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Detection of Representative Enteropathogenic Bacteria, *Vibrio* spp., Pathogenic *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Yersinia enterocolitica*, Using a Virulence Factor Gene-Based Oligonucleotide Microarray[§]

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Rapid identification of enteropathogenic bacteria in stool samples is critical for clinical diagnosis and antimicrobial therapy. In this study, we describe the development of an approach that couples multiplex PCR with hybridization to a DNA microarray, to allow the simultaneous detection of the 10 pathogens. The microarray was synthesized with 20-mer oligonucleotide probes that were designed to be specific for virulence-factor genes of each strain. The detection limit for genomic DNA from a single strain was approximately 10 fg. In the presence of heterogeneous non-target DNA, the detection sensitivity of the array decreased to approximately 100 fg. We did not observe any non-specific hybridization. In addition, we successfully used this oligonucleotide-based DNA microarray to identify the causative agents in clinical stool samples from patients with food-borne enteritis.

***Keywords*:** multiplex PCR, microarray, oligonucleotide probes, enteropathogenic bacteria

Enteropathogenic bacteria represent a serious public health threat worldwide. Hundreds of outbreaks of cases of food-borne bacterial infection occur around the world and the species of causative bacteria are continuously diversifying (Hong *et al.*, 2004; Jin *et al.*, 2005). Thus, the ability to detect and identify enteropathogenic bacteria accurately has become increasingly important for environmental surveillance, clinical medicine, and biodefense (Vora *et al.*, 2004; Li *et al.*, 2006; Sergeev *et al.*, 2006).

Traditional methods for the identification of pathogenic bacteria include enrichment culture, microscopy, serology, and biochemical assays. However, these methods have several limitations, such as time-consuming, dependency on enrichment and selective culture, and difficulties with quantitative analysis (de Boer and Beumer, 1999; Eom *et al.*, 2007). Many modified PCR methods have been developed to overcome the need for prior cultivation. These technologies enable rapid detection of pathogenic bacteria in food, soil and water, with high degrees of sensitivity and specificity. Many of these approaches are based on the amplification of pathogen-specific rRNA sequences (Warsen *et al.*, 2004; Maynard *et al.*, 2005; Chiang *et al.*, 2006; Wang *et al.*, 2007) or virulence-factor genes (Bej *et al.*, 1999; Kong *et al.*, 2002; Burton *et al.*, 2005; Thong *et al.*, 2005; Park *et al.*, 2006). However, after amplification, the sizes of the PCR products and their sequences must be checked in order to remove the possibility of false positive results. Another limiting factor is that the PCR products must be visualized by gel electrophoresis and staining, which limits the sensitivity of detection to approximately 10^{3-4} copies per reaction (Maynard *et al.*, 2005).

Research on microarrays as multiple analytic systems has generated increased interest in the last decade. Microarrays provide a powerful analytical tool for the simultaneous detection of multiple analytes in a single experiment (Seidel and Niessner, 2008). The use of DNA microarrays has been shown to be effective for the high-throughput detection of pathogenic microorganisms in clinical and environmental samples (Wilson *et al.*, 2002; Bekal *et al.*, 2003; Panicker *et al.*, 2004; Call, 2005; Jin *et al.*, 2006; Jordan, 2007; Kostic *et al.*, 2007). The DNA microarrays that are used consist of numerous oligonucleotide probes that have been immobilized at high densities on a solid support. Fluorescently-labeled PCR-amplified DNA fragments can then be hybridized to the oligonucleotides. PCR amplification prior to the labeling and hybridization reactions increases the sensitivity of the technique, one of the potential strategies for increasing sensitivity of DNA microarrays (Gonzalez *et al.*, 2004; Call, 2005; Loy and Bodrossy, 2006). By combining DNA microarray technology with the amplification of different virulence-factor genes by multiplex PCR, it has been possible to detect simultaneously several fish (Gonzalez *et al.*, 2004; Warsen *et al.*, 2004) and waterborne (Panicker *et al.*, 2004; Maynard *et al.*, 2005) pathogenic bacteria.

Rapid detection of enteropathogenic bacteria from stool samples is very important for the effective treatment of patients suffering from gastrointestinal disease. To date, the combination of multiplex PCR and DNA microarrays has not been applied to the detection of enteropathogenic bacteria in

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stool samples. In this study, we developed and evaluated a multiplex PCR-oligonucleotide-based microarray system for the detection of enteropathogenic bacteria from stool samples of patients with enteritis. We designed and tested oligonucleotide probes that targeted the virulence-factor genes of the 10 enteropathogenic bacteria that are frequently responsible for outbreaks of enteritis in Korea (Kong *et al.*, 2002; Kim *et al*., 2003). We demonstrated that this microarray approach has the potential to be used for the comprehensive detection of enteropathogenic bacteria in stool samples from patients.

Materials and Methods

Bacterial strains and genomic DNA extraction

The strains used in this study are shown in Table 1. The bacterial strains or DNAs were obtained from the Center for Infectious Diseases, Korea National Institute of Health. Genomic DNA was extracted using a PrimePrep™ Genomic DNA Isolation kit (GeNet Bio, Korea). The genomic DNA extracted from the bacteria was quantified by ND-1000 spectrophotometer (Nanodrop Technologies, USA).

Human stool samples were collected from healthy individuals. One gram (wet weight) of fresh stool sample was mixed with 9 ml of 0.85% NaCl in a 15 ml conical tube (Axygen, USA), and then centrifuged at a low speed (300×g) for 10 min to remove large particles. This centrifugation step was repeated twice, and then the upper phase was centrifuged at 7,000×g for 10 min to collect the pellets (Wang *et al.*, 2002, 2004). The aspirated pellets were transferred to 1.5-ml microfuge tubes, and then genomic DNA was extracted using an *AccuPower*® Stool DNA Extraction kit (Bioneer, Korea). The mock-infected stool specimens were prepared as follows. A serial dilution, in the range of 100 ng to 1 fg, of bacterial genomic DNA from each of the 10 strains was prepared and mixed with 100 ng of extracted stool genomic DNA.

Oligonucleotide primers and probes

The targeted genes, oligonucleotide primer sets, and DNA probes used for the detection of each of the 10 pathogens are listed in Supplementary data Table 1. All primer sets and probe sequences were designed at the conserved regions by using the Primer3 program (http://primer3.sourceforge.net). The number of the gene sequences retrieved from GenBank database for designing of probes and primers were shown in the Table 1. PCR products ranged from 105 to 710 bp in length. The oligonucleotide probes were designed to be complementary to nucleotide sequences within the amplified segments of the targeted genes. The oligonucleotides were synthesized at Digital Genomics (Korea). A T-spacer, which comprised 15 dTTP molecules, was added to the 5' end of each oligonucleotide, in order to improve the accessibility of the probes on the array to the target DNA. In addition, an amino group was added to the 5′ end of each probe during the synthesis to increase the efficiency of immobilization of the oligonucleotides to silylated slides (CEL Associates, USA). The specificity of potential primer and probe sequences was analyzed by comparing them with known gene sequences in GenBank, using the BLAST N search program provided by the National Center for Biotechnology Information.

PCR amplification conditions

Supplementary data Table 1 lists the primers used to amplify the specific virulence-factor genes in the different pathogens. Each individual PCR and multiplex PCR amplification was performed in a 50-μl reaction mixture that contained 1 fg to 100 ng of purified genomic DNA, each dNTP at a concentration of 200 μM, 10-20 pmol of each primer, 1× reaction buffer [Tris-HCl (pH 9.0), $(NH_4)_2SO_4$, 20 mM $MgCl_2$], and 1-3 U of *Prime Taq* DNA polymerase (GeNet Bio). The following PCR amplification conditions were used: 5 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C; and 5 min at 72°C. All PCR products were electrophoresed through 2% (w/v) agarose gels and stained with ethidium bromide (0.5 μg/ml). DNA bands were visualized under UV light. Products were purified using the GeneAll® Expin™ PCR SV purification kit (GeneAll Biotechnology, Korea) according to the manufacturer's instructions, and then labeled as described below.

Microarray construction

Our pathogen-detection microarray contained a combination of short, 20-mer oligonucleotide probes that were specific for the virulence-factor genes of each pathogen. In preparation for printing, lyophilized oligonucleotides were dissolved in distilled water to obtain a stock concentration of 100 pmol/μl. These solutions were diluted in 4× ArrayIt® Micro Spotting Solution (TeleChem International, USA) to a final concentration of 50 pmol/μl. Ten microliters of each probe were transferred into a 384-well microplate (Matrix Technologies, USA) and stored at -20°C until printed onto a 25×75-mm silylated slide (CEL Associates) with a MicroGrid II Arrayer (BioRobotics, USA), using a quill pin (Matrix Technologies). The distance between spots within an array set was 400 μm, which was sufficient to minimize the cross-talk

Table 1. Bacterial strains and target genes used in this study

Organisms	ATCC no.	Target genes	GenBank accession no.[a]	Distribution[b]
Vibrio cholerae O1 Inaba	ATCC 9459	*tcpI*	X74730 (8)	species-specific
V. parahaemolyticus O3:K6	ATCC 17802	*tlh*	AY829372 (11)	species-specific
V. vulnificus	ATCC 29307	*vvhA*	AB124802 (13)	species-specific
Salmonella enterica serovar Typhimurium	ATCC 14028	*fliC*	AE008787 (20)	serotype-specific
S. enterica serovar Enteritidis	ATCC 13076	*sen*	AF370707 (3)	serotype-specific
Shigella flexneri 2a	ATCC 25876	*S0745*	AE005674 (3)	species-specific
Shigella sonnei	ATCC 9290	*bcv*	D00660 (1)	species-specific
Enterotoxigenic *E. coli* (ETEC)	ATCC 43896	*cooA*	M58550 (6)	ETEC-specific
Enterohemorrhagic *E. coli* (EHEC)	ATCC 43895	*rfbE*	AF163336 (18)	EHEC(serotype O157)-specific
Yersinia enterocolitica O:8	ATCC 9610	*invA*	Z48169 (3)	species-specific

[a] The number in the parenthesis after the accession number of target gene indicates that total number of gene sequences used for primer and probe design.
[b] This indicates the range of the occurrence of the gene sequences in the taxonomic hierarchy.

effect between spots. Each probe was printed in triplicate within each array. The printed slides were dried for 12 h at room temperature. The slides were then washed once for 1 min with 0.2% SDS in water and twice for 1 min each time with distilled water to remove unbound oligonucleotides. To reduce the nonspecific adsorption of fluorescent probe to the surface of the slide, printed slides were blocked with BlockIt™ Microarray Blocking Buffer (TeleChem International) for 12 h at room temperature, rinsed with distilled water, air dried, and stored in a clean slide box at room temperature until used.

Amplicon labeling and hybridization

The purified PCR products were chemically labeled using the BioPrime Array CGH Genomic Labeling System (Invitrogen, USA) according to the manufacturer's instructions. The labeled target DNA was purified using a BioPrime Array CGH Genomic Labeling System purification module (Invitrogen), and dissolved in an appropriate volume of distilled water.

All hybridizations were carried out in triplicate. The fluorescently-labeled target DNA was completely dried under vacuum and hybridized with microarray slides according to the manufacturer's instructions (TeleChem International). The microarray slides were placed into a hybridization cassette (TeleChem International) and immediately plunged into a 42°C water bath, in which they were incubated for 4 h. After hybridization, the time that the slide remained at room temperature was minimized in order to prevent cross-hybridization. Each microarray slide was taken out, and the coverslips were immediately removed by dipping the slides into wash buffer I (0.5× SSC, 0.01% SDS). The slides were washed using pre-warmed (50°C) wash buffer I, wash buffer II (0.5× SSC), wash buffer III (0.1× SSC), and wash buffer IV (0.01× SSC), each for 5 min at ambient temperature, prior to being dried by centrifugation as described above.

Image processing and data analysis

The microarrays were scanned using a ScanArray 5000 Microarray Analysis System (Perkin-Elmer, USA) at a resolution of 5 μm. For all experiments, the laser power and photomultiplier tube gain were adjusted to avoid saturation of spots (70-100%). The scanned images were saved as 16-bit TIFF format files, and each spot was quantified using GenePix® Pro 6.1 software (Molecular Devices, USA). The signal-to-noise ratio (SNR) was also calculated based on the following formula: SNR=(signal intensity – background)/standard deviation (SD) of background, in which the background measurement refers to the local spot background intensity and the standard deviation of background was calculated across all pixels measured by the GenePix® Pro 6.1 software (Rhee *et al.*, 2004). The SNRs from three replicate data sets were then averaged to give the mean SNR for a particular probe. Spots that appeared to be lower than the threshold value were removed from the data set. The cutoff value for determining whether or not a target was positive was assigned as the average signal intensity of the negative control plus 3×standard deviation. An oligonucleotide that was complementary to the 16S rRNA gene was included within the array as a positive control, and an oligonucleotide that did not recognize any bacterial genes as a negative control, so as to avoid false-positive and -negative results (Supplementary data Table 1).

Application of oligonucleotide-based DNA microarrays to the analysis of clinical samples

To evaluate such potential, stool specimens from 82 patients with food-borne enteritis were kindly provided by the Chungbuk Institute of Health and Environmental Research and tested using the DNA microarray method. Stool genomic DNA was extracted as described above. All clinical samples were amplified using multiplex primers, labeled, and hybridized to the DNA microarrays, as described above. However, false-positive signals were observed with enterotoxigenic *Escherichia coli* (ETEC)-specific probes. To confirm the results, the PCR products were re-amplified with ETEC-specific nested PCR primers (cooAII_F: 5′-AGTGTTGACCCGACTGTTGA-3′, and cooAII_R: 5′-CTGCATGGATTGAAAGTTTC-3′). The size of the re-amplified PCR products, 337 bp, was confirmed by agarose gel electrophoresis.

Results and Discussion

Multiplex PCR of bacterial virulence-factor genes

The sensitivity of oligonucleotide-based DNA microarrays hybridized with total genomic DNA from microbial communities (Rhee *et al.*, 2004) is usually inadequate to allow the detection of low level of populations in the same microbial communities. However, the labeling and hybridization of amplified PCR products, instead of genomic DNA, increases the sensitivity of microarrays. This approach to detect microbes necessitates the combination of a number of PCR products prior to their hybridization on microarrays (Maynard *et al.*, 2005). As the first step in developing our multiplex PCR-DNA microarray approach, we designed PCR primers against the virulence-factor genes of each of our target enteropathogenic bacteria. Ten pairs of PCR primers were designed that allowed the simultaneous amplification of virulence-factor genes from 10 different food-borne pathogens in a single tube (Supplementary data Table 1). As shown in Fig. 1, PCR amplification of target genes from genomic DNAs, extracted from individual strains or a mixture of strains, generated specific DNA fragments that ranged in size from 105 to 710 bp. This result shows that multiplex PCR for simultaneous and specific amplification from 10 different pathogenic strains can be successfully performed.

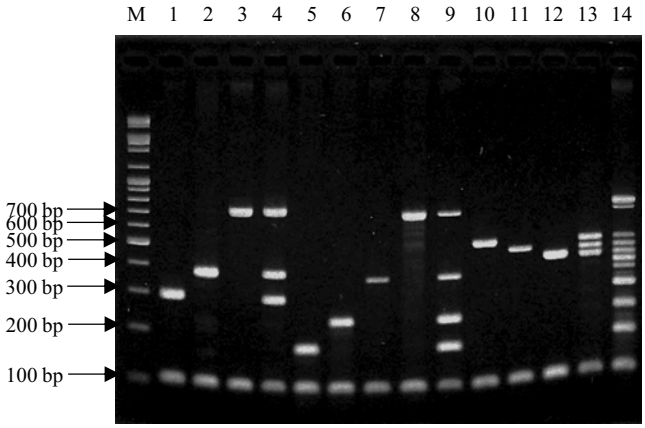

Fig. 1. Agarose gel electrophoresis showing the results of individual and multiplex PCR amplification of target genes. Lanes: M, DNA size standard (Bioneer, Korea); 1, 292-bp *tcpI* fragment; 2, 380-bp *tlh* fragment; lane 3, 710-bp *vvhA* fragment; 4, multiplex PCR of *tcpI*, *tlh* and *vvhA*; 5, 172-bp *fliC* fragment; 6, 232-bp *sen* fragment; 7, 350-bp *S0745* fragment; 8, 662-bp *bcv* fragment; 9, multiplex PCR of *fliC*, *sen*, *S0745* and *bcv*; 10, 480-bp *cooA* fragment; 11, 436-bp *rfbE* fragment; 12, 410-bp *invA* fragment; 13, multiplex PCR of *cooA*, *rfbE*, and *invA*; 14, multiplex PCR amplification of all 10 targeted genes. 16S rDNA (105 bp) was co-amplified as a positive control.

The PCR amplification thresholds for three different virulence genes (*Vibrio cholerae*: *tcpI*, *Vibrio parahaemolyticus*: *tlh*, and *Vibrio vulnificus*: *vvhA*) were estimated using diluted genomic DNA ranging from 100 ng to 1 fg as a PCR template. The minimum amount of genomic DNA template required to allow detection by visualization of the appropriately sized product after agarose gel electrophoresis was approximately 10 pg (data not shown). If we assume that 4 fg of genomic DNA represent approximately one bacterial genome (Gonzalez *et al.*, 2004; Burton *et al.*, 2005; Maynard *et al.*, 2005), the PCR detection limit for these targets corresponds to approximately 10^{3-4} bacterial genomes. The PCR detection sensitivity for the target enteropathogenic strains was also tested using genomic DNA extracted from these strains, which had been combined with 100 ng of stool genomic DNA extracted from healthy humans. The detection limits were similar to those that had been obtained by the dilution of pure genomic DNA in distilled water (data not shown). This result also indicates that our PCR primers were very specific, even in the presence of DNA from diverse stool microorganisms.

Specificity of microarray hybridization

To synthesize an oligonucleotide- probe-based microarray, three 20-mer probes against each target virulence-factor gene were designed, which would bind within the amplified PCR fragments (Supplementary data Table 1). When PCR fragments that had been amplified from the genomic DNA of each pathogenic strain were labeled, both individually, and multiplex-amplified products only hybridized to their complementary probes under our hybridization conditions (Fig. 2). PCR amplification using stool DNA, with no added pathogenic bacterial DNA, showed no significant signal (SNR≤4.26) with any of the probes (data not shown). Taken together, these results indicated that the designed probes were specific for their corresponding target genes in the pathogenic strains examined. The method described here, which combines multiplex PCR and microarray, overcomes the problems of non-specific PCR amplification that is often observed in highly sensitive PCR assays (Gonzalez *et al.*, 2004; Sergeev *et al.*, 2004; Vora *et al.*, 2004; Call, 2005). One of the major advantages of the microarray assay over agarose gel analysis of the PCR products was that detection did not rely solely on the length of the PCR products, but also required the fragments to contain sequences that were complementary to the oligonucleotide probes on the microarray.

Determination sensitivity of DNA microarray

The detection sensitivity of the DNA microarray was determined using genomic DNA that was extracted from the 10 pathogenic strains. Diluted genomic DNA (100 ng to 1 fg) from the pathogens was amplified by multiplex PCR and then randomly labeled with Cy3. Ten femtograms of genomic DNA was the minimum amount that gave a signal intensity that was significantly higher than the background level, when compared with the signal intensity of the negative control (no template DNA) (Fig. 3A). Therefore, the detection sensitivity with multiplex PCR and our hybridization conditions was estimated to be ≥10 fg, which corresponds to approximately 2 or 3 copies of a bacterial genome.

In stool samples, other diverse non-pathogenic bacteria are

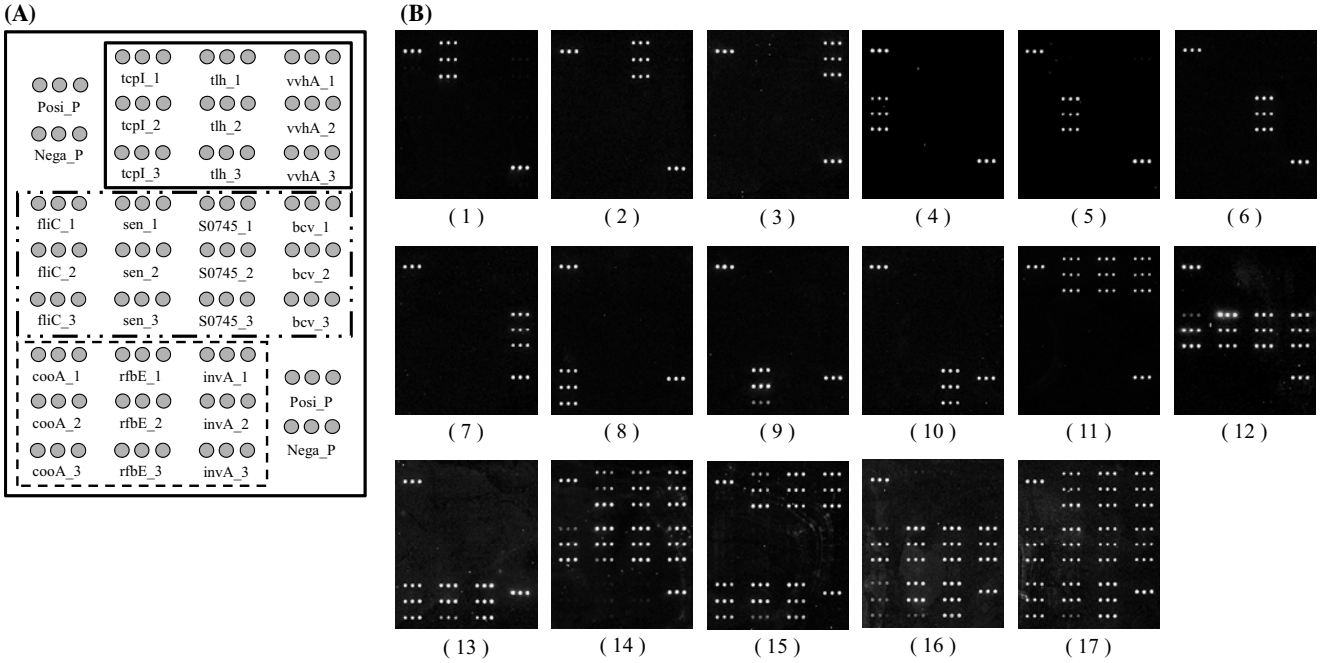

Fig. 2. Specificity of DNA microarray hybridization. (A) Schematic diagram showing the position of the probes spotted in triplicate on glass slides. (B) Specific hybridization of individual or multiplex PCR amplification products to the DNA microarray. (1) *V. cholerae*, (2) *V. parahaemolyticus*, (3) *V. vulnificus*, (4) *S. typhimurium*, (5) *S.* enteritidis, (6) *S. flexneri*, (7) *S. sonnei*, (8) ETEC, (9) EHEC, (10) *Y. enterocolitica*, (11) *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, (12) *S. typhimurium*, *S. enteritidis*, *S. flexneri*, and *S. sonnei*, (13) ETEC, EHEC, and *Y. enterocolitica*, (14) Combination of strains used in lanes 11 and 12, (15) Combination of strains used in lanes 11 and 13, (16) Combination of strains used in lanes 12 and 13, (17) Combination of strains used in lanes 11-13.

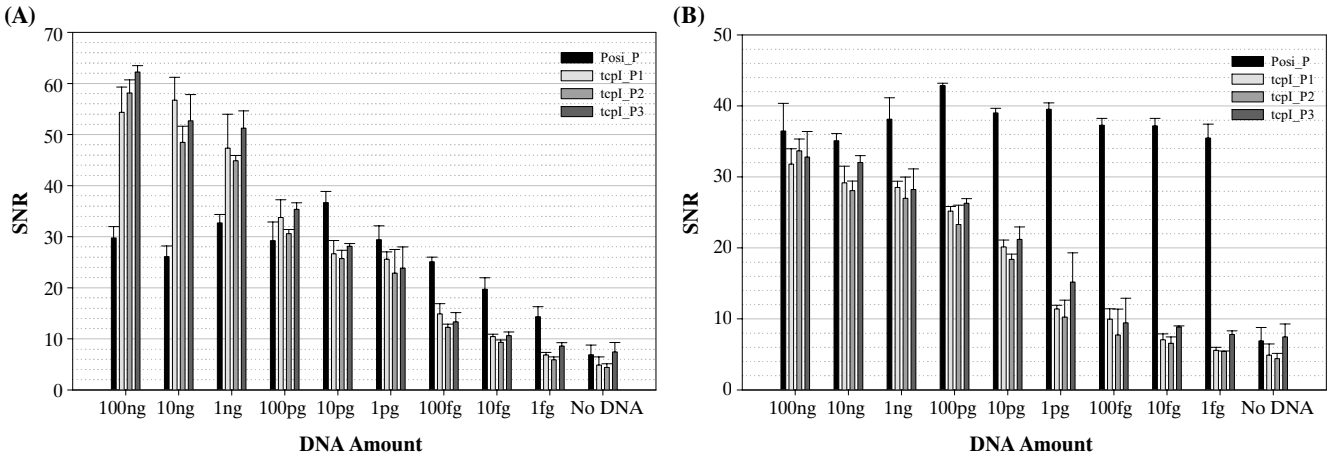

Fig. 3. Detection sensitivity of DNA microarray hybridization for *V. cholerae*. The signal intensities obtained with the probes for a representative gene, *tcpI*, were calculated in this experiment. The signal intensities with positive control probes (Posi_P) were calculated to verify the validity of the results. (A) Detection sensitivity of 10-fold serially diluted genomic DNA extracted from *V. cholera*, (B) Diluted genomic DNA from *V. cholerae* was mixed with 100 ng of stool DNA and hybridized to the DNA microarray.

present, in addition to the pathogens of interest. There is a possibility that the presence of non-target DNAs may interfere with the amplification and/or hybridization of the target DNAs and hence affect the detection sensitivity. There are several studies on detection of pathogens in the samples in which known amounts of genomic DNA of pathogen were added (Wilson *et al.*, 2002; Gonzalez *et al.*, 2004; Panicker *et al.*, 2004; Burton *et al.*, 2005; Maynard *et al.*, 2005). Panicker *et al*. (2004) demonstrated that similar quantitative results could be obtained between spiking pathogens to samples before extraction of DNA and spiking corresponding amount of genomic DNA of pathogens to the sample DNA. In this study, various amounts of pathogenic bacterial DNA (100 ng to 1 fg) were mixed with 100 ng of stool genomic DNA prior to performing multiplex PCR. The hybridization signals were significantly higher than the background when ≥100 fg of genomic DNA were present (Fig. 3B), which is approximately 10^{2-3} times higher than the sensitivity for purified genomic DNA alone. These results suggest that the detection limit for pathogenic bacteria is influenced by the presence of non-target genomic DNAs in the stool. A similar range of detection sensitivities was observed with other microarray approaches to detecting target strains in environmental samples (Wilson *et al.*, 2002; Gonzalez *et al.*, 2004; Panicker *et al.*, 2004; Maynard *et al.*, 2005). This confirmed that our multiplex PCR-DNA microarray method is suitable for analyzing low levels of bacteria, which is a common problem in many different samples including stool samples (Sergeev *et al.*, 2004).

We examined signal intensities of the three different probes for each virulence- factor genes. Although, overall probes intensities were similar, the vvhA_P2, vvhA_P3, and fliC_P1 probes were lower than those with the other probes (data not shown). While the variations in probe sensitivity are difficult to predict, there are many possible explanations for their observation. For example, the local secondary structure of ssDNA could interfere with the hybridization process and cause a lower signal intensity (Myers *et al.*, 2006). Therefore, to increase the sensitivity of probes for detection of virulence-factor genes, and to improve reliability and confidence in the microarray method, the number of oligonucleotide probes should be designed and used for each target gene (Sergeev *et al.*, 2004, 2006; Myers *et al.*, 2006).

Analysis of clinical stool samples using the multiplex PCR-DNA microarray approach

We showed that our microarray method could potentially provide a highly specific and sensitive tool for the detection and identification of pathogens. To evaluate such potential, clinical stool samples from 82 patients with food-borne enteritis were analyzed to identify the causative agents. Possible causative agents of the samples were preliminarily verified using selective cultivation and PCR amplification approaches. The results obtained using our multiplex PCR-DNA microarray approach coincided with those obtained by the cultivation and PCR approaches (Table 2). From our clinical samples, 10 different target enteropathogens could be specifically detected. Seven samples used in this study supposed to contain enteropathogenic viruses based on symptoms and epidemiological data showed no hybridization signal. A PCR amplification approach confirmed the presence of these viruses (data not shown). With non-target clinical sample 1 which is known to contain *E. coli* EAEC strain, one probe, cooA_P2, of the three redundant probes for ETEC weakly hybridized (SNR=4.91) (data not shown). We tried to confirm the results from the microarray by detecting the target genes in the stool samples using a nested PCR approach. When an aliquot of the multiplex PCR product was re-amplified with ETEC-specific nested PCR primers, a band of the expected size was obtained only with target clinical sample 11 and the sequence of the product was identical to the target gene sequence (GenBank accession no. M58550). Unspecific PCR amplification is not unusual especially in microbial community genomic DNAs due to the tremendous unexplored genetic diversity (Peplies *et al.*, 2003; Call, 2005). Our result demonstrates that multiple probe approach is necessary to avoid possible false positive signals (Peplies *et al.*, 2003; Kim *et al.*, 2008). However, increasing the number of target genes in a

Table 2. Results of DNA microarray hybridization of clinical samples

Sample	Multiplex PCR-oligonucleotide-based DNA microarray hybridization[a]												Identification by conventional method[c]
	Probe name										Target fluorescence value	Cutoff value[b]	
	tcpI	tlh	vvhA	fliC	sen	S0745	bcv	cooA	rfbE	invA			
Target													
1	+	-	-	-	-	-	-	-	-	-	4941.9±542.4	184.9	*Vibrio cholerae*
2	-	+	-	-	-	-	-	-	-	-	3706.4±647.6	28.8	*V. parahaemolyticus*
3	-	-	-	+	-	-	-	-	-	-	3469.6±162.1	582.7	*Salmonella enterica* serovar Typhimurium
4	-	-	-	-	+	-	-	-	-	-	2882.8±379.2	41.3	*S. enterica* serovar Enteritidis
5	-	-	-	-	-	-	+	-	-	-	20515.4±384.8	888.6	*Shigella sonnei*
6	-	-	-	-	-	-	-	+	-	-	13986.7±670.4	96.9	Enterotoxigenic *E. coli* (ETEC)
7	-	-	-	-	-	-	-	+	-	-	10145.8±469.8	96.9	ETEC
8	-	-	-	-	-	-	-	+	-	-	8094.2±810.0	96.9	ETEC
9	-	-	-	-	-	-	-	+	-	-	4297.3±131.2	96.9	ETEC
10	-	-	-	-	-	-	-	+	-	-	5847.0±427.5	96.9	ETEC
11	-	-	-	-	-	-	-	+	-	-	8300.0±925.2	96.9	ETEC
12	-	-	-	-	-	-	-	-	+	-	2368.0±168.8	966.0	Enterohemorrhagic *E. coli* (EHEC)
13	-	-	-	-	-	-	-	-	-	+	6436.8±118.9	191.1	*Yersinia enterocolitica*
Non-Target													
1	-	-	-	-	-	-	-	Δ	-	-	3791.4±167.0	96.9	Enteroaggregative *E. coli* (EAEC)
2	-	-	-	-	-	-	-	-	-	-	-	-	*Clostridium perfringens*
3	-	-	-	-	-	-	-	-	-	-	-	-	Rotavirus
4	-	-	-	-	-	-	-	-	-	-	-	-	Rotavirus
5	-	-	-	-	-	-	-	-	-	-	-	-	Rotavirus
6	-	-	-	-	-	-	-	-	-	-	-	-	Rotavirus
7	-	-	-	-	-	-	-	-	-	-	-	-	Norovirus
8	-	-	-	-	-	-	-	-	-	-	-	-	Norovirus
9	-	-	-	-	-	-	-	-	-	-	-	-	Norovirus
10 - 69	-	-	-	-	-	-	-	-	-	-	-	-	Unidentified

[a] +, positive; -, negative; Δ, weak signal
[b] Average signal intensity of the negative control plus 3×standard deviation.
[c] Selective cultivation for enteropathogenic bacteria and PCR methods for virus were used.

multiplex PCR is known to decrease the sensitivity of the PCR amplification (Chizhikov *et al.*, 2001; Volokhov *et al.*, 2002; Chiang *et al.*, 2006). Thus, to increase the specificity and sensitivity of multiplex PCR–DNA microarray for entropathogenic bacteria, we need to determine how many genes (i.e. pathogens) can be used for multiplex PCR in a single tube without sacrificing the sensitivity of hybridization to the microarray.

In conclusion, we have described the use of an oligonucleotide-based DNA microarray for identification of the 10 pathogens that cause enteritis. Although the time of detection of pathogen needs additional 10 hours for labeling and hybridization compared with conventional PCR method, the analysis time is shorter than that of traditional cultivation approaches (Call, 2005; Abubakar *et al.*, 2007; Jordan, 2007). The most important advantages of our protocol are the high sensitivity and sequence-based detection of target genes compared with conventional PCR method. We suggest that a slight modification at general molecular clinical laboratories is enough for accommodating our protocol.

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