Rapid Discrimination of Fish Pathogenic *Vibrio* and *Photobacterium* Species by Oligonucleotide DNA Array

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ABSTRACT—We developed a low-density oligonucleotide DNA array for the discrimination of 17 *Vibrio* and 1 *Photobacterium* species that are pathogenic to aquatic animals. We designed oligonucleotide probes targeting non-coding regions of the intergenic transcribed spacers (ITS) between 16S and 23S ribosomal DNA. Based on the alignment of ITS sequences obtained from the database and the results in this study, three oligonucleotide probes from a bacterial species were designed and immobilized on a nylon membrane. The ITS regions were PCR-amplified using a pair of universal primers, followed by hybridization of the digoxigenin (DIG)-labeled PCR products to the membrane. Specific signals were produced with alkaline phosphatase-conjugated anti-DIG antibody and chemiluminescent substrate. Although cross hybridization was observed, the patterns from each species were different among the species. This DNA array is useful for a rapid and easy discrimination of *Vibrio* species. The total process, including PCR amplification, can be carried out within a day. Our method enables a global screening of fish pathogenic *Vibrio* and *Photobacterium* species

Key words: DNA array, discrimination, *Vibrio*, *Photobacterium*

Vibrio species are widely distributed in aquatic environments and many of the species are causative agents of infection in humans and aquatic animals (Buller, 2004). In fish pathology, the identification of *Vibrio*-like organisms depends first on their growth on a selective medium: thiosulphate citrate bile salt sucrose (TCBS). Generally, further identification depends on their physiological and biochemical characteristics. However, commercial bacterial identification systems based on the characteristics need extreme care to take correct answers, because misidentification by these systems has been reported (Pillot *et al.*, 2002; O'Hara *et al.*, 2003). Alternative serological identification of *Vibrio* species with polyclonal antisera (Tajima *et al.*, 1987; Martin and Siebeling, 1991; Mutharia *et al.*, 1993; Martinez-Govea *et al.*, 2001) and monoclonal antibodies (Ito and Yokota, 1987; Chen *et al.*, 1992; Sung *et al.*, 1993) has been reported, but the number of detectable species by the serological method is limited. Although some species-specific PCR primers has also been

reported (Buller, 2004), they are not suitable for exhaustive screening.

DNA array allows the simultaneous screening of a large amount of genes in a short assay time. Because of their usefulness, DNA arrays have been utilized for bacterial identification (Call, 2005). Warsen *et al.* (2004) developed a DNA array based on 16S ribosomal RNA gene for the discrimination of fish pathogens. Gonzalez *et al.* (2004) reported the detection of marine fish pathogenic bacteria using multiplex PCR and DNA microarray. They targeted the genes encoding cytolysin, DNA gyrase B subunit, urease, phospholipase and plasmid borne-loci. Yet, their method is not enough for an exhaustive screening of *Vibrio* species, because *Listonella anguillarum*, *V. parahaemolyticus* and *V. vulnificus* has only been targeted.

Intergenic transcribed spacers (ITS) between 16S and 23S rRNA gene were thought to be under less selective constraints and, therefore, to provide higher genetic variation than rRNA gene sequences (Gürtler and Stanisich, 1996). ITS have been utilized to differentiate various bacteria (Gürtler and Stanisich, 1996; Kong *et al.*, 1999; Chen *et al.*, 2005) including *Vibrio*

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species (Chun *et al.*, 1999; Maeda *et al.*, 2000; Simon *et al.*, 2002). In this study, we utilized species-specific sequences in the ITS non-coding region to design oligonucleotide probes for DNA array for the discrimination of genus *Vibrio* at the species level.

Materials and Methods

PCR amplification of ITS non-coding region and sequence analysis

The bacterial strains used in this study are listed in Table 1. Bacteria were cultured on Marine agar 2216 (Difco, USA). Chromosomal DNA was extracted by a method using proteinase K and phenol-chloroform (Sambrook *et al.*, 1989). The PCR primer pair used for amplification of the ITS non-coding region consisted of VITSF, 5' - wrctcttaacaatttga-3' (designed based on the consensus sequence found in the box A (Simon *et al.*, 2002) of ITS non-coding region) and VITSR, 5' - cgstkagycacttaaccata-3' (designed based on the consensus sequence in 23S rRNA gene). The expression of the primer sequence is based on the IUPAC code. Temperature cycling on an iCycler (Bio-Rad, USA) included an initial denaturation step (94°C for 4 min), 30 cycles of core PCR reaction (30 s at 94°C, 30 s at 55°C, 60 s at 72°C), and a final elongation step (7 min at 72°C). PCR products were cloned into the plasmid vector pDrive (Qiagen, Germany), and amplified for nucleotide sequencing. Sequence was performed using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) and analyzed in the ABI 377 automated DNA sequencer (Applied Biosystems, USA). The collected sequencing data were aligned using the Genetyx software package (version 6, Genetyx, Japan).

DNA array composed with oligonucleotides

The sequences of oligonucleotide probes are listed in Table 2. The unique regions to one particular *Vibrio* and *Photobacterium* species were identified using the sequence alignment from the sequences obtained in this study and the sequences from a GenBank database. Oligonucleotides were commercially synthesized. Because most *Vibrio* and *Photobacterium* species were hardly identified with a single probe, we used a combination of three probes to identify individual species. The positive control probe was designed based on the 5'-end of the 23S rRNA gene sequence. The oligonucleotide probes (0.1 µM in sterilized DW) were transferred onto a positively charged nylon membrane using multi-pin-blotter (Atto, Japan) according to the layout illustrated in Fig. 1. The spotted nylon membrane was baked at 120°C for 20 min and stored at room temperature.

Sample labeling and hybridization with DNA array

The ITS non-coding region of sample DNA was

Table 1. Strains used in this study and results of PCR amplification

Species	Strain numbers used for sequence analysis and/or PCR	Results of PCR amplification
<i>P. damsela</i> subsp. damsela	ATCC33539	+
<i>P. damsela</i> subsp. piscicida	ATCC17911	+
<i>V. alginolyticus</i>	IID950	+
<i>V. anguillarum</i> (<i>Listonella anguillarum</i>)	NCMB6	+
<i>V. harveyi</i>	ATCC35084	+
	HUFP9111	+
<i>V. cholerae</i>	WA1	+
<i>V. fluvialis</i>	IAM14403	+
<i>V. ichthyenteri</i>	IFO15847	+
<i>V. nigripulchritudo</i>	ATCC27043	+
<i>V. ordalii</i>	ATCC33509	+
<i>V. parahaemolyticus</i>	IFO12711	+
<i>V. pelagius</i> (<i>L. pelagia</i>)	ATCC25916	+
<i>V. penaeicida</i>	KH1	+
<i>V. salmonicida</i>	NCIMB2262	+
<i>V. splendidus</i>	IAM14411	+
<i>V. tapetis</i>	NCIMB13622	+
<i>V. tubiashii</i>	ATCC19109	+
<i>V. vulnificus</i>	ATCC27562	+
<i>Vibrio</i> sp. *	—	+
<i>Aeromonas caviae</i>	JCM1060	+
<i>A. hydrophila</i>	IAM12337	+
<i>A. jandaei</i>	JCM8316	+
<i>A. salmonicida</i>	JCM7874	—
<i>A. sobria</i>	JCM2139	+
<i>Bacillus subtilis</i>	IAM1069	—
<i>Citrobacter freundii</i>	FPC349	—
<i>Edwardsiella ictaluri</i>	JCM1679	—
<i>E. tarda</i>	ATCC15947	—
<i>Escherichia coli</i>	IAM1239	—
<i>E. vulneris</i>	IAM14239	—
<i>Flavobacterium branchiophilum</i>	FPC520	—
<i>F. columnare</i>	ATCC43622	—
<i>F. psychrophilum</i>	NCMB1947	—
<i>Hafnia alvei</i>	JCM1666	—
<i>Klebsiella pneumoniae</i>	IAM12015	—
<i>Lactococcus garvieae</i>	FPC136	—
<i>Micrococcus luteus</i>	IAM1056	—
<i>Nocardia seriolae</i>	TC 258	—
<i>Pseudomonas anguilliseptica</i>	NCMB 1949	—
<i>P. chlororaphis</i>	IAM12354	—
<i>P. fluorescens</i>	IAM12022	—
<i>P. plecoglossicida</i>	ATCC700383	—
<i>P. putida</i>	ATCC12633	—
<i>Serratia marcescens</i>	IAM12142	—
<i>S. plymuthica</i>	IAM13543	—
<i>Staphylococcus aureus</i>	IAM1011	—
<i>S. epidermidis</i>	IAM12012	—
<i>Streptococcus iniae</i>	ATCC29178	—
<i>Tenacibaculum maritimum</i>	ATCC43397	—
<i>Yersinia ruckeri</i>	ATCC29473	—

*: Isolated from diseased striped jack *Pseudocaranx dentex* (unpublished).

Only extracted genomic DNA exist.

Table 2. Oligonucleotide probes used for DNA hybridization

Species	Probe	Sequence
<i>V. alginolyticus</i>	A	TGGTGTGTTGTCATAAAGACAAACATTGAGAAGCTTTACAAACAATCTTAAA
	B	GATTTTGCCGGACTCAATTTTGAATAATCACTGTAAAAGTGATATTCCTCA
	C	AACAAGTACCAAAGTTGTCTCTCATTATTTGAATGAGCGAGAGACATTC
<i>V. anguillarum</i> (<i>L. anguillarum</i>)	A	TATCAATACGTTAAACGTATTGATGTTGAGAAGCTTTTATTTGACAACAAC
	B	TTGATTTTGCCGGACTCAATTTTGAAAAGCTTACCGTAGTAAGCTTTCCCA
	C	CAGACAAGTACCAAAGTTGTCTCTCATTATTTGAATGAGCGAGAGACGT
<i>V. harveyi</i> (ATCC35084)	A	TGTATTGGTGTGTTGTCATAAAGACAAACATTGAGAAGCTTTACAAACAAAC
	B	ACTCGATTTTGCCGGACTCAAATTTCCAAGAACACTTGAATGTGTTGATT
	C	TCAAATCACCAAAGTTGTCTCTCATTATTTGAATGAGCGAGAGACATATC
<i>V. cholerae</i>	A	TGTTGGTGTGTTATCTTTGATAAAGACATTGAGAAGCTTTACAAACAACAATA
	B	GATTTTGCCGGACTCAAATTTGAACAAGACCGAAGTCTTATTCGATACCA
	C	CTGTTTAAACAACCTAAGTTGTCTCGCGTTTAAATTTACATGAGTGCGAGA
<i>V. fluvialis</i>	A	TTTGTTGGTGTATTCCATTGAGGAATACATTGAGAAGCTTTACAAACAACA
	B	GTGTGACTGATTTTGCCGGACTCAATTTGAAGACTTACTGAATCAGTAAG
	C	TTGGGTTGCTGTTAAACAACCTAGGTGTACACATTTATTTTTTCATGAGT
<i>V. harveyi</i> (HUF9111)	A	GGTGTGTTGTCGTAAGACAAACATTGAGAAGCTTTACAAACAAAGCTTAAAA
	B	The same sequence as <i>V. harveyi</i> (ATCC35084).
	C	ATGAACAAGTACCAAAGTTGTCTGCAATTTTTATACATGATGCAGACTC
<i>V. ichthyenteri</i>	A	TTTTTGGTGTGTTACCTTAAAGGTAACATTGAGAAGCTTTTTTCGATAACA
	B	ATTTTGCCGGACTCAATTTTGAATAATCATCATAAATGATGATATTGCCA
	C	TTTCGTTAAACAACCAAGTTGTCTCTCTATTTGATGAGTGAGAGACGTTT
<i>V. nigripulchritudo</i>	A	GTTGGTACCTAAAACACAGTTTTAGGATTTGAGAAGCTTTTAAATTGAATAACAACGCTTAT
	B	AAGAGGTCTTGTATGTTCAAACAACCAAGGTTTGTGTCTCTCATTATTGA
	C	AGAGACATTCGATTTTGCCGGACTCAAATATAACATCACAAGGATGTTT
<i>V. ordalii</i>	A	ATATTGGTGTGTTGTCATCAAGACAAGCATTGAGAAGCTTTACAAACAACAA
	B	CGGACTCAATTTTTCTTCTCCCTTACTTTAAAAAAGTAAGACAAAAGCCA
	C	CGAATCTTGAGGATGTAGTTAAACAACCAAGTTGTCTGCAATTATTTAA
<i>V. parahaemolyticus</i>	A	GTTTTGTGAGCTTTTCAAATTTGTTAAAGAGCTATGTTAGCTATGTTAGCT
	B	CTCAAATTCGAAGAACACTTGAATGTGTTATTTTGGTGTGTTGCTTAAAG
	C	The same sequence as <i>V. harveyi</i> (HUF9111)-C.
<i>V. pelagius</i> (<i>L. pelagia</i>)	A	TTTACTGTTCTTAATAAATAAGAGCAGATAAACATTGAGAAGCTTTTAAATT
	B	GTGTTTGCCGGACTCAATGGTGAATCAATGTAACATTGATTGCAATACA
	C	GTAACAACCAAGGTTTTTGGTTGTAACAAGAAGGGTTAATTCCTATTAC
<i>V. penaeicida</i>	A	ACCTAAATGAACAAGTCATCTAGGATTTGAGAAGCTTTTAAATTTGAATAAC
	B	GGGTTTTACGAAAGTGAAAACATATTTGAGTCCGGCAAAATCGAAGTGT
	C	AACAACCAAGGTTTATCGTTGTCTCTTTATTATGAGCGAGACAACACTTC
<i>V. salmonicida</i>	A	TAACTTAATTACAGCCTGAGCTGTTTTGTTTTACTTTTTAAAAAGTAAAGATAAATTAAAGC
	B	CGCCGGATTCAAAATACAAGAACACTTGAATGTGTTTTAATTTGTTTAT
	C	GGACGTTTGCTTTCACTTTTCAAAAATGAAGACAAAACAGCAACCAAGGT
<i>V. splendidus</i>	A	TTTATCCAAAGGATAAACATTGAGAAGCTTTTAAATTTGATTAAGTCTTAGA
	B	GTGTTTGCCGGACTCAATGTGATTCAAATTAGATTTGAATCGAATACAAG
	C	TAATGTATGGCAACTAACCAAGGTTTTTGGTTGTAATAAGAAGGGTTAGT
<i>V. tapetis</i>	A	GTATAGGATTTGAGAAGCTTTTAAATTTGAATAACAACATATCACAGTCG
	B	TATGTTTTTATTTCGATGAAAACCCAAGAACACTTGAATGTGTGTTGGTA
	C	ATGTATGGCAACAACCAAGGTTGTCTCTCTATTTTATGAGTGAGAGACG
<i>V. tubiashii</i>	A	AATTCATAGAATTAGGATTTGAGAAGCTTTTAAATTTGAATAACAACGCATCTCAAAGAGAT
	B	ATACATGTGCAGACACGATTTTGCCGGACTCAAATATTAACATCGCAAG
	C	CTCTGTTTAAACAACCAAGTTGTCTGCATTTTATACATGTGCAGACAC
<i>V. vulnificus</i>	A	TGTTGGTGTGTTGTCATAAAGACAAACATTGAGAAGCTTTACAAACAACAAT
	B	GATTTTGCCGGACTCAATTTTGAATAAGTCACTTAACAGTGAATATTCCCA
	C	TTAAACAACCAAGTTGCTATCTCATTATTTGAATGAGCGAGATAGCGTT
<i>P. damsela</i>	A	GAGAAGCTGTTTTTATCTTTGATAAAAAGCTTAAATTAATCAATCATTGATTGAATTT
	B	GCCGGACTCACTATTTGCTTTCACTTTTAAAAAGTGAAGACAAAAAATAC
	C	TTTCGTATGTTCAAACAACCAAGGTTGTCTATCTGAGTTGTGATAGACGTT
<i>Vibrio</i> sp.	A	GTGTTTACAAGTCATGAAAATTCATGAAAAGTAAACTTGAGAAGCTTTTAAATAAAATTTGA
	B	AATCAAACAACCAAGTTGTCTCTCATTAAATTTTTTCATGAGCGAGAGAC
	C	TTTGCTTGCTACTTTTCACTTATGCTGCGTTAGCTTATAAATTTGCTTGG
Positive control		TGCCAAGGCATCCACCGTGTACGCTTAGTCACTTAACCATACAACCCGAA

Fig. 1. Positions of *Vibrio* and *Photobacterium*-specific oligonucleotides on the DNA array. Spots 1: *V. alginolyticus*, 2: *V. anguillarum*, 3: *V. harveyi* (ATCC35084), 4: *V. cholerae*, 5: *V. fluvialis*, 6: *V. harveyi* (HUF9111), 7: *V. ichthyenteri*, 8: *V. nigrilulchritudo*, 9: *V. ordalii*, 10: *V. parahaemolyticus*, 11: *V. pelagius*, 12: *V. penaeicida*, 13: *V. salmonicida*, 14: *V. splendidus*, 15: *V. tapetis*, 16: *V. tubiashii*, 17: *V. vulnificus*, 18: *P. damsela*, 19: *Vibrio* sp., 20: positive control.

labeled with digoxigenin (DIG) during PCR amplification. The PCR mixture (10 μ L) contains DIG-UTP (Roche Diagnostics, Switzerland), *Taq* DNA polymerase (Takara, Japan), the primers (VITSF and VITSR), buffer and sample DNA. PCR was performed as described above to incorporate DIG into PCR amplicons. Hybridization was performed as follows: A 0.5 μ L of denatured DIG-labeled PCR product in 500 μ L of hybridization buffer (5 $\times$ SSC containing 0.1% N-lauroylsarcosine, 1% blocking reagent, 0.02% SDS and 20% formamide) was incubated with DNA array at 42°C for 2 h. Hybridization temperature and formamide concentration to obtain high signal intensity was determined in the preliminary examination (data not shown). The membrane was washed 3 times for 5 min each with 2 $\times$ SSC-0.1% SDS, followed by 5 times for 10 min each

with 0.1 $\times$ SSC-0.1% SDS at room temperature. The membrane was then processed with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics, Switzerland). Signals were produced with the chemiluminescent substrate CDP-Star (Amersham Biosciences, USA). To detect the signal, instant films were exposed for 1 to 5 minutes to the arrays using an ECL Mini-Camera (Amersham Biosciences, USA).

Diagnosis using oligonucleotide DNA array

Ayu (*Plecoglossus altivelis*), which were suspected of being infected with *Vibrio* organisms, were diagnosed using oligonucleotide DNA array. Bacteria were isolated from the diseased fish on brain heart infusion agar (Difco) with 1.5% NaCl. DNA was extracted from the isolated bacterium and the spleen of the fish, and then subjected to oligonucleotide DNA array analysis. API 20E strip (bioMérieux, France) was used to identify the biochemical characteristics of the isolated bacterium.

Results

PCR amplification of the ITS non-coding region and sequence analysis

Our newly designed PCR primers amplified the ITS non-coding region from all *Vibrio* and *Photobacterium* strains analyzed in this study (Table 1). However, no amplicons were obtained from non-*Vibrio* and non-*Photobacterium* specimens, except for *A. caviae*, *A. hydrophila*, *A. jandaei* and *A. sobria*. The amplified segment had a length of 240 to 431 bp and included the 5' end of the 23S rRNA gene sequence. The nucleotide sequence data obtained in this study appear in the GenBank database under accession numbers AB255703 to AB255738. Because differences were seen in the ITS sequences of two strains of *V. harveyi* ATCC35084 and HUF9111, oligonucleotide probes were designed to each strains.

Hybridization with DNA array

Fig. 2 shows the prototypes of hybridization images. Number of strains applied to hybridization was shown in Table 3. Although the cross hybridization was observed, all of the tested strains reacted with the corresponding 3 spots and positive controls. The profiles obtained from each species were reproducible and different among the species. *V. nigrilulchritudo*, *V. penaeicida* and *V. vulnificus* also hybridized with the three spots corresponding to the *V. tapetis*, *V. pelagius* and *V. alginolyticus* respectively. Among 18 species, *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* displayed a similar hybridization image. *Aeromonas* species were weakly hybridized only to the positive control spots.

Fig. 2. Hybridization images (A) and photographs (B) of 17 *Vibrio*, 1 *Photobacterium* and 1 *Aeromonas* species to the oligonucleotide DNA arrays. Black circle: strong reaction, gray circle: medium reaction, white circle: weak reaction. Image and photograph 1: *V. alginolyticus*, 2: *V. anguillarum*, 3: *V. harveyi* (ATCC35084), 4: *V. cholerae*, 5: *V. fluvialis*, 6: *V. harveyi* (HUP9111), 7: *V. ichthyenteri*, 8: *V. nigripulchritudo*, 9: *V. ordalii*, 10: *V. parahaemolyticus*, 11: *V. pelagius*, 12: *V. penaeicida*, 13: *V. salmonicida*, 14: *V. splendidus*, 15: *V. tapetis*, 16: *V. tubiashii*, 17: *V. vulnificus*, 18: *P. damsela*, 19: *Vibrio* sp., 20: *A. jandaei*.

Table 3. Number of strains applied for hybridization

Species	Number of strains
<i>P. damsela</i> subsp. <i>damsela</i>	2
<i>P. damsela</i> subsp. <i>piscicida</i>	2
<i>V. alginolyticus</i>	1
<i>V. anguillarum</i> (<i>Listonella anguillarum</i>)	13
<i>V. harveyi</i>	3
<i>V. cholerae</i>	2
<i>V. fluvialis</i>	1
<i>V. ichthyenteri</i>	2
<i>V. nigripulchritudo</i>	1
<i>V. ordalii</i>	1
<i>V. parahaemolyticus</i>	1
<i>V. pelagius</i> (<i>L. pelagia</i>)	1
<i>V. penaeicida</i>	1
<i>V. salmonicida</i>	1
<i>V. splendidus</i>	1
<i>V. tapetis</i>	1
<i>V. tubiashii</i>	1
<i>V. vulnificus</i>	4
<i>Vibrio</i> sp.*	1
<i>Aeromonas caviae</i>	1
<i>A. hydrophila</i>	1
<i>A. jandaei</i>	1
<i>A. salmonicida</i>	1
<i>A. sobria</i>	1

*: Isolated from diseased striped jack *Pseudocaranx dentex* (unpublished).

Diagnosis using oligonucleotide DNA array

Both hybridization images obtained from extracted DNA from the isolated bacteria and the spleen of infected fish represented signals at the three spots corresponding to *V. anguillarum* as well as the hybridization image that obtained from the type strain of the bacterium (Fig. 3). The bacteria isolated from the fish were also

identified as *V. anguillarum* from their biochemical characteristics (data not shown).

Discussion

In this study, we developed a low-density microarray for the rapid discrimination of *Vibrio* and *Photobacterium* pathogens. Among 18 species tested in this study, 12 species: *V. anguillarum*, *V. cholerae*, *V. fluvialis*, *V. ichthyenteri*, *V. ordalii*, *V. pelagius*, *V. salmonicida*, *V. splendidus*, *V. tapetis*, *V. tubiashii*, *P. damsela* and *Vibrio* sp. isolated from diseased striped jack were clearly discriminated by hybridizing with the three spots corresponding to each species. On the contrary, *V. nigripulchritudo*, *V. penaeicida* and *V. vulnificus* also hybridized with the three spots corresponding to the *V. tapetis*, *V. pelagius* and *V. alginolyticus* respectively. However, reverse was not happened, i. e. *V. tapetis*, *V. pelagius* and *V. alginolyticus* was not hybridized with the three spots corresponding to the *V. nigripulchritudo*, *V. penaeicida* and *V. vulnificus*. Different patterns of cross-hybridization to other species make it possible to distinguish these species. *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* displayed similar hybridization images. The similarity of hybridization images was predictable, since three species have similar ITS sequences. Close relationship between these three species has been reported (Kita-Tsukamoto *et al.*, 1993). Therefore, biochemical tests or species-specific PCR (Venkateswaran *et al.*, 1998; Kim *et al.*, 1999; Pillot *et al.*, 2002; Oakey *et al.*, 2003; Conejero and Hedreya 2004; Di Pinto *et al.*, 2005) should be used to differentiate these three species.

Fig. 3. Hybridization results obtained from DNA extracted from isolated bacteria (A), spleen of infected fish (B) and *V. anguillarum* type strain NCMB 6 (C).

Because of their high variability and ease of PCR amplification, we targeted the ITS non-coding region for the discrimination of *Vibrio* species. It has been reported that the ITS regions shows larger variations than rRNA gene (Gürtler and Stanisich, 1996), which allows designing species-specific probes to distinguish closely related bacterial species. Usage of ITS for microarray-based bacterial identification has additional advantages, because this region is surrounded by conserved genomic regions and can be easily PCR-amplified from a broad spectrum of bacteria using universal primers. The method consisted of PCR amplification of the ITS non-coding region using a pair of universal primers, followed by hybridization of the DIG-labeled PCR products to a panel of oligonucleotides immobilized on a nylon membrane. This method enabled the identification of *Vibrio* and *Photobacterium* at the species level within a day including the PCR amplification step. Warsen *et al.* (2004) developed a DNA array based on 16S ribosomal RNA gene for the discrimination of fish pathogens. By this method, 15 fish pathogens were simultaneously detectable, but only *V. salmonicida* was included in the experiment. The poor variation in 16S rRNA gene sequences among *Vibrio* species makes identification at the species level by 16S rRNA gene-targeted hybridization difficult. Gonzalez *et al.* (2004) reported the detection of marine fish pathogenic bacteria including *Vibrio* and *Photobacterium* species using multiplex PCR and DNA microarray. They targeted the genes coding cytolysin, DNA gyrase B subunit, urease, phospholipase and plasmid. Five fish pathogens (*V. vulnificus*, *V. anguillarum*, *P. damsela* subsp. *damsela*, *V. parahaemolyticus* and *A. salmonicida* subsp. *salmonicida*) can be detected by their method. Our method has greatly increased the number of species that can be detected.

Without culture-based techniques, *V. anguillarum* was directly detected from infected ayu. In the case of diagnosis of infected juvenile fish, from which it is usually difficult to isolate the causative agent without contamination, our method is more useful than a classic culture-based method. Unfortunately, because of cross-hybridization, samples with multi-*Vibrio* infections or from sites containing multiple *Vibrio* species cannot be directly analyzed by our method. On the other hand, false-positive reactions from multiple infection or contamination of non-*Vibrio* and non-*Photobacterium* bacteria can be eliminated by using our newly designed PCR primer pair. Because of their high specificity, no PCR product was obtained from non-*Vibrio* and non-*Photobacterium* specimens, except for some species belonging to the genus *Aeromonas*. However, amplicons obtained from *Aeromonas* species were not hybridized with any oligonucleotide except positive controls.

Differences were seen in the ITS sequences of two strains of *V. harveyi* ATCC35084 and HUF9111.

Intraspecific variations of the ITS sequence might exist in other *Vibrio* species. In such case, the intraspecific variation may lead to a false negative result. False positive results may also derive from the samples that are not listed under coverage but share the same sequence identity within the probe region. In fact, two distinct species *V. cholerae* and *V. mimicus* cannot be distinguished, because the ITS sequences for the two species are very similar (Chun *et al.*, 1999). When using the method described here, care must be taken at the points described above for the species identification. Our data is insufficient to proof the identification accuracy. More number of strains should be tested to confirm the accuracy of our method. Nevertheless, if un-detectable species or strains were found, the assay can be extended by adding specific oligonucleotide probes to the array to identify more microorganisms.

In conclusion, our method is useful for a rapid and easy discrimination of *Vibrio* species. The total process, including PCR amplification, can be carried out within a day. Our method enables a global screening of *Vibrio* and *Photobacterium* species without special techniques and equipment.

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