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Irregular virulence genes expression of *Vibrio parahaemolyticus* in shrimp or seawater matrix

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ABSTRACT

The three virulence genes (*tlh, tdh* and *trh*) expression of five *Vibrio parahaemolyticus* strains (ATCC33847, ATCC17802, VP1, VP2 and VP3) in shrimp or seawater matrix at 9°C and 25°C were detected by the quantitative reverse transcription-PCR (qRT-PCR). The results showed that the transcription levels of *tlh* in ATCC17802 and VP3, *tdh* in VP1 were up-regulated, but the transcription levels of *tlh* and *tdh* in ATCC33847 were significantly ($p < 0.05$) down-regulated in shrimp samples compared with that in seawater samples. Additionally, low-temperature had a positive effect on genes expression, including *tlh* and *tdh* in ATCC33847, *trh* in ATCC17802, *tlh* in VP2. Meanwhile, low-temperature had a negative effect on the expression of *tlh* in ATCC17802 and *tdh* in VP1. Interestingly, the transcription levels of *tdh* in VP1 and *tlh* in VP3 cultured at 9°C and 25°C, respectively, were significantly ($p < 0.05$) higher in shrimp samples than that in other samples. These results indicated the expression of virulence genes in *V. parahaemolyticus* were irregular with the varying of environmental conditions.

INTRODUCTION

Vibrio parahaemolyticus is a gram-negative marine bacterium which is a natural inhabitant of marine and estuarine environments around the world. It has been perceived as a major cause of food-borne illness characterized by acute gastroenteritis, mainly due to consumption of raw or undercooked contaminated seafood [1,2]. Furthermore, *V. parahaemolyticus* infection is a growing public health concern because of the emergence of pandemic strains that have caused severe outbreaks worldwide [3].

It is well known that *V. parahaemolyticus* has some major virulence genes contributing to the virulence of the *V. parahaemolyticus*-mediated disease, such as *tlh*, coding thermolabile haemolysin (TLH); *tdh*, coding thermostable direct haemolysin (TDH); and *trh*, coding TDH-related haemolysin. Many factors may increase or decrease the virulence genes expression of *V. parahaemolyticus*. For instance, some physical and chemical factors, Gamma-irradiation, UVC-irradiation, N-acetyl-D-glucosamine (GlcNAc), may lead to the variation in some genes expression of *V. parahaemolyticus* [4-6]. Additionally, bile acids played a critical role in the expression of *tdh* virulence gene of *V. parahaemolyticus* [7-9]. And Nakano et al. [10] showed that *Hfq* gene could regulate the expression of virulence-associated factors such as TDH and may be involved in the pathogenicity of *V. parahaemolyticus*. Exposure *V. parahaemolyticus* to seawater for 8 months decreased the expression level of virulence pathogenicity island (VPI) gene [11]. Moreover, after the recovery of culturability, the viable but non-culture (VBNC) cells of *V. parahaemolyticus* did not significantly induce the transcription of the main virulence genes in the environmental conditions [12]. The expression of *tlh* gene was strongly up-regulated under conditions simulating those in the human intestine [9,13]. Most reports on *V. parahaemolyticus*

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virulence were carried out with cells cultivating in growth medium.

In this study, we firstly evaluated virulence genes expression of *V. parahaemolyticus* strains responding to the shrimp (*Litopenaeus vannamei*) or seawater matrix, which is crucial to understand and control the impact of this pathogen on public health. Shrimps and seawater were chosen as test models because *V. parahaemolyticus* was the natural inhabitant of seafood and the marine environment of coastal areas [14,15].

MATERIALS AND METHODS

Bacterial strains and inoculums preparation

Five different *V. parahaemolyticus* strains were used for the virulence genes expression analysis in this study, including strain ATCC33847 (*tlh*$^+$, *tdh*$^+$ and *trh*$^-$), ATCC17802 (*tlh*$^+$, *tdh*$^-$ and *trh*$^+$), VP1 (*tlh*$^+$, *tdh*$^+$ and *trh*$^-$), VP2 (*tlh*$^+$, *tdh*$^-$ and *trh*$^+$) and VP3 (*tlh*$^+$, *tdh*$^-$ and *trh*$^-$). ATCC33847 and ATCC17802 were acquired from the Institute of Microbiology of Chinese Academy of Sciences (AS,IMCAS); VP1 were isolated from a Qingdao clinical specimen in China and confirmed by 16S sequencing analysis; VP2 and VP3 were isolated from shrimp samples in Shanghai, identified by PCR molecular methods and kept in our laboratory. The strains were maintained in glycerol broth at −80°C. All bacteria were recovered in 5 mL of tryptone soy broth (TSB, Beijing Land Bridge Technology Co., Ltd., China) supplemented with 3% (w/v) NaCl and incubated overnight at 37°C with shaking at 180 rpm.

Shrimp samples (13 ± 1 g per sample) were purchased from a Nanhui Famers Markets in Shanghai, China. The shrimp samples were kept in sterile homogeneous bags and frozen immediately at -80°C until the experiment started. Seawater samples were collected from Shanghai coast of the East China Sea and filtered to remove the sediment by an ordinary filter paper. Then 9 mL of seawater was kept in 15 mL sterilized tubes at -80°C before using. No background *V. parahaemolyticus* was detected in shrimp or seawater samples.

Artificial contamination

Bacteria used for inoculation were cultured in TSB (plus 3% NaCl) at 37°C for nearly 10 h until late log growth phase (~10^7 CFU/mL). One milliliter of each *V. parahaemolyticus* strain was transferred to 9 mL of seawater and TSB (plus 3% NaCl) samples to achieve a final concentration of approximately 10^6 CFU/mL per sample. A 100 µL culture suspension (10^7 CFU/mL) of each *V. parahaemolyticus* strain was inoculated gently spreading onto the abdominal region of each one shrimp sample. The final concentration of *V. parahaemolyticus* inoculated on shrimp was 10^6 CFU/g on average. The inoculated shrimp or seawater samples were incubated at 9°C and 25°C for 12 h, and the control samples were incubated at 37°C. Samples for each treatment were performed in duplicate. The 9°C and 25°C treatments were chosen with the reason that they represent the mean temperatures of seawater in winter and summer in Shanghai coast of the East China, respectively. Meanwhile, the 37°C was applied in this study as it was the optimum growth temperature to *V. parahaemolyticus* in pure cultures.

RNA-based standard curve

RNA standards of *V. parahaemolyticus* which bore the *tlh, tdh and trh* 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 (10^3–10^8 copies/mL) and Ct values were determined. Correlation coefficients (R^2) and efficiencies (E) of amplification were calculated ($E=10^{-1/slope}-1$).

RNA extraction and cDNA synthesis

The 10 mL seawater and 1 mL control samples were collected following the above step. Seawater samples were centrifuged at 10,000 rpm for 10 min at 4°C and control samples were centrifuged at 12,000 rpm for 2 min at 4°C, then the supernatant was removed and the pellets were kept in -80°C before RNA extraction. Shrimps were homogenized manually for 10 min with saline solution containing 0.85% (w/v) NaCl. Shrimp homogenates (approximately 30 mL) were collected into 50 mL sterilized tubes and then centrifuged (10,000 rpm, 10 min, 4°C). The supernatant was removed and pellets were kept in -80°C for RNA extraction. Three independent replicates were performed for each sample.

RNA was extracted using TRIzol reagent (Invitrogen) according to manufacturer's instructions with slight modifications. Aliquots of 1 mL TRIzol® Reagent (Invitrogen Co., Ltd., China) was added to the sterilized tubes containing the bacterial cells and mixed repeatedly for 15 min and then incubated 20 min on ice. Then 200 µl of mixture (chloroform:phenol:isopropanol=24:25:1) (Sangon Biotech Co., Ltd., Shanghai, China) was added and mixed well. Immediately after extraction for 2 min on ice, the tubes were centrifuged (12,000 rpm, 15 min, 4°C). A 358 µL of the supernatant was transferred into a new sterilized 1.5 mL tube and 358 µL of isopropanol (Sangon Biotech Co., Ltd., Shanghai China) was added, incubated at -20°C for 20 min, and then centrifuged (12,000 rpm, 15 min, 4°C). The supernatant was removed and 1 mL 75% (v/v) cooled ethanol was added and the tube inverted several times and centrifuged (10,000 rpm, 5 min, 4°C). The supernatant was removed and dried on ice for 2-3min to make ethanol volatilization. Then RNAs were suspended with 30 mL RNase Free dH_2O. The quantity of extracted RNA was determined by Biotek SynrgyTM2 (Gene Co., Ltd., USA), and the integrity of RNA was confirmed by gel electrophoresis.

The cDNA synthesis was performed 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). PCR conditions: 37°C for 15 min, 85°C for 5 s and cooling to 4°C. The synthesized cDNAs were stored at -20ºC.

qRT-PCR analysis of *V. parahaemolyticus* virulence genes *tlh, tdh* and *trh*

The reverse transcription quantitative real time polymerase chain reaction (qRT-PCR) was applied to monitor the virulence genes expression of *V. parahaemolyticus* in various environments because of its specificity and sensitivity [16]. qRT-PCR was carried out with the ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using FastStart Universal SYBR Green Master (Rox) (Roche Co., Switzerland). Primers used in this study were listed in Table 1 [12,17-19]. The accuracy of measurements of gene transcription levels based on qRT-PCR assays relies on the use of housekeeping genes as internal controls. Two genes *pvsA* and *pvuA* had been identified as optimal housekeeping genes for virulence expression analysis of *V. parahaemolyticus* in different samples (pure culture, shrimps and seawater) in our laboratory.

Table 1: Primers used for the study.

Gene	Primer[a] 5′→3′	Amplicon size (bp)	References
*pvs*A	F-pvsA: CTCCTTCATCCAACACGAT R-pvsA: GGGCGAGATAATCCTTGT	104	[12,17]
pvuA	F-pvuA: CAAACTCACTCAGACTCCA R-pvuA: CGAACCGATTCAACACG	156	[12,17]
tlh	F-tlh: AGCTGGTTCTTAGGTCACTTCTCC R-tlh: GGTTTGTAGTTCTTCGCCAGTTTT	191	[19]
*td*h	F-tdh: GTAAAGGTCTCTGACTTTTGGAC R-tdh: TGGAATAGAACCTTCATCTTCACC	269	[18]
trh	F-trh: TTGGCTTCGATATTTTCAGTATCT R-trh: CATAACAAACATATGCCCATTTCC	500	[18]

[a]F: Forward; R: Reverse.

PCR was performed in a final volume of 20 µL including 2 µL of template DNA or cDNA, 10 µL of SYBR Green PCR Mix (Rox), 1.5 µL (10 mM concentration) of each primer, and 5 µL RNase Free dH_2O (Shanghai Lifefeng Biotechnology Co., Ltd., China). The cycling profile involved initial PCR activation step at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s. Fluorescent signals were collected at the extension step. A no-template negative control was included in each run. Melting curve was acquired to ensure that a single product was amplified in each reaction.

Statistical analysis

Statistical analysis was performed using SPSS statistical package 19.0 (SPSS Inc., Chicago, IL). The least significant difference (LSD) test was used to determine differences at α=0.05. The figure was performed using Origin pro 8.0 (Origin Lab Corp., Northampton, USA).

RESULTS

Standard curves and amplification efficiency of *tlh, tdh* and *trh* for *V. parahaemolyticus*

There was a linear relationship over the range of 10^3–10^8 CFU/mL. The resulting equation of this straight line for *tlh* gene was: y=47.14-3.45x, which showed a good correlation between the threshold cycle (Ct) values and Log_{10} CFU/mL with R^2 value of 0.994 (Figure 1). Meanwhile, the amplification efficiency was 95% (Figure 1). The equation of standard curve for *tdh* gene was y=44.52-3.43x with R^2 value of 0.990, which indicated an efficiency of 96% (Figure 1). In addition, the equation of standard curve for *trh* gene was y=46.99-3.73x with R^2 value of 0.996. The amplification efficiency was 85%. Based on the information as described above, the R^2 values were acceptable in each of the three standard curves, and the real-time PCR assay could therefore be used to detect the target bacteria.

Irregular expression of virulence genes of *V. parahaemolyticus* in shrimp or seawater matrix

The results for transcription levels of target genes after *V. parahaemolyticus* inoculated in shrimp or seawater samples were shown in Figures 2A-2C. The transcription levels of *tlh* gene in these five strains differed from one another (Figure 2A). The *tlh* expression in strain ATCC17802 and VP3 were higher in shrimp samples than that in seawater samples. Whereas, strain ATCC33847 had significantly ($p < 0.05$) lower *tlh* expression in shrimp samples compared with that in seawater samples. Besides, strain VP1 in shrimp samples at 9°C showed a significant increase in *tlh* expression compared with other samples. For strain VP2, highest *tlh* expression was displayed in the seawater samples at 9°C. Interestingly, the highest *tlh* gene expression in strain VP3 was observed in shrimp samples at 9°C, and was 67.9-fold higher than that in seawater samples.

Significant differences of *tdh* transcription levels were observed between shrimp or seawater samples except those in strain VP1 at 9°C (Figure 2B). Strain VP1 demonstrated higher *tdh* expression in shrimp samples compared with that in seawater

samples. The increased transcription levels in shrimp samples at 25°C were 10.9-fold the transcription levels measured in seawater samples. However, the expression of strain ATCC33847 was lower in shrimp samples, which was similar to *tlh* gene expression.

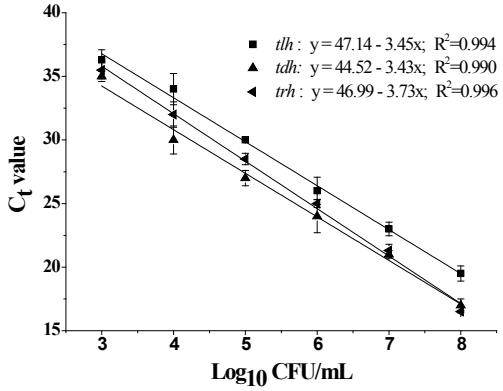

Figure 1: Standard curve of RNA-based qRT-PCR for *tlh, tdh and trh* genes of *V. parahaemolyticus.*

With respect to *trh* gene, the transcription levels showed significant deviations between shrimp and seawater samples except those in strain ATCC17802 at 25°C. In strains ATCC 17802 and VP2, significantly lower *trh* expression was discovered in shrimp samples than that in seawater samples at 9°C (Figure 2C). These results showed that virulence gene expression of these five strains were unstable in shrimp or seawater samples.

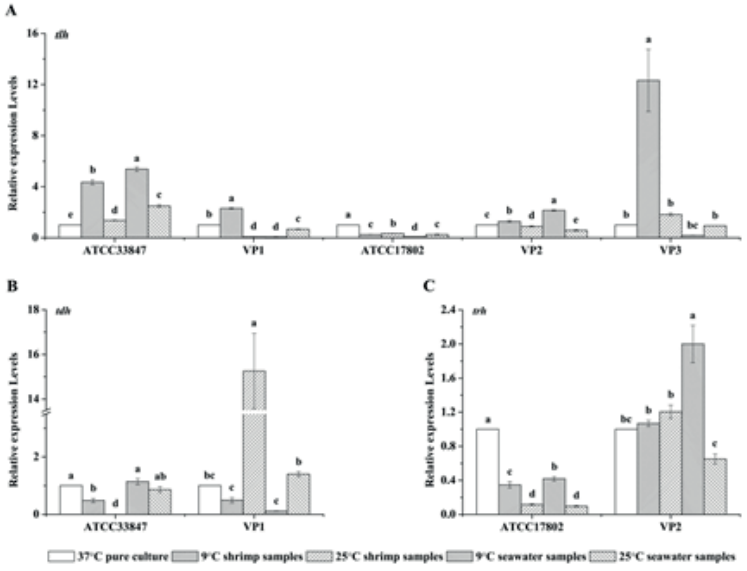

Figure 2: Transcription levels of selected virulence genes (A: *tlh*, B: *tdh* and C: *trh*) of each *V. parahaemolyticus* strain incubated in various environmental conditions. Values shown are the means from three independent RNA collections. Standard deviations are indicated as vertical bars. a-e: between different environmental conditions, for each strain, values not having the same superscript are statistically significant different ($p < 0.05$).

Irregular expression of virulence genes of *V. parahaemolyticus* at 9°C and 25°C

The *tlh* transcription levels of strain ATCC33847 and VP2 in shrimp and seawater samples, VP1 and VP3 in shrimp samples were enhanced clearly at low-temperature. With regard to strain ATCC17802 in all samples, strain VP1 and VP3 in seawater samples, the *tlh* transcription levels were inhibited at low-temperature (Figure 2A). The low-temperature significantly ($p < 0.05$) increased *tdh* transcription levels of strain ATCC33847 in shrimp and seawater samples, which were contrary when compared to strain VP1 (Figure 2B). Meanwhile, the low-temperature also played a positive role in *trh* transcription levels of strain ATCC17802 in all samples and strain VP2 in seawater samples (Figure 2C). Interestingly, *tdh* gene expression of VP1 in shrimp samples at 25°C was 31.13-fold the transcription levels observed at 9°C. Through above analysis, virulence genes expression of *V. parahaemolyticus* responded differently to temperatures.

DISCUSSION

V. parahaemolyticus-mediated disease has traditionally been associated with two virulence genes, *tdh* and *trh* [20,21]. Meanwhile, *V. parahaemolyticus* also has an additional hemolysin gene, *tlh*, coding thermolabile haemolysin (TLH), which is

widely considered to be a marker for *V. parahaemolyticus* [22,23]. The results of this study showed that genes expression of different strains in various conditions were irregular.

The data indicated that *tlh* expression in strain ATCC17802 and VP3 were higher than that in seawater samples. Interestingly, the transcription level of *tdh* gene in strain VP1 (*tlh*$^+$, *tdh*$^+$, *trh*$^-$ and serotype O3:K6) isolated from a patient, were higher in shrimps, especially at 25°C, which corresponded to the room temperature, than in other conditions (Figure 2B). Cabanillas-Beltrán et al. [24] reported that the *V. parahaemolyticus* O3:K6 (*tlh*$^+$, *tdh*$^+$, *trh*$^-$) strain in raw or undercooked shrimps caused more than 1230 cases of gastroenteritis in the south of Sinaloa State, north-western Mexico. The *tdh* overexpression of VP1 in shrimp samples at 25°C was coincident with this disease outbreak. In general, these genes overexpression in shrimps may be explained that *V. parahaemolyticus* strain would produce more TDH to strive against shrimp lysozyme. Shrimp lysozyme was reported to have antibacterial activity against *V. parahaemolyticus* [25,26]. We hypothesized that shrimp lysozyme may stimulate *tdh* expression of strain VP1 (O3:K6) in shrimps at 25°C. In contrast, compared with seawater samples, *tlh* and *tdh* expression of strain ATCC33847 were lower in shrimp samples, indicating that these two genes in ATCC33847 had a stronger ability to against the poor nutrition conditions. These results were in accordance with those developed by Asakura et al. [27], who demonstrated that the transcription level of selected genes in *Vibrio cholerae* were up-regulated under starvation conditions in seawater. Based on the above analysis, the virulence genes transcription levels of *V. parahaemolyticus* in shrimp or seawater samples were irregular.

The transcription levels of *tlh* and *tdh* in strain ATCC33847, *trh* in strain ATCC17802 and *tlh* in VP2 were up-regulated at low-temperature (Figure 2A and 2B). Their overexpression could be linked to stimulation of low-temperature. Similar observation has been reported that some target genes were detected at higher transcription levels in Vibrio vulnificus bacteria that had entered the VBNC state induced at 4°C [28]. In addition, strain VP3 presented the highest transcription level of *tlh* in shrimp samples at 9°C and the expression was nearly 6.75-fold higher than that at 25°C. These results showed that *tlh* expression of VP3 was significantly stimulated in shrimp sample at low-temperature. Some reports indicated that the *tdh* expression of Ure+ strain without *trh* was much higher than those of other Ure+ strains carrying the *trh* gene, which suggested that the *trh* regulate the expression of *tdh* [29]. Likewise, *tlh* expression in strains without *tdh* and *trh* genes was much higher than those carrying *tdh* or *trh* genes, which suggested that *tdh* or *trh* may regulate *tlh* expression of VP1 in shrimp at low-temperature. Further studies are needed to examine this hypothesis. Additionally, *tlh* in strain ATCC17802 and *tdh* of strain VP1 demonstrated lower expression at 9°C, which indicated that these genes expression at low-temperature were inhibited in parallel with its growth. Previous studies demonstrated that incubation temperature significantly affects the gene expression profile of *Vibrio cholerae*. These authors pointed that the transcription of some target genes in the VBNC induced at 4°C were detected at higher levels in bacteria [30]. In summary, *tlh*, *tdh*, *trh* expression of *V. parahaemolyticus* were irregular under various environments.

In conclusion, we have firstly demonstrated the transcription levels of *V. parahaemolyticus* virulence genes (*tlh*, *tdh*, *trh*) in shrimp or seawater samples under 9 and 25°C using the qRT-PCR assay. These results showed that virulence genes expression of *V. parahaemolyticus* did not make unifying changes in the shrimp or seawater matrix, which may be related to their actual virulence. Therefore, more strains of different origins should be investigated to better understand the complex expression pattern of *V. parahaemolyticus* virulence genes in actual matrices at various temperatures.

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REFERENCES

1. Morris JG and Black RE. Cholera and other *vibrioses* in the United States. N Engl J Med. 1985;312:343-350.
2. Drake SL, et al. An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Compr Rev Food SCI F. 2007;6:120-144.
3. Nair GB, et al. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clin Microbiol Rev. 2007;20:39-48.
4. Abdallah FB, et al. Alterations of Outer Membrane Proteins and Virulence Genes Expression in Gamma-Irradiated *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. Foodborne Pathog Dis. 2009;6:1171-1176.
5. Lagha R, et al. Effect of Ultraviolet-C (UV-C) irradiation on the virulence genes expression in *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. Afr J Microbiol Res. 2012;6:4256-4260.
6. Thompson FL, et al. Effect of N-acetyl-D-glucosamine on gene expression in *Vibrio parahaemolyticus*. Microbes Environ. 2011;26:61-66.
7. Osawa R and Yamai S. Production of thermostable direct hemolysin by *Vibrio parahaemolyticus* enhanced by conjugated bile acids. Appl Environ Microbiol. 1996;62: 3023-3025.
8. Osawa R, et al. Levels of thermostable direct hemolysin produced by *Vibrio parahaemolyticus* O3:K6 and other serovars grown anaerobically with the presence of a bile acid. Curr Microbiol. 2002;44:302-305.

9. Gotoh K, et al. Bile acid-induced virulence gene expression of *Vibrio parahaemolyticus* reveals a novel therapeutic potential for bile acid sequestrants. PLoS One. 2010;5:e13365.

10. Nakano M, et al. *Hfq* regulates the expression of the thermostable direct hemolysin gene in *Vibrio parahaemolyticus*. BMC microbiol. 2008;8:155.

11. Abdallah FB, et al. Virulence gene expression, proteins secreted and morphological alterations of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in response to long-term starvation in seawater. Afr J Microbiol Res. 2011;5:792-801.

12. Coutard F, et al. Real-time reverse transcription-PCR for transcriptional expression analysis of virulence and housekeeping genes in viable but nonculturable *Vibrio parahaemolyticus* after recovery of culturability. Appl Environ Microbiol. 2007;73:5183-5189.

13. Broberg CA, et al. *Vibrio parahaemolyticus* cell biology and pathogenicity determinants. Microbes Infect. 2011;13:992-1001.

14. Martinez-Urtaza J, et al. Environmental determinants of the occurrence and distribution of *Vibrio parahaemolyticus* in the Rias of Galicia, Spain. Appl Environ Microbiol. 2008;74:265-274.

15. Caburlotto G, et al. Serological and molecular characterization of *Vibrio parahaemolyticus* marine strains carrying pandemic genetic markers. ISME J. 2010;4:1071-1074.

16. Nolan T, et al. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 2006;1:1559-1582.

17. Jin MT, et al. Difference of three standard curves of real-time reverse transcriptase PCR in viable *Vibrio parahaemolyticus* quantification. Wei Sheng Wu Xue Bao, 2014;54:383-390.

18. Luan XY, et al. Comparison of different primers for rapid detection of *Vibrio parahaemolyticus* using the polymerase chain reaction. Lett Appl Microbiol. 2007;44:242-247.

19. Li Q, et al. Selection of reference genes for virulence gene expression in *Vibrio parahaemolyticus*. Wei Sheng Wu Xue Bao. 2013;53:306-312.

20. Iida T, et al. Close proximity of the *tdh*, *trh* and *ure* genes on the chromosome of *Vibrio parahaemolyticus*. Microbiology. 1998;144:2517-2523.

21. Caburlotto G, et al. Presence of T3SS2 and other virulence-related genes in *tdh*-negative *Vibrio parahaemolyticus* environmental strains isolated from marine samples in the area of the Venetian Lagoon, Italy. FEMS Microbiol Ecol. 2009;70:506-514.

22. 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 Methods. 1999;36:215-225.

23. DePaola A, et al. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Appl Environ Microbiol. 2003;69:1521-1526.

24. Cabanillas-Beltrán H, et al. Outbreak of gastroenteritis caused by the pandemic *Vibrio parahaemolyticus* O3:K6 in Mexico. FEMS Microbiol Lett. 2006;265:76-80.

25. Hikima S, et al. Characterization and function of kuruma shrimp lysozyme possessing lytic activity against *Vibrio species*. Gene. 2003;316:187-195.

26. de-la-Re-Vega E, et al. White shrimp (*Litopenaeus Vannamei*) recombinant lysozyme has antibacterial activity against gram negative bacteria: *Vibrio alginolyticus*, *Vibrio parahemolyticus* and *Vibrio cholera.* Fish Shellfish Immunol. 2006;20:405-408.

27. Asakura H, et al. Gene expression profile of *Vibrio cholerae* in the cold stress-induced viable but non-culturable state. Environ Microbiol. 2007;9:869-879.

28. Rao NV, et al. Induction, resuscitation and quantitative real-time polymerase chain reaction analyses of viable but nonculturable *Vibrio vulnificus* in artificial sea water. World J Microbiol Biotechnol. 2014;30:2205-2212.

29. Okuda J, et al. Analysis of the thermostable direct hemolysin (*tdh*) gene and the *tdh*-related hemolysin (*trh*) genes in urease-positive strains of *Vibrio parahaemolyticus* isolated on the West Coast of the United States. J Clin Microbiol. 1997;35:1965-1971.

30. González-Escalona N, et al. Quantitative reverse transcription polymerase chain reaction analysis of *Vibrio cholerae* cells entering the viable but non-culturable state and starvation in response to cold shock. Environ Microbiol. 2006;8:658-666.