Korean J. Malacol. 35(3): 275-289 2019
https://doi.org/10.9710/kjm.2019.35.3.275

Characterization and species-specific detection of *Vibrio parahaemolyticus*-related species associated with mass mortality of ark shell (*Scapharca broughtonii*)

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ABSTRACT

Ark shell (*Scapharca broughtonii*) is cultured in southern coast of Korea and is a commercially important shell in aquaculture. However its productivity has decreased rapidly in the last decade due to mass mortality. Various microbial investigations were performed to identify the caused bacterial species for economic losses. Using cumulative mortality analysis, 16S rDNA sequence analysis, and API 20 kit assay, *Vibrio parahaemolyticus*-related species (8M 4-1) was identified as an etiologic bacterium. The LD_{50} value of the isolated bacterium to ark shell was lower than 10^6 CFU/mL. After infection, histological diagnosis from hepatopancreatic tissue also provided evidence for pathogenicity. To monitor this ark shell pathogenic bacterium, we designed the PCR detection method with the *Vibrio* species *groEL* gene. We also tested various virulence gene-targeted detection methods, but amplified products were not suitable for *V. parahaemolyticus* recognition. In this study, we identified *V. parahaemolyticus*-related species as a causative organism of mass mortality, and demonstrated that PCR detection assay could successfully detect *V. parahaemolyticus* strains from infected tissues with high sensitivity. Using these results, we identified *V. parahaemolyticus*-related 8M 4-1 species as the causative agent of ark shell mortality and showed that newly developed PCR detection method was useful for pathogen monitoring.

Key words: Ark shell (*Scapharca broughtonii*), mass mortality, *Vibrio parahaemolyticus*, PCR

Introduction

Several Anadarinae subfamilies such as Arcinae, Noetinae, and Anadarinae have been reported, and some species of Anadarinae are important in South Korea (Yoo, 1986). Among the nine species of Anadarinae, six species (*Anadara granosa*, *Anadara ogawai*, *Anadara subcrenata*, *Anadara daitokudoensis*, *Anadara satowi*, and *Scapharca broughtonii*) are distributed in Korea, and only three species (*A. subcrenata*, *A. satowi*, and *S. broughtonii*) are important for the coastal aquaculture industry of South Korea (Chun *et al*., 1991; Koh *et al*., 1997). The ark shell (*S. broughtonii*) is considered as a healthy seafood in Korea; the numerous dramatic bays along the southern coast of Korea are important for ark shell aquaculture (Chun *et al*., 1991; Koh *et al*., 1997). In the last decade, the productivity of ark shell has decreased rapidly due to mass mortality, resulting in economic loss. However, the reason for this increased mortality is unknown (Kim *et al*., 2008; Shin *et al*., 2008). To overcome this economic loss, various research have focused on only environmental factors, such as temperature, salinity, and oxygen levels, which

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affect the physiology of *S. broughtonii*. However, few microbiological investigations have been performed (Kim *et al*., 2008; Shin *et al*., 2008).

Recently, metagenomic analysis with pyro-sequencing was found to be useful for investigating environmental microbiota and the distribution of specific strains. This method can identify non-culturable microorganisms that are viable but non-culturable (VBNC) (Hoff *et al*., 2008; Schmidt *et al*., 1991). However, this method requires isolation, identification, and incubation steps for biochemical and/or microbiological research, such as functional metabolites screening and determination of the virulence (Kim *et al*., 2012; Sorokin *et al*., 2010; Suzuki *et al*., 1997; Woo *et al*., 2003). For these reasons, metagenomic analysis is inappropriate for certain investigations in which culturable microorganisms are essential.

In marine mollusks such as bivalves, only the ark shell has hemoglobin as a blood pigment. Microbial toxins and metabolites can cause blood pigment hemolysis, leading to mass ark shell mortality. Therefore, we focused on hemolytic activity as the main factor causing pathogenicity. In previous studies, we isolated and identified bacteria with alpha- or beta-hemolysis activity, from ark shell bodies and aquafarm sediment, and compared the difference of microflora isolated from regions with high and low mortality occurred (Gwon *et al*., 2013; Kim *et al*., 2012).

Kim *et al*. (2012) and Gwon *et al*. (2013) isolated, analyzed, and identified microbiota for 4 months, and identified 53 species of 140 isolates from Gang-Jin Bay, South Korea, where ark shell mass mortality was reported. In this study, based on these results, we performed an artificial challenge test with live healthy ark shells to determine the mortality associated with different types of bacteria and selected six unique specimens among the 140 isolates. However, the cumulative mortalities of six isolates were 20%–80%. Only one bacterium (8M 4-1), which was isolated from an ark shell (*S. broughtonii*) body in August, was associated with a mortality rate over 80%. Therefore, we confirmed 8M 4-1 as a mass mortality-associated microorganism and decided to characterize it. And we confirmed and characterized the pathogenic effect of 8M 4-1, and developed a PCR monitoring assay to prevent the mass mortality of ark shells.

Materials and methods

1. Isolation and identification of pathogenic bacterial strains

Ark shells (*S. broughtonii*) and sediment were collected from two regions of ark shell culture farms for 5 months (June to October). Samples of homogenized ark shell tissue and sediment were plated on marine agar (Difco, USA), R2A agar (Difco, USA), and diluted marine and R2A agar plates. The inoculated plates were incubated at 25°C for 7 days for colony growth. The colonies were transferred and streaked onto new plates (marine, R2A, or diluted marine and R2A agar plates) to obtain single colonies. Isolated single colonies were grown in corresponding liquid media and then dropped onto blood agar plates (Micromedia, Korea) to test for hemolytic activity (Mann *et al*, 1994). Isolates, which make a halo or change color (green or dark red) on blood agar plates, were transferred to isolated media and collected for identification. Each hemolytic bacterium was cultured and collected, and chromosomal DNA was purified using a MagExtractor system (Toyobo, Japan) with a magnetic isolation kit (Toyobo, Japan) and an automated DNA extraction system (MFX-6100; Toyobo, Japan). The purity of the extracted total DNA was determined using a NanoVue plus spectrophotometer (GE Life Science, USA) and used as the PCR template for 16S rDNA amplification. Using a 27F and 1492R (Table 1) primer pair, approximately 1.5-kbp amplicons were analyzed by agarose gel electrophoresis and purified using a QIAquick PCR purification kit (Qiagen, Germany). After determining the purity and concentration of the purified PCR products, an ABI 3130XL automatic DNA sequencer (Applied Biosystems, USA) was using for sequencing.

From the bacteria isolated from ark shell and sediment at Gang-Jin Bay, only the bacteria associated with mass mortality underwent further microbiological,

Table 1. Primer sequences used for the amplification of the *toxR, tlh, tdh, trh* and 16S rDNA genes in 8M 4-1

Target gene	Primer sequence (5'-3')	Product size	Reference
toxR	toxR-1F: CATTACTCCCGCTTGCTTCTG toxR-1R: ATACGAGTGGTTGCTGTCATG	350 bp	Kim *et al.* (1999)
tlh	tlh-F: CATTACTCCCGCTTGCTTCTG tlh-R: GCGAACATAGGTATAGGTTTGGTT	113 bp	Feng *et al.* (2007)
tdh	tdh-1F: AATGGTTGACATCCTACATGACTG Tdh-1R: ACTTGACCTGATTTTACGAACACA	112 bp	Feng *et al.* (2007)
toxR	toxR-2F: GTCTTCTGACGCAATCGTTG toxR-2R: ATACGAGTGGTTGCTGTCATG	368 bp	Suthienkul *et al.* (1995)
tdh	tdh-2F: GTAAAGGTCTCTGACTTTTGGAC tdh-2R: TGGAATAGAACCTTCATCTTCACC	269 bp	Bej *et al.* (1999)
trh	trh-F: TTGGCTTCGATATTTTCAGTATCT trh-R: CATAACAAACATATGCCCATTTCCG	500 bp	Bej *et al.* (1999)
16S rDNA	27F: AGAGTTTGATCCTGGCTCAG 1492R: GGTTACCTTGTTACGACTT	1.5 kbp	

biochemical, and molecular biological testing. Diverse candidate species were cultured and collected, and approximately 10^9 colony-forming units (CFU)/mL of bacteria were injected into ark shells (shell length, height, width, and weight was 7.3 ± 4.2 cm, 5.7 ± 4.0 cm, 4.3 ± 3.8 cm, and 88.4 ±16.6 g, respectively), which were acclimated to 25°C, to measure the pathogenicity (Yue *et al*., 2010). To identify the most pathogenic specimen, the cumulative mortality and physical changes of infected ark shells were observed for 2 weeks. Among the 140 isolates, strong candidates (those that caused the highest mortality) were analyzed with an API kit (BioMérieux, France) for biochemical identification and characterization. Among the API kits, the API 20 NE V7.0 strip was used for strain identification, and the results were analyzed by comparing biochemical profile patterns from the ApiwebTM program (https://apiweb.biomerieux.com).

2. 8M 4-1 bacterial challenge test

After infection test to identify virulent strains among the 140 isolates, the specimen 8M 4-1, which caused the highest mortality rate, was selected and cultured to quantify the mortality. The sample was grown on marine medium and then plated on marine agar plates for further incubation overnight. Bacteria were isolated, collected from plates, and washed with phosphate-buffered saline (PBS) for counting. To measure the optical density at 600 nm, the harvested cells were serially diluted 10-fold with PBS. The diluted bacterial suspensions were dropped onto marine plates and the number of viable cell was determined. Based on the OD_{600} values, 10^9-10^6 CFU/mL bacteria were injected into 10 ark shells, respectively. Control ark shells were injected with PBS. Five replicated infection sample containers (10 ark shells per container) of four concentrations and three control samples were retained for 2 weeks under continuous aeration and circulation with sea water at 25°C. When the gill of an ark shell dissolved, this was an indication of infection and recorded to quantify the mortality rate. Infected ark shells were removed from containers, and hepatopancreatic samples were spread-plated on thiosulfate-citrate-bile salts-sucrose (TCBS; Difco, USA) agar plates for pathogen isolation and prepared for histology.

3. Histology of infected ark shell tissues

Infected and control hepatopancreatic tissue samples were fixed using Bouin's fixative solution for 24 hours for histological examination. Post-fixation, the samples were cut diagonally to include various visceral organs in 2-mm-thick slices, and the same fixative solution was not used for more than 12 hours during pre-fixation. Subsequently, the fixed tissues were washed with fresh water and dehydrated through a serially prepared alcohol series (70%-100%). Dehydrated tissues were clarified with xylene, penetrated with pre-warmed paraffin (55-60°C), and then embedded in paraffin using a stainless mold. The prepared paraffin blocks were sectioned into 4-㎛ m-thick sections using a rotary microtome (Leica, Germany) and attached on clean glass slides. The samples were stained with hematoxylin and eosin (H&E) using an Auto Stain System (Leica, Germany). The samples were observed with a light microscope (Zeiss, Germany) and viewfinder images were captured with a digital camera and Zen2012 software (Zeiss, Germany).

4. Amplification of virulence genes

8M 4-1 isolate was the strongest candidate to be the causative agent causing ark shell mass mortality, and was identified as *V. parahaemolyticus* (3.1). To identify the virulence and suitable monitoring marker, toxR, thermolabile direct hemolysin (*tlh*), thermostable direct hemolysin (*tdh*), tdh-related hemolysin (*trh*), and 16S ribosomal DNA genes were amplified with gene-specific primer sets (Table 2). These genes were reported as pathogenicity regulator genes and pathogenic factors in *V. parahaemolyticus*. 16S rDNA was amplified as a positive control in the PCR (Asim *et al*., 1999; Feng *et al*., 2007; Kim *et al*., 1999; Suthienkul *et al*., 1995). The PCRs were performed using conditions reported in reference papers, and the sizes of the PCR products were confirmed by agarose gel electrophoresis.

5. Development of a monitoring method

1) Identification and analysis of the *groEL* gene

To monitor pathogenic bacteria, we selected the *groEL* gene as the target gene for species-specific PCR. The *groEL* gene sequences from 27 *Vibrio* species were obtained from the National Center for Biotechnology Informatics (NCBI) GenBank database and sequences were aligned using the BioEdit sequence alignment editor program (Version 7.2.3). Conserved regions were selected and used to design degenerate universal primers for *Vibrio* species. Oligonucleotide primers UPgEL-Vibrio-F: 5'-ATGGCTGCTAAAGACGTTMRDTT-3' and UPgEL-Vibrio-R: 5'-CATCATRCCDCCCATRCCRCCC-3' were designed and used to amplify the *groEL* gene of 8M 4-1. PCR thermocycling was performed with 50 μL of reaction mixture containing 250 μM of each deoxyribonucleoside triphosphate (dNTP), 10 pM of each primer, 5 μL of 10× Taq buffer with MgCl2, 0.5 U of Ex-Taq DNA polymerase (Takara, Japan), and distilled water to a final volume of 50 μL. The PCR amplification cycling program consisted of denaturation at 95°C for 5 min, followed by 25 cycles at 95°C for 30 sec, 55°C for 1 min, 72°C for 30 sec, and a final 7 min extension at 72°C. The size of the PCR amplicon was confirmed by 1% agarose gel electrophoresis. The amplicon was cloned into a pGEM-T easy vector system (Promega, USA) and then sequenced with the 3130XL Genetic analyzer (Applied Biosystems, USA)

2) Species-specific detection and sensitivity of the PCR assay

The *groEL* gene sequence of 8M 4-1 was aligned with 27 species of the *groEL* genes from different *Vibrio* species and species-specific detection primers were designed. The nucleotide regions that were not conserved and had high nucleotide variability were selected and compared with the *groEL* sequence of *V. parahaemolyticus* for the specificity of detection. Among the primers gEL-8M 4-1-F3 (5'-TCTAGTTGATAAGAAGATCTCA-3') and gEL-8M 4-1-R2 (5'-GAGACCAGCGACAGACGCTGCA-3') were selected from the 629–649 bp and 1517–1538 bp regions of the 8M 4-1 *groEL* sequence, respectively. The species-specific PCR test consisted of one initial cycle of denaturation at 95°C for 5 min, followed by 25 cycles at 95°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec, and finally one cycle at 72°C for 7 min. The species specificity of the PCR assay was tested using purified chromosomal DNA from 28 strains of 21

Table 2. Biochemical analysis of 8M 4-1 with an API NE20 kit

Test	Substrate	Reaction	8M 4-1
NO_3	Potassium nitrate	Reduction of nitrates to nitrites	+
TRP	Tryptophan	Reduction of nitrates to nitrogen	+
GLU	Glucose	Indole production	+
ADH	Arginine	Acidification	-
URE	Urea	Arginine dihydrolase	-
ESC	Esculin	Urease	-
GEL	Gelatin (with India ink)	Hydrolysis (b-glucosidase)	+
PNPG	p-Nitrophenyl-b-D-galactopyranoside	Hydrolysis (protease)	+
GLU	Glucose	b-Galactosidase	+
ARA	Arabinose	Assimilation	+
MNE	Mannose	Assimilation	+
MAN	Mannitol	Assimilation	+
NAG	N-Acetyl-glucosamine	Assimilation	+
MAL	Maltose	Assimilation	+
GNT	Gluconate	Assimilation	+
CAP	Caproate	Assimilation	-
ADI	Adipate	Assimilation	-
MLT	Malate	Assimilation	+
CIT	Citrate	Assimilation	-
PAC	Phenyl-acetate	Assimilation	-
OX	Tetramethyl-p-phenylene diamine	Cytochrome oxidase	+

Vibrio species, 21 non-*Vibrio* species, and 6 environmental isolates (Table 4). Using the designed gEL-8M 4-1-F3 and gEL-8M 4-1-R2 primers, species specificity was confirmed by the detection of amplified products on an agarose gel. 16S rDNA was amplified as a control. The primer pairs are listed in Table 1. The detection sensitivity of the PCR monitoring method was evaluated using different concentrations of purified total DNA from 8M 4-1. The DNA was serially diluted 10-fold with distilled water (4.1 μg to 4.1 pg) and the PCR was performed under the same conditions.

3) Monitoring of the 8M 4-1 isolate from artificially infected samples

The total tissue from artificially infected ark shells was isolated and homogenized with the same volume of PBS, and chromosomal DNA was purified from the tissue lysate using a QIAamp DNA Mini kit (Qiagen, Germany). The purity and concentration of the total DNA from the infected samples were measured and used as a template for the PCR assay described above.

Results

1. Isolation, identification, and characterization of pathogenic bacterial strains

Among the 140 isolates that were found to be associated with ark shell mass mortality, only six bacterial species caused >20% mortality after 2 weeks. The mortalities associated with 7M 1-25 (isolated from sediment), 7M 3-18 (isolated from sediment), 7M 19-22 (isolated from sediment), 7M 26-3 (isolated from ark shell), and 8M 28-7 (isolated from ark shell) were

20%-30%, but 8M 4-1 (isolated from ark shell) caused a cumulative mortality of 80% (Data not shown). These results suggest that 8M 4-1 is a virulent bacterial strain that can cause ark shell mass mortality.

The 16S rDNA sequence was compared with the NCBI GenBank database using a BLASTN search. 7M 1-25 (*V. harveyi*, 98%), 7M 3-18 (*V. harveyi*, 99.8%), 7M 19-22 (*V. harveyi*, 99.7%), 7M 26-3 (*V. alginolyticus*, 98%), 8M 28-7 (*V. alginolyticus*, 99%), and 8M 4-1 (*V. parahaemolyticus*, 98.2%) were identified as *Vibrio* species-related strains.

Biochemical and bacteriological characteristics of 8M 4-1, as determined with an API 20 NE kit, are listed in Table 2, and the results are recorded as numerical patterns: 7077744 (Table 2). Using the API kit, the determined profile of 8M 4-1 was *V. parahaemolyticus* with 99.1% identity. This was considered a very good identification with no against test. When 8M 4-1 isolated from infected ark shell tissue was cultured in TCBS, a selective medium for *Vibrio* species, it grew into a single green colony, consistent with the morphological character of *V. parahaemolyticus*.

Based on the high induced mortality rate, similarity of the 16S rDNA sequence, and the identified biochemical profiles from the API kit analysis, 8M 4-1 was determined to be a mortality-causing *V. parahaemolyticus* allied invader. Next, we conducted further investigations on virulence factors and histopathological changes, and developed a PCR detection assay.

2. Experimental infection test

Ark shell mass mortality in the south coast of Korea was highest between September and October, and the sea water temperature during this period was approximately 25°C. Therefore, an infection test was performed at 25°C for 2 weeks. 8M 4-1 was harvested with PBS from cultured agar plates and serially diluted from 1×10^9 CFU/mL to 1×10^6 CFU/mL and 100 μL was injected into ark shell bodies.

After injection, the reaction to closing the shell was slowed, and the mantle and gills were dissolved. Afterwards, we observed increased mucus, tissue edema at the foot, and a change in tissue color (the foot organ changed color from red to yellow or purple). Simultaneously, epidemic mortality occurred the next day. On the third day post-infection, moribund and dead ark shells were detected at all concentrations in the infection group samples. In each group, the highest mortality was reached between days 5 and 8 post-infection (Fig. 1). In the preliminary infection challenge with six isolates, ark shells in the non-infected control group had a 0% mortality rate; however, in the present test, ark shells in the PBS-injected control group had a 30% death rate. In addition, the infected group also had a higher cumulative mortality rate. However, the artificial infection test results and the cumulative mortality rates clearly demonstrate that 8M 4-1 can produce virulence factors to ark shell, resulting in mortality rate in the infection group.

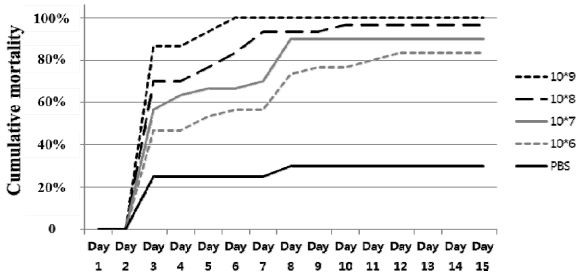

Fig. 1. Cumulative mortality of ark shell at different concentrations of 8M 4-1 10^9-10^6 CFU/mL).

3. Histological analysis of infected ark shell tissues

In histological examinations, changes were observed in various tissues. In particular, there was predominant degeneration of the digestive gland of the hepatopancreas. Compared with control clams (Fig. 2A), obvious cell degeneration was observed at epithelial cells of the digestive gland in infected clams, and the necrotic cell debris was scattered in the intraluminal space of digestive tubules (DT). Degenerate epithelial cells are desquamated from the basement membrane in infected clams at the middle phase of infection (Fig. 2B). In addition, there was degeneration of hemocytes in infected clams. Normal hemocytes were distributed in connective tissue that linked and surrounded DT (Fig. 2A), whereas pale

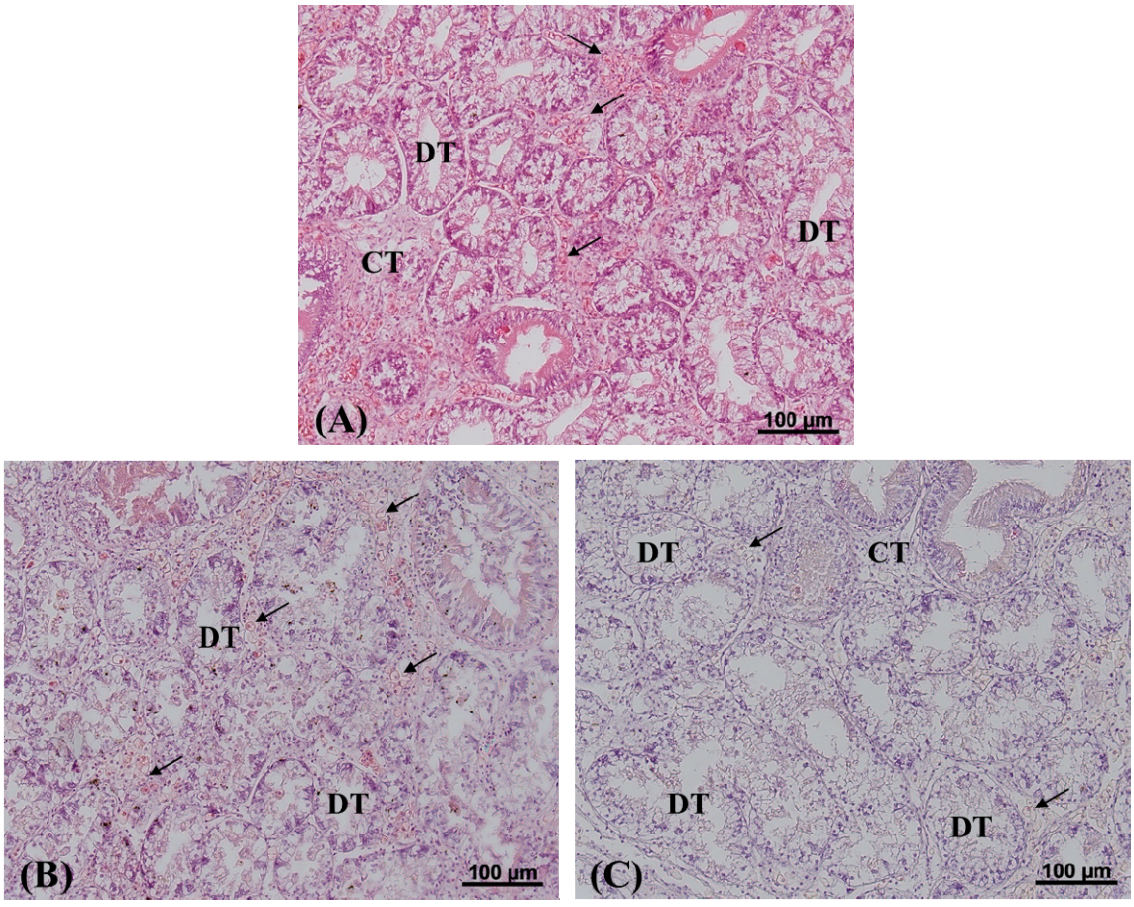

Fig. 2. Histological analysis and changes in the hepatopancreas after infection. (**A**) Digestive gland of a control clam. The arrows indicate hemocytes. DT, digestive tubule; CT, connective tissue. (**B**) Digestive gland of an infected clam in the middle of infection. (**C**) Digestive gland of an infected clam at the end of infection.

hemocytes increased as infection progressed (Fig. 2C).

4. Detection of virulence genes

Using results from 16S rDNA sequence analysis and biochemical characterization, 8M 4-1 was identified as a *V. parahaemolyticus*-related strain. Previously identified *V. parahaemolyticus* species-specific pathogenic genes were amplified to identify virulence and monitoring markers. Two primer sets targeting the *toxR* gene, two primer sets targeting the *tdh* gene, one primer set targeting the *tlh* gene, and primers targeting the *trh* gene (Table 1) were used for target gene amplification (Fig. 3). However, only primers targeting the *tlh* and *toxR*-2 gene regions produced amplicons of the predicted size (Fig. 3). PCRs targeting the *toxR*-1, *tdh*-1, *tdh*-2, and *trh* genes generated products of the wrong size or no products (Fig. 3). 16S rDNA was amplified as a positive control for PCR amplification and generated a single 1.5-kbp band on an agarose gel (Fig. 3).

5. Species-specific PCR detection assay for 8M 4-1 monitoring

1) Identification and analysis of the 8M 4-1 groEL gene

A total of 1625 bp of the 8M 4-1 *groEL* gene was amplified, cloned, analyzed, and compared with 27 species of other *groEL* genes from *Vibrio* species. Sequence homology was 97.4%–53.1% and *V. parahaemolyticus* had the highest identity (Table 3). Except in the case of *V. parahaemolyticus*, *V. rotiferianus* (90.4%), and *V. harveyi* (88.3%), which had the first, second, and third highest similarity,

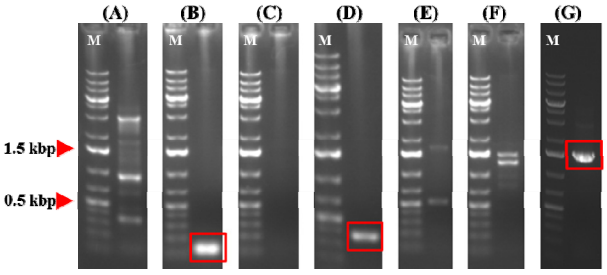

Fig. 3. Virulence gene amplification and electrophoresis. M: DNA marker, (**A**) *toxR*-1, (**B**) *tlh*, (**C**) *tdh*-1, (**D**) *toxR*-2, (**E**) *tdh*-2, (**F**) *trh*, and (**G**) 16S rDNA (positive control).

respectively, the rest of the species had approximately 80.7% identity on average. These results demonstrate that 8M 4-1 is closely related to *V. parahaemolyticus*.

2) Specificity and sensitivity of PCR detection

The sequence of the 8M 4-1 *groEL* gene was aligned with 27 species of *groEL* genes and the locations of the *V. parahaemolyticus* species-specific detection primer markers were determined (Fig. 4). The 5' and 3' regions of the *groEL* gene were selected for the forward and reverse primers, respectively, and the

Table 3. The *groEL* sequence identity between *Vibrio* species and 8M 4-1 (*V. parahaemolyticus*)

Species	Identity (%)	Length (bp)
8M 4-1	100	1625
V. parahaemolyticus	97.4	1644
V. anguillarum	80.4	1635
V. furnissii	82.3	1638
V. cholera	80.3	1635
V. splendidus	82.6	1647
V. vulnificus	83.2	1641
V. fischeri	82.8	1647
V. harveyi	88.3	1660
V. ordalii	79.4	1635
Allivibrio salmonicida	80.8	1638
V. alginolyticus	72.5	1599
V. rotiferianus	90.4	1647
Pseudomonas fluorescens	68.6	1647
V. scophthalmi	84.0	1647
V. ichthyoenteri	84.3	1647
V. tubiashii	84.8	1647
V. nigripulchritudo	85.6	1647
V. orientalis	86.4	1647
V. sinaloensis	84.7	1647
V. brasiliensis	85.8	1647
V. mimicus	80.7	1635
V. caribbenthicus	82.5	1647
Photobacterium damselae	82.2	1644
V. coralliilyticus	84.3	1647
V. metschnikovii	80.9	1638
V. shilonii	84.7	1636
Grimontia hollisae	53.1	1494

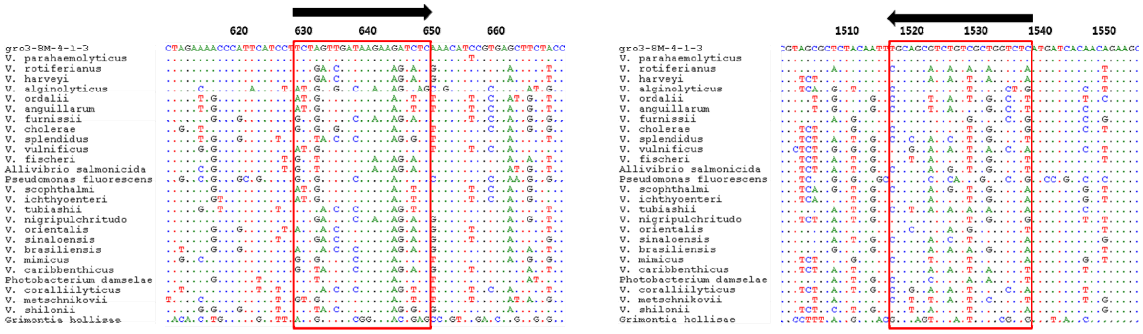

Fig. 4. Locations of species-specific detection primer sequences in the *groEL* gene. Nucleotide identity is indicated by dots. Boxes indicate primer regions and arrows indicate primers.

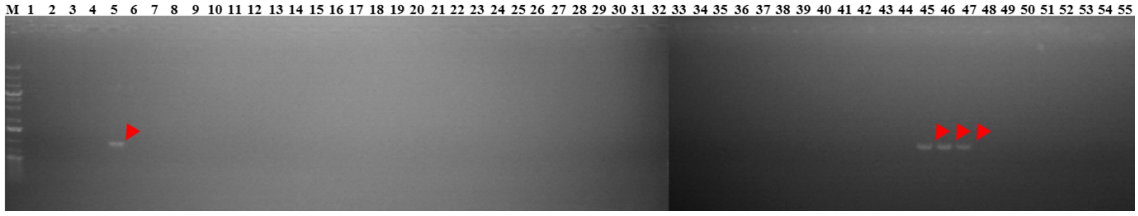

Fig. 5. Agarose gel electrophoresis of *groEL*-targeted species-specific PCR products. Lane 1, 7M 1-25; lane 2, 7M 3-18; lane 3, 7M 19-22; lane 4, 7M 26-3; lane 5, 8M 4-1; lane 6, 8M 28-7; lane 7, *Bacillus cereus*; lane 8, *Edwardsiella tarda*; lane 9, *Enterobacter cloacae*; lane 10, *Enterococcus faecalis*; lanes 11–12, *Escherichia coli*; lane 13, *Klebsiella pneumoniae*; lane 14, *Klebsiella pneumoniae*; lane 15, *Providencia stuartii*; lane 16, *Proteus mirabilis*; lane 17, *P. stuartii*; lane 18, *Pseudomonas aeruginosa*; lanes 19 and 20, *Staphylococcus aureus*; lane 21, *Staphylococcus aureus*; lane 22, *Staphylococcus haemolyticus*; lane 23, *Streptococcus iniae*; lane 24, *Streptococcus mutans*; lane 25, *Streptococcus parauberis*; lane 26, *Streptococcus vestibularis*; lane 27, *S. iniae*; lane 28, *Vibrio aestuarianus*; lane 29, *Vibrio alginolyticus*; lane 30, *Vibrio anguillarum*; lane 31, *V. anguillarum* 01 type; lane 32, *Vibrio campbellii*; lane 33, *Vibrio carchariae*; lane 34, *Vibrio cincinnatiensis*; lane 35, *Vibrio fluvialis*; lane 36, *Vibrio furnissii*; lane 37, *Vibrio harveyi*; lane 38, *Vibrio hollisae*; lanes 39 and 40, *Vibrio ichthyoenteri*; lane 41, *Vibrio mediterranei*; lane 42, *Vibrio mimicus*; lane 43, *Vibrio natriegens*; lane 44, *Vibrio nereis*; lanes 45–47, *Vibrio parahaemolyticus*; lane 48, *Vibrio proteolyticus*; lane 49, *Vibrio rotiferianus*; lane 50, *Vibrio splendidus*; lane 51, *Vibrio tubiashii*; and lanes 52–55, *Vibrio vulnificus*.

specificity and annealing temperatures of 15 combinations of primers (5 forward primers with 3 reverse primers) were analyzed to determine a suitable marker for detection PCR. Among the paired primers, both gEL-8M 4-1-F3 and gEL-8M 4-1-R2 had an annealing temperature of 65°C and led to the production of a band of the predicted length (909 bp) using templates from 8M 4-1 and *V. parahaemolyticus*. Other *Vibrio* species and environmental isolates (Table 4) did not produce the bands (Fig. 5). For the positive control, PCR reactions with primers targeting 16S rDNA were conducted with the same templates. As expected, the positive control PCRs produced a single 1.5-kbp band in every strain (Fig. 6).

Purified 8M 4-1 chromosomal DNA was serially diluted from 4.1 μg to 4.1 pg and used as a template for PCR sensitivity testing. The *groEL*-targeted gene-specific detection PCR produced clear bands of the expected size on agarose gels using as little as 4.1 pg of DNA (Fig. 7).

3) Detection of 8M 4-1 from infected ark shells

To estimate the accuracy and sensitivity of the detection PCR method from infected ark shell samples, PCR reactions were performed with total genomic DNA from moribund ark shell specimens. Purified DNA from control non-infected ark shells was used as the template in negative control PCR reactions. The template DNA from infected samples produced specific amplicons up to 10 ng, but DNA from non-infected

Table 4. Strains used in this study for the PCR detection assay

	Species	Reference	PCR amplification
1	7M 1-25 (*Vibrio harveyi*)	Environmental source	-
2	7M 3-18 (*Vibrio harveyi*)	Environmental source	-
3	7M 19-22 (*Vibrio harveyi*)	Environmental source	-
4	7M 26-3 (*Vibrio alginolyticus*)	Environmental source	-
5	8M 4-1 (*Vibrio parahaemolyticus*)	Environmental source	+
6	8M 28-7 (*Vibrio alginolyticus*)	Environmental source	-
7	*Bacillus cereus*	KCTC 1012	-
8	*Edwardsiella tarda*	Environmental source	-
9	*Enterobacter cloacae*	KCTC 2361	-
10	*Enterococcus faecalis*	KCTC 3206	-
11	*Escherichia coli*	Environmental source	-
12	*Escherichia coli*	ATCC 8739	-
13	*Klebsiella pneumoniae*	KCTC 12385	-
14	*Klebsiella pneumoniae*	KCTC 2208	-
15	*Providencia stuartii*	Environmental source	-
16	*Proteus mirabilis*	KCTC 2510	-
17	*Providencia stuartii*	KCTC 2568	-
18	*Pseudomonas aeruginosa*	ATCC 15522	-
19	*Staphylococcus aureus*	ATCC 6538	-
20	*Staphylococcus aureus*	KCTC 3881	-
21	*Staphylococcus aureus*	KCTC 1928	-
22	*Staphylococcus haemolyticus*	KCTC 3341	-
23	*Streptococcus iniae*	FP5228	-
24	*Streptococcus mutans*	KCTC 3065	-
25	*Streptococcus parauberis*	KCTC 3651	-
26	*Streptococcus vestibularis*	KCTC 3650	-
27	*Streptococcus iniae*	Environmental source	-
28	*Vibrio aestuarianus*	KCCM 40863	-
29	*Vibrio alginolyticus*	KCCM 40513	-
30	*Vibrio anguillarum*	Environmental source	-
31	*Vibrio anguillarum* (Type O1)	Environmental source	-
32	*Vibrio campbellii*	KCCM 41986	-
33	*Vibrio carchariae*	KCCM 40865	-
34	*Vibrio cincinnatiensis*	KCCM 41683	-
35	*Vibrio fluvialis*	KCCM 40827	-
36	*Vibrio furnissii*	KCCM 41679	-
37	*Vibrio harveyi*	KCCM 40866	-
38	*Vibrio hollisae*	KCCM 41680	-
39	*Vibrio ichthyoenteri*	FB 4004	-
40	*Vibrio ichthyoenteri*	KCCM 40870	-
41	*Vibrio mediterranei*	KCCM 40867	-
42	*Vibrio mimicus*	KCCM 42257	-
43	*Vibrio natriegens*	KCCM 40868	-
44	*Vibrio nereis*	KCTC 2722	-
45	*Vibrio parahaemolyticus*	KCCM 11965	+
46	*Vibrio parahaemolyticus*	KCCM 41664	+
47	*Vibrio parahaemolyticus*	KCTC 2471	+
48	*Vibrio proteolyticus*	KCCM 11992	-
49	*Vibrio rotiferianus*	KCTC 12125	-
50	*Vibrio splendidus*	KCTC 12679	-
51	*Vibrio tubiashii*	KCTC 12729	-
52	*Vibrio vulnificus*	KCCM 41665	-
53	*Vibrio vulnificus*	KCTC 2962	-
54	*Vibrio vulnificus*	KCTC 2980	-
55	*Vibrio vulnificus*	KCTC 2982	-

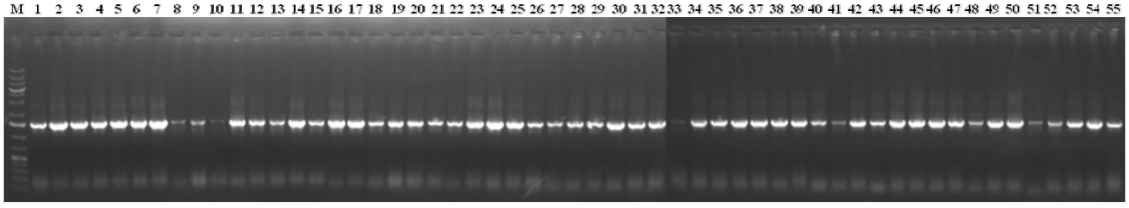

Fig. 6. Agarose gel electrophoresis of 16S rDNA targeted PCR products. Lane 1, 7M 1-25; lane 2, 7M 3-18; lane 3, 7M 19-22; lane 4, 7M 26-3; lane 5, 8M 4-1; lane 6, 8M 28-7; lane 7, *Bacillus cereus*; lane 8, *Edwardsiella tarda*; lane 9, *Enterobacter cloacae*; lane 10, *Enterococcus faecalis*; lanes 11–12, *Escherichia coli*; lane 13, *Klebsiella pneumoniae*; lane 14, *Klebsiella pneumoniae*; lane 15, *Providencia stuartii*; lane 16, *Proteus mirabilis*; lane 17, *P. stuartii*; lane 18, *Pseudomonas aeruginosa*; lanes 19 and 20, *Staphylococcus aureus*; lane 21, *Staphylococcus aureus*; lane 22, *Staphylococcus haemolyticus*; lane 23, *Streptococcus iniae*; lane 24, *Streptococcus mutans*; lane 25, *Streptococcus parauberis*; lane 26, *Streptococcus vestibularis*; lane 27, *S. iniae*; lane 28, *Vibrio aestuarianus*; lane 29, *Vibrio alginolyticus*; lane 30, *Vibrio anguillarum*; lane 31, *V. anguillarum* 01 type; lane 32, *Vibrio campbellii*; lane 33, *Vibrio carchariae*; lane 34, *Vibrio cincinnatiensis*; lane 35, *Vibrio fluvialis*; lane 36, *Vibrio furnissii*; lane 37, *Vibrio harveyi*; lane 38, *Vibrio hollisae*; lanes 39 and 40, *Vibrio ichthyoenteri*; lane 41, *Vibrio mediterranei*; lane 42, *Vibrio mimicus*; lane 43, *Vibrio natriegens*; lane 44, *Vibrio nereis*; lanes 45–47, *Vibrio parahaemolyticus*; lane 48, *Vibrio proteolyticus*; lane 49, *Vibrio rotiferianus*; lane 50, *Vibrio splendidus*; lane 51, *Vibrio tubiashii*; and lanes 52–55, *Vibrio vulnificus*.

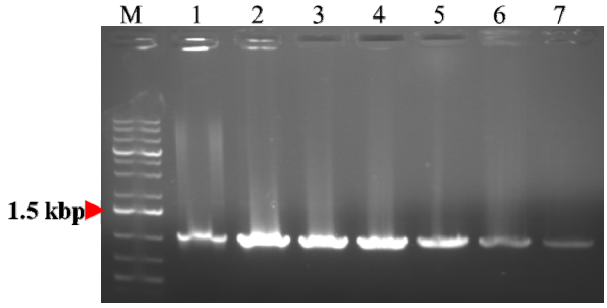

Fig. 7. Sensitivity of the detection PCR assay using purified chromosomal DNA from 8M 4-1. M, DNA ladder; lane 1, 4.15 μg; lane 2, 0.41 μg; lane 3, 0.04 μg; lane 4, 4.15 ng; lane 5, 0.41 ng; lane 6, 0.04 ng; and lane 7, 4.15 pg.

samples did not produce amplicons (Fig. 8). Unfortunately, the level of detection was lower than with purified bacterial DNA.

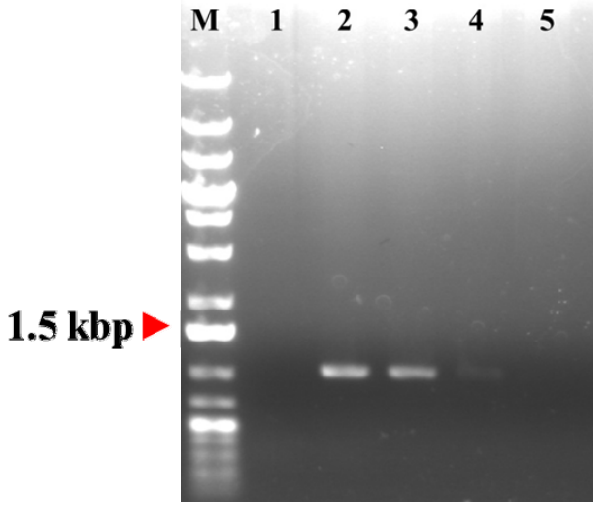

Fig. 8. Detection and sensitivity of PCR with infected ark shells. M, DNA ladder; lane 1, DNA from non-infected ark shell tissue; lane 2, 100 ng of DNA from infected ark shell tissue; lane 3, 10 ng of infected ark shell tissue; lane 4, 1 ng of infected ark shell tissue; lane 5, 0.1 ng of infected ark shell tissue.

Discussion

Through continuous studies, we selected 140 isolates as potential mass mortality-associated pathogens, and identified virulent isolates using mortality tests. Among the 140 isolates, six isolates revealed virulence to ark shell, and 8M 4-1 had the highest cumulative mortality. Therefore, 8M 4-1 is probably a major causative organism of ark shell mass mortality. Using various investigations including molecular biological, biochemical, and microbiological analyse, such as 16S rDNA sequence identity testing, API kit analysis, virulence gene amplification, morphological characteristics, and the *groEL* gene identification, we showed that 8M 4-1 was most closely related to *V. parahaemolyticus*.

Vibrio species are Gram-negative, curved rod-like microorganisms found in various marine environments,

which have been shown to be pathogen to marine organisms (Afi et. al., 2010; Cabral, 2010; Chatterjee and Halder, 2012; Cheng *et al*., 2004; Lacoste *et al*., 2001; Paillard *et al*., 2004; Travers *et al*., 2008). It has been reported that these *Vibrio* species induce vibriosis disease in a variety of aquaculture organisms such as bivalves, fish, and shrimp, resulting in enormous economic loss (Afi et. al., 2010; Frans *et al*., 2011; Paillard *et al*., 2004; Park, 2009). Among *Vibrio* species, *V. parahaemolyticus* is well known to cause mass mortality in various marine organisms including oysters, scallops, clams, fish, and shrimp (Cai *et al*., 2006; Cheng *et al*., 2008, Liu *et al*., 2000; Ruangpan and Kitao, 1991; Torkildsen *et al*., 2005, Yue *et al*., 2010). In addition, the results for the cumulative mortality rate after infection study showed that the 8M 4-1 strain could induce mortality. At 3 days post-injection, moribund ark shell specimens were observed at concentrations from 10^9 to 10^6 CFU/mL, but there were some differences in the number of dead ark shells depending on the concentration. However, almost all of the experimental organisms died within 1 week after infection. Therefore, the LD50 value was found to be lower than 10^6 CFU/mL. Furthermore, the LD50 values of diverse environmentally isolated *V. parahaemolyticus* pathogens were reported to be 10^9-10^6 CFU/mL from various aquaculture organisms (Cheng *et al*., 2008). Therefore, from the LD_{50} value of 8M 4-1 identified in this study, we conclude that 8M 4-1 is a causative strain of mass mortality.

In the preliminary screening test of the primary pathogenic strains, the 8M 4-1 strain had 80% mortality rate with 10^9 CFU/mL among 140 strains. However, in the experiment to determine cumulative mortality through secondary anthropogenic infection, mortality rates of 90–100% were observed at all concentrations (10^9-10^6 CFU/mL), providing further support of the pathogenicity of 8M 4-1. However, the positive control group injected with PBS during the secondary infection test also had increased 30% of mortality rate. Therefore, it is possible that the increased mortality was not due to the virulence of the 8M 4-1 strain, but rather the poor physiological condition of the ark shell at the beginning of the experiment.

After artificial infection, we found physical changes in the ark shells that were consistent with degeneration. The difference was even more obvious when the infected ark shells were compared with those in the non-infected group (Fig. 2.). Moreover, optical microscopy of the ark shell tissues after infection showed histopathological changes that confirmed that the 8M 4-1 strain was pathogenic and the causative agent of mass mortality.

In many studies, *V. parahaemolyticus* and related bacterial strains were identified as causative agents of disease and death in aquatic organisms (Cai *et al*., 2006; Cheng *et al*., 2008, Liu *et al*., 2000; Ruangpan and Kitao, 1991; Torkildsen *et al*., 2005, Yue *et al*., 2010). The characteristics of these microorganisms were reported; however, these investigations were limited to verification of the pathogenic strain and experiments with diverse features. Unfortunately, these studies did not provide solutions to protect against these microorganisms. The use of chemicals such as antibiotics is believed to be the fastest and most reliable prophylactic and therapeutic method, but these strategies have been restricted by many limitations in recent years. For this reason, the goal of our study was to develop a method for monitoring the causative strain of mass mortality and to identify it rapidly before the onset of such mortality events.

Molecular biological methods have made it possible to detect various target microorganisms easily, quickly, and accurately (Hong *et al*., 2007; Hossain *et al*., 2011; Jo *et al*., 2013; Kim *et al*., 2008). The method of gene amplification using PCR is the most suitable and widely used method to obtain accurate and rapid diagnosis results (Hong *et al*., 2007; Hossain *et al*., 2011; Jo *et al*., 2013; Kim *et al*., 2008). Substantial information on genes has also been obtained using these methods (Hong *et al*., 2007; Hossain *et al*., 2011; Kim *et al*., 2008). For PCR detection of *V. parahaemolyticus*, various gene-targeted detection methods have been reported, and the target genes have primarily been virulence genes such as *toxR*, *tlh*, *tdh*, and *trh*, and housekeeping genes such as *rpoS*, *gyrB*, and *groESL* (Coutard *et al*., 2005; Feng *et al*.,

2007; Hossain *et al*., 2011; Kim *et al*., 1999; González *et al*., 2004). However, as shown from the results of this study, we found that some virulence genes in 8M 4-1 had different lengths, non-specific amplification, and no amplification (Fig. 3.). These instances are the result of gene deletions and mutations, which are characteristics of virulence genes (Hossain *et al*., 2011; Kim *et al*., 2008). Therefore, a housekeeping gene was chosen as the target gene for detection. The *groEL* gene, which encodes the chaperonin *groEL* (a 60-kDa heat shock protein), a recently investigated protein, was selected as the target gene.

Because the 8M 4-1 strain is not *V. parahaemolyticus*, but a species closely related to it, the *groEL* gene of 8M 4-1 was amplified and analyzed for species-specific detection PCR with a universal degenerated primer set. Analysis of the 8M 4-1 *groEL* gene showed an average of 80% similarity with other *Vibrio* species, but 97%, 90%, and 88% similarities with *V. parahaemolyticus*, *V. rotiferianus*, and *V. harveyi*, respectively. The lowest identity (54%) was with *V. hollisae* (Grimontia hollisae), but this may have been due to the fact that the known sequence length of *V. hollisae* was too short (Table 3). Among the *Vibrio* species, *V. parahaemolyticus*, *V. alginolyticus*, and *V. harveyi* are closely related strains with similar microbiological characteristics, consistent with similarities in the *groEL* gene in these species. Therefore, the similarities between the *groEL* genes can fully explain microbiological relatedness. A 3% difference in identification also suggests that the taxa are sero-types of the same species, but more research is needed to confirm this.

The PCR reactions using purified bacterial DNA as the template successfully identified only the targeted 8M 4-1 strain and *V. parahaemolyticus* strains listed in Table 4, and generated no false-positive or false-negative amplicons with environmental isolates (Fig. 5.). The sensitivity of the developed assay was found to be similar to that of other PCR-based detection methods (Hossain *et al*., 2011). Using total DNA from infected and control ark shells, selective detection was observed from infected ark shell tissues, but the sensitivity using ark shell total DNA was lower than with purified bacterial DNA. This phenomenon is often reported and occurs because the PCR template consists of total DNA, which includes chromosomal DNA from ark shell tissue and 8M 4-1 (Kim *et al*., 2008). Therefore, the genomic DNA of 8M 4-1 is actually a small part of the total, or the extracts inhibit PCR amplification. These results showed that the monitoring method, which targeted the *groEL* gene, was suitable and useful for detecting *V. parahaemolyticus*-related species. This rapid, simple, and accurate method can be used to predict *V. parahaemolyticus*-associated risks for the aquaculture industry, and can be used in microbiology research in the seafood and diagnosis industry.

In conclusion, the microbiological, molecular-biological, biochemical, and histopathological findings in this study show that the *V. parahaemolyticus*-related 8M 4-1 isolate is associated with ark shell mass mortality. However, increased cumulative mortality was found to be associated with ark shells in poor physical condition; this suggests that ark shells in poor physical condition are more prone to infection by pathogenic bacteria. After spawning, the physical condition of ark shells starts to deteriorate and then, the invasion of pathogenic bacteria increases the probability of mass mortality. Further research on the correlations between the physiological activities of ark shells, various environmental factors, and the presence of pathogenic bacteria is essential to clarify the results of this study.

Acknowledgment

This work was supported by a grant from the National Institute of Fisheries Science (R2019016).

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