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Full length Research Paper

Differentiation of *Salmonella enterica* based on PCR detection of selected somatic and flagellar antigens

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Serotyping is the basis of *Salmonella* surveillance. However, the limitations of the traditional serotyping have stimulated rapid research and development in DNA-based serotyping. The aim of this study was to apply a combination of sequential multiplex PCRs targeting the O, H and Vi antigens to serotype a panel of 122 recently isolated human- and foodborne- *Salmonella* strains. The mPCR that targets the o (genes *wzxC2, rfbJ, prt, tyv, wzxE, wzxC1, prt*) and Vi (*via*B) antigens successfully subtyped the strains into serogroups C2 (n = 35, 28.7%), B (n = 33, 27.1%), D9 (n = 29, 22.9%), E (n = 21, 17.2%), C1 (n = 2, 64%) and A (n = 2, 1.64%). Eight of the *Salmonella* strains from serogroup D were positive for Vi antigen. Two multiplex PCRs were optimized for detection of H1 antigens (Ha, Hb, Hd, r, z_{10}, z_6, g and m) and H2 antigens (1.5, 1.2, 1.6 and enx). Overall, the multiplex PCRs of O, H and Vi antigens results correctly serotyped 94 of 122 strains (77%). The most frequent serovars encountered were *Salmonella weltevrerden*, *Salmonella enteritidis, Salmonella typhimurium, Salmonella hadar* and *Salmonella typhi*. Application of DNA based technique for serogrouping and serotyping of the selected *Salmonella enterica* was found to be robust, quick, specific and reliable for the specific antigenic targets and is useful in the study area which lack complete serotyping facilities.

Key words: *Salmonella*, somatic antigens (O), flagella antigens (H), serotyping, multiplex PCR.

INTRODUCTION

Serotyping is the fundamental approach in the epidemiological surveillance of *Salmonella* (Fitzgerald et al., 2006). *Salmonella* serotype is based on immuno-reactions of three antigens which are the O (somatic), H (flagellar) and Vi (capsular) antigens in Kauffman White scheme. Over 2,500 *Salmonella* serovars have been identified by using more than 150 somatic (O) and flagella (H) antigens (Popoff, 2001).

The O-antigen of *Salmonella* is a polysaccharide, one of the major components of the bacterial cell surface. O-antigen biosynthesis is encoded by a number of genes arranged in a large regulon known as the *rfb* gene cluster. Serogroups A, B, C2, D and E of *Salmonella* are composed of *rfb* regions which encode for the biosynthesis of rhamnose, galactose and mannose, while serogroup C1 contains four mannose residues (Lee et al., 1992). Paratose synthase (*prt*) and CDP-tyvelose-2-epimerase (*tyv*) genes are encoded in serogroups A and D. However, *tyv* gene of serogroup A is a mutant form which does not produce active CDP-tyvelose epimerase due to a deletion in the fourth codon (Verma and Reeves, 1989). Serogroups B and C2 have the *rfbJ* genes which encode for abequose synthase. However, the *rfbJ* gene of serogroup C2 differs from that of group B in a central region of 12.4 kb (Brown et al., 1992). There are 12 segments of potential transmembrane proteins encoded by the *wzx* gene. This gene is found in all *Salmonella* O-antigen gene clusters. It has been proposed that the proteins produced by *wzx* gene are involved in transfer of O antigen subunits across the cytoplasmic membrane to the periplasmic side (Liu et al., 1996).

The flagella antigens H1 (phase 1) and H2 (phase 2) are encoded by *fliC* and *fljB* genes, respectively. With several monophasic exceptions, these two genes are present at two different locations on the *Salmonella* chromosome, but either one of them is expressed at one time due to phase variation (McQuiston et al., 2004). The virulence capsular polysaccharide (Vi) antigen is expressed in certain *Salmonella* serovars such as *Salmonella typhi,*

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Salmonella paratyphi C *and Salmonella dublin*. The biosynthesis of Vi antigen is controlled by chromosomal loci *viaA* and *viaB* (Vilolgeux et al., 1995).

The traditional method of serotyping *Salmonella* is time consuming, tedious and costly as it requires hundreds of antisera as well as well-trained technicians (Lim and Thong, 2009). Hence, there is an interest to develop DNA based serotyping which is more specific and rapid. Fitzgerald et al. (2007) developed a multiplex, bead-based suspension array for molecular determination of common *Salmonella* serogroups B, C1, C2, D, E and O13 targeting *rfb* gene clusters. Herrera-Leon et al. (2007) developed multiplex PCRs for serogroups B, C1, C2, E1 and D and flagella antigens by targeting the *wzx, tyv, fliC* and *fljB* genes. Levy et al. (2008) developed a multiplex PCR for A, B, D and Vi antigen by using primers for *rfbJ* and *viaB* genes.

Lim and Thong (2009) optimized a combination of primers developed by Herrera-Leon et al. (2007) and Levy et al. (2008) to identify serogroups A, B, C1, E, D and Vi antigen and the flagella antigens of *Salmonella*. However the H targets described by Lim and Thong (2009) were limited. Recently, Cardona-Castro et al. (2009) developed multiplex PCRs for serotyping *Salmonella* serogroups B, C2, D and E targeting *rfb, wzx, fliC* and *fljB* genes, however the authors noted a lack of concordance in the mPCR and traditional serotyping.

The aim of the study was to improve on the previous work done by Lim and Thong (2009) to apply and optimize three multiplex PCRs to serogroup and serotype the common *Salmonella* serovars in Malaysia. Complete *Salmonella* serotyping facility is lacking and is not easily available in Malaysia and it is usually limited to the Salmonella Reference Centre, Institute for Medical Research, Kuala Lumpur. Hence the utility and development of this alternative approach will enhance *Salmonella* surveillance in the country.

MATERIAL AND METHODS

Bacterial strains

A total of 122 *Salmonella* strains were obtained from the culture collection of Laboratory of Biomedical Science and Molecular Microbiology, University Malaya. An additional 14 *Salmonella* strains of known serovars were used as positive controls in evaluation of all multiplex PCR and these include the representatives from group A (*S. paratyphi* A, n = 2), group B (*S. paratyphi* B, n = 2, *Salmonella typhimurium*, n = 2), group D (*Salmonella enteritidis*, n = 1), group D (Vi) (*S. typhi*, n = 2), group E (*Salmonella weltevreden*, n = 2), group C1 (*Salmonella braenderup*, n = 1) and group C2 (*Salmonella hadar*, n = 1, *Salmonella corvallis*, n = 1).

Multiplex PCR for O and Vi antigens

A multiplex PCR (mPCR) for simultaneous differentiation of serogroups A (*prt*), B (*rfbJ*), C1 (*wzxC1*), C2 (*wzxC2*), D (*prt, tyv*), E (*wzxE*), Vi (*via*B) and *ori*C region previously described by Lim and Thong (2009) were adopted. The primers used for this mPCR have been reported by Hirose et al. (2002), Lim et al. (2003), Herrera-Leon et al. (2007) and Levy et al. (2008). DNA amplification was performed in a reaction volume of 25 µl. Each reaction contained 1X PCR buffer, 2.5 mM $MgCl_2$, 250 µM dNTPs, 1.75 U *Taq* DNA (Promega), 0.4 µM (each) of primers (F/R-rfbJ, F/R-prt, F/R-tyv, F/R-wzxC1, F/R-wzxC2, F/R-wzxE, F/R-Vi) and 0.2 µM P1/P2 primers. Five microliters of boiled cell suspension (approximately, 100 ng of crude DNA) was used as a template for each reaction. The PCR conditions consisted of denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 40 s, 50°C for 30 s, 68°C for 30 s and a final extension at 68°C for 7 min (Lim and Thong, 2009).

Multiplex PCRs for H antigens

A multiplex PCR for simultaneous detection of *fliC* gene alleles (a, b and d) using primers and conditions previously described by Lim and Thong (2009) was followed. Due to the limited information provided by these primers, additional primers as described by Cardona-Castro et al. (2009) were incorporated in this study to detect more alleles in the flagella antigens H1 (*fliC* gene) and H2 (*fliB* gene). The PCR conditions were as previously described by Cardona-Castro et al. (2009).

All PCR products were analyzed in a 1.5% agarose gel stained using ethidium bromide (0.5 µg/ml) and then visualized using Gel Doc (Bio-rad, Molecular Imager, Gel Doc™, XR Imaging system). A DNA standard, 100 bp ladder (Promega) was used as the molecular weight marker.

DNA sequencing of selected amplicons

Amplified DNA products from selected targets from monoplex PCRs representing O, H1 and H2 flagella antigens were verified by DNA sequencing. The amplicons were purified using a DNA purification kit (Qiagen) and sent to commercial facility (Research Biolabs, Singapore) for sequencing. The results of DNA sequence data were compared to the available sequences in the GenBank using the BLAST algorithm available on National Center for Biotechnology website (http://www.ncbi.nlm.nih.gov).

Validation of the multiplex PCRs

To validate the optimized multiplex PCRs, the test was conducted on 31 coded *Salmonella* strains of known serogroups (A, B, C1, C2, D and E) and Vi positive strains. *Escherichia coli* and *Vibrio parahaemolyticus* were included as negative controls (Table 1).

RESULTS

Multiplex PCR for O grouping and detection of Vi antigen

All positive control strains produced amplicons of expected bands size for the specific serogroups; group A (*prt* gene, 256 bp), group B (*rfbJ* gene, 662 bp), group C1 (*wzxC1* gene, 483 bp), group C2 (*wzxC2* gene, 154 bp), group D (*prt* gene, 256 bp and *tyv* gene, 615 bp), group E (*wzxE* gene, 345 bp) and the expected band size of Vi antigen (*viaB* gene, 439 bp) (Figure 1). The data corroborated the work previously published by Lim and Thong (2009) indicating that the PCR conditions were robust and reproducible. Application of the multiplex PCR

Table 1. *Salmonella* strains of known serovars used for blind testing and verification of the sequential Multiplex PCRs results compared to conventional serotyping.

Serotypes (conventional)	Source (no)	O-multiplex PCR	H-multiplex PCR	mPCR results
S. paratyphi A	Human (n = 2)	A	a, 1,5	*S. paratyphi* A
S. paratyphi B	Human (n = 2)	B	b, 1,2	*S. paratyphi* B
S. typhimurium	Human (n = 2), Food (n = 10)	B	i, 1,2	*S. typhimurim*
S. enteritidis	Human (n = 4), Env. (n = 1)	D	g,m	*S. enteritidis*
S. weltevreden	Human (n = 2), Food (n = 3)	E	r, z6	*S. weltevreden*
S. hadar	Human (n = 2), Env. (n = 1)	C2	z10, enx	*S. hadar*
S. muenchen	Food (n = 1)	C2	d, 1.2	*S. muenchen*
S. typhi	Human (n = 3)	D	d	*S. typhi*
S. braenderup	Food (n = 2)	C1	e,h	*S. braenderup*
E. coli	Food (n = 1)	-	-	-
V. parahaemolyticus	Food (n = 1)	-	-	-

Env. = environmental samples.

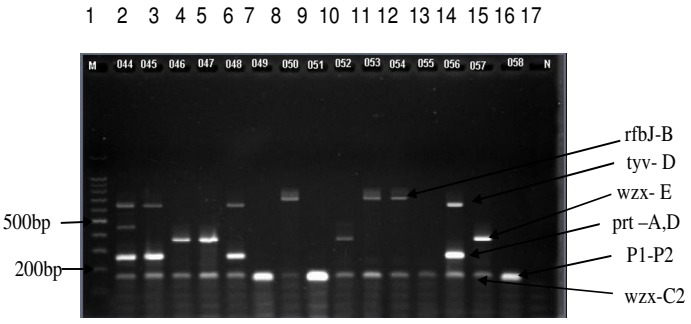

Figure 1. Multiplex PCR for O serogrouping. Lane 1, DNA marker 100 bp; lane 2, *S. typhi* (group D (Vi); lanes 3, 6 and 14, *S. enteritidis* (group D); lanes 4, 5, 10 and 15, *S. weltevreden* (group E); lanes 7,9 and 16, *S. hadar*; lanes 8, *S. stanley*; lanes 11 and 12, *S. typhimurium*; lane 17, negative control (H_2O).

for O serogrouping on 122 *Salmonella* strains produced the common serogroups: C2 (n = 35, 28.7%), B (n = 33, 27.1%), D9 (n = 29, 22.9%), E (n = 21, 17.2%), C1 (n = 2, 64%) and A (n = 2, 1.64%). Eight of the *Salmonella* strains from serogroup D were positive for Vi antigen.

Multiplex PCRs for flagella antigens (H typing)

The H1 multiplex PCR previously reported by Lim and Thong (2009) produced the expected bands of flagella antigens among the positive control strains for *S. paratyphi* A (Ha-423 bp), *S. paratyphi* B (Hb-551 bp) and *S. typhi* (Hd-763 bp) (Figure 2). When this PCR was applied on the 122 *Salmonella* strains, only 18.1% produced the expected bands size of flagella antigens (H1: a, b and d) while 81.9% of them were not amplified. Therefore, due to the limited information provided by H1 mPCR assay, additional primers for more flagella antigens (both H1 and H2) as described by Cardono-Castro et al. (2009) were incorporated. Initial application of the PCR conditions did not yield any specific bands and there were many spurious bands (data not shown). Hence, the PCR conditions were re-optimized. The optimized PCR components comprised of 1.2X PCR buffer, 1.8 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM (each) of flagella antigens primers, 0.14 µM of the positive control primers (P1-P2), 1 U *Taq* DNA polymerase (Promega) and 100 ng DNA. The cycling parameters of this multiplex PCR consisted of denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min.

The optimized PCR conditions produced the expected sized amplicons corresponding to the H1 (*fliC*) antigen with the positive control strains: *S. typhimurium* (*fliC*-I, 185 bp), *S. braenderup* (*fliC*-e,h, 324 bp), *S. enteritidis* (*fliC*-g,m,153 bp), *S. weltevreden* (*fliC*-z6, 240 bp and *fliC*-r,175 bp) and *S. hadar* (*fliC*-z10, 100 bp) (Figure 3). Multiplex PCR for the H2 (*fljB*) flagella antigen was done concurrently with H1- typing. The results of H2 multiplex PCR produced amplicons with expected bands size with positive control strains for *S. paratyphi* A (*fljB*-1.5, 190 bp), *S. paratyphi* B (*fljB*-1.2, 185 bp), *S. typhimurium* (*fljB*-1.2, 185 bp), *Salmonella stanley* (*fljB*-1.2, 185 bp) and *S. hadar* (*fljB*-enx, 120 bp) (Figure 3).

The accuracy of the amplification was confirmed by sequencing the amplicons generated by both HI and H2 mPCRs. DNA sequence analysis of the amplicons corresponding to the different regions of *fli*C and *flj*B genes had sequence identify of 97 - 100% with published sequences in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

Validation of the multiplex PCRs

Thirty one of *Salmonella* strains previously isolated and identified from various sources (clinical, food and

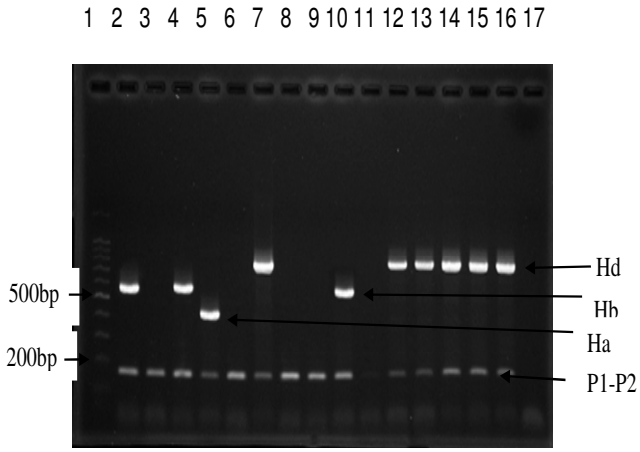

Figure 2. Multiplex PCR for *Salmonella* flagella antigens (H1). Lane 1, DNA marker 100 bp; lanes 2, 4 and 10, *S. paratyphi* B (group B); lane 5, *S. paratyphi* A (group A); lane 7, *S. stanley*; lanes 12 and 16, *S. typhi* (group D(Vi); lane 17, negative control (H_2O).

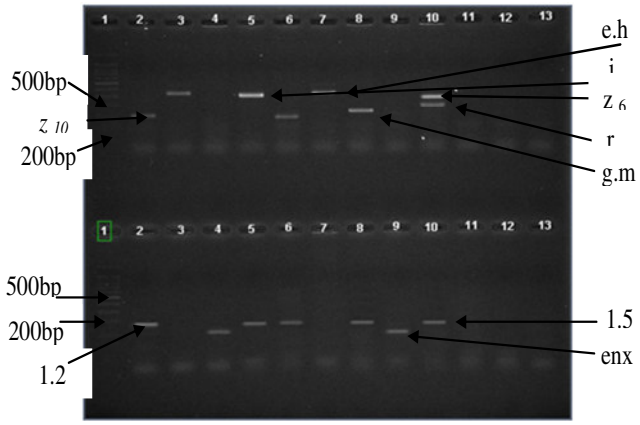

Figure 3. Multiplex PCR for flagella antigens using optimized PCR condition. Upper panel (H1); Lane 1, DNA marker 100 bp; lanes 2 and 6, *S. hadar* (*fli*C- z_{10}); lane 5, *S. typhimurium* (*fli*C- i); lanes 3 and 7, *S. braenderup* (*fli*C- e,h); lane 8, *S. enteritidis* (*fli*C- g,m); lane 10, *S. weltevreden* (*fli*C- r and z_6); lanes 11 – 13, negative control (H_2O). Lower panel (H2); Lane, 1 DNA marker 100 bp; lanes 2 and 5, *S. typhimurium* (*flj*B- 1.2); lanes 4 and 9 *S. hadar* (*flj*B- enx); lanes 6, 8 and 10, *S. paratyphi* A (*flj*B- 1.5); lanes 11 – 13, negative control (H_2O).

environmental samples) were blind-coded and tested with the multiplex PCRs for O and H antigens (Table 1). The multiplex PCR for O-serogrouping produced amplicons corresponding to O antigens targets from the 31 isolates and correctly identified serogroups A, B, C1, C2, D and E. The multiplex PCR for H1 and H2 serotyping produced corresponding amplicons to H1 antigens (Ha, Hb, Hd, r, i, g,m, e,h, z10 and z6) and H2 antigens (1.5, 1.2, 1.6 and enx). Overall, this DNA serotyping correctly identified *S. weltevreden*, *S. enteritidis*, *S. typhimurium*, *S. hadar*, *S. paratyphi* B, *S. typhi* and *S. paratyphi* A. There was a 100% concordance between mPCR and traditional serotyping data.

In summary, the combination of O and H multiplex PCRs results for identification *Salmonella* serovars enabled molecular serotyping of 94/122 (77%) of *Salmonella* serovars. The most frequently encountered serotypes were *S. welterverden* (n = 21), *S. enteritidis* (n = 18), *S. typhimurium* (n = 19), *S. hadar* (n = 14) and *S. typhi* (n = 8). The least frequent were *S. paratyphi* B (n = 2), *S. paratyphi* A (n = 1), *Salmonella duisburg* (n = 1), *Salmonella sandiego* (n = 1) and *Salmonella muenchen* (n = 1) (Table 2). Some of the tested strains were biphasic which have phase 1 (H1) and phase 2 (H2) flagella antigens such as *S. paratyphi* A (Ha and *fljB*-1.5), *S. typhimurium* (*fliC*-i and fljB-1.2) and *S. hadar* (*fliC*-z10) and *fljB*-enx). The rest (23%) of tested strains could not be identified due to the limitation of the PCR targets.

DISCUSSION

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, D and E1 based on different regions of *wzx, tyv, fliC* and *fljB* genes. Even though this approach defined a wide range of *Salmonella* serovars, it could not differentiate serovars that belong to serogroup A and the strains that possess capsular antigens. Levy et al. (2008) developed two multiplex PCRs for serogroups A (*prt*), B (*rfb*) and D (*prt* and *tyv*), as well as Vi antigen (*viaB*) and flagella antigen phase1 (*fliC*). This approach was very useful for identifying *S. typhi* and *S. paratyphi* A which are associated with typhoid and paratyphoid fever, respectively, 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 re-optimized a multiplex PCR to simultaneously differentiate A, B, C1, E, D and Vi positive strains. The present study further applied this mPCR developed by Lim and Thong (2009) on a larger set of unknown *Salmonella* strains. The specificity of the primers for serogroups A, B, C1, D, and E and Vi strains was 100%. In addition, this mPCR could differentiate *S. typhi* and *S. paratyphi* A. However, this approach did not identify serogroup C2, in particular *S. hadar*, which is commonly reported (Herikstad et al., 2002). Moreover, no phase 2 genes were included and detection of phase1 was limited to a few alleles. Recently Cardona-Castro et al. (2009) reported sequential multiplex PCRs for serotyping *Salmonella* serovars (B, C2, D and E) targeting *rfb, wzx, fliC* and *fljB* genes. Therefore, these

Table 2. Overall results of DNA typing *Salmonella* based on mPCR detection O, H and Vi genes.

Sero-group	*Salmonella* serotypes	Antigenic formula	No tested (%)	O Genes	H Genes
A	*paratyphi* A	1,2,12:a:[1,5]	1 (0.8%)	*prt*	*a, 1.5*
	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*
B	*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%)	*wzxC1*	*e,h*
	hadar	6,8:z10:e,n,x	14 (11.4%)	*wzxC2*	*z10, enx*
C2	*bovismorbificans*	6,8:r:1.5	1 (0.8%)	*wzxC2*	*r, 1.5*
	muenchen	6,8:d:1,2	1 (0.8%)	*wzxC2*	*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* *viaB*	*d*
E	*weltevreden*	3,10[15]:r:z6	21 (17.2%)	*wzxE*	*r, z6*

primers were incorporated in the present study to serotype a wider range of *Salmonella* strains in Malaysia. Unspecific PCR amplicons were produced by using PCR conditions described by Cardona-Castro et al. (2009). This could be due to variations in source and quality of reagents, quality of DNA template and different models of thermocylers used. Hence, even though the primers information is publicly available, one needs to optimize the conditions for positive results and the validity of the amplicons be confirmed by DNA sequencing.

Group C2 primers were incorporated in mPCR for somatic (O) antigen typing. The multiplex PCRs described in this study successfully identified the common serogroups A, B, C1, C2, D and E, in addition to Vi positive strains and selected alleles of flagella antigens H1 and H2. There was a complete concordance of the serotypes of the 31 positive control strains determined by conventional and PCR serotyping. Overall, the conesquence of all sequential multiplex PCRs for O and H was that only 77% of total tested *Salmonella* strains could be identified, that is, *S. welterverden*, *S. enteritidis*, *S. typhimurium*, *S. hadar*, *S. paratyphi* B, *S. paratyphi* A and *S. typhi*. These are the more common and important serovars in Malaysia. Multiplex PCRs for H antigens detected two phases of flagella antigens in some *Salmonella* strains such as *S. typhimurium* and *S. hadar* which are known as biphasic (Hong et al., 2008). Furthermore, the assay could detect one phase of flagella antigens such *S. typhi* and *S. enteritidis* which are known as monophasic (Burnens et al., 1996). The rest of tested strains (23%) could not be completely serotyped. This may be due to limited primers for detection of *fliC* and *fljB* genes, or the strains may be converted into monophasic (Burnens et al., 1996; Herrera-Leon et al., 2007).

In conclusion, this approach of sequential multiplex PCRs to serotype *Salmonella* is especially useful for laboratories that lack complete serotyping facilities. It is very quick, specific and reliable for selected *Salmonella* serovars. The present study concurred with previous researchers (Fitzgerald et al., 2007; Herrera-Leon et al., 2007) that DNA-based approach cannot yet be used as substitute for traditional serotyping method. The two methods could be complementary, whereby the main *Salmonella* serotypes can be identified by multiplex PCR serotyping, while less common serotypes can be identified by traditional serotyping method by the reference centre.

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