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Elution Buffers for Human Enteric Viruses in Vegetables with Applications to Norovirus Detection

Aerie Moon, Jaehyun Ahn[1], and Weon Sang Choi*

Department of Biotechnology, College of Science and Technology, Dongguk University-Gyeongju, Gyeongju, Gyeongbuk, Korea
[1]Ourhome Food Research Institute, Seongnam, Gyeonggi, Korea
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ABSTRACT - The efficient elution of viruses from contaminated food is a critical step for its detection. In this study, conditions optimal for enteric viral recovery from three leafy vegetables (cabbage, lettuce, sesame leaf) and three root vegetables (carrot, onion, mooli) were analyzed to find common buffers (statistically not different) applicable to the leafy and root vegetables. Viral recovery varied depending on the food matrices or elution buffers. Buffer solutions containing 0.25M threonine / 0.3M NaCl (pH 9.5) or 0.25M glycine / 0.14M NaCl (pH 9.5) could efficiently recover poliovirus from five out of six vegetables. The threonine buffer was applied to one leafy vegetable (sesame) and one root vegetable (carrot) for genogroup II norovirus (NoV) detection. The detection sensitivity was significantly higher from the leafy vegetable compared to the root vegetable. The use of these common elution buffers should facilitate the detection of low levels of NoV and other enteric viruses in a wide range of vegetables.

Key words: *poliovirus, norovirus, elution, detection*

Introduction

The role of food in the transmission of viral gastroenteritis has been increasingly recognized, including foods such as vegetables and mollusks. The transmission of enteric viruses typically occurs through foods via a fecal-oral route. Enteric viruses can persist in the environment for long periods and are highly resistant to disinfectants, heat, pressure, and temperature[1)]. In addition, a few infectious viral particles are sufficient for inducing gastroenteritis[2)].

Fresh vegetables may be exposed to fecal contamination from numerous methods: irrigation with contaminated water, fertilization with inadequately composed manure, or handling by infected persons with poor hygiene[3)]. Salad vegetables are usually consumed raw after being washed with water; however, enteric viruses can survive this minimal treatment[4,5)]. Although procedures are available for the detection of enteric viruses (including norovirus (NoV)) in vegetables[3-9)], one of the most critical requirements for effective detection is the elution of viruses from vegetables. Once viral particles are dried on the surface of food or absorbed into the food, elution is difficult. The recovery can vary enormously depending on the food matrices and elution buffers used. Various kinds of buffers are used alone or combined for the elution step; however, elution buffers applicable to all kinds of vegetables have not been optimized. The CDC has recently developed a basic list of food commodities for outbreak analysis (http://www.cdc.gov/foodborneburden/attribution.html), with foods categorized according to the nature of the food source. In contrast, it is necessary to categorize foods in ways helpful for suggesting the elution buffers and methods required for the sensitive detection of enteric viruses in fresh vegetables. As sesame leaves, carrot, lettuce, onion, cabbage, and mooli are considerably consumed raw after or minimally cooked in many restaurants and homes in Korea, these vegetables represent a potential hazard for consumers. Therefore, it will be very useful to develop an efficient viral elution procedure for these vegetables according to the food source.

Poliovirus, a member of the *Picornaviridae* family, is a non-enveloped icosahedral virus containing a single positive-strand RNA genome. Poliovirus is frequently used as a surrogate, due to its similar size and morphology to NoV[3,8-13)], and the plaque assay is more convenient for the evaluation of viral recovery than RT-PCR. In this report, we describe common elution buffers for detecting enteric viruses in leafy (cabbage, lettuce, sesame leaf) and root (carrot, onion, mooli) vegetables and its application for detecting NoV in one leafy and one root vegetable.

*Correspondence to: Weon Sang Choi, Department of Biotechnology, College of Science and Technology, Dongguk University-Gyeongju, Gyeongju, Gyeongbuk, Korea
Tel: 82-54-770-2227, Fax: 82-54-770-2386
E-mail: weonsang@dongguk.ac.kr

Materials and Methods

Cell culture and viruses

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, and poliovirus titer was determined by plaque assays using HeLa cells[9]. The NoV GII used in this study was obtained from the Division of Enteric and Hepatitis Viruses at the National Institute of Health, (Seoul, Korea). The titer of NoV GII stock was 128 RT-PCR units/ml. The NoV titer in RT-PCR units was determined by endpoint dilutions. RT-PCR was performed in a MyCycler (Bio-Rad, USA). One RT-PCR unit was defined as the last dilution from which NoV RNA could be amplified and visualized from gel electrophoresis (Mupid, Takara) with ethidium bromide[9,14].

Reagents

One -step RT-PCR premix and PCR reagents were obtained from the Intron Biotechnology (Seongnam, Korea). Primers were purchased from Genotech (Daejeon, Korea). All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, USA).

Inoculation of vegetables

Sesame leaves, carrots, onions, and mooli were purchased at a local supermarket (Gyeongju, Korea) and tested negative for poliovirus and NoV through with PCR. An individual carrot (25 g), onion (25 g), and mooli (25 g) were cut into pieces (about 2 × 3 cm), rinsed in water, and allowed to dry for 1 hr in a laminar flow chamber. Individual sesame leaves (10 g) were also cut into pieces (about 1 × 1 cm) and were washed and dried in the same manner. A portion (10-100 μL) of a known plaque-forming unit (PFU) of poliovirus (10^5-10^6 PFU) or NoV (10-250 RT-PCR units) was spread over the vegetable surface and allowed to dry for 3 hr in a laminar flow chamber.

Viral recovery in the poliovirus plaque assay

Virus inoculated on the vegetables was eluted with elution buffer (150 mL) in a centrifuge tube and placed into a shaking incubator (20-23°C, 150 rpm) for 3 hr. There was very little difference in viral recovery between 3 and 16 incubations (data not shown). The aqueous phase was decanted into sterile 250 mL centrifuge bottles and five mL was used for the plaque assay after filtering with a syringe filter (0.45 μm porosity; Pall Corporation, USA). All experiments were performed at least three times independently and recovery were calculated based on the titer of the added poliovirus stock (set at 100%) counted on every trial.

Viral recovery and RNA extraction for RT-PCR

10-25 g of virus-inoculated vegetables were eluted with buffers, and the suspension was further purified to remove RT-PCR inhibitors. 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, polyethylene glycol (PEG) 8000 and NaCl were added to achieve a final concentration of 11% PEG and 0.3M 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 11,000 × g for 20 min and the pellets were resuspended in 1-3 mL of diethyl pyrocarbonate (DEPC)-treated water. The QIAmp Viral RNA mini kit (Qiagen, USA) was used to extract RNA from the concentrated viruses according to the manufacturer's instructions with minor modification. Instead of 140 μL of viral concentrates, 280 μL was used for RNA extraction.

RT-PCR and RT-PCR combined with nested PCR

Reaction conditions and the primers (DG172, DG173, DG213, DG214, GII-F1M, GII-R1M, and GII-F3M) for RT-PCR, or RT-PCR combined with nested PCR, have previously been described[9,11,15]. The 340 and 524 bp RT-PCR products for GII NoV and poliovirus, respectively, and the 310 and 506 bp RT-PCR (combined with nested PCR) products for GII and poliovirus, respectively, were separated by electrophoresis (Mupid, Takara) on 1.5% agarose gels containing ethidium bromide and visualized with ultraviolet (UV) light.

Statistical analysis

Results obtained were subjected to *F*-test using SPSS version 18. A *P* value of 0.05 was considered to represent the minimum level of significance.

Results and Discussion

Poliovirus recovery from vegetables by elution

The elution of viruses from contaminated food is a critical step for efficient viral detection. Elution efficiency can vary according to the food matrices, isoelectric point of viral surface proteins, electrostatic interactions between viruses and food surface, different stabilities of viruses under drying conditions, smoothness of the food surface, and viral resistance at low pH on the food surface[3,10,16-20]. Beef extract[4,21], amino acid buffer[3,8,22], Tris buffer[23], amino acid-NaCl buffer[3,9,14,19,24], Tris-glycine buffer[3,25], or a combination of these buffers[6,12,19,26] were examined for their ability to elute virus from produce in several studies. In this study, several buffers including PBS, beef extract, glycine buffers, and threonine buffers were compared for viral recovery from one

Table 1. Recoveries of poliovirus from vegetables using different elution buffers[a] (Unit: %)

Buffer numbers	Elution buffers	pH	cabbage[b]	lettuce[b]	sesame leaves	carrot	onion	mooli
1	PBS	7.4	5.7 ± 0.9	5.0 ± 1.6	3.7 ± 1.4	17.1 ± 1.7	11.8 ± 7.3	33.4 ± 9.3
2	3% beef extract	9.5	7.4 ± 1.4	4.8 ± 1.3	5.5 ± 0.6	71.5 ± 2.1	7.3 ± 1.9	58.3 ± 15.7
3	0.05M Gly-0.14M NaCl	7.5	46.6 ± 40.3	7.8 ± 0.8	10.3 ± 3.5	25.2 ± 6.1	7.5 ± 4.7	12.9 ± 5.1
4	0.05M Gly-0.14M NaCl	9.5	73.4 ± 15.1	42.2 ± 7.5	40.2 ± 16.9	49.9 ± 15.19	N/D[c]	N/D
5	0.05M Gly-0.3M NaCl	7.5	37.6 ± 21.9	5.8 ± 0.8	23.8 ± 7.9	N/D	N/D	N/D
6	0.05M Gly-0.3M NaCl	9.5	50.8 ± 28.9	48.5 ± 11.2	57.5 ± 30.4	N/D	10.3 ± 4.3	78.2 ± 7.9
7	0.25M Gly-0.14M NaCl	7.5	50.1 ± 21.0	8.1 ± 0.9	31.3 ± 14.9	49.3 ± 24.4	N/D	30.6 ± 16.0
8	0.25M Gly-0.14M NaCl	9.5	61.9 ± 5.5	46.0 ± 19.1	44.6 ± 13.2	54.3 ± 16.1	25.6 ± 5.5	55.4 ± 11.2
9	0.25M Gly-0.3M NaCl	7.5	61.1 ± 41.0	27.3 ± 16.2	45.7 ± 22.2	N/D	N/D	41.8 ± 16.1
10	0.25M Gly-0.3M NaCl	9.5	68.3 ± 43.9	34.9 ± 19.6	30.9 ± 12.8	N/D	N/D	N/D
11	0.05M Thr-0.14M NaCl	7.5	20.0 ± 4.5	8.3 ± 2.2	9.7 ± 2.6	11.6 ± 6.2	6.6 ± 2.3	37.7 ± 7.9
12	0.05M Thr-0.14M NaCl	9.5	54.2 ± 24.3	42.6 ± 19.2	33.6 ± 12.8	93.3 ± 9.5	8.5 ± 5.6	43.1 ± 10.1
13	0.05M Thr-0.3M NaCl	7.5	59.0 ± 7.9	29.2 ± 17.8	17.0 ± 7.7	N/D	N/D	53.1 ± 3.0
14	0.05M Thr-0.3M NaCl	9.5	82.6 ± 13.3	50.3 ± 13.2	33.6 ± 13.5	51.1 ± 8.1	25.5 ± 3.2	N/D
15	0.25M Thr-0.14M NaCl	7.5	82.3 ± 8.1	53.3 ± 11.3	33.3 ± 11.5	53.0 ± 20.7	N/D	29.1 ± 10.0
16	0.25M Thr-0.14M NaCl	9.5	77.2 ± 11.9	46.1 ± 23.7	28.4 ± 8.4	43.3 ± 26.3	N/D	47.9 ± 15.4
17	0.25M Thr-0.3M NaCl	7.5	89.1 ± 13.3	56.2 ± 13.3	37.1 ± 13.0	N/D	N/D	35.1 ± 6.2
18	0.25M Thr-0.3M NaCl	9.5	90.0 ± 13.3	62.7 ± 19.7	44.7 ± 8.6	80.3 ± 10.1	35.2 ± 5.0	33.5 ± 9.3

a: recovery was calculated based on the titer of the added poliovirus stock (set as 100%) counted on every trial (average ± standard deviation)
b: modified data from Moon et al. 2009[9)]
c: N/D; not tested

Table 2. Selected buffers for the efficient elution of poliovirus from vegetables

	Leafy vegetables			Root vegetables		
	cabbage	lettuce	sesame leaf	carrot	onion	mooli
Selected buffer numbers from Table 1[a]	4,8,9,10,14,15,16,17,18 $P = 0.1688$ ≥ 61.1%[b]	4,6,8,12,14,15,16,17,18 $P = 0.3728$ ≥ 42.2%[b]	4,6,8,9,18 $P = 0.4449$ ≥ 40.2%[b]	2,12,18 $P = 0.0706$ ≥ 71.5%[b]	8,14,18 $P = 0.0802$ ≥ 25.5%[b]	2,6,8,13 $P = 0.0674$ ≥ 53.1%[b]
Top 3 buffers	14,17,18 $P = 0.5710$	15,17,18 $P = 0.5472$	6,9,18 $P = 0.4379$	2,12,18 $P = 0.0706$	8,14,18 $P = 0.0802$	2,6,8 $P = 0.1437$

a: Buffers tested were numbered at Table 1. High recovery buffers, statistically not different, were selected.
b: Minimum recovery of the selected buffers within group is shown.

leafy vegetable (sesame leaf) and three root vegetables (carrot, onion, mooli) (Table 1). The recovery data for the leafy vegetables (cabbage and lettuce) from Moon et al.[9)] were analyzed together with the data of this study for finding common elution buffers. The efficacy of a buffer widely varied according to the food matrices. All the tested buffers only eluted limited amounts of virus from onions (6.6-35.2%). Phosphate buffered saline (PBS), previously used to elute viruses from raw vegetables[3,5,27-29)], was generally inefficient, compared with other buffers, at recovering poliovirus. In our study, only 3.7-33.4% of polioviruses were recovered with PBS (pH 7.4) from sesame leaf, carrot, onion and mooli. In the previous study, 5.0-5.7% of polioviruses were eluted from cabbage and lettuce[9)]. Beef extract (3%, pH 9.5) was highly efficient for elution from two root vegetables (71.5% in carrot, 58.3% in moolis), whereas only 4.8-7.4% was recovered from sesame leaf (Table 1). Beef extract was also not efficient from two leafy vegetables (cabbage, lettuce) in the previous report[9)]. To find common buffers (statistically not different) applicable to all the vegetables tested, buffers that had a good and consistent elution capacity were selected from Table 1 (summarized in Table 2). Buffers at pH 9.5 generally had a tendency to recover more poliovirus particles from food surfaces, probably due to protection against the acidic pH on the food surface[26)]. In addition, a better recovery was generally observed when glycine or threonine buffers with a concentration of 0.25M were used (compared to buffers with a concentration of 0.05M). Two buffers, 0.25M

glycine and 0.14M NaCl (pH 9.5) (#8), or 0.25M threonine and 0.3M NaCl (pH 9.5) (#18) could efficiently recover poliovirus from five out of six vegetables (Table 2; three leafy and two root vegetables). Based on the results, we deduced that buffers containing 0.25M threonine / 0.3M NaCl (pH 9.5) (#18) or 0.25M glycine /0.14M NaCl (pH 9.5) (#8) are good common viral detection buffers applicable to leafy and root vegetables.

Detection sensitivity

The sensitivity of NoV GII detection by RT-PCR combined with nested PCR was 0.2 RT-PCR units, which was determined using serial dilutions of stool samples (data not shown). The level of NoV GII detection for the procedure was determined by inoculating serially-diluted NoV GII on sesame leaves (leafy vegetables) or carrots (root vegetables), processing with 0.25M threonine/0.3M NaCl (pH 9.5), and then examining final RNA concentrates by RT-PCR combined with semi-nested PCR (NoV GII). Poliovirus (10,000 pfu) was used as a positive control to monitor proper processing. With the initial seeding levels, 100 RT-PCR units of GII NoV/10 g sesame leaves were detected, whereas 50 RT-PCR units of GII NoV/25 g carrot were detected (Fig. 1). No sample inhibition was observed in any of the samples tested, as dilution prior to amplification didn't improve viral detection[14,30] (data not shown). Detection limits can widely differ according to the analytical method used (i.e., RT-PCR, RT-PCR with nested PCR, or real-time PCR), sample amount tested, virus strains, and the primer sets used[8]. Therefore, direct comparison of analyses is difficult due to variations in food matrices and the methods employed[26]. The detection limits obtained with our procedure are roughly similar to the detection limits previously reported (10-1,500 RT-PCR units/ 6-50 g food)[6,14,19,25,30,31], with the exception of work that reported as little as 1 RT-PCRU of NoV in green onions[7]. Nevertheless, that work was not validated for other food matrices. The number of NoV genome copies per RT-PCR unit has been estimated to be as low as 10-50[32,33]. Therefore, about 500-5,000 GII NoV particles, detected by RT-combined with semi-nested PCR, can be detected in 10-25 g of leafy or root vegetables with this procedure.

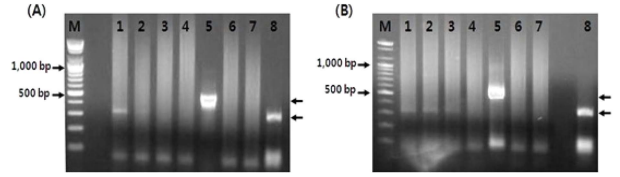

Fig. 1. Detection of noroviruses in sesame leaves and carrots by RT-PCR combined with semi-nested PCR. (A) 10 g of sesame leaves or (B) 25 g of carrots were artificially contaminated with GII NoV. Lane M, molecular size marker (100 bp ladder); lane 1, 250 RT-PCR units of NoV; lane 2, 100 RT-PCR units of NoV; lane 3, 50 RT-PCR units of NoV; lane 4, 10 RT-PCR units of NoV; lane 5, 10,000 PFU of poliovirus as a positive control to monitor proper processing; lanes 6 & 7, negative control; lane 8, positive control (GII NoV cDNA was amplified by PCR).

In summary, 18 buffers were compared for eluting viruses in vegetables. Our results suggest that buffers with 0.25M threonine / 0.3M NaCl (pH 9.5) or 0.25M glycine / 0.14M NaCl (pH 9.5) can serve as common buffers useful for eluting viruses in leafy and root vegetables. The use of these common elution buffers should facilitate the detection of low levels of NoV and other kinds of enteric viruses found in a wide range of vegetables.

요 약

오염된 식품으로부터 바이러스를 효과적으로 검출하기 위해서는 식품에 부착된 바이러스를 효과적으로 elute시키는 것이 결정적으로 중요하다. 본 연구에서는 채소류에 공통적으로 적용할 수 있는 elution용액을 찾기 위해 폴리오바이러스를 인위적으로 오염시킨 1가지 엽채류 (깻잎) 와 3가지 근채류 (당근, 양파, 무) 로부터 바이러스 회수율을 조사한 후, 최적의 바이러스 회수조건을 찾기 위해 기보고된 상추와 양배추의 회수율을 함께 분석하였다. 바이러스의 회수율은 식품의 matrix와 사용된 elution용액의 종류에 따라 차이가 컸으나 0.25M threonine / 0.3M NaCl (pH 9.5) 또는 0.25M glycine / 0.14M NaCl (pH 9.5)을 사용하였을 때 6가지 채소 중 5가지로부터 폴리오바이러스를 효과적으로 elute 할 수 있었다. 0.25M threonine / 0.3M NaCl (pH 9.5)를 노로바이러스 검출에 적용해 본 결과 근채류인 당근보다 엽채류인 깻잎으로부터 노로바이러스 GII를 더 잘 검출할 수 있었다. 이 같은 공통 elution용액을 사용할 경우 다양한 종류의 채소류에 오염된 노로바이러스를 포함한 소화기바이러스의 검출을 용이하게 해 줄 것이다.

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