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Detection of Norovirus in Contaminated Ham by Reverse Transcriptase-PCR and Nested PCR

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Abstract In order to enhance the efficacy of norovirus detection by reverse transcriptase-polymerase chain reaction (RT-PCR) and nested PCR, this study developed a norovirus mRNA concentration method using poly oligo dT-conjugated magnetic beads. An efficient norovirus detection protocol was performed on commercial ham using 2 viral elution buffers (glycine buffer and Tris beef extract buffer) and 2 concentration solutions [polyethylene glycol (PEG) and zirconium hydroxide]. The different approaches were verified by RT-PCR and nested PCR. This method was performed on ham in less than 8 hr by artificial inoculation of serial dilutions of the virus ranging from 1,000 to 1 RT-PCR unit/mL. The viral extraction and concentration method had 10-fold higher sensitivity using the combination of Tris beef extract buffer and PEG as compared to glycine buffer and zirconium hydroxide. This method proved that RT-PCR and nested PCR have the sensitive ability to detect norovirus in commercial ham, in that norovirus was successfully detected in artificially contaminated samples at a detection level as low as 1-10 RT-PCR unit/mL. Overall, such a detection limit suggests this protocol is both quick and efficient in terms of its potential use for detecting norovirus in meat products.

Keywords: norovirus, reverse transcriptase-polymerase chain reaction (RT-PCR), ham, magnetic bead

Introduction

Noroviruses (genus *Norovirus*, family Caliciviridae) are a group of related, single stranded RNA nonenveloped viruses that cause acute gastroenteritis in humans. Considerable attention has focused on the development of a novel, efficient, cost effective, and easily replicable norovirus detection method (1-5). Large outbreaks of norovirus are commonly associated with closed or semi closed communities such as hospitals, age care facilities, and other institutional settings where food and water are centrally administered.

Norovirus transmission occurs via the fecal-oral route through contaminated food and water and also through person to person contact (6). Because outbreaks are generally associated with the winter months, norovirus-associated illnesses are sometimes referred to as the 'winter vomiting disease'.

To establish food hygiene quality, incidences of fecal contamination in foods are monitored by counting fecal coliform bacteria (7). However, human pathogenic viruses have been isolated from sampling sites with acceptable fecal coliform counts, indicating coliform standards may be unreliable for monitoring foodborne viral pathogens from fecal contamination (7-9).

Recently, molecular methods using nucleic acid amplication by polymerase chain reaction (PCR) have been proposed as more effective tools for the identification of viral pathogens in food and water (3,8,10-13). However, PCR based methods are highly dependent on the methods of virus concentration and nucleic acid purification that use polyethylene glycol (PEG) 8000, zirconium chloride, and Freon (trichlorotrifluoroethane) (14,15). This is due to the low sensitivity of PCR in the presence of PCR inhibitors such as polysaccharides and humic acids, which are particularly abundant in food samples.

In order to detect norovirus inoculated in hams, this study developed a Tris elution buffer PEG TRIzol poly (dT) magnetic bead (TPTT) protocol that was modified from the glycine PEG Tri reagent poly (dT) (GPTT) procedures described by Kingsley and Richards (12) that included Tris elution buffer and PEG for the elution and concentration steps, and TRIzol reagent and poly (dT) beads for the rapid extraction and purification steps.

Materials and Methods

Virus The norovirus Hu/GII-4/C5-159/South Korea strain used in this study was obtained from the Korea Food & Drug Administration (KFDA), and had been isolated from patient fecal samples.

Virus extraction and concentration Viral extraction and concentration were performed according to procedures that were modified from Kingsley and Richards (12). Briefly, 30 g of ham were homogenized in 270 mL of glycine elution buffer (0.1 M glycine, 0.3 M NaCl, pH 9.5) or Tris elution buffer (100 mM Tris HCl, 50 mM glycine, 1% beef extract, pH 9.5) at 20°C using a homogenizer (Omni Macro Homogenizer, Marietta, GA, USA) at the high setting for 2 min. One-hundred mL of ham extract was seeded with serial 10-fold dilutions of the norovirus that ranged from 10^{-1} to 10^{-4} dilution/mL. The seeded extract was then incubated for 30 min at 37°C. After centrifugation at 15,000×g at 4°C, the norovirus in the

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supernatant was mixed with an equal volume of concentration buffer [16% PEG (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 0.525 M NaCl] and incubated for 1 hr on ice. The norovirus was then centrifuged at 10,000×g for 5 min at 4°C and resuspended in 300 μL of RNase-free H_2O. The experiments were performed in triplicate.

Extraction of viral RNA The total RNAs were extracted with 3 different methods: TRIzol, a viral RNA extraction kit, and TRIzol+Dynabeads, according to protocols modified from Kingsley and Richards (12). Briefly, 150 μL of norovirus that was resuspended in 300 μL of diethyl pyrocarbonate (DEPC) treated water was mixed with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and incubated at 20°C for 5 min. After adding 0.2 mL of chloroform, the sample was incubated at 20°C for 3 min and then centrifuged at 12,000×g for 15 min. The total RNA in a top aqueous layer was precipitated by adding 0.5 volume of isopropanol for 10 min at 20°C, followed by centrifugation at 12,000×g for 15 min. The resulting white pellets were washed with 1 mL of a cold 75% ethanol and then centrifuged at 7,500×g for 5 min. The pellets were then resuspended in 100 μL of RNase free water and were mixed with 100 μL of 1×RNA binding buffer (20 mM Tris HCl, pH 7.5, 1.0 M LiCl, 2 mM ethylendiamine tetraacetic acid, EDTA). After vortex mixing for 30 sec, the samples were heated to 65°C for 2 min and then placed on ice. After adding 100 μL of Dynabeads oligo$(dT)_{25}$ (Dynal, Oslo, Norway) to the resuspended pellet, the samples were mixed using a rotating shaker at 8 rpm (FinePCR, Rose Scientific Ltd., Edmonton, ABT, Canada) for 5 min at room temperature. Next, the samples were placed on the magnetic bead attractor (Dynal) for 1 min, after which the supernatant was removed, and were then resuspended in a washing buffer (10 mM Tris HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA). The samples were again resuspended in 100 μL of RNase free H_2O and heated to 90°C for 2 min to liberate the viral RNA from the Dynabeads, followed by magnetic extraction to pellet the Dynabeads. RT-PCR was performed with 10 μL aliquots of the eluate. One RT-PCR unit was the amount of virus genome needed to yield a positive result (16).

Primers and PCR Using a one step RT-PCR kit from Qiagen (Valencia, Carlsbad, CA, USA) and primers (0.2 μM of primer GII-FIM, 5'-TTGTGAATGAAGATGG CGTCGART-3'; and 0.2 μM of primer GII-R1M, 5'-CC RCCIGCATRICCRTTRTACAT-3') as suggested by the KFDA, RT-PCR was performed to produce a 343 bp amplicon with 20 μL of PCR reaction mixture containing 5 mM of dNTP and 25 mM of $MgSO_4$, in accordance with the procedures recommended by the manufacturer. RT-PCR amplification was carried out at 50°C for 30 min, followed by a 15 min *Taq* activation step at 95°C. Forty cycles were performed using a 56°C annealing temperature for 1 min, 1 min of extension at 72°C, and 30 sec of denaturation at 95°C. For the final cycle, the annealing time was extended to 2 min and the final extension was performed for 10 min. The PCR products were separated by electrophoresis at 70 V for 70 min on 1% agarose gels using Tris acetate/ethylenediamine tetraacetic acid electrophoresis (TAE) buffer. To verify the positive norovirus, semi nested PCR was performed using a premix PCR kit (AccuPower PCR Pre-Mix, Bioneer, Korea) as well as primer GII-F3M (5'-GGGAGGGCGATCGCAATCT-3') and primer GII-R1M, which generated a 310 bp amplicon. Semi nested PCR amplification was carried out at 95°C for an initial *Taq* activation step of 15 min, followed by 30 cycles of annealing at 60°C for 1 min, extension for 1 min at 72°C, and denaturation at 95°C for 30 sec.

Results and Discussion

RNA purification and optimal PCR conditions Comparative analysis of the 3 RNA purification methods (TRIzol, viral RNA extraction kit, and TRIzol+Dynabeads) highlighted there was varying efficiency in extracting the total RNAs and removing the inhibitors involved in PCR amplification. The results of RT-PCR demonstrated that the RNA purification method using TRIzol and the magnetic poly (dT) beads successfully produced a 310 bp amplicon from 1,000-fold diluted norovirus. In contrast, the RNA purification methods of using TRIzol alone or the commercial RNA extraction kit only showed amplicons from the 10-fold diluted norovirus (Fig. 1). The TRIzol+Dynabeads method was 100-fold higher in detection as compared to the TRIzol and viral RNA extraction kit methods (Fig. 1). For the TRIzol+Dynabeads method, detectable amplicons were observed in the 100-fold diluted samples in one step PCR, and in the 1,000-fold diluted samples in semi nested PCR. However, the TRIzol and viral RNA extraction kit methods only produced amplicons for the undiluted norovirus in one step PCR, and for the 10- fold diluted samples in semi nested PCR.

To evaluate the effects of $MgSO_4$ concentration on norovirus detection, this study used homogenized hams artificially seeded with a 10-fold serial dilution of norovirus, in which 4 mM of $MgSO_4$ showed a 10-fold

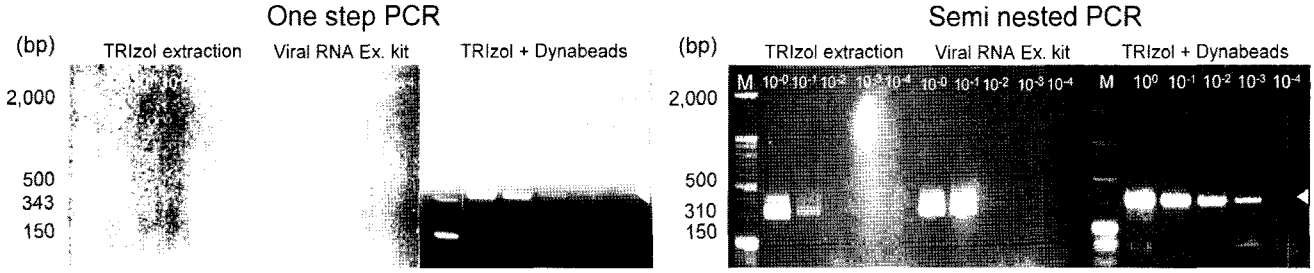

Fig. 1. Comparison of RNA extraction methods for the detection of norovirus by nested PCR. M, 100-bp molecular size ladder; 10^0, undilution; 10^{-1}, 10-fold; 10^{-2}, 100-fold; 10^{-3}, 1,000-fold; and 10^{-4}, 10,000-fold dilutions.

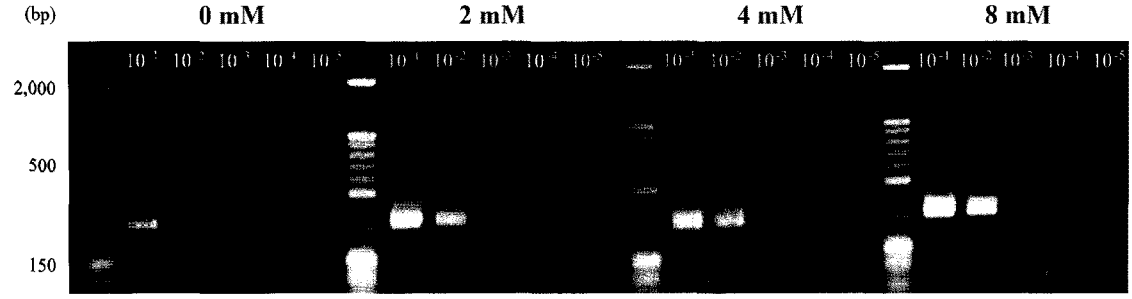

Fig. 2. Comparisons of $MgSO_4$ concentrations for norovirus detection. M, 100-bp molecular size ladder; 10^0, undilution; 10^{-1}, 10-fold; 10^{-2}, 100-fold; 10^{-3}, 1,000-fold; 10^{-4}, 10,000-fold dilutions; and 10^{-5}, 100,000-fold dilutions.

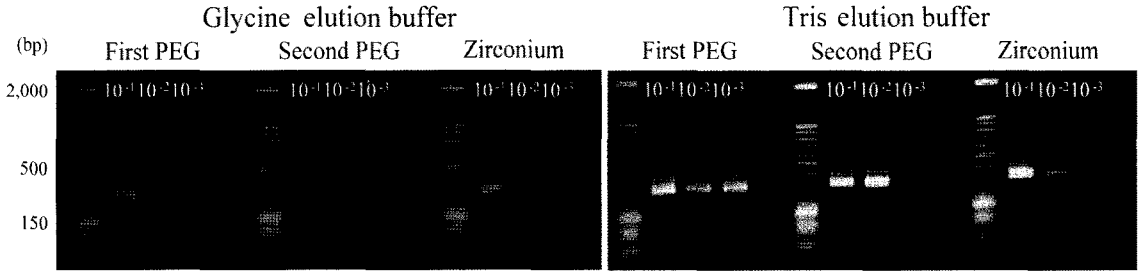

Fig. 3. Effects of elution buffers and polyethylene glycol (PEG)/zirconium hydroxide (zirconium) treatments in the detection of norovirus in infected ham by nested PCR. M, 100-bp molecular size ladder; 10^0, undilution; 10^{-1}, 10-fold; 10^{-2}, 100-fold; and 10^{-3}, 1,000-fold dilutions.

higher sensitivity in RT-PCR than 0, 2, and 8 mM (Fig. 2).

There have been numerous RNA virus extraction and detection methods described previously (3,8-13). In this study, to detect norovirus from contaminated ham, this study demonstrated a nested RT-PCR protocol called TPTT that consists of rapid and efficient RNA extraction, concentration, and purification procedures. The TPTT protocol involved: homogenization of the ham in tris NaCl buffer at pH 9.5 to elute the virus from the solids, followed by precipitation of the virus by PEG, and finally, TRIzol reagent and chloroform was used to extract the RNA. The norovirus total RNA layer in the aqueous phase was precipitated by the addition of isopropyl alcohol and was then dissolved in RNase free H_2O. Poly (dT) magnetic beads were employed to remove RT-PCR inhibitors. The total time required to perform the TPTT extraction procedure was approximately 8 hr when coupled with one step RT-PCR and the TRIzol reagent and Dynabead steps.

Concentration of norovirus This study evaluated the effects of 2 concentration solutions, PEG and zirconium hydroxide, on norovirus concentration when combined with 2 elution buffers (glycine and Tris elution buffer). There was no difference observed between the glycine and Tris elution buffers for the PEG treated samples (Fig. 3). In semi nested PCR, for the first 1 hr PEG treatment using Tris elution buffer, detectable amplicons were found in the 1,000-fold diluted norovirus inoculated samples, while the second 1 hr PEG treatment using Tris elution buffer produced an amplicon for the 100-fold diluted norovirus seeded samples. However, for the zirconium hydroxide treated samples and the first 1 hr PEG treatment combined with tris elution buffer, a 10- fold higher sensitivity was shown as compared to the respective combinations with glycine elution buffer (Fig. 3). Regarding the overall effects of the PEG and zirconium hydroxide treatments on norovirus concentration, the first 1 hr PEG treatment showed a 10-fold higher nested PCR detection limit than the second 1 hr PEG treatment (Fig. 3), while it showed a 100-fold higher detection limit than the zirconium hydroxide treatment.

Earlier studies have applied Tris elution buffer in artificially inoculated fruits and vegetable samples to efficiently extract norovirus (17,18). And glycine elution buffer has also been used as a virus elution buffer in contaminated samples (12,18,19). Using both glycine and Tris elution buffers, this study evaluated elution efficiency after incubating norovirus seeded hams for 30 min at 37°C. The use of zirconium hydroxide facilitates the separation of foodborne bacteria and viruses (hepatitis A virus and norovirus) through interaction between ligands (free amino, hydroxyl, and carboxyl residues) on the virion surface and the hydroxyl groups of zirconium hydroxide, thus facilitating precipitation (14,20). Previously, D'Souza and Jaykus (14) tested a zirconium hydroxide suspension in terms of its efficiency for norovirus concentration. However, in our study, the zirconium hydroxide treatment showed a 10-fold lower sensitivity than the second 1 hr PEG treatment in semi nested PCR. In conclusion, TPTT provided rapid and efficient detection of 1 RT-PCR unit/mL (approximately 0.1 g of ham) of norovirus. This method, by showing detection of 1 RT-PCR unit/mL of norovirus seeded in 3.75 g of ham homogenate, is more sensitive than most currently published trials that have been based on the GPTT method (12).

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