International Food Research Journal 21(3): 921-927 (2014)
Journal homepage: *http://www.ifrj.upm.edu.my*

Development of a SYBR green based real-time polymerase chain reaction assay for specific detection and quantification of *Vibrio parahaemolyticus* from food and environmental samples

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Article history

Received: 10 December 2013
Received in revised form: 21 January 2014
Accepted: 22 January 2014

Keywords

Real-time Polymerase Chain Reaction (real-time PCR)
SYBR Green
Thermo labile (tl) gene
Vibrio parahaemolyticus

Abstract

Vibrio parahaemolyticus is a foodborne pathogen and their human infection is regularly associated with the consumption of raw or undercooked seafood and contaminated water supplies. Many conventional biochemical identification and confirmation procedures are performed to detect the presence of this pathogen, both from seafood or environmental samples. However, these procedures not only require two or more days to complete, they do not have the capabilities to determine the number of *V. parahaemolyticus* cells in any given samples. Thus, in this study we describe the development of a rapid SYBR green based real-time PCR assay, targeting the thermo labile (*tl*) gene of *V. parahaemolyticus* for the detection and enumeration of this bacterium from seafood and environmental samples. We report that the real-time PCR assay and the primers designed are highly specific, and only generated the desired amplicons with *V. parahaemolyticus* DNA samples against other bacteria and fungi species. Our assay is also highly sensitive, and, is able to detect *V. parahaemolyticus* with high coefficient values in concentrations as low as 1.0 pg/µl DNA for pure genomic DNA solutions and 10 cells/ml in serially diluted cell suspension and spiked samples. This assay can be completed in less than 3 hours and may be used as a tool for rapid determination of *V. parahaemolyticus* densities in the food industries, environmental risk assessment and for clinical diagnostics purposes.

Introduction

Vibrio spp. have been recognized as one of the leading cause of foodborne outbreaks in the Asian region countries such as Malaysia, Japan, China, India, Taiwan and Korea (Lesley *et al.*, 2011; Nelapati *et al.*, 2012; Theethakaew *et al.*, 2013). Among the genus *Vibrio*, *Vibrio parahaemolyticus* is one of the most prominent species associated with human illness (Lesley *et al*., 2011). Formerly known as *Pasteurella parahaemolytica*, this bacterium was first isolated in Japan in 1950 and has since been detected all around the world (Lesley *et al.*, 2011; Bechlars *et al.*, 2013; Stephens *et al.*, 2013).

V. parahaemolyticus is an enteric pathogen that can cause gastroenteritis in human, usually after the ingestion of undercooked or mishandled seafood (Stephens *et al.*, 2013). It is a halophilic (salt-requiring), non-sporing, Gram-negative facultatively anaerobic bacterium that lives naturally in brackish saltwater worldwide (Johnson *et al.*, 2010; Broberg *et al*., 2011; Quiroz-Guzmán *et al*., 2013). This bacterium is an invasive organism affecting primarily the colon. *V. parahaemolyticus* grows optimally in 37°C in which it can reach generation time up to 8 or 9 times in water and 12 to 18 times in seafood (Nelapati *et al*., 2012). Symptoms of gastroenteritis caused by *V. parahaemolyticus* include watery diarrhea, abdominal cramps, vomiting, headache, chills, nausea and fever (Sakata *et al*., 2012). The pathogenesis of *V. parahaemolyticus* is based on the presence of several commonly documented virulence factors, namely thermo labile (*tl*), thermo stable direct hemolysin (*tdh*) and tdh-related hemolysin (*trh*) genes (Kim *et al*., 2008; Rizvi and Bej, 2010; Theethakaew *et al*., 2013).

To date, many biochemical identification and confirmation procedures are performed to detect and confirm the presence of this pathogen, especially from seafood samples. However, these procedures require 2 or more days to complete (Roman *et al.*, 2011; Sakata *et al*., 2012). Standard PCR is also a method of choice, in which rapid detection of pathogens in biological materials can be achieved. However, it does not allow quantitative analysis of template DNA or cell concentrations (Gao *et al*., 2004). More recently, real-time Polymerase Chain Reaction (real-time PCR) have been applied on the studies of *V. parahaemolyticus* (Cai *et al*., 2006; Rizvi and Bej, 2010). Real-time PCR is not only more sensitive,

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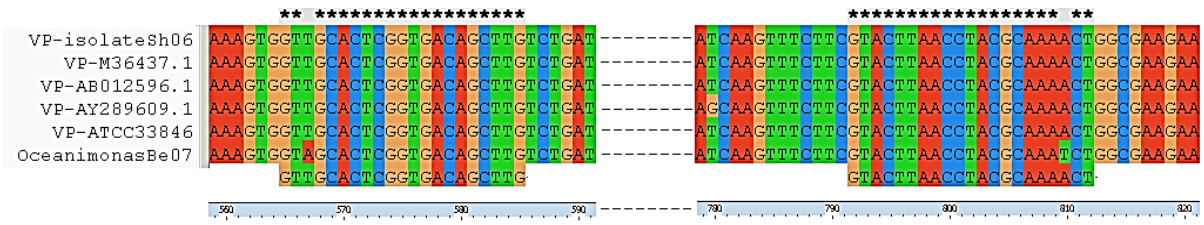

Figure 1. Alignments of the partial thermo labile (*tl*) gene region used for primer design. The illustration demonstrates primer specificity is aligned with sequences of *tl* sequences from Oceanimonas Be07, *V. parahaemolyticus* isolate Sh06, *V. parahaemolyticus* isolate ATCC33846 and other *V. parahaemolyticus* isolates taken form the GenBank (Accession numbers M36437.1, AB012596.1 and AY289609.1). Nucleotide polymorphisms are marked with an asterisk and gaps are indicated by dashes

but, more samples can be processed in a short period of time compared to conventional PCR (Cai *et al.*, 2006).

Previous real-time PCR assays conducted to detect and quantify *V. parahaemolyticus* from various food samples were based on the presence of the *tdh*, *trh*, *tl* and *toxR* genes (Kim *et al.*, 2008; Rizvi and Bej, 2010). However, these *tdh* and *trh* based assays only target pathogenic strains and do not offer a complete representation of total *V. parahaemolyticus* population, while other attempts to perform real-time PCR based on *tl* is shown to be unspecific, with in silico analysis showing the target locus to be conserved between *V. parahaemolyticus* and *Aeromonas* spp. Therefore in this study, we describe the development of a specific, rapid and easy SYBR green-based real-time PCR assay for the specific detection and quantification of *V. parahaemolyticus* DNA particularly targeting the thermo labile hemolysin (*tl*) gene. The thermolabile hemolysin gene in *V. parahaemolyticus* is species-specific, and can be used as a specific marker for PCR amplification detection of this pathogen. We further validated the designed primers and real-time PCR assay by detecting and enumerating *V. parahaemolyticus* population from fish, cockle and shrimp pond samples.

Materials and Methods

Design of primers

Real-time PCR primers were designed based on the thermo labile hemolysin (*tl*) gene region from several *V. parahaemolyticus* strains (Accession No. VP-isolateSh06, M36437.1, AB012596.1, AY289609.1 and ATCC33846) retrieved from the GenBank (Figure 1). The BLASTN algorithms were used to validate the designed primers for significant homology with other DNA sequences, following sequence alignment with the software ClustalX2 (Larkin *et al*., 2007). Two regions with single nucleotide polymorphism that are able to differentiate between *V. parahaemolyticus* and *Oceanimonas* sp. Be07 were identified and used to design the forward and reverse primers, designated as MV2B-TLF (5'-GTT GCA CTC GGT GAC AGC TTG-3') and MV2B-TLR (5'- AGT TTT GCG TAG GTT AAG TAC-3') to amplify a 248 bp PCR product. Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA).

Specificity and sensitivity of the designed primers

The specificity of the designed primers, were determined by using several organisms obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and laboratory collection. The list of the tested organisms is shown in Table 1. The glycerol stocks containing the test cultures were transferred into sterile Luria Broth for overnight culturing. The DNA of the test cultures were then extracted using cell boil DNA extraction method as described by Noorlis *et al.* (2011).

The sensitivity of the real-time PCR conditions and the primers (MV2B-TLF and MV2B-TLR) were determined by performing assays on *V. parahaemolyticus* DNA from different sources. The real-time PCR assays were performed on pure genomic DNA extracted using a commercial kit (PowerSoil® DNA Isolation Kit, MO BIO laboratories Inc., CA, US) with concentrations varying from 10 ng/µl to 1 pg/µl. All DNA concentrations were determined by absorbance at 260/280 nm using the NanoDrop system (Thermo-Fisher Scientific, DE, US). The real-time PCR assay was also conducted on DNA extracted from serially diluted (10^1–10^7 cells/ml) *V. parahaemolyticus* cells. Besides determining the sensitivity, the linearity of the assay over the template dilution range was also tested. In addition to the DNA templates, non-template controls (NTC) were processed in each PCR run. The cycle number at which the amplification curves crossed the specified threshold value was determined.

Real-time PCR reaction conditions and cycling parameters

All real-time PCR reactions were performed using a Rotor Gene 6000 (Corbett Research,

Table 1. List of organisms used for testing primers specificity of the real-time PCR assay designed from the thermo labile (*tl*) gene.

	Species	real-time PCR assay[a]
V. parahaemolyticus	*V. parahaemolyticus* (lab collection – environmental)	+
	V. parahaemolyticus (lab collection – food)	+
	V. parahaemolyticus (shrimp sample)	+
	V. parahaemolyticus (cockle sample)	+
	V. parahaemolyticus (fish sample)	+
	V. parahaemolyticus (shrimp pond water sample)	+
Other *Vibrio* species	*Vibrio cholerae* (lab collection)	-
Non-*Vibrio* species	*Escherichia coli* (lab collection)	-
	Escherichia coli K011 (ATCC 55124)	-
	Staphylococcus aureus (lab collection)	-
	Shigella sonnei (ATCC 25931)	-
	Shigella flexneri (ATCC 12022)	-
Fungi	*Phanerochaete chrysosporium* (ATCC 24725)	-
	Gloeophyllum trabeum (ATCC 11539)	-
	Cryptococcus curvatus (ATCC 20509)	-
	Trichoderma reesei (ATCC 13631)	-
Yeast	*Saccharomyces cerevisiae* (ATCC 24859)	-

[a]Symbols: – not detected, + detected

Sydney, Australia) system under the following cycling conditions: one cycle of 5 min at 95°C to activate DNA polymerase, followed by 50 cycles of 5 s at 95°C, 10 s at 60°C, and 25 s at 72°C. Real-time PCR amplification was run in a 25 µl volume using the following reaction components; 12.5 µl of SYBR supermix (QIAGEN, Hilden, Germany), 2.0 µl of each 10 µM forward and reverse primers, 7.5 µl of deionized ultrapure water and 1.0 µl of DNA template.

To evaluate amplification specificity, melting curve analysis was performed at the end of each PCR run by slowly increasing the temperature from low temperature (60°C) to high temperature (90°C) while continuously monitoring the fluorescence.

Standard curve construction

In order to quantify the *V. parahaemolyticus* population in the tested samples, a standard curve was constructed for the mathematical conversion of Cycle Threshold (Ct) values into bacterial cell number. To produce the standard curve, the log value of the calculated number of cells added to each reaction tube (derived from artificially spiked seafood) was plotted against the Ct value.

DNA extraction from fish sample spiked with V. parahaemolyticus

Prior to performing the spiking experiment, serial dilution of the *V. parahaemolyticus* cells were prepared. Tilapia (*Oreochromis mossambica*) flesh was used to perform the spiking. The fish samples were spiked with 1 ml portion of 10^1–10^7 cells/ml *V. parahaemolyticus* cells in alkaline peptone water. Then 1.5 ml aliquots of the sample broth were subjected to centrifugation at 800 rpm for 3 minutes to enable separation of the meat particles. Then, the supernatant obtained were re-centrifuged at 12 000 rpm for 3 min to pellet the bacterial cells. The pellet were resuspended in 500 µl of sterile distilled water

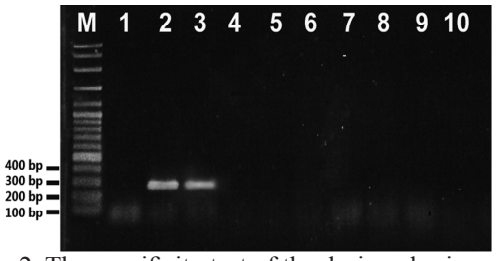

Figure 2. The specificity test of the designed primers with the purified DNA from various strains of bacteria and fungi. Lane M:100 bp DNA ladder, Lane 1: NTC, Lane 2: positive strain, Lane 3: cockle sample, Lane 4: *E. coli*, Lane 5: *S. cerevisiea*, Lane 6: *C. curvatus*, Lane 7: *G. trabeum*, Lane 8: *P. chrysosporium*, Lane 9: *S. flexneri*

and boiled for 10 min. The boiled cell lysate were cooled immediately at -20°C for 10 min, followed by centrifugation at 13 000 rpm for 3 min (Noorlis *et al.*, 2011). The lysate were used as DNA templates in the real-time PCR assay to generate standard curves. The standard curve obtained was used as a reference for the quantification of samples in subsequent real-time PCR assays. The fish flesh was previously tested by standard PCR and was found to be negative for *V. parahaemolyticus*. This preliminary step is important to ensure that the amount of *V. parahaemolyticus* detected in the spiked samples have no outside influence.

Validation of the real-time PCR assay

A total of 6 fresh seafood samples (3 cockles and 3 fish samples) were obtained from wet markets in Kuching, Sarawak. The samples were collected in sterile, sealed bags and placed in crushed ice prior to transportation. The samples were analyzed within 24 hours of collection. A 25 g-portion of each sample were homogenized with 225 ml of alkaline peptone water, in a sterile stomacher bag for 60 s (Noorlis *et al.*, 2011). Aliquots of the homogenized mixtures were used for DNA extraction by the boil cell method as described above. The cell lysate were used as the DNA template for the real-time PCR assays. In addition to the seafood samples, 8 water samples (week 1, week 4, week 8 and week 12) from 2 shrimp ponds (Pond 1 and Pond 14) were also tested to validate the real-time PCR assay. DNA isolation for each sample was also performed via the boil cell method.

Results

Evaluation of the designed primers and the real-time PCR assays

The specificity of the designed primer set was confirmed via BLASTN analyses. From the output of the in silico analysis, 100% query coverage was only observed for *V. parahaemolyticus* strains. The same

Table 2. Ct values and absolute quantification of V. parahaemolyticus cells of the seafood and shrimp pond samples

		Ct Value	*V. parahaemolyticus* cell density
Seafood samples	Cockle Sample I	26.17 ±0.39	6.7×10^4 cells/g
	Cockle Sample II	27.25 ±0.18	2.6×10^4 cells/g
	Cockle Sample III	24.39 ±0.36	3.3×10^5 cells/g
	Fish Sample I	24.96 ±0.22	2.0×10^5 cells/g
	Fish Sample II	28.53 ±0.48	8.1×10^3 cells/g
	Fish Sample III	27.64 ±0.16	1.8×10^4 cells/g
Shrimp pond samples	Pond 1 – Week 1	22.25 ±0.28	7.9×10^2 cells/ml
	Pond 1 – Week 4	24.02 ±0.38	8.3×10^1 cells/ml
	Pond 1 – Week 8	25.80 ±0.25	9.0×10^0 cells/ml
	Pond 1 – Week 12	25.68 ±0.18	1.0×10^1 cells/ml
	Pond 14 – Week 1	21.17 ±0.01	3.1×10^3 cells/ml
	Pond 14 – Week 4	20.86 ±0.35	4.6×10^3 cells/ml
	Pond 14 – Week 8	21.21 ±0.50	3.0×10^3 cells/ml
	Pond 14 – Week 12	23.72 ±0.27	1.2×10^2 cells/ml

analyses also indicated that the expected amplicon to be 248 bp in size. In addition, conventional PCR was performed on a referral *V. parahaemolyticus* strain, other vibrio and non-vibrio species, fungi as well as yeast to ensure a specific amplification process. This was done before conducting the real-time PCR assay. From the conventional PCR assay, all non *V. parahaemolyticus* isolates were tested negative. Figure 2 shows the result of the PCR on several representative microorganisms. From the agarose gel analysis, the amplicon was shown to be approximately 250 bp in size, confirming the BLASTN result.

The dynamic range for the primers were assessed with 10-fold dilution series of *V. parahaemolyticus* DNA (100 ng/ul - 1 pg/ul) which were extracted using a commercial kit and the extracted DNA from a series of *V. parahaemolyticus* cell dilutions. For the pure genomic DNA assay, correlation coefficient for the standard curve was 0.99 and the assay was able to detect as low as 1.0 pg/µl of DNA (Figure 3). Meanwhile for the dilution series of *V. parahaemolyticus* cells, the assay showed linear relationship across 5 orders of magnitude (10^1-10^5 cells/ml) with $R^2 = 0.96$ (Figure 4).

The real-time PCR result for the spiking experiment is shown in Figure 5. From the graph, a linear correlation was obtained ($R^2 = 0.97$), demonstrating the accuracy and feasibility of our assay to quantify *V. parahaemolyticus* density in complex seafood samples, with the smallest detectable amount of 10 cell/ml sample broth.

Validation of the real-time PCR assay and V. parahaemolyticus density determination

In order to validate the real-time PCR assay, 6 seafood (3 cockles and 3 fish) and 8 shrimp pond water samples were tested. Separate standard curves (Figure 4 and Figure 5) were constructed to quantify the amount of *V. parahaemolyticus* in the different samples of various origins and characteristics. *V. parahaemolyticus* was detected in all the samples. The Ct values and the corresponding absolute quantification of *V. parahaemolyticus* cells from the seafood and shrimp pond water samples are shown in Table 2.

Melting curve analysis

Melting curve analysis was conducted at the end of each PCR run, as SYBR green based PCR detection require an accurate T_m value to confirm the amplification of the targeted gene segment. At low temperature, all PCR products are double stranded, therefore the SYBR green dye will bind to them and increase the fluorescence intensity. Whereas, in high temperature, the PCR products are denatured and degraded, resulting in rapid decrease in fluorescence intensity. All positive PCR amplifications exhibited a single dissociation peak in the melting curve analysis (Figure 6), with a Tm value of 85.6°C. This single peak suggests that only one product is amplified, thus validating the specificity and reliability of the real-time PCR assay developed in this study.

Discussion

Real-time PCR has been shown to be useful in rapid detection and accurate enumeration of pathogenic bacteria in food products (Malorny *et al.*, 2008; Macé *et al.*, 2013). The progression of the real-time PCR reaction may be monitored after each cycle rather than at the end, providing a much better quantification assay. This method does not require post PCR sample handling, thus avoiding potential PCR carryover contamination as well as time saving (Tyagi *et al*., 2009). Quantitative real-time PCR is highly recommended in directly detecting and enumerating specific bacteria from highly complex microbial communities without conducting enrichment of samples in order to save time, effort and cost (Sakata *et al*., 2012).

In this study, we have demonstrated that our SYBR Green based real-time PCR assay developed can specifically and accurately quantify *V. parahaemolyticus* from genomic DNA preparation of various sources, including artificially and naturally infested samples over a large concentration range. SYBR Green fluorescence based real-time PCR assay was chosen as this system is an excellent and rapid alternative compared to the other kinds of labeled hybridization probes (Rizvi and Bej, 2010; Ponchel *et al*., 2003).

Since we have developed new primer sets in this study, it was necessary to test the specificity of the assay. Real-time PCR efficiency can be affected by amplicon size as long amplicons will lead to decreased real-time PCR efficiency (Wang and Seed,

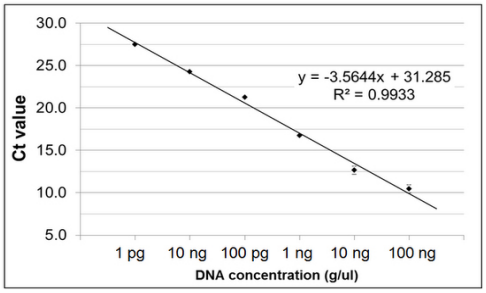

Figure 3. Standard curve for absolute quantification of genomic DNA generated with 10-fold serial dilutions of genomic DNA isolated from pure cultures of *V. parahaemolyticus*. The error bars indicate the standard deviation from 3 independent experiments

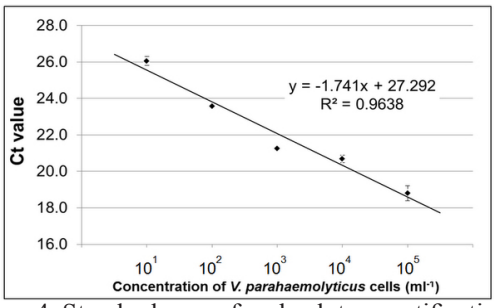

Figure 4. Standard curve for absolute quantification of *V. parahaemolyticus* cell suspensions. The error bars indicate the standard deviation from 2 independent experiments

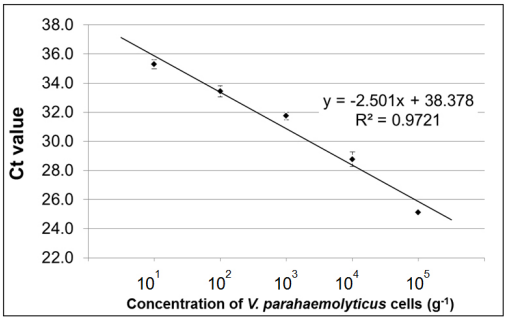

Figure 5. Standard curve for absolute quantification of *V. parahaemolyticus* cell suspensions in spiked fish sample. The error bars indicate the standard deviation from 2 independent experiments

2006). Since PCR efficiency is one of the major factor in ensuring accurate quantification process, it is recommended that the amplicon size to be between 100-250 bp. From the analyses of agarose gel electrophoresis and BLASTN comparisons, the targeted gene sequence was confirmed to be 248 bp in length. Thus, our targeted amplicon size fulfills this criterion. Based on the results obtained from the conventional PCR tested against varieties of other vibrio and non-vibrio species, fungi and also yeast, there is no non-specific amplification occurred. Thus, the designed primers are highly specific, and, this can avoid false positive detection.

The reliability of our protocol in generating standards curves was established by performing real-

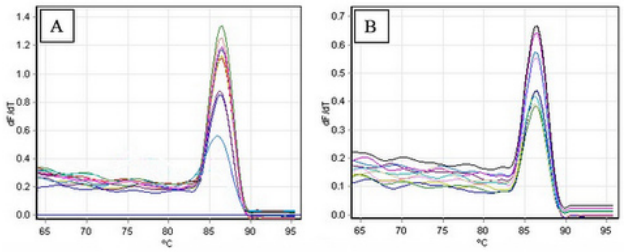

Figure 6. Melting curve produced from (A) pure genomic DNA, (B) 10 fold serial dilution of *V. parahaemolyticus* cells concentration, c sample analysis

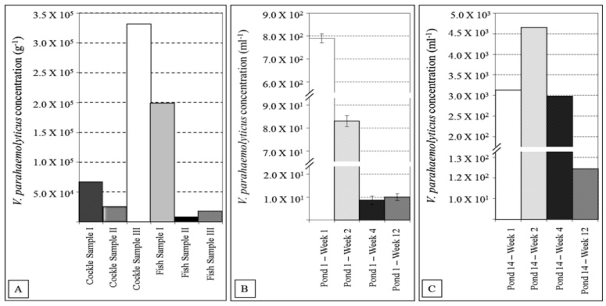

Figure 7. *V. parahaemolyticus* density in 14 samples tested. (A) 6 from food samples (Cockle Sample I, Cockle Sample II, Cockle Sample III, Fish Sample I, Fish Sample II and Fish Sample III), (B) and (C) 8 from shrimp pond samples (Pond 1 Week 1, Pond 1 Week 4, Pond 1 Week 8, Pond 1 Week 12, Pond 14 Week 1, Pond 14 Week 4, Pond 14 Week 8, Pond 14 Week 12). Cell densities detected in each sample are the means of three replications. Error bars represent standard error of the mean

time PCR on three different *V. parahaemolyticus* DNA sources; pure genomic DNA from a referral strain, serially diluted *V. parahaemolyticus* cell cultures and spiked fish samples. The linear graph was used to provide a reliable mean to compare Ct values and correlate the amount of *V. parahaemolyticus* DNA present in samples to that of colony counting. In the amplification plot of the serial dilution of DNA, the Ct value is inversely proportional to the initial template of DNA concentration. In all three sample types, linear graphs (Figure 3, Figure 4, Figure 5) over several orders of magnitude with high R^2 values were generated, indicating that the assay retains its reliability and sensitivity over a broad range of template DNA concentrations (Blackstone *et al.*, 2003). For pure genomic DNA, our assay was able to quantify as little as 1.0 pg/µl of *V. parahaemolyticus* DNA. The same sensitivity level was also demonstrated in the detection of *V. parahaemolyticus* cells from culture suspensions and serially spiked fish samples. For the culture suspensions, *V. parahaemolyticus* was detected at 10 CFU/ml, while in the serially spiked fish samples, *V. parahaemolyticus* was detected 10 CFU/g sample.

To further evaluate the ability of our real-time PCR assays, different types of naturally *V.*

parahaemolyticus infested samples (seafood and water samples) were analyzed. From the results obtained, *V. parahaemolyticus* was detected in all the processed samples (Figure 7), with the seafood samples showing higher *V. parahaemolyticus* cells concentrations (8.1×10^3 - 3.3×10^5 cells/g) as compared to the shrimp pond water samples (9.0×10^0 – 4.6×10^3 cells/g). The results are in agreement with other studies that indicated the higher concentrations of *V. parahaemolyticus* cells in seafood samples as compared to water or environmental origins, mainly due to the ability of marine organisms to accumulate *V. parahaemolyticus* cells within their tissues (Nelapati *et al*., 2012; Quiroz-Guzmán *et al.*, 2013).

Our real-time PCR assay was developed to target the thermo labile (*tl*) gene. Thermolabile hemolysin (*tl*) gene is also known as the lecithin-dependent hemolysin (*ldh*) gene (Zhang *et al*., 2005). These genes produce a heat labile protein. According to Zhang *et al.* (2005), this type of hemolysin is an exotoxin that attacks red blood cell membranes and causes cell to rupture. However, the role of *tl* gene in the pathogenicity of *V. parahaemolyticus* is still unclear (Shinoda *et al.*, 1991). According to studies conducted by Taniguchi *et al.* (1985) and Rizvi and Bej (2010), this gene was shown to be present in all of the *V. parahaemolyticus* strains tested. However, other commonly used marker genes such as *tdh*, *trh* and *toxR* are only present in clinical and pathogenic *V. parahaemolyticus* strains, rendering them unsuitable for quantification of total non-pathogenic and environmental *V. parahaemolyticus* population (Rosec *et al.*, 2009; Theethakaew *et al*., 2013). Thus, our assay is not only applicable for clinical diagnosis but also for environmental monitoring. For the seafood industries, our assay can be used for routine and rapid monitoring of the prevalence of *V. parahaemolyticus* in food products without compromising their market cost. This step will not only assist in quality control of the food products, but also monitor for potential excessive *V. parahaemolyticus* contamination.

We conclude that our real-time protocol provides specific, accurate and fast quantitation of *V. parahaemolyticus* from seafood, environmental and clinical samples over several orders of magnitude in a sensitive and rapid way, with all assays being carried out and accomplished in less than 3 hours.

Acknowledgments

We thank Ma Xiang Ru for her valuable technical assistance. We also thank Bong Chun Yong for providing the shrimp pond water samples. This study was supported by the Malaysian Ministry of Higher Education (MOHE) Fundamental Research Grant Scheme grant FRGS/01(15)/744/2010(30).

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