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Original Article

Occurrence of *Vibrio parahaemolyticus* in cockle (*Anadara granosa*) harvested from the south coast of Thailand

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Abstract

Vibrio parahaemolyticus is a seafood-borne pathogen that is widely distributed in the marine environment. The occurrence of *Vibrio parahaemolyticus* in cockle (*Anadara granosa*) harvested at the south coast of Thailand was determined. A total of 65 samples were collected monthly. They were examined by polymerase chain reaction (PCR). *Vibrio parahaemolyticus* was found in all samples. The virulence strain carrying the thermostable direct hemolysin (*tdh*) and TDH-related hemolysin gene (*trh*) was found in samples collected in July. This information can be used for the risk-assessment plan and eating cooked cockle is recommended.

Keywords: cockle, *Vibrio parahaemolyticus*, *tdh*, *trh*, *toxR*

1. Introduction

Vibrio parahaemolyticus is widely spread in marine environments and can be detected in various types of seafood throughout the world (Tepliski *et al*., 2009). It is also known as a common food-borne pathogen in Asia (Cheng *et al*., 2008). It is a food borne-pathogen and a major cause of gastroenteritis particularly from traditional consumption of raw or undercooked seafood (Mead *et al*., 1999). However, not all the environmental strains are considered pathogenic. Clinical isolates of *V. parahaemolyticus* most often produce either the thermostable direct haemolysin (TDH) or TDH-related haemolysin (TRH) encoded by *tdh* and *trh* genes, respectively (Zhang and Austin, 2005). Therefore, for disease prevention, after isolation of *V. parahaemolyticus* in seafood, isolated strains were characterized to fully evaluate the risk posed by the implicated food. The detection of *V. parahaemolyticus* in seafood product is mostly performed by conventional cultural methods followed by biochemical identification of the isolated strains. To date, miniature biochemical tests API 20E, API 20NE and Alsina's scheme systems have been developed for identification of some isolates of the Vibrionaceae family. However, they resulted in false-positives or false-negatives in all the biochemical identification methods (Croci *et al*., 2007).

The conventional method to detect pathogenic *V. parahaemolyticus* carrying *tdh* exploits their ability to produce β-haemolysis on a high-salt blood agar called Wagatsuma agar (Wagatsuma, 1968). The reaction is called Kanagawa phenomenon (KP). It requires fresh human or rabbit blood and tends to produce false-positive reactions (Chun *et al*., 1975); furthermore, some of KP-negative strains on Watgatsuma agar were found to carry the *trh* gene (Nishibuchi *et al*., 1985). However, there is no commercially

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available detection method for TRH-producing strains. Therefore, DNA-based molecular technique using polymerase chain reaction (PCR) was applied to detect the virulence gene of *V. parahaemolyticus* in seafood products as it is a rapid test and is highly specific. Among the different PCR methods developed in recent years for *V. parahaemolyticus* identification, several researchers have proposed a PCR method targeted to *toxR*, a gene involved in the regulation in many *Vibrio*s (Kim *et al*., 1999), pR72H *Hind*III DNA fragment of *V. parahaemolyticus* (Robert-Pillot *et al*., 2002) and a thermolabile haemolysin gene (*tl*) which is not associated with illness in humans. This *toxR* targeting gene was shown to be present in all of the *V. parahaemolyticus* tested (Taniguchi *et al*., 1986) and was therefore used to detect this species in shellfish (Bej *et al*., 1999). The identification protocol of *V. parahaemolyticus* through the detection of *toxR* gene was able to specifically identify this strain, and did not produce any false-positive result on the strains belonging to other *Vibrio* species (Croci *et al*., 2007). PCR-based methods targeting *tdh* and *trh* genes have been developed for more sensitive detection of pathogenic *V. parahaemolyticus* (Karunasagar *et al*., 1996).

Previous conventional methods for the detection of *V. parahaemolyticus* and virulence strains have been restricted to labor- and time-intensive testing of individual isolates by phenotypic assays, which restricts the number of isolates that can be obtained and tested from a given sample. Therefore, the aims of this study were to determine the occurrence of *V. parahaemolyticus* presence in cockles at a harvesting area and to detect the presence of virulent isolates carrying the *tdh* and *trh* genes by a PCR-based method.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

The bacterial strains used in this experiment were obtained from department of medical science, Thailand. *Vibrio* species (non-virulence and virulence *V. parahaemolyticus*, *V. alginolyticus, V. cholerae*), and other bacteria (*E. coli* O 157:H7, *Shigella* spp. and *Salmonella typhi*, *S. paratyphi* and *S. enteritidis*) were used as reference strains. Tryptic soy agar (TSA) (Himedia, India) or tryptic soy broth (TSB) (Himedia, India) supplemented with 2% NaCl was used for growth of all *Vibrio* species. Other bacterial cultures were grown on TSA or TSB medium.

2.2 Specificity and sensitivity of PCR analysis

The DNA prepared from reference strains by modified Sambrook method (1989) were used as templates to evaluate the specificity and sensitivity of the PCR assay. For sensitivity experiments, DNA of *V. parahaemolyticus* (tdh^+, trh^+) was serially diluted with TE buffer and subjected to PCR amplification. At the same time, *V. parahaemolyticus* (tdh^+, trh^+) culture was grown overnight at 37°C, washed by 0.85% (w/v) sterile physiological saline, and diluted serially to 10^8-10^1 CFU ml^{-1} to determine the detection limit of the PCR.

2.3 DNA extraction

Total genomic DNA was extracted by modified Sambrook method (1989) as follows: 1 ml of cell culture was centrifuged at 8,000 g for 5 min and suspended the pellet with 450 µl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA); 25 µl of 10% SDS was added into the reaction and mixed well by inverting, and the tube was incubated at room temperature for 30 min to lyse the cells. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed vigorously. The mixer was centrifuged at 8,000 g 10 min and 400 µl of clear supernatant was transferred to a new tube to which 40 µl of 3M sodium acetate pH 5.5 was then added. After gentle mixing, DNA was precipitated by adding 2 volumes of absolute ethanol and 70% ethanol. Purified DNA was suspended in 100 µl sterilized distilled water and measured for DNA quality by spectrophotometer at 260 and 280 nm. One microlitre of purified DNA was used for PCR amplification.

2.4 Detection of *toxR*, *tdh* and *trh* genes by PCR reaction

PCR reaction for detection of *V. parahaemolyticus* was performed separately for *toxR*, *tdh* and *trh* genes. DNA amplification was performed in a final volume of 25 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 50 pmole of each primer, 2.5 Unit of *Taq* DNA polymerase (Promega, USA), and 1 µl of supernatant containing DNA. The presence of the *toxR* gene was determined by using following primers described by Kim *et al*. (1999): 5' -GTC TTC TGA CGC AAT CGT TG-3' (forward) and 5' -ATA CGA GTG GTT GCT GTC ATG-3' (reverse), which produced a 368 bp amplicon. The reactions were performed with a PTC 100 thermocycler (M.J. Research, Watertown, MA, USA) as follows: 1 min of initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, alignment at 63°C for 1.5 min, and extension at 72°C for 1.5 min and final extension at 72°C for 7 min. The *tdh* and *trh* gene were amplified with the following primer sets: 5' -CCA CTA CCA CTC TCA TAT GC-3' (forward) and 5' -GGT ACT AAA TGG CTG ACA TC-3' (reverse) for *tdh*; and 5' -GGC TCA AAA TGG TTA AGG G-3' (forward) and 5' -CAT TTC CGC TCT CAT ATG C-3' (reverse) for *trh* (Tada *et al*., 1992). These primer sets produced 251 and 250 bp amplicons, respectively. The reaction was performed as follows: 1 min of initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, alignment at 55°C for 1 min, and extension at 72°C for 1 min and final extension at 72°C for 7 min. The PCR products were separately resolved by electrophoresis in 1.5% agarose gel in Tris-borate-EDTA buffer at 100 V for 20 min. The gels were visualized with a UV transilluminator.

2.5 Sample processing and detection of *V. parahaemolyticus*

A total of 65 samples of cockles (*Anadara granosa*) obtained from coastal sites of Ampur Kanchanadit, Surat Thani Province, Thailand, was examined for the presence of *V. parahaemolyticus*. Cockles were placed into sterile bags and transported to the laboratory under refrigerated condition (4°C). All specimens were examined within 2 h after collection. Each sample was scrubbed under tap water to remove debris, allowed to dry, disinfected with 70% ethanol, and opened aseptically using a sterilized knife. Blended samples (25 g) were inoculated in 225 ml alkaline peptone water (APW; 10g of peptone and 10 g of NaCl in 1,000 ml of distilled water, pH 8) and cultured by shaking for 12 h at 37°C. A loopfull of enrichment broth was streaked onto Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar containing 2% of NaCl. After 24 h of incubation at 37°C, suspect sucrose-negative 5 colonies were subcultured in APW with 2% of NaCl and cultured with shaking overnight. Extracted DNA of suspect strains was tested by polymerase chain reaction (PCR) amplification of *V. parahaemolyticus* specific target gene (Bej *et al*., 1999).

3. Results and Discussion

3.1 Specificity and sensitivity of the PCR assay

Primer pairs using in this experiment to identify *V. parahaemolyticus* to species level was designed on the basis of *toxR* gene that encoded regulatory protein in many *Vibrio* (Kim *et al*., 1999). To detect the virulence phenotype, primer pairs were designed from *tdh* and *trh* gene that encoded thermostable direct haemolysin (TDH) or TDH-related haemolysin (TRH), respectively. To evaluate the specific PCR assay, PCR amplification was performed with non-pathogenic and pathogenic strains of *V. parahaemolyticus*, *V. alginolyticus* and other genera. Non-pathogenic and pathogenic strain of *V. parahaemolyticus* was confirmed by the biochemical test from Department of Medical Science, Thailand. It was reported that non-virulent strain was not amplified PCR products by virulent primer (Tada *et al*., 1992). From this study, a product with a length of 386 bp from the *toxR* primer resulted from the two strains of *V. parahaemolyticus*, while no amplification was apparent for non - *V. parahaemolyticus* bacterial strains. However, PCR amplification was detected in the virulence strain with a length of 250 bp by *tdh* and *trh* primer as shown in Figure 1.

A dilution series of genomic DNA of *V. parahaemolyticus* was prepared as described in above. Aliquots of each dilution were used as templates for PCR. Amplification using *toxR* primers resulted in a product with aliquots containing 0.6 ng of genomic DNA, while detection limiting using *tdh* and *trh* was 6 ng. More than 6 ng of DNA was required when using multiplex PCR in order to detect virulence strain. The sensitivity of cell culture was determined using a series of 10 fold dilutions of virulence *V. parahaemolyticus* cells. The minimal cell concentration detectable by *toxR* was 10^5 CFU ml^{-1} or 10^3 cells per PCR reaction using 20 cycles of amplification. Whereas, increasing amplification to 30 cycles decreased detection limiting to 10 CFU ml^{-1} per PCR reaction. When using *tdh* and *trh*, the sensitivity is 100 CFU ml^{-1}.

Several primer pairs designed from targeted genes of *V. parahaemolyticus* were described previously, such as *toxR*, *tl*, *vmp*, *gyrB* and pR72H DNA fragment. Among these primers, *toxR* show a high efficiency and reliabilities due to its not producing any false-positive result on the strains belonging to other Vibrio species (Croci *et al*., 2007). Although, sensitivity of *toxR* of this experiment (0.6 ng) was lower than previously published with the same target researches (Rosac *et al*., 2009; Venkateswaran *et al*., 1998; Luan *et al*., 2007). The sensitivity of detection limit by *toxR* probably depended on type of *V. parahaemolyticus* strain. However, the sensitivity of detection limit of *toxR* was higher than using multiplex PCR (*toxR*, *tdh*, *trh)*, *tl*, *vmp* and *gyrB* (Luan *et al*., 2007). In addition, primer targeting *tlh* gene was able to detect at 200 pg of *V. parahaemolyticus* DNA or 10^4 CFU/ml by real-time PCR (Ward and Bej, 2006)

3.2 Detection of *V. parahaemolyticus* by conventional and PCR method

Sixty-five cockle samples collected from the south coast of Thailand during September, 2007 to October, 2008 were monitored (Table 1). There were fifty-six samples (86.2%) contaminated with *V. parahaemolyticus*. In July, 2008, the percentage of *Vibrio parahaemolyticus* contamination was 40%, which was the lowest, and one contaminated sample (1.8%) producing both TDH and TRH was observed in monsoon season (Table 1 and Figure 3). In contrast, higher densities of *V. parahaemolyticus* were observed in samples collected in the dry season. Non-virulence strain was not detected. This might be due the influence of seasonal change. The prevalence of *V. parahaemolyticus* in the environment is assumed to be correlated to parameters such as temperature and salinity. Total and pathogenic *V. parahaemolyticus* were presumed in higher densities with higher water temperatures (Yeung and Boor, 2004). Regarding salinity, its increase is negatively correlated to the presence of *V. parahaemolyticus*, but studies diverged regarding the magnitude of its effect. DePaola *et al*. (2000) observed that salinity affected

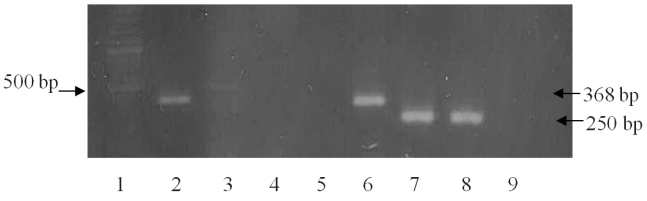

Figure 1. Amplification product of *toxR, tdh* and *trh* primer, respectively in non-virulence (lane 2-4) and virulence *V. parahaemolyticus* (lane 6-8). Lane 1 showed as DNA marker 100 bp. H_2O is negative control (lane 5 and 9).

Table 1. Occurrence of *toxR*-, *tdh*- and *trh*- positives strains in the south coast of Thailand in 2007-2008.

Date	Detected no. of positive samples as determined by PCR (%)*		
	toxR	*tdh*	*trh*
August, 2007	5 (100%)	-	-
September, 2007	5 (100%)	-	-
October, 2007	5 (100%)	-	-
November, 2007	3 (60%)	-	-
December, 2007	5 (100%)	-	-
January, 2008	3 (60%)	-	-
February, 2008	5 (100%)	-	-
March, 2008	5 (100%)	-	-
April, 2008	4 (80%)	-	-
May, 2008	5 (100%)	-	-
June, 2008	4 (80%)	-	-
July, 2008	2 (40%)	1	1
August, 2008	5 (100%)	-	-
Total	56 (86.2%)	1 (1.8%)	1 (1.8%)

* Five samples were collected monthly

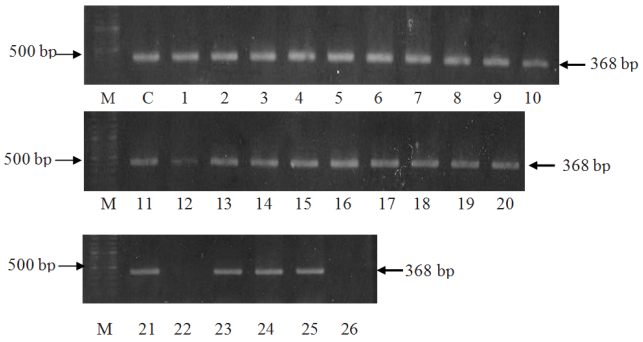

Figure 2. Detection of presumptive colonies grown on TCBS plate by *toxR* primer (lane 1-25). 100 bp marker and positive control (*V. parahaemolyticus*) was marked at lane M and C, respectively. Distilled water is used negative control (lane 26).

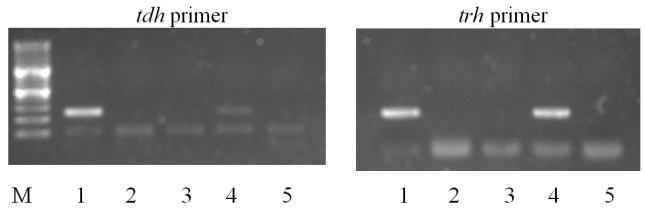

Figure 3. PCR detection of cockle samples collected in July, 2008 detected by *tdh* and *trh* primers. Virulence strain showed both TDH and TRH producing (lane 4). The 100 bp marker was marked in lane M. Positive (virulence *V. parahaemolyticus*) and negative control (distilled water) showed in lane (1) and lane (5), respectively.

the *V. parahaemolyticus* level less than temperature. In contrast, Martinez-Urtaza *et al.* (2008), found that salinity was the primary factor.

Some blue-green colony on selective media could not produced a PCR product because it was not a *V. parahaemolyticus* (Figure 2, lane 22). Several limitations associated with the use of TCBS agar for the correct identification of *V. parahaemolyticus* colonies have been reported in recent years (Hara-Kudo *et al*., 2003). *V. parahemolyticus* grown on TCBS agar showed similar morphology and characteristics to those of other *Vibrio* spp. (*V. vunificus*, *V. mimicus*, *V. alginolyticus*, *V. fluvialis*), *Photobacterium* spp., *Chryseomonas* spp. or *Shewanella* spp. The limited selectivity of the medium allows for the over growth of other bacteria that predominate in the environment, particularly *V. alginolyticus* that easily grows on this media and may limit the detection of *V. parahaemolyticus* colonies, thereby masking the presence of *V. parahaemolyticus* colonies and complicating the identification of Vibrio colonies (Martinez-Urtaza *et al*., 2008).

Some researchers reported that the number of total *V. parahaemolyticus* cells did not reflect that of pathogenic strain in seafood. They mostly detected tdh^+ *V. parahaemolyticus* when the levels of total *V. parahaemolyticus* were less than the hazardous limit of 10^4 bacteria per gram indicated by USFDA (Raghunath *et al.*, 2008). Thereby, occurrence of pathogenic strain did not correlate with the higher number of total *V. parahaemolyticus*. This suggests that seafood with total *V. parahaemolyticus* count less than hazardous limit are also not completely safe and total *V. parahaemolyticus* count is not a reliable indicator for pathogenic *V. parahaemolyticus*.

Normally, only 1-2% of environmental strains process the *tdh* gene. In recent years, some investigators (Depaola *et al*., 2003; Deepanijali *et al*., 2005) have reported a higher prevalence of 12.8 and 10.2%, respectively. In our study, only 1.8% of pathogenic strains were detected. It seemed that the number of virulent positive strains present in our study was lower than that of negative strains for these genes in the enrichment broth. Thus, the chance of detecting virulent strains was low by conventional method where 5 colonies were tested. In addition, TCBS agar being a highly inhibitory selective medium may inhibit growth of some strains of pathogenic *V. parahaemolyticus*. Some laboratory used direct PCR and colony hybridization technique in order to obtain accurate detection of total and pathogenic *V. parahaemolyticus* (Deepanijali *et al*., 2005; Raghunath *et al*., 2008). Their results showed that a great numbers of both *tdh* and *trh* genes were detected in samples after 18 h enrichment and hence demonstrate the necessities for enrichment for detection of pathogenic *V. parahaemolyticus* in seafood by PCR. Therefore, PCR and colony hybridization of enrichment broth seem to be very useful in detecting the presence of these virulent strains.

Virulence of *V. parahaemolyticus* has been found to correlate with the production of the hemolytic toxin TDH and

TRH (Iida *et al*., 1998). However, this bacterium can adhere to various cell lines, and exhibit cytotoxic activity and mouse lethality even in the absence of this hemolysins (Bhattacharjee *et al*., 2005; Lynch *et al*., 2005). Recently, Vongxay *et al*. (2008) isolated *V. parahaemolyticus* from clinical and seafood sources. They found that the *tdh*-positives of clinical isolates were generally of higher cytotoxicity and enteropathogenicity than the *tdh*-positives and negatives isolated from seafood. This indicates that there were some virulence factors or mechanisms other than TDH or TRH that are involved in pathogenesis of *V. parahaemolyticus*.

4. Conclusion

The results illustrate that PCR assay is efficient to detect the occurrence of *V. parahaemolyticus* by *toxR* primer. Virulent strain of *V. parahaemolyticus* was detected with *tdh* and *trh* primer. Sensitivity of *toxR* primer was studied. It was found that the detection limit was 0.6 ng while sensitivity of *tdh* and *trh* primer enabled detection at 6 ng DNA. PCR technique was used for detection of *V. parahaemolyticus* in cockles harvested from the south coast of Thailand during September, 2007 to February, 2008. Sixty-five samples were examined. It was found that 86.2% of samples were contaminated. Virulent strain of *V. parahaemolyticus* was also found in samples (1.8%). Contamination of virulent strain of *V. parahaemolyticus* was found in July when contamination was lower than in summer.

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