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Development and evaluation of a Multiplex Polymerase Chain Reaction for the detection of *Salmonella* species

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Abstract. The present study aims to develop a system which consists of four pairs of primers that specifically detects *Salmonella* spp., *Salmonella* serovar Typhi and *Salmonella* serovar Paratyphi A with an internal amplification control. The system, when applied in Polymerase Chain Reaction (PCR) under specific conditions, reaction mixture and cycling temperatures produced four bands; 784 bp, 496 bp, 332 bp and 187 bp. The DNA band 784 bp is present in all *Salmonella* spp., while the bands of 496 bp and 332 bp are only present in *S.* Paratyphi A and *S.* Typhi, respectively. An internal amplification control as indicated by the 187 bp shows the system is working in optimum condition in all the tests. This multiplex PCR was evaluated on 241 bacterial cultures and 691 naturally contaminated samples. Overall, this multiplex PCR detection system provides a single step for simultaneous detection of DNAs of *Salmonella* spp., *S.* Typhi and *S.* Paratyphi A.

INTRODUCTION

Salmonella infections and enteric fever remain an important public health problem in many parts of the world, especially in developing countries. Every year, non-typhoidal *Salmonella* causes 93.8 million human infections and 155,000 deaths worldwide (Majowicz *et al.*, 2010). Gastroenteritis is commonly caused by non-typhoidal *Salmonella* such as *Salmonella enterica* serovar Typhimurium, serovar Enteritidis, serovar Stanley and serovar Weltevreden. The distribution of different *Salmonella* serovars varies among countries and regions (Hendriksen *et al.*, 2011). On the other hand, WHO estimated 17 million cases of typhoid fever annually, where *Salmonella enterica* serovar Typhi (*S.* Typhi) is the predominant organism isolated over the past decades, however, in some provinces in China and Pakistan, there is an increasing numbers of enteric fever cases caused by *Salmonella* Paratyphi A (*S.* Paratyphi A) (Girard *et al.*, 2006).

These pathogens are acquired following ingestion of faecally-contaminated food or water or directly from asymptomatic carriers, and outbreaks are frequent (Girard *et al.*, 2006). Important vehicles of transmission in some countries include shellfish taken from sewage-contaminated beds, raw fruits, vegetables fertilized by night soil and eaten raw, milk and milk products, or during preparation of food by hands (Riyaz-Ul Hassan *et al.*, 2004). Given the similar presentations of typhoid and paratyphoid fevers, these conditions cannot be reliably distinguished on clinical grounds alone (Vollaard *et al.*, 2005). Although several assays for detecting *S.* Typhi antigens or antibodies have been used for their rapidity and simplicity, no non-culture tests for typhoid fever have shown to be highly specific and/or sensitive (Rasoul *et al.*, 2007). Confirmation of typhoid fever requires the

identification of *S.* Typhi in clinical specimens (Bhan *et al.*, 2005).

Early diagnosis and prompt treatment with appropriate antimicrobial agents are crucial to reduce the morbidity and mortality associated with enteric fever. Clinical diagnosis of typhoid fever is difficult due to the overlapping of the signs and symptoms with other common febrile illness (Crump *et al.*, 2004). Laboratory diagnosis of typhoid fever is conventionally based on culture and serological methods. However, these methods lack sensitivity and/or specificity (Wain *et al.*, 2001), and as a result of this, they are of little diagnostic value. Blood culture can only detect about 45% to 70% of patients with typhoid fever, depending on the amount of blood sampled, the bacteremic level of *S.* Typhi, the type of culture medium used and the length of incubation period (Parry *et al.*, 2002). The clinical usefulness of culture method is further restricted due to the fact that it takes at least two days to identify the microorganism. Furthermore, the likelihood of a positive blood culture and serology diagnosis declines significantly with delayed presentation or prior antibiotic use (Parry *et al.*, 2002). Therefore, we aim to develop a reliable, sensitive and cost effective conventional multiplex system for simultaneous detection of *Salmonella* spp., *S.* Typhi and *S.* Paratyphi A in this study.

MATERIALS AND METHODS

Primer design for *Salmonella* species

For primer design, gene sequences of *Salmonella* species were extracted from Genbank followed by multiple sequence alignment using Mega 4 program (Tamura *et al.*, 2007). The regions which potentially differentiate individual species were selected for bacterial species confirmation while the conserved region was also selected for genus identification. Primers were designed using the Primer 3 software and evaluated with *in-silico* PCR as reported previously (Ou *et al.*, 2007) prior to actual PCR evaluation in the laboratory. Besides the 3 primer sets that target Salmonellae, a pair of primers targeting plasmid pQE-30 (Qiagen, Venlo, Limburg), which originally used for expressing N-terminally 6xHis-tagged proteins was also tested to be suitable act as the non-competitive internal amplification control (IAC) in this system. The plasmid pQE30 is transformed in *E. coli* host strain. The PCR primers and the expected band sizes are listed in Table 1.

Bacterial Strains

Salmonella species including *Salmonella* Typhi (n=120), *Salmonella* Paratyphi A (n=56), other *Salmonella* species (n=120) and 55 non-*Salmonella* strains were retrieved

Table 1. Primers used in this study and the targeted band sizes

Primer	Sequence 5' to 3'	Band size
hil 1	CGG AAC GTT ATT TGC GCC ATG CTG AGG TAG	784 bp
hil 2	GCA TGG ATC CCC GCC GGC GAG ATT GTG	
spa 1[a]	NNCCC AAT ATC GTC TCC CGC CTC GCCNN	496 bp
spa 2	CTT GTA CTT CGA GCC ATG GAG TGT CG	
typ 1[b]	NNCAT CCT GCC GGA AAG TGA ACNN	332 bp
typ 2	NGAC GAA TTT GCT CCA TCN	
IAC-R[c]	NNGTT CTG AGG TCA TTA CTG GNN	187 bp
IAC-F	CGG ATA ACA ATT TCA CAC AG	

[a] Patent field IP20072043 2009: A pair of specific oligonucleotide sequences for detecting of DNA of *Salmonella enterica* serovar Paratyphi A and A corresponding pair of primers as internal control amplification.
[b] MY-135531-A·DNA primers for specific detection of *Salmonella enterica* serovar Typhi. Malaysian patent.
[c] Patent filed P120110054142: One Step Multiplex System for the Detection of *Salmonella* spp., *S.* Typhi, and *S.* Paratyphi A with an Internal Control.

from the culture collection of the laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduates Studies, University Malaya. The strains were screened on selective media, confirmed using biochemical tests, API20E (Biomerieux, France) and PFGE as previously described (Thong *et al.*, 2002).

Template DNA preparation

Template DNA preparation was carried out using the crude lysate method. Basically, a bacterial colony was picked and suspended in 100 µl of ddH_2O. The DNA suspension was boiled at 99°C for 5 min and snapped cold on ice for 10 min. The cell lysate was then quickly centrifuged (1 min) at maximum speed and 5 µl of the supernatant (~50 ng) containing the bacterial genomic DNA was used for PCR reaction. For IAC, the plasmid DNA was extracted by using QIAprep Spin Miniprep Kit (Qiagen, Venlo, Limburg) according to the instruction of the manufacturer.

Polymerase Chain Reaction and Sequencing

PCR was carried out in a total volume of 25 µl, including 1X PCR buffer, 1.2 mM MgCl2, 0.8 µM dNTP, 0.4 µM primers, and 0.75 U Taq DNA Polymerase (Promega, Madison, WI) and ~50ng of DNA. The PCR program was set at 95°C for 5 min (1 cycle); 95°C for 1 min, 55°C for 1 min, 72°C for 1 min (30 cycles); 72°C for 5 min (1 cycle) and the reaction mixture was electrophoresed on a 1% (w/v) agarose gel. The representative amplicons for each target were purified and sent to a commercial facility for sequencing to confirm the identity.

Detection limit of Multiplex PCR system on artificially contaminated samples

Sensitivity of the PCR system was evaluated on stool, blood and food samples. Briefly, the overnight culture of *S.* Typhi, *S.* Paratyphi A and *S.* Typhimurium were serially diluted and spiked into the stool, blood and food samples. The samples which were artificially contaminated by 10^2 to 10^8 CFU/ml or g of the bacteria were left at room temperature for half an hour, followed by centrifugation and washing (twice) with Tris-EDTA (TE) buffer. Finally, the suspension was boiled at 99°C and subjected to multiplex PCR detection.

Application of the multiplex PCR system on naturally contaminated samples

This field evaluation of the multiplex PCR was carried out from 2007 to 2009 and conducted by independently by 9 individual researchers. A total of 691 samples comprising food (n=415), flies (n=40), environmental water (n=50), stools samples from human (n=55) and animals (n=131) were collected.

Briefly, the samples were homogenized with buffered peptone water (BPW) and enriched with Rappaport-Vassiliadis (RV) broth overnight at 37°C. Two hundred µl of the enriched culture was subjected to template preparation and PCR confirmation. On the other hand, the enriched culture was also analyzed on XLD agar, followed by biochemical tests such as triple sugar iron (TSI), Lysine Iron agar (LIA), methyl red, Voges Proskauer (VP), Simmons citrate, oxidase, urease and Sulfide-Indole-Motility (SIM) tests. The results obtained from multiplex PCR and conventional methods were compared.

Specificity and sensitivity values were calculated using the formula described by Altman and Bland (1994):

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{Number of false positives}}$$

$$\text{Specificity} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{Number of false negatives}}$$

In this study,

True positive = positive by PCR and selective media + biochemical tests

False positive = positive by PCR but negative by selective media + biochemical tests

True negative = negative by PCR and selective media + biochemical tests

False negative = negative by PCR but positive by selective media + biochemical tests

RESULTS AND DISCUSSION

The newly developed multiplex PCR correctly identified 120 *S.* Typhi and 56 *S.* paratyphi A strains. All *Salmonella* strains that were non-Typhi/Paratyphi A were confirmed as *Salmonella* spp. All non-*Salmonella* strains showed 187 bp amplicon only and this indicated that the system is functional and it is specific with no false negative was being observed (Table 2). The representative gel picture is shown in Figure 1. The detection limit of the multiplex PCR on spiked blood and stools were 10^5 CFU/ml (~500 CFU per PCR) while the multiplex PCR was more sensitive when applied on food samples where 10^4 CFU/ml (~50 CFU per PCR) could be detected.

The results obtained for stools and flies samples based on multiplex PCR were in concordance with the biochemical tests (Table 3). Out of the 55 human stool samples, only 6 were *Salmonella* positive and no *S.* Paratyphi A or *S.* Typhi was detected. *Salmonella* spp. was isolated from 5 stool samples obtained from reptiles (two turtles, two lizards and one snake) where one *S.* Typhi and one *S.* Paratyphi A were detected. On the other hand, results of biochemical tests revealed that 15 out of 40 flies collected were harboring *Salmonella* spp. From these 15 biochemical confirmed isolates, 12 were confirmed as *Salmonella*, of which one was *S.* Paratyphi A positive and two were not *Salmonella* spp. based on the multiplex PCR results.

As the isolation of typhoidal strains from animal and insect reservoirs is rare, the *S.* Typhi and *S.* Paratyphi A isolates obtained from reptiles and fly were subjected to molecular serotyped as previously described (Lim and Thong, 2009). As a result, the *S.* Typhi isolates was of D serogroup harbouring Vi antigens while the two *S.* Paratyphi A isolates was of A serogroup. The molecular serogrouping confirmed that the multiplex PCR is specific and indicates the threats that animal to be possible reservoirs of typhoidal strains of *Salmonella* other than human. As the study of flies was based on the whole-body testing, it is unable to define the role of flies as the carrier or as a mechanical vector. Based on previous studies, *Salmonella* could be found in both internal and external of the flies but is much more readily to be recovered from the interior (Sulaiman *et al.*, 2000; Holt *et al.*, 2007).

Seventy water samples were obtained from various states across Malaysia and the sources included ponds, lakes, rivers, sea water, swimming pools and monsoon drains. Fifty water samples were collected and only 2 were *Salmonella* positive (confirmed by biochemical and multiplex PCR). On the other hand, the multiplex PCR confirmed the presence of *Salmonella* spp. in one of the water samples obtained from river, which was not detected by the selective media (Table 3). The PCR results were validated by using an alternative PCR detection targeting *invA* gene as previously described (Rahn *et al.*, 1992). As *Salmonella* may enter a 'viable but nonculturable' physiological state for survival purpose in the environment, the failure of culturing *Salmonella* on selective media was not surprising. Gross under-estimation of the true extent of *Salmonella* viability in the environment is possible and it can be resolved by using molecular methods.

A total of 415 food samples comprising 240 raw food and 175 ready-to-eat (RTE) food were collected. Among the raw food samples, 40 (16.7%) were contaminated with *Salmonella* as confirmed by biochemical tests, while multiplex PCR confirmed that only 38 (15.8%) were contaminated. By using the biochemical tests and multiplex PCR, we confirmed that 23 (13.1%) RTE foods were *Salmonella*-contaminated (Table 3).

In general, the discovery of DNA in the 1950s and advances in the field of molecular biology and immunology has provided new powerful tools to enhance laboratory diagnosis. The basic principle underlining DNA analysis is that all living organisms contain DNA or RNA whose nucleotide sequences differ among species and it is this species-specific sequence that can be detected. It is known that a DNA probe specific to the Vi antigen of *Salmonella* Typhi has been used to detect the microorganism in blood of patients with typhoid fever (Rubin *et al.*, 1985; Rubin *et al.*, 1990). This hybridization method, however, requires

Table 2. Specificity and sensitivity test of the multiplex PCR

Species	Total of Strains Tested	Multiplex Result			
		784bp	496bp	332bp	187bp
Salmonella Typhi	120	+	–	+	+
Salmonella Paratyphi A	56	+	+	–	+
Salmonella Paratyphi B	15	+	–	–	+
Salmonella Typhimurium	25	+	–	–	+
Salmonella Enteritidis	20	+	–	–	+
Salmonella Kentucky	8	+	–	–	+
Salmonella Weltevreden	6	+	–	–	+
Salmonella Albany	5	+	–	–	+
Salmonella Virchow	5	+	–	–	+
Salmonella Paratyphi C	4	+	–	–	+
Salmonella Corvalis	4	+	–	–	+
Salmonella Dublin	4	+	–	–	+
Salmonella Bereilly	3	+	–	–	+
Salmonella Matopeni	3	+	–	–	+
Salmonella Muenchen	2	+	–	–	+
Salmonella Blockley	1	+	–	–	+
Salmonella Hadar	1	+	–	–	+
Salmonella Haifa	1	+	–	–	+
Salmonella Houten	1	+	–	–	+
Salmonella Hvittingfoss	1	+	–	–	+
Salmonella Infantis	1	+	–	–	+
Salmonella Bovismorficans	1	+	–	–	+
Salmonella Lomita	1	+	–	–	+
Salmonella Newport	1	+	–	–	+
Salmonella Okerara	1	+	–	–	+
Salmonella Raus	1	+	–	–	+
Salmonella Tshiongwe	1	+	–	–	+
Salmonella Thompson	1	+	–	–	+
Salmonella Agona	1	+	–	–	+
Salmonella Braenderup	1	+	–	–	+
Salmonella Chingola	1	+	–	–	+
Burkholderia pseudomallei	2	–	–	–	+
Escherichia coli	10	–	–	–	+
Klebsiella pneumoniae	10	–	–	–	+
Listeria monocytogenes	2	–	–	–	+
Pseudomonas aeruginosa	8	–	–	–	+
Shigella bodyii	5	–	–	–	+
Shigella dysentery 2	5	–	–	–	+
Shigella flexneri	5	–	–	–	+
Shigella sonnei	5	–	–	–	+
Staphylococcus aureus	3	–	–	–	+

* '+' indicates presence of band and '–' indicates absence of band.

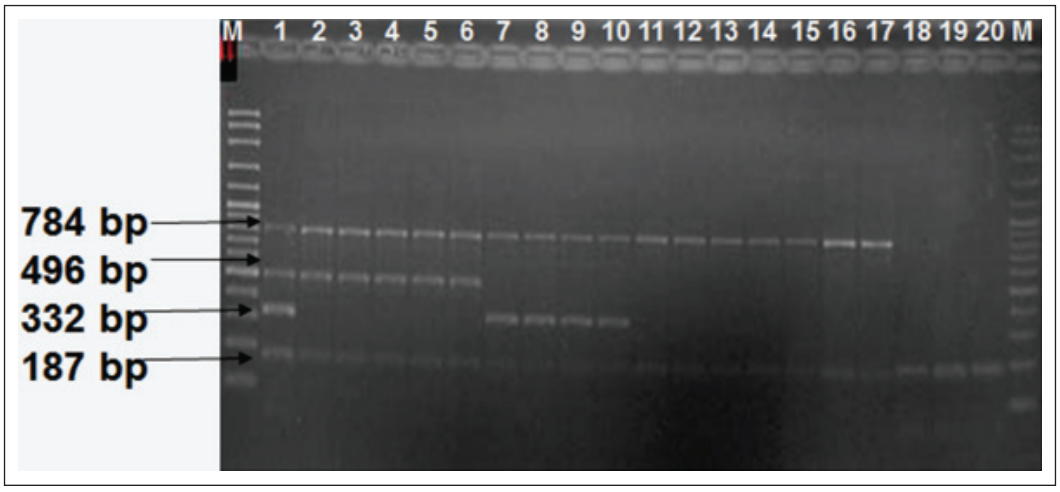

Figure 1. Detection of the *Salmonella* species using multiplex PCR
Lane M: 500 bp molecular ladder; lane 1: In-house *Salmonella* species ladder; lane 2-6: *S.* Paratyphi A; lane 7-10: *S.* Typhi; lane 11-17: Non non-Typhi/Paratyphi A *Salmonella* strains; lane 18-20: Other non-Salmonellae bacteria

Table 3. Detection of *Salmonella* spp. from different sources using the multiplex PCR

Sources	No of samples	No. of food contaminated by *Salmonella* serovars as confirmed by PCR	No. of food contaminated by *Salmonella* serovars as confirmed by selective media + biochemical tests	Specificity/sensitivity
Food*	415	38	40	Specificity = 1.0 Sensitivity = 0.95
Flies	40	15	15	Specificity = 1.0 Sensitivity = 1.0
Water*	50	2	2	Specificity = 1.0 Sensitivity = 1.0
Human stools	55	6	6	Specificity = 1.0 Sensitivity = 1.0
Animal stools	131	5	5	Specificity = 1.0 Sensitivity = 1.0
Total	691	66	68	Overall specificity = 1.0 Overall sensitivity = 0.97

* The samples which harboured "viable but nonculturable" *Salmonella* spp. were excluded in this analysis.

concentration of bacteria from the blood samples and amplification of total bacterial DNA by overnight incubation of the bacteria on nylon filters to increase the sensitivity of the probe. This process of concentration is difficult to achieve because patients with typhoid fever usually have less than 15 *Salmonella* Typhi cells per ml of blood and the probe is not able to detect when the specimen is less than 500 bacterial cells. The

problem of sensitivity of DNA probe or specificity of the immuno-detection method could be circumvented by polymerase chain reaction (PCR), which can detect very little amount of DNA by way of enzymatic amplification, particularly in culture-negative cases.

Several PCR assays using different sets of primers targeting different sites of the *S.* Typhi genome have been developed to detect the microorganism. However, these methods are not fully specific for *S.* Typhi or involve multiple primers that increase the complexity of PCR systems. A PCR-based assay that can detect *S.* Typhi DNA by amplification of flagelin gene of S. Typhi in the blood of typhoid patients has been developed (Song *et al.*, 1993). In this process, two pairs of oligonucleotide primers were designed to amplify a 343 bp fragment of the flagelin gene. This flagelin gene targeting primers was subsequently used by many other researchers (Liu *et al.*, 1996; Kumar *et al.*, 2002). However, this PCR assay was not completely specific for *S.* Typhi as it also detects other *Salmonella* strains containing the dH flagelin such as *S.* Stanley, *S.* Livingston and *S.* Schwarzengrund. On the other hand, a nested PCR based on the *viaB* sequence to detect *S.* Typhi has been described (Hashimoto *et al.*, 1995). All the *S.* Typhi strains isolated from blood specimens possess the Vi antigens and the DNA sequence encoding the Vi antigen that is called the *viaB* regions. However, nested PCR method is time consuming, laborious and the major drawback of this method is the carryover contamination which often results in false positives. More recently, a multiplex PCR targeting the *tyv*, *prt*, *viaB* and *fliC* genes was developed for the simultaneous detection of *S.* Typhi and *S.* Paratyphi A (Hirose *et al.*, 2002). The *fliC- a* gene was probably selected because of its presumed restricted distribution to Paratyphi A strains However, the *prt* gene had been reported and was found to be more widely distributed (Hirose *et al.*, 2002). However, this method utilizes five pairs of primers in one assay and therefore is complex and requires optimization of each amplified product.

The infections of *S.* Typhi and *S.* Paratyphi A certainly can be distinguished effectively through the genomic approach. The application of this approach directly to various specimen dramatically reduces the time to final identification. Overall, the newly developed multiplex PCR detection system in this study has high specificity and sensitivity for a better control and surveillance of *S.* Typhi, *S.* Paratyphi A and other *Salmonella* spp.

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