

Research & Reviews: Journal of Microbiology and Biotechnology

A Novel Reverse-Transcriptase Real-Time PCR Method for Quantification of Viable *Vibrio Parahaemolyticus* in Raw Shrimp Based on a Rapid Construction of Standard Curve Method

Mengtong Jin^{1#}, Haiquan Liu^{1,2,3#}, Wenshuo Sun¹, Qin Li¹, Zhaohuan Zhang¹, Jibing Li¹, Yingjie Pan^{1,2,3},

Yong Zhao^{1,2,3*}

¹College of Food Science and Technology, Shanghai Ocean University, Shanghai, 201306, China

²Laboratory of Quality and Safety Risk Assessment for Aquatic Products on Storage and Preservation (Shanghai), Ministry of Agriculture, Shanghai, 201306, China

³Shanghai Engineering Research Center of Aquatic-Product Processing and Preservation, Shanghai, 201306, China

Research Article

Received date: 10/03/2015

Accepted date: 15/03/2015

Published date: 19/03/2015

#These authors contributed equally to this study

*For Correspondence

Yong Zhao, College of Food Science and Technology, Shanghai Ocean University, Shanghai 201306, China, Tel: +86-21- 61900503; Fax: +86-21-61900503

Email: yzhao@shou.edu.cn

Keywords: *Vibrio parahaemolyticus*, RNA standards, viable, RT-qPCR, shrimp

ABSTRACT

Vibrio parahaemolyticus is an important pathogen that leads to food illness associated seafood. Therefore, rapid and reliable methods to detect and quantify the total viable *V. parahaemolyticus* in seafood are needed. In this assay, a RNA-based real-time reverse-transcriptase PCR (RT-qPCR) without an enrichment step has been developed for detection and quantification of the total viable *V. parahaemolyticus* in shrimp. RNA standards with the target segments were synthesized in vitro with T7 RNA polymerase and subsequently converted to cDNA for establishing the standard curve to quantify *V. parahaemolyticus*. The reaction efficiency was 94.56%, and was in the acceptable range. Without pre-enrichment, the sensitivity of RT-qPCR on quantifying *V. parahaemolyticus* in shrimp was 58 cfu/g, approximately. In addition, the survival of *V. parahaemolyticus* in spiked shrimp samples was quantified by RT-qPCR, DNA-based qPCR and standard plate count method. The quantification results of RT-qPCR have shown a good statistical correlation ($R^2=0.96$) when compared to the standard plate count method. However, the correlation coefficient between plate count method and DNA-based qPCR was only 0.85 for quantifying *V. parahaemolyticus*. Based on the results, this new method could be an effective way for providing reliable quantitative data on viable *V. parahaemolyticus* in shrimp.

INTRODUCTION

Vibrio parahaemolyticus (*V. parahaemolyticus*), a Gram-negative halophilic bacterium, is a common pathogenic bacteria causing food poisoning, such as gastroenteritis illnesses or septicemia. *V. parahaemolyticus* with the highest infection rate in marine animals are widespread in coastal and marine waters and mainly live in their intestines [1].

In previous studies, the standard plate count method has been used to detect *V. parahaemolyticus*. However, this method is time consumption including three sequential steps: cultural enrichment, selective plating and a biochemical test, usually taking 3-4 days to complete the whole process [2]. Therefore, a fast and accurate detection method is urgent to be established for quantifying *V. parahaemolyticus* in seafood. Meanwhile, real-time PCR methods with high detection sensitivity compared to standard PCR show great potential, due to that Real-time PCR can monitor the reaction products in real time without going through electrophoresis. By virtue of those numerous advantages, Real-time PCR has been used increasingly in many environments and

food samples for the detection and quantification of pathogenic microorganisms [3].

Actually, real-time methods are also utilized in detecting of *V. parahaemolyticus* [4,5]. But most methods using DNA quantification could overestimate the number of bacteria because all the lived, dead, and non-culturable cells can be detected [6,7]. Studies demonstrated that the amounts of bacterial messenger RNA (mRNA) in cells are strongly associated with the cell activity [8]. The resulting quantitative reverse transcription PCR (RT-qPCR) technique which utilizes the mRNA template other than DNA to specifically detect the viable cells will have more potential applications in the future [9]. In addition, the half-life period of mRNA is much shorter than that of DNA [10]. Thus, using mRNA template can largely reduce the probability of false-positive test. By employing this approach, more accurate risk data of samples can be obtained, hence, proposing a more accurate and effective risk avoidance measures.

Many studies have focused on the distribution of total and pathogenic *V. parahaemolyticus* in oysters and mussels [1,3,4,11]. However, there is very few data about crustaceans, despite that the popularity of crabs and shrimps as well as their consumption are worldwide [12-14]. Indeed, shrimps are one of the major aquatic products exported in large quantities in tropics. More and more people consume shrimps which might increase the risk of food borne outbreaks. Here, we investigate the detection and quantification of viable *V. parahaemolyticus* method in shrimp samples. The aim of the present study is to establish a RNA-based approach which can be used to detect and quantify viable *V. parahaemolyticus* in shrimp.

MATERIALS AND METHODS

Bacterial strains and culture conditions

V. parahaemolyticus QD was a pandemic O3:K6 strain isolated from food samples. It has been identified using molecular methods and verified by DNA sequencing. The strain was grown in 5 ml of tryptic soy broth (TSB; Land Bridge, Beijing, China) supplemented with 2.5% NaCl with constant shaking (170 r/min) at 37 °C for 8-12 h.

Artificial contamination of shrimp samples

Live shrimp samples were purchased from a local supermarket in Shanghai. Twenty-four shrimp samples were put in two aseptic plastic bags, averagely. The full shrimps were placed under ultraviolet light for 20 min, and then 400 ml alkaline peptone water with 5 ml of different concentration (10^7 cfu/ml, 10^8 cfu/ml) of *V. parahaemolyticus* were added into sterile plastic bags, respectively. The plastic bags were shaken for 10 min. After inoculating, shrimps were transferred into new sterile plastic bags. Twenty-four shrimps placed in twenty-four plastic bags (Twelve shrimps were inoculated by 10^7 cfu/ml *V. parahaemolyticus*. The others were inoculated by 10^8 cfu/ml *V. parahaemolyticus*). These shrimp samples were divided into three groups, averagely (Each group included four shrimps inoculated by 10^7 cfu/ml *V. parahaemolyticus* and four shrimps inoculated by 10^8 cfu/ml *V. parahaemolyticus*).

Detection of *V. parahaemolyticus* by bacterial culture method

One group of shrimp samples were homogenized in 30 ml of saline water (0.85% NaCl) and homogenized for 2 min in a stomacher (BagMixer400VW, Interscience, France) at room temperature. One milliliter of the suspension in the plastic bag was ten-fold serially diluted and 100 µl of appropriate dilutions was spread onto TCBS (thiosulfate-citrate-bile salts-sucrose, TCBS, Beijing Land Bridge Technology Company Ltd., Beijing, China) plates and incubated for 16 h at 37 °C for enumerating the total number of bacteria.

Detection of *V. parahaemolyticus* by real-time reverse-transcriptase PCR

Optimization of Real-time PCR Conditions

The SYBR Green I based real-time PCR assay was performed on an ABI Prism 7500 Fast Sequence Detection System (Applied Bio-systems, Foster City, CA, USA) using FastStart Universal SYBR Green Master (Rox) (Roche, Germany). The tlh primers used in this study were designed with eprimer3 software (Whitehead Institute, Cambridge, MA, USA), based on sequences from the NCBI database. The primers were listed in Table 1. Reactions were carried out in MicroAmp optical eight-tube strips using ABI Prism 7500 (Applied Biosystems, USA) sequence detection system. PCR was performed in a final volume of 20 µl including 2 µl of template DNA or cDNA, 10 µl of FastStart Universal SYBR Green Master (Rox) (Roche, Germany), 1.5 µl (10 mM concentration) of each primer, and 5 µl RNase Free dH₂O.

The cycling profile included the initial PCR activation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and primers annealing at 60 °C for 1 min. Fluorescent signals were collected at the extension step. A no-template negative control was included in each run.

Specificity of primers

The specificity of the primers was tested using *V. parahaemolyticus* QD strain as a positive control and 22 bacterial strains as negative controls (two *V. cholerae* strains, one *V. alginolyticus* strain, one *V. natriegen* strain, seven *L. monocytogenes* strains, one *L. innocua* strain, three *Pseudomonas* strains, one *Yersinia* strain, one *Escherichia coli* strain, one *Staphylococcus aureus* strain, one *Salmonella* strain, one *Macroccoccus caseolyticus* strain, one *Pseudomonas fluorescens* strain and one *Pseudomonas putida* strain), involving the main spoilage and pathogenic bacteria found in food. DNA were extracted from these bacteria and

used as templates for PCR reaction. The specificity of primers was established by performing agarose gel electrophoresis of final PCR product.

Table 1: List of primers used in this study, with their sequences, length, T_m, the percentage of GC and product size.

Primer Name	Sequence	Length	T _m (°C)	GC (%)	Product Size (bp)
Forward primer	5'-agctggttcttaggtcacttctcc-3'	24	61.47	50	191
Reverse primer	5'-ggtttgtagttcttcgccagtttt-3'	24	61.96	41.67	

Sensitivity of RT-qPCR

The detection and quantification threshold of the RT-qPCR assays were determined by inoculating 100 µl of different concentrations of *V. parahaemolyticus* on shrimp samples. Shrimp samples then mixed with 30 ml of sterile 0.85% NaCl. Shrimp samples were immersed in the inoculum solution and shaken for 10 min. Then all the aliquots of each shrimp were transferred into 50 ml sterile tubes. The tubes were centrifuged at 10000 g (4 °C) for 5 min. After removal of the supernatant, the pellet was used to extract RNA. The RNA was subsequently subjected to real-time RT-PCR.

RNA-based standard curve

RNA standards of *V. parahaemolyticus* which bore the tlh target gene constructed in vitro transcription were synthesized by TaKaRa Biotechnology Dalian Co., Ltd., China. Then the RNA standards were reverse-transcribed by using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology Dalian Co., Ltd., China) according to the manufacturer's guidelines. The total RNA was purified twice by using DNase I. After purification, the purity and the number of constructed copies of the RNA standards were calculated through its OD value. A standard curve for the RT-qPCR assays was generated with ten-fold serial dilutions of standard cDNA (10²-10⁷ copies/ml) and CT values were determined. Correlation coefficients (R²) and efficiencies (E) of amplification were calculated.

RNA extraction and cDNA synthesis

Total bacterial RNA from shrimp was extracted according the method used by Sirsat [45]. The concentration of RNA was determined by Biotek Synergy™ 2 (Gene Co., Ltd., USA) and the integrity of RNA was confirmed by gel electrophoresis. Then the cDNA was synthesized by using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology Dalian Co., Ltd., China) according to the manufacturer's guidelines. Reactions were carried out in a Mastercycler ep cycler (Eppendorf, Germany), following PCR conditions: 37 °C for 15 min, 85 °C for 5 s and cooling to 4 °C. The synthesized cDNAs were stored at -20 °C before subsequent use. One group of shrimp samples mentioned in section 2.2 were homogenized in 30 ml of saline water (0.85% NaCl) and shaken for 10 min at room temperature. All the aliquots were transferred into one 50 ml sterile tube. The tubes were centrifuged at 10000 g (4 °C) for 5 min. The pellet was stored at -80 °C until extracting RNA as mentioned above.

Detection of *V. parahaemolyticus* by real-time PCR

DNA-based standard curve

To construct standard curve, DNA were extracted from of ten-fold serial dilutions of suspensions of pure *V. parahaemolyticus* (10²-10⁷ cfu/ml) and were amplified as described above. CT values were generated by real-time PCR. In parallel, the same concentrations of suspensions of *V. parahaemolyticus* were counted for determination of viable cell counts on selective agar (TCBS). A linear relationship was produced by plotting the number of bacteria against the CT values. Correlation coefficients (R²) and efficiencies (E) of amplification were calculated.

DNA extraction

Bacterial DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech Beijing Co., Ltd., China) according to the manufacturer's instruction. Finally, DNA was eluted with TE buffer (Tiangen) and stored at -20 °C.

One group of shrimp samples were homogenized in 30 ml of saline water (0.85% NaCl) and homogenized for 2 min in a stomacher at room temperature. All the aliquots were transferred into one 50 ml sterile tube and centrifuged at 10000 g (4 °C) for 5 min. The pellet was stored at -20 °C until extracting DNA as mentioned above.

Shrimp samples for quantitative testing

RNA and DNA were extracted from artificial contamination shrimp samples (see section 2.2) respectively, and then subjected to RT-qPCR or qPCR. Quantitative results were obtained from standard curves. Simultaneously, the traditional plating methods were performed for bacterial enumeration.

Statistical analysis

The values were expressed as mean ± standard deviation (SD). Statistical analysis was performed by using the Origin 8.0 to determine the statistical differences between the plate counting and the RNA- /DNA-based qPCR detection methods.

RESULTS

Specificity of quantitative real-time PCR assays

The specificity of the assays was determined by PCR assay with the DNA targets extracted from pure cultures of *V. parahaemolyticus* and non-*parahaemolyticus* strains. Agarose gel electrophoresis showed a single band (data not shown) when testing DNA templates from *V. parahaemolyticus* strain. None of the PCR amplified products was observed from the strains of non-*parahaemolyticus* species indicating a good specificity of primers. The specificity was further detected by the melting curves of qRT-PCR assay. Also negative controls did not show any melt temperature (T_m) at $\sim 81.53^\circ\text{C}$ indicating the primers had significant specificity (data not shown).

Determination of the sensitivity of quantitative real-time PCR assays

The sensitivity of real-time PCR was tested as described in materials and methods by using serial dilutions of mRNA extracted from the known number of *V. parahaemolyticus* which spiked in shrimp. For the spiked shrimp, the sensitivity of the RT-qPCR assay was 58 cfu/g.

RNA-based standard curve

The RNA standard was synthesized by TaKaRa Biotechnology Dalian Co., Ltd., China and the results had a good quality (Table 2). Standard curve was generated by RNA-based qRT-PCR amplification of serial dilutions of cDNA standard (10^2 - 10^7 copies/ μl) in EASY Dilution Buffer. The equation of the copies/ μl versus the threshold cycle (CT) values was obtained as $y = -3.46x + 36.00$ with R^2 of 0.998; the amplification efficiency (E) was 94.56%ve information indicated that the R_2 and E value were acceptable in this method.

Table 2: Quality parameters of standard RNA.

Standard RNA	A260	A280	A260/280	Concentration (ng/ μl)	Copies (copies/ μl)
tlh	0.64	0.30	2.09	25.43	1×10^{11}

DNA-based standard curve

DNA was extracted from ten-fold dilutions of pure *V. parahaemolyticus* to construct DNA-based standard curve. Each CT value was determined by DNA-based real-time PCR assay. CT values were plotted as a function of the cell concentration and the plot showed the expected linear relationship between the Log cfu/ml and CT values (Figure 2). The slope of the DNA-based standard curve was -3.50, which corresponded to 93.03% efficiency for qPCR assay. The R^2 and E value were acceptable in the method.

Quantitative detection of *V. parahaemolyticus* in spiked shrimp samples

The survival of *V. parahaemolyticus* in artificially contaminated shrimp was detected and quantified by plate count method, DNA-based qPCR and RT-qPCR method. The results of RT-qPCR assay and the plate count method for spiked shrimp samples had a good statistical correlation ($R^2=0.96$), while the correlation coefficient for plate count method and DNA-based qPCR just reached $R^2=0.85$ (Figure 3).

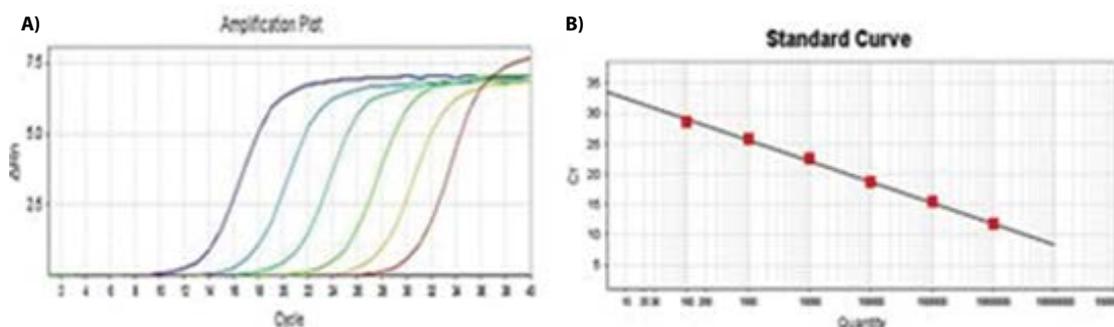


Figure 1: The amplification and standard curve of RNA-based qRT-PCR. (A) Amplification of the tenfold dilution of cDNA standards ranging from 10^7 copies/ μl to 10^2 copies/ μl (B) The standard curve was generated by plotting the mean Ct values versus the ten-fold serial dilutions of RNA standard over a range of concentrations from 10^7 copies/ μl to 10^2 copies/ μl .

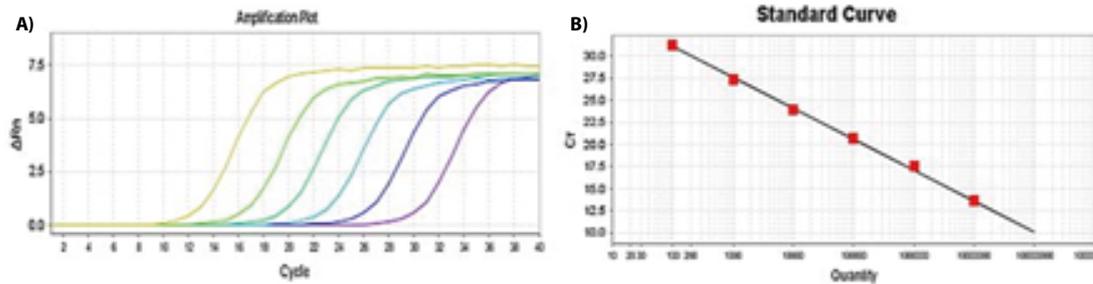


Figure 2: The amplification and standard curve of DNA-based RT-qPCR. (A) Amplification of the tenfold dilutions of standard DNA ranging from 10^7 Log cfu/ml to 10^2 Logcfu/ml. (B) The standard curve was generated by plotting the mean Ct values versus the tenfold serial dilutions of standard DNA over a range of concentrations from 10^7 Logcfu/ml to 10^2 Logcfu/ml.

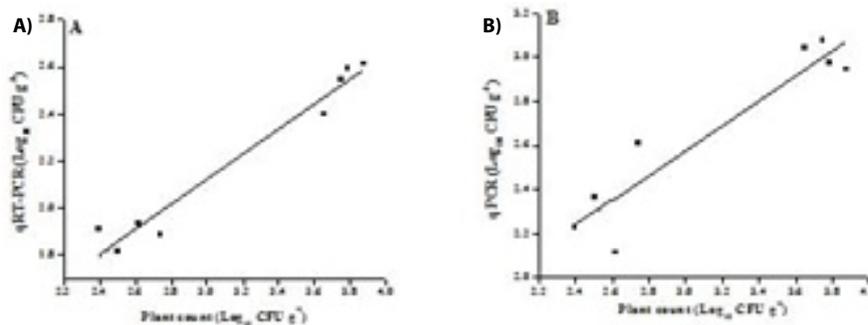


Figure 3: Quantification of *V. parahaemolyticus* in spiked shrimp samples by RT-qPCR, DNA-based qPCR and plate count technique. (A) Comparison of plate count and RT-qPCR, $y=0.53x+0.51$, $R^2=0.96$ (B) Comparison of plate count and DNA-based qPCR, $y=0.56x+0.89$, $R^2=0.85$.

DISCUSSION

In recent years, real-time PCR for detecting and quantifying the pathogens has been increasingly used in food and environmental samples by targeting DNA^[7,16,17], including qPCR employed DNA standards for detecting *V. parahaemolyticus*. However, the main drawback of these methods is that DNA in dead cells could be still detected and cause false positive results. It was reported that DNA could exist for up to three weeks after bacterial cells death^[18]. Since RNA has a shorter half-life than DNA in dead bacteria, our study established an RNA-based real-time RT-PCR, which used in vitro-transcribed RNA standards. The method was developed to quantify viable *V. parahaemolyticus* in shrimp. To our knowledge, this was the first time that a real-time PCR was employed to quantify viable *V. parahaemolyticus* from the viewpoint of RNA standards obtaining by in vitro transcription. A similar strategy constructed RNA standards for quantification of mRNA corresponding to the *InvA* and 16S *rRNA* genes of *Salmonella*^[19]. In vitro-transcribed RNA standards has been proved well for quantification. The thermolabile hemolysin gene (*tlh*), which was regarded as a species-specific marker for *V. Parahaemolyticus*^[20], was selected as targets for this study. The *tlh* gene has been used in previous study for the quantification of *V. parahaemolyticus*^[21,22].

This assay established RNA standards by in-vitro transcription which is the highlight of the study. However, in published studies about real-time RT-PCR, most of the methods employed DNA-based standard curves to quantifying bacteria, rather than RNA-based standard curves. For example Bui et al.^[23] quantified *Campylobacter jejuni* by the DNA standard curve of real-time RT-PCR and investigated the survival of *Campylobacter* in chicken faecal samples. These methods are easier to optimize because of the absence of the efficiency of reverse-transcription. Yet, these methods ignored the difference between DNA and RNA extraction, which might cause inaccurate quantification.

What's more, some earlier researches required pre-enrichment procedures before starting real-time RT-PCR assay. For instance, Miller et al.^[24] used real-time RT-PCR to detect *Salmonella enterica* in Serrano peppers, Jalapeno, lettuce and tomatoes with a short period of pre-enrichment (6 h), and they demonstrated that this method rapidly and successfully detected *Salmonella* in food.

The important characters of quantitative PCR assay are its specificity, sensitivity and linear ranges. After optimization, the protocol was found to be highly specific for *V. parahaemolyticus* indicated by that no amplification signal of DNA or RNA from non-*parahaemolyticus* was obtained by the qPCR protocol (data not shown). In addition, the specificity of the qPCR was further tested by the melting temperature (Tm). Results showed that the amplification efficiency of both RNA-based (E=94.56%) and DNA-based (E=93.03%) methods were within the acceptable range (90%-110%)^[25] and sensitivity testing showed that the detection limit level for shrimp sample detected by RNA-based method was 58 cfu/g. In a word, the RNA-based real-time RT-PCR assay was characterized by its high specificity and sensitivity. Furthermore, this method had advantages over bacterial culture method due to without pre-enrichment.

Nowadays, some researches have focused on viability-PCR because of that the viability dye could penetrate into cell membrane of all dead bacteria except for live cells. Once entry in the dead cells, the DNA-binding dye induced irreversible DNA modifications, produce the inhibition of DNA amplification [26]. However v-PCR currently suffered from some practical limitations [27]. In case of the complexity of sample matrix, the signals of dead cell might be inhibited, resulting in the overestimation of intact cells. Because of those, this study employing RNA, as the basis of quantification ensures that only viable bacteria will be quantified.

The difference on the quantification between real-time RT-PCR and the traditional method could be attributed to many factors. Firstly, there was a heavy amount of matrix debris from shrimp, which might have interfered with the following processes, such as RNA isolation and reverse-transcription. Secondly, the colony-forming unit is not an exact standard unit and could contain from 10 to 100 bacteria, while molecular-based methods could quantify the bacteria on a single cell count basis [28]. Finally, the efficiency of RNA extraction as well as reverse-transcription may have the lower quantification results than traditional method. The highly stable DNA of dead bacterial cells may result in an overestimation of bacterial cells. As such, the quantification results of DNA-based real-time PCR were higher than that of RT-qPCR. However, RNA extraction procedures were rather complex and the instability of RNA may also cause the lower quantification results compared with DNA-based RT-PCR.

The different molecular techniques, especially real-time PCR method, offer an attractive alternative for quantifying total bacteria. This method is widely applied in various studies, which requires reproducible and accurate bacterial quantification [29-32]. Afterwards, the novel method in this study utilized mRNA standard curve to quantify the *V. parahaemolyticus* in shrimp. The purpose of this study was tried to mimic the bacterial culture method as close as possible, for culture method is convention and standard quantification method.

Whereas, considering what is said above, it is difficult to create a new rapid molecular method which is independent of culture. The new molecular method has its advantages over traditional method, such as, detecting viable but non-culture bacteria in samples which can't be quantified by bacterial culture method. In summary, the novel method has the potential to be used as an alternative bacterial quantitation method. However, this method is not applied to test the shrimp samples in market. Further studies are required to validate the practicability and accuracy of this novel method.

ACKNOWLEDGEMENT

This research was supported by the National Natural Science Foundation of China (31271870), the project of Science and Technology Commission of Shanghai Municipality (14DZ1205100, 14320502100), Key Project of Shanghai Agriculture Prosperity through Science and Technology (2014, 3-5 and 2015, 4-8) Shanghai Engineering Research Center of Aquatic-Product Processing and Preservation (11DZ2280300).

REFERENCES

1. Broberg CA, et al. *Vibrio parahaemolyticus* cell biology and pathogenicity determinants. *Microbes Infect.* 2011; 13: 992-1001.
2. Kim HJ, et al. Simultaneous detection of Pathogenic *Vibrio* species using multiplex real-time PCR. *Food Control.* 2012; 23: 491-498.
3. Klein D. Quantification using real-time PCR technology: applications and limitations. *Trends Mol Med.* 2002; 8:257-260.
4. Blackstone GM, et al. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. *J Microbiol Meth.* 2003; 53: 149-155.
5. Robert-Pillot A, et al. Total and pathogenic *Vibrio parahaemolyticus* in shrimp: Fast and reliable quantification by real-time PCR. *Int J Food Microbiol.* 2010; 143: 190-197.
6. Flekna G, et al. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Res Microbiol.* 2007; 158: 405-412.
7. Wolffs P, et al. Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. *J Microbiol Meth.* 2005; 60: 315-323.
8. Kobayashi H, et al. Limiting false-positive polymerase chain reaction results: detection of DNA and mRNA to differentiate viable from dead bacteria. *Diagn Microb Infec Dis.* 2009; 64: 445-447.
9. MaurerJJ. 2006. *The mythology of PCR: a warning to the wise.* Springer US. 2006; 27-40.
10. Lin S, et al. Preliminary development and validation of a real-time reverse transcription PCR assay for the semi-quantification of viable *Campylobacter jejuni* in water samples. *Water Sci. Technol.* 2009; 60: 3151-3158.
11. Ottaviani D, et al. Presence of pathogenic *Vibrio parahaemolyticus* strains in mussels from the Adriatic Sea, Italy. *Food microbiology.* 2005; 22: 585-590.
12. GopalS, et al. The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. *Int J Food Microbiol.* 2005; 102: 151-159.

13. Jeyasekaran G, et al. Quantitative and qualitative studies on the bacteriological quality of Indian white shrimp (*Penaeus indicus*) stored in dry ice. *Food Microbiol.* 2006; 23: 526-533.
14. Yano Y, et al. Occurrence and density of *Vibrio parahaemolyticus* in live edible crustaceans from markets in China. *J Food Protect.* 2006; 69: 2742-2746.
15. Sirsat SA, et al. Optimization of the RNA extraction method for transcriptome studies of *Salmonella* inoculated on commercial raw chicken breast samples. *BMC research notes.* 2011; 4: 60.
16. Lund M, et al. Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR. *J Clin Microbiol.* 2004; 42: 5125-5132.
17. Ronner AC and Lindmark H. Quantitative detection of *Campylobacter jejuni* on fresh chicken carcasses by real-time PCR. *J. Food Protect.* 2007; 70: 1373-1378.
18. Josephson K, et al. Polymerase chain reaction detection of nonviable bacterial pathogens. *Appl Environ Microbiol.* 1993; 59: 3513-3515.
19. Fey A, et al. Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism. *Appl Environ Microbiol.* 2004; 70: 3618-3623.
20. Bej AK, et al. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tlh*, *tdh* and *trh*. *J Microbiol Meth.* 1999; 36: 215-225.
21. Park JY, et al. Multiplex Real-time Polymerase Chain Reaction Assays for Simultaneous Detection of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. *Osong Public Health and Research Perspectives.* 2013; 4: 133-139.
22. Yi M, et al. 2014. Real time loop-mediated isothermal amplification using a portable fluorescence scanner for rapid and simple detection of *Vibrio parahaemolyticus*. *Food Control.* 2014; 41: 91-95.
23. Bui XT, et al. Reverse transcriptase real-time PCR for detection and quantification of viable *Campylobacter jejuni* directly from poultry faecal samples. *Res microbiol.* 2012; 163: 64-72.
24. Miller ND, et al. Real-time reverse-transcriptase PCR for *Salmonella* Typhimurium detection from lettuce and tomatoes. *LWT-Food Science and Technology.* 2011; 44: 1088-1097.
25. Lutfalla G and Uze G. Performing quantitative reverse-transcribed polymerase chain reaction experiments. *Method Enzymol.* 2006; 4: 386-400.
26. Soejima T, et al. Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. *Microbiol Immunol.* 2007; 51: 763-775.
27. Fittipaldi M, et al. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *J. Microbiol Meth.* 2012; 91: 276-289.
28. Dolan A, et al. A novel quantitative reverse-transcription PCR (qRT-PCR) for the enumeration of total bacteria, using meat micro-flora as a model. *J Microbiol Meth.* 2009; 77: 1-7.
29. Oppliger A, et al. Exposure to bioaerosols in poultry houses at different stages of fattening use of real-time PCR for airborne bacterial quantification. *Ann Occup Hyg.* 2008; 52: 405-412.
30. Aagesen AM and Hase CC. Seasonal effects of heat shock on bacterial populations, including artificial *Vibrio parahaemolyticus* exposure, in the Pacific oyster, *Crassostrea gigas*. *Food Microbiol.* 2014; 38: 93-103.
31. Blanco-Abad V, et al. Evaluation of different procedures for the optimized detection of *Vibrio parahaemolyticus* in mussels and environmental samples. *Int J Food Microbiol.* 2009; 129: 229-236.
32. ChurrucaE, et al. Detection of *Campylobacter jejuni* and *Campylobacter coli* in chicken meat samples by real-time nucleic acid sequence-based amplification with molecular beacons. *Int J Food Microbiol.* 2007; 117: 85-90.