P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : *https://tci-thaijo.org/index.php/SciTechAsia* **Science & Technology Asia**
Vol.22 No.4 October - December 2017 Page: [11 – 18]

Original research article

Detection of Three Pathogenic *Vibrio* Species Using Multiplex Polymerase Chain Reaction (Multiplex-PCR)

Umaporn Khimmakthong[1,*] and Supaporn Noochu[1]

[1]*Faculty of Veterinary Science, Rajamangala University of Technology Srivijaya, Thungyai, Nakhon Si Thammarat 80240, Thailand*

Received 17 July 2017; Received in revised form 13 September 2017
Accepted 16 November 2017; Available online 1 December 2017

ABSTRACT

Vibrio cholera, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are opportunistic pathogens causing disease in weak shrimp and possible food poisoning in shrimp consumers. In this study, three pairs of primers were designed to amplify the target DNA fragments of three *Vibrio* spp. and together with one pair for internal amplification control. The PCR condition was optimized to detect three *Vibrio* spp. in one reaction tube. The specificity and sensitivity of the reaction were evaluated. The results showed that this technique can detect *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* in the same reaction tube with high specificity. Sensitivity is moderate, 0.5 ng-10 pg. In the future, this technique can be used to detect these bacteria in shrimp. It is potentially useful for shrimp farmers and shrimp consumers.

Keywords: Bacteria; Multiplex; PCR; *Vibrio*

1. Introduction

Thailand is one of the main producers of shrimp and is the largest exporter of shrimp in the world [1]. However, shrimp farmers have also faced with the spread of the disease. It was found that the disease is caused by viruses and bacteria [2]. Bacteria genus *Vibrio*, gram-negative bacteria predominantly found in seawater, some can cause disease in marine animals such as shrimp, shellfish and some fish species [3, 4]. *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* are opportunistic pathogens. They do not cause disease in healthy shrimp but cause the disease when the immune system of shrimp is disturbed until a level lower than normal. Moreover, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* have been recognized as important food-borne pathogens, which can cause human diseases [5,6] and are responsible for most cases of seafood-related human illness caused by *Vibrio* species [7].

A traditional method used to detect bacteria of

***Corresponding author:** tal_biot@hotmail.com **doi:** 10.14456/scitechasia.2017.14

the genus *Vibrio* is a biochemical method. This method is cumbersome and time-consuming, it takes about three days. Later, the application of molecular techniques is used such as polymerase chain reaction (PCR), which is a simple, cheap and fast [8]. Multiplex PCR refers to the use of more than one pair of primer to amplify several different DNA sequences simultaneously in one reaction (one tube). This technique has been used for the detection of bacteria in seafood, especially for detection of *Vibrio* spp. [9-14]. There are many research reports on the use of multiplex PCR for detection of *Vibrio* spp. [15-17]. Multiplex PCR has the potential to save time compared to traditional PCR [18]. However, there were some studies that reported on the shortcomings of this technique. Some research suggested that multiplex PCR could not differentiate the similar bacteria such as between *V. parahaemolyticus* and *V. alginolyticus*. Sometimes the results of the experiment may be false-negative because of the PCR inhibitors contained in the sample [19]. The method of preventing false-negative results is achieved by using an internal amplification control (IAC) in every reaction tube [20].

The purpose of this study is to develop multiplex PCR techniques that can detect three *Vibrio* spp. simultaneously in a single reaction tube with good specificity and high sensitivity. In this study, three pairs of primers were designed to detect three *Vibrio* spp. including *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, and together with one pair for internal amplification control. The PCR condition was optimized to detect three *Vibrio* spp. in one reaction tube. The specificity and sensitivity of the reaction were evaluated. This technique will help save money and will be useful both for shrimp farmers and shrimp consumers.

2. Materials and Methods

2.1 Materials

All *Vibrio* bacteria were cultured in Tryptic Soy Broth (TSB) purchased from HiMedia (India) contained 1.5% NaCl other bacteria were cultured in Nutrient Broth (NB) purchased from BD Difco™ (USA). Primers used in this study were purchased from Eurofins Genomics (Germany). MaestroGen Nano-Drop spectrophotometer (Taiwan) was used to measure the concentration and purity of extracted DNA. PCR was run in FlexCycler^2, Biometra GmbH (Germany). Electrophoresis was run in Wide Mini-Sub Cell GT, Bio-Rad (USA) and used 100bp marker purchased from GeneDireX (China).

2.2 Bacteria

Most of the bacteria used in this study including *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Vibrio alginolyticus*, *Vibrio fluvialis*, *Vibrio mimicus*, *Aeromonas hydrophila*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* were purchased from the Department of Medical Sciences, Thailand. *Vibrio harveyi* was donated from Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, Prince of Songkla University. *Klebsiella* spp., *Bacillus subtilis* and *Escherichia coli* were isolated in the laboratory of microbiology, Faculty of Veterinary Science, Rajamangala University of Technology Srivijaya, Thailand.

2.3 DNA template preparation

All *Vibrio* bacteria were cultured in 10 ml of TSB contained 1.5% NaCl, incubated at 28°C in a shaking incubating machine at the speed of 180 rpm for 16 h. Other bacteria (*A. hydrophila*, *S. typhimurium*, *P. aeruginosa*, *Klebsiella* spp., *B. subtilis* and *E. coli*) were cultured in 10 ml of NB, incubated at 37°C in a shaking incubating machine at the speed of 180 rpm for 16 h. Then, 1 ml of cell suspensions were centrifuged at a speed of 10,000 rpm for 1 min in order to separate the cell pellets. The bacterial DNA was extracted from the cell pellets by boiling method, modified from [21]. Briefly, the cell pellets were suspended in 200 µL of distilled water. The cell suspensions were boiled for 10 min and immediately cooled on ice for 5 min. After centrifuged at a speed of 10,000 rpm for 1 min, the supernatant was used as DNA template. For sensitivity experiment, the concentration and purity of extracted DNA were quantified using

a Nano-Drop spectrophotometer.

2.4 Design and synthesis of primers

The sequence of bacteria genes was brought from the GenBank database. The GenBank accession numbers of *collagenase* gene of *V. parahaemolyticus* and *vvhA* of *V. vulnificus* are AF326572.1 and KC821520.1, respectively. The multiple alignments were done to find a specific region, which found in any individual bacteria only, not found in other bacteria. Then, that DNA region was applied to design primers by using Primer3 Input (version 0.4.0) (Source: http://bioinfo.ut.ee/ primer3-0.4.0/). The received primers must have similar the melting temperature (Tm) values in the range of 55-65 degrees Celsius. In addition, the size of PCR product received from each pair of primers must be different at least 50 bp.

2.5 Optimal condition for multiplex PCR

To test the accuracy of the primer pairs, single PCR was done. PCR reaction (25 µL) including 1 µL of DNA template, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer and 5 µL of PCR Master Mix II (5X) (GeneMark, Taiwan). The single PCR was run at different annealing temperature including 62 or 63 ºC for 30 s, and 72 ºC for 45 s. The cycling was initiated at 94 ºC for 5 min and final extension was done at 72 ºC for 10 min. Then, four primer pairs were put together in a single tube to make multiplex PCR. Multiplex PCR reaction (25 µL) including 3 µL of DNA template (including 1 µL of *V. cholerae*, 1 µL of *V. parahaemolyticus*, and 1 µL of *V. vulnificus*), 4 µL of 10 µM forward primer (including 1 µL of VC-F, 1 µL of VP-F, 1 µL of VV-F and 1 µL of 16S-F), 4 µL of 10 µM reverse primer (including 1 µL of VC-R, 1 µL of VP-R, 1 µL of VV-R and 1 µL of 16S-R) and 5 µL of PCR Master Mix II (5X) (GeneMark, Taiwan). The reaction was amplified for 30 cycles at 94 ºC for 30 s, 62 ºC for 30 s, and 72 ºC for 45 s. The cycling was initiated at 94 ºC for 5 min and final extension was done at 72 ºC for 10 min. PCR product was checked by agarose gel electrophoresis.

2.6 The specificity of multiplex PCR

The specificity of multiplex PCR was determined by applying multiplex PCR with all bacteria in this study, including *V. chole-rae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. mimicus*, *V. harveyi*, *A. hydrophila*, *S. typhimurium*, *P. aeruginosa*, *Klebsiella* spp., *B. subtilis* and *E. coli***.** DNA of bacteria was extracted by a

Table 1. Target gene of bacteria species and primer sequence.

Target genes and species	Primer sequence (5′-3′)	Tm (ºC)	PCR products size (bp)	Reference
ompW	VC-F : CACCAAGAAGGTGACTTTATTGTG	58.2	427	[22]
V. cholerae	VC-R : CGTTAGCAGCAAGTCCCCAT	60.4		
Collagenase	VP-F : GCAAATATGCGGGGCCAATCTT	62.2	281	[23]
V. parahaemolyticus	VP-R : CGCCGCTTGATTGTCTTTTTCG	61.5		
vvhA	VV-F : TCCATCGATGTTCGCGTCAA	60.1	213	This study
V. vulnificus	VV-R : ACTGCTGGCGAATGGACCAA	62.4		
16S rRNA	16S-F : CCTGGTAGTCCACGCCGTAA	61.6	168	[8]
	16S-R : CGAATTAAACCACATGCTCCA	56.9		

Fig. 1. The sequence regions of *vvhA* used for primer design.

boiling method and subjected as DNA template. PCR reaction (25 µL) including 1 µL of DNA template, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer and 5 µL of PCR Master Mix II (5X) (GeneMark, Taiwan). Each reaction was amplified for 30 cycles at 94 ºC for 30 s, 62 ºC for 30 s, and 72 ºC for 45 s. The cycling was initiated at 94 ºC for 5 min and final extension was done at 72 ºC for 10 min. PCR product was checked by agarose gel electrophoresis.

2.7 The sensitivity of multiplex PCR

V. cholerae, *V. parahaemolyticus* and *V. vulnificus* were cultured and adjusted to concentrations of 500 ng/µL, 300 ng/µL, 150 ng/µL, 75 ng/µL, 50 ng/µL, 25 ng/µL, 12.5 ng/µL, 5 ng/µL, 2.5 ng/µL, 1 ng/µL, 0.5 ng/µL, 0.1 ng/µL, 50 pg/µL, 25 pg/µL, 10 pg/µL, 1 pg/µL and 0.1 pg/µL, respectively. The sensitivity was tested by added 1 µL of each DNA at a various concentration to the reaction total volume of 25 µL. Then, run multiplex PCR to determine the lowest bacteria concentration that it could be detected.

2.8 Data Analysis

The results of single PCR and multiplex PCR, as well as sensitivity test and specificity test, were showed in a form of the PCR product compared with 100 bp markers.

3. Results and Discussion

3.1 Design and synthesis of primers

All pairs of primers used in this study were shown in **Table 1**. The PCR primers for detection of *16s rRNA*, *V. cholerae* and *V. parahaemolyticus* were synthesized according to the trial of Wei et al. (2014), Nandi et al. (2000) and Khimmakthong and Sukkarun (2017), respectively [8,22-23]. The PCR primers for detection of *V. vulnificus* were designed in this study. The PCR primers for detection of *V. parahaemolyticus* were designed from the sequence of *collagenase* gene. However, this gene can found in all *Vibrio* spp., it has the high diversity between *Vibrio* spp. [24], so this gene is considered to be a good choice. The *vvhA* gene is responsible for haemolytic and cytolytic activity of *V. vulnificus* [8]. The average T_m of all the primers used in this study was 60.41 ± 1.97 ºC. The sequence regions of *collagenase* and *vvhA* used for primer design were shown in Fig. 1.

3.2 Optimal condition for multiplexPCR

Single PCR result showed the amplified fragments of 427 bp for *ompW* of *V. cholerae*, 2 8 1 bp for *collagenase* of *V. parahaemolyticus*, 2 1 3 bp for *vvhA* of *V. vulnificus* and 1 6 8 bp for *1 6 S rRNA*. At various annealing temperature, 62 ºC showed the sharp single band as showed in Fig. 2 while 60 ºC and 63 ºC showed non-

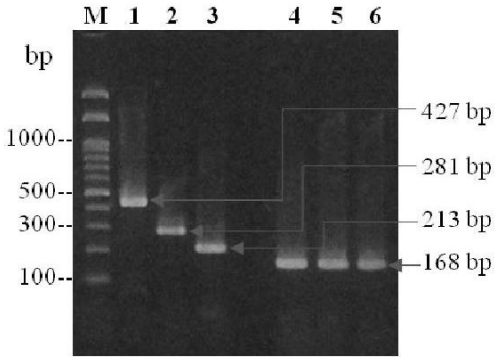

Fig. 2. PCR product of single PCR at annealing temperature 62 ºC. Lane M: 100 bp markers, Lane 1: 427 bp for *ompW* of *V. cholera*, Lane 2: 281 bp for *collagenase* of *V. parahaemolyticus*, Lane 3: 213 bp for *vvhA* of *V. vulnificus* and Lane 4, 5 and 6: 168 bp for *16S rRNA* of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, respectively.

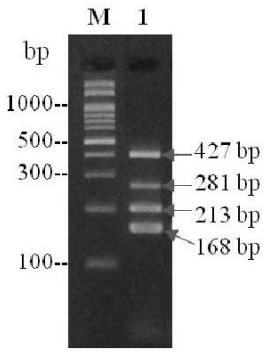

Fig. 3. PCR product of multiplex PCR. Lane M: 100 bp markers, Lane 1: 427 bp, 281 bp, 213 bp and 168 bp of *V. cholera*e, *V. parahaemolyticus*, *V. vulnificus* and *16S rRNA*, respectively.

specific band and cloudy band for some gene, respectively (data not shown). So, the optimal annealing temperature for single PCR is 62 ºC and this temperature was used for multiplex PCR.

When run multiplex PCR by add four pairs of primer in a single reaction tube, the result showed four bands of four genes in that single tube. Each band is clear and not overlapped as showed in Fig. 3.

3.3 The specificity of multiplex PCR

After applied multiplex PCR with other bacteria that not are *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* including *V. alginolyticus*, *V. fluvialis*, *V. mimicus*, *V. harveyi*, *A. hydrophila*, *S. typhimurium*, *P. aeruginosa*, *Klebsiella* spp., *B. subtilis* and *E. coli*, the result showed that no band of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* but have only the band of *16S rRNA* that appears as showed in Fig. 4. This indicates that the techniques used are very specific.

3.4 The sensitivity of multiplex PCR

The sensitivity of the multiplex PCR was evaluated using DNA template concentrations from 500 ng-0.1 pg. The result showed that minimum DNA concentrations of *V. cholera* and *V. parahaemolyticus* that target gene can be detected was 0.5 ng/µL while a minimum concentration of *V. vulnificus* that target gene can be detected was 10 pg/µL. At the same time, *16S rRNA* was detected at all concentration used this study and is still likely to be detected at concentrations below 0.1 pg/µL as showed in Fig. 5.

Mata et al. (2004) detected of 4 pathogens in fish including *Streptococcus iniae*, *Streptococcus difficilis*, *Streptococcus parauberis* and *Lactococcus garvieae* using multiplex PCR. The results showed that the sensitivity can detect the amount of 25 pg for *S. iniae*, 12.5 pg for *S. difficilis*, 30 pg for *S. parauberis*, and 50 pg for *L. garvieae* [25]. Cho et al. (2016) used multiplex PCR for detection of 4 fungal pathogens including *Fusarium oxysporum*, *Bipolaris cactivora*, *Phytophthora* spp. and *Collectotrichum* spp. The results showed that *B. cactivora* and *F. oxysporum* were detected in the at a sensitivity level of only 0.01 ng. *P. nicotianae* sensitivity was 0.1 ng and 1 ng of DNA for *P. cactorum* [26]. In this study, the sensitivity of multiplex PCR technique is moderate that was 10 pg for *V. vulnificus*, 0.5 ng for *V. cholera* and *V. parahaemolyticus*. This may be due to

several reasons, including the extraction yield of target DNA [27], the physicochemical conditions of the reaction, the nature of the (microorganism) DNA target, and the selected PCR primers and probes [28]. In this experiment, DNA was extracted by a boiling method not by using the extraction kit, the DNA content obtained maybe less but this method is simple and takes less time. In addition, PCR reagents used in this study was master mix reagent that easy to use and cheap, but performance also will decrease.

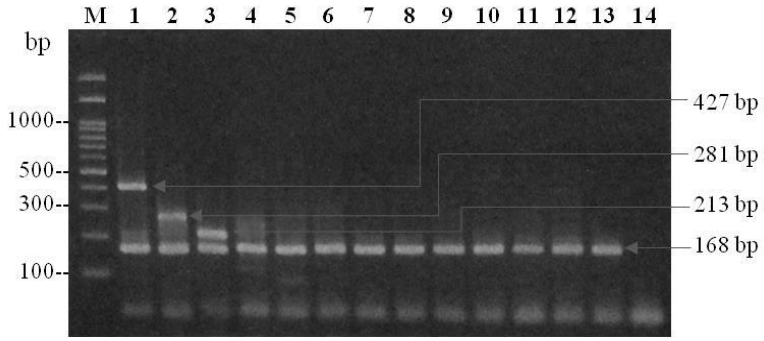

Fig. 4. PCR product of multiplex PCR that used DNA template from various bacteria. Lane M: 100 bp markers, Lane 1: 427 bp and 168 bp for *ompW* and *16s rRNA* of *V. cholera*, Lane 2: 281 bp and 168 bp for *callagenase* and *16s rRNA* of *V. parahaemolyticus*, Lane 3: 213 bp and 168 bp for *vvhA* and *16s rRNA* of *V. vulnificus*, Lane 4-13: 168 bp for *16S rRNA* of *V. alginolyticus*, *V. fluvialis*, *V. mimicus*, *V. harveyi*, *A. hydrophila*, *S. typhimurium*, *P. aeruginosa*, *Klebsiella* spp., *B. subtilis* and *E. coli*, respectively, Lane 14: negative control.

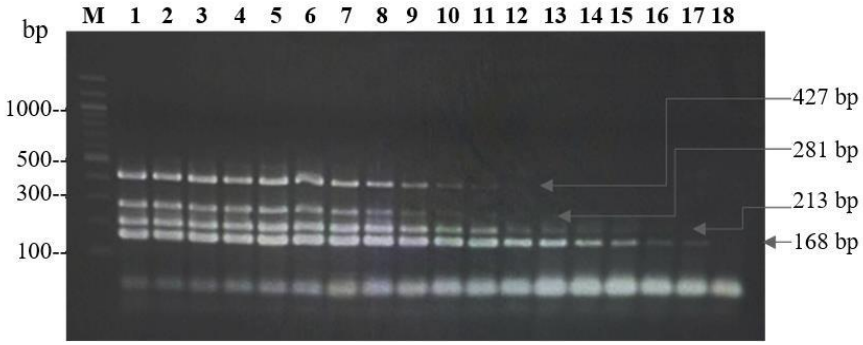

Fig. 5. PCR product of multiplex PCR that used DNA template at various concentrations. Lane M: 100 bp markers, Lane 1-18: PCR product of multiplex PCR that used DNA template extracted from *V. cholera*, *V. parahaemolyticus*, and *V. vulnificus* at the amount of 500 ng, 300 ng, 150 ng, 75 ng, 50 ng, 25 ng, 12.5 ng, 5 ng, 2.5 ng, 1 ng, 0.5 ng, 0.1 ng, 50 pg, 25 pg, 10 pg, 1 pg, 0.1 pg and no DNA template, respectively.

4. Conclusion

Multiplex PCR technique in this study was able to detect of *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* in the same reaction tube with high specificity and sensitivity at a moderate level. It can be used to detect bacteria at a dangerous level. Moreover, Compared to ordinary PCR, it saves time and cost. In the future, if this technique is used to detect these bacteria in

shrimp. It is very useful for shrimp farmers because it led to the monitoring outbreak and management planning of pond and water to make the shrimp is always healthy and for shrimp consumers to avoid eating shrimp contaminated with these bacteria.

Acknowledgements

The authors need to thanks, Rajamangala University of Technology Srivijaya for research funding. Thank you, Faculty of Veterinary Science, for a place to do research. And finally, thank you Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, Prince of Songkla University for *V. harveyi.*

References

[1] Tanticharoen M, Flegel TW, Meerod W, Grudloyma U, Pisamai N. Aquacultural biotechnology in Thailand: the case of the shrimp industry. Int J Biotechnol 2008;10: 588-603.

[2] Flegel TW, Lightner DV, Lo CF, Owens L. Shrimp disease control: past, present and future. Dis Asian Aquac 2008;6:355-78.

[3] Baffone W, Citterio B, Vittoria E, Casaroli A, Pianetti A, Campana R. Determination of several potential virulence factors in *Vibrio* spp. isolated from sea water. Food Microbiol 2001;18(5):479-88.

[4] Toranzo AE, Magarino B, Romalde JL. A review of the main bacterial fish diseases in mariculture systems. Aquaculture 2005;246(1-4):37-61.

[5] Zhang XH, Austin B. Haemolysins in *Vibrio* species. J Appl Microbiol 2005; 98(5):1011-19.

[6] Su YC, Liu CC. *Vibrio parahaemo-lyticus*: a concern of seafood safety. Food Microbiol 2007; 24(6):549-58.

[7] Yano Y, Hamano K, Satomi M, Tsutsui I, Ban M, Aueumneoy D. Prevalence and antimicrobial susceptibility of *Vibrio* species related to food safety isolated from shrimp cultured at inland ponds in Thailand. Food Control 2014; 38:30-36.

[8] Wei S, Zhao H, Xian Y, Hussain AM, Wu X. Multiplex PCR assays for the detection of *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae* with an internal amplification control. Diagn Microbiol Infect Dis 2014;79:115-18.

[9] Espiñeira M, Atanassova M, Vieites JM, Santaclara FJ. Validation of a method for the detection of five species, serogroups, biotypes and virulence factors of *Vibrio* by multiplex PCR in fish and seafood. Food Microbiol 2010;27(1):122-31.

[10] Izumiya H, Matsumoto K, Yahiro S, Lee J, Morita M, Yamamoto S, Arakawa E, Ohnishi M. Multiplex PCR assay for identification of three major pathogenic *Vibrio* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus.* Mol Cell Probes 2011;25(4):174-76.

[11] Hossain MT, Kim YO, Kong IS. Multiplex PCR for the detection and differentiation of *Vibrio parahaemo-lyticus* strains using the *groEL*, *tdh* and *trh* genes. Mol Cell Probes 2013;27(5-6):171-75.

[12] Yáñez R, Bastías R, Higuera G, Salgado O, Katharios P, Romero J, Espejo R, Garcia K. Amplification of *tlh* gene in other Vibrionaceae specie by specie-specific multiplex PCR of *Vibrio parahaemolyticus*. Electron J Biotechnol 2015;18(6):459-63.

[13] Cano-Gomez A, Høj L, Owens L, Baillie BK, Andreakis N. A multiplex PCR-based protocol for identification and quantification of *Vibrio harveyi*-related species. Aquaculture 2015;437:195-200.

[14] Malcolm TTH, Cheah YK, Radzi CWJWM, Kasim FA, Kantilal HK, John TYH, Martinez-Urtaza J, Nakaguchi Y, Nishibuchi M, Son R. Detection and quantification of pathogenic *Vibrio parahaemolyticus* in shellfish by using multiplex PCR and loop-mediated isothermal amplification assay. Food Control 2015;47:664-71.

[15] Nhung PH, Ohkusu K, Miyasaka J, Sun XS, Ezaki T. Rapid and specific identification of 5 human pathogenic Vibrio species by multiplex polymerase chain reaction targeted to dnaJ gene. Diagn Microbiol Infect Dis;59(3):271-75.

[16] Tarr CL, Patel JS, Puhr ND, Sowers EG, Bopp CA, Strockbine NA. Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. J Clin Microbiol 2006;45(1):134-40.

[17] Teh CSJ, Chua KH, Thong KL. Simultaneous differential detection of human pathogenic and nonpathogenic *Vibrio* species using a multiplex PCR based on *gyrB* and *pntA* genes. J Appl Microbiol 2010;108(6):1940-45.

[18] Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE. Multiplex PCR: optimization and application in diagnostic virology. Clin Microbiol Rev 2000;13(4):559-70.

[19] Al-Soud WA, Rådström P. Purification and characterization of PCR-inhibitory components in blood cells. J Clin Microbiol 2001;39(2):485-93.

[20] Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J Clin Microbiol 2004;42(5):1863-68.

[21] Pathmanathan SG, Cardona-Castro N, Sanchez-Jimenez MM, Correa-Ochoa MM, Puthucheary SD, Thong KL. Simple and rapid detection of *Salmonella* strains by direct PCR amplification of the *hilA* gene. J Med Microbiol 2003;52:773-76.

[22] Nandi B, Nandy RK, Mukhopadhyay S, Nair GB, Shimada T, Ghose AC. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein ompW. J Clin Microbiol 2000;38(11):4145-51.

[23] Khimmakthong U, Sukkarun P, The spread of *Vibrio parahaemolyticus* in tissues of the Pacific white shrimp *Litopenaeus vannamei* analyzed by PCR and histopathology. Microb Pathog 2017;113:107-12.

[24] Di Pinto A, Ciccarese G, Tantillo G, Catalano D, Forte VT. A collagenase-targeted multiplex PCR assay for identification of *Vibrio alginolyticus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus*. J Food Prot 2005;68(1):150-53.

[25] Mata AI, Gibello A, Casamayor A, Blanco MM, Domínguez L, Fernández - Garayzábal J F. Multiplex PCR assay for detection of bacterial pathogens associated with warm-water Streptococcosis in fish. Appl Environ Microbiol 2004;70(5):3183-87.

[26] Cho H, Hong SW, Kim H, Kwak YS. Development of a multiplex PCR method to detect fungal pathogens for quarantine on exported Cacti. Plant Pathol J 2016;32(1):53-57.

[27] Yamamoto Y. PCR in diagnosis of infection: detection of bacteria in cerebrospinal fluids. Clin Diagn Lab Immunol 2001;9(3):508-14.

[28] Bastien P, Procop GW, Reischl U. Quantitative real-time PCR is not more sensitive than "conventional" PCR. J Clin Microbiol 2008;46(6):1897-900.