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RESEARCH PAPER

A Novel PCR Detection Method for Major Fish Pathogenic Bacteria of *Vibrio anguillarum*

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Abstract

For the rapid identification and detection of *Vibrio anguillarum*, we have PCR amplification technique targeting *gyrB* region has been evaluated. We have designed twosets of PCR primers for the specific amplification of *gyrB* in *V. anguillarum* using single and nested PCR. PCRs specificity was demonstrated by successful amplicon from *V. anguillarum* DNA. The detection limit of the single PCRs and the nested PCR were 4.0 pg and 40 fg of *V. anguillarum* DNA, respectively. Using the nested PCR, the direct sensitive detection of *V. anguillarum* from organs of diseased fishes is possible.

Keywords: *Vibrio anguillarum*, PCR detection, *gyrB.*

Introduction

Vibrioanguillarum (syn. *Listonellaanguillarum*) is a gram-negative, short, rod-shaped bacterium with motility primarily enabled by a single polar flagella. This bacterium has been identified as the main cause of fish vibriosis, which has caused severe economic losses in the fish farming industry (Ganzhorn, 2005; Actis *et al.*, 2011).

To control and monitor the outbreak of vibriosis in fish farms, development of a method for rapid diagnosis is important. Several methods have been previously developed to identify *V. anguillarum*, such as the selective medium method(Alsina*et al*., 1994), API 20E system (Grisez*et al*., 1991), fluorescent antibody technique (Miyamoto and Eguchi, 1997), pulsed-field gel electrophoresis analysis (Skov *et al*., 1995), and DNA hybridization (Martinez-Picado*et al*., 1996). However, these methods require considerable time and effort to identify *V. anguillarum*.On the other hand, several scientists have presented a method using PCR primers for identification, detection, and functional analysis of *V. anguillarum*. This method is desirable because PCR is a simple, sensitive, and efficient method for the detection of pathogenic bacteria from domesticated or wild animals including fish. The targeted genomic regions they used as primers were 16S rDNA (Kita-Tsukamoto *et al.*, 1993; Urakawa *et al*., 1997),*rpoN* gene (Gonzalez *et al.*, 2003), hemolysin gene (Hirono *et al.*, 1996; Rodkhum *et al.*, 2006),*amiB* gene (Hong *et al*., 2007), *rpoS* gene (Kim *et al*., 2008), *empA* gene (Xiao*et al.*, 2009). However, it is generally believed that the evolutionary rate of non-protein-coding regions, such as 16S rDNA, is slower than that of protein-coding regions and that the phylogenetic resolution of 16S rDNA is sometimes not sufficient to design specific PCR primers (Yamamoto and Harayama, 1998; Küpfer*et al.*, 2006).In addition,*V. anguillarum*has a very close phylogenetic relationshipwith other *Vibrio* species based on genetic analysis of 16S rDNA and *recA* regions (Kita-Tsukamoto *et al.*, 1993; Urakawa *et al.*, 1997; Thompson *et al.*, 2004).Thus, the 16S rDNA region may not be the most suitable for designing specific PCR primers to detect and identify *V. anguillarum*. Moreover, in the case of *rpoN*and thehemolysin gene, it is reported that these PCRs amplify the false positive band from *V. ordalii* in some conditions (Hirono *et al.*, 1996; Gonzalez *et al.*, 2003; Rodkhum *et al.*, 2006). Thus, it is necessary to check the functionality and specificity of primers to ensure that they are of sufficiently high quality to avoid a critical problem associated with PCR, which is the detection of pseudogene sequences with primers that are not optimal for the target gene (Izumi *et al.*, 2005).We suggest that the chromosomal DNA coding B subunit of the DNA gyrase (*gyrB*), which plays a role in the detection of pathogenic bacteria in aquatic animals, is the superior region to make highly specific PCR primers(Venkateswaran *et al*., 1998; Izumi and

Wakabayashi, 2000; Izumi *et al*., 2007a;Lan*et al*., 2008; Persson*et al*., 2015).

In the present study, we evaluated the sensitivity and specificity of PCR amplification techniques to identify *V. anguillarum*. The target region of the PCR primers was located in *gyrB* of *V. anguillarum*. Further, we also describe the application of this PCR technique for the direct detection of *V. anguillarum* from the gills, kidneys, andbody surface lesionsof the rainbow trout (*Oncorhynchusmykiss*) and ayu(*Plecoglossusaltivelisaltivelis*).

Materials and Methods

Bacterial Strains and Growth Conditions

Seven isolates of *Vibrio anguillarum* including type strain (strain no. ATCC19264; derived from cod) and unidentified 15 white-pigmented bacterial isolates from the gill, kidney, and lesion of various fishes were used for determination of *gyrB* sequences (Table 1). To identify these white-pigmented bacterial isolates, of those *gyrB* sequences were compared with the NCBI GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), and the biochemical properties were inspected using the API 20 NE system (bioMérieux, Marcy l'Etoile, France). Twenty-five strains of other *Vibrio* species and related 7 bacterial strains were used to verify the PCR specificity (Table 2). All the strains were routinely cultured at their optimum temperatures on heart-infusion agar, Luria-Bertani ager, or tryptic-soy agar supplemented with 1.5% NaCl.

DNA Extraction

TemplateDNA of bacterial isolates for the *gyrB* sequence determination and specificity of the PCRs was prepared according to previous studies (Walsh *et al*., 1991; Izumi and Wakabayashi, 1997). Briefly, one loop of bacterial pellet was mixed with 300 µL of 5% Chelex100 (Sigma, MO, USA) and incubated at 55°C for 30 min. Following mixing by vortex at high speed for 5-10 s, the mixture was boiled for 20 min and then centrifuged for 10 min at 10,000 g. Without further purification, an aliquot of the supernatant containing DNA was used as the template for PCR amplification.

To determine the sensitivity of the PCR, DNA of *V. anguillarum* (strain no. ATCC19264) was prepared with PureLink DNA Extraction Kit (Invitrogen/Thermo Fisher Scientific, MA, USA). DNA concentration was measured spectrophotometrically at 260 nm using a UV-1650PC spectrophotometer (Shimadzu, Kyoto, Japan).

The *Gyrb* Sequence Determination

The *gyrB* region was amplified by PCR using the

Table 1. *Vibrio angillarum* and White-pigmented bacterial isolates used for the determination of *gyrB* sequences and the biochemical property inspection with API 20 NE

Bacterial species and strain number	Isolation year	Host fish	Isolated from	Identification by *gyrB* sequencing (Identities%)[*1]	API 20 NE result	Accession number
Vibrio angillarum[*2]						
ATCC19264	1956	Cod	Lesion	*V. angillarum* NCMB6 (100)	N.D.[*3]	AB373053
GMA5-5	2005	Ayu	Kidney	*V. angillarum* NB10 (100)	N.D.	AB373054
GMA5-80	2005	Ayu	Kidney	*V. angillarum* NCMB6 (98.9)	N.D.	AB373055
GMA5-144	2005	Rainbow trout	Kidney	*V. angillarum* NCMB6 (98.9)	N.D.	AB373056
GMW-45	2006	Rainbow trout	Kidney	*V. angillarum* NCMB6 (98.9)	N.D.	AB373057
GMW-48	2006	Rainbow trout	Kidney	*V. angillarum* NCMB6 (98.8)	N.D.	AB373058
GMW-51	2006	Char	Kidney	*V. angillarum* NCMB6 (98.8)	N.D.	AB373059
White-pigmented bacterial isolates						
GM2311	1998	Ayu	Kidney	*Pseudomonas fluorescens* (96.7)	No identification	AB373060
GMW-4	2004	Carp	Gill	*Aeromonas hydrophila* (98.1)	*A. hydrophila*	AB373061
GMW-5	2004	Carp	Kidney	*Shewanella xiamenensis* (99.3)	No identification	AB373062
GMW-10	2004	Crucian carp	Kidney	*A. hydrophila dhakensis* (98.7)	*A. hydrophila*	AB373063
GMW-12	2004	Carp	Kidney	*A. hydrophila* (98.9)	*A. hydrophila*	AB373064
GMW-15	2004	Carp	Kidney	*Klebsiella oxytoca* (99.6)	No identification	AB373065
GMW-20	2004	Carp	Kidney	*A. sobria* (98.4)	*A. hydrophila*	AB373066
GMW-23	2004	Carp	Lesion	*Aeromonas sp* (98.4)	*A. hydrophila*	AB373067
GMW-27	2005	Motsugo	Kidney	*S. baltica* (97.5)	No identification	AB373068
GMW-31	2006	Yamame	Kidney	*A. salmonicida salmonicida* (100)	No identification	AB373069
GMW-33	2006	Char	Kidney	*A. salmonicida salmonicida* (100)	No identification	AB373070
GMW-35	2006	Char	Kidney	*A. bestiarum* (100)	*A. hydrophila*	AB373071
GMW-37	2006	Goldfish	Kidney	*P. putida* (99.8)	*P. putida*	AB373072
GMW-38	2006	Char	Kidney	*A. salmonicida salmonicida* (100)	No identification	AB373073
GMW-40	2006	Rainbow trout	Kidney	*Aeromonas sp* (98.4)	*A. hydrophila* or *sobria*	AB373074

[*1]Result of BLAST programs search nucleotide databases using determined the *gyrB* sequences in this study.
[*2]*Vibrio angillarum* isolates except for ATCC19264 were from Gunma Prefecture, Japan.
[*3]N.D.: Not done.
All isolates were grown on heart-infusion ager at 18°C.

Table 2. Bacterial strains used for PCR specificity

Bacterial species	Used culture ager	Strain number
Vibrio parahaemolyticus	TS[*1]	NBRC12711
V. aestuarianus	TS	NBRC15629
V. alginolyticus	TS	NBRC15630
V. campbellii	TS	NBRC15631
Photobacterium damselae subsp. *damselae*	TS	NBRC15633
V. harveyi	TS	NBRC15634
V. mediterranei	TS	NBRC15635
V. natriegens	TS	NBRC15636
V. orientalis	TS	NBRC15638
V. penaeicida	TS	NBRC15640
V. splendidus	TS	NBRC15643
V. tubiashii	TS	NBRC15644
V. vulnificus	TS	NBRC15645
V. ichthyoenteri	TS	NBRC15847
V. diazotrophicus	TS	IAM14402
V. fluvialis	TS	IAM14403
V. gazogennes	TS	IAM14404
V. metschnikovii	TS	IAM14406
V. nereis	TS	IAM14407
Listonella pelagia	TS	IAM14408
V. proteolyticua	TS	IAM14410
V. haliotico	TS	IAM14596
V. equitatus	TS	IAM14957
V. superstes	TS	IAM15009
V. ordalii	TS	ATCC33509
Aeromonas salmonicida masoucida	TS	1-a-1
A. salmonicida salmonicida	TS	FPC367
Edwardsiella tarda	LB[*2]	JCM1656
Escherichia coli	TS	IAM1239
Pseudomonas aeruginosa	LB	IAM1514
P. fluorescens	LB	IAM12022
P. putida	LB	FPC333

[*1]TS=tryptic-soy agar. [*2]LB=Luria-Bertani agar.
All isolates were grown at 25°C.

degenerated primers UP1 (5'-CAYGCNGGNGGNAARTTYGA-3') and UP2r(5'-TCNACRTCNGCRTCNGTCAT-3') designed by Yamamoto and Harayama (Yamamoto and Harayama, 1995). PCR amplification was performed in a total reaction volume of 10 µL with a GeneAmpPCR System 9700 (Applied Biosystems/Thermo Fisher Scientific,MA, USA). The reaction mixture contained 1 µL of template DNA, 2 nmol of each dNTP, 10 pmol of each primer, and 0.25 unit of EX*Taq* DNA polymerase (Takara Bio, Shiga, Japan). The PCR conditions were 35 cycles of amplification consisting of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 90 sec, followed by one cycle of 72°C for 5 min. The amplicon of 1.2 kb was analyzed by direct sequencing using the PCR primers UP1 and UP2r with an Applied Biosystems 3730*xl* DNA Analyzer and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems/Thermo Fisher Scientific) atTakara Bio. DNA sequence data were analyzed with the computer software of CHROMAS LITE(http://www.technelysium.com.au/).

Design of Specific PCR Primers and PCR Amplification of *V. anguillarum*

To design specific oligonucleotide primers for *V. anguillarum*, the *gyrB* sequence of 7 isolates of *V. anguillarum* and 15 white-pigmented bacterial isolates in Gunma Prefecture, 2 strains of *V. anguillarum*and 24 strains of other *Vibrio* specieson the GenBank database, were used (Table 1 and 3). Two sets of PCR primers, Va-GBF2/Va-GBR1 and Va-GBF1/Va-GBR1, were designed for the specific amplification of *V. anguillarum*. The nucleotide sequences and locations of these 3 primers in Table 4. PCR amplification was performed in a total reaction volume of 10 µL with a GeneAmpPCR System 9700 (Applied Biosystems/Thermo Fisher Scientific). The reaction mixture contained 1 µL of template DNA, 2 nmol of each dNTP, 10 pmol of each primer, and 0.25 unit of EX*Taq* DNApolymerase (Takara Bio). In the case of nested PCR, after the first PCR amplification with the external primers set, Va-GBF2/Va-GBR1, each PCR product was diluted to 5% with 1/10 TE buffer (1 mMTris-HCl, 0.1 mM EDTA, pH 8.0), and

Table 3. The g*yrB* sequences of *Vibrio* species used for PCR primer design

Species name	Strain number	GenBank accession no.
V. angillarum	610	AM162569
V. angillarum	06/09/23	EF064158
V. aestuarianus	01/32	AJ582818
V. campbellii	NBRC15631	AY946040
V. chagasii	LMG21353^T	AJ577820
V. cholerae	323	DQ316974
V. cholerae	O395	NC_009457
V. crassostreae	LGP7	AJ582799
V. cyclitrophicus	LMG21359	AM162562
V. diazotrophicus	IAM1442	AY988154
V. fischeri	ATCC7744	AY455874
V. gigantis	LGP13	AJ577817
V. harveyi	ATCC14126^T	DQ648280
V. kanaloae	LMG20539	AM162563
V. lentus	CIP107166	AM162564
V. natriegens	IFO15635	AY988156
V. parahaemolyticusl	NCMB1902	AM235735
V. pomeroyi	LMG20537^T	AJ577822
V. proteolyticus	IFO13287	AY988157
V. splendidus	ATCC33125	EF380261
V. tapetis	GDEI	AM118101
V. tasmaniensis	LMG20012^T	AJ577823
V. vulnificus	ATCC27562	AY705491
V. orientalis	ATCC33934	EF380260
V. fischeri	MJ-1	EF380254
V. salmonicida	ATCC43839	EF380256

Table 4. PCR conditions and oligonucleotide sequences of primers used in this study

Primer name	Sequence(5'to3')	Location*
Va-GBF1	CGTAACGGCGCTATTCACACA	64 to 84
Va-GBR1	TCCCATCGTCACGCTCAGAGC	409 to 389
Va-GBF2	GTGTCGGTGTGTCCGTCGTC	8 to 27

* The locations represent *gyrB* region of seven isolates of *Vibrio anguillarum* using in this study (GenBank accession no. AB373053-AB373059).

then used as the template of the second PCR amplification with the internal primers set, Va-GBF1/Va-GBR1. The PCR cycling protocols for amplification were carried out at 94°C for 5min, followed by 35 cycles of at 94°C for 30 s, at 68°C for 30 s, at 72°C for 60 s and a final extension step at 72°C for 5 min. The presence of amplified product was confirmed by 2% L03 agarose gel (Takara Bio) electrophoresis in Tris-acetate-EDTA buffer with ethidium bromide stain.

Specificity and Sensitivity of the PCRs

The specificity of two sets of PCR primers, Va-GBF2/Va-GBR1 and Va-GBF1/Va-GBR1, were evaluated with 7 isolates of *V. anguillarum*, 15 white-pigmented bacterial isolates, 25 strains of other *Vibrio* species, and related 7 bacterial strains listed in Table 1 and 2.The specificity of PCR experiments were repeated two times for the assessment of technological reproducibility and stability.

The sensitivity of the PCRswere evaluated with high purity extracted DNA of *V. anguillarum* using PureLink DNA Extraction Kit (Invitrogen/Thermo Fisher Scientific)..A serial 10-fold dilution of extracted DNA was used as templates ranging from 40 ng to 40 ag per PCR tube. The sensitivity of single PCR with the external primers, single PCR with the internal primers, and nested PCR with the external and internal primer set were compared.

PCR Detection from Diseased Fish

To detect *V. anguillarum* directly from the diseased fish by the PCR, two types of samples were prepared. The one was the gill, kidney, and body surface lesion of moribund rainbow trout from the farm where the outbreak of vibriosis had been confirmed by the culture method. The other was the gill washings of ayu that had been challenged experimentally with *V. anguillarum*. In short, the cultured *V. anguillarum*was diluted to $1.5.\times10^5$ CFU/ml with well freshwater in the 500 Lfiber reinforced plastics (FRP) tank. Ayu were entered into theFRP tank for 24 hours. After exposure to *V. anguillarum*, ayu was reared withrunning well freshwater at 16°C. Thereafter, the gill of ayuwhich died of infection challenge was used as gill washing sample.The templates of

rainbow trout were prepared with PureLink DNA Extraction Kit (Invitrogen/Thermo Fisher Scientific). The templates of ayu were prepared according to the previous paper (Izumi *et al.*, 2005). Using these templates DNA, we analyzed single PCR with the internal primer set and nested PCR with the external and internal primer sets.

Results

The *Gyrb* Sequences

Approximately 1.2 kb amplicons were observed by PCR with the universal primers of UP1 and UP2r from 7 isolates of *V. anguillarum* and 15 white-pigmented bacterial isolates. The nucleotide sequences of these PCR products were determined and deposited in DDBJ under the accession numbers listed in Table 1. The lengths of partially determined sequences of *gyrB* were 1107 bp in *V. anguillarum*isolates, and 1041 or 1044 bp in white-pigmented isolates. The sequence identities of7 isolates *V. anguillarum* were from 98.8 to 100%,relative to *V. anguillarum*NCMB6 or NB10 on the GenBankdatabase.Homology searches using BLAST revealed that 15 white-pigmented bacterial isolates belong to the genus *Aeromonas* (n=10), *Pseudomonas* (n=2), *Shewanella* (n=2), *Klebsiella* (n=1). As a result of the biochemical inspections using API 20 NE system (bioMérieux), 7 isolates were identified as the genus *Aeromonas*, 1 isolate as the genus *Pseudomonas*, and 7 isolates were not identified (Table 1).

Specificity and Sensitivity of PCR Detection of *V. Anguillarum*

Both the external primer set, Va-GBF2/Va-GBR1, and the internal primer set, Va-GBF1/Va-GBR1, were able to amplify the expected sized PCR product (402 bp and 346 bp, respectively) from 7 isolatesof *V. anguillarum*, while no PCR amplification was observed from 15 white-pigmented isolates, 25 strains of other *Vibrio* species, and related 7 bacterial strains listed in Table 1 and 2.In addition,the specificity of PCR experimentsof two timesgavethe same result.
In the sensitivity, the detection limit of single PCR with the external primers, theinternal primers, and thenested PCR were 4.0 pg, 4.0 pg, and 40fg of *V. anguillarum*DNA per PCR tube, respectively (Figure 1).

Detection from the Diseased Fish

The detection limit of the single PCRs with the external and internal primer set are approximately equal. Therefore, analysis of detection from the diseased fish was performed except the single PCR with the external primer set.In the case of rainbow trout which were diagnosed as vibriosis bythe culture method, PCR products sized at 346 bp were obtained from the gill, kidney, and body surface lesion by the single PCR with the internal primers and the nested PCR (Figure 2 sample no. 1 to 6). Detection of *V. anguillarum* from the gill washings of ayu was also possible by the nested PCR (Fig. 2 sample no. 7 and 8).

Discussion

In this study, we used the *gyrB* region to design specific PCR primers to identify and detect *V. anguillarum* isolates. Recently, the *gyrB* region has been used instead of 16S rDNAfor phylogenetic analysis among closely related bacterial taxa, as the sequence database of *gyrB*has become substantial (Yamamoto and Harayama, 1995; Watanabe *et al.*, 2001; Parkinson *et al.*, 2007). In addition,*gyrB* has been successfully used to detect specific pathogenic bacteria directly from fish samples that were heavily contaminatedwith other environmental bacteria (Venkateswaran *et al.*, 1998; Izumi and Wakabayashi, 2000; Izumi *et al.*, 2007b).
Tomake this study practical, unidentified white-pigmented bacterial isolates were collected and used as bacteria that can cause a false positive reaction using the PCR method. The same strategy was undertaken in the PCR detection of*Flavobacteriumpsychrophilum*, the etiological agent of bacterial cold-water disease, by Wiklund*et al*(Wiklund *et al.*, 2000). They successfully examined the specificity of their PCR detection using unidentified yellow-pigmented isolates as bacteria that resemble *F. psychrophilum*. In their case, they used yellow-pigmented isolates because *F. psychrophilum* forms a yellow-pigmented colony on agar plates.
The percent of nucleotide identityin the determined sequences of seven*V. anguillarum*isolates were 98.8%–100%, relative to *V. anguillarum*in the GenBank database.This higher percent identity suggests that the *V. anguillarum*has a very close phylogeneticintra-species relationship. In contrast, the *gyrB*nucleotide sequences of three *Pseudomonas putida*isolates havea percent identity of 91.6%–97.6% (Yamamoto and Harayama, 1995).
Althoughbiochemical inspection could not identify the seven white-pigmented bacterial isolates, all of them (n=15) were identified with sufficient percent identity of their *gyrB* sequences. They were classified intogenera *Aeromonas*(n=10), *Pseudomonas* (n=2), *Shewanella* (n=2), and *Klebsiella* (n=1).This result is consistent with a previous report by Kozińska (Kozińska, 2007)that many *Aeromonas* isolates were obtained from diseased and healthy fish in carp and trout farms.Further, this indicates that the *gyrB* sequence determination has a greater potential for the identification of genetically close bacterium genera, such as the genus *Vibrio*,than does conventional biochemical assays.

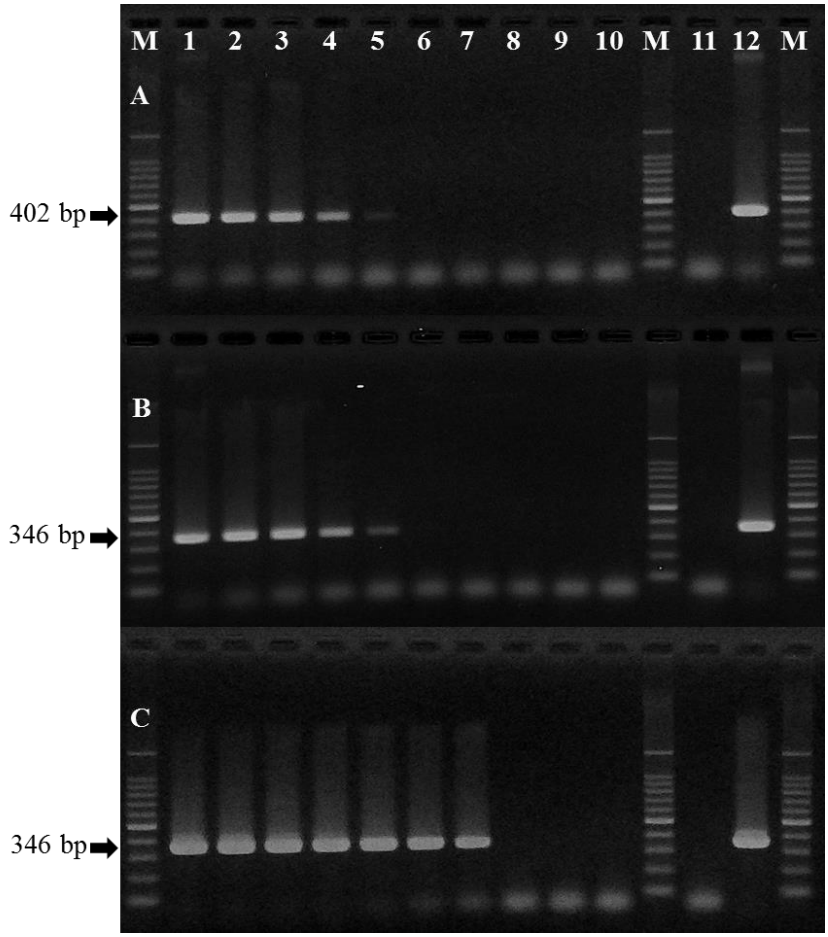

Figure 1. The sensitivity of PCRs to detect *Vibrio anguillarum* (strain no. ATCC19264). (A) Single PCR with the external primers Va-GBF2/Va-GBR1. (B) Single PCR with the internal primers Va-GBF1/Va-GBR1. (C) Nested PCR with the external primers Va-GBF2/Va-GBR1 and the internal primers Va-GBF1/Va-GBR1. Lane: M, 100 bp DNA marker;1, 40 ng; 2, 4.0 ng; 3, 400 pg; 4, 40 pg; 5, 4.0 pg; 6, 400 fg; 7, 40 fg; 8, 4.0 fg; 9, 400 ag; 10, 40 ag; 11, Negative control (sterilized H_2O) ; 12, Positive control.

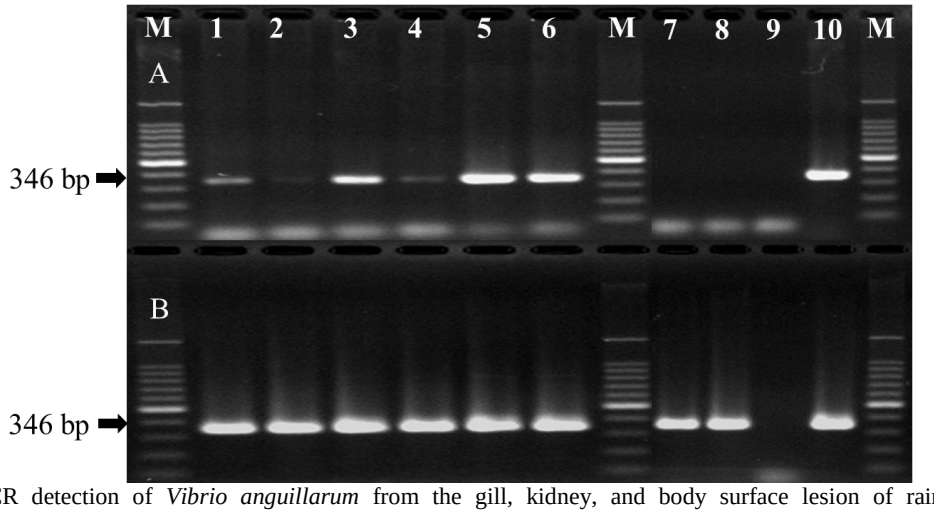

Figure 2. PCR detection of *Vibrio anguillarum* from the gill, kidney, and body surface lesion of rainbow trout (*Oncorhynchusmykiss*), and the gill washings of ayu(*Plecoglossusaltivelisaltivelis*) by single PCR with the internal primers (A) and the nested PCR (B). Sample M was 100 bp DNA marker. Samples no. 1 to 6 were from rainbow trout. Samples no. 7 and 8 were from ayu. Sample no. 9 and 10 were negative (sterilized H_2O) and positive control, respectively. The template DNA of samples no. 1, 2, 7, and 8 were prepared from gill, no. 3 and 4 were prepared from kidney, no. 5 and 6 were prepared from body surface lesion. Samples no. 1 to 6 were positive for *V. anguillarum* by single PCR with the internal primers and nested PCR method. Sample no. 7 and 8 were positive for *V. anguillarum* by nested PCR.

The detection limit of a single PCR is 4.0 pgof *V. anguillarum* DNA per PCR tube, comparable to previously reported data (Hong *et al*., 2007; Kim *et al*., 2008).The nested PCRswere at least 100 times more sensitive than single PCRs and achieved good performance in field samples using gill washings of ayu. Thus, the result of the nested PCRs in this study is more sensitive than the PCR method previouslyreported (Hong *et al*., 2007; Kim *et al*., 2008), it is a useful method for detecting *V. anguillarum*.Furthermore, assuming the length of *V. anguillarum* chromosomal DNA is 4.0–4.1Mbp(Naka *et al*.,2011),the limit of sensitivity of nested PCR amplification is calculated to be approximately 10 cells per PCR reaction tube. This is a sufficient value comparedwith those of previous studies using PCR for the detection of pathogenic bacteria.(Hong *et al*., 2007; Kim*et al.*, 2008; Teh*et al*., 2010; Payattikul *et al*., 2015).

In summary, the PCR method described in the present study allows for the specific detection of *V. anguillarum* from the tissue of diseased fish, rapidly and with sufficient sensitivity, and without isolating disease-causing bacteria using culture method. Our results suggest that the PCR technique with primers based on the *gyrB* sequence is a useful and powerful tool for the diagnosis and understanding of the epidemiology of fish vibriosis.

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