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Development of a Virus Elution and Concentration Procedure for Detecting Norovirus in Cabbage and Lettuce

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Abstract In this study, a rapid and efficient concentrating procedure that can be used for detecting viruses in vegetables was developed. The Sabin strain of poliovirus type 1 was used to evaluate the efficiency of virus recovery. The procedure included: (a) elution with 0.25 M threonine-0.3 M NaCl pH 9.5; (b) polyethylene glycol (PEG) 8000 precipitation; (c) chloroform extraction; (d) 2nd PEG precipitation; (f) RNA extraction; (g) reverse transcription-polymerase chain reaction (RT-PCR) combined with semi-nested PCR. The overall recoveries by elution/concentration were 29.0% from cabbage and 13.7% from lettuce. The whole procedure usually takes 18 hr. The overall detection sensitivity was 100 RT-PCR units of genogroup II norovirus (GII NoV)/25 g cabbage and 100 RT-PCR units of GII NoV/10 g lettuce. The virus detecting method developed in this study should facilitate the detection of low levels of NoV in cabbage and lettuce.

Key words: norovirus, poliovirus, detection, reverse transcription-polymerase chain reaction (RT-PCR) inhibitor, elution

Introduction

Norovirus (NoV), are the most common agents of acute viral gastroenteritis, and NoV transmission may be associated with infected people and contaminated food or water (1,2). NoVs, previously known as small round-structured viruses (SRSV) or Norwalk-like viruses (NLV) or human caliciviruses, are non-enveloped positive stranded RNA viruses and constitute a genus in the *Caliciviridae* family (1,3). Currently, there are at least 5 NoV genogroups (GI, GII, GIII, GIV, and GV), which are, in turn, divided into at least 25 genetic clusters based on the diversity of their genomes (4-6). NoVs that infect human (GI, GII, and GIV) do not grow in cell or organ culture, and there is no small animal model for infection and gastrointestinal disease (2,7,8). Outbreaks of food-borne diseases mediated by these viruses have been associated with shellfish, ice, water, bakery products (frosting), various types of salads (vegetable, chicken, fruit, and tossed), and cold foods (celery, melon, vermicelli consommé, sandwiches, and cold cooked ham) (9,10). However, direct virus strain identification in foods that are implicated in outbreaks has rarely been achieved due to food matrix complexity, low levels of contamination, and genetic diversity (10-12).

Though the exact infectious dose of NoV is not defined yet, it is estimated that about 10-100 NoV particles may be enough to cause gastroenteritis (5,13,14). Various methods have been developed to concentrate viruses prior to their detection. These applications, however, have been limited to the concentration of these viruses from clinical, environmental, meat, and shellfish samples (15). Recently, sensitive methods have been described for analysis of foods other than shellfish (10-12,14,16-24). However, several factors affect the specificity and sensitivity of reverse transcription-polymerase chain reaction (RT-PCR) assays, including the sample quality, RNA extraction and purification methods, the primers, RT-PCR conditions, and the detection methods for virus-specific amplicons (8,12). In addition, analysis of food samples can result in false-negative PCR results when the level of virus contamination is low (25) and when a larger sample (at least 10-25 g food) needs to be examined. Therefore, applying an RT-PCR-based technique for routine detection of NoVs in food samples requires the development of an efficient, simple, and reproducible procedure for eluting and concentrating contaminating viruses and the removal of food-related RT-PCR inhibitors (25).

In this study, we adapted the procedures developed for detecting NoV in oysters (26-28) and modified the procedures using poliovirus as a surrogate for NoV (12,16,18,22). Although the plaque assay is more convenient for the evaluation of virus recoveries than RT-PCR, there is no plaque assay available for NoV. Here, we propose a method for detecting viruses in cabbage and lettuce.

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Materials and Methods

Cell culture and viruses 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 (11). The stock of the Sabin strain of poliovirus type 1 used in this study was derived from transfection of the full-length viral cDNA to COS-1 cells. The poliovirus titer was determined by plaque assays using HeLa cells.

Reagents One-step RT-PCR premix and PCR reagents were purchased from Intron Biotechnology (Seongnam, Korea). The QIAamp® Viral RNA Mini Kit was obtained

from Qiagen (Hilden, Germany). All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Inoculation of cabbage and lettuce Cabbage and lettuce were purchased at a local supermarket (Samsung TESCO Home Plus, Gyeongju, Korea) and tested negative for the poliovirus and NoV with PCR. Individual cabbage leaves (ca. 25 g) were cut into pieces of about 2×3 cm, rinsed in water, and allowed to dry for 1 hr in a laminar flow hood. Individual lettuce leaves (ca.10 g) were also cut into pieces of about 1×1 cm and were washed and dried in the same manner. Instead of 25 g, 10 g of lettuce leaves were used because the volume of 25 g lettuce was too large to be washed efficiently in a 250-mL centrifuge bottle. A portion (10-100 μL) of a known plaque forming unit (PFU) of poliovirus (10^5-10^6 PFU) or NoV was spread over the vegetable surface and was allowed to dry for 3 hr in a laminar flow.

Virus recovery and concentration The inoculated virus on the cabbage or lettuce was washed with elution buffer (150 mL) in a centrifuge tube in a shaking incubator (20-23°C, 150 rpm) for 3 hr. There was very little difference in virus recoveries between 3 and 16 hr incubations (data not shown). The aqueous phase was decanted into sterile 250-mL centrifuge bottles. After measuring the volume of the wash solution, polyethylene glycol (PEG) 8000, and NaCl were added in order to achieve a final concentration 14% PEG and 0.3 M NaCl. The viral suspensions were stored at 4°C for 3 hr to allow precipitation of the viral particles. Viruses were concentrated by centrifugation at 18,000×g for 20 min and the last about 12 mL of residual with the pellet were kept to avoid its loss. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the suspension, vigorously shaken for 20 min, and centrifuged at 11,000×g for 20 min at 4°C. After collecting the aqueous layer, the virus was precipitated again by the addition of PEG 8000 and NaCl (final conc. 14% PEG and 0.3 M NaCl), and incubation at 4°C for 3 hr. Viruses were concentrated by centrifugation at 18,000×g for 20 min, the supernatant was discarded, and the pellet was suspended in 1 mL diethyl pyrocarbonate (DEPC) treated water.

RNA extraction from concentrated viruses The QIAamp® viral RNA extraction kit (Qiagen) was used to extract RNA from the concentrated viruses, according to the manufacturer's instructions with minor modification. Instead of 140 μL of virus concentrates, 280 μL was used for RNA extraction.

RT-PCR and RT-PCR combined with semi-nested PCR RT-PCR conditions (27) and the primers used (DG172, DG173, DG213, DG214, GII-F1M, GII-R1M, and GII-F3M) were described previously (23,27,28). Primers for NoVs (GII-F1M, GII-R1M, and GII-F3M) are based on previously designed primers (29,30), which have been modified according to sequences identified from patient stools samples collected in Korea (28). Five μL of extracted viral RNA was reverse transcribed in a total volume of 20 μL. The RT-PCR mixture contained 8 μL of one-step RT-PCR premix, 100 pmol sense primer (GII-F1M: GGGAG GGCGATCGCAATCT for GII NoV; DG172: GATTACA AGGATGGTACGCTTACA for poliovirus) and 200 pmol antisense primer (GII-R1M: CCRCCIGCATRICCRTTRT ACAT for GII NoV; DG173: GACTCTATGTAATTGGT GATGCCT for poliovirus). The RT step (45°C, 30 min) was followed by 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 RT-PCR combined with semi-nested or nested PCR mixture included 1 μL of RT-PCR reaction product, 2.5 units of *i-max Taq* DNA polymerase (Intron Biotechnology, Seoul, Korea), and 100 pmol of each primer (GII-F3M: TTGTGAATGAAGATGGCGTCGART and GII-R1M for GII NoV; DG213: CTTACACCCCTCTCCACCAAGGAT and DG214: TTGTTCCATGGCTTCTTCTTCGTA for poliovirus). The 340, 524 bp RT-PCR products for GII NoV, and poliovirus, respectively and 310, and 506 bp RT-PCR combined with semi-nested or nested PCR products for GII, and poliovirus, respectively, were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide and visualized with ultraviolet (UV) light.

Results and Discussion

Poliovirus recovery from cabbage and lettuce by elution and concentrating procedure Poliovirus recoveries were evaluated by plaque assay. The recovery procedure included elution, 1st PEG precipitation, solvent extraction (chloroform: isoamyl alcohol 24:1), and 2nd PEG precipitation. All steps were performed at least 3 times as independent trials and recoveries were calculated based on the titer of the added poliovirus stock as 100%. The most important modification was made to the elution stage of virus recovery from vegetable surfaces. Eighteen buffer combinations were compared for virus elution from the vegetable leaves (Table 1, 2). Phosphate buffered saline (PBS) has been used to wash intact raw vegetables (15,20,31,32). However, poliovirus recovery by eluting with PBS was inefficient. In our study, only 5.7% (4.8-6.7% range from cabbage) and 5.0% (4.0-6.2% range from lettuce) of poliovirus were recovered by using PBS (pH 7.4). An average of 7.4% (6.1-8.7% range from cabbage) and 4.8% (3.2-5.3% range from lettuce) of poliovirus were recovered by elution with beef extract (3%, pH 9.5). However, poliovirus recoveries when eluting with glycine buffers and threonine buffers were much better than PBS and beef extract. We recovered 37.6-73.3% from cabbage and 5.8-48.5% from lettuce using glycine buffers. We recovered 20.0-90.0% from cabbage and 8.6-62.5% from lettuce by using threonine buffers. Generally, better recoveries from cabbage and lettuce were observed when using threonine buffers and buffers with a concentration of 0.25 M than when using glycine buffers and buffers with a concentration of 0.05 M. We recovered more viruses using buffers containing 0.3 M NaCl than 0.14 M NaCl (except when they contained 0.05 M glycine). The performance of pH 9.5 buffers was better than pH 7.5 buffers in cabbage samples (except when they contained 0.25 M threonine and 0.14 M NaCl). Interestingly, in the case of lettuce, we couldn't find any dependency on NaCl concentration in glycine buffers. However, we recovered more viruses by using threonine buffers with 0.3 M NaCl than threonine buffers with 0.14 M NaCl. We recovered more viruses by using pH 9.5 buffers than pH 7.5 buffers

Table 1. Recoveries of poliovirus from cabbage using different buffers for elution[1)]

Elution buffers	pH	Recovery (%)[2)]			
		Mean		SD[3)]	Range
Phosphate buffered saline (PBS)	7.4	5.7		0.9	4.8-6.7
3% Beef extract	9.5	7.4		1.4	6.1-8.7
0.05 M Glycine-0.14 M NaCl	7.5	47.5	52.3 (37.6-73.3)	48.2	5.3-100
0.05 M Glycine-0.14 M NaCl	9.5	73.3		15.1	62.0-90.5
0.05 M Glycine-0.3 M NaCl	7.5	37.6		21.9	15.4-75.5
0.05 M Glycine-0.3 M NaCl	9.5	50.8		28.9	9.7-71.7
0.25 M Glycine-0.14 M NaCl	7.5	50.1	60.3 (50.1-68.3)	21.0	19.8-66.8
0.25 M Glycine-0.14 M NaCl	9.5	61.9		5.5	56.3-68.5
0.25 M Glycine-0.3 M NaCl	7.5	61.1		41.0	18.2-100
0.25 M Glycine-0.3 M NaCl	9.5	68.3		43.9	18.1-100
0.05 M Threonine-0.14 M NaCl	7.5	20.0	53.9 (20.0-82.6)	4.5	15.1-24.0
0.05 M Threonine-0.14 M NaCl	9.5	53.8		21.7	25.8-85.2
0.05 M Threonine-0.3 M NaCl	7.5	59.0		7.9	52.0-69.4
0.05 M Threonine-0.3 M NaCl	9.5	82.6		13.3	66.2-100
0.25 M Threonine-0.14 M NaCl	7.5	82.3	84.7 (77.2-90.0)	8.1	72.9-89.5
0.25 M Threonine-0.14 M NaCl	9.5	77.2		11.9	71.2-95.0
0.25 M Threonine-0.3 M NaCl	7.5	89.1		13.3	69.3-100
0.25 M Threonine-0.3 M NaCl	9.5	90.0		13.3	67.5-100

[1)]Results are the mean of at least 3 independent trials.
[2)]Recovery (%) was calculated based on the poliovirus plaque assay count of the initial seeding as 100%.
[3)]Standard deviation.

Table 2. Recoveries of poliovirus from lettuce using different buffers for elution[1)]

Elution buffers	pH	Recovery[2)]			
		Mean		SD[3)]	Range
Phosphate buffered saline (PBS)	7.4	5.0		1.6	4.0-6.2
3% Beef extract	9.5	4.8		1.3	3.2-5.3
0.05 M Glycine-0.14 M NaCl	7.5	7.8	26.1 (5.8-48.5)	0.8	7.1-8.5
0.05 M Glycine-0.14 M NaCl	9.5	42.2		7.5	31.8-49.5
0.05 M Glycine-0.3 M NaCl	7.5	5.8		0.8	5.0-6.6
0.05 M Glycine-0.3 M NaCl	9.5	48.5		11.2	35.7-64.8
0.25 M Glycine-0.14 M NaCl	7.5	8.1	29.1 (8.1-46.0)	0.9	7.2-9.5
0.25 M Glycine-0.14 M NaCl	9.5	46.0		19.1	21.1-65.1
0.25 M Glycine-0.3 M NaCl	7.5	27.3		16.2	7.5-47.1
0.25 M Glycine-0.3 M NaCl	9.5	34.9		19.6	7.9-51.1
0.05 M Threonine-0.14 M NaCl	7.5	8.3	32.6 (8.3-50.3)	2.2	5.8-10.9
0.05 M Threonine-0.14 M NaCl	9.5	42.6		19.2	24.0-71.1
0.05 M Threonine-0.3 M NaCl	7.5	29.2		17.8	9.4-43.7
0.05 M Threonine-0.3 M NaCl	9.5	50.3		13.2	28.5-63.9
0.25 M Threonine-0.14 M NaCl	7.5	53.3	54.5 (46.1-62.5)	11.3	41.4-65.6
0.25 M Threonine-0.14 M NaCl	9.5	46.1		23.7	18.9-61.8
0.25 M Threonine-0.3 M NaCl	7.5	56.2		13.3	28.7-71.7
0.25 M Threonine-0.3 M NaCl	9.5	62.5		19.6	23.8-89.3

[1)] Results are the mean of at least 3 independent trials.
[2)]Recovery (%) was calculated based on the poliovirus plaque assay count of the initial seeding as 100%.
[3)] Standard deviation.

from lettuce (except when they contained 0.25 M threonine and 0.14 M NaCl). In total, 0.25 M threonine-0.3 M NaCl (pH 9.5) was the best buffer among those tested for the elution of poliovirus from cabbage and lettuce. However, further experiments on the effect of ionic strength, pH, and the presence of Mg^{2+} ions in buffers are needed for more efficient elution of viruses from various food matrices.

The recoveries after the 1st PEG precipitation and chloroform treatment ranged from 23.3-62.7% (average 41.7±16.8%) of the remaining poliovirus in the eluates

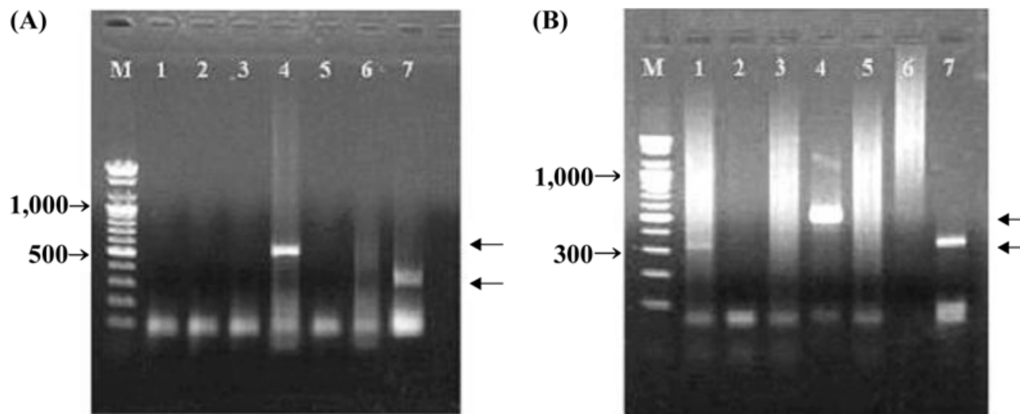

Fig. 1. Detection of norovirus in cabbage by RT-PCR and RT-PCR combined with semi-nested PCR. Twenty-five g of the cabbage were artificially contaminated with GII NoV. (A) RT-PCR. (B) RT-PCR combined with semi-nested PCR. Lane M, molecular size marker (100 bp ladder); lane 1, 100 RT-PCR units of NoV; lane 2, 50 RT-PCR units of NoV; lane 3, 10 RT-PCR units of GII NoV; lane 4, 1,000 PFU of poliovirus; lane 5 and 6, negative control; lane 7, positive control (NoV GII cDNA was amplified using PCR).

from the cabbage leaves. The recoveries after 1st PEG precipitation and chloroform treatment ranged from 22.0-45.3% (average 33.9±9.2%) of the remaining poliovirus in the eluates from the lettuce. We recovered 18.8-35.1% (average 32.2±4.1%) of the poliovirus remaining in the eluates from cabbage by using a 2nd PEG precipitation. In the case of lettuce, we recovered 21.4-22.4% (average 21.9 ±0.7%) of the poliovirus remaining in the eluates by precipitating the samples a 2nd time with PEG. We could concentrate the viruses 150 fold (from 150 to 1 mL) and the cumulative recovery of the whole procedure was 29.0% for cabbage and 13.7% for lettuce. These viral recovery rates were comparable with previously reported rates: 16% of hepatitis A virus (HAV) were recovered from salad vegetables by PEG precipitation and chloroform/butanol extraction (19); 18% of poliovirus from lettuce samples by elution and filtration (18); 15% of poliovirus from frozen strawberry (16); 10-53% of poliovirus and 2-4% of HAV from lettuce samples by sequential steps of homogenization, filtration and 2 steps of PEG precipitation (33); and 32% of HAV from vegetable surfaces by filtration (15).

Detection sensitivity Several groups have reported the sensitivities of NoV detection techniques in food samples other than shellfish. For example, Leggitt and Jaykus (33) and Dubois *et al.* (19) were able to detect 1.5×10^3 RT-PCR units/30-50 g of food sample. Sair *et al.* (11), Schwab *et al.* (10), Butot *et al.* (34), and Baert *et al.* (24) reported similar sensitivities (i.e., 10-100 RT-PCRU/6-50 g of food sample). Le Guyader *et al.* (12) detected 10 RT-PCRU NoV/g lettuce. However, we could not directly compare our methods with these reported procedures because the detection limit can differ dramatically depending on the analytical method (i.e., RT-PCR only, or RT-PCR and nested PCR combined, or RT-PCR and hybridization combined, or real-time PCR), sample amount tested, virus strains, and the primer sets used (12,28). In our study, the overall detection sensitivity was determined by inoculating viruses on 25 g cabbage leaves or 10 g lettuce leaves, processing the seeded vegetables, and then examining final RNA concentrates by RT-PCR (poliovirus) or RT-PCR combined with semi-nested PCR (NoV GII). With the initial seeding levels, we were able to detect 100 PFU poliovirus or 100 RT-PCR units GII NoV/25 g cabbage or 10 g lettuce (Fig. 1-3). However, we could not detect GII NoV when we inoculated less amount of NoV on the cabbage (Fig. 1, lane 2 and 3) or the lettuce (data not shown). No sample inhibition was observed in any of the samples tested, since dilution prior to amplification didn't help the detection of viruses (data not shown). The number of poliovirus particles per PFU has been estimated to be about 50-1,000 (35) and the number of NoV genome copies per RT-PCR unit has been estimated to be as low as 10-50 (36,37) so that about 5,000-100,000 poliovirus particles (by RT-PCR) or 1,000-5,000 GII NoV particles (by RT-combined with semi-nested PCR) can be detected in 25 g of cabbage or in 10 g of lettuce with this procedure.

Comparison of 2 concentration procedure prior to RNA amplification Based on the initial procedural results, additional procedures to elute and concentrate viruses from the vegetable leaves were developed. The established procedure in this study was adapted from the method applied to the shellfish (26,28), that is, elution-PEG precipitation-chloroform-PEG precipitation (EPCP). The procedure includes 2 PEG precipitation steps, and we could not recover all the viruses after PEG precipitation. Therefore, we expected more virus could be concentrated if one PEG precipitation step can be eliminated. The new modified procedure is as follows: 1) virus elution with 75 mL 0.25 M threonine-0.3 M NaCl (pH 9.5); 2) extraction with 75 mL of chloroform:isoamyl alcohol (24:1); 3) virus precipitation with PEG 8000 (ECP). Poliovirus was inoculated on 25 g of cabbage or 10 g of lettuce, processed by 2 procedures and then evaluated by RT-PCR. As indicated by the results, an initial seeding with as few as 100 PFU poliovirus were consistently detected from cabbage leaves by the 2 described methods, but a much stronger band was observed by EPCP (Fig. 3). As few as 100 PFU poliovirus were repeatedly detected (though it is a faint band) on lettuce by EPCP, but 100 PFU poliovirus was not detected by ECP, meaning the performance of EPCP is better than that of ECP (Fig. 3). One possible explanation might be differences in volume of eluant used,

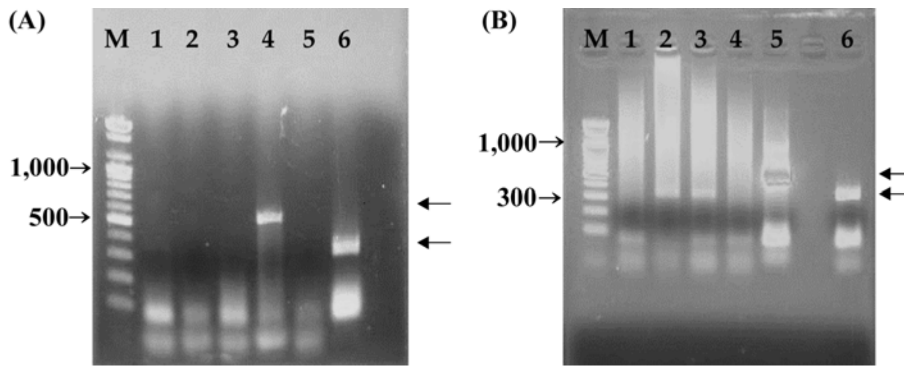

Fig. 2. Detection of norovirus in lettuce by RT-PCR and RT-PCR combined with semi-nested PCR. Ten g of lettuce were artificially contaminated with GII NoV. (A) RT-PCR. Lane M, molecular size marker (100 bp ladder); lane 1, negative control; lane 2, 250 RT-PCR units of NoV; lane 3, 100 RT-PCR units of NoV; lane 4, 1,000 PFU of poliovirus; lane 5, negative control; lane 6, positive control (GII NoV cDNA was amplified by PCR). (B) RT-PCR combined with semi-nested PCR. Lane M, molecular size marker (100 bp ladder); lane 1, negative control; lane 2, 250 RT-PCR units of NoV; lane 3, 100 RT-PCR units of NoV; lane 4, negative control; lane 5, 1,000 PFU of poliovirus; lane 6, positive control (GII NoV cDNA was amplified by PCR).

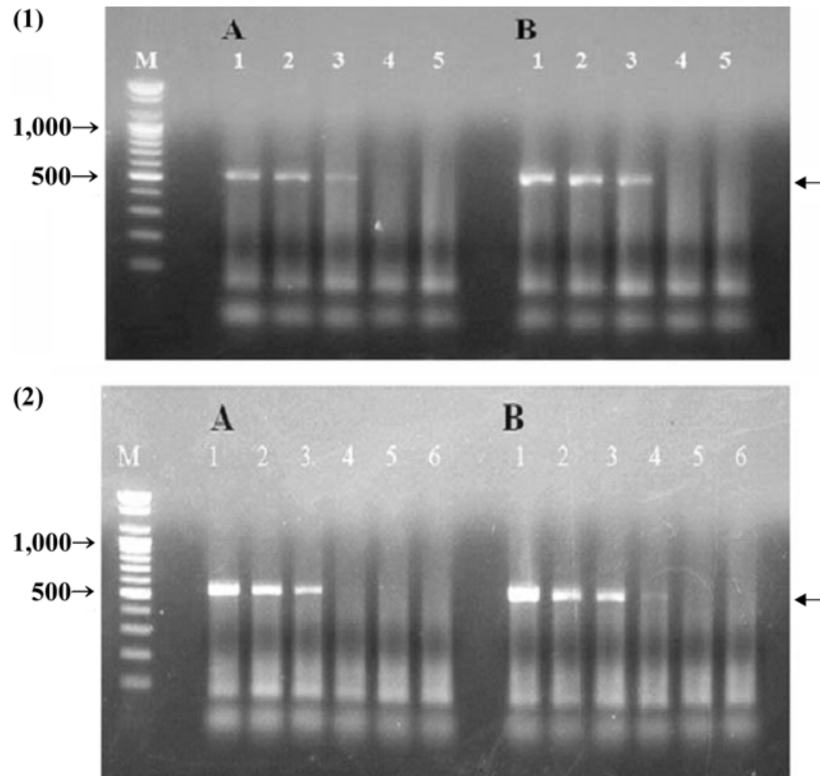

Fig. 3. Comparison of 2 concentration procedures by RT-PCR. 1. Cabbage (A) Elution/chloroform/PEG/RNA extraction. (B) Elution/1st PEG/chloroform/2nd PEG/RNA extraction. Twenty-five g of cabbage were artificially inoculated with poliovirus. Lane M, molecular size marker (100 bp ladder); lane 1, 10,000 PFU; lane 2, 1,000 PFU; lane 3, 100 PFU; lane 4, 10 PFU; lane 5, negative control. 2. Lettuce (A) Elution/chloroform/PEG/RNA extraction. (B) Elution/1st PEG/chloroform/2nd PEG/RNA extraction. Ten g of lettuce were artificially inoculated with poliovirus. Lane M, molecular size marker (100 bp ladder); lane 1, 100,000 PFU; lane 2, 10,000 PFU; lane 3, 1,000 PFU; lane 4, 100 PFU; lane 5, 10 PFU; lane 6, negative control.

that is, 150 mL buffer was used for elution in ECPC, but considering the chloroform extraction step, only 75 mL buffer was used in ECP. Therefore, it is possible that 75 mL buffer was not enough for efficient recovery of virus from vegetable surfaces.

In this study, we adapted a previously developed procedure for detection of virus from oyster samples and modified the eluting and concentrating conditions to allow for efficient recovery of virus from vegetables. The elution and concentration procedures developed in this study can be performed in less than 18 hr and when coupled with real-time RT-PCR can yield quantitative results within 24 hr. The virus-detection method developed in this study should facilitate detection of low levels of NoV in cabbage and lettuce. This method should also be applicable to other kinds of enteric viruses, including HAV, in cabbage and lettuce.

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