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Detection of Oyster-Associated Norovirus by Microchip Electrophoresis of an Amplified cDNA

- Research Note -

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Abstract

Noroviruses, members of the family *Caliciviridae*, are often found in shellfish grown in polluted water and are emerging as a leading cause of foodborne disease worldwide. As the presence of norovirus in food commodities becomes an important medical and social issue, there are increasing needs for designing improved detection methods for the virus. In this study, we tested the Agilent 2100 Bioanalyzer for the analysis of norovirus DNA amplified from oyster samples. Microchip electrophoresis provided us with more accurate information, compared to conventional agarose gel electrophoresis, in the resolution and quantification of amplified products. The development of an improved method for food-associated noroviruses would contribute to a rapid identification of contaminated food and improve our understanding of the modes of food contamination and norovirus transmission.

Key words: norovirus, viral gastroenteritis, RT-PCR, bioanalyzer, microchip electrophoresis, oysters

INTRODUCTION

Noroviruses, which belong to the family *Caliciviridae*, have emerged as a major cause of recent outbreaks of acute viral gastroenteritis worldwide (1,2). Molluscan shellfish are filter-feeders and can concentrate environmentally stable, positive-stranded RNA viruses, such as norovirus, hepatitis A virus and enterovirus, present in coastal waters (2).

Genetically, noroviruses are subdivided into five genogroups. Among them, GI, GII, and GIV genogroups have been detected in humans (3). Within GI and GII genogroups, noroviruses can be further delineated into at least 31 genotypes, based on genetic divergence in the polymerase and capsid gene sequences (4,5).

Since no infection of cell culture has been developed yet (6), detection of noroviruses has relied mainly on reverse transcription-PCR (RT-PCR) methods (6,7). However, international consensus for a standardized method has not emerged yet. Because of genome variety, it may be difficult to design broadly reactive PCR primers.

Agarose gel electrophoresis and polyacrylamide gel electrophoresis have been standard methods for DNA analysis based on the size and purity of DNA. Recently, a microchip-based capillary gel electrophoresis system has been introduced. The bioanalyzer is a capillary electrophoresis instrument that separates nucleic acids using microfluidics (8). In this study, the microchip capillary electrophoresis and conventional agarose gel electrophoresis methods were compared, primarily for the size resolution of the amplified DNA.

A rapid and convenient diagnostic procedure is needed for the prevention of foodborne infection caused by noroviruses and for determining norovirus-related contamination index of food samples. The goal of this study was to find a method suitable for quantitative detection of norovirus contamination in food commodities.

MATERIALS AND METHODS

Samples

Fresh oysters were obtained from local commercial harvesters in the coastal area of Gyeongsangnam-do during January 2007 to march 2007. For each sample, a minimum of ten shellfish were opened and animals were separated from the shell. Peripheral flesh and organs were removed, and digestive glands were finely chopped as described previously (9).

Sample processing

To allow release of viruses into solution, 10 g of the digestive glands were placed in 75 mL of 0.25 M glycine-0.14 M NaCl (pH 9.0) with addition of 0.05 mL of antifoam B (Sigma, St. Louis, MO). The samples were homogenized at 9,500 rpm for 1min and centrifuged at

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4,000×*g* for 20 min. Equal volumes of 18% PEG 6000 with 0.525 M NaCl were added to the supernatant and the mixtures were incubated overnight at 4°C. After centrifugation at 8,000×*g* for 20 min at 4°C, pellets were resuspended in 5 mL of 50 mM Tris-0.2% Tween 20 (pH 8.0) with vigorous vortexing, and supernatants were mixed with the same volume of chloroform, and centrifuged at 4,000×*g* for 20 min at 4°C. For precipitation of virus, an equal volume of 16% PCG 6000 and 0.525 M NaCl solution was added to the aqueous phase and it was incubated at 4°C for 4 hr. Precipitates were recovered by centrifugation at 8,000×*g* for 25 min at 4°C and resuspended in 250 μL of 50 mM Tris-0.2% Tween 20 (pH 8.0).

RNA extraction

RNA extraction was performed by using the TRIzol Reagent (Gibco BRL, Rockville, MD), a commercially guanidinium-phenol-solution. In brief, 1 mL of TRIzol was added to 200 μL of each virus suspension, vigorously mixed for 30 sec and incubated at room temperature for 3 min. Residual protein was removed by adding of 200 μL of chloroform, mixing for 30 sec, and centrifugation for 10 min at 12,000×*g* and 4°C. RNA in the aqueous phase was precipitated by mixing with the same volume of isopropanol for 15 sec, incubation for 10 min at 4°C, and centrifugation for 20 min at 12,000 ×*g* and 4°C. The RNA pellet was then washed with 0.7 mL of 75% ethanol and centrifuged for 3 min at 12,000×*g* and 4°C. The pellet was air-dried, resuspended in 100 μL of DEPC-treated water, and stored at -80°C.

Primers

To amplify the ORF1-ORF2 junction region, combination of the primer pairs shown in Table 1 was customized (Bionics) and used (10,11).

Reverse transcription and semi-nested PCR

RT-PCR was carried out as described previously (12) with minor modification. Briefly, 5 μL of RNA solution was added to 20 μL of an RT mixture containing 10 U of avian myeloblastosis virus RT (Promega, Madison, Wis.), 5 μL of 5×enzyme buffer (Promega), 2 μL of 10 mM dNTP, and 50 pmole of random hexamers (Pharmacia Biotech), and incubated for 1 hr at 42°C. PCR was performed by using 5 μL of cDNA along with 0.5 U of rTaq DNA polymerase (Takara) and 25 pmol of both forward and reverse primers (primer set A or B) in a final volume of 50 μL. Amplification was performed for 30 cycles of denaturation for 30 sec at 94°C, primer annealing for 30 sec at 54°C, and elongation for 1.5 min at 72°C with the GeneAmp PCR System 9700 (Applied Biosystems). The semi-nested PCR was performed in the same condition with 2.5 U of rTaq DNA polymerase (Takara) and primer set C or D in a 100 μL reaction. PCR products were separated by electrophoresis in a 2% agarose gel or in a microchip.

Microchip electrophoresis

PCR products were analyzed by using the commercial bioanalyzer Agilent 2100 (Agilent Technologies) as described (8). Briefly, 9 μL of gel-dye mixture was placed into the appropriate wells of a microchannel by applying pressure with a 1-mL syringe. Wells containing 5 μL of DNA size marker were subsequently loaded with 1 μL of sample. After mixing by vortex, chips were immediately placed into the bioanalyzer and run for 30 min according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Shellfish are commonly implicated as vehicles for the transmission of noroviruses. Noroviruses have been detected in oysters and clams in the United States (13,14),

Table 1. Primers used in this study

Primer		Sequence (5' to 3')	Polarity	Location
1st PCR	Primer set A (GI-specific)			
	COG1F	CGYTGGATGCGNTTYCATGA	sense	5291
	G1SKR	CCAACCCARCCATTRTACA	antisense	5671
	Primer set B (GII-specific)			
	COG2F	CARGARBCNATGTTYAGRTGGATGAG	sense	5003
	G2SKR	CCRCCNGCATRHCCRTTRTACAT	antisense	5401
Nested PCR	Primer set C (GI-specific)			
	G1SKF	CTGCCCGAATTYGTAAATGA	sense	5342
	G1SKR	CCAACCCARCCATTRTACA	antisense	5671
	Primer set D (GII-specific)			
	G2SKF	CNTGGGAGGGCGATCGCAA	sense	5058
	G2SKR	CCRCCNGCATRHCCRTTRTACAT	antisense	5401

Y=C or T; N=any; R=A or G; B=G or T or C; H=A or T or C.

United Kingdom (15), and Japan (16). Although cell culture systems for noroviruses are not yet available, other detection methods, such as RT-PCR, quantitative real-time PCR and NASBA methods have been developed (17,10). As these amplification-based methods emerge as important tools for norovirus detection, broadly working primers become a key component. The PCR amplicons in these methods generally range from 100 to 400 bp in size. On the other hand, agarose gel electrophoresis is one of the conventional methods for separating DNA based on size differences. However, agarose gel electrophoresis provides poor resolution for DNA in this range. In this study, we compared the microchip and agarose gel for electrophoretic performance. For this purpose, we chose oyster samples that were proven positive for GI or GII types of norovirus. As shown in Fig. 1, major bands with expected size for GI or GII type were seen in both electrophoresis methods. Apparently, the microchip method provided better resolution of GI and GII type fragments than the agarose gel method. As expected, only the type-specific products were amplified in a primer-dependent manner from samples positive for both GI- and GII-type viruses.

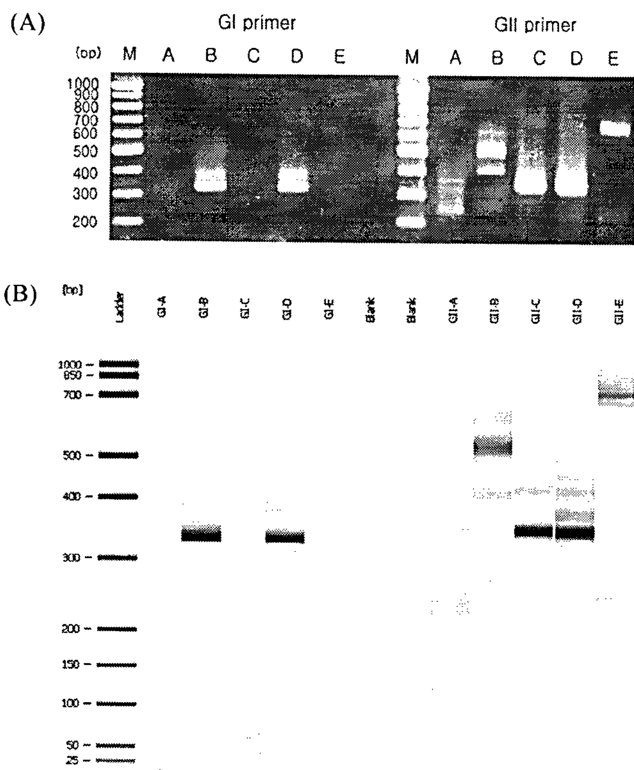

Fig. 1. Electrophoresis of noroviral DNA amplified from oyster samples. Products of RT-PCR by using GI-specific primers are separated in the left panel and those with GII-specific primers are on the right panel. Separation in 2% agarose gel (Panel A) and Agilent 2100 bioanalyzer microchip (Panel B). Lanes M and Ladder, a ladder of DNA size markers; lane A, from norovirus-free oysters; lane B, from GI-positive oysters; lane C, from GII-positive oysters; lane D, cDNAs of GI and GII type norovirus; lane E, negative control. Note that there are two empty lanes in (B).

Fig. 2 shows the profiles of electropherogram by the Agilent 2100 bioanalyzer. The peak number, migration time, peak area, and the estimated size of DNA fragments are presented for the corresponding lanes of Fig. 1 (B). The estimated DNA concentration of each peak was also given based on its peak area compared with that of markers. The upper and lower markers, of known size and concentration, served as an internal standard. The size of GI (334~335 bp) or GII (347~349 bp) fragments estimated in this method was very close to their expected values (336 and 344 bp).

As shown above, microchip electrophoresis has higher sensitivity and better resolution than agarose gel, especially in resolving DNA of small size and gives more accurate information on size. These advantages may contribute to designating internationally acceptable primers. The higher sensitivity would also be advantageous for norovirus detection. Although real-time PCR may provide higher sensitivity than microchip electrophoresis, the latter method certainly costs less and does not require expensive instruments.

In conclusion, this study provides a rapid, accurate, semi-quantitative tool for norovirus detection in foods. The assay can be easily performed by local laboratories for detecting viral agents of food-borne disease. Development of a sensitive method to detect noroviruses from food will improve our understanding of the modes of food contamination and norovirus transmission, thereby contributing to better public health policy and safety.

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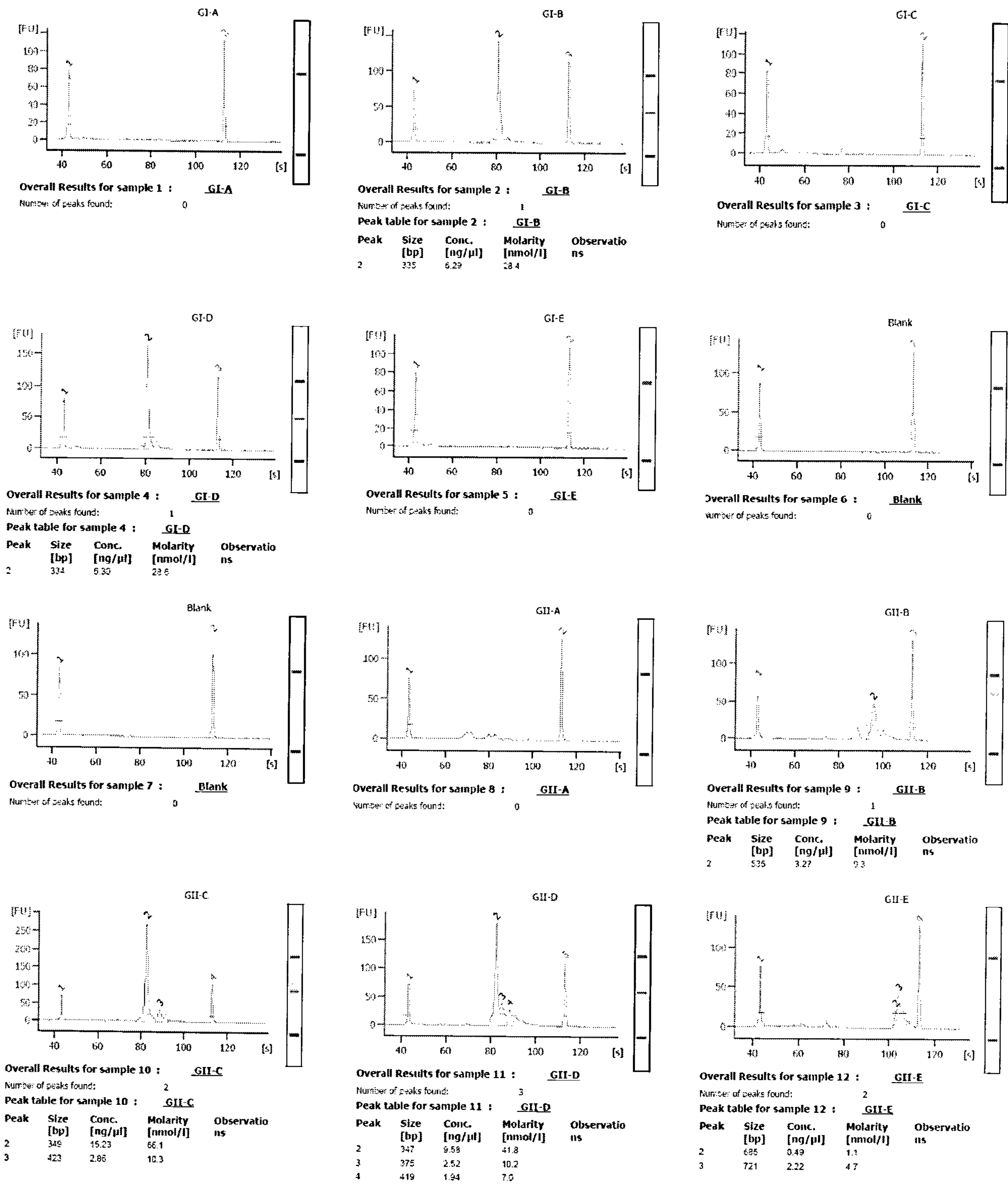

Fig. 2. Profiles of electropherogram for RT-PCR products separated by the Agilent 2100 bioanalyzer in Fig. 1. Each panel corresponds to lanes of Fig. 1 (B).

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