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Evaluation of the Sensitivity and Specificity of Primer Pairs and the Efficiency of RNA Extraction Procedures to Improve Noroviral Detection from Oysters by Nested Reverse Transcription-Polymerase Chain Reaction

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Noroviruses (NoV) are the key cause of acute epidemic gastroenteritis, and oysters harvested from NoV-polluted sea areas are considered as the significant vectors of viral transmission. To improve NoV detection from oyster using nested reverse transcription-polymerase chain reaction (RT-PCR), we evaluated the sensitivity and specificity of previously published primer pairs and the efficiency of different RNA extraction procedures. Among the primer pairs used for RT-PCR, the sensitivity of GIF1/GIR1-GIF2/GIR1 and GIIF1/GIIR1-GIIF2/GIIR1 was higher than that of other primer pairs used in nested RT-PCR for the detection of NoV genogroup I (NoV GI) and NoV GII from both NoV-positive stool suspension and NoV-seeded oyster concentrates, respectively; the resulting products showed neither unspecific bands in the positive samples nor false-positive bands in the negative controls. The extraction of NoV RNA from oyster samples using a QIAamp® Viral RNA Mini kit with a QIAshredder™ Homogenizer pretreatment afforded more efficient recovery (mean recovery for NoV GI and GII, 6.4%) and the procedure was less time consuming (<30 min) than most other RNA extraction procedures. The results of RNA extraction procedure and primer pairs evaluated by nested RT-PCR assay in this study can be useful for monitoring NoV contamination in oysters, which is an indicator of possible public health risks.

Keywords: norovirus, oyster, nested RT-PCR, primer sensitivity and specificity, RNA extraction efficiency

Noroviruses (NoV) are known to cause acute epidemic gastroenteritis worldwide. Five genogroups (GI-GV) of NoV have been established thus far. NoV of GI and GII have been associated with many viral outbreaks (Mounts *et al.*, 2000; Kim *et al.*, 2005; Doyle *et al.*, 2009). NoV are excreted in human feces and contaminate seawater in areas such as shellfish-growing areas (Sugiyama *et al.*, 2002; La Rosa *et al.*, 2007). Since NoV have long survival periods (few weeks or months) (Nappier *et al.*, 2008), the shellfish harvested from NoV-polluted sea areas may be contaminated with NoV. Therefore, shellfish, such as oysters, are considered to be significant vectors of NoV transmission (Huppatz *et al.*, 2008; Sala *et al.*, 2009).

Since no effective cell culture assay-based techniques have been developed for NoV detection thus far (Duizer *et al.*, 2004; Straub *et al.*, 2007), molecular methods such as nested reverse transcription-polymerase chain reaction (RT-PCR) have been used to detect human NoV from oysters (Myrmel *et al.*, 2004; Boxman *et al.*, 2006; Choo and Kim, 2006). To improve the sensitivity of nested RT-PCR for detecting human NoV from oysters, it is necessary to increase the speed, sensitivity, and specificity of this assay. Moreover, efficient RNA extraction procedures would be required to increase RNA recovery and to decrease the level of nested RT-PCR inhibitors co-extracted from oyster matrices (Sair *et al.*, 2002). In addition, it is necessary to identify highly sensitive and specific primer pairs that can be optimized for the detection of NoV genetic materials. In this study, we evaluated the sensitivity and specificity of primer pairs and the RNA extraction efficiency of different RNA extraction procedures to improve NoV detection from oysters by using nested RT-PCR.

Materials and Methods

Virus samples and most probable number determination

NoV-positive stool samples [GI/5, GI/6, GII4, and GII/16; GI and GII were classified according to the previous studies of Kageyama *et al.* (2004) and Okada *et al.* (2005)], kindly provided by the Korea Center for Disease Control and Prevention (KCDC), Seoul, South Korea, were diluted to make a 20% suspension in phosphate-buffered saline (PBS; pH 7.4). NoV GI and GII titers were calculated by performing nested RT-PCR with GIF1/GIR1-GIF2/GIR1 (Table 1) and GIIF1/GIIR1-GIIF2/GIIR1 (Table 2) primer pairs, respectively, by using the most probable number (MPN) method (U.S. Food and Drug Administration, 1992). All NoV suspensions were dispensed into 1 ml aliquots and stored at -70°C until used in this study.

Virus concentration in oyster samples

Shucked oyster samples (*Crassostrea gigas*) were obtained from the

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Table 1. Primers and amplification conditions for NoV GI nested RT-PCR

Region	Name	Sequence (5'→3')[c]	Polarity	Position[d]	Amplification condition[e]	Reference
RNA polymerase (ORF1)	NV36[a]	ATAAAAGTTGGCATGAACA	Sense	4487-4505	94°C 3 min; 94°C 90 sec, 52°C 90 sec, 72°C 90 sec, 35 cycles; 72°C 7min	Naitou and Morita (2001)
	NV35[a]	CTTGTTGGTTTGAGGCCCATA	Antisense	4956-4936	94°C 3 min; 94°C 1 min, 50°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	
	NV82[b]	TCATTTGATGCAGATTA	Sense	4555-4572		
	SM82[b]	CCACTATGATGCAGATTA	Sense	4555-4572		
	NV81[b]	ACAATCTCATCATCACCATA	Antisense	4884-4865		
	JV12Y[a]	ATACCACTATGATGCAGAYTA	Sense	4552-4572	94°C 3 min; 94°C 30 sec, 37°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	Saito *et al.* (1998)
	JV13I[ab]	TCATCATCACCATAGAAIGAG	Antisense	4878-4858	94°C 3 min; 94°C 30 sec, 37°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	
	G1[b]	TCNGAAATGGATGTTGG	Sense	4691-4707		
Capsid (ORF 2)	SRI1[a]	CGCCATCTTCATTCACAAA	Antisense	5671-5652	94°C 3 min; 94°C 30 sec, 37°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	Häfliger *et al.* (1997)
	SRI2[ab]	AAATGATGATGGCGTCTA	Sense	5356-5373	94°C 3 min; 94°C 30 sec, 37°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	
	SRI3[b]	AAAAYRTCACCGGGKGTAT	Antisense	5596-5578		
	COG1F[a]	CGYTGGATGCGNTTYCATGA	Sense	5291-5310	94°C 3 min; 94°C 1 min, 37°C 1 min, 72°C 2 min, 35 cycles; 72°C 15 min	Nishida *et al.* (2003)
	G1SKR[ab]	CCAACCCARCCATTRTACA	Antisense	5671-5653	94°C 3 min; 94°C 1 min, 37°C 1 min, 72°C 2 min, 35 cycles; 72°C 15 min	
	G1SKF[b]	CTGCCCGAATTYGTAAATGA	Sense	5342-5365		
Capsid (ORF 2)	G1FF1[a]	ATHGAACGYCAAATYTTCTGGAC	Sense	5075-5097	95°C 2 min; 94°C 30 sec, 48°C 30 sec, 72°C 2 min, 35 cycles; 72°C 5 min	Kageyama *et al.* (2003) Gallimore *et al.* (2005)
	G1FF2[a]	ATHGAAAGACAAATCTACTGGAC	Sense	5075-5097	95°C 2 min; 94°C 30 sec, 48°C 30 sec, 72°C 2 min, 35 cycles; 72°C 5 min	
	G1FF3[a]	ATHGARAGRCARCTNGGTGGAC	Sense	5075-5097		
	G1SKR[ab]	CCAACCCARCCATTRTACA	Antisense	5671-5653		
	G1FFN[b]	GGAGATCGCAATCTCCTGCCC	Sense	5313-5330		
	GIF1[a]	CTGCCCGAATTYGTAAATGATGAT	Sense	5342-5365	94°C 3 min; 94°C 30 sec, 54°C 30 sec, 72°C 45 sec, 35 cycles; 72°C 7 min	Kim *et al.* (2005)
	GIR1[ab]	CCAACCCARCCATTRTACATYTG	Antisense	5671-5649	94°C 3 min; 94°C 30 sec, 56°C 30 sec, 72°C 45 sec, 35 cycles; 72°C 7 min	
	GIF2[b]	ATGATGATGGCGTCTAAGGACGC	Sense	5358-5380		

[a] Primers for first PCR.
[b] Primer for nested PCR.
[c] Degenerate positions H:A/T/C, K:T/G, N:A/T/G/C, R:A/G, Y:T/C.
[d] Relative positions of primers in Norwalk/68/US (accession no. M87661).
[e] Temperature and time of initial denatuation step; temperatures and times of denaturation, annealing, and extension, and cycles; temperature and time of final extension step.

Noryangjin fishery wholesale market, which supplies oysters from Tongyeong oyster beds. All oyster samples were placed on ice, transported to the laboratory within 1 h, and washed twice externally with sterilized distilled water. The digestive glands were cut, and divided into 25 g samples that were stored at -70°C until processing. All oyster samples were analyzed using a modified version of the protocol described by Mendez *et al*. (2000) and Mullendore *et al*. (2001). Briefly, NoV GI/6 or GII/16 suspensions with MPN of 13,500 were seeded in the 25 g oyster (digestive glands) samples in triplicate (3 trials). Then, 7 volumes of chilled sterile distilled water were added to the 25 g virus-seeded oyster samples and 1 NoV-unseeded oyster sample (negative control). Subsequently, the samples were homogenized twice for 60 sec in an OmniMixer Waring blender (Omni International) at 25,000 rpm. Acid adsorption of viruses to oyster homogenates was performed by adding distilled water to the homogenized samples and adjusting the pH to 5.0 with 1 M HCl to reduce the conductivity to <2,000 μS/cm. After allowing adsorption for 15 min with gentle stirring, the oyster solids with the adsorbed viruses were centrifuged for 20 min at 2,000×g and eluted with 7 volumes of chilled 0.05 M glycine/0.14 M NaCl (pH 7.5); subsequently the pH was adjusted to 7.5. The eluates were gently stirred for 15 min, and the oyster solids were pelleted by centrifugation (5,000×g for 20 min at 4°C); the supernatant was collected in a second tube and stored at 4°C. The pellet was resuspended by dissolving in 15 ml of 0.5 M threonine/0.14 M NaCl (pH 7.5) and then vortexed for 60 sec. This resuspended solution was centrifuged (5,000×g for 20 min at 4°C), and then the supernatants were combined and the pellets were discarded. The viruses in the supernatant were precipitated using polyethylene glycol 8,000 (PEG 8,000)-NaCl solution [final concentration of each solution was 8% (w/v) and 0.3 M, respectively] at 4°C for 2 h or overnight, sedimented (6,700×g for 30 min at 4°C), and resuspended in 15 ml of PBS (pH 7.5). Then, 15 ml of Vertrel XF

Table 2. Primers and amplification conditions for NoV GII nested RT-PCR

Region	Name	Sequence (5'→3')[c]	Polarity	Position[d]	Amplification condition[e]	Reference
RNA polymerase (ORF1)	NV36[a]	ATAAAAGTTGGCATGAACA	Sense	4487-4505	94°C 3 min; 94°C 90 sec, 52°C 90 sec, 72°C 90 sec, 35 cycles; 72°C 7min	Naitou and Morita (2001)
	NV35[a]	CTTGTTGGTTTGAGGCCCATA	Antisense	4956-4936		
	NV82[b]	TCATTTTGATGCAGATTA	Sense	4555-4572	94°C 3 min; 94°C 1 min, 50°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	
	SM82[b]	CCACTATGATGCAGATTA	Sense	4555-4572		
	NV81[b]	ACAATCTCATCATCACCATA	Antisense	4884-4865		
	SRII1[a]	CGCCATCTTCATTCACAAA	Antisense	5096-5078	94°C 3 min; 94°C 30 sec, 55°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	Häfliger *et al.* (1997)
	SRII2[ab]	TWCTCYTTYTATGGTGATGATGA	Sense	4583-4605	94°C 3 min; 94°C 30 sec, 50°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	
	SRII3[b]	TTWCCAAACCAACCWGCTG	Antisense	4767-4785		
	JV13I[a]	TCATCATCACCATAGAAIGAG	Antisense	4605-4585	94°C 3 min; 94°C 30 sec, 37°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	Vennema *et al.* (2002) Boxman *et al.* (2006)
	JV12Y[ab]	ATACCACTATGATGCAGAYTA	Sense	4279-4299	94°C 3 min; 94°C 30 sec, 37°C 1 min, 72°C 1 min, 35 cycles; 72°C 7 min	
	NoroIIR[b]	AGCCAGTGGGCGATGGAATTC	Antisense	4515-4495		
Capsid (ORF2)	COG2F[a]	CCARGARBCNATGTTYAGRTGGATGAG	Sense	5003-5028	94°C 3 min; 94°C 1 min, 37°C 1 min, 72°C 2 min, 35 cycles; 72°C 15 min	Nishida *et al.* (2003)
	G2SKR[ab]	CCRCCNGCATRHCCRTTRTACAT	Antisense	5389-5367	94°C 3 min; 94°C 1 min, 37°C 1 min, 72°C 2 min, 35 cycles; 72°C 15 min	
	G2SKF[b]	CNTGGGAGGGCGATCGCAA	Sense	5047-5065		
	G2FB1[a]	GGHCCMBMDTTYTACAGCAA	Sense	4922-4941	95°C 2 min; 95°C 30 sec, 48°C 30 sec, 72°C 2 min, 35 cycles; 72°C 5 min	Gallimore *et al.* (2005) Kageyama *et al.* (2003)
	G2FB2[a]	GGHCCMBMDTTYTACAAGAA	Sense	4922-4941		
	G2FB3[a]	GGHCCMBMDTTYTACARNAA	Sense	4922-4941		
	G2SKR[ab]	CCRCCNGCATRHCCRTTRTACAT	Antisense	5389-5367	95°C 2 min; 95°C 30 sec, 48°C 30 sec, 72°C 2 min, 35 cycles; 72°C 5 min	
	G2FBN[b]	TGGGAGGGCGATCGCAATCT	Sense	5048-5067		
	NV2oF2[a]	GGAGGGCGATCGCAATC	Sense	5050-5066	95°C 5 min; 95°C 30 sec, 48°C 30 sec, 72°C 1 min, 35 cycles; 72°C 7 min	Bull *et al.* (2006)
	NV2oR[a]	GTRAACGCRTTYCCMGC	Antisense	5428-5412		
	G2F3[b]	TTGTGAATGAAGATGGCGTCGA	Sense	5079-5100	95°C 5 min; 95°C 30 sec, 48°C 30 sec, 72°C 1 min, 35 cycles; 72°C 7 min	
	G2SKR[b]	CCRCCNGCATRHCCRTTRTACAT	Antisense	5389-5367		
	GIIF1[a]	GGGAGGGCGATCGCAATCT	Sense	5049-5067	94°C 3 min; 94°C 30 sec, 54°C 30 sec, 72°C 45 sec, 35 cycles; 72°C 7 min	Kim *et al.* (2005)
	GIIR1[ab]	CCRCCIGCATRICCRTTRTACAT	Antisense	5389-5367	94°C 3 min; 94°C 30 sec, 56°C 30 sec, 72°C 45 sec, 35 cycles; 72°C 7 min	
	GIIF2[b]	TTGTGAATGAAGATGGCGTCGA	Sense	5079-5100		

[a] Primers for first PCR.
[b] Primer for nested PCR.
[c] Degenerate positions B:T/G/C, D:A/G/T, H:A/T/C, K:T/G, M:A/C, N:A/T/G/C, R:A/G, W:A/T, Y:T/C.
[d] Relative positions of primers in Lordsdale/93/UK (accession no. X86557).
[e] Temperature and time of initial denatuation step; temperatures and times of denaturation, annealing, and extension, and cycles; temperature and time of final extension step.

(DuPont, USA) was added to this solution, which was then vortexed for 2 min, and then centrifuged at 2,000×g for 30 min at 4°C. The supernatant was separated and precipitated again using PEG 8,000-NaCl solution, as mentioned above, and centrifuged (14,000×g for 15 min at 4°C); finally, each pellet was resuspended with 2.5 ml of PBS. The final concentrates were stored at -70°C until used.

RNA extraction

To evaluate the sensitivity and specificity of the primer pairs of NoV GI and GII, we diluted the NoV GI/6 and GII/16 suspensions, with MPN of 1,620 each, and the NoV GI/5 and GII/4 suspensions, with MPN of 4,800 each, to 140 μl with RNase-free distilled water. In addition, NoV GI/5 and GII/4 suspensions with MPN of 4,800 were diluted to 500 μl (5 g samples) of oyster concentrates. Viral RNA was extracted from the samples using a QIAamp® Viral RNA Mini kit (QIAGEN, Germany) according to the manufacturer's instructions. The final concentrates (30 μl) were stored at -70°C until used.

To evaluate the efficiency of the methods for extracting NoV RNA from oyster, we extracted viral RNA from 5 g samples (500 μl) of the concentrates by using 6 different extraction procedures (Methods A-

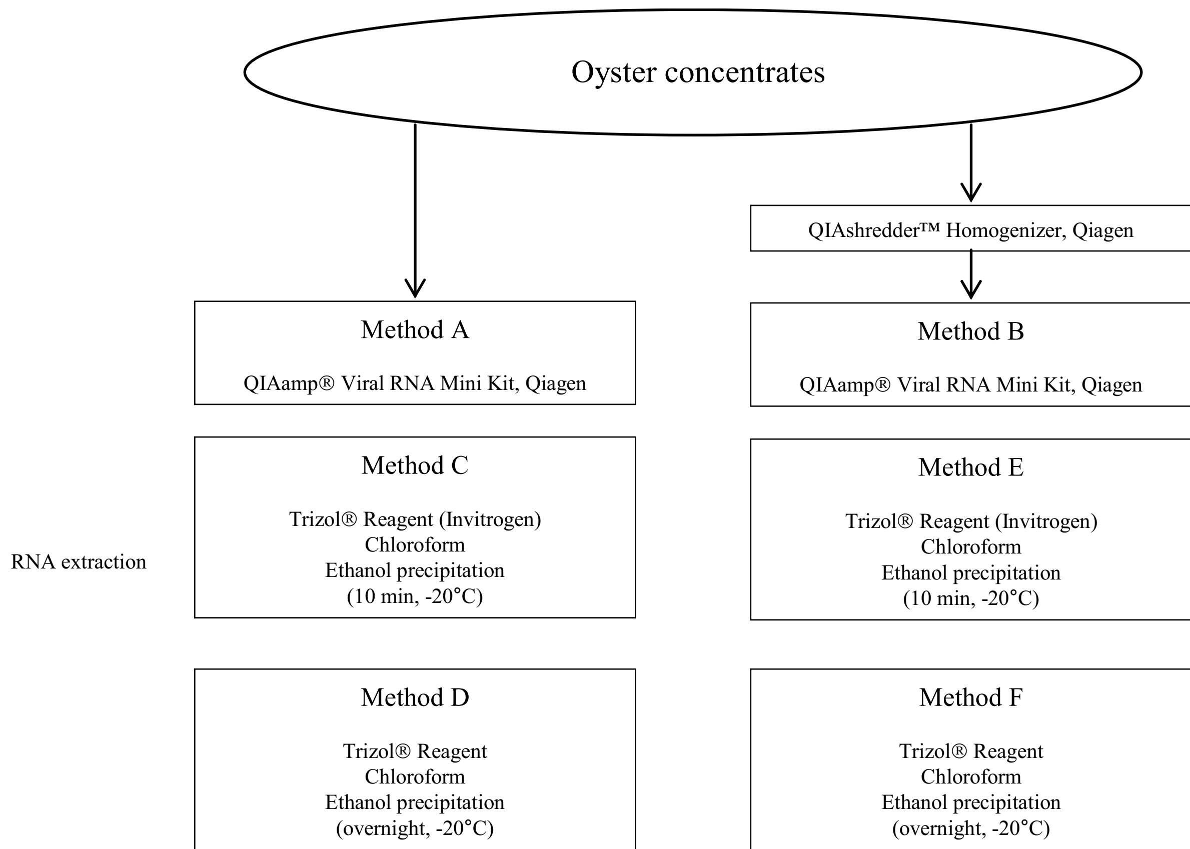

Fig. 1. Flow diagram showing the principles of the six different procedures for extracting NoV RNA in oysters.

F; Fig. 1). For the methods B, E, and F, the final concentrate was loaded into a QIAshredder™ Homogenizer (QIAGEN, Germany) and centrifuged at 16,000×g for 2 min and the resulting supernatant was recovered. If the volume of the resulting supernatant was less than 500 μl, RNase-free water was added to the supernatant to make the final volume up to 500 μl. In methods A and B, viral RNA was extracted from the final concentrate with a QIAamp® Viral RNA Mini kit according to the manufacturer's instructions, and the final concentrates (50 μl) were stored at -70°C until used. In methods C-F, 3 volumes (1.5 ml) of Trizol® Reagent (Gibco BRL, USA) were added to the final concentrate that was vortexed for 30 sec, incubated at room temperature for 10 min, and centrifuged (16,000×g for 30 min at 4°C). Then, 500 μl of chloroform was added to the resulting supernatant that was vortexed for 30 sec, incubated at room temperature for 10 min, and centrifuged (16,000×g for 30 min at 4°C). The aqueous phase of this solution was added to 500 μl of isopropanol, and this mixture was vortexed for 30 sec, incubated for 10 min (Methods C and E) or overnight (Methods D and F) at -20°C, and centrifuged (16,000×g for 30 min at 4°C). The pellet was washed with 1 ml of 75% cold ethanol and centrifuged (10,000×g for 10 min at 4°C). The pellet obtained in this step was air-dried, resuspended in 50 μl of diethyl pyrocarbonate-treated water by incubation for 10 min at 60°C, and stored at -70°C.

RT and nested RT-PCR

To determine the sensitivity and specificity of primer pairs for NoV GI and GII, we performed RT on a PTC-100 thermal cycler (Bio-Rad Laboratories Inc., USA) using SuperScript™ II Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 25 μl mixtures containing 100 nM of random primer (TaKaRa Bio Inc., Japan), 2.5 μl of deoxynucleoside triphosphates (dNTP; 2.5 mM each), and 5.0 μl, 0.5 μl, or 0.05 μl of RNA were incubated at 65°C for 5 min and quickly chilled on ice. Then, 5 μl of 5× First-Strand Buffer, 2.5 μl of 100 mM dithiothreitol, and 25 U of RNasin® Ribonuclease Inhibitor (Promega, USA) were added to these mixtures that were incubated at 42°C for 2 min; then, 250 U of SuperScript™ II Reverse Transcriptase (Invitrogen, USA) was added to this mixture. cDNA was synthesized at 42°C for 50 min, and the mixture was incubated at 70°C for 15 min to denature the enzyme. Then, 5 μl of each mixture (NoV GI/6 and GII/16 cDNA, with an MPN of 54, 5.4, and 0.54 each, and NoV GI/5 and GII/4 cDNA, with an MPN of 160, 16, and 1.6 each) was added to 15 μl of PCR mixture [final concentration: 10× buffer, 200 μM of each dNTP, 1.25 U of *Ex Taq* Polymerase, and 10 pmole of forward and reverse primers (Tables 1 and 2)]. The PCR protocol is described in Tables 1 and 2. For nested PCR amplification, 1 μl of each RT-PCR product was added to 20 μl (final volume) of PCR reaction mixture containing 10 pmole of primers. This protocol is also described in Tables 1 and 2. Thermal cycling was performed on a PTC-100 thermal cycler. The results were analyzed by electrophoresis with a 1.5% agarose gel and subsequent staining with ethidium bromide.

To evaluate the efficiency of the methods for extracting NoV RNA from oyster, three 15 μl mixtures containing 100 nM of random primer (TaKaRa Bio Inc., Japan); 1.5 μl of dNTP (2.5 mM each); and 3.0 μl (MPN=162), 0.3 μl (MPN=16.2), or 0.03 μl (MPN=1.62) of RNA, were subjected to RT as mentioned above. The oligonucleotide

primer sequences used to amplify the partial capsid region of NoV GI (Table 1) and GII (Table 2) were identical to those described by Kim *et al*. (2005). We added 5 μl of each mixture (MPN=54, 5.4, and 0.54) to 15 μl of PCR mixture (final concentration: 10× buffer, 200 μM of each dNTP, 1.25 U of *Ex Taq* Polymerase, and 10 pmole of forward and reverse primers) in triplicate. The first PCR and nested PCR were performed as mentioned above. The results were analyzed by electrophoresis with a 1.5% agarose gel and subsequent staining with ethidium bromide.

Quality control of the amplification method

To avoid false-positive results due to contamination with amplified DNA of previous PCR assays, we conducted experiments in separate areas and used different sets of apparatus for sample preparation, reagent preparation, and sample amplification. Each cabinet was equipped with an independent batch of reagents, micropipettes, pipette tips, and sterile reagent tubes. NoV-seeded positive controls as well as negative controls were incorporated into all PCR assays to ensure the propriety of the PCR assay.

Results

Sensitivity and specificity of the nested RT-PCR for detecting NoV

We evaluated the sensitivity of 6 and 7 different primer pairs for detecting NoV GI and GII, respectively. For NoV GI detection from stool samples, GIF1/GIR1-GIF2/GIR1 primer pair was more sensitive (mean recovery, 100%) than the other 5 primer pairs (mean recovery, <64%; Table 3). In addition, the amplification of NoV GI by nested RT-PCR yielded false-positive bands in negative controls (i.e., SRI1/SRI2-SRI2/SRI3 primer pair; Table 3) and unspecific bands in test samples (e.g., JV13I/JV12Y-JV12Y/G1 primer pair; data not shown) with other primer pairs but not with NV36/NV35-NV82/SM82/NV81. For detecting NoV GII from stool samples, GIIF1/GIIR1-GIIF2/GIIR1 primer pair was more sensitive (mean recovery, 100%) than the other 7 primer pairs (mean recovery, <57%; Table 4). The amplification of NoV GII by nested RT-PCR revealed unspecific bands in test samples (e.g., JV13I/JV12Y-JV12Y/G1 primer pair; data not shown) with other primer pairs but not with the NV2oF2/NV2oR-G2F3/G2SKR primer pair (data not shown). Our results indicated that the sensitivity of NoV detection from NoV-seeded oyster concentrates using each primer pair was similar or lower as compared to that from the stool samples (Tables 3 and 4), indicating that the materials co-extracted from oyster matrices considerably inhibited the nested RT-PCR amplification. On the basis of these results, we used GIF1/GIR1-GIF2/GIR1 and GIIF1/GIIR1-GIIF2/GIIR1 primer pairs as the standard primer pairs to determine the RNA extraction efficiency of 6 different RNA extraction procedures.

RNA extraction efficiency

Viral RNA was extracted from 500 μl (5 g samples) of NoV-seeded oyster concentrates by 6 different extraction procedures. The NoV recovery achieved with these procedures was relatively low, approximately less than 8.0% (Table 5). For NoV GI detection, RNA extraction with QIAamp® Viral RNA Mini kit with a QIAshredder™ Homogenizer pretreatment (method B) was more efficient (mean recovery, 6.4%) than other extraction methods (mean recovery, <3.5%) (Table 5). Using method B, up to NoV GI/6 with MPN of 5.4 could be obtained by PCR amplification. For NoV GII detection, the most efficient method was method F (mean recovery, 7.9%; Table 5). However, the methods A and B were also more efficient (mean recovery, 6.4%) than the other 3 methods (mean recovery, <2.2%), and NoV GII/16 recovery for up to MPN of 5.4 was possible with the methods A, B, and F. Overall, the method B appeared to be the most effective for extracting NoV RNA from oyster samples.

Discussion

Nested RT-PCR has been used to detect NoV from oysters because this method is more sensitive as compared to the conventional RT-PCR approach (Myrmel *et al.*, 2004; Choo and Kim, 2006). To improve the efficiency of nested RT-PCR for NoV detection from oysters, it is necessary to develop efficient methods for extracting NoV RNA from oysters and to optimize the sensitivity and specificity of the primer pairs used for NoV RNA amplification by nested RT-PCR. In this study, we determined the most specific and sensitive primer pairs for amplifying NoV RNA by nested PCR and the most efficient methods for extracting NoV RNA from oysters.

To select the most specific and sensitive primer pair(s) for nested RT-PCR, we examined 6 and 7 different primer pairs for NoV GI and GII, respectively. The sensitivity and specificity of oligonucleotide primers used for NoV GI and GII amplification have been initially described in previously published reports (Häfliger *et al.*, 1997; Saito *et al.*, 1998; Naitou and Morita, 2001; Vennenma *et al.*, 2002; Kageyama *et al.*, 2003; Nishida *et al.*, 2003; Gallimore *et al.*, 2005; Kim *et al.*, 2005; Bull *et al.*, 2006). However, it is difficult to select the best primer pairs for PCR amplification of NoV RNA from oysters from the published protocols because different methods have used different oyster matrices, genotypes of NoV, reagents, and equipment, etc. To overcome these problems, we analyzed the sensitivity and specificity of the published primer pairs under same conditions of oyster samples, NoV genotypes, reagents, and equipment, and selected the best primer pairs for nested RT-PCR. The primer pairs, GIF1/GIR1-GIF2/GIR1 for NoV GI and GIIF1/GIIR1-GIIF2/GIIR1 for NoV GII (Kim *et al*., 2005) were more sensitive than other primers (Tables 3 and 4); these pairs did not show unspecific bands in positive samples and false-positive bands in negative controls (data not shown). In addition, these PCR primer pairs were specifically targeted at the capsid genes that can be classified on the basis of their genetic relatedness (Ando *et al*., 2000). It is important to clarify the genetic diversity of NoV in NoV-contaminated oysters because these viruses may cause disease outbreaks as suggested previously (Ueki *et al*., 2005; Nishida *et al*., 2007).

We compared 6 different procedures for extracting NoV RNA from NoV-seeded oyster concentrates. Efficient extraction of NoV RNA was achieved with method B (QIAshredder™ Homogenizer pretreatment+QIAamp® Viral RNA Mini kit) and this method yielded up to NoV GI and GII with MPN of 5.4 (Table 5). This result illustrates that pretreatment with QIAshredder™ Homogenizer and the QIAamp® Viral RNA spin column used in method B effectively facilitated RNA

Table 3. Primers sensitivity for NoV GI nested RT-PCR

Region	Primer names	Number of positive samples/ number of tested samples											
		Stool								Oyster			
		GI/5				GI/6				GI/5			
		160 MPN	16 MPN	1.6 MPN	Mean% recovery[c]	54 MPN	5.4 MPN	0.54 MPN	Mean% recovery[c]	160 MPN	16 MPN	1.6 MPN	Mean% recovery[c]
RNA polymerase (ORF1)	NV36/NV35-NV82/SM82/NV81[a]	0/5	0/5	0/5	0.0 (0.0-0.0)	5/5	4/5	1/5	32 (12-84)	0/5	0/5	0/5	0.0 (0.0-0.0)
	SRI1/SRI2-SRI2/SRI3	NA[b]	NA	NA	-	NA	NA	NA	-	NA	NA	NA	-
	JV13I/JV12Y-JV12Y/G1	5/5	5/5	3/5	57 (18-180)	5/5	5/5	1/5	64 (21-200)	5/5	5/5	0/5	15 (0.0-45)
Capsid (ORF2)	COG1F/G1SKR-G1SKF/G1SKR	5/5	5/5	0/5	15 (0.0-45)	5/5	0/5	0/5	4.3 (0.0-13)	5/5	4/5	0/5	8.1 (2.8-23)
	G1FF1/G1FF2/G1FF3/G1SKR-G2FFN/G1SKR	5/5	5/5	3/5	57 (18-180)	5/5	3/5	0/5	15 (5.0-47)	5/5	5/5	0/5	15 (0.0-45)
	GIF1/GIR1-GIF2/GIR1	5/5	5/5	4/5	100 (34-340)	5/5	5/5	2/5	100 (30-340)	5/5	5/5	2/5	34 (11-110)

[a] Primer set for first PCR-primers set for nested PCR
[b] Primers were not available for RT-nested PCR amplification since PCR bands were shown in negative controls.
[c] Percentage of NoVs recovered was calculated using the initial MPN of inoculated NoVs as 100%. The numbers in parentheses are the range between low and high 95% confidence limits.

Table 4. Primers sensitivity for NoV GII nested RT-PCR

Region	Primer names	Number of positive samples/ number of tested samples											
		Stool								Oyster			
		GII/4				GII/16				GI/5			
		160 MPN	16 MPN	1.6 MPN	Mean% recovery[c]	54 MPN	5.4 MPN	0.54 MPN	Mean% recovery[c]	160 MPN	16 MPN	1.6 MPN	Mean% recovery[c]
RNA polymerase (ORF1)	NV36/NV35-NV82/SM82/NV81[a]	0/5	0/5	0/5	0.0 (0.0-0.0)	5/5	3/5	1/5	20 (7.3-56)	0/5	0/5	0/5	0.0 (0.0-0.0)
	SRII1/SRII2-SRII2/SRII-3	5/5	2/5	2/5	5.9 (2.2-16)	5/5	3/5	3/5	32 (14-76)	5/5	5/5	0/5	15 (0.0-45)
	JV13I/JV12Y-JV12Y/NoroIIR	5/5	5/5	3/5	57 (18-180)	5/5	2/5	0/5	8.4 (2.1-34)	5/5	5/5	2/5	34 (11-110)
Capsid (ORF2)	COG2F/G2SKR-G2SKF/G2SKR	5/5	5/5	0/5	15 (0.0-45)	5/5	0/5	0/5	4.3 (0.0-13)	5/5	4/5	0/5	8.1 (2.8-23)
	G2FB1/G2FB2/G2FB3/G2SKR-G2FBN/G2SKR	5/5	5/5	3/5	57 (18-180)	5/5	3/5	1/5	20 (7.3-56)	5/5	5/5	2/5	34 (11-110)
	NV2oF2/NV2oR-G2F3/G2SKR	5/5	2/5	0/5	3.1 (1.0-9.8)	5/5	5/5	0/5	44 (15-130)	0/5	0/5	0/5	0.0 (0.0-0.0)
	GIIF1/GIIR1-GIIF2/GIIR1	5/5	5/5	4/5	100 (34-340)	5/5	5/5	2/5	100 (30-340)	5/5	5/5	2/5	34 (11-110)

[a] Primer set for first PCR-primer set for nested PCR
[b] Percentage of NoVs recovered was calculated using the initial MPN of inoculated NoVs as 100%. The numbers in parentheses are the range between low and high 95% confidence limits.

Table 5. Recovery and detection limit of NoV GI and GII in oyster samples using different RNA extraction procedures

Extraction method	Trial	GI/6				GII/16			
		Number of positive samples/number of tested samples			Mean% recovery[b]	Number of positive samples/number of tested samples			Mean% recovery[b]
		54 U	5.4 U	0.54 U		54 U	5.4 U	0.54 U	
Method A	1	3/3	1/3	0/3		3/3	1/3	0/3	
	2	2/3	0/3	0/3		2/3	1/3	0/3	
	3	3/3	0/3	0/3		3/3	3/3	0/3	
	Total	8/9	1/9	0/9	3.5 (1.7-7.4)	8/9	5/9	0/9	6.4 (3.2-13)
Method B	1	3/3	1/3	0/3		3/3	1/3	0/3	
	2	3/3	1/3	0/3		2/3	2/3	0/3	
	3	3/3	0/3	0/3		3/3	2/3	0/3	
	Total	9/9	2/9	0/9	6.4 (2.8-15)	8/9	5/9	0/9	6.4 (3.2-13)
Method C	1	0/3	0/3	0/3		0/3	0/3	0/3	
	2	3/3	0/3	0/3		0/3	0/3	0/3	
	3	0/3	0/3	0/3		0/3	0/3	0/3	
	Total	3/9	0/9	0/9	0.70 (0.20-2.1)	0/9	0/9	0/9	0.0 (0.0-0.0)
Method D	1	0/3	0/3	0/3		3/3	0/3	0/3	
	2	3/3	0/3	0/3		1/3	0/3	0/3	
	3	3/3	0/3	0/3		3/3	0/3	0/3	
	Total	6/9	0/9	0/9	1.7 (0.70-3.9)	7/9	0/9	0/9	2.2 (1.0-4.9)
Method E	1	0/3	0/3	0/3		0/3	0/3	0/3	
	2	0/3	0/3	0/3		2/3	3/3	0/3	
	3	2/3	0/3	0/3		0/3	0/3	0/3	
	Total	2/9	0/9	0/9	0.41 (0.10-1.7)	2/9	3/9	0/9	1.1 (0.40-2.6)
Method F	1	0/3	0/3	0/3		3/3	0/3	0/3	
	2	3/3	0/3	0/3		3/3	3/3	0/3	
	3	3/3	0/3	0/3		3/3	0/3	0/3	
	Total	6/9	0/9	0/9	1.7 (0.70-3.9)	9/9	3/9	0/9	7.9 (3.4-19)

[a] Not assayed

[b] Percentage of NoVs recovered was calculated using the initial MPN of inoculated NoVs as 100%. The numbers in parentheses are the range between low and high 95% confidence limits.

resuspension in the elution buffer and decreased the level of inhibitors in the final resuspension as reported previously (Sair *et al*., 2002). Although the extraction of NoV GII RNA from oyster concentrates using method F (QIAshredder™ Homogenizer pretreatment+Trizol® Reagent with overnight isopropanol precipitation; mean recovery, 7.9%) was slightly more effective than that by method B (mean recovery, 6.4%), the method F may not be feasible for extracting NoV RNA from oyster concentrates because the mean recovery of NoV GI RNA by this method is very low (1.7%; Table 5) and this method is more time consuming (about 1.5 day) as compared with method B (less than 30 min).

In this study, less than 10% of NoV RNA was recovered from all the procedures (Table 5). These results agreed with those described by Schultz *et al*. (2007), who reported that the efficiency of NoV RNA extraction from NoV-spiked oyster samples was 10- to 100-fold lower than NoV RNA extracted from stool samples using 4 sample treatment procedures and 3 RNA isolation methods. It appears that the loss of NoV particles during the concentration of oyster solids and the high level of nested RT-PCR inhibitors co-extracted from oyster matrices are the main causes of low recovery of NoV RNA from oyster samples (Tables 3 and 4).

Overall, by using the RNA extraction procedure and primer pairs showing the best results in this study, we can improve the sensitivity and specificity of the detection of NoV in oysters by nested RT-PCR assay. This improved nested RT-PCR assay can be used to monitor NoV contamination in Korean oysters, which can be considered as an indicator of possible public health risks.

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