Original Article

Application of PCR-based serogrouping of selected *Salmonella* serotypes in Malaysia

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Abstract

Background: Differentiation of *Salmonella enterica* into its serogroups is important for epidemiological study. The objective of the study was to apply a multiplex PCR targeting serogroups A, B, C1, D, E and Vi-positive strains of *Salmonella enterica* commonly found in Malaysia. A separate H-typing multiplex PCR which identified flagellar antigen "a", "b" or "d" was also optimized to confirm clinical serotypes, *S.* Paratyphi A and *S.* Typhi.

Methodology: Sixty-seven laboratory *Salmonella enterica* strains were tested. Six sets of primers targeting defined regions of the O antigen synthesis genes (*rfb* gene cluster*)* and Vi antigen gene (*viaB)* were selected and combined into a multiplex PCR for O-grouping. Four primers (H-for, Ha-rev, Hb-rev and Hd-rev) were used in the second step multiplex PCR for H-typing. The optimized mPCR assays were further evaluated with 58 blind-coded *Salmonella* strains.

Results: The multiplex PCR results obtained showed 100% concordance to the conventionally typed serogroups. Validation with 58 blind coded *Salmonella* strains yield 100% accuracy and specificity.

Conclusion: Based on this study, PCR serogrouping proved to be a rapid, alternative method for further differentiation of *Salmonella enterica*.

Keywords: multiplex PCR, *Salmonella*, serogroup, serotype

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Introduction

Salmonella are facultative anaerobic Gram-negative bacilli belonging to the Family *Enterobacteriaceae*. The genus of *Salmonella* is very polymorphic and comprises a number of closely related serotypes, many of which are gastrointestinal pathogens for human and animals [1]. In many countries including Malaysia, *Salmonella* is the leading cause of food-borne outbreaks and infections. *Salmonella* may be transmitted by a wide variety of agricultural products and processed foods. Most *Salmonella* infections in humans are zoonotic in origin, but some Typhi and Paratyphi serotypes are human restricted [2].

Salmonella isolates are serotyped according to the Kaufmann-White scheme using somatic (O), capsular (Vi) and flagellar (H) antigens that are present in the cell surface of *Salmonella*. The O factors determine the serogroup and the H factors define the serotype of a *Salmonella* strain [3, 4]. O antigen is extremely polymorphic. It is a polymer with repeating units of three to six sugars in *Salmonella* serogroups A to E. The basis of the variation in O antigen structure is represented by the different types of sugar present, the arrangement of sugars, the addition of branch sugars, and modifying side groups; such variation is used to serogroup *Salmonella* isolates [4-6].

Salmonella comprise two species which are *S. enterica* and *S. bongori*. The species *Salmonella enterica* comprise six subgroups which are groups I, II, IIIa, IIIb, IV and VI. *S. enterica* subsp. I (*S. enterica* subsp. Enterica) is usually isolated from humans and warm-blooded animals and the majority of the serotypes isolated in clinical laboratories belong to this subspecies [7]. The other subspecies and *S. bongori* are usually isolated from either the environment or reptiles and therefore are not clinically important [8]. To date, more than 2,500 different serotypes have been described, of which 59% belong to *S. enterica* subsp. I [3,4]. Over 95% of the strains that cause infections in humans and animals belong to serogroups A to E [9,10].

Identification of *Salmonella* is important for surveillance, improving prevention and control of food-borne diseases. It has allowed rapid detection, identification of sources, control of outbreaks, and also identification of emerging serotypes and new mechanisms of transmission [4].

Although the conventional serotyping method based on the Kauffmann-White scheme is widely used, there are some limitations; for example, the method is time-consuming and tedious, as well as subjective in interpretation. Furthermore, it requires well-trained technicians and high-quality anti-sera, both of which could be difficult to obtain consistently and are very costly in resource-limited settings. Such limitations have encouraged development of several molecular methods based on the amplification of DNA for identification of *Salmonella* serotypes. These molecular methods, such as multiplex PCR, are highly sensitive, very specific, fast and reproducible. Such approaches have previously been reported by others [1,4,6,7,14,19,22,24-27,29]. In *Salmonella*, the genes responsible for biosynthesis of the O antigens are normally grouped together on the chromosome in a gene cluster called *rfb* [6]. This *rfb* gene cluster that encodes many enzymes for O antigen biosynthesis and assembly is highly polymorphic and has been targeted as a molecular marker for detection of *Salmonella* serotypes. The *rfb* gene cluster of the more common *Salmonella* serogroups have been studied extensively at the molecular level to date [4,6,11-14].

Differences in the genes responsible for the final steps in dideoxyhexose synthesis provided opportunities for design of serogroup-specific probes. Groups A and D have *prt* (previously known as *rfbS*) and *tyv* (previously known as *rfbE*) in place of *rfbJ*. The gene *prt* encodes paratose synthase that converts CDP-4-keto-3,6-dideoxyglucose to CDP-paratose. The *tyv* gene encodes CDP-tyvelose epimerase, which further converts CDP-paratose to CDP-tyvelose. However, *tyv* gene of group A does not produce active CDP-tyvelose epimerase due to the 1-bp deletion which causes the frameshift mutation and converts the fourth codon to an amber stop codon [15]. *Salmonella* groups B and C2 were found to have *rfbJ* genes which encode abequose synthase, converting CDP-4-keto-3,6-dideoxyglucose to CDP-abequose. The *rfbJ* genes of groups B and C2 are very different, and there are differences in *rfbD* and *rfbN* of E1 and other groups [12]. The *rfb* regions from serogroups A, B, C2, D, and E, which all have a trisaccharide O subunit containing mannose, rhamnose, and galactose, are related. The *rfb* gene cluster from serogroup C1, whose O subunit is composed of four mannose residues, one *N*-acetylglucosamine residue, and a glucose side branch, shows little homology to them [16].

The *wzx* gene (previously *rfbX*), which encodes a protein consisting of 12 potential transmembrane segments, is found in all of these *Salmonella* O-antigen gene clusters. It has been proposed that wzx proteins are involved in transferring the completed O antigen subunits across the cytoplasmic membrane to the periplasmic side [17]. The wzx proteins from different O antigen clusters have little similarity among them, even at the amino acid sequence level, thus making this gene an excellent target for serogroup-specific primers [18].

The flagellar antigens H1 and H2 are encoded by the *fliC* and *fljB* genes, respectively. Either one of them is expressed at one time due to a mechanism regulated by the operon *fljBA*. *Salmonella* serotyping methods recognize 63 distinct phase 1 flagellar antigenic factors and 37 phase 2 flagellar antigenic factors although the latter are not always present [19].

The virulence capsular polysaccharide (Vi antigen), a homopolymer of N-acetylgalactosamine uronic acid that forms a coat on the external surface of the bacterial cell, is expressed by *S.* Typhi, *S.* Paratyphi C, some of the strains of *S*. Dublin, and a few strains of *Citrobacter freundii* [20]. Production of the Vi antigen is controlled by two chromosomal loci, *viaA* and *viaB*. The *viaB* region is believed to consist of the structural genes specific for Vi antigen expression. Vi antigen is an essential factor for *S.* Typhi survival in human serum [21].

The objective of this paper is to develop and apply 2-sequential multiplex PCRs that enable rapid identification of the major serogroups of *Salmonella* that are commonly encountered in Malaysia and also to confirm clinically important *Salmonella* serotypes Paratyphi A, C and Typhi that cause enteric fever. The first multiplex PCR identifies groups A, B, C1, D, E and Vi positive strains. The second multiplex PCR was optimized to confirm H flagellar antigens "a', "b" and "d".

Materials and Methods

Bacterial Strains

Sixty-seven laboratory strains with known serogroups representing the commonly isolated *Salmonella* from humans and food in Malaysia were used in optimization and verification of the O-grouping multiplex PCR. Conventional serotyping

was carried out by the *Salmonella* Reference Centre at the Institute of Medical Research, Malaysia. These strains included: *S. enterica* ser Paratyphi A in serogroup A(O:2) (n= 4); ser Paratyphi B (n=6), ser Typhimurium (n=10), ser Agona (n=1) and ser Stanley (n=4) in serogroup B (O:4); ser Virchow (n=1), ser Infantis (n=1), and ser Braenderup (n=3) in serogroup C1 (O:7); ser Enteritidis (n=16) and ser Typhi (expressed Vi-antigen) (n=12) in serogroup D (O: 9; O:9,46; O:9,46,27), ser Weltevreden (n=4) and ser Senftenberg (n=1) in serogroup E (O:3,10; O:1,3,19); and *Salmonella* serotypes Muenchen (n=1), Newport (n=1), Corvallis (n=1) and Hadar (n=1) in serogroup C2-C3 (O:8). The same group of strains were also used in optimization and verification of the H-typing multiplex PCR that identified H antigen 'a', 'b', and 'd'. Ten non-*Salmonella* strains, *Klebsiella pneumoniae* (n=2), *Escherichia coli* (n=2), *Vibrio cholerae* (n=1), *V. parahaemoliticus* (n=2) and *Acinetobacter baumanii* (n=3), were evaluated with the multiplex PCR method to test for cross-reactivity with *Salmonella* primers.

Primers

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 designed by Hirose *et al.* (2002) [25], Lim *et al.* (2003) [26], Levy *et al.* (2008) [22] and Herrera-Leon *et al.* (2007) [7] (Table 1). Primers used in H typing multiplex PCR were from Levy *et al.* (2008) [22] to detect H1 antigen 'a', 'b' and 'd' (Table 1). Internal controls (P1-P2 primers that amplify *oriC*) were incorporated to avoid false negative result [22, 33].

DNA extraction

A single colony of bacterial cells was suspended in 50 µl of double-distilled water. The suspension was heated at 99^{o}C for 5 min, chilled in ice, and the cell debris was pelleted by centrifugation at 13,400g for 2 min and 5 µl (approx. 10 ng DNA) of clear supernatant was used as the DNA template in a PCR.

O-Serogrouping multiplex PCR

DNA amplification was performed in a reaction volume of 25 µl. Each reaction contained 1X PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 µM (each) of primers F-prt, R-prt, F-rfbJ, R-rfbJ, F-vi, R-vi, F-wzxC1, R-wzxC1, F-tyvD, R-tyvD, F-wzxE, and R-wzxE, 0.2 µM of the positive control primers (P1-P2) and 1.75 U Promega *Taq* DNA polymerase. Five microliters of a briefly centrifuged, boiled strain suspension was used as a template. PCR was performed in a Mastercycler (Eppendorf). Fragments were separated in 1.5% agarose gel by unidirectional electrophoresis using 0.5X TBE buffer and visualized by staining with ethidium bromide. Fragment size was determined by comparison with 100 bp DNA ladders (Promega).

The cycling parameters of the O grouping multiplex PCR 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.

Generally, for each PCR experiment, DNA from *S.* Paratyphi A (representative of serogroup A); *S.* Agona, *S.* Typhimurium, or *S.* Paratyphi B (representative of serogroup B), *S.* Virchow or *S.* Infantis (representative of serogroup C1), *S.* Enteritidis or *S.* Typhi (representative of serogroup D), and *S.* Senftenberg or *S.* Weltevreden (representative of serogroup E) were used as positive controls. A negative control (using sterile water as template) was included in each run.

H-typing Multiplex PCR

The H-typing multiplex PCR comprised of a denaturation step of 2 min at 95°C, followed by 35 cycles of the following three steps: 95°C for 30 s and 55°C for 30 s and 72°C for 30s and 1 final extension step at 72°C for 5 min. PCR products were separated on 1.5% (wt/vol) agarose gels, stained with ethidium bromide and visualized on a UV transilluminator.

Validation of the Multiplex PCRs

A blind testing for validation of the optimized multiplex PCRs was conducted on 58 blind-coded *Salmonella* known strains which included negative control strains from serogroups other than A, B, C1, D and E (Table 2). All strains were from our laboratory collection from various sources, such as human, food and animal samples.

Sequence Analysis

Twenty PCR products obtained with the primers representing each serogroup, Vi-positive, and each H1 antigen were sequenced to validate their identities. PCR products were purified using Intron DNA extraction kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's protocol and

then submitted to a commercial laboratory (Research Biolabs, Singapore) for sequencing.

Table 1. Oligonucleotide primers used for *Salmonella* multiplex PCRs.

Target	Primers	Sequence (5' to 3')	Amplicon size (bp)	Reference
B group	F-rfbJ	CCAGCACCAGTTCCAACTTGATAC	662	[26]
	R-rfbJ	GGCTTCCGGCTTTATTGGTAAGCA		
D group	F-tyv	GAGGAAGGGAAATGAAGCTTTT	614	[25]
	R-tyv	TAGCAAACTGTCTCCCACCATAC		
Vi strains	F-vi	GTTATTCAGCATAAGGAG	439	[25]
	R-vi	CTTCCATACCACTTTCCG		
A & D group	F-prt	CTTGCTATGGAAGACATAACGAACC	256	[25]
	R-prt	CGTCTCCATCAAAAGCTCCATAGA		
C1 group	F-wzxC1	CAGTAGTCCGTAAAATACAGGGTGG	483	[7]
	R-wzxC1	GGGGCTATAAATACTGTGTTAAATTCC		
E group	F-wzxE1	TAAAGTATATGGTGCTGATTTAACC	345	[7]
	R-wzxE1	GTTAAAATGACAGATTGAGCAGAG		
H:a, H:b and H:d	H-for	ACTCAGGCTTCCCGTAACGC		[22]
	Ha-rev	GAGGCCAGCACCATCAAGTGC	423	
	Hb-rev	GCTTCATACAGACCATCTTTAGTTG	551	
	Hd-rev	GGCTAGTATTGTCCTTATCGG	763 (d) or 502 (j)	
oriC	P1	TTATTAGGATCGCGCCAGGC	163	[33]
	P2	AAAGAATAACCGTTGTTCAC		

Results

The serogroup-specific primers were initially tested individually to determine the specificity. DNA sequences analysis of the representative amplicons produced by each primer pair showed high identity levels ranging from 97% to 99% to the GenBank sequence database, confirming the specificity of the primers (data not shown). When negative control strains of *Salmonella* were tested, no amplification was obtained. All the tested primers were completely specific for the targeted serogroups.

A multiplex PCR using all the primers set was then optimized. Both extension and annealing temperatures were altered gradually to allow optimal annealing for all primers in the mixture. The annealing temperature suitable for all reactions was 50°C and the optimal extension temperature was 68°C. The magnesium concentration was varied from 2 to 3.5 mM. With low magnesium concentration some of the expected PCR fragments were faint or non-detectable, while with high magnesium concentration nonspecific PCR products of various sizes were amplified. Based on empirical experiments, the optimum magnesium concentration for the assay was determined to be 2.5 mM.

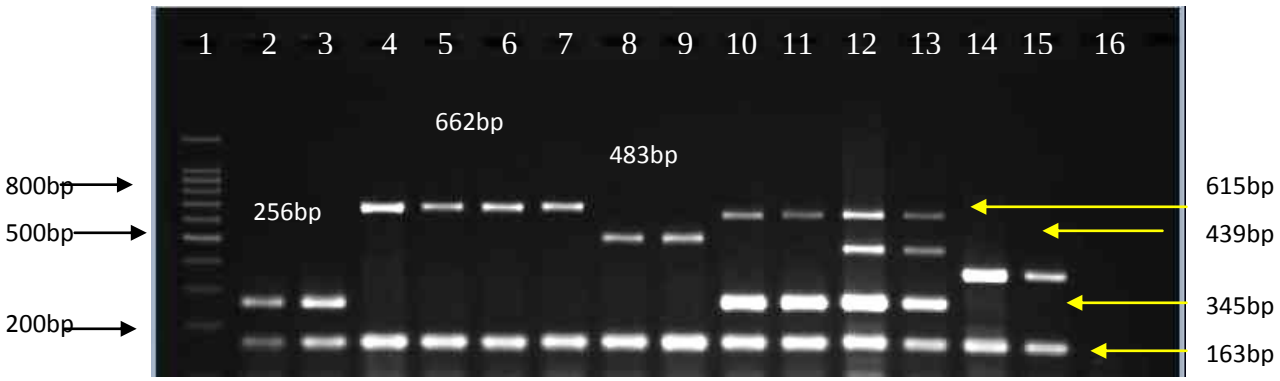
The optimized multiplex PCR was evaluated with 67 known bacterial species and strains. Representative agarose gels of PCR products are shown in Figures 1 and 2. Each serogroup was identified by its specific amplicon: serogroup A- 256 bp band which contains the *prt* genes, serogroup B - 662 bp band, serogroup C1- 483bp band, serogroup D – 256 bp and 615 bp bands and serogroup E – 345 bp band. As expected, *S.* Typhi from serogroup D, which carries a virulence capsular antigen, showed 3 bands with corresponding sizes of 256 bp, 439 bp and 615 bp. Overall, the multiplex PCR correctly identified 4 isolates of serogroup A, 21 isolates of serogroup B, 5 isolates of serogroup C1, 28 isolates of serogroup D in which 12 isolates were Vi positive and presumptively identified as *S.* Typhi, and 5 isolates of serogroup E. The specificity of the primers were tested with non-targeted serogroup C2-C3 and showed no cross-reactivity.

The PCR amplified product of 163 bp by P1-P2 primer was present in all the *Salmonella* isolates. However, these internal control *Salmonella*-specific

Table 2. *Salmonella* Strains used for blind testing and the PCRs results.

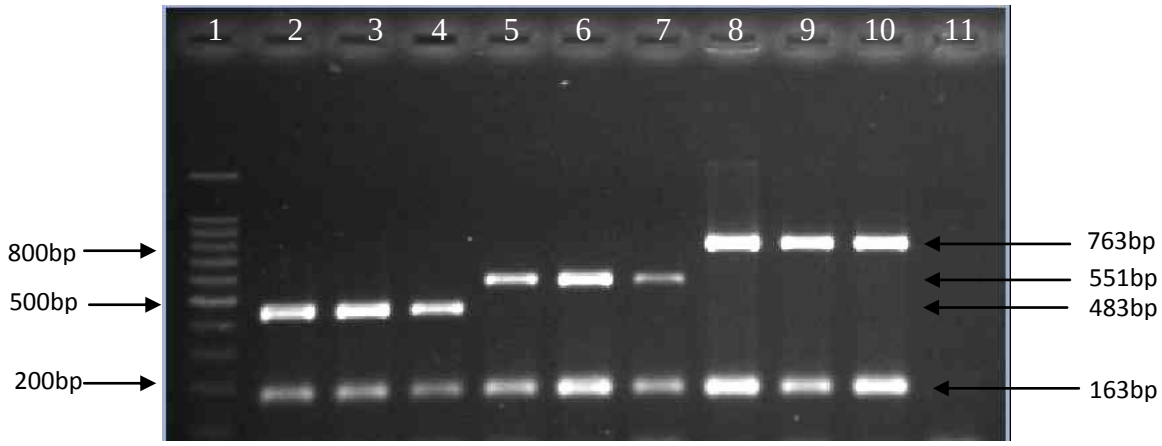
Serotype	O group	H 1 antigen	Total no. of strains	No. of strains tested positive									
				Prt	rfbJ	wzxC1	Tyv	Vi	wzxE	Ha	Hb	Hd	P1-P2
Paratyphi A	A	a	6	6	-	-	-	-	-	6	-	-	6
Paratyphi B	B	b	1	-	1	-	-	-	-	-	1	-	1
Heidelberg	B	-	1	-	1	-	-	-	-	-	-	-	1
Sandiego	B	-	2	-	2	-	-	-	-	-	-	-	2
Typhimurium var. Copenhagen	B	-	1	-	1	-	-	-	-	-	-	-	1
Agona	B	-	1	-	1	-	-	-	-	-	-	-	1
Typhimurium	B	-	1	-	1	-	-	-	-	-	-	-	1
Indiana	B	-	1	-	1	-	-	-	-	-	-	-	1
Thompson	C1	-	2	-	-	2	-	-	-	-	-	-	2
Oranienburg	C1	-	1	-	-	1	-	-	-	-	-	-	1
Braenderup	C1	-	2	-	-	2	-	-	-	-	-	-	2
Augustenberg	C1	-	1	-	-	1	-	-	-	-	-	-	1
Infantis	C1	-	2	-	-	2	-	-	-	-	-	-	2
Virchow	C1	-	1	-	-	1	-	-	-	-	-	-	1
Typhi	D (Vi)	d	13	13	-	-	13	13	-	-	-	13	13
Enteritidis	D	-	5	5	-	-	5	-	-	-	-	-	5
Javiana	D	-	1	1	-	-	1	-	-	-	-	-	1
Meleagridis	E	-	1	-	-	-	-	-	1	-	-	-	1
Amsterdam	E	-	2	-	-	-	-	-	2	-	-	-	2
Biafra	E	-	1	-	-	-	-	-	1	-	-	-	1
Weltevreden	E	-	1	-	-	-	-	-	1	-	-	-	1
Blockley	C2-C3	-	2	-	-	-	-	-	-	-	-	-	2
Hiduddify	C2-C3	-	1	-	-	-	-	-	-	-	-	-	1
Corvallis	C2-C3	-	1	-	-	-	-	-	-	-	-	-	1
Kentucky	C3	-	1	-	-	-	-	-	-	-	-	-	1
Worthington	G	-	3	-	-	-	-	-	-	-	-	-	3
Havana	G	-	1	-	-	-	-	-	-	-	-	-	1
Ajiobo	G2	-	1	-	-	-	-	-	-	-	-	-	1
Alachua	O	-	1	-	-	-	-	-	-	-	-	-	1
Total			**58**	**25**	**8**	**9**	**19**	**13**	**5**	**6**	**1**	**13**	**58**

Figure 1. O-grouping Multiplex PCR amplification of *Salmonella* groups A, B, C1, D, E and Vi positive strains.



All strains yielded an internal control *Salmonella*-specific band of approximately 163bp, in addition to serogroup-specific bands: approximately 256bp (serogroup A), approximately 662 bp (serogroup B), approximately 483bp (serogroup C1), approximately 615 bp (serogroup D and *S.* Typhi with addition Vi band size of 439bp), and approximately 345bp (serogroup E). Lane 1, 100bp ladder; lanes 2-3, *S.* Paratyphi A; lane 4, *S.* Typhimurium; lane 5, *S.* Agona; lanes 6-7, *S.* Paratyphi B; lane 8, *S.* Infantis; lane 9, *S.* Virchow; lanes 10-11, *S.* Enteritidis; lanes 12-13, *S.* Typhi; lane 14, *S.* Senftenberg; lane 15, *S.* Weltevreden; lane 16, negative

Figure 2. H-typing multiplex PCR amplification of *Salmonella*.



Lane 1, 100bp ladder; lane 2, *S.* Paratyphi A; lane 3, *S.* Paratyphi A ; lane 4; *S.* Paratyphi A; lane 5, *S.* Paratyphi B ; lane 6, *S.* Paratyphi B ; lane 7, *S.* Paratyphi B ; lane 8, *S.* Typhi; lane 9, *S.* Typhi; lane 10, *S. Muenchen* ; lane 11negative.

primers did not amplify any specific fragment from the 10 non-*Salmonella* isolates; instead, there were faint multiple unspecific fragments. When all these *Salmonella* strains were tested with the H-antigen multiplex PCR, all the *Salmonella* serotypes Typhi, Muenchen and Stanley strains yielded amplicon of flagellar antigen d, all the *S.* Paratyphi A isolates yielded amplicon of flagellar antigen a, and all the *S.* Paratyphi B isolates exhibited amplicon denoting flagellar antigen b (Fig.2).

Fifty-eight *Salmonella* isolates from various sources such clinical, food and environmental samples were blind-coded and tested with the two multiplex PCRs (Table 2). The multiplex PCRs produced PCR products corresponding to all the antigen targets from these 58 isolates (100%). The H-typing multiplex PCR correctly detected the *a* allele of the serotype Paratyphi A, *b* allele for serotype Paratyphi B and *d* allele of the serotype Typhi strains.

The multiplex PCRs proved 100% sensitivity and specificity in detecting all the tested true strains. The O-grouping multiplex PCR was also 100% sensitive in detecting other serotypes within groups A, B, C1, D and E. The specificity of the multiplex PCRs for serotypes *Salmonella* Typhi and *Salmonella* Paratyphi A was also 100%. Both positive and negative predictive values of the multiplex PCRs were 100%.

Discussion

Cross-reactions between serotypes occurred when traditional serological assays are used for the serotyping of *Salmonella* [23]. One way to avoid this serological cross-reactivity is to use DNA-based methods for serotyping, such as PCR, which does not involve antigens or antibodies. Various PCR tests have been developed for species identification of *Salmonella enterica* [1,4,6,7,22,24-27].

Herrera-Leon *et al.* (2007) [7] had developed a multiplex PCR identifying serogroups B, C1, C2, D and E. In the O-grouping multiplex PCR developed by Levy *et al.* (2008) [22], only serogroups A, B, D and Vi-positive strains were identified. We combined both these systems to cover a wider range of major serogroups (A, B, C1, D and E as well as Vi-positive strains) to enhance the versatility of the system. However, primers wzxC2 described by Herrera-Leon *et al.* (2007) [7] that determined C2 group was not included as the amplicon (154 bp) was too close to the internal control amplicon of 163 bp. In addition, H-typing multiplex PCR which was developed by Levy *et al*. (2008) [22] to further identify enteric fever pathogens *Salmonella* Typhi and *Salmonella* Paratyphi A was also re-optimized in this work as the published conditions gave unspecific binding.

Multiplex PCR is a demanding technique that requires extensive optimization of *Taq* DNA polymerase, $MgCl_2$, additional reagents, primers and PCR cycling parameters such as annealing temperature [28, 29]. In this study, the PCR conditions were optimized by adjusting the concentrations of $MgCl_2$, primers and *Taq* polymerase as well as the cycling parameters which were denaturation time, annealing and extension temperature.

Human-specific pathogens *S.* Typhi and *S.* Paratyphi are the etiologic agents of enteric fever. Therefore, when *a Salmonella* bacterium is identified, a further test should be performed to determine whether the isolate is *S.* Typhi or *S.* Paratyphi. *S.* Typhi and *S.* Paratyphi C carry a Vi antigen. Since our first-step multiplex assay included

both O group and Vi antigen detection, *S.* Paratyphi C could be detected (as it is the only serotype in serogroup C1 that expresses Vi antigen) and *S.* Typhi can be presumptively identified at this stage. In *Salmonella* group D, only serotype Typhi and a subset of serotype Dublin expresses the Vi antigen. If the O-grouping multiplex PCR identified Vi-positive strain in serogroup D, by using a second-step H multiplex PCR, *S.* Typhi could be confirmed by positive result of H antigen d whereas serotype Dublin will show a negative result of H:d. Among all non-*Salmonella Enterobacteriaceae*, only *Citrobacter freundii* carries the Vi antigen. The chosen *viaB* gene primers in our study detected only *Salmonella* Vi antigen as described by Hirose *et al.* (2002) [25].

Some unusual Typhi strains express *fliC-j* gene, which is an alternate phase of serotype Typhi H-1 antigen genes. The *fliC-j* gene is a 261 bp deletion derivative of the *fli-d* gene [25]. The O-grouping multiplex PCR could not distinguish the *Salmonella* serotype Typhi j strains from serotype Typhi strains that possess *fliC-d* gene. The H-for and Hd-rev primers used in the H-typing multiplex PCR could differentiate serotype Typhi d and j strains as the primers could also bind to DNA external to the deletion of a 261bp in *fliC-d* to amplify a 502bp fragment rather than the 763bp d amplicon [22]. *Salmonella* serotype Typhi strains that express j flagellar antigen were not available in this study; therefore, the priming of the *fliC-j* allele could not be determined in this work. In our 2-sequential multiplex PCRs, strains identified as serogroup A that showed flagellar antigen a would be classified as *Salmonella* Paratyphi A, as only this serotype in serogroup A expressed this H1 antigen a. Besides serotype Paratyphi B, other serotypes express flagellar antigen b, such as Abony in serogroup B. Therefore this system could not be used as definitive detection of serotype Paratyphi B.

The use of an internal amplification control (IAC) in diagnostic PCR is becoming mandatory. An IAC is a non-target DNA sequence present in the same sample reaction tube which is co-amplified simultaneously with the target sequence. In a PCR without an IAC, a negative result could either mean that that there was no target sequence present in the reaction, or that reaction was inhibited due to incorrect mixture, poor polymerase activity, error caused by the PCR components or malfunction of the thermal cycler, or the inhibitory substances present in the sample matrix [30]. To avoid false-negative results, an IAC is incorporated into the system where a control signal will always be produced when there is no target sequence present. In this study, the P1-P2 primer pair targeting the *oriC* gene, a ubiquitous component of all *Salmonella*, was included as an internal control in all multiplex reactions. The PCR amplified product of 163 bp by P1-P2 primer was present in samples from all the tested *Salmonella* isolates. However, the primers did not amplify any specific fragment from the 10 non-*Salmonella* isolates; instead, there were multiple unspecific fragments. These data agreed with the study conducted by Marlony *et al.* (2003) [31] that primer set P1-P2 (*oriC*) produced multiple, non-target-sized fragments of 0.2 to 3 kb in several non-*Salmonella* strains, confirming that P1-P2 primer could be unique to *Salmonella* sequences, which are not conserved in non-*Salmonella* organisms. The inclusion of the internal control primers in each reaction reliably identified sample preparation problems so that reactions that failed to amplify a product could be repeated. Besides, this strategy could also serve as exclusivity test for non-*Salmonella* isolates.

There are many advantages in using this multiplex PCR system. The technique is straightforward and easily established in laboratories which are equipped for DNA template preparation and PCR. The use of crude DNA extracts eliminates the need for more specialized template preparation protocols. Serogrouping could be carried out using a minimal number of reactions, and products can be detected easily by gel electrophoresis. It is reliable and reproducible as the interpretation of the results is not subjective. It provides a fast and cost-effective way of analyzing large number of samples.

In this study, the detection of Vi antigen by O-grouping multiplex PCR provides a very efficient and important means of studying the salmonellosis surveillance and epidemiological distribution of the predominant *Salmonella* serotypes. As this 2-sequential multiplex PCRs system could identify some enteric fever pathogens (*S.* Typhi and *S.* Paratyphi), appropriate medical treatment could be initiated without further delay.

Nonetheless, conventional serotyping still must be performed as the mPCR system could not differentiate all the serogroups; however, the information gathered can serve as a guide in conventional serotyping.

This study also demonstrates the adaptability of the PCR method for grouping alternative combinations of serogroup-specific primer sets.

Moreover, there is the possibility of expanding the approach to detect more serogroups/serotypes as with more new publications of the partial and/or complete sequence of the bacilli, new primers could be designed to allow the identification of more serogroups and/or serotypes and the expansion of the typing scheme.

However, multiplex PCR has inherent limitations, such as the limit in the number of different amplicons that could be amplified due to primer-primer interactions and nonspecific amplifications that reduce the amplification efficiency [32]. Overall, the optimization of multiplex PCR was very challenging and highly empirical.

In conclusion, the results obtained in this study indicate that the multiplex PCR test is a sensitive, reliable, specific, and highly effective diagnostic tool for the simultaneous identification of *Salmonella* serogroups A, B, C1, D, E and Vi positive strains. This system could greatly reduce our reliance upon the costly and tedious conventional serotyping. It could be applied by any facility that lacks the expensive typing sera and expertise needed for conventional serotyping but is equipped with basic PCR facilities.

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