



# The Optimization of Methods for the Collection of Aerosolized Murine Norovirus

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## Abstract

Globally, norovirus is the most common gastroenteritis causing pathogen. Annually, norovirus causes 685 million cases of acute gastroenteritis and 200,000 deaths, worldwide. Recent evidence has suggested that norovirus can also be spread via aerosolization; however, an indoor generation source has yet to be determined. We optimized a sampling method for the collection of aerosolized norovirus using murine norovirus (MNV) as a surrogate. Optimization of the sampling method was performed using two bioaerosol samplers (SKC BioSampler and the NIOSH Bioaerosol Cyclone Sampler 251) and two sampling media (Hanks Balanced Salt Solution [HBSS] and Phosphate Buffered Saline [PBS]). Murine norovirus was aerosolized in a bioaerosol chamber and later collected using each sampler/media combination. Collected MNV was quantified using quantitative polymerase chain reaction (qPCR). Intact capsids of MNV were assessed using propidium monoazide dye in combination with qPCR and confirmed with transmission electron microscopy. Ten trials were conducted, with each trial lasting for 30 min. The SKC BioSampler collected a significantly higher concentration of MNV than the NIOSH-251 sampler did ( $p$ -value  $< 0.0001$ ). However, there were no significant differences in the relative percent of MNV that remained viable between both samplers ( $p$ -value = 0.2215). The use of HBSS sampling media yielded a higher concentration of MNV than PBS media ( $p$ -value = 0.0125). However, PBS media maintained viability at a significantly higher percentage than HBSS media ( $p$ -value  $< 0.0001$ ). The results support the optimization of a sampling method for the collection of aerosolized MNV and possibly norovirus in different sampling environments.

**Keywords** Bioaerosol · Norovirus · Aerosol · Environmental health · Exposure science · Industrial hygiene

## Introduction

The United States (U.S.) has approximately 179 million cases of acute gastroenteritis (AGE) each year (Hall et al. 2013). Acute gastroenteritis is characterized by vomiting or diarrhea, which results from inflammation of the mucous membrane in the gastrointestinal tract (Chow et al. 2010). There are several causes for AGE, including contact with infectious bacteria and viruses. Among the infectious diseases that can cause AGE, norovirus is the leading cause (Patel et al. 2008). Human norovirus (NV) causes between 19 and 21 million cases of AGE, 1.7–1.9 million outpatient visits, 400,000 emergency department visits, 56,000–71,000 hospitalizations, and 570–800 deaths, each year (Gregoricus et al. 2011). Globally, NV is estimated to be the cause for over 200,000 deaths annually in developing countries (Lopman et al. 2016). Therefore, NV is a significant public health problem and there is a need to identify and control routes of NV transmission.

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Norovirus is transmitted through direct or indirect contact via contaminated surfaces. However, there are four studies which provide evidence that NV can become aerosolized (Bonifait et al. 2015; Brooks et al. 2005; Uhrbrand et al. 2017, 2011). Norovirus was hypothesized to be transmitted via an airborne route (Marks et al. 2000), however, a consistent source for indoor aerosol generation has yet to be determined. One study was able to detect NV in healthcare settings around workers and patients (Bonifait et al. 2015). Based on the findings of Bonifait et al., it is probable that an indoor aerosolization source for NV is the flushing of toilets due to the high concentrations of bioaerosols that are known to be generated via this mechanism (Knowlton et al. 2018). Two of the four studies that detected aerosolized NV at two different wastewater treatment plants (Uhrbrand et al. 2017, 2011). The fourth study was able to detect aerosolized NV near a biosolids application site (Brooks et al. 2005). Although all four of these studies were able to detect aerosolized NV, they all used different sampling methodology. The methodologies differed by sampler type (i.e., filter, impaction, impingement), specific sampler, and sampling media that was used. Therefore, there is a need to optimize a sampling method specifically for aerosolized NV to increase the likelihood of detection in future investigations.

Several factors should be considered before selecting a type of sampler for bioaerosols. Specifically, collection efficiency for target aerosol, area vs. personal sampling, downstream analysis of biological sample, sampling flow rate, sampling time, and the specific microbial target are factors that should be considered when selecting a bioaerosol sampler (Macher and Willeke 1992; Nevalainen et al. 1992; Lin and Li 1998; Verreault et al. 2008; Uhrbrand et al. 2018). Sampling approaches commonly used for the collection of aerosolized viruses are filtration, impingement, and impaction. Each one of these methods has been used to collect aerosolized virus (Verreault et al. 2008). Filtration and impingement have been used for collection in previous investigations of aerosolized NV (Bonifait et al. 2015; Brooks et al. 2005; Uhrbrand et al. 2017, 2011).

One of the most common liquid impingers used for bioaerosol collection is the SKC BioSampler. The SKC BioSampler has been used extensively to study various targets including fungi, bacteria, and virus (Farnsworth et al. 2006; Lindsley et al. 2010; Willeke et al. 1998; Hermann et al. 2006; Fabian et al. 2005). However, while the SKC BioSampler has been successfully used to collect NV, the sampler's collection efficiency is < 10% for recovery of submicrometer (i.e., 100–1000 nm) and ultrafine particles (i.e., < 100 nm), which typically contain virus (Hogan Jr et al. 2005; Brooks et al. 2005). Other than a decrease in collection efficiency for submicrometer and ultrafine particles, the primary weakness associated with the use of liquid impingers involves the loss of liquid media due to evaporation. Prolonged sampling

with liquid media samplers at a high airflow could lead to cell damage or desiccation if too much of the liquid media evaporates, which could affect viability of microorganisms (Stewart et al. 1995; Wang et al. 2001).

Filtration samplers operate by directing airflow through a membrane filter. The filter collects particles over a specified size range that is impacted by the sampler design and membrane filter being used. Filtration samplers can be used with personal or area samplers to collect several types of bioaerosols including fungi, bacteria, virus, and organic particles (e.g., endotoxin). Membrane filters have been previously used to collect aerosolized virus (Blachere et al. 2009; Lindsley et al. 2010, 2006). Two previous studies have collected aerosolized NV using filtration samplers (Uhrbrand et al. 2017, 2011). The use of membrane filters allows for longer sampling times (e.g., greater than 8-h) compared to impingement methods without the worry of liquid media evaporation. However, when microorganisms are collected on a membrane filter, they are exposed to constant airflow resulting in desiccation effects (e.g., viability disruption). Therefore, using filtration as a sampling method may result in the viability of the target organism being compromised.

The United States National Institute for Occupational Safety and Health (NIOSH) has designed a sampler for collecting bioaerosols that combines filtration with cyclonic technology. The NIOSH bioaerosol sampler size-fractionates aerosols and collects them in centrifuge tubes, facilitating direct sample processing (Cao et al. 2011). Ambient air is drawn into an inlet at 3.5 l/min, the first stage of the NIOSH bioaerosol sampler deposits aerosol particles that are > 4 µm on the wall of a 15 ml centrifuge tube (Cao et al. 2011). In the second stage, 1 to 4 µm particles are deposited on the wall of a 1.5 ml microcentrifuge tube, and particles that are < 1 µm are collected on a 37 mm filter. The first stage of the sampler collects the non-respirable size fraction, while the second stage and filter collect the respirable fraction (Cao et al. 2011). This sampler has been used in both laboratory chamber trials and environmental settings to sample for not only NV, but respiratory viruses as well (Bailey et al. 2018; Bonifait et al. 2015). Therefore, this type of filtration sampler is considered ideal for the collection of aerosolized NV.

The media used should be based on sampler type and target organism. When using liquid impingement samplers, such as the SKC BioSampler or Coriolis µ sampler, a liquid media must be selected. Buffers are among the most common type of liquids chosen for bioaerosol sampling; however, the type of buffer chosen can vary greatly. In previous studies sampling for virus using liquid impingers, buffers such as distilled water, phosphate buffered saline (PBS), Hanks balanced salt solution (HBSS), universal transport media nutrient broth, and peptone broth have been used successfully (Chang et al. 2010; Fabian et al. ; Bonifait et al.

2015; Lindsley et al. 2010; Tseng and Li 2005). Each one of these sampling media provides a liquid solution that maintains osmotic balance and offers nutrients to the collected targets. The use of liquid sampling medium more effectively maintains viability of the target organism during sampling compared to filter-based media. Viability assessment techniques are essential in the assessment of the ability of the target organism to result in an infection. The use of liquid sampling media eliminates the need for filter extraction, allows for immediate sample analyses and enumeration of target organisms.

The assessment of virus viability can include performing a plaque assay or a qualitative assessment of the intact viral capsid. An intact viral capsid suggests viability, while a compromised capsid suggests that the virion is non-viable or unable to result in infection. One method in particular to investigate this directly is through the combination of using propidium monoazide dye (PMA) with polymerase chain reaction (PCR). Propidium monoazide dye is a DNA/RNA intercalating dye that is activated upon photolysis. The dye is only able to attach to DNA/RNA if the viral capsid is compromised, thus halting amplification by PCR. The combination of PMA with PCR allows for the differentiation of nucleic acid from organisms with an intact capsid (i.e., viable) from organisms where the capsid is not intact (i.e., non-viable). Several studies have already investigated the use of PMA dye with PCR quantification of MNV. These studies found that the PMA: PCR method correlated well with plaque assays detecting viable MNV (Lee et al. 2015; Kim and Ko 2012; Randazzo et al. 2018, 2016). However, only one study has attempted to combine this new technique to investigate the viability of aerosolized MNV (Bonifait et al. 2015).

The aim of this study was to optimize a sampling method for the collection of aerosolized MNV. To achieve this aim, two objectives were designed: (1) to compare viral concentrations of collected aerosolized MNV across two bioaerosol sampler and sampling media; and (2) to compare the effect of sampler and sampling media on the viral capsid integrity of MNV. Two sampler types were used for this experiment; a liquid impinger and a filter-based sampler.

## Materials and Methods

### Sample Handling

Murine norovirus (MNV, Dr. Skip Virgin's Laboratory, Washington University, St. Louis, MO) was used as a human norovirus surrogate for this project. Stock concentrations of MNV were  $10^7$  plaque forming units (PFU)/ml stored in Dulbecco's Modified Eagle Medium (DMEM). Upon arrival,

stock concentration of MNV was divided into 50  $\mu$ l aliquots and stored in a  $-80$  °C freezer until needed.

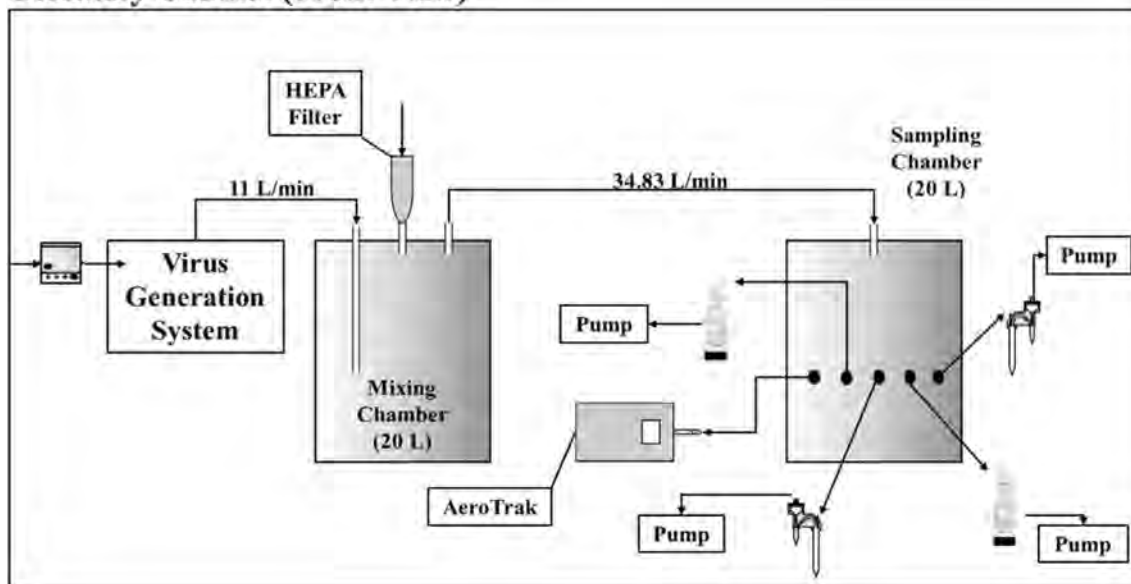
### Aerosolization Chamber

Aerosolization of MNV was performed inside of an aerosol chamber within a biosafety cabinet. A known concentration of MNV was diluted in DMEM to a final concentration of  $10^5$  PFU/ml. The diluted MNV solution was aerosolized using an Aeroneb Solo nebulizer (Model Aeroneb Solo System, Aerogen, Ireland) into dry, HEPA filtered air. The generated bioaerosol entered a mixing chamber, and was then drawn into a sampling chamber. Bioaerosol samplers were used to collect the generated bioaerosol from the sampling chamber. An optical particle counter (OPC, AeroTrak handheld particle counter 9306-V2; TSI Inc., USA) was used to monitor particle size and number concentration of the aerosols generated during sampling. The chamber schematic and sampler layout, along with airflow and flow rates, in the chamber are shown in Fig. 1.

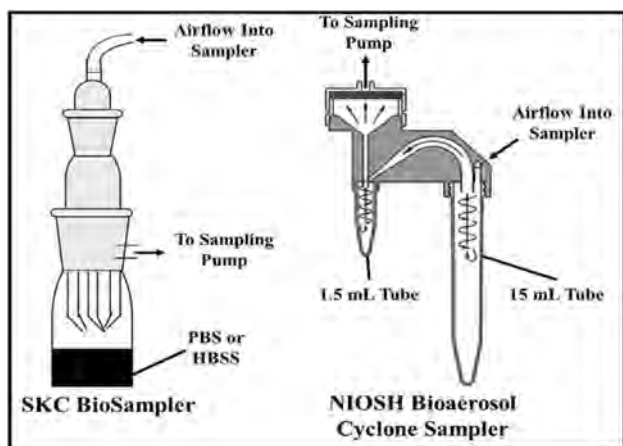
Four bioaerosol samplers were used in this experiment; two SKC BioSamplers (SKC Inc., PA, USA) and two National Institute for Occupational Safety and Health (NIOSH) Bioaerosol Cyclone Samplers (NIOSH-251, BC251, NIOSH, Morgantown, WV, USA). The SKC BioSamplers contained 20 ml of either HBSS or PBS. The BioSamplers were operated at 12.5 l per minute (l/min). The NIOSH-251 samplers contained a 37-mm Polyvinylchloride (PVC) filter (SKC Inc., PA, USA) and were operated at 3.5 l/min. Diagrams of the SKC BioSampler and NIOSH-251 sampler are shown in Fig. 2. Prior to activation of sampling, the bioaerosol was generated for 5 min to allow the mixing chamber to become saturated with aerosolized MNV. After 5 min, the air samples were collected for 30 min simultaneously with all samplers.

Ten aerosolization experiments were performed for a sample size of  $n = 40$  (20 samples for each liquid media). The viral particles collected using the NIOSH-251 sampler were eluted using 4 ml from the first stage, 1 ml from the second stage, and 5 ml from the filter of either HBSS or PBS media. A total of 10 ml was eluted from the entire sampler. This method was lab optimized for other viral targets (i.e., swine and human influenza). After sampling, the remaining volume of liquid sampling media was recorded from each sampler (i.e., SKC BioSampler) and aliquoted. The aliquots were stored at  $-80$  °C until further analysis. Airflow calibration of all samplers were performed prior to aerosol sampling using a primary airflow standard (BGI tetraCal, Mesa Labs, Butler, NJ, USA), and verified post sampling using the same primary airflow standard. Temperature and relative humidity were monitored throughout all trials during each experiment using a Hygro-Thermometer (Extech Instruments, Waltham, MA, USA).

### Biosafety Cabinet (Front View)



**Fig. 1** Schematic of bioaerosol chamber setup inside of biosafety cabinet. Arrows indicate airflow direction. Two NIOSH-251 samplers and SKC BioSamplers are shown in the schematic



**Fig. 2** Diagram depicting the SKC BioSampler and NIOSH-251 sampler. Arrows indicate airflow direction

### RNA Isolation

Total MNV RNA collected using the bioaerosol samplers was extracted using the Qiagen QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Samples were extracted following manufacturer's instructions using the spin method. Total RNA extraction occurred with 140  $\mu$ l from each sample and was eluted in 90  $\mu$ l of elution buffer.

### RT-qPCR

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was used to quantify MNV from collected aerosolized samples. Viral RNA was converted to complementary DNA (cDNA) using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase Kit (Thermo Fisher Scientific, Waltham, MA, USA) per the manufacturer's instructions. The PCR reactions were performed on a qPCR system (QuantStudio 7 Flex Real-Time PCR System, Applied Biosystems, Waltham, MA, USA). The mastermix volume for each well was 20  $\mu$ l, with 5  $\mu$ l of sample added to the appropriate wells for a total volume of 25  $\mu$ l per well. The following primers and probe were used to complete RT-qPCR for collected MNV aerosol samples:

- MNV Forward: 5'-GCC CTT GTA CCA CCC TAT TT-3'.
- MNV Probe: 5'-56-FAM-CGC TTT GGA ACA ATG-MGBNFQ.
- MNV Reverse: 5'-CTC GAC GCA CAT CAA GAA GA-3'.

Primers and probe were designed in consultation with Dr. Skip Virgin's Laboratory at Washington University School of Medicine. Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and the probe was purchased from Thermo Fisher Scientific. Reverse transcription-qPCR was performed with the following cycling conditions: 1 cycle at 50 °C for 15 min, 1 cycle at 95 °C for 10 min,

and 45 cycles of 95 °C for 15 s, 60 °C for 1 min. Samples from each experiment were analyzed with RT-qPCR, run in triplicates, and the results averaged. A standard curve was generated by performing a ten-fold dilution series using IDT gBlocks (IDT, Coralville, IA), which contained gene fragments of the same amplicon sequence used by the primers/probe. The curve contained seven-points, as well as, a negative control. MNV in liquid solution was evaluated prior to aerosolization to ensure a baseline concentration.

### PMA Assay

Intact viral capsids were quantified using propidium monoazide dye (PMA, Biotium, Fremont, CA, USA). The 20 mM stock solution of PMAxx dye was diluted in molecular water to 5 mM. Prior to extraction, 1.05 µl of 20 mM PMAxx dye was added to 140 µl of sample in 1.5-ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) for a final concentration of 150 µM. The samples were then incubated in the dark at room temperature for 5 min and were inverted every 30 s for mixing. Upon completion of incubation in the dark, the samples were exposed to an LED light with a wavelength of 465–475 nm for 10 min inside of a PMA-Lite™ LED Photolysis Device (Biotium, Fremont, CA, USA). Following photolysis, the extraction of MNV was completed using the same Qiagen kit as before. PMA: RT-qPCR was completed using the same primers/probe and one-step kit as mentioned before. A standard curve was generated by performing a ten-fold dilution series of using IDT gBlocks of the same amplicon sequence used by the primers/probe. Samples from each experiment were analyzed with PMA: RT-qPCR, run in triplicates, and the results averaged. Viability of MNV in liquid solution was evaluated prior to aerosolization to ensure a baseline concentration.

### Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to qualitatively assess the integrity of MNV capsids. Due to limited resources, only one sample collected from the SKC BioSampler, NIOSH-251 sampler, and Nebulizer stock were analyzed. In addition, only samples collected with PBS media were analyzed due to the statistically significant higher concentration of viable MNV virions compared to HBSS media. Samples were fixed with paraformaldehyde, 4% in PBS solution (Alfa Aesar, MA, USA). Post-fixation, MNV samples were transferred to the University of Iowa Central Microscopy Research Facility (CMRF) (Iowa City, IA, USA). Once samples arrived at CMRF, they were negative stained with uranyl acetate. An aliquot of the sample was applied to a TEM carbon coated grid. A JEOL JEM 1230 (JOEL USA, Inc., Peabody, MA, USA) was used to capture images of MNV virions for collected samples from the SKC

BioSampler, NIOSH-251 sampler, and the stock solution. Images were captured digitally using Gatan UltraScan 1000 2 K × 2 K CCD camera (Gatan, Inc., Pleasanton, CA, USA).

### Data Analysis

The distribution of the data was evaluated graphically. Viral RNA concentration was adjusted for airflow (e.g., RNA copies/m<sup>3</sup>) using the pre- and post-calibration averages for each sampler per trial. Viral concentration (e.g., RNA copies/m<sup>3</sup>) was compared across sampler and sampling media using multiple linear regression analysis in R (R Core Team 2018). A statistically significant threshold of  $p \leq 0.05$  across study variables was set for all analyses. Multiple linear regression models were fit considering log-RNA concentration as a function of media, sampler, temperature, relative humidity, and log-nebulizer concentration. The final model was selected using AIC via backward stepwise selection.

The relative percent of intact MNV virion capsid from the PMA assay was calculated using Eq. 1 with total RNA copies collected for each sampler per trial. The treated PMA sample was divided by the untreated PMA sample and then multiplied by 100 to convert to a percentage.

$$\frac{\text{Intact MNV virion capsids}_{\text{PMA Treated}}}{\text{Total MNV virions}_{\text{PMA Untreated}}} \times 100 \quad (1)$$

As with viral concentration, the relative percent of intact capsids was compared across sampler and sampling media using multiple linear regression analysis in R (R Core Team 2018). A statistically significant threshold of  $p \leq 0.05$  across study variables was set for all analyses. Multiple linear regression models were fit considering log-RNA concentration as a function of media, sampler, temperature, relative humidity, and log-nebulizer concentration. The final model was selected using AIC via backward stepwise selection.

## Results

### Viral Collection

The LOD for qPCR was 5 RNA copies/µL based on gBlock analysis. The mean viral concentration using the SKC BioSampler using HBSS or PBS was  $8.78 \times 10^4$  and  $4.75 \times 10^4$  RNA copies/m<sup>3</sup>, respectively (Table 1). The mean viral concentration using the NIOSH-251 using HBSS and PBS media was  $1.66 \times 10^4$  and  $1.16 \times 10^4$  RNA copies/m<sup>3</sup>, respectively. Viral concentration was found to be log normal through graphical evaluation, therefore, log-RNA concentration (RNA copies/m<sup>3</sup>) were analyzed. The model with the lowest Akaike information criterion (AIC) estimator-modeled RNA concentration on the log

**Table 1** Airborne viral concentration (RNA copies/m<sup>3</sup>) from the qPCR for each collected sample across all trials for sampler and sampling media type

Trial	PBS		HBSS	
	SKC	NIOSH	SKC	NIOSH
1	6.56 × 10 <sup>4</sup>	1.51 × 10 <sup>4</sup>	3.96 × 10 <sup>4</sup>	1.82 × 10 <sup>4</sup>
2	4.44 × 10 <sup>4</sup>	2.93 × 10 <sup>4</sup>	5.52 × 10 <sup>4</sup>	2.38 × 10 <sup>4</sup>
3	9.81 × 10 <sup>4</sup>	1.17 × 10 <sup>4</sup>	9.71 × 10 <sup>4</sup>	1.16 × 10 <sup>4</sup>
4	2.78 × 10 <sup>4</sup>	1.78 × 10 <sup>4</sup>	7.82 × 10 <sup>4</sup>	1.22 × 10 <sup>4</sup>
5	4.84 × 10 <sup>4</sup>	8.57 × 10 <sup>3</sup>	5.56 × 10 <sup>4</sup>	1.03 × 10 <sup>4</sup>
6	4.71 × 10 <sup>4</sup>	4.79 × 10 <sup>3</sup>	2.80 × 10 <sup>5</sup>	1.49 × 10 <sup>4</sup>
7	1.72 × 10 <sup>4</sup>	5.01 × 10 <sup>3</sup>	3.96 × 10 <sup>4</sup>	1.04 × 10 <sup>4</sup>
8	4.41 × 10 <sup>4</sup>	4.05 × 10 <sup>3</sup>	1.16 × 10 <sup>5</sup>	1.39 × 10 <sup>4</sup>
9	1.80 × 10 <sup>4</sup>	1.27 × 10 <sup>4</sup>	2.02 × 10 <sup>4</sup>	3.42 × 10 <sup>4</sup>
10	6.46 × 10 <sup>4</sup>	5.95 × 10 <sup>3</sup>	9.68 × 10 <sup>4</sup>	1.65 × 10 <sup>4</sup>
Mean	4.75 × 10 <sup>4</sup>	1.16 × 10 <sup>4</sup>	8.78 × 10 <sup>4</sup>	1.66 × 10 <sup>4</sup>
SD	2.45 × 10 <sup>4</sup>	7.87 × 10 <sup>3</sup>	7.41 × 10 <sup>4</sup>	7.43 × 10 <sup>3</sup>

The mean and standard deviation for each column are provided at the bottom of the table

scale as a function of media, sampler, temperature, relative humidity, and the log nebulizer concentration. Regardless of sampler type, the use of HBSS sampling media resulted in significantly higher viral concentrations than PBS media ( $p$ -value = 0.0125). Using the SKC BioSampler resulted in significantly higher viral concentrations than the NIOSH-251 sampler, across both media ( $p$ -value < 0.0001).

### PMA Analysis

The mean relative percent of MNV with intact capsids using the SKC BioSampler using HBSS and PBS media was 36.1% and 59%, respectively. The mean relative percent of MNV with intact capsids collected with the NIOSH-251 sampler using HBSS and PBS media was 38.2% and 79.4%, respectively. The mean relative percent of MNV with intact capsids for the starting nebulizer viral solution was 46.7% (Table 2). Relative percent of intact capsids data was found to be normally distributed through graphical evaluation. The model with the lowest AIC estimate modeled the relative percent of MNV with intact capsids as a function of media and sampler. The relative percent of MNV with intact capsids was significantly higher when PBS media was used for sampling or washing ( $p$ -value < 0.0001), regardless of sampler. There was no significant difference between the SKC BioSampler and the NIOSH-251 sampler with respect to relative percent of MNV with intact capsids ( $p$  value = 0.2215), regardless of media.

**Table 2** Relative percent of MNV with intact capsids from the qPCR for each sample across all trials for sampler, media, and nebulizer

Trial	PBS		HBSS		NEB
	SKC	NIOSH	SKC	NIOSH	
1	21.5	13.2	56.1	19.7	52.3
2	77.0	85.6	63.6	50.7	43.3
3	20.7	100.0	25.1	44.4	44.3
4	43.2	76.9	11.7	22.9	23.8
5	92.6	97.8	41.5	10.6	21.9
6	94.5	94.5	42.6	28.1	47.2
7	60.9	82.0	26.6	85.0	55.0
8	30.2	92.6	16.4	14.2	89.6
9	81.5	51.6	47.5	19.1	37.8
10	67.9	100.0	29.8	87.4	51.8
Mean	59.0	79.4	36.1	38.2	46.7
SD	28.4	27.5	17.0	28.3	18.9

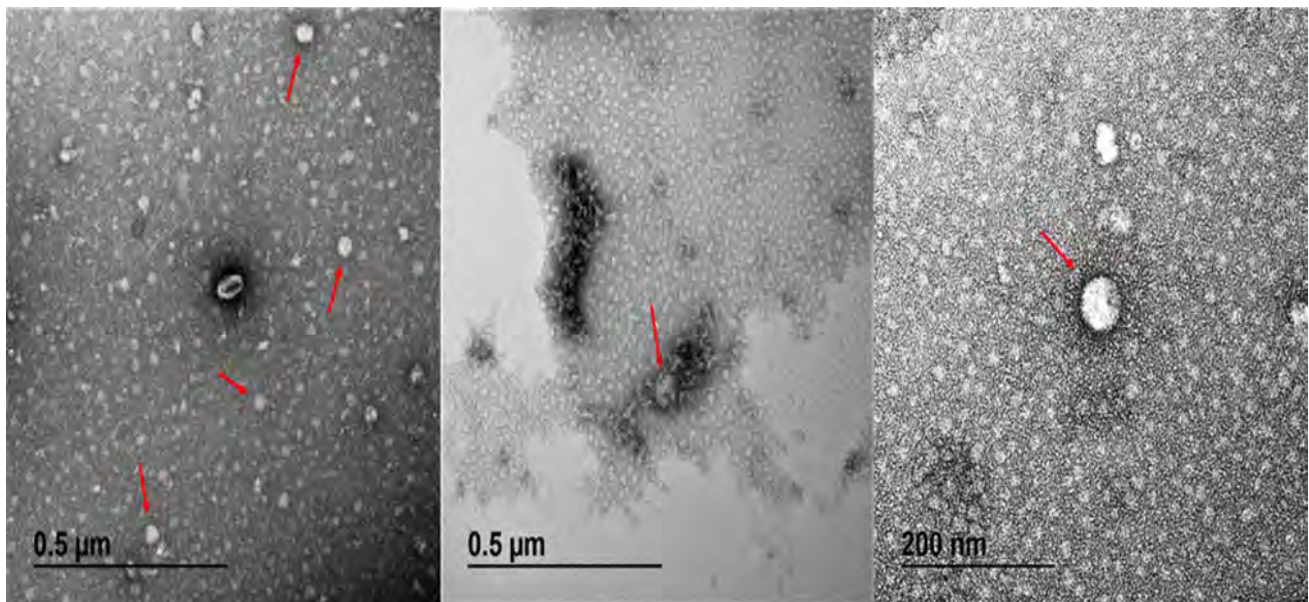
The arithmetic mean and standard deviation are shown at the bottom

### Transmission Electron Microscopy Analysis

Using data from the PMA: RT-qPCR analysis, the overall media with the highest relative percent of intact capsids was selected for TEM analysis. Samples collected using the SKC BioSampler and NIOSH-251 sampler with PBS media, and a nebulizer sample, were used to performed TEM analysis. All samples analyzed using TEM were successful and MNV virions were identified. Murine norovirus virions capsids appeared intact for all three samples analyzed (Fig. 3).

### Discussion

The SKC BioSampler had significantly higher concentration of aerosolized MNV when compared to the NIOSH-251 sampler ( $p$ -value < 0.0001) when sampling in a controlled laboratory setting. This observation differs from the results of previous studies that have compared viral collection between the SKC BioSampler and NIOSH-251 sampler in a controlled laboratory setting (Kienlen 2015; Girlando 2014). The results of this study also differed from Cao et al., who found that the SKC BioSampler and NIOSH-251 sampler were able to collect similar concentrations of aerosolized virus (Cao et al. 2011). The viral concentrations were significantly different across the samplers used in this study. We suspect that the observed difference in concentration was due to multiple factors including loss of virus. The differences in viral concentration between the SKC BioSampler and NIOSH-251 sampler were an unexpected result of this study. The differences could have resulted from a few sources. First, it was assumed that MNV was evenly distributed in the sampling chamber which was connected to



**Fig. 3** TEM images of murine norovirus samples. The left image is of viral stock, the center image is from a SKC BioSampler using PBS, and the right image is from a NIOSH-251 sampler extracted with PBS. Red arrows denote MNV virions with intact capsids (Color figure online)

the SKC BioSampler and NIOSH-251 sampler. There could have been uneven mixing in the sampling chamber which would lead to gradients in concentration of airborne MNV within the chamber. However, the laboratory setup included a “mixing chamber” for the aerosol to decrease the likelihood of unequal mixing prior to sampling. Second, the collection efficiency of the SKC BioSampler may have been higher for larger droplets than the NIOSH-251 sampler. If this were the case, then the SKC BioSampler would have collected more of the larger droplets containing MNV than the NIOSH-251 sampler. Third, it is possible that some of the collected MNV adhered to the interior surfaces of the NIOSH-251 sampler. This error has been suggested previously by NIOSH since it operates as a dry sampler. The SKC BioSampler contains liquid media during operation; however, the NIOSH-251 sampler is washed with liquid media after sampling. Therefore, it is possible that the lower concentration of MNV from the NIOSH-251 sampler could have resulted from inadequate washing and removal of all MNV particles. In future investigations, surfaces of the NIOSH-251 sampler should be evaluated for viral losses after sample extraction. After elution, all samples from each stage within sampler and sample number were pooled to determine an overall concentration. The authors did not evaluate each stage separately. Loss of viral particles could be explained if the stages were evaluated separately, therefore the authors recognize this as a limitation in the study.

When HBSS was used for sampling media, viral concentration, regardless of sampler, was significantly and substantially higher than PBS sampling media ( $p$ -value = 0.0125).

Across both bioaerosol samplers, the viral concentration of MNV was two times higher when HBSS sampling media was used compared to the viral concentration when PBS sampling media was used. This result concurs with a previous study that found influenza viral recovery was significantly higher when HBSS media was used compared to PBS when using Qiagen QIAamp Viral RNA Mini Kit, regardless of all other investigated variables (i.e., air and filter type) (Thedell et al. 2018). However, that study also found that influenza viral recovery was slightly higher when PBS sampling media was used with an extraction method that required Trizol reagent. Therefore, it is possible that the sampling media used for bioaerosol collection could have an unforeseen interaction with different extraction methods, but this requires further investigation. The use of HBSS and PBS sampling media for the collection of virus is supported by this study along with others (Thedell et al. 2018; Bonifait et al. 2015; Lindsley et al. 2010; Fabian et al. 2009a, b; Kienlen 2015).

While the use of HBSS sampling media resulted in higher airborne concentrations of MNV collected, the opposite was discovered when determining the sampling media associated with the highest relative percent of intact virion capsids. As such, it was found that regardless of sampler, the use of PBS sampling media resulted in a significantly higher relative percentage of virions with intact capsids ( $p$ -value < 0.0001). Phosphate-buffered saline sampling media resulted in a higher concentration of viable MNV virions was an unexpected result. Across both bioaerosol samplers, the relative percent of MNV was two times higher when PBS sampling

media was used compared to when HBSS sampling media was used. With the use of HBSS resulting in higher collected amounts of aerosolized MNV, and the use of PBS resulting in higher numbers of viable virions, careful consideration should be given to the research question being posed. If the aim of bioaerosol sampling microbial risk is to only assess the amount of aerosolized NV, then HBSS should be selected as the sampling media. However, if the aim of the bioaerosol sampling includes an assessment of virion viability, then PBS should be strongly considered.

Phosphate-buffered saline and HBSS are relatively similar solutions. Both solutions act as a pH buffer and maintain osmotic balance. Our expectation was that HBSS would have yielded a higher amount of viable virions due to added supplements of glucose, calcium chloride, sodium bicarbonate, and magnesium sulfate; however, this was not our observation in this study. In fact, the use of HBSS sampling media is recommended when viability assays will be performed while using the NIOSH-251 sampler. The results of this study do not agree with the results of a previous study which found that the addition of magnesium and calcium increased MNV infectivity (Nelson et al. 2018). These cations appear to interact with a binding site found on the capsid protein. Therefore, if differences are present between MNV and NV regarding cation binding sites, then there could be differences in the effect sampling media has on each viral strain. At this time, there is not enough evidence to determine the reason why the use of PBS sampling media resulted in higher concentrations of MNV virions with intact capsids. Future investigations are required to determine the effect sampling medium has on MNV viability.

Regardless of sampling media, there was no significant difference in the relative percent of MNV with intact capsids between the SKC BioSampler and the NIOSH-251 sampler ( $p$ -value = 0.2215). The effect of sampling on viability between the two samplers has yet to be published for aerosolized MNV. Bonifait et al. found that the NIOSH-251 sampler preserved MNV at 76–86%. Regardless of sampling media, we found that the NIOSH-251 sampler maintained MNV viability over a wider range, 10.6–100%. However, Bonifait et al. only used PBS sampling media with the NIOSH-251 sampler (Bonifait et al. 2015). Therefore, when looking only at the results when using PBS sampling media and the NIOSH-251 sampler, the range of viable virions changes to 51.6–100%, with an outlier at 13.2%. These observations more closely agree with the results reported by Bonifait et al., while also showing the effect that sampling media choice has on maintaining viral viability during sampling of aerosolized MNV. Overall, it appears that NIOSH-251 and SKC BioSampler are able to maintain the viability of aerosolized MNV during sample collection.

There was a large amount of variability among reported viral collection and the relative percent of MNV with intact

capsids. This large variability could be due to experimental setup, virus handling, and analytical methods used. Similar issues were previously reported with similar experimental setups and analytical methods of influenza virus (Kienlen 2015; Thedell et al. 2018). In addition, the final volume after sampling when using the SKC BioSampler was 15 ml compared to the final volume of the sample collected with the NIOSH-251 being 10 ml resulting in fewer sample aliquots. Only 140  $\mu$ l was needed for extraction and PCR analyses, therefore, it was possible that variability between the aliquots could have resulted in variability in downstream analysis results.

One major limitation of this study was the inability to verify the PMA results using plaque assays. However, due to the number of published studies with strong correlations between plaque assay and PMA assay for MNV viability assessment, the authors of this study felt that performing plaque assays was not needed. This was the first study to verify viability of MNV using PMA across multiple samplers and media. One study analyzed MNV viability using PMA for samples collected using one wash media and a NIOSH-251 sampler, however, they also verified the results using plaque assays (Bonifait et al. 2015).

Prior to the onset of this project, extraction, reverse-transcription, and qPCR were performed with spiked MNV samples to ensure that extraction and analysis methods were within the acceptable parameters. In addition, extraction and analysis performed during the project included a negative control. However, a potential limitation was the lack of an internal control used in this study to account for viral loss during extraction, reverse transcription, and qPCR. In attempt to control variability introduced by experiment design, extraction kits, RT-qPCR kits, and gBlocks were used to reduce variation of viral concentration between samples due to minor process or analytical error. In addition, this experiment was carried out in a controlled laboratory setting, reducing the potential for environmental contamination. Although error may be present in this experiment, we do not expect error to be differentially distributed among our comparison group.

Transmission electron microscopy was used as an alternative, qualitative approach to assess the presence of intact virions among some of the samples. However, due to limited resources, only one sample collected from the SKC BioSampler, NIOSH-251 sampler, and Nebulizer stock were analyzed. In addition, only samples collected with PBS media were analyzed due to the statistically significant higher concentration of viable MNV virions compared to HBSS media. MNV virions were imaged using negative staining. The authors assumed that all virions present in the collected samples were from MNV. However, the MNV TEM samples were not labeled prior to imaging, therefore, it is possible that virions present were not MNV, though unlikely.

Therefore, for future TEM and SEM imaging we recommend labeling of intact virions.

## Conclusion

In conclusion, collection of aerosolized MNV does appear to have a significant effect on viral collection and viability of MNV virions during the sampling process. The use of HBSS sampling media was found to yield significantly higher concentrations of collected virus, whereas the use of PBS sampling media was able to maintain a significantly higher percentage of viable MNV virions during sampling. Using the NIOSH-251 sampler resulted in samples collected with a similar proportion of MNV virion viability compared to the SKC BioSampler. The method of choice to characterize MNV aerosol should consider the question the investigator is attempting to answer. If an investigator is primarily interested in the concentration of aerosolized MNV, then HBSS should be used. However, if assessing MNV viability is the objective, then PBS may be more appropriate. Regarding sampler selection and viability, there were similar proportions of viable viral particles detected across both samplers. Therefore, both samplers can be used to maintain viral viability, and the selection of sampler may depend upon if a personal or area sampling is needed. We believe that this study provides guidance for an sampling method optimized for the collection of aerosolized MNV and our approach can be used to perform a microbial risk assessment targeting NV in occupational and environmental settings.

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