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Simultaneous Detection of Major Enteric Viruses Using a Combimatrix Microarray[§]

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Various enteric viruses including norovirus, rotavirus, adenovirus, and astrovirus are the major etiological agents of food-borne and water-borne disease outbreaks and frequently cause non-bacterial gastroenteritis worldwide. Sensitive and high-throughput detection methods for these viral pathogens are compulsory for diagnosing viral pathogens and subsequently improving public health. Hence, we developed a sensitive, specific, and high-throughput analytical assay to detect most major enteric viral pathogens using "Combimatrix" platform oligonucleotide probes. In order to detect four different enteric viral pathogens in a sensitive and simultaneous manner, we first developed a multiplex RT-PCR assay targeting partial gene sequences of these viruses with fluorescent labeling for the subsequent microarray. Then, five olignonucleotides specific to each of the four major enteric viruses were selected for the microarray from the oligonulceotide pools targeting the specific genes obtained by multiplex PCR of these viruses. The oligonucleotide microarray was evaluated against stool specimens containing single or mixed viral species. As a result, we demonstrated that the multiplex RT-PCR assay specifically amplified partial sequences of four enteric viruses and the subsequent microarray assay was capable of sensitive and simultaneous detection of those viruses. The developed method could be useful for diagnosing enteric viruses in both clinical and environmental specimens.

Keywords: enteric viruses, microarray, DNA chip

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Introduction

The public health burden of acute non-bacterial gastroenteritis, which is primarily caused by enteric viral pathogens such as norovirus (NoV), rotavirus (RoV), adenovirus (AdV), and astrovirus (AstV) (Oishi *et al.*, 1994; Glass *et al.*, 2000; Pang *et al.*, 2000) is substantial. Epidemics of these viruses have been commonly reported worldwide. The viruses can survive for prolonged periods of time in water and on surfaces, which facilitates viral transmission, making them important food-borne and water-borne pathogens (Ansari *et al.*, 1991; Dennehy *et al.*, 2000). The impact of those highly contagious viruses in both developing and developed countries is enormous; however, the lack of, or the fastidiousness of, conventional cultivation methods for NoV and enteric AdV has lead to a limitation in analytical tools. Therefore, various epidemiological studies were largely performed using non-cultural analytical methods (Karamoko *et al.*, 2005; Verma and Arankalle, 2010; Hamza *et al.*, 2011).

Non-cultural methods that include electron microscopy (EM), enzyme-linked immunosorbent assay (ELISA), molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR) or PCR, and nucleic acid hybridization, are commonly applied techniques for diagnosing common enteric viruses (Shastri *et al.*, 1998; Lin *et al.*, 2004; Jaaskelainen and Maunula, 2006; Park *et al.*, 2008). Among them, PCR-based methods are highly sensitive and specific in detecting viruses and thus it is most commonly used throughout the world. The PCR products are generally verified by the amplicon size as well as their intensity using conventional gel electrophoresis, or in some cases, by colorimetric or fluorometric methods using appropriate chemicals (Clemence *et al.*, 1999). The urgent need to rapidly and simultaneously detect more than one virus in a sample led to multiplex PCR, employing multiple sets of species/strain-specific primers (Jaaskelainen and Maunula, 2006). However, the design of compatible sets of primers and the difficulty in correctly detecting related viral sequences present in the same specimen, can restrict the number of detectable viruses in a single assay (Bressoud *et al.*, 1990; Wright *et al.*, 1995; Atmar *et al.*, 1996; Broude *et al.*, 2001). In addition, the confirmation of the virus requires additional steps such as nucleic acid sequencing, hybridization, and so on (Chizhikov *et al.*, 2002). Real-time PCR assays using different fluorescent probes can also be applied for the specific detection of the amplified products in multiplex methods (Khodakov *et al.*, 2008). However, the limitation in the number of fluorescent chemicals and the potential optical interference between a subset of fluorescent chemicals can restrict the utility of real-time PCR-based, simultaneous, detection methods (Morrison *et al.*, 1998; Vet

Table 1. Primers used in the uniplex and multiplex RT-PCR for viral detection

Name (genome)	Gene	Primer	Sequence (5′ → 3′)	Location	Polarity	Amplicon size (bp)	Reference
Astrovirus (ssRNA)	ORF1a	MON340	CGTCATTATTTGTTGTCATACT	1182–1203	+	288	Belliot *et al.* (1997)
		MON348	ACATGTGCTGCTGTTACTATG	1450–1470	-		
Norovirus (ssRNA)	RdRp	G2SKF	CNTGGGAGGGCGATCGCAA	5003–5028	+	386	Kojima *et al.* (2002)
		G2SKR	CCRCCNGCATRHCCRTTRTACAT	5367–5389	-		
Adenovirus (dsDNA)	Fiber	AdF1	ACTTAATGCTGACACGGGCAC	30397–30418	+	541	Xu *et al.* (2000)
		AdF2	TAATGTTTGTGTTACTCCGCTC	30916–30938	-		
Rotavirus (dsRNA)	VP4	CON3	TGGCTTCGCCATTTTATAGACA	11–32	+	876	Gentsch *et al.* (1992)
		CON2	ATTTCGGACCATTTATAACC	868–887	-		

et al., 1999).

Microarray-based detection methods are highly attractive due to their high sensitivity, rapidity, and high-throughput detection of multiple PCR amplicons. Despite the relatively high cost of equipment and labeling reagents in microarray experiments, it generally provides a sensitive, specific, and high-throughput detection platform for simultaneous detection (Park *et al.*, 2006). The basic principle of microarray technology is the selective hybridization of sequences from target genes to the specific capture probes embedded on a microarray (Hacia, 1999). The most widely used method is fluorescent labeling for targets, which enables the direct visualization of labeled genes on the array. Furthermore, the explosive growth of viral genomic sequences available in public databases allows this method to be more flexibly applied for thousands of different viral species/strains. In fact, many novel infectious viruses have recently been diagnosed by microarray-based detection methods (Wang *et al.*, 2002). Therefore, microarray technology for the simultaneous detection of enteric viruses from environmental and/or clinical samples would meet the needs for high-throughput detection and possible genotyping of multiple enteric viruses (Urisman *et al.*, 2006; Kistler *et al.*, 2008). In the present study, we developed a Combimatrix microarray for simultaneous detection of various enteric viral pathogens.

Materials and Methods

Stool specimen and extraction of viral nucleic acids

Adenoviruses (AdV), astroviruses (AstV), noroviruses (NoV), and rotaviruses (RoV) were obtained from the infected human patients' stool samples in Korea, which were given by the Korea Centers for Disease Control and Prevention (KCDC) and Water-borne Virus Bank (WAVA, Korea). Viral nucleic acids from 140 μl of 10% fecal suspension in phosphate-buffered saline (PBS) (2.7 M NaCl, 54 mM KCl, 86 mM Na_2HPO_4, 28 mM KH_2PO_4) were extracted using a QIAamp viral RNA mini kit (QIAGEN, USA) following the manufacturer's protocol and stored at –80°C.

(RT)-PCR amplification of partial genes of four enteric viruses

Viral nucleic acids (RNA or DNA) from clinical samples were quantified by RT-PCR assay using a One-Step RT-PCR kit (QIAGEN). Table 1 summarizes the primers used in

Table 2. Summary of sequences of the selected specific microarray probes

Virus	Probe name	Probe Sequence (5′→3′)	Probe type[a]
Astrovirus	Ast_1	TGGCAATGACATAGTAACAGCAGCACAT	S
	Ast_2	AAACCAGGTGCATTATGTGTTATAGACACCC	D
	Ast_3	GTTGGAGTCAACGAATTTGTTGTCATAAAACCA	D
	Ast_4	TAAAACCAGGTGCATTATGTGTTATAGACACCC	D
	Ast_5	GAAGGAAAAGGGACAGGTTTCTTTTCTGGC	D
Norovirus	No_1	GTCCAGAATGTACAATGGTTATGCAGGTGG	S
	No_2	GCCCTGATCTGAACCCCTATCTTTCTCAT	S
	No_3	TGGAGCGCGCCCTTGGGCCCTGATCTG	D
	No_4	GGCCCTGATCTGAACCCCTATCTTTCTC	D
	No_5	CTCATTTGTCCAGAATGTACAATGGTTATGCAG	D
Adenovirus	Ad_1	AGGTGGCTCTAAGTTAATAATCAATCTTGGTCC	S
	Ad_2	AGCTATGGACTTAGGAGACGGTCTTGC	S
	Ad_3	GCGTCTGCTTTAATTATGAGCGGAGTAACA	S
	Ad_4	AACAACCAACTTCAACTCAGAATTGGCT	S
	Ad_5	GGAGCTATTACTTTAGCACTAGATGCAGCG	S
Rotavirus	Ro_1	AGGAGAGCACAAGTTAATGAAGATATTACAATT	S
	Ro_2	GAGAGCACAAGTTAATGAAGATATTACAATTTC	D
	Ro_3	ACCAATTCAAAATACTAGAAATGTAGTTCCATT	D
	Ro_4	TAGGAGAGCACAAGTTAATGAAGATATTACAAT	D
	Ro_5	GGAAGGAAATGCAATATAATAGAGATATTATAA	D

[a] Designed probe types are designated: S, standard type; D, tile discrete type.

this study, along with their sequences, positions, and amplicon sizes.

Uniplex RT-PCR: The uniplex RT-PCR reaction mixture (25 μl) contains 5 μl of 5× Qiagen RT-PCR buffer, 0.4 mM dNTP mix, RT-PCR enzyme mix, 50 pmol of primers F and R, 20 U of RNase inhibitor (TaKaRa Bio Inc., Japan) with 2.5 μl of viral RNA or DNA template. The RT-PCR reaction was performed using a 2720 Thermal Cycler (Applied Biosystems, USA). The viral RNA was reverse-transcribed for 60 min at 42°C and then incubated for 15 min at 95°C. PCR reactions were done using 45 cycles of 30 sec at 94°C, 30 sec at 47.5°C, and 45 sec at 72°C followed by the final extension at 72°C for 10 min. Negative controls were included in all RT-PCR reactions.

Multiplex RT-PCR: The multiplex RT-PCR reaction mixture (50 μl) contains 10.0 μl 5× Qiagen RT-PCR buffer, 0.4 mM dNTP mix, RT-PCR enzyme mix, 20 U of RNase inhibitor (TaKaRa Bio Inc.), 50 pmol/each of four primer sets, 1 μl/each of the four viral RNA/DNA templates. The RT-PCR reaction was performed under the same conditions as for the uniplex RT-PCR.

Design of oligonucleotide array chips

Both conserved and variable sequences were used for probe design using a software package (Premiere Biosoft ArrayDesigner Software ver.4.0). Probes of standard types, of discrete tiling types and of overlapping types were selected as follows: The AdV probes included 15 standard, 15 discrete tiling, and 103 overlapping tiling types; The AstV probes included 11 standard, 13 discrete tiling, and 92 overlapping tiling types; The NoV probes targeting genogroup II (GII-4) included 9 standard, 11 discrete tiling, and 73 overlapping tiling types; The RoV probes included 17 standard, 22 discrete tiling, and 170 overlapping tiling types. A total of 551 oligonucleotide probes were synthesized and printed onto the CombiMatrix CustomArray^{TM} 4×2K microarray support (Macrogen, Korea) to generate the prototype chips for the four viruses. Likewise, the optimized chips were designed, using a subset of 5 probes of either standard type or discrete tiling type for each of the viruses that had been selected based on the specificity and intensity of the positive signals. The oligonucleotide sequences of the selected probes are shown in Table 2. Each 4×2K microarray chip has four identical array sectors that can be hybridized with different target samples using the sectored hybridization cap provided.

Labeling and purification of the PCR products

The fluorescently labeled DNA samples were generated by nested PCR using a G-taq PCR kit (LaboPass, Korea). The reaction was performed in 50 μl of a reaction mixture containing 50 pmol of forward and reverse primers, 5 U of G-taq polymerase, 250 mM of dATP, dGTP, and dTTP, 125 mM dCTP, 25 mM Cy3-dCTP and templates. The PCR amplification and labeling were performed under the following condition: denaturation for 1 min at 94°C; 45 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec; and final extension at 72°C for 10 min. The Cy3-labelled amplicon DNAs were purified using a QIAquick PCR Purification Kit (QIAGEN), and then reconstituted in 30 μl of water. The quantity and quality of the Cy3-labelled DNAs were determined using an ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., USA).

Hybridization on microarray chips

The chips were activated at 65°C for 10 min with nuclease-free water (30 μl) added to each of the sectors, pre-incubated at the desired hybridization temperature for 30 min with gentle rotation, and then subjected to pre-hybridization at 50°C for 30 min in pre-hybridization solution (6× SSPE, 0.05% Tween-20, 20 mM EDTA, 5× Denhardt's solution, 100 ng/μl denatured salmon sperm DNA, and 0.05% SDS). Then, the oligonucleotide microarrays were hybridized for 16 h at 50°C in hybridization solution (6× SSPE, 0.05% Tween 20, 20 mM EDTA, 100 ng/μl denatured salmon sperm DNA, 0.04% SDS) containing labeled probes (50 ng/μl).

Microarray scanning and data analysis

After hybridization, the slides were washed according to the manufacturer's instructions. The slides were centrifuged briefly and then air-dried. Then the arrays were imaged with a fluorescence scanner (GenePix 4000B; Axon Instruments, USA) containing a 532 nm laser for Cy3 and analyzed using GenePix Pro 5.0 software (Axon). All subsequent statistical analyses were conducted using Microsoft Excel. The data were analyzed independently in comparison with different types of viruses assuming that each virus is unique. The averages of all normalized probe intensities within the same probe sets and their standard deviations were calculated for each probe.

Results

Virus-specific uniplex RT-PCR and preparation of the Cy3-labeled probes for the prototype microarray experiments

In order to obtain the viral particles of prevalent strains in Korea, we obtained the stool samples from the Korea Centers

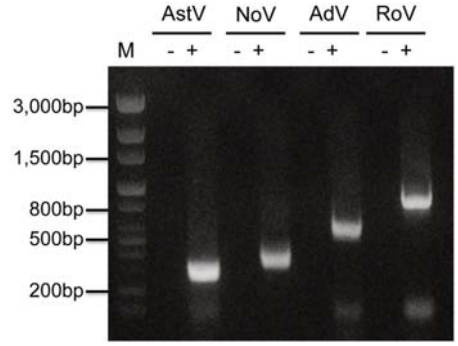

Fig. 1. PCR amplification of viral amplicon by monoplex RT-PCR. Virus-specific RT-PCR was performed for each of the four foodborne viruses, using primer sets listed in Table 1, in the presence (+) or absence (-) of the corresponding viral particles; AstV, astrovirus serotype 1 (288 bp); NoV, norovirus GII-4 (386 bp); AdV, adenovirus serotype 41 (541 bp); RoV, rotavirus (876 bp). M represents the 100-bp ladder size marker (Cosmo, Korea) with the sizes designated in the left.

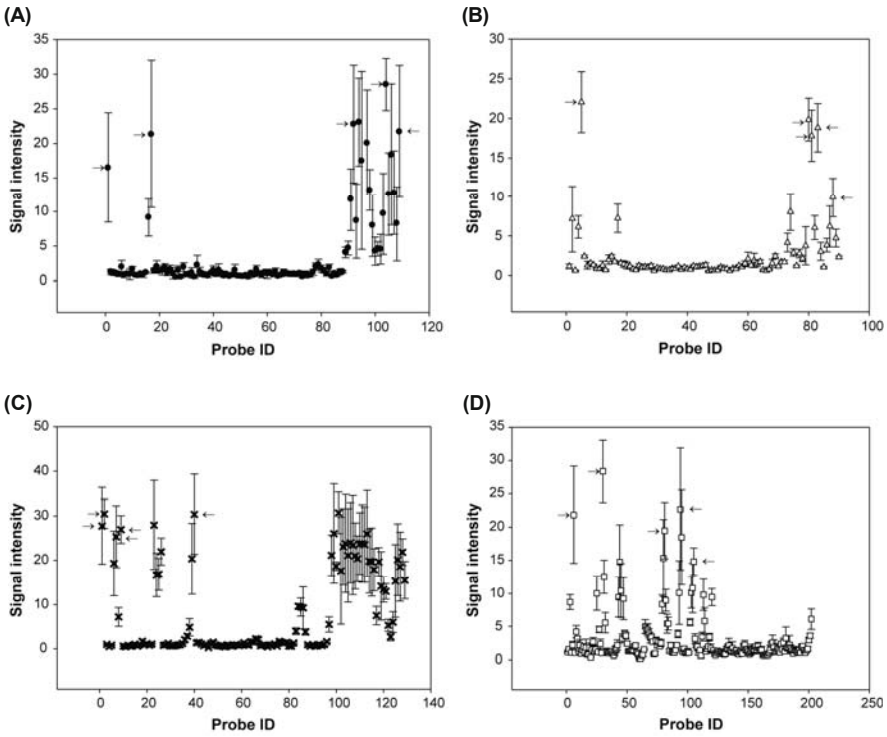

Fig. 2. Results of hybridization using the prototype microarray chip. The average signal intensities of the probes for astrovirus (A), norovirus (B), adenovirus (C), and rotavirus (D) printed in the prototype microarray chip are shown with the error bars representing standard deviations. Five arrows in each panel indicate the selected probes for the further study, based on the high intensity and the low variation of the positive signals.

for Disease Control and Prevention (KCDC) and waterborne virus bank (WAVA, Korea) that were known to be positive for one of the aforementioned four viruses including AstV, NoV, enteric AdV, and RoV. First, as listed in Table 1, we generated PCR primers for individual uniplex RT-PCR amplification, based on the conserved sequences of each viral genome as previously described (Gentsch *et al*., 1992; Belliot *et al*., 1997; Xu *et al*., 2000; Kojima *et al*., 2002). The primer sets were successful in amplifying the corresponding amplicons (Fig. 1), which were verified by nucleotide sequencing (data not shown). We also confirmed the specificity of the primer sets by the absence of cross-reactivity toward the non-cognate amplicons, since we observed the single, desired PCR amplification products from the mixture of all the four viral amplicons (data not shown). These amplified products were purified and used for fluorescence labeling using Cy3 for microarray experiments as described in 'Materials and Methods'.

Development of the prototype microarray and initial validation of the probes

All the microarrays used in this study were generated using CombiMatrix CustomArray^{TM} 4×2K microarray systems. Each 4×2K microarray has four identical array sectors that can be hybridized with four different types of probes using the sectored hybridization caps provided, which enables simultaneous hybridization of these probes.

To determine the selectivity and the specificity of the probes to detect the viruses in the microarray platform, initial validation was performed by designing the prototype oligonucleotide microarrays containing the oligonucleotide probes with variable sequences that were randomly designed using Premiere Biosoft Array Designer Software ver.4.0. A total of 551 probes with the average length of 30 nucleotides were printed on the microarray. Five identical oligonucleotides for each probe were spotted for consistency validation as well as statistical analysis. The prototype microarray chip was subjected to hybridization with the fluorescently labeled, virus-specific, amplicon DNAs obtained from each uniplex RT-PCR as described in Fig. 1. After hybridization, high-resolution scan images were first obtained (Supplementary data Fig. S1) and the signal intensities of each probe were measured for subsequent statistical analyses. The hybridizations were repeated and analysed three times. The calculated aver-

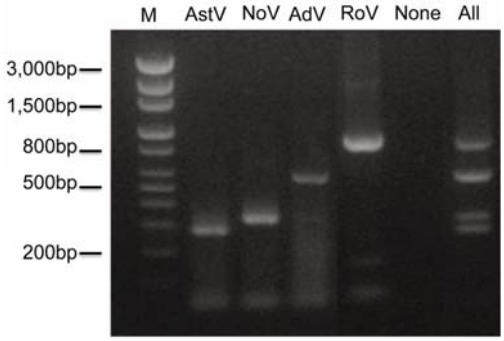

Fig. 3. Detection of foodborne viruses by multiplex RT-PCR. Multiplex RT-PCR was performed using four primer sets against the nucleic acid extracts from the stool specimen containing nothing (none), one or all of the four viral particles as in Fig. 1; AstV, astrovirus serotype 1 (288 bp); NoV, norovirus GII-4 (386 bp); AdV, adenovirus serotype 41 (541 bp); RoV, rotavirus (876 bp). M represents the 100-bp ladder size marker (Cosmo, Korea) with the sizes designated in the left.

ages of all the normalized probe intensities within the same probe sets and their standard deviations from three independent hybridization experiments are shown in Fig. 2, which enables us to determine the sensitivity of the probes. Based primarily on the signal intensities, the five probes were of either the standard type or the discrete tile type of probes and these were chosen to design the microarray (Table 2). The sequences of these probes are within the highly conserved stretches of the viral amplicons (Supplementary data Fig. S2).

Multiplex RT-PCR assay against the clinical samples

To facilitate the detection of the four viruses from the clinical samples, we first spiked a specific type of one or all of the isolated viruses (at 10^2–10^4 (RT)-PCR units for each virus) into the stool specimens from an uninfected human subject. The uniplex PCR against the viral nucleic extracts from the spiked stool specimens was successful under the same conditions as in Fig. 1 (data not shown). Furthermore, we tried to optimize the multiplex RT-PCR assay for simultaneous amplification of the four viral amplicons in a single reaction, using the mixture of the same primer sets used in the uniplex RT-PCR for the AdV, AstV, NoV, and RoV. Successful amplification of all the four amplicons from the spiked stool specimens was obtained (Fig. 3) under our previously described optimized multiplex RT-PCR conditions. We also confirmed that the primer sets in their uniplex format displayed no cross-reactivity even against the mixed viral nucleic acids extracted from the stool specimen (data not shown). Thus, the multiplex RT-PCR could be used for RT-PCR-based detection of the viruses by gel electrophoresis and fluorescent labeling, and for the microarray-based detection and confirmation as well.

Development of the microarray chip-based simultaneous detection method for the four enteric viruses

The microarray for the four food-borne viruses that had been created using the selected oligonucleotide probes listed in Table 2 was used for hybridization with the Cy3-labeled probes from the uniplex RT-PCR as in Fig. 2 (Fig. 4A), as well as with the labeled probes from the multiplex RT-PCR against the stool samples (Fig. 4B). All the microarray experiments were performed under the same conditions as for the prototype microarray, according to the manufacturer's suggested protocols. The scanned images of the microarray chips that had been hybridized to the labeled PCR products were generated (Supplementary data Fig. S3) and the signal intensities were obtained and treated as in Fig. 2, resulting in the data shown in Fig. 4. These results verified the sensitivity as well as the specificity of the selected probes. For example, the signal intensities of probes for AstV (Ast_1 to Ast_5) shown in Fig. 4A were unambiguously higher than the other probes with no cross-hybridization. Slight quantitative differences between the results in Figs. 4A and 4B were observed, which might be attributed to the difference in the sample type (viral amplicon DNA vs viral RNA/DNA extracted from stool specimens), or else the PCR format (uniplex vs. multiplex). These results suggested that the oligonucleotide microarray combined with the optimized multiplex RT-PCR amplification/labeling could unambiguously detect and distinguish one or more viruses of the four major enteric viruses in the stool specimens.

Discussion

Microarray technology is very useful and applicable for a number of genome-wide analyses to identify genomic islands, gene expression patterns, gene dosages, single nucleotide polymorphisms and so on (Gunderson *et al.*, 2005). It is also useful for more practical applications, such as the detection of specific pathogens from clinical and environmental specimens, which has rapidly increased during the past decade, largely due to its high-throughput capability. Since microarray technology is essentially based on nucleic acid hybridization, the platform is particularly useful for detection of viral pathogens that are fastidious or unculturable under laboratory culture conditions. Furthermore, the common food-borne gastroenteritis outbreaks worldwide are caused primarily by four enteric viruses that include NoV, AsV, RoV, and enteric AdV, some of whose cultivation methods remain to be further developed. The currently used detection methods such as microscopy, immunodetection, conventional RT-PCR, and TaqMan real-time RT-PCR have been developed for the detection of such viruses (Lemarchand *et al.*, 2004; Jaaskelainen and Maunula, 2006). To the best of our knowledge, this study presents the first detection method based on microarray chips targeting all of the four major enteric viruses, using highly species-specific oligonucleotide probes.

The development of a simple molecular assay for simultaneous detection of multiple viruses is highly challenging, although it may facilitate the detection of various viruses with regard to cost and speed. However, it is not currently available, primarily due to the significant genetic diversity even in a single viral species. Furthermore, viral genomes do not have universal genes such as the 16S or 18S rRNA, which serve as phylogenetic probe targets in bacterial and fungal pathogens, respectively. Thus, the molecular targets will differ, depending on the conserved genes of each viral group/species. Such information is largely dependent on the amount of sequence data available for the given species. Based on the increasing amount of sequence data and number of literature reports for the four major viruses, we were able to design target amplicons that could serve as species-specific probes without cross-reactivity toward the other viruses.

Although microarray detection of various viruses such as adenovirus, astrovirus, norovirus, and influenza virus has been reported, most of these reports were focused on genotyping of one or two viral species (Jacobi *et al.*, 1998; Jaaskelainen and Maunula, 2006; Quan *et al.*, 2007; Wang *et al.*, 2008) or on comprehensive detection of a similar group of viruses (Pasquini *et al.*, 2008; Chung *et al.*, 2010; Engel *et al.*, 2010). However, the quintessential advantage of microarrays is that a large panel of probes can be hybridized without additional effort and cost in a single reaction, which makes microarrays particularly suitable for detection of multiple viruses and subtyping of a group of viruses, based on their genetic diversity. Since viruses do not have universal

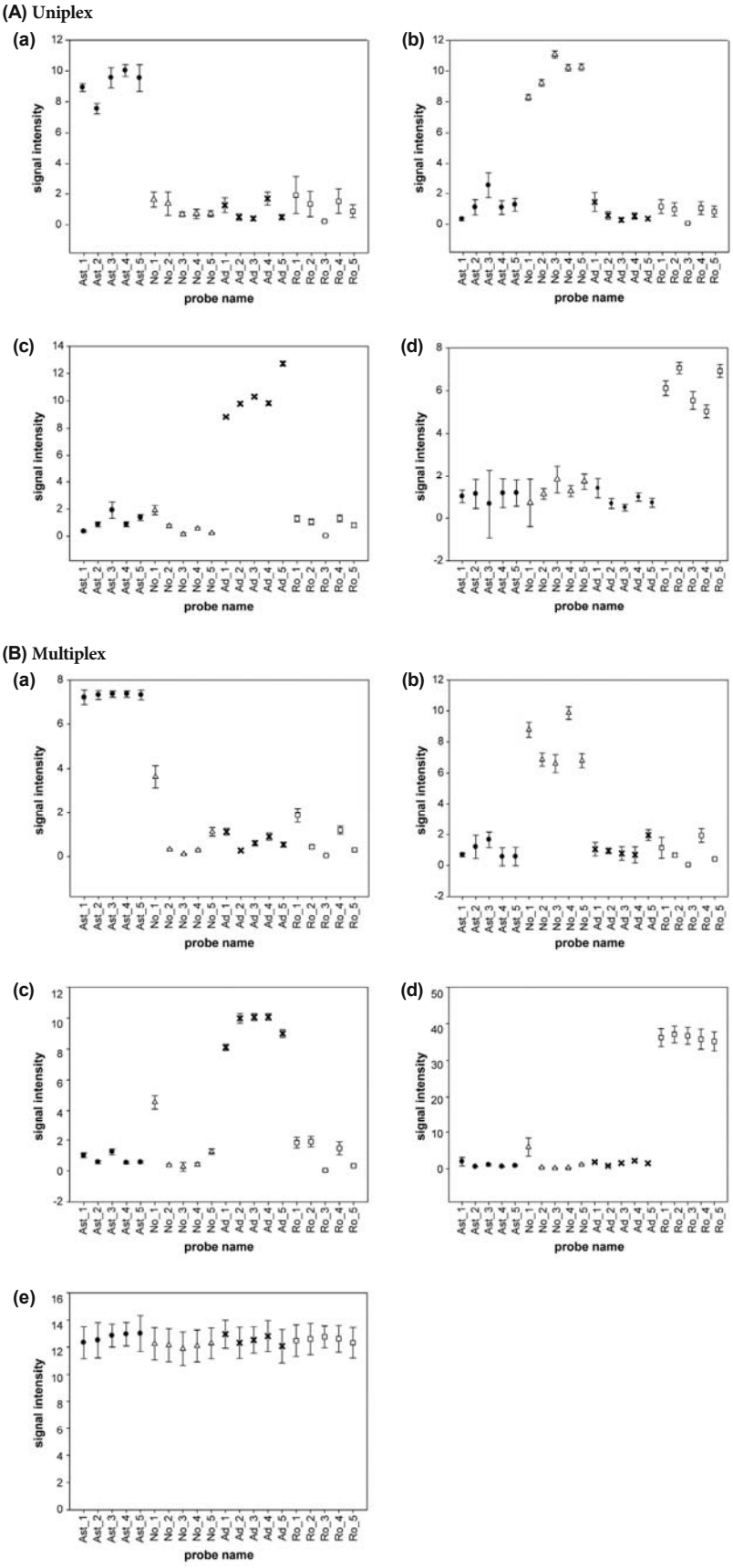

Fig. 4. Hybrdization using the microarray chip developed for simultaneous detection. The average signal intensities of the selected probes in the developed microarray chip are shown with the error bars representing standard deviations. The microarray chip was hybridized using the probes labeled by uniplex RT-PCR as in Fig. 1 (A) or multiplex RT-PCR (B) against the nucleic acids extracted from stool samples containing astrovirus (a), norovirus (b), adenovirus (c), rotavirus (d), and all four viruses (e).

phylogenetic markers, the development of simultaneous detection methods based on different oligonucleotide probes and their reference hybridization profiles with various viral nucleic acids would greatly expand the spectrum of detectable viruses, as demonstrated by Wang *et al.* (2008).

The present study was focused on the experimental optimization for simultaneous detection of four major enteric viruses with high sensitivity and selectivity. However, there is some limitation in this study. Although the most prevalent serotypes of each virus were selected for this study, tests were performed for only a limited number of isolates of each virus. There could be variation in the viral nucleic acid sequences in the regions used for both the primers and probes. Therefore, a panel of different strains of viruses should be tested, and the future primer and probe sequences could be adjusted as needed, based on these. Additionally, we did not fully characterize the limit of detection of our developed assay in this study. Approximate 10^2–10^4 (RT)-PCR units for each virus were inoculated and successfully detected. Viral concentrations in clinical specimens such as stool were typically much higher than this study (Rohayem *et al*., 2004). Therefore, it should be possible to apply our developed assay to diagnose clinical specimens. However, viral concentrations in environmental samples are known to be much lower (Meritxell *et al*., 2005). Therefore, the limit of detection should be fully characterized prior to applying this microarray system to detect enteric viruses in environmental samples such as surface water.

The absence of cross-reactivity in these experiments, as well as apparent sequence homology, between the oligonucleotide probes and the selected amplicons of the four viruses were verified by the specificity of the multiplex RT-PCR, probe labeling and hybridization detection and thus may also indicate the potential function of the probes in genotyping given viral species in the microarray platforms, since a few nucleotide variations can be introduced to genotype the subtypes of each viral species, without loss of species-specificity. It is still possible to test additional specificity of our assay with not only negative stool samples but also with positive stool samples for other diarrheal microbial pathogens (e.g., bacteria, etc). However, it is believed that PCR and subsequent hybridization with 5 independent probes should provide good specificity with reasonably good repeatability.

In future, this study could be extended to both simultaneous detection and subtyping of tested viruses, based on current knowledge of the sequence variations in the selected amplicons of those viruses. The clearer benefit of arrays is for strain discrimination or identification of more than just 4 viral targets. This study is a good, early demonstration that sets the stage for future research, such as strain typing for outbreak tracking or identifying the source of an epidemic. Although there exists a limitation if the viral detection necessitates the identification of novel sequences, which are currently unknown, and thus are not represented in the designed microarray chips, microarray-based detection and subtyping nevertheless allows rapid and extensive diagnosing and monitoring of a large repertoire of the four viral genotypes, which will contribute to better human health risk assessment for enteric viruses in clinical and possibly environmental settings.

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References

Ansari, S.A., Springthorpe, V.S., and Sattar, S.A. 1991. Survival and vehicular spread of human rotaviruses: Possible relation to seasonality of outbreaks. *Rev. Infect. Dis.* **13**, 448–461.

Atmar, R., Barbarad, B., Edward, A.D., and Taber, L.H. 1996. Comparison of reverse transcription-PCR with tissue culture and other rapid diagnostic assays for detection of type A influenza virus. *J. Clin. Microbiol.* **34**, 2604–2606.

Belliot, G., Laveran, H., and Monroe, S.S. 1997. Detection and genetic differentiation of human astroviruses: phylogenetic grouping varies by coding region. *Arch. Virol.* **142**, 1323–1334.

Bressoud, A., Whitcomb, J., Pourzand, C., Ealler, O., and Cerutti, P. 1990. Rapid detection of influenza virus Hl by the polymerase chain reaction. *Biochem. Biophy. Res. Commun.* **167**, 425–430.

Broude, N.E., Driscoll, K., and Cantor, C.R. 2001. High-level multiplex DNA amplification. *Antisense Nucleic Acid Drug Dev.* **11**, 327–332.

Chizhikov, V., Wagner, M., Ivshina, A., Hoshino, Y., Kapikian, A.Z., and Chumakov, K. 2002. Detection and genotyping of human group A rotaviruses by oligonucleotide microarray hybridization. *J. Clin. Microbiol.* **40**, 2398–2407.

Chung, I.H., Yoo, H.S., Eah, J.Y., Yoon, H.K., Jung, J.W., Hwang, S.Y., and Kim, C.B. 2010. A DNA microarray for identification of selected Korean birds based on mitochondrial cytochrome c oxidase I gene sequences. *Mol. Cell.* **30**, 295–301.

Clemence, M., Martine, V., Michele, A., and Bruno, L. 1999. Comparison of two nested PCR, cell culture, and antigen detection for the diagnosis of upper respiratory tract infections due to influenza viruses. *J. Med. Virol.* **59**, 215–220.

Dennehy, P.H. 2000. Transmission of rotavirus and other enteric pathogens in the home. *Pediatr. Infect. Dis. J.* **19**, S103–105.

Engel, E.A., Escobar, P.F., Rojas, L.A., Rivera, P.A., Fiore, N., and Valenzuela, P.D. 2010. A diagnostic oligonucleotide microarray for simultaneous detection of grapevine viruses. *J. Virol. Methods* **163**, 445–451.

Gentsch, J.R., Glass, R.I., Woods, P., Gouvea, V., Gorziglia, M., Flores, J., Das, B.K., and Bhan, M.K. 1992. Identification of group A rotavirus gene 4 types by polymerase chain reaction. *J. Clin. Microbiol.* **30**, 1365–1373.

Glass, R.I., Noel, J., Ando, T., Fankhauser, R., Belliot, G., Mounts, A., Parashar, U.D., Bresee, J.S., and Monroe, S.S. 2000. The epidemiology of enteric caliciviruses from humans: A reassessment using new diagnostics. *J. Infect. Dis.* **181**(S2), S254–261.

Gunderson, K.L., Steemers, F.J., Lee, G., Mendoza, L.G., and Chee, M.S. 2005. A genome wide scalable SNP genotyping assay using microarray technology. *Nat. Genet.* **37**, 549–554.

Hacia, J.G. 1999. Resequencing and mutational analysis using oligonucleotide microarrays. *Nat. Genet.* **21**, 42–47.

Hamza, I.A., Jurzik, L., Uberla, K., and Wilhelm, M. 2011. Methods to detect infectious human entericviruses in environmental water samples. *Int. J. Hyg. Environ. Health* **214**, 424–436.

Jaaskelainen, A.J. and Maunula, L. 2006. Applicability of microarray technique for the detection of noro-and astroviruses. *J. Virol. Methods* **136**, 210–216.

Jacobi, A., Bachand, G.D., Hamelin, R.C., and Castello, J.D. 1998. Development of a multiplex immunocapture RT-PCR assay for detection and differentiation of tomato and tobacco mosaic

tobamoviruses. *J. Virol. Methods* **74**, 167–178.

Karamoko, Y., Ibenyassine, K., Aitmhand, R., Idaomar, M., and Ennaji, M.M. 2005. Adenovirus detection in shellfish and urban sewage in Morocco (Casablanca region) by the polymerase chain reaction. *J .Virol. Methods* **126**, 135–137.

Khodakov, D.A., Zakharova, N.V., Gryadunov, D.A., Filatov, F.P., Zasedatelev, A.S., and Mikhailovich, V.M. 2008. An oligonucleotide microarray for multiplex real-time PCR identification of HIV-1, HBV, and HCV. *BioTechniques* **44**, 241–248.

Kistler, A.L., Gancz, A., Clubb, S., Skewes-Cox, P., Fischer, K., Sorber, K., Chiu, C.Y., Lublin, A., Mechani, S., Farnoushi, Y., and *et al.* 2008. Recovery of divergent avian bornaviruses from cases of proventricular dilatation disease: Identification of a candidate etiologic agent. *Virology* **5**, 88.

Kojima, S., Kageyama, T., Fukushi, S., Hoshino, F.B., Shinohara, M., Uchida, K., Natori, K., Takeda, N., and Katayama, K. 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J. Virol. Methods* **100**, 107–114.

Lemarchand, K., Masson, L., and Brousseau, R. 2004. Molecular biology and DNA microarray technology for microbial quality monitoring of water. *Cri. Rev. Microbiol.* **30**, 145–172.

Lin, B., Vora, G.J., Thach, D., Walter, E., Metzgar, D., Tibbetts, C., and Stenger, D.A. 2004. Use of oligonucleotide microarrays for rapid detection and serotyping of acute respiratory disease-associated adenoviruses. *J. Clin. Microbiol.* **42**, 3232–3239.

Meritxell, F.C., Ayalkibet, H., Pilar, C.C., Nestor, A.G., Annika, A., and Rosina, G. 2005. Nested multiplex PCR assay for detection of human enteric viruses in shellfish and sewage. *J. Virol. Methods* **125**, 111–118.

Morrison, T.B., Weis, J.J., and Wittwer, C.T. 1998. Quantification of low-copy transcripts by continuous SYBR® Green I monitoring during amplification. *Biotechniques* **24**, 954–962.

Oishi, I., Yamazaki, K., Kimoto, T., Minekawa, Y., Utagawa, E., Yamazaki, S., Inouye, S., Grohmann, G.S., Monroe, S.S., Stine, S.E., and *et al.* 1994. A large outbreak of acute gastroenteritis associated with astrovirus among students and teachers in Osaka, Japan. *J. Infect. Dis.* **170**, 439–443.

Pang, X.L., Honma, S., Nakata, S., and Vesikari, T. 2000. Human caliciviruses in acute gastroenteritis of young children in the community. *J. Infect. Dis.* **181**(S2), S288–294.

Park, Y., Cho, Y.H., Jee, Y., and Ko, G. 2008. Immunomagnetic separation combined with real-time reverse transcriptase PCR assays for detection of norovirus in contaminated food. *Appl. Environ. Microbiol.* **74**, 4226–4230.

Park, H.G., Song, J.Y., Park, K.H., and Kim, M.H. 2006. Fluorescent-based assay formats and signal amplification strategies for DNA microarray analsis. *Chem. Eng. Sci.* **61**, 954–965.

Pasquini, G., Barba, M., Hadidi, A., Francesco, F., Rodolfo, N., Sobol, I., Tiberini, A., Caglayan, T., Hamed, M., Anfoka, G., and *et al.* 2008. Oligonucleotide microarray-based detection and genotyping of *Plum pox virus*. *J. Virol. Methods* **147**, 118–126.

Quan, P.L., Palacios, G., Jabado, O.J., Conlan, S., Hirschberg, D.L., Pozo, F., Jack, P.J.M., Cisterna, D., Renwick, N., Hui, J., and *et al.* 2007. Detection of respiratory viruses and subtype identification of influenza A viruses by GreeneChipResp oligonucleotide microarray. *J. Clin. Microbiol.* **45**, 2359–2364.

Rohayem, J., Berger, S., Juretzek, T., Herchenrodera, O., Mogel, M., Poppe, M., Henker, J., and Rethwilm, A. 2004. A simple and rapid single-step multiplex RT-PCR to detect Norovirus, Astrovirus and Adenovirus in clinical stool samples. *J. Virol. Methods* **118**, 49–59.

Shastri, S., Doane, A.M., Gonzales, J., Upadhyayula, U., and Bass, D.M. 1998. Prevalence of astroviruses in a children's hospital. *J. Clin. Microbiol.* **36**, 2571–2574.

Urisman, A., Molinaro, R.J., Fischer, N., Plummer, S.J., Casey, G., Klein, E.A., Malathi, K., Magi-Galluzzi, C., Tubbs, R.R., Ganem, D., Silverman, R.H., and Derisi, J.L. 2006. Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. *PLoS Pathogen.* **2**, 211–225.

Verma, V. and Arankalle, V.A. 2010. Hepatitis E virus-based evaluation of a virion concentration method and detection of enteric viruses in environmental samples by multiplex nested RT-PCR. *J. Appl. Microbiol.* **108**, 1630–1641.

Vet, J.A.M., Majithia, A.R., Marras, S.A.E., Tyagi, S., Dube, S., Poiesz, B.J., and Kramer, F.R. 1999. Multiplex detection of four pathogenic retroviruses using molecular beacons. *Proc. Natl. Acad. Sci. USA* **96**, 6394–6399.

Wang, D., Coscoy, L., Zylberberg, M., Avila, P.C., Boushey, H.A., Ganem, D., and Derisi, J.L. 2002. Microarray-based detection and genotyping of viral pathogens. *Proc. Natl. Acad. Sci. USA* **99**, 15687–15692.

Wang, L.C., Pan, C.H., Severinghaus, L.L., Liu, L.Y., Chen, C.T., Pu, C.E., Huang, D., Lir, J.T., Chin, S.C., Cheng, M.C., and *et al.* 2008. Simultaneous detection and differentiation of Newcastle disease and avian influenza viruses using oligonucleotide microarrays. *Vet. Microbiol.* **127**, 217–226.

Wright, K.E., Wilson, G.A.R., Novosad, D., Dimock, C., Tan, D., and Weber, J.M. 1995. Typing and subtyping of influenza viruses in clinical samples by PCR. *J. Clin. Microbiol.* **33**, 1180–1184.

Xu, W., McDonough, M.C., and Erdman, D. 2000. Species-specific identification of human adenoviruses by a multiplex PCR assay. *J. Clin. Microbiol.* **38**, 4114–4120.