

Detection and quantification of airborne norovirus during outbreaks in healthcare facilities

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40-WORD SUMMARY

This study investigates the presence of norovirus bioaerosols during gastroenteritis outbreaks in healthcare facilities. It shows the presence and the resistance of bioaerosols to the stress of aerosolization, suggesting a potential mode of transmission for norovirus.

ABSTRACT

Background. Noroviruses are responsible for at least 50% of all gastroenteritis outbreaks worldwide. Noroviruses GII can infect humans via multiple routes including direct contact with an infected person, contact with fecal matter or vomitus, and with contaminated surfaces. Though norovirus is an intestinal pathogen, aerosols could, if inhaled, settle in the pharynx and later be swallowed. The aims of this study were to investigate the presence of norovirus GII bioaerosols during gastroenteritis outbreaks in healthcare facilities as well as studying the *in vitro* effects of aerosolization and air sampling on the noroviruses using murine norovirus as a surrogate.

Methods. A total of 48 air samples were collected during norovirus outbreaks in 8 healthcare facilities. Samples were taken 1 m away from each patient, in front of the patient's room and at the nurses' station. The resistance to aerosolization stress of murine norovirus MNV-1 bioaerosols was also tested *in vitro* using an aerosol chamber.

Results. Norovirus genomes were detected in 6/8 healthcare centers. The concentrations ranged from 1.35×10^1 to 2.35×10^3 genomes per m^3 in 47% of air samples. Norovirus MNV-1 preserved its infectivity and integrity during *in vitro* aerosol studies.

Conclusion. Norovirus genomes are frequently detected in the air of healthcare facilities during outbreaks, even outside patients' rooms. In addition, *in vitro* models suggest this virus may withstand aerosolization.

INTRODUCTION

Noroviruses are non-enveloped, single-stranded RNA viruses, belonging to the Caliciviridae family. They are the most common cause of epidemic gastroenteritis, responsible for at least 50% of all gastroenteritis outbreaks worldwide [1]. They are a major cause of foodborne illnesses and one of the major pathogens responsible for nosocomial infections [2-4]. Gastroenteritis outbreaks mostly occur in facilities where hygiene is compromised and contact between infected patients and personnel is intense, such as hospitals and nursing homes [7]. In the United-States, norovirus infections represent 2 millions of outpatient visits, 414 000 emergency room visits, 56 000 -71 000 hospitalizations and up to 800 deaths each year [8]. Children, elderly, immunocompromised persons and people living/working in healthcare facilities are at higher risk of contracting the disease [5].

Noroviruses are highly contagious, with an infectious dose ranging from 18 to 2 800 particles, making their spread difficult to prevent [10]. A descriptive study performed in 2011-2012 to estimate the incidence of norovirus outbreaks in hospitals and nursing homes in Catalonia demonstrated the occurrence of norovirus to be very high and associated with significant mortality [11] and that even small amount of contamination can lead to a potential risk to public health [10, 12]. Multiple routes of infection transmission have been documented including: direct contact with an infected person and/or fecal matter and vomitus droplets, and contact with contaminated surfaces [5, 9]. Indirect evidence suggests that norovirus could be transmitted through the airborne route and this route of transmission has already been suggested in literature [13-16]. Nenonen *et al.* showed high nucleotide similarity between norovirus GII.4 strains present in the dust of rooms of patients infected by norovirus [17]. However, norovirus has never

been detected in the air of hospitals outside patients' rooms and the infectious potential of airborne noroviruses has never been studied since this virus was, until very recently, not culturable [18]. Assessing the norovirus' capacity to withstand the stress associated with aerosolization is essential to investigate its potential for airborne dissemination. Several models have been developed to assess the persistence of norovirus infectivity in the environment and surrogates for human noroviruses are used: feline calicivirus, bacteriophages MS2 and murine norovirus (MNV) [12]. Murine norovirus (MNV-1) shares similar genetic and structural features with the human norovirus therefore is a culturable surrogate [19] and used to study the resistance to environmental stress of human norovirus [12].

A virus is generally considered infective if its integrity is documented. In recent years, a new technique, propidium monoazide (PMA), has been developed to assess the structural integrity of microorganisms and differentiate intact and membrane compromised microorganisms. It is a DNA/RNA intercalating dye with a photo-inducible azide group, which allows covalently cross-links with RNA after an exposure to bright light. In virology studies, PMA only penetrates viruses with damaged capsid and can hence differentiate intact from compromised virions that will not be amplified by PCR. This method was previously used to determine the integrity of norovirus particles [20].

The general aim of this study was to investigate the potential for airborne transmission of human norovirus. To achieve this goal, two distinct and complementary objectives were designed: 1) quantify the presence of norovirus GII in air samples during gastroenteritis outbreaks in healthcare facilities and 2) study the virus' resistance to aerosolization by assessing its integrity when subjected to *in vitro* aerosolization stress using murine norovirus as surrogate. Integrity

preservation was determined by culture and PMA. The use of PMA qPCR method as an indicator of murine norovirus integrity was also validated.

MATERIALS AND METHODS

Field study

Sampling human norovirus in health-care facilities

Air sampling was performed in 8 healthcare facilities of the Quebec City area (Canada) when viral gastroenteritis outbreaks occurred. Norovirus was established as the causal agent of the gastroenteritis outbreaks (PCR) by the public health laboratory of the Quebec Province (LSPQ). Air samples were taken in 3 distinct locations on patients wards: (1) inside the room of patients with gastroenteritis symptoms (<24h); (2) in the hallways or the common room outside of the rooms of patients with symptoms; and (3) at the nurses' station. A total of 48 air samples were collected: 26 from patient rooms, 16 from hallways/common areas and 6 from nurse's stations. Air samples were taken with the Coriolis μ ® (Bertin Technologies, St-Berthely, France) set at 200 L/min for 10 minutes (sampler D50 <0,5 μ m) and 15 mL of phosphate buffered solution (PBS) was used for fluid collection (Lonza, Bâle, Switzerland). Samples were concentrated on Amicon Ultra-15 centrifugal filter unit (porosity of 50 kDa (Millipore, Billerica, MA)) to a final volume of 400 μ l. Concentrated air samples were spiked with 1 μ l of a MS2 bacteriophage suspension (10^6 ge/ml) as an internal control for RNA extraction and qPCR.

RNA isolation of human norovirus

Viral RNA was isolated using the MagMax® Viral RNA Isolation Kit (Life Technologies, Carlsbad, CA). Total RNA was eluted and immediately transcribed into complementary DNA or frozen at -80°C until RT-PCR was performed.

***In vitro* experiments**

Murine norovirus and cells

MNV-1 and macrophages RAW 264.7 cells were cultivated as mentioned by Wobus *et al.*, in presence of macrophages RAW 264.7 in DMEM (Cellgro, Mediatech, Herndon, Virginia, United States) [21]. An initial stock at 10^7 PFU/ml was prepared, divided into subsamples (70 ml of MNV-1 at 10^7 PFU/ml) for each experiment and then kept at -80°C .

MNV-1 aerosolization

Aerosolization was performed in an aerosol chamber (GenaMini, SCL Medtech Inc., Montreal, Canada). Sixty-five mL of MNV-1 (10^7 PFU/ml) in DMEM were nebulized (Single-Jet Atomizer, model 9302, TSI Inc., Shoreview, MN) at a rate of 3 L/min using HEPA filtered air. The average liquid flow rate of the nebulizer was of 0.18 ± 0.2 mL per min. Aerosols were dried through a desiccator (EMD Chemicals Inc., Gibbstown, NJ,) allowing the formation of droplet nuclei before entering the chamber and were diluted with HEPA-filtered dry air at a rate of 23 L/min [22]. An Aerodynamic Particle Sizer (APS; model 3321, TSI Inc.) was used to monitor particle size distribution and concentration during aerosol sampling. Temperature and relative humidity were also measured.

Aerosols sampling

Air samples were collected using National Institute for Occupational Safety and Health (NIOSH) 2-stage cyclone aerosol sampler prototype (NIOSH-251, CDC/NIOSH, Morgantown, WV) [23, 24] for 25 min at 10 L/min then particles were eluted from the first stage and from the filter using 4 ml PBS and from the second stage with 1 ml PBS using an orbital shaker (WIS Biomed, San

Mateo, CA) for 15 min at room temperature. All eluents were pooled together. Aerosolization experiments were performed five times. Viral culture, RNA extraction and cDNA synthesis were performed the same day as the aerosolization experiments took place.

Quantification by plaque assay

The plaque assays were performed as previously described by Gonzalez-Hernandez *et al.* [25] except that plaques were visualized by crystal violet staining after fixation with formaldehyde. Each plaque assay had one negative control well.

RNA isolation of murine norovirus

Total viral RNA was extracted using the QIAamp viral RNA Mini kit (Qiagen, Mississauga, Ontario, Canada). Total RNA was eluted in 80 µl of elution buffer, supplied with the kit.

Viral genome quantification

Viral genomes cDNA synthesis

RNA was converted to cDNA using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA), following manufacturer instructions.

Quantification of viruses by qPCR

Separate reactions were performed for the detection of MS2 (internal control), human norovirus GII from field air samples and MNV-1 from *in vitro* study air samples. MS2 genomes were detected using qPCR described by [22]. Every sample was positive for MS2, which shows that the RNA extraction and cDNA synthesis were efficient.

Detection of norovirus GII cDNA and MNV-1 was done using qPCR as described by Kageyama *et al.* [26] and Girard *et al.* [27] respectively. Quantification was performed using a standard curve of a ten-fold dilution series of MNV-1 plasmid DNA preparation or norovirus GII plasmid DNA preparation [27]. Serial 10-fold dilutions from 10^1 to 10^7 molecules per reaction tube were used. The curve was prepared using the pGC™ Blue Cloning & amplification kits (Lucigen, Middleton, WI). The DNA plasmids were purified using the Qiagen plasmid mini kit and were quantified with a NanoDrop™ 2000 spectrophotometer (Thermo scientific, Waltham, MA).

PMA- qPCR

This method, previously used with norovirus by Parshionikar *et al.* [20] allows quantification of virus with an intact capsid. Propidium monoazide (PMA, Biotium Inc., Hayward, CA, USA) was dissolved in 20% dimethyl sulfoxide to create a 5 mM stock solution and stored at -20°C in the dark. $4.2\ \mu\text{l}$ of PMA™ was added to $140\ \mu\text{l}$ of air samples aliquots to a final concentration $150\ \mu\text{M}$ in light-transparent 1.5 ml tubes (Fisher Scientific Co., Ottawa, ON). Following an incubation period of 5 min in the dark with occasional mixing, samples were exposed to light for 10 min using a PMA-Lite LED Photolysis Device (a long-lasting LED Lights with 465-475 nm emission for PMA™ activation; Biotium Inc.). Viral RNA extraction was performed as mentioned previously.

RESULTS

Field study

Norovirus GII genomes were detected in air samples from six of the eight healthcare facilities (75%) and in 23/48 air samples. Norovirus RNA was detected in fourteen symptomatic patient's

rooms out of twenty-six (54%), six hallways out of sixteen (38%) and three nurse stations out of six (50%). Positive samples concentrations ranged from 1.35×10^1 to 2.35×10^3 genomes per m^3 (Table 1).

***In vitro* experiments**

For all experiments, the aerosols median mass aerodynamic diameter in the GenaMini chamber ranged from 0.89 to 1.08 μm , and the total particles concentration was from 2.42×10^4 to 5.37×10^4 particles per cm^3 . The RH and temperature inside the chamber fluctuated between $5.9 \pm 1.9\%$ and $24.1 \pm 0.9^\circ C$. The concentration of infectious viruses, total genomes and intact viruses into the nebulizer of the aerosol chamber did not vary significantly between the beginning and the end of the aerosolization process (Figure 1). The concentrations of norovirus MNV-1 in the nebulizer were 1×10^7 infectious virus/ml (Figure 1A), $2-4 \times 10^9$ intact viruses/ml (Figure 1C), and $6-8 \times 10^{10}$ genomes/ml (Figure 1B) as determined by plaque assay, qPCR and PMA-qPCR, respectively.

Using PMA qPCR, it has been possible to determine the relative percentage of intact norovirus MNV-1 within the NIOSH-251. Figure 2 shows that the NIOSH-251 recovered more than 89% intact viruses. The cultivable-to-genome ratio in the nebuliser and the sampler was calculated and the result was converted into a percentage to determine the norovirus resistance to aerosolization and air sampling. The relative percentage of norovirus MNV-1 infectivity varied from 76% to 86%. The NIOSH-251 was efficient in preserving MNV-1 infectivity (Figure 2).

DISCUSSION

This study provides original quantitative data regarding the airborne dissemination of norovirus in healthcare facilities and documents for the first time widespread dissemination of human norovirus GII in the air of healthcare facilities during gastroenteritis outbreaks. The lack of positive norovirus detection does not necessarily mean there was no human norovirus in the air but simply that the detection limit of the test was reached. The air from patient rooms may contain up to 2000 genomes/m³, and considering that an average human breaths approximately 6 liters of air per minute, a healthcare worker could inhale up to 60 copies of human norovirus during a 5-minute stay in the room of a symptomatic patient. For some individuals, this quantity could be sufficient to cause the disease.

Many processes can lead to the creation of norovirus aerosols and several sources can be identified such as resuspension from fomites [28-32], flushing toilets [33, 34], vomit droplets [16] and healthcare workers (serving as vector for aerosolized particles) [3]. All of these sources need to be considered to avoid epidemics. Overall, the detection of significant concentrations of human norovirus genomes in the air of corridors and nursing stations suggests that they can remain suspended in the air for prolonged periods of time. This provides additional support to the hypothesis that human norovirus may be an airborne disease as suspected by Sawyer *et al.* [35].

Although norovirus is an intestinal pathogen, noroviruses could be transmitted through the airborne route and subsequently could, if inhaled, settle in the pharynx and later be swallowed.

Hence, *in vitro* studies were performed to evaluate the preservation of the aerosolized norovirus infectious potential using MNV-1 as a surrogate and a NIOSH-251 air sampler. Noroviruses

could withstand aerosolization with no significant loss of infectivity. The difference between the concentration of infectious viruses and intact viruses might be explained by the presence of damaged receptors, making their attachment impossible, but also by the fact that aggregated viruses can only be detected as a single plaque-forming unit. Since culture methods for human norovirus were only recently published (after this study was completed), we suggest that NIOSH-251 sampler could be used in the field to evaluate culturability and infectivity of airborne human viruses. These results may explain in part the propensity of this virus to cause abrupt and widespread outbreaks in healthcare settings and confined environments such as aircrafts [36] and cruise ships [37]. A few years ago, Marks *et al.* also raised the possibility of an airborne spread of norovirus following infections by inhalation in hotels, restaurants and schools [14, 15].

The findings presented in this report could have an important impact on the infection control practices and recommendations for managing norovirus outbreaks in healthcare facilities. They suggest that air may be an important but yet underappreciated mode of transmission of norovirus and may explain in part the well-known difficulty of controlling norovirus outbreaks. Currently, the US Centers for Diseases Control recommends the implementation of contact precautions only when caring for patients with norovirus gastroenteritis [38]. This recommendation is based on the belief that norovirus are unlikely to remain viable on air currents that travel long distances. There is a need for identifying the optimal infection prevention measures required to ensure a safe hospital environment; for example, the use of full airborne precautions (including the use of respirators, the closing of patient rooms' doors and the use of negative pressure rooms) could help prevent transmission of this troublesome virus.

CONCLUSION

This study detected high concentrations of infectious norovirus GII in the air of healthcare facilities during outbreaks. *In vitro* models suggest this virus may withstand aerosolization, supporting a probable mode of transmission for norovirus.

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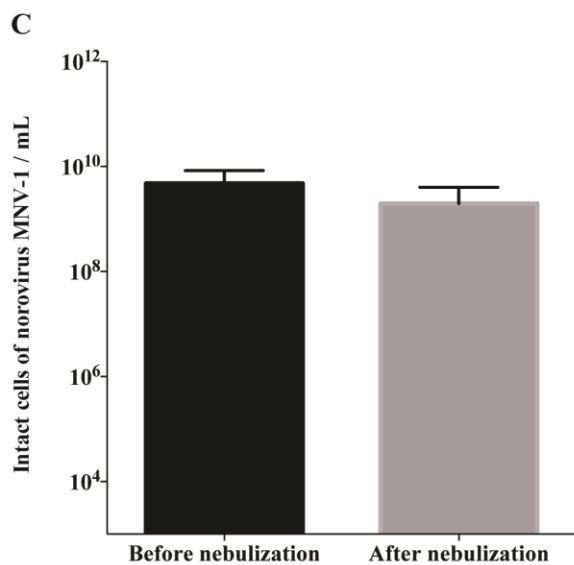
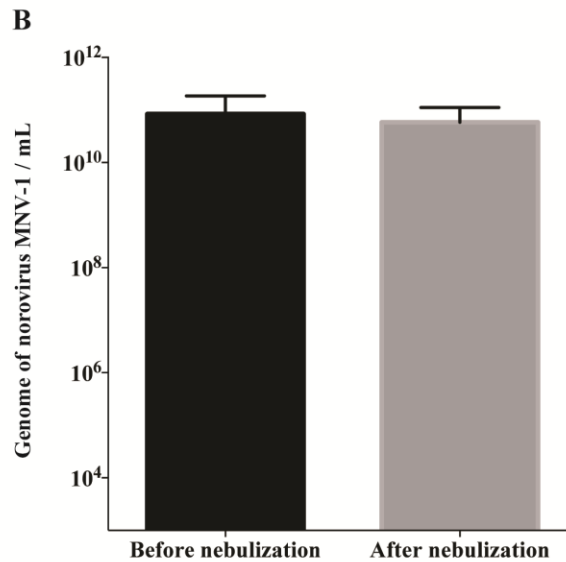
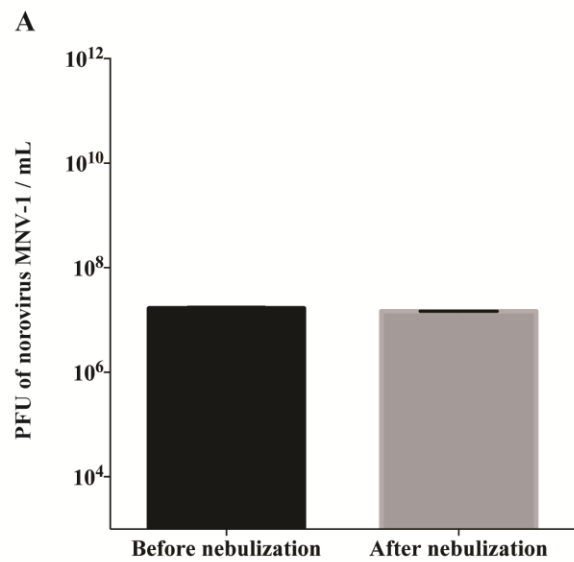
TABLE 1: Detection and concentration of norovirus GII RNA recovered from the air in patient rooms, hallways and nursing stations during 8 confirmed norovirus outbreaks, Quebec, 2012. Air samples were taken with the Coriolis μ ® set at 200 L/min for 10 minutes.

Healthcare centers	Number of positive sample	Range of Norovirus GII
Location	detected in the air	(Genome/m³)
Patient room	14/26	$1.46 \times 10^1 - 2.35 \times 10^3$
Nurse station	3/6	$1.35 \times 10^1 - 1.22 \times 10^2$
Hallway/Common area	6/16	$1.54 \times 10^1 - 5.43 \times 10^2$

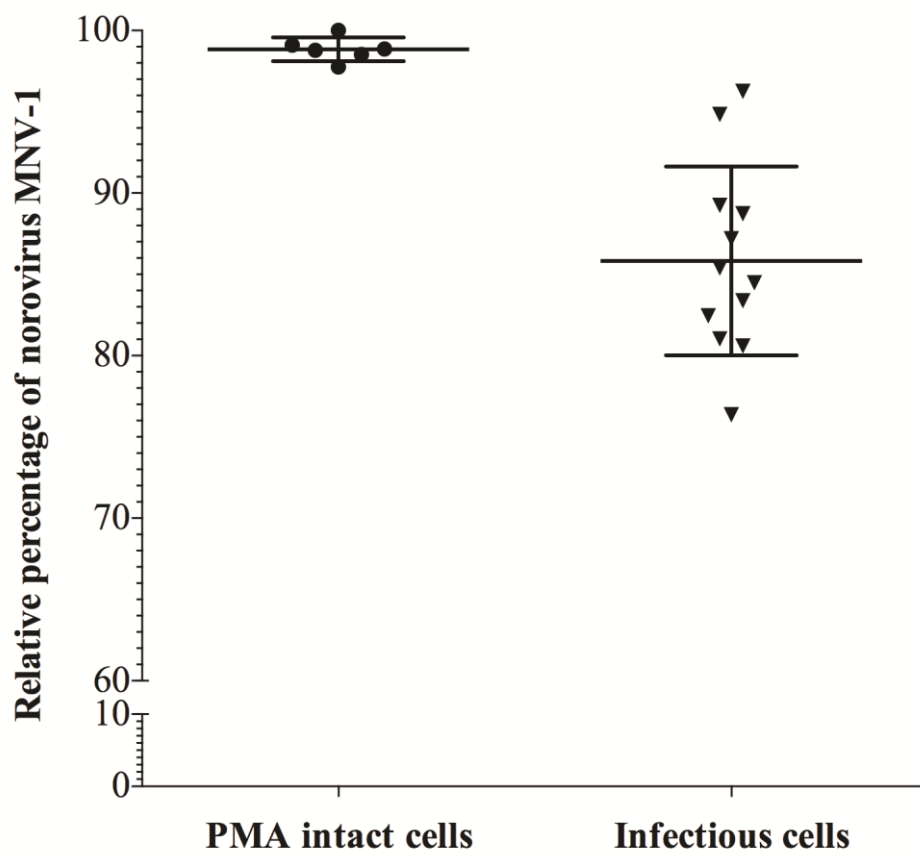
FIGURE LEGENDS

Figure 1: MNV-1 concentration in the nebulizer at the beginning (black bar) and at the end (grey bar) of the aerosolization. 2A: Infectious MNV-1 concentration, 2B: MNV-1 genome concentration, 2C: concentration of MNV-1 with intact viral capsid. There is no significant difference between virus concentration at the beginning and at the end of the aerosolization.

Figure 2: Relative percentage on MNV-1 with intact capsid after aerosolization and sampling with the NIOSH-251 (black round) as determined using PMA-qPCR assay. Relative MNV-1 infectious percentage after aerosolization and sampling with the NIOSH-251 (black triangle). Horizontal bars represent the mean of experiments with standard deviation.



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