmonella*. Gastroenteritis is characterized by diarrhoea, fever and abdominal pain. However it is an infection remain localized to the intestine and mesenteric lymph nodes, the infection is usually self-limiting, lasting 2–5 days (Heithoff *et al.*, 2008).

2.5 Serotyping of *Salmonella*

Serotyping is the fundamental approach in epidemiological surveillance and outbreak investigations for *Salmonella*. The most common method of subtyping *Salmonella* is the Kauffmann – White (KW) scheme which classifies *Salmonella* into different serotypes based on the immunoreactions of three different kinds of antigens which are O (somatic), H (flagellar) and Vi (capsular) antigens (Lindberg *et al.*, 1984). Somatic antigen (O) typing denotes the serogroup, and the flagellar H typing denotes the serotype. The Vi or capsular antigens are specific to some of serovars such as *S*. Typhi. Currently, KW scheme classification recognized 2,541 *Salmonella enterica* serovars based on antigenic differences

in the Lipopolysaccharide (LPS) O-antigen and phase 1 (H1) and phase 2 (H2) flagellar antigens (Soliman *et al*., 2009).

2.6 LPS Structure of *Salmonella*

Lipopolysaccharide (LPS) is considered as the major components of the outer membrane of Gram-negative bacteria, which is differentiate between the enteric and non enteric organisms. Lipopolysaccharide molecule has two different affinities (polar and non polar) consisting of three regions: the O specific polysaccharide (OPS), the core region and the lipid A (Jin *et al.,* 2001). Knowledge of the fine structure of the LPS O-chains is essential for understanding of the molecular basis of antigenic serospecific factors. The O-antigen of *Salmonella* is a polysaccharide (OPS), which is a branch of the lipopolysaccharide (LPS) (Kumriska *et al.*, 2007).

The composition of O-antigen is varied between *Salmonella*, which is composed of an oligosaccharide unit. The diversity is represented by variation in sugar constituents, sugars arrangement, addition of branch sugars, modifying side groups and specific linkages between O units. These variations provide the basis of serotyping of *Salmonella* (Fitzgerald *et al.*, 2003).

In biosynthesis of O-antigen a lot of enzymes are involved, and they encoded by a number of genes arranged in a large regulon named as *rfb* gene clusters which vary in size for each serogroup depending on the sugar component and complexity of the O antigen. *rfb* gene clusters have been characterized from a growing number of Gram-negative bacteria. The *rfb* gene cluster sited at about 42 min and connected to the operon on the chromosome

of *Salmonella enterica*, this operon is located between *galF* and *gnd* in *Salmonella enterica.* The sequences of *rfb* genes have low content of GC (normally less than 40%), the deviation in GC content from that of typical *S. enterica* genes (51%) increase the idea of *rfb* DNA is originated in species other than *S. enterica* and was detained by lateral gene transfer (Fitzgerald *et al.*, 2006).

Serogroups A, B, C2, D, and E of *Salmonella* are composed of *rfb* regions which contain rhamnose, galactose, and mannose, while four mannose residues introduce in composition of serogroup C1, one glucose side branch and *N*-acetylglucosamine residue (Lee *et al*., 1992).

The somatic (O) antigen in groups A, B, and D is composed of *rfb* regions contain repeat unit of different four sugars, all of them have general structure of a backbone sugars which are mannosyl, rhamnosyl and galactose, while the last type of sugar is a dideoxyhexose associated with mannose residue, the dideoxyhexose is paratose, abequose and tyvelose. The diversion of the biosynthesis for these three sugars only occurs at the last steps. Serogroups A and D have the gene known as *rfbS* which is encoding for paratose synthase currently named as (*prt)* gene, which converts CDP-4-keto-3, 6-dideoxyglucose to CDP-paratose, moreover the gene known as *rfbE* and encoded for CDPtyvelose- 2-epimerase currently renamed as (*tyv)* gene, it responsible for converting CDP-paratose to CDP-tyvelose. However, serogroup A is contained mutant form of (*tyv)* gene and does not produce active CDP-tyvelose epimerase due to the removal of 1-bp which causes the frameshift mutation and converts the fourth codon to an amber stop codon (Verma & Reeves, 1989; Lim & Thong, 2009).

Serogroups B and C2 have *rfbJ* genes which encode abequose synthase, converting CDP-4-keto-3, 6-dideoxyglucose to CDP- abequose. However, the *rfbJ* genes of serogroups B are very varied from serogroup C2, the variation occurs at nucleotide stage as well as amino acid stage by 44% and 64% respectively (Luk *et al*., 1993), and there are variation in *rfbD* and *rfbN* of group E1 and other serogroups. The genes encoded the mannose pathway of serogroup C1 and C2 are varied from other serogroups (Xiang *et al*., 1993).

There are 12 segments of potential transmembrane protein encoded by gene named *wzx* gene. This gene is found in all of *Salmonella* O-antigen gene clusters. It has been proposed that the proteins produced by *wzx* gene are involved in transferring the total of O antigen subunits across the cytoplasmic membrane to the periplasmic side (Liu et al., 1996). The Wzx proteins represent as mechanism of O antigen export in *Salmonella enterica.* One of Wzx proteins is O antigen polymerase which is functioned in polymerization of O units to form O antigen. Wzx proteins of different O antigen clusters of *Salmonella* are different, and there is little similarity even at the amino acid sequence level. It has been proposed that Wzx is responsible for the transfer of O units from the cytoplasmic to the periplasmic side of the cytoplasmic membrane (Liu *et al*., 1996; Marolda *et al*., 2004).

2.7 Flagella antigens (H)

The motility of microorganisms is carried out by flagellum which is the primary organelle for cellular movement. The constitution of bacterium flagellum distinguished a complex structure which consists of more than 20 component proteins and has numerous main characters such as basal body, transmembrane motor, hook structure, and an elongated helical filament (Malapaka *et al*., 2007).

Flagellin is a globular protein which is a monomeric protein produced by arranging and aggregation of flagellar filaments, the composition and characteristics of flagellar filaments effect the composition of flagellin. On the contrary, there are differences of the flagellin at the molecular weight level between the bacteria, and there is a large diversity in flagellar antigens has been verified (Joys *et al.,* 1985).

Salmonella serotypes typically have two flagella antigens that are expressed in two phases; specifically, H phase1 and H phase2, the genes of which are *fliC* and *fljB*, respectively. The two genes are located at different positions on the chromosome, and these can be expressed as major flagellins but not at same time in the bacterium. These two flagellin are different in the antigenic specificity which resulting in two types of cells with totally different flagella antigens (McQuiston *et al*., 2004).

Some of H antigens are composed of multiple factors; for example, H:e,n,x is the designation for a flagellar antigen that consists of three different parts of factors, e, n, and x. There are 114 H antigens composed of combinations of 99 distinct antigenic factors in *Salmonella*. Flagellar antigens that are immunologically related together are known as complexes. For instance, the G complex includes all flagellar antigen types that contain antigenic factor g (e.g., g,m; f,g; g,z51), plus flagellar antigen m,t. Flagellar antigen types that include antigen H:z4 are considered the Z4 complex (McQuiston *et al.*, 2004).

2.8 Capsular antigen (Vi)

Vi antigen stand for capsule antigen of *Salmonella*, which is polysaccharide antigen produced by *Salmonella* strains in the outer surface, and it is the only true capsule that

produced by *Salmonella* strains. It was discovered in 1934, and is associated with virulence, therefore it termed as Vi antigen. It is produced by some of *Salmonella* strains such as *S.* Typhi, *S.* Paratyphi C, *S.* Dublin and a few strains of *Citrobacter freundii* (Selander *et al.,* 1992). Two independent loci of genes are required for biosynthesis of Vi antigen, which are *via*A and *viaB.*

Some of *Salmonella enterica* serovars such as Paratyphi A and Paratyphi B do not possess the locus of *via*B in their chromosome, even though both of which are pathogenic and cause enteric fever (paratyphoid fever) in humans (McClelland *et al.*, 2004). Vi antigen as in *S.*Typhi and *S*. Paratyphi C prevent the agglutination in O antisera. Serotyping by agglutination tests considered have quite a lot of drawbacks such as the difficulty to maintain the complexity of the serotyping scheme. Commercial antisera often are unavailable for less common antigens or, if available, are of variable quality. To get around the problems associated with traditional serotyping, molecular techniques have been developed such as DNA probes. DNA based methods have the potential to be faster and able to be automated, and generally, they are more precise than traditional serological typing (McQuiston *et al.,* 2004).

2.9 Molecular approaches for serotyping of *Salmonella*

The molecular approaches of serotyping were developed and applied to overcome the limitations of the traditional method. The technology for DNA-based assays is fairly widespread, making it quite accessible to laboratories more than traditional serotyping. Furthermore DNA based methods have the potential to be faster and able to be automated, and generally, there are more precise than traditional serological typing, in addition these

methods appear to support and provide a lot of information in epidemiological cases. After all DNA – based methods are highly sensitive, highly degree of specificity, reliable and reproducible (Herrera-loan *et al*., 2004; McQuiston *et al*., 2004, Lim & Thong, 2009).

2.10 Multiplex Polymerase chain reaction

Multiplex polymerase chain reaction (MPCR) is a modification of the standard PCR and is described as the simultaneous amplification of numerous and various regions of genetic material by using more than one primer pair. Detection of multiple targets reduces the costs and time (Markoulatos *et al.*, 2002). The success of multiplex PCR technique is affected by some factors such as the concentrations of the primers for different loci, the concentration of the PCR buffer, the cycling temperatures, the equilibrium between the magnesium chloride and deoxynucleotide concentrations, and concentration of DNA and Taq polymerase (Henegariu *et al*., 1997).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacteria strains

A total of 126 strains obtained from the culture collection of Laboratory of Biomedical Science and Molecular Microbiology, University of Malaya, were used (Appendix 1). *Salmonella* strains of known serovars used as positive controls in all multiplex PCR and these include the following groups; group A (*S.* Paratyphi A (O:2) (n=2); group B (*S.* Paratyphi B in (n=2), *S.* Typhimurium (n=2); group D (*S.* Enteritidis (n=1); group D (Vi) (*S.* Typhi) (n=2); group E (*S.* Weltevreden (n=2); group C1 *S.* Braenderup (n=1); group C2 (*S.* Hadar (n=1), *S.* Corvallis (n=1) (Table 3.1).

Table 3.1: Known bacterial strains used as positive and negative controls

No	Strains	Source	Serotypes	O antigen	H antigen
1	SPA 02/08	Human	Paratyphi A	A	a, 1.5
2	TA 531/04	Human	Paratyphi A	A	a, 1.5
3	SPB 04/08	Human	Paratyphi B	B	b, 1.2
4	SPB 02/07	Human	Paratyphi B	B	b, 1.2
5	STM 03/07	Human	Typhimurium	B	i, 1.2
6	STM 48/07	Human	Typhimurium	B	i, 1.2

7	SBR 50/07	Human	Braenderup	C1	e.h, enz
8	S.Hadar (29)	Human	Hadar	C2	z_{10}, enx
9	R696k/08	Food	Corvallis	C2	z_4
10	ST 02/08	Human	Typhi	D (Vi)	d
11	ST 01/07	Human	Typhi	D (Vi)	d
12	SE 22/08	Human	Enteritidis	D	g,m
13	SWE 32/07	Human	Weltevreden	E	r, z_6
14	TF7/01	Food	Weltevreden	E	r, z_6

3.1.2 Preparation of media and solutions

The culture media are provided by commercial company (Oxoid England) and sterilized by autoclave at 121ºC for 15 min.

3.1.2.1 Luria-Bertani (LB) agar (Oxoid England)

Tryptone	1.0 g
Yeast extracts	0.5 g
NaCl	0.5g
dH2O	100 ml
Bacteriological agar	1.5 g

3.1.2.2 Luria-Bertani (LB) Broth (Oxoid England)

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

3.1.2.3 Bismuth Sulphite agar (BSA) (Oxoid England)

BSA base	4.0g
dH_2O	100ml

BSA base was weighed and suspended with 100 ml of dH_2O. Then it was boiled to dissolve completely by microwave. It was cooled to approximately 50ºC and poured into sterile petri dishes.

3.1.2.4 50% Glycerol

Ultra pure glycerol	25 ml
ddH_2O	25 ml

3.1.3 Reagents for polymerase chain reaction (PCR)

All the solutions and reagents of PCR such as *Taq* DNA polymerase (5 U/µl), magnesium chloride (25 mM), PCR nucleotide Mix (40 mM, pH 7.5) were commercially available from (Promega Inc., Madison, WI, USA).

3.1.3.1 Primers

The primer sequences are as listed in Table 3.2 and Table 3.3. They were commercially synthesized by QIAGEN Operon and Bioneer. The primers were dissolved in ddH_2O according to the instruction provided by the manufacturer. Primers for O-serogrouping multiplex PCR were selected based on the *rfb* gene clusters specific for *Salmonella* serogroups A, B, C1, D and E, as well as primers based on *viaB* gene to detect Vi-positive strains as reported by Levy *et al*. (2008) and Herrera- Leon *et al.* (2007). The

primers mixture of multiplex PCR for O antigen included rfbJ (F, R), tyv (F, R), vi (F, R), prt (F, R), wzx C1 (F, R), wzx C2 (F, R), wzx E1 (F, R) and P1-P2 primers (Table 3.2).

Primers that detected the H1 antigen 'a', 'b' and 'd' which target the *fli*C gene of flagella. The primers mixture of multiplex PCR for H1 antigen included H- for, Ha- rev, Hb- rev, Hd- rev and P1-P2 primers (Table 3.2).

P1-P2 primer for amplification the *oriC* region of *Salmonella* spp. (Levy et al., 2008) was used as an internal amplification control (IAC). In most of diagnostic PCR using of IAC becoming obligator, any PCR reaction lacking an IAC, the result could either mean that there was no presence of target sequence inside the reaction (Lim & Thong, 2009). The *oriC* region is located in the chromosome of *Salmonella* and encodes the replication origin, (Zskind & Smith, 1980), therefore *Salmonella* strains contain specific sequences which can be detected by specific PCR to differentiate them from non- *Salmonella* strains.

Table 3.2: Oligonucleotide primers used for O- multiplex PCR and H1- multiplex PCR

T[1]	**P**[2]	**Sequence (5 to 3)**	**size (bp)**	**Reference**
B	F-rfbJ	CCAGCACCAGTTCCAACTTGATAC	662	Lim *et al.* (2003)
	R-rfbJ	GGCTTCCGGCTTTATTGGTAAGCA		
D	F-tyv	GAGGAAGGGAAATGAAGCTTTT	614	Hirose *et al.* (2002)
	R-tyv	TAGCAAACTGTCTCCCACCATAC		
Vi	F-vi	GTTATTCAGCATAAGGAG	439	
	R-vi	CTTCCATACCACTTTCCG		

A and D	F-prt	CTTGCTATGGAAGACATAACGAACC	256	
	R-prt	CGTCTCCATCAAAAGCTCCATAGA		
C1	F-wzxC1	CAGTAGTCCGTAAAATACAGGGTGG	483	Herrera-Leon *et al.* (2007)
	R-wzxC1	GGGGCTATAAATACTGTGTTAAATTCC		
C2	F-wzxC2	ACTGAAGGTGGTATTTCATGGG	154	
	R-wzxC2	AAGACATCCCTAACTGCCCTGC		
E	F-wzxE1	TAAAGTATATGGTGCTGATTTAACC	345	
	R-wzxE1	GTTAAAATGACAGATTGAGCAGAG		
H	H-for	ACTCAGGCTTCCCGTAACGC		Levy *et al.* (2008)
	Ha-rev	GAGGCCAGCACCATCAAGTGC	423	
	Hb- rev	GCTTCATACAGACCATCTTTAGTTG	551	
	Hd-rev	GGCTAGTATTGTCCTTATCGG	763	
oriC	P1	TTATTAGGATCGCGCCAGGC	163	
	P2	AAAGAATAACCGTTGTTCAC		

T[1] Target

P[2] Primer

Additional new primers described by Cardono-Castro *et al.* (2009) were used for detection more flagella antigens H1 and, H2. All flagella antigens target *fliC*, and *fliB* genes (flagellins H1, H2) (Table 3.3).

Table 3.3: Additional oligonucleotide primers used for H1 and H2- multiplex PCR

Target	No access	Sequence (5 to 3)	Size bp
fliC-i	fliC-i D13689	F-TACGCCAAAGTTACCGTTACGG	304bp
		R-AATCATCGCCTACCTTAACTGCTAA	
fljB-1,2	fljB-1,2 AF045151	F-GAATGGTACGGCTTCTGTAACC	185bp
		R-CCGTCAGCAGCAGTATAACTTG	
fliC-f,g	fliC-f,g AY649707	F-GAAGCTACAGTGGGTGATCTGA	210bp
		R-CTTAAAGAGGTCAACCGCAGTG	
fliC-g,m	fliC-g,m AY649709	F-CTGTCTCAGGACAACCAGATGA	153bp
		R-GGATTTCAGATCACCCACTGTC	
fljB-1,6	fljB-1,6 AY353295	F-CTTGGCACGGCTTCTGTAAC	257bp
		R-GCATCTGCTGAAACAACTGTCG	
fliC-e,h	fliC-e,h AY649703	F – GCTAAATTAGTAAAAATGTCTTATACCGA	324bp
		R-TTAGCCAGCGGGTTTTCG	
fljB-1.5	fljB-1.5 AY353287	F-GCTGTAACGGGTGGTACAGTTA	190bp
		R-CTGTTTTAGTTGCCGCACCAG	
fliC-I,z13	fliC-I,z13 AY353450	F-GATCAGTTGACCGACAGTGGTA	102bp
		R-ACCGCCATCAATAGTCTTACCG	
fliC-z6	fliC-Z6 AY353353	F-GCTGTGACAGTAGCTGCCAAT	240bp
		R-CGTACCAGCGGTCATAGACAC	
fljB-enx	fljB-enx AY353305	F-TGTAAGTGGTTATACCGATG	120bp
		R-CCTGTAACAGTAGATTTAGTTG	
fliC-z10	fliC-Z10 AY353472	F-ATCAAGTAGTGTTCAGGATG	100bp
		R-ACCATTCTTATCAGTGTACG	

fliC-r	fliC-r AY434701	F-TGCTGCATCAGCAACTACCT R-TCCTGTTGGTGTGCCAGTTA	175bp

- Primers for H1- M.PCR; fliC-e,h, fliC-g,m, fliC-f,g, fliC-i, fliC-Z6, fliC-I,z13, fliC-Z10 and fliC-r, primers for H2-M.PCR; fljB-1.5, fljB-enx, fljB-1,2, fljB-1,6 described Cardono-Castro *et al*. (2009).

3.1.4 Solutions and reagents used for agarose gel electrophoresis

3.1.4.1 Agarose gel (1.5%)

0.5X TBE buffer	100 ml
Agarose powder	1.5 g

3.1.4.2 TBE buffer (10x)

Tris base	121.2 g
Orthoboric acid	61.8 g
EDTA	0.745 g
ddH2O	1000 ml

All of the ingredients of TBE buffer (10x) were weighed and suspended in 500 ml of ddH_2O by stirring with magnetic stirrer on the hot plate. pH of the buffer was measured and adjusted to pH 8.0 by adding additional NaOH or HCL. Then ddH_2O was added to top it up until 1000 ml and sterilized by autoclave at 121ºC for 15 min.

3.1.4.3 TBE buffer (0.5x)

10x TBE buffer	50 ml
ddH_2O	950 ml

All the ingredients were measured and mixed together thoroughly. It was stored at room temperature.

3.1.4.4 Ethidium bromide (10 mg/ml)

Ethidium bromide	100 mg
Deionised water	10 ml

The solution was then stored at room temperature in a dark container and diluted to 1.0 μg/ml with distilled water before use.

3.2 Methods

3.2.1 Purity and Storage of Strains

All the strains were labeled properly and purified before analysis. The purity of the strains was checked on selected media (Bismult Sulphite agar) and incubated at 37 ºC for overnight. The pure colonies were streaked on to a new plate for further testing, after purity was achieved, Then the confirmed strains were kept as glycerol stock (25% glucerol at -20ºC) and in stab culture at room temperature. Furthermore the confirmation of *Salmonella* was done by PCR targeting *hil*A gene to differentiate the *Salmonella* genus from the non-*Salmonella* genus (Pathmanathan, 2003). All the strains were subjected to PCR as preliminary screening of *Salmonella*, which is developed to detect *Salmonella* serovars and to distinguish *Salmonella* spp. from non- *Salmonella* spp. The multiplex PCR was needed primers targeting the *hil*A gene of *Salmonella* spp. to obtain a789 bp product. The *hil*A gene is an important feature of *Salmonella* pathogenesis, as it is required for bacterial colonization of the extracellular, luminal compartment of the host intestine (Murray & Lee, 2000), it is located and encoded in *Salmonella* pathogenicity island 1 (SPI1) (Mills *et al*., 1995), the *hil*A gene is present in all invasive strains of *Salmonella* (Galan, 1996) and absent from closely related genera such as Escherichia (Baumler *et al*.,

1998; Mills *et al*., 1995). Internal amplification control (IAC) is mandatory for diagnosis the PCR. An IAC is a non target DNA sequence present in the same sample reaction tube which is co-amplified simultaneously with the target sequence (Anonymous, 2002). In this study, the IAC was a synthetic DNA derived from plasmid pBR 322. The sequence is proprietor information (unpublished date, patent field).

3.2.2 Polymerase Chain Reactions

3.2.2.1 DNA Template Preparation

The template DNA of the strains was prepared by direct cell lysis using boiling method. A few single colonies of each strain were picked up from the LBA plate and suspended into a 500µl microcenterifuge tube containing 100µl of ddH_2O. This cell suspension mixed well, and boiled at 99 °C for 5 minutes in Perkin Elmer (DNA thermal cycler 480). After boiling, they were kept on ice for 10 minutes. The cell lysate was centrifuged at 13400 rpm for 3min. Then the supernatant was transferred to a fresh microfuge tube and an aliquote (5µl) ≈ 100 ng was used for PCR.

3.2.2.3 Multiplex PCR for serogrouping (O antigens) and detection of Vi antigen

Multiplex PCR for simultaneous detection of serogroup A, B, C1, C2, D, and E and *via*B genes and *oriC* region which were described by Hirose *et al.* (2002) , Lim *et al*. (2003), and Herrera-Leon *et al*. (2007), Levy *et al.* (2008).The evaluation and specificity of the primers were previously carried by (Lim and Thong, 2009). The optimized PCR

condition optimized by Lim and Thong (2009) reconfirmed and tested in this study. PCR reaction mixture and cycling parameters are shown in Table 3.4 and 3.5 respectively.

Table 3.4: PCR reaction mixture for multiplex PCR serogrouping of A, B, C1, C2, D, E and Vi (Lim and Thong, 2009)

Component	**Stock concentration**	**Reaction concentration**	**Volume (µl)**
Green GoTaq® FlexiBuffer	5X	1X	5
$MgCl_2$	25 mM	2.5 mM	2.5
dNTPs	10 mM	250 µM	0.625
Primers[1]	100 µM	0.4 µM	0.1
P1-P2[2]	10 µM	0.2 µM	0.5
Taq DNA polymerase	5U/µl	1.75 U	0.35
ddH_2O	-	-	Make to25
DNA template	-	-	5
Total			25

Primers[1]: (F-rfbJ, R-rfbJ, F-prt, R-prt, F-tyv, R-tyv, F-wzxC1, R-wzxC1, F-wzxC2, R-wzxC2, F-wzxE, R-wzxE, F-vi, R-vi). P1-P2[2]**:** primers for *oriC* region. Source Promega.

Table 3.5: PCR cycling parameters for multiplex serogrouping of A, B, C1, C2, D, E and Vi (Lim and Thong, 2009)

Step	**Conditions**	**Optimized parameters**
1	Initial denaturation	95ºC – 5min
2	Denaturation	95ºC – 40 sec
	Annealing	50ºC – 30 sec
	Extension	68ºC – 30 sec
	Cycles numbers	35
3	Further extension	68ºC – 7 min

3.2.2.4 Multiplex PCR for serotyping (H antigens)

Multiplex PCR for simultaneous detection of flagella antigens phase one (H1) were done by applying specific primers for Ha, Hb, and Hd, which were described by Levy *et al.* (2008) and Lim & Thong (2009). The present study followed the optimized conditions described by Lim & Thong (2009). The optimized reaction mixture and cycling parameters are shown in Table 3.6 and 3.7.

Table 3.6: PCR reaction mixture for multiplex PCR serotyping for Ha, Hb, and Hd flagella antigens (Lim and Thong 2009)

Component	**Stock concentration**	**Reaction concentration**	**Volume (µl)**
Green GoTaq® FlexiBuffer	5X	1X	5
$MgCl_2$	25 mM	1.8 mM	1.8
dNTPs	10 mM	200 µM	0.5
Primers[1]	10 µM	0.2 µM	0.5
P1-P2	10 µM	0.14 µM	0.35
Taq DNA polymerase	5U/µl	1.75 U	0.2
ddH_2O	-	-	Make to25
DNA template	-	-	5
Total			25

Primers[1]: H- for, Ha- rev, Hb- rev, and Hd- rev

Table 3.7: PCR cycling parameters for multiplex PCR serotyping for Ha, Hb, and Hd flagella antigens (Lim and Thong 2009)

Step	**Conditions**	**Optimized parameters**
1	Initial denaturation	95ºC – 2min
2	Denaturation	95ºC – 30 sec
	Annealing	55ºC – 30 sec
	Extension	72ºC – 30 sec
	Cycles numbers	35
3	Further extension	72ºC – 5min

3.2.2.5 Optimization of Multiplex PCR using additional flagella antigens primers

Additional new primers for detection more flagella antigens H1 and, H2 (Table 2) as described by Cardono-Castro *et al.* (2009) were evaluated to overcome the limitation of Lim and Thong (2009) and to identify more serotypes. All flagella antigens target *fliC* and *fliB* genes (flagellins H1, H2).

3.2.3 Analysis of PCR products

Gels of 1.5 % agarose were prepared for electrophoresis. A DNA ladder 100bp (Promega) was used as the molecular weight marker. An aliquot 5 µl of the PCR products were loaded in to the wells. Gel electrophoresis was run at the voltage of 100 to separate the PCR products for 30min. The gel was stained using EtBr (0.5µg/ml) for 10 minutes and the water used for washing the stain for 5min.

3.2.4 Gel documentation

After destaining, the gel was visualized by UV transilluminator. The photo of the gel was taken using Gel Doc (Bio-rad, Molecular Imager, Gel Doc ^{TM}, XR Imaging system - Laboratory of Biomedical Science and Molecular Microbiology, University of Malaya).

3.2.5 Validation of the Multiplex PCRs

A blind testing for validation of the optimized multiplex PCRs was conducted on 37 coded *Salmonella* strains of known serogroups A, B, C1, C2, D, and E, and Vi positive strains. *E. coli* and *V.paraheamolyticus* were included as negative control.

3.2.6 Sequence Analysis

Selected products from monoplex PCRs representing phase 1, 2 flagella antigens were purified by using Intron DNA extraction kit according to the manufacturer's protocol and sequenced by a commercial sequencing facility (First base Sequencing) to verify the amplicons.

CHAPTER 4

RESULTS

4.1 Purity and Storage of strains

All the strains were obtained after purifying them on Bismult Sulphite agar (BSA). *Salmonella* bacteria gave black colonies with or without metallic sheen on BSA as *Salmonella* produce hydrogen sulphide, the H_2S interact with iron that involved in the medium formula then iron precipitation give the characteristic of brown to black colonies. The *Salmonella* strains were further confirmed by the multiplex PCR that targets the *hil*A gene which has been reported to be very specific for *Salmonella* (Pathmanathan *et al.*, 2003). 122 out 126 strains produced the expected band size of *hil*A at 789bp and all the tested strains showed expected band at 164bp for internal amplification control (IAC) (Fig 4.1 and 4.2). Repeating PCR amplifications gave similar reproducible results.

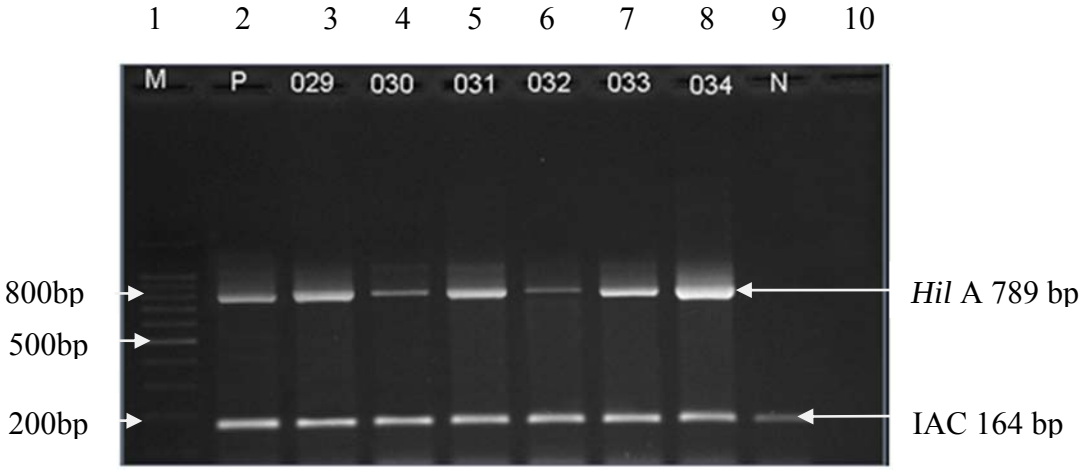

Fig 4.1: A representative photo of multiplex PCR for confirmation *Salmonella*. Lane 1 DNA marker 100bp ladder; lane 2 positive control; lanes 3, 4, 5, 6, 7, 8, *Salmonella* spp. show *hil*A gene at 789 bp ; lane 9 Negative control (H_2O). IAC is shown in all lanes indicating of the success of the PCR.

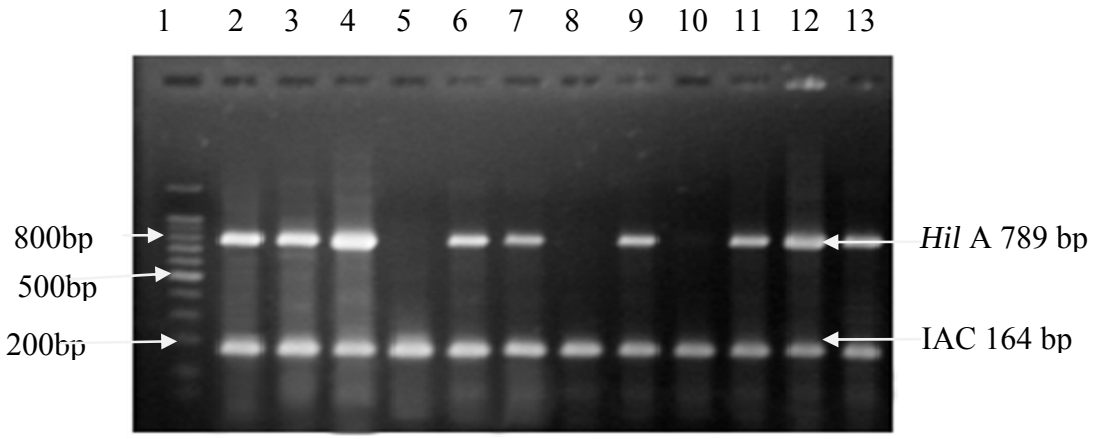

Fig 4.2: Second replicate gel photo of multiplex PCR for confirmation *Salmonella*. Lane 1 DNA marker 100bp ladder; lane 2 positive control; lanes 3, 4, 6, 7, 9,10, 11, 12, 13, *Salmonella* spp. show *hil*A gene at 789 bp ; lanes 5, 8, Negative control (H_2O). IAC is shown in all lanes indicating of the success of the PCR.

4.2 Multiplex PCR for serogrouping (O antigens) and detection of Vi antigen

All positive control strains produced amplicons of expected band sizes for the specific group; group A (*prt* gene - 256bp), group B (*rfbJ* gene - 662bp), group D (*prt* gene - 256bp and *tyv* gene - 615bp), group E (*wzx*E gene - 345bp), group C1 (*wzx*C1 gene - 483bp), group C2 (*wzx*C2 gene - 154bp) and Vi antigen (*viaB* gene, 439bp) (Fig 4.3). The data corroborated with the work previously published by Lim and Thong (2009) indicating that the previously developed PCR conditions were robust and reproducible.

The multiplex PCR was then tested on the 122 strains. Among them, 28.7% (n=35), 27.1% (n=33), 22.9% (n=29), 17.2% (n=21), 1.64% (n=2), and 1.64% (n=2) were of C2, B, D, E, C1 and A, respectively. Eight of the *Salmonella* strains from group D have Vi antigen. All the results of multiplex PCR for O and Vi antigens showed in Appendix 2. Repeating PCR amplifications gave similar reproducible results

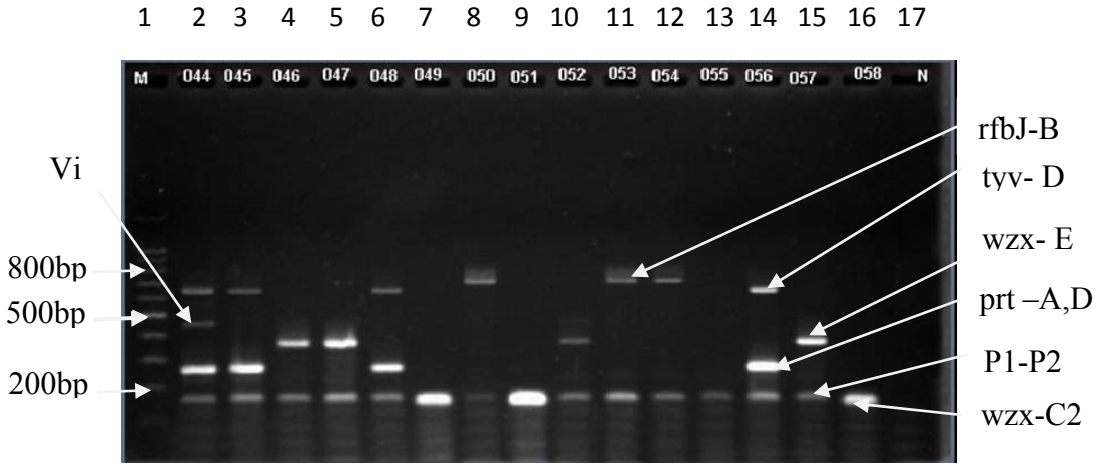

Fig 4.3: Multiplex PCR for O serogrouping. Lane 1 DNA marker 100bp ladder; lane 2 *S.*Typhi (group D (Vi); lanes 3, 6, 14, *S.* Enteritidis (group D); lanes 4, 5, 10, 15, *S.* Weltevreden (group E); lanes 7,9,16, *S.* Hadar; lanes 8, *S.* Stanely; lane11, 12 *S.*Typhimurium; lane 17 Negative control (H_2O).

The *prt* gene (256bp) is present both group A and D. Therefore *tyv* gene (614 bp) is needed to differentiate group D from group A. therefore members of group A showed the amplicon of *prt* alone while members of group D have both the *prt* and *tyv* amplicons. Although the size of IAC (*oriC*- 163bp) is very close to wzx C2 targets (154bp) strains which are of C2, the C2 strains have a thicker and darker band as unappeared to non- C2 strains (Fig 4.3). For further confirmation the PCR was repeated without using the P1-P2 primers (*oriC).*

4.3 Multiplex PCR for serotyping (H1 antigens a, b,and d)

The multiplex PCR for H1 produced the expected bands of flagella antigens among the positive control strains for *S*. Paratyphi A (Ha - 423bp), *S*. Paratyphi B (Hb-551bp), *S*. Typhi and *S*. Stanley (Hd-763bp) (Fig 4.4).

The multiplex PCR for HI typing was applied on the 122 strains. One strain produced expected band size for Ha (423bp), two strains produced expected band size for Hb (551bp), and sixteen strains produced expected band size for Hd (763bp). The multiplex PCR for H1 typing was robust and reproducible for serotyping of limited number of serovars such as such as *S.* Paratyphi A, and *S.* Typhi. The percentage of the strains produced flagella antigens (H1; a, b, and d) was 18.1% while the percentage of non-expressed strains was 81.9%. Therefore, due to the limited information provided by H1 multiplex PCR assay, additional primers for flagella antigens (both H1 and H2) as described by Cardono-Castro *et al.* (2009) were used. Repeating PCR amplifications gave similar reproducible results.

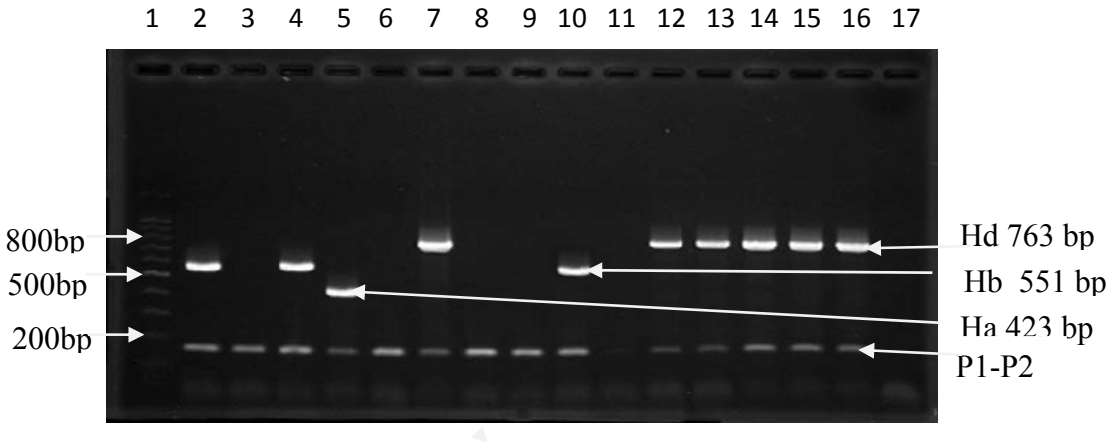

Fig 4.4: Multiplex PCR for *Salmonella* flagella antigens (H1). Lane1 DNA marker 100bp ladder; lane 2, 4, 10, *S.* Paratyphi B (group B); lane 5, *S.* Paratyphi A (group A); lane 7, *S.* Stanley; lane 12, 16, *S.*Typhi (group D (Vi); Lane 17, Negative control

4.4 Optimization of Multiplex PCR by using additional flagella antigens primers

The H-multiplex PCR of the new flagella antigens primers (Table 3.3) was applied by using PCR conditions recommended by Cardona-Castro *et al.* (2009). The positive control strains were used for optimization the H multiplex PCR of the new primers. The

PCR conditions and cycling parameters used by Cardona-Castro *et al.* (2009) are shown in Table 4.1 and 4.2 respectively.

Table 4.1: Optimized PCR condition recommended by Cardona-Castro *et al.* (2009)

Step	**Condition**	**Optimization**
1	Initial denaturation	94°C – 5min
2	Denaturation	94°C – 1min
	Anealing	58°C – 1min
	Extension	72°C – 1min
	Cycles	35cycle
3	Further extension	72°C –10min

Table 4.2: PCR cycling parameters for multiplex PCR serotyping of flagella antigens (H1, H2)

Component	**Reaction Concentration**
5x Green GoTaq® FlexiBuffer	1x
$MgCl_2$ (25 mM)	1.8mM
dNTPs (10 mM)	250µM
Primers[1] (100µl)	0.2µM
Taq® DNA polymerase (5U/µl)	1U

Primers[1]**:** fliC-i, fliC-f.g, fliC-g.m, fliC-e.h, fliC-I, z13, fliC-z6, fliC- z10, fliC-r, fljB-1.2, fljB-1.6, fljB-1.5, and fljB-enx which are described by Cardona-Castro et al., (2009).

Analysis of the amplicons using 2.5% of agarose gel showed unsatisfactory results. Non- specific bands were produced (Fig 4.5).

In Fig 4.5, lanes 2, 4 are *S*. Hadar which produced faint and an unspecific band size approximately at 450bp. The flagella antigens encoded by *S*.Hadar [*fli*C- z_{10} (100bp) and *flj*B- enx (120bp)] did not obtain. Lanes 3, 5, 8, 9, 10 are *S.* Weltevreden, some of this strain produced faint and an unspecific bands size at 450bp and 400bp, and other did not produced any amplicon. The flagella antigens encoded by *S.* Weltevreden [*fli*C- z_6, r, 240bp, 175bp) also did not recognize. *S*.Typhi at lane 6, 7 is used as a nagetive control and showed no amplicons.

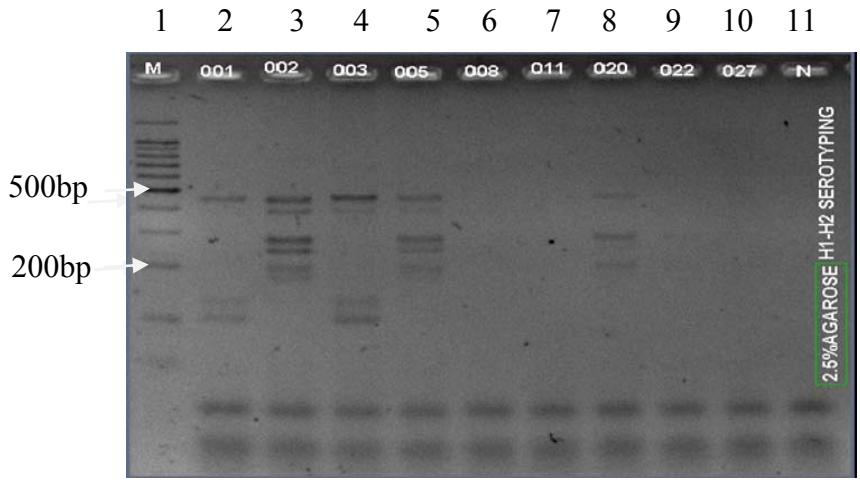

Fig 4.5: Multiplex PCR for flagella antigens (H1, H2) using recommended PCR conditions described by Cardona-Castro et al., (2009). Lane1 DNA marker 100 bp ladder; lane 2, 4, *S.* Hadar; lane 3, 5, 8, 9, 10, *S.* Weltevreden; lane 6, 7, *S.* Typhi; lane 11, Negative control (H_2O).

Since there were non-specific bands produced, further optimization had to be carried out. The primers were separated in two sets in accordance with the phase 1 and phase 2, PCRs conditions were re-optimized according to Lim and Thong (2009) (Table 4.3). In addition the PCR reaction volume was reduced to 25µl.

Table 4.3: PCR conditions for multiplex PCR serotyping H1, H2 according to Lim and Thong (2009) optimized conditions

Step	Condition	Recommended[1]	**Optimized**
1	Predenaturation	94°C – 5min	**95°C – 2min**
2	Denaturation	94°C – 1min	**95°C – 30sec**
	Annealing	58°C – 1min	**55°C – 30sec**
	Extension	72°C – 1min	**72°C – 30sec**
	Cycles	35cycle	**35cycle**
3	Final extension	72°C –10min	**72°C –5 min**

[1] Recommended condition by Cardona-Castro *et al.* (2009)

The optimized multiplex PCR for H antigens applied on the tested strains. The strains produced specific and non specific bands, some of the specific bands were faint (Fig 4.6).

In (Fig 4.6), the upper panel showed the amplicons of multiplex PCR for H1 antigens. *S.* Hadar at Lanes 2, 4 produced the expected band size of *fli*C-z_{10} at 100bp and an unspecific band size at 450bp. *S.* Weltevreden at lanes 3, 5, 7, 8 and10 produced the expected bands size of *fli*C-r and *fli*C- z_6 at 175bp and 240 bp respectively. There is an unspecific band size at 450bp among *S.* Weltevreden. The lower panel showed the amplicons of multiplex PCR for H2 antigens. *S.* Hadar at Lanes 2, 4 produced the expected band size of *flj*B- enx at 120bp in addition to an unspecific band at 450bp. *S.* Weltevreden at lanes 3, 5, 7, 8 and 10 did not produce any amplicons for H2 antigens as well as *S.* typhi in lanes 6, 9 and 11.

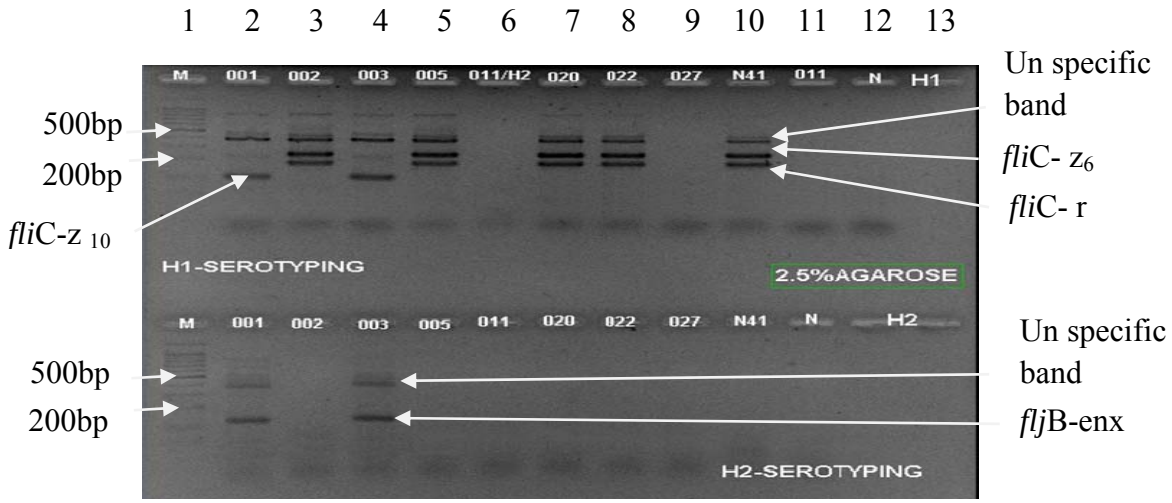

Fig 4.6: Multiplex PCR for flagella antigens using PCR conditions described by Lim and Thong (2009). Upper panel (H1); Lane1 DNA marker 100 bp ladder; lane 2, 4, *S.* Hadar; lane 3, 5, 7, 8, 10, *S.* Weltevreden; lane 6, 9, 11, *S.* Typhi; lane 12, Negative control (H_2O); Lower panel (H2); Lane1, DNA marker 100 bp ladder; lane 2, 4, *S.* Hadar; lane 3, 5, 7, 8, 9, 10, *S.* Weltevreden; lane 6, *S.* Typhi; lane 11 Negative control (H_2O)

Further optimization was done by increasing the initial denaturation time from 2 min to 5 min, as well as the buffer concentration was changed from 1X to 1.2X. The PCR conditions and other cycling parameters were constant as are shown in Table 4.4 and 4.5.

Table 4.4: The optimized PCR conditions for multiplex PCR serotyping H1 and H2

Step	**Condition**	**Optimization**
1	Predenaturation	95°C **–* 5min**
2	Denaturation	95°C – 30sec
	Annealing	55°C – 30sec
	Extension	72°C – 30sec
	Cycles	35cycle
3	Final extension	72°C –5 min
4	Hold	4°C

* Predenaturation time changed to 5min

Table 4.5: Optimized PCR reaction cycling parameters for multiplex PCR serotyping

Component	**Reaction Concentration**
5x Green GoTaq® FlexiBuffer	1x to* **1.2x**
$MgCl_2$, 25 mM	1.8mM
dNTPs, 10 mM	200µM
Primers, 100µl	0.2µM
Taq® DNA polymerase,5u/µl	1U

* Buffer concentration changed to1.2X

The optimized PCR conditions were applied on the tested strains (Table 4.4 and 4.5). Specific bands size for the new targets and elimination of unspecific bands were achieved. Representative gel pictures of the multiplex PCR are shown in Fig 4.7 and 4.8.

S. Hadar produced specific band at 100bp for *fli*C-z10 (Fig 4.6: lane 2, 4). *S.* Weltevreden produced specific bands at 175bp for *fli*C-r and 240bp for *fli*C-z6 (Lane 3, 5, 7, 8, and 10). *S.* Typhi did not produce any amplicon (Lane 6, 9). There were no unspecific bands among the tested strains.

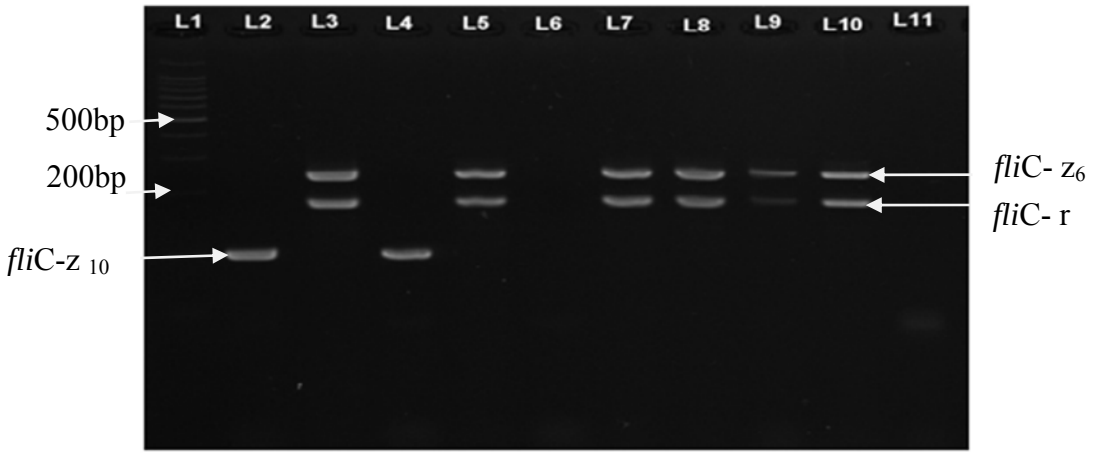

Fig 4.7: Multiplex PCR for flagella antigens (H1) using optimized PCR conditions. L1 DNA marker of 100 bp ladder; lane 2, 4, *S.* Hadar; lane 6, *S.* Typhi; lane 3, 5, 7, 8, 9, 10, *S.* Weltevreden; lane 11, Negative control (H_2O).

S. Hadar produced the expected band size at 120bp indicating for flagellar antigrn *flj*B- enx (lane 2, 4), other tested strains did not produce any amplicons, There were no unspecific bands among the tested strains (Fig 4.8).

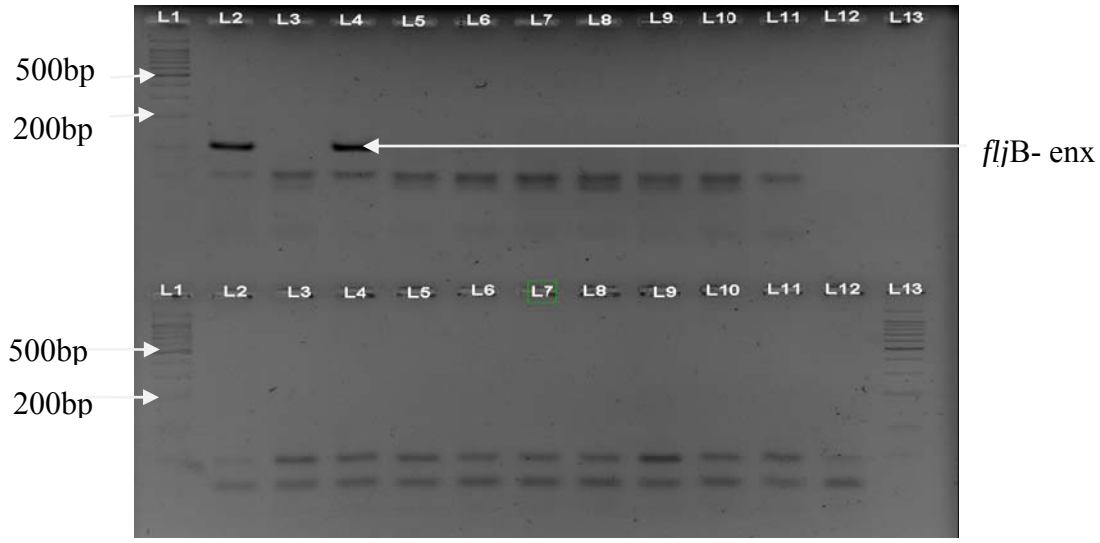

Fig 4.8: Multiplex PCR for flagella antigens (H2) using optimized PCR. Upper panel; Lane1 DNA marker of 100 bp ladder; lane 2, 4, *S.* Hadar; lane 6, *S.* Typhi; lane 3, 5, 7, 8, 9, 10, *S.* Weltevreden; lane 11, Negative control (H_2O). Lower panel; Lane1 DNA marker of 100 bp ladder ; lane 2, 4, 6, *S.* Braenderup; lane 3, 5, 7, *S.* Enteritidis ; lane 8, 9, *S.* Weltevreden ; lane 11, 12, Negative control (H_2O).

4.5 Confirmation of primers amplicons by DNA sequencing

The amplicons generated by primers *fli*C (r, z_6, z_{10}, g.m, e.h and i) and *flj*B (1.5, 1.2, enx and 1.6) were verified by DNA sequencing. The results of DNA sequences were compared to the National Center for Biotechnology Information (NCBI) database by using Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST). The Biosystems Sequence Scanner software v1.0 used for obtains the nucleotide sequence. The blast search showed 97%-100% similarity level for all amplicons generated by the primers to the reported serotypes or serogroups in the GenBank. All DNA sequences successfully recognized different regions of *fli*C and *flj*B genes (Table 4.6). In Table 4.6

the score ranged 134- 496 bits, the score indicates of how good the alignment is, and the higher score the better alignment, and the expected value (E value) showed the statistical significance. Lower E value, the more significant the hit. All the results obtained from NCBI are shown in Appendix 5 to Appendix 14.

Table 4.6: Results of blast search of DNA sequences showing the percentage of identity, score and E value

Primers	**Size (bp)**	**Score (bit)**	**E (value)**	**Percentage of Identity**
fliC-e.h	324	496	4e-137	100%
fljB-enx	120	134	9e-29	100%
fliC-z_6	240	355	5e-95	98%
fliC-r	175	243	3e-61	97%
fljB-1.5	190	268	5e-69	98%
fljB-1.6	257	364	8e-98	97%
fliC-g.m	153	193	2e-46	100%
fliC-z10	100	315	6e-83	99%
fljB-1.2	185	259	3e-66	98%
fliC-i	304	460	1e-126	98%

4.6 Application of optimized Multiplex PCR for serotyping of *Salmonella* strains using additional primers for H1 and H2 antigens

After optimization, two H multiplex PCRs were applied. New flagella antigen primers for H1 [*fli*C-e,h (324bp), *fli*C-g,m (153bp), *fli*C-f,g(210bp), *fli*C-i,(304bp) *fli*C -Z6 (240bp), *fli*C-I,z13 (102bp), *fli*C-Z10 (100bp) and *fli*C-r (175bp)] were used on the positive control strains. The multiplex PCR produced the expected bands size for *S.* Typhimurium (*fli*C-i -185bp), *S.* Enteritidis (*fli*C-g.m-153bp), *S.* Weltevreden (*fli*C-z6-240bp, r, 175bp) and *S.* Hadar (*fli*C-z10 -100bp) (Fig 4.9).

Multiplex PCR of H2 flagella antigens [*flj*B-1.5 (190bp), *flj*B-enx (120bp), *flj*B-1.2 (185bp) *flj*B-1.6 (257)] were done concurrently with H1- typing. The results of H2 multiplex PCR produced expected bands size with positive control strains for *S.* Paratyphi A (*flj*B-1.5 -190bp), *S*. Paratyphi B, *S.* Typhimurium, *S.* Stanley (*flj*B-1.2 - 185bp), and *S.* Hadar (*flj*B-enx - 120bp) (Fig 4.9).

Some of the tested strains were biphasic which have phase1 (H1) and phase 2(H2) flagella antigens such as *S.* Paratyphi A (Ha - 423bp and fljB-1,5 -190bp), *S.* Typhimurium (*fli*C-i -185bp and *flj*B-1,2 - 185bp), and *S.* Hadar (*fli*C-z10 -100bp and *flj*B-enx - 120bp). The specificity of the new primers described by Cardona-Castro *et al.* (2009) was confirmed by DNA sequencing (section 4.5).

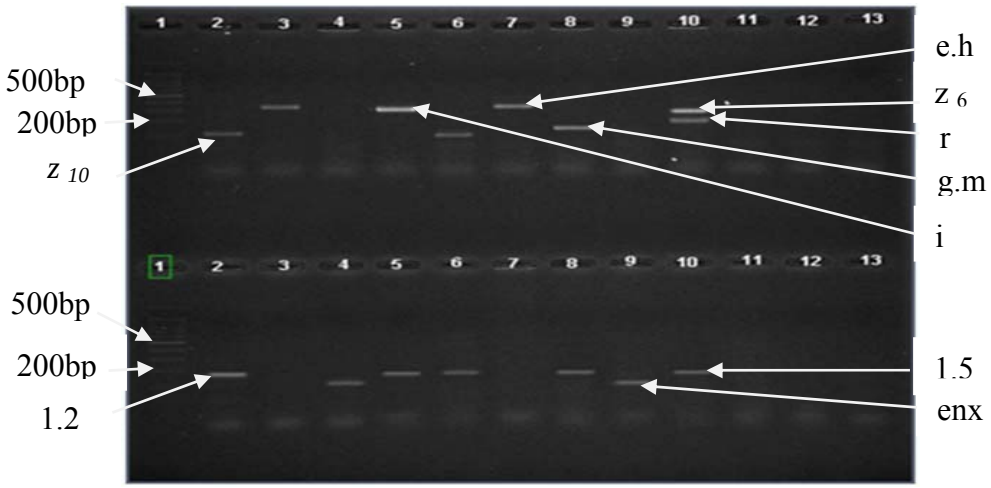

Fig 4.9: Multiplex PCR for flagella antigens using optimized PCR condition. Upper panel (H1); Lane1 DNA marker of 100 bp ladder; lane 2, 6, *S.* Hadar (*fli*C- z_{10}); lane 5 *S.* Typhimurium (*fli*C- i); lane 3, 7, *S.* Braenderup (*fli*C- e.h); lane 8, *S.* Enteritidis (*fli*C- g.m); lane 10, *S.* Weltevreden (*fli*C- r and z_6); lane 11-13 Negative control (H_2O). Lower panel (H2); Lane1 DNA marker of 100 bp ladder; lane 2, 5, *S.* Typhimurium (*flj*B- 1.2); lane 4, 9, *S.* Hadar (*flj*B- enx); lane 6, 8, 10, *S.* Paratyphi A (*flj*B- 1.5); lane 11-13 Negative control (H_2O).

In Fig 4.9, Strains of group C2 at upper panel (lane 2, 6) produced expected band size for *fli*C- z_{10} (100bp), presumptively identified as *S.* Hadar, and confirmed by production of band size for *flj*B- enx (120bp) at lower panel (lane 4, 9). Strain of group B at upper panel (lane 5) produced band size for *fli*C- i (304bp) and at lower panel (lane2) band size for *flj*B- 1.2 (185bp) and confirmed as *S.* Typhimiurim. *S.* Enteritidis confirmed by producing *fli*C- g.m (153bp) at upper panel (lane 8). Strains of group C1 at upper panel (lane 3, 7) produced band size for *fli*C- e.h (324bp) and confirmed as *S.* Braenderup. Band size for *flj*B- 1.5 (190bp) at lower panel (lane 6, 8, 10) produced by strains of group A and confirmed as *S.* Paratyphi A. All the results of multiplex PCR for H antigens showed in Appendices 3 and 4.

4.8 Validation of the Multiplex PCRs

Thirty seven of *Salmonella* strains of known serovars previously isolated and identified from various sources such clinical, food and environmental samples were blind-coded and tested with the multiplex PCRs for O and H antigens (Table 4.7). The multiplex PCR of O serogrouping produced PCR products corresponding to O antigens targets from the 37 isolates and correctly identified serogroups A, B, C1, C2, D, and E. Two of non *Salmonella* used (*E.* coli and *V*. parahaemolyticus) and could not produce any amplicons for the serogrouping. The multiplex PCR of H1 and H2 serotyping produced corresponding amplicons to H1 antigens (Ha, Hb, Hd, r, i, g.m, e.h, z_{10} and z_6) and H2 antigens (1.5, 1.2, 1.6 and enx). Overall of serotyping correctly identified *S.* Weltevreden, *S.* Enteritidis, *S*. Typhimurium, *S.* Hadar, *S.* Paratyphi B, *S.* Typhi and *S.* Paratyphi A. There is 100% of concordance between MPCR and traditional serotyping (Table 4.7).

Table 4.7: *Salmonella* strains used for blind testing and verification of the sequential multiplex PCRs results compared to conventional serotyping

No	Strains	Source	O-MPCR	H-MPCR	PCR Serotypes	Traditional serotyping
1	023/09 Sal	Human	A	a, 1.5	Paratyphi A	Paratyphi A
2	R911k/09	Food	B	i, 1.2	Typhimurim	Typhimurim
3	R441k/09	Food	B	i, 1.2	Typhimurim	Typhimurim
4	328.0193	Human	B	i, 1.2	Typhimurim	Typhimurim
5	R128/09	Food	B	i, 1.2	Typhimurim	Typhimurim
6	328.0193	Human	B	i, 1.2	Typhimurim	Typhimurim
7	R913k/09	Food	B	i, 1.2	Typhimurim	Typhimurim
8	R1167,E	Food	B	i, 1.2	Typhimurim	Typhimurim

9	R1458k	Food	B	i, 1.2	Typhimurim	Typhimurim
10	R1438k	Food	B	i, 1.2	Typhimurim	Typhimurim
11	R259k/09	Food	B	i, 1.2	Typhimurim	Typhimurim
12	R2798/07	Food	B	i, 1.2	Typhimurim	Typhimurim
13	R1167	Food	B	i, 1.2	Typhimurim	Typhimurim
14	R128k/09	Food	B	i, 1.2	Typhimurim	Typhimurim
15	R359k/09	Food	B	i, 1.2	Typhimurim	Typhimurim
16	R2792k/07	Food	B	e.h	Sandiego	Sandiego
17	SPB 02/07	Human	B	i, 1.2	Paratyphi B	Paratyphi B
18	018/09 Sal	Human	B	i, 1.2	Paratyphi B	Paratyphi B
19	R1843k/09	Food	B	d, enx	Sarajane	Sarajane
20	R776k	Food	B	d	Duisburg	Duisburg
21	11983/06	Food	C1	e.h	Braenderup	Braenderup
22	15376/05	Food	C2	d	Muenchen	Muenchen
23	R3802k/08	Food	C2	z_{10}, enx	Hadar	Hadar
24	R2803k/08	Enviro[1]	C2	z_{10}, enx	Hadar	Hadar
25	R908k/09	Enviro[1]	D	g.m	Enteritidis	Enteritidis
26	253.0374	Human	D	g.m	Enteritidis	Enteritidis
27	292-0820	Human	D	g.m	Enteritidis	Enteritidis
28	280-0374	Human	D	g.m	Enteritidis	Enteritidis
29	280/286	Human	D	g.m	Enteritidis	Enteritidis
30	039/09 Sal	Human	D	d	Typhi	Typhi

31	040/09 Sal	Human	D	d	Typhi	Typhi
32	041/09 Sal	Human	D	d	Typhi	Typhi
33	R1907/09	Food	E	r, z_6	Weltevreden	Weltevreden
34	R2571k/08	Food	E	r, z_6	Weltevreden	Weltevreden
35	7752/06	Food	E	r, z_6	Weltevreden	Weltevreden
36	08-287-1652	Human	E	r, z_6	Weltevreden	Weltevreden
37	276.46	Human	E	r, z_6	. Weltevreden	Weltevreden
38	259	Human	*E. coli*	-	-	-
39	VC 2-2	Envi	V.P[2]	-	-	-

[1] Enviromental strains
[2] *V. parahaemolyticus*

4.9 Outcome of multiplex PCRs in differentiation of *Salmonella enterica* based on O and H antigens

The combination of O and H multiplex PCRs results for identification *Salmonella* serovars enabled molecular serotypting of 94 of 122 *Salmonella* strains (77%). The most frequently encountered serotypes were *S.* Welterverden (n=21), *S.* Enteritidis (n=18), *S.* Typhimurium (n=19) and *S.* Hadar (n=14), the least frequent were *S.* Paratyphi B (n=2), *S.* Paratyphi A (n=1), *S.* Duisburg (n=1), *S.* Sandiego (n=1), and *S.* Muenchen (n=1) (Table 4.8). The rest (23%) of tested strains could not be identified due to the limitation of PCR targets.

Table 4.8: Overall results of *Salmonella* typing based on multiplex PCR detection O, H and Vi genes

Serogroup	**Serotypes**	**Antigenic formula**	**No test (%)**	**O genes**	**H genes**
A	Paratyphi A	1,2,12:a:[1,5]	1 (0.8%)	*Prt*	*a, 1.5*
B	Typhimurium	1,4,[5],12:i:1,2	19 (15.5%)	*rfbJ*	*i, 1.2*
	Stanley	1,4,[5],12,[27]	5 (4.1%)	*rfbJ*	*d, 1.2*
	Paratyphi B	1 ,4,[5],12:b:1.2	2 (1.6%)	*rfbJ*	*b, 1.2*
	Sandiego	1,4,[5],12:e,h:1,2	1 (0.8%)	*rfbJ*	*e.h,*
	Duisburg	1,4,12,[27]:d:enz	1 (0.8%)	*rfbJ*	*d*
	Sarajane	1,4,[5],12,[27]:d:enx	1 (0.8%)	*rfbJ*	*d, enx*
C1	Braenderup	6,7 : e,h : e,n,z15	1 (0.8%)	*wzx*C1	*e.h*
C2	Hadar	6,8:z10:e,n,x	14 (11.4%)	*wzx*C2	*z $_{10}$, enx*
	Bovismorbificans	6,8:r:1.5	1 (0.8%)	*wzx*C2	*r, 1.5*
	Muenchen	6,8:d:1,2	1 (0.8%)	*wzx*C2	*d, 1.2*
D	Enteritidis	9,12:g,m	18 (14.8%)	*prt, tyv*.	*g.m*
	Typhi	9,12[Vi]:d	8 (6.6%)	*prt , tyv via*B	*d*
E	Weltevreden	3,10[15]:r:z6	21 (17.2%)	*wzx*E	*r, z6*

CHAPTER 5

DISCUSSION

Facilities to serotype members of the *Salmonella* genus are limited in Malaysia. Hence, the main aim of this study was to apply DNA based typing (PCR) to differentiate *Salmonella enterica* based on the O and H antigens. The multiplex PCR described in this study will enable one to rapidly identify the major serogroups of *Salmonella* (A, B, C1, C2, D, and E) and Vi positive strains that are commonly encountered in Malaysia and also to confirm clinically important *Salmonella* serotypes such as *S.* Typhi, *S.* Paratyphi A, *S.* Welterverden, *S.* Enteritidis and *S.* Hadar (Bulletin of Ministry of Health Malaysia (2007). These serotypes are main causes of enteric fever and gastrointestinal infections (Mead *et al.*, 1999). In order to achieve the goal, further improvement were made to overcome the limitations of the previous studies (Herrera- Leon *et al*., 2007; Levy *et al*., 2008; Lim and Thong, 2009) for differentiation and serotyping *Salmonella enterica* strains. This was carried out by combining the sequential multiplex PCRs and addition of new primers recently described by Cardona-Castro *et al*. (2009).

Confirmation of the *Salmonella* genus was initially carried using the *hil*A target as it was shown to be very specific for *Salmonella* (Pathmanathan *et al*., 2003). Multiplex PCR for *Salmonella* spp. confirmation produced an intense band of the expected 784 bp while non - *Salmonella* strains did not give any of target amplicon. PCR amplifications gave similar reproducible results and the specificity of PCR was 100% and concordant with Pathmanathan *et al.* (2003).

The success of optimizing multiplex PCR assay is influenced by concentrations of the primers at the different loci, the concentration of the PCR buffer, *Taq* polymerase, amount $MgCl_2$, additional reagents and the cycling temperatures and the equilibrium between the magnesium chloride and deoxynucleotide concentrations (Schoske *et al*., 2003). Direct application of MPCR of H serotyping using recommended PCR conditions described by Cardona-Castro *et al*., (2009) produced unspecific products. This could due to variations in quality of reagents, quality of DNA template, and different models of thermocylers used. The unspecific bands could produce due to using of many primers pairs. The possibility of producing spurious amplification products increases when more than one pair of primer is used in multiplex PCR, primarily because of the formation of primer dimers (Elnifro *et al*., 2000). The primers were separated in two sets in accordance with the phase 1 and phase 2, and PCRs conditions were re-optimized according to Lim and Thong (2009). Correctly the tested strains produced the specific products but there was an unspecific band. The probability of producing unspecific products increases when more than one pair of primer is used in multiplex PCR, primarily because of the formation of primer dimmers (Brownie *et al*., 1997; Elnifro *et al.,* 2000). Furthermore optimization was done in the PCR conditions described by Lim and Thong (2009) which the initial denaturation time changed from 2 min to 5 min, and the buffer concentration changed from 1X to 1.2X. Increasing of buffer concentration reduce the appearance of unwanted, long, non- specific products and increase the desired products (http://www.highveld.com/pages/pcr-troubleshooting.html).

From the results obtained after optimization, all the tested strains produced specific bands size for the targeted flagella antigens without any unspecific bands. The big change

made by this study was decreasing the time of denaturation, annealing and extension. The decreasing of time in this case succeeded in elimination the production of unspecific bands.

Many multiplex PCR assays have been described for serotyping *Salmonella* based on O and H antigens (Hirose *et al*., 2002; Lim *et al*., 2003; Herrera-Leon *et al*., 2007; Levy *et al*., 2008). Herrera- Leon *et al*. (2007) developed three multiplex PCRs for serogroups B, C1, C2, E1, and D based on different regions of *wzx, tyv, fliC* and *fljB* genes. Even though this approach defined some of common *Salmonella* serovars, it could not differentiate serovars that belong to group A and the strains with capsular antigens. Levy *et al*. (2008) developed two multiplex PCRs for groups A (*prt),* B (*rfb)*, and D (*prt and tyv)*, in addition to Vi antigen (*via*B) and flagella antigen phase1 *(fliC*). This approach was very useful for detection of enteric fever pathogens (*S.* Typhi, *S.* ParatyphiA) but it did not distinguish the common groups of *Salmonella* C1, C2 and E. Subsequently, Lim and Thong (2009) successfully combined the different primers reported by Herrera- Leon *et al.* (2007) and Levy *et al*. (2008) and reoptimised a multiplex PCR to simultaneously differentiate A, B, C1, E, D and Vi. The detection of flagella antigens H1 (a, b, and d) enabled the detection of *S.* Typhi and *S.* Paratyphi A which cause enteric fever with 100% specificity. However this approach did not identify serogroup C2 which include some of the common *Salmonella* pathogens such as *S*. Hadar (Herikstad *et al*., 2002). Moreover there was no detection of flagella phase 2 (*flj*B gene) and the detection of flagella antigens phase 1 (*fliC* gene) targeted a few antigens alleles (*a, b, d).*

The present study further applied mPCR developed by Lim and Thong (2009). The specificity of detection groups A, B, C1, D, and E and Vi positive strains all were 100%. In this study group C2 were incorporated to multiplex PCR for somatic antigen typing. The

multiplex PCR in this study identified the common serogroups A, B, C1, C2, D, and E, with Vi positive strains and flagella antigens H1, H2 and achieved 100% concordance to the identified strains by traditional serotyping. Application of multiplex PCRs for O, H and Vi antigens were reproducible and robust results. Recently Cardona-Castro *et al.* (2009) reported developed multiplex PCR for serotyping *Salmonella* serovars (B, C2, D, and E) targeting *rfb, wzx, fliC* and *fljB* genes. Due to limitation of Lim and Thong (2009), the primers targeting (*fli*C and *flj*B genes) that reported by Cardona-Castro *et al*. (2009) were incorporated in the multiplex PCR for flagella antigens typing to serotype a wide range of *Salmonella* strains. The H- typing multiplex PCR described by Lim and Thong (2009) identified presumptively *S.* Paratyphi A and *S.* Typhi by detection of H1 antigens (a and d). *S.* Paratyphi A is only serotype expressed (a) antigen among group A. The present study confirmed serotyping of *S.* Paratyphi A by detection of H1 (Ha) and H2 (1.5) antigens. There are some serotypes express H1 antigen (b) among serogroup B such as *S.* Abony and *S.* paratyphi B. Therefore this system cannot be used as confirmative detection of *S.* paratyphi B. The optimized multiplex PCR of H1 and H2 serotyping produced corresponding amplicons for (Hb, 1.2) to confirm *S.* paratyphi B. It was very important to apply the second phase flagella antigens multiplex PCR.

The primers of A, B, D, were designed targeting defined regions of the abequose and paratose synthase genes: *rfbJ* (662bp) of *Salmonella* serogroup B and *prt* (256bp) of *Salmonella* serogroup A and D, *tyv* (614bp) gene of serogroups A and D, but there is a difference between the nucleotide bases, which creates an early codon stop in serogroup A, indicated that the detection of group A only depend upon paratose which is incorporated in its O-antigen. These primers were designed based on this difference, which were described by Levy *et al*. (2008). Serogroups C1, C2 and E primers designed by Herrera-Leon *et al*.

(2007) targetted gene wzx. The *wzx* gene encodes a flippase for O-unit translocation across the cell membrane. Low similarity at the amino acid sequence in the Wzx protein from different O antigen provide a mean of differentiation of *Salmonella* based on the *wzx* gene (Marolda *et al,* 2004). In order to better differentiate among these major serogroups, the primers designed gave precise specificity in priming and gave DNA products with different sizes in polymerase chain reactions (483 bp for serogroup C1, 154bp for serogroup C2, 345 bp for serogroup E)

The biosynthesis of the Vi antigens is controlled by two independent loci of genes which are *via*A and *via*B (Vilolgeux *et al*., 1995). In order to detect capsular antigen the specific primers described by Levy *et al.* (2008) target *via*B gene. These primers were incorporated in the O serogrouping multiplex PCR by using same PCR conditions and cycling parameters (439bp for Vi antigen). The incorporation of Vi primers in multiplex PCR for serogrouping, enabled the detection of *S.* Paratyphi C (only serovar carries Vi antigen among group C1). Moreover, this PCR could presumptively identifiy *S.* Typhi and *S.* Dublin which belong to group D (Hirose *et al*., 2002). In this study eight strains of group D produced specific band size of Vi antigen (439bp) and presumptively detected as *S.*Typhi. Later *S*.Typhi was confirmed by multiplex PCR for H typing and produced d antigen. The identification of Vi antigen provides a very efficient and vital for studying the salmonellosis surveillance and epidemiological distribution of the major *Salmonella* serovars.

P1-P2 primers for amplification the *ori*C region of *Salmonella* spp (Levy *et al*., 2008) was used as an internal amplification control (IAC). In most of diagnostic PCR using of IAC is essential. The use of an IAC is to ensure that negative results are the negative. In

this study IAC primers produced amplicon size of 163bp in all confirmed *Salmonella* strains, and the primers did not amplify any specific fragment from the non-*Salmonella* strain.

Overall, the multiplex PCR assays identified 94 of 122 (77%) of *Salmonella* strains. These include *S.* Welterverden, *S.* Enteritidis, *S.* Typhimurium, *S.* Hadar, *S.* Paratyphi B, *S.* Paratyphi A and *S.* Typhi, these are the most common and important serovars in Malaysia according to buletin of Ministry of Health Malaysia (2007). H multiplex PCRs detected two phase of flagella antigens in some *Salmonella* strains such as *S.* Typhimurium, and *S.* Hadar which are known as biphasic (Hong *et al*., 2008). Furthermore it succeeded the detection of monophasic flagella antigens such *S.* Typhi and *S.* Enteritidis which are known as monophasic (Burnens *et al*., 1996).

Twenty nine (23%) of tested strains could not be serotyped. This may due to deficiency of the primers used for detection *fli*C and *flj*B genes, or the strains may be converted into monophasic or become motionless. The feature of monophasic could either to missing of switching mechanism or disability to express the second flagellar antigen. Furthermore the feature of monophasic could be natural (Burnens *et al*., 1996; Herrera-Leon *et al*., 2007).

The sequential multiplex PCRs approach used in this study was a specific, fast, reliable and cost-effective method. This DNA based technique would extensively reduce the reliance on the traditional time-consuming methods and reduce the amount of sera needed for *Salmonella* identification.

The present study concurred with previous researchers (Herrera-Leon *et al*., 2007), that DNA based approach cannot use as substitute of traditional serotyping method. The two methods could be complementary, therefore the main *Salmonella* serotypes can be identified by multiplex PCR serotyping, while less common serotypes can be identified by traditional serotyping method.

CHAPTER 6

CONCLUSION

The objective of the study was achieved. The main findings are:

The major *Salmonella* serogroups A, B, C1, C2, D, and E, and Vi positive strains were successfully identified. This multiplex PCR will serve as a rapid detection of *Salmonella* serogrouping commonly encountered in Malaysia. The PCR was specific and reproducible.

The Multiplex PCR for H antigens was successfully applied and detected H1 flagella antigens (H a, H b, H d, r, z_6, z_{10}, g.m, e.h, and i) and H2 flagella antigens (1.5, 1.2, 1.6, and enx) for most tested strains.

The common serovars completely identified were *S.* Welterverden, *S.* Enteritidis, *S.* Typhimurium, *S.* Hadar, *S.* Paratyphi B, *S.* Paratyphi A and *S.* Typhi.

Serotyping of *Salmonella* is the cornerstone of study of epidemiology of *Salmonella.* Since the conventional serotyping is time - consuming and lack specificity. The multiplex PCR will greatly enhance the quality and quantity of serotyping.

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APPENDIX

Appendix A

Information of tested *Salmonella* strains

No	Code	Sex	Date	City	Source	Sample
1	001/09 Sal	-	2003	Kota Bharu	Human	Stool
2	002/09 Sal	Male	2002	Kota Bharu	Human	Stool
3	003/09 Sal	Male	2002	Kota Bharu	Human	Stool
4	004/09 Sal	Male	2004	Kubang Kerian	Human	Stool
5	005/09 Sal	Female	2003	Gurun	Human	Stool
6	007/09 Sal	Female	2004	Kota Bharu	Human	Stool
7	008/09 Sal	Male	2004	Kota Bharu	Human	Stool
8	009/09 Sal	Female	2004	Kota Bharu	Human	Stool
9	010/09 Sal	Male	2005	Bachok	Human	Blood
10	011/09 Sal	Male	2005	Kuala Krai	Human	Blood
11	012/09 Sal	Male	2004	Kota Bharu	Human	Stool
12	013/09 Sal	Male	2004	Kota Bharu	Human	Stool
13	014/09 Sal	Male	2002	Kota Bharu	Human	Stool
14	015/09 Sal	Female	2004	Bachok	Human	Stool
15	016/09 Sal	Male	2002	Kuala Krai	Human	Stool
16	017/09 Sal	Male	2004	Kota Bharu	Human	Stool
17	018/09 Sal	Male	2005	Kota Bharu	Human	Stool
18	019/09 Sal	Female	2003	Kota Bharu	Human	Stool
19	020/09 Sal	Male	2003	Kota Bharu	Human	Stool
20	021/09 Sal	Female	2005	Kota Bharu	Human	Stool
21	022/09 Sal	Female	2005	Kota Bharu	Human	Stool
22	023/09 Sal	Male	2003	Pasir Puteh	Human	Stool
23	024/09 Sal	Female	2003	Kota Bharu	Human	Stool
24	025/09 Sal	Male	2003	Kuala Krai	Human	Stool
25	026/09 Sal	Male	2002	Besut	Human	Stool
26	027/09 Sal	Male	2002	Besut	Human	Stool
27	028/09 Sal	Female	2008	Kuala Krai	Human	Blood
28	029/09 Sal	Male	2002	Kota Bharu	Human	Blood
29	030/09 Sal	Male	2009	Kota Bharu	Human	Stool
30	031/09 Sal	Male	2009	Kota Bharu	Human	Stool
31	032/09 Sal	Female	2009	Kota Bharu	Human	Stool
32	033/09 Sal	Male	2009	Bachok	Human	Stool
33	034/09 Sal	Male	2009	Paris Mas	Human	Stool
34	035/09 Sal	Male	2009	Rantau Panjang	Human	Body fluid
35	036/09 Sal	Male	2009	Rantau Panjang	Human	Blood
36	037/09 Sal	Male	2009	Pengkalan Chepa	Human	Stool
37	038/09 Sal	Female	2009	Muar	Human	Stool

38	039/09 Sal	Male	2005	Bachok	Human	Blood
39	040/09 Sal	Male	2009	Kota Bharu	Human	Stool
40	041/09 Sal	Male	2009	Kota Bharu	Human	Blood
41	042/09 Sal	Female	2009	Kota Bharu	Human	Blood
42	043/09 Sal	Male	2009	Kota Bharu	Human	Stool
43	044/09 Sal	Male	2005	Bachok	Human	Blood
44	045/09 Sal	Male	2007	Kuala Terengganu	Human	Blood
45	046/09 Sal	Male	2006	Bachok	Human	Stool
46	047/09 Sal	Female	2006	Kota Bharu	Human	Stool
47	048/09 Sal	Male	2007	Kota Bharu	Human	Blood
48	049/09 Sal	Female	2002	Kubang Kerian	Human	Stool
49	050/09 Sal	Male	2003	Bachok	Human	Stool
50	051/09 Sal	Male	2002	Peringat	Human	Stool
51	052/09 Sal	Male	2002	Kota Bharu	Human	Stool
52	053/09 Sal	Male	2006	Kota Bharu	Human	Stool
53	054/09 Sal	Female	2003	Kubang Kerian	Human	Stool
54	055/09 Sal	Male	2002	Kota Bharu	Human	Stool
55	056/09 Sal	Male	2006	Tumpat	Human	Blood
56	057/09 Sal	Male	2002	Kota Bharu	Human	Stool
57	058/09 Sal	Male	2002	Kota Bharu	Human	Blood
58	059/09 Sal	Male	2006	Kota Bharu	Human	Stool
59	060/09 Sal	Male	2002	Kubang Kerian	Human	Stool
60	061/09 Sal	Female	2006	Jertih	Human	Blood
61	062/09 Sal	Male	2006	Kota Bharu	Human	Blood
62	063/09 Sal	Male	2007	Kota Bharu	Human	Blood
63	064/09 Sal	Male	2002	Pasir Puteh	Human	Stool
64	065/09 Sal	Male	2003	Kubang Kerian	Human	Stool
65	066/09 Sal	Male	2007	Kuala Terengganu	Human	Blood
66	067/09 Sal	Male	2006	Kota Bharu	Human	Stool
67	068/09 Sal	Male	2002	Besut	Human	Stool
68	069/09 Sal	Female	2002	Kota Bharu	Human	Stool
69	070/09 Sal	Male	2007	Kota Bharu	Human	Stool
70	071/09 Sal	Female	2003	Gurun	Human	Stool
71	072/09 Sal	Male	2007	Kota Bharu	Human	Stool
72	073/09 Sal	Female	2006	Jertih	Human	Blood
73	074/09 Sal	Female	2002	Puchong	Human	Stool
74	075/09 Sal	Male	2003	Bachok	Human	Stool
75	076/09 Sal	Male	2006	Tumpat	Human	Stool
76	317-0325	-	2006	Selayang	Human	Stool
77	328.0193	-	2006	Selayang	Human	Stool
78	2801 286	-	2006	Selayang	Human	Stool
79	276.46	-	2006	Selayang	Human	Stool
80	253.0374	-	2006	Selayang	Human	Stool
81	292-0820	-	2006	Selayang	Human	Stool
82	280-0374	-	2006	Selayang	Human	Stool

83	281-1339	-	2006	Selayang	Human	Stool
84	319-680	-	2006	Selayang	Human	Stool
85	289.235	-	2006	Selayang	Human	Stool
86	08-287-1652	-	2006	Selayang	Human	Stool
87	13896/06	-	2006	Petaling	Food	Fried fish
88	15416/06	-	2005	Port Dickson	Food	Fried egg
89	633/06	-	2006	Port Dickson	Food	Fried noodles
90	11983/06	-	2006	Jasin	Food	Chicken rice
91	15376/05	-	2005	Port Dickson	Food	Rice
92	3277/06	-	2006	MBPJ	Food	Fried rice
93	7752/06	-	2006	Port Dickson	Food	Chicken Rendang
94	R1352/09	-	2009	Pedang Besar	Food	Squid
95	R1244k/09	-	2009	Perlis	Food	Chicken sausage
96	R3802k/08	-	2008		Food	cow meat
97	R908k/09	-	2009	Kubang Pasu	Enviro	sink swab
98	R1167	-	2009		Food	Canned Sardines
99	R128k/09	-	2009	Negeri Paris	Food	Burger
100	R259k/09	-	2009	Kota Star	Food	Pizza
101	R128/09	-	09	Negeri Paris	Food	Burger
102	R1244k/09	-	2009	Perlis	Food	Chicken sausage
103	R913k/09	-	2009	Pedang Besar	Food	Preserved Olives
104	R1167,E	-	2009		Food	Canned Sardines
105	R1458k	-	2009	Bukit Kayu Hitam	Food	Fruit juice
106	R696k/08	-	2008	Langkawi	Food	Fried rice
107	R1438k	-	2009	Bukit Kayu Hitam	Food	Fruit juice
108	R911k/09	-	2009	Pedang Besar	Food	Crispy Fish
109	R2571k/08	-	2008	Sik	Food	Spinach
110	R776k	-	2009	Perlis	Food	Preserved fish
111	R2480k/08	-	2008	Kota Star	Food	Chicken entries
112	R2803k/08	-	2008		Enviro	toilet bowl
113	R1907/09	-	2009	Kangar	Food	Food
114	R444k/09	-	2009	Kubang Pasu	Enviro	Hand Swab
115	R441k/09	-	2009	Kubang Pasu	Enviro	Hand Swab
116	R259k/09	-	2009	Kota Star	Food	Pizza
117	R2480k/08	-	2008	Kota Star	Food	Chicken entries
118	R2792k/07	-	2007	Bukit Kayu Hitam	Food	Soybean soya
119	R2571/08	-	2008	Sik	Food	Spinach
120	R3801k/08	-	2008	Kangar/ Perlis	Food	Cow meat
121	R2798/07	-	2007	Bukit Kayu Hitam	Food	Spinach
122	R1843k/09	-	2009	Kota Star	Food	Food

Appendix B

The results of multiplex PCR for O and Vi antigens

code	*prt*	*tyv*	*Vi*	*rfbJ*	*wzxC1*	*wzxC2*	*wzxE*	P1-P2	Serogroup
001/09 Sal	-	-	-	-	-	+	-	+	C2
002/09 Sal	-	-	-	-	-	-	+	+	E
003/09 Sal	-	-	-	-	-	+	-	+	C2
004/09 Sal	-	-	-	-	-	+	-	+	C2
005/09 Sal	-	-	-	-	-	-	+	+	E
007/09 Sal	-	-	-	+	-	-	-	+	B
008/09 Sal	+	-	-	-	-	-	-	+	A
009/09 Sal	-	-	-	+	-	-	-	+	B
010/09 Sal	-	-	-	-	-	-	+	+	E
011/09 Sal	+	+	+	-	-	-	-	+	D(Vi)
012/09 Sal	-	-	-	-	-	-	+	+	E
013/09 Sal	+	+	-	-	-	-	-	+	D
014/09 Sal	-	-	-	-	-	+	-	+	C2
015/09 Sal	-	-	-	-	-	+	-	+	C2
016/09 Sal	-	-	-	-	-	+	-	+	C2
017/09 Sal	+	+	-	-	-	-	-	+	D
018/09 Sal	-	-	-	+	-	-	-	+	B
019/09 Sal	-	-	-	-	-	+	-	+	C2
020/09 Sal	-	-	-	-	-	-	+	+	E
021/09 Sal	-	-	-	+	-	-	-	+	B
022/09 Sal	-	-	-	-	-	-	+	+	E
023/09 Sal	-	-	-	-	-	+	-	+	C2
024/09 Sal	-	-	-	-	-	-	+	+	E
025/09 Sal	-	-	-	-	-	+	-	+	C2
026/09 Sal	-	-	-	+	-	-	-	+	B
027/09 Sal	-	-	-	-	-	+	-	+	C2
028/09 Sal	+	+	+	-	-	-	-	+	D(vi)
029/09 Sal	-	-	-	-	-	+	-	+	C2
030/09 Sal	-	-	-	-	-	+	-	+	C2
031/09 Sal	-	-	-	+	-	-	-	+	B
032/09 Sal	+	-	-	-	-	-	-	+	A
033/09 Sal	+	+	-	-	-	-	-	+	D
034/09 Sal	-	-	-	+	-	-	-	+	B
035/09 Sal	+	-	-	-	-	+	-	+	C2
036/09 Sal	-	-	-	-	-	+	-	+	C2
037/09 Sal	-	-	-	-	-	-	+	+	E
038/09 Sal	-	-	-	-	-	-	+	+	E
039/09 Sal	+	+	+	-	-	-	-	+	D(vi)

040/09 Sal	+	+	+	-	-	-	-	+	D (vi)
041/09 Sal	+	+	+	-	-	-	-	+	D (vi)
042/09 Sal	+	+	+	-	-	-	-	+	D (vi)
043/09 Sal	+	+	+	-	-	-	-	+	D (vi)
044/09 Sal	+	+	+	-	-	-	-	+	D (vi)
045/09 Sal	+	+	-	-	-	-	-	+	D
046/09 Sal	-	-	-	-	-	-	+	+	E
047/09 Sal	-	-	-	-	-	-	+	+	E
048/09 Sal	+	+	-	-	-	-	-	+	D
049/09 Sal	-	-	-	-	-	+	-	+	C2
050/09 Sal		-	-	+	-	-	-	+	B
051/09 Sal	-	-	-	-	-	+	-	+	C2
052/09 Sal	-	-	-	-	-	-	+	+	E
053/09 Sal	-	-	-	+	-	-	-	+	B
054/09 Sal	-	-	-	+	-	-	-	+	B
055/09 Sal	-	-	-	-	-	-	-	+	-
056/09 Sal	+	+	-	-	-	-	-	+	D
057/09 Sal	-	-	-	-	-	-	+	+	E
058/09 Sal	-	-	-	-	-	+	-	+	C2
059/09 Sal	-	-	-	+	-	-	-	+	B
060/09 Sal	-	-	-	-	-	+	-	+	C2
061/09 Sal	+	+	-	-	-	-	-	+	D
062/09 Sal	+	+	-	-	-	-	-	+	D
063/09 Sal	+	+	-	-	-	-	-	+	D
064/09 Sal	-	-	-	+	-	-	-	+	B
065/09 Sal	-	-	-	-	-	-	+	+	E
066/09 Sal	+	+	-	-	-	-	-	+	D
067/09 Sal	+	+	-	-	-	-	-	+	D
068/09 Sal	-	-	-	-	-	+	-	+	C2
069/09 Sal	-	-	-	-	-	+	-	+	C2
070/09 Sal	+	+	-	-	-	-	-	+	D
071/09 Sal	-	-	-	-	-	-	+	+	E
072/09 Sal	-	-	-	+	-	-	-	+	B
073/09 Sal	+	+	-	-	-	-	-	+	D
074/09 Sal	-	-	-	-	-	+	-	+	C2
075/09 Sal	-	-	-	-	-	+	-	+	C2
076/09 Sal	-	-	-	-	-	+	-	+	C2
317-0325	-	-	-	+	-	-	-	+	B
328.0193	-	-	-	+	-	-	-	+	B
2801 286	+	+	-	-	-	-	-	+	D
276.46	-	-	-	-	-	-	+	+	E
253.0374	+	+	-	-	-	-	-	+	D
292-0820	+	+	-	-	-	-	-	+	D
280-0374	+	+	-	-	-	-	-	+	D
281-1339	-	-	-	-	-	+	-	+	C2

319-680	-	-	-	+	-	-	-	+	B
289.235	+	+	-	-	-	-	-	+	D
08-287-1652	-	-	-	-	-	-	+	+	E
13896/06	-	-	-	-	+	-	-	+	C1
15416/06	-	-	-	-	-	+	-	+	C2
633/06	-	-	-	-	-	+	-	+	C2
11983/06	-	-	-	-	+	-	-	+	C1
15376/05	-	-	-	-	-	+	-	+	C2
3277/06	+	+	-	-	-	-	-	+	D
7752/06	-	-	-	-	-	-	+	+	E
R1352/09	-	-	-	+	-	-	-	+	B
R1244k/09	-	-	-	-	-	+	-	+	C2
R3802k/08	-	-	-	-	-	+	-	+	C2
R908k/09	+	+	-	-	-	-	-	+	D
R1167	-	-	-	+	-	-	-	+	B
R128k/09	-	-	-	+	-	-	-	+	B
R359k/09	-	-	-	+	-	-	-	+	B
R128/09	-	-	-	+	-	-		+	B
R1244k/09	-	-	-	-	-	+	-	+	C2
R913k/09	-	-	-	+	-	-	-	+	B
R1167,E	-	-	-	+	-	-	-	+	B
R1458k	-	-	-	+	-	-	-	--	B
R696k/08	-	-	-	-	-	-	-	+	B
R1438k	-	-	-	+	-	-	-	+	B
R911k/09	-	-	-	+	-	-	-	+	B
R2571k/08	-	-	-	-	-	-	+	+	E
R776k	-	-	-	+	-	-	-	+	B
R2480k/08	-	-	-	-	-	+	-	+	C2
R2803k/08	-	-	-	-	-	+	-	+	C2
R1907/09	-	-	-	-	-	-	+	+	E
R444k/09	-	-	-	-	-	+	-	+	C2
R441k/09	-	-	-	+	-	-	-	+	B
R259k/09	-	-	-	+	-	-	-	+	B
R2480k/08	-	-	-	-	-	+	-	+	C2
R2792k/07	-	-	-	+	-	-	-	+	B
R2571/08	-	-	-	-	-	-	+	+	E
R380k/08	-	-	-	+	-	-	-	+	C2
R2798/07	-	-	-	+	-	-	-	+	B
R1843k/09	-	-	-	+	-	-	-	+	B

Appendix C

The results of multiplex PCR for H antigens (Ha, Hb, Hd, fliC-r, fliC-i, fliC- g.m and fliC-e.h)

				H1			
code	**Ha**	**Hb**	**Hd**	**fliC-r**	**fliC-i**	**fliC-g.m**	**fliC-e.h**
001/09 Sal	-	-	-	-	-	-	+
002/09 Sal	-	-	-	+	-	-	-
003/09 Sal	-	-	-	-	-	-	+
004/09 Sal	-	-	-	-	-	-	-
005/09 Sal	-	-	-	+	-	-	-
007/09 Sal	-	-	+	-	-	-	-
008/09 Sal	-	-	-	-	-	-	-
009/09 Sal	-	-	+	-	-	-	-
010/09 Sal	-	-	-	+	-	-	-
011/09 Sal	-	-	+	-	-	-	-
012/09 Sal	-	-	-	+	-	-	-
013/09 Sal	-	-	-	-	-	+	-
014/09 Sal	-	-	-	-	-	-	+
015/09 Sal	-	-	-	+	-	-	-
016/09 Sal	-	-	-	-	-	-	+
017/09 Sal	-	-	-	-	-	+	-
018/09 Sal	-	+	-	-	-	-	-
019/09 Sal	-	-	-	-	-	-	-
020/09 Sal	-	-	-	+	-	-	-
021/09 Sal	-	-	-	-	+	-	-
022/09 Sal	-	-	-	+	-	-	-
023/09 Sal	-	-	-	-	-	-	-
024/09 Sal	-	-	-	+	-	-	-
025/09 Sal	-	-	-	+	-	-	-
026/09 Sal	-	-	+	-	-	-	-
027/09 Sal	-	-	-	+	-	-	-
028/09 Sal	-	-	+	-	-	-	-
029/09 Sal	-	-	-	-	-	-	+
030/09 Sal	-	-	-	-	-	-	-
031/09 Sal	-	-	-	-	-	-	-
032/09 Sal	-	+	-	-	-	-	-
033/09 Sal	+	-	-	-	-	+	-
034/09 Sal	-	-	-	-	-	-	-
035/09 Sal	-	-	-	-	-	-	-
036/09 Sal	-	-	-	-	-	-	-
037/09 Sal	-	-	-	+	-	-	-

038/09 Sal	-	-	-	+	-	-	-
039/09 Sal	-	-		-	-	-	-
040/09 Sal	-	-	+	-	-	-	-
041/09 Sal	-	-	+	-	-	-	-
042/09 Sal	-	-	+	-	-	-	-
043/09 Sal	-	-	+	-	-	-	-
044/09 Sal	-	-	+	-	-	-	-
045/09 Sal	-	-	+	-	-	+	-
046/09 Sal	-	-	-	+	-	-	-
047/09 Sal	-	-	-	+	-	-	-
048/09 Sal	-	-	-	-	-	+	-
049/09 Sal	-	-	-	-	-	-	+
050/09 Sal	-	-	-	-	-	-	-
051/09 Sal	-	-	-	-	-	-	+
052/09 Sal	-	-	-	+	-	-	-
053/09 Sal	-	-	-	-	+	-	-
054/09 Sal	-	-	-	-	+	-	-
055/09 Sal	-	-	-	-	-	-	-
056/09 Sal	-	-	-	-	-	+	-
057/09 Sal	-	-	-	+	-	-	-
058/09 Sal	-	-	-		-	-	+
059/09 Sal	-	-	-	-	+	-	-
060/09 Sal	-	-	-	-	-	-	+
061/09 Sal	-	-	-	-	-	+	-
062/09 Sal	-	-	-	-	-	+	-
063/09 Sal	-	-	-	-	-	+	-
064/09 Sal	-	-	-	-	+	-	-
065/09 Sal	-	-	-	+	-	-	-
066/09 Sal	-	-	-	-	-	+	-
067/09 Sal	-	-	-	-	-	+	-
068/09 Sal	-	-	-	+	-	-	-
069/09 Sal	-	-	-	-	-	-	+
070/09 Sal	-	-	-	-	-	+	-
071/09 Sal	-	-	-	+	-	-	-
072/09 Sal	-	-	-	-	+	-	-
073/09 Sal	-	-	-	-	-	+	-
074/09 Sal	-	-	-	-	-	-	+
075/09 Sal	-	-	-	+	-	-	-
076/09 Sal	-	-	-	+	-	-	-
317-0325	-	-	+	-	-	-	-
328.0193	-	-	-	-	+	-	-
2801 286	-	-	-	-	-	+	-
276.46	-	-	-	+	-	-	-
253.0374	-	-	-	-	-	+	-
292-0820	-	-	-	-	-	+	-

280-0374	-	-	-	-	-	+	-
281-1339	-	-	-	-	-	-	-
319-680	-	-	+	-	-	-	-
289.235	-	-	-	-	-	-	-
08-287-1652	-	-	-	+	-	-	-
13896/06	-	-	-	-	-	-	-
15416/06	-	-	-	-	-	-	-
633/06	-	-	-	-	-	-	-
11983/06	-	-	-	-	-	-	+
15376/05	-	-	+	-	-	-	-
3277/06	-	-	-	-	-	-	-
7752/06	-	-	-	+	-	-	-
R1352/09	-	-	-	-	-	-	-
R1244k/09	-	-	-	-	-	-	+
R3802k/08	-	-	-	-	-	-	+
R908k/09	-	-	-	-	-	+	-
R1167	-	-	-	-	+	-	-
R128k/09	-	-	-	-	+	-	-
R259k/09	-	-	-	-	+	-	-
R128/09	-	-	-	-	+	-	-
R1244k/09	-	-	-	-	-	-	+
R913k/09	-	-	-	-	+	-	-
R1167,E	-	-	-	-	+	-	-
R1458k	-	--	-	-	+	-	-
R696k/08	-	-	-	-	-	-	-
R1438k	-	--	-	-	+	-	-
R911k/09	-	-	-	-	+	-	-
R2571k/08	-	-	-	+	-	-	-
R776k	-	-	+	-	-	-	-
R2480k/08	-	-	-	-	-	-	-
R2803k/08	-	-	-	-	-	-	+
R1907/09	-	-	-	+	-	-	-
R444k/09	-	-	-	-	-	-	-
R441k/09	-	-	-	-	+	-	-
R259k/09	-	-	-	-	+	-	-
R2480k/08	-	-	-	-	-	-	-
R2792k/07	-	-	-	-	-	-	+
R2571/08	-	-	-	+	-	-	-
R3801k/08	-	-	-	-	-	-	+
R2798/07	-	-	-	-	+	-	-
R1843k/09	-	-	+	-	-	-	-

Appendix D

Continue to the results of multiplex PCR for H antigens (fliC-z10, fliC-z6, fliC-i, z13, fljB-1.5, fljB-1.2, fljB-1.6, and fljB-enx)

	H1				H2			
code	**fliC-z_{10}**	**fliC-z_6**	**fliC-I,z_{13}**	**fljB-1.5**	**fljB-1.2**	**fljB-1.6**	**fljB-enx**	**Serovars**
001/09 Sal	+	-	-	-	-	-	+	Hadar
002/09 Sal	-	+	-	-	-	-	-	Weltevreden
003/09 Sal	+	-	-	-	-	-	+	Hadar
004/09 Sal	-	-	-	-	-	-	-	-
005/09 Sal	-	+	-	-	-	-	-	Weltevreden
007/09 Sal	-	-	-	-	+	-	-	Stanley
008/09 Sal	-	-	-	-	-	-	-	-
009/09 Sal	-	-	-	-	+	-	-	Stanley
010/09 Sal	-	+	-	-	-	-	-	Weltevreden
011/09 Sal	-	-	-	-	+	-	-	Typhi
012/09 Sal	-	+	-	-	-	-	-	Weltevreden
013/09 Sal	-	-	-	-	-	-	-	Enteritidis
014/09 Sal	+	-	-	-	-	-	+	Hadar
015/09 Sal	-	-	-	+	-	-	-	Bovismorbificans
016/09 Sal	+	-	-	-	-	-	+	Hadar
017/09 Sal	-	-	-	-	-	-	-	Enteritidis
018/09 Sal	-	-	-	-	+	-	-	ParatyphiB
019/09 Sal	-	-	-	-	-	-	-	-
020/09 Sal	-	+	-	-	-	-	-	Weltevreden
021/09 Sal	-	-	-	-	+	-	-	Typhimurim
022/09 Sal	-	+	-	-	-	-	-	Weltevreden
023/09 Sal	-	-	-	-	-	-	-	-
024/09 Sal	-	+	-	-	-	-	-	Weltevreden
025/09 Sal	-	+	-	-	-	-	-	-
026/09 Sal	-	-	-	-	+	-	-	Stanley
027/09 Sal	-	+	-	-	-	-	-	-
028/09 Sal	-	-	-	-	+	-	-	Typhi
029/09 Sal	+	-	-	-	-	-	+	Hadar
030/09 Sal	-	-	-	-	-	-	-	-
031/09 Sal	-	-	-	-	+	-	-	ParatyphiB
032/09 Sal	-	-	-	+	-	-	-	ParatyphiA
033/09 Sal	-	-	-	-	-	-	-	Enteritidis
034/09 Sal	-	-	-	-	+	-	-	Stanley
035/09 Sal	-	-	-	-	-	-	-	-
036/09 Sal	-	-	-	-	-	-	-	-
037/09 Sal	-	+	-	-	-	-	-	Weltevreden

038/09 Sal	-	+	-	-	-	-	-	Weltevreden
039/09 Sal	-	-	-	-	+	-	-	Typhi
040/09 Sal	-	-	-	-	+	-	-	Typhi
041/09 Sal	-	-	-	-	+	-	-	Typhi
042/09 Sal	-	-	-	-	+	-	-	Typhi
043/09 Sal	-	-	-	-	+	-	-	Typhi
044/09 Sal	-	-	-	-	+	-	-	Typhi
045/09 Sal	-	-	-	-	-	-	-	Enteritidis
046/09 Sal	-	+	-	-	-	-	-	Weltevreden
047/09 Sal	-	+	-	-	-	-	-	Weltevreden
048/09 Sal	-	-	-	-	-	-	-	Enteritidis
049/09 Sal	+	-	-	-	-	-	+	Hadar
050/09 Sal	-	-	-	-	+	-	-	Stanley
051/09 Sal	+	-	-	-	-	-	+	Hadar
052/09 Sal	-	+	-	-	-	-	-	Weltevreden
053/09 Sal	-	-	-	-	+	-	-	Typhimurim
054/09 Sal	-	-	-	-	+	-	-	Typhimurim
055/09 Sal	-	-	-	-	-	-	-	-
056/09 Sal	-	-	-	-	-	-	-	Enteritidis
057/09 Sal	-	+	-	-	-	-	-	Weltevreden
058/09 Sal	+	-	-	-	-	-	+	Hadar
059/09 Sal	-	-	-	-	+	-	-	Typhimurim
060/09 Sal	+	-	-	-	-	-	+	Hadar
061/09 Sal	-	-	-	-	-	-	-	Enteritidis
062/09 Sal	-	-	-	-	-	-	-	Enteritidis
063/09 Sal	-	-	-	-	-	-	-	Enteritidis
064/09 Sal	-	-	-	-	+	-	-	Typhimurim
065/09 Sal	-	+	-	-	-	-	-	Weltevreden
066/09 Sal	-	-	-	-	-	-	-	Enteritidis
067/09 Sal	-	-	-	-	-	-	-	Enteritidis
068/09 Sal	-	+	-	-	-	-	-	-
069/09 Sal	+	-	-	-	-	-	+	Hadar
070/09 Sal	-	-	-	-	-	-	-	Enteritidis
071/09 Sal	-	+	-	-	-	-	-	Weltevreden
072/09 Sal	-	-	-	-	+	-	-	Typhimurim
073/09 Sal	-	-	-	-	-	-	-	Enteritidis
074/09 Sal	+	-	-	-	-	-	+	Hadar
075/09 Sal	-	+	-	-	-	-	-	-
076/09 Sal	-	+	-	-	-	-	-	-
317-0325	-	-	-	-	-	-	-	-
328.0193	-	-	-	-	+	-	-	Typhimurim
2801 286	-	-	-	-	-	-	-	Enteritidis
276.46	-	+	-	-	-	-	-	Weltevreden
253.0374	-	-	-	-	-	-	-	Enteritidis
292-0820	-	-	-	-	-	-	-	Enteritidis

280-0374	-	-	-	-	-	-	-	Enteritidis
281-1339	-	-	-	-	-	-	-	-
319-680	-	-	-	-	-	-	-	-
289.235	-	-	-	-	-	-	-	-
08-287-1652	-	+	-	-	-	-	-	Weltevreden
13896/06	-	-	-	-	-	-	-	-
15416/06	-	-	-	-	-	-	-	-
633/06	-	-	-	-	-	-	-	-
11983/06	-	-	-	-	-	-	-	Braenderup
15376/05	-	-	-	-	-	-	-	Muenchen
3277/06	-	-	-	-	-	-	-	-
7752/06	-	+	-	-	-	-	-	Weltevreden
R1352/09	-	-	-	-	-	-	-	-
R1244k/09	-	-	-	-	-	+	-	
R3802k/08	+	-	-	-	-	-	+	Hadar
R908k/09	-	-	-	-	-	-	-	Enteritidis
R1167	-	-	-	-	+	-	-	Typhimurim
R128k/09	-	-	-	-	+	-	-	Typhimurim
R259k/09	-	-	-	-	+	-	-	Typhimurim
R128/09	-	-	-	-	+	-	-	Typhimurim
R1244k/09	-	-	-	-	-	+	-	
R913k/09	-	-	-	-	+	-	-	Typhimurim
R1167,E	-	-	-	-	+	-	-	Typhimurim
R1458k	-	-	-	-	+	-	-	Typhimurim
R696k/08	-	-	-	-	-	-	-	-
R1438k	-	-	-	-	+	-	-	Typhimurim
R911k/09	-	-	-	-	+	-	-	Weltevreden
R2571k/08	-	+	-	-	-	-	-	Weltevreden
R776k	-	-	-	-	-	-	-	Duisburg
R2480k/08	-	-	-	-	-	-	-	-
R2803k/08	+	-	-	-	-	-	+	Hadar
R1907/09	-	+	-	-	-	-	-	Weltevreden
R444k/09	-	-	-	-	-	-	-	-
R441k/09	-	-	-	-	+	-	-	Typhimurim
R259k/09	-	-	-	-	+	-	-	Typhimurim
R2480k/08	-	-	-	-	-	-	-	-
R2792k/07	-	-	-	-	-	-	-	Sandiego
R2571/08	-	+	-	-	-	-	-	Weltevreden
R3801k/08	+	-	-	-	-	-	+	Hadar
R2798/07	-	-	-	-	+	-	-	Typhimurim
R1843k/09	-	-	-	-	-	-	+	Sarajane

Appendix E

PCR Product of Primers (*fli*C e.h) - DNA sequencing blast result

```
>    gb|AY353327.1|  Salmonella enterica strain CDC str.1466 phase 2 flagellin
(fljB)
gene, complete cds
Length=1506

 Score =  496 bits (268),  Expect = 4e-137
 Identities = 268/268 (100%), Gaps = 0/268 (0%)
 Strand=Plus/Plus

Query  15    ATTGAAGGCGGTTATGCGCTTAAAGCTGGCGATAAGTATTACGCTGCAGATTACGATGAA  74
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  973   ATTGAAGGCGGTTATGCGCTTAAAGCTGGCGATAAGTATTACGCTGCAGATTACGATGAA  1032

Query  75    GCGACAGGAGCAATTAAAGCTAAAACCACAAGTTATACTGCTGCTGACGGCACTACCAAA  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1033  GCGACAGGAGCAATTAAAGCTAAAACCACAAGTTATACTGCTGCTGACGGCACTACCAAA  1092

Query  135   ACAGCGGCTAACCAACTGGGTGGTGTAGACGGTAAAACCGAAGTCGTTACTATCGACGGT  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1093  ACAGCGGCTAACCAACTGGGTGGTGTAGACGGTAAAACCGAAGTCGTTACTATCGACGGT  1152

Query  195   AAAACCTACAATGCCAGCAAAGCCGCTGGTCATGATTTCAAAGCACAACCAGAGCTGGCT  254
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1153  AAAACCTACAATGCCAGCAAAGCCGCTGGTCATGATTTCAAAGCACAACCAGAGCTGGCT  1212

Query  255   GAAGCAGCCGCTAAAACCACCGAAAACC  282
             ||||||||||||||||||||||||||||
Sbjct  1213  GAAGCAGCCGCTAAAACCACCGAAAACC  1240


>    gb|AY353319.1|  Salmonella enterica strain CDC str.93-0004 phase 2 flagellin
(fljB) gene, complete cds
Length=1506

 Score =  496 bits (268),  Expect = 4e-137
 Identities = 268/268 (100%), Gaps = 0/268 (0%)
 Strand=Plus/Plus

Query  15    ATTGAAGGCGGTTATGCGCTTAAAGCTGGCGATAAGTATTACGCTGCAGATTACGATGAA  74
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  973   ATTGAAGGCGGTTATGCGCTTAAAGCTGGCGATAAGTATTACGCTGCAGATTACGATGAA  1032

Query  75    GCGACAGGAGCAATTAAAGCTAAAACCACAAGTTATACTGCTGCTGACGGCACTACCAAA  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1033  GCGACAGGAGCAATTAAAGCTAAAACCACAAGTTATACTGCTGCTGACGGCACTACCAAA  1092

Query  135   ACAGCGGCTAACCAACTGGGTGGTGTAGACGGTAAAACCGAAGTCGTTACTATCGACGGT  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1093  ACAGCGGCTAACCAACTGGGTGGTGTAGACGGTAAAACCGAAGTCGTTACTATCGACGGT  1152

Query  195   AAAACCTACAATGCCAGCAAAGCCGCTGGTCATGATTTCAAAGCACAACCAGAGCTGGCT  254
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1153  AAAACCTACAATGCCAGCAAAGCCGCTGGTCATGATTTCAAAGCACAACCAGAGCTGGCT  1212

Query  255   GAAGCAGCCGCTAAAACCACCGAAAACC  282
             ||||||||||||||||||||||||||||
Sbjct  1213  GAAGCAGCCGCTAAAACCACCGAAAACC  1240
```

```
Sbjct  1213  GAAGCAGCCGCTAAAACCACCGAAAACC  1240
```

> gb|AF425736.1|AF425736 Salmonella enterica subsp. enterica serovar Hadar phase-2 flagellin (fljB) gene, partial cds
Length=1351

```
 Score =  496 bits (268),  Expect = 4e-137
 Identities = 268/268 (100%), Gaps = 0/268 (0%)
 Strand=Plus/Plus

Query  15    ATTGAAGGCGGTTATGCGCTTAAAGCTGGCGATAAGTATTACGCTGCAGATTACGATGAA  74
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  928   ATTGAAGGCGGTTATGCGCTTAAAGCTGGCGATAAGTATTACGCTGCAGATTACGATGAA  987

Query  75    GCGACAGGAGCAATTAAAGCTAAAACCACAAGTTATACTGCTGCTGACGGCACTACCAAA  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  988   GCGACAGGAGCAATTAAAGCTAAAACCACAAGTTATACTGCTGCTGACGGCACTACCAAA  1047

Query  135   ACAGCGGCTAACCAACTGGGTGGTGTAGACGGTAAAACCGAAGTCGTTACTATCGACGGT  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1048  ACAGCGGCTAACCAACTGGGTGGTGTAGACGGTAAAACCGAAGTCGTTACTATCGACGGT  1107

Query  195   AAAACCTACAATGCCAGCAAAGCCGCTGGTCATGATTTCAAAGCACAACCAGAGCTGGCT  254
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1108  AAAACCTACAATGCCAGCAAAGCCGCTGGTCATGATTTCAAAGCACAACCAGAGCTGGCT  1167

Query  255   GAAGCAGCCGCTAAAACCACCGAAAACC  282
             ||||||||||||||||||||||||||||
Sbjct  1168  GAAGCAGCCGCTAAAACCACCGAAAACC 1195
```

Appendix F

PCR Product of Primers (*flj* B *enx*) - DNA sequencing blast result

>gb|AY353327.1| Salmonella enterica strain CDC str.1466 phase 2 flagellin (fljB)
gene, complete cds
Length=1506

```
 Score =  134 bits (72),  Expect = 9e-29
 Identities = 72/72 (100%), Gaps = 0/72 (0%)
 Strand=Plus/Plus

Query  21   TGAAGTTACCGTTGATAGTGCTACAGGGGCTGTATCATTCGGTGCTACCCCAACTAAATC  80
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  750  TGAAGTTACCGTTGATAGTGCTACAGGGGCTGTATCATTCGGTGCTACCCCAACTAAATC  809

Query  81   TACTGTTACAGG  92
            ||||||||||||
Sbjct  810  TACTGTTACAGG  821
```

>gb|AY353326.1| Salmonella enterica strain CDC str.1698 phase 2 flagellin (fljB)
gene, complete cds
Length=1506

```
 Score =  134 bits (72),  Expect = 9e-29
 Identities = 72/72 (100%), Gaps = 0/72 (0%)
 Strand=Plus/Plus

Query  21   TGAAGTTACCGTTGATAGTGCTACAGGGGCTGTATCATTCGGTGCTACCCCAACTAAATC  80
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  750  TGAAGTTACCGTTGATAGTGCTACAGGGGCTGTATCATTCGGTGCTACCCCAACTAAATC  809

Query  81   TACTGTTACAGG  92
            ||||||||||||
Sbjct  810  TACTGTTACAGG  821
```

gb|AF425736.1|AF425736 Salmonella enterica subsp. enterica serovar Hadar phase-2 flagellin
(fljB) gene, partial cds
Length=1351

```
 Score =  134 bits (72),  Expect = 9e-29
 Identities = 72/72 (100%), Gaps = 0/72 (0%)
 Strand=Plus/Plus

Query  21   TGAAGTTACCGTTGATAGTGCTACAGGGGCTGTATCATTCGGTGCTACCCCAACTAAATC  80
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  705  TGAAGTTACCGTTGATAGTGCTACAGGGGCTGTATCATTCGGTGCTACCCCAACTAAATC  764

Query  81   TACTGTTACAGG  92
            ||||||||||||
Sbjct  765  TACTGTTACAGG  776
```

Appendix G

PCR Product of Primers (*fli*C z6) - DNA sequencing blast result

gb|AY353353.1| Salmonella enterica strain CDC str.147 phase 2 flagellin (fljB)
gene, complete cds
Length=1503

```
 Score =  355 bits (192),  Expect = 5e-95
 Identities = 198/201 (98%), Gaps = 2/201 (0%)
 Strand=Plus/Minus

Query  11   ATTTACTTTATAGAAGCCATTTTTACCGTCATCAGGGGTGGTCAGACCGGTAACTTCAAC  70
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753  ATTTACTTTATAGAAGCCATTTTTACCGTCATCAGGGGTGGTCAGACCGGTAACTTCAAC  694

Query  71   ATAGTAGTTTTTGCTAGCACTATCATAATAAACTTTGCCATCTTTTATTGCTGCAGTACC  130
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  693  ATAGTAGTTTTTGCTAGCACTATCATAATAAACTTTGCCATCTTTTATTGCTGCAGTACC  634

Query  131  TGTTGTAGCACCAGTGCCTGCTTTAAGGGCTGCGTCTTTATTAAGATCAGTAATATCTAA  190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  633  TGTTGTAGCACCAGTGCCTGCTTTAAGGGCTGCGTCTTTATTAAGATCAGTAATATCTAA  574

Query  191  ATTGGCAGC-ANNTGTCACAG  210
            |||||||| |  ||||||||
Sbjct  573  ATTGGCAGCTAC-TGTCACAG  554
```

> gb|AY353352.1| Salmonella enterica strain CDC str.01-263 phase 2 flagellin (fljB)
gene, complete cds
Length=1503

```
 Score =  355 bits (192),  Expect = 5e-95
 Identities = 198/201 (98%), Gaps = 2/201 (0%)
 Strand=Plus/Minus

Query  11   ATTTACTTTATAGAAGCCATTTTTACCGTCATCAGGGGTGGTCAGACCGGTAACTTCAAC  70
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753  ATTTACTTTATAGAAGCCATTTTTACCGTCATCAGGGGTGGTCAGACCGGTAACTTCAAC  694

Query  71   ATAGTAGTTTTTGCTAGCACTATCATAATAAACTTTGCCATCTTTTATTGCTGCAGTACC  130
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  693  ATAGTAGTTTTTGCTAGCACTATCATAATAAACTTTGCCATCTTTTATTGCTGCAGTACC  634

Query  131  TGTTGTAGCACCAGTGCCTGCTTTAAGGGCTGCGTCTTTATTAAGATCAGTAATATCTAA  190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  633  TGTTGTAGCACCAGTGCCTGCTTTAAGGGCTGCGTCTTTATTAAGATCAGTAATATCTAA  574

Query  191  ATTGGCAGC-ANNTGTCACAG  210
            |||||||| |  ||||||||
Sbjct  573  ATTGGCAGCTAC-TGTCACAG  554
```

> gb|AY353519.1| Salmonella enterica strain CDC str.994 phase 1 flagellin (fliC)
gene, complete cds
Length=1503

 Score = 311 bits (168), Expect = 1e-81
 Identities = 182/189 (96%), Gaps = 0/189 (0%)
 Strand=Plus/Minus

```
Query  11   ATTTACTTTATAGAAGCCATTTTTACCGTCATCAGGGGTGGTCAGACCGGTAACTTCAAC  70
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  753  ATTTACTTTATAGAAGCCATTTTTACCGTCATCAGGGGTAGTCAGACCGGTAACTTCAAC  694

Query  71   ATAGTAGTTTTTGCTAGCACTATCATAATAAACTTTGCCATCTTTTATTGCTGCAGTACC  130
             |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  693  GTAGTAGTTTTTGCTAGCACTATCATAATAAACTTTGTCATCTTTTATTGCTGCAGTACC  634

Query  131  TGTTGTAGCACCAGTGCCTGCTTTAAGGGCTGCGTCTTTATTAAGATCAGTAATATCTAA  190
            |||||||||||||| |||||||||||||| || ||||||||||||||||||||||||||
Sbjct  633  TGTTGTAGCACCAGTCCCTGCTTTAAGGGCCGCATCTTTATTAAGATCAGTAATATCTAA  574

Query  191  ATTGGCAGC  199
            ||| |||||
Sbjct  573  ATTAGCAGC  565
```

Appendix H

PCR Product of Primers (*fli*C r) - DNA sequencing blast result

> gb|AY353463.1| Salmonella enterica strain CDC str.2201 phase 1 flagellin (fliC)
gene, complete cds
Length=1476

```
 Score =  243 bits (131),  Expect = 3e-61
 Identities = 139/143 (97%), Gaps = 2/143 (1%)
 Strand=Plus/Minus

Query  14   TCGGCTGC-ACTGTTACTTCATAAACACCATCTTTAGCCGTAGTACCTGAAACATCAGCG  72
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  749  TCGGCTGCAACTGTTACTTCATAAACACCATCTTTAGCCGTAGTACCTGAAACATCAGCG  690

Query  73   TAATATTTTCNATTTAGTATCATCAAACTTCAGATCACCAGTAATAGCAGGAGTACCACC  132
            |||||||||| |  ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  689  TAATATTTTCCA-GTAGTATCATCAAACTTCAGATCACCAGTAATAGCAGGAGTACCACC  631

Query  133  TAAGGTAGTTGCTGATGCAGCAA  155
            |||||||||||||||||||||||
Sbjct  630  TAAGGTAGTTGCTGATGCAGCAA  608
```

> gb|AY353459.1| Salmonella enterica strain CDC str.16 phase 1 flagellin (fliC)
gene, complete cds
Length=1482

```
 Score =  243 bits (131),  Expect = 3e-61
 Identities = 139/143 (97%), Gaps = 2/143 (1%)
 Strand=Plus/Minus

Query  14   TCGGCTGC-ACTGTTACTTCATAAACACCATCTTTAGCCGTAGTACCTGAAACATCAGCG  72
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  749  TCGGCTGCAACTGTTACTTCATAAACACCATCTTTAGCCGTAGTACCTGAAACATCAGCG  690

Query  73   TAATATTTTCNATTTAGTATCATCAAACTTCAGATCACCAGTAATAGCAGGAGTACCACC  132
            |||||||||| |  ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  689  TAATATTTTCCA-GTAGTATCATCAAACTTCAGATCACCAGTAATAGCAGGAGTACCACC  631

Query  133  TAAGGTAGTTGCTGATGCAGCAA  155
            |||||||||||||||||||||||
Sbjct  630  TAAGGTAGTTGCTGATGCAGCAA  608
```

> gb|AF420426.1|AF420426 Salmonella enterica subsp. enterica serotype Heidelburg phase-1 flagellin (fliC) gene, partial cds
Length=1364

 Score = 243 bits (131), Expect = 3e-61
 Identities = 139/143 (97%), Gaps = 2/143 (1%)
 Strand=Plus/Minus

```
Query  14   TCGGCTGC-ACTGTTACTTCATAAACACCATCTTTAGCCGTAGTACCTGAAACATCAGCG  72
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  695  TCGGCTGCAACTGTTACTTCATAAACACCATCTTTAGCCGTAGTACCTGAAACATCAGCG  636

Query  73   TAATATTTTCNATTTAGTATCATCAAACTTCAGATCACCAGTAATAGCAGGAGTACCACC  132
            |||||||||| |  ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  635  TAATATTTTCCA-GTAGTATCATCAAACTTCAGATCACCAGTAATAGCAGGAGTACCACC  577

Query  133  TAAGGTAGTTGCTGATGCAGCAA  155
            |||||||||||||||||||||||
Sbjct  576  TAAGGTAGTTGCTGATGCAGCAA  554
```

Appendix I

PCR Product of Primers (*fli*C- i) - DNA sequencing blast result

> gb|CP001363.1| Salmonella enterica subsp. enterica serovar Typhimurium str.
14028S, complete genome
Length=4870265

Features in this part of subject sequence:
flagellin

Score = 460 bits (249), Expect = 1e-126
Identities = 259/264 (98%), Gaps = 3/264 (1%)
Strand=Plus/Plus

```
Query  14       ACCGTTNTTATCAGTATAAGACATCTTAACAACAGATGCTGTGCCGGTAACACCTGCTGC  73
                |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2059600  ACCGTTATTATCAGTATAAGACATCTTAACAACAGATGCTGTGCCGGTAACACCTGCTGC
2059659

Query  74       TGTCAATGCGGCTTTAGCCTCTGTCAAATCAGCATTTGCAACTTGTACATTTTTCACATC  133
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2059660  TGTCAATGCGGCTTTAGCCTCTGTCAAATCAGCATTTGCAACTTGTACATTTTTCACATC
2059719

Query  134      CTCAGTTGCTGTCGCAGGTAGTCCACCTGTAAGCGGGGAAGTCGCACCGCCAGCAAGAGT  193
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2059720  CTCAGTTGCTGTCGCAGGTAGTCCACCTGTAAGCGGGGAAGTCGCACCGCCAGCAAGAGT
2059779

Query  194      CACCTCACCGTTCGTCTTATCAACGGAAACTTCATAATAGCCATCTTTACCAGTTCCCCC  253
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2059780  CACCTCACCGTTCGTCTTATCAACGGAAACTTCATAATAGCCATCTTTACCAGTTCCCCC
2059839

Query  254      CGTAACGGTA-CNNNTTGGCGTAA  276
                |||||||||| |   |||||||||
Sbjct  2059840  CGTAACGGTAACT--TTGGCGTAA  2059861
```

> emb|FN424405.1| Salmonella enterica subsp. enterica serovar Typhimurium str.
D23580 complete genome
Length=4879400

Features in this part of subject sequence:
flagellin

Score = 460 bits (249), Expect = 1e-126
Identities = 259/264 (98%), Gaps = 3/264 (1%)
Strand=Plus/Plus

```
Query  14       ACCGTTNTTATCAGTATAAGACATCTTAACAACAGATGCTGTGCCGGTAACACCTGCTGC  73
                |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2031705  ACCGTTATTATCAGTATAAGACATCTTAACAACAGATGCTGTGCCGGTAACACCTGCTGC
2031764

Query  74       TGTCAATGCGGCTTTAGCCTCTGTCAAATCAGCATTTGCAACTTGTACATTTTTCACATC  133
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2031765  TGTCAATGCGGCTTTAGCCTCTGTCAAATCAGCATTTGCAACTTGTACATTTTTCACATC
2031824
```

```
Query  134      CTCAGTTGCTGTCGCAGGTAGTCCACCTGTAAGCGGGGAAGTCGCACCGCCAGCAAGAGT  193
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2031825  CTCAGTTGCTGTCGCAGGTAGTCCACCTGTAAGCGGGGAAGTCGCACCGCCAGCAAGAGT
2031884

Query  194      CACCTCACCGTTCGTCTTATCAACGGAAACTTCATAATAGCCATCTTTACCAGTTCCCCC  253
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2031885  CACCTCACCGTTCGTCTTATCAACGGAAACTTCATAATAGCCATCTTTACCAGTTCCCCC
2031944

Query  254      CGTAACGGTA-CNNNTTGGCGTAA  276
                |||||||||| |   |||||||||
Sbjct  2031945  CGTAACGGTAACT--TTGGCGTAA  2031966
```

> gb|AE006468.1| Salmonella enterica subsp. enterica serovar Typhimurium str.
LT2, complete genome
Length=4857432

Features in this part of subject sequence:
flagellar biosynthesis; flagellin

Score = 460 bits (249), Expect = 1e-126
Identities = 259/264 (98%), Gaps = 3/264 (1%)
Strand=Plus/Plus

```
Query  14       ACCGTTNTTATCAGTATAAGACATCTTAACAACAGATGCTGTGCCGGTAACACCTGCTGC  73
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2048195  ACCGTTATTATCAGTATAAGACATCTTAACAACAGATGCTGTGCCGGTAACACCTGCTGC
2048254

Query  74       TGTCAATGCGGCTTTAGCCTCTGTCAAATCAGCATTTGCAACTTGTACATTTTTCACATC  133
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2048255  TGTCAATGCGGCTTTAGCCTCTGTCAAATCAGCATTTGCAACTTGTACATTTTTCACATC
2048314

Query  134      CTCAGTTGCTGTCGCAGGTAGTCCACCTGTAAGCGGGGAAGTCGCACCGCCAGCAAGAGT  193
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2048315  CTCAGTTGCTGTCGCAGGTAGTCCACCTGTAAGCGGGGAAGTCGCACCGCCAGCAAGAGT
2048374

Query  194      CACCTCACCGTTCGTCTTATCAACGGAAACTTCATAATAGCCATCTTTACCAGTTCCCCC  253
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2048375  CACCTCACCGTTCGTCTTATCAACGGAAACTTCATAATAGCCATCTTTACCAGTTCCCCC
2048434

Query  254      CGTAACGGTA-CNNNTTGGCGTAA  276
                |||||||||| |   |||||||||
Sbjct  2048435  CGTAACGGTAACT--TTGGCGTAA  2048456
```

Appendix J

PCR Product of Primers (*fljB-1.5*) - DNA sequencing blast result

```
>  gb|AY353276.1|  Salmonella enterica strain CDC str.1967 phase 2 flagellin
(fljB)
gene, complete cds
Length=1521

 Score =  268 bits (145),  Expect = 5e-69
 Identities = 147/149 (98%), Gaps = 0/149 (0%)
 Strand=Plus/Plus

Query  20   AAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGCCGCCAAAAATGGCGATTAT  79
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  700  AAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGCCGCCAAAAATGGCGATTAT  759

Query  80   GAAGTTAACGTTGCTACTGACGGTAAAGTTACACTTGCTACGGGTGCAACTAAAACCACA  139
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  760  GAAGTTAACGTTGCTACTGACGGTAAAGTTACACTTGCTACGGGTGCAACTAAAACCACA  819

Query  140  ATGCCTGCTGGTGCGGNNACTAAAACAGA  168
            ||||||||||||||||  ||||||||||
Sbjct  820  ATGCCTGCTGGTGCGGCAACTAAAACAGA  848


>  emb|FM200053.1|  Salmonella enterica subsp. enterica serovar Paratyphi A
str.
AKU_12601 complete genome, strain AKU_12601
Length=4581797

 Features in this part of subject sequence:
   Flagellar synthesis: phase 2 flagellin (filament structur...

 Score =  267 bits (144),  Expect = 2e-68
 Identities = 148/151 (98%), Gaps = 0/151 (0%)
 Strand=Plus/Minus

Query  18       ATAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGCCGCCAAAAATGGCGATT  77
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2725412  ATAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGCCGCCAAAAATGGCGATT
2725353

Query  78       ATGAAGTTAACGTTGCTACTGACGGTAAAGTTACACTTGCTACGGGTGCAACTAAAACCA  137
                |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  2725352  ATGAAGTTAACGTTGCTACTGACGGTAAAGTTACACTTGCTACGAGTGCAACTAAAACCA
2725293

Query  138      CAATGCCTGCTGGTGCGGNNACTAAAACAGA  168
                ||||||||||||||||  ||||||||||
Sbjct  2725292  CAATGCCTGCTGGTGCGGCAACTAAAACAGA  2725262
```

> gb|DQ838330.1| Salmonella paratyphi strain N1996_04 phase 2 flagellin (fljB) gene, partial cds
Length=1393

 Score = 267 bits (144), Expect = 2e-68
 Identities = 148/151 (98%), Gaps = 0/151 (0%)
 Strand=Plus/Plus

```
Query  18   ATAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGCCGCCAAAAATGGCGATT  77
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  637  ATAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGCCGCCAAAAATGGCGATT  696

Query  78   ATGAAGTTAACGTTGCTACTGACGGTAAAGTTACACTTGCTACGGGTGCAACTAAAACCA  137
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  697  ATGAAGTTAACGTTGCTACTGACGGTAAAGTTACACTTGCTACGAGTGCAACTAAAACCA  756

Query  138  CAATGCCTGCTGGTGCGGNNACTAAAACAGA  168
            |||||||||||||||||  ||||||||||||
Sbjct  757  CAATGCCTGCTGGTGCGGCAACTAAAACAGA  787
```

Appendix K

PCR Product of Primers (*flj*B- 1.6) - DNA sequencing blast result

```
>  gb|AY353272.1|  Salmonella enterica strain CDC str.02-296 phase 2 flagellin
(fljB)
gene, complete cds
Length=1521

 Score =  364 bits (197),  Expect = 8e-98
 Identities = 208/214 (97%), Gaps = 2/214 (0%)
 Strand=Plus/Plus

Query  11   ATTNGA-GCAGANAATAACAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGC  69
            ||| || ||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  681  ATTTGACGCAGATAATAACAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGC  740

Query  70   CGCCAAAAATGGCGATTATGAAGTTAACGTTGCTACTGACGGTACAGTAACCCTTGCGGC  129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  741  CGCCAAAAATGGCGATTATGAAGTTAACGTTGCTACTGACGGTACAGTAACCCTTGCGGC  800

Query  130  TGGCGCAACTAAAACCACAATGCCTGCTGGTGCGACAACTAAAACAGAAGTACAGGAGTT  189
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  801  TGGCGCAACTAAAACCACAATGCCTGCTGGTGCGACAACTAAAACAGAAGTACAGGAGTT  860

Query  190  AAAAGATACACCGACAGTTGTTCCCAGCAGATGC  223
            |||||||||||||| |||||||| | |||||||||
Sbjct  861  AAAAGATACACCGGCAGTTGTTTC-AGCAGATGC  893


>  gb|AY353271.1|  Salmonella enterica strain CDC str.00-0462 phase 2 flagellin
(fljB) gene, complete cds
Length=1521

 Score =  364 bits (197),  Expect = 8e-98
 Identities = 208/214 (97%), Gaps = 2/214 (0%)
 Strand=Plus/Plus

Query  11   ATTNGA-GCAGANAATAACAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGC  69
            ||| || ||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  681  ATTTGACGCAGATAATAACAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGC  740

Query  70   CGCCAAAAATGGCGATTATGAAGTTAACGTTGCTACTGACGGTACAGTAACCCTTGCGGC  129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  741  CGCCAAAAATGGCGATTATGAAGTTAACGTTGCTACTGACGGTACAGTAACCCTTGCGGC  800

Query  130  TGGCGCAACTAAAACCACAATGCCTGCTGGTGCGACAACTAAAACAGAAGTACAGGAGTT  189
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  801  TGGCGCAACTAAAACCACAATGCCTGCTGGTGCGACAACTAAAACAGAAGTACAGGAGTT  860

Query  190  AAAAGATACACCGACAGTTGTTCCCAGCAGATGC  223
            |||||||||||||| |||||||| | |||||||||
Sbjct  861  AAAAGATACACCGGCAGTTGTTTC-AGCAGATGC  893


>  gb|AY353269.1|  Salmonella enterica strain CDC str.00-0377 phase 2 flagellin
(fljB) gene, complete cds
Length=1521

 Score =  364 bits (197),  Expect = 8e-98
 Identities = 208/214 (97%), Gaps = 2/214 (0%)
 Strand=Plus/Plus
```

```
Query  11   ATTNGA-GCAGANAATAACAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGC  69
            ||| || ||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  681  ATTTGACGCAGATAATAACAAGTACTTTGTTACTATTGGTGGCTTTACTGGTGCTGATGC  740

Query  70   CGCCAAAAATGGCGATTATGAAGTTAACGTTGCTACTGACGGTACAGTAACCCTTGCGGC  129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  741  CGCCAAAAATGGCGATTATGAAGTTAACGTTGCTACTGACGGTACAGTAACCCTTGCGGC  800

Query  130  TGGCGCAACTAAAACCACAATGCCTGCTGGTGCGACAACTAAAACAGAAGTACAGGAGTT  189
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  801  TGGCGCAACTAAAACCACAATGCCTGCTGGTGCGACAACTAAAACAGAAGTACAGGAGTT  860

Query  190  AAAAGATACACCGACAGTTGTTCCCAGCAGATGC  223
            |||||||||||||| |||||||| | |||||||||
Sbjct  861  AAAAGATACACCGGCAGTTGTTTC-AGCAGATGC  893
```

Appendix L

PCR Product of Primers (*fli*C- g.m) - DNA sequencing blast result

```
>  emb|AM933172.1|  Salmonella enterica subsp. enterica serovar Enteritidis
str.
P125109 complete genome
Length=4685848

 Features in this part of subject sequence:
   threonine operon leader peptide (artificial fragment)
   flagellin

 Score =  193 bits (104),  Expect = 2e-46
 Identities = 104/104 (100%), Gaps = 0/104 (0%)
 Strand=Plus/Plus

Query  21       AACCATTACCATCGATCTGCAAAAAATTGATGTGAAAAGCCTTGGCCTTGATGGGTTCAA  80
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1147061  AACCATTACCATCGATCTGCAAAAAATTGATGTGAAAAGCCTTGGCCTTGATGGGTTCAA
1147120

Query  81       TGTTAATGGGCCAAAAGAAGCGACAGTGGGTGATCTGAAATCCA  124
                ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1147121  TGTTAATGGGCCAAAAGAAGCGACAGTGGGTGATCTGAAATCCA  1147164


>  emb|AM933173.1|  Salmonella enterica subsp. enterica serovar Gallinarum
str. 287/91
complete genome
Length=4658697

 Features in this part of subject sequence:
   flagellin

 Score =  193 bits (104),  Expect = 2e-46
 Identities = 104/104 (100%), Gaps = 0/104 (0%)
 Strand=Plus/Plus

Query  21         AACCATTACCATCGATCTGCAAAAAATTGATGTGAAAAGCCTTGGCCTTGATGGGTTCAA
80
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1183736  AACCATTACCATCGATCTGCAAAAAATTGATGTGAAAAGCCTTGGCCTTGATGGGTTCAA
1183795

Query  81       TGTTAATGGGCCAAAAGAAGCGACAGTGGGTGATCTGAAATCCA  124
                ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1183796  TGTTAATGGGCCAAAAGAAGCGACAGTGGGTGATCTGAAATCCA  1183839
```

> gb|CP001144.1| Salmonella enterica subsp. enterica serovar Dublin str. CT_02021853, complete genome
Length=4842908

Features in this part of subject sequence:
phase-1 flagellin

Score = 193 bits (104), Expect = 2e-46
Identities = 104/104 (100%), Gaps = 0/104 (0%)
Strand=Plus/Plus

```
Query  21       AACCATTACCATCGATCTGCAAAAAATTGATGTGAAAAGCCTTGGCCTTGATGGGTTCAA  80
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270416  AACCATTACCATCGATCTGCAAAAAATTGATGTGAAAAGCCTTGGCCTTGATGGGTTCAA
1270475

Query  81       TGTTAATGGGCCAAAAGAAGCGACAGTGGGTGATCTGAAATCCA  124
                ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270476  TGTTAATGGGCCAAAAGAAGCGACAGTGGGTGATCTGAAATCCA  1270519
```

Appendix M

PCR Product of Primers (*fli*C- z_{10}) - DNA sequencing blast result

> gb|DQ838269.1| Salmonella enterica subsp. enterica serovar Hadar strain S06071_03
phase 1 flagellin (fliC) gene, partial cds
Length=1335

Score = 315 bits (170), Expect = 6e-83
Identities = 173/174 (99%), Gaps = 1/174 (0%)
Strand=Plus/Plus

```
Query  7    ATCAACTCTCAGACCCTGGGTCTGGATACGCTGAATGTGCAGAAAAAATATGATGTTGAT  66
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  ATCAACTCTCAGACCCTGGGTCTGGATACGCTGAATGTGCAGAAAAAATATGATGTTGAT  457

Query  67   AGCACTGGCG-TACTCAATCTCTTGATTTAAAAACTGCTGGAATTACTGGTGCTACATTA  125
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  458  AGCACTGGCGTTACTCAATCTCTTGATTTAAAAACTGCTGGAATTACTGGTGCTACATTA  517

Query  126  AAAGCTGGTATTACTGGTACAACGACAGAAACCGGTAGCGTTAAAGATGGTAAA  179
            |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518  AAAGCTGGTATTACTGGTACAACGACAGAAACCGGTAGCGTTAAAGATGGTAAA  571
```

> gb|DQ838261.1| Salmonella enterica subsp. enterica serovar Hadar strain 85_03
phase 1 flagellin (fliC) gene, partial cds
Length=1335

Score = 315 bits (170), Expect = 6e-83
Identities = 173/174 (99%), Gaps = 1/174 (0%)
Strand=Plus/Plus

```
Query  7    ATCAACTCTCAGACCCTGGGTCTGGATACGCTGAATGTGCAGAAAAAATATGATGTTGAT  66
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  ATCAACTCTCAGACCCTGGGTCTGGATACGCTGAATGTGCAGAAAAAATATGATGTTGAT  457

Query  67   AGCACTGGCG-TACTCAATCTCTTGATTTAAAAACTGCTGGAATTACTGGTGCTACATTA  125
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  458  AGCACTGGCGTTACTCAATCTCTTGATTTAAAAACTGCTGGAATTACTGGTGCTACATTA  517

Query  126  AAAGCTGGTATTACTGGTACAACGACAGAAACCGGTAGCGTTAAAGATGGTAAA  179
            |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518  AAAGCTGGTATTACTGGTACAACGACAGAAACCGGTAGCGTTAAAGATGGTAAA  571
```

> gb|DQ838258.1| Salmonella enterica subsp. enterica serovar Haifa strain ST174
phase 1 flagellin (fliC) gene, partial cds
Length=1335

 Score = 315 bits (170), Expect = 6e-83
 Identities = 173/174 (99%), Gaps = 1/174 (0%)
 Strand=Plus/Plus

```
Query  7    ATCAACTCTCAGACCCTGGGTCTGGATACGCTGAATGTGCAGAAAAAATATGATGTTGAT  66
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  ATCAACTCTCAGACCCTGGGTCTGGATACGCTGAATGTGCAGAAAAAATATGATGTTGAT  457

Query  67   AGCACTGGCG-TACTCAATCTCTTGATTTAAAAACTGCTGGAATTACTGGTGCTACATTA  125
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  458  AGCACTGGCGTTACTCAATCTCTTGATTTAAAAACTGCTGGAATTACTGGTGCTACATTA  517

Query  126  AAAGCTGGTATTACTGGTACAACGACAGAAACCGGTAGCGTTAAAGATGGTAAA  179
            |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518  AAAGCTGGTATTACTGGTACAACGACAGAAACCGGTAGCGTTAAAGATGGTAAA  571
```

Appendix N

PCR Product of Primers (*flj*B-1.2) - DNA sequencing blast result

```
>  gb|CP001363.1|  Salmonella enterica subsp. enterica serovar Typhimurium
str.
14028S, complete genome
Length=4870265

Features in this part of subject sequence:
   flagellin

 Score = 259 bits (140), Expect = 3e-66
 Identities = 148/151 (98%), Gaps = 3/151 (1%)
 Strand=Plus/Plus

Query  7        CGTTCTGCGGGACCTGGTTAGCCTGCGCCAGAACGGAAGTACCGGCCTGCTGCAGAATCT  66
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2933485  CGTTCTGCGGGACCTGGTTAGCCTGCGCCAGAACGGAAGTACCGGCCTGCTGCAGAATCT
2933544

Query  67       GCGCGCGAGACATGTTGGAAACTTCGGTCGCGTAG-CGGAATCTTCGATACG-CTACGCG  124
                ||||||||||||||||||||||||||||||||| ||||||||||||||| |||||||
Sbjct  2933545  GCGCGCGAGACATGTTGGAAACTTCGGTCGCGTAGTCGGAATCTTCGATACGGCTACGCG
2933604

Query  125      CTTCAGACAGATTGTT-ACGGTATTGCCCAG  154
                ||||||||||||||| |||||||||||||
Sbjct  2933605  CTTCAGACAGATTGTTTACGGTATTGCCCAG  2933635


 Features in this part of subject sequence:
   flagellin

 Score =  198 bits (107),  Expect = 6e-48
 Identities = 137/151 (90%), Gaps = 3/151 (1%)
 Strand=Plus/Plus

Query  7        CGTTCTGCGGGACCTGGTTAGCCTGCGCCAGAACGGAAGTACCGGCCTGCTGCAGAATCT  66
                |||| ||||| |||||||| |||||||||||||||| ||||||||||||||||||||||
Sbjct  2059083  CGTTTTGCGGAACCTGGTTCGCCTGCGCCAGAACGGAGGTACCGGCCTGCTGCAGAATCT
2059142

Query  67       GCGCGCGAGACATGTTGGAAACTTCGGTCGCGTAG-CGGAATCTTCGATACG-CTACGCG  124
                ||||||||||||||||||||||||||||||||| ||||||||||||||| ||||| |
Sbjct  2059143  GCGCGCGAGACATGTTGGAAACTTCGGTCGCGTAGTCGGAATCTTCGATACGGCTACGGG
2059202

Query  125      CTTCAGACAGATTGTT-ACGGTATTGCCCAG  154
                |   || ||| ||||| ||||| ||||||||
Sbjct  2059203  CAGAAGTCAGGTTGTTTACGGTGTTGCCCAG  2059233
```

RESEARCH OUTPUT

Mohd Elbagir Elhassan Nori and Kwai lin Thong (2010) Differentiation of *Salmonella enterica* based on PCR detection of selected somatic and flagella antigens. African Journal of Microbiology Research. Accepted (ISI, IP 0.5). *ISSN 1996-0808© 2010 Academic Journals*.