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Development of oligonucleotide microarray system for differential diagnosis of enteric viruses in diarrheic fecal samples in pigs

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Abstract

An oligonucleotide microarray system was developed for the simultaneous detection of porcine epidemic diarrhea virus, transmissible gastroenteritis virus, porcine enteric calicivirus, porcine group A and C rotavirus. RNAs of the reference viruses and porcine diarrhea samples were extracted and amplified using one-step multiplex RT-PCR in the presence of cyanine 5-dCTP and hybridized on the microarray chip that spotted the virus-specific oligonucleotides. This system were approximately 10- to 100-fold higher in sensitivity than conventional RT-PCR, and the assay time was less than 3 hours. The relative sensitivity and specificity were 92% and 72.2%, respectively, based on 102 porcine diarrhea samples using RT-PCR as gold standard. These results suggested that the oligonucleotide microarray system in this study be probably more reliable and reproducible means for detecting porcine enteric viruses and that it could be of substantial use in routine diagnostic laboratories.

Key words : Microarray, porcine enteric viruses, DNA chip

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Introduction

Enteric diseases in suckling and weaned piglets are often multifactorial[1)]. Intestinal infections are common in growing pigs and can be caused by many pathogens, and various environmental and managemental factors[2)]. Either singly or in combination, porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus

(TGEV), porcine enteric calicivirus (PECV), porcine group A rotavirus (PRV gp A), and porcine group C rotavirus (PRV gp C) are identified as the major viral enteropathogens in swine[2]. These viruses have a highly conserved and specific RNA genome, thus it can be useful for molecular identification and diagnosis[3].

The main focus for the rapid and reliable diagnosis of porcine viral enteritis over the last decade has shifted from conventional laboratory techniques such as antigen detection, electron microscopy and culture to molecular methods based on biotechnology protocols[4]. The conventional polymerase chain reaction (PCR) is one of the most well known and successful molecular methods for detecting specific porcine enteropathogenic viruses (PEVs), and is better in speed, in sensitivity and specificity than in other conventional microbiological assays. However, a second round of nested amplification or Southern hybridization is required in some circumstances[5]. Therefore, there is a need for a more specific, highly sensitive, and rapid detection method that can more reliably diagnose porcine viral enteric diseases. The microarray is powerful tool that can be used to detect thousands of gene sequences simultaneously on a glass slide[1]. In addition, it can be used to detect bacteria and to perform DNA-based typing of specific pathogenic bacterial strains and viruses[6-9]. Moreover, the combination of PCR with microarray technology will provide a novel means for identifying PEVs, and it could be expected to revolutionize the diagnosis and management of disease. The aim of this study was to develop an oligonucleotide microarray system for the rapid and reliable detection of 5 major PEVs.

Materials and Methods

The tissue culture fluid used in this study contained TGEV(Miller strain), PRCV (KPRCV strain), PEDV (KPEDV-9), PRV gp A (Gottfried strain) and PRV gp C (Cowden strain), all of which were obtained from Choong Ang Vaccine Lab (Daejeon, Korea), and PECV identified by RT-PCR and sequencing analysis from diarrheic fecal samples of pigs. The RNAs from mock infected swine testicular cells and from Newcastle disease virus B1 strain were extracted and used as negative and positive controls, respectively.

Two hundred and forty five fecal samples were obtained from 245 piglets of both genders (various breeds and ages), which were suspected of being infected with enteropathogenic viruses during 2004-2006. Feces were sampled using a sterile swab, immersed immediately in a 1.5㎖ tube containing 400㎕ of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.4) and stored at -70℃ until needed.

The viral RNA was extracted using Tri Reagent (Molecular Research Center, Inc, Ohio) according to the manufacturer' s protocol. In order to compare the sensitivity of the microarray system with RT-PCR, TGEV (1×10^4 $TCID_{50}$/0.1㎖), PRCV (1×10^4 $TCID_{50}$/0.1㎖), PEDV (1×10^4 $TCID_{50}$/0.1㎖), PRV gp A (1×10 $TCID_{50}$/0.1㎖), or PRV gp C (3×10 $TCID_{50}$/0.1㎖) were serially diluted 10-fold with maintenance medium. In case of PECV, 1 ng/ℓ of RNA

was serially diluted 10-fold with RNase-free water.

The primers were designed from published sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov) and were analyzed using the Primer 3 algorithm (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)[10]. Table 1 and 2 gave complete list of the microarray primers and probes. During the automated oligonucleotide synthesis, the 5' end of each probe was modified by adding an amino-link group (TFA aminolink CE reagent, PE Applied Biosystems, CA) to facilitate covalent immobilization on the aldehyde-coated glass surface[7].

Table 1. List of porcine enteric viruse-specific oligonucleotide probes for the oligonucleotide microarray system

Type and name	Sequence[a] (5'-3')	Position	Tm (℃)	GenBank[b]
TGEV_1	GATAGAAGTCACGTTCACACACAAATACCACT	1236-1267	62.48	AJ271965
TGEV_2	GCATTGTATTGGGATTATGCTACAGAAAAT	253-282	60.65	
PEDV_1	CTTGCATCACTCTTATGCTGTGGATAATGT	254-283	62.38	AF015888
PEDV_2	CACTACAACAATTGTCTACGGACGTGTTGGT	522-552	65.39	
PECV_1	GAGGAATTCAAGGTGCCTTATATGGGGAAC	4579-4608	65.79	AF182760
PECV_2	TCAAGAACCTGACTAGCTATGGGTTGGTCC	4697-4726	65.80	
PRV_A_1	CGGATAGCTCCTTTTAATGTATGGTATTGAA	33-63	61.57	X04613
PRV_A_2	ACATGGAGGTTCTGTACTCWTTRTCAAAAAC	22-52	62.44	AF317123
PRV_C_1	AATGTTCCACCTTATAGCAGACGCACTAGT	123-152	62.32	M61101
PRV_C_2	TGACTACTCAATTGTTGCTAACTGGAGAAG	1038-1067	60.30	M29287
Control	TTGGACTTATGACCACCGTAGATAGGAAGG	725-754	63.97	AF309418

[a] Nucleotide abbreviations are according to the IUPAC system of nomenclature : R, A+G; Y, C+T; M, A+C; K, G+T; S, G+C; W, A+T.

[b] Accession numbers for GenBank sequences that were used to design probes.

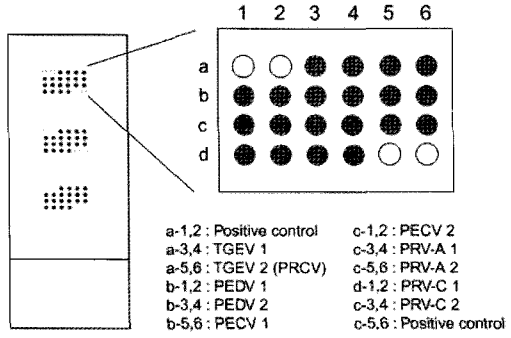

Fig 1. Diagram of the oligonucleotide microarray chip design

Oligonucleotides were spotted at 1 μM in spotting buffer (350 mM sodium bicarbonate, pH 9.0) on a glass slide coated with an aldehyde group (CEL Associates, Inc, Pearland, TX) using a MicroGrid II (BioRobotics, London, UK). Amine groups in DNAs were coupled with the aldehyde groups on the slide by the Schiff's reaction. $NaBH_4$ was used to reduce remaining aldehyde groups. Oligonucleotide probes were spotted in duplicate in a 6 × 5 matrix (Fig 1).

Fluorescently labeled samples for hybridization were generated by multiplex PCR with primers complementary to target regions of each viral genes (Table 2). Briefly, 25 $\mu\ell$ of a reaction mixture containing 1× PCR buffer with 1.5 mM $MgCl_2$, 300 nM each primer, 20 μM Cy5-dCTP,

100 μM each dATP, dGTP, and dTTP, 1 U of Taq polymerase (Takara, Bio Inc, Otsu, Japan), and 5μℓ of cDNA from the reverse transcription was subjected to 25 cycles of PCR. The multiplex PCR cycle included 30 sec at 94℃, 30 sec at 50℃, and 60 sec at 72℃. Finally, the fluorescent probe was served oligonucleotide microarray chip hybridization. Cy5-labeled porcine enteric virus probe and positive control probe were mixed in a 25 μℓ of 1× hybridization buffer, containing 6× SSC (1× SSC is 0.15M NaCl plus 0.015 M sodium citrate), 0.2% SDS, 5× Denhardt's solution, and 50% formamide and were denatured for 3 min at 95℃, followed by chilling on ice for 1 min. The final concentration of each fluorescent probe in the hybridization solution was usually above 0.1 μM. This concentration ensures rapid and efficient hybridization of a fluorescent sample with immobilized

Table 2. Designed primers used for the amplification of porcine enteric viruses by RT-PCR

Name	Sequence[a] (5′ 3′)	Product size	Position	GenBank[b]
TGEV_N_F	GAG AAC TGC AGG TAA AGG TG	306 bp	27819 27850	AJ271965
TGEV_N_R	CTC TGT TCT TTT GCC ACT TC		27935 27916	
TGEV_S_F	ACC ACC TAA TTC AGA TGT GG	334 bp	20505 20524	AJ271965
TGEV_S_R	ATG GTT TAA CCT GCA CTC AC		20838 20819	
PEDV_M_F	AAC TGG GTC TTT TTC GCT	355 bp	220 237	AF015888
PEDV_M_R	CAG ATG AAG CAT TGA CTG AA		574 555	
PECV_F	CTC CTA TGC TGA GGA CAC AC	380 bp	4392 4411	AF182760
PECV_R	GAG TGT CTG TTG GCT CAA TG		4771 4752	
PRV_7_F	AAA RAR RRA RWW KCY GWC TG	376 bp	8 27	X04613,
PRV_7_R	GGM MAW CCY TTW GTT WMR AA		383 364	M61101
PRV_6_F	GAG ARK CWY MAM GAA ATG GA	295 bp	328 347	AF317123,
PRV_6_R	GTT GAA TGT TTG CTG GTG C		642 622	M29287
Control_F	GGT TTT CTC AGT AGT GCA GG	425 bp	3670 3689	AF309418
Control_R	CGA CAG ATA GAT CAA GGC TC		4094 4075	

[a] Nucleotide abbreviations are according to the IUPAC system of nomenclature : R, A+G; Y, C+T; M, A+C; K, G+T; S, G+C; W, A+T.

[b] Accession numbers for GenBank sequences that were used to design primers.

probes. Aliquots (10μℓ) from sample were applied to each microarray area and covered with a plastic coverslip (Sigma, USA) to prevent evaporation of the sample during hybridization in the incubation chamber (GenomicTree, Daejeon, Korea). Hybridization was performed for 2 hrs at 42℃. Slides were then washed with wash buffer I (2× SSC, 0.1% SDS) for 5 min, and with 1×SSC 5 min, and with 0.5× SSC for 5 min at room temperature and dried by an air stream to remove any remaining solution completely.

The slides were then dried under an air stream to completely remove any remaining solution. The microarray images were captured using a confocal fluorescent scanner (ScanArray 4000, Packard Biochip Technologies, MA, USA) using a red He/Ne laser (632㎚ for cy5). Fluorescent images obtained at 694㎚ (cy5) were analyzed using QuantArray software (Packard BioScience, MA, USA).

Conventional one-step RT-PCR was carried out using the primers listed in Table 1. The total volume of 25μℓ of the reaction

mixture containing 1× RT-PCR buffer (Takara Bio, Inc, Otsu, Japan) containing 1.5mM $MgCl_2$, 500nM of each primer, 100 μM of each dNTP, 1U of Taq polymerase (Takara Bio, Inc, Otsu, Japan), 5 U of RNA inhibitor (Promega, Madison, WI), 5U of avian myeloblastoma virus reverse transcriptase (Takara Bio, Inc, Otsu, Japan), and 5$\mu\ell$ of RNA. The one-step multiplex RT-PCR conditions used were: 42℃ for 45 min and 25 amplification cycles of 30 sec at 94℃, 30 sec at 50℃ and 60 sec at 72℃ in a PE 2400 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). PCR products were run in 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

The sensitivities and specificities of the two assays used in this study were compared, and agreements between the oligonucleotide microarray and RT-PCR were assessed using kappa statistics[11].

Results and Discussion

The lowest RNA detection limits for the oligonucleotide microarray were 1 x 10^2 $TCID_{50}/m\ell$ for TGEV, PRCV, and PEDV, 1 x 10^{-4} $TCID_{50}/m\ell$ for PRV gp A and PRV gp C, and 100 fg/$\mu\ell$ feces for PECV. On the other hand, the lowest detection limit for RT-PCR was 1 x 10^3 $TCID_{50}/m\ell$ for TGEV, PRCV and PEDV, and 1× 10^{-2} $TCID_{50}/m\ell$ for PRV gp A and PRV gp C, and 10fg/$\mu\ell$ feces for PECV (data not shown). Thus the sensitivity of oligonucleotide microarray was 10- to 100-fold higher than that of conventional RT-PCR.

Of the 102 samples tested, positive signals were detected for TGEV (9.8%), PEDV (24.5%), PECV (16.6%), PRV group A (6.8 %), and PRV group C (1.9%). Of these, concurrent infection was also detected in 3 cases (2 cases of TGEV and PEDV; one case of PEDV and PECV). Compared with RT-PCR, the sensitivity and specificity of the oligonucleotide microarray were 92.0% and 72.2%. The oligonucleotide microarray and RT-PCR disagreed in 17 samples, but the overall agreement between them was significant (Cohen's kappa 0.67, $P< 0.001$). The detection rate of oligonucleotide microarray and RT-PCR were 59.8% and 22.6%, respectively.

Table 3. Analysis of sensitivity and specificity of the oligonucleotide microarray in comparison with RT-PCR for the detection of porcine enteric viruses from swine fecal samples

		RT-PCR		Total
		+	-	
DNA chip	+	46	15	61
	-	2	39	41
	Total	48	54	102

* Sensitivity = 46/(48+2) x 100 = 92.0%
Specificity = 39/(15+39) x 100 = 72.2%
Cohen's kappa = 0.67
Percent observed agreement (Po) = (46+39)/102 x 100 = 83.3%.

Of the 61 samples found to be positive using the oligonucleotide microarray, 15 samples were negative by RT-PCR. These were confirmed as positive by nested PCR. While, of the 48 positive samples by RT-PCR, two samples were negative by oligonucleotide microarray (Table 3). This results suggested that this oligonucleotide microarray can be a valuable method for the differential diagnosis of porcine enteric viruses.

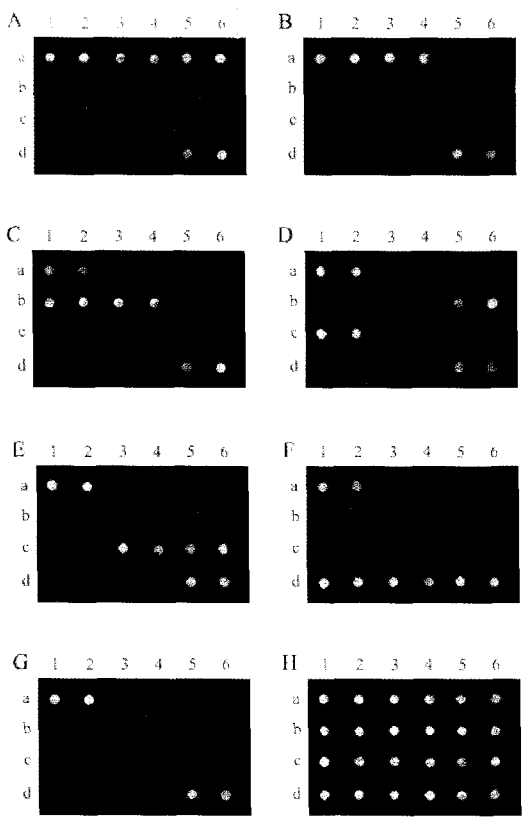

Fig 2. Laser scan images of the oligonucleotide microarray system for porcine enteric viruses. The cy5-labeled RT-PCR amplified products of the reference viruses positive control and each of the porcine enteric virus are hybridized. Each of the porcine enteric viral and reference viral DNA were labeled with cy5 by multiplex RT-PCR. The DNA chips images were obtained by scanning at LP = 75 and PMT = 75. The labeled porcine enteric viruses are (A) TGEV, (B) PRCV, (C) PECV, (D) PECV, (E) PRV gp A, (F) PRV gp C, (G) positive control, (H) all viruses, respectively.

In this study, the oligonucleotide microarray system was developed to detect TGEV, PEDV, PECV, and PRV gp A and C. Due to the high morbidity and rapid spread of disease, more rapid assays that

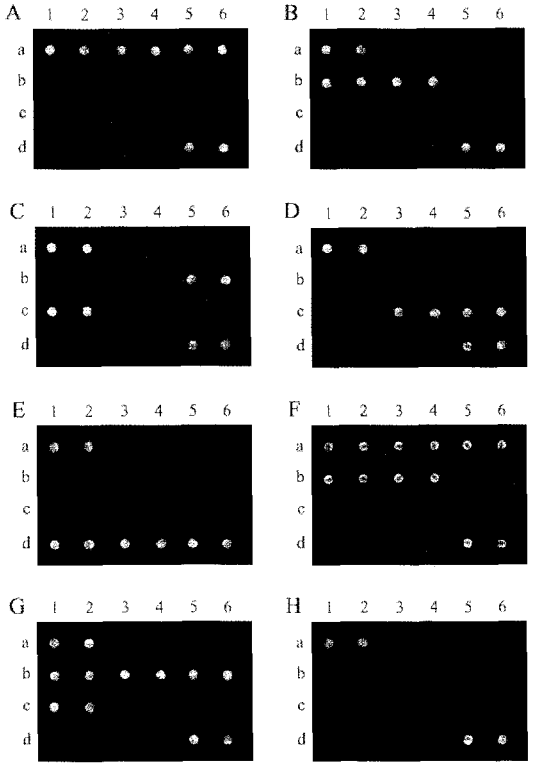

Fig 3. Results of laser scan images of the oligonucleotide microarray to which the cy5-labeled RT-PCR amplified products of the positive control and each of the fecal samples are hybridized. Each of the porcine enteric viruses and positive control DNA are labeled with cy5 by multiplex RT-PCR. The oligonucleotide microarray images are obtained by scanning at LP = 75 and PMT = 75. The labeled porcine enteric viruses were (A) TGEV, (B) PEDV, (C) PECV, (D) PRV gp A, (E) PRV gp C, (F) TGEV and PEDV, (G) PEDV and PECV, (H) no infection, respectively.

can detect individual viruses are essential for the prompt control and treatment of their respective diseases. One of the advantages of the developed procedure is that viruses can be detected without the need for conventional isolation methods. Therefore, the results cab be confirmed quickly and the problems associated with

isolation processes can be avoided. Furthermore, the oligonucleotide microarray system saves time and reagents because only one reaction needs to be set up, which is ideal for conserving limited samples. For example, whereas one-step RT-PCR amplification takes approximately 4 hrs, it can be completed in less than two hours. Moreover, more than three independent assays can be carried out on a single glass slide, making this method as a high-throughput tool for the detection of PEVs. Oligonucleotides were arrayed twice on chip to increase accuracy, and a set of oligonucleotides were also spotted in triplicate to reduce costs.

In conclusion, five major porcine enteric viruses could be identified simultaneously by oligonucleotide microarray system modified in this study. Because it can be a rapid and reliable method for simultaneously detecting various porcine enteric viruses in fecal samples in a single reaction, clinical laboratory can identify enteric viruses in a rapid and sensitive manner. It can also allow veterinarians to treat porcine enteric viral disease promptly and take appropriate preventive measures.

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