— **Research Letter** —

Oligonucleotide Probes for Phylogenetic Detection of Waterborne Bacteria

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Waterborne diseases were globally occurred, and many people were suffered from and often killed by them. In order to prevent outbreaks of waterborne diseases, rapid detection of pathogenic microbes in aquatic environment is the important strategy in addition to the construction of water supply and vaccination. The 16S ribosomal RNA (rRNA) gene is often used as a target gene for bacterial detection using hybridization techniques. In this study, we aimed to design the oligonucleotide probes that could be used for the detection of waterborne bacteria with hybridization techniques because design of specific probes is important to assure for the precise detection of target bacteria. We then evaluated the specificities of designed probes by using an oligonucleotide microarray. In conclusion, we confirmed that seven designed oligonucleotide probes were suitable for the specific detection of waterborne bacteria. These probes appear to be used for 16S rRNA targeted hybridization techniques such as fluorescence *in situ* hybridization (FISH) and oligonucleotide microarray.

Key words —— waterborne bacteria, oligonucleotide probe, 16S ribosomal RNA gene, microarray

INTRODUCTION

Waterborne diseases occur worldwide, and as a consequence many people suffer from serious illness that may result in death. In order to prevent outbreaks of waterborne disease, rapid detection of pathogenic microbes in aquatic environments as well as the construction of water supplies and sufficient vaccination regimes is required.

Conventional culture methods are widely used for the detection of waterborne bacteria, due to their relatively simple and cost-effective use. However, the abundance of aquatic bacteria is often underestimated because many of them are "hard-to-culture" under conventional conditions. In addition, relatively long periods of time are required for the formation of visible bacterial colonies on the media. Therefore, in environmental microbiology, many molecular biological tools, which are culture independent, have been developed and used for bacterial detection in addition to conventional culture methods.[1)]

The ribosomal RNA (rRNA) gene is often used as a target gene for bacterial detection with hybridization techniques.[2)] Viable *Escherichia coli* cells in cow manure can be detected selectively by using fluorescence *in situ* hybridization (FISH) following direct viable count procedure.[3)] Phylogenetic oligonucleotide microarray has great potential for monitoring predominant bacterial populations in wastewater.[4)] 16S rRNA gene sequences have highly conserved regions in identical bacterial species, and the databases of 16S rRNA gene sequences are available on the internet. Ribosomal Database Project (RDP) release 10 (http://rdp.cme.msu.edu/) have 1358426 16S rRNA gene sequences as of 25th January 2010. We can easily access the database and use for our purpose, such as DNA sequencing and design of gene probes and PCR primers.

In this study, we aimed to design the oligonucleotide probes for detection of waterborne bacteria, that are described in World health Organization (WHO) Guidelines for drinking-water quality (http://www.who.int/water_sanitation_health/dwq/guidelines/en/index.html). We also evaluated the specificities of the designed probes by using oligonucleotide microarray, because an oligonucleotide microarray is a powerful tool that can be used for the simultaneous detection of various genes.[5)]

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MATERIALS AND METHODS

Bacterial Strains —— The following strains were selected and used as representative waterborne pathogenic bacterial strains: *Acinetobacter baumannii* (*Ac. baumannii*) ATCC 19606, *Aeromonas caviae* ATCC 15468, *Aeromonas hydrophilia* (*Aer. hydrophila*) ATCC 7966, *Bacillus cereus* (*B. cereus*) ATCC 14579, *Escherichia coli* (*E. coli*) ATCC 11775, *Pleshiomonas shigelloides* (*Ple. shigelloides*) ATCC 14079, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* (*Staph. aureus*) ATCC 12600, *Tukamurella inchonensis* ATCC 700082, *Vibrio fluvialis* (*V. fluvialis*) ATCC 33809, *Vibrio parahaemolyticus* (*V. parahaemolyticus*) ATCC 17802 and *Vibrio vulnificus* (*V. vulnificus*) ATCC 27562. Each bacterium was grown following American Type Culture Collection guidelines.

Oligonucleotide Probe Design —— The 11 test probes were selected to detect waterborne bacteria. It included three published probes and eight newly designed probes. The latter were designed by using the phylogenetic software package ARB.[6] The Basic Local Alignment Search Tool (BLAST) search (National Center for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov/BLAST/) was used to predict probe specificity. Probes with a Tm of 60 ± 5°C, a %G+C of 50 ± 10%, and a length of 20 ± 2 mer were accepted.

To analyze the oligonucleotide microarray data, we also designed mismatched (non-targeted) probes. All 11 test probes had 11 mismatched probes, respectively. A mismatch probe have two nucleotides mismatches against corresponding a test probe, and the mismatched probe have no complementary sequences in the 16S rRNA gene sequence database.

Microarray Fabrication —— Oligonucleotide probes were attached to a slide glass by the procedure described previously.[7] All oligonucleotide probes modified with 5′-terminal thiol group were synthesized by Hokkaido System Science (Hokkaido, Japan). The probes dissolved in 50 mM Tris-HCl (pH 8.0) were diluted to 50 μM in the same buffer. An aliquot (10 μl) of each probe and 10 μl of 50 mM Tris-HCl (pH 8.0) containing 20% (w/v) trehalose were mixed. The mixture were applied in duplicate to a clean, dry amino group-derivatized glass slide (SD00011, Matsunami Glass, Osaka, Japan) treated with *N*-(6-Maleimidocaproyloxy)succinimide (EMCS) (Dojindo Laboratories, Kumamoto, Japan). Volumes of deposited probe solutions were about 250 nl, resulting in spots with a diameter of approximately 900 μm. The slides were kept in a humidity chamber at room temperature (approximately 25°C) for 16 hr, and then washed with 2 × Sodium Chloride-Sodium citrate (SSC) at room temperature for 5 min to remove unbound oligonucleotides. To block unbound EMCS, the slides were soaked in 50 mM phosphate buffer, 1 M NaCl (pH 7.0) containing 2% bovine serum albumin (BSA; Wako Pure Chemical Industries, Osaka, Japan) at room temperature for 2 hr, and rinsed several times with distilled water, then dried in air.

Genomic DNA Extraction, Gene Amplification and Hybridization —— Genomic DNA of cultured cells was extracted as described previously.[7] Briefly, cells were disrupted mechanically with glass beads, and phenol purification and ethanol precipitation were performed. Primers used for amplification of 16S rRNA gene fragments are listed in Table 1. PCR and transcription by the T7 RNA polymerase, from a T7 RiboMAX express kit (Promega, Madison, WI, U.S.A.), were carried out

Table 1. Oligonucleotide Primers Used in this Study

Primer	Position[a]	Sequence (5′-3′)[b]
p46f	46– 65	T7 promoter[c]-GCCTAAYACATGCAAGTCGA
p329r	311– 329	GTGTCTCAGTYCCAGTGTG
p410f	410– 429	T7 promoter-AAGAAGGCCTTCGGGTTGTA
p702r	683– 702	CTACGCATTTCACCGCTACA
p711f	711– 728	T7 promoter-AGGAACACCRGTGGCGAA
p1069r	1055–1069	AGCTGACGACAGCCA
p1193f	1193–1212	T7 promoter-GTCAARTCATCATGSCCCTT
p1512r	1498–1512	ACGGYTACCTTGTTA

a) *E. coli* numbering. *b*) Mixture: Y, C or T; R, A or G; S, C or G. *c*) A 24-bp T7 promoter sequence (5′-ATTGTAATACGACTCACTATAGGG-3′) was attached to the 5′ end.

in vitro.[7)]

The purified was labeled with Alexa Fluor 546 at guanine by using the ULYSIS Alexa Fluor 546 nucleic acid labeling kit (Invitrogen, Carlsbad, CA, U.S.A.) and the manufacturer's recommended procedures. After removing of unreacted fluorophore with the Cleanup Kit, the hybridization solution was prepared as follows: Fluorescently labeled RNA, 15 µl of 20 × Saline-Sodium Phosphate-EDTA (SSPE) [1 × SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA (pH 7.7)], and 5 µl of 5% (w/v) sodium dodecyl sulfate solution were mixed, and adjusted to a total volume of 50 µl with the nucleases-free water. Afterwards, the hybridization solution was heated at 95°C for 5 min and subsequently cooled on ice. 40 µl of hybridization solution was poured on the DNA microarray and the hybridization was performed for 30 min at 45°C. Then, the DNA microarray was washed with 2 × SSPE for 20 min at 48°C and rinsed with distilled water.

Data Acquisition and Analysis —— Air-dried slides were imaged at a resolution of 10 µm with a GenePix4000 microarray scanner (Axon, Sunnyvale, CA, U.S.A.) at the same laser power and sensitivity level of the photomultiplier for each slide. Fluorescent spot intensities were quantified using GenePix Pro 5.1 software (Axon). The average value for each set of duplicate spotted probes was compared to the corresponding mismatched probes, and probes that had a signal-to-noise fluorescence ratio of greater than 2.0 were defined as positive.

RESULTS AND DISCUSSON

Eight oligonucleotide probes and nine mismatched probes were newly designed to detect waterborne pathogenic bacteria (Table 2). Finally, 22 probes were used in this study. To evaluate the specificities of the oligonucleotide probes, 1 µg of fluorescently labeled transcripts of 16S rRNA gene fragments, which were generated from the rRNA gene of each bacterium via PCR, were individually hybridized to the oligonucleotide microarrays. In many cases, hybridization with transcripts of bacterial 16S rDNAs resulted in positive signals with their specific probes as well as the positive control probe EUB1387 (Table 3). Unexpectedly, *Ple. shigelloides*, *V. fluvialis* and *V. vulnifics* showed positive hybridization signals against ES (signal ratio; 4.93), ACINE (13.1) and ES (8.76), respectively. In contrast, *Staph. aureus* showed a weak hybridiza-

Table 2. Oligonucleotide Probes Designed in this Study

Probe[a)]	Target	Position[b)]	Sequence (5′-3′)	Reference
VFLUV	*Vibrio* spp.	69– 89	AAACAAGTTTCTCTGTCGCTG	This study
M-VFLUV	Mismatched		AAAGAAGTTTCTCTGTCCCTG	This study
BCEAN	*Bacillus* spp.	195– 209	GCCGCCTTTCAATTTCGAAC	This study
M-BCEAN	Mismatched		GCGGCCTTTCAATTTCCAAC	This study
STAAU	*Staphylococcus* spp.	195– 210	GACCGTCTTTCACTTTTGAAC	This study
M-STAAU	Mismatched		GAGCGTCTTTCACTTTTCAAC	This study
ES	*E. coli*, *Shigella* spp.	444– 462	CTTTACTCCCTTCCTCCCC	This study
M-ES	Mismatched		CTTAACTCCCTTCCTGCCC	This study
PSHIGE	*Plesiomonas* spp.	465– 485	ACGTCAATGCCACTAGGTATT	This study
M-PSHIGE	Mismatched		ACGACAATGCCACTAGGAATT	This study
VVUPA	*V. vulnificus*, *V. parahaemolyticus*	470– 490	CGCTAACGTCAAATGATAGTG	8
M-VVUPA	Mismatched		CGCAAACGTCAAATGATTGTG	8
AERO	*Aeromonas* spp.	585– 605	CACATCTAACTTATCCAACCGC	8
M-AERO	Mismatched		CACTTCTAACTTATCCAAGCGC	8
PSUDO	*Pseudomonas* spp.	736– 758	TCAGTATCAGTCCAGGTGGT	This study
M-PSUDO	Mismatched		TCACTATCAGTCCAGGAGGT	This study
TSUKA	*Tsukamurella* spp.	991–1009	CGGCGATCCTCTATATGTCA	This study
M-TSUKA	Mismatched		CGCCGATCCTCTATATCTCA	This study
ACINE	*Ac. baumannii*	1274–1295	GATCGGCTTTTTGAGATTAGCA	This study
M-ACINE	Mismatched		GATGGGCTTTTTGAGATTTGCA	This study
EUB1387	Most *Bacteria*	1369–1386	CCGGGAACGTATTCACCG	10
M-EUB	Mismatched		CCGCGAACGTATTCAGCG	This study

a) Mismatched probes have "M-" at the beginning of their names. *b*) *E. coli* numbering.

Table 3. Specificity of Oligonucleotide Probes for Detection of Waterborne Bacteria

Probe	Bacterial strains[a)]											
	Ab	Ac	Ah	Bc	Ec	Ps	Pa	Sa	Ti	Vf	Vv	Vp
ACINE	**20.7**	0.87	0.96	0.90	1.06	1.66	1.16	0.67	1.68	*13.1*	0.84	0.89
AERO	1.24	**7.75**	**9.10**	0.92	0.12	1.30	1.12	0.52	0.88	1.24	0.92	0.88
BCEAN	0.62	0.10	1.32	**2.42**	1.00	0.40	1.50	0.93	0.89	0.62	1.15	0.12
ES	1.53	1.22	1.68	1.57	**5.46**	*4.93*	1.42	1.60	1.28	1.27	*8.76*	1.11
PSHIGE	0.93	0.19	1.15	0.99	1.06	**3.42**	0.94	1.09	0.97	0.97	1.15	0.93
PSUDO	0.99	1.18	1.09	1.14	0.92	1.04	**6.20**	1.16	1.05	0.99	0.99	1.04
STAAU	0.97	0.44	0.50	0.99	0.85	0.83	0.38	1.16	0.32	1.01	1.01	1.00
TSUKA	1.03	0.53	1.26	1.00	0.82	0.77	0.57	1.05	**2.51**	1.02	0.89	0.97
VFLUV	0.65	0.50	0.83	0.50	0.83	0.94	0.84	0.42	1.02	**2.04**	**2.02**	**2.93**
VVUPA	1.22	0.80	1.00	0.86	1.11	0.41	1.25	0.97	1.18	1.40	**2.58**	**2.47**
EUB1387	**8.37**	**6.77**	**5.50**	**10.4**	**5.25**	**8.11**	**4.46**	**10.7**	**2.26**	**8.37**	**3.38**	**3.15**

The values indicate the ratios of signal intensity (test probe/mismatched probe). Experiments were repeated twice.
Bold, positive; *italic*, false positive; underlined, false negative.
a) Ab, *Aci. baumannii*; Ac, *Aeromonas caviae*; Ah, *Aer. hydrophila*; Bc, *B. cereus*; Ec, *E. coli*; Ps, *Ple. shigelloides*; Pa, *Pseudomonas aeruginosa*; Sa, *Staph. aureus*; Ti, *Tsukamurella inchonensis*; Vf, *V. fluvialis*; Vv, *V. vulnificus*; Vp, *V. parahaemolyticus*.

tion signal against STAAU probe (intensity ratio; 1.16), a result that was graded as negative (ratio < 2.0, Table 2).

Analysis of the specificities of the designed probes using the BLAST and Probe Match Program (the Ribosomal Database Project II) indicated that each probe would only show the ideal hybridization result. Two probes (ES and ACINE), however, showed positive results against non-targeted bacterial 16S rRNAs. The number of mismatches to the 16S rRNA gene sequences of the targeted bacteria were five (73.7% similarity of ES to *Ple. shigelloides* sequence), seven (68.2%; ACINE to *V. fluvialis*) and six (68.4%; ES to *V. vulnificus*). The longest identical sequence stretch between the probe and target sequences was 12, 7 and 8 bases, respectively. Liebich *et al.*[10)] reported that nonspecific hybridization could be avoided by implementing simultaneous threshold criteria for 50-mer oligonucleotide probes of $\leq$ 90% similarity, $\leq$ 20-base stretches, and a free energy release of ≥ -35 kcal/mol. Using the Mfold web server for nucleic acid folding and hybridization prediction,[11)] the free energies of the mismatch probes-16S rRNAs were estimated to be as -20.2 kcal/mol (ES to *Ple. shigelloides* sequence), -11.8 kcal/mol (ACINE to *V. fluvialis*) and -14.5 kcal/mol (ES to *V. vulnificus*). These values were implemented with the threshold determined by Liebich *et al.*,[10)] with the exception that our newly designed probes were short oligonucleotides of approximately 20 bases.

In conclusion, we confirmed that seven oligonucleotide probes, AERO, BCEAN, PSHIGE, PSUDO, TSUKA, VFLUV and VVUPA, were suitable for specific detection of waterborne bacteria such as *Aeromonas* spp., *B. cereus*, *Ple. shigelloides*, *Pseudomonas* spp., *Tsukamurella* spp., *V. fluvialis*, *V. vulnificus* and *V. parahaemolyticus*. These selected probes appeared to be potentially useful for 16S rRNA targeted hybridization techniques such as FISH and oligonucleotide microarray.

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