

Establishment of Real-time Quantitative PCR Method for *Vibrio parahaemolyticus* in Sediment of the *Stichopus japonicus* Culture Pond

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A rapid real-time quantitative PCR method was developed for *V. parahaemolyticus* in sediment of the *Stichopus japonicus* culture pond by using specific primers targeted *toxR* gene. The known number *V. parahaemolyticus* cells were spiked into sterile sediment served as the simulative sediment samples. Three modified sediment DNA extraction methods were tried, it was proved that modified lysozyme-SDS gentle lyse method is feasible for the sediment DNA extraction. The standard curves was made by using serial diluted total DNA extracted from the simulated sediment and which from plasmid of pure culture. Besides, sensitivity, specificity and repeatability of the detection method were demonstrated well. The quantification limit was found to be 10^2 cfu g⁻¹ for *V. parahaemolyticus* in sediment, and have a high repeatability as a result of all the coefficient of variation (CV) values between 0.31% and 0.92%, less than 5%. Development of the real-time PCR quantification method for *V. parahaemolyticus* in the sediment was important for disease prevention the cultured *Stichopus japonicus*.

Keywords: *toxR*; *Vibrio parahaemolyticus*; Real-time Quantitative PCR; *Stichopus japonicus* culture pond; Sediment DNA extraction method.

Vibrio parahaemolyticus is a gram-negative halophilic bacterium spreading over coastal and estuarine waters^{1, 2}, recognized as a leading food-borne pathogen to cause infections in humans³. Such organisms have been considered to be an agent of gastroenteritis associated with consumption of seafood⁴, with the symptom of abdominal pain, diarrhea, vomiting, shock or even death. In recent years, it was reported⁵ that *V. parahaemolyticus* was the pathogen of off-plate syndrome in Liaoning Province. Off-plate syndrome is one of the severe infectious diseases

of *Stichopus japonicus* during nursery period, whose high mortality has a severe threat to the development of *Stichopus japonicus* aquaculture industry⁶. Therefore, it is essential to detect the number of *V. parahaemolyticus* in the eco-system of *Stichopus japonicus* culture pond, which can prevent against the off-plate syndrome as well, to some extent.

The conventional microbiological enumeration methods, such as agar-plate culture method, most-probable-number (MPN) method and direct microscopic count method, ect., are not only cockamamie, time-consuming or error-prone, but also the specificity is poor. While, real-time PCR method, built on the probes⁷ or dyes⁸ to quantify the copies of target DNA in a sample, has the advantages of strong specificity, high sensitivity, good repeatability, and convenient manipulation.

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The method has been successfully used for quantifying the number of target gene as a marker for specific bacteria in the environment⁹. The validity of using these molecular biological techniques to quantify the organisms in the environmental sample depends on obtaining as much as possible pure DNA. In the existing literatures, the standard curves were set up based on DNA extracted from water samples¹⁰ and that from plasmid of pure culture to detect the bacteria in soil¹¹. However, it is inevitable that some DNA is lost and PCR inhibitors exist when extracted from sediment samples. So, it is necessary to use a new standard curve based on DNA extracted directly from sediment environment besides improve the yield and purity of the DNA extraction when detecting the number of *V. parahaemolyticus* in sediment environment of *Stichopus japonicus* culture.

A PCR method could distinguish *V. parahaemolyticus* from others closely related *Vibrio* species by designing species-specific primers. Several studies suggested that the *toxR* gene, proposed as an ancestral gene of *Vibrionaceae* family, is a useful target for *Vibrio* species identification^{12,13}. To develop a reliable, rapid, real-time PCR quantification of *V. parahaemolyticus* in sediment of *Stichopus japonicus* culture pond, *toxR* gene was used as primers, three sediment DNA extraction methods were compared, the PCR reaction conditions and system were optimized, and the standard curve based on the DNA extracted directly from sediment environment was set up. The result showed the specification, validity, quantification limit, repeatability of the protocol were high, was compared with the standard curve based on plasmid DNA.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The strain (*V. parahaemolyticus*) for development and optimization of the real-time quantitative PCR protocol was isolated from diseased sea cucumber and confirmed to be the pathogen of the off-plate syndrome by artificial infection. The non-*V. parahaemolyticus* strains used to assess the specificities of the primers were *Nitrobacter* sp., *Nitrosomonas* sp., *Pseudomonas*

sp., *Thalassobacillus* sp. and *Escherichia coli*. The stains were incubated in marine broth 2216E (MB) or MA at 28°C overnight and then the concentration of bacteria (cfu ml⁻¹) was calculated by dilution plate counting method. Other species served as negative controls were cultured on selective medium based on specific conditions.

Primers

The species-specific primers for detection total *V. parahaemolyticus* in this study was based on conserved regions of *toxR* gene as described in reference 14, which could amplify a 180-bp specific segment¹⁴. The sequences of primers were as follows: PA-Forward 52 -ATT GAC GCC TCT GCTAATGAG-32, PA-Reverse 52 -TAC GCAAAT CGG TAG TAA TAG TG-32. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Real-time PCR Assay

The PCR amplification reactions were performed by using the Real-Time Detection system CFX96 (Bio-Rad, USA). Real-time PCR reaction mixture (20µl) consisted of DNA sample (1.0µl), 2×Ultra SYRB Mixture (10µl), 0.4µl each of the forward and reverse primers (10µM). The amplification mixtures were loaded into capillary-type reaction tubes. The optimized two-step PCR thermal cycling conditions included 95°C for 10min, followed by 40 cycles of 95°C for 15s, 60°C for 10min. In order to check and confirm the PCR amplicons and specificity of the primers, melting curve program was followed by the thermal cycling conditions with 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, 60°C for 15 s. All real-time PCR reactions were performed in triplicate. Conventional PCR amplification was performed with Mastercycler gradient (Eppendorf, Germany). Reaction mixture (50µl) contained the DNA sample (2µl), 10×PCR buffer (5µl), dNTPs (5µl), MgCl₂ (4µl), *Taq* DNA polymerase (0.8µl), and Sterile MilliQ water (27.2µl). The PCR cycling conditions included an initial melting cycle of 97°C for 5 min, followed by 30 cycles of 94°C for 1 min (denaturation) ÷ 51°C for 1 min (annealing), 72°C for 2 min (elongation) ÷ and a final extension cycle of 72°C for 10 min. The PCR products were verified with electrophoresis in 2% agarose gel (wt/v) using 100bp ladder marker, stained with GelGreen, and photographed using DNR MF-chemiBIS (DNR, Israel).

Standard Curve Based on Plasmid

The purified PCR products were connected with PMD18-T vector, transferred to TOP10 competent cells, and selected through blue-white plaque on LB plate with ampicillin. The validated white plaque were cultured in ampicillin LB media overnight, and sent to Sangon Biotech (Shanghai) Co., Ltd for sequencing. The positive recombinant plasmids were extracted by using Plasmid Mini Preparation Kit (Sangon Biotech (Shanghai) Co., Ltd., China), the concentration of plasmid were measured by NanoDrop 1000 (Nanodrop, USA). After the adequately dilution, standard curve of the threshold cycle (C_T) vs copies μl^{-1} of the recombinant plasmid was established.

Standard Curve Based on Simulated Sediment Samples

DNA Removed Sediment Samples

The sediment samples were collected from *Stichopus japonicus* culture pond in Qingdao City, Shandong Province, ground into powder after dried at 80°C overnight, and sieved on a 200 mesh screen, subsequently. The pre-handling samples were autoclaved for 2h to destroy the DNA and stored at -20°C until use.

DNA Extraction and Purification

The simulated samples were prepared by adding known-concentration bacteria to the autoclaved sediment, the DNA removed sediment samples served as negative control. Three sediment DNA extraction procedures, as described by Nathalie Fortin *et al.*¹⁵ (method 1), Fu *et al.*¹⁶ (method 2), Huang *et al.*¹⁷ (method 3), respectively, were prepared. The crude DNA was purified with Clean&Concentrator™-25 kit (Sangon Biotech (Shanghai) Co., Ltd., China). The concentrations, purity, quality of the crude extracted DNA were detected by electrophoresis (60 V for 1 h) on a 0.8% (wt/v) agarose gel with Gelgreen staining. The suitable approach applied to environmental samples could be found by analyzing the follow

experiment results.

Standard Curve Based on Simulated Environmental Samples

The DNA extracted from the simulated environmental samples with the known *V. parahaemolyticus* number was used as after a series of dilution. Then a standard curve of the C_T versus cfu g^{-1} of sediment samples was obtained, through which the cell number of *V. parahaemolyticus* in the unknown environmental samples can be estimated.

Statistical Analysis

The cycle threshold (C_T) was recorded by the Real-Time Detection system and the software in it. The standard curve of the C_T vs log was plotted by using Microsoft Excel. The coefficient of variation (CV) was analyzed by using One-way ANOVA in SPSS version 16.0.

RESULTS

Establishment of Standard Curve Based on Plasmid

The density of extracted standard positive plasmid was 1.94×10^9 copies μl^{-1} , measured by NanoDrop 1000. Standard curve based on positive plasmid was made by using serial dilution of the known concentration as a template for real-time PCR amplification with varying copies of plasmid ($1.94 \times 10^4 \sim 1.94$ copies μl^{-1}). Then a curve of C_T values vs log copies μl^{-1} of plasmid standards was successfully established with the detection limit up to 1.94 copies μl^{-1} by using the optimized reaction system (Fig. 1). As a standard reflecting the relationship between varying copies of plasmid (represents the number of targeted segments) and the threshold cycle (C_T), it could show the seasonal variation of *V. parahaemolyticus* in sediment of the *Stichopus japonicus* culture pond simply and rapidly.

Table 1. The results of reproducibility assay for real-time PCR

concentration(cfu g^{-1})	Ct1	Ct2	Ct3	\bar{X}	s	CV(%)
105	24.14	24.11	24.00	24.0833	0.0737	0.3061
104	28.18	28.37	28.37	28.3067	0.1097	0.3875
103	31.70	31.60	32.15	31.8167	0.2930	0.9208
102	34.66	34.09	34.25	34.3333	0.2940	0.8563

Development of Real-Time PCR Protocol Based on Simulated Sediment Samples DNA Extraction Methods

The total DNA templates were extracted from simulated sediment samples and negative controls by using three methods, respectively. Results showed that, except the negative controls, all of the three DNA extraction methods could yield DNA bands in agarose gel, indicating the low DNA content in the negative controls and without contamination in the process of DNA extraction artificially.

Development of Standard Curve Based on Simulated Sediment Samples

As shown in Fig.2, When using crude DNA extracted from simulated sediment samples with varying number of bacteria cell ($10^8 \sim 10^1$ cfu g^{-1}) with 3 methods as template, the positive signal did not appear with $10^8 \sim 10^7$ cfu g^{-1} bacteria cells, and appeared with $10^6 \sim 10^4$ cfu g^{-1} bacteria cells, indicating the real-time PCR protocol had been

greatly improved in terms of accuracy and reliability through a serial dilution to remove the PCR inhibitors, the C_T values appeared at the same position when the concentrations were under 10^4 cfu g^{-1} due to few number of *V. parahaemolyticus* (Fig. 2). Better amplification curves appeared in real-time PCR reaction when using purified DNA extracted with method 2 as template, compared with those with method 1 and 3.

The amplification signals detected the varying number of *V. parahaemolyticus* in simulated sediment samples could be given by the system automatically. A standard curve of the C_T vs log cfu g^{-1} in sediment samples with varying the bacteria equivalents number per germ ($3.1 \times 10^5 \sim 3.1 \times 10^2$ cfu g^{-1}) was obtained (Fig. 3a and 3b). The specific PCR products were verified by melting curve analysis of the typical melt profiles at $81.5^\circ C$, while the negative controls did not show any peaks in melting temperature (T_m) at $81.5^\circ C$ (Fig. 3c).

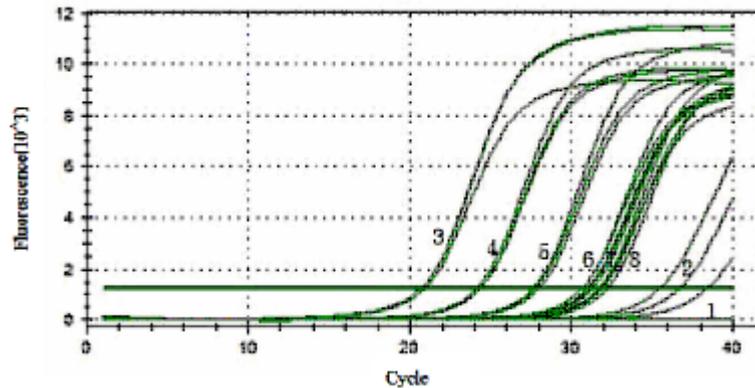


Fig. 1. Real-time PCR amplification signals detecting simulated sediment samples of the varying number of *V. parahaemolyticus* cells with sediment DNA extraction of method 1. 1: 10^8 cfu g^{-1} , 2: 10^7 cfu g^{-1} , 3: 10^6 cfu g^{-1} , 4: 10^5 cfu g^{-1} , 5: 10^4 cfu g^{-1} , 6: 10^3 cfu g^{-1} , 7: 10^2 cfu g^{-1} , 8: 10^1 cfu g^{-1}

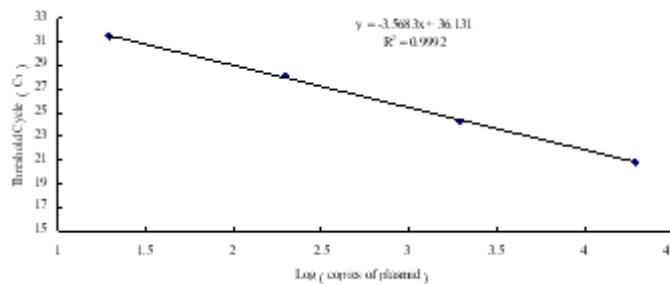


Fig. 2. Standard curve based on plasmid with the varying copies ($1.94 \times 10^4 \sim 1.94$ copies ul^{-1}) plotted vs the cycle threshold (C_t) values

Specificity Assay

To evaluate the species specificity of the established real-time PCR method, 5 non-*parahaemolyticus* strains (*Nitrobacter* sp., *Nitrosomonas* sp., *Pseudomonas* sp.,

Thalassobacillus sp. and *Escherichia coli*.) and *V. parahaemolyticus* were examined simultaneously. The *V. parahaemolyticus* strains showed positive signals in the real-time PCR assay, however, real-time PCR amplification of DNA from five other

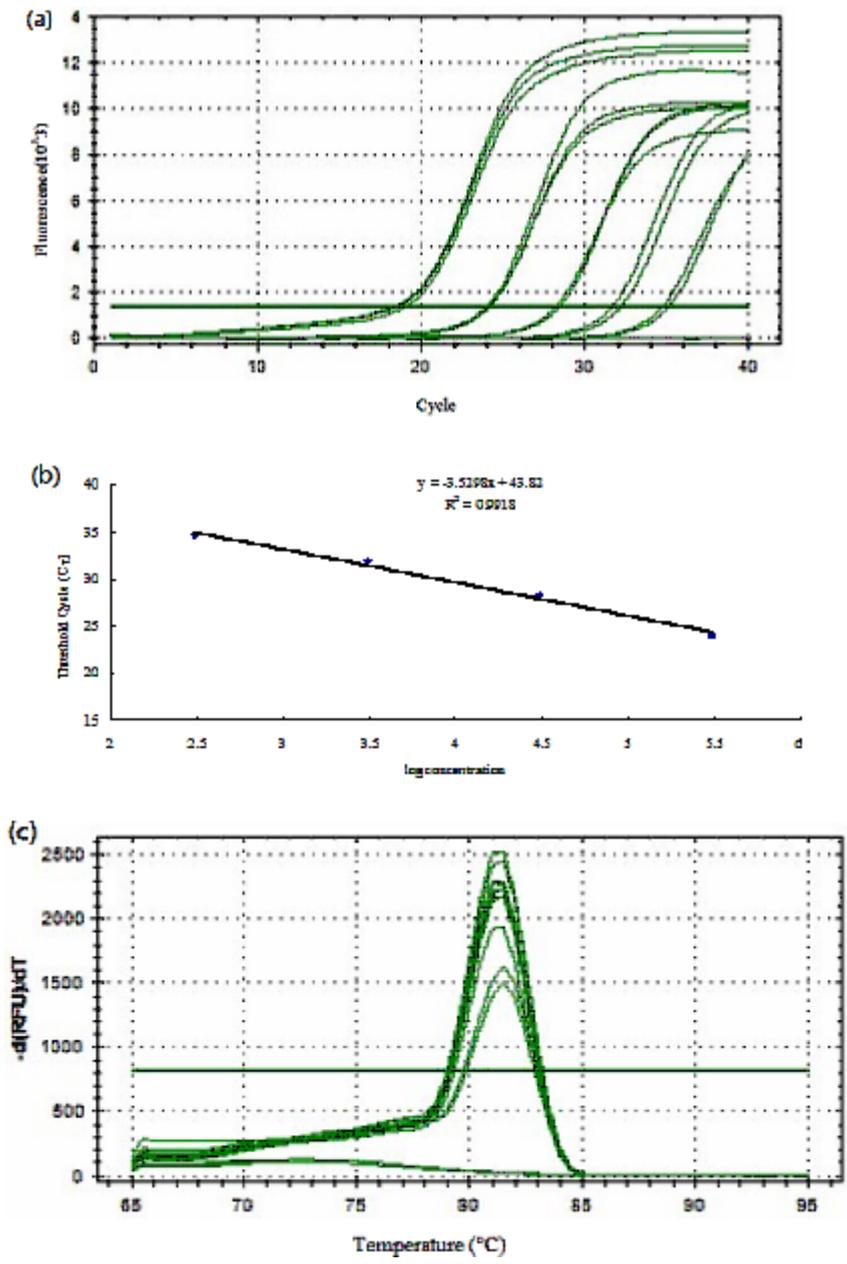


Fig. 3. (a) Real-time PCR amplification signals detecting simulated sediment samples with the varying number of *V. parahaemolyticus* cells per germ (10⁶, 10⁵, 10⁴, 10³, 10²) with negative controls; (b) standard curve based on simulated sediment samples with known number of cell equivalents per germ (10⁵, 10⁴, 10³, 10²) plotted vs the cycle threshold (Ct) values; (c) the melting curve for the amplicons showing a specific peak at 81.5°C against the negative controls

strains did not produce any PCR products in the same size of the products generated by *parahaemolyticus* (Figure 4).

The melting curve analysis showed a reproducible unique melting peak at 81.5°C, excluded the possibility of non-specific amplification products or primer dimers. The verified specific primers could be used to quantify the *V. parahaemolyticus* in the unknown environmental samples.

Reproducibility Assay

The various C_T values were produced by using the simulated sediment DNA as templates for the real-time PCR amplification with the varying concentration of *V. parahaemolyticus* (10⁵ cfu g⁻¹~ 10² cfu g⁻¹). Each reaction was performed in

triplicate. Reproducibility of the protocol was confirmed by One-way ANOVA with calculating the standard deviation (s) and Coefficient of Variation (CV). Results indicated that the established real-time PCR method for *V. parahaemolyticus* has a good repeatability with all the CV values varying between 0.31% and 0.92%, less than 5% (Table 1).

Sensitivity and Detection Limits

The specific primers was tested on DNA templates extracted from simulated environmental samples, succeed in producing typical PCR products of 180bp size. a serial dilution DNA serving as templates with varying number of *V. parahaemolyticus* equivalents per gram (3.1×10⁷ ~ 3.1×10² cfu g⁻¹) was used for the real-time PCR and traditional PCR amplification, respectively. It

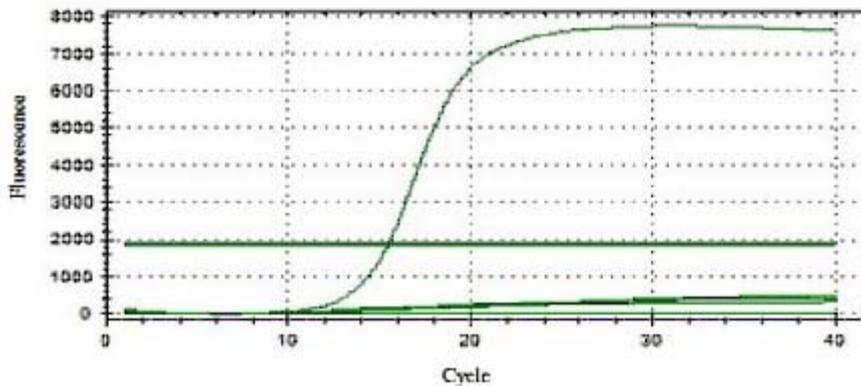


Fig. 4. Real-time PCR amplification positive signals detecting *V. parahaemolyticus* against the non-*parahaemolyticus* stains and negative controls

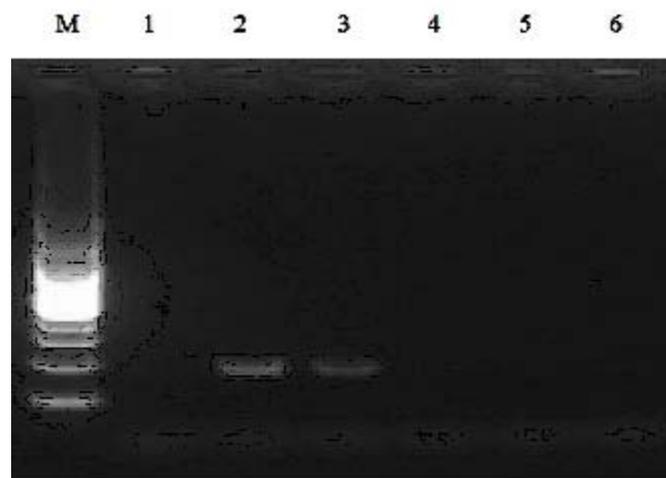


Fig. 5. Conventional PCR gel (2% wt/v) for *V. parahaemolyticus* in simulated sample dilutions, the original concentration in the simulated sample was estimated to be 3.1×10⁷ cfu g⁻¹.

M: 100bp Marker; 1: a negative control; 2~ 6 lanes: 3.1×10⁷ ~ 3.1×10² cfu g⁻¹

showed that the sensitivity of real-time PCR method was much higher than the conventional PCR with the quantification limit up to 10^2 cfu g^{-1} (Figure 3a), while the latter detection limit was up to 10^6 cfu g^{-1} (Figure 5). In fact, the real-time PCR could show positive weak signals with $3.1 \times 10^1 \sim 3.1$ cfu g^{-1} of cell for detecting, though not enough for quantification precisely.

DISCUSSION

The pathogenic bacteria in the environment have close relationship with those in the body of farmed animals, high concentration of pathogens in environments may cause diseases. So, a simple, rapid, accurate protocol to quantify the pathogens in the environment is needed to give early warning for disease occurring. PCR-based molecular biology methods are widely employed in detection and identification of specific microbes with the feature of high sensitivity, specificity and speed. In particular, real-time PCR technology could offer an innovative protocol for detecting and enumerating the specific pathogenic microorganism in the environment¹⁸, with higher efficiency by amplified short fragment (between 150 bp and 250 bp) than conventional PCR. However, as an approach to quantify the original bacterial DNA in the environment samples, the real-time PCR method has a higher requirement high for the yield and quality of DNA templates. The key to success of the real-time PCR protocol was based on the efficient extraction and purification of DNA. Factors, such as humic acids, heavy metals, phenolic compounds, widely existing in the sediment that inhibit the amplification for nucleic acids of the real-time PCR¹⁹. Due to the similar electric charges and alike size of humic acids to DNA, it is crucial to remove the inhibitors before cells lysis. Besides, the efficiency and purity of DNA extractions are mainly affected by two parts, the way of cell lysis and buffer handling. In this paper, the high quality DNA template was extracted by the method of lysozyme-SDS gentle lyse (method 2) with TENP buffer washing more than 2 times and being purified by the Purification Kit.

In most existing literatures, the standard curves were set up based on DNA from plasmid of pure culture to detect the bacteria in soil¹¹, which can only relatively reflect the abundance of the

bacteria. Though above efforts was made in DNA extraction in this paper, It is still inevitable that some DNA was lost and PCR inhibitors residue can't be removed thoroughly especially from sediment samples. So, a standard curve based on DNA extracted directly from sediment environment was set up to reflect the real number of *V. parahaemolyticus* in sediment environment of *Stichopus japonicus* culture more accurately.

Several molecular biology methods were developed for diagnosis and detection of *V. parahaemolyticus*. The PCR method for specific detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin gene (*trh*) of pathogenic *V. parahaemolyticus* was established by Tada, J. *et al*²⁰ with the detection limit to 400 fg (100 cells). *V. parahaemolyticus* was detected by using the DNA-DNA hybridization assay of synthesizing *tdh*-gene targeted oligodeoxyribonucleotide probes, as described by Nishibuchi, M. *et al*²¹. However, this method of targeted the hemolysin gene had failed in detecting all of the *V. parahaemolyticus*²². Therefore, we tried to establish the method of real-time fluorescence quantitative PCR targeting specific sequence of the *toxR* gene. The gene could be used in developing a PCR protocol to identify the *V. parahaemolyticus* from the environment²³, because it is a highly conserved gene among *V. parahaemolyticus*. In this study, the assay described the development of the real-time PCR assay based on SYBR Green I by using the specific primers to quantify *V. parahaemolyticus* in sediment from *Stichopus japonicus* culture pond targeted *toxR* gene. Meanwhile, specificity, reproducibility and sensitivity of the developed real-time PCR quantification method of *V. parahaemolyticus* was demonstrated well. The melting curve was obtained by amplification of the unique peak at 81.5°C, while the non-*Parahaemolyticus* strains without the melting temperature as well as negative controls, showed high specificity of the protocol.

Process of the established standard curve based on simulated sediment samples, was almost identical with detection procedures for *V. parahaemolyticus* in the environment. The standard curve showed a high correlation between C_t and log density of *V. parahaemolyticus* in the

sediment (cfu g⁻¹), and the amplification efficiency was 92%. The detection limit up to 10² cfu g⁻¹ was verified by simulated sediment samples spiked with the known concentration of bacteria cells, which was not available for the conventional PCR. A rapid real-time PCR detection system for *V. parahaemolyticus* in the seafood was described by Kim, J.S. *et al*²⁴ with detection limit up to 1.5 cfu g⁻¹. Probability because the aims to this method for detection, rather than quantification. The detection limit could be improved significantly without the establishment of standard curve based on simulated sample. A multiplexed real-time PCR method for *V. parahaemolyticus* with TaqMan Fluorescent Probes in Shellfish was developed by Linda N *et al*²⁵. The minimum detection level of purified *V. parahaemolyticus* genomic DNA was approximately 10⁴ cfu ml⁻¹ in pure cultures. The reduced sensitivity of the real-time PCR may be caused by the increased fluorescence background that is an unavoidable by-product of the presence of multiple TaqMan fluorescent probes.

In conclusion, The real-time quantitative PCR method is a feasible protocol for quantification of *V. parahaemolyticus* in sediment from the *Stichopus japonicus* culture pond with strong specificity, high sensitivity and good reproducibility. Our further research include detecting the number of the *V. parahaemolyticus* in the *Stichopus japonicus* culture eco-system and taking precautions against off-plate syndrome. Establishment of the real-time PCR detection method for the *V. parahaemolyticus* in sediment is crucial to the *Stichopus japonicus* culture industry, or even the aquaculture industry.

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