



Comparison of Swab Sampling Methods for Norovirus Recovery on Surfaces

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Abstract

Human noroviruses (HuNoVs) can be easily transferred by the contacts of humans or fomites. Swab sampling methods are widely used for recovering HuNoVs from small surfaces of various fomites or hard-to-reach locations and swab sampling conditions are important for the accurate detection of HuNoVs, which have a low infectious dose and relatively long persistence under a range of environmental conditions. Therefore, to determine the suitable swab sampling method for recovering HuNoVs from various surfaces, we evaluated combinations of four swab materials (cotton, microdenier polyester [a type of microfiber], polyurethane foam, and rayon) and three elution buffer solutions (phosphate-buffered saline [PBS], PBS with 0.2% Tween-80, and 3% beef extract-50 mM glycine [pH 9.5]). First, we inoculated HuNoVs or murine noroviruses (MuNoVs), the surrogate of HuNoVs, onto test coupons (10 × 10 cm) consisting of three common surface materials (high-density polyethylene, stainless steel, and wood). Coupons were swabbed using a combination of each swab material and elution buffer, and the viral recovery was measured by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) or plaque assay. By RT-qPCR, we confirmed that the cotton swab–PBS and microdenier polyester–PBS combinations had recovery efficiencies greater than 80% for viruses on plastic and stainless steel surfaces. The cotton swab–PBS combination had the highest recovery efficiency on all surface materials via the plaque assay. Therefore, a cotton or a microdenier polyester swab with PBS could be a useful method for sampling HuNoVs on various surfaces.

Keywords Human norovirus · Murine norovirus · Swab sampling method · Surface · Recovery

Introduction

Human noroviruses (HuNoVs), belonging to the family *Caliciviridae*, are nonenveloped icosahedral virions (27–40 nm), with a linear, positive-sense, and single-stranded RNA genome (~7.6 kb in length) (Kapikian et al. 1972; Jiang et al. 1993). HuNoVs are one of the most common causative agents of nonbacterial gastroenteritis in all age groups (Ahmed et al. 2014). Many studies have found that HuNoVs are important etiological agents of food and waterborne gastroenteritis via the fecal–oral route (Patel et al. 2009; Giammanco et al. 2014).

Infection by HuNoVs can occur at low infectious doses (Teunis et al. 2008) and HuNoVs have a relatively long persistence under various environmental conditions (Cheesbrough et al. 2000; Jones et al. 2007; Lamhoujeb et al. 2008). Several studies have demonstrated that HuNoVs are easily transferred from surfaces to the hand (Barker et al. 2004; Otter et al. 2011). Moreover, Wikswo et al. (2015) found that HuNoV was the most frequently reported cause of

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acute gastroenteritis outbreaks in the United States and can be transmitted in various ways, including person-to-person contact and environmental contamination.

Therefore, accurate detection and sampling methods are important in the management of HuNoV outbreaks. HuNoV detection has mainly relied on molecular methods, including nucleic acid amplification and real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) has become the gold standard for HuNoV detection due to its accuracy and rapidity (Vinjé 2015). The swab sampling method is appropriate for small surfaces or hard-to-reach locations (Centers for Disease Control and Prevention 2012). Therefore, in general, the swab sampling method is used for collecting pathogens such as HuNoVs on surfaces (Boxman et al. 2009; Centers for Disease Control and Prevention 2012; Piedrahita et al. 2017). Even though swab sampling conditions are significant for accurate HuNoV detection, previous studies have suggested the use of different swab sampling methods under different test conditions, including virus types (HuNoV or HuNoV surrogates), surface material types, elution buffers, and the area used for swab sampling with different detection methods (Julian et al. 2011; Rönqvist et al. 2013; Park et al. 2015; Ibfelt et al. 2016).

The aim of this study was to elucidate the suitable combinations of both the swab material and the elution buffer for swab sampling to recover HuNoVs from various surfaces. We evaluated various combinations of swab materials and elution buffers using HuNoVs and murine noroviruses (MuNoVs), which are a surrogate of HuNoVs, inoculated onto three common surface materials (wood, plastic, and stainless steel). The recovery efficiencies of viruses were measured by RT-qPCR or plaque assay.

Materials and Methods

Cells and Viruses

RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) containing 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) as previously described (Lee et al. 2008). The cells were maintained in 5% CO₂ in an incubator at 37 °C. MuNoVs were kindly provided by Dr. Herbert W. Virgin of the Washington University School of Medicine, USA, and were inoculated in a confluent monolayer of RAW 264.7 cells for 3 days for virus propagation (Lee et al. 2008). Infected cells were subjected to three cycles of freeze–thawing and MuNoVs were purified from the supernatant via centrifugation with chloroform (AMRESCO, Solon, OH, USA) at 5000×g for 20 min at 4 °C and concentrated using an Amicon Ultra-15 centrifugal

filter unit with an Ultracel-10 membrane (nominal molecular weight limit: 10 kDa; Millipore, Billerica, MA, USA). A HuNoV genogroup II genotype 4 (GII/4) stool sample was kindly provided by Dr. In-Soo Choi of Konkuk University, Republic of Korea, and was suspended in 10% of 1× phosphate-buffered saline (PBS). The sample was centrifuged at 20,000×g for 20 min at 4 °C and the concentrated supernatant, including HuNoVs, was collected. The virus stocks were stored at –80 °C until use.

Preparation of Surface Coupons

The coupons (10×10 cm) were prepared using three common surface materials (high-density polyethylene [HDPE], stainless steel, and wood [*Chamaecyparis obtusa*]). To eliminate the possible contamination of viruses on coupons, they were washed using deionized water, dried in a fume hood, and autoclaved for 30 min at 121 °C. The sterilized coupons were stored in a dry oven at 60 °C until use.

Swabs and Elution Buffers

Four sterilized swabs were used in this study: (1) a cotton swab (head width [W] 11.5 mm × head thickness [T] 10.0 mm × head length [L] 24.0 mm; Dae Han Medical Supply Co., Ltd., Seoul, Korea), (2) a microdenier (microfiber) polyester swab (W 13.0 mm × T 4.2 mm × L 25.7 mm; TX714MD; Texwipe, Kernersville, NC, USA), (3) a polyurethane foam swab (W 13.0 mm × T 7.8 mm × L 25.7 mm; STX712A; Texwipe), and (4) a rayon swab (W 4.5 mm × T 4.0 mm × L 15.0 mm; 3M™ Quick Swab; 3M Microbiology, St. Paul, MN, USA). Three autoclaved-elution buffers (PBS, PBS with 0.2% Tween-80 [PT], and 3% beef extract-50 mM glycine [pH 9.5; BG]) were also prepared before the experiments.

Swab Sampling of Viruses on Surface Coupons

Ten microliters (µL) of virus stocks, including 1.6×10^5 plaque forming units (PFU) of MuNoVs or 3.6×10^5 genomic copies of HuNoVs, were inoculated onto the sterilized coupons using the spike method. Little droplets (~0.5 µL per droplet) of virus stock droplets were randomly spotted on the surface of coupons. The spiked coupons were dried in a biosafety cabinet for 15 min at room temperature and a relative humidity of 20–40%. Then, the coupon surfaces were swabbed horizontally, vertically, and diagonally using swabs completely moistened with 5 mL elution buffer in a 15-mL conical tube. Each experiment was independently performed three times with duplicate swab samples and swabs moistened with 10 µL of sterilized PBS were used as negative controls.

After sampling, the swabs were subjected to six dipping and pressing cycles and vortexing for 15 s in a conical tube. The eluted swabs were removed, and the tubes were held at 4 °C for 15 min. When the BG buffer was used as the elution buffer, the eluate was immediately adjusted to neutral pH (7.0–7.5) using 1.0 M HCl. The final eluate was stored at –80 °C until use.

Viral RNA Extraction and RT-qPCR

Viral RNA was extracted from each sample using a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The final elution volume was 50 µL and the extracted viral RNA was stored at –80 °C until use. An RT-qPCR assay was conducted with a 7300 Real-Time System (Applied Biosystems, Foster City, CA, USA) using the extracted viral RNA. Subsequently, TaqMan® based RT-qPCR assays were performed in duplicate targeting the capsid regions of MuNoVs (Lee et al. 2008) and HuNoVs (Ministry of Food and Drug Safety 2013), with some modifications (Table 1). The RT-qPCR mixture had a total volume of 25 µL consisting of 5 µL of RNA, 13.5 µL of AgPath-ID™ One-Step RT-PCR reagents (12.5 µL of 2× RT-PCR buffer and 1 µL of 25× RT-PCR enzyme mix; Thermo Fisher Scientific, Waltham, MA, USA), forward and reverse primers (1 µM each for MuNoV and 400 nM for HuNoV) (Table 1), and a probe (240 nM for MuNoV and 200 nM for HuNoV) (Table 1). The RT-qPCR included reverse transcription at 48 °C for 30 min, followed by heat inactivation of the reverse transcriptase and initial denaturation at 95 °C for 15 min. Amplification was conducted with 45 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Fluorescence data were acquired at the extension step and the threshold cycle (*Ct*). RNAs from MuNoV and HuNoV GII/4, and distilled water, were used as positive and negative controls, respectively. The cycle number corresponding to

an increase in fluorescence over the threshold was calculated with threshold auto. To generate the standard curves, 10-fold serial dilutions of plasmids containing the capsid regions of MuNoV and HuNoV GII were prepared (Lee et al. 2008; Park et al. 2008), ranging from 5.45×10^6 to 5.45×10^{-1} genomic copies for MuNoV and from 4.43×10^6 to 4.4×10^{-1} genomic copies for HuNoV. Serial dilutions were included in duplicate in every RT-qPCR amplification. Standard curves were created by plotting *Ct* values versus the log number of genomic copies within the linear range of quantification and a trend line was generated through these points. The limit of quantification (LOQ) was based on the lowest range of standards that contributed to the linear part of the standard curve (Kephart and Bushon 2010). Negative controls were used to measure a limit of detection (LOD). If *Ct* values were undetermined in the negative controls, the values were set to the end cycle of thermal cycling (45). A cycle threshold higher than the LOD was considered to be “non-detected” (ND). Results between the LOQ and the LOD were qualified as “detected but not quantified” (DNQ).

MuNoV Plaque Assay

A MuNoV plaque assay for the eluates was performed as previously described with some modifications (Lee et al. 2008). Briefly, RAW 264.7 cells were seeded on 6-well plates (3×10^6 cells per well) and incubated at 37 °C with 5% CO₂ until a cell monolayer developed. The eluates were serially diluted and 500 µL of samples were inoculated into the wells with a fully developed cell monolayer. The inoculated plates were incubated for 1 h at 37 °C with 5% CO₂ and rocked every 15 min. Subsequently, the cells were overlaid with 3 mL of a 1:1 (v/v) mixture (37 °C) of 1.5% SeaPlaque agarose (Lonza, Rockland, ME, USA) and 2× minimum essential medium (Gibco), containing 10% fetal bovine serum (Gibco), 10 mM nonessential amino acids (Gibco), 10 mM sodium bicarbonate (Gibco), 10 mM

Table 1 Primers and probes used in this study

Assay name	Primer or probe (polarity)	Sequence (5'–3') ^a	Location ^b	Target	References
MuNoV-1	MNV1F (+)	ACGCCACTCCGCACAAA	5614–5630	Capsid gene of murine norovirus 1	Lee et al. (2008)
	MNV1R (–)	GCGGCCAGAGACCACAAA	5657–5649		
	MNV1P (+)	VIC-AGCCCGGGTGATGAG-MGB	5632–5646		
HuNoV	BPO-13 (+)	AICCIATGTTYAGITGGATGAG	5007–5028	Capsid gene of human norovirus genogroup II	Ministry of Food and Drug Safety (2013)
	BPO-13N (+)	AGTCAATGTTTAGTGGATGAG	5007–5028		
	BPO-14 (–)	TCGACGCCATCTTCATTCACA	5100–5080		
	BPO-18 (+)	VIC-CACRTGGGAGGGCGATCG CAATC-TAMRA	5044–5066		

^aMGB minor groove binder, TAMRA 6-carboxy-tetramethylrhodamine, I inosine, Y C or T, R A or G

^bRelative positions of primers and probe in murine norovirus 1 clone CW1 (accession no. DQ285629) and Lordsdale virus (accession no. X86557) for MuNoV-1 and HuNoV, respectively

HEPES (Gibco), and 50 µg/µL gentamicin (Gibco). After 4 days of incubation, MuNoV plaques were counted. Approximately 100 PFU/500 µL of MuNoVs and 500 µL of PBS were used as the positive and negative controls, respectively.

Statistical Analysis

All statistical analyses were performed with the IBM® SPSS® Statistics (Release ver. 23.0.0.2; IBM Corporation, Armonk, NY, USA) package. For the calculation of means, DNQs were assigned a value of half the limit of quantification (LOQ/2). The recovery efficiency of the swab sampling method was calculated using the following equation: virus recovery efficiency (%) = (PFU [or genomic copies] of recovered viruses/PFU [or genomic copies] of initially inoculated viruses) × 100. The normality of data was verified using the Shapiro–Wilk test, and the equality of variances was tested using Levene’s test. When the data displayed normality and the equality of variances, a one-way analysis of variance (ANOVA) with a Bonferroni post hoc test was performed to define significant differences. When the data were not normal, or the variances of the data were not equal,

a Kruskal–Wallis (KW) test or a Mann–Whitney (MW) test with a Bonferroni correction was used.

Results

Quantitative Evaluation of the MuNoV Recovery Efficiencies for Swab Material–Elution Buffer Combinations on Surface Coupons

Figure 1 shows the mean MuNoV recovery efficiencies of each combination of swab material–elution buffer on three different coupons using RT-qPCR. There were significant differences in recovery efficiencies depending on the swab material type and elution buffer used on three coupon materials according to the KW test ($P < 0.05$). MuNoV was more efficiently recovered with PBS buffer when the same swab material type was tested on each coupon material (MW test with a Bonferroni correction; $P < 0.016$) (Fig. 1). The cotton and microdenier polyester swabs with PBS had an MuNoV recovery efficiency of more than 80% on HDPE coupons (Fig. 1a) and all swab materials with PBS resulted in a large

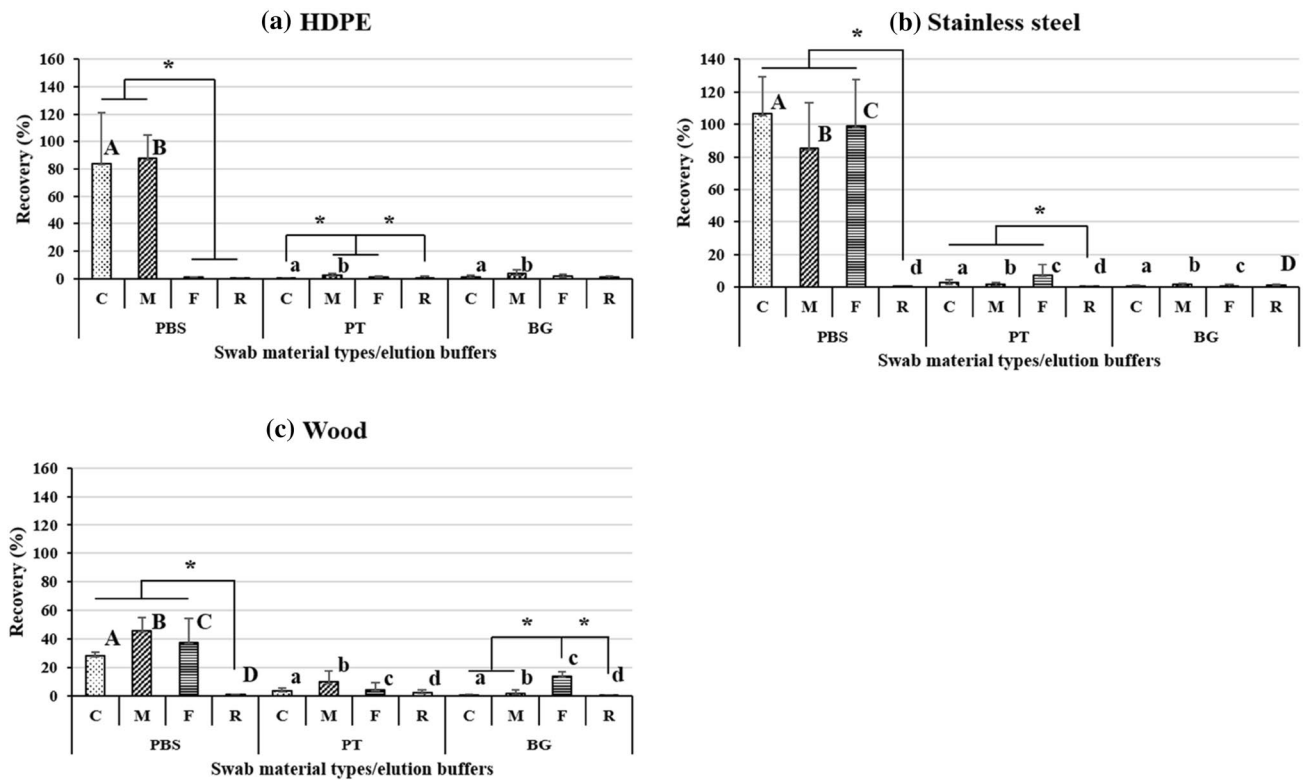


Fig. 1 Mean murine norovirus (MuNoV) recovery efficiencies with standard deviation determined by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) on **a** high-density polyethylene (HDPE), **b** stainless steel, and **c** wood surfaces. C, M, F, and R represent cotton, microdenier polyester, polyurethane foam, and rayon, respectively. Three independent experiments were per-

formed for each combination of swab material and elution buffer. The different upper (A, B, C and D) or lower cases (a, b, c and d) indicate significant differences in the results via the swab materials ($P < 0.05$). Asterisks indicate significant differences in the results via the elution buffers ($P < 0.05$)

recovery of MuNoVs for stainless steel and wood coupons, with the exception of the rayon swabs (Fig. 1b, c) (MW test with a Bonferroni correction; $P < 0.008$). The recovery efficiencies of MuNoVs on wood coupons were generally lower compared with the other surface materials (Fig. 1c).

Figure 2 shows the mean MuNoV recovery efficiencies of each combination of swab material–elution buffer on three different types of coupon using a plaque assay. The recovery efficiencies of MuNoVs from all combinations were below 8%. Only the cotton swabs with PBS had a recovery efficiency greater than 3% for all coupons and the microdenier polyester swabs with PT especially had a significant recovery efficiency for an HDPE surface ($7.10 \pm 3.51\%$) according to the MW test with a Bonferroni correction ($P < 0.016$).

Quantitative Evaluation of HuNoV Recovery Efficiencies Using Cotton or Microdenier Polyester Swabs with PBS on Surface Coupons

Figure 3 shows the recovery efficiencies of HuNoVs on surface coupons using cotton or microdenier polyester swabs with PBS. Both combinations resulted in HuNoV removal efficiencies of more than 80% from HDPE and

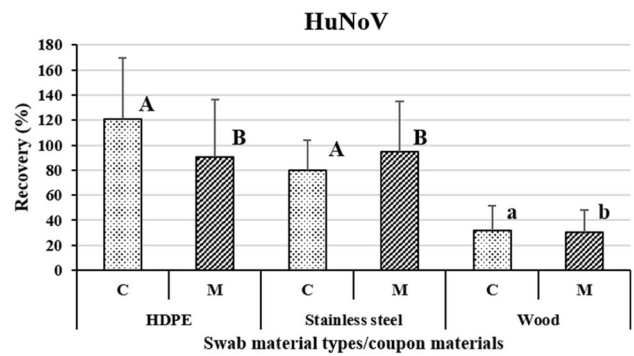


Fig. 3 Mean human norovirus (HuNoV) recovery efficiencies with the standard deviation determined by RT-qPCR on HDPE, stainless steel, and wood surfaces. C and M represent cotton and microdenier polyester, respectively. Three independent experiments were performed for each combination of swab material and PBS. The different upper (A and B) or lower cases (a and b) indicate significant differences in the results via the swab materials ($P < 0.05$)

stainless steel coupons. However, the efficiencies of both combinations for wood coupons (cotton swabs: $31.9 \pm 19.3\%$; microdenier polyester swabs: $30.3 \pm 19.3\%$) were significantly lower than the other surface materials

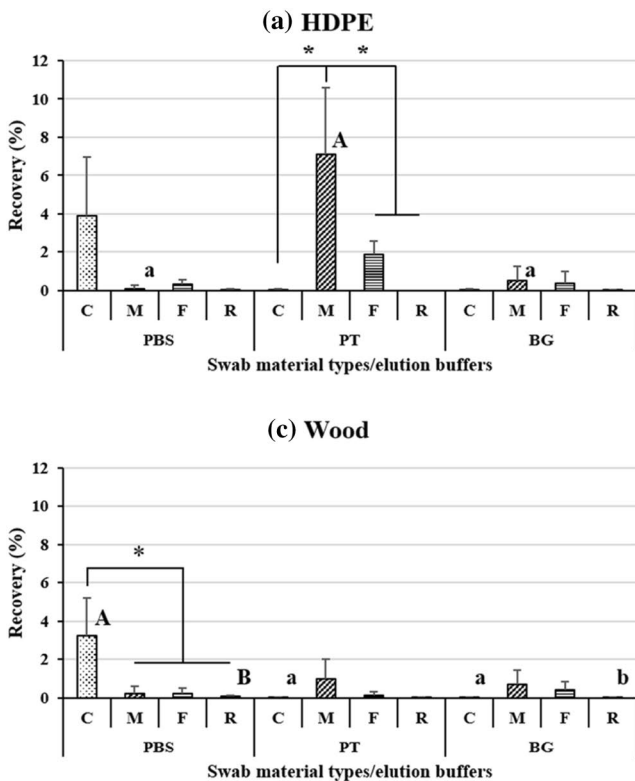


Fig. 2 Mean MuNoV recovery efficiencies with the standard deviation determined by plaque assay on **a** HDPE, **b** stainless steel, and **c** wood surfaces. C, M, F, and R represent cotton, microdenier polyester, polyurethane foam, and rayon, respectively. Three independent experiments were performed for each combination of swab mate-

rial and elution buffer. The different upper (A and B) or lower cases (a and b) indicate significant differences in the results via the swab materials ($P < 0.05$). Asterisks indicate significant differences in the results via the elution buffers ($P < 0.05$)

(ANOVA or the MW test with a Bonferroni correction; $P < 0.016$).

Discussion

Surfaces contaminated by humans or fomites are an important transmission route of enteric viruses, including HuNoVs (Rusin et al. 2002; D'Souza et al. 2006). In many studies, swab sampling has been suggested as an efficient sampling method for recovering HuNoVs or HuNoV surrogates from surfaces (Rönnqvist et al. 2013; De Keuckelaere et al. 2014; Granime et al. 2015). Therefore, swab sampling has been widely applied in HuNoV outbreak investigations (Boxman et al. 2009; Wadl et al. 2010; Nenonen et al. 2014). To determine the ideal swab sampling method for HuNoV detection, we evaluated various combinations of swab materials and elution buffers for MuNoVs or HuNoVs on three surface materials using RT-qPCR or plaque assay as the cultivation method.

Previous studies have reported that the type of swab material and elution buffer are important factors for the effective recovery of HuNoV from surfaces (Rönnqvist et al. 2013; De Keuckelaere et al. 2014; Ibfelt et al. 2016; Turnage and Gibson 2017). Our results showed that both MuNoVs and HuNoVs were effectively recovered using cotton and microdenier polyester swabs with PBS (> 80% on HDPE and stainless steel coupons; > 20% on wood coupons) when RT-qPCR was used as the detection method (Figs. 1, 3). De Keuckelaere et al. (2014) reported that the combination of a microfiber material and PBS achieved an HuNoV recovery efficiency greater than 80% from HDPE surfaces via RT-qPCR. On the other hand, Rönnqvist et al. (2013) reported that a glycine buffer (pH 9.5) with a microfiber swab resulted in better HuNoV recovery from low-density polyethylene plastic ($89 \pm 2\%$) and stainless steel ($79 \pm 10\%$) surfaces compared with PBS. Moreover, Park et al. (2015) reported that macrofoam swabs resulted in good HuNoV recovery efficiencies from stainless steel surfaces compared with cotton, rayon, or polyester swabs and, especially, the cotton swab did not show strong efficiencies in comparison with our data. Differences in the production process, including the dyeing phase, could affect the virus recovery efficiency of each swab (Rönnqvist et al. 2013). Rönnqvist et al. (2013) reported that two microfiber swabs had remarkable differences in their recovery efficiencies for HuNoVs and found that the properties of each swab, including net surface charge, were altered during the production process. The production process may determine the properties of swabs irrespective of the material type. Moreover, the properties of the elution buffer could also affect the recovery efficiencies achieved during swab sampling. For example, a surfactant like Tween-80 can increase the water content

of the target surface and facilitate the solubilization of cell surfaces, while a high pH of the buffer (e.g., pH 9.5) could change the net surface charge of HuNoVs (Rönnqvist et al. 2013; Park et al. 2015). However, our study found a lower MuNoV and HuNoV recovery efficiency when PT or BG was used as the elution buffer (data not shown), indicating that they would be suitable for certain types of swab materials. In consideration of various factors, which could affect the recovery efficiencies of swab sampling directly, it is recommended that further studies and discussions for the combination of swab material and elution buffer for HuNoVs and their surrogates should be performed for a precise HuNoV sampling from various surfaces.

The results of this study indicated that the differences in the recovery efficiencies of MuNoVs could be due to the detection method used regardless of whether cotton or microdenier polyester swabs with PBS were used for the sampling of surfaces (Figs. 1, 2). Only 2–4% of MuNoVs were recovered using cotton swabs with PBS from the three different surface types when a plaque assay was used (Fig. 2). Previous studies have reported that a less than 1 \log_{10} reduction of MuNoVs can occur within 1 h following exposure at pH 7.0 and room temperature (Cannon et al. 2006; Lee et al. 2008). In this study, the average time elapsed, including the inoculation of MuNoVs on coupons to the swab, was less than 40 min, which could not have significantly affected the total concentration of MuNoVs. The properties of each swab material and the interactions between the swab material and viruses during sampling could affect the results of a plaque assay. The cotton swab and PBS resulted in excellent recovery efficiencies for the various surface materials compared with the other combinations. This combination could therefore be useful for the accurate detection of HuNoVs using RT-qPCR or cultivation methods.

Our study resulted in poor MuNoV or HuNoV recovery efficiencies from wood surfaces compared to the HDPE and stainless steel surfaces (Figs. 1, 2, 3). Scherer et al. (2009) reported that the physical properties of a surface could affect the recovery efficiency of a virus when using swab sampling. Wood has many crevices and pores, which can result in viruses becoming trapped within the matrix. Therefore, compared to the HDPE or stainless steel surfaces, viruses were not easily removed from wood surfaces, even when the cotton swab and PBS combination was used for sampling.

Previous studies have indicated that swab materials can absorb potential PCR inhibitors in the environment, such as heavy metals and humic acids (Deng and Bai 2003; Gilbert et al. 2014). The use of internal process control (Granime et al. 2015; Park et al. 2017) and/or dilution of extracted RNA (Lowther et al. 2017) have been suggested to control the effects of PCR inhibitors and further studies should be performed with the consideration of various conditions in

fields. In conclusion, a cotton or microdenier polyester swab with PBS could be a useful method for the efficient detection of HuNoVs on various surfaces.

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