Genes & Genomics (2019) 41:35–42
https://doi.org/10.1007/s13258-018-0736-7

Online ISSN 2092-9293
Print ISSN 1976-9571

RESEARCH ARTICLE

Multiplex PCR using *YeaD* and 16S rRNA gene to identify major pathogens in vibriosis of *Litopenaeus vannamei*

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Received: 16 July 2018 / Accepted: 30 August 2018 / Published online: 4 September 2018

Abstract

The *Vibrio* species causing major diseases in *Litopenaeus vannamei* are *Vibrio harveyi, Vibrio alginolyticus*, and *Vibrio parahaemolyticus*. For multiplex PCR primers, *YeaD* was used to detect the three *Vibrio* species. Bioinformatic analysis such as MultiPLX and primer-BLAST was used to design stable and species-specific multiplex PCR primers. Multiplex PCR results showed clear band patterns with bands at 185 bp for *V. alginolyticus*, 396 bp for *V. harveyi*, 805 bp for *V. arahaemolyticus*, and 596 bp for common *Vibrio* species. The minimum concentration of DNA was measured by PCR; the value for *V. alginolyticus* was 0.1 ng, that of *V. harveyi* was 0.03 ng, and that of *V. parahaemolyticus* was 0.003 ng. Taken together, *YeaD* showed stability and specificity in identifying *Vibrio* species. Our multiplex PCR amplification method is an effective and inexpensive tool for identifying V*ibrio* species.

Keywords *Litopenaeus vannamei* · Multiplex polymerase chain reaction · *Vibrio harveyi* · *Vibrio alginolyticus* · *Vibrio parahaemolyticus*

Introduction

Shrimp accounts for 15.3% of internationally traded seafood, comprising the largest share of trades after tuna. There are various species of shrimp including Black Tiger Shrimp (*Penaeus monodon*), Pacific white shrimp (*Litopenaeus vannamei*), mantis shrimp (*Oratosquilla oratoria*), *Alvinocaridid shrimps* (Zhao et al. 2018; Xue et al. 2018; Wang et al. 2017a; Zhang et al. 2018). Although shrimp are extensively marketed worldwide, they have serious disease problem because antibiotics are not available for all crustaceans that don't have specific immune system (Lu et al. 2017; Pourmozaffar et al. 2017). For example, Thailand was the world's largest shrimp producer in 1992, but China, Norway, and Vietnam currently dominate the market because of continuous disease outbreaks since 1997 (FAO 2016). One of the most important diseases caused by gram-negative bacteria is vibriosis, a septicaemic disease transmitted by *Vibrio* species (Kinne 1993; Gomez-Gil et al. 1998; Soto-Rodriguez et al. 2010). *Vibrio* species have been studied, for example Pinctada fucata, before and after infection with *Vibrio alginolyticus* (Wang et al. 2017b). In Korea, *Vibrio* species were detected in 30% of sick shrimp *Litopenaeus vannamei* in 2008 (Jung

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13258-018-0736-7) contains supplementary material, which is available to authorized users.

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et al. 2012). The *Vibrio* species causing major vibriosis in *L. vannamei* are *Vibrio harveyi, Vibrio alginolyticus*, and *Vibrio parahaemolyticus* (Vandenberghe et al. 1999; Shanmugasundaram et al. 2015; Min et al. 2015; Heenatigala and Fernando 2016).

These three species cause symptoms such as loose shell syndrome and red disease (Jayasree et al. 2006; Aftabuddin et al. 2017; Biju and Gunalan 2016). However, predominant symptoms caused by the three species differ. *Vibrio harveyi* predominantly causes loose shell syndrome and white gut syndrome, while *V. alginolyticus* causes shell disease with black spots. *V. parahaemolyticus* is responsible for red disease as well as tail necrosis (Jayasree et al. 2006). *Vibrio harveyi* typically infects adult and post-larval stage shrimp, while *V. alginolyticus* infects hatchery and larval stage shrimp. *V. parahaemolyticus* infects juvenile and adult stage shrimp (Vandenberghe et al. 1999). All three *Vibrio* species fall into the Harveyi clade, whose members are phylogenetically similar, but genetically little different (Sawabe et al. 2013; Sun et al. 2009). Different species can be identified by real-time PCR, microarray, and multiplex PCR methods (Lee et al. 2016; Lan et al. 2016). Multiplex PCR has advantages because it can distinguish species easily and quickly; limitations of the other methods include their costs and technical difficulties (Hossain et al. 2012).

An important aspect of multiplex PCR is selecting a target gene, which should be species-specific, widely distributed, and stable in the genome (Halder et al. 2009). The 16S ribosomal RNA (16S rRNA) gene is commonly used as a target gene for identification. However, sequencing of the 16S rRNA gene cannot distinguish between closely related *Vibrio* species (Gomez-Gil et al. 2004; Haldar et al. 2007). Therefore, another suitable gene in *Vibrio* species must be identified for multiplex PCR (Castroverde et al. 2006; Goarant et al. 2007; Kim et al. 2015; Pinto et al. 2005). Among *Vibrio* species chromosomes, putative D-hexose-6-phosphate mutarotase (*YeaD*) gene is not registered in the nucleotide database and is only registered in the protein database of NCBI.

YeaD encodes the important enzyme D-hexose-6-phosphate epimerase-like protein, which is involved in galactose metabolism (You et al. 2010). This enzyme converts α-galactose to β-galactose. The *YeaD* protein shows some differences in the substrate binding pocket, which may be important for enzyme specificity, but the catalytic residues and a few substrate-binding residues in the protein are conserved (Chittori et al. 2007; You et al. 2010).

In this study, *YeaD* was analysed by bioinformatics methods to evaluate both species-specificity and stability in *Vibrio* species. Next, *YeaD* was selected to design multiplex PCR primers for distinguishing *V. harveyi, V. alginolyticus*, and *V. parahaemolyticus*, while 16S rRNA gene-based primers were used to detect various bacteria including all *Vibrio* species. 16S rRNA-based primers reacted to various bacteria, including non-*Vibrio* species.

Materials and methods

Bacterial strains

Vibrio harveyi (PK-PVH), *V. alginolyticus* (PK-PVAL), and *V. parahaemolyticus* (PK-PVP) strains were used in this study. *Vibrio vulnificus* (PK-PW) and *V. mimicus* (PK-PVM) were used as controls. DNA of the strains were sourced and extracted from the Department of Fisheries Science, Pukyong National University. DNA were classified by direct sequencing for a specific region; cholera toxin transcriptional activator (*toxR*) gene for *V. harveyi*, thermolabile hemolysin (*tlh*) gene for *V. alginolyticus* and *V. parahaemolyticus*, cytolysin (*vvh*) gene for *V. vulnificus. Vibrio parahaemolyticus, V. vulnificus*, and *V. mimicus* were classified again using an API 20E kit (BioMérieux, Marcy-l'Étoile, France). Six unknown *Vibrio* species were additionally used and classified through direct sequencing (Supplementary Fig. 1).

Primer design

Four primer sets were designed using *YeaD* and the 16S rRNA gene. *YeaD*-based primers were used to identify the three major *Vibrio* species: *V. harveyi, V. alginolyticus*, and *V. parahaemolyticus*. The 16S rRNA gene-based primers were designed to detect various bacteria including all *Vibrio* species. Primers harv-F (5′-GTT CTG CAA GTA TCG ACA ACG-3′) and harv-R (5′-TAG AGC GAG TTC AAC GAT CAC-3′) generated a 396-bp (bp) product. Primers algi-F (5′-CGC CTG AAG GTC AAG AT-3′) and algi-R (5′-CAC ACC GTT ATG GTT CTC AC-3′) generated a 185-bp product. Primers para-F (5′-AAA GAG GCG GCT TGA TAG TC-3′) and para-R (5′-CAA CGT GCG GTT AAG AAC AG-3′) generated an 805-bp product. 16S rRNA gene-based primers universal-F (5′-CCA CAC TGG AAC TGA GAC A-3′) and universal-R (5′-TAA TCT TGC GAC CGT ACT CC-3′) generated a 596-bp product.

PCR assay and sensitivity test

PCR conditions were optimized as follows: 2.5 μL 10× Ex Taq buffer (Takara, Shiga, Japan), 10 pM of each primer (in case of Multiplex PCR, 5 pM of each primer), 2.5 mM each dNTP (Takara), 1.0 U Taq High-fidelity DNA polymerase (Takara), and 10 ng template DNA. PCR amplifications were performed in a thermal cycler (Eppendorf, Hamburg, Germany). Samples were heated to 94 °C for 3 min, followed by 30 cycles as follows: 94 °C for 40 s, 57 °C for

40 s, and 72 °C for 40 s. Cycling was followed by a final extension step at 72 °C for 3 min. The PCR products were subjected to 1.5% agarose gel electrophoresis. Additionally, PCR for checking the minimum concentration of templates was performed using 3, 1, 0.3, 0.1, 0.03, 0.01, and 0.003 ng template. PCR products were analysed quantitatively using the Image-J program (Fig. 1).

Targeting sequence *gapA, YeaD*, and 16 s rRNA gene

Sequences of chromosome 1 of *V. harveyi* (strain QT520), *V. parahaemolyticus* (strain K01M1), and *V. alginolyticus* (strain CHN25) from NCBI were used to design primers. The location of glyceraldehyde-3-phosphate dehydrogenase A (*gapA*) gene in chromosome 1 was obtained using another *gapA* from the three *Vibrio* species (strains LMG4044, ANC5-1, and LMG2850 in NCBI). Open Reading Frame Finder (ORF Finder) in NCBI was used to identify ORF regions in the *gapA* sequence (https://www.ncbi.nlm.nih.gov/orffinder/). An ORF of 850–900 bp was identified at the 3′ end of the *gapA* sequence. SmartBLAST was used to determine which protein is expressed by the ORF. The ORF showed a 100% match to *YeaD* protein. A region from *gapA* to *YeaD* in each *Vibrio* species was cut out and aligned using BioEdit for primer design. This was also conducted for *V. anguillarum* (strain NB10), *V. campbellii* (strain LMB29), *V. cholera* (strain E1162), *V. fischeri* (strain MJ11), *V. fluvialis* (strain ATCC 33809), *V. mimicus* (strain MB-451_contig43), *V. splendidus* (strain 5S-101_contig_13), and *V. vulnificus* (strain FORC_036) for comparison. For 16 s rRNA primer design, *V. harveyi* (strain LDP 1-1-10), *V. alginolyticus* (CECT 43), and *V. parahaemolyticus* (CECT 5305) were aligned by BioEdit.

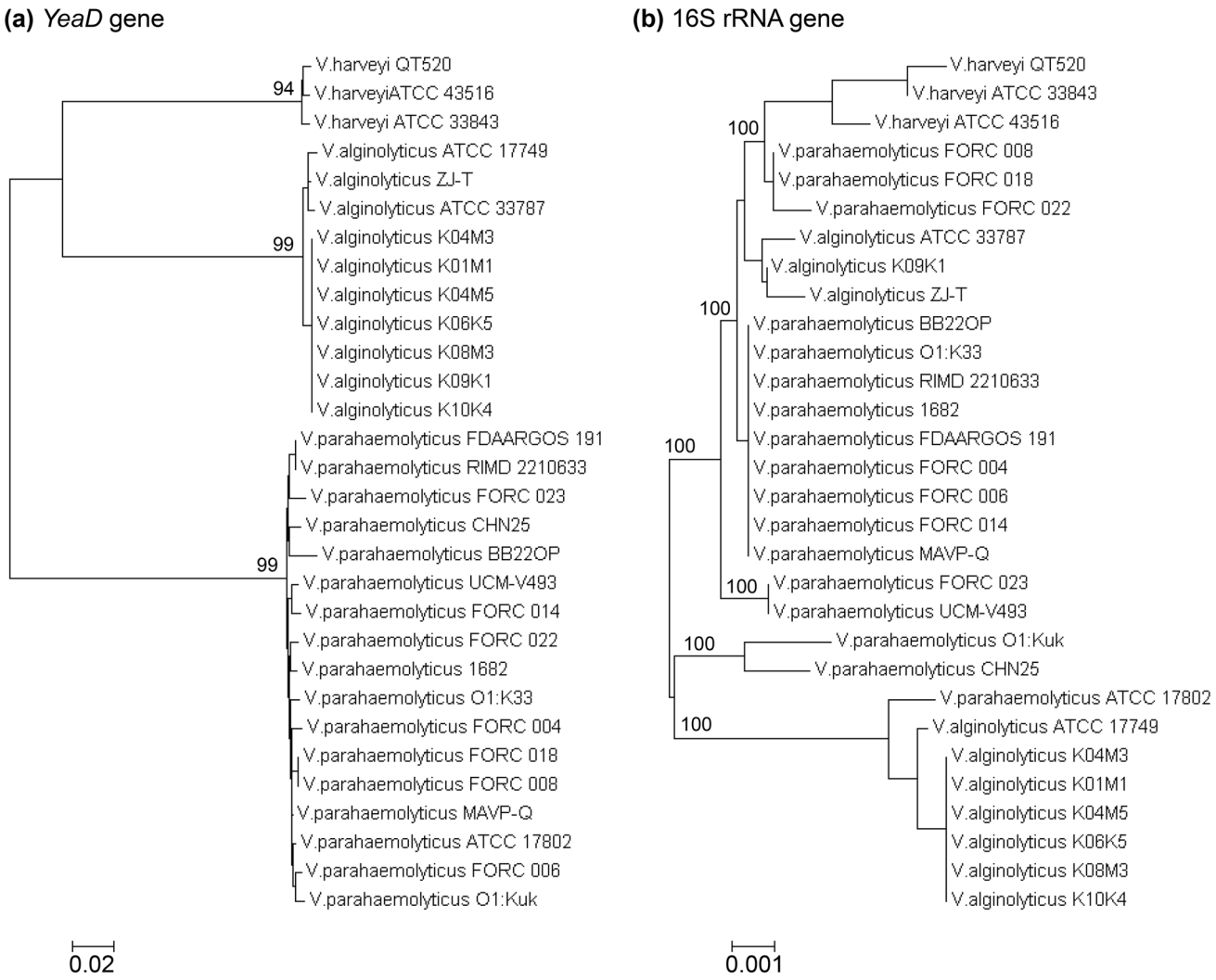

Fig. 1 Phylogenetic tree for *YeaD* (**a**) and 16S rRNA gene (**b**). The phylogenetic tree is made by genes of 30 *Vibrio* strains in NCBI recognized by designed primers through primer-BLAST

Bioinformatics analysis

Targeted genes were compared using ClustalW in BioEdit software (http://www.mbio.ncsu.edu/BioEdit/page1.html) (Thompson et al. 1994). Primers were designed by comparing the three *Vibrio* species (*Vibrio harveyi, V. alginolyticus*, and *V. parahaemolyticus*) and eight control *Vibrio* species (*V. anguillarum, V. campbellii, V. cholera, V. fischeri, V. fluvialis, V. mimicus, V. splendidus*, and *V. vulnificus*). All primers for *YeaD* showed species-specificity. When comparing the strains of three *Vibrio* species in the *YeaD* gene sequences, the *YeaD* gene mutation rate in the homologous strains is low enough to design the primer (Supplementary Fig. 2). Targeted sequences for primer-design were the same in homologous strains. 16S rRNA gene-based primers were used for all strains.

Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) is used to identify the species detected by primers (Ye et al. 2012). Each *YeaD* primer detected only target species in the program. Each primer corresponds to the sequence of *Vibrio harveyi, V. alginolyticus, V. parahaemolyticus* registered in NCBI (Supplementary Fig. 3). 16S rRNA-based primers detected various bacteria including 27 *Vibrio* species, Photobacterium species, and *Escherichia coli*. Primer-BLAST parameters were changed; Database to 'nr' and Organism to '*Vibrio* (taxid: 662)' or 'Bacteria (taxid: 2)'.

AutoDimer and MultiPLX 2.1 software (http://bioinfo.ebc.ee/multiplx/) were used to evaluate primer stability such as hairpin and dimer formation (Vallone and Butler 2004; Kaplinski et al. 2004). All designed primers were stable according to the results and could form multiplex primers.

The *YeaD* and 16S rRNA genes were analyzed by the neighbour-joining method using the MEGA7 program (Saitou and Nei 1987; Kumar et al. 2016). Thirty strains were detected by Primer-BLAST analysis from NCBI (Fig. 2). The nucleotides examined included approximately 1000 bp of 16S rRNA and *YeaD*. The phylogenetic tree of 30 strains revealed different stability and specificity characteristics between the two genes (Fig. 3).

Results

PCR amplification and multiplex PCR

PCR was performed with each primer set according to the PCR assay conditions. PCR analysis of the genomic

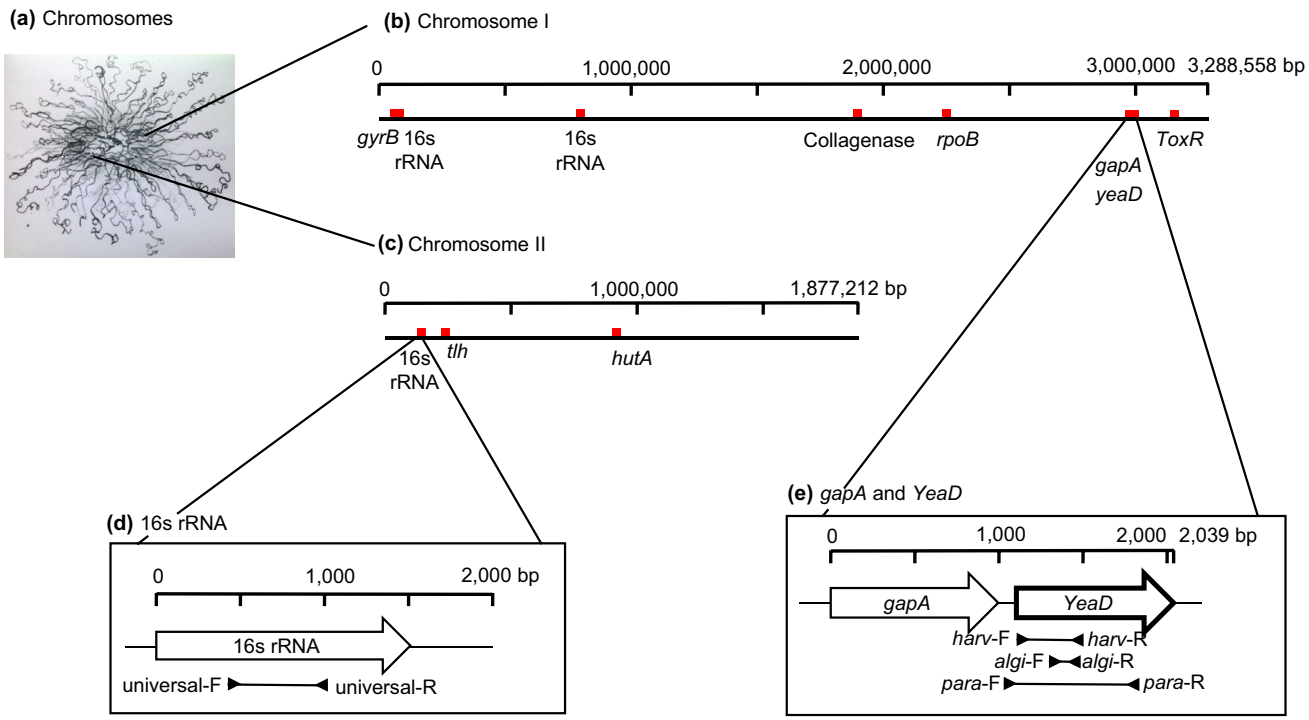

Fig. 2 *Vibrio* genes for multiplex PCR primers. **a** Chromosomes photograph of bacteria (Mašková 2011). **b** and **c** Two chromosomes present in *Vibrio* species. One is composed of approximately 3 million bp, while the other is composed of approximately 2 million bp. Other studies examining *Vibrio* detected gyrB, 16 s rRNA, collagenase, rpoB, toxR, tlh, and hutA. Their approximate locations on the chromosomes are indicated. The 16S rRNA gene and *YeaD* used in the study are shown on the chromosomes. 16S rRNA has several homologous genes. **d** The sizes of 16S rRNA and positions of the universal F and universal R primers. **e** The harv-F, harv-R, algi-F, algi-R, para-F, and para-R primers indicate the position detected by each. Para-F is located slightly outside of *YeaD*

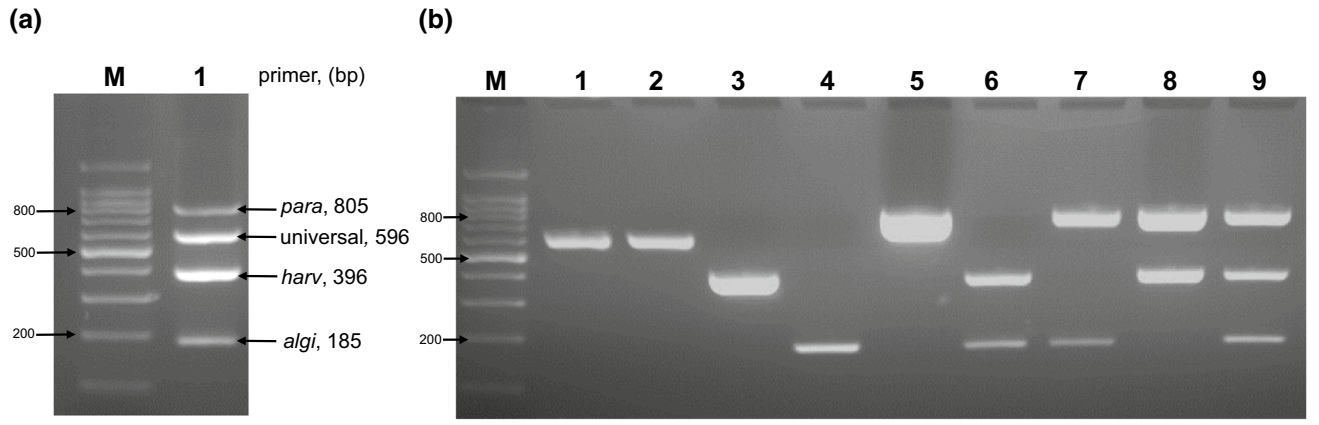

Fig. 3 Multiplex PCR with multiple samples and primer sets. **a** Lane M—100-bp marker; lane 1—*V. harveyi, V. alginolyticus*, and *V. parahaemolyticus* with all primers (harv-F, harv-R, algi-F, algi-R, para-F, para-R, universal-F, and universal-R). **b** Lane M—100-bp; lane 1—*Vibrio vulnificus* with all primers; lane 2—*V. mimicus* with all primers; lane 3—*V. harveyi* with harv-F, and R; lane 4—*V. alginolyticus* with algi-F, and R; lane 5—*V. parahaemolyticus* with para-F, and R; lane 6—*V. harveyi* and *V. alginolyticus* with harv-F, R, algi-F, and R; lane 7—*V. alginolyticus* and *V. parahaemolyticus* with algi-F,R, para-F, and R; lane 8—*V. harveyi* and *V. parahaemolyticus* with harv-F, R, para-F, and R; lane 9 - *V. harveyi, V. alginolyticus*, and *V. parahaemolyticus* with harv-F, R, algi-F, R, para-F, and R

DNA of *V. alginolyticus, V. harveyi*, and *V. parahaemolyticus* indicated fragment sizes of 185 bp, 396 bp and 805 bp, respectively. A band at 596 bp appeared when using the universal primers for the 16S rRNA gene in all three *Vibrio* species.

Multiplex PCR was performed with multiple samples and primers (Fig. 4). Three *Vibrio* species with all primer sets obtained clear 396-bp, 185-bp, 805-bp, and 596-bp bands (Fig. 4a, lane 1). *V. vulnificus* and *V. mimicus* control samples were inserted with all primer sets (harv-F, harv-R, algi-F, algi-R, para-F, para-R, universal-F, and universal-R), but only a 596-bp band was observed for the 16S rRNA gene to detect common bacteria (Fig. 4b, lanes 1 and 2). Additional multiplex PCR was performed on unknown *Vibrio* species along with identification by direct sequencing, and the same results were obtained (Supplementary Fig. 1).

Sensitivity test for PCR

PCR was performed to determine how many nanograms of template could be recognized. PCR detected up to 0.1 ng in *V. alginolyticus*, 0.03 ng in *V. harveyi*, and 0.003 ng in *V. parahaemolyticus* (Fig. 1).

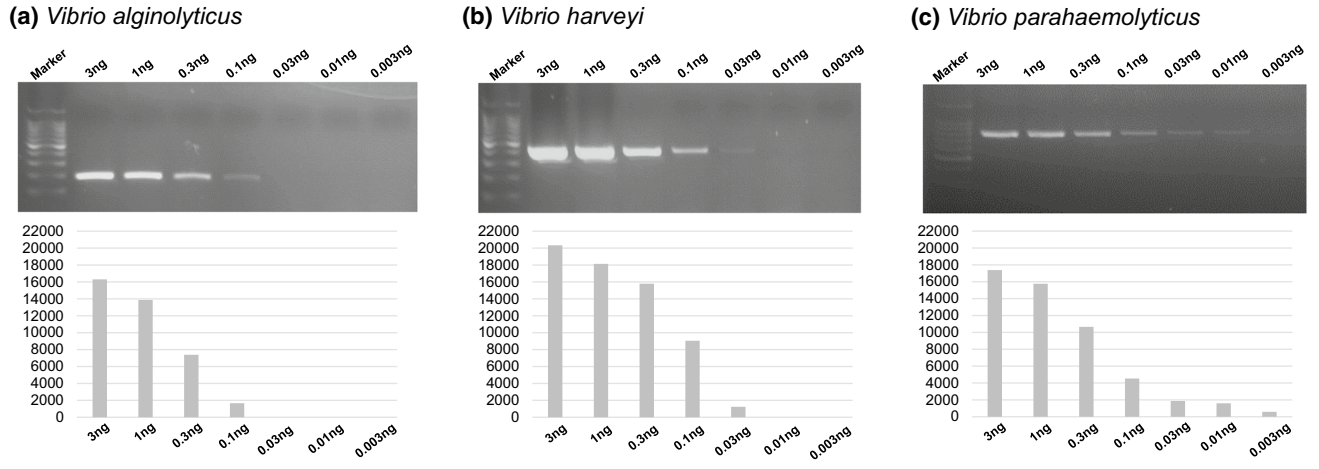

Fig. 4 Sensitivity test by PCR to determine the template concentration detection limit of *V. alginolyticus* (**a**), *Vibrio harveyi* (**b**), and *Vibrio parahaemolyticus* (**c**). Each lane represents sample concentration as follows; lane M—100-bp marker; lane 1—3 ng; lane 2—1 ng; lane 3—0.3 ng; lane 4—0.1 ng; lane 5—0.03 ng; lane 6—0.01 ng; 7—0.003 ng. PCR products were analysed quantitatively using the Image-J program

Phylogenetic analysis

The phylogenetic tree for the 16S rRNA gene and *YeaD* was constructed with MEGA 7 software (Fig. 2). Sequences for phylogenetic tree contained approximately 1000 bp from the 16S rRNA gene and *YeaD*. All samples were registered in NCBI and recognized by designed primers using primer-BLAST. For the 16S rRNA gene, it was difficult to classify *V. parahaemolyticus* ATCC 17802 or *V. alginolyticus* ATCC 33787, K09K1, and ZJ-T from other species. In contrast, for *YeaD*, the sequence difference between species of *V. alginolyticus, V. harveyi*, and *V. parahaemolyticus* was remarkable, indicating that three species samples showed clear separation in phylogeny tree.

Discussion

Multiplex PCR is useful for rapid and accurate measurement if reliable primers are used (Wei et al. 2014). Multiplex PCR can detect template concentrations as low as 0.1 ng in *Vibrio alginolyticus*, 0.03 ng in *V. harveyi*, 0.003 ng in *V. parahaemolyticus*. The copy numbers of a template can be calculated through nanograms of DNA considering the 5.2 million bp of *Vibrio* chromosomes and 650 daltons per base pair (Staroscik 2004; Danna et al. 1973). 0.1 ng means 17,800 templates, 0.03 ng means 5340 templates, and 0.003 ng means 534 templates. Since *Vibrio* has a generation time of 27 min in the glucose medium and 19 min in the LB medium, 2.15×29 *Vibrio* species in the LB medium and 4.20×26 *Vibrio* species in the glucose medium are obtained in 1 colony after 10 h of culture (Stokke et al. 2011). Therefore, a single colony is fully identifiable when extracted from *Litopenaeus vannamei*. In preceding experiments, *V. harveyi, V. alginolyticus*, and *V. parahaemolyticus* were extracted and cultured from *L. vannamei* showing vibriosis symptoms. The *Vibrio* species is extractable from stomach, hepatopancreas, haemolymph, muscle, and gut of *L. vannamei* (Sirirustananun et al. 2011; Yatip et al. 2018; Ananda et al. 2017). In the case of *V. alginolyticus*, 6×106 cfu shrimp-1 was obtained in the ventral sinus of *L. vannamei* on a challenge test (Sirirustananun et al. 2011).

To be reliable, the primers must recognize all strains of the same species without detecting other species. Bioinformatics analysis by primer-BLAST in NCBI supported the reliability of our primers (Supplementary Fig. 3). No *Vibrio* species were identified other than the target species among all NCBI strains using the designed-primers. Only a few other *Vibrio* species were searched but not identified because they differed by more than five nucleotides from the primers. In addition, all but one of the 17 *V. parahaemolyticus*, 11 *V. alginolyticus*, and 3 *V. harveyi* strains registered in NCBI were detected, except for 1 *V. alginolyticus*. However, the 1 *V. alginolyticus* (strain K08M4) is registered as *V. splendidus* in the BioProject section of NCBI. Thus, except for the confounding strain, 100% matching was achieved. This suggests that the designed primers are reliable and induce low mutation rate.

For *YeaD*, there was a distinct sequence difference between all *Vibrio* species used for comparison (Supplementary Fig. 2). However, *YeaD* gene sequences of the same species were well-conserved. Although *YeaD* is the essential gene involved in galactose metabolism, it remains unclear how *YeaD* functions using different sequences for each species (You et al. 2010). We initially attempted to construct primers for *gapA*. Although the three species were distinguished from each other using *gapA*, it was more convenient to construct primers for *YeaD* to compare all *Vibrio* species than *gapA*. Primers for *YeaD* clearly identified the different species. Stability of *YeaD* gene suggests to be genetic marker to identify gram-negative bacteria.

Litopenaeus vannamei is predominantly related to *V. harveyi, V. alginolyticus*, and *V. parahaemolyticus* based on previous infection rates and association studies. In the past, Anguirre-Guzmán et al., selected *V. harveyi, V. alginolyticus, V. parahaemolyticus*, and *V. penaeicida* as potential pathogenic *Vibrio* species of *L. vannamei* (Aguirre-Guzmán et al. 2001). In Ecuador and Mexico research, 90 isolates of diseased *L. vannamei* were found to have 42 *Vibrio harveyi* (46.7%), 14 *V. alginolyticus* (15.6%), and 8 *V. parahaemolyticus* (8.9%). 4 *P. damselae* (4.4%), 1 *V. mimicus* (1.1%) were also found in small amounts (Johannessen et al. 1999). In India, *V. parahaemolyticus* (83.4%) was found in the hepatopancreas of infected *L. vannamei* without identification of *V. alginolyticus* and *V. harveyi* (Shanmugasundaram et al. 2015). In shrimp culture ponds of Sri Lanka, *V. parahaemolyticus* (55.5%), *V. alginolyticus* (27.7%), *V. damsela* (5.6%), *V. anguillarum* (5.6%), unidentified *Vibrio* (5.6%) were found without identification of *V. harveyi* (Heenatigala and Fernando 2016). To find out association between *L. vannamei* and *Vibrio* species in research, we counted the number of research. The number of research associated with *Vibrio* and *L. vannamei* were 262 in PubMed. Among them, 45 papers related to *V. harveyi* (17.2%), 106 papers related to *V. alginolyticus* (40.5%), and 80 papers related to *V. parahaemolyticus* (30.5%). There were also 15 papaers of *V. anguillarum* (5.7%), 11 papers of *V. campbellii* (4.2%), 4 papers of *V. penaeicida* (1.5%). So, *V. harveyi, V. alginolyticus* and *V. parahaemolyticus* are major pathogens and high association with *L. vannamei*.

In addition to *Vibrio harveyi, V. alginolyticus* and *V. parahaemolyticus*, the other *Vibrio* species also exist in diseased *Litopenaeus vannamei*. Fourteen *Vibrio* species are reported to be infected in cultured shrimp; *Vibrio harveyi, V.*

splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbellii, V. fischeri, V. damsella, V. pelagicus, V. orientalis, V. ordalii, V. menditerrani, V. logei (Annam 2015). According to a infect rate research in Ecuador and Mexico, the other *Vibrio* species can be detected with a probability of 28.9%. 16S rRNA gene-based primers only detect the other *Vibrio* species without identification. If 16S rRNA gene-based primers detect bacteria, direct sequencing or other PCR techniques can identify the undefined *Vibrio* species.

Acknowledgements This research was a part of the project titled "Omics based on fishery disease control technology development and industrialization (20150242)," funded by the Ministry of Oceans and Fisheries, Korea.

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