Food Sci. Biotechnol. Vol. 18, No. 5, pp. 1150 ~ 1154 (2009)

Food Science and Biotechnology

ⓒ The Korean Society of Food Science and Technology

# Development of a Virus Elution and Concentration Procedure for Detecting Norovirus in Oysters

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**Abstract** Low levels of virus contamination and naturally occurring reverse transcription-polymerase chain reaction (RT-PCR) inhibitors restrain virus detection in oysters. A rapid and efficient oyster-processing procedure that can be used for sensitive virus detection in oysters was developed. Poliovirus type 1 Sabin strain was used to evaluate the efficacy of virus recovery. The procedure included (a) acid-adsorption and elution with buffers (0.25 M glycine-0.14 M NaCl, pH 7.5; 0.25 M threonine-0.14 M NaCl, pH 7.5); (b) polyethylene glycol (PEG) precipitation; (c) resuspension in Tween 80/Tris solution and chloroform extraction; (d) the second PEG precipitation; (e) viral RNA extraction with TRIzol and isopropanol precipitation; and (f) RT-PCR combined with semi-nested PCR. The overall recovery of elution/concentration was 19.5% with poliovirus. The whole procedure usually takes 19 hr. The overall detection sensitivity was 4 RT-PCR units of genogroup I norovirus (NoV) and 6.4 RT-PCR units of genogroup II Nov/25 g of oysters initially seeded. The virus-detecting method developed in this study should facilitate the detection of low levels of NoV in oysters.

**Keywords:** elution, concentration, norovirus, poliovirus, oyster

## Introduction

Food-borne viral infections, especially with norovirus (NoV), are now recognized as a major cause of gastroenteritis in all age groups. NoV transmission occurs by the fecal-oral route and may be associated with infected people, contaminated food or water (1). Bivalve shellfish is well-known as a major vector of NoV transmission, and the estimated infectious dose of NoV to cause gastroenteritis is approximately 10-100 particles (2,3). NoVs (genus, *Norovirus*; family, Caliciviridae) are non-enveloped viruses that have single-stranded positive sense RNA (1). They are a group of genetically diverse viruses. Currently, based on the diversity of the genome sequences, there are at least 5 NoV genogroups (GI, GII, GIII, GIV, and GV), which are, in turn, divided into at least 25 genetic clusters (4,5). GI, GII, and GIV have been found in humans. NoVs that infect human do not grow in cells or organ culture, and there is no small animal model for infection and gastrointestinal disease (1).

Many analytical methods to detect NoV in shellfish using reverse transcription-polymerase chain reaction (RT-PCR) have been reported. However, there is no standardized methodology (3) because the sample quality, the RNA extraction and purification methods, the primers, the RT-PCR conditions, and the detection methods for virus-specific amplicons affect the sensitivity and specificity of RT-PCR assays (5). Analysis of small samples (0.1 g oysters meat) usually does not require extensive processing steps (6) but can result in false-negative PCR results when the level of virus is low (7), and larger sample size (at least 25 g oysters meat) needs to be examined. For the application of RT-PCR for detection of NoVs in shellfish sample, an efficient procedure for eluting, concentrating viruses from bivalve shellfish, and removal of food-related RT-PCR inhibitors are prerequisites (7). Detection methods for NoV have been reported (6-10), however, detection sensitivity is not enough for field application. To improve the detection sensitivity, the previous procedures (7,8) were adapted for the elution and concentration of viruses, and were modified using poliovirus as a surrogate for NoV (7,9,10) because plaque assay is not available for NoV, and plaque assay is more convenient for the evaluation of virus recoveries than RT-PCR. Here, an improved method for virus detection in oysters is proposed.

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Received February 17, 2009; Revised July 7, 2009;
Accepted July 9, 2009

## Materials and Methods

**Cell culture and viruses** The stock of poliovirus type 1 Sabin was used in this study and the poliovirus titer was determined by plaque assays using HeLa cells (11). The NoVs used in this study were obtained from the Division of Enteric and Hepatitis Viruses, National Institute of Health, Seoul, Korea. The NoV titer in RT-PCR units was determined by endpoint dilutions. One RT-PCR unit was defined as the last dilution from which NoV RNA could be amplified using RT-PCR and detected by electrophoresis on agarose gel containing ethidium bromide (12).

**Oyster samples** Oysters for the procedure development were shipped from Tongyeong, South Korea, in chilled containers and homogenized with a Waring blender immediately after receipt. Oyster homogenates were divided into 25 g portions and stored at -70°C until further use. Oyster homogenates were tested in each elution and

concentration steps, and were confirmed that they were not naturally contaminated by poliovirus using plaque assay.

**Reagents** One step RT-PCR premix and PCR reagents were obtained from Intron Biotechnology (Seongnam, Korea). TRIzol was purchased from Invitrogen (Carlsbad, CA, USA). All chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Modified procedures to elute and concentrate viruses from oysters** Twenty-five g of oyster meat were seeded with 5,000 plaque forming units (PFU) of poliovirus by acid-adsorption. Seven volumes of distilled water were added to the oyster homogenates, and the pH was adjusted to pH 4.8 (8). Viruses were acid-adsorbed to the oysters by stirring at room temperature for 20 min. The virus-adsorbed oysters were pelleted by 20 min of centrifugation at 6,000×g, and then eluted with 3 volumes of the first buffer (Table 1). After 20 min of stirring, supernatants were collected by centrifugation at 6,000×g at 4°C for 20 min. The oyster solids were re-eluted with 3 volumes of the second buffer (Table 1), and this supernatant was combined with elutes from the first buffer. After measuring the volume of the combined supernatants, virus was precipitated by addition of polyethylene glycol (PEG) 8,000 and NaCl (final conc.11% PEG and 0.3M NaCl) with incubation at 4°C for 3 hr. The resulting floc was sedimented by centrifugation at 16,000×g at 4°C for 20 min, and resuspended in 10 mL Tween 80/Tris solution (final conc. 0.04% Tween 80/10 mM Tris). An equal volume of chloroform (chloroform: isoamyl alcohol 24:1) was added to the suspension, which was then vigorously shaken for 20 min and centrifuged at 11,000×g at 4°C for 20 min. After collecting the aqueous layer, the interphase was extracted one more time with equal volume of chloroform, and then virus was precipitated by addition of PEG 8,000 and NaCl (final conc. 13% PEG and 0.3 M NaCl) at 4°C for 3 hr. The resulting flocs were centrifuged at 35,000×g at 4°C for 20 min, the supernatant was discarded, and the pellet was resuspended in 600 μL diethyl pyrocarbonate (DEPC) treated water.

**RNA extraction from concentrated viruses** The suspension was divided, and each half was extracted with 800 μL TRIzol, and 200 μL chloroform for 5 min, and centrifugation at 12,000×g and 4°C for 15 min. The aqueous phase was precipitated with isopropanol by centrifugation at 12,000×g and 4°C for 15 min. The resulting pellet was washed with 70% ethanol, dried, and solubilized in 50 μL DEPC-treated water. RNAs from both tubes were combined, and 5 μL RNA was used for RT-PCR.

**Primers, RT-PCR, semi-nested PCR** RT-PCR conditions were described previously (6,13,14). Briefly, 5 μL of extracted viral RNA was reverse transcribed and amplified in a total volume of 20 μL. The reaction mixture contained 8 μL of One-step RT-PCR premix, 100 pmol sense primer (GI-F1M: CTGCCCGAATTYGTAAATGATGAT for GI; GII-F1M: GGGAGGGCGATCGCAATCT for GII), and 200 pmol antisense primer (GI-R1M: CCAACCCARCCA TTRTACATYTG for GI; GII-R1M: CCRCCIGCATRICC RTTRTACAT for GII). The reverse-transcription (RT) step (45°C, 30 min) was followed by initial denaturation at 94°C for 5 min, and PCR amplification (30 cycles of 45 sec at 94°C, 45 sec at 55°C, 45 sec at 72°C, and a final elongation of 7 min at 72°C). The 50 μL semi-nested PCR reaction mixture included 1 μL of RT-PCR reaction product, 2.5 units of *i-max Taq* DNA polymerase (Intron Biotechnology, Seongnam, Korea), and 100 pmol of each primer (GI-F2: ATGATGATGGCGTCTAAGGACGC and GI-R1M for GI; GII-F3M: TTGTGAATGAAGATGGCGTCGART and GII-R1M for GII). The 329 and 340 bp RT-PCR products for GI and GII respectively, and 313 and 310 bp semi-nested PCR products for GI and GII respectively were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide and visualized under UV light.

**Table 1. Poliovirus recoveries from oysters after elution with buffers**

| | Buffers used[1)] | | Recovery[2)] (mean±SD, %) |
|---|---|---|---|
| | 1st Elution | 2nd Elution | |
| 1 | 0.05 M Gly-0.14 M NaCl | 0.05 M Gly-0.14 M NaCl | 30.4±11.7 |
| 2 | 0.1 M Gly-0.14 M NaCl | 0.1 M Gly-0.14 M NaCl | 43.6±11.5 |
| 3 | 0.25 M Gly-0.14 M NaCl | 0.25 M Gly-0.14 M NaCl | 49.5±13.3 |
| 4 | 0.5 M Gly-0.14 M NaCl | 0.5 M Gly-0.14 M NaCl | 79.9±13.7 |
| 5 | 0.05 M Thr-0.14 M NaCl | 0.05 M Thr-0.14 M NaCl | 30.5±10.7 |
| 6 | 0.1 M Thr-0.14 M NaCl | 0.1 M Thr-0.14 M NaCl | 45.7±12.3 |
| 7 | 0.25 M Thr-0.14 M NaCl | 0.25 M Thr-0.14 M NaCl | 81.3±13.5 |
| 8 | 0.5 M Thr-0.14 M NaCl | 0.5 M Thr-0.14 M NaCl | 79.3±9.2 |
| 9 | 0.1 M Tris-HCl | 0.1 M Tris-HCl | 62.5±15.6 |
| 10 | Phosphate buffered saline[3)] | Phosphate buffered saline | 65.2±14.9 |
| 11 | 0.25 M Gly-0.14 M NaCl | 0.25 M Thr-0.14 M NaCl | 81.9±10.3 |
| 12 | 0.25 M Gly-0.14 M NaCl | 0.5 M Thr-0.14 M NaCl | 82.3±10.2 |
| 13 | 0.5 M Gly-0.14 M NaCl | 0.25 M Thr-0.14 M NaCl | 82.1±15.3 |
| 14 | 0.5 M Gly-0.14 M NaCl | 0.5 M Thr-0.14 M NaCl | 85.0±17.3 |

[1)]The pH of all the buffers is 7.5 except PBS.
[2)]Results are the mean of at least 3 independent trials; SD is standard deviation.
[3)]0.01M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4, at 25°C.

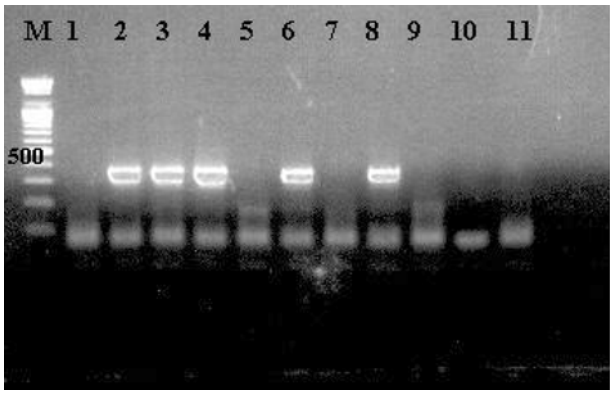


**Fig. 1. Detection of GI norovirus (NoV) in seeded oysters (25 g) by RT-PCR combined with semi-nested PCR.** Lane M, molecular weight marker (100 bp ladder); lane 1, negative control (oysters that were not seeded with NoV); lane 2, oysters seeded with 56 RT-PCR units; lane 3, 10 fold dilution of lane 2; lane 4, oysters seeded with 24 RT-PCR units; lane 5, 10 fold dilution of lane 4;, lane 6, oysters seeded with 8 RT-PCR units; lane 7, 10 fold dilution of lane 6; lane 8, oysters seeded with 4 RT-PCR units; lane 9, 10 fold dilution of lane 8; lane 10, oysters seeded with 1.6 RT-PCR units; lane 11, 10 fold dilution of lane 10.

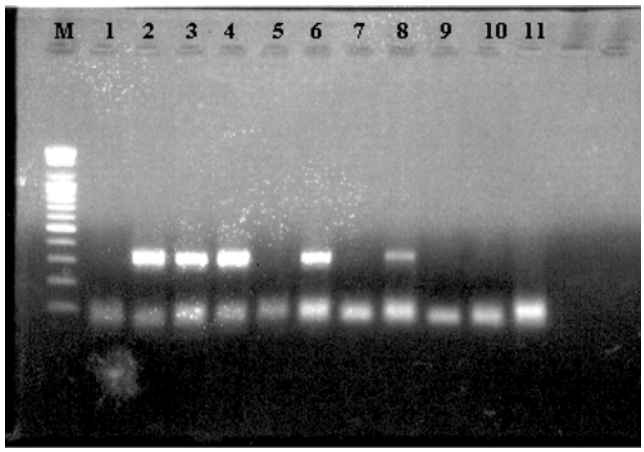


**Fig. 2. Detection of GII Nov in seeded oysters (25 g) by RT-PCR combined with semi-nested PCR.** Lane M, molecular weight marker (100 bp ladder); lane 1, negative control (oysters that were not seeded with NoV); lane 2, oysters seeded with 64 RT-PCR units; lane 3, 10 fold dilution of lane 2; lane 4, oysters seeded with 19.2 RT-PCR units; lane 5, 10 fold dilution of lane 4;, lane 6, oysters seeded with 12.8 RT-PCR units; lane 7, 10 fold dilution of lane 6; lane 8, oysters seeded with 6.4 RT-PCR units; lane 9, 10 fold dilution of lane 8; lane 10, oysters seeded with 3.2 RT-PCR units; lane 11, 10 fold dilution of lane 10.

## Results and Discussion

**Poliovirus recovery from oysters** Several earlier works have utilized poliovirus for the development of virus detection methods because of the ease of laboratory manipulation, relative similarity to the other human enteric RNA viruses such as hepatitis A virus (HAV), and NoV. In this work poliovirus was recruited in the elution and concentration steps because plaque assay is not available for NoV. The recovery procedure included acid-adsorption, elution with buffers, PEG precipitation, solvent extraction, and the second PEG precipitation. All recoveries were calculated based on the titer of the added poliovirus stock as 100%. The previous report (7) mentioned that acid adsorption-elution with glycine buffer (pH 7.5) retained fewer inhibitors than direct elution with glycine buffer (pH 9.5). Because of cell death due to the low pH (pH 4.8 was used for acid-adsorption), plaque assay was not performed for evaluating virus-elution. Based on the more than 85% poliovirus recovery, depending on the buffers used in the next step (Table 1), it was deduced that most of the viruses were adsorbed to the oysters in the acid-adsorption step. An earlier report (8) also mentioned that 98% of HAV was adsorbed to the oyster's solid in the acid-adsorption step.

Fourteen buffers were compared for virus elution from the acid-adsorbed oyster solids (Table 1). Tris-HCL buffer and phosphate-buffered saline (PBS) recovered 62.5-65.2% of the virus from acid-adsorbed oysters. Poliovirus recovery by eluting twice with 0.05 M glycine-0.14 M NaCl (pH 7.5) or 0.05 M threonine-0.14 M NaCl (pH 7.5) was inefficient, with only 30.4-30.5% recovery. However, when the concentration of the buffers was increased, more viruses from the acid-adsorbed oyster solids were recovered. An earlier report showed that 0.5 M threonine (pH 7.5) was effective for the elution of viruses from sewage (15), tomato sauce, and blended strawberry (16). Glycine and threonine (0.5 M, pH 7.5) buffers were also tested and was found that the use of glycine and threoninine at 0.5 M was not desirable because the eluates were too dense and included too many oyster components that negatively influenced the next step. Therefore, 0.25 M glycine-0.14 M NaCl (pH 7.5) was used in the 1st elution and 0.25 M threonine-0.14 M NaCl (pH 7.5) was used in the 2nd elution in the subsequent experiment. Cumulative recovery by this buffer combination was 81.9±10.3% (Table 1).

Previous studies have shown that virus recovery by PEG precipitation was dependent upon PEG concentration (10) and averaged 58.1-60% for poliovirus (7,10) and 71% for HAV (8). In this study, the 1st PEG precipitation recoveries ranged between 59.35 (48.6/81.9 in 8 % PEG) and 70.9% (58.1/81.9 in 16% PEG) of the remaining poliovirus in the eluates (Table 2). However, the pellet from 16% PEG precipitation was too dense and sticky and made the next step difficult. In addition, various nonspecific bands appeared after RT-PCR (data not shown). The virus recovery from the eluates was 64.7% (53.0/81.9) when 11% PEG 8000/0.3M NaCl was used (Table 2). There was not much difference between 3 and 16 hr incubation in virus recoveries (6,13). Therefore, virus was precipitated using 11% PEG (0.3M NaCl) at 4°C for 3 hr in the subsequent experiments.

The effect of Tween 80 for virus recovery in the resuspended solution before chloroform extraction was also evaluated. An earlier report showed that addition 0.1% Tween 80 to the PBS increased virus yield from sewage (15). Our result confirmed this. The addition of Tween 80 (final conc. 0.04%/10 mM Tris) easily resuspended the pellet and increased virus recovery yields after chloroform treatment.

The poliovirus recoveries of 3 solvents (Freon, chloroform, and dichloromethane) using the poliovirus-oyster concentrates recovered from the 1st PEG treatment

**Table 2. Poliovirus recoveries by the processing steps**

| Step | | Poliovirus recoveries[1)] (mean±SD %) |
|---|---|---|
| Elution | | 81.9±10.3 |
| 1st PEG precipitation | PEG 8% | 48.6±8.1 |
| | PEG 11% | 53.0±10.5 |
| | PEG 16% | 58.1±11.7 |
| Chloroform | Tween 80 not added | 29.9±4.3 |
| | Tween 80 added | 37.6±8.4 |
| 2nd PEG precipitation | PEG 13% | 19.5±6.3 |

[1)]Recovery based on percentage of amount of virus initially added. Results are the mean of at least 2 independent trials. SD is standard deviation.

were also compared. Previous literature showed that better HAV detection sensitivity was observed when chloroform was used instead of Freon (8,10), though chloroform recovered less HAV viruses than Freon when measured by plaque assay (8). In our study, recovery (calculated based on the poliovirus recovered from the 1st PEG as 100%) was the highest (78.6±15.1%) when Freon (1,1,2-trichloro-1,2,2-trifluoroethane) was used and recovery was the lowest (36.5±10.7%) when dichloromethane was used. Intermediate recovery (61.5±10.4%) was observed when chloroform was used for extraction. However, RT-PCR band intensity from chloroform extraction was similar to the Freon extraction (6, data not shown) and Freon is not legally available. Therefore, chloroform was used in the subsequent experiment. The effect of chloroform for removing RT-PCR inhibitors from oysters was evident because if chloroform extraction step was eliminated after PEG precipitation, the band after RT-PCR or RT-nested PCR was not observed (data not shown).

The 2nd PEG precipitation with 13% PEG 8000/0.3 M NaCl recovered 51.9% (19.5/37.6) of the poliovirus remaining after chloroform extraction (Table 2).

Based on the procedural results, a procedure to elute and concentrate viruses from the oysters was developed. Three major modifications were made to the original previous procedure (8): (i) elution buffers combination, (ii) PEG concentration and incubation time, and (iii) Tween 80 addition before chloroform extraction. Cumulative recovery of the whole procedure was 19.5±6.3%. This viral recovery rate in oysters was much higher than those of previous studies: i.e. 5.53% of HAV (7) and 10.3% of poliovirus (8) from 25 g oysters. However, it was comparable with the recoveries from vegetables: 16% of HAV from salad vegetables (17); 18% poliovirus from lettuce samples (18); 15% of poliovirus from frozen strawberry (19); 29.0% of poliovirus from cabbage, and 13.7% of poliovirus from lettuce (13).

**Virus detection sensitivity in oysters processed by the modified procedure** Several groups have reported the sensitivities of NoV detection in oysters, i.e., 5-1,000 RT-PCR units per 1.5-50 g oysters (10,12,20-22). In this study, with initial seeding levels, 4 RT-PCR units of GI NoV (Fig. 1) and/or 6.4 RT-PCR units of GII NoV (Fig. 2) in 25 g oysters were detected. Dilution of viral RNA didn't affect the detection, meaning RT-PCR inhibitors almost completely removed during processing (data not shown). We could not directly compare our methods with these reported procedures, because the detection limit can differ dramatically depending on the analytical method (for example, RT-PCR only, or RT-PCR and nested PCR combined, or RT-PCR and hybridization combined, or real-time PCR), oysters amount tested, kinds of oysters tissues (digestive or whole tissues), virus strains tested, and the primers used (23). Nevertheless, if the detection limit is compared in terms of initial seeding level per 25 g oyster meat, the sensitivity using this procedure is significantly higher than these previous reports (10,12, 20-22). The number of genome copies per RT-PCR unit has been estimated to be as low as 10-50 (12,24,25) so that 40-200 particles GI or 64-320 particles GII/25 g oyster meat can be detected with this procedure. The sensitivity increase seemed to be associated with improvements to viral elution (pH and concentration of buffers), PEG concentration (higher PEG concentration and Tween 80), RNA extraction (TRIzol), and viral RNA detection analysis (RT-semi-nested PCR). While whole oyster tissues were used in this study, dissection of bivalve stomach and digestive diverticula from more oysters could potentially result in an even more sensitive test for the virus (12,26).

Recently, several real-time RT-PCR methods for the detection of NoV in shellfish or stool samples have been reported (22,27-35). The elution and concentration procedure developed in this study can be performed in less than 19 hr in oysters and when coupled with real-time RT-PCR based detection, can yield quantitative results within 24 hr.

In summary, a previously developed procedure was adapted, and modified for eluting and concentrating virus. The virus-detection method developed in this study should facilitate detection of low levels of NoV in oysters, and could be used for monitoring Nov. This method should also be applicable to other kinds of enteric viruses, including HAV, in oysters.

## Acknowledgments

This work was supported by research grant from Korea Food & Drug Administration (L-05-02-2-FOD-056-A, S-09-02-2-FOD-028-0-D). This work was also supported by the Dongguk University Research Fund of 2009.

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