jmb

Simultaneous Detection of Four Foodborne Viruses in Food Samples Using a One-Step Multiplex Reverse Transcription PCR

Shin-Young Lee[1], Mi-Ju Kim[1], Hyun-Joong Kim[1], KwangCheol Casey Jeong[1,2], and Hae-Yeong Kim[1]*

[1]Institute of Life Sciences & Resources and Department of Food Science & Biotechnology, Kyung Hee University, Yongin 17104, Republic of Korea
[2]Department of Animal Sciences and Emerging Pathogens Institute, University of Florida, Gainesville, FL 32601, USA

A one-step multiplex reverse transcription PCR (RT-PCR) method comprising six primer sets (for the detection of norovirus GI and GII, hepatitis A virus, rotavirus, and astrovirus) was developed to simultaneously detect four kinds of pathogenic viruses. The size of the PCR products for norovirus GI and GII, hepatitis A virus (VP3/VP1 and P2A regions), rotavirus, and astrovirus were 330, 164, 244, 198, 629, and 449 bp, respectively. The RT-PCR with the six primer sets showed specificity for the pathogenic viruses. The detection limit of the developed multiplex RT-PCR, as evaluated using serially diluted viral RNAs, was comparable to that of one-step single RT-PCR. Moreover, this multiplex RT-PCR was evaluated using food samples such as water, oysters, lettuce, and vegetable product. These food samples were artificially spiked with the four kinds of viruses in diverse combinations, and the spiked viruses in all food samples were detected successfully.

Keywords: One-step multiplex reverse transcription PCR, simultaneous detection, norovirus, hepatitis A virus, rotavirus, astrovirus

Introduction

Acute gastroenteritis related to viral food poisoning is a major human health problem worldwide. Although the mortality of viral gastroenteritis is low, its influence is significant in terms of the economy and its high morbidity rates [1]. The symptoms of viral foodborne disease/poisoning are often vomiting, diarrhea, nausea, headache, and low-level fever [2]. One characteristic of viral food poisoning is that a small amount of virus particles can lead to illness in humans [3]. Moreover, these viruses are stable in the environment, transmitted via the fecal-oral route, and rapidly spread, and contribute to secondary infections [2, 3]. Major viruses causing gastroenteritis in humans through food consumption include the norovirus (NoV) GI and GII genogroups, hepatitis a virus (HAV), astrovirus (AstV), and rotavirus (RoV) [2, 3]. Representative viral-contaminated foods include water, shellfish (oysters), and fresh vegetables [4–10]. Prior reports of viral outbreaks from foods have inspired many additional studies related to viral contamination in foods [4, 7, 11, 12].

Several common detection methods for foodborne viruses include electron microscopy (EM) examination, virus isolation techniques, the enzyme-linked immunosorbent assay (ELISA), and molecular methodologies such as dot blot hybridization and reverse transcription PCR (RT-PCR) [13, 14]. Each method has its own disadvantages concerning sensitivity and reproducibility. In the case of EM analysis and the direct immunoassay methods, there are limitations in that these methods need highly concentrated samples of virus (at least 10^6 particles per milliliter) to allow detection. In contrast, molecular-based methods such as conventional PCR or quantitative reverse transcription PCR (RT-qPCR) exhibit high specificity and sensitivity and can quickly detect small amounts of virus with accuracy [15].

Although quantitative methods like RT-qPCR are available for the detection of viruses, the application of conventional RT-PCR combined with sequencing analysis is still important as a standard method for epidemiological studies of viral outbreaks. This is because viruses mutate quickly to adapt to their surroundings, causing various genotypes with different sequences.

For this reason, conventional multiplex RT-PCR could be a useful method for the specific and rapid diagnostic monitoring and simultaneous detection of foodborne pathogenic viruses. This could positively impact on future epidemiological studies and the food industry [14, 16].

In this study, four kinds of RNA viruses (NoV GI and GII, HAV, AstV, and RoV) were selected because of their frequent contributions to viral food poisoning in Korea. A total of six primer sets for these four viruses were selected, designed to amplify specific target regions. Target regions on capsid proteins with low mutation rates were selected. The one-step multiplex RT-PCR method was developed to simultaneously analyze these primer sets, and may serve as a rapid and accurate improvement to existing methods like sequencing analysis. The method could also be applied in the clinic or in the food industry and could contribute to food safety management.

Materials and Methods

Viruses and Clinical Samples

NoV GI, NoV GII, and AstV were provided by the Waterborne Virus Bank at Catholic University in Korea. The HAV HM175 strain, grown in fetal rhesus kidney-derived (FRhK-4) cells, and group A human RoV, grown in a monkey kidney cell line (MA104), were obtained from the Ministry of Food and Drug Safety (MFDS) in Korea. All the specimens were stored at –80°C until use.

Extraction of Viral RNA

Fecal specimens of NoV GI, NoV GII, and AstV were weighed to 0.1 g, and 600 µl of phosphate-buffered saline was added to the samples in sterile microcentrifuge tubes. After vortexing for 30 min, the stool samples were centrifuged at 16,200 ×g for 30 min at 4°C and the supernatants were used for RNA extraction. In the case of HAV and RoV, cell-cultured liquid samples were directly harvested for viral nucleic acids after mixing well. Total RNA was extracted from 140 µl of the fecal suspensions of NoV and AstV and from the cell culture samples of HAV and RoV using a QIAamp Viral RNA kit (Qiagen, USA) according to the manufacturer's instructions. The extracts were eluted in 60 µl of diethylpyrocarbonate (DEPC)-treated water that was certified as RNase- and DNase-free, and the eluted RNA was stored at –80°C until use.

PCR Primers

Six sets of primers for specific viruses were used in this study (Table 1). All of the primer sets targeted a gene for the capsid protein of the relevant virus. A pair of primers (GI-FIM and GI-RIM) was used for detecting NoV GI, generating a 330 bp PCR product [17]. To detect HAV, a 244 bp PCR product of the VP3/VP1 junction region was generated using the two primers HAV1 and HAV2 [18]. Primers named MON269 and MON270 were used for amplifying AstV, generating an amplicon size of 449 bp [19]. The two primer sets for NoV GII and HAV (P2A region) were newly designed in this study using the Primer Designer program, ver. 3.0 (Scientific and Educational Software, USA) based on conserved sequences of reference strains of NoV and HAV obtained from the GenBank database. These two pairs of primers generated 164 bp and 198 bp PCR amplicons for NoV GII and HAV, respectively. In the case of RoV, the reverse primer, originally designed on the basis of the VP7 gene, was modified in this study

Table 1. Primer sequences used in this study.

Target virus	Primer	Final concentration (µM)	Sequence (5′ → 3′)[a]	Polarity[b]	Amplicon size (bp)	Reference
Norovirus GI	GI-F1M	0.8	CTG CCC GAA TTY GTA AAT GAT GAT	+	330	Kojima *et al.* [17]
	GI-R1M		CCA ACC CAR CCA TTR TAC ATY TG	-		
Norovirus GII	GII-2F3	0.4	CAC CCC TCA CTG GTC AGC AAA AT	+	164	This study
	GII-2R3		AGC AAG ATG GGC CAA ATA GGG AT	-		
HAV (VP3/VP1)	HAV1	0.4	GCT CCT CTT TAT CAT GCT ATG GAT	+	244	Bower *et al.* [18]
	HAV2		CAG GAA ATG TCT CAG GTA CTT TCT	-		
HAV (P2A)	HAV-F2	0.4	GCT GGA GAC TTG GAG TCA TC	+	198	This study
	HAV-R2		GGC TTG TGA AAA CAG TCC CT	-		
Rotavirus	VP7-F	0.4	ATG TAT GGT ATT GAA TAT ACC AC	+	629	Iturriza-Gomara *et al.* [20]
	Rota-R2		GTT GTT TGA CAA CCT ATC CCT AAC G	-		
Astrovirus	Mon 269	0.5	CAA CTC AGG AAA CAG GGT GT	+	449	Noel *et al.* [19]
	Mon 270		TCA GAT GCA TTG TCA TTG GT	-		

[a]Mixed based; Y = T + C

[b] +, Forward primer; -, Reverse primer.

[20]. All primer sets were synthesized by BIONICS Inc., (Korea) and all information for the primers is provided in Table 1.

One-Step Single RT-PCR

One-step single RT-PCR was performed with 5 μl of total extracted RNA, 4 μl of dNTPs (each dNTP at 2.5 mM; Bioneer, Korea), 5 μl of 10× reaction buffer, 1 μl of Top DNA polymerase (5 U/μl; Bioneer), 1 μl of avian myeloblastosis virus (AMV) reverse transcriptase (10 U/μl; Progema, USA), 1 μl of each forward/reverse primer, and DEPC-treated water in a final reaction volume of 50 μl. The optimal concentration of each primer set is shown in Table 1.

The RT-PCR amplification was performed in a thermocycler (ASTEC, Japan) with an initial cDNA synthesis (reverse transcription) step at 42°C for 40 min, followed by pre-denaturation for 2 min at 94°C. Next, the samples underwent 45 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C. Post-elongation was performed for 7 min at 72°C. The single RT-PCR products were electrophoresed on a 2% agarose gel in 0.5× TAE buffer containing ethidium bromide (0.5 μg/ml) and were visualized under ultraviolet light.

One-Step Multiplex RT-PCR

In the one-step multiplex RT-PCR, 5 μl of mixed RNA (each RNA was mixed, then serially diluted) was used to perform the assay with 8 μl of dNTPs (each dNTP at 2.5 mM), 5 μl of 10× reaction buffer, 1 μl of Top DNA polymerase (5 U/μl), 1 μl of AMV reverse transcriptase (10 U/μl), and DEPC-treated water to 50 μl. The six primer pairs were mixed in a single tube. The same concentrations of primers and the same thermal cycler program described above were used. The multiplex RT-PCR amplicons were analyzed on a 3% agarose gel in 0.5× TAE buffer containing ethidium bromide, and the results were recorded by photography under UV light.

Limit of Detection for One-Step Single and Multiplex RT-PCRs

To compare the detection limits of the one-step single and multiplex RT-PCR methods, 10-fold serial dilutions (10^0 to 10^{-5}) of five different viral RNA samples in DEPC-treated water were tested using both assays. In the one-step single RT-PCR, the PCR mixture conditions were the same as those of the one-step multiplex RT-PCR except that a single, specific primer set for each target virus was contained in a separate reaction tube. The one-step single and multiplex RT-PCR methods were performed using the same dilution series, the same PCR machine, and the same thermal cycling conditions.

Assays Using Artificially Contaminated Food and Water

On the basis of the frequency of viral food poisoning, the one-step multiplex RT-PCR method was performed with representative water and food samples, including oysters, lettuce, and vegetable product. After artificially inoculating viruses into samples that tested negative for each virus, the samples were handled with different methods depending on the sample type. All of the procedures were conducted according to the recommended guidelines established by the MFDS for the detection of foodborne viruses, with minor modifications.

For the contaminated water sample, virus adsorption-elution techniques were used [5]. First, the four kinds of viruses were mixed in water, and the solution was allowed to flow through a water sampling device to adsorb on a positively charged filter. After that, 1.5% beef extract buffer was used to extract viruses from the charged filter, and the solution was adjusted to pH 7.0–7.5 with 1 M HCl. Next, the extract was adjusted to pH 3.5, stirred at room temperature for 30 min, and centrifuged at 16,200 ×*g* for 15 min. After centrifugation, the supernatant was removed, and the precipitate was eluted with 20–30 ml of 0.15 M Na_2HPO_4. The final solution was adjusted to pH 7.0–7.5 with 1 M HCl. The solution was used for viral RNA purification.

In the case of the oyster samples, the midgut glands were separated with autoclaved tweezers and scissors and mixed with the prepared virus samples in 50 ml tubes. Proteinase K (25 mg/ml) was added to the tubes at a 1:1 ratio, and the samples were homogenized completely. The resulting solutions were incubated at 37°C on a shaker for 60 min and placed in a water bath at 60°C for 15 min. After that, the solutions were centrifuged twice at 3,400 ×*g* for 5 min. The final supernatants were used for viral RNA extraction.

For the lettuce samples, a pre-mixed virus solution was spread onto lettuce purchased at a market, and the leaves were dried at room temperature for 60 min. The artificially contaminated lettuce leaves were cut with autoclaved tweezers and scissors. The lettuce slices were put in 50 ml tubes and used as pretreated samples. The extract solution (pH 9.5) contained 0.25 M threonine and 0.3 M NaCl, and a volume of 9.5 times the quantity of each lettuce sample was added. In a shaking incubator, the solutions were shaken for 1 h and stored at 4°C for 1 h to remove bubbles. The liquid from each sample was transferred to 50 ml tubes and centrifuged at 3,400 ×*g* for 30 min, and the supernatants were transferred to new 50 ml tubes. To concentrate the viruses in the lettuce samples, 40% PEG 8000 (polyethylene glycol) and 3 M NaCl were added at volumes equaling 45% and 15% of each supernatant, respectively. These solutions were precipitated at 4°C for 16 h in an incubator with stirring. After the primary precipitation, the concentrates were transferred to autoclaved tubes and centrifuged at 16,200 ×*g* for 20 min. Leaving a small quantity, most of each supernatant was removed, and the precipitate on the wall of each 50 ml tube was dissolved in the remaining liquid. The resulting solutions were purified using chloroform, and a secondary concentration procedure was conducted using the same method described above. After the second concentration, the concentrates were centrifuged at 16,200 ×*g* for 20 min, and all of the supernatants were eliminated. For the final elution, precipitates on the walls of the tubes were eluted with DEPC-treated water.

For the vegetable product samples, virus solutions and

uncontaminated liquid from the vegetable product were combined in 50 ml tubes. The samples were centrifuged at 3,400 ×g for 30 min, and the supernatants were re-centrifuged under the same conditions to remove floating particles of vegetable product. After centrifugation, the supernatants were used to extract viral nucleic acids.

Sequence Analysis

The PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen). Sequencing was performed using an automated DNA sequencer (Applied Biosystems, USA). Sequence analysis of the PCR products was carried out using specific forward and reverse primers. The results were compared with the sequences in GenBank of the National Center for Biotechnology Information using the Basic Local Alignment Search Tool.

Results and Discussion

Specificity of the Six Primer Sets

A specificity analysis of the six primer sets was conducted with other kinds of viruses and foodborne pathogenic bacteria. A specific PCR amplicon was obtained from the single virus samples and from a mixture of five viral RNA samples, generating bands of the expected size without nonspecific bands. There were no cross-contamination cases, as shown in Fig. 1. To further confirm the specificity of the primers, different pathogenic bacteria (*Bacillus cereus, Campylobacter jejuni, Shigella flexneri, Staphylococcus aureus*, and *Escherichia coli* O157:H7) were used and no amplicons were generated with these five pathogens (data not shown).

Limit of Detection Test for the One-Step Single and Multiplex RT-PCRs

To compare the sensitivity of the one-step single and multiplex RT-PCR methods, the same 10-fold diluted samples were utilized. In the one-step single RT-PCR, NoV GI, NoV GII, HAV(VP3/VP1), HAV(P2A), RoV, and AstV showed a positive result at the 10^{-2}, 10^{-2}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-3} dilution, respectively (Figs. 2A−2F). The detection limits of the one-step multiplex RT-PCR were seen at the 10^{-1} dilution for NoV GI, NoV GII, RoV, and AstV, and at the 10^{-2} dilution for HAV(VP3/VP1) and HAV(P2A) (Fig. 2G). Overall, for NoV GI, NoV GII, HAV (P2A region), and RoV, the sensitivity of the multiplex RT-PCR was reduced 10-fold compared with the single RT-PCR. For HAV (VP3/VP1 junction region) and AstV, the detection limits were reduced 100-fold.
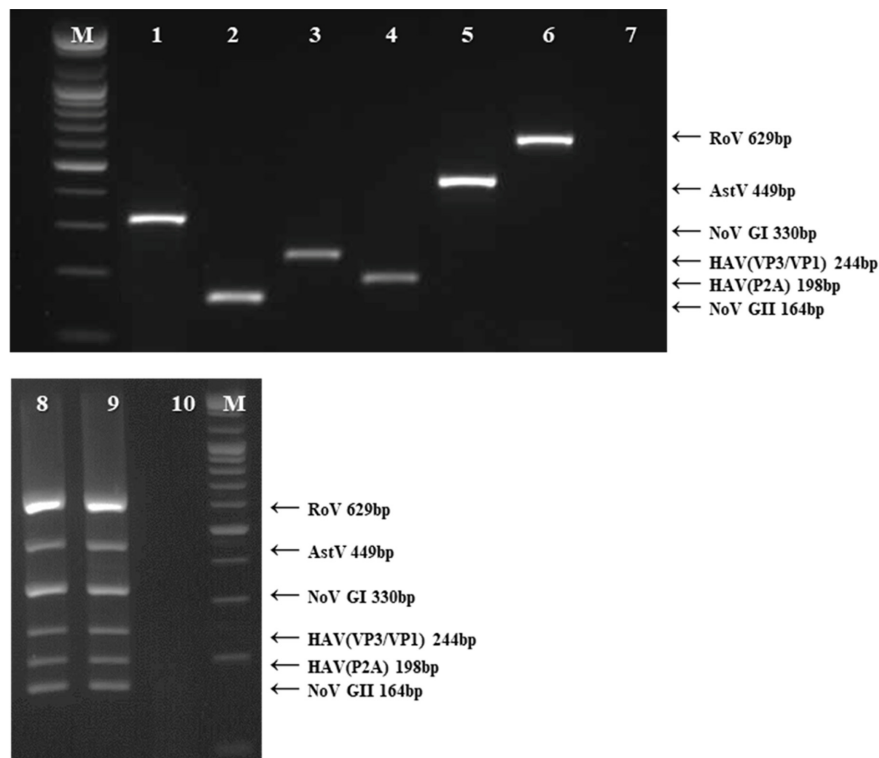
Fig. 1. Specificity testing for the one-step multiplex PCR assay using all of the primer pairs for six positive controls.
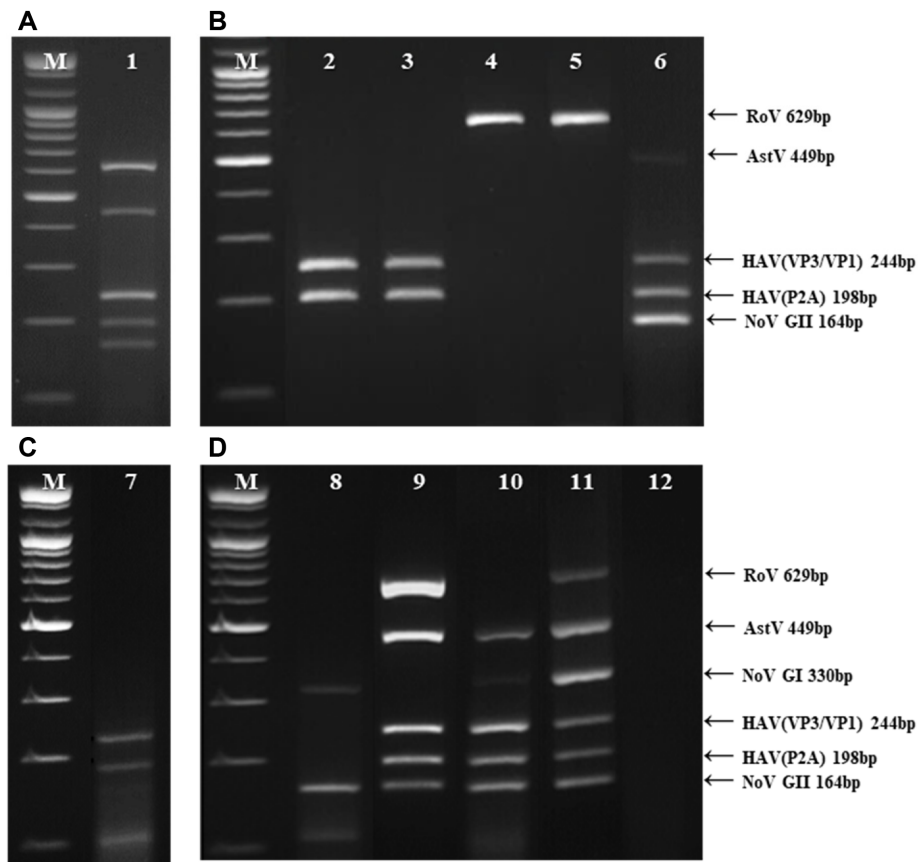Lanes: M, 100 bp DNA ladder; 1, NoV GI; 2, NoV GII; 3, HAV(VP3/VP1 region); 4, HAV(P2A region); 5, AstV; 6, RoV; 7, no template; 8-9, NoV GI, NoV GII, HAV, AstV, and RoV; 10, no template. AstV, astrovirus; HAV, hepatitis A virus; NoV, norovirus; RoV, rotovirus.
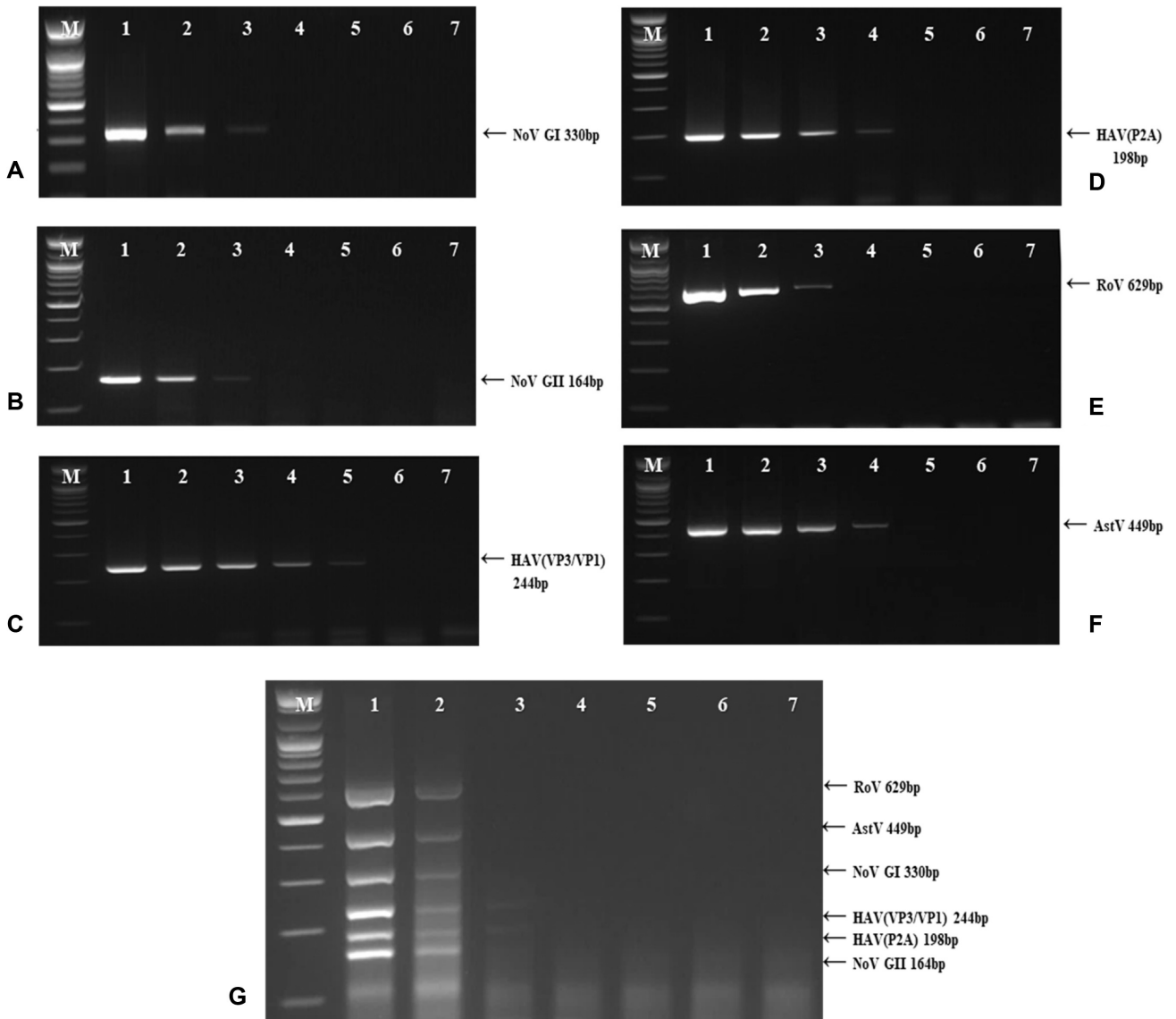
Fig. 2. Comparison of the sensitivity between one-step single and multiplex PCRs using 10-fold serial dilutions of total viral RNA. (**A**) NoV GI, (**B**) NoV GII, (**C**) HAV (VP3/VP1), (**D**) HAV (P2A), (**E**) RoV, (**F**) AstV, (**G**) NoV GI, NoV GII, HAV, RoV, and AstV. Lanes: M, 100 bp DNA ladder; 1, undiluted; 2, 10-fold; 3, 100-fold; 4, 1,000-fold; 5, 10,000-fold; 6, 100,000-fold; and 7, no template. AstV, astrovirus; HAV, hepatitis A virus; NoV, norovirus; RoV, rotovirus.

The major disadvantage of a multiplex PCR method is decreased sensitivity, caused by increased competition between reagents when multiple templates are combined in a single tube [16]. However, multiplex PCR is able to detect various targets simultaneously in a single reaction, and the method can discriminate between specific viruses on the basis of PCR product size. Thus, despite a relatively low sensitivity, multiplex PCR could be a more effective method to analyze viral food poisoning because of cost efficiency, high reproducibility, and reliability. Currently, there are many studies related to viruses in foods [7, 12, 21–24]. Certain problems inherent to the analysis methods common in these studies could be solved with the adoption of multiplex PCR. To understand the extent of the interference between primers and nucleic acids, two primer sets were used with all of the target viral RNA samples, and the primer concentrations were adjusted. Using these assays, the one-step multiplex RT-PCR method was optimized. RoV has a double-stranded RNA, unlike the other viruses [25]. Thus, when the RNA of RoV was pre-heated, the

Fig. 3. Multiplex PCR using artificially contaminated water and food samples.
(**A**) Water, (**B**) Oysters, (**C**) Lettuce, (**D**) vegetable product. Lanes: M, 100 bp DNA ladder; 1, water sample with NoV GII, HAV, AstV, and RoV; 2-3, oysters with HAV; 4-5, oysters with RoV; 6, oysters with HAV, NoV GII, and AstV; 7, lettuce with HAV; 8, vegetable product with NoV GI and NoV GII; 9, vegetable product with NoV GII, HAV, RoV, and AstV; 10, vegetable product with NoV GI, NoV GII, HAV, and AstV; 11, vegetable product with NoV GI, NoV GII, HAV, RoV, and AstV; 12, no template. AstV, astrovirus; HAV, hepatitis A virus; NoV, norovirus; RoV, rotovirus.

intensity of the PCR amplicon for RoV was improved.

Tests of the Artificially Contaminated Water and Food Samples

The developed assay was applied to water, oysters, lettuce, and vegetable product samples that were artificially inoculated with the viruses. After the inoculated samples were processed (using different methods, depending on the sample type), the resulting solutions were used to extract viral RNA. The extracts were then tested using the developed method. All samples yielded specific detection with the expected amplicon for the relevant virus (Fig. 3).

Virus adsorption-elution techniques have commonly been used for the concentration of viruses from large amounts of water. Assays for virus adsorption have used positively charged filters because viruses in natural water present negative charges on their surfaces [21, 26, 27]. Thus, a

positively charged filter was also used in this study. In the case of oysters, it is known that the midgut can specifically bind virus particles [11]. Fresh lettuce and frozen berries are problematic because they can harbor HAV [4, 28–30]. In Korea, a less fermented vegetable product is infrequently associated with acute gastroenteritis in humans, likely due to the use of untreated groundwater [9]. Focusing on these foods, we evaluated whether our multiplex PCR method could be applied. All of the samples tested were analyzed successfully. However, it might be necessary to validate the method with other types of foods, with naturally contaminated foods, and on a larger scale.

Because RNA viruses mutate easily, they often have diverse sequences and generate variants able to adapt to the surrounding environment. Thus, molecular epidemiological studies of viral foodborne infections are important and should include an analysis of phylogenetic trees. By its

nature, a real-time RT-PCR assay yields small amplicons of approximately 85−150 bp, and these amplicons are difficult to sequence and analyze for the construction of phylogenetic trees [31]. For this reason, conventional RT-PCR methods are still required for a thorough sequence analysis. In this study, to make it possible to do direct sequencing on the multiplex RT-PCR amplicons, a one-step multiplex RT-PCR method was developed with NoV GI, NoV GII, HAV, AstV, and RoV. On the basis of existing primer sets, specific primer pairs were selected, and some primer sets were re-designed to prevent PCR amplicons from overlapping (and to increase sensitivity). Although HAV has low heterogeneity, it can be divided into variants causing human or non-human infection [32, 33]. Thus, in this study, two primer sets were used to amplify different regions of HAV. Finally, this method was applied to water and various foods, and it was possible to detect the specific viruses in the different samples. Based on these results, this assay has the potential to serve as an effective diagnostic tool for identifying four kinds of RNA viruses (norovirus, hepatitis A virus, rotavirus, and astrovirus) that cause gastroenteritis. The multiplex RT-PCR assay developed in this study could be a reliable method for the rapid, accurate, and simultaneous analysis of foodborne viruses relevant to food safety.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Van Maarseveen NM, Wessels E, de Brouwer CS, Vossen AC, Claas EC. 2010. Diagnosis of viral gastroenteritis by simultaneous detection of adenovirus group f, astrovirus, rotavirus group A, norovirus genogroups I and II, and sapovirus in two internally controlled multiplex real-time PCR assays. *J. Clin. Virol.* **49:** 205-210.
2. Clark B, McKendrick M. 2004. A review of viral gastroenteritis. *Curr. Opin. Infect. Dis.* **17:** 461-469.
3. Koopmans M, Duizer E. 2004. Foodborne viruses: an emerging problem. *Int. J. Food Microbiol.* **90:** 23-41.
4. Butot S, Putallaz T, Sanchez G. 2007. Procedure for rapid concentration and detection of enteric viruses from berries and vegetables. *Appl. Environ. Microbiol.* **73:** 186-192.
5. Ikner LA, Soto-Beltran M, Bright KR. 2011. New method using a positively charged microporous filter and ultrafiltration for concentration of viruses from tap water. *Appl. Environ. Microbiol.* **77:** 3500-3506.
6. Kim MJ, Lee SY, Kim HJ, Lee JS, Joo IS, Kwak HS, *et al*. 2016. Development of a one-step duplex RT-PCR method for the simultaneous detection of VP3/VP1 and VP1/P2B regions of the hepatitis A virus. *J. Microbiol. Biotechnol.* **26:** 1398-1403.
7. Kittigul L, Singhaboot Y, Chavalitshewinkoon-Petmitr P, Pombubpa K, Hirunpetcharat C. 2015. A comparison of virus concentration methods for molecular detection and characterization of rotavirus in bivalve shellfish species. *Food Microbiol.* **46:** 161-167.
8. Lee MJ, Kim WH, Cho HG, Lee SS. 2012. Epidemiological study of ground-waterborne norovirus GI.3-associated gastroenteritis outbreaks in Gyeonggi province of South Korea in May 2011. *J. Bacteriol. Virol.* **42:** 232-241.
9. Lee SG, Cho HG, Paik SY. 2015. Molecular epidemiology of norovirus in South Korea. *BMB Rep.* **48:** 61-67.
10. Wang D, Wu Q, Kou X, Yao L, Zhang J. 2008. Distribution of norovirus in oyster tissues. *J. Appl. Microbiol.* **105:** 1966-1972.
11. Le Guyader FS, Loisy F, Atmar RL, Hutson AM, Estes MK, Ruvoën-Clouet N, *et al*. 2006. Norwalk virus-specific binding to oyster digestive tissues. *Emerg. Infect. Dis.* **12:** 931-936.
12. Gentry-Shields J, Jaykus LA. 2015. Comparison of process control viruses for use in extraction and detection of human norovirus from food matrices. *Food Res. Int.* **77**: 320-325.
13. Vinje J. 2015. Advances in laboratory methods for detection and typing of norovirus. *J. Clin. Microbiol.* **53:** 373-381.
14. Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima, H. 2003. Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. *J. Virol. Methods* **114:** 37-44.
15. Atmar RL, Estes MK. 2001. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. *Clin. Microbiol. Rev.* **14:** 15-37.
16. Khamrin P, Okame M, Thongprachum A, Nantachit N, Nishimura S, Okitsu S, *et al*. 2011. A single-tube multiplex PCR for rapid detection in feces of 10 viruses causing diarrhea. *J. Virol. Methods* **173:** 390-393.
17. Kojima S, Kageyama T, Fukushi S, Hoshino FB, Shinohara M, Uchida K, *et al*. 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J. Virol. Methods* **100:** 107-114.
18. Bower WA, Nainan OV, Han X, Margolis HS. 2000. Duration of viremia in hepatitis A virus infection. *J. Infect. Dis.* **182:** 12-17.
19. Noel JS, Lee TW, Kurtz JB, Glass RI, Monroe SS. 1995. Typing of human astroviruses from clinical isolates by enzyme immunoassay and nucleotide sequencing. *J. Clin. Microbiol.* **33:** 797-801.
20. Iturriza-Gomara M, Kang G, Gray J. 2004. Rotavirus genotyping: keeping up with an evolving population of

human rotaviruses. *J. Clin. Virol.* **31:** 259-265.

21. Karim MR, Rhodes ER, Brinkman N, Wymer L, Fout GS. 2009. New electropositive filter for concentrating enteroviruses and noroviruses from large volumes of water. *Appl. Environ. Microbiol.* **75:** 2393-2399.

22. Woods JW, Calci KR, Marchant-Tambone JG, Burkhardt W III. 2016. Detection and molecular characterization of norovirus from oysters implicated in outbreaks in the US. *Food Microbiol.* **59:** 76-84.

23. Randazzo W, Piqueras J, Rodríguez-Díaz J, Aznar R, Sánchez G. 2017. Improving efficiency of viability-qPCR for selective detection of infectious HAV in food and water samples. *J. Appl. Microbiol.* DOI: 10.1111/jam.13519. [*In Press*].

24. Hennechart-Collette C, Martin-Latil S, Fraisse A, Perelle S. 2017. Comparison of three extraction methods to detect noroviruses in dairy products. *Food Microbiol.* **61:** 113-119.

25. Gouvea V, Glass RI, Woods P, Taniguchi K, Clark HF, Forrester B, *et al*. 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J. Clin. Microbiol.* **28:** 276-282.

26. Lee HT, Kim MS, Paik SY, Lee CH, Jheong WH, Kim JM, *et al*. 2011. Evaluation of electropositive filtration for recovering norovirus in water. *J. Water Health* **9:** 27-36.

27. Wyn-Jones AP, Sellwood J. 2001. Enteric viruses in the aquatic environment. *J. Appl. Microbiol.* **91:** 945-962.

28. Coudray-Meunier C, Fraisse A, Martin-Latil S, Guillier L, Delannoy S, Fach P, *et al.* 2015. A comparative study of digital RT-PCR and RT-qPCR for quantification of hepatitis A virus and norovirus in lettuce and water samples. *Int. J. Food Microbiol.* **201:** 17-26.

29. Bartsch C, Szabo K, Dinh-Thanh M, Schrader C, Trojnar E, Johne R. 2016. Comparison and optimization of detection methods for noroviruses in frozen strawberries containing different amounts of RT-PCR inhibitors. *Food Microbiol.* **60:** 124-130.

30. Fraisse A, Coudray-Meunier C, Martin-Latil S, Hennechart-Collette C, Delannoy S, Fach P, *et al*. 2017. Digital RT-PCR method for hepatitis A virus and norovirus quantification in soft berries. *Int. J. Food Microbiol.* **243:** 36-45.

31. Williams-Woods J, Gonzalez-Escalona N, Burkhardt W III. 2011. Direct sequencing of hepatitis A virus and norovirus RT-PCR products from environmentally contaminated oyster using M13-tailed primers. *J. Virol. Methods* **178:** 253-257.

32. Manso CF, Romalde JL. 2013. Detection and characterization of hepatitis A virus and norovirus in mussels from Galicia (NW Spain). *Food Environ. Virol.* **5:** 110-118.

33. Said R, Wolfaardt M, Taylor MB. 2014. Molecular characterisation of hepatitis A virus strains from water sources in South Africa. *Water Sci. Technol.* **69:** 923-933.