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Two Rapid Methods to Identify Three Species of Pathogenic Vibrio in *Penaeus vannamei*

Kai Luo[1], Hanwen Yuan[1], Qiaoqing Xu[1,3*], Wenbing Zhang[2,*]

[1]*School of Animal Science, Yangtze University, Jingzhou 434020, China;*

[2]*The Key Laboratory of Aquaculture Nutrition and Feed (Ministry of Agriculture), The Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao, China;*

[3]*Engineering Research Center of Ecology and Agricultural Use of Wetland, Ministry of Education, Yangtze University, Jingzhou 434024, China.*

Keywords: *Penaeus vannamei*; *Vibrio fluvialis*; *Vibrio anguillarum*; *Vibrio alginolyticus*; multiplex PCR; qPCR

Abstract

Vibrio species frequently infect *Penaeus vannamei* causing great economic losses to the Whiteleg shrimp industry. Rapid detection of pathogenic *Vibrio* infection would improve the fight against these diseases. In this study, single and multiple polymerase chain reaction (PCR) methods were developed to detect three species of pathogenic *Vibrio*: *Vibrio fluvialis, Vibrio anguillarum*, and *Vibrio alginolyticus*. Specific primers were designed for the *toxR* gene of *V. fluvialis*, the *flaA* gene of *V. anguillarum*, and the *pyrH* gene of *V. alginolyticus*. The bacteria were used as templates to establish a 25 μL reaction system for PCR amplification. The results showed that single and specific PCR amplification products of expected sizes were obtained (228bp, 1665bp, and 383bp, respectively). The lowest concentration detected for the three *Vibrio* species were 5.21×10^{2}, 2.70×10^{4}, and 2.48×10^{2} colony forming units (cfu)/mL, respectively. We also developed a multiplex PCR method to identify the three *Vibrio* species accurately, and with improved identification efficiency. In addition, quantitative real-time PCR (qPCR) was used to identify the minimum detectable DNA concentration for the three *Vibrio* species (1.0×10^{-6} nmol/L for *V. fluvialis*, 1.0×10^{-7} nmol/L for *V. anguillarum*, and 1.0×10^{-8} nmol/L for *V. alginolyticus*). Technical requirements for ordinary PCR are low, therefore PCR is a feasible technique to detect and diagnose *Penaeus vannamei* bacterial disease.

* Corresponding author. e-mail: xuqiaoqing@yangtzeu.edu.cn

Introduction

Among the three varieties of shrimp in aquaculture, *Penaeus vannamei* (Whiteleg shrimp) is the most cultivated worldwide. It is native to South America's Pacific coast and is distributed mainly from northern Peru to the gulf coast. The excellent yield of *P. vannamei* varieties has led to its extensive and wide-ranging cultivation in China. *P. vannamei* is tasty, well-adapted, and shows strong disease resistance, fast growth rate, low demands on feed protein, and a high processing dressing percentage. However, expansion and increased intensity of cultivation have caused problems resulting in large losses to the aquaculture industry. Among them bacterial disease is the most important, in particular infections caused by *Vibrio parahaemolyticus, V. anguillarum,* and *V. alginolyticus* (W. Y. Sheng et al., 2004). The *toxR* gene of *V. fluvialis* is the ancestral gene of *Vibrio* (Rupa C *et al*., 2006). The flagellum is the virulence organelle that infects fish and plays a vital role in attacking the host (Ge L, 2007). *V. alginolyticus* is a conditional pathogenic bacterium that is prone to infect its hosts in water of 25-32°C (Q. Cheng *et al*., 2006). The *pyrH* gene of *V. alginolyticus* is also one of the main pathogenic genes.

Currently, bacterial pathogen detection requires biochemical tests (R. X. Zhang *et al*., 2013), automatic microorganism identification instruments (J. T. Jia *et al*., 2012), polymerase chain reaction (PCR) (J. T. Rao, 2007), probe techniques (J. T. Jia *et al*., 2012), immunofluorescence techniques (Banner, C.R *et al*., 1992), and monoclonal antibody techniques (X. L. Li *et al*., 2009). These methods can identify different pathogenic bacteria quickly and accurately. In the present study, we developed PCR-based tests to identify *V. parahaemolyticus, V. anguillarum,* and *V. alginolyticus*. Quantitative real-time PCR (qPCR) was used to authenticate the three bacteria simultaneously. The advantages and disadvantages of the PCR technique were compared. Thus, we established a fast and accurate method to identify three species of *Vibrio*.

Materials and methods

Source of the bacteria. V. parahaemolyticus, V. anguillarum, V. alginolyticus, V. fluvialis were purchased from the national aquatic pathogen library, at Shanghai Ocean University.

Primer design. The target sequences of the *toxR* gene of *V. fluvialis*, the *flaA* gene of *V. anguillarum*, and the *pyrH* gene of *V. alginolyticus* were downloaded from the National Centre for Biotechnology Information (NCBI). Specific primers corresponding to each bacterial gene that were designed for primer premier 5.0. Primers are shown in table 1. The *V. anguillarum* F_1/R_1 primer pair was used for PCR, and the *V. anguillarum* F_2/R_2 primer pair was used for qPCR. The primers were pre-tested to ensure that each primer pair could not amplify the DNA of *P. vannamei* organization.

Table 1 Primers used for PCR amplification

Bacteria	*Virulence gene*	*Primer*	*Primer sequences*	*length(bp)*	*GenBank*
V .fluvialis	*toxR*	*toxR*-F	TGCAAGTAAAGATCCTGATG	228	AF170885
		toxR-R	GTCGTAAACAAAATGACACAA		
V. anguillarum	*flaA*	*flaA*-F_1	TTACGCAGAAGCGGTGAT	1665	L47122
		flaA-R_1	GCTGTTGGATGAAGGGTC		
V. anguillarum	*flaA*	*flaA*-F_2	TAGCGGATTTAGCAAGTTCAC	95	L47122
		flaA-R_2	TGGTCATAGTTTGCTCTCCT		
V. alginolyticus	*pyrH*	*pyrH*-F	AAAGAACTGGTTGAACTGGGTG	383	KC954181
		pyrH-R	CCATCAACTTTCGTCGCTTT		

Genomic DNA extraction. V. anguillarum, V. alginolyticus, and *V. fluvialis* were inoculated into nutrient Luria-Bertani (LB) medium, incubated, and shaken (200rpm) at 37°C for 12 h. The bacteria were harvested by centrifugation at 12000 g for 1 min, and the supernatant was discarded. The bacterial DNA was extracted from the cell pellets of all samples following the instructions in a bacteria genomic DNA extraction kit (TIANGEN, China).

Reaction conditions. The optimized PCR was set up in a 25-μL reaction mixture comprising 2.5 μL of 10× Taq Buffer, 2 μL of $MgCl_2$ (25 mM), 2 μL of dNTPs (2.5 mM), 1 μL (20 Pico moles) each of the forward and reverse primers, 0.25μL of rTaq DNA polymerase (5 U/μL, NovoGene, China), 1 μL of template comprising living bacteria, and nuclease free water to 25 μL. The qPCR reaction was set up in a 20-μL reaction mixture

comprising 0.5 μL (20 Pico moles) each of forward and reverse primers, 10 μL of SYBR® Green Mix, 4 μL of DNA, and nuclease free water to 20 μL.

In the general PCR assay, the cycling conditions comprised an initial denaturation (95°C for 5 min), followed by 35 cycles of denaturation (95°C, 30 s), primer annealing (55°C for 30 s), and extension (72°C, 1 min 30 s). After a final extension (72°C, 10 min), the PCR products were held at 12°C. The reaction products were checked using 2% agarose gel electrophoresis. The qPCR cycling conditions were one cycle at 95°C, 3 min, followed by 40 cycles at 95°C for 3 s, 60°C for 25 s. Dissociation analysis was performed by incubating the reaction at 95°C for 15 s, 60°C for 1 min, and 95°C for 15s.

Specificity analysis. The template used was single, or multiple bacteria, to test their specificity within the same reaction system.

Sensitivity analysis. V. anguillarum, V. alginolyticus, and *V. fluvialis* were inoculated into nutrient Luria-Bertani (LB) medium, and cultivated by shaking overnight (37°C, 200 rpm). The 1.5 mL liquid cultures were moved to an EP tube (1.5 mL) and centrifuged (12000 g, 1 min). The bacteria were diluted with deionized water by 10 X dilution method to 10^{-8}–10^{-9} to test the sensitivity of multiplex PCR detection. Samples at dilutions of 10^{-5}–10^{-7} were plated on LB agar for platecounting.

Results

Single PCR assay. V. anguillarum, V. fluvialis, *V. alginolyticus* were subjected to PCR amplification and gel imaging. Single bright bands of 1665 bp (*V. alginolyticus* F_1/R_1), 228 bp (*V. fluvialis* F/R), and 383bp (*V. alginolyticus* F/R) corresponded to the expected sizes (Fig. 1).

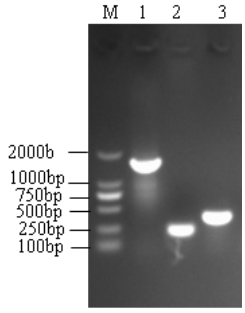

Fig.1 Schematic figure for PCR amplification of three kind of vibrio. Lane M, 2000bp maker molecular maker. Lane 1, *V. alginolyticus* F_1/R_1 (1665bp). Lane 2, *V. fluvialis* (228bp). Lane 3, *V. alginolyticus* (383bp).

Primer specificity. Based on the designed primer sets, the specificities of each set against *V. parahaemolyticus, V. anguillarum, V. alginolyticus, V. fluvialis* were examined. For all primer pairs, PCR amplicons were only produced from the bacterium against which the primers were designed and from no other bacteria (Fig.2)

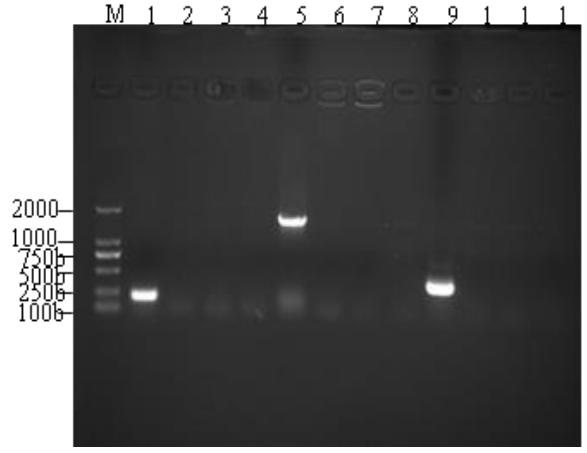

Fig.2 Schematic figure of the primers specificity assay for three kinds of vibrio. Lane M, 2000bp maker molecular maker. Lane 1, *V. fluvialis* (228bp). Lane 2-4, *V. fluvialis* F/R with the bacteria of *V. parahaemolyticus, V. anguillarum, V. alginolyticus.* Lane 5, *V. alginolyticus* F_1/R_1 (1665bp). Lane 6-8, *V. alginolyticus* F_1/R_1 with the bacteria of *V. parahaemolyticus, V.anguillarum, V. fluvialis*. Lane 9, *V. alginolyticus* (383bp). Lane 10-12, *V. alginolyticus* F/R with the bacteria of *V. parahaemolyticus, V. fluvialis*, *V. anguillarum*.

Detection sensitivity. After diluting the bacteria by 10 times, the minimum detectable amounts of bacteria were determined as 5.21×10^2 colony forming units (cfu)/mL for *V. fluvialis* (Fig. 3), 2.70×10^4 cfu/mL for *V. anguillarum* (Fig. 4), and 2.48×10^2 cfu/mL for *V. alginolyticus* (Fig. 5), with high sensitivity.

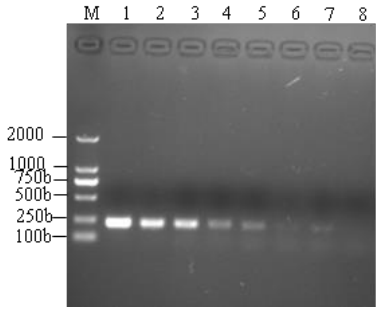

Fig.3 Schematic figure for the *Vibrio fluvialis* sensitivity test. Lane M, 2000bp maker molecular maker. Lane 1, 5.21×10^{6} cfu/mL. Lane 2, 5.21×10^{5} cfu/mL. Lane 3, 5.21×10^{4} cfu/mL. Lane 4, 5.21×10^{3} cfu/mL. Lane 5, 5.21×10^{2} cfu/mL. Lane 6, 5.21×10 cfu/mL. Lane 7, 5.21 cfu/mL. Lane 8, negative control.

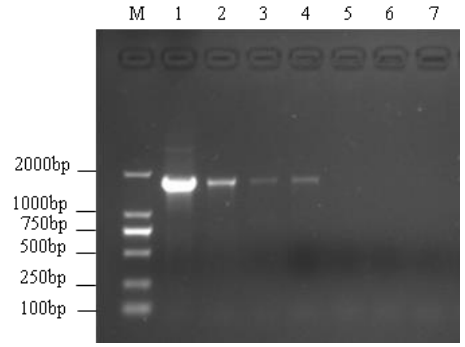

Fig.4 Schematic figure for the *Vibrio anguillarum* sensitivity test. Lane M, 2000bp maker molecular maker. Lane 1, 2.70×10^{7} cfu/mL. Lane 2, 2.70×10^{6} cfu/mL. Lane 3, 2.70×10^{5} cfu/mL. Lane 4, 2.70×10^{4} cfu/mL. Lane 5, 2.70×10^{3} cfu/mL. Lane 6, 2.70×10^{2} cfu/mL. Lane 7, negative control.

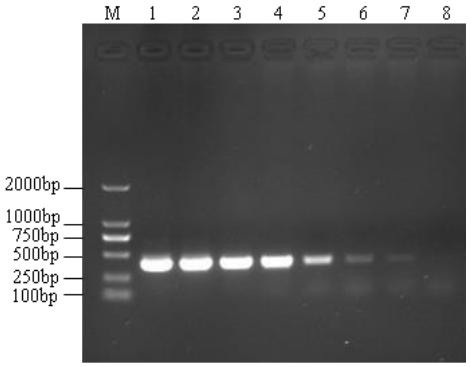

Fig. 5 Schematic figure for the *Vibrio alginolyticus* sensitivity test. Lane M, 2000bp maker molecular maker. Lane 1, 2.48×10^{7} cfu/mL. Lane 2, 2.48×10^{6} cfu/mL. Lane 3, 2.48×10^{5} cfu/mL. Lane 4, 2.48×10^{4} cfu/mL. Lane 5, 2.48×10^{3} cfu/mL. Lane 6, 2.48×10^{2} cfu/mL. Lane 7, 2.48×10 cfu/mL. Lane 8, negative control.

Multiplex PCR assay. The three kinds of bacterial pathogens were mixed to determine whether single or multiple species of pathogen could be amplified effectively. The results demonstrated that all combinations of the pathogenic bacteria could be detected successfully (Fig 6).

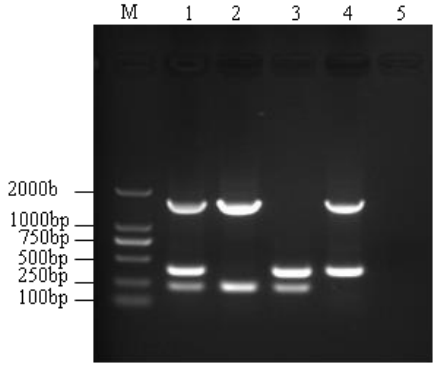

Fig.6 Schematic figure for multiplex PCR amplification of three kinds of vibrio. Lane M, 2000bp maker molecular maker. Lane 1, *V.anguillarum* (1665bp), *V. alginolyticus* (383bp) and *V. fluvialis* (228bp). Lane 2, *V. anguillarum* (1665bp) and *V. fluvialis* (228bp). Lane 3, *V. alginolyticus* (383bp) and *V. fluvialis* (228bp). Lane 4, *V. anguillarum* (1665bp) and *V. alginolyticus* (383bp).

Real-time PCR assay. DNA was extracted from the cell pellets of all samples following the instructions of a bacteria genomic DNA extraction kit. The genomic DNA contents were diluted according to formula of 0.66 × N/C (N, the length of primer; C, the DNA concentration). The minimum detectable DNA contents were determined as 1.0×10^{-6} nmol/mL for *V. fluvialis* (Fig. 7, Table 2, Ref_6: represent the *V. fluvialis* DNA concentration 1.0×10^{-6} nmol/mL), 1.0×10^{-7} nmol/mL for *V. anguillarum* (Fig. 8, Table 3, Ref_7: represent the *V. anguillarum* DNA concentration 1.0×10^{-7} nmol/mL), and 1.0×10^{-8} nmol/mL of *V. alginolyticus* (Fig. 9, Table 4, Ref_8: represent the *V. alginolyticus* DNA concentration 1.0×10^{-8} nmol/mL).

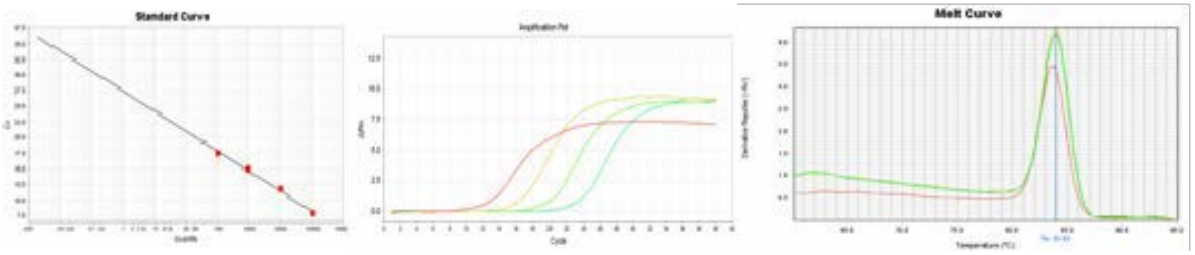

Fig.7 The standard curve, amplification plot and melt curve of *Vibrio fluvialis* quantitative polymerase chain reaction (qPCR). From down to up is Ref_3, Ref_4, Ref_5, Ref_6; R^2=0.992.

Table 2 The qPCR data of Vibrio fluvialis.

DNA concentration (nmol/L)	*Sample*	*CT*	C_{T+1}-C_T
1.0×10^{-3}	Ref_3	8.004	
1.0×10^{-4}	Ref_4	11.804	3.799
1.0×10^{-5}	Ref_5	14.996	3.192
1.0×10^{-6}	Ref_6	17.572	2.575
1.0×10^{-7}	Ref_7	16.981	-0.591

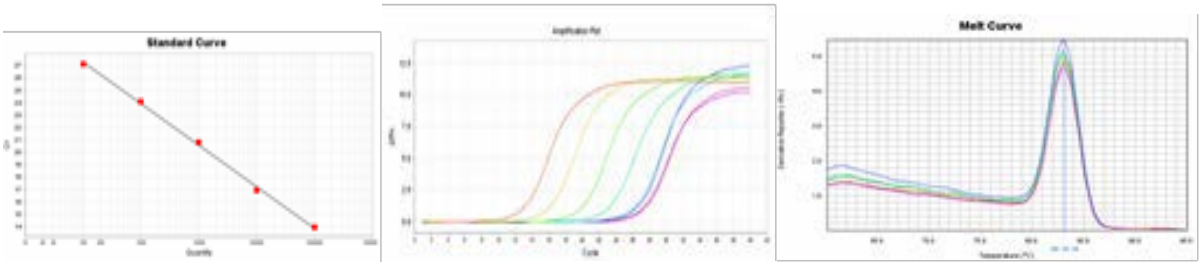

Fig.8 The standard curve, amplification plot and melt curve of *Vibrio anguillarum* quantitative polymerase chain reaction (qPCR). From down to up is Ref_3, Ref_4, Ref_5, Ref_6, Ref_7; R^2=0.998.

Table 3 The qPCR data of Vibrio anguillarum

DNA concentration (nmol/L)	*Sample*	C_T	C_{T+1}-C_T
1.0×10^{-3}	Ref_3	13.941	
1.0×10^{-4}	Ref_4	16.931	2.989
1.0×10^{-5}	Ref_5	20.828	3.897
1.0×10^{-6}	Ref_6	24.110	3.281
1.0×10^{-7}	Ref_7	27.120	3.009
1.0×10^{-8}	Ref_8	27.422	0.302
1.0×10^{-9}	Ref_9	28.163	0.741
1.0×10^{-10}	Ref_{10}	28.409	0.246

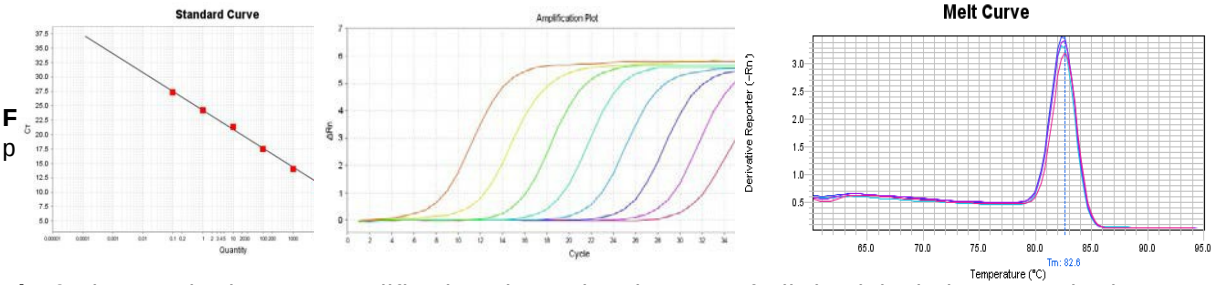

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Fig.9 The standard curve, amplification plot and melt curve of Vibrio alginolyticus quantitative polymerase chain reaction (qPCR). From down to up is Ref3, Ref4, Ref, Ref6, Ref7, Ref8; R2=0.998.

Table 4 The qPCR data of Vibrio alginolyticus

DNA concentration (nmol/L)	*Sample*	*CT*	C_{T+1}-C_T
1.0×10^{-3}	Ref_3	11.239	
1.0×10^{-4}	Ref_4	14.045	2.806
1.0×10^{-5}	Ref_5	17.466	3.421
1.0×10^{-6}	Ref_6	21.355	3.889
1.0×10^{-7}	Ref_7	24.265	2.911
1.0×10^{-8}	Ref_8	27.343	3.078

Discussion

Vibrio spp. are gram-negative bacilli that are distributed widely and abundantly in the sea (Thompson F *et al*., 2004). Currently, more than 10 kinds of pathogenic *Vibrio* species have been detected. These infect *P. vannamei*, and cause great economic losses to the aquaculture industry (H. B. Wu *et al*., 2004; Kushmaro A *et al*., 2001). *V. fluvialis* is distributed globally and has been detected in oyster, clam, conch, shrimp, and fish (X. J. Zhang, 2006). The bacteria can also cause diarrhea in humans (C. H. Lai *et al*., 2006). *V. fluvialis* can survive in different environments and can develop drug resistance to multiple antibiotics. Therefore, it is necessary to establish a rapid and effective method to detect the pathogen rapidly and treat using drugs in the early stage of the disease.

In the present study, two methods were established to identify three kinds of pathogenic vibrio in *P. vannamei*. The results showed that single and specific PCR amplification products of expected sizes were obtained (228bp, 1665bp, and 383bp, respectively). The lowest concentration that could be detected for the three *Vibrio* species were 5.21×10^{2}, 2.70×10^{4}, and 2.48×10^{2} cfu/mL, respectively. A multiplex PCR method that could accurately identify the three *Vibrio* species, and improve identification efficiency, was also developed. The other method is qPCR to identify the minimum detectable DNA concentration for the three *Vibrio* species (1.0×10^{-6} nmol/L for *V. fluvialis*, 1.0×10^{-7} nmol/L for *V. anguillarum*, and 1.0×10^{-8} nmol/L for *V. alginolyticus*). Traditional methods of diagnosis are carried out by culturing bacteria on agar plates, followed by biochemical characterization (Q. Zhang *et al*., 2010). These techniques have some disadvantages, particularly because of their time-consuming nature and low accuracy, thus delaying the effective treatment of fish diseases. *V. alginolyticus* and *V. fluvialis* had been identified by sequencing 16S rRNA and *hsp60* (P. P. He *et al*., 2013), *Vibrio rotiferianus* h by observing the morphology of the pathogen, biochemical characterization, sequencing of the 16S rRNA, and drug sensibility tests (C. Y. Jin, 2013). PCR is applied widely to detect various bacteria and viruses (P. P. He *et al*., 2013; S. S. He, 2012). *V. fluvialis* can be identified by single PCR of the *toxR* gene (W. Y. Wen *et al*., 2009) and *V. anguillarum* by single PCR for a metalloprotease gene (J. H. Yu *et al*., 2002). The *toxR* gene (161bp) of *V. alginolyticus* was also detected successfully using PCR (Y. F. Han *et al*., 2009). It is difficult to identify *Vibrio* species using the 16S rRNA because the homology of the 16S rRNA is high in Vibrios. For instance, the homology of the 16S rRNA is 99.6%, as high as *V. choerae* and *V. mimicus*. Compared with the above methods, the present study designed specific primers (for the *toxR* gene of *V. fluvialis*, the *flaA* gene of *V. anguillarum*, and the *pyrH* gene of *V. alginolyticus*), which are more rapid, efficient, sensitive, and cheaper than previous methods. We also developed a multiplex PCR method to identify the three kinds of Vibrios and calculated the minimum detectable concentration of bacteria. At the same time, we used qPCR to authenticate the sensitivity of the primers for these bacteria. qPCR is a rapid and sensitive diagnostic tool to detect pathogens (Bilodeau A. L. *et al*., 2003; Griffin M. J. *et al*., 2013). However, it requires high quality equipment, and is expensive and complex to operate; this is less optimal for the detection of bacterial pathogens. Therefore, we believe that the general PCR method is useful to authenticate vibriosis in *P. vannamei,* allowing the aquaculture industry to use appropriate coping and control measures, such as detecting bacterial concentration in the water and disinfecting the water environment to reduce incidence of disease.

Acknowledgment

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