Research Article
Construction of Method for Rapid Detection of *Vibrio Parahaemolyticus* Using the Quantitative Real-Time PCR Based on the ToxR Gene

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Abstract: In this research, a sensitive, rapid and highly reproducible SYBR green based real-time PCR assay was developed for detection of toxR positive pathogenic Vibrio parahaemolyticus. To establish a real-time PCR assay for accurate and rapid detection of *Vibrio parahaemolyticus*. The special target sequence of toxR gene of *Vibrio parahaemolyticus* was amplified and characterized with a pair of primes. Reaction system and determination approach of real-time PCR were established for detection of *Vibrio parahaemolyticus*. The foodborne pathogen of *Staphylococcus aureus, Salmonella* were used as specificity reference for the method, the amplification curves were observed as a typical "S" curve, other pathogens were also tested and no amplification was observed. In addition, the results of melting curve analysis showed only a specific peak with a melting temperature of 86.33°C and no primer-dimers peak was observed. These findings indicated that the PCR primers had high specificity. Analysis of standard curves revealed excellent correlation between the number of copies (in the range of 4×10^9 to 4×10^1 and PCR threshold cycle (Ct) with a correlation coefficient of 0.992 ($R^2 = 0.992$). It was found that the limit of this assay was 15 CFU/mL for pure culture and 1.535pg for genomic DNA. The total detection assay could be completed in 2 h. Results indicated that real-time PCR detection methods established in this research was accurate, sensitive, rapid, reproducible for the quantitative detection of environmental *Vibrio parahaemolyticus*.

Keywords: Rapid detection, real-time PCR, toxR gene, *Vibrio parahaemolyticus*

INTRODUCTION

Vibrio parahaemolyticus is a gram-negative halophilic bacterium that naturally inhabits marine and estuarine environments (Blanco *et al*., 2009; Tyagi *et al*., 2009). The organism can cause potentially serious infections in humans when contaminated drinking water or food is consumed. *V. parahaemolyticus* was the first time identified as a cause of food-borne illness in Japan in 1950, after an outbreak of food poisoning which affected 272 patients (Zen *et al*., 1965). In recent years, *V. parahaemolyticus* has become widely disseminated throughout the world at locations that include Spain, Asia, Russia, South America, Africa and the United States (Nair and Hormazabal, 2005; Ward and Bej, 2006; Wong *et al*., 2006; Wong *et al*., 2000; Yu *et al*., 2011; Chen *et al*., 2012). Thus, *Vibrio parahaemolyticus* has been considered as one of the most important food-borne bacterial pathogens. Because of the safety concerns, detection and characterization of *V. parahaemolyticus* have attracted much attention.

The detection of pathogenic bacteria is important for human health and safety. The development of rapid and specific methods of detecting pathogenic bacteria in fields such as the food industry, clinical diagnosis and environmental control is required.

Conventional methods of for isolation and identification of *Vibrio parahaemolyticus* involve a culture procedure using enrichment media and subsequent isolation on in selective plating media. The traditional and standard bacterial detection methods may take up to 7 days to yield an result and the operation processes are very complex (Ray *et al*., 1978).

In recent years, modern molecular biology methods have been more and more used in pathogenic bacteria detecting due to their high sensitivity and specificity. Polymerase Chain Reaction (PCR) is clearly rapid and highly specific for detection of *V. parahaemolyticus* (Wei *et al*., 2010; Luan *et al*., 2008). Compared with the time consuming culturing process, bacterial genome DNA can be amplified by PCR in a short time. The PCR detection takes much less time than traditional detection methods. Thus, PCR technology has the potential to enable the rapid and specific detection of pathogenic bacteria via specific amplification.

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Real-time PCR has been successfully used to detect and quantify pathogens. It has the advantage of high sensitivity, specificity and rapidity of assay, such as SYBR Green (Molecular Probes, Eugene, OR), that will bind to amplified cDNA or with florescence -tagged probe that fluoresce when bound to complementary sequences in the amplified region.

In comparison to TaqMan PCR, SYBR Green real-time PCR procedure has the advantages of being easy to design, relatively low setup and running costs (Arikawa *et al*., 2008; Barkallah *et al*., 2013) and possibly more accurate results and linearity. Moreover, SYBR Green I has the advantage of being a more flexible method without the need for individual probe design. To evaluate the specificity of SYBR Green real-time PCR, DNA melting curves of amplified products can distinguish false positive signals due to primer-dimers or non-specific amplification.

To detect *Vibrio parahaemolyticus*, several target genes that reflect phylogenetic relationship such as the 16S rRNA, tdh, gyrB and toxR are selected. The toxR gene which codes for a trans-membrane DNA binding regulatory protein is present on the ancestral chromosome (Provenzano *et al*., 2000) and is known as a general gene in the Vibrio species (Osorio and Klose, 2000; Takahashia *et al*., 2005).

In this research, a SYBR Green real-time PCR detection of the toxR gene was established and evaluated to develop a highly specific, sensitive and rapid approach for the detection and quantification of the *Vibrio parahaemolyticus.*

MATERIALS AND METHODS

- **Main experimental materials and instruments: Standard bacterial strains:** The standard bacterial strains including *Vibrio parahaemolyticus* (ATCC17802), Staphylococcus aureus (ATCC6538) and Salmonella enteritidis (ATCC13076) used in this research were purchased from American type culture collection (ATCC).

Reagents: The nutrient broth was bought from Land Bridge Technology (Beijing) Co. Ltd., China. Bacterial genomic DNA extraction kit was purchased from Tiangen Technology (Beijing) Co. Ltd., China. Premix Prime STAR HS conventional PCR kit, r-Taq enzyme, dNTP Mix, pMD19-T vector kit, BamHI, Hind Ⅲ digestion enzymes kit and 10×Loading Buffer were purchased from Takara Biotechnology (Dalian) Co. Ltd., China. SYBR Green Real-time PCR Master Mix kit was bought from TOYOBO CO., LTD. Life Science Department OSAKA, JAPAN. Gel Extraction Kit and Plasmid Extraction Kit were purchased from OMEGA Co. Ltd., USA. DH5α super competent cells kit were purchased from Dingguo Technology (Beijing) Co. Ltd., China.

Experiment instruments: MX3000 real-time PCR instrument was purchased from Agilent, USA, 2720 PCR was purchased from ABI, USA, GS-800 gel image system and electrophoresis apparatus were purchased from Bio-Rad. 5415D trace tabletop centrifuges was purchased from Eppendorf, Germany. ND-1000 nucleic acid protein analyzer was purchased from NanaDrop Technologies, USA.

- **Bacterial cultures and preparation of template DNA:** The standard strains of Vibrio parahaemolyticus stored at -70°C was thawed through water bath, then enriched and incubated in nutrient broth overnight at 37°C in a rotary shaker (220 rpm). Then 1mL overnight broth culture was added into a 1.5 mL EP tube, the bacterial genomic DNA was extracted and purified through Bacterial DNA Extraction Kit (TIANGEN) according to the manufacturer's instructions and then the extracted DNA concentration and the OD260/OD280 ratio was measured on a NanoDrop 1000 spectrophotometer (Thermo scientific) with software ND-1000 v3.7.1. The extracted DNA was stored at -20°C until use.

Primer design and synthesis: Currently, the pR72H (Rosec *et al*., 2009), tdh (Blackstone *et al*., 2003), trh (Garrido *et al*., 2012), gyrB (Teh *et al*., 2010), toxR (Rosec *et al*., 2009) and tlh (Wang *et al*., 2011) gene could be used as the target gene for detection of Vibrio parahaemolyticus. In this research, we selected the toxR gene (GB L11929.1) of Vibrio parahaemolyticus as the target gene. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for database searching in the GenBank (www.ncbi.nlm.nih.gov/genbank). Sequences were aligned and the conserved and specific regions were screened using the Clustal W program (www.clustal.org). All primers were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). A pair of specific primers was designed to amplify a 257 bp segment from the toxR gene. The forward primer was 5'-GAACCAGAAG CGCCAGTAGT -3', the reverse primer was 5'-GCATGGTGCTTAACGTAGCG-3'. The specificity of the primers was confirmed using the BLAST program and subsequently validated by amplification of the genomic DNA. We checked out that the primer designed were specific for V. parahaemolyticus. Then the primers were synthesized by Shanghai Invitrogen Biological Engineering and Technology Services Co. Ltd.

Preparation and purification of toxR gene target fragment: In order to obtain the target fragment, the PCR was carried out in 50 μL mixtures using PrimeSTAR HS conventional PCR kit, following reaction mixture components (final concentrations): containing 1.5 μL Vibrio parahaemolyticus template genomic DNA, 25 μL PrimeSTAR enzyme, 0.75 μL 20

μmol/L forward primer, 0.75 μL 20 μmol/L reverse primer, 22 μL double-distilled water.

Amplification was performed with a thermal cycler, ABI 2720 (Applied Biosystems, Foster City, USA) using the following procedure: 35 cycles of 98°C for 10s, 56°C for 15s and 72°C for 3 min 50s and a final extension step of 72°C for 10 min. Then, 2 μL r-Taq enzyme and 3 μL dNTP Mix were added and incubated for 20 min at 72°C. Then T A cloning was performed.

The amplified PCR products were analyzed by electrophoresis through a 1% agarose gel followed by staining with Gold view™ and observed under the gel image system (Quantity One™, Bio-RAD, CA, USA). The target fragment were recovered and purified by Gel Extraction kit (OMEGA) following the manufacturer's instructions.

- **Preparation of toxR plasmid standard:**
  T A cloning: A recombinant plasmid containing the target gene was constructed. The A-tailed DNA fragments were ligated to pMD-19-T vector at 4°C for 16 h in a total volume of 10 μL using the DNA Ligation Kit (TaKaRa). Then the recombinant plasmid was transformed into DH5α-competent cells and the positive clones were selected by LB media containing 100 μg/mL ampicillin.

Plasmid DNA extraction and enzyme digestion: Six colonies were picked up from LB plates and transferred into 6 mL of LB medium containing 100 μg/mL ampicillin and incubated at 37°C in a rotary shaker (200 rpm) 12 h. Then the plasmid DNA was extracted using Plasmid Extraction Kit (OMEGA) according to the manufacturer's instructions, the concentration and the OD260/OD280 ratio of the plasmid DNA were measured on a NanoDrop 1000 spectrophotometer (Thermo scientific) with software ND-1000 v3.7.1. The recombinant plasmid was double-digested with Hind III and BamH I.

The recombinant plasmid which showed correct digested results in gel image system was sent to Shanghai Invitrogen Biological for sequencing.

Construction of standard curve for fluorescence quantitative PCR: According to the average molecular weight formula (Godornes *et al.*, 2007): dsDNA = (number of bases) × (660 Dalton / base) (6.022×10^{23} (molecules/mole) Avogadro's number 660 Da Average weight of a single base pair), based on the length of each cloned circular plasmid 2949bp calculated and determined that the copy number of standard containing the recombinant plasmid of Vibrio parahaemolyticus toxR gene 10^{10}copies/μL, then the recombinant plasmid was 10-fold serially diluted and stored at -20°C as a quantitative standard samples.

Quantitative PCR was performed in an Agilent MX3000 cycler (Applied Biosystems, Foster City,

USA). PCR was performed in a 20 μL volume using the following reaction mixture components (final concentrations): SYBR Green Real-time PCR Master Mix (TOYOBO, JAPAN), 20 μmol each of the toxR forward and reverse primers (Invitrogen, Shanghai, China). The remainder of the reaction mixtures consisted of double-distilled water and 0.5 μL DNA template. Thermo cycling was performed according to the specifications for the primers using the following conditions: 5 min at 95°C followed by 40 cycles of 10 s at 95°C, 20 s at 60°C and elongation at 72°C for 30 s. Data acquisition was performed by MXpro software at the end of each elongation step. Each sample was analyzed for three times.

The PCR amplicons were confirmed by electrophoretic (1% agarose gel) separation of reaction products (2 μL). The images were captured digitally and analyzed using the Gel Image system (GS-800, Bio-Rad, USA).

- **Specificity of detection:** Specificity was assessed by carrying out assays with purified DNA from 2 non target bacterial species, including Staphylococcus aureus (ATCC6538) and Salmonella enteritidis (ATCC13076). Genomic DNA was extracted from pure cultures of each bacteria. The real-time PCR reaction system and reaction conditions were the same as described above.
- **Sensitivity of detection:** The real-time PCR assay established in this study should have high sensitivity, when the sample concentration of the *Vibrio parahaemolyticus* was low, they could also be accurately detected.

To determine the sensitivity of the PCR assay for pure cultures, *V. parahaemolyticus* cultures were 10-fold serially diluted to the range from 10^7 to 10^1 CFU/mL and estimated by plate-counting method. A sample of each diluted specimen was added to 4 mL nutrient broth and cultured at 37°C for 12 h. Subsequently, 1 mL aliquot of the culture and each serial dilution were used to prepare DNA template by DNA extraction kit as described above.

To determine the minimum detection level of purified *V. parahaemolyticus* DNA, serial dilutions of genomic DNA (ranging from 1 pg to 100 ng) from *V. parahaemolyticus* were prepared. Aliquots of each 10-fold serial dilution were used as templates for real-time PCR detection.

RESULTS AND DISCUSSION

- **Evaluation of enzyme digestion:** The amplified PCR products were analyzed by electrophoresis through a 1% agarose gel followed by staining with Goldview™ and observed under the gel image system.
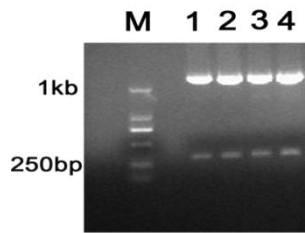
Fig. 1: Agarose gel shows the products obtained from recombinant plasmid-after-digestion. Lane M: D2000 DNA ladder, lane 1-4: products of enzyme digestion

The recombinant plasmid which were extracted from the positive TA clones were double-digested with Hind III and BamH, the results were shown in Fig. 1. It indicated that the target fragment was successfully ligated into the vector.

Sequencing analysis of plasmid DNA: The presence of the correct insert was confirmed by sequencing. The TA clones which were shown positive through for restriction enzyme digestion were sequenced, the sequencing results further confirmed that the target
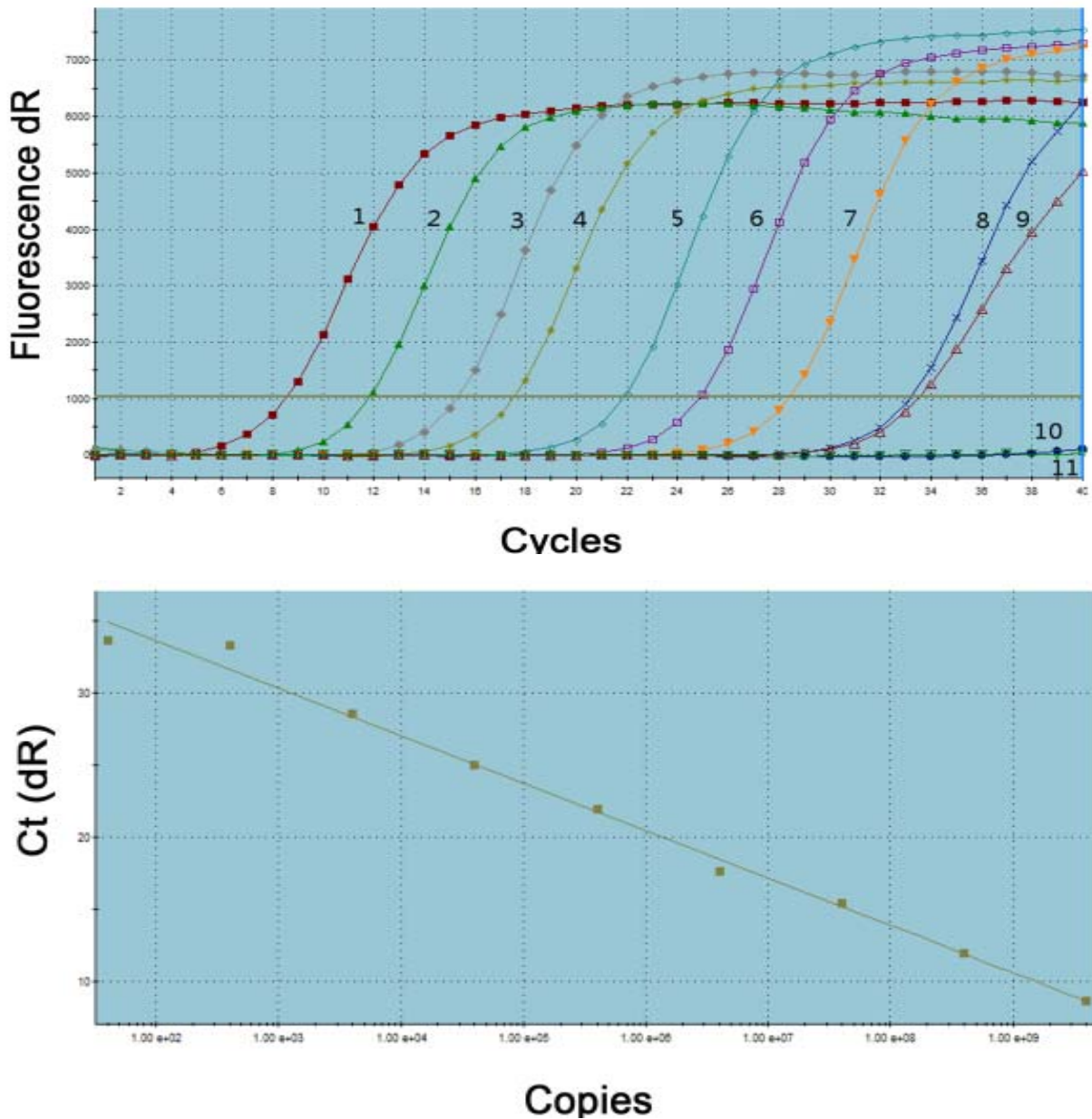

Fig. 2: Amplification and standard curves of the real-time PCR assay Amplification curves were generated by fluorescence data collected at each cycle during the final phase of the PCR. Values are triplicates of different dilutions of the control plasmid used as standard. Control plasmid copy numbers per sample were in the range of $4\times10^9\sim4\times10^0$ copies/μL and negative control (NTC). The R2 linearity value from linear regression is 0.992
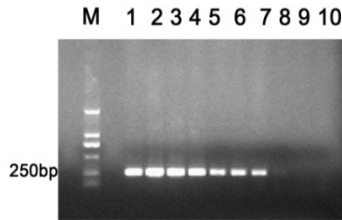
Fig. 3: Real-time PCR reaction products checked on agarose gel 1%: serial 10-fold dilutions of control plasmid. Specific bands of approximately 257 bp were visualized for all triplicates of recombinant plasmid dilutions. M: D2000 DNA Ladder. 1-10: control plasmid dilutions with concentrations of $4 \times 10^9 \sim 4 \times 10^0$ copies/µL

fragments had been successfully ligatited into the pMD-19-T vector; BLAST (http:// blast.ncbi. nlm.nih. gov/Blast.cgi) was used for analysising the sequencing results, meanwhile the Vector NTI software (Invitrogen) was used for aligning the sequencing results. Compared with the toxR gene of the GenBank, it showed that the homology between the sequencing results and the toxR gene was 99%. The results indicated that positive toxR gene recombinant plasmid standard was successful created in this research, data and figure of the sequencing results were not shown.

- **Linearity results of gradient diluted sample of toxR standard:** Each dilution was loaded in triplicate. After analysis, Cycle threshold (Ct) values were plotted against the known copy numbers of the recombinant plasmid DNA and correlation coefficient and slope of the curve were

determined. Amplification curves were obtained in the range of 4×10^9 to 4×10^0 copies/µL of plasmid DNA. When the quantitative PCR reaction was done, the MxPro software was used for data analysis. The critical numbers of cycles (Threshold Cycle, CT) was taken as the ordinate and the copy numbers of standard serial dilutions was taken as abscissa to establish a standard curve of the real-time quantitative PCR. The Ct value may be defined as ''the first cycle at which there is a significant increase in fluorescent signal over the background signal or a specified threshold'' (Smart Cycler Operator Manual, 2001, Cepheid). The linear regression equation y = -3.293x+40.20, the slope of the standard curve was -3.293, with a regression coefficient of $R^2 = 0.992$ (Fig. 2). The strong correlation between the copy numbers of initial recombinant plasmid in the PCR reaction and the associated Ct values illustrated the quantitative potential for real time PCR.

Furthermore, amplification products were also checked on agarose gel 1% stained with Goldview™ and observed under the gel image system. A clear and well-defined specific band of approximately 257 bp was visualized with all triplicates of recombinant plasmid dilutions, except the concentration of 4×10^2, 4×10^1 and 4×10^0 copies/µL (Fig. 3).

- **Detection specificity:** In order to evaluate the specificity of the developed assay, the specificity of each primer set was tested against non target organisms, including Staphylococcus aureus
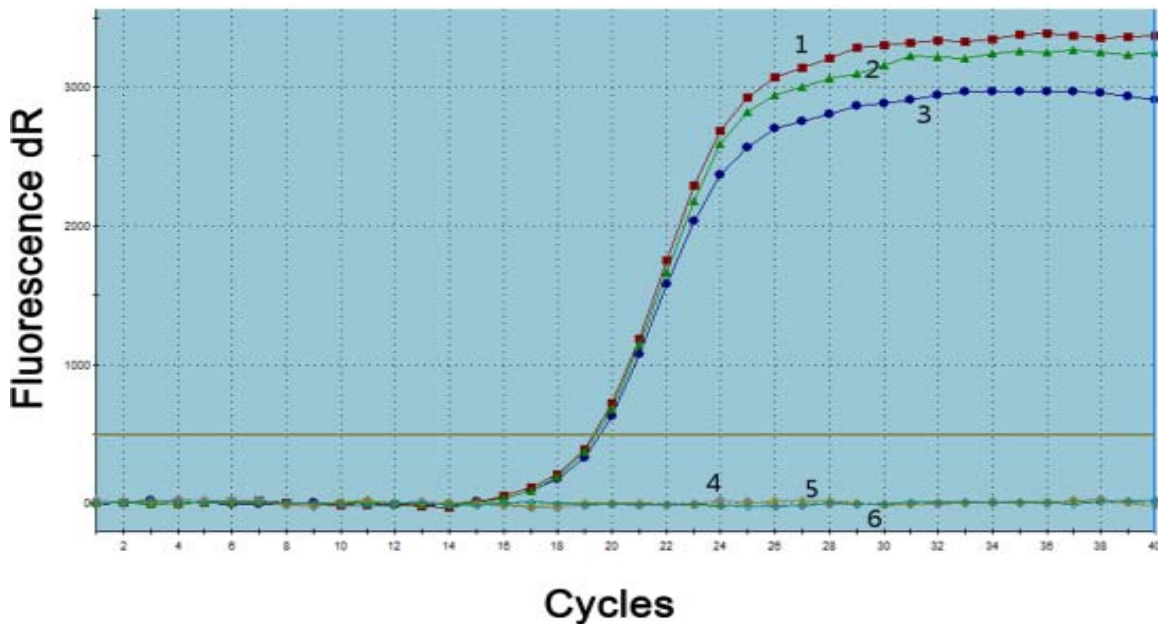

Fig.4: SYBR Green real-time PCR amplification curve analysis of V. parahaemolyticus reference and non- V. parahaemolyticus reference strains after 40 cycles. 1-3: Vibrio parahaemolyticus, 4: Staphylococcus aureus, 5: Salmonella, 6: negative control
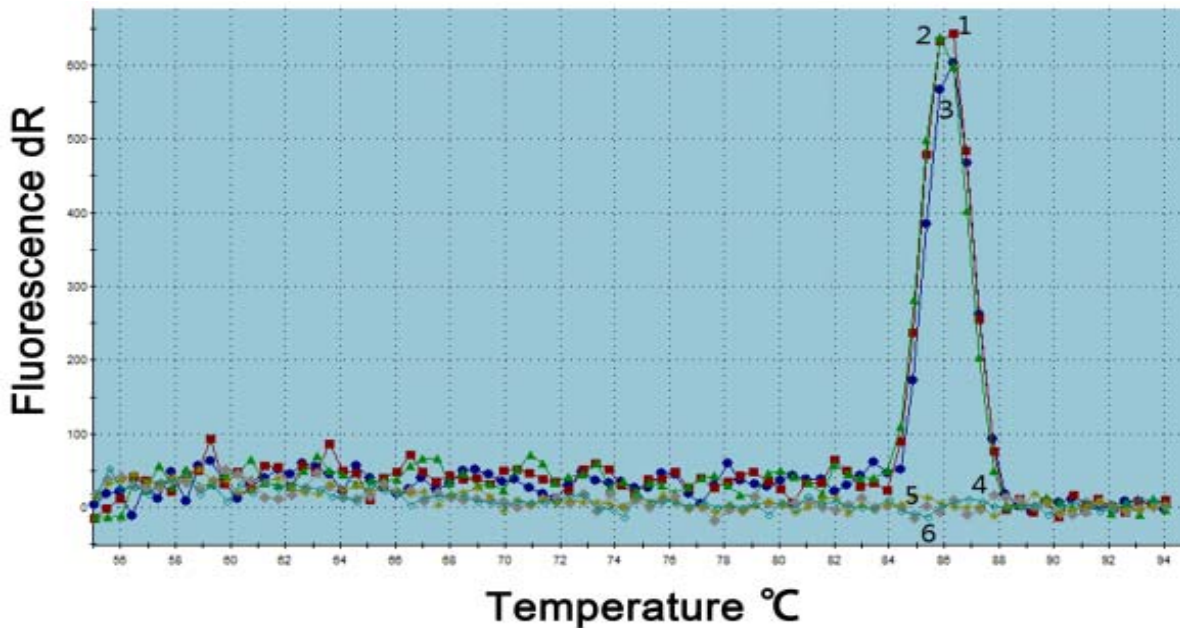
Fig. 5: Dissociation curve analysis of SYBR Green real-time PCR products of V. parahaemolyticus reference and non- V. parahaemolyticus reference strains after 40 cycles. 1-3: Vibrio parahaemolyticus, 4: Staphylococcus aureus, 5: Salmonella, 6: Negative control
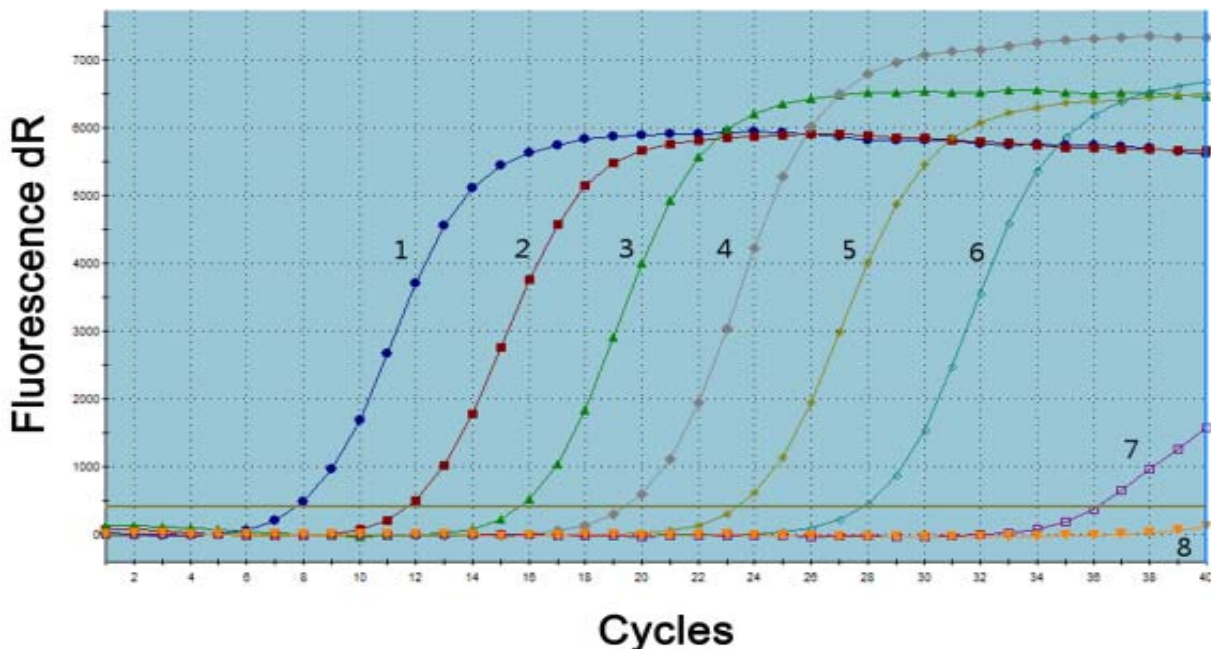

Fig. 6: Sensitivity of SYBR Green real-time PCR detection for serially 10-fold diluted culture of Vibrio parahaemolyticus, 1-7: 1.5×107～1.5×101cfu/mL, 8: negative control

and Salmonella enteritidis. No fluorescent signal was observed after 40 cycles of amplification with any of the non target bacteria tested (Fig.4). The DNA from the *V. parahaemolyticus* strains gave the expected positive signal and generated high C_T values, well above background levels.

The specificity of the real-time PCR reaction was confirmed by melting curve analysis and a reproducible distinct melting point (Tm) of 86.33°C was observed for all *V. parahaemolyticus* amplicons (Fig.5), indicating the formation of a single PCR product with no artifacts, such as nonspecific amplification products or primer.
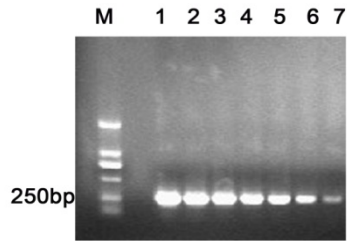
Fig. 7: Real-time PCR reaction products checked on agarose gel 1%: 10-fold diluted culture of Vibrio parahaemolyticus. Specific bands of approximately 257 bp were visualized for all triplicates of recombinant plasmid dilutions. M: D2000 DNA Ladder. 1-7: $1.5 \times 10^7 \sim 1.5 \times 10^1$ cfu/mL

- **Detection sensitivity:** First, the sensitivity of the assay was detected by using pure *V. parahaemolyticus* culture with 10-fold serially diluted to the range from 10^7 to 10^1 CFU/mL. Each level was tested in triplicate. The results showed that the limits of detection of the real-time PCR were 10 cfu/mL in the pure *V. parahaemolyticus* culture (Fig.6 and 7).

Then, the sensitivity of the assay was determined by using purified genomic DNA. Genomic DNA extracted from *V. parahaemolyticus* with 10-fold serially diluted in double-distilled water to obtain amounts ranging from 153.5ng to 1.535 pg. Each level was tested in triplicate. As shown in Fig.8, the minimum level of detection of purified *V. parahaemolyticus* genomic DNA was 1.5 pg/µL. Data and figure of real-time PCR reaction products checked on agarose gel 1% were not shown.

- **Evaluation of repeatability and stability:** The repeatability and stability of built quantitative PCR method were analyzed through detection of different gradient dilution *V. parahaemolyticus* culture. Each dilution was tested in triplicate experiments. The experimental results showed that the coefficient of variation (CV values) of the three independent experiments was less than 1.5%, it indicated that quantitative PCR reaction system designed in this research had good stability and repeatability, The results were shown in Table1.
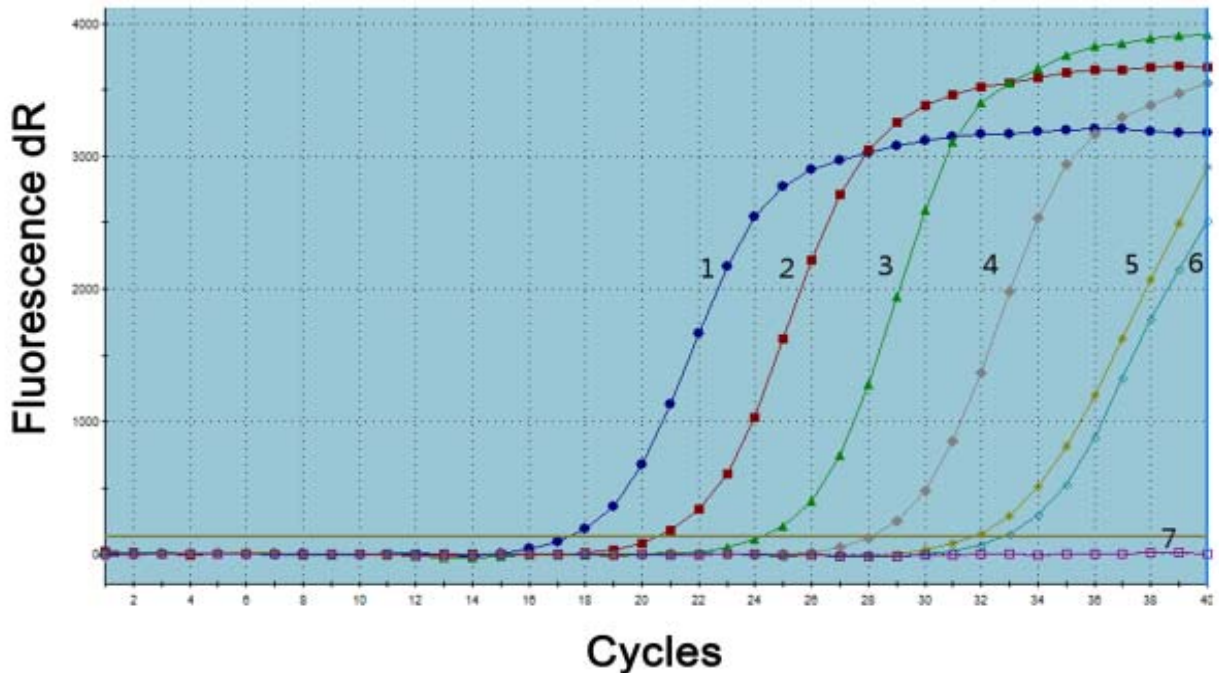

Fig. 8: Real-time PCR amplification plot for serially 10-fold diluted V. parahaemolyticus pure genomic DNA, 1: 153.5ng/µL, 2: 15.35 ng/µL, 3: 1.535 ng/µL, 4: 153.5 pg/µL, 5: 15.35 pg/µL, 6: 1.535 pg/µL, 7: negative control

Table 1: Detection of the reproducibility and stability of diluted pure culture using the real-time PCR

Concentration (cfu/mL)	Ct				
	1	2	3	Mean±SD	CV%
1.5×10^5	16.74	17.05	16.85	16.88±0.16	0.9
1.5×10^4	20.72	20.68	20.91	20.77±0.12	0.6
1.5×10^3	24.7	24.8	24.66	24.72±0.07	0.3
1.5×10^2	29.53	29.15	28.88	29.19±0.33	1.1

CONCLUSION

Vibrio parahaemolyticus has been considered as one of the most important food-borne bacterial pathogens. Because of its safety concerns, detection and characterization of *V. parahaemolyticus* have attracted much attention. The toxR gene that we selected in this research was first discovered as the regulatory gene of the cholera toxin operon, but it was later shown to be involved in the regulation of many other genes in Vibrio cholera (DiRita, 1992), Kim *et al* found that the degree of homology of the toxR gene between *V. parahaemolyticus* and V. cholerae (52% identity) is much lower than that of the rRNA gene (91 to 92% identity) (Kim *et al*., 1999). Then, they therefore investigated in their study the toxR gene sequence could be used to develop a PCR method for the specific identification of V. *parahaemolyticus*.

In the present study we report a useful SYBR Green real-time PCR method based on the specific primer to quantify toxR positive viable cells of *V. parahaemolyticus*.

The standard bacterial strains of *V. parahaemolyticus* and non-*V. parahaemolyticus* strains were used to evaluate the specificity of the primer designed for this research.

The results showed that the primer was selective, obtaining highly specificity.

To determine the linearity of the real-time PCR assay and to establish a standard curve used for quantification, serial 10-fold dilutions of the plasmid DNA templates at a copy number from 4×10^9 to 4×10^0 copies/μL of PCR reaction were analyzed using the real-time PCR. The standard curve showed a linear range across at least 9 logs of copies where the correlation coefficient was highly significant at 0.992. The slope value of the standard curves was -3.293.

To detect the sensitivity of the real-time PCR assay, serial 10-fold dilutions of *V. parahaemolyticus* pure culture and purified genomic DNA extracted from the pure culture were analyzed using the real-time PCR. In this study, after proper optimization, the limit of the detection was 10cfu/mL in the pure *V. parahaemolyticus* culture and 1.5 pg/μL of genomic DNA. Furthermore, this method could be completed within 1 working day, indicating a substantial gain of time over conventional culture methods, which took more than 7 days. It may be used in the future for further epidemiological investigations of *V. parahaemolyticus* spread.

In conclusion, this study demonstrated that the real-time PCR assay was a highly specific (detected only toxR positive *V. parahaemolyticus* strains), sensitive (10 CFU/mL per reaction limit of detection), rapid (time consuming was <24 h for total assay) and efficient method for the detection of pathogenic *V. parahaemolyticus*.

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