Concentration Method for the Detection of Enteric Viruses from Large Volumes of Foods

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ABSTRACT

Enteric viruses are the major cause of outbreaks of foodborne viral disease worldwide, and vegetables and fruits are considered significant vectors of virus transmission. In this study, we compared viral elution concentration methods in strawberry and lettuce and tested the secondary concentration step for concentrating viruses from large volumes of lettuce samples. Among the tested procedures, the combination of a 0.05 M glycine plus 100 mM Tris elution buffer (pH 9.5) and a polyethylene glycol precipitation concentration was most efficient for the detection of norovirus genogroup II from strawberries (50% of samples) and lettuce (2.9% of samples). The secondary concentration step using ultrafiltration devices could be applied to large lettuce samples without any decrease in detection limit and efficiency, and other cultivable enteric viruses including enteroviruses, adenoviruses, and rotaviruses were recovered from lettuce at efficiencies of 11.4, 9.05, and 11.3%, respectively. This method could be useful for detecting enteric viruses in fresh foods.

The consumption of enteric virus–contaminated foods such as vegetables, fruits, shellfish, and ready-to-eat foods can cause viral gastroenteritis and has become an emerging issue in public health (7, 22, 28). The U.S. Centers for Disease Control and Prevention reported that noroviruses (NoVs) and hepatitis A virus are major agents of foodborne outbreaks, and a variety of salads and cakes contaminated with these enteric viruses have been frequently associated with foodborne viral disease outbreaks (19). In Korea, school lunches have been implicated as a source of NoV in several outbreaks (15).

Commonly studied groups of enteric viruses in foods are NoVs, enteroviruses (EVs), adenoviruses (AdVs), rotaviruses (RVs), and hepatitis A virus (1, 16, 17, 23, 25, 26). These viruses are frequently detected in contaminated water and can be transferred onto food surfaces by contaminated irrigation water (9). All of these viruses are environmentally resistant, persist for a long period on food surfaces (20), and cause serious gastroenteritis with a low infectious dose (27). However, the levels of enteric viruses present in contaminated foods are usually very low, making it difficult to recover and detect these viruses (4).

To reduce the potential risk of foodborne viral disease outbreaks, investigators have developed many sensitive and useful methods for detecting enteric viruses from various food items (2, 26, 27, 29). However, vegetables and fruits have not been studied as intensively as other foods in spite of recent concerns about salad-associated outbreaks (2, 3, 11).

To establish a useful method for detecting enteric viruses from vegetables and fruits, the following processes should be standardized: (i) virus elution and concentration from foods, (ii) viral RNA extraction from concentrates, and (iii) the detection efficiency of molecular and cell culture techniques. The elution and concentration step is the most crucial step for detection of viruses in foods, and several protocols have been applied with various foods (3, 10, 23, 26, 29). However, existing studies of enteric viruses on vegetables and fruits have been limited to small volumes of food samples (5 to 20 g) (5) and only a few virus types (2, 29, 31). To improve such limited approaches, we evaluated a procedure for detecting various enteric viruses (NoVs, EVs, AdVs, and RVs) on contaminated strawberries and lettuces in large volumes of food by reverse transcription (RT) PCR and cell culture assay.

MATERIALS AND METHODS

Cells and viruses. NoV-positive stool samples (Korea Center for Disease Control and Prevention, Seoul, South Korea) were diluted to a 20% suspension in phosphate-buffered saline (PBS), pH 7.4. The titer of NoV and EV suspensions was determined by RT-PCR amplifiable unit (RTPCR(U) endpoint dilution of stool suspension. Human RV strain Wa (VR-2018), AdV type 5 (ATCC VR-5), and coxsackievirus type B5 Faulkner (ATCC VR-185) were propagated in MA-104, A549, and Buffalo green monkey kidney (BGMK) cell monolayers, respectively. The titers of RV, AdV, and EV were calculated based on the 50% tissue culture infectious dose (TCID50) method (24). All virus
Suspensions were aliquoted in 1-ml volumes and stored at –270°C until used.

**Sample processing for virus recovery.** Strawberries and lettuce were purchased from retail markets, stored at 4°C, and used within 24 h. The procedure for testing virus elution and concentration methods is shown in Figure 1. The samples of whole strawberries (5 g) and lettuce (25 g) were placed in sterile polypropylene containers and exposed to UV radiation for 10 min to reduce contaminating microorganisms that might affect cell culture assays. The samples were inoculated with a portion of 10-fold serial dilutions of virus suspension ranging from 4,800 to 0.48 RTPCRU for NoV, 10⁵.30 to 10⁵.80 TCID₅₀ for EV, 10⁵.90 to 10⁶.05 TCID₅₀ for AdV, and 10⁴.55 to 10⁴.78 TCID₅₀ for RV on the food surface, followed by air drying for 1 h at room temperature. The elution buffer was PBS (pH 7.4), 0.5 M threonine–0.14 N NaCl (pH 8.5), and 0.05 M glycine–100 mM Tris (pH 9.5); 25 ml (for strawberry) or 250 ml (for lettuce) was added to the samples to elute viruses from the food surface and was gently mixed for 30 min at room temperature. After removing the samples, the eluates were immediately adjusted to neutral pH (pH 7.4 to 7.2) with 1.0 N HCl, transferred to a sterile centrifuge bottle, and centrifuged at 3,500 x g for 15 min to remove food debris. For method P (17), the resulting supernatants were mixed with 8% (wt/vol) polyethylene glycol (PEG) 8,000 (plus 0.3 M NaCl) and incubated for 2 h at 4°C to precipitate viruses. The samples were centrifuged at 10,000 x g for 30 min at 4°C, and the pellet was suspended with sterile PBS. For method F (6), the eluates were adjusted to pH 3.5 with 1.0 N HCl and filtered through a negatively charged membrane prefilter (5.0-μm pore size; SVPP, Millipore Co., Billerica, MA) and filter (3.0-μm pore size; SSWP, Millipore). After washing with 10 ml of 0.14 N NaCl (pH 3.5), 15% beef extract–0.05 M glycine solution (pH 9.5) was added to elute viruses, and concentrates were collected. For method U (3), the eluates were transferred to a concentrator (strawberries: 50,000 nominal molecular weight limit Centriprep YM-50, Millipore; lettuces: 100,000 nominal molecular weight limit Centricon Plus-70, Millipore) and centrifuged at 1,500 to 3,500 x g for 10 to 40 min to obtain concentrates.

To recover NoV from 100 g of lettuce, 500 to 600 ml of 0.05 M glycine–100 mM Tris (pH 9.5) and method P were used as described above. A secondary concentration was performed to reduce concentrate volumes further. Five milliliters of suspended PEG pellets were transferred to an Ultracel-50k (Amicon, Millipore) concentrator and centrifuged at 3,500 x g for 10 min to obtain concentrates up to a volume of 140 μl.

After all steps, the collected concentrates, which would be used for RT-PCR, were treated with the Plant RNA Isolation Aid (Ambion, Huntingdon, UK) as previously described by Butot et al. (3) to reduce potential PCR inhibitors. For cell culture assays, the concentrates were filtered through a 0.2-μm-pore-size filter. The final concentrates were stored at –70°C until used.

All tests for the RT-PCR analysis contained a negative control inoculated with only sterile PBS and a process control inoculated with coxsackievirus B5 Faulkner (ATCC VR-185) to confirm successful virus concentration. After all procedures done, the percentage of virus recovered was calculated by dividing the RTPCRU of NoV recovered by the RTPCRU of NoV inoculated. Each test was analyzed in triplicate in 1 day, and we repeated each test three times.
Extraction of viral RNA. RNA was extracted from each concentrate of methods P, F, and U using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The final elution of RNA was performed using 30 μl of elution buffers. The final eluates (30 μl) were used immediately or were stored at −70°C until used.

RT-PCR. RT was performed as described by Kojima et al. (14) with minor modifications. Ten microliters of extracted RNA was mixed with 10 μl of a reaction mixture containing 5× RT buffer, 200 μM concentrations of each deoxynucleoside triphosphate (dNTP), 80 pmol of random primers (Takara Bio Inc., Shiga, Japan), 100 U of Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Madison, WI), and 10 U of RNAsin (Promega). RT was carried out at 42°C for 1 h after the tubes were heated to 95°C for 5 min to inactivate enzymes. Twelve microliters of the RT mixture was mixed with the PCR mixture containing 10 mM Tris-Cl (pH 8.8), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 200 μM concentrations of each dNTP, 2.5 U of Taq polymerase (Takara Bio), and 0.15 μM concentrations of each primer (forward primer G2-F1M: 5′-GGGAGGGC-GATGCACATCT-3′; reverse primer G2-R1M: 5′-CC(A/G)CCIG-CAT(A/G)ICC(A/G)TT(A/G)TACAT-3′) (13) in a total volume of 20 μl. The PCR protocol was an initial denaturation step at 94°C for 3 min, 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min. Amplified PCR products were visualized under UV light after electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

Cell culture assay. We prepared 10-fold dilutions of the final concentrates of EV, AdV, and RV from food samples and inoculated them into 96-well tissue culture dishes containing confluent monolayers of BGMK, A549, and MA 104 cells, respectively. All the dishes were incubated for 90 min at 37°C in an atmosphere of 5% CO2 until used. The cells were washed twice with sterile 10 mM PBS, and 200 μl of minimal essential medium (for EV and RV) or Dulbecco’s minimal essential medium (for AdV) containing 2% (vol/vol) fetal bovine serum was added to each dish containing cell monolayers. The tissue culture dishes were incubated at 37°C in an atmosphere of 5% CO2 and observed for 5 days with phase contrast microscopy for evidence of cytopathic effects. For analysis of RV, all the dishes containing MA 104 cells were maintained by mixing 8 μg/ml trypsin (Difco, Becton Dickinson, Sparks, MD). All tests included a negative control inoculated with sterile PBS and a positive control inoculated with each tested virus stock already prepared. Virus recovery (as a percentage) was calculated using the initial TCID50 value of the inoculated viruses as 100%. Each trial was conducted in duplicate.

RESULTS

Virus recovery using elution and concentration methods with strawberry and lettuce samples. Five grams of strawberry samples inoculated with serially diluted NoV were processed with three elution buffers and three concentration methods. Using 0.05 M glycine–100 mM Tris (pH 9.5) buffer with PEG precipitation or ultrafiltration was more effective than other combinations for NoV detection from inoculated strawberries (Table 1). NoV was detected at up to 10−3 dilution (corresponding to 4.8 RTPCRU) with these combinations (data not shown). The recovery achieved with other combinations was relatively low, ca. less than 5.0%. Overall, 0.05 M glycine–0.1 M Tris buffer (pH 9.5) was the most efficient buffer system for eluting NoV from strawberry surfaces (3.9 to 50% of virus recovered), and PEG precipitation was better for concentrating NoV than was filtration or ultrafiltration (Table 1). Filtration was the least effective of the three concentration methods we tested.

The most efficient elution buffer for strawberries, 0.05 M glycine–100 mM Tris buffer (pH 9.5), was applied to 25-g lettuce samples to compare virus recovery after PEG precipitation and ultrafiltration. Both methods produced similar NoV recovery, and NoV was detected at dilutions of 10−2 (corresponding to 48 RTPCRU) (Table 2).

SECONDARY CONCENTRATION STEP USED FOR LARGE SAMPLES OF LETTUCE AND STRAWBERRIES. We tested a commercially available ultrafiltration device, the Ultracel-50k, to reduce the volumes for large samples of lettuce (100 g) and strawberries (100 g). The ultrafiltration device successfully concentrated the lettuce samples to a concentrate of 140 μl. Addition of the secondary concentration step to the existing procedure did not decrease the NoV detection limit and recovery efficiency compared with the results from 25-g samples of lettuce and strawberries (Table 2).

RECOVERY OF INFECTIOUS ENTERIC VIRUS IN LETTUCE SAMPLES. Inoculated cultivable enteric viruses, 105–108 TCID50 of EV, 105–106 TCID50 of AdV, and 104–107 TCID50 of RV, were recovered from 25-g lettuce samples using PEG precipitation and ultrafiltration and were reinoculated into appropriate cell lines. After 5 days, the results were recorded and analyzed according to the Reed-Muench method (24). Approximately 6.3 to 16% of infectious viruses were recovered from lettuce surfaces (Table 3), and the recoveries were differed according to virus type and concentration method. Overall, virus recovery with PEG precipitation was higher than that with ultrafiltration.

DISCUSSION

Enteric viruses associated with foodborne outbreaks are a major health problem worldwide, and vegetables and fruits...
are frequently identified as possible vehicles of virus transmission because fecally contaminated irrigation waters may be used for cultivation (23) and these products may be consumed raw (3). Several methodological studies have been conducted with rapid and sensitive detection methods, but the information they provided is not enough to monitor the presence of enteric viruses in vegetables and fruits. In this study, we described the usefulness of a secondary concentration step for the virus recovery from lettuce.

We tested three elution buffers and three concentration methods as previously used by other researchers (3, 6, 16, 21) to select the most efficient combinations of methods for virus recovery. Buffer with high pH was more effective for eluting NoV from strawberries (Table 1), and this result is consistent with those of other studies (1, 3). Overall, the efficiency of PEG precipitation was higher than that of other concentration methods (Tables 1 through 3). However, conflicting results were obtained in other studies with PEG precipitation and ultrafiltration (3), possibly because of differences in the type of food matrix or experimental conditions for RNA extraction and RT-PCR assay (25). Similarly, the different detection limits for strawberry and lettuce may be the result of the type of food matrix, drying conditions, viral strain, sample size, and debris such as seeds or leaves (8). Lettuce leaves have many wrinkles, and inoculated viral particles may persist on these leaves more readily than on strawberry surfaces.

Cell culture and RT-PCR assays are widely used for the detection of enteric viruses in foods (9, 29). Despite the many advantages of RT-PCR analysis, it does not allow differentiation between noninfectious and infectious viruses, making it necessary to test the efficiency of elution and concentration methods for the detection of infectious enteric viruses in food samples. Many investigators have provided information on the detection limits of infectious enteric viruses by using PCR-based assays (3, 29). However, few studies have been performed to determine the efficiency of several concentration methods using cell culture analysis (6). Using EV, AdV, and RV, we found that 6.30 to 15.7% of infectious viruses could be recovered from lettuces. These efficiencies were higher than those obtained with RT-PCR analysis. This difference is probably due to the fact that the initial inoculation volume of the AdV, EV, and RV was much higher than that of the NoV. In their preliminary study, Butot et al. (3) reported that 1 TCID$_{50}$ corresponds to 6,000 RTPCRU, and Senouci et al. (30) found that the recovery rate increased with the concentration of the virus in the samples.

Recently developed detection methods for viruses on vegetables and fruits have been restricted to relatively small food samples (5 to 25 g) (3, 10, 23, 25), corresponding to fewer than two strawberries or five lettuce leaves. Because the levels of enteric viruses on food surfaces are expected to be very low (26, 27) and consumption of vegetables and fruits per day can reach 100 g, improved methods for processing large food samples are needed. In the present study, we concentrated NoV from large lettuce samples (100 g). Because of the increased buffer volumes (500 to 600 ml), an additional concentration step was required for volume reductions. Secondary concentration steps have been used to concentrate large volumes of environmental water samples for the detection of enteric viruses (12, 18). The ultrafiltration-based secondary concentration step was used to reduce the sample volumes up to 140 μl and worked with the other procedures without any decrease of detection limit and recovery in lettuce samples (Table 2). This approach provides advantages such as the ability to process large food samples and to increase the detection possibility.

### Table 2. Recovery and detection limit of NoV from lettuce using different viral concentration methods

<table>
<thead>
<tr>
<th>No. of tests yielding virus at each limit of detection</th>
<th>Mean (range) % virus recovery $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,800</td>
<td>480</td>
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<tr>
<td>25 g of lettuce</td>
<td></td>
</tr>
<tr>
<td>PEG precipitation</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>100 g of lettuce</td>
<td></td>
</tr>
<tr>
<td>PEG precipitation with second concn</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$ Each concentration method was tested three times for each trial and each detection limit (0.48 to 4,800 RTPCRU).

$^b$ Percentage of virus recovered was calculated using the initial concentration of inoculated virus as 100%.

### Table 3. Recovery of cultivable enteric viruses from 25-g lettuce samples using two virus concentration methods

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean ± SD recovery (%) $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEG precipitation</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>9.1 ± 3.0</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>11 ± 4.9</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>11 ± 6.0</td>
</tr>
</tbody>
</table>

$^c$ Percentage of virus recovered was calculated using the initial TCID$_{50}$ value of inoculated virus as 100%. Each test was done in duplicate.
There is no standardized methodology for detecting a broad range of enteric viruses in vegetables and fruits. The modified procedure reported herein makes it possible to detect low levels of enteric viruses naturally present on raw vegetables. With this approach, more direct information on the potential presence of viral pathogens in foods will be provided to public health officials.

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REFERENCES


