Int Aquat Res (2022) 14:241–250
https://doi.org/10.22034/IAR.2022.1958494.1275

ORIGINAL RESEARCH

Development of a method of sensitively and specifically detecting a *Vibrio* sp. strain MA3 associated with mass mortalities of the pearl oyster *Pinctada fucata martensii* using quantitative PCR

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Received: 10 May 2022 / Accepted: 03 November 2022 / Published online: 22 November 2022

Abstract The mass mortality of the Japanese pearl oyster *Pinctada fucata martensii* from 2019 to 2021 had a serious impact on Japan's pearl oyster industry. We previously reported that the possible cause of mass mortality was bacterial infection and that a new strain of *Vibrio* sp. (strain MA3) closely related to *Vibrio alginolyticus* was isolated from dead pearl oysters. Therefore, the development of a method for specifically detecting only strain MA3 as distinguished from other genus *Vibrio* species has been eagerly desired. In the present study, a successful quantitative PCR method was established with primers that specifically amplify the intergenic spacer (IGS) region of the 16S–23S rDNA genes of strain MA3. The specificity of this quantitative PCR was high, and it was possible to amplify only IGS genes prepared from strain MA3; however, it could not amplify IGS genes prepared from *V. alginolyticus* (NBRC15630) of species closely related to strain MA3. Furthermore, this method was highly sensitive and was able to detect up to 10^1 copies of the IGS gene. Using this newly developed quantitative PCR method, strain MA3 in tissues such as the adductor muscles and gills of dead pearl oysters in infection experiments was detected, although strain MA3 could not be detected in non-infected pearl oysters. Additionally, this PCR method was utilized for detecting strain MA3 in natural seawater because it could be detected in natural seawater to which a bacterial solution of strain MA3 had been added. By using the specific detection methods developed in the present study, it was possible to detect strain MA3 in seawater and diagnose infected pearl oysters, which will lead to preventing the mass mortality of pearl oysters.

Keywords Marine bacterium . Bivalves . Infection experiment . Detection method . IGS region

Introduction

The pearl oyster is the bivalve mollusk most commonly used for making high quality pearls, and Japanese pearl oysters (*Pinctada fucata martensii*) are highly regarded around the world (Nagai 2013). However, the mass mortalities of Japanese pearl oysters that occurred in the summers of 2019 and 2020 had a serious impact on Japan's pearl oyster industry. A *Vibrio* sp. strain, MA3, a suspected causative bacterium, was

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detected in dead pearl oysters collected from Ago Bay, Mie Prefecture, where mass mortality has occurred (Sakatoku et al. 2021). This bacterium was not detected in healthy pearl oysters and was a new strain specifically found in this disease. The characterization of this strain indicated that the bacterium was closely related to *V. alginolyticus* (Sakatoku et al. 2021). The gene for hemolysin, which destroys the blood cells of the host, was also found in the *Vibrio* sp. strain MA3 (Sakatoku et al. 2021). Since pearl oyster mass mortality occurred again in 2021, developing a method specifically for detecting and diagnosing infected pearl oysters is eagerly desired.

Bivalve farming is constantly under threat of mass mortality from infection with marine bacteria that inhabit the ocean (for a review, see Destoumieux-Garzón et al. 2020). Bivalves are known to accumulate pathogenic bacteria in their bodies because they feed on planktons by filtering large amounts of seawater containing marine bacteria (Vezzulli et al. 2018; Pierce and Ward 2019). Previously cases of mass mortality of bivalves reportedly have been due to bacterial infections including brown ring disease of the Japanese littleneck clam (*Ruditapes philippinarum*) by *V. tapetis* (Borrego et al. 1996), juvenile oyster disease (*Crassostrea virginica*) by *Roseovarius crassostreae* (Boettcher et al. 2005), and nocardiosis of the pacific oyster (*Crassostrea gigas*) by *Nocardia crassostreae* (Friedman et al. 1998). Only two previous bacterial infections of pearl oysters have been reported: Akoya oyster disease by Spirochaeta (Matsuyama et al. 2017) and black-spot shell disease by *Tenacibaculum* sp. (Sakatoku et al. 2018). Mass mortality caused by bacterial infections is believed to be the result of complex interactions between hosts, environmental factors, and pathogens (Destoumieux-Garzón et al. 2020). The prompt detection of infected individuals and early treatment are very important for preventing disease spread (Adams and Thompson 2012; Leigh et al. 2019). However, a method of detecting strain MA3 has not yet been developed. Thus, in this study, the intergenic spacer (IGS) region from 16S to 23S rRNA was sequenced, and specific quantitative PCR methods were developed using a primer set designed in this region. Using this newly developed quantitative PCR method, strain MA3 could be detected in infected pearl oysters and in natural seawater to which a small amount of bacterial solution of strain MA3 had been added.

Materials and methods

Bacterial strains

Strain MA3 isolated from pearl oysters that died in the mass mortality incident of 2019 was used in the present study (Sakatoku et al. 2021). The 15 type strains of the genus *Vibrio* (*V. alginolyticus* NBRC15630, *V. alginolyticus* NBRC15930, *V. harveyi* NBRC15632, *V. harveyi* NBRC15634, *V. harveyi* NBRC101064, *V. harveyi* NBRC113563, *V. parahaemolyticus* NBRC12711, *V. proteolyticus* NBRC13287, *V. aestuarianus* NBRC15629, *V. campbellii* NBRC15631, *V. natriegens* NBRC15636, *V. azureus* NBRC104587, *V. sagamiensis* NBRC104589, *V. hyugaensis* NBRC110633, and *V. jasicida* NBRC110949) were purchased from the National Institute of Technology and Evaluation (Tokyo, Japan). Primer sets specific to strain MA3 were developed using the above type strains and strain MA3.

DNA extraction from each bacterial strain

Strain MA3 and the 15 type strains were cultured in a liquid medium (0.5% tryptone, 0.5% yeast extract, 0.2% beef extract, and 0.2% sodium acetate prepared in 70% seawater) at 25˚C for 24 hours (Sakatoku et al. 2021). After the bacteria were collected by centrifuging, DNA extraction from the bacteria was performed with the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The extracted genomic DNA was refrigerated at -30˚C for a month until use and utilized as a template for PCR amplification.

Sequence analysis of the IGS region

The IGS region was amplified by PCR (Mastercycler® Nexus, Eppendorf Japan, Tokyo, Japan) using IGSF (5'-TGGGGTGAAGTCGTAACAAGG-3') and IGSR (5'-TCCTTCATCGCCTCTGACTG-3') primer sets (Li et al. 2018). PCR amplification was performed using an initial denaturation step of 95˚C for 1 min, followed by 30 cycles at 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 2 min; the sample was then incubated at 72˚C for 1 min to perform a final extension. The PCR products were purified using a QIAquick PCR

purification kit (QIAGEN, Hilden, Germany) and then inserted into the pGEM®-T Easy Vector (Promega Corporation, Madison, WI, USA) and transformed into *E. coli* DH5α (Takara Bio Inc., Shiga, Japan). The inserted PCR products were confirmed by 2% gel electrophoresis after PCR amplification with M13 primers (Forward primer: 5'-GTAAAACGACGGCCAGT-3'; Reverse primer: 5'-CAGGAAACAGC-TATGAC-3'). The inserted clones were outsourced to a vendor (Macrogen Japan Corp., Tokyo, Japan) to determine their nucleotide sequences. The obtained sequences were classified by the types of tRNA they possessed based on sequence analysis of the tRNA region using software (Ver.12, GENETYX Co., Tokyo, Japan). Specific primers for strain MA3 were designed according to these sequences.

Specificity of primers that amplify the IGS gene of strain MA3

Genomic DNAs extracted from the above 15 species of *Vibrio* bacteria were used as templates to investigate the specificity of the primers. The primer (0.5 μM), template DNA (1 ng), reaction buffer (1x), dNTP (200 mM), and sterilized water were added (total volume 10 ml) in accordance with the manufacturer's instructions (Takara Bio Inc., Shiga, Japan). The PCR reaction consisted of initial denaturation at 95°C for 1 min, 30 cycles of 94°C for 1 min and at 68°C for 1 min, and a final extension at 72°C for 1 minute. The PCR products were analyzed using 2% gel electrophoresis in order to amplify the desired products. After cloning the amplified products by the same method described above, the nucleotide sequences were determined by entrusting them to a vendor (Macrogen Japan Corp., Tokyo, Japan).

Development of a quantitative PCR method

The IGS gene of strain MA3 was amplified using a specific primer set. The PCR products were inserted into pGEM®-T Easy Vector (Promega Corporation, Madison, WI, USA) by the method described above. This plasmid was diluted to 10^1-10^9 copies/μl, and PCR was performed to make a standard curve. The copy number of the IGS gene was calculated according to the method of Tang et al. (2017). The probe was designed by a contractor (Integrated DNA Technologies, Tokyo, Japan). The PCR reaction was performed with the Light-Cycler® 96 System (Roche Diagnostics K.K., Tokyo, Japan) and analyzed using the same company's analysis software. All qPCR reactions were performed in triplicate. The reaction mixture was adjusted in accordance with the kit's manual (Takara Bio Inc., Shiga, Japan); the reaction cycle included initial denaturation at 95°C for 1 min, 60 cycles of 94°C for 1 min and 68°C for 1 min, followed by maintaining at 4°C. Using both 1 pg and 1 ng of the genomic DNAs of strain MA3 and *V. alginolyticus* NBRC15630 as templates, PCR was performed using the procedures described above to confirm the specificity of the developed quantitative PCR method.

Detection of strain MA3 in infected pearl oysters and natural seawater with a bacterial solution added

The infected pearl oysters used in the infection experiment by Sakatoku et al. (2021) were utilized in the present study. In the experimental group, the culture medium of strain MA3 was injected into the adductor muscle (1.7×10^8 CFU/oyster), and the oyster was then held at 28°C for 14 days without feeding. In the control group, sterilized culture medium was injected instead of the bacterial solution. Genomic DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) from 25 mg of adductor muscle, gill, and mantle tissue in the dead pearl oysters (n = 9) by infection with strain MA3 and in the surviving pearl oysters (control group) (n = 4). A sample diluted to a DNA concentration of 10 ng/ml was used as a template and analyzed by quantitative PCR.

Next, strain MA3 was added to the above-mentioned liquid medium (6 ml) at a concentration of 10^6 cells/ml, and it was shake-flask cultured at 25°C for 9 hours. Then, the bacterial solution of 10^9 cells/ml was serially diluted from 10^1, 10^3, 10^5, 10^7, and 10^9 cells/ml. One ml of each diluted bacterial solution was added to 1 L of natural seawater (including some marine bacteria) collected from Tsukumo Bay on the Noto Peninsula and suspended. Thereafter, bacterial cells were collected by filtration, and genomic DNA was extracted by a kit (MP Biomedicals, Santa Ana, CA, USA). Then, the DNA concentration diluted to 1 ng was used as a template and analyzed by quantitative PCR. On the other hand, a sterilized culture medium was added to natural seawater. Thereafter, the seawater sample was analyzed by quantitative PCR as a negative control.

Table 1 Sequence analysis of intergenic (IGS) region of strain MA3

Type[*1]	tRNA genes	Identity[*2]	Length
IGS^{0}		92.24%	325bp
			329bp
IGS^{G}	Glu (UUC)	99.40%	506bp type 1
			506bp type 2
IGS^{I}	Ile (GAU)		657bp
IGS^{IA}	Ile (GAU), Ala (GGC)	90.71% - 97.51%	575bp type 1
			575bp type 2
			599bp
			602bp
IGS^{AG}	Ala (GGC), Glu (UUC)		597bp
IGS^{GLV}	Glu (UUC), Lys (UUU),	99.59%	743bp type 1
	Val (UAC)		743bp type 2

[*1]: Types were classified into 6 types according to the types of tRNA contained in the sequences.
[*2]: Nucleotide sequence identity among the obtained sequences in the same group.

Results

Sequence analysis of the IGS region of strain MA3

The genomic DNA of strain MA3 was amplified by PCR using IGS primers. After that, the PCR products were analyzed by electrophoresis. Several bands were obtained by PCR. These amplified PCR products were sequenced by TA cloning. As a result, 35 types of sequences were obtained and were classified into 6 types according to the types of tRNA contained in the sequences (Table 1). The types of sequences obtained are tRNA-free: IGS^{0}; tRNAGlu-encoding: IGS^{G}; tRNAIle-encoding: IGS^{I}; tRNAIle- and tRNAAla-encoding: IGS^{IA}; tRNAAla- and tRNAGlu-encoding: IGS^{AG}; tRNAGlu-, tRNALys-, and tRNAVal-encoding: IGS^{GLV}.

Design of a specific primer set for strain MA3

In the present study, the sequences in the IGS region of 325 bp to 743 bp were determined. On the basis of those sequences, the primers that specifically amplified the nucleotide sequence of strain MA3 were designed. As a result of comparing the sequences of the IGS region in the purchased *Vibrio* spices with the sequences of 6 types of IGS in strain MA3, it was found that the sequence of IGS^{GLV} type 1 of the MA3 strain possessed a specific sequence in strain MA3. Fig. 1 shows the sequence of IGS^{GLV} type 1 of *V. alginolyticus* NBRC15630, *V. campbellii* NBRC15631, *V. natriegens* NBRC15636, and *V. jasicida* NBRC110949 together with the sequence of IGS^{GLV} type 1 of strain MA3. The designed forward primer (MA3IGSGLVF) and the reverse primer (MA3IGSGLVR) correspond to sequences 435 to 457 and 707 to 729 in the IGS of the strain MA3, respectively. No sequence completely matched the designed primer sequences, although a BLAST search was performed on the sequences of primers MA3IGSGLVF and MA3IGSGLVR.

Confirmation of the specificity of a primer set designed by PCR

Using the designed primers, PCR was performed with DNA extracted from *Vibrio* sp. strain MA3 and the purchased 15 types of *Vibrio* bacteria. Thereafter, the PCR products were analyzed by gel electrophoresis (Fig. 2). As a result, specific amplification could be observed only in strain MA3, and the specificity of the designed primer set could be confirmed.

Development of a quantitative PCR method

The plasmids inserted in genes of strain MA3 were serially diluted from 10^{1} to 10^{9} copies and amplified by quantitative PCR using the specific primer set and a specific probe (Fig. 3) to make a standard curve.

As a result, the coefficient of determination (R^{2}) was 0.9939, showing a strong negative correlation between Cq values and the number of plasmid copies (Fig. 4).

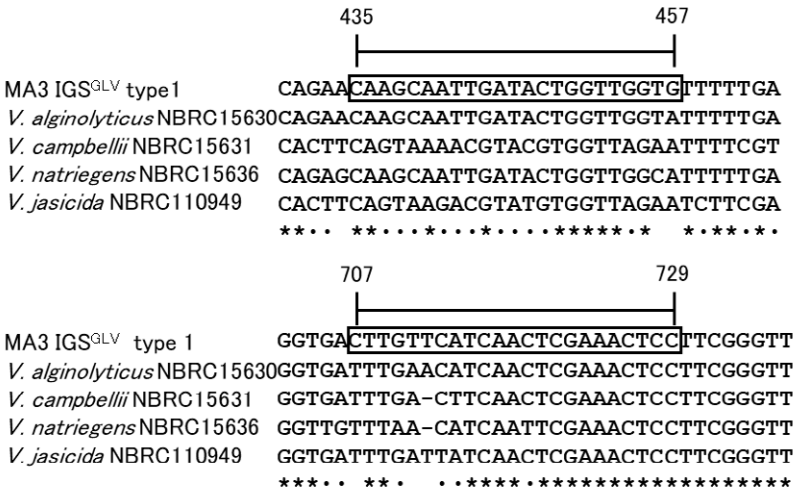

Fig. 1 Multiple alignments of IGS nucleotide sequences from *Vibrio* sp. MA3 and from 4 species of *Vibrio* strains. Forward primer (MA3IGSGLVF) and reverse primer (MA3IGSGLVR) were located in the IGS region at 435 to 457 and 707 to 729, respectively. Each specific primer is surrounded by a square. Dots indicate that the nucleotide sequences of 3 to 4 strains are homologous among 5 strains. The asterisk indicates that the nucleotide sequences of all strains are homologous among the 5 strains.

Fig. 2 Confirmation of the specificity of the designed primers for detecting strain MA3 by PCR. Lane M: DNA ladder marker; Lane 1: *Vibrio* sp. MA3; Lane 2: *V. alginolyticus* NBRC15630; Lane 3: *V. alginolyticus* NBRC15930; Lane 4: *V. harveyi* NBRC15632; Lane 5: *V. harveyi* NBRC15634; Lane 6: *V. harveyi* NBRC101064; Lane 7: *V. harveyi* NBRC113563; Lane 8: *V. parahaemolyticus* NBRC12711; Lane 9: *V. proteolyticus* NBRC13287; Lane 10: *V. aestuarianus* NBRC15629; Lane 11: *V. campbellii* NBRC15631; Lane 12: *V. natriegens* NBRC15636; Lane 13: *V. azureus* NBRC104587; Lane 14: *V. sagamiensis* NBRC104589; Lane 15: *V. hyugaensis* NBRC110633; Lane 16: *V. jasicida* NBRC110949.

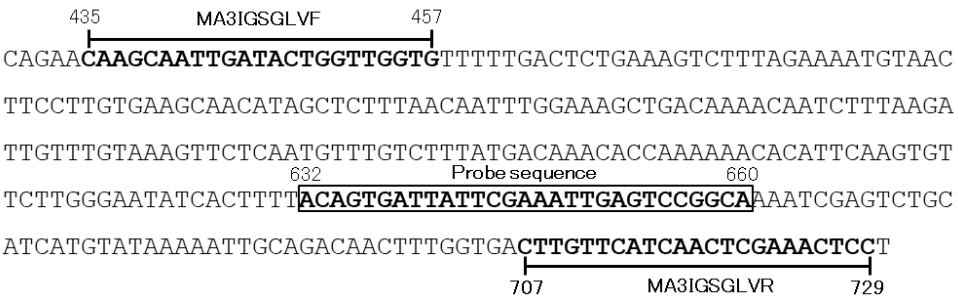

Fig. 3 Location of the probe for quantitative PCR amplification. The probe was located in the IGS region at 632 to 660, which is indicated by the surrounding square box and bold characters. The specific primer set (MA3IGSGLVF: 435 to 457; MA3IGSGLVR: 707 to 729) is shown in bold type.

It was possible to develop a highly sensitive quantitative PCR method because the detection limit was 10^1 copies.

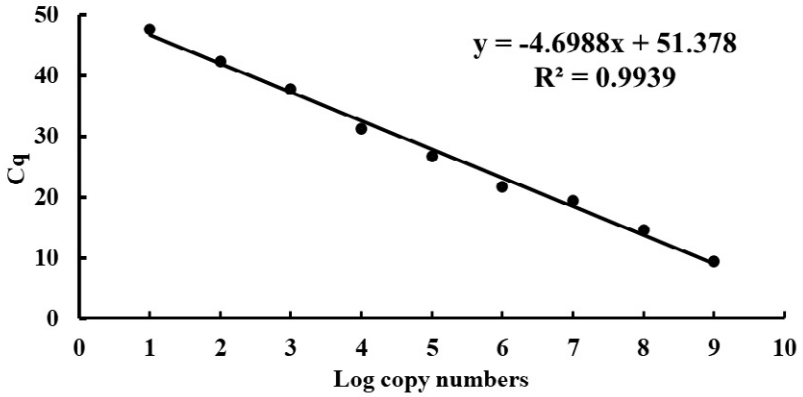

Fig. 4 The standard curve of quantitative PCR for detecting *Vibrio* sp. strain MA3. There was a strong negative correlation between Cq values and the number of plasmid copies.

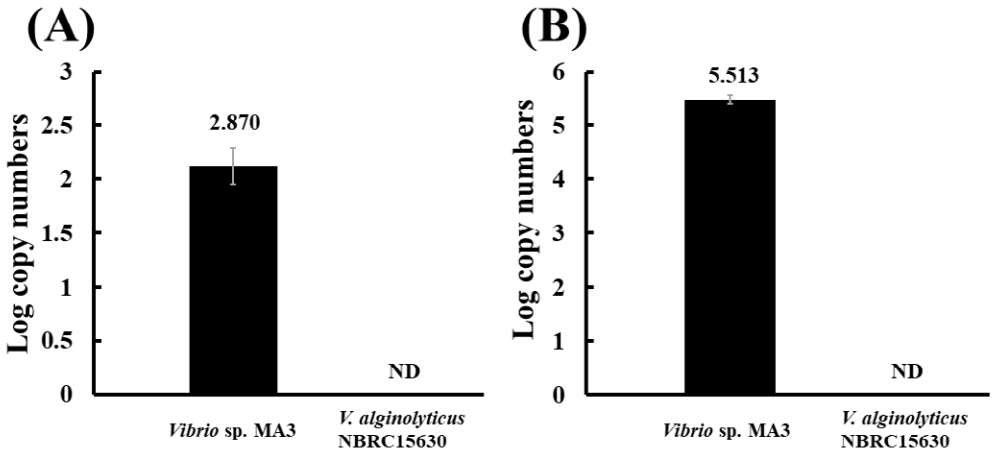

Fig. 5 Specificity of real-time PCR with specific primers for detection in the genomic DNA (A: 1 pg/ml; B: 1 ng/ml) of *Vibrio* sp. strain MA3 and *Vibrio alginolyticus* NBRC15630.

Using the genomic DNA of *Vibrio* sp. strain MA3 and *V. alginolyticus* NBRC15630 as templates, quantitative PCR was performed by the same methods as described above. As a result, at 1 pg and 1 ng, the IGS gene in strain MA3 could be amplified; however, the IGS gene in *V. alginolyticus* NBRC15630 could not be amplified (Fig. 5).

Detection of MA3 strains in the adductor muscles, gills, and mantles of pearl oysters infected and uninfected with strain MA3

All infected pearl oysters died. Blackening of the gills and atrophy of the mantle were observed in the infected oysters as compared with the gills and mantles of the control oysters (Fig. 6).

The IGS genes in the strain MA3 collected from the adductor muscle, gills, and mantle tissues of individuals that died in the infection experiment were analyzed by quantitative PCR. The copy number of the IGS gene of strain MA3 in each tissue is shown in a box plot (Fig. 7). IGS genes could be quantified in the adductor muscle and gills, but only one sample could be detected in the mantle (Fig. 7). On the other hand, IGS genes of strain MA3 were not detected in the control pearl oysters.

Detection of strain MA3 in natural seawater with a bacterial solution added

The results are shown in Fig. 8. IGS genes of strain MA3 were detected by adding strain MA3 to natural seawater containing other marine bacteria. A strong correlation (coefficient of determination: 0.9971) was obtained between the concentration of the added bacterial cells and the copy number of the IGS gene in

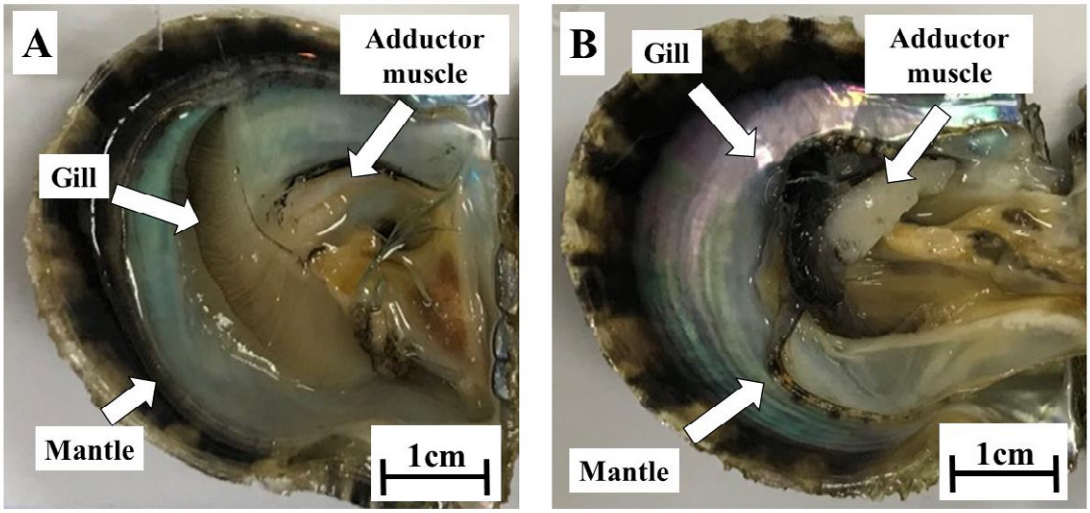

Fig. 6 Photographs of dissected pearl oysters (A: non-infected pearl oyster; B: infected pearl oyster). Remarkable atrophy of the mantle and blackish discoloration of the gill were caused in the experimental group as compared with non-infected pearl oysters.

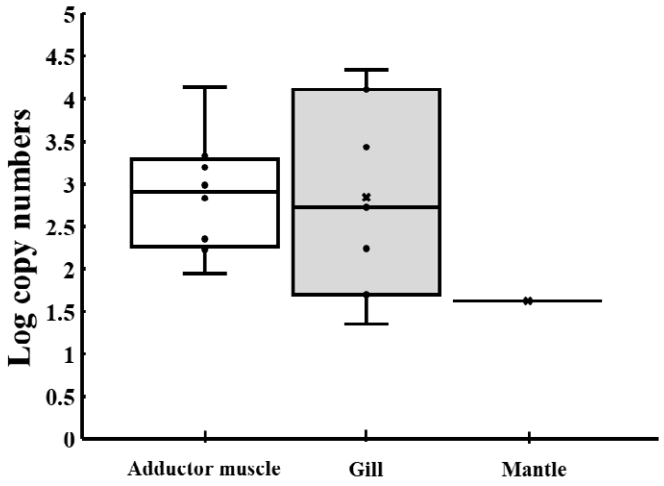

Fig. 7 The copy numbers of IGS genes in the adductor muscle, gills, and mantles of dead individuals in the infection experiment. The DNA samples (10 ng) extracted from the adductor muscle, gills, and mantle were used for quantitative PCR. In non-infected pearl oysters, the IGS gene was not detected. Control group: n = 4; Experimental group: n = 9.

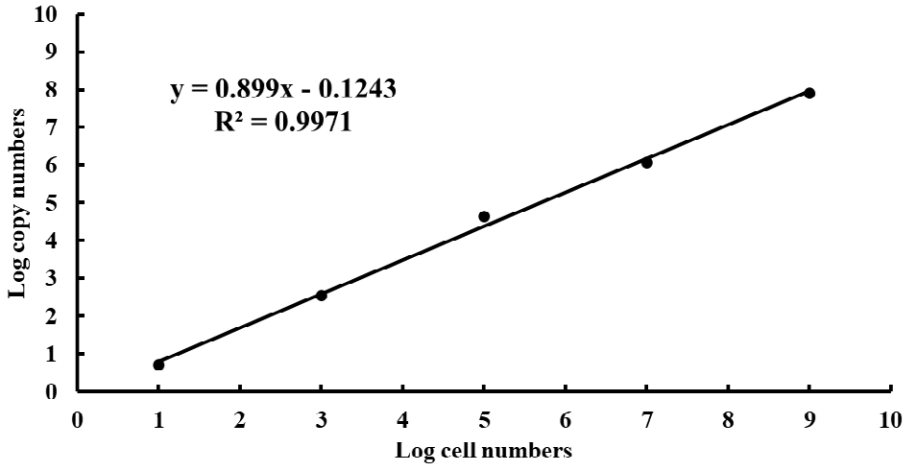

Fig. 8 Specific detection of strain MA3 in natural seawater to which the bacterial solution was added. There was a strong positive correlation between the number of bacterial cells and the copy numbers of the IGS gene in strain MA3. Each value is the mean of triplicates.

strain MA3. On the other hand, IGS genes of strain MA3 could not be detected in the negative control without the addition of the strain MA3 bacterial solution.

Discussion

In the present study, a quantitative PCR method was developed for strain MA3, which is highly associated with the mass mortality of pearl oysters. Quantitative PCR has been used for the detection and quantification of pathogens in shellfish due to its higher specificity and sensitivity as compared with other diagnostic methods (Campbell and Wright, 2003; Carrasco et al. 2013; López-Sanmartín et al. 2019). The common method for detecting and identifying bacteria is amplifying and analyzing the 16S rRNA gene by PCR. The 16S rRNAs of *Vibrio* bacteria such as *V. cholerae*, *V. mimicus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, and *V. campbellii* have very high sequence similarity (Hoffmann et al. 2010; Neogi et al. 2010; Sakatoku et al. 2021); however, it is inappropriate to use this gene in the specific detection of strains. The 16S–23S rRNA gene region contains the tRNA gene and a non-cording region, and the gene exists between the 16S rRNA gene and the 23S rRNA gene. Since the majority of this gene is a non-coding amino acid, it evolves faster and has a higher mutation rate than 16S rRNA. Therefore, the IGS region can be recognized as a characteristic of individual strains (Maeda et al. 2000; Carrasco et al. 2013; Pourahmad et al. 2019). In this study, primers were designed to target the IGS region, and a specific quantitative PCR method was developed that can amplify the IGS region separately from that of the closely related species *V. alginolyticus* NBRC15630. Furthermore, the IGS gene in the MA3 strain could be detected in several tissues of pearl oysters experimentally infected with strain MA3 and in natural seawater to which the bacterial solution of strain MA3 had been added. Therefore, it is highly possible that the quantitative PCR method developed will lead to the early detection of MA3 strain infections in pearl oysters and likely will prevent the mass mortality of pearl oysters due to MA3 strains.

In pearl oysters that died due to infection, the adductor muscle, gills, and mantles were analyzed by quantitative PCR. Strain MA3 was detected in the adductor muscles and gills of most individuals but in the mantle of only one individual. Since strain MA3 was injected into the adductor muscle, the detection of strain MA3 in the adductor muscle is reasonable. The detection of strain MA3 in the gills suggests that the gill is an appropriate tissue for strain MA3 detection. After strain MA3 was injected, it was expected that the bacteria would be hematogenously disseminated into the gills. The gills, which act as bivalves' main filter for feeding and breathing, are tissues known to accumulate pathogens such as bacteria and viruses (Wang et al. 2014). Therefore, the gills seem to be the best choice for detecting strain MA3 in oysters. However, it is unclear whether the MA3 strains injected were growing in the host. Therefore, the MA3 strain in each tissue of the pearl oyster during the infection experiment will be quantified over time in order to clarify the growth of the MA3 strain within the host.

Contracted mantles and gills that have turned black are symptoms reportedly associated with this mass mortality (Sakatoku et al. 2021). In the infected pearl oysters studied as well as those victims of mass mortality, the blackening of gills and atrophy of the mantle were observed. Since strain MA3 could not be detected from the mantle, it is estimated that the number of strain MA3 present in the mantle is quite small. Therefore, it is possible that strain MA3 does not directly contribute to the contraction of the mantle. On the other hand, since a considerable amount of strain MA3 was detected in the gills, it is possible that gill blackening itself is directly caused by the bacteria. The contraction of the mantle in dead pearl oysters is expected to be associated with toxins produced by strain MA3.

Hemolysin, the most common virulence factor of *Vibrio* species, seems to be one of the toxins produced by the MA3 strain suspected of causing mass mortality (Avendaño-Herrera et al. 2014; Zhang and Austin 2005; Sakatoku et al. 2021). Five types of hemolysins, including thermolabile hemolysin (TLH), have been reported in *V. alginolyticus*, a species closely related to strain MA3 (Jia et al. 2010). In this study, the total nucleotide sequence of TLH hemolysin, which exhibits hemolytic activity, in strain MA3 was determined (Sakatoku et al. 2021). Future plans involve inserting this hemolysin gene into *Escherichia coli* so that it will express hemolysin, after which the function of hemolysin at the protein level will be analyzed.

V. alginolyticus is a bacterium that proliferates actively when seawater temperatures rise. It has been reported to be an opportunistic pathogen caused by co-infection with other pathogens and by a decrease in the host's immune capacity due to environmental stress (Li et al. 2019; Destoumieux-Garzón et al. 2020). In

infection experiments, pearl oysters did not die at a temperature of 25°C, but they did die at a temperature of 28°C, which caused mantle contraction (Sakatoku et al. 2021). Since elevated seawater temperature is known to significantly affect bivalve immunity (Matozzo et al. 2012; Meng et al. 2017; Rahman et al. 2019; Trigg et al. 2020), it may be necessary to investigate the relationship between water temperature and the immune system in pearl oysters.

Conclusion

The present study involved the successful development of a quantitative PCR method with primers that specifically amplify the IGS region of the 16S–23S rDNA genes of strain MA3. The specificity of this quantitative PCR was high, and it was possible to amplify only IGS gene prepared from strain MA3, but it could not amplify IGS gene prepared from *V. alginolyticus* (NBRC15630) of a species closely related to strain MA3. Furthermore, this method was highly sensitive and was able to detect up to 10^1 copies of the IGS gene. Using the developed quantitative PCR method, strain MA3 was detected in tissues such as the adductor muscles and gills of dead pearl oysters in infection experiments, although strain MA3 could not be detected in non-infected pearl oysters. Since the detection of strain MA3 depends on the concentration of the bacterial solution added to natural seawater, the developed PCR method was utilized for detecting strain MA3 in natural seawater. Thus, the specific detection method developed made it possible to detect strain MA3 in seawater and to diagnose infected pearl oysters. This accomplishment will contribute to preventing the mass mortality of pearl oysters.

Competing interests The authors have no competing interests to declare.

Acknowledgments This study was supported in part by grants to A.S. (Grant-in-Aid for Scientific Research [C] No. 20K06203 by JSPS and Nos. 20E003, 21E018 and 22K008 by Kurita Water and Environment Foundation). This work was partly supported by the cooperative research program of the Institute of Nature and Environmental Technology, Kanazawa University, Accept Number 22040.

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