APPLICATION OF TETRAPLEX PCR FOR DETECTION OF *VIBRIO CHOLERAE*, *V. PARAHAEMOLYTICUS*, *V. VULNIFICUS* AND *V. MIMICUS* IN COCKLE

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Abstract. A tetraplex PCR method was developed for simultaneous detection of *Vibrio cholerae, V. parahaemolyticus, V. vulnificus* and *V. mimicus* in cockle samples in comparison with conventional culture method. Specific primers targeting *omp*W of *V. cholerae*, *tl* of *V. parahaemolyticus, hsp*60 of *V. vulnificus* and *sod*B of *V. mimicus* were employed in the same PCR. Detection limit of the tetraplex PCR assay was 10^4 cfu/ml (400 cfu/PCR reaction) for pure cultures of all four species of *Vibrio*. In *Vibrio* spiked cockle samples, the limit of detection after 6 hours enrichment in alkaline peptone water was 1 cfu/10 g of cockle tissue for three *Vibrio* spp, except for *V. mimicus* that was 10^2 cfu/10 g of cockle tissue. When the tetraplex PCR and culture methods were applied to 100 cockle samples, *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae* and *V. mimicus* were detected in 100, 98, 80 and 9% of the samples by tetraplex PCR and in 76, 42, 0 and 0% by the culture method, respectively. This developed tetraplex PCR method should be suitable for simultaneous and rapid detection of *Vibrio* species in food samples and for food safety assessment.

Keywords: *Vibrio cholerae, V. parahaemolyticus, V. vulnificus, V. mimicus*, tetraplex PCR, cockle

INTRODUCTION

The genus *Vibrio* is small, comma-shaped bacterium, which occurs naturally in aquatic environment as commensal and symbiont in estuarine and marine animals (including zooplankton, crustaceans and molluscs) (Colwell and Hug, 1994; Thompson *et al*, 2004). Among the different *Vibrio* spp, *V. cholerae*, *V. parahaemolyticus, V. vulnificus* and *V. mimicus* are the species linked to human diseases especially gastroenteritis (Chakraborty *et al*, 1997; Panicker *et al*, 2004a; Shinoda and Miyoshi, 2011). *V. cholerae* O1/O139 is the causative agent of cholera which is endemic in less developed and developing countries (Wiwanitkit, 2008; Nguyen *et al*, 2009). *V. parahaemolyticus* is a gastroenteritis pathogen associated with consumption of raw or uncooked seafood especially shellfish (Robert-Pillot *et al*, 2004;

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McLaughlin *et al*, 2005); *V. vulnificus* is the cause of septicemia, wound infection and gastroenteritis associated with consumption of raw shellfish and exposure to contaminated water (Penman *et al*, 1995; Linkous and Oliver, 1999), causing high morbidity among immunocompromized patients (Jones and Oliver, 2009); and *V. mimicus* is also one of the causative agents of gastroenteritis (Takahashi *et al*, 2007) associated with eating raw shellfish (Hlady and Klontz, 1996) causing sporadic diarrhea in many countries including Japan (Shinoda *et al*, 2004) and Thailand (Chitov *et al*, 2009). As infection with these four *Vibrio* spp is commonly associated with gastroenteritis, a rapid and sensitive detection is essential both from food safety and from epidemiologic perspectives.

Currently, the conventional standard microbiological method is based on phenotypic identification, which requires several days to carry out the enrichment step, cultivation and biochemical tests (Huq *et al*, 2006). Some *Vibrio* spp can cause problems owing to variability in biochemical characteristics within species (Kwok *et al*, 2002; Thompson *et al*, 2004), and can become a "viable but non-culturable" (VBNC) organism resulting in unsuccessful isolation of some *Vibrio* spp (Binsztein *et al*, 2004; Trevors, 2011).

A molecular biological method, such as polymerase chain reaction (PCR), is more rapid, sensitive and specific than standard culturing methods for detection of low microbial concentrations and detection of VBNC pathogens (Binsztein *et al*, 2004; Gugliandolo *et al*, 2010). Moreover, multiplex PCR (m-PCR) can simultaneously detect several targets in a single reaction. Additionally, PCR can be used for large-scale screening detection of several pathogens, such as *Aeromonas* spp, *Salmonella* spp, *V. cholerae, V. parahaemolyticus* and *V. vulnificus* (Gugliandolo *et al*, 2010). A number of multiplex PCR assays have been developed for detection of *Vibrio* spp; however, certain complications have not been resolved, *eg* differentiation of closely related species and simultaneous detection of many species in the same sample (Nhung *et al*, 2007; Tarr *et al*, 2007). Moreover, sources and methods used in these studies were varied, depending on the types of samples.

We, therefore, developed a tetraplex PCR method for detection of *Vibrio* spp in cockle samples in comparison with conventional culture method. For specific identification of a bacterial species by PCR, selection of an appropriate target is vital. We designed primers specific for *hsp*60, encoding heat shock protein 60 (Kwok *et al*, 2002), to target *V. vulnificus*; modified primers for *sod*B, encoding iron superoxide dismutase (Tarr *et al*, 2007) to target *V. mimicus* (Goel *et al*, 2007); primers for *omp*W, encoding the outer membrane protein, to target specific species of *V. cholerae* (Goel *et al*, 2007); and primers for *tl*, encoding thermolabile hemolysin to target *V. parahaemolyticus* (Pinto *et al*, 2007). The tetraplex PCR was compared with the conventional culture method used for detection of the four *Vibrio* spp in cockle samples.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were obtained from various sources, including American Type Culture Collection (ATCC), Department of Medical Sciences Thailand Culture Collection (DMSC), and clinical and environmental sources at Srinagarind Hospital and Department of Microbiology Laboratory, Faculty of Medicine, Khon Kaen University, Thailand (Table 1).

Table 1
Specificity of PCR assay for *Vibrio* spp.

Strain	Source	No.of strains	*V. cholerae*	*V. parahaemolyticus*	*V. vulnificus*	*V. mimicus*
			*omp*W	*tl*	*hsp*60	*sod*B
V. cholerae O1	Clinical strain	5	5		0	0
V. cholerae O139	Environmental strain	1	1	0	0	0
V. cholerae non O1/O139	Clinical strain	1	1	0	0	0
	environmental strain	1	1	0	0	0
V. parahaemolyticus	ATCC 17802	1	0	1	0	0
V. vulnificus	ATCC 27562	1	0	0	1	0
V. mimicus	ATCC 33653	1	0	0	0	1
V. alginolyticus	DMST 14800	1	0	0	0	0
V. fluvialis	DMST 19347	1	0	0	0	0
Aeromonas hydrophila	Clinical strain	1	0	0	0	0
Plesiomonas shigelloides	Clinical strain	1	0	0	0	0
Salmonella spp	Clinical strain	8	0	0	0	0
Shigella dysenteriae	DMST 15111	1	0	0	0	0
Shigella flexneri	DMST 4423	1	0	0	0	0
Shigella boydii	DMST 28180	1	0	0	0	0
Shigella sonnei	ATCC 11060	1	0	0	0	0
Enterobacter spp	Clinical strain	1	0	0	0	0
Escherichia coli	ATCC 25922	1	0	0	0	0
Proteus vulgaris	Clinical strain	1	0	0	0	0
Klebsiella spp	Clinical strain	1	0	0	0	0
Pseudomonas aeruginosa	Clinical strain	1	0	0	0	0
Staphylococcus aureus	ATCC 25923	1	0	0	0	0
Staphylococcus epidermidis	Clinical strain	1	0	0	0	0
Listeria monocytogenes	Clinical strain	1	0	0	0	0
Micrococcus spp	Clinical strain	1	0	0	0	0
Bacillus spp	Clinical strain	1	0	0	0	0

ATCC, American Type Culture Collection; DMST, Department of Medical Sciences Thailand Culture Collection

Preparation of cockle samples

Cockle sample preparation was modified from that of Blackstrone *et al* (2003) and Canigral *et al* (2009). In brief, 250 g of each cockle sample were cut into small pieces and suspended in 250 ml of phosphate-buffered saline (PBS).

PCR primers specificity and sensitivity assay

Primer pair of *hsp*60 for identification of *V. vulnificus* (Accession no. FJ646619) was newly designed and primer pair of *sod*B for identification of *V. mimicus* (Accession no. AB050800) was modified from those of Tarr *et al* (2007). Specificity of these two new primer pairs was tested using BLAST of NCBI nucleotide public database. Specificity of the primers was also evaluated by PCR assay using 100 ng of DNA template of the strains listed in Table 1. Specificity of primers used for *V. cholerae* and *V. parahaemolyticus* had previously been verified by Wongboot

et al (unpublished). The target genes and oligonucleotide primers used in tetraplex PCR for detection of *Vibrio* spp are listed in Table 2.

Sensitivity of tetraplex PCR for detection of *Vibrio* spp was performed according to Kong *et al* (2002). In brief, a mid-log phase culture of four reference strains of *Vibrio* spp mixed in equal numbers was serially diluted ten-fold in sterile saline to obtain 10^7-10^0 cfu/ml. DNA from 1 ml of each dilution was extracted using DNA purification kit (Puregene DNA Purification System, Gentra Systems, Big Lake, MN) according to the manufacturer's instructions and 2 l used as template DNA for tetraplex PCR. Each experiment was performed in triplet.

Bacterial suspensions containing approximately 10^0-10^3 cfu were added to 20 ml (10 g) of sterile cockle suspension in PBS, and the cockle suspension was homogenized in 80 ml of alkaline peptone water (APW), pH 8.6 (Oxoid, Hampshire, England) and incubated at 37ºC for 0, 3, 6 and 18 hours. Each sample was analyzed by both tetraplex PCR and culture methods. Three independent experiments were performed.

Tetraplex PCR assay

DNA template for tetraplex PCR reaction was extracted using a genomic DNA purification kit (Puregene DNA Purification System, Gentra Systems, Big Lake, MN) according to the manufacturer's instructions. Amplification reaction of the target genes for *Vibrio* spp was conducted in a 25- l reaction volume containing 0.3 mM of each dNTP, 2.0 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 U *Taq* polymerase (RBC Bioscience, San Diego, CA), 500 ng (3 l) of target DNA and primers at concentrations listed in Table 2. PCR thermocycling performed using a thermocycler (Veriti Thermal Cycler, Applied Biosystems, Foster City, CA) was as follows: 94ºC for 10 minutes; 35 cycles of 94ºC for 40 seconds, 60ºC for 1 minute, 72ºC for 1 minute; and a final heating at 72ºC for 7 minute. Amplified DNA was analyzed by 3% agarose gel-electrophoresis and visualized under UV light transilluminator after ethidium bromide staining.

DNA probe hybridization

To confirm the specificity of PCR amplicons produced by tetraplex PCR, amplicons were probed with specific oligonucleotides prepared from the control strains. The DNA probe hybridization was performed using DIG high prime DNA labeling and detection starter kit I (Roche Diagnostic, Manntein, Germany) according to the manufacturer's instructions.

Culture method

A loopful (5 l) of enrichment culture was streaked onto thiosulfate-citrate-bile-salt-sucrose (TCBS) agar (Eiken, Japan) and incubated at 37ºC for 18-24 hours. Colonies were identified using standard biochemical tests (Ramamurthy and Nair, 2007).

RESULTS

Specificity of PCR primers

Specificity of all four pairs of primers was evaluated in the present work using the conditions optimized for tetraplex PCR. The results revealed that no amplicon was produced using DNA template from other bacteria (Table 1). The tetraplex PCR yielded amplicon size of 307, 211, 150 and 123 bp for *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. mimicus*, respectively (Fig 1A). Tetraplex PCR amplicons of *Vibrio* spp were confirmed

Table 2
Primers used in this study.

Identification	Target gene	Primer sequence (5′→3′)	Amplicon size (bp)	Primer (M)	Reference
V. cholerae	Outer membrane protein (*omp*W)	F-GTACTTGCAGCCCTAAGCTC R-GGACCATAAAGGTAGGTGGC	307	0.6	Wongboot W, unpublished
V. parahaemolyticus	Thermolabile hemolysin *(tl)*	F-CCACATTAGATTTGGCGAACGA R-CAGACAAGCTGTCACCGAGT	150	0.4	Wongboot W, unpublished
V. vulnificus	Heat shock protein 60 *(hsp*60*)*	F-ACGCTGCCAGACTCTTGATT R-AAATCGAGCAAGTAGGCAC	211	0.4	This study
V. mimicus	Iron-cofactored superoxide dismutase (*sod*B)	F-GCATTCGGTTCTTTCGCTGAT R-TGAAGTGTTAGTGATTGCTAGAGAT	123	1.0	Modified from Tarr *et al*, 2007

Table 3
Detection using tetraplex PCR and culture methods of spiked *Vibrio* spp in cockle samples after various enrichment time.

Vibrio spp	Amount of *Vibrio* spp (cfu/10 g)	Tetraplex PCR Enrichment in APW (h)				Culture method Enrichment in APW (h)			
		0	3	6	18	0	3	6	18
V. cholerae	0	-	-	-	-	-	-	-	-
	10^0	-	+	+	+	-	-	+	+
	10^1	-	+	+	+	-	+	+	+
	10^2	-	+	+	+	-	+	+	+
	10^3	-	+	+	+	-	+	+	+
V. parahaemolyticus	0	-	-	-	-	-	-	-	-
	10^0	-	-	+	+	-	-	-	+
	10^1	-	+	+	+	-	-	+	+
	10^2	-	+	+	+	-	+	+	+
	10^3	-	+	+	+	-	+	+	+
V. vulnificus	0	-	-	-	-	-	-	-	-
	10^0	-	-	+	+	-	-	-	+
	10^1	-	-	+	+	-	-	+	+
	10^2	-	+	+	+	-	+	+	+
	10^3	-	+	+	+	-	+	+	+
V. mimicus	0	-	-	-	-	-	-	-	-
	10^0	-	-	-	-	-	-	-	-
	10^1	-	-	-	-	-	-	-	-
	10^2	-	+	+	+	-	-	+	+
	10^3	-	+	+	+	-	+	+	+

by DNA hybridization.

Sensitivity of tetraplex PCR assay of pure bacterial culture

The sensitivity of tetraplex PCR for detection of the four *Vibrio* spp was determined using 10-fold serial dilutions of bacterial mixtures in combination with the plate counting method to determine the detection limit. The detection limit of tetraplex PCR assay was 10^4 cfu/ml (400 cfu/PCR reaction) for all four *Vibrio* spp (Fig 1A).

Sensitivity of tetraplex PCR assay of spiked *Vibrio* spp in cockle samples

The detection limits of the tetraplex PCR and culture methods were tested with inocula at levels ranging from 10^0-10^3 cfu/10 g of cockle tissue. The results showed that the detection limit of tetraplex PCR for detection of all 4 *Vibrio* spp spiked in cockle samples after 3 hours enrichment was 10^2 cfu/10 g of cockle tissue for *V. vulnificus* and *V. mimicus*, whereas *V. cholerae* and *V. parahaemolyticus* was detected at 1 and 10 cfu/10 g of cockle tissue. After 6 hours enrichment, tetraplex PCR detected 1 cfu/10 g of cockle tissue for three *Vibrio* spp but not for *V. mimicus*, whereas the culture method was able to detect all 4 *Vibrio* spp at 10^2 cfu/10 g of cockle tissue. After 18 hour enrichment, both tetraplex PCR and cuture methods detected 1 cfu/10 g of cockle tissue for three *Vibrio* spp but not for *V. mimicus* (Table 3).

Detection of *Vibrio* spp in cockle samples

A total of 100 cockle samples were analyzed for *Vibrio* spp in 6 hours enrichment broth by both tetraplex PCR and culture methods. Using culture method, *V. parahaemolyticus* was detected in 76% and *V. vulnificus* in 42% of cockle samples and none for *V. cholerae* and *V. mimicus*. Using tetraplex PCR, *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae* and *V. mimicus* was detected in 100, 98, 80 and 9% of cockle samples, respectively (Table 4). Mixed *Vibrio* spp contaminations were detected (Fig 1B). Mixed *Vibrio* spp were found in quadruple, triple, and double infections in 19, 59, and 21 samples, respectively.

Table 4
Detection of *Vibrio* spp in 100 cockle samples by tetraplex PCR and culture methods after 6 hour enrichment.

Vibrio spp	Number of samples positive for *Vibrio* spp	
	Tetraplex PCR	Culture method
V. cholerae	80	0
V. parahaemolyticus	100	76
V. vulnificus	98	42
V. mimicus	9	0

DISCUSSION

The culture method has long been accepted as the gold standard for detecting *Vibrio* spp; however, the method is time-consuming, labor-intensive and unable to detect VBNC *Vibrio* spp, leading to false negative results (Oliver, 1995; Chomvarin *et al*, 2007). A previous study showed that multiplex PCR for detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. mimicus* was possible but sensitivity was not determined nor was the method applied to specimens (Tarr *et al*, 2007). In the current study, we developed a tetraplex PCR method for direct detection and differentiation of four human pathogenic *Vibrio* spp in cockle samples and compared it with the standard culture method.

Several studies reported that sensitivity of pentaplex PCR for detection of *Vibrio*

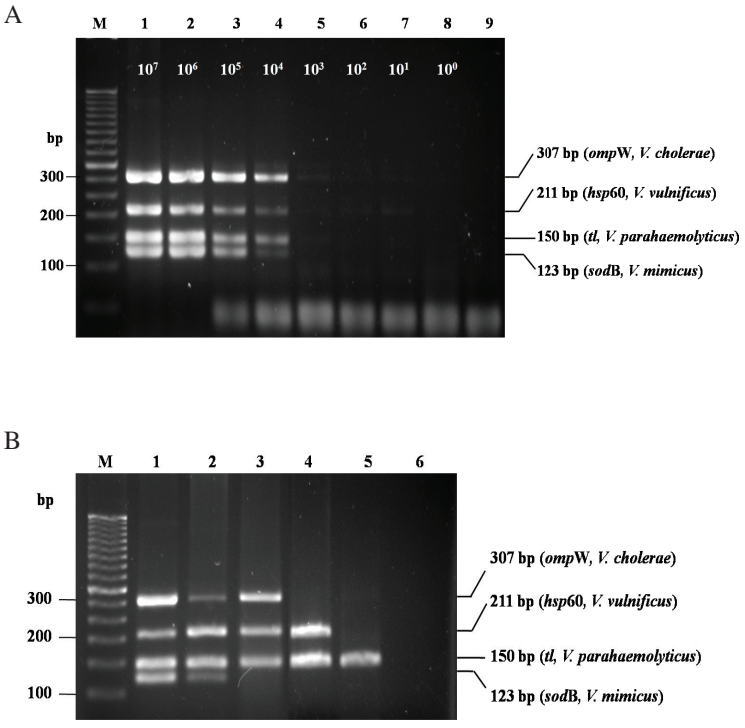

Fig 1- Gel electrophoresis of amplicons from tetraplex PCR. A. Detection of *V. cholerae, V. parahaemolyticus, V. vulnificus* and *V. mimicus* cultures. Lanes 1-8, bacterial culture at 10^7,10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 cfu/ml, respectively; lane 9, negative control; lane M, 50 bp DNA markers. B. Detection of *Vibrio* spp in natural cockle samples. Lane 1, positive control of four *Vibrio* spp; lane 2, cockle sample positive for *V. cholerae, V. parahaemolyticus, V. vulnificus* and *V. mimicus*; lane 3, cockle sample positive for *V. cholerae, V. parahaemolyticus* and *V. vulnificus*; lane 4, cockle sample positive for *V. parahaemolyticus* and *V. vulnificus*; lane 5, cockle sample positive for *V. parahaemolyticus*; lane 6, negative control; lane M, 50 bp DNA markers.

spp was 10^5 cfu/ml for *V. cholerae* and *V. vulnificus* and 10^6 cfu/ml for *V. parahaemolyticus, V. alginolyticus* and *V. mimicus* in stool samples (Nhung *et al*, 2007). After enrichment for 6 hours, our optimized tetraplex PCR condition achieved a detection limit of 1 cfu/10 g of spiked cockle tissue for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, and 10^2 cfu/10 g for *V. mimicus*.

Sensitivity of a detection method depends on several factors, such as the target gene, amount of template DNA, amount of *Taq* DNA polymerase, relative primer concentrations, and balance between $MgCl_2$ and dNTPs concentrations. Optimization of the PCR is essential in order to reduce the competition for the PCR reagents by individual targets before applying the assay to specimens (Markoulatos *et al*, 2002; Nordstrom *et al*, 2007; Elizaquivel *et al*, 2008).

The enrichment process is also a very important step to increase the numbers of *Vibrio* spp and it helps the recovery of injured cells (Panicker *et al*, 2004b). In addition, the enrichment step helps to dilute inhibitors of PCR, thereby reducing the number of false negative results (Chomvarin *et al*, 2007). Previous studies have reported that enrichment for 5 hours was sufficient for multiplex PCR detection coupled with microarray hybridization of 1 cfu/g of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in oyster samples (Panicker *et al*, 2004b). Tyagi *et al* (2009) reported that after 6 hour enrichment, *V. parahaemolyticus* could be detected by SYBR green quantitative PCR at 1 cfu/g of shrimp homogenate. Our findings showed that 6 hours of enrichment prior to tetraplex PCR is sufficient

for detection of 1 cfu/10g of three *Vibrio* spp in cockle samples except for *V. mimicus* that required 10^2 cfu/10g. The culture method was 10-fold less sensitive. The explanation for the low sensitivity of *V. mimicus* in both tetraplex PCR and culture methods may be due to overgrowth of the other *Vibrio* spp, thereby resulting in the lower ability to compete for PCR primer reagents or to grow on TCBS plate.

The higher sensitivity of PCR over culture method may be the result of bacteria entering into VBNC state (Canigral *et al*, 2009; Vezzulli *et al*, 2009): this is not a limitation for the PCR assay as it is able to detect both culturable and VBNC cells (Colwell, 2000; Lipp *et al*, 2003). It is interesting to note that most of the *V. cholerae* detected in cockle samples belong to *V. cholerae* O1 serogroup (data not shown), suggesting that the risk for cholera disease is because VBNC bacteria are transmitted to humans without being detected by the culture method. Therefore cleaning and heat cooking of cockles should be performed before consumption in order to reduce foodborne illness, and consumption of uncooked cockles should be avoided.

In conclusion, the tetraplex PCR developed in this study is a specific, sensitive and rapid method for simultaneous and direct detection of *V. cholerae, V. parahaemolyticus, V. vulnificus* and *V. mimicus* in cockle samples. The method can be used for identifying the causative agents of gastroenteritis in suspected food samples and for rapid monitoring of transmission of these bacteria in food procedures, thereby enabling appropriate prevention measures to be taken to prevent the occurrence of diarrheal diseases.

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