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4 **Comparison of Process Control Viruses for use in Extraction and Detection of Human**
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6 **Norovirus from Food Matrices**
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Abstract

Although RT-qPCR is a powerful tool for human norovirus (HuNoV) detection, low virus concentrations in potentially large sample volumes necessitate the use of inefficient sample processing step(s) prior to detection. Process control viruses (PCVs) are used to monitor the efficiency of these virus concentration steps. This study compared five PCVs [Mengovirus (Mengo), murine norovirus (MNV-1), MS2 coliphage, Tulane virus, and turnip crinkle virus (TCV)] to two HuNoV strains for recovery during the steps of elution, polyethylene glycol precipitation (PEG), and RNA extraction from select foods (lettuce and sliced deli ham). Results demonstrate high recovery efficiencies of HuNoV GI.6 and GII.4 using the methods described in this study: combined (sequential) losses during processing from sliced deli ham and lettuce were $<1 \log_{10}$ genome equivalent copies (GEC). When considering the processing steps separately, HuNoV loss was negligible after elution, and low after PEG precipitation (mean $0.5 \log_{10}$ GEC) and RNA extraction (mean $0.1 \log_{10}$ GEC). The virus that least mimicked the behavior of HuNoV during sample processing was MNV-1. Of the viruses tested, a commercial mengovirus strain gave recovery efficiencies closest to HuNoV, showing combined losses from sliced deli ham and lettuce of $<1 \log_{10}$ GEC and $\sim 1 \log_{10}$ GEC, respectively. All PCVs do not behave equivalently and validation of their performance is recommended before their routine use on an application-by-application basis.

Keywords: norovirus, process control virus, RT-qPCR, processing efficiency

Abbreviations: HuNoV, human norovirus; PCV, process control virus; MNV-1, murine norovirus; TCV, turnip crinkle virus; PEG, polyethylene glycol; GEC, genome equivalent copies; RT, room temperature; RT-qPCR, real-time quantitative polymerase chain reaction;

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BSA, bovine serum albumin; TCID₅₀, median tissue culture infectious dose; CPE, cytopathic effect; PBS, phosphate buffered saline; FBS, fetal bovine serum.

1. Introduction

Human noroviruses (HuNoV) are the leading cause of foodborne disease in the United States (Scallan et al., 2011) and perhaps worldwide (Glass, Parashar, & Estes, 2009). Despite their public health significance, routine detection of HuNoV in food and environmental samples has been historically difficult to quantify, in part due to the potentially low densities of the virus in foods and the lack of a cell culture system. Molecular amplification methods, specifically reverse transcription quantitative PCR (RT-qPCR), offer powerful tools for rapid detection of HuNoV in complex matrices. In addition to avoiding the need for cultivation, RT-qPCR can save both time and expenses and has the potential to be sensitive, inexpensive, quantitative, and amenable to automation. However, low virus concentrations in potentially large sample volumes necessitates the use of labor intensive and potentially inefficient concentration step(s) prior to detection (Knight, Li, Uyttendaele, & Jaykus, 2013). Loss during these processing procedures can result in an underestimation of HuNoV load or false negative results.

To ensure accurate quantitation and interpretation of molecular data for HuNoV in various food matrices, controls are needed to determine the efficiency of various processing steps, including virus recovery, concentration, RNA extraction, and RT-PCR. Nucleic acid controls have been developed to quantify the RT-qPCR efficiency of HuNoV (Gregory, Webster, Griffith, & Stewart, 2011; Hata et al., 2011; Lee et al., 2011; Liu et al., 2013; Stals et al., 2009), as well as to quantify the recovery efficiency of viral nucleic acid during extraction procedures (da Silva et al., 2007; Hata et al., 2011; Mormann, Dabisch, & Becker, 2010). A few studies have attempted to quantify the recovery efficiencies of concentration procedures utilizing process control viruses (PCVs). For example,

mengovirus has been utilized as a PCV for HuNoV in wastewater (da Silva et al., 2007) and in shellfish (Le Guyader et al., 2009), and MS2 has been used to monitor recovery of HuNoV from experimentally contaminated foods (Mormann et al., 2010). However, at present, there are no published studies evaluating potential PCVs for their similarity to HuNoV during processing; yet proven controls are imperative for enhancing the detection and accurate quantitation of HuNoV from complex sample matrices.

An ideal PCV for HuNoV would be similar morphologically and physiochemically to HuNoV, i.e. a non-enveloped positive-sense, single stranded RNA virus; sufficiently genetically distinct from HuNoV; and not normally expected to occur naturally in the foodstuffs being analyzed (“Microbiology of food and animal feed - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR - Part 1: Method for quantification,” 2013). Unfortunately, no one PCV is ideal. The goal of this study was to evaluate five viruses for use as PCVs when recovering and detecting HuNoV in foods: Mengovirus (Mengo), murine norovirus (MNV-1), MS2 coliphage, Tulane virus, and turnip crinkle virus (TCV). All viruses are icosahedral, non-enveloped, positive-sense single-stranded RNA viruses with similar size, genome, and isoelectric point to HuNoV (Table 1). Mengo is a member of the *Picorniviridae* family and a commonly used PCV (da Silva et al., 2007; Le Guyader et al., 2009). MS2 is a virus in the family *Leviviridae* that infects the bacterium *Escherichia coli* and other members of the *Enterobacteriaceae*. MNV-1 and Tulane are culturable HuNoV surrogates in the family *Caliciviridae* utilized in HuNoV inactivation and persistence studies (Cromeans et al., 2014), and MNV-1 has been used as a PCV for hepatitis A virus (Coudray, Merle, Martin-Latil, Guillier, & Perelle, 2013; Martin-Latil, Hennechart-Collette, Guillier, & Perelle, 2012). TCV is a plant pathogen in the family

Tombusviridae first isolated from turnip and limited in spread to the UK and Yugoslavia (http://www.dpvweb.net/dpv/showdpv.php?dpvno=109). These potential PCVs were evaluated against HuNoV GI.6, a commonly implicated foodborne outbreak strain, and GII.4, the most common cause of HuNoV outbreaks, during virus recovery (elution), concentration, and RNA extraction procedures commonly used for foods (Baert, Uyttendaele, & Debevere, 2008; Fraisse et al., 2011; “Microbiology of food and animal feed - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR - Part 1: Method for quantification,” 2013; Park, Kim, & Ko, 2010; Summa, Bonsdorff, & Maunula, 2012).

2. Methods

2.1 Viruses and virus propagation

HuNoV strains GI.6 and GII.4 were obtained as clinical stool specimens from HuNoV outbreaks (provided by S. R. Greene NCPH, Raleigh, NC). Stool samples were suspended 20% in phosphate buffered saline (PBS, pH 7.4) and estimated to have a threshold cycle (CT) of 25 and 21, respectively (personal communication, S. R. Green), equivalent to a titer of 7.8×10^7 (GI.6) and 2.7×10^7 (GII.4) genome equivalent copies (GEC)/ml by RT-qPCR (Section 2.3.1).

Mengovirus strain MC₀, an avirulent strain lacking the poly(C) tract, was purchased from American Type Culture Collection (ATCC VR-1597) with a concentration of 6.2×10^8 PFU/ml. Due to a shortage by the manufacturer, additional mengo was obtained as a part of an RNA extraction control kit (developed and manufactured by CEERAM SAS and distributed by Life Technologies™) with a concentration of 1.6×10^8 GEC/ml.

MS2 coliphage (ATCC 15597-B1) was purchased from ATCC (Manassas, VA). The virus stock was quantified using the Single Agar Layer method 1602 (USEPA, 2001) using *Escherichia coli* F+ cells purchased from ATCC (15597) and cultured in tryptic soy broth (Fisher Scientific, Waltham, MA). Stock titer used in experiments was 7.7×10^7 PFU/ml.

MNV-1 was obtained from the laboratory of Dr. Howard Virgin, (Washington University, St. Louis, MO). The virus stock was propagated in RAW 264.7 cells, a mouse monocyte macrophage line purchased from ATCC (TIB-71), and the cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10% low endotoxin fetal bovine serum (FBS, HyClone, Logan, UT) at 37°C under a 5% CO₂ atmosphere as described previously (Tung, Macinga, Arbogast, & Jaykus, 2013). MNV-1 stock titers were 1.3×10^6 PFU/ml.

A purified stock of TCV was obtained from the laboratory of Dr. Steve Lommel (North Carolina State University, Raleigh, NC). Virus concentration was determined using spectroscopy as described previously (Gentry-Shields & Stewart, 2013) at 2.0×10^{15} virus particles/ml.

Tulane virus was obtained from the laboratory of Dr. Jason Jiang (Cincinnati Children's Hospital, Cincinnati, OH) and cultivated in LLC-MK2 cells (Farkas, Sestak, Wei, & Jiang, 2008), a rhesus monkey kidney epithelial line, that were purchased from ATCC (CCL-7). The cells were cultured in M199 medium (Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Cambrex Bio Science Walkersville Inc., Walkersville, MD) and 1% Penicillin-streptomycin (Life Technologies) at 37°C under a 5% CO₂ atmosphere. Virus titer was determined by the 50% cell culture infectious dose (TCID₅₀) assay. Briefly, LLC-MK2 cells were grown on 48-well cell culture plates (BD, Franklin Lakes,

NJ) until 90% confluent (~24 h). Eluates were serially diluted 10^{-1} to 10^{-6} in M199 media. Each well (6 per dilution) was inoculated with 50 μ l of diluted sample. The inoculated plates were incubated at 37°C in 5% CO₂ for 1 h with periodic rocking, followed by the addition of 1 ml maintenance media (M199 media supplemented with 2% FBS and 1% Pen-strep). Starting 24 h after inoculation, the wells were visually examined for cytopathic effect (CPE) using an inverted microscope (Nikon Inc. Melville, NY). CPE was generally fully developed 5 d post-inoculation. The stock titer was calculated using the Reed-Muench Calculator Spreadsheet (<http://www.med.yale.edu/micropath/pdf/infectivity%20calculator.xls>) to be 1.3×10^7 TCID₅₀/ml.

The concentrations of all viruses in genome equivalent copies (GEC) were determined as described previously (Gentry-Shields & Stewart, 2013). All viruses were stored at -80°C.

2.2 Experimental Procedures

All five potential PCVs were evaluated against HuNoV GI.6 and GII.4 for the steps of virus recovery (elution), concentration, and RNA extraction. These processes are described below.

2.2.1 Elution

Fifteen g of romaine lettuce or 25 g of sliced deli ham were seeded with 20 μ l of each virus as several small drops across the food sample (total inoculum concentration ranging from 7-10 log₁₀ GEC) and dried for 30 min at room temperature (RT). The virus-seeded food sample was placed in a polypropylene bag containing a filter compartment (Nasco

Whirl-Pak, Fort Atkinson, WI) and soaked with 25 ml elution buffer (0.04M glycine, 0.15M NaCl, 0.01M NaOH [all from Fisher Scientific], pH 9.0). The sample was stomached for 1 min at 230 rpm using a stomacher (Seward, Davie, FL). The rinse fluid was removed via the filter compartment of the bag to a 50-ml centrifuge tube and adjusted to pH 7.0 ± 0.5 with 0.1 M HCl. To quantify virus recovery during elution, un-inoculated samples were processed in the same manner as above then this set of control samples was inoculated with the same quantity of virus *after* the elution step. All samples were stored at -20°C before polyethylene glycol (PEG) precipitation and chloroform:butanol purification, RNA extraction, and RT-qPCR (described below). Elution recovery was quantified as the difference in virus quantity detected by RT-qPCR from samples spiked pre-elution to samples spiked post-elution. For each virus spike concentration, experiments were performed in triplicate with three independent samples processed per replicate.

2.2.2 Concentration

Viruses were concentrated from lettuce and ham eluates using polyethylene glycol (PEG) precipitation. Twenty-five ml “mock” eluates obtained from un-inoculated foods were seeded with 50 μl of each virus (total inoculum concentration ranging from 5-10 $\log_{10}$ GEC), and subjected to precipitation by the addition of PEG MW 8000 (Sigma–Aldrich, Steinheim, Germany), NaCl, and bovine serum albumin (BSA, Fisher Scientific) in a final concentration of 12% (w/v), 5%, and 3%, respectively (Liu et al., 2013). Samples were incubated on a shaking platform at 4°C for 15 min or 2 h and centrifuged at $18,500 \times g$ for 20 min at 4°C . The pellet was suspended in 1 ml of PBS and stored at -20°C . To remove residual inhibitory substances, the virus concentrates were further subjected to a

chloroform:butanol purification step. Briefly, 1 ml of the suspended pellet was treated with one volume of chloroform:butanol (1:1, v/v). The mixture was vortexed for 2 min at RT and centrifuged again at $18,500 \times g$ for 20 min. The aqueous phase (supernatant) was isolated and stored at -80°C until RNA extraction and RT-qPCR (described below). Similar to the elution experiments, recovery was determined as the difference in virus quantity detected by RT-qPCR from samples spiked pre-concentration to samples spiked post-concentration. For each virus spike concentration, all experiments were performed in triplicate with three independent samples processed per replicate.

2.2.3 RNA Extraction

One ml of PBS was seeded with 100 μl of each virus (total concentration ranging from 2-8 $\log_{10}$ GEC) and nucleic acid extraction was performed using the automated NucliSENS® easyMag® system (bioMérieux, Durham, NC) as per manufacturer instructions. The final purified RNA was eluted into 40 μl of proprietary buffer. RNA extracts were stored at -80°C until analysis by RT-qPCR. Viral recovery during RNA extraction was estimated by comparing virus quantity pre-RNA extraction to that post-extraction. For each virus spike concentration, all experiments were performed in triplicate with three independent samples processed per replicate.

2.3 Quantification of virus recovery

2.3.1 RT-qPCR

RNA isolations were diluted 1:10 and 1:100, and all three dilutions were subjected to RT-qPCR amplification in duplicate using primers described in Table 2. The 25 μl RT-

qPCR mixture consisted of 2.5 µl of RNA, 400 nM of forward and reverse primers, 200 nM of fluorescently labeled TaqMan probe, 12.5 µl 2× reaction buffer (SuperScript® III One-Step qRT-PCR Kit, Invitrogen, Grand Island, NY), and 0.5 µl RT/Platinum® Taq Mix. The reaction mixture was subjected to a one-step thermal cycling profile using a CFX96 Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA) under the following amplification conditions: (i) reverse transcription for 30 min at 50°C; (ii) initial denaturation for 15 min at 95°C; and (iii) 45 cycles of 15 s at 95°C and 30 s at 60°C.

Virus concentration ($\log_{10}$ GEC) was estimated by comparison with standard curves for each virus generated using RNA transcripts serially diluted 1:10 in DEPC-treated water and subjected to RT-qPCR in triplicate. The $\log_{10}$ -transformed RNA GEC was plotted against the CT value and analyzed by linear regression to make the standard curves. RT-qPCR assays were repeated 3 times over non-consecutive days and combined to create one standard curve.

2.3.2 Cell Culture

Recovery of MNV-1, MS2, and Tulane virus after elution and concentration was also quantified by cell culture using the methods described in Section 2.1.

2.4 Statistical Analyses

All statistical analyses were performed using JMP Pro 11 (SAS Institute Inc., Cary, NC). The Shapiro-Wilk test found that virus losses were not normally distributed (data not shown) so all statistical tests were non-parametric. The impact of initial spike concentration on virus recovery was evaluated using the Kruskal-Wallis test for significance. Losses experienced by each virus during RNA extraction, PEG precipitation,

and elution (expressed as log₁₀ GEC, PFU, or TCID₅₀) were also compared using the Kruskal-Wallis test and followed by the Steel-Dwass with control test to compare individual virus losses to HuNoV GI.6 and GII.4. All tests were conducted with $\alpha = 0.05$, and associations with a $p < 0.05$ were considered significant.

3. Results

3.1 Elution

All virus losses were $<1 \log_{10}$ GEC (Figure 1) during elution, regardless of initial spike concentration. All PCVs had statistically similar recoveries to HuNoV GI.6 and GII.4 from both lettuce and sliced deli ham ($p > 0.05$).

3.2 Concentration

Mean HuNoV losses during concentration from sliced deli ham were 0.6 ± 0.8 and 0.4 ± 0.6 GEC for GI.6 and GII.4, respectively, and losses from lettuce were 0.5 ± 0.8 and 0.7 ± 1.3 GEC, respectively. All PCVs had statistically similar recoveries to HuNoV GI.6 and GII.4 in both lettuce and ham ($p > 0.05$, Figure 2). The recovery efficiency was not statistically different when varying the PEG precipitation incubation period from 2 h to 15 min or when varying the initial spike concentration of virus ($p > 0.05$).

3.3 RNA Extraction

Viral RNA extraction efficiencies varied widely by virus type and by initial spike concentration ($p < 0.05$, Figure 3). HuNoV GI.6 and GII.4 experienced low losses during RNA extraction, $<1 \log_{10}$ GEC across all initial spike concentrations evaluated. No PCV had a

statistically similar recovery to HuNoV GI.6 or GII.4 at medium (4-5.9 log₁₀ GEC) and high (6-7.9 log₁₀ GEC) inoculum levels. At a low inoculum level (2-3.9 log₁₀ GEC), TCV and Tulane virus had statistically similar recoveries to both HuNoV GI.6 and GII.4 (p > 0.05), while Mengo (Ceeram) had a statistically similar recovery to HuNoV GI.6.

3.4 Cell Culture

MNV-1, MS2, and Tulane virus losses occurring during elution and concentration steps were quantified by cell culture in addition to RT-qPCR. MS2 losses during both elution and PEG precipitation were similar when quantified by infectivity assays or by RT-qPCR (Table 3). Similarly, MNV-1 losses during PEG precipitation were similar when quantified by infectivity assays or by RT-qPCR (MNV-1 could not be detected by cell culture after elution experiments). However, Tulane losses quantified by TCID₅₀ assays were much higher than losses quantified by RT-qPCR for elution from lettuce (loss of 3.2 ± 0.2 log₁₀ TCID₅₀/ml) and PEG precipitation from sliced deli ham (2.7 ± 0.7 log₁₀ TCID₅₀/ml).

4. Discussion

Since the concept of PCVs was posed almost a decade ago, various viruses have been used to serve this role (e.g., da Silva et al., 2007; Le Guyader et al., 2009). However, in most cases high titers of PCVs are spiked into the food to be processed for virus concentration and purification, with little knowledge of how efficiently those PCVs are being recovered. Nonetheless, the use of a validated PCV is a requirement for detection of HuNoV in food matrices ("Microbiology of food and animal feed - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR - Part 1: Method for

quantification,” 2013). To our knowledge, this is the first study of its kind in which head-to-head comparisons of PCVs were made for the steps of elution, concentration, and RNA extraction as applied to recovery of HuNoV in select foods.

High recovery efficiencies were observed for both HuNoV strains tested using the sample processing methods. Specifically, losses were negligible during elution, averaged 0.5 log₁₀ GEC for the concentration step, and averaged 0.1 log₁₀ GEC for RNA extraction. Combined, this equates to about a 1 log₁₀ GEC loss in recovery of HuNoV using this particular virus extraction/detection protocol, with little difference between GI.6 and GII.4 strains or spike level. These recovery efficiencies are similar to those reported by others using similar methods (Baert et al., 2008; Griffin, Brinkman, Hedrick, Rhodes, & Fout, 2014; Park et al., 2010; Summa et al., 2012). For example, Summa et al. (2012) reported recovery efficiencies of 19% and 47% from lettuce and ham (including elution and PEG precipitation). Park et al. (2010) reported elution recovery efficiencies of 65% and 40% from lettuce and ham using a similar elution buffer to this study.

When examining the separate steps of elution and concentration, most PCVs showed virtually no virus loss during elution and losses <1.5 log₁₀ GEC for the concentration step, although some variability was observed with the latter. Comparatively speaking, losses during RNA extraction were higher than the previous two steps. RNA recoveries for the PCVs varied considerably, but in general they were similar to those reported in previous studies. Stals et al. (2011) reported recoveries of 1-12% for HuNoV GI, GII, and MNV-1 using the easyMAG RNA purification system, and Costafreda et al. (2006) reported recoveries of 1.5-11.6% for mengo and hepatitis E viruses using a Qiagen RNeasy kit. Inoculum level influenced RNA recovery for most viruses, including mengo, MS2, MNV-1,

HuNoV GII.4, and Tulane. Variance associated with inoculum level has been demonstrated for this system previously; Stals et al. (2011) reported greater recovery with a higher inoculum level of HuNoV GI and GII vs. a lower inoculum level. Surprisingly, viral RNA recovery in our study waffled with initial spike concentration – HuNoV GII.4 RNA recovery decreased with higher inoculum levels while MS2 RNA recovery increased.

Keeping in mind the specific parameters of this study, we can conclude that, based on combined losses, the PCV with the most dissimilar recovery to HuNoV was MNV-1, with ~2-3 log₁₀ GEC combined losses from sliced deli ham and lettuce. In fact, MNV-1 losses were so high during RNA extraction ($2.0 \pm 0.5 \log_{10}$ GEC) that MNV-1 could not be detected when low and medium spike volumes were used, and thus recovery could only be evaluated using an initial spike concentration $\geq 6 \log_{10}$ GEC. We suspect that the reason for this low recovery may be related to a high concentration of extra-viral RNA or a low ratio of infectious to noninfectious virus particles. This is supported by the difference in MNV-1 stock titer as evaluated by cell culture ($6.1 \log_{10}$ PFU/ml) versus by RT-qPCR ($11.8 \log_{10}$ GEC/ml). One way to avoid this problem in the future may be the use of an RNase pre-treatment to digest free viral RNA and, theoretically, the RNA associated with non-intact capsids (Li et al., 2012; Topping et al., 2009).

Choosing a single PCV that most mimicked HuNoV during processing and detection is more difficult. Based on the combined recoveries in elution, concentration, and RNA extraction, MS2 and Tulane virus performed relatively well across all spike levels. As a bacteriophage, MS2 is easy to work with and can be produced at high concentration, but may have chemical and/or structural features that impact its usefulness for all applications (“Microbiology of food and animal feed - Horizontal method for determination of hepatitis

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4 298 A virus and norovirus in food using real-time RT-PCR - Part 1: Method for quantification,”
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7 299 2013). Tulane virus is closer to HuNoV structurally, but is difficult to obtain in high
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9 300 concentrations and is not yet widely used. Mengovirus (purchased as part of a kit from Life
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11 301 Technologies), with combined losses from sliced deli ham and lettuce of $<1 \log_{10}$ GEC and
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14 302 $\sim 1 \log_{10}$ GEC, respectively, is a good PVC candidate. In addition to its similar recovery to
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16 303 HuNoV during processing, an additional benefit to the use of Mengo is that it can be grown
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19 304 *in vitro*, allowing for in-house propagation, quantification, and purification. Interestingly, in
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24 306 which produced significantly lower RNA extraction efficiency (mean loss = $2.0 \pm 0.8 \log_{10}$
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26 307 GEC) compared to the strain obtained from Life Technologies (mean loss = $0.6 \pm 0.4 \log_{10}$
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29 308 GEC). While the reason for such a discrepancy is unknown, it is clear that differences in
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32 309 virus source can drastically impact the efficiency of RNA extraction, and perhaps other
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34 310 steps in virus concentration and purification schemes. These findings reiterate the
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36 311 importance of using high quality, standardized virus stocks as PCVs.

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39 312 This study provides evidence of the virus recovery efficiencies for commonly used
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41 313 concentration and purification steps for HuNoV extraction from foods. For all PCVs, the
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44 314 combined steps of elution and PEG precipitation usually resulted in $<1.5 \log_{10}$ GEC losses,
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46 315 but RNA extraction losses were higher and more variable. This suggests that the efficiency
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49 316 of this latter step must be carefully considered when designing virus concentration and
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52 317 purification methods for foods. In fact, it appears that small adjustments in processing
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54 318 methods can have significant effects on virus recovery efficiency and reiterates the
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56 319 requirement for a PCV. While recovery efficiencies varied by virus and by processing step,
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59 320 most of the viruses evaluated would be suitable PCVs for monitoring the efficiency of

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4 321 HuNoV GI and GII extraction and detection using the methods described in this study.
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7 322 Consideration for choice of PCV may take into account availability of virus stocks,
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9 323 laboratory constraints (e.g., for cell culture), and food/environmental matrix to be
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11 324 analyzed. Regardless of the choice of PCV, matrices should be tested with the PCV
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14 325 beforehand to determine extraction efficiency and optimize input virus levels.
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19 327 Acknowledgements
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22 328 The authors would like to thank Dr. Tim Sit at N.C. State University for providing the
23
24 329 turnip crinkle virus stocks. We would like to thank Kathy Latham at Thermo-Fisher and Dr.
25
26 330 Fabienne Loisy at Ceeram for providing the mengovirus control. Funding was provided by a
27
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29 331 Food Virology Collaborative grant (no. 1111-2011-0494) from the National Institute of
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32 332 Food and Agriculture (NIFA) at the U.S. Department of Agriculture (USDA).
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Figures

Figure 1. Box and whisker plot depicting the losses of each virus (in $\log_{10}$ GEC) during elution from sliced deli ham and lettuce. The lower boundary of the box indicates the 25th percentile, the line within the box represents the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers below and above the box indicate the 10th and 90th percentiles, respectively. All viruses were spiked onto triplicate samples and experiments were performed in triplicate using varying concentrations (7-10 $\log_{10}$ GEC) of virus. Input concentration of virus did not make a significant difference in recovery efficiency ($p > 0.05$) so box and whisker plots represent all spike concentrations of an individual virus.

Figure 2. Box and whisker plot depicting the losses of each virus (in $\log_{10}$ GEC) during PEG concentration from sliced deli ham and lettuce. The lower boundary of the box indicates the 25th percentile, the line within the box represents the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers below and above the box indicate the 10th and 90th percentiles, respectively. All viruses were spiked into triplicate samples and experiments were performed in triplicate using varying concentrations of virus (5-10 $\log_{10}$ GEC). Input concentration of virus did not make a significant difference in recovery efficiency ($p > 0.05$) so box and whisker plots represent all spike concentrations of an individual virus.

Figure 3. Box and whisker plot depicting the losses experienced by each virus (in $\log_{10}$ GEC) during RNA extraction. The lower boundary of the box indicates the 25th percentile, the

line within the box represents the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers below and above the box indicate the 10th and 90th percentiles, respectively, and points represent outliers. All viruses were spiked into triplicate samples and experiments were performed in triplicate using varying concentrations of virus (low, 2-3.9 log₁₀ GEC; medium, 4-5.9 log₁₀ GEC; and high, 6-7.9 log₁₀ GEC). Input concentration of virus did make a significant difference in recovery efficiency ($p < 0.05$) so box and whisker plots are presented for low, medium, and high inoculum levels of virus.

TABLES

Table 1. Human norovirus and potential process control virus size, genome length, and isoelectric point.

Virus	Particle Size (nm. diam.)	Genome Length (kb)	Isoelectric point (pI)
Human norovirus (HuNoV)	38 – 40	7.6	5.5 – 6.0
Mengovirus (Mengo)	30	8.4	Unknown
Murine norovirus (MNV-1)	28 – 35	7.4	4.8
MS2 coliphage	27	3.6	3.9
Tulane virus	40	6.7	10
Turnip crinkle virus (TCV)	28 – 35	4.0	5.0 – 6.0

Table 2. Primer and probe identities, sequences, oligo, and source for RT-qPCR assays used in this study.

Virus	Name	Sequence (5'-3')	Location	Reference
Norovirus GI.6 (HuNoV GI.6)	COG1F	CGYTGGATGCGNTTYCATGA	5291	(Kageyama et al., 2003)
	COG1R	CTTAGACGCCATCATCATTYAC	5375	
	Ring1a	6-FAM-AGATYGGGATCYCCTGTCCA-BHQ1	5340	
	Ring1b	6-FAM-AGATCGCGGTCTCCTGTCCA-BHQ1	5340	
Norovirus GII.4 (HuNoV GII.4)	JJV2F	CAAGAGTCAATGTTTAGGTGGATGAG	5003	(Jothikumar et al., 2005)
	COG2R	TCGACGCCATCTTCATTACACA	5080	
	RING2P	6-FAM-TGGGAGGGCGATCGCAATCT-BHQ1	5048	
Murine norovirus-1 (MNV-1)	G54763F	TGATCGTGCCAGCATCGA	4763	(Tung et al., 2013)
	G54863R	GTTGGGAGGGTCTCTGAGCAT	4884	
		6-FAM-		
	G54808P	CTACCCACCAGAACCCCTTTGAGACTC-BHQ1	4808	
Turnip crinkle virus (TCV)	TCV900F	GTTTCGACGCATCTTCCATATCT	908	This study
	TCV900R	CTCTTTCCATCAACCCTCTTCTC	1008	
		6-FAM-		
	TCV900Probe	TGGGCAATGGTTTAGACTTTGGAGTCC-BHQ1	936	
Mengovirus (Mengo)	Mengo 110	GCGGGTCCTGCCGAAAGT	110	(Pintó, Costafreda, & Bosch, 2009)
	Mengo 209	GAAGTAACATATAGACAGACGCACAC	209	
	Mengo 147	6-FAM-ATCACATTACTGGCCGAAGC-BHQ1	147	
MS2 coliphage	MS2qfor	ATTCCGACTGCGAGCTTATT	1630	(Conn, Habteselassie, Blackwood, & Noble, 2012)
	MS2qrev	TTCGACATGGGTAATCCTCA	1758	
		6-FAM-		
	MS2qProbe	ATTCCCTCAGCAATCGCAGCAAACT-BHQ1	1689	
Tulane virus	FW	GAGATTGGTGTCAAAACACTCTTTG	3645	(Sestak et al., 2012)
	RV	ATCCAGTGGCACACACAATTT	3800	
	Probe	6-FAM-AGTTGATTGACCTGCTGTGTCA-BHQ1	3697	

Table 3. Losses of MNV-1, MS2, and Tulane during elution and PEG precipitation (in log₁₀ PFU for MNV-1 and MS2 and log₁₀ TCID₅₀ for Tulane).

Processing Step	Virus	Ham			Lettuce		
		N	Mean	Std Dev	N	Mean	Std Dev
Elution	MNV-1	3	ND	ND	5	ND	ND
	MS2	3	0.0	0.0	5	0.2	0.2
	Tulane	3	2.0	1.8	5	3.2	0.2
PEG	MNV-1	4	0.3	0.0	4	0.3	0.0
	MS2	4	0.1	0.2	4	0.1	0.2
	Tulane	4	2.7	0.7	3	1.0	1.7

N = number of samples; ND = not detected

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Figure 1

Figures

Figure 2

Figure 3