Detection and Quantification of Airborne Norovirus During Outbreaks in Healthcare Facilities

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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, fecal matter, or vomitus, and contact with contaminated surfaces. Although 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 and to study 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 type 1 (MNV-1) bioaerosols was also tested in vitro using an aerosol chamber.

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

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

Keywords. norovirus; airborne transmission; GenaMini chamber; nosocomial infection.

Noroviruses are nonenveloped, 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 where contact between infected patients and personnel is intense, such as hospitals and nursing homes [5]. In the United States, norovirus infections represent 2 million outpatient visits, 414 000 emergency room visits, 56 000–71 000 hospitalizations, and up to 800 deaths each year [6]. Children, elderly persons, immunocompromised persons, and people living or working in healthcare facilities are at higher risk of contracting the disease [7].

Noroviruses are highly contagious, with an infectious dose ranging from 18 to 2800 particles, making their spread difficult to prevent [8]. A descriptive study performed in 2011–2012 to estimate the incidence of norovirus outbreaks in hospitals and nursing homes in Catalonia, Spain, demonstrated the occurrence of norovirus to be very high and associated with significant mortality [9], and showed that even a small amount of contamination can lead to a potential risk to public...
health [8, 10]. Multiple routes of infection transmission have been documented, including direct contact with an infected person, fecal matter, and/or vomitus droplets, and contact with contaminated surfaces [7, 11]. Indirect evidence suggests that norovirus could be transmitted through the airborne route, and this route of transmission has already been suggested in literature [12–15]. Nenonen et al showed high nucleotide similarity between norovirus GII.4 strains present in the dust of rooms of patients infected by norovirus [16]. 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 as this virus was, until very recently, not culturable [17]. Assessing the capacity of norovirus 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 calcivirus, bacteriophage MS2, and murine norovirus (MNV) [10]. Murine norovirus type 1 (MNV-1) shares similar genetic and structural features with the human norovirus and, therefore, is a culturable surrogate [18] and is used to study the resistance to environmental stress of human norovirus [10].

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 photoinducible azide group, which allows covalent cross-links 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 polymerase chain reaction (PCR). This method was previously used to determine the integrity of norovirus particles [19].

The general aim of this study was to investigate the potential for airborne transmission of human norovirus. To achieve this goal, 2 distinct and complementary objectives were designed: (1) to quantify the presence of norovirus GII in air samples during gastroenteritis outbreaks in healthcare facilities and (2) to study the virus’ resistance to aerosolization by assessing its integrity when subjected to in vitro aerosolization stress using MNV as a surrogate. Integrity preservation was determined by culture and PMA. The use of a PMA quantitative PCR (qPCR) method as an indicator of MNV integrity was also validated.

**MATERIALS AND METHODS**

**Field Study**

**Sampling Human Norovirus in Healthcare Facilities**

Air sampling was performed in 8 healthcare facilities of the Quebec City area (Canada) when viral gastroenteritis outbreaks occurred. Norovirus was established via PCR as the causal agent of the gastroenteritis outbreaks by the public health laboratory of the Quebec Province.

Air samples were taken in 3 distinct locations on patient wards: (1) inside the room of patients with gastroenteritis symptoms (<24 hours), (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 nurses’ stations.

Air samples were taken with the Coriolis μ (Bertin Technologies, St-Berthely, France) set at 200 L/minute for 10 minutes (sampler D50 <0.5 µm) and 15 mL of phosphate-buffered saline (PBS) was used for fluid collection (Lonza, Bâle, Switzerland). Samples were concentrated on an Amicon Ultra-15 centrifugal filter unit (porosity of 50 kDa; Millipore, Billerica, Massachusetts) to a final volume of 400 µL. Concentrated air samples were spiked with 1 µL of an MS2 bacteriophage suspension (10⁶ genome/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, California). Total RNA was eluted and immediately transcribed into complementary DNA (cDNA) or frozen at −80°C until reverse transcription PCR was performed.

**In Vitro Experiments**

**Murine Norovirus and Cells**

MNV-1 and macrophage RAW 264.7 cells were cultivated as mentioned by Wobus et al, in presence of macrophage RAW 264.7 in Dulbecco modified Eagle’s medium (DMEM; Cellgro, Mediatech, Herndon, Virginia) [20]. An initial stock at 10⁷ plaque-forming units (PFU)/mL was prepared, divided into subsamples (70 mL of MNV-1 at 10⁷ 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 milliliters of MNV-1 (10⁷ PFU/mL) in DMEM was nebulized (Single-Jet Atomizer, model 9302; TSI Inc, Shoreview, Minnesota) at a rate of 3 L/minute using high-efficiency particulate air (HEPA)–filtered air. The average liquid flow rate of the nebulizer was 0.18 ± 0.2 mL per minute. Aerosols were dried through a desiccator (EMD Chemicals Inc, Gibbstown, New Jersey), allowing the formation of droplet nuclei before entering the chamber, and were diluted with HEPA-filtered dry air at a rate of 23 L/minute [21]. An Aerodynamic Particle Sizer (model 3321; TSI Inc) was used to monitor particle size distribution and concentration during aerosol sampling. Temperature and relative humidity (RH) were also measured.
**Aerosol Sampling**

Air samples were collected using the National Institute for Occupational Safety and Health (NIOSH) 2-stage cyclone aerosol sampler prototype (NIOSH-251; Centers for Disease Control and Prevention [CDC]/NIOSH, Morgantown, West Virginia) [22, 23] for 25 minutes at 10 L/minute, 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, California) for 15 minutes at room temperature. All eluents were pooled together. Aerosolization experiments were performed 5 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 [24], except that plaques were visualized by crystal violet staining after fixation with formaldehyde. Each plaque assay had one negative control well.

**RNA Isolation of MNV**

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 Genome cDNA Synthesis**

RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California), 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 elsewhere [21]. 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 [25] and Girard et al [26], respectively. Quantification was performed using a standard curve of a 10-fold dilution series of MNV-1 plasmid DNA preparation or norovirus GII plasmid DNA preparation [26]. Serial 10-fold dilutions from 10^6 to 10^9 molecules per reaction tube were used. The curve was prepared using the pGC Blue Cloning and Amplification kits (Lucigen, Middleton, Wisconsin). The DNA plasmids were purified using the Qiagen Plasmid Mini Kit and were quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts).

**PMA-qPCR Assay**

The PMA-qPCR method, previously used with norovirus by Parshionikar et al [19], allows quantification of virus with an intact capsid. PMA (Biotium Inc, Hayward, California) was dissolved in 20% dimethyl sulfoxide to create a 5 mM stock solution and stored at −20°C in the dark. A total of 4.2 µL of PMA was added to 140 µL of air sample aliquots to a final concentration of 150 µM in light-transparent 1.5-mL tubes (Fisher Scientific, Ottawa, Ontario, Canada). Following an incubation period of 5 minutes in the dark with occasional mixing, samples were exposed to light for 10 minutes using a PMA-Lite light-emitting diode (LED) Photolysis Device (a long-lasting LED light with 465–475 nm emissions for PMA activation; Biotium Inc). Viral RNA extraction was performed as mentioned previously using the QIAamp viral RNA Mini Kit (Qiagen, Mississauga, Ontario, Canada).

**RESULTS**

**Field Study**

Norovirus GII genomes were detected in air samples from 6 of the 8 healthcare facilities (75%) and in 23 of 48 air samples. Norovirus RNA was detected in 14 of 26 symptomatic patients’ rooms (54%), 6 of 16 hallways (38%), and 3 of 6 nurses’ stations (50%). Positive sample concentrations ranged from 1.35 × 10^1 to 2.35 × 10^3 genomes/m^3 (Table 1).

**In Vitro Experiments**

For all experiments, the aerosol median mass aerodynamic diameter in the GenaMini chamber ranged from 0.89 µm to 1.08 µm, and the total particle concentration was from 2.42 × 10^4 to 5.37 × 10^4 particles/cm^3. The RH and the temperature varies inside the chamber between 5.9% ± 1.9% and 24.1°C ± 0.9°C respectively. The concentration of infectious viruses, total genomes, and viruses with intact structure 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 MNV-1 in the nebulizer were 1 × 10^7 infectious virus particles/mL (Figure 1A), 2–4 × 10^9 intact viruses particles/mL (Figure 1C), and 6–8 × 10^10 genomes/mL (Figure 1B) as determined by plaque assay, qPCR, and PMA-qPCR, respectively.

Using PMA-qPCR, it is possible to determine the relative percentage of intact MNV-1 within the NIOSH-251. Figure 2

**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**

<table>
<thead>
<tr>
<th>Healthcare Center Location</th>
<th>No. of Positive Samples Detected in Air</th>
<th>Range of Norovirus GII Genomes/m^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients’ rooms</td>
<td>14/26</td>
<td>1.46 × 10^1–2.35 × 10^2</td>
</tr>
<tr>
<td>Nurses’ stations</td>
<td>3/6</td>
<td>1.25 × 10^1–1.12 × 10^2</td>
</tr>
<tr>
<td>Hallway/common areas</td>
<td>6/16</td>
<td>1.54 × 10^1–5.43 × 10^2</td>
</tr>
</tbody>
</table>

Air samples were taken with the Coriolis μ, set at 200 L/minute for 10 minutes.
shows that the NIOSH-251 recovered >89% intact viruses particles. The cultivable-to-genome ratio in the nebulizer and the sampler was calculated, and the result was converted into a percentage to determine the resistance of norovirus to aerosolization and air sampling. The relative percentage of 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 breathes approximately 6 L 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 [27–31], flushing toilets [32, 33], vomitus droplets [15],

![Figure 1. Murine norovirus type 1 (MNV-1) concentrations (plaque-forming units [PFU]) in the nebulizer at the beginning (black bars) and at the end (gray bars) of the aerosolization. A, Infectious MNV-1 concentration. B, MNV-1 genome concentration. C, 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.](image-url)
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 [34]. 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, we performed in vitro studies 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 concentrations 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 PFU. As culture methods for human norovirus were only recently published (after this study was completed), we suggest that the 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 [35] and cruise ships [36]. 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 [13, 14].

The findings presented in this report could have an important impact on infection control practices and recommendations for managing norovirus outbreaks in healthcare facilities. They suggest that air may be an important yet underappreciated mode of transmission of norovirus and may explain in part the well-known difficulty of controlling norovirus outbreaks. Currently, the CDC recommends the implementation of contact precautions only when caring for patients with norovirus gastroenteritis [37]. This recommendation is based on the belief that noroviruses 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.

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

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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