DUPLEX PCR FOR DETECTION OF *SALMONELLA* AND *SHIGELLA* SPP IN COCKLE SAMPLES

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Abstract. *Salmonella* and *Shigella* spp are important causative agents of foodborne diseases. A sensitive, specific and rapid method is essential for detection of these pathogens. In this study, a duplex PCR method was developed for simultaneous detection of *Salmonella* and *Shigella* spp in cockle samples and compared with the traditional culture method. Enrichment broths for *Salmonella* spp recovery were also compared. Sensitivity of the duplex PCR for simultaneous detection of *Salmonella* and *Shigella* spp from pure culture was 10^3 CFU/ml (40 CFU/PCR reaction), and that of sterile cockle samples spiked with these two pathogens was 1 CFU/10 g of cockle tissue after 9 hours enrichment [3 hours in buffered peptone water (BPW), followed by 6 hours in Rappaport Vasiliadis (RV) broth or tetrathionate (TT) broth for *Salmonella* spp and 6 hours enrichment in *Shigella* broth (SB) for *Shigella* spp]. There was no significant difference in detection sensitivity between enrichment in RV and TT broths. *Salmonella* spp detected in cockles in Khon Kaen, Thailand by duplex PCR and culture method was 17% and 13%, respectively but *Shigella* spp was not detected. The duplex PCR technique developed for simultaneous detection of *Salmonella* and *Shigella* spp in cockle samples was highly sensitive, specific and rapid and could serve as a suitable method for food safety assessment.

Keywords: duplex PCR, culture method, *Salmonella* spp, *Shigella* spp, cockles

INTRODUCTION

Salmonella spp is one of the most important pathogens in foodborne illness worldwide (Guzman-Herrador *et al*, 2011; Lal *et al*, 2012; Severi *et al*, 2012). More than 2,500 serovars of *Salmonella* spp are considered pathogenic in humans and animals (Popoff *et al*, 2004). *Salmonella* spp are the causative agent of non-typhoid salmonellosis (gastroenteritis) and typhoid salmonellosis (enteric fever) (Portillo, 2000). *Shigella* spp is another group of foodborne pathogens responsible bacillary dysentery (shigellosis) (Warren *et al*, 2006). *Shigella* spp consists of four species: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* (Niyogi, 2005). *Salmonella* and *Shigella* spp commonly contaminate a variety of food including seafood (Heinitz *et al*,

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2000; Uyttendaele *et al*, 2001; Shabarinath *et al*, 2007), which is a common source of foodborne illness worldwide (Vaillant *et al*, 2005; Heier *et al*, 2009; Much *et al*, 2009) including Thailand (Chanachai *et al*, 2008). *Salmonella* spp is the most common causative agent of gastroenteritis associated with the consumption of contaminated shellfish (Archer and Young, 1988; Miescier *et al*, 1992; Wilson and More, 1996) and cockles eaten raw in Thailand constitute a reservoir of pathogens.

Culture method is the recommended method for detection of *Salmonella* and *Shigella* spp in food products but it is labor intensive, time consuming requiring several days to complete (Otero *et al*, 1998). Polymerase chain reaction (PCR) is a highly sensitive, highly specific and rapid method for detection of pathogens in food samples (Agarwal *et al*, 2002; Markoulatos *et al*, 2002; Senachai *et al*, 2013). Moreover, PCR can provide simultaneous detection of multiple pathogens in a single PCR reaction (Vantarakis *et al*, 2000; Li *et al*, 2005; Senachai *et al*, 2013). However, the PCR technique requires an enrichment step in a non-selective and/or selective medium in order to enhance sensitivity by increasing the number of cells and diluting PCR-inhibitory substances in food samples (Fluit *et al*, 1993; Vantarakis *et al*, 2000; Myint *et al*, 2006). The selective enrichment broth for *Salmonella* spp, Rappaport Vasiliadis (RV) and tetrathionate (TT) broths, are both recommended by the US Food and Drug Administration (US FDA, 2007). As multiple enrichment broths increases the cost of *Salmonella* detection, it would be useful if the use of enrichment broth is minimized.

In this study, *inv*A (encoding invasion protein A) (Shannon *et al*, 2007) and *ipa*H (encoding the invasion plasmid antigen H) (Vu *et al*, 2004) were selected for identification of *Salmonella* and *Shigella* spp, respectively, using a duplex PCR method for simultaneous detection of both pathogens in cockle samples and compared with two selective enrichment media in enhancing recovery of *Salmonella* spp in cockle samples.

MATERIALS AND METHODS

Preparation of pure culture

Salmonella and *Shigella* spp were separately grown in brain heart infusion (BHI) broth. An equal aliquot of mid log-phase culture of *Salmonella* and *Shigella* spp were combined and serially diluted ten-fold in sterile saline to obtain 10^0-10^7 CFU/ml. One ml aliquot of each dilution was used for extraction of DNA for use as template for duplex PCR. A mid log-phase culture of each bacterium was also serially diluted ten-fold in sterile saline and 0.1 ml aliquot of each dilution was used to determine the numbers of bacteria by plate counting. Experiments were performed three times independently.

Preparation of *Salmonella* and *Shigella* spp-spiked cockle samples

Cockle samples were prepared according to Agarwal *et al* (2002) and Blackstone *et al* (2003). In brief, 250 g of cockle samples were added to 250 ml of phosphate-buffered saline (PBS), and then 20 ml (10 g) aliquot of cockle suspension was homogenized in 80 ml of buffered peptone water (BPW) and 80 ml of *Shigella* broth (SB) for enrichment of *Salmonella* and *Shigella* spp, respectively. *Salmonella* and *Shigella* spp containing approximately 10^0-10^3 CFU from pure culture were added to the cockle homogenate in BPW and SB, respectively. The BPW samples were incubated at 37°C for 0, 3, 6, 12 and 24 hours, and each sample was subjected

to analysis using both duplex PCR and culture techniques. One ml aliquot of each suspension was transferred to 10 ml of tetrathionate (TT) broth and another 0.1 ml aliquot to 10 ml of Rappaport Vassiliadis (RV) broth and were incubated at 43ºC and 42ºC, respectively, for 6, 12 and 24 hours. The SB samples were incubated at 42ºC for 0, 3, 6, 12 and 20 hours; and each sample was used for analysis by both duplex PCR and culture methods. Experiments were conducted three times independently.

Duplex PCR

One ml aliquot of cockle suspension was centrifuged at 13,000*g* for 15 minutes. The pellet was washed twice with PBS and DNA was extracted using Puregene DNA purification kit according to the manufacturer's instructions (Puregene DNA Purification System, Gentra System, Big Lake, CA). DNA was extracted from pellets of TT and RV broths using the method of Myint *et al* (2006). In brief, the pellet was re-suspended in 100 l of sterile distilled water and heated at 95ºC for 10 minutes. Then the lysate was immediately placed on ice for 10 minutes and was used as the DNA template for the PCR assays.

New primers pair for amplification of *Salmonella* spp *inv*A (640 bp) and *Shigella* spp *ipa*H (232 bp) were 5'-GGCACTAATCGCAATCAACAATT-3' and 5'-CCTGATCGCACTGAATATCGTAC-3'; and'5'-AGTGCCTCTGCGGAGCTTCG-3' and 5'-GGAGAGTTCTGACTTTATCCCG-3', respectively. The reaction mixture (25 l) contained 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM of each dNTP, 1.5 M of primers for *Salmonella* spp and 0.1 M for *Shigella* spp, 1 U *Taq* polymerase and 500 ng (2 l) of DNA template in a total volume of 25 l. PCR thermocycling (Veriti Thermal Cycler, Applied Biosystems, Foster City, CA) conditions were: 94ºC for 10 minutes; 35 cycles of 94ºC for 30 seconds, 60ºC for 1 minute and 72ºC for 1 minute, and a final heating at 72ºC for 10 minutes. Amplicons were separated by 1.5% agarose gel electrophoresis at 100 V for 40 minutes and visualized under a UV transilluminator after ethidium bromide staining.

Culture method

A loopful of each enrichment broth prepared as described above was streaked on Salmonella-Shigella (SS) agar and xylose lysine deoxycholate (XLD) agar plates, which then were incubated at 37ºC for 24 hours. Colonies of *Salmonella* or *Shigella* spp were identified using biochemical tests (Washington, 2006).

Detection of *Salmonella* and *Shigella* spp in cockle samples

A total of 30 cockle samples obtained from markets and supermarkets in Khon Kaen were investigated for the presence of *Salmonella* and *Shigella* spp by both duplex PCR and culture methods. Cockle samples were prepared as described above and incubated in pre-enrichment BPW (24 hours) followed by selective RV/TT broth for *Salmonella* spp (24 hours) and SB broth for *Shigella* spp (20 hours). One ml aliquot and a loopful of BPW, RV/TT broth and SB was removed for analysis by duplex PCR and culture methods, respectively.

RESULTS

Sensitivity of duplex PCR in detecting pure cultures of *Salmonella* and *Shigella* spp

The limit of detection of *inv*A and *ipa*H duplex PCR from pure culture of *Salmonella* and *Shigella* spp was 10^3 CFU/ml (40 CFU/PCR) and 10^2 CFU/ml (4 CFU/PCR), respectively (Fig 1).

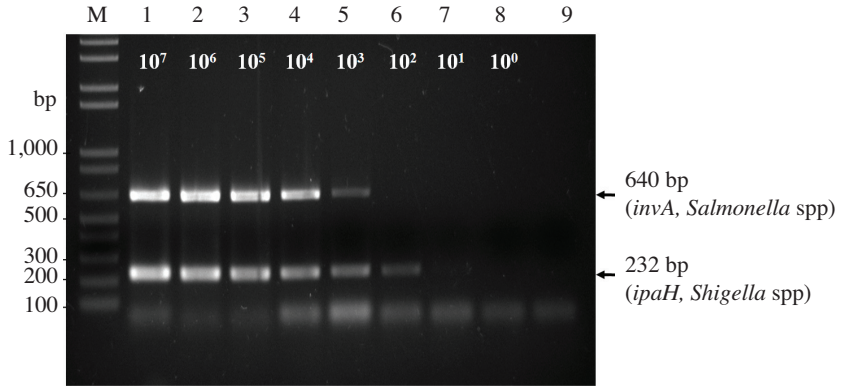

Fig 1–Sensitivity of duplex PCR for detection of *Salmonella* and *Shigella* spp from pure culture. A mid log-phase culture of *Salmonella* and *Shigella* spp were combined and serially diluted ten-fold to obtain 10^7-10^0 CFU/ml. Lane M, 100 bp DNA ladder; lanes 1-8, amplicons from 10^7, 10^6, 10^5, 10^4, 10^3,10^2, 10^1 and 10^0 CFU, respectively; lane 9, negative control.

Sensitivity of duplex PCR in detecting *Salmonella* and *Shigella* spp-spiked sterile cockle samples

The sensitivity of duplex PCR to detect *Salmonella* spp and *Shigella* spp in cockle samples was evaluated by spiking sterile cockle tissues with 10^0-10^3 CFU of *Salmonella* and *Shigella* spp with detection limit of 1 CFU/10 g of cockle tissues following 9 hours of enrichment (3 hours in BPW followed by 6 hours in RT/TT broth for *Salmonella* spp and 6 hours in SB for *Shigella* spp) (Table 1).

Comparison of effect of RV and TT enrichment broths on detection of *Salmonella* spp in cockle samples using PCR and culture methods

TT and RV broths were compared as selective enrichment broths after pre-enrichment in BPW using *Salmonella* spp-spiked cockle samples. After 15 hours enrichment (3 hours in BPW followed by 12 hours in TT or RV broth), *Salmonella* spp could be detected by PCR but not by culture method (Table 2), but after 36 hours enrichment (12 hours in BPW followed by 24 hours in TT or RV broth), *Salmonella* spp were detected in 2 of 10 samples by both PCR and culture methods (Table 2). RV broth seemed to be slightly better than TT broth when PCR was used, as after 12 hours in BPW followed by 12 hours or 24 hours in TT or RV broth, there were 3 more *Salmonella* spp- positive samples (30%) compared to 1 more sample (10%) using TT broth.

Detection of *Salmonella* and *Shigella* spp in cockle samples

All 30 cockle samples were tested for *Salmonella* and *Shigella* spp by both duplex PCR and culture methods using 24 hour incubation in BPW followed by 24 hours in RV broth and 20 hours in SB broth. *Shigella* spp were not detected in any of the 30 cockle samples using either method. *Salmonella* spp were detected in 17% (5/30) of cockle samples by duplex PCR and in 13% (4/30) by the culture method. *Salmonella* spp detected in cockle samples were *Salmonella* serovar Rissen, *Salmonella* serovar Mgulani, *Salmonella* serovar Typhimurium and *Salmonella enterica* subspecies *enterica*

Table 1
Sensitivity of duplex PCR in detecting *Salmonella* and *Shigella* spp spiked in sterile cockle samples after various enrichment conditions.

Enrichment condition	Presence of *Salmonella* and *Shigella* spp															
	Condition of *Shigella* spp enrichment (time and CFU)															
	SB 3 h				SB 6 h				SB 12 h				SB 20 h			
	10^0	10^1	10^2	10^3	10^0	10^1	10^2	10^3	10^0	10^1	10^2	10^3	10^0	10^1	10^2	10^3
BPW 3 h + TT 6 h	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
BPW 3 h + RV 6 h	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
BPW 3 h + TT 12 h																
BPW 3 h + RV 12 h																
BPW 3 h + TT 24 h	+/-								+/+							
BPW 3 h + RV 24 h																
BPW 6, 12, 24 h + TT 6, 12, 24 h																
BPW 6, 12 ,24 h + RV 6, 12, 24 h																

+/-, presence of *Salmonella* spp/absence of *Shigella* spp; +/+, presence of *Salmonella* spp/presence of *Shigella* spp; BPW, buffered peptone water; RV, Rappaport Vassiliadis; SB, *Shigella* broth.

serovar 4, 5, 12 :i :- (a serotype antigenically similar to *Salmonella enterica* serotype Typhimurium) (Torre *et al*, 2003).

DISCUSSION

Sensitivity of duplex PCR for simultaneous detection of *Salmonella* and *Shigella* spp was evaluated using both pure culture and spiking sterile cockle samples with the two pathogens. For pure culture, duplex PCR had a sensitivity of 10^3 CFU/ml or 40 CFU/PCR reaction compared with a previous study of 10^4 CFU/ml *Salmonella* spp from pure culture (Upadhyay *et al*, 2010), whereas Rahn *et al* (1992) reported a sensitivity of $3x10^2$ CFU/ml *Salmonella* spp by uniplex PCR**.** Sensitivity of duplex PCR was better than the culture method in sterile cockle samples spiked with *Salmonella* and *Shigella* spp. After 9 hour enrichment, *Salmonella* and *Shigella* spp were detected by duplex PCR at 1 CFU/10 g of cockle, a shorter period of time than that reported by Vantarakis *et al* (2000), which required a 22 hour enrichment before multiplex PCR could detect *Salmonella* and *Shigella* spp in 10-100 cells/ml of mussel homogenate, or that of Li *et al* (2005) requiring 24 hour enrichment for multiplex PCR to detect *Salmonella* and *Shigella* spp at approximately 1 CFU/g of raw and ready-to- eat meat products.

Salmonella spp was not found in the pre-enrichment broth (BPW), as previously reported but could be detected in the selective enrichment broth (Upadhyay *et al*, 2010). In the current study, we compared both TT and RV as selective enrichment broths of *Salmonella* spp spiked sterile cockle and unspiked cockle samples. The sensitivity in detecting

Table 2
Detection by PCR and culture methods of *Salmonella* spp in 10 cockle samples after various enrichment conditions.

Enrichment conditions	Number of positive samples		
	PCR +/culture -	PCR -/culture +	PCR +/culture +
BPW 3 h + TT 12 h	1	0	0
BPW 3 h + RV 12 h	1	0	0
BPW 3 h + TT 24 h	2	0	0
BPW 3 h + RV 24 h	2	0	0
BPW 6 h + TT 12 h	1	0	1
BPW 6 h + RV 12 h	1	0	1
BPW 6 h + TT 24 h	1	0	1
BPW 6 h + RV 24 h	1	0	1
BPW 12 h + TT 12 h	1	0	1
BPW 12 h + RV 12 h	1	0	1
BPW 12 h + TT 24 h	1	0	1
BPW 12 h + RV 24 h	1	0	1
BPW 24 h + TT 12 h	1	0	1
BPW 24 h + RV 12 h	**2**	0	1
BPW 24 h + TT 24 h	0	0	2
BPW 24 h + RV 24 h	**1**	0	2
Total	18	0	14

BPW, buffered peptone water; RV, Rappaport Vassiliadis; SB, *Shigella* broth.

Salmonella spp grown in the two types of selective enrichment broths was not significantly different using either PCR or culture methods. Although the number of cockle samples employed for detecting *Salmonella* spp was small, RV enrichment broth appeared slightly better than TT broth in agreement with previous reports (Schlundt *et al*, 1993; Kumar *et al*, 2010). In order to reduce the cost and effort of *Salmonella* spp detection, we suggest that RV broth be only used for high recovery of *Salmonella* spp, while both selective enrichment broths be used together for recovery of all *Salmonella* strains. The duration of enrichment resulting in the highest sensitivity for detecting *Salmonella* and *Shigella* spp in cockle samples by both duplex PCR and culture methods was pre-enrichment in BPW for 24 hours and in RV or TT for 24 hours. Using these condition, percent *Salmonella* spp detected by duplex PCR was higher (17%) than by culture (13%) methods, consistent with the report of the prevalence of *Salmonella* in shrimp samples collected from four different shrimp farms and fresh food markets around Bangkok of 24% and 19% by PCR and culture method, respectively (Upadhyay *et al*, 2010). Similarly the prevalence of *Salmonella* in oysters and clams in India was 33.3% and 11.1% detected by PCR and culture methods, respectively (Shabarinath *et al*, 2007). In cold temperature seawater, prevalence of *Salmonella* detected in oysters from both coasts of the United States is lower (7.4%) (Brands *et al*, 2005).

Shigella spp were not found in the cockle samples in the current study using all three methods. A multiplex PCR method for detection of *Salmonella* and *Shigella* spp in mussels could only detect *Salmonella* spp and not *Shigella* spp (Vantarakis *et al*, 2000).

In summary, duplex PCR method was more sensitive and rapid than the traditional culture method in cockle samples contaminating with *Salmonell*a and *Shigella* spp. In spiked sterile cockle samples, 9 hour enrichment (BPW 3 hours + TT/RV broth 6 hours) and 18 hours (BPW 6 hours + TT/RV broth 12 hours) was sufficient for detection of those pathogens by PCR and culture method, respectively. As there is a highly competitive suite of microbiota in natural cockles compared to spiked sterile cockles, a longer selective enrichment time was needed, namely 36 hours (24 hours BPW + 12 hours RV broth) and 48 hours (24 hours BPW + 24 hours RV broth) by duplex PCR and culture method, respectively. Extrapolation of results from spiked sterile cockle samples to those in unspiked cockles should be done with caution.

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