Heliyon 7 (2021) e07560

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Improved real-time quantitative reverse transcription PCR detection of norovirus following removal of inhibitors

SungJun Park [a,b], Cheonghoon Lee [a,c,*], Kyuseon Cho [a], Hye Young Ko [a], Sung Jae Jang [a], GwangPyo Ko [a,b,c,**]

[a] Department of Environmental Health Sciences, Graduate School of Public Health, Seoul National University, Seoul, Republic of Korea
[b] N-Bio, Seoul National University, Seoul, Republic of Korea
[c] Institute of Health and Environment, Seoul National University, Seoul, Republic of Korea

ARTICLE INFO

Keywords:
Norovirus
Real-time quantitative reverse transcription PCR
Inhibitor
Water
Vegetable
Berry

ABSTRACT

Human norovirus (HuNoV) is an important enteric virus that can cause large gastroenteritis outbreaks via the fecal-oral route from contaminated water and produce. Real-time quantitative reverse transcription PCR (RT-qPCR) is the only method to apply the routine detection of HuNoV in various samples, however, inhibitors present in the samples can affect the accuracy and sensitivity of RT-qPCR results. Here, we suggest an inhibitor-removal treatment for two types of noroviruses using two commercial kits. Two types of water sample (surface and seawater) and four types of produce (green onions, lettuces, radishes, and strawberries) were evaluated. The recovery efficiencies of noroviruses in water samples clearly increased in surface and seawater samples with the inhibitor-removal treatment compared to untreated samples. Moreover, murine norovirus-1 was well recovered from the four types of produce with the inhibitor-removal treatment. The mean recovery efficiencies of HuNoV genogroup II genotype 4 in lettuces and strawberries were also increased in the treated samples. Therefore, we suggest that the inhibitor-removal treatment could be useful for improving the accuracy and sensitivity of RT-qPCR methods for noroviruses in water and produce.

1. Introduction

Human norovirus (HuNoV) is a major enteric virus that is mainly transferred via the fecal-oral route [1, 2]. HuNoV can be transmitted via various types of fecal-contaminated water [3, 4, 5, 6]. Moreover, produce, including vegetables and berries, is also the major carrier of HuNoV and can cause large gastroenteritis outbreaks [7, 8, 9, 10]. Because of the lower minimum infectious dose of HuNoV, below 100 viral particles, accurate and sensitive detection methods for detecting HuNoV in environmental and food samples are important to prevent the socio-economical risks due to the outbreaks of HuNoV [1].

Real-time quantitative reverse transcription PCR (RT-qPCR) is the only method for routine detection of HuNoV in various samples [1, 11, 12]. However, inhibitors present in samples, including debris, lipids, metal ions, organic acids, and polysaccharides, can affect RT-qPCR results [13, 14]. Typically, elution and concentration process are necessary for detecting HuHoV because samples may contain only a few HuHoVs. During these processes, inhibitors present in samples can also be concentrated and result in the misdetection of HuNoV. Therefore, the inhibitor-removal treatment for environmental and food samples should be considered to improve RT-qPCR results for HuHoVs.

In this study, we suggested an inhibitor-removal treatment using commercial kits and evaluated using two types of water (surface water and seawater) and four types of produce (green onions, lettuces, radishes, and strawberries), which were selected because previous studies have reported that the norovirus outbreaks have occurred via the consumption of those produce [15, 16, 17]. The treatment has two steps: elution/concentration of samples and RNA extraction for noroviruses. Two types of noroviruses, murine norovirus-1 (MuNoV-1), as the major surrogate for HuNoV, and HuNoV genogroup II genotype 4 (HuNoV GII) were applied for evaluation of our inhibitor-removal treatment.

2. Materials and methods

2.1. Preparation of viruses

MuNoV-1s were provided by Dr. Herbert W. Virgin in Washington University School of Medicine (St. Louis, MO, USA) and proliferated using RAW 264.7 cells as previously described with some modification [18]. First, RAW 264.7 cells were cultured in Dulbecco's modified Eagle's

* Corresponding author.
** Corresponding author.
E-mail addresses: shota2@snu.ac.kr (C. Lee), gko@snu.ac.kr (G. Ko).

https://doi.org/10.1016/j.heliyon.2021.e07560
Received 28 December 2020; Received in revised form 8 February 2021; Accepted 9 July 2021

medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Gibco), 10 mM non-essential amino acids (Gibco), 10 mM sodium bicarbonate (Gibco), 10 mM HEPES buffer (Gibco), and 50 μg/μL gentamicin (Gibco) in an incubator with 5% CO_2 atmosphere at 37 °C. MuNoV-1s were inoculated in monolayer of RAW 264.7 cells for 3 days and three cycles of freezing-thawing procedure for infected cells were performed to obtain proliferated MuNoV-1s. Then, the supernatant was collected and purified using centrifugation with chloroform (AMRESCO, Solon, OH, USA) at 5,000 ×g for 20 min at 4 °C. The supernatant with MuNoV-1s was concentrated using centrifugation in an Amicon Ultra-15 centrifugal filter unit with an Ultracel-10 membrane (Millipore, Billerica, MA, USA). To measure the MuNoV-1 concentration in the supernatant, a plaque assay was performed as described previously [18]. First, 3×10^6 RAW 264.7 cells were inoculated in each well of six-well plates in an incubator with 5% CO_2 atmosphere at 37 °C. Following development of cell monolayers, serial diluted samples were applied to the wells and incubated for 1 h with 5% CO_2 atmosphere at 37 °C. During incubation, the plates were rocked every 15 min to facilitate infection of MuNoV-1. Then, the medium in the plate was discarded and 3 mL of 1:1 (v/v) mixture of 1.5% SeaPlaque agarose (Lonza, Rockland, ME, USA) and 2× minimum essential medium (Gibco) with 10% fetal bovine serum (Gibco), 10 mM nonessential amino acids (Gibco), 10 mM sodium bicarbonate (Gibco), 10 mM HEPES (Gibco), and 50 μg/μL gentamicin (Gibco) was added to each well. MuNoV plaques were counted after 4 days of incubation.

HuNoV GII (GII/Hu/KR/2014/GII.4/SeoulGJ1) was obtained from a patient and provided by Dr. In-Soo Choi in Konkuk University, Republic of Korea. The stool sample contained HuNoV GII was initially suspended using 1× phosphate-buffered saline (PBS) and centrifuged at 20,000 ×g for 20 min at 4 °C to collect the debris. Then, the debris was subjected to sequential dilution using 1× PBS for proper concentration for further experiments. All concentrated viruses were stored at -80 °C until use.

2.2. Preparation of water and produce

Two types of water (surface water and seawater) samples were collected in Gomso Bay, Jeollabuk-do, Republic of Korea, or near upstream to establish different water conditions. The salinity and turbidity, which are the environmental parameters related to inherent inhibitors such as suspended solids, metals and various ions, were measured using a YSI multi parameter instrument (Professional Plus; Yellow Springs Instruments, Yellow Springs, OH, USA) as previously described [3, 19]. Each water sample was stored in 1-L sterilized bottle and transported to the laboratory at 4 °C. Green onions, lettuces, radishes, and strawberries were purchased in a local market in Seoul, Republic of Korea, and transported to the laboratory at 4 °C. All samples were stored at 4 °C until use.

2.3. Inhibitor-removal treatment for water and produce

First, 1.7×10^4 plaque forming units of MuNoV-1s or 5.0×10^4 genomic copies (copies) of HuNoV GIIs were inoculated in 300 μL of each water or the eluted solution from the produce. To make eluted solution, 90 mL 0.25 M Threonine-0.3 M NaCl solution (pH 9.5) or 3% beef extract solution with 100 mM Tris-HCl, and 50 mM glycine were applied to 25 g of produce, as described previously with some modification [20]. To confirm the effect of inherent inhibitors, samples were serially ten-fold diluted for twice. The solution with produce was agitated in a 3D-shaker at 150 rpm for 1 h and the eluted solution was collected for further analyses.

Subsequently, as the first step of the inhibitor-removal treatment (pre-treatment), a QIAshredder (Qiagen, Hilden, Germany) was applied in 300 μL norovirus-inoculated samples according to the manufacturer's instructions. Viral RNA was extracted from 140 μL of samples following pre-treatment using a Quick RNA MiniPrep (Zymo Research, Irvine, CA, USA), an easy-spin Total RNA Extraction Kit (iNtRON biotechnology Inc., Gyeonggi-do, Republic of Korea) or a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The final eluate volume was 50 μL. Then, as the second step of the inhibitor-removal treatment (post-treatment), an OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA) was used according to the manufacturer's instructions. The final eluate was stored at -80 °C until use. Samples without inhibitor-removal treatment (untreated samples) were used as a control.

2.4. Measurement of noroviruses using RT-qPCR

RT-qPCR analyses for noroviruses were performed using a 7300 Real-Time System (Applied Biosystems, Thermo Fisher Scientific) as described previously with some modification (Table 1) [18,20]. An AgPath-ID One-Step RT-PCR reagent (Thermo Fisher Scientific) was used for RT-qPCR analyses with 25 μL reaction mixture containing 2.5 μL eluate sample, forward and reverse primers (1 μM each for MuNoV-1 and 400 nM each for HuNoV GII, respectively), and probe (240 nM for MuNoV-1 and 200 nM for HuNoV GII, respectively). RT-qPCR assays were performed with the following conditions: a reverse transcription of RNA at 48 °C for 30 min, an initial denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation of 95 °C for 15 s and annealing and extension at 60 °C for 1 min. RNA extracted from noroviruses or distilled water were used as the positive or negative control, respectively. Plasmids containing capsid region of MuNoV-1 or HuNoV GII, as described in our previous studies [18, 21], were used to generate the standard curves for RT-qPCR assays. The limit of quantification (LOQ) was established using the lowest range of standards, as previously suggested [22]. The limit of detection (LOD) was also established using negative controls and the "not detected" (ND) and "detected but not quantified" (DNQ) samples were determined as our previous study suggested [23]. The recovery of viruses for each sample was calculated via this formula: Virus concentration after viral RNA extraction with or without the inhibitor-removal treatment/Inoculated (initial) concentration of viruses (%).

2.5. Statistical analyses

The data are expressed as the means ± standard deviation (SD) of three independent experiments and properly analyzed using independent *t*-test. $P < 0.05$ were considered statistically significant. SPSS statistics for Windows (ver. 21.0; IBM Corp, Armonk, New York, USA) and SigmaPlot for Windows (ver. 12.0; Systat Software Inc., San Jose, CA, USA) were used to perform the statistical analyses and visualization.

3. Results

3.1. Environmental parameters of water samples

Table 2 summarizes the average environmental parameters of water samples. Surface water samples showed lower salinity (0.29 psu) than seawater. Seawater showed lower average turbidity (5.16 NTU) than surface water.

3.2. Evaluation of norovirus recovery efficiencies in water samples with the inhibitor-removal treatment

Initially, we confirmed that a QIAamp Viral RNA Mini Kit (Qiagen) is the suitable nucleic acid extraction kit for our inhibition-removal treatment, exhibited the highest recovery efficiency for both MuNoV-1 and HuNoV GII in water samples and green onion (Figure. S1 and S2). Figure 1 shows the recovery efficiencies of noroviruses in water samples with or without the inhibitor-removal treatment measured by RT-qPCR. The LOD was 2.0×10^1 copies/reaction for both RT-qPCR analyses for MuNoV-1 and HuNoV GII. MuNoV-1s were recovered significantly from surface water with the treatment (60.55 ± 8.00%) compared to untreated samples (36.81 ± 15.74%) ($P < 0.05$) (Figure 1a). Near 70% mean

Table 1. Primers used to analyze murine or human norovirus in various samples.

Virus	Primer or probe (polarity)	Sequence (5′ – 3′)[a]	Location[b]	Reference
Murine norovirus-1 (MuNoV-1)	MNV1F (+)	ACGCCACTCCGCACAAA	5614–5630	[15]
	MNV1R (-)	GCGGCCAGAGACCACAAA	5667–5649	
	MNV1P (+)	VIC-AGCCCGGGTGATGAG-MGB	5632–5646	
Human norovirus (HuNoV; GII)	BPO-13 (+)	AICCIATGTTYAGITGGATGAG	5007–5028	[17]
	BPO-13N (+)	AGTCAATGTTTAGGTGGATGAG	5007–5028	
	BPO-14 (-)	TCGACGCCATCTTCATTCACA	5100–5080	
	BPO-18 (+)	VIC-CACRTGGGAGGGCGATCGCAATC-TAMRA	5044–5066	

[a] MGB: minor groove binder; TAMRA: 6-carboxy-tetramethyl-rhodamine; VIC: 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein; I: inosine; Y: C or T; R: A or G.
[b] Relative positions of primers and probe in MuNoV-1 clone CW1 (accession no. DQ285629) or Lordsdale virus (accession no. X86557) for HuNoV GII.

Table 2. Environmental parameters of the water samples.[a]

Parameter	Water type	
	Surface water (N = 3)	Seawater (N = 3)
Salinity (psu)[b]	0.29 ± 0.31 (0.20)	31.83 ± 0.01 (31.83)
Turbidity (NTU)[c]	7.23 ± 4.28 (5.94)	5.16 ± 0.66 (5.52)

[a] Data are presented as the means ± standard deviation (median).
[b] Practical salinity unit.
[c] Nephelometric turbidity unit.

recovery efficiency for MuNoV-1 was achieved with seawater samples after the inhibitor-removal treatment (Figure 1a). Overall, recovery efficiencies of HuNoV GII were relatively lower than those of MuNoV-1 in water samples (Figure 1b). Nevertheless, the mean recovery efficiency for HuNoV GII from surface water was clearly increased with the treatment (52.22%). In seawater samples with the treatment, HuNoV GIIs were also highly recovered with the treatment (48.89 ± 4.09%) compared to untreated samples (25.55 ± 2.04%) ($P < 0.05$).

3.3. Evaluation of norovirus recovery efficiencies in produce with the inhibitor-removal treatment

Figure 2 shows the recovery efficiencies of noroviruses in produce with or without the inhibitor-removal treatment measured by RT-qPCR. The LOD was 2.0×10^1 copies/reaction for both RT-qPCR analyses for MuNoV-1 and HuNoV GII. Recovery efficiencies for MuNoV-1 were clearly increased in all produce with the inhibitor-removal treatment (Figure 2a). Especially, MuNoV-1s were well recovered in strawberries with treatment (66.16 ± 5.42%) compared to untreated samples (45.72 ± 8.99%) ($P < 0.05$). Mean recovery efficiencies of HuNoV GII in green onions and radishes after treatment were relatively lower than untreated samples (Figure 2b). However, over 30% mean recovery efficiencies of HuNoV GII was achieved in all four produce with inhibitor-removal treatment, especially in lettuces and strawberries, compared to untreated samples.

4. Discussion

In this study, we evaluated the recovery efficacies of noroviruses using RT-qPCR in water and produce with or without our inhibitor-removal treatment. Both MuNoV-1 and HuNoV GII were well recovered from surface and seawater samples with the inhibitor-removal treatment (Figure 1). Especially, surface water samples were very turbid (7.23 ± 4.28 NTU) and seawater samples showed high salinity (31.83 ± 0.01 psu) (Table 2), indicating that these samples had large amounts of debris, ions, and salts, which are potential RT-qPCR inhibitors [13, 14]. Therefore, the inhibitor-removal treatment improved the RT-qPCR results for detecting of noroviruses in water samples.

Moreover, the inhibitor-removal treatment increased the recovery efficiencies of noroviruses from produce (Figure 2). The accurate and sensitive methods for detecting noroviruses are important in the prevention of HuNoV transmission because various points in the produce supply chain, such as irrigation, contact by harvesters or food handlers, conveyor belts, and rinsing, could be the major routes for HuNoV

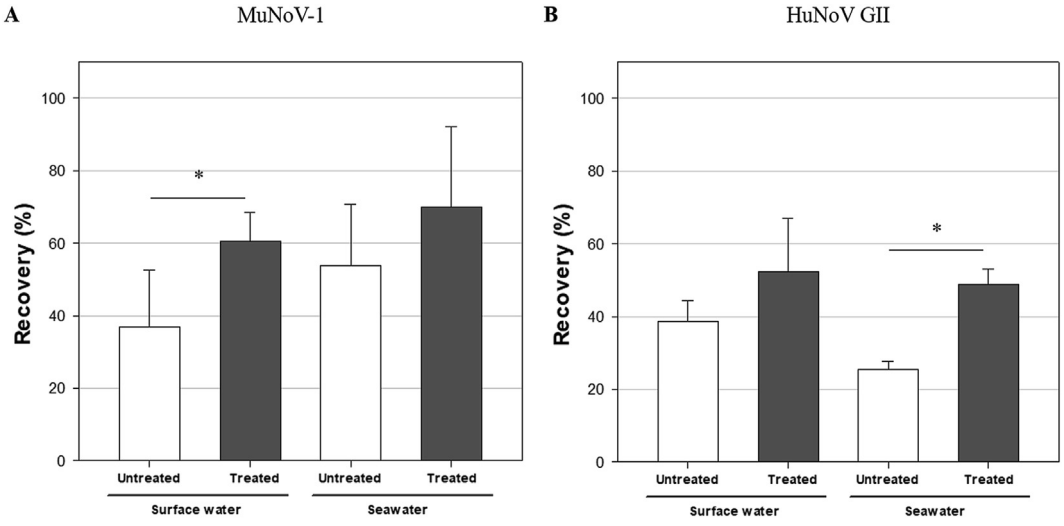

Figure 1. Recovery efficiencies of noroviruses in surface and seawater samples with or without the inhibitor-removal treatment. (A) MuNoV-1, (B) HuNoV GII. Data were measured by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR), expressed as the means ± standard deviation (SD) of three independent experiments. Asterisks indicate statistical significance ($P < 0.05$; independent *t*-test).

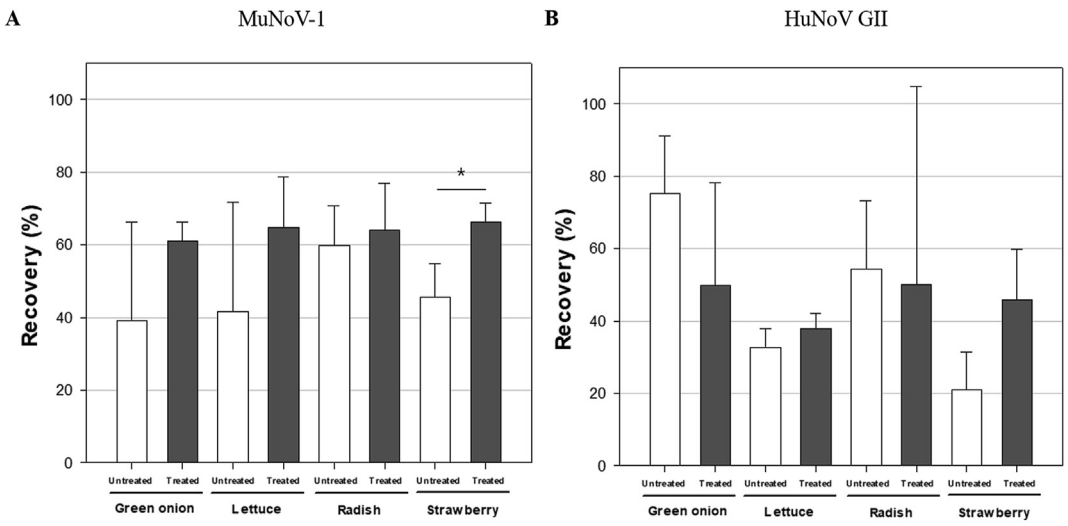

Figure 2. Recovery efficiencies of noroviruses in various produce with or without the inhibitor-removal treatment. (A) MuNoV-1, (B) HuNoV GII. Data were measured by RT-qPCR, expressed as the means ± SD of three independent experiments. Asterisks indicate a statistical significance ($P < 0.05$; independent *t*-test).

infection [24]. Therefore, as Figure 2 suggests, HuNoV can be reliably detected after removing potent inhibitors originating from produce, including polysaccharides, phenols, and polyphenols, which can affect the RT-qPCR results [14].

With the inhibitor-removal treatment, the mean recovery efficiencies of noroviruses improved for all kinds of samples (Figure 1 and Figure 2). The mean recovery efficiencies of MuNoV-1 and HuNoV in produce were constant (Figure 2). We established an inhibitor-removal treatment using two well-known commercial kits with clear instructions that showed the best performance to increase recovery efficiencies of noroviruses among various nucleic acid extraction kits. Our inhibitor-removal treatment could potentially be used to detect various enteric viruses in environmental samples or fresh produce with a well-structured manual. However, we confirmed the differences in recovery efficiencies between two types of noroviruses, even though MuNoV-1 is the well-known surrogate for HuNoVs. There are various factors such as the inherent differences between noroviruses, including capsid structures and genes, and extrinsic differences, including kit efficiencies and effects of inhibitors, can affect the recovery efficiencies of the inhibitor-removal treatment. Therefore, the further evaluations using different enteric viruses is important to confirm the suitability of our inhibitor-removal treatment. Moreover, internal PCR controls such as armored RNA and mengovirus are valuable to find the effects of PCR inhibitors [25, 26]. Therefore, further studies should be performed using the large scales of samples with internal PCR controls before application of this inhibitor-removal treatment under different conditions.

In conclusion, this inhibitor-removal treatment improved RT-qPCR detection of noroviruses in various water samples and produce. Considering the low infectious dose and high infectivity of HuNoV infection, this method could be useful to prevent HuNoV transmission.

Declarations

Author contribution statement

SungJun Park, Cheonghoon Lee: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kyuseon Cho: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Hye Young Ko: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Sung Jae Jang: Analyzed and interpreted the data; Wrote the paper.

GwangPyo Ko: Conceived and designed the experiments; Wrote the paper.

Funding statement

This work was supported by a grant (14162MFDS973) from Ministry of Food and Drug Safety in 2016 and a National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (no. 2018R1D1A1B07050085).

Data availability statement

Data included in article/supp. material/referenced in article.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e07560.

References

[1] E. Robilotti, S. Deresinski, B.A. Pinsky, Norovirus, Clin. Microbiol. Rev. 28 (1) (2015) 134–164.
[2] A.N. Roth, K.R. Grau, S.M. Karst, Diverse mechanisms underlie enhancement of enteric viruses by the mammalian intestinal microbiota, Viruses 11 (8) (2019) 760.
[3] K. Cho, C. Lee, S. Park, J.H. Kim, Y.S. Choi, M.S. Kim, et al., Use of coliphages to investigate norovirus contamination in a shellfish growing area in Republic of Korea, Environ. Sci. Pollut. Res. 25 (30) (2018) 30044–30055.
[4] C.J.P.A. Hoebe, H. Vennema, A.M. De Roda Husman, Y.T.H.P. Van Duynhoven, Norovirus outbreak among primary schoolchildren who had played in a recreational water fountain, J. Infect. Dis. 189 (4) (2004) 699–705.
[5] M. Kitajima, E. Haramoto, C. Phanuwan, H. Katayama, S. Ohgaki, Detection of genogroup IV norovirus in wastewater and river water in Japan, Lett. Appl. Microbiol. 49 (5) (2009) 655–658.
[6] M. Riera-Montes, K. Brus Sjölander, G. Allestam, E. Hallin, K.O. Hedlund, M. Löfdahl, Waterborne norovirus outbreak in a municipal drinking-water supply in Sweden, Epidemiol. Infect. 139 (12) (2011) 1928–1935.
[7] K.M. Herman, A.J. Hall, L.H. Gould, Outbreaks attributed to fresh leafy vegetables, United States, 1973-2012, Epidemiol. Infect. 143 (14) (2015) 3011–3021.

[8] J. Loutreul, C. Cazeaux, D. Levert, A. Nicolas, S. Vautier, A.L. Le Sauvage, et al., Prevalence of human noroviruses in frozen marketed shellfish, red fruits and fresh vegetables, Food Environ. Virol. 6 (3) (2014) 157–168.
[9] D. Mäde, K. Trübner, E. Neubert, M. Höhne, R. Johne, Detection and typing of norovirus from frozen strawberries involved in a large-scale gastroenteritis outbreak in Germany, Food Environ. Virol. 5 (3) (2013) 162–168.
[10] V. Terio, M. Bottaro, E. Pavoni, M.N. Losio, A. Serraino, F. Giacometti, et al., Occurrence of hepatitis A and E and norovirus GI and GII in ready-to-eat vegetables in Italy, Int. J. Food Microbiol. 249 (2017) 61–65.
[11] T. Kageyama, S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F.B. Hoshino, et al., Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR, J. Clin. Microbiol. 41 (4) (2003) 1548–1557.
[12] K.J. Rolfe, S. Parmar, D. Mururi, T.G. Wreghitt, H. Jalal, H. Zhang, M.D. Curran, An internally controlled, one-step, real-time RT-PCR assay for norovirus detection and genogrouping, J. Clin. Virol. 39 (4) (2007) 318–321.
[13] M. Kim, G. Ko, Quantitative characterization of the inhibitory effects of salt, humic acid, and heavy metals on the recovery of waterborne norovirus by electropositive filters, J. Water Health 11 (4) (2013) 613–622.
[14] C. Schrader, A. Schielke, L. Ellerbroek, R. Johne, PCR inhibitors - occurrence, properties and removal, J. Appl. Microbiol. 113 (5) (2012) 1014–1026.
[15] M.S. Noor Hidayah, C. Tuan Zainazor, C.F. Pui, A. Noorlis, M.R. Noor Eliza, M.D. Naziehah, et al., Occurrence of norovirus GI in green and red onion, Int. Food Res. J. 18 (2) (2011) 677–681.
[16] A. Predmore, J. Li, Enhanced removal of a human norovirus surrogate from fresh vegetables and fruits by a combination of surfactants and sanitizers, Appl. Environ. Microbiol. 77 (14) (2011) 4829–4838.
[17] J.H. Yu, N.Y. Kim, Y.J. Koh, H.J. Lee, Epidemiology of foodborne norovirus outbreak in Incheon, Korea, J. Kor. Med. Sci. 25 (8) (2010) 1128–1133.
[18] J. Lee, K. Zoh, G. Ko, Inactivation and UV disinfection of murine norovirus with TiO_2 under various environmental conditions, Appl. Environ. Microbiol. 74 (7) (2008) 2111–2117.
[19] H.Y. Ko, K. Cho, S. Park, J.H. Kim, J.H. Kang, Y.S. Jeong, et al., Host-specific *Bacteroides* markers-based microbial source tracking in aquaculture areas, Microb. Environ. 33 (2) (2018) 151–161.
[20] Ministry of Food and Drug Safety, Guideline for Investigation on the Cause of Food Poisoning. Chapter 5. Ministry of Food and Drug Safety, Chungcheonbuk-Do, Republic of Korea, 2013 (Korean).
[21] Y. Park, Y.-H. Cho, Y. Jee, G. Ko, Immunomagnetic separation combined with real-time reverse transcriptase PCR assays for detection of norovirus in contaminated food, Appl. Environ. Microbiol. 74 (13) (2008) 4226–4230.
[22] C.M. Kephart, R.N. Bushon, Utility of Microbial Source Tracking Markers for Assessing Fecal Contamination in the Portage River Watershed, Northwestern Ohio, 2008, US Geological Survey, 2010. https://pubs.usgs.gov/sir/2010/5036/. (Accessed 20 November 2020).
[23] C. Lee, S. Park, K. Cho, J.E. Yoo, S. Lee, G. Ko, Comparison of swab sampling methods for norovirus recovery on surfaces, Food Environ. Virol. 10 (4) (2018) 378–385.
[24] M. Bouwknegt, K. Verhaelen, A. Rzeżutka, I. Kozyra, L. Maunula, C.-H. von Bonsdorff, et al., Quantitative farm-to-fork risk assessment model for norovirus and hepatitis A virus in European leafy green vegetable and berry fruit supply chains, Int. J. Food Microbiol. 198 (2015) 50–58.
[25] M.I. Costafreda, A. Bosch, R.M. Pintó, Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples, Appl. Environ. Microbiol. 72 (6) (2006) 3846–3855.
[26] J. Stevenson, W. Hymas, D. Hillyard, The use of Armored RNA as a multi-purpose internal control for RT-PCR, J. Virol. Methods 150 (1) (2008) 73–76.