

## Detection of marine pathogenic bacterial *Vibrio* species by multiplex polymerase chain reaction (PCR)

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**Abstract:** Polymerase chain reactions (PCR) were performed to accomplish quick and accurate detection of *Vibrio* species. The primers prepared with 16S-23S rDNA intergenic spacer (IGS) region exhibited an excellent species-specificity for *Vibrio* sp and detected *Vibrio* sp more successfully than the conventional culture method. Multiplex PCR was also fruitful not only for the identification of the 5 standard *Vibrio* sp simultaneously, but also for the detection of *Vibrio* sp in the samples collected from the natural environment.

**Key words:** *Vibrio* sp, Multiplex PCR, Species-specific primer  
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### Introduction

Seawater is highly susceptible to contamination due to nearby municipal waste water. Pathogens originating from the contamination frequently provoke lots of disease and sometimes menace even human life (Elmanama *et al.*, 2005). The more serious the contamination, the more abundant the distribution of the pathogens (Sharma and Chaturvedi, 2007; Williams *et al.*, 2007).

*Vibrio* sp are Gram-negative rod-shaped bacteria and are pathogenic to vertebrate as well as invertebrate animals (Ramalingam and Shyamala, 2006).

The conventional methods for identification of *Vibrio* sp such as the culture method and the biochemical method are usually time-consuming and often yield false results especially when a limited number of cells exist in the sample (Brasher *et al.*, 1998).

Polymerase chain reaction (PCR) quickly detects pathogenic microorganisms without the need of the cultivation process through the oligonucleotide primer-direction method (Brasher *et al.*, 1998) by way of DNA amplification. Primers synthesized from 16S rDNA gene can be used for the species-specific detection by using PCR for the purpose of phylogenetic classification of microorganisms (Kang *et al.*, 2003). However the highly stable gene sequence limits the applicability of this method for the precise identification of the microorganisms. Hence intergenic spacer (IGS) region between 16S and 23S rDNA gene has been used preferentially for the detection of the microorganisms (Kang *et al.*, 2003). The IGS between 16S and 23S rDNA gene is usually composed of tRNA gene and non-coding region. As the 16S rDNA gene is highly stable, it is used as a tool for the information of phylogenetic classification (Darwish *et al.*, 2005).

In this study, species-specific primers against *V. parahaemolyticus*, *V. fluvialis*, *V. vulnificus*, *V. cholerae*, and *V. proteolyticus* were prepared from their 16S-23S IGS regions. The 5

*Vibrio* sp have been reported to cause serious diseases such as cholera, septicemia and gastroenteritis (Yoon *et al.*, 2003). The species-specific primers thus made were subjected to the experiments to confirm whether they detect the same strains in the samples collected from the environmental samples such as oyster, shellfish, octopus, flounder fish and seawater collected from Yellow Sea of Korean peninsula.

### Materials and Methods

**Bacterial strains and DNA extraction:** The bacterial strains investigated in this study were purchased from Korea Culture Center of Microorganisms. *Vibrio* sp were grown on brain heart infusion broth (BHI; Difco) supplemented with 1% (w/v) NaCl at 37°C for 24hr. The cell pellet, from 10 ml overnight culture, was used for extraction of DNA. Genomic DNA was extracted using Bio 101 systems GNOME® kit (Q-Bio gene) and the DNA concentration was determined using a spectrophotometer (Shimadzu) at 260 nm wavelength.

**16S-23S IGS region amplification of *Vibrio* sp:** All oligonucleotide primers used in this study were synthesized by Bionics, Korea. The PCR primers used for amplification of DNA fragments spanning the 16S-23S IGS regions were targeted at highly conserved regions of the 3' and 5' ends of the 16S and 23S rDNAs respectively. 16S-23S IGS regions was amplified and analyzed according to Lee *et al.* (2002). Species-specific primers for detection of the 5 *Vibrio* sp are given in Table 1.

**Multiplex PCR amplification:** Approximately 50 ng of bacterial DNA was used for multiplex PCR amplification. 20 µl PCR reaction mixture contained 2 µl 10X PCR buffer, 2.5 µl dNTP mixture, 2 mM MgCl<sub>2</sub>, 25 pmol of each primer, 0.1 µl DNA templates and 2U *Taq* plus polymerase. PCR amplification reactions were performed in a GeneAmp® PCR system 2400 (Applied biosystem); preincubation step at 94°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min and extension at 72°C for 2.5 min followed by final incubation at 72°C for 10 min.



**Sensitivity of detection of multiplex PCR:** The lowest amount of DNA of each targeted strains was tested in the multiplex PCR. 100 pg of purified genomic DNA from each of the 5 *Vibrio* sp was serially diluted in sterile distilled water up to 10 fg to measure the detection sensitivity of multiplex PCR. DNA extraction was carried out according to the procedure described by Lee and Choi (1995). The sensitivity varying the cell number of the 5 targeted strains from oyster tissue homogenates was also measured in the range between 10 cfu g<sup>-1</sup> and 10<sup>6</sup> cfu g<sup>-1</sup> (Brasher *et al.*, 1998). The detection capability of PCR was tested with the samples collected from the sea, *i.e.*, oyster (*Crassostrea gigas*), arkshell (*Scapharca broughtonii*), octopus (*Octopus dofleini*), flounder fish (*Paralichthys olivaceus*) and seawater collected from Hwasung, Anmyendo and Incheon, locating by Yellow Sea of Korean peninsula. Oyster, arkshell, octopus and flounder fish were shucked following the standard method (APHA, 2005).

### Results and Discussion

**Preparation of species-specific primers of *Vibrio* sp:** PCR reactions were performed to amplify the 16 S-23 S rDNA IGS regions of *Vibrio* sp by using 16 S-23S/F and 16 S-23S/R primers.

Primers were prepared by using the sequence of the non-coding region because the non-coding region did not show any

similarity except tRNA gene and Box A element. The sequence homology of the primers was confirmed with the BLAST program of Gene Bank, and it was found to be specific to each *Vibrio* sp. Sequences and target positions of the species-specific primers of the *Vibrio* sp are summarized in Table 1.

**Detection of 5 *Vibrio* sp:** In order to substantiate the assessment of the primer, the IGS sequence of PCR was compared with those of the other *Vibrio* species. Genomic DNA was extracted from 7 strains of *Vibrio* sp and 23 strains other than *Vibrio* sp and it was used for

**Table - 1:** Sequences of the species-specific primers of *Vibrio* species and their PCR product size. Primers are targeted for 16S-23S IGS region

Strain	Products (bp)	Primer	Sequence (3'→5')
<i>V. parahaemolyticus</i> ATCC17802	400	VPAR-F VPAR-R	CACTCACCTCTCTCGAACTC TAGCGTTGTTCCGGAATTACT
<i>V. fluvialis</i> ATCC33089	250	VFLU-F VFLU-R	TATACTATCCGACGACTGGC TTCGTAGATATTCGGAGGAA
<i>V. vulnificus</i> ATCC25922	350	VVUL-F VVUL-R	ATTTCACCTCTACACTCGGA TAGCGTTGTTCCGGAATTACT
<i>V. cholerae</i> ATCC25920	600	VCHO-F VCHO-R	TATACTATCCCGGAGAACAC ATATTTACCTCTCTCGAACTC
<i>V. proteolyticus</i> ATCC15338	150	VPRO-F VPRO-R	CCTCTCTCGAACTCCGGAG GTTGTGGAATTACTCGGAGGAA

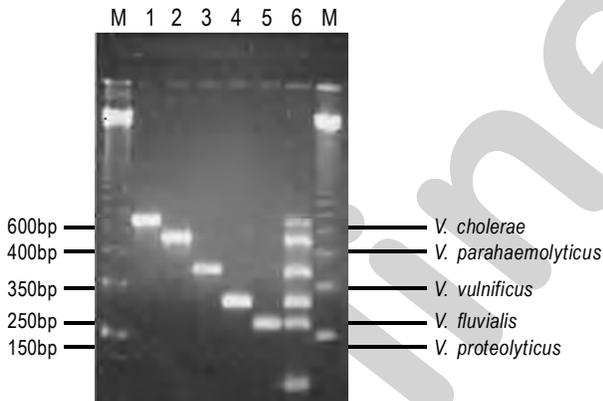
**Table - 2:** Bacterial strains for the evaluation of the species-specific primers of *Vibrio* species

Strain	Source	VPAR	VFLU	VVUL	VCHO	VPRO
<i>Acinetobacter lwoffii</i>	ATCC15309	-	-	-	-	-
<i>Aeromonas sobria</i>	ATCC43979	-	-	-	-	-
<i>Bacillus megaterium</i>	ATCC14581	-	-	-	-	-
<i>Bacillus polymyxa</i>	KCCM3541	-	-	-	-	-
<i>Bacillus subtilis</i>	KCCM11779	-	-	-	-	-
<i>Enterobacter aerogenes</i>	ATCC13048	-	-	-	-	-
<i>Enterococcus faecalis</i>	ATCC29212	-	-	-	-	-
<i>Escherichia coli</i>	IFO3806	-	-	-	-	-
<i>Escherichia coli</i>	ATCC11775	-	-	-	-	-
<i>Klebsiella pneumoniae</i>	ATCC10031	-	-	-	-	-
<i>Micrococcus luteus</i>	ATCC4698	-	-	-	-	-
<i>Proteus vulgaris</i>	ATCC6059	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	ATCC15522	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	ATCC15523	-	-	-	-	-
<i>Pseudomonas fluorescens</i>	ATCC13525	-	-	-	-	-
<i>Pseudomonas stutzeri</i>	ATCC17588	-	-	-	-	-
<i>Salmonella enteritidis</i>	ATCC13076	-	-	-	-	-
<i>Shigella boydii</i>	ATCC8700	-	-	-	-	-
<i>Shigella flexneri</i>	ATCC29903	-	-	-	-	-
<i>Staphylococcus aureus</i>	ATCC62738	-	-	-	-	-
<i>Staphylococcus aureus</i>	ATCC65389	-	-	-	-	-
<i>Staphylococcus aureus</i>	ATCC27735	-	-	-	-	-
<i>Staphylococcus epidermidis</i>	ATCC12228	-	-	-	-	-
<i>Vibrio alginolyticus</i>	ATCC17749	-	-	-	-	-
<i>Vibrio cholerae</i>	ATCC25920	-	-	-	+	-
<i>Vibrio diazotrophicus</i>	ATCC 33466	-	-	-	-	-
<i>Vibrio parahaemolyticus</i>	ATCC17802	+	-	-	-	-
<i>Vibrio proteolyticus</i>	ATCC15338	-	-	-	-	+
<i>Vibrio fluvialis</i>	ATCC33809	-	+	-	-	-
<i>Vibrio vulnificus</i>	ATCC25922	-	-	+	-	-

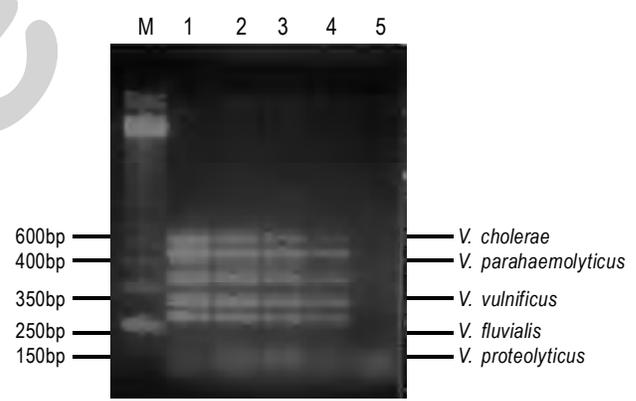
VPAR, VFLU, VVUL, VCHO and VPRO are primers for *V. parahaemolyticus*, *V. fluvialis*, *V. vulnificus*, *V. Cholerae* and *V. proteolyticus*, respectively

**Table - 3:** Comparison of detection capability of the PCR method with the conventional culture method

Sampling site	Sample	<i>V.parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. fluvialis</i>	<i>V. cholerae</i>	<i>V. proteolyticus</i>
		PCR method / Culture method				
Yellow sea (Gingok-ri, Hwasung, Korea)	Seawater	+/+	-/-	+/+	+/+	-/-
	Arkshell( <i>Scapharca broughtonii</i> )	+/+	+/+	+/-	+/+	-/-
	Oyster( <i>Crassostrea gigas</i> )	+/+	+/+	+/+	+/+	+/-
	Octopus( <i>Octopus dofeini</i> )	+/+	-/-	-/-	-/-	-/-
	Skin and intestine tissue mixture of flounder fish( <i>Paralichthys olivaceus</i> )	+/+	+/-	+/-	-/-	-/-
	Gill of flounder fish ( <i>Paralichthys olivaceus</i> )	+/+	+/-	+/-	-/-	-/-
Yellow sea (Anmyen Tae-an-kun, Korea)	Seawater	+/+	+/+	+/+	+/-	-/-
	Arkshell( <i>Scapharca broughtonii</i> )	+/+	+/+	+/-	+/-	-/-
	Oyster( <i>Crassostrea gigas</i> )	+/+	+/+	+/+	+/-	+/-
	Octopus( <i>Octopus dofeini</i> )	+/+	+/-	+/-	+/-	-/-
	Skin and intestine tissue mixture of flounder fish( <i>Paralichthys olivaceus</i> )	+/+	+/-	+/-	-/-	-/-
	Gill of flounder fish ( <i>Paralichthys olivaceus</i> )	+/+	+/-	-/-	-/-	-/-
Yellow sea (Yongdo- dong, Incheon, Korea)	Seawater	+/+	+/+	+/+	+/+	-/-
	Arkshell( <i>Scapharca broughtonii</i> )	+/+	+/+	+/-	+/+	-/-
	Oyster( <i>Crassostrea gigas</i> )	+/+	+/+	+/+	+/+	+/-
	Octopus( <i>Octopus dofeini</i> )	+/+	+/-	+/-	+/-	-/-
	Skin and intestine tissue mixture of flounder fish( <i>Paralichthys olivaceus</i> )	+/+	+/-	+/-	+/-	-/-
	Gill of flounder fish ( <i>Paralichthys olivaceus</i> )	+/+	+/-	+/-	+/-	-/-
Pure culture (Korea Center of Disease Control and Prevention)		+/+	+/+		+/+	



**Fig. 1:** Electrophoresis of multiplex PCR products from 5 *Vibrio* species. Lane M: 50 bp DNA step ladder(Promega), Lane 1: *V. cholerae*, Lane 2: *V. parahaemolyticus*, Lane 3: *V. vulnificus*, Lane 4: *V. fluvialis*, Lane 5: *V. proteolyticus*



**Fig. 2:** Detectable sensitivity of multiplex PCR with DNA isolated from 5 *Vibrio* species. Lane M: 50 bp DNA step ladder(Promega), Lane 1: 100 pg ml<sup>-1</sup> DNA, Lane 2: 10 pg ml<sup>-1</sup> DNA, Lane 3: 1 pg ml<sup>-1</sup> DNA, Lane 4: 0.1 pg ml<sup>-1</sup> DNA, Lane 5: 10 fg ml<sup>-1</sup> DNA

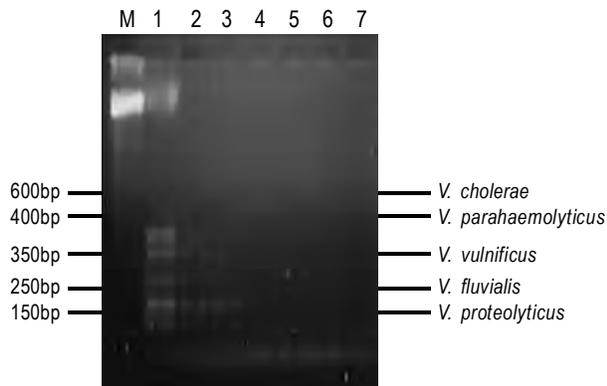
PCR reaction to prove the species-specificity of the *Vibrio* species-specific primer. The results indicated that the primers prepared with IGS sequence exhibited an excellent *Vibrio* species-specificity and thereby they could be successfully used for the detection of *Vibrio* sp as demonstrated in Table 2.

Multiplex PCR was attempted to detect different kinds of *Vibrio* sp at the same time. The multiplex PCR attempt was successful using

5 species-specific primers, and the results are displayed in Fig. 1.

Detection sensitivity on the DNA content was investigated in the range of 100 pg ml<sup>-1</sup> -10 fg ml<sup>-1</sup>, and the results are disclosed in Fig. 2. The detection was successful down to 0.1 pg ml<sup>-1</sup> in the test with the 1/10 diluted total DNA of the 5 *Vibrio* sp. Bands were not observed when the DNA content was less than 10 fg ml<sup>-1</sup>.





**Fig. 3:** Detectable sensitivity of multiplex PCR with different cell numbers of each *Vibrio* species in oyster tissue homogenates. Lane M: 50 bp DNA step ladder (Promega), Lane 1:  $10^6$  cfu  $g^{-1}$ , Lane 2:  $10^5$  cfu  $g^{-1}$ , Lane 3:  $10^4$  cfu  $g^{-1}$ , Lane 4:  $10^3$  cfu  $g^{-1}$ , Lane 5:  $10^2$  cfu  $g^{-1}$ , Lane 6: 10 cfu  $g^{-1}$

The primer prepared by Kim and Jeong (2001) using the 16S rRNA region of *V. fluvialis* was sensitive down to 5 pg  $ml^{-1}$ , while the PCR using the specific genes of *E. coli*, *Salmonella typhimurium*, *V. cholerae* and *V. parahaemolyticus* respectively was sensitive down to 100 fg  $ml^{-1}$ . Hence it can be said that the PCR sensitivity of the present study is far more outstanding.

Detection of multiplex PCR against 5 target *Vibrio* sp was performed at different cell numbers from oyster tissue homogenates as shown in Fig. 3. When less than  $10^3$  cfu  $g^{-1}$  of the 5 *Vibrio* sp were present in oyster, all the 5 species were not detected at the same time. Since Bej *et al.* (1999) and Lee and Choi (1995) reported the threshold cell number for the detection to be  $10^3$  cfu  $g^{-1}$ , the performance of the primer of the present study can be said to be almost equivalent to that of the previous studies.

**Detection of *Vibrio* sp in natural resources:** Genomic DNA was extracted from oyster, arkshell, octopus, flounder fish and seawater collected from three different places in Yellow Sea, and amplified as shown in Table 3. The PCR was more sensitive than the conventional culture method, because the latter could not detect the bacteria in some samples, while PCR proved the existence of the bacteria. *V. parahaemolyticus* was detected from all samples. *V. vulnificus* was detected from all samples except sea water and octopus from Hwasung. *V. fluvialis* was not detected from the octopus of Hwasung and flounder fish gill of Anmyendo. *V. cholerae* was not detected from the octopus and flounder fish tissue mixture (skin and intestine tissue mixture) and gill of Hwasung as well as from flounder fish tissue mixture and gill of Anmyendo. *V. proteolyticus* was found only in the oysters collected from Hwasung, Anmyendo and Incheon. *V. parahaemolyticus* causing fish disease was detected from all samples.

Lee *et al.* (2002) centrifuged the sea water contaminated with *Vibrio* sp and washed with sterilized distilled water to prepare salt-free cell suspension. They performed the multiplex PCR to explore the field applicability of the species-specific primer, and observed that all the target genes were detected. Their primers were detectable for *V. vulnificus* from octopus infected with the strain. However, in this study, even the DNA extracted from the samples collected from the nature, showed no detection from octopus. It might be due to negligible numbers of the strain in samples.

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