Thesis for the Degree of Master of Engineering

Application of the specific gene for the detection of *Vibrio anguillarum* by Polymerase Chain Reaction

by

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(특정유전자를 이용한 *Vibrio anguillarum*의 신속·정확한 PCR 검출 방법)

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A dissertation

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Application of the specific gene for the detection of *Vibrio anguillarun* by Polymerase Chain Reaction

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Abstract

Vibrio anguillarum, an opportunistic fish pathogen, is the main species responsible for vibriosis, a disease that affects feral fish, farmed fish, and causes considerable economic losses in marine aquaculture. Several studies aimed at detecting *V. anguillarum* have targeted ribosomal DNA belong to the bacterium. In this study, we used polymerase chain reaction (PCR) and targeted the *amiB* gene, encodes the peptidoglycan hydrolase N-acetylmuramoyl-L-alanine amidase, and *rpoS* gene, a general stress regulator, to detect *V. anguillarum*. PCR specificity was identified by 429-bp and 689-bp amplification of 6 strains of *V. anguillarum* and by the loss of a PCR product with 36 other bacterial species. The PCR produced a 429-bp amplified fragment from as little as 1 pg of *V. anguillarum* DNA. The limit of detection for this PCR technique was approximately 20 bacterial colonies in 25 mg of infected flounder tissue. And the PCR assay was sensitive enough to detect *rpoS* expression from 3 pg of genomic DNA, or from 6 colony-forming units mL^{-1} (cfu) of cultured bacterium. However, the assay was less sensitive when genomic DNA from infected founder and prawn was used (limit of detection, 50 ng and 10 ng per gram tissue, respectively). These data demonstrated that PCR amplification of *amiB* and *rpoS* gene is a sensitive, species-specific and rapid method to detect *V. anguillarum* in practical applications.

Chapter I.

Species-specific PCR detection of the fish pathogen, *Vibrio anguillarum*, using the *amiB* gene, which encodes N-acetylmuramoyl-L-alanine amidase

ABSTRACT

Vibrio anguillarum is the causative agent of the fish disease vibriosis and is the most intensely studied species of *Vibrio*. In the present study, we designed specific primers and a PCR assay to detect *V. anguillarum*. The primers were designed to amplify a 429-bp internal region of the *V. anguillarum amiB* gene, which encodes the peptidoglycan hydrolase N-acetylmuramoyl-L-alanine amidase. PCR specificity was demonstrated by successful amplification of DNA from *V. anguillarum* and by the absence of a PCR product from 25 other *Vibrio* strains and various enteric bacteria. The PCR produced a 429-bp amplified fragment from as little as 1 pg of *V. anguillarum* DNA. The limit of detection for this PCR technique was approximately 20 bacterial colonies in 25 mg of infected flounder tissue. These results suggest that this PCR system is a sensitive and species-specific detection method, and is possible to use as a diagnostic tool to detect *V. anguillarum*.

INTRODUCTION

Vibrio anguillarum is a Gram-negative bacterium that causes hemorrhagic septicemia in fish, a disease that leads to great economic losses in fish farming worldwide. Although this bacterium has been reclassified as *Listonella anguillarum* based on 5S rRNA sequence analysis, it is still commonly referred to as *V. anguillarum* (MacDonell & Colwell, 1984).

To date, our knowledge of the biochemical and molecular biological aspects of *V. anguillarum* pathogenesis have been rather limited compared to that for *Vibrio* species that cause human illnesses, such as *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. The major virulence factors of *V. anguillarum* are the hemolysin, protease, outer membrane protein, flagellin, and hemagglutinating proteins (Toranzo *et al*., 1983, 1993; Kodama *et al*., 1984). Recently, putative virulence genes of *V. anguillarum* were identified by random genome sequencing (Rodkhum *et al*., 2006). Lorenzo *et al* (2003) have also determined the complete sequence of the virulence plasmid pJM1 from *V. anguillarum*, which affects marine fish.

The morphological and biochemical identification of *V. anguillarum* from colonies of mixed *Vibrio* species isolated from marine environments is both time-consuming and inaccurate. Several methods are commonly used to identify *V. anguillarum* in environmental samples, including the API 20E system, BioLOG fingerprinting, and Biotype-100 biotyping, although it has been reported that diverse *V. anguillarum* strains are misidentified as other bacteria when these methods are used (Grisez *et al.,* 1991; Austin *et al*., 1995; Kühn *et al.,* 1996). Other methods, which include plasmid analysis, ribotyping characterization, pulsed-field gel electrophoresis analysis, and

DNA hybridization, are also used to identify *V. anguillarum* (Marianne *et al*., 1995; Skov *et al., 1995*; Martinez-Picado *et al*., 1996; Austin *et al*., 1997; Thompson *et al*., 2004). PCR methods, because of their relative rapidity and simplicity, have also been developed to identify and detect *V. anguillarum*. The phylogenetic relationships between *V. anguillarum* and other *Vibrio* species have been reported, based on comparisons of the DNA sequences of PCR amplicons generated using specific primers that target the *recA* and 16S rRNA genes (Dorsch *et al*., 1992; Kita-Tsukamoto *et al*., 1993; Urakawa *et al*., 1997; Thompson *et al*., 2004). However, these genes are not useful in discriminating between closely related strains, due to the very high degrees of sequence identity among these strains.

Recently, two PCR methods that target the sigma factor σ^{54}(*rpoN*) and the *hemolysin* genes were reported for the specific identification of *V. anguillarum* (Gonzalez *et al*., 2003; Rodkhum *et al*., 2006). Unfortunately, these methods are also not absolutely specific. Gonzalez *et al.* (2003) have demonstrated that the annealing temperature is very important in detecting the PCR product using specific primers for the *rpoN* gene. The expected band also appeared at the normal annealing temperature with *V. ordalii*, which is known to be a very difficult strain to differentiate from *V. anguillarum*. A multiplex PCR has been reported for the specific detection of *V. anguillarum* using primers that target five *hemolysin* genes (Rodkhum *et al*., 2006). This method also fails to discriminate reliably *V. ordalii* from *V. anguillarum*. Consequently, there remains a need for specific primers for PCR detection of *V. anguillarum* strains.

The present study aimed to evaluate the sensitivity and specificity of PCR primers

designed to target the *amiB* gene for the detection of *V. anguillarum*. In our previous study, the *amiB* gene, which encodes N-acetylmuramoyl-L-alanine amidase, was isolated from *V. anguillarum* and characterized (Ahn *et al*., 2006). This enzyme catalyzes the cleavage of peptide bonds in peptidoglycan. By comparing our sequence with the *amiB* sequences reported for other *Vibrio* species, we identified a variable region that should enable specific detection of *V. anguillarum*. We also conducted fish infection studies to evaluate our method.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

In total, 41 *Vibrio* strains from 36 bacterial species were used in this study (Table 1). All of the strains were routinely cultured at their optimum temperatures on Brain Heart Infusion (BHI; Difco) and Luria-Bertani agar (LB; Difco). The strains were stored in BHI and LB with 20% glycerol at -70°C.

Table 1. Strains used in this study

Species	Source[a] or reference
Vibrio alginolyticus	KCTC 2472
Vibrio anguillarum (O1 type)	Holmstrøm and Gram, 2003.
Vibrio anguillarum (NB)	Milton et al., 1997.
Vibrio anguillarum J-0-3	[b] E
Vibrio anguillarum J-0-2	[b] E
Vibrio anguillarum	KCTC 2711(corresponded to ATCC 19264)
Vibrio anguillarum YT	E
Vibrio cambellii	KCCM 40864
Vibrio carchariae	KCCM 40865
Vibrio cholerae	ATCC 14547
Vibrio cincinnatiensis	KCTC 2733
Vibrio damselae	E
Vibrio diazotrophicus	KCCM 41666
Vibrio fluvialis	KCTC 2473
Vibrio furnissii	KCTC 2731
Vibrio harveyi	KCCM 40866
Vibrio hollisae	KCCM 41680
Vibrio logei	KCTC 12281
Vibrio mediterranei	KCCM 40867
Vibrio metschnikovii	KCTC 2736
Vibrio mimicus	KCTC 2737
Vibrio natriegens	KCCM 40868
Vibrio navarrensis	KCCM 41682
Vibrio nereis	KCTC 2722
Vibrio ordalii	KCCM 41669
Vibrio orientalis	KCTC 2725
Vibrio parahaemolyticus	KCCM 11965
Vibrio proteolyticus	KCTC 2730
Vibrio salmonicida	KCCM 41663
Vibrio tubiashii	KCTC 2728
Vibrio vulnificus	KCTC 2959 (corresponded to ATCC 27562)
Aeromonas hydrophila	KCTC 2358
Escherichia coli BL21(DE3)	L
Escherichia coli XLI-blue	L
Enterobacter cloacae	E
Edwardsiella tarda	E
Klebsiella oxytoca	E
Klebsiella pneumoniae	E
Salmonella typhi	E
Shigella flexneri	E
Shigella sonnei	E

Note: L, laboratory collection; E, environmental source; and [a]E, environmental strain kindly provided by Dr. S. I. Park (Pukyong National University, Busan, Korea)

Design of specific primers for *V. anguillarum* detection

We used a segment of the *amiB* gene sequence as the PCR target for the specific detection of *V. anguillarum*. To design a primer pair, a sequence comparison was made using the known N-acetylmuramoyl-L-alanine amidase genes of *Vibrio* spp. (*cholerae* O1, *parahaemolyticus*, *alginolyticus*, *fischeri*, *splendidus* and *angustum*), which were retrieved from the Entrez database using the National Center for Biotechnology Information GenBank database and the BLAST search program. The forward primer van-ami8 (5′-ACATCATCCATTTGTTAC-3′, positions 8 to 25 in the *V. anguillarum amiB* gene), and the reverse primer van-ami417 (5′-CCTTATCACTATCCAAATTG-3′, positions 417 to 436 in the *V. anguillarum amiB* gene) were used (Fig. 1). And we used 16S rRNA- and the *rpoN* gene-specific primers for positive controls (Gonzalez *et al*., 2003).

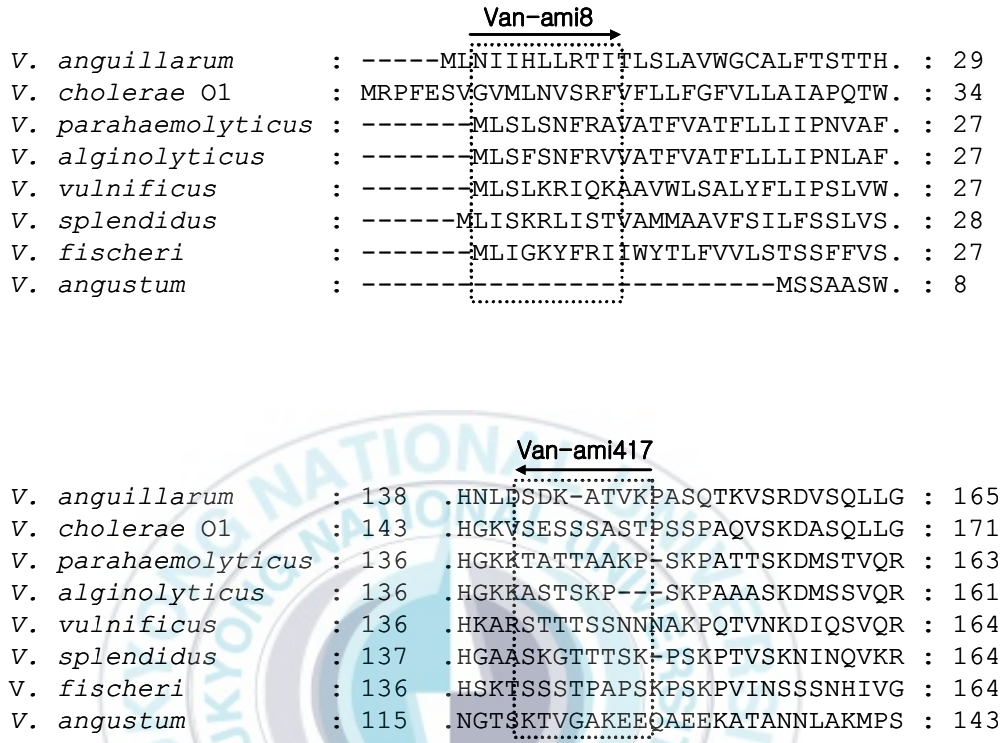

Fig. 1. Alignment of N-acetylmuramoyl-L-alanine amidase amino acid sequences (*amiB* sequences) of *V. anguillarum* (accession no. ABD85291), *V. cholerae* O1 (AAF93517), *V. parahaemolyticus* (BAC61083), *V. alginolyticus* (ZP01261820), *V. fischeri* (YP205709), *V. vulnificus* (AA009746), *V. splendidus* (ZP00991953), and *V. angustum* (ZP01237176).

Primer regions are indicated by boxes and primers were indicated by arrows.

PCR analysis

PCR amplification was performed on colonies of bacteria and DNA purified from each strain as templates. The PCR was carried out in a 50-μL reaction mixture that contained 250 μM of each deoxyribonucleoside triphosphate (dNTP), 10 pmol of each primer, 5 μL of 10× Taq buffer with $MgCl_2$, 0.5 U of Taq DNA polymerase (Takara Bio, Japan), and distilled water up to 50 μL. The PCR thermocycling with van-ami8 and 417 primer pair, involved one initial cycle of denaturation at 95°C for 10 min, followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and finally, one cycle of 72°C for 7 min. When the PCR assay was conducted with a primer set corresponding to the *rpoN* gene, the annealing temperature was 62°C. The PCR products were analyzed by 1.5% agarose gel electrophoresis in 1× TAE buffer. A 100-bp DNA ladder (Cosmo Genetech, Korea) was used as the molecular weight marker.

Fish infection and DNA isolation from tissues

100 μL of *V. anguillarum* O1 suspension (6.4×10^9 CFU/mL) was injected into the abdomen of healthy flounders (*Paralichthys olivaceus*) with body weight of 100 g. Viable cells were determined by cell counting from the internal organs of the fish after injection. The tissues (kidney, liver and intestine) were removed, homogenized in sterile phosphate-buffered saline (PBS) using a homogenizer at 9700 rpm for 20~30 seconds, serially diluted 10-fold in saline solution, and dropped onto TCBS (Difco) and BHI agar plates. Colonies were counted after incubation at 25°C for 24 h. For PCR amplification, total DNA was purified from 25 mg of each tissue using the QIAamp DNA Mini Kit (Qiagen, Germany), and 0.2 μg DNA was used as template.

RESULTS

Sequence analysis and specific primer design

The *amiB* gene from serotype O1 of *V. anguillarum* has recently been cloned and sequenced by Ahn *et al.* (2006). Alignment of the encoded amino acid sequence with the N-acetylmuramoyl-L-alanine amidases from *V. cholerae* O1, *V. parahaemolyticus*, *V. alginolyticus*, *V. fischeri*, *V. vulnificus*, *V. splendidus* and *V. angustum* showed 77, 67, 67, 66, 65, 65 and 56% sequence identity, respectively. To design a primer pair, the nucleotide and amino acid sequences were compared with other *amiB* gene sequences described for the genus *Vibrio* using the CLUSTAL W program (Fig. 1). Regions of high sequence variability among the *amiB* genes were used for the design of specific primer sets. Among the several primer pairs tested, the van-ami8 and van-ami417 primer pair, which is located between nucleotides 8 and 417 of the *amiB* gene, showed the highest specificity in the PCR assay.

Specificity of PCR detection of *V. anguillarum*

PCR amplification with colonies of six strains of *V. anguillarum* using the van-ami8 and van-ami417 primers resulted in a product of the predicted length (429 bp), while no products were obtained from other *Vibrio* strains (Fig. 2A). When we conducted the same experiment with the enteric bacteria listed in Table 1, no amplified band was seen. 16S rRNA-and the *rpoN* gene-specific primers were selected for positive controls. The expected 1465-bp amplicon for 16s rRNA was seen with every strain used in this study (data not shown). However, in case of the *rpoN* gene, a 519 bp amplicon only appeared in 6 strains of *V. anguillarum* (Fig. 2B).

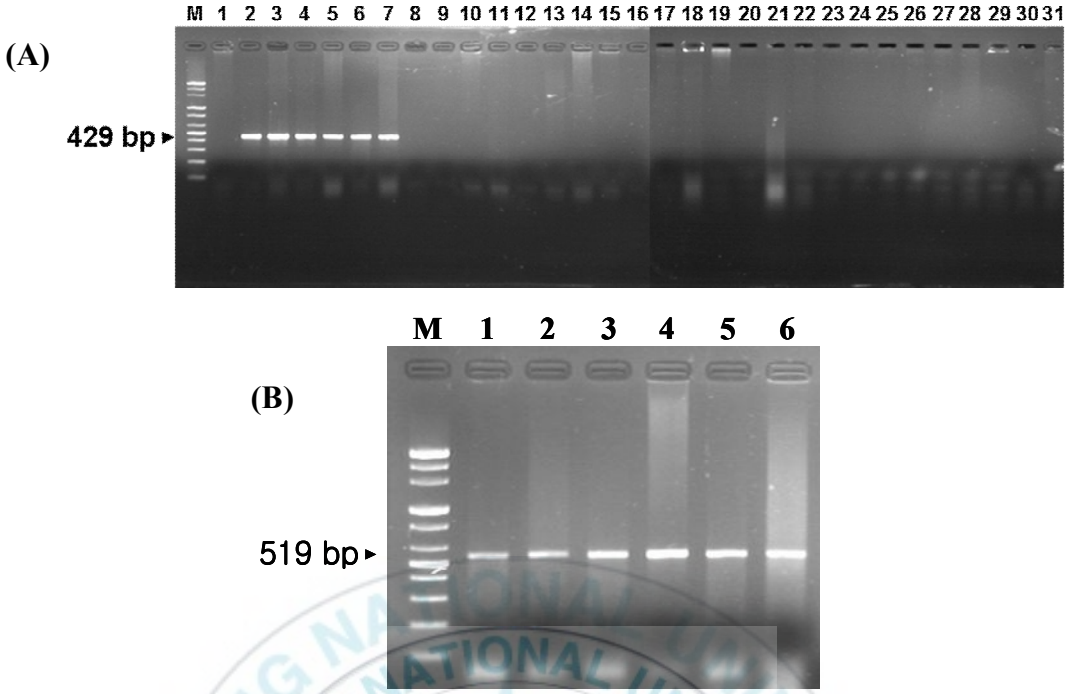

Fig. 2A. Agarose gel electrophoresis of *V. anguillarum*-specific DNA products amplified by PCR using the van-ami8 and van-ami417 primers.

M, 100-bp DNA ladder; lane 1, *V. alginolyticus* (ATCC 17749); lanes 2-7, *V. anguillarum* (O1 type, NB10, J-O-3, J-O-2, ATCC 19264, and YT); lane 8, *V. campbellii* (ATCC 25920); lane 9, *V. carchariae* (LMG 7890); lane 10, *V. cholerae* (ATCC 14035); lane 11, *V. cincinnatiensis* (NCTC 12012); lane 12, *V. damselae*; lane 13, *V. diazotrophicus* (ATCC 33466); lane 14, *V. fluvialis* (ATCC 33809); lane 15, *V. furnissii* (ATCC 35016); lane 16, *V. harveyi* (NCMB 1280); lane 17, *V. hollisae* (LMG 17719); lane 18, *V. logei* (ATCC 29985); lane 19, *V. mediterranei* (NCTC 11946); lane 20, *V. metschnikovii* (LMG 11664); lane 21, *V. mimicus* (ATCC 33653); lane 22, *V. natriegens* (ATCC 14048); lane 23, *V. navarrensis* (ATCC 51183); lane 24, *V. nereis* (ATCC 25917); lane 25, *V. ordalii* (ATCC 33509); lane 26, *V. orientalis* (NCMB 2195); lane 27, *V. pharahaemolyticus* (ATCC 17802); lane 28, *V. proteolyticus* (NCMB 1326); lane 29, *V. salmonicida* (NCMB 2262); lane 30, *V. tubiashii* (ATCC 19109); lane 31, *V. vulnificus* (ATCC 27562).

Fig. 2B. Agarose gel electrophoresis of *V. anguillarum*-specific DNA products amplified by PCR with the *rpoN* gene-specific primers.

M, 100-bp DNA ladder; lane 1-6, *V. anguillarum* (O1 type, NB10, J-O-3, J-O-2, ATCC 19264, and YT)

Sensitivity of PCR detection of *V. anguillarum*

Serially diluted samples were used as templates for 30 cycles of PCR amplification. The van-ami8 and van-ami417 primers were tested for amplification while varying the DNA template concentrations from 1 μg/μL to 0.1 pg/μL. We tested the level of product detectable by agarose gel electrophoresis of an amplified *amiB* gene band of the expected size (429 bp). The expected PCR product was seen for reactions that contained as little as 1 pg of genomic DNA template (Fig. 3).

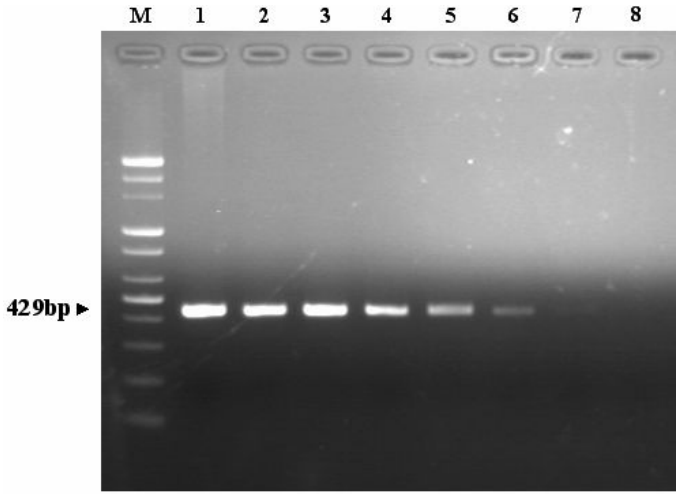

Fig. 3. Sensitivity of the van-ami8 and van-ami417 PCR primer pair for amplification.

M, 100-bp DNA ladder; the following lanes contain *V. anguillarum* DNA: lane 1, 1 μg; lane 2, 0.1 μg; lane 3, 0.01 μg; lane 4, 1 ng; lane 5, 0.1 ng; lane 6, 0.01 ng; lane 7, 0.001 ng; lane 8, 0.0001 ng.

Bacterial cell counting in flounder tissues

Detection of *V. anguillarum* from internal organs was attempted after injecting the fish with *V. anguillarum*. A liquid extract of the homogenized tissue was dropped onto TCBS and BHI agar plates and the plates were incubated. The colony counts are expressed as the logarithm of CFU/mg of tissue. In the control group, no bacteria were seen on the TCBS plates, whereas colonies, ranging from 1.0 to 1.5 log CFU/mg of tissue, did appear on the BHI plates (Table 2). At 24 h post-injection, the numbers of bacterial colonies were 3.9, 4.2, and 3.7 log CFU/mg of kidney, intestine and liver, respectively, on TCBS plates. To evaluate whether specific detection could be achieved with tissues contaminated by *V. anguillarum*, PCR amplification was performed using total DNA purified from each infected organ. As shown in Figure. 4, all of the DNA samples purified from infected tissues generated a clear band of 429 bp.

Table. 2. *V. anguillarum* CFU counts in homogenates of infected organs.

Sample group	Sample from	Plate culture (log CFU/mg)	
		BHI	TCBS
Control fish (PBS treatment)	kidney	1.4	0
	intestine	1.0	0
	liver	1.5	0
Infected fish (after 24hr)	kidney	5.8	3.6
	intestine	5.4	4.2
	liver	5.6	3.7

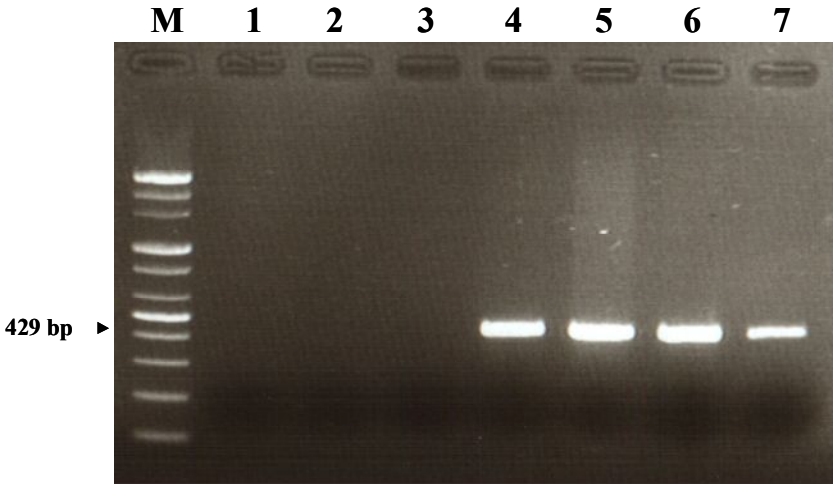

Fig. 4. Agarose gel electrophoresis of PCR products from chromosomal DNA recovered from non-injected or infected tissues of flounder.

M, 100-bp ladder; lane 1, 2, and 3, liver, kidney and intestine tissues from normal flounder without infection; lane 4, 5, and 6, liver, kidney and intestine tissues from flounder infected with *V. anguillarum*; lane 7, PCR product with chromosomal DNA purified from *V. anguillarum* for a positive control.

DISCUSSION

The availability of rapid and specific diagnostic methods for the detection of the fish pathogen *V. anguillarum* is important in aquaculture because *V. anguillarum* causes vibriosis in fish, which results in large economic losses. Many attempts have been made to develop methods for the specific detection of *V. anguillarum*.

To date, all of the reported methods, including various PCR protocols, have certain problems in specifically detecting *V. anguillarum*. An important issue is discrimination between *V. anguillarum* and *V. ordalii*, which is the most closely related strain to *V. anguillarum*. The API 20E system has been used in biochemical and serological analyses to type *Vibrio* species. However, it has been reported that this system cannot distinguish *V. anguillarum* from *V. ordalii*. Austin *et al.* (1995) have reported that *V. anguillarum* can be distinguished from *V. ordalii* by ribotyping with an acetylaminofluorene-labeled ribosomal probe. However, in plasmid profiling, *V. ordalii* has a 32-kb plasmid that corresponds to pHJ101, which is present in *V. anguillarum* biotype 2. Urakawa *et al.* (1997) have analyzed 35 *Vibrio* strains by 16S rDNA genotyping and they have reported that the 16S rDNA sequences of the *Vibrionaceae* family show more than 90% homology. In particular, *V. anguillarum* (ATCC 19264) and *V. ordalii* (ATCC 35509) share 97% identity. Thompson *et al.* (2004) have performed a phylogenetic analysis of the *Vibrionaceae* family that involved the genera *Vibrio*, *Photobacterium*, and *Grimontia* using the *recA* gene sequence. Their results show that the *recA* sequence similarity of *V. anguillarum* (LMG4437) and *V. ordalii* (LMG13544) is almost 98%. This high degree of sequence identity does not allow reliable discrimination of specific strains using PCR. To

address this problem, the *rpoN* gene, which encodes sigma factor σ^{54}, can be used to identify *V. anguillarum* in turbot tissue and blood (Gonzalez *et al*., 2003). However, even using that sequence, *V. anguillarum* could not be distinguished from *V. ordalii* at all annealing temperatures. Using an annealing temperature of 58°C, a band was detectable not only in *V. anguillarum*, but also in *V. ordalii* (NCIMB 2167). Thus, a better technique is required for the reliable identification of *V. anguillarum*.

In this study, we examined whether the *amiB* gene, which encodes the enzyme N-acetylmuramoyl-L-alanine amidase, could be used to design PCR primers to detect *V. anguillarum* in a specific fashion. By comparing the available *amiB* gene sequences from the GenBank database, we identified as a target the sequence that encodes the N-terminal region of the AmiB protein, which shows high variability among strains (Fig. 1). The primers we designed could be used to generate successfully the predicted PCR product from six strains of *V. anguillarum*, but not from the other strains listed in Table 1. As mentioned above, it has been reported that when using PCR primers that target the *rpoN* gene, the annealing temperature of 62°C is very important, to exclude detection of a specific band from *V. ordalii*. However, in our study, the predicted PCR product was produced only from *V. anguillarum*, even using an annealing temperature of 55°C (Fig. 2). This result suggests that the sequence similarity of the N-terminal region may be low, although no *amiB* gene sequence from *V. ordalii* is yet available.

To investigate whether this PCR system could be used in practical situations, we injected the *V. anguillarum* O1 strain into flounders. The colony number injected to fish was 6.4×10^9 CFU/mL, which is close to the value of LD_{50} (5.4×10^9

CFU/mL). After 24 h, the kidney, intestine and liver were removed from the injected fish, homogenized, and plated onto BHI and TCBS plates to recover *V. anguillarum*. Our results showed that *V. anguillarum* proliferates well in these fish organs. The PCR assays were performed using 0.2 μg of purified total DNA extracted from 25 mg of the infected tissues. The 429-bp amplicon was obtained for every samples of infected tissue (Fig. 4). Furthermore, the PCR products were cleanly amplified without interference from other sequences in the total DNA of the fish tissues. To determine the detection limit of the PCR assay, 0.2 μg of purified DNA from the infected flounder tissue was serially diluted and performed PCR. After PCR amplification, the detectable bands were obtained from 0.2 μg to 1ng of DNA (data not shown). Templates used for this study contained chromosomal DNAs of *V. anguillarum* together with flounder tissue. We could estimate that the amount of chromosomal DNA of *V. anguillarum* in 1 ng of template was correspond to that of about 20 colonies. Gonzalez *et al*. (2003) reported that the detection limit of PCR using the *rpoN* gene was 50-500 colonies/ 25 mg of tissue. Thus, our result was more sensitive than the PCR assay with the *rpoN* gene to detect *V. anguillarum*.

In conclusion, it is possible to apply the described PCR method for the direct detection of *V. anguillarum* in marine flounder without the time-consuming bacterial cultivation step. We are continuing to investigate the suitability of this assay system for the analysis of other marine organisms and seafood.

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Chapter II.

Application of the *rpoS* gene for the detection for the detection of *Vibrio anguillarum* in flounder and prawn by polymerase chain reaction

ABSTRACT

Vibrio anguillarum, an opportunistic fish pathogen, is the main species responsible for vibriosis, a disease that affects feral fish, farmed fish, and shellfish, and causes considerable economic losses in marine aquaculture. In this study, we used polymerase chain reaction (PCR) to detect *V. anguillarum*. PCR specificity was evaluated by amplifying the *rpoS* gene, a general stress regulator, in six strains of *V. anguillarum* and 36 other bacterial species. PCR amplified a species-specific fragment (689 bp) from *V. anguillarum*. Furthermore, the PCR assay was sensitive enough to detect *rpoS* expression from 3 pg of genomic DNA, or from 6 colony-forming units mL^{-1} (cfu) of cultured *V. anguillarum*. However, the assay was less sensitive when genomic DNA from infected flounder and prawn was used (limit of detection, 50 ng and 10 ng per gram tissue, respectively). These data demonstrate that PCR amplification of the *rpoS* gene is a sensitive and species-specific method to detect *V. anguillarum* in practical applications.

INTRODUCTION

As the global consumption of seafood increases and wild stocks decline, the supply of seafood is becoming increasingly dependent on aquaculture. In Korea and adjacent countries, increased interest in health, changing dietary habits, and economic growth has caused a sharp increase in the consumption of fresh seafood [Yano, Yokoyama, Satomi, Oikawa & Chen 2004].

Vibriosis is a devastating disease that affects both wild stocks and cultured fish and shellfish. This disease is severely damaging to the aquaculture industry and exacerbates the shortage of fresh fish and shellfish. *Vibrio anguillarum,* a gram-negative marine bacterium is the main causative agent of vibriosis [Austin & Austin 1999]. The development of a rapid and accurate method to detect *V. anguillarum* is critical for the control of this disease and the protection of stocks.

Typical methods of bacterial pathogen detection use microbiological or biochemical methods, such as the most-probable-number test, the use of selective agar media, and DNA–DNA hybridization for specific genes. However, these methods are labor-intensive, time-consuming, and require the analysis of a great number of samples [Peeler, Houghtby & Rainosek 1992; Kaysner & DePaola, 2001]. Therefore, research has focused on developing faster and more efficient detection methods. The PCR method for detecting microbial pathogens in food, clinical, and environmental samples is highly specific and relatively less time-consuming than classical methods. Furthermore, multiplex PCR assays using several target genes have been successful in detecting multiple organisms and in differentiating related strains. However, multiple primer sets can produce identical PCR amplicons and low

amplification efficiency, and false-positive bands can be caused by modified annealing temperatures [Wilson, Strout, DeSantis, Stilwell, Carrano & Andersen 2002; Chiang, Yang, Li, Ho, Lin & Tsen 2006].

Several PCR methods targeting various genes have been developed to identify and detect *V. anguillarum*. Specific primers for the *recA*, 16S rRNA, *rpoN*, and *hemolysin* genes have been used [Dorsch, Lane & Stackebrandt 1992; Kita-Tsukamoto, Oyaizu, Nanba & Simidu 1993; Urakawa, Kita-Tsukamoto & Ohwada 1997; Gonzalez, Osorio & Santos 2003; Thompson, Thompson, Vandemeulebroecke, Hoste, Dawyndt & Swings 2004; Rodkhum, Hirono, Crosa & Aoki 2006]. However, the *recA* and 16S rRNA genes have a high degree of sequence homology and thus are not effective in discriminating among related strains. It has been also reported that detection assays using the *rpoN* and *hemolysin* genes are not absolutely species-specific [Gonzalez, Osorio & Santos 2003]. Research using the *rpoN* gene has demonstrated that the annealing temperature is very important for species-specific PCR amplification. Using the annealing temperature at 58°C, the same amplicon was produced in *V. anguillarum* and *V. ordalii* [Gonzalez, Osorio & Santos 2003]. Likewise, multiplex PCR using primer sets targeting five *hemolysin* genes was unable to reliably differentiate between *V. ordalii* and *V. anguillarum* [Rodkhum, Hirono, Crosa & Aoki 2006].

In this investigation, we used PCR to amplify the *rpoS* gene as a method to detect *V. anguillarum*. The *rpoS* gene is a housekeeping gene for the general regulation of stress. The *rpoS* gene controls the expression of various virulence genes [Fanget, Libbey, Buchmeier, Loewen, Switala, Harwood & Guiney 1992; Kowarz, Coynault,

Robbes-Saule & Norel 1994; Beltrametti, Kresse & Guzmán 1999; Hengge-Aronis 2000] and is involved in the pathogenic colonization of host tissue [Olsén, Arnqvist, Hammer, Sukupolvi & Normark 1993; Römling, Livers, Austin, Kaiser, Jensen, Burgess & Blum 1998; Merrell, Tischler, Lee & Camilli 2000; Hülsmann, Rosche, Kong, Hassan, Beam & Oliver 2003].

This report describes the isolation of the *rpoS* gene from *V. anguillarum* and the evaluation of species-specific PCR assays to detect this *Vibrio* species. PCR primer pairs based on various regions of the *rpoS* gene from several *Vibrio* species were designed and evaluated. Specificity of the PCR assay was verified by testing various bacterial strains. PCR detection of *V. anguillarum* using primers for the *rpoS* gene was also examined after infecting flounder and prawn with *V. anguillarum*.

MATERIALS AND METHODS

Bacterial strains and growth medium

In total, 37 bacterial species were used in this study (Table 3). All *Vibrio* and non-*Vibrio* species were routinely grown at their optimum temperatures on brain heart infusion agar (BHI; Difco, Detroit, MI, USA), heart infusion agar (HI; Difco), and Luria–Bertani agar (LB; Difco).

Table 3. Strains used in this study.

Species	Source[a] or reference
Vibrio alginolyticus	KCTC 2472
Vibrio anguillarum (O1 type)	Holmstrøm and Gram, 2003.
Vibrio anguillarum (NB)	Milton et al., 1997.
Vibrio anguillarum J-0-3	[b] E
Vibrio anguillarum J-0-2	[b] E
Vibrio anguillarum	KCTC 2711(corresponded to ATCC 19264)
Vibrio anguillarum YT	E
Vibrio cambellii	KCCM 40864
Vibrio carchariae	KCCM 40865
Vibrio cholerae	ATCC 14547
Vibrio cincinnatiensis	KCTC 2733
Vibrio damselae	E
Vibrio diazotrophicus	KCCM 41666
Vibrio fisheri	KCCM 41685
Vibrio fluvialis	KCTC 2473
Vibrio furnissii	KCTC 2731
Vibrio harveyi	KCCM 40866
Vibrio hollisae	KCCM 41680
Vibrio logei	KCTC 12281
Vibrio mediterranei	KCCM 40867
Vibrio metschnikovii	KCTC 2736
Vibrio mimicus	KCTC 2737
Vibrio natriegens	KCCM 40868
Vibrio navarrensis	KCCM 41682
Vibrio nereis	KCTC 2722
Vibrio ordalii	KCCM 41669
Vibrio orientalis	KCTC 2725
Vibrio parahaemolyticus	KCCM 11965
Vibrio proteolyticus	KCTC 2730
Vibrio salmonicida	KCCM 41663
Vibrio tubiashii	KCTC 2728
Vibrio vulnificus	KCTC 2959 (corresponded to ATCC 27562)
Aeromonas hydrophila	KCTC 2358
Escherichia coli BL21(DE3)	L
Escherichia coli XLI-blue	L
Enterobacter cloacae	E
Edwardsiella tarda	E
Klebsiella oxytoca	E
Klebsiella pneumoniae	E
Salmonella typhi	E
Shigella flexneri	E
Shigella sonnei	E

[a] L, laboratory collection; E, environmental source.

[b] Environmental isolate kindly provided by Prof. S. I. Park (Dept. of Aquatic Life Medicine, Pukyong National University, Busan, Korea).

Isolation of the *rpoS* gene from *V. anguillarum*

DNA purification, endonuclease restriction, ligation, and gel electrophoresis were performed as previously described by Sambrook & Russell (2001). The cloning vectors pGEM-4Z and pUC19 were used to generate a genomic DNA library from *V. anguillarum*. Primers for the T7 and SP6 promoter regions were used to sequence cloned DNA fragments using a PRISM 377 automated DNA sequencer (Perkin–Elmer Applied Biosystems, Foster City, CA, USA). To determine the transcriptional start site of the *rpoS* gene, we used the 5′-rapid amplification of cDNA ends (5′ RACE) system, version 2.0 (RACE: Invitrogen, San Diego, CA, USA). We used BLAST and FASTA (National Center for Biotechnology Information) to analyze the similarity of *rpoS* sequences from various species of *Vibrio*.

DNA purification and PCR analysis

Genomic DNA was purified as described by Ausubel, Brent, Kingston Moore, Smith, Seidman & Struhl (1987). PCR was performed using colonies of bacteria or purified genomic DNA from the specified strains. The PCR amplification was carried out using a single colony or 1 μg of purified DNA as a template in a reaction mixture containing ExTaq (Takara Bio, Tokyo, Japan). PCR using a single colony (*rpoS* and 16S rRNA expression) consisted of an initial denaturation at 94°C for 10 min, followed by 25 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 51°C, and extension for 30 s at 72°C. Final elongation was carried out for 7 min at 72°C. PCR with genomic DNA consisted of an initial denaturation for 5 min at 94°C, followed by the 25 cycles of the program described above. The PCR amplicons were

confirmed by electrophoresis using a 1.5% agarose gel in 1× TAE (Tris-acetate-EDTA) buffer. A 100-bp Labo DNA ladder (Cosmo Genetech, Seoul, Korea) was used as the molecular weight marker.

Specificity and sensitivity of *rpoS*-targeted primers

The selected primers were evaluated for species-specificity by amplifying purified genomic DNA from all the strains listed in Table 4. An oligonucleotide primer pair, the forward primer VARPO1 and the reverse primer VARPO2 (Table 4) was used in each PCR to test the specificity and sensitivity of detection for all bacterial strains used in this study. Two other primers, 27F and 1492R, located between nt 27 and 1492 of the 16S rRNA sequence, were used as a positive control (Table 4). To determine sensitivity, genomic DNA was serially diluted in sterile distilled water (0.0003–3.0 ng/sample) and amplified by PCR under optimal conditions. Experiments were performed at least three times using duplicate samples.

Table 4. Description of the PCR primer sequences and amplicon sizes used in this study

Target	Primer	Sequence	Positions within *rpoS* ORF (bp)	Amplicon size (bp)
RpoS	VARPO1	AGACCAAGAGATCATGGATT	42~61	689 bp
	VARPO2	AGTTGTTCGTATCTGGGATG	711~730	
16S rDNA	27F	AGAGTTTGATCMTGGCTCAG	27~46	1466 bp
	1492R	TACGGYTACCTTGTTACGACTT	1471~1492	

Detection of *V. anguillarum* in infected flounder (*Paralichthys olivaceus*) and prawn (*Penaeus monodon*)

Experimental infections were performed in aerated plastic containers (15 L), using healthy flounder and prawn ranging in weight from 144 to 170 g and 21 to 27 g, respectively. Flounder were given an intraperitoneal injection of 0.1 mL phosphate-buffered saline (PBS) containing 2.7×10^9 colony-forming units (cfu) of *V. anguillarum*. Each prawn was injected with 2.6×10^6 cfu of *V. anguillarum* re-suspended in 0.1 mL PBS. The second group of flounder and prawn were injected with 0.1 mL of sterile PBS. The third group was not manipulated at all. The internal organs from infected and healthy fish were removed under aseptic conditions and homogenized in PBS. For prawn, the entire body (including the carapace) was homogenized in PBS. The bacterial count of *V. anguillarum* was determined by plating the appropriate dilutions of homogenized tissue on BHI and thiosulphate citrate bile-salts sucrose (TCBS) agar. Finally, total DNA was extracted from each sample of infected and noninfected tissue using a DNA extraction kit (Qiagen GmbH, Hilden, Germany).

Nucleotide sequence accession number

The nucleotide sequences of the *rpoS* gene from the *V. anguillarum* strains YT and ATCC 19264 have been deposited in GenBank under the accession numbers AY695433 and AY695434, respectively.

RESULTS

Sequence analysis of the *rpoS* gene

We determined the entire sequence of the *rpoS* gene isolated from two strains of *V. anguillarum* (YT and ATCC 19264). The nucleotide sequence of the *rpoS* gene had an open reading frame of 1002 base pairs (bp) and consisted of 333 amino acids, including the initiation and stop codons. The estimated molecular mass of RpoS protein is 38 kDa. The *rpoS* gene sequences isolated from the yellowtail fish strain (YT) and the ATCC 19264 strain were almost identical except for two bases, located at 738 bp and 897 bp. The nucleotide sequences of the *rpoS* gene were compared to four other *Vibrio* species using ClustalW. The sequences varied in length, but the alignment of sequences from *V. cholerae, V. harveyi, V. parahaemolyticus,* and *V. vulnificus* showed 81, 79, 81, and 79% sequence identity, respectively, to the *rpoS* sequence from *V. anguillarum*.

Design of *V. anguillarum*-specific primers

To specifically detect *V. anguillarum* by PCR, the *rpoS* sequences from *Vibrio* species including *V. cholerae*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* were retrieved from the NCBI GenBank database (accession number: AE004139, AF321124, AF144608, AY187681, respectively) and compared the homology using the BLAST search program with the *rpoS* gene sequence of *V. anguillarum* determined in this investigation. We found regions of high sequence variability in the *rpoS* gene among the five *Vibrio* species, and specific primer pairs for *V. anguillarum* were designed based on these regions (Fig. 5). Among the primers tested, the forward primer VARPO1 and the reverse primer VARPO2, which amplify a segment between the nucleotide positions (nt) 42–730 of the *rpoS* gene from *V. anguillarum* (Fig. 5 and Table 4) showed the highest specificity and sensitivity in the PCR assay.

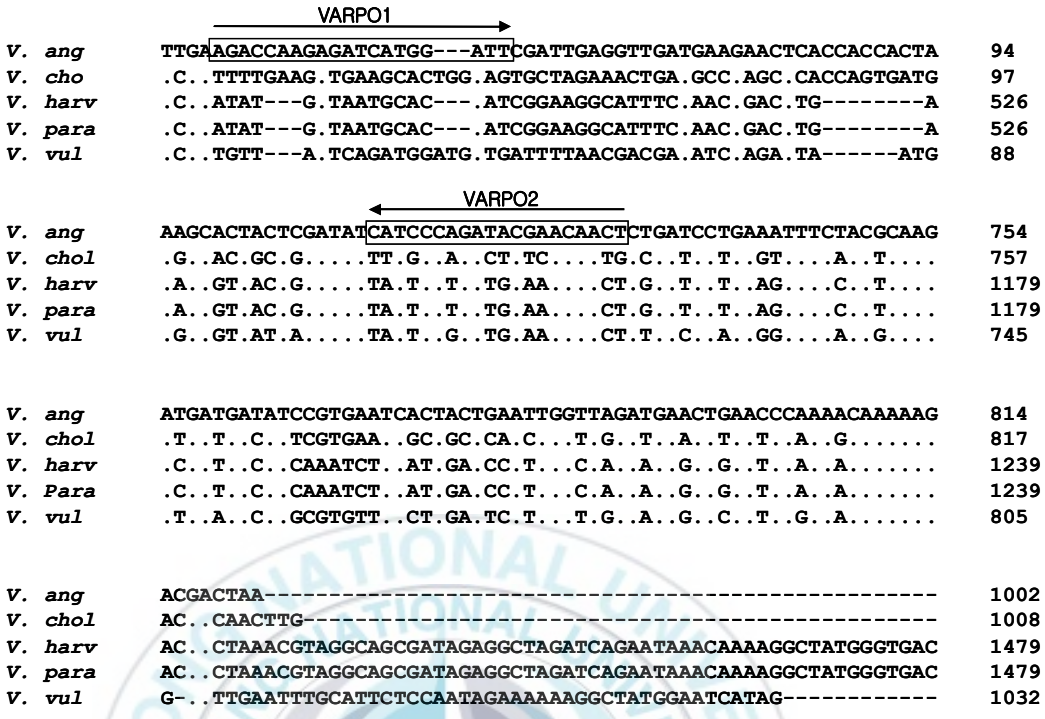

Figure 5. Nucleotide sequence alignment of RNA polymerase sigma factor (*rpoS*) from *V. anguillarum* (accession no. AY695434), *V. cholerae* (AE004139), *V. harveyii* (AF321124), *V. parahaemolyticus* (AF144608), and *V. vulnificus* (AY187681).

Nucleotides identical to those of the *V. anguillarum* sequence are indicated by dots.

Specificity of detection

We expected the *rpoS*-specific primers designed for this investigation to produce a single PCR amplicon of 689 bp for *V. anguillarum*. PCR amplification of *rpoS* using colonies and purified genomic DNA from six strains of *V. anguillarum* yielded a product of the predicted length, but no PCR products were obtained from species other than *V. anguillarum*. No amplification was observed in the same experiment using the enteric bacteria listed in Table 1 (Fig. 6A). Primers for 16S rRNA were used as a positive control for PCR assay, and the expected 1466-bp product was amplified from every strain (Fig. 6B). These results were consistent within all three replicates for each extraction method.

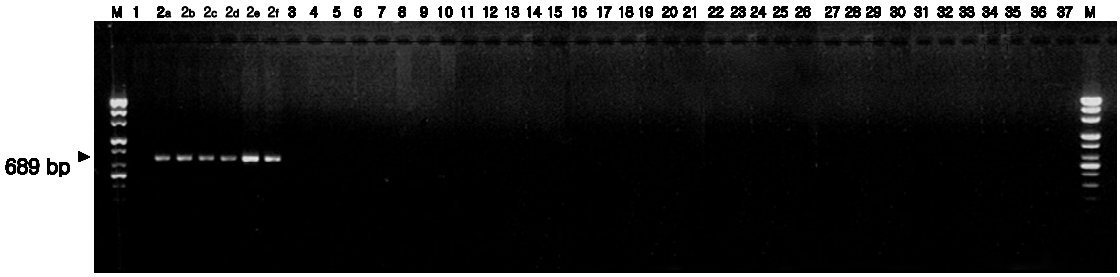

Fig. 6. (A) Agarose gel electrophoresis of *V. anguillarum*-specific DNA products amplified by PCR using the primers VARPO1 and VARPO2.

M, 100-bp DNA ladder lane 1; *V. alginolyticus* (KCTC 2472); lanes 2a–2f, *V. anguillarum* (O1 type, NB 10, J-O-3, J-O-2, ATCC 19264, and YT); lane 3, *V. campbellii* (KCCM 40864); lane 4, *V. carchariae* (KCCM 40865); lane 5, *V. cholerae* (ATCC 14547); lane 6, *V. cincinnatiensis* (KCTC 2733); lane 7, *V. damselae*; lane 8, *V. diazotrophicus* (KCCM 41666); lane 9, *V. fischeri* (KCCM 41685); lane 10, *V. fluvialis* (KCTC 2473); lane 11, *V. furnissii* (KCTC 2731); lane 12, *V. harveyi* (KCCM 40866); lane 13, *V. hollisae* (KCCM 41680); lane 14, *V. logei* (KCTC 12281); lane 15, *V. mediterranei* (KCCM 40867); lane 16, *V. metschnikovii* (KCTC 2736); lane 17, *V. mimicus* (KCTC 2737); lane 18, *V. natriegens* (KCCM 40868); lane 19, *V. navarrensis* (KCCM 41682); lane 20, *V. nereis* (KCTC 2722); lane 21, *V. ordalii* (KCCM 41669); lane 22, *V. orientalis* (KCTC 2725); lane 23, *V. pharahaemolyticus* (KCCM 11965); lane 24, *V. proteolyticus* (KCTC 2730); lane 25, *V. salmonicida* (KCCM 41663); lane 26, *V. tubiashii* (KCTC 2728); lane 27, *V. vulnificus* (ATCC 27562); lane 28, *Aeromonas hydrophila* (KCTC 2358); lane 29, *Escherichia coli* BL21 (DE3); lane 30, *Enterobacter cloacae*; lane 31, *Edwardsiella tarda*; lane 32, *Klebsiella oxytoca*; lane 33, *K. pneumoniae*; lane 34, *Salmonella typhi*; lane 35, *Shigella flexneri*; lane 36, *S. sonnei*; lane 37, *E. coli* XL1-blue.

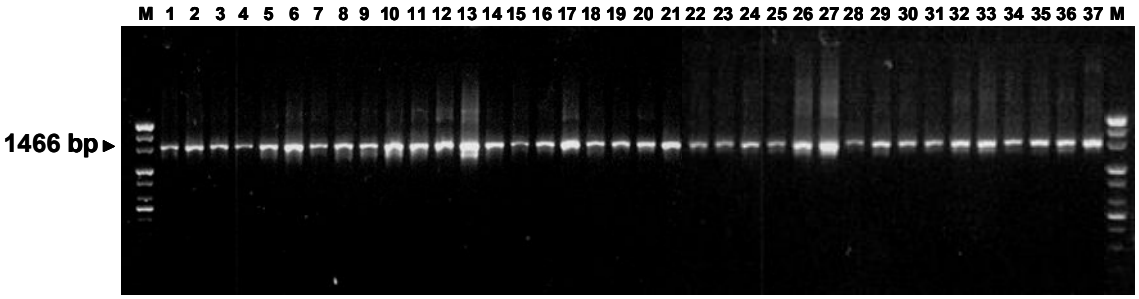

Fig. 6(B) Agarose gel electrophoresis of 16S rRNA products amplified by PCR using the primers 27F and 1492R (positive control).

Lanes were loaded with samples from the same organisms as in Fig. 2A, with the exception of lane 2, which was V. anguillarum serotype O1.

Sensitivity of PCR detection of *V. anguillarum*

Serially diluted genomic DNA samples were prepared as described in the Materials and Methods. Aliquots of diluted DNA, ranging from 0.003 to 3 ng/sample, were amplified by PCR amplification using the primers VARPO1 and VARPO2. PCR amplified the *rpoS* product (689 bp) from as little as 3 pg of purified genomic DNA or 6 cfu mL^{-1} from colony PCR, which was visualized by agarose gel electrophoresis (Fig. 7). All reactions were performed in triplicate.

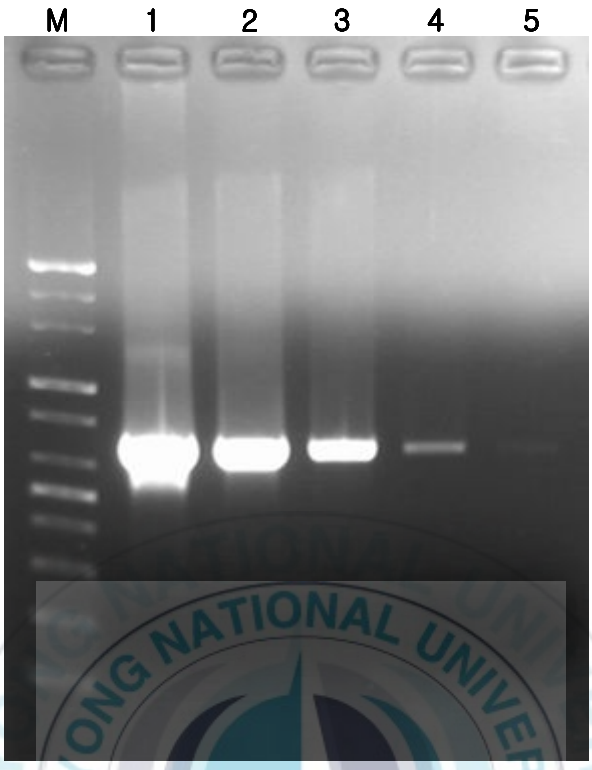

Fig. 7. Sensitivity of VARPO1/VARPO2 primers in amplifying the *V. anguillarum*-specific amplicon at various DNA concentrations.

M, 100-bp DNA ladder. The remaining following lanes contain *V. anguillarum* DNA.

Lane 1, 3 ng; lane 2, 300 pg; lane 3, 30 pg; lane 4, 3 pg; lane 5, 0.3 pg.

Detection of *V. anguillarum* from infected flounder and prawn

To study the detection and sensitivity of this method in fish, we injected flounder with 2.7×10^9 cfu of *V. anguillarum*. After injection, liquid extracts of homogenized liver, kidney, and intestine were serially diluted in saline buffer, and dropped onto BHI and TCBS agar plates. The plates were incubated for 24 h at 25°C. In the control group, few bacterial colonies were detected on the TCBS plates (Table 5). The bacterial population of infected flounder tissue on the TCBS plates was 1.8, 1.7, and 2.3 log cfu mg^{-1} of liver, kidney, and intestine, respectively (Table 5). To analyze the specific detection of *V. anguillarum* from infected flounder tissues, PCR amplification was performed using DNA isolated from infected tissue samples. The PCR assay produced a clear band (689 bp) from infected samples (Fig. 8). When DNA from noninfected flounder tissue was amplified (negative control), no band was observed (Fig. 8). To evaluate the detection limit of this method, serially diluted DNA from flounder tissue was examined. PCR using *rpoS*-specific primers was able to amplify *rpoS* from as little as 50 ng of purified DNA in infected flounder tissue (data not shown).

To investigate the specificity and sensitivity of this method in prawn, we injected animals with *V. anguillarum* and then homogenized the entire animal after 24 h. Homogenized tissues were serially diluted (10×) in saline solution, plated on BHI agar, and then incubated at 25°C for 24 h. The number of bacteria in the control group, used as a negative control for PCR, was between 1.4 and 1.5 log cfu g^{-1} of homogenized tissue. In samples from infected prawn, the number of bacteria was 6.2 log cfu g^{-1} of homogenized tissue. Purified DNA was used as a template for the

specific detection of *V. anguillarum* by PCR. PCR using DNA from infected prawn produced the predicted 689-bp amplicon (Fig. 9). To determine the detection limit for the PCR reaction, serially diluted DNA purified from infected prawn was tested. PCR using *rpoS*-specific primers was able to produce an amplicon from as little as 10 ng of purified DNA in prawn tissue (data not shown).

Table. 5. CFU counts from homogenates of flounder organs on BHI and TCBS plates.

Sample group	Sample from	Plate culture (log CFU/mg)	
		BHI	TCBS
Control fish (PBS treatment)	liver	2.1 ± 0.1	0 ± 0.1
	kidney	2.8 ± 0.2	0.6 ± 0.3
	intestine	2.1 ± 0.1	0.2 ± 0.1
Infected fish (after 24 hr)	liver	5.9 ± 0.2	1.8 ± 0.4
	kidney	6.5 ± 0.2	1.7 ± 0.9
	intestine	5.7 ± 0.3	2.3 ± 1.9

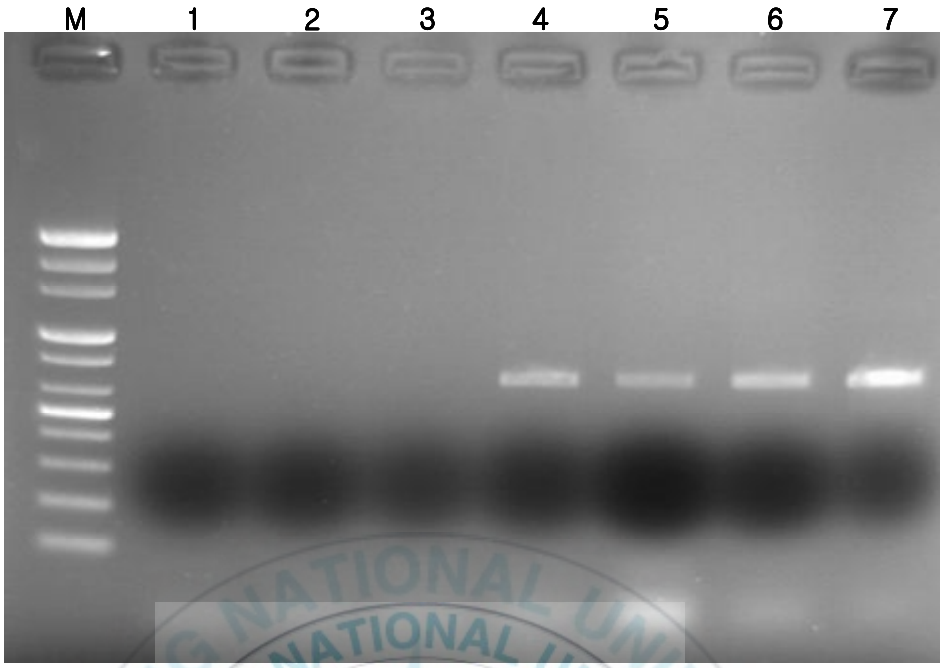

Fig. 8. Agarose gel electrophoresis of PCR products from genomic DNA recovered from noninfected or infected flounder tissues.

M, 100-bp ladder; lanes 1, 2, and 3, liver, kidney, and intestine, respectively, from noninfected flounder; lane 4, 5, and 6, liver, kidney, and intestine, respectively, from flounder infected with *V. anguillarum*; lane 7, PCR product from purified *V. anguillarum* genomic DNA (positive control).

Table 6. CFU counts in homogenates of injected prawns and controls on BHI plates.

Sample group	Samples	Plate culture (log CFU/g)
Control	Untreated	1.5 ± 0.1
	PBS treated	1.5 ± 0.1
Infected prawn (after 24 hr)	*V. anguillarum* injected	6.3 ± 0.1

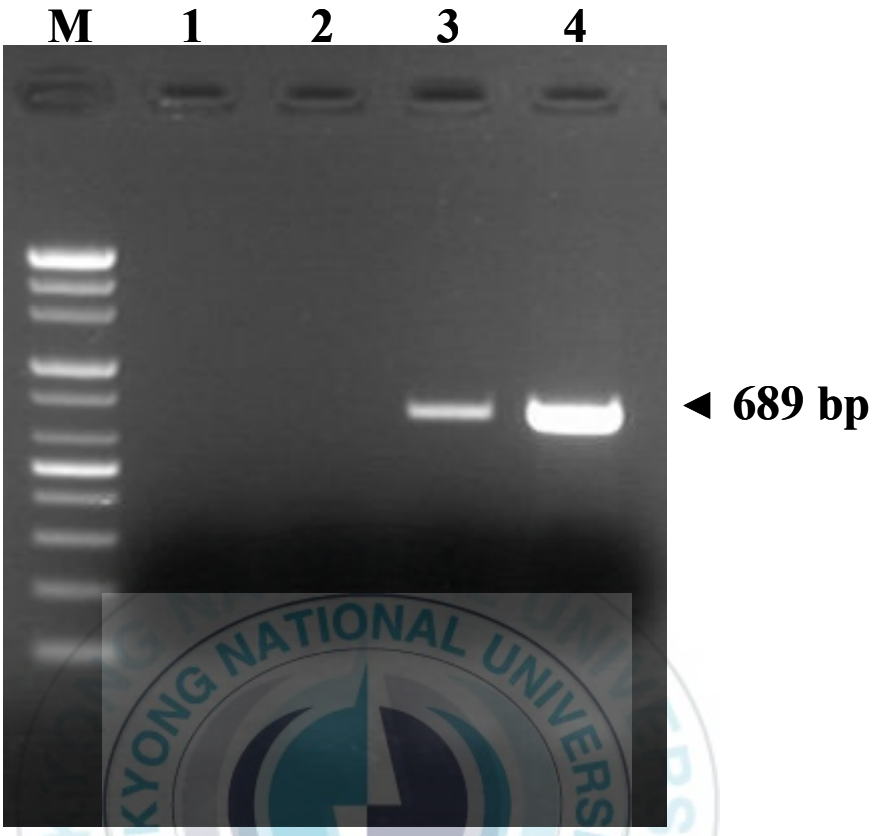

Fig. 9. Agarose gel electrophoresis of PCR products from *V. anguillarum* genomic DNA isolated from noninfected or infected prawn.

M, 100-bp ladder; lane 1, noninfected prawn; lane 2, PBS-injected prawn; lane 3, prawn infected with *V. anguillarum*; lane 4, PCR product from purified *V. anguillarum* genomic DNA (positive control).

Discussion

The detection of *Vibrio* species is important to the marine aquaculture industry because of the large financial losses caused by vibriosis. Among the *Vibrio* species, *V. anguillarum* is the main causative agent of vibriosis. Therefore, sensitive, specific, simple, and rapid methods to detect *V. anguillarum* infection are invaluable to the seafood industry.

To date, various methods have been developed to detect pathogenic bacteria. PCR-based diagnostic methods are more sensitive, specific, and relatively less time-consuming than classical methods. Other research groups have also developed PCR-based methods for the detection of *V. anguillarum* (Hirono, Masuda & Aoki 1996; Gonzalez, Osorio & Santos 2003; Rodkhum, Hirono, Crosa & Aoki 2006). However, the methods used in these investigations were not able to differentiate between *V. anguillarum* and the closely related species *V. ordalii*, which is biochemically and genetically similar. In addition, the methods used in these studies did not differentiate between the various strains of *V. anguillarum*. Hirono, Masuda & Aoki (1996) cloned the *hemolysin* (*vah*1) gene from *V. anguillarum* and designed a DNA probe based on this gene sequence. Unfortunately, this method could not detect *V.*

anguillarum strains that did not possess the *vah*1 gene. Rodkhum, Hirono, Crosa & Aoki (2006) used multiplex PCR for five *hemolysin* genes to detect *V. anguillarum*; however, this method amplified the same products from *V. ordalii*. PCR-based detection assays using virulence genes are also unsuitable; such a method would lack specificity because of the frequent mutations in virulence genes and the absence of this nonessential gene in some species (Brauns, Hudson & Oliver 1991; Kumar, Parvathi, Karunasagar & Karunasagar 2006; Gonzalez, Osorio & Santos 2003). Gonzalez, Osorio & Santos (2003) amplified the sigma factor δ^{54} (*rpoN*) gene to detect *V. anguillarum*. A specific amplicon was produced in all strains of *V. anguillarum*, but the same band appeared during the amplification of *V. ordalii* DNA when the annealing temperature was set at 58°C. Increasing the annealing temperature to 62°C eliminated this band. These results suggest that primers specific to *V. anguillarum* genes are necessary for the development of reliable PCR-based detection assays.

We sequenced the *rpoS* genes from two strains of *V. anguillarum*, and compared these sequences to the *rpoS* gene sequences from five other *Vibrio* species. This comparison allowed us to design specific primers for the *rpoS* gene in *V. anguillarum*,

which successfully detected *rpoS* from pure bacterial cultures and infected tissues from flounder and prawn. The *rpoS* gene encodes the RNA polymerase sigma factor (RpoS), which regulates the expression of genes involved in the stress response (Hülsmann, Rosche, Kong, Hassan, Beam & Oliver 2003). Stressors may include high or low temperature, osmotic shock, nutrient starvation, and oxidative damage. Furthermore, RpoS regulates the expression of virulence genes and plays a role during the colonization of host tissue (Fanget, Libbey, Buchmeier, Loewen, Switala, Harwood & Guiney 1992; Olsén, Arnqvist, Hammer, Sukupolvi & Normark 1993; Kowarz, Coynault, Robbes-Saule & Norel 1994; Römling, Livers, Austin, Kaiser, Jensen, Burgess & Blum 1998; Beltrametti, Kresse & Guzmán 1999; Hengge-Aronis et al., 2000; Merrell, Tischler, Lee & Camilli 2000; Hülsmann, Rosche, Kong, Hassan, Beam & Oliver 2003). For these reasons, the *rpoS* gene is important for adaptation to numerous environmental changes. The *rpoS* gene is particularly important after infection, when the bacterium must cope with the adverse environment within the host. Therefore, we used the *rpoS* gene for species-specific detection of *V. anguillarum*.

We cloned the *rpoS* gene from *V. anguillarum* (1002 bp) and compared its nucleotide

sequence with *rpoS* sequences from other *Vibrio* species, including *V. cholerae* (1008 bp), *V. harveyi* (1787 bp), *V. parahaemolyticus* (966 bp), and *V. vulnificus* (1032 bp). The *rpoS* sequence from *V. harveyi* was substantially longer than *V. anguillarum*, whereas the sequence from *V. parahaemolyticus* was shorter. Thus, it appears that *V. harveyi* and *V. parahaemolyticus* are not as closely related to *V. anguillarum* as other *Vibrio* species. This has also been noted in other studies (Thompson, Gevers, Tompson, Dawyndt, Naser, Hoste, Munn & Swings 2005; Nhung, Shah, Ohkusu, Noda, Hata, Sun, Iihara, Goto, Masaki, Miyasaka & Ezaki 2006). Multiple alignment of the *rpoS* sequences from *Vibrio* species showed that the amino- and carboxy-terminal regions were more variable among species (data not shown). Therefore, we focused on these regions to design specific primers. After PCR specificity and sensitivity were analyzed using various bacterial strains and DNA dilutions, the optimal primer pair was used to detect *V. anguillarum* from infected tissues. The results show that our PCR-based detection assay is sensitive, species-specific, and is able to differentiate between *V. anguillarum* and *V. ordalii* in pure cultures.

We tested the specificity and sensitivity of our method using infected tissue from flounder and prawn, two of the most commonly cultured seafoods in Korea. *V.*

anguillarum was injected into the hosts and the infected organs were isolated. In this investigation, we saved time by performing a direct PCR assay with purified DNA. Without the process of bacterial cultivation, we reduced the time required and successfully obtained a sensitive and species-specific result. However, sensitivity was lower using this method than when we used pure cultured samples. This change in the detection limit may have resulted from the early infection phase of *V. anguillarum* and inhibition by the host tissue matrix (Gonzalez, Osorio & Santos 2003; Rodkhum, Hirono, Crosa & Aoki 2006).

In conclusion, PCR amplification of the *rpoS* gene is a sensitive, species-specific method for the detection of *V. anguillarum* in aquaculture farms. We will continue to evaluate the effectiveness of this method in other marine organisms.

특정유전자를 이용한 *Vibrio anguillarum*의 신속·정확한 PCR 검출 방법

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요약

*Vibrio anguillarum*은 어류에 패혈증은 유발하는 대표적인 어류 병원성 균주로써 야생 또는 양식 어류에 발병하며 특히 이로 인한 양식산업의 막대한 경제적 손실을 야기한다. 이러한 *V. anguillarum*을 검출하기 위하여 ribosomal DNA를 표적으로 한 연구가 주로 발표 되었다. 하지만 본 연구에서는 *amiB*라는 세포벽 분해효소를 encoding하고 있는 유전자와 *rpoS*라는 스트레스 조절 유전자를 표적으로 한 PCR 방법으로써 *V. anguillarum*를 검출하는 실험을 수행하였다. PCR 검출의 특이성은 429-bp 또는 698-bp의 증폭된 밴드가 6 계통의 *V. anguillarum*에서는 모두 검출되는 반면 *V. anguillarum*가 아닌 36종의 다른 미생물에서는 어떠한 밴드가 검출되지 않음으로써 확인 되었다. 또한 PCR을 통한 검출 방법은 *amiB* gene을 표적으로 한 경우 *V. anguillarum*의 chromosomal DNA가 1 pg만 있더라도 가능 하였으며, 감염 실험의 경우 25 mg의 감염어류의 조직 내에 20 CFU의 *V. anguillarum*만이 있더라도 가능하였다. 그리고 *rpoS* gene을 표적으로 한 실험의 경우 3 pg의 genomic DNA, ml 당 6 CFU (colony-forming unit)의 *V. anguillarum*만이 존재 하더라도 검출이 가능하였다. 하지만 감염된 넙치 또는 새우에서는 실험 결과가 위의 결과보다는 조금 덜 민감하여 각각 1 g의 숙주세포 조직 내에 넙치의 경우에는 50 pg, 새우의 경우에는 10 pg의 genomic DNA가 있어야 검출이 가능 하였다. 이러한 실험 결과는 *amiB* 또는*rpoS* 유전자를 표적으로 한 PCR 검출법이 실생활에서 민감하고, 종특이적이며, 빠른 시간 내에 *V. anguillarum*을 검출할 수 있음을 증명한다.

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