Thesis for the Degree of Master of Fisheries Science

SYBR Green I-based real-time PCR assay targeting *groEL* gene for the detection and quantification of *Vibrio alginolyticus* from shellfish and shrimp

by

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KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

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(조개류와 새우로부터 *Vibrio alginolyticus* 의 검출과 정량을 위한 *groEL* 유전자 표적 real-time PCR 분석)

Advisor: Professor In-Soo Kong

by

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Abstract

V. alginolyticus is an important opportunistic pathogen for humans and marine animals. Culture-based methods and the traditional polymerase chain reaction (PCR) can not quantify the pathogen with sufficient sensitivity. Thus, reliable, rapid, and accurate detection and quantification methods are essential to prevent and control *V. alginolyticus*. A real-time PCR assay was developed by using SYBR Green I targeting *groEL* gene to detect and quantify *V. alginolyticus*. A species-specific primer was designed based on *groEL* gene. Specificity of the primer was confirmed by using three *V. alginolyticus* strains and 32 other *Vibrio* and non-*Vibrio* strains. Only the *V. alginolyticus* strain showed a positive result in the specificity test. A melting curve analysis showed a specific peak with a melting temperature of 85.80 ± 0.15ºC. A standard curve was produced to permit quantification of the target organism. Detection sensitivity was 0.14 pg of genomic DNA (equivalent to 10 cells/ml) for a pure culture of *V. alginolyticus*. *V. alginolyticus* was also quantified in artificially inoculated shellfish and shrimp. The results indicated that SYBR

Green I-based quantitative real-time polymerase chain reaction (qRT-PCR) targeting the *groEL* gene enabled accurate, sensitive, and rapid quantitative detection of *V. alginolyticus* in shellfish, shrimp and seawater.

Keywords: *groEL* gene, quantitative detection, real-time PCR, SYBR Green I, *V. alginolyticus*, specificity, sensitivity.

Introduction

Vibrio alginolyticus is a Gram-negative halophilic bacterium distributed worldwide, and it has been considered as an important opportunistic pathogen of humans, fish, shrimp and shellfish (Hervio-Heath et al., 2002; George et al., 2005; Gómez-León et al., 2005; Xie et al., 2005; Gonzalez-Escalona et al., 2006; Oksuz and Gurler, 2013). *V. alginolyticus* infection often causes severe mortality in fish and shrimp and has become a major problem in aquaculture globally (Liu et al., 2004; Gómez-León et al., 2005).

Human diseases caused by *V. alginolyticus* include gastroenteritis, soft tissue wounds (superficial wounds), otitis media, food intoxication, and septicaemia (Taylor et al., 1981; Reina et al., 1995; Gomathi et al., 2013). *V. alginolyticus* can cause mortality in immune-compromised patients (Campanelli et al., 2008). Septic shock due to *V. alginolyticus* in a Korean patient with cirrhosis was reported after ingestion of raw fish (Lee et al., 2008). *V. alginolyticus* infection in humans is acquired either through ingestion of contaminated seafood or contact of traumatised skin with seawater or brackish water; thus, it is gaining attention as a public health issue.

Effective prevention, control, and treatment of these diseases require rapid, reliable and highly sensitive diagnostic techniques. A number of microbiological techniques based on cultivation, isolation and pure cultures and serotyping of organisms have been used to identify pathogenic microorganisms. These methods are often time consuming and have limited discriminatory power (Aznar et al., 1993; Austin et al., 1995). Mustafa et al. (2013) reported that conventional methods of bacterial identification are less specific and cannot distinguish between species, particularly *V. alginolyticus*, which is biochemically very similar to *V. parahaemolyticus*. Molecular methods based on DNA probes or the polymerase chain reaction (PCR) have been developed to overcome these limitations, offering a rapid, reliable and sensitive alternative to culture-based methods (Vantarakis et al., 2000; Del Cero et al., 2002; Bader et al., 2003; Bilodeau et al., 2003).

Many PCR-based methods have been described for detecting *Vibrio* species. These methods often use primers for specific or universal genes such as those encoding toxins, or the *16S sRNA* or *23S sRNA* gene as a target marker. However, the nucleotide sequences of these genes are similar among bacterial species, particularly those within the same genus, and the absence

of toxin genes in non-virulent strains precludes their use as targets for species-specific identification of bacterial pathogens (Chizhikov et al., 2001). Izumiya et al. (2011) reported that a virulence gene can be exchanged among *Vibrio* species, or a particular gene can disappear, resulting in false-positive or -negative results when such genes are targeted. To overcome this problem, PCR methods that target housekeeping genes such as 16S sRNA, *23S sRNA*, *amiB*, *dnaJ*, *gyrB*, *pho*, *rpoA*, *groEL*, and *rpoB* (Thomson et al. 2005; Nhung et al. 2007) have been developed. But, house-keeping genes have also some limitation to be used as genetic marker because of its high sequence similarity among bacterial species.

The *groEL* gene, a house-keeping gene, encodes the GroEL chaperone (also known as Hsp60, Cpn60, GroL and Mop A), which plays a vital role in the control of cellular stress in bacterial cells. When bacterial cells are exposed to environmental stress or enter into host tissue, the *groEL* gene induces to express at markedly higher level to protect their cells from damage. The *groEL* gene has the potential to serve as genetic marker because it has been well documented that this gene is one of the most conserved systems in nature. Despite of conserved nature of the *groEL* gene, the level of

interspecies variation of *groEL* sequence is greater (Nishibuchi, 2006; Yushan et al., 2010). Due to the superiority of *groEL* over other house-keeping genes, we demonstrated that the *groEL* gene is a suitable genetic marker for detection of many *Vibrio* species using single PCR and Restriction Fragment Length Polymorphism (RFLP) analysis (Kim et al., 2010, 2012; Yushan et al., 2010; Hossain et al., 2011, 2012, 2013).

Traditional PCR cannot directly quantify a target organism. Quantitative detection is necessary to determine the intensity of infection in a sample or the presence of an organism in the environment. Real-time quantitative PCR facilitates detection and quantification of pathogenic microorganisms. Another advantage of real-time PCR over traditional PCR is the ability to detect target DNA of very low copy number, allowing detection of the infection at an earlier stage. For this reason, real-time quantitative PCR has revolutionised molecular diagnostics, and the technique is being used in a rapidly expanding number of applications (Arya et al., 2005). Real-time quantitative PCR is operated on the basis of two approaches: dye and probe. SYBR Green I is an intercalating dye that binds to the minor groove of double-stranded DNA and emits a fluorescent signal. The advantages to

using SYBR Green I for real-time PCR detection includes high detection sensitivity and low reaction cost compared to probe based fluorescent. It allows specificity of amplicons by melting curve analysis, producing a characteristic melting temperature (Tm). The use of melting curve analysis eliminates the necessity for agarose gel electrophoresis, because the melting temperature (Tm) of the specific amplicon is analogous to the detection of an electrophoretic band.

Therefore, the aim of the study was to detect *V. alginolyticus* by real-time PCR using *groEL* gene as a target marker. Sensitivity of qRT-PCR assays was also determined for quantification of *V. alginolyticus* and verified the efficacy of the developed method using artificially inoculated shellfish, shrimp and seawater.

Materials and Methods

Bacterial culture and DNA template preparation:

Thirty-five bacterial strains were used in this study (Table 1), including three *V. alginolyticus* strains and three *V. parahaemolyticus* strains. All *Vibrio species* were cultured in brain–heart infusion (BHI; BD, Franklin Lakes, NJ, USA) broth with 2.5% sodium chloride, whereas the other bacterial strains were cultured in luria–bertani broth (LB, USB, Cleveland, OH, USA). Template DNA was extracted from overnight culture of all bacteria by the phenol-chloroform extraction and ethanol precipitation method described by Ausubel et al. (1998). Briefly, one milliliter of overnight bacterial culture was taken in eppendorf tube and centrifuged at 12000 rpm for 3 minutes. The supernatant was discarded and pellet was resuspended with 600 µl distilled water (DW). 17.5 µl of 20% SDS and 4 µl of protease K (20 mg/ml) was added and mixed thoroughly, and left it for incubated at 37ºC for 1 hour. After incubation, 100 µl of 5M sodium chloride solution was added and mixed by inversion. Then, 350 µl phenol and 350 µl chloroform were added, mixed by vortexing and centrifuged at 12000 rpm for 10 minutes. The supernatant was collected and added with

Table 1. Strains used in this study

	Microorganisms	Source of reference	qPCR
1	*V. aestuarianus*	KCCM 40863	-
2	*V. alginolyticus*	KCCM 40513, 2 E	+
3	*V. anguillarum*	KCTC 2711	-
4	*V. campbellii*	KCCM 40864	-
5	*V. cholerae*	KCTC 2715	-
6	*V. cincinnatiensis*	KCTC 2733	-
7	*V. damsella*	E	-
8	*V. diazotrophicus*	KCCM 41606	-
9	*V. fisheri*	KCCM 41685	-
10	*V. fluvialis*	KCTC 2473	-
11	*V. furnissii*	KCTC 2731	-
12	*V. harveyi*	KCCM 40866	-
13	*V. logei*	KCTC 2721	-
14	*V. mediterranei*	KCCM 40867	-
15	*V. metschnikovii*	KCTC 2736	-
16	*V. mimicus*	ATCC 33653	-
17	*V. natriegens*	KCCM 40868	-
18	*V. navarrensis*	KCCM 41682	-
19	*V. nereis*	KCCM 41667	-
20	*V. ordalii*	KCCM 41669	-
21	*V. parahaemolyticus*	KCCM 11965, KCCM 41664, KCTC 2471	-
22	*V. proteolyticus*	KCTC 2730	-
23	*V. tubiashii*	KCTC 2728	-
24	*V. vulnificus*	KCTC 2980	-
25	*Aeromonas hydrophila*	KCTC 2358	-
26	*A. salmonicida*	KCCM 40239	-
27	*Edwardsiella tarda*	KCTC 12267	-
28	*Enterobacter cloacae*	E	-
29	*Escherichia coli*	E	-
30	*Salmonella typhi*	E	-
31	*Shigella flexneri*	E	-

KCCM, Korean Culture Centre of Microorganisms, Korea; E, Environmental strain; KCTC, Korean Collection for Type Culture, Korea; ATCC, American Type Culture Collection, USA; +, Positive result; -, Negative result.

equal amount of phenol and chloroform and then centrifuged. This procedure was repeated for three times and finally supernatant was collected. The supernatant was added with double volume of 100% ethanol and centrifuged (at 12000 rpm for 10 min) at 4ºC. After centrifuge, the supernatant was discarded. Then 70% ethanol washing was performed by centrifuging at 12000 rpm for 10 minutes maintaining 4ºC. Finally, supernatant was discarded, vacuum dried for 15 minutes and stored at –20ºC until use. DNA concentration was measured by a spectrophotometer (Eppendorf BioPhotometer 6131, Hamburg, Germany) at a wavelength of 260 nm. Genomic DNAs of all *Vibrio* and non-*Vibrio* strains purified and identified by *16S rRNA* were used as templates for PCR.

Primer design:

A species-specific primer set for detecting *V. alginolyticus* was designed based on the *groEL* gene. A number of available sequences of the *groEL* gene of *Vibrio* and non-*Vibrio species* were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) using the BLASTN search program provided by the National Center for Biotechnology Information (NCBI). The nucleotide sequences were aligned using the ClustalW program. The most conserved regions of the *groEL* gene for target species

were selected and a specific primer set was designed (Table 2 and Figure 1). The primer set was commercially synthesized by Cosmo Genetech, Seoul, Korea. Amplicon size of the primer was 301 bp. A universal *16S rRNA* primer was also used in order to cheek the purity of all *Vibrio* and non-*Vibrio* strains by traditional polymerase chain reaction (PCR) (Kim et al., 2008) (Table 2).

SYBR Green real-time PCR assay:

Real-time PCR and data analysis were performed by Thermal Cycler Disc™ Real Time System Lite (model TP700/760, software version V5.0x) (TaKaRA Bio INC.) using the SYBR Premix Ex Taq™ (Tli RNaseH Plus). The PCR mixture (25 µl) contained 12.5 µl 2x SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa Bio INC.), 0.5 µl (15 µM/L) of each primer (*groEL*-up, *groEL*-rp), 9.5 µl of sterile distilled water and 2.0 µl of extracted DNA was used as template. The two-steps shuttle PCR protocol was optimized that included initial denaturation of 95ºC for 30 s followed by 35 cycles each having 05 s at 95ºC for denaturation, 15 s at 66ºC for annealing and extension.

Table 2. Description of primers

Target	Primer	Sequence	Size
groEL	up	5´-GATTCGGTGAAGAAGAGATGATCTC-3´	301 bp
	rp	5´-TCTTCGTTGTCACCCGTTAGGTGA -3´	
16S rRNA	up	5´-AGAGTTTGATCMTGGCTCAG-3´	1466 bp
	rp	5´-TACGGYTACCTTGTTACGACTT-3´	

Figure 1. Location of primer sequences in *groEL* gene. Primer regions are indicated by underlines. Nucleotides sequence identical to those of the *V. alginolyticus* are indicated by dots.

A melting curve analysis of the amplified DNA was performed by slow heating from 60ºC to 95ºC to assess the specificity of the PCR product. The threshold was set automatically by which the cycle threshold (Ct) or quantification cycle (Cq) was determined. The amplified product was further analyzed by 1.2% agarose gel electrophoresis. To determine sensitivity of the real-time PCR assay, purified genomic DNA of *V. alginolyticus* KCCM 40863 was serially diluted 10-fold from 140 ng to 0.014 pg/μl, which was equivalent to approximately 10^7 to 1 CFU/ml, in sterile distilled water and subject to real-time PCR amplification using the protocol described above.

Quantification of *V. alginolyticus* from artificially inoculated shellfishes:

To quantify the *V. alginolyticus* from shellfish, tissues of oyster (*Crassostrea gigas*), thick shell mussel (*Mytilus coruscus*) and Manila clam (*Tapes philippinarum*) were used in a spiking test as described by Hossain et al. (2012). Briefly, 200 µl of overnight cultured bacterial cells were added to 3-ml fresh BHI media, followed by a 2–3-h incubation at 37ºC until optical density (OD) reached 1 (equivalent to approximately 10^8 CFU/ml). Then, 1-ml bacterial suspension was added to an Eppendorf tube and centrifuged at 10000 × g for 3 min. The pellet was suspended in 1-ml physiological saline (0.85%). A 200-µl aliquot of this bacterial suspension was added to 1 g of shellfish tissue. Total DNA was extracted from tissues before and after 5-h enrichment (incubation) using a DNA extraction kit (NucleoGen Biotech, Siheung, Korea). Briefly, small amount of tissue (upto 25 mg) was taken in a 1.5 ml appendorf tube. 180 µl buffer NCL and 20 µl proteinase K was mixed with it and incubated at 56ºC until the tissue was completely lysed. After incubation, 200 µl buffer NL solution was added to the tube, mixed by pulse-vortexing for 15 s and incubated at 70ºC for 10 minutes. Then, 400 µl ethanol (96-100%) was added to the solution and mixed by pulse-vortexing. This mixture was placed to a spin column (in a 2

ml collection tube) and centrifuged it at 6000 x g for one minute. The tube containing the filtrate was discarded and the spin column was placed in a clean 2 ml collection tube. Wash C solution of 700 µl was placed in the spin column and centrifuged at 6000 x g for one minute. The collection tube was discarded and the spin column was placed in another clean collection tube. The spin column was added with 700 µl wash D solution and centrifuged at 6000 x g for one minute. Finally, it was centrifuged for 2 minutes at maximum speed to remove residual wash solution. The spin column was again placed in clean tube, and 200 µl elution buffer or distilled water was added and hold it for one minute. Then it was centrifuged at 6000 x g for one minute. Lastly, this step was repeated with 5 minutes incubation of the spin column loaded with elution buffer or distilled water. DNA was also extracted from fresh shellfish that were not inoculated with bacteria. Real-time PCR was performed to quantify the CFU and corresponding DNA levels in fresh and inoculated shellfish.

Quantification of *V. alginolyticus* in artificially inoculated shrimp:

Twenty-one live shrimp (*Trachysalambria curvirostris*) (22 ± 2 g) were collected from a local market. They were divided into three groups and maintained in 15-L plastic seawater tanks at 4°C with aeration. The shrimp were artificially inoculated using the method described by Tran et al. (2013). Briefly, one group of shrimp was inoculated by intramuscular injection. One millilitre of bacterial suspension was centrifuged at 10000 × g for 3 min. The pellet was resuspended in 100-µl physiological saline (0.85%). Each shrimp was injected with 100-µl bacterial suspension and maintained in 15-L plastic seawater tanks with aeration. Another group of shrimp was immersed in 150-ml bacterial suspension (OD of 1, 1.5×10^7 CFU/ml) for 10 min in a beaker with aeration. Then, the shrimp and the bacterial suspension were released in a 15-L plastic tank to a bacterial density of 1.5×10^6 CFU/ml. Samples were collected from shrimp muscle, hepatopancreas and water of each group after 24, 48, and 120 h. DNA was extracted from shrimp tissue and hepatopancreas using an extraction kit (NucleoGen Biotech), and the simple boiling method was used to extract DNA from water, according to Zhou et al. (2007). Then, DNA was quantified by real-time PCR. The numbers of viable bacteria in shrimp tissue, hepatopancreas,

and water were determined by the plate-count method. Samples collected from non-inoculated shrimp were also analysed by real-time PCR.

Results

Specificity of detection:

To evaluate the specificity of developed assay, DNA isolated from pure culture of three *V. alginolyticus* strains and 32 other *Vibrio* and non-*Vibrio* species were examined. A band of 1466 bp was found from all *Vibrio* and non-*Vibrio* species when traditional PCR was performed with *16S rRNA* universal primer (Figure 2). All *V. alginolyticus* strains tested showed positive results, with a mean Ct values of 14±0.35, whereas other *Vibrio* and non- *Vibrio* strains showed negative result in real-time PCR (Figure 3). The specificities of the PCR products were determined by melting curve analysis; a reproducible distinct melting temperature (Tm) of 85.80±0.15ºC was observed for all target strains (Figure 4). Primer specificity was confirmed by resolution of the PCR products in 2% agarose gel (Figure 5).

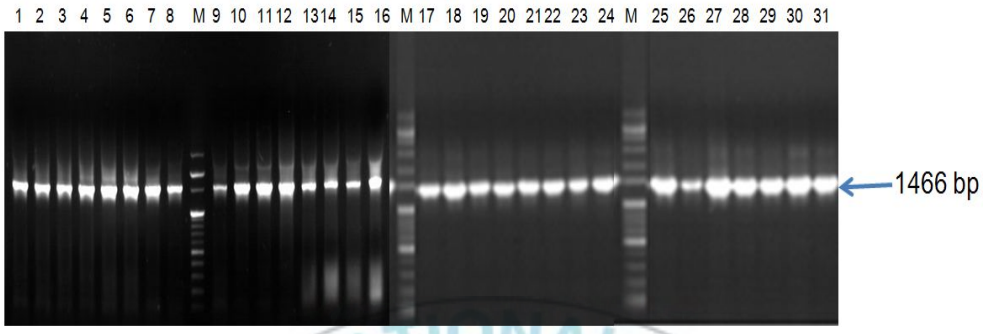

Figure 2. Agarose (1.2%) gel electrophoresis of DNA products amplified from *Vibrio* and non-*Vibrio* species by traditional PCR using the *16S rRNA* universal primer set. Lane M; 100 bp DNA ladder, Lanes 1-31; Corresponding number of microorganisms shown in Table 1.

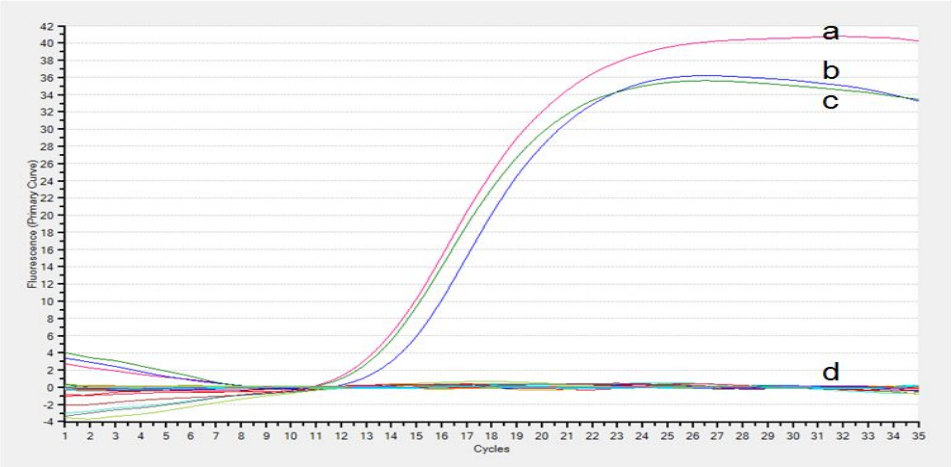

Figure 3. Real-time PCR amplification of *V. alginolyticus* strains, other *Vibrio spp.* and *non-Vibrio spp.* for specificity test. a-c, V. alginolyticus strains; d, *V. aestuariansus, V. anguillarum, V. campbellii, V. cholera, V. cincinnatiensis, V. damsella, V. diazotrophicus, V. fisheri, V. fluvialis, V. furnissii, V. harveyi, V. logei, V. mediterranei, V. metschnikovii, V. mimicus, V. natriegens, V. navarrensis, V. nereis, V. ordalii, V. parahaemolyticus, V. proteolyticus, V. tubiashii, V. vulnificus, Aeromonas hydrophila, A. salmonicida, Edwardsiella tarda, Enterobacter cloacae, Escherichia coli, Salmonella typhi, Shigella flexneri,* NTC (No template control) and NAC (No amplicon control).

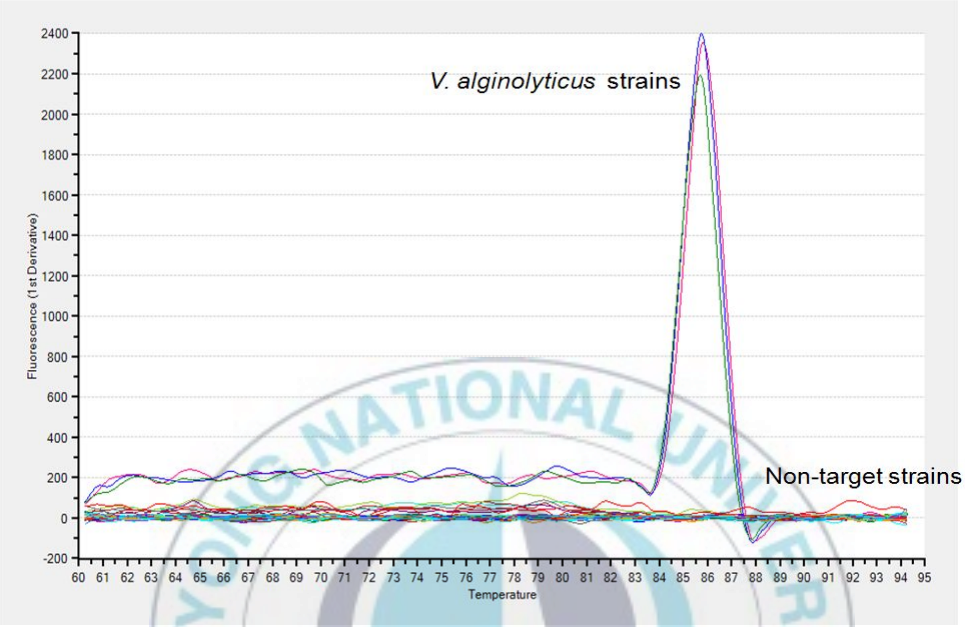

Figure 4. Melting curve analysis of *V. alginolyticus* strains, other *Vibrio spp.* and non-*Vibrio. spp.* for the specificity test.

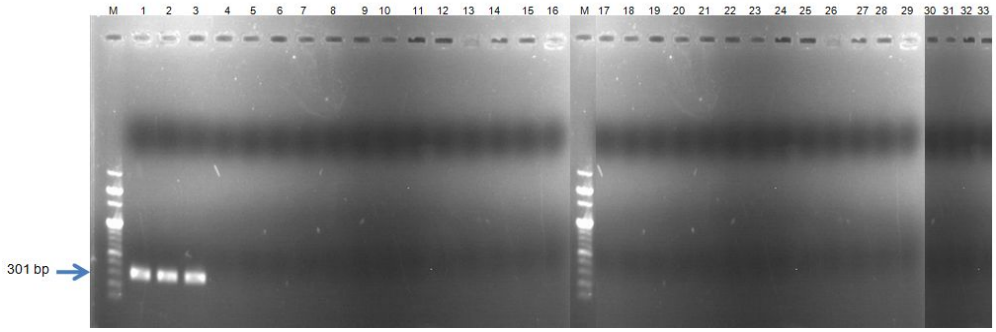

Figure 5. Corresponding agarose gel electrophoresis of real-time PCR products. Lane M, 100-bp DNA ladder; Lanes 1-3, *V. alginolyticus* strains; Lanes 4-33, *V. aestuariansus, V. anguillarum, V. campbellii, V. cholera, V. cincinnatiensis, V. damsella, V. diazotrophicus, V. fisheri, V. fluvialis, V. furnissii, V. harveyi, V. logei, V. mediterranei, V. metschnikovii, V. mimicus, V. natriegens, V. navarrensis, V. nereis, V. ordalii, V. parahaemolyticus, V. proteolyticus, V. tubiashii, V. vulnificus, Aeromonas hydrophila, A. salmonicida, Edwardsiella tarda, Enterobacter cloacae, Escherichia coli, Salmonella typhi, Shigella flexneri,* respectively.

Sensitivity:

A standard curve using various concentrations of purified *V. alginolyticus* DNA was constructed to determine assay sensitivity. The results showed a linear relationship between Ct and log input CFU per ml. The detection limit was 0.14 pg/µl (equivalent to 10^1 CFU/ml) for purified DNA with a Ct value of 33.28 (Figure 6, Table 3), and the expected dissociation temperature of 85.80 ± 0.15°C (Figure 7). The linear regression equation was Y = −3.276 + 38.02, the slope of the standard curve was −3.293 and efficiency of the assay was 102% with R^2 = 0.982 (Figure 8). The strong correlation between the Ct values and log CFU inputs indicated the quantitative potential for real time PCR. The real-time PCR product was also examined in agarose gel where sensitivity was found at 10^2 CFU/ml (Figure 9).

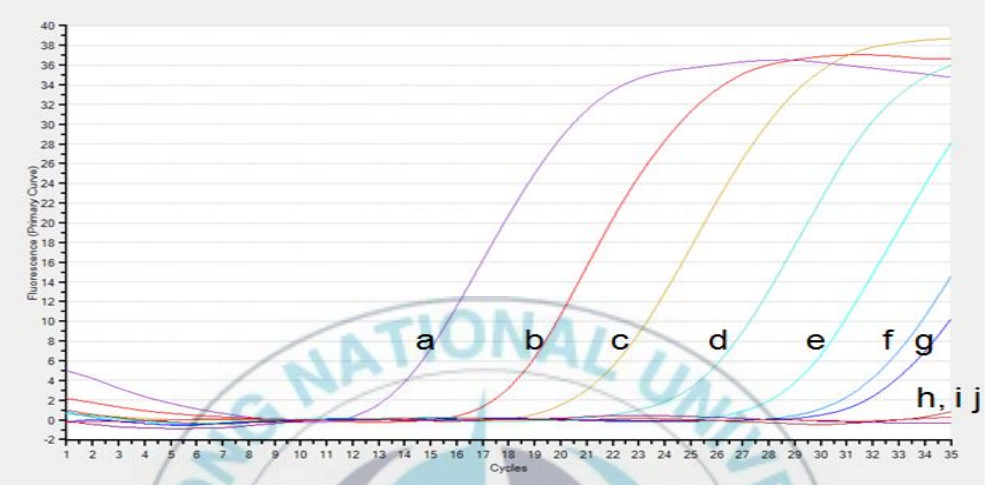

Figure 6. Amplification plot of primary curve for sensitivity test. a-h, *V. alginolyticus* 10^7 CFU/ml (140 ng/µl DNA), 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 CFU/ml (0.014 pg/µl DNA), respectively; i, NTC (No template control); j, NAC (No amplicon control).

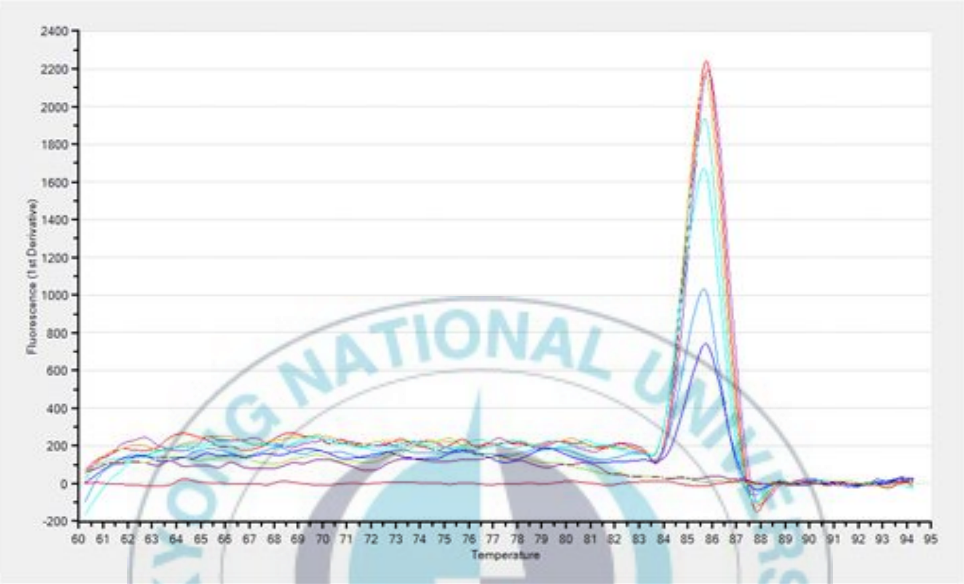

Figure 7. Corresponding melting curve analysis for the sensitivity test.

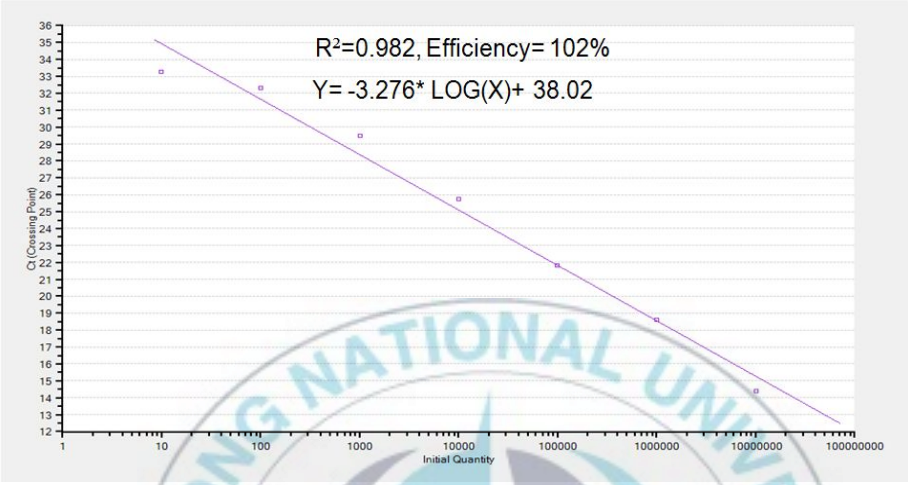

Figure 8. Standard curve analysis for the sensitivity test.

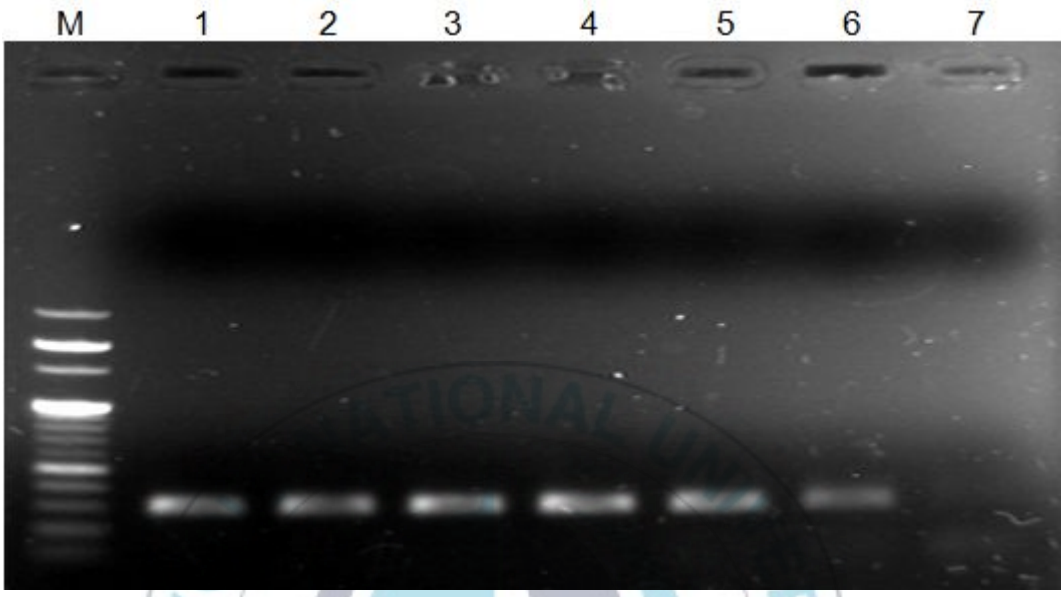

Figure 9. Corresponding agarose gel electrophoresis of real-time PCR products for sensitivity test. Lane M, DNA ladder; Lane 1, 10^7 CFU/ml; Lane 2, 10^6 CFU/ml; Lane 3, 10^5 CFU/ml; Lane 4, 10^4 CFU/ml; Lane 5, 10^3 CFU/ml; Lane 6, 10^2 CFU/ml; Lane 7, 10^1 CFU/ml .

Table 3. Sensitivity of real-time PCR assay of pure culture of bacteria

No.	Amount of DNA	CFU/ml	Ct (Cp)	Dissociation temp. (°C)
1	140 ng/μl	10^7	14.38	85.84
2	14.0 ng/μl	10^6	18.62	85.76
3	1.4 0 ng/μl	10^5	21.84	85.70
4	0.14 ng/ul	10^4	25.77	85.68
5	14.0 pg/μl	10^3	29.49	85.65
6	1.40 pg/μl	10^2	32.30	85.65
7	0.14 pg/μl	10^1	33.28	85.73
8	0.014 pg/μl	1	-	79.86

Detection and quantification of *V. alginolyticus* from artificially inoculated shellfishes:

V. alginolyticus was detected and quantified in artificially inoculated shellfish by real-time PCR (Table 4). The fresh shellfish yielded a negative result, whereas the inoculated shellfish after 0- (zero) and 5-h enrichment showed positive results. Shellfish after 5-h enrichment exhibited a greater number of *V. alginolyticus* than those after 0-h enrichment.

Table 4. Quantification of *V. alginolyticus* from artificially inoculated shellfishes

No.	Samples	Ct value (mean)	Amount of DNA (μg/g)	CFU/g
1	Fresh Oyster	-	-	-
2	Fresh Mussel	-	-	-
3	Fresh Clam	-	-	-
4	Oyster 0-h enrichment	23.46	443.72	4.4×10^5
5	Mussel 0-h enrichment	22.94	642.86	6.4×10^5
6	Clam 0-h enrichment	23.11	570.32	5.7×10^5
7	Oyster 5-h enrichment	22.50	877.23	8.7×10^5
8	Mussel 5-h enrichment	20.95	2594.89	2.5×10^6
9	Clam 5-h enrichment	20.49	3590.20	3.5×10^6

Detection and quantification of target species from artificially inoculated shrimp and seawater:

To assess the applicability of the developed assay, *V. alginolyticus* was quantified in artificially inoculated live shrimp and seawater (Table 5). The shrimp that were inoculated by intramuscular injection contained higher amount of bacteria than the shrimp that were inoculated by immersion. The *V. alginolyticus* level was higher in pancreatic cells than in muscle tissue. *V. alginolyticus* was found in higher level in water of immersion test than that of injection test. The *V. alginolyticus* did not increase significantly with time in alive shrimp. The numbers of bacteria determined by real-time PCR were slightly higher than those by plate counts in most samples.

Table 5. Quantification of *V. alginolyticus* from artificially inoculated shrimp and seawater

Treatm-ents	Incubation period (hours)	Item	Ct (mean)	Calculated by qPCR (CFU/ml)	Calculated by qPCR (CFU/g)	Plate counts by culture-based method
IJ-1	24	MT	26.91	2.4×10^3	1.9×10^4	8.5×10^3 CFU/g
		H	26.38	3.5×10^3	2.8×10^4	8.6×10^3 CFU/g
IJ-2	48	MT	30.34	1.4×10^2	1.1×10^3	7.2×10^2 CFU/g
		H	26.03	4.5×10^3	3.6×10^4	1.3×10^4 CFU/g
IJ-3	120	MT	29.24	4.7×10^2	3.8×10^3	5.7×10^2 CFU/g
		H	25.62	6.0×10^3	4.8×10^4	3.6×10^3 CFU/g
IM-1	24	MT	34.07	1.6×10	1.2×10^2	1.0×10^2 CFU/g
		H	33.43	2.5×10	2.0×10^2	1.3×10^2 CFU/g
IM-2	48	MT	33.62	2.1×10	2.5×10^2	1.2×10^2 CFU/g
		H	33.46	2.4×10	1.9×10^2	1.0×10^2 CFU/g
IM-3	120	MT	32.56	4.6×10	3.7×10^2	2.0×10^2 CFU/g
		H	32.27	5.7x10	4.5×10^2	3.0×10^2 CFU/g
IM-1	24	SW	21.70	9.5×10^4	N/A	2.0×10^4 CFU/ml
IJ-1		SW	31.21	1.2×10^2	N/A	1.0×10^2 CFU/ml
IM-2	48	SW	21.47	1.1×10^5	N/A	7.6×10^4 CFU/ml
IJ-2		SW	31.23	1.1×10^3	N/A	8.4×10^2 CFU/ml
IM-3	120	SW	22.00	7.7×10^4	N/A	2.3×10^4 CFU/ml
IJ-3		SW	32.26	5.7×10^3	N/A	2.0×10^3 CFU/ml

IJ- Injection, IM- Immersion, MT- Muscle tissue, H- Hepatopancreas tissue, SW- Seawater

Discussion

V. alginolyticus is an important opportunistic pathogen causing disease in human and aquatic animals. Quantifying target microorganisms is essential to determine the intensity of infection in a host or concentration in the environment. Because traditional PCR is not quantitative, real-time PCR was used in this study to detect and quantify *V. alginolyticus* from pure culture, and artificially inoculated shellfish and shrimp. Real-time PCR is a more accurate, sensitive, reliable and rapid method than traditional PCR for quantitative detection of a pathogen (Mokhtari et al., 2013). SYBR Green I is a less-costly option than probe-based real-time PCR. It also exhibits specificity by producing a distinctive Tm for a specific primer. Several PCR-based methods have been developed for detecting *V. alginolyticus*; these have targeted the *16S rRNA, ompK, toxR, gyrB, rpoX*, and collagenase genes (Gómez-León et al., 2005; Zhou et al., 2007; Luo and Hu, 2008; Jing-jing et al., 2011). However, the *16S rRNA* gene failed to discriminate *V. alginolyticus* from other *Vibrio* species because of their high sequence similarity (Lalitha et al., 2008). Toxin genes can be used as identification markers for virulent strains. However, there is a risk of misidentification

because such genes can be transferred among bacteria (Neogi et al., 2010). Xie et al. (2005) reported that there is no correlation between the pathogenecity and the presence of virulent genes. *V. alginolyticus* possesses various pathogenic properties that are used to adapt to different hosts and environments (Ren et al., 2013; Zhao et al., 2010, 2011). Therefore, it is important to conduct surveillance of the total (both toxigenic and non-toxigenic) *V. alginolyticus* population to increase our understanding of the role of this microorganism in the environment. Thus, we selected the *groEL* gene, which was used to detect many *Vibrio* species, with the exception of *V. alginolyticus*, in our previous studies (Hossain et al., 2011, 2012, 2013, 2013a).

SYBR Green I-based real-time PCR methods to quantitatively detect many *Vibrio* species, including *V. alginolyticus*, have been developed. However, no real-time PCR assay targeting the *groEL* gene for quantification of *V. alginolyticus* has been reported. SYBR Green I binds double-stranded DNA and emits light upon excitation. Thus, as PCR product accumulates, fluorescence emission increases. The advantages of SYBR Green I are that it is inexpensive, easy to use, and sensitive. Malinen et al. (2003) compared

SYBR Green I-based real-time PCR with the TaqMan assay for detection of faecal pathogens; the two assays exhibited identical detection sensitivity. The disadvantages of SYBR Green I is that it binds to any double-stranded DNA, having a chances to produce primer-dimer and other non-specific amplification, which results in an overestimation of the target DNA. This problem can be resolved by designing a good primer and optimising the PCR conditions. In this study, we designed a species-specific primer, optimised the reaction conditions and reagent concentrations, and achieved good results. A two-step shuttle PCR assay using a high annealing temperature (66ºC) was required for specific amplification. Similar annealing temperatures, such as 67ºC and 69ºC, have been used for detection of *V. anguillarum* and *V. parahaemolyticus*, respectively (Kim et al., 2010; Hossain et al., 2011). The use of a high annealing temperature reduced the PCR reaction time to only 55 min. In specificity test, three *V. alginolyticus* strains showed positive result and thirty-two non-target strains showed negative result in real-time and traditional PCR. This result indicates that the primer set based on *groEL* gene was specific for *V. alginolyticus* and suits one of the objectives of this study.

The detection limit of this study was found to be 0.14 pg/μl for purified DNA which was equivalent to 10^1 CFU/ml bacterial cell. The standard curve suggested marked efficiency, and the Ct values and log CFU numbers showed good correlation, indicating the quantitative potential of the real-time PCR assay. The detection limit was similar to those reported by Jing-jing et al. (2011) and Zhou et al. (2007). In contrast, the sensitivity of the traditional PCR method was 100 pg; i.e. 1000 times less sensitive than the assay developed herein (Hossain et al., 2011, 2012, 2013). The real-time PCR product examined in agarose gel showed ten times less sensitivity, indicating that agarose gel hinders the sensitivity and shows lower level of sensitivity than actual one.

To assess the applicability of the developed assay, artificially inoculated shellfishes and shrimps were quantified in this study. The fresh oyster just after collection from market did not show positive result. The quantity of *V. alginolyticus* increased with the increase of enrichment period. This result indicates that the real-time PCR can successfully quantify the bacterial load from shellfishes. In the shrimp inoculation test, the level of *V. alginolyticus* was higher in pancreatic cell than that of muscle tissue which justifies the

study since hepatopancreatic cell contains higher level of pathogenic microorganisms (Janakirarm et al., 2000). The shrimp that were inoculated by intramuscular injection contained higher amount of bacteria than the shrimp that were inoculated by immersion. The amount of *V. alginolyticus* was steady with time which indicates that it could not make infection in alive shrimp. The lower temperature and/or inappropriate host may be the cause. The numbers calculated by real-time PCR are a little higher than by plate counts in most of the samples. It indicates that real-time PCR cannot discriminate between live and dead cells while only live cells could be counted by plate culture. In general, the number of CFU calculated by real-time and by culture-based method correlated with each other.

Conclusion

This study was conducted to develop a real-time PCR assay for the quantitative and specific detection of *V. alginolyticus* from shellfish, shrimp and seawater. The *groEL* gene provided a suitable phylogenetic marker for the detection of *V. alginolyticus* by real-time PCR. The primer set designed for this study was specific and sensitive. This real-time PCR assay also quantified *V. alginolyticus* from artificially inoculated shellfishes, shrimps and seawater. In summary, SYBR Green I real-time PCR targeting *groEL* gene is specific, sensitive, rapid and reliable for quantitative detection of *V. alginolyticus* from pure culture of bacteria, shellfishes, shrimps and seawater. This real-time PCR assay may provide a helpful tool for the diagnosis and quantification of *V. alginolyticus* from marine aquaculture animals, shellfishes and environment.

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