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# Application of Buoyant Density Centrifugation Method for the Rapid Detection of Feline Calicivirus in Oyster and Lettuce as Norovirus Surrogate

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**Abstract** Norovirus has become the most common cause of human gastroenteritis in developed countries. Detection procedures of foodborne viruses from foods require several steps. The concentration step using polyethylene glycol (PEG) is time-consuming and the detection efficiency of reverse transcription-polymerase chain reaction (RT-PCR) is affected by inhibitors from food components. In this study, a rapid detection method based on buoyant density centrifugation was developed to replace the time-consuming chloroform-polyethylene glycol-Tris·Tween method. Feline calicivirus that belongs to the family Caliciviridae was used as a surrogate model for norovirus. After artificial inoculation of feline calcivirus (FCV) to oyster and lettuce, 830 μL of homogenized sample suspension was layered on the top of 670 μL 20% percoll and centrifuged. Then RNA extraction step was proceeded with the supernatant. By varying several physical conditions, the detection limits were lowered to $2.4\times10^2$ PFU per 1 g in oyster and $2.4\times10^0$ PFU per 1 g in lettuce. The protocol obtained in this study could be used to develop new detection method for norovirus in foods.

**Keywords:** norovirus, feline calicivirus, reverse transcription-polymerase chain reaction (RT-PCR), rapid detection, percoll

## Introduction

Noroviruses are positive-sense single-stranded RNA viruses that belong to the Caliciviridae family based on both phylogeny and morphology. They were formerly called Norwalk-like viruses (NLVs), calicivirus, small round structures viruses (SRSV) and named as norovirus recently (1-3). Noroviruses are the major cause of nonbacterial gastroenteritis in humans. The virus can be transmitted via fecal-oral route, person-to-person transmission, and ingestion of contaminated foods (3,4). The symptoms of norovirus illness include vomiting, diarrhea, and some stomach cramping. Sometimes people additionally have a low-grade fever, chills, headaches, and muscle pains. These symptoms usually appear within 24-48 hr after ingestion of the virus, but they can appear as early as 12 hr after exposure. Most of the people are completely recovered without complications, but the very young, the elderly, and the persons with compromised immune systems may need some special cares (5). The sewage-contaminated shellfish like oysters, fruits, and ground-water are the vectors of norovirus. Especially when eating raw foods like oysters and salads, more attentions are needed. In case of shellfish, it is more dangerous because of their filter-feeding characteristics. In recent years, norovirus food poisoning has been rapidly increasing in Korea. According to the report of Division of Enteric Hepatitis Viruses, about 3.5% of acute diarrhea and 19% of viral diarrhea were occurred by norovirus in 2000-2006 (6). Korea Food & Drug Administration reported that 51 of 259 food poisoning and 51 of 3,338 cases occurred in 2006 were caused by norovirus (7).

Recently, there have been various studies on the detection techniques of norovirus. But, it is difficult to detect them because they are infected at very low levels, have environment-resistance and the patients who doesn't have symptoms can discharge viruses. Also, NLVs cannot be grown in cell culture, so cultivable feline calcivirus (FCV) was used as a surrogate model. Both FCV and NLVs belong to the same family Caliciviridae and due to their similar genetic and morphological properties, many researchers have been using FCV as a surrogate for NLV (3-5,8). The study to detect virus at low levels, reduce detection time, and reduce loss of virus by complicated steps has been in progress. In this study, buoyant density centrifugation (BDC) method was applied after inoculating FCV to oyster and lettuce as norovirus surrogate. The basic principle of BDC method is that percoll (Sigma-Aldrich, St. Louis, MO, USA) and sample components are separated by density gradient during centrifugation, so its detection steps are very simple. The objective of this study was to develop a rapid and simple method to detect FCV in oyster and lettuce.

## Materials and Methods

**Oyster and lettuce** Oyster and lettuce samples, collected randomly, were obtained from the market in Cheongju area. Each of the 25 g sample was put into the sterilized filter bag and artificially inoculated with FCV before the experiments. The oyster was stored at –20°C and lettuce was stored at 4°C prior to use.

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**Virus and cell** The FCV VR-782 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and was propagated in Crandell-Reese feline kidney (CRFK) cells from Korean cell line bank (KCLB, Seoul, Korea). The cells were grown in Dulbecco's modified Eagle medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone), 1% of 10 mM non-essential amino acids (NEAA, Gibco, Grand Island, NY, USA), and 1% penicillin-streptomycin (Gibco). They were incubated at 37°C in humidified 5% $CO_2$ incubator and splitted when proliferating over 90% of all of the cells (9,10).

The FCV was inoculated to the monolayers of CRFK cells at 90% confluency and incubated at 37°C in the maintenance medium (DMEM, 5% FBS, 1% penicillin-streptomycin, 1% NEAA) for 3-4 days. The virus stocks were put into cryogenic vial (Nalgene, Rochester, NY, USA) and stored in the liquid nitrogen. They were defrosted at 37°C in the water bath for 10 min prior to the artificial inoculation.

**Plaque assay** Five-tenth mL of serial 10-fold dilutions from $10^{-2}$-$10^{-6}$ of virus stock using Dulbecco's phosphate buffered saline (DPBS, Gibco, pH 7.4) were inoculated to the CRFK monolayers in 25 $cm^2$-tissue flasks (Corning, Corning, NY, USA). The overlay medium composed of equal volumes of 2× DMEM (10% FBS, 1% penicillin-streptomycin, 1% NEAA) and agarose gel (Junsei, Tokyo, Japan) was used as a culture medium. The flasks were incubated at 37°C until the plaque formation. For absorption of FCV, 18.5% formaldehyde was added for 20 min. To remove agar, the cell layers were washed with running water carefully and then dyed with crystal violet for 5 min. The monolayers were washed with running water again. After drying, plaques were counted to get plaque forming units (PFU) per mL (10,11).

**Detection of FCV using chloroform-polyethylene glycol-Tris · Tween (CPT) method** To concentrate and detect virus from foods, CPT method is broadly used. But this method delays the detection time due to complicated steps. One mL of FCV suspensions at a final concentration of $3.3\times10^6$ PFU/mL were inoculated to the 25 g of oyster or lettuce samples, respectively. To allow absorption of FCV to sample tissues, the inoculated oyster or lettuce samples were incubated at 37°C for 20 min, and 125 mL of PBS (Gibco, pH 7.4) was added, followed by homogenization at medium speed for 2 min in a Stomacher (Seward, London, UK).

Fifteen mL of sample homogenates mixed with 9 mL of chloroform (Sigma-Aldrich, St. Louis, MO, USA) were vortexed and then centrifuged at 3,000×g for 30 min. The resulting supernatant was precipitated with 16% polyethylene glycol (PEG) 8000 (Sigma-Aldrich), 0.3 M NaCl (DC Chemical, Seoul, Korea) through overnight incubation at 4°C. The sample was centrifuged at 6,700×g for 30 min, and the pellet was resuspended in 2 mL of 50 mM Tris-0.2% Tween 80 (Junsei, Chemical Co., Tokyo, Japan). Resuspended pellet was held at room temperature for 1 hr and centrifuged at 7,500×g for 15 min. The secondary PEG precipitation was proceeded by using 10% PEG 8000, 0.3 M NaCl with incubation at 4°C for 2 hr. The precipitate was recovered by centrifugation at 7,500×g for 15 min and resuspended in 0.5 mL of 50 mM Tris-0.2% Tween 80. After another centrifugation at 7,500×g for 15 min, RNA was extracted from the supernatant (12).

**Detection of FCV based on BDC method** One mL of FCV suspension was inoculated to food samples and homogenized using same procedure as in CPT method. A sample volume of 830 μL of the homogenate was layered on the top of 670 μL 20%(v/v) Percoll (Sigma-Aldrich), which showed the most stable result among the tested combinations (13). The tube was centrifuged at 15,000×g for 15 min. A 800 μL of resulting supernatant was collected and then vortexed with 700 μL of PBS. The mixture was centrifuged at 12,000×g for 30 min. A 300 μL of supernatant was transferred into another tube and 140 μL was used for RNA extraction.

**Improvement of detection efficiency using PEG precipitation** In case of BDC method, while the detection time is shortened because of the simple detection steps, the detection efficiency is lower than CPT method. Therefore, we added PEG concentration of FCV to improve its detection efficiency.

After inoculation of FCV to food samples, a sample volume of 830 μL was layered on the top of 670 μL 20%(v/v) percoll and then centrifuged at 15,000×g for 15 min. A 800 μL of resulting supernatant was vortexed with 700 μL of PBS. The mixture was centrifuged at 12,000×g for 30 min and 1 mL of resulting supernatant was precipitated with 16% PEG 8000, 0.3 M NaCl by incubating at 4°C for 2 hr. Then it was centrifuged at 6,700×g for 30 min and the pellet was resuspended in 0.5 mL of 50 mM Tris-0.2% Tween 80. Resuspended pellet was kept at room temperature for 1 hr and centrifuged at 7,500×g for 15 min after vortexing slightly. The resulting supernatant was used for RNA extraction.

**Improvement of detection efficiency by diluting reverse transcription-polymerase chain reaction (RT-PCR) mixture** Dilution of RNA extracts with DEPC-DW (Bioneer, Daejon, Korea) was performed to improve detection efficiency by decreasing PCR inhibitors existing in RNA extracts. 2, 5, 10, 20, 50, and 100-fold dilutions were carried out and their detection efficiency was compared using RT-PCR.

**RNA extraction** The QIAamp viral mini kit (Qiagen, Hilden, Germany) was used for RNA extraction by following the manufacturer's instructions. Brifely, 560 μL buffer AVL was mixed with 140 μL of sample for lysis. A 560 μL of 99% ethanol was added and mixed by vortexing, and droplets from the tube cap were removed by slight centrifugation. The QIAamp mini spin column was loaded with 630 μL of this mixture and centrifuged. The collection tube was replaced and centrifuged after adding remaining 630 μL of mixture. The collection tube was replaced, and the spin column was washed with 500 μL of buffer AW2 subsequent to wash with 500 μL of buffer AW1. The ingredient was centrifuged and then collection tube was replaced once again. Viral elution buffer was added and incubated for 1 min. Extracted RNA was either used directly in RT-PCR or stored at −70°C (14).

**RT-PCR assay** Two primers calcapF (5'-TTCGGCCTT TTGTGTTCC-3') and calcapR (5'-TTGAGAATTGAACA CATCAATAGATC-3') target on capsid protein gene of FCV were used in the RT-PCR assay (16). RT-PCR was performed using Accupower RT-PCR Premix (Bioneer). A 13 μL of RNA was mixed with 0.4 μL of calcapR primer, and then 0.4 μL of calcapF primer was added. The 20 μL of final volume that 6.2 μL of DEPC-DW was added was mixed with RT-PCR premix. RT was performed at 42°C for 1 hr (synthesis of cDNA), 94°C for 5 min (inactivation of RTase). PCR was performed with an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 91°C for 1 min, annealing at 56°C for 1min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The final products of RT-PCR were analyzed by electrophoresis using 2% agarose (SeaKem, Rockland, ME, USA).

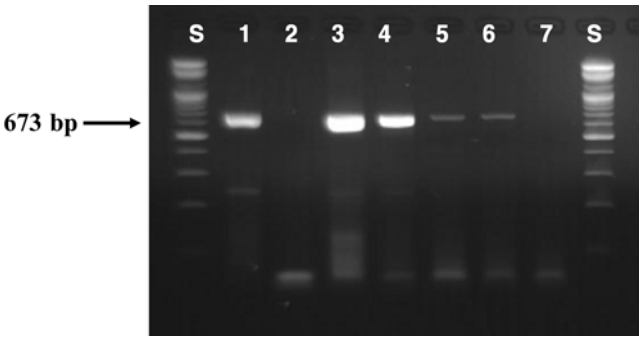


**Fig. 1. Detection limit of FCV by RT-PCR.** S, size marker (100 bp); lane 1, positive control; lane 2, negative control; lane 3, $3.01\times10^{2}$ PFU/RT-PCR mixture; lane 4, $3.01\times10^{1}$ PFU/RT-PCR mixture lane 5, $3.01\times10^{0}$ PFU/RT-PCR mixture; lane 6, $3.01\times10^{-1}$ PFU/RT-PCR mixture; lane 7, $3.01\times10^{-2}$ PFU/RT-PCR mixture.

## Results and Discussion

**Quantification of FCV and RT-PCR assay** The cultured FCV cells were quantified by plaque assay and the FCV concentration used for the artificial inoculation was $3.3\times10^{6}$ PFU /mL. The detection limit without food component was 0.31 PFU per RT-PCR mixture (Fig. 1).

**Comparison of CPT and BDC method to detect FCV in oyster and lettuce** The efficiencies of CPT and BDC methods were compared. One mL of FCV suspension was inoculated to food samples and homogenized in PBS. PEG precipitation was carried out at the conditions of 16 and 10% PEG and 20% percoll, which showed the most stable result among the tested combinations and then RT-PCR was performed. In CPT method, the detection limits were $2.4\times10^{3}$ PFU per 1 g oyster and $2.4\times10^{2}$ PFU per 1 g lettuce (Fig. 2).

BDC method could detect FCV as low as $4.02\times10^{3}$ PFU per 1 g oyster and $4.02\times10^{3}$ PFU per 1 g lettuce, respectively (Fig. 3). Although the detection efficiency in BDC method was not higher than CPT method, the steps were much simpler and therefore the detection time was greatly reduced in BDC method.

**Improvement of detection efficiency using PEG precipitation** BDC method could reduce detection time greatly but the detection efficiency was little lower than CPT method. To improve detection efficiency, PEG precipitation was applied to the BDC method. Percoll was mixed with the homogenates and 16% PEG 8000 was added to 1 mL of the resulting supernatant after centrifugation.

After trials with several different PEG concentrations coupled with BDC method, the condition of 16% PEG 8000 precipitaiton at 4°C for 2 min was selected. This improved protocol could detect the presence of FCV at the original inoculation level of $3.3\times10^{6}$ PFU in 25 g of oyster and $3.3\times10^{5}$ PFU in 25 g of lettuce, respectively. Therefore, the detection limits of improved method were $2.4\times10^{3}$ PFU per 1 g oyster and $2.4\times10^{2}$ PFU per 1 g lettuce (Fig. 4).

Percoll has been applied to isolate bacterial cells from food samples by precipitating them after centrifugation. In this study, we take advantage of an idea to precipitate food components instead of bacterial cells and take virus particles in the supernatent. The results in Fig. 4 showed possibility of developing simple and sensitive detection method by modifying BDC method in further study.

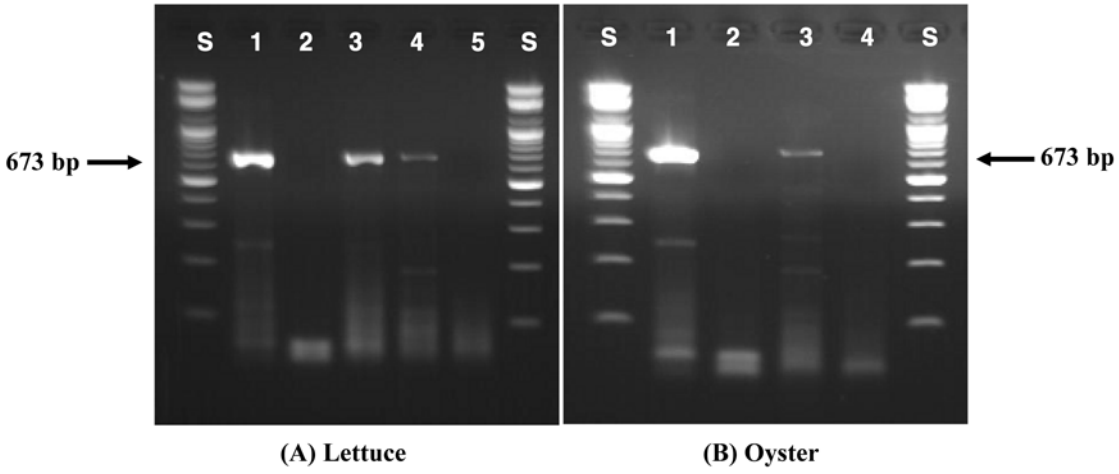


**Fig. 2. Comparison of detection limit from 25 g lettuce (A) and oyster (B) sample using chloroform-PEG-Tris · Tween (CPT) method.** S, size marker (100 bp); lane 1, positive control; lane 2, negative control; lane 3, $6.02\times10^{3}$ PFU; lane 4, $6.02\times10^{2}$ PFU; lane 5, $6.02\times10^{1}$ PFU.

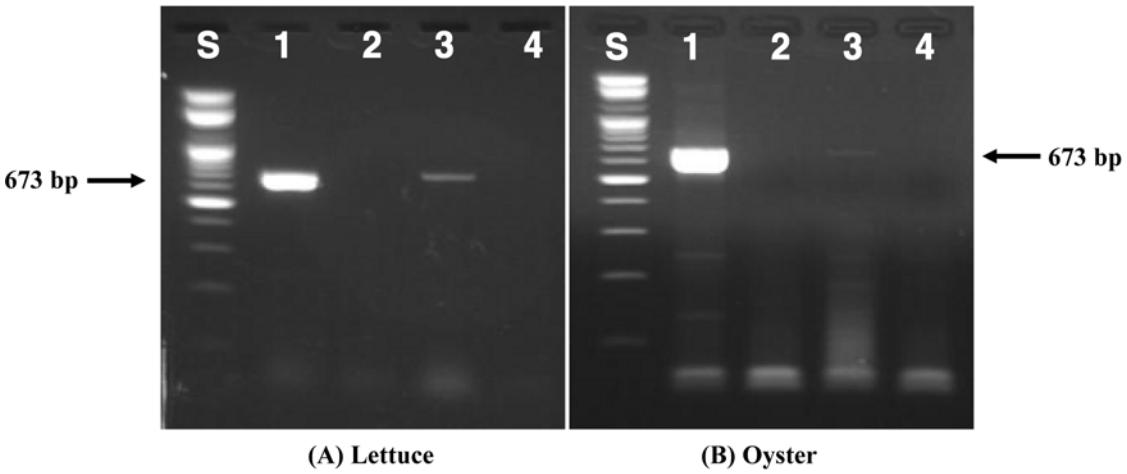


**Fig. 3. Comparison of detection limit from 25 g lettuce (A) and oyster (B) sample using BDC method.** S, size marker (100 bp); lane 1, positive control; lane 2, negative control; lane 3, $1.0\times10^4$ PFU; lane 4, $1.0\times10^3$ PFU.

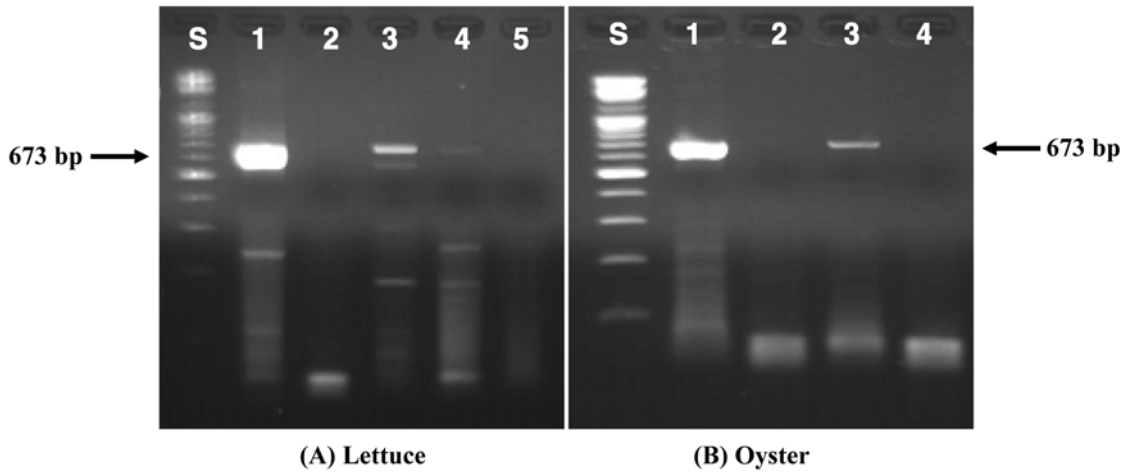


**Fig. 4. Comparison of detection limit from 25 g lettuce (A) and oyster (B) sample using BDC method and PEG precipitation.** S, size marker (100 bp); lane 1, positive control; lane 2, negative control; lane 3, $6.02\times10^3$ PFU; lane 4, $6.02\times10^2$ PFU; lane 5, $6.02\times10^1$ PFU.

**Improvement of detection efficiency by diluting RT-PCR mixture** To improve detection efficiency by reducing inhibitors, dilutions of RT-PCR mixture were carried out. In case of oyster, 10-fold dilution of original RT-PCR mixture was most efficient and in lettuce 5-fold dilution was most efficient (Fig. 5). These results are due to the decrease of PCR inhibitors composed of food components by diluting RT-PCR mixtures, inspite of the decrease of nucleic acid concentration.

**Assessment of the developed protocol** In this study, an improved method to detect FCV as norovirus surrogate in oyster and lettuce was developed by utilizing BDC, by improving concentration efficiency of virus particles with 16% PEG 8000, and by diluting RT-PCR mixture. Instead of previous 19 hr consuming CPT method, this new method needed only 5 hr. The detection limits of improved method were $2.4\times10^2$ PFU per 1 g oyster (Fig. 6B) and $2.4\times10^0$ PFU per 1 g lettuce (Fig. 6A), respectively.

There have been many studies to develop efficient protocols for the isolation and concentration of foodborne virus from foods. General procedures for the detection of foodborne virus from foods include several steps such as homogenization of food materials (23), removal of food components (12), concentration of virus particles using PEG (18,19), extraction of RNA (4), and RT-PCR (18,19). However the PEG concentration step is time-consuming and causes damages to virus due to the complicated procedures. In our newly developed protocol, detection steps are simplified and the detection efficiency was also enhanced than CPT method. For the enhancement of detection efficiency, concentration step and removal of PCR inhibitors are very crucial. Although the sensitivity of this new BDC method was not the best compared with the previous studies (12,21,22), this is the simplest and shortest method ever known. Although many researchers have been using FCV as norovirus surrogate because of their genetic and morphological similarities, there still exist some differences between two of them. Application of this protocol using buoyant density centrifugation method should need some modifications to detect norovirus in oyster and lettuce samples. However the results obtained in this study

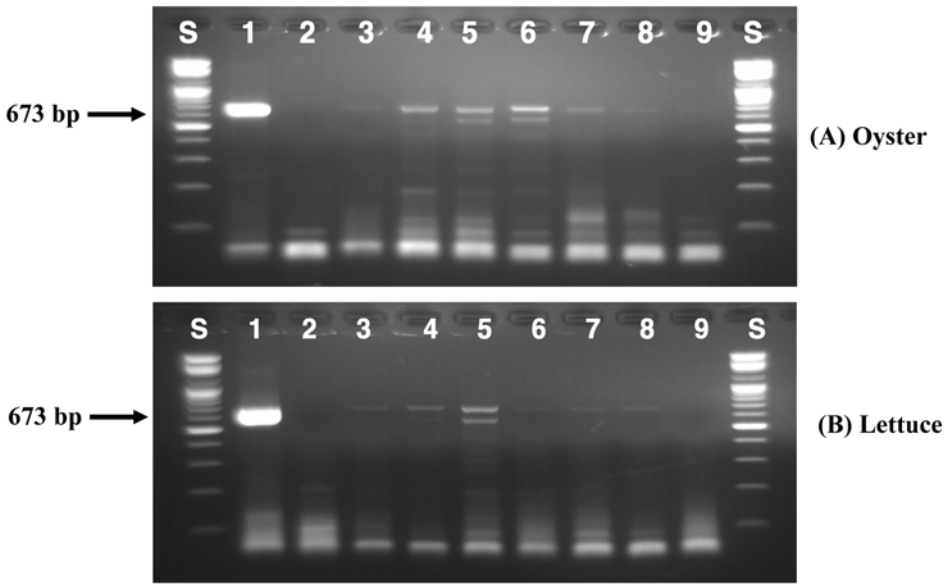


**Fig. 5. Detection limits of FCV after dilution of RT-PCR mixtures.** S, size marker (100 bp); lane 1, positive control; lane 2, negative control; lane 3, $3.3\times10^{6}$ PFU/mL; lane 4, 2-fold dilution of lane 3 RNA; lane 5, 5-fold dilution of lane 3 RNA, lane 6, 10-fold dilution of lane 3 RNA; lane 7, 20-fold dilution of lane 3 RNA; lane 8, 50-fold dilution of lane 3 RNA; lane 9, 100-fold dilution of lane 3 RNA.

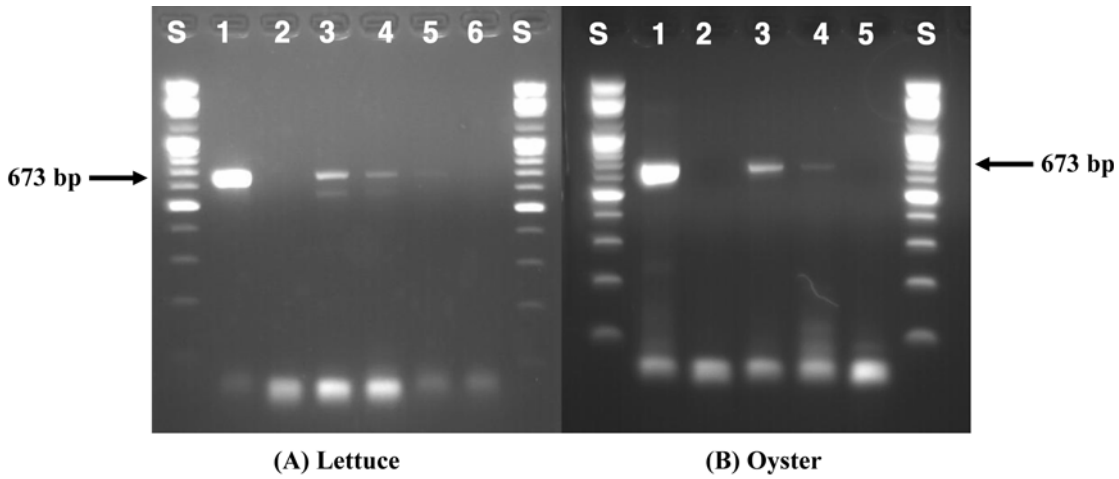


**Fig. 6. Detection limits of FCV by newly developed protocol.** S, size marker (100 bp); lane 1, positive control; lane 2, negative control; lane 3, $6.02\times10^{2}$ PFU; lane 4, $6.02\times10^{1}$ PFU, lane 5, $6.02\times10^{0}$ PFU, lane 6, $6.02\times10^{-1}$ PFU.

could be efficiently used to develop new detection method for norovirus in food samples.

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## References

1. Junyi L, Qingping W, Xiaoxia K. Development of a concentration method and its application for the detection of noroviruses in drinking water in China. J. Microbiol. 45: 48-52 (2007)
2. Sarbelio M, Espinosa M, Farkas T, Jiang X. Human caliciviruses and pediatric gastroenteritis. Semin. Pediatr. Infect. Dis. 15: 237-245 (2004)
3. Woo GJ, Hwang IG, Kwak HS, Kim MG, Park JS, Lee GY, Koh YH. Application of detection method and evaluation for foodborne virus. Annual Report Korea Food & Drug Administration 8-1: 569-575 (2004)
4. Taku A, Gulati BR, Allwood PB, Palazzi K, Hedberg CW, Goyal SM. Concentration and detection of caliciviruses from food contact surfaces. J. Food Protect. 65: 999-1004 (2002)
5. Koopmans M, Durzer E. Foodborne viruses: An emerging problem. Int. J. Food Microbiol. 90: 23-41 (2004)
6. Jee YM. Norovirus food poisoning and laboratory surveillance for viral gastroenteritis. Food Ind. Nutr. 11: 6-11 (2006)
7. Korea Food & Drug Administration. Present conditions of foodborne viruses. Available from: http://fm. kfda. go. kr. Accessed Aug. 04, 2007.
8. Claarke IN, Lambden PR, Organization and expression of calicivirus gene. J. Infec. Dis. 181: 309-316 (2000)
9. Chi HY, Lee CH, Kim KH, Kim SL, Chung IM. Induction of apoptotic cell death by red pericarp rice ('Jakwangchalbyeo') extracts. Food Sci. Biotechnol. 15: 534-542 (2006)

10. Bidawid S, Malika N, Adegbunrinb O, Sattarb SA, Farber JM. A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. J. Virol. Methods 107: 163-167 (2003)
11. D'Souza DH, Sair A, Williams K, Papafragkou E, Jean J, Moore C, Jaykus LA. Persistence of caliciviruses on environmental surfaces and their transfer to food. Int. J. Food Microbiol. 108: 84-91 (2006)
12. Green DH, Lewis GD. Comparative detection of enteric viruses in wastewaters, sediments, and oysters by reverse transcriotion-PCR and cell culture. Water Res. 33: 1195-1200 (1999)
13. Lantz PG, Knutsson K, Blixt Y, Al-Soud WA, Borch E, Radstrom P. Detection of pathogenic *Yersinia enterocolitica* in enrichment media and pork by a multiplex PCR: A study of sample preparation and PCR-inhibitory components. Int. J. Food Microbiol. 45: 93-105 (1998)
14. Bidawid S, Farber JM, Sattar SA. Rapid concentration and detection of hepatitis A virus from lettuce and strawberries. J. Virol. Methods 88: 175-185 (2000)
15. Boxman ILA, Tilburg JJHC, Loeke NAJM, Vennema H, Jonker K, Boer E, Koopmans M. Detection of noroviruses in shellfish in the Netherlands. Int. J. Food Microbiol. 108: 391-396 (2006)
16. Sykes JE, Allen JL. Studdert P, Browning GF. Detection of feline calicivirus, feline herpesvirus 1, and *Chlamydia psittaci* mucosal swabs by multiplex RT-PCR/PCR. Vet. Microbiol. 81: 95-108 (2001)
17. Fleet GH, Heiskanen P, Reid I, Buckle KA. Foodborne viral illness - status in Australia. Int. J. Food Microbiol. 59: 127-136 (2000)
18. Mullendore JL, Sobsey MD, Shieh YSC. Improved method for the recovery of hepatitis A virus from oysters. J. Virol. Methods 94: 25-35 (2001)
19. Leggitt PR, Jaykus LA. Detection methods for human enteric viruses in representative foods. J. Food Protect. 63: 1738-1744 (2000)
20. Lees DN, Henshilwood K, Dor WJ. Development of a method for detection of entericviruses in shellfish by PCR with poliovirus as a model. Appl. Environ. Microb. 60: 2999-3005 (1994)
21. Kohno T, Mohan S, Gooto T, Morita C, Nakano T, Hong W, Sangco JCE, Morimatsu S, Sano K. A new improved method for the concentration of HIV-1 infective particles. J. Virol. Methods 106: 167-173 (2002)
22. Lee SY, Jang KI, Woo GJ, Kwak HS, Kim KY. Development of protocol for the effective detection of feline calicivirus as norovirus surrogate in oyster and lettuce. Korean J. Food Sci. Technol. 39: 71-76 (2007)
23. Kingsley DH, Richards GP. Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like virus in shellfish. Appl. Environ. Microb. 67: 4152-4157 (2001)
24. Chang KH, Park JS, Choi JH, Kim CJ, Paik HD. Cytotoxic effects of partially purified substance from *Bacillus polyfermenticus* SCD supernatant toward a variety of tumor cell lines. Food Sci. Biotechnol. 16: 163-166 (2007)
25. Choi SJ, Yoon KY, Choi SG, Kim DO, Oh S, Jun WJ, Shin DH, Cho SH, Heo HJ. Protective effect of *Acanthopanax senticosus* on oxidative stress induced PC12 cell death. Food Sci. Biotechnol. 16: 1035-1040 (2007)