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ORIGINAL PAPER

Multiplexed real-time PCR amplification of *tlh*, *tdh* and *trh* genes in *Vibrio parahaemolyticus* and its rapid detection in shellfish and Gulf of Mexico water

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Abstract In this study, we have developed a SYBR Green^{TM} I-based real-time multiplexed PCR assay for the detection of *Vibrio parahaemolyticus* in Gulf of Mexico water (gulf water), artificially seeded and natural oysters targeting three hemolysin genes, *tlh*, *tdh* and *trh* in a single reaction. Post-amplification melt-temperature analysis confirmed the amplification of all three targeted genes with high specificity. The detection sensitivity was 10 cfu (initial inoculum) in 1 ml of gulf water or oyster tissue homogenate, following 5 h enrichment. The results showed 58% of the oysters to be positive for *tlh,* indicating the presence of *V. parahaemolyticus*; of which 21% were positive for *tdh*; and 0.7% for *trh,* signifying the presence of pathogenic strains. The C_t values showed that oyster tissue matrix had some level of inhibition, whereas the gulf water had negligible effect on PCR amplification. The assay was rapid ($\sim$8 h), specific and sensitive, meeting the ISSC guidelines. Rapid detection using real-time multiplexed PCR will help reduce *V. parahaemolyticus*-related disease outbreaks, thereby increasing consumer confidence and economic success of the seafood industry.

Keywords *Vibrio* · Real-time PCR · SYBR Green I · Shellfish · Gulf of Mexico

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Introduction

Vibrio parahaemolyticus, a Gram-negative, facultative halophilic bacterium is commonly found in warm coastal waters worldwide (Twedt 1989). Shellfish and other bivalves accumulate this pathogen in their tissue during filter-feeding, and could cause gastroenteritis in humans who consumed raw or poorly cooked seafood (DePaola et al. 1990). Although the illnesses caused by this organism are usually self-limiting, in some severe cases dehydration or bloody diarrhea may persist, resulting in hospitalization of the patient and/or prolonged antibiotic treatment (Shevchuk et al. 1986; Miyamoto et al. 1969; Morris 2003). Sporadic cases of *V. parahaemolyticus*-related gastroenteritis due to the consumption of raw oysters in humans primarily along the coastal states in the United States and other countries such as Japan and South East Asia have been documented (Alam and Miyoshi 2003; DePaola et al. 1990, 2003; Hara-Kudo et al. 2003; Ijumba 1984; Robert-Pillot et al. 2004). The first recorded outbreak in the United States was reported in 1971 in Maryland (CFSAN 2000)

including 10 cases followed by a relatively larger outbreak in 1998 in the Pacific Northwest United States affecting 209 individuals and including 1 death (CDC 1999). This has led to the Interstate Shellfish Sanitation Conference (ISSC 2000), FDA and seafood industry to seriously consider the development of a nucleic acid-based rapid detection of this pathogen to ensure the safety of post-harvest processed (PHP) shellfish for human consumption at the highest level, thereby protecting consumer health. Real-time polymerase chain reaction (real-time PCR) methods of pathogen detection, using fluorogenic DNA-binding dyes or fluorescent-labeled oligonucleotide probes, have been shown to be effective, rapid and sensitive method for detection of vibrios (Fukushima et al. 2003; Kim et al. 2008; Lo et al. 2008; Panicker et al. 2004; Qin et al. 2008; Rosec et al. 2009; Wittwer et al. 2001). Using this approach, recently a nucleic acid-based real-time PCR with Taqman™ fluorescence probes targeting *V. parahaemolyticus* three hemolysin genes, the thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*) (Tada et al. 1992; Takeda 1982; Taniguchi et al. 1985, 1986) and thermostable-related hemolysin (*trh*) genes have been described for rapid detection of total and pathogenic strains of this pathogen in shellfish (Membrillo-Hernandez 1997; Nordstrom et al. 2007; Ward and Bej 2006). In another study, Taqman™ probe-based real-time PCR was developed for the detection of a newly emerged pandemic *V. parahaemolyticus* O3:K6 in Gulf of Mexico water targeting a segment of the ORF8 DNA (Okuda et al. 1997; Myers et al. 2003 Rizvi et al. 2006). However, since the first appearance in 1996 in the U.S. causing an outbreak of gastroenteritis disease, the pandemic *V. parahaemolyticus* O3:K6 serogroup strain has not been further isolated. Therefore, the importance of this pathogen in the U.S. coastal waters and seafood harvested from these areas has become less significant. Although the Taqman™ probe-based real-time PCR has been shown to be effective for rapid and specific detection of this pathogen, this approach could be relatively costly. Therefore a cost-effective detection method for routine monitoring of PHP oysters that will maintain the highest level of product safety without compromising the market cost of the product is desirable. Recently, real-time PCR using DNA-binding fluorescent dye SYBR green has been reported for the detection of pathogenic *V. parahaemolyticus* in shellfish targeting a single gene *tdh* (Tyagi et al. 2009). In addition, conventional PCR detection of *V. parahaemolyticus* in Middle Black Sea Coast of Turkey by targeting the *tlh*, *tdh* and *trh* genes has been reported (Terzi et al. 2009). Here we describe a rapid, specific, sensitive and cost-effective multiplexed SYBR Green™ I-based real-time PCR detection of total and pathogenic *V. parahaemolyticus* in shellfish and gulf water targeting all three targeted genes *tlh*, *tdh,* and *trh* simultaneously in a single reaction in an effort to reduce shellfish-related *V. parahaemolyticus*-illnesses.

Materials and methods

Bacterial strains and microbiological media

All *V. parahaemolyticus* strains used in this study, along with other various *Vibrio* strains, are listed in Table 1. *V. parahaemolyticus* was cultured in T_1N_1 broth medium [10% (w/v) tryptone, 1% (w/v) NaCl] or on T_1N_3 agar plates (10% tryptone, 3% NaCl) (Atlas 1993) at 35°C overnight. All other strains were grown and maintained as follows: all *V. vulnificus* strains were grown on one-half strength marine agar or broth (Becton–Dickinson, Franklin Lakes, NJ); *V. cholera* was grown on Luria–Bertani (LB) agar or broth (Atlas and Bej 1990); *V. alginolyticus, V. campbelli, V. furnisii,* and *V. mimicus* were all grown on full strength marine agar or broth.

DNA purification for PCR optimization

Genomic DNA from an overnight (1.5 ml) culture of *V. parahaemolyticus* F113A was purified by following method described by Ausubel et al. (1987). Purified DNA was resuspended in Tris–EDTA (pH 8.0) buffer (Ausubel et al. 1987) and the concentration measured in a Lambda II spectrophotometer (Perkin-Elmer, Shelton, CT) at a wavelength of 260 nm.

Oligonucleotide primers

A segment of the thermolabile hemolysin encoding gene, *tlh,* was targeted for the detection of all *V. parahaemolyticus;* thermostable direct hemolysin encoding gene, *tdh,* and the *tdh* related gene, *trh* were

Table 1 List of strains used in this study and the results from the specificity of the multiplexed real-time PCR targeting the *tlh*, *tdh*, and *trh* genes

Strain and source	*tlh*	*tdh*	*trh*
V. parahaemolyticus			
T3980 (human patient)	+	+	−
901128 (human patient)	+	−	−
VP-3V0C (unknown)[a]	+	+	+
VP-855329-2 (oyster)	+	−	−
JJ51A (oyster plant)	+	+	+
VP-JJ2J1C (oyster plant)	+	+	+
VP-N7 (oyster plant)	+	+	+
48262 (human patient)	+	+	+
8332924 (oyster)	+	−	−
35V0A (sea water)	+	−	−
852850 (unknown)	+	+	+
NY477 (human patient)	+	+	−
AQ4037 (unknown)	+	−	+
v9401392 (human patient)	+	+	+
48057 (human patient)	+	+	+
14D1 (unknown)	+	−	−
VP89-1B (sea water)	+	−	−
JJ41B2 (oyster plant)	+	+	+
8338335 (oyster)	+	+	+
48256 (human patient)	+	−	−
WR2 (sea water)	+	−	−
VP43-1A (sea water)	+	+	+
VP53 (sea water)	+	−	−
30V10A (oyster)	+	+	+
ATCC17802 (human patient)	+	−	−
96736341 (oyster)	+	−	−
48291(human patient)	+	+	+
47583 (human patient)	+	+	+
T3979 (human patient)	+	+	−
9401078 (human patient)	+	+	+
VP-8657 (human patient)	+	+	−
47977 (human patient)	+	+	−
48432 (human patient)	+	−	−
VPSAK5 (human patient)	+	−	−
VP8659 (human patient)	+	+	−
AOC3 (oyster)	+	−	−
VP-48215 (human patient)	+	+	+
KCHD613 (human patient)	+	−	−
9200713 (oyster)	+	−	−
SAK11 (human patient)	+	+	−
VP41977 (Human patient)	+	+	+
553-14 (human patient)	+	−	−
F11-3A (oyster)	+	+	+
TX2071 (unknown)	+	+	−
V. alginolyticus ATCC17749	−	−	−
V. campbelli ATCC25920	−	−	−
V. vulnificus LAM-624	−	−	−
*V. furnisii*CDC195883	−	−	−
V. mimicus ATCC33653	−	−	−
V. cholerae 145A	−	−	−
Escherichia coli ATCC	−	−	−

[a] Unknown = Strains obtained from various laboratories, but the source of isolation could not be traced

also targeted for the pathogenic strains of *V. parahaemolyticus*. The description of the primers including the nucleotide sequence, position within the gene, length and the T_m values are presented in Table 2. The T_m values of all of the primers were determined using the equation $[T_m\ (°C) = 2(A + T) + 4(G + C)]$ (Bej et al. 1991; Rychlik and Rhoads 1989). All primers were custom synthesized by Integrated DNA Technology, Inc., Coralville, IA.

Optimization of PCR

Purified DNA from *V. parahaemolyticus* F113A was used for the optimization of the PCR (Bej et al. 1999). The PCR consisted of 1× PCR buffer [10× buffer consisted of 200 mM Tris–Cl (pH 8.4), 500 mM KCl], 1.5, 2.5 or 3.5 µl of PCR Enhancer mixture (Invitrogen, Inc., Carlsbad, CA), 2.5, 3 or 4 mM $MgCl_2$; 0.2, 0.6 or 1 µM each of the *tlh*, *tdh* and *trh* primers, 200 µM of each dNTP (Sigma-Aldrich), 1×, 2× or 4× SYBR Green™ I nucleic acid fluorescent dye (Roche, Basel, Switzerland), three units of thermostable DNA polymerase (New England Biolabs, Beverly, MA) and autoclaved (121°C under 15 psi for 20 min) MilliQ water (Millipore, Bedford, MA) to bring the total reaction volume to 25 µl.

The PCR temperature cycling parameters used for the optimization of the amplification reaction are described below: initial denaturation of the template DNA at 95°C for 120 s followed by ample number of amplification cycles to surpass the threshold cycle by six cycles. These amplification cycles consisted of denaturation of the template DNA at 94°C for 15 or

Table 2 Description of oligonucleotide primers, amplicon size and the melt temperature of the amplicons observed after real-time PCR amplification with SYBR Green™ I

Target gene	Primer	Sequence	Position within gene (bp[a])	Length (nt[b])	Primer T_m (°C) (calculated)[c]	Amplicon size (bp[a])	Amplicon T_m (°C) (melt temperature observed)
tl	L-TLH-781	5′-AAAGCGGATTATGCAGAAGCACTG-3′	781–804	24	70	173	~85
	R-TLH-933	5′-TGTGCCTTGATGAACTCGTTC-3′	933–953	21	62		
tdh	L-TDH-169	5′-GTAAAGGTCTCTGACTTTTGGAC-3′	169–191	23	68	270	~82
	R-TDH-415	5′-TGGAATATGAACCTTCATCTTCACC-3′	415–438	24	66		
trh	L-TRH-355	5′-TTCACAAAATCAGAAAAAACAAGA-3′	355–378	24	62	217	~81
	R-TRH-547	5′-TTTAATTTTGTGACATACATTCATC-3′	547–571	25	62		

[a] Base pair of DNA

[b] Nucleotide

[c] T_m (°C) = 2(A+T) + 4(G+C)

20 s, primer annealing at 52°C, 56°C or 60°C for 15 or 30 s, and primer extension at 72°C for 25 or 45 s. All PCR amplification reactions were performed in a Cepheid™ Smart Cycler instrument (Cepheid, Sunnyvale, CA). Following PCR amplification, the sizes of the amplicons were confirmed by 2% (w/v) NuSeive 3:1 (FMC Bioproducts, Philadelphia, PA) agarose gel (Ausubel et al. 1987), and the results were documented digitally using a Kodak *ds120* photo documentation system and 1D image analysis software (ver. 3) (Kodak, Rochester, NY).

Specificity of the PCR

The specificity of the primers to their respective targeted genes was confirmed by using the BLAST search program (www.ncbi.nlm.nih.gov) and by PCR amplification in multiplexed format on 44 *V. parahaemolyticus* strains and other *Vibrio* spp. using the PCR parameters described above (Table 1).

Sensitivity of the PCR

Purified genomic DNA from *V. parahaemolyticus* F113A was tenfold serially diluted (1.0 µg/µl to 0.1 pg/µl) in sterile distilled water. PCR amplifications were performed using optimized concentrations of reagents and temperature cycling parameters as described in the results section. Positive amplifications of the targeted gene segments were identified by the melt temperatures, which were generated by the melt curve analysis by the Cepheid™ Smart Cycler software (ver. 2d). The amplicon sizes were confirmed by gel electrophoresis using a 2% (w/v) NuSeive 3:1 agarose gel. This experiment was performed in triplicate to ensure a consistent level of detection.

Detection of *V. parahaemolyticus* in pure culture

A stationary-phase *V. parahaemolyticus* F113A culture (4.6×10^7 cfu/ml) grown overnight culture was tenfold serially diluted to extinction. Then cells were collected by centrifugation at 9,300×*g* for 10 min. The supernatant was discarded, and the cells were resuspended in 100 µl of Instagene™ Matrix consisting of specially formulated Chelex™ resin (BioRad Laboratories, Hercules, CA). The samples were then incubated at 56°C for 10 min, vortexed intermittently for 1 min and then boiled for 6 min. For each 25 µl PCR, 3 µl of the Instagene™ treated boiled sample was used as a source of the template DNA. All experiments were conducted in triplicate in order to evaluate the reliability of the sensitivity of detection.

Detection of *V. parahaemolyticus* in gulf water

An overnight culture of *V. parahaemolyticus* F113A was inoculated in fresh T_1N_1 broth in 1:10 ratio, and grown at 37°C until the OD_{450nm} reached 0.2. The culture was centrifuged and cell pellet was resuspended in autoclaved Gulf of Mexico water (gulf water from herein). The viable plate count on T_1N_3 agar plate was determined to be 4.6×10^7 cfu/ml. The culture was then subjected to tenfold serial dilution to

extinction in 10 ml of autoclaved gulf water and enriched at 37°C for 5 h in a rotary shaker incubator set at 150 rpm. The gulf water was collected from Dauphin Island, AL and the salinity was determined to be 27 ± 3 ppt ($n = 5$) using a refractometer (Reichert Scientific Instruments, Buffalo, NY). The cells were then collected by centrifugation; supernatant was carefully discarded, and the cells were resuspended in 100 µl of Instagene™ Matrix (BioRad) and used for PCR amplification as described above. To determine the consistency of the level of detection, all experiments were conducted in triplicate.

Detection of *V. parahaemolyticus* in seeded oysters

All oyster samples were obtained from a local seafood store in Birmingham, AL. A pure culture of *V. parahaemolyticus* F113A was grown in T_1N_1 broth until the OD_{450nm} reached to 0.2. The viable plate count of 10^7 cfu/ml was determined on T_1N_3 agar plates (Atlas 1993). The culture was then tenfold serially diluted in T_1N_1 broth to extinction and 10 ml of each dilution was added to an additional 50 ml of T_1N_1 broth and 1 ml of homogenized oyster shell stock (ISSC, 2000). After 5 h of enrichment in a rotary shaker incubator (Innova 4000) set at 150 rpm and 37°C, 1 ml aliquots were collected by centrifugation at 9,500×*g* for 10 min. The supernatant was carefully discarded using a micropipette, and the cell pellet was resuspended in 100 µl of Instagene™ Matrix (BioRad) and treated to release template DNA as described above. For each SYBR Green™ I PCR, 3 µl of the supernatant of the boiled samples were used as a source of template DNA. Unseeded oyster homogenate shell stock (1 ml) was enriched and treated in Instagene™ matrix as described above, and used as a control. All experiments were conducted in triplicate in order to evaluate the reliability of the sensitivity of detection.

Detection of *V. parahaemolyticus* in natural oysters

A total of 24 oysters were collected from the Gulf of Mexico water near Dauphin Island, Alabama during the month of June 2007. Collected oysters were immediately chilled and cleaned with 70% (v/v) alcohol with a scrubbing brush using standard method (Kaysner et al. 1992; NSSP 1997). The oysters were shucked, and the shell stock homogenized in an autoclaved Waring Blender (Fisher Scientific). An aliquot (30 ml) of the samples from each group was then enriched for 5 h in T_1N_1 broth as described above. Enriched samples were aliquoted and stored in −80°C until used for PCR. DNA from the enriched samples were purified using the Instagene™ Matrix as described above and 3 µl was used for the multiplexed PCR amplification. Purified genomic DNA from *V. parahaemolyticus* F113A strain consisting of all three hemolysin genes were spiked into 3 µl Instagene™ treated oyster homogenate and PCR amplified to determine that negative detection of the samples was not due to the inhibition of the reaction by the oyster tissue matrix.

Results

Primer selection and optimization of real-time PCR

The primers for the *tlh* gene target amplified the expected 173 bp DNA fragment for each of the 44 *V. parahaemolyticus* strains tested, however *tlh* amplification was negative for the rest of the non-*parahaemolyticus* vibrios and non-vibrio species. This suggests that the R-TLH-731 and L-TLH-933 primers are specific for the detection of genus *V. parahaemolyticus.* Only 26 (∼59%) of the *V. parahaemolyticus* strains tested positive for *tdh* and only 19 (∼ 43%) for *trh*, showing that not all of the isolates tested were pathogenic. Amplification of *tlh, tdh* and *trh* was evident by the fluorescent detection displaying the expected amplicon melt temperature values (Table 2) and was confirmed by gel electrophoresis (not shown).

To achieve consistent and detectable level of fluorescence during the melt-temperature analysis for all amplicons, the *tlh* primer was sufficient at 0.2 µM concentration, whereas the *tdh* and *trh* primer needed to be 0.6 µM. The SYBR Green™ I dye was most favorable at a 2× concentration. The $MgCl_2$ concentration of 3 mM along with 2.5 µl of enhancer mixture was found to be optimum for the assay.

The following PCR cycling parameters were used for efficient and consistent amplification of the targeted gene sequences: initial denaturation of the template DNA for 120 s at 95°C followed by 6 cycles of amplification cycles surpassing the threshold cycle

Table 3 Sensitivity of PCR detection on purified *Vibrio parahemolyticus* DNA

DNA concentration	PCR results (all three targeted genes)	C_t value	Melt temperatures (°C)		
			tlh	*tdh*	*trh*
100 ng	+	12.16 ± 0.09	85.24 ± 0.06	81.57 ± 0.26	80.43 ± 0.01
10 ng	+	16.02 ± 1.17	85.33 ± 0.13	82.20 ± 0.05	81.22 ± 0.34
1 ng	+	20.77 ± 0.40	84.92 ± 0.33	81.17 ± 0.32	80.86 ± 0.67
100 pg	+	24.99 ± 1.11	84.72 ± 0.26	81.71 ± 0.22	80.74 ± 0.42
10 pg	+	27.36 ± 1.53	84.29 ± 0.26	81.81 ± 0.21	80.62 ± 0.42
1 pg	−	32.87 ± 1.15	75.62 ± 0.13	75.17 ± 0.25	74.89 ± 0.32
100 fg	−	33.21 ± 0.04	75.24 ± 0.07	74.03 ± 0.64	73.37 ± 0.54
Negative control	−	38.69 ± 0.03	74.48 ± 0.06	75.27 ± 0.08	75.67 ± 0.04

(C_t). The amplification cycles consisted of a denaturation of the template DNA at 94°C for 15 s, annealing at 56°C for 15 s, and extension for 25 s at 72°C for consistent results.

Sensitivity of detection

A minimum of 10 pg of purified genomic DNA from *V. parahaemolyticus* F113A displayed a detectable level of positive amplification for all three gene targets with the C_t value of 27.36 ± 1.53 (Table 3). The amplification was evident with the DNA melt temperatures (Fig. 1) and confirmed by gel electrophoresis displaying the DNA bands of expected size of 173 bp, 217 bp, and 270 bp for *tlh, trh* and *tdh,* respectively (data not shown). Increasing C_t values with the expected melt temperatures were observed as the amount of initial template DNA increased (Table 3). This level of detection of genomic DNA is comparable to approximately 10^3 cfu *V. parahaemolyticus* (Atlas and Bej 1990). A good linear correlation of the samples with positive PCR amplification was found between the C_t values and the concentration of purified DNA ($n = 3$; $r^2 = 0.99$) (Fig. 2).

Detection of *V. parahaemolyticus* in pure cultures

The level of detection of an unenriched *V. parahaemolyticus* in pure culture was 10^7 cfu/ml with a C_t value of 24.56 ± 1.53 and amplicon melt temperatures of 84.45 ± 0.13°C (*tlh*), 81.83 ± 0.24°C (*tdh*), and 80.08 ± 0.06°C (*trh*) (Table 4). This level of detection was consistent with three individual reactions.

Detection of *V. parahaemolyticus* in seeded gulf water

The minimum level of detection of all three hemolysin genes following 5 h enrichment of gulf waters samples of a serial dilution consisted of an inoculum of 10 cfu/ml prior to enrichment. The observed C_t value for this sample was 25.35 ± 0.06 and the amplicon melt temperatures were approximately 84.46 ± 0.04, 81.72 ± 0.14, and 81.43 ± 0.2°C for targeted genes *tlh, tdh*, and *trh*, respectively (Table 5). This level of detection was found to be consistent in all replicates.

Detection of *V. parahaemolyticus* in seeded oysters

The sensitivity of detection of *V. parahaemolyticus* in 1.0 ml of oyster shell stock homogenate with initial inoculum of 10^1 cfu *V. parahaemolyticus* exhibited positive results of C_t value of 27.42 ± 0.18 and T_m values of 84.19 ± 0.52 (*tlh*), 81.32 ± 0.67 (*tdh*) and 80.70 ± 0.23 (*trh*) following 5 h of enrichment (Table 6). This detection level was reproducible for three individual samples tested. The positive results were confirmed by the post-amplification melt temperature analysis and gel electrophoresis (not shown). Oyster tissue samples without any added *V. parahaemolyticus* exhibited negative amplification results confirming that the positive amplifications and sensitivity of detection were a direct result of the seeded culture and not pre-existing *V. parahaemolyticus* in the oyster samples.

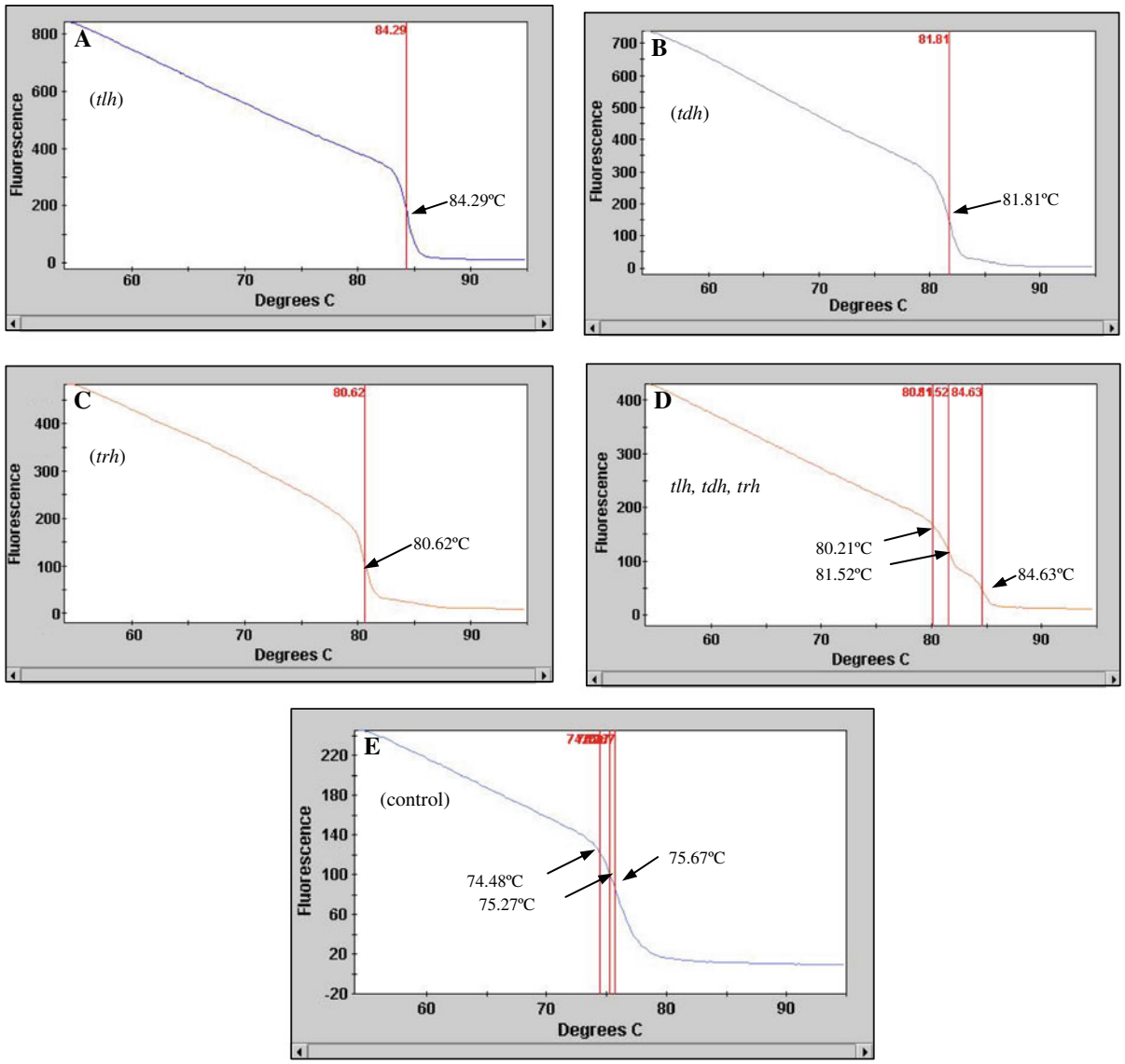

Fig. 1 Optics graphs generated by the Cepheid™ Smart Cycler displaying the amplicon melt curves and temperatures of the hemolysin genes following real time PCR of the purified genomic DNA from *V. parahaemolyticus* with SYBR Green™ I dye. The decline of the fluorescent signals with increasing temperatures indicates the dissociation of SYBR Green™ I dye from amplicons. The melt temperature values were generated by the Smart Cycler software when one-half of the double-stranded amplicons were denatured. **a** The melt temperature value of the *tlh* gene, **b** the melt temperature value of the *tdh* gene, **c** the melt temperature value of the *trh* gene, **d** the melt temperatures following multiplexed PCR targeting *tlh*, *tdh* and *trh* genes, **e** the melt temperatures following multiplexed PCR without any DNA added in the reaction (negative control)

Detection of *V. parahaemolyticus* in natural oysters

Multiplexed PCR amplification on enriched 24 oyster homogenates exhibited positive amplification of *tlh* on 14 samples (~58%); three of these 14 *tlh*-positive samples (~21%) were positive for *tdh*. Also, one of the 14 *tlh*-positive samples (0.7%) amplified for the *trh*, but was negative for the *tdh* gene. The PCR amplified DNA were confirmed for the *tlh* (174 bp); *tdh* (270 bp); and *trh* (217 bp) by agarose gel (data not shown). Oyster samples spiked with *V. parahaemolyticus* F113A

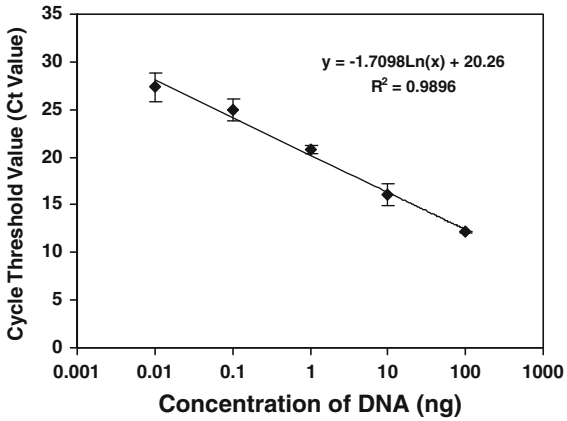

Fig. 2 Standard curve for the amount of purified DNA versus C_t values. The error bars indicate the standard deviation from three independent experiments

exhibited amplification of all three targeted hemolysin genes suggesting that negative amplification was not due to the inhibition by oyster tissue matrix.

Discussion

In this study, we have developed and optimized a multiplexed real-time PCR assay using SYBR Green™ I DNA-binding fluorescent dye targeting hemolysin genes for the detection of *V. parahaemolyticus* in gulf water and shellfish. For the detection of total *V. parahemolyticus,* a species-specific gene *tlh* was targeted; and for virulent strains two other hemolysin genes, *tdh* and *trh* were used (Bej et al. 1999; Terzi et al. 2009; Tyagi et al. 2009; Ward and Bej 2006). These 2 hemolysin genes specifically detect the pathogenic strains of *V. parahaemolyticus* that has recently been validated by Espiñeira et al. (2010). Real-time PCR with SYBR Green™ I DNA-binding fluorescent dye targeting *tdh* or the *trh* gene of *V. parahaemolyticus* has been reported (Fukushima et al. 2003). However, a comprehensive detection of all three hemolysin genes using SYBR Green™ I have not been described. In fact, to the best of our

Table 4 Sensitivity of PCR detection of *Vibrio parahemolyticus* in pure cultures without enrichment

V. parahaemolyticus (cfu/ml)	PCR results (all 3 targeted genes)	C_t value (unenriched samples)	Melt temperatures (°C) (unenriched samples)		
			tlh	*tdh*	*trh*
10^6	+	16.14 ± 0.08	84.53 ± 0.05	81.41 ± 0.30	80.60 ± 0.56
10^5	−	24.56 ± 1.53	84.45 ± 0.13	81.83 ± 0.24	80.08 ± 0.06
10^4	−	37.12 ± 1.18	75.47 ± 0.03	75.18 ± 0.71	74.29 ± 0.12
Positive control[a]	+	20.61 ± 0.27	84.62 ± 0.70	81.56 ± 0.76	80.76 ± 0.11
Negative control[b]	−	36.98 ± 0.31	73.03 ± 0.54	72.89 ± 0.28	72.39 ± 0.02

[a] Purified DNA (1 ng), [b] no DNA added

Table 5 Sensitivity of PCR detection of *Vibrio parahemolyticus* in gulf water after 5 h enrichment

V. parahaemolyticus initial inoculum (cfu/ml)	PCR results (all three targeted genes after 5 h enrichment)	C_t value (on enriched samples)	Melt temperatures (°C) (on enriched samples)		
			tlh	*tdh*	*trh*
10^6	+	14.70 ± 0.04	84.89 ± 0.21	81.84 ± 0.87	80.44 ± 0.80
10^5	+	15.88 ± 1.08	84.45 ± 0.37	81.66 ± 0.13	80.63 ± 0.23
10^4	+	17.16 ± 1.28	84.87 ± 0.07	81.86 ± 0.19	81.09 ± 0.16
10^3	+	18.23 ± 0.34	84.53 ± 0.14	81.72 ± 0.04	81.05 ± 0.23
10^2	+	20.64 ± 1.01	84.86 ± 0.20	81.84 ± 0.05	81.26 ± 0.21
10^1	+	25.35 ± 0.06	84.46 ± 0.04	81.72 ± 0.14	81.43 ± 0.20
10^0	−	38.56 ± 0.08	74.39 ± 0.06	72.89 ± 0.12	73.26 ± 0.06
Positive control[a]	+	12.86 ± 0.71	85.06 ± 0.80	81.93 ± 0.63	80.19 ± 0.56
Negative control[b]	−	37.90 ± 0.05	72.93 ± 0.02	72.39 ± 0.02	73.39 ± 0.08

[a] Purified DNA (1 ng); [b] no DNA added

Table 6 Sensitivity of PCR detection of *Vibrio parahemolyticus* in oyster homogenate after 5 h enrichment

V. parahaemolyticus initial inoculum (cfu/ml)	PCR results (all three targeted genes after 5 h enrichment)	C_t value (on enriched samples)	Melt temperatures (°C) (on enriched samples)		
			tlh	*tdh*	*trh*
10^3	+	19.97 ± 0.35	84.30 ± 0.11	81.46 ± 0.08	80.13 ± 0.15
10^2	+	22.29 ± 0.59	84.49 ± 0.14	81.49 ± 0.78	80.04 ± 0.62
10^1	+	27.42 ± 0.18	84.19 ± 0.52	81.32 ± 0.67	80.10 ± 0.23
10^0	−	32.65 ± 0.14	73.23 ± 0.21	73.18 ± 0.43	72.47 ± 0.08
Positive control[a]	+	12.49 ± 0.35	84.92 ± 0.49	81.81 ± 0.24	80.56 ± 0.72
Negative control[b]	−	33.45 ± 0.19	74.69 ± 0.34	75.03 ± 0.23	75.62 ± 0.15

[a] Purified DNA (1 ng); [b] no DNA added

knowledge, this is the first study in which a SYBR Green™ I-based real-time multiplexed PCR amplification targeting three genes segments simultaneously has been optimized and applied in shellfish (shell stock) and in gulf water. The strategy for the primer selection for the multiplexed PCR assay was to choose segments of the targeted genes (*tlh, tdh* and *trh*) that will generate distinguishable melt temperature values enabling us to identify the unlike fragments upon completion of the PCR. In our experience, at least 1°C difference between two targeted genes was necessary for the Cepheid™ Smart Cycler system to distinguish unlike fragments and reliably display distinct melt temperature values. Additionally, the differences in the amplicon lengths were kept within 100 bp so that the amplification of all three genes remains equally efficient. The use of the enhancer reagent in the reaction helps keep the formation of primer artifacts to a minimum level. Although in previous studies *tlh, tdh* and *trh* genes were shown to be specific for *V. parahaemolyticus* (Bej et al. 1999; Terzi et al. 2009; Tyagi et al. 2009; Ward and Bej 2006), however, since we have selected new primer sets in this study, it was necessary to test the specificity of the assay. The selected primers amplified only *V. parahaemolyticus*. It helped avoid false negative detection and generated realistic C_t values for DNA and cfu. Multiplexed PCR with SYBR Green™ I enabled us to establish reliable and specific detection of this pathogen in shellfish and gulf water. Multiplexed PCR amplification of the *V. parahaemolyicus* culture without enrichment exhibited detection level of 10^6 cfu. A recent study by Terzi et al. (2009) also showed similar level of detection in pure cultures *V. parahaemolyicus* for all three targeted genes in single-tube reaction. However, 5 h enrichment of the oyster homogenate shell stock significantly improved the sensitivity of detection to 10 cfu in 10 ml of gulf water and 1 g of oyster tissue homogenate. Detection of 1 cfu of *V. parahaemolyticus* in oyster tissue homogenate following either 8 h (Rizvi et al. 2006) or an overnight (Ward and Bej 2006) enrichment by real-time PCR and Taqman™ probes has been reported. Haldar et al. (2010) has reported similar level (10–100 cells) of detection of several pathogenic *Vibrio* including *V. parahaemlytics.* In another study by Nordstrom et al. (2007), *V. parahaemolyticus* was detected at <10 cfu following MPN enrichment. In this study, we were able to achieve detection of initial inoculum (prior to enrichment) of 10 cfu of *V. parahaemolyticus* in 10 ml Gulf of Mexico water or 1 ml homogenized shell stock after 5 h of enrichment, which is well within the minimum level of detection recommended by the ISSC for safe consumption of oysters. Also <10 cfu *V. parahaemolyticus* in 1 g of oyster tissue homogenate is considered as "non-detectable" level and therefore safe for human consumption (ISSC 2000). A biochemical test by the identification of a β-type hemolysis on Wagatsuma blood agar referred as the Kanagawa phenomenon was considered to be closely associated with the pathogenicity of *V. parahaemolyticus* (Miyamoto et al. 1969; Wagatsuma 1968). However, this method of detection is laborious and lengthy. Current real-time PCR assay with SYBR Green™ I is rapid (~8 h to complete), specific to the targeted genes and reliable for the detection of *V. parahaemolyticus* in shellfish and gulf water. The standard curve from the multiplexed PCR amplification on purified *V. parahaemolyticus* DNA

exhibited a good linear correlation among serially diluted samples. This was used to provide us with a reliable means to compare C_t values and correlate the amount of *V. parahaemolyticus* DNA present in samples to that with cfu. Also, the levels of inhibition of PCR by the shellfish or gulf water samples matrix could be estimated. In this study, the C_t values for enriched oyster tissue homogenate was approximately 1.5 cycles higher than gulf water samples indicating that oyster tissue components may have had an inhibitory effect on the PCR amplification. The C_t values for gulf water samples were <1 cycle higher than *V. parahaemolyticus* in pure cultures, suggesting that gulf water had a negligible inhibitory effect on the real-time PCR. However, for SYBR Green I-based PCR detection requires an accurate T_m value to confirm the amplification of a targeted gene segment. Therefore, the C_t values may not be essential for confirming detection, but are indicative of the presence of the targeted amplicons. All positive PCR amplifications exhibited expected T_m values suggesting that the multiplexed PCR assay developed in this study is specific, reliable and reproducible. Enrichment of *V. parahaemolyticus* in oyster tissue homogenate and in gulf water allowed us to ensure detection of viable cells. In addition, an enrichment period enabled us to achieve the sensitivity of detection at a level that is in compliance with the current ISSC guidelines. In this study, the oyster shell stock was enriched in the ISSC-recommended T_1N_1 growth medium whereas no recommendation was specified for testing the presence of this pathogen in gulf water. It has been documented that the gulf water is a nutrient-rich environment mostly due ot the agricultural run-offs (U.S. Geological Survey and U.S. Government Department of Interior, http://water.usgs.gov/nawqa/sparrow/gulf_findings/primary_sources.html). The high level of nutrient and warm summer temperature often leads to eutrophication followed by hypoxic conditions in the coastal gulf water spreading miles offshore (Boesch et al. 2009). Due to the high nutrient content, the gulf water was used in this study for enrichment of *V. parahaemolytius* without addition of any nutrient medium. Moreover, Julie et al. (2010) correlated the importance of ecological parameters such as nutrient, salinity, turbidity, chlorophyll A and temperature and the level of total and pathogenic *V. parahaemolyticus* in sediment, water, and mussels prior to and day of collection from the French Atlantic coast. They have used the PCR method targeting the hemolysin genes to assess the level of this pathogen. The relevance of the study by Julie et al. (2010) to this report is that the level of the *V. parahaemolyticus* in environmental water modulates with the changing environmental parameters therefore enrichment step is crucial for samples with low counts on the targeted pathogen. The results from this study suggests that multiplexed real-time PCR with SYBR Green^{TM} I is cost-effective, specific and sensitive and capable of simultaneously detecting species-specific and pathogenic strains of *V. parahaemolyticus* in shellfish and gulf water. This means of rapid detection will help the shellfish industry implement routine monitoring of oysters and keep track of global transmission of this pathogen, which often occurs through cargo ballast water. The current study offers a workable rapid detection assay for total species and pathogenic strains of *V. parahaemolyticus,* which will help seafood industry maintain a steady supply of safe oysters, thus prevent disease outbreaks and protect consumer health.

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