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Inactivation of Norovirus by ozone gas in conditions relevant to healthcare

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Summary We evaluated the ability of ozone gas to inactivate Norovirus and its animal surrogate feline calicivirus (FCV) in dried samples placed at various locations within a hotel room, a cruise liner cabin and an office. Norovirus was measured by quantitative reverse transcriptase real-time polymerase chain reaction (QRT-PCR) assay, and FCV by a combination of QRT-PCR and virus infectivity assays. We were able to reduce the concentration of infectious FCV by a factor of more than 10^3, and in some cases beyond detection, under optimal conditions of ozone exposure with less than an hour of total operation. QRT-PCR assays indicated similar decreases in both viral RNAs. Virus-containing samples dried onto hard surfaces (plastic, steel and glass), and soft surfaces such as fabric, cotton and carpet, were equally vulnerable to the treatment. Our results show that Norovirus can be inactivated by exposure to ozone gas from a portable commercial generator in settings such as hotel rooms, cruise ship cabins and healthcare facilities.

Introduction

Noroviruses (NVs, calicivirus family) are increasingly recognized as the main cause of gastroenteritis outbreaks in health care facilities, senior citizens' homes and cruise ships. A challenge to developing effective disinfectants against these environmentally resistant agents has been the lack of a sensitive methodology to quantify their infectivity. This has been resolved in part by the use of the surrogate feline calicivirus (FCV), which can be readily grown and assayed in cell culture.[1] Moreover, it is now feasible to correlate the virus titre with virus RNA concentration as determined by quantitative reverse transcriptase real-time polymerase chain reaction (QRT-PCR) assays.[2–6] Consequently, it is possible to assess definitively the effect of antiviral

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agents by comparing FCV and NV under similar inactivation conditions.[2,4] In addition it is also feasible to use FCV as a surrogate virus for NV in field conditions where the experimental use of NV itself would not be acceptable due to its infectious risk.[7] The use of these techniques has allowed for significant advances in our understanding of the epidemiology and economic impact of NV infections.[8–11]

NVs are very stable in the environment; hence, agents such as bleach and peroxides are required for disinfection, although the efficacy of such agents against NV has been questioned.[2] More recently it was shown that some of them were less effective against calicivirus-contaminated fabrics and carpet.[12] In addition, these agents are used in liquid form, may not completely penetrate contaminated areas within a room and are labour-intensive to use. Furthermore, they are difficult to apply to surfaces such as bedding and curtains where they may have a bleaching effect.

A recent study showed that ozone dissolved in water could inactivate water-borne NV.[2] We therefore decided to evaluate the possibility of using ozone gas as a virucidal agent against NV. This was based on the premise that in view of the superior penetrability of gases, it should be feasible to inactivate the virus in any location and on any surface within a contaminated room. We therefore tested the efficacy of ozone gas, from a proprietary portable ozone generator, against FCV- and NV-containing specimens, in an office, a hotel room and a cruise liner cabin. We expected that the findings from such tests would be relevant to any healthcare facility, which could be temporarily isolated for treatment with ozone gas.

Methods

Equipment

The prototype Viroforce ozone generator was constructed as a portable module containing multiple corona discharge units, a circulating fan and an efficient catalytic converter (scrubber) to reconvert ozone to oxygen at the termination of the ozone exposure period. The unit was controlled remotely from outside the test room. In addition, a portable custom-made rapid humidifying device (RHD) was used to provide a burst of water vapour when required. Preliminary tests in our laboratory had indicated that an ozone level of 25 ppm, at high relative humidity (in excess of 70%), resulted in more than 99.9% inactivation of several viruses, including feline calicivirus (unpublished data).

Ozone concentration was monitored continuously by means of an Advanced Pollution Instrumentation Inc. model 450 system (from Teledyne, San Diego, CA, USA), which measured samples of the ozonated air and passed them through a UV spectrometer. The input teflon sampling tube was taped in an appropriate location for the duration of the experiment.

Relative humidity and temperature were recorded by a portable hygrometer (VWR Scientific, Ontario, Canada). The probe was taped in a convenient location inside the test room. Temperature did not exceed 23 °C.

Test rooms and protocol

Most tests were carried out in an office (volume 34 m^3) containing normal office furniture and adjacent to the laboratory. Additional tests were conducted in a local standard hotel room (volume 47.6 m^3) containing double bed, table, chairs, open closet and adjoining bathroom, and in a standard cabin (volume 36.4 m^3) of a cruise liner which was docked in Vancouver for half a day. Vents, windows, and doors were sealed with tape.

The standard protocol was based upon preliminary laboratory tests: virus samples (50–100 μL) were dried onto sterile plastic or other surfaces in duplicate in the Viroforce Laboratory. When dry, the samples were transported quickly to the test site in sterile containers. The samples were placed at various locations in the test room, and the ozone generator and rapid humidifying device (RHD) were placed in a central location. These units were operated remotely from outside the room. At the commencement of the test, the samples were uncovered, the door closed and sealed, and the generator switched on.

The ozone level reached 20–25 ppm within several minutes and was maintained at this level for 20 min. This time was determined from consultation with cruise liner staff, who indicated the desirability of a short treatment time compatible with the rapid turnover of passengers. The RHD was then activated to produce a burst of water vapour for 5 min. Both generator and RHD were then switched off for another 10 min to allow 'incubation' in the humid atmosphere. The scrubber was then turned on to remove all ozone gas. Ozone levels decreased to less than 1 ppm within 15 min, at which point the door was opened and the test samples retrieved for transport back to the laboratory. The samples were reconstituted in 1.0 mL medium and kept frozen at −80 °C until ready for assay.

In all experiments, control samples of the dried viruses were either transported to the test site, and

stored away from the ozone, or were stored in the Viroforce laboratory for the duration of the tests.

Materials

The lids of sterile polystyrene tissue culture trays were used as plastic surfaces. Samples of fabrics and carpet (typical of those used in hotel rooms) were cut into small pieces, cleaned in detergent, washed, dried, and sterilized by autoclaving. Cotton tips were heated for 2 min in a microwave oven.

All media, serum and other reagents used in cell and virus work were obtained from Invitrogen (Gibco, Ontario, Canada). Sterile plastic culture vessels and other supplies were BD-Falcon brand obtained from VWR Scientific. Molecular biology reagents and kits were obtained from Qiagen (Missisauga, Ontario, Canada) and PCR primers from Operon (Huntsville, AL, USA).

Cell lines and viruses

FCV and feline kidney cell line (FK cells) were obtained from BC-Centre for Disease Control (BCCDC). The cells were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, without antibiotics or antimycotics. Stock virus, obtained as clarified supernatants from FCV-infected flasks, had titres of $1-2 \times 10^7$ Pfu (plaque forming units)/mL.

NV specimens, in the form of stools in which NV had been diagnosed by RT-PCR, were obtained from BC-CDC, along with NV-negative stool samples.

Virus assays

For the quantitative measurement of infectious FCV, plaque assays were conducted in duplicate, according to standard techniques making use of agarose overlays.[1] Very low numbers of infectious virus were detected by incubating undiluted samples and serial two-fold dilutions along rows of cells cultured in 96-well trays (without overlays). The end-point was the dilution of sample that no longer gave rise to characteristic viral CPE (cytopathic effects). If no viral CPE were seen in any culture wells after 5 days of incubation, the sample was considered completely free of infectious virus.[13]

Quantitative RT-PCR (reverse transcriptase real-time PCR) measurements

Treated and control samples were removed from frozen storage and their RNA extracted and purified by means of Qiagen RNA extraction kits. Quantitative RT-PCR measurements were made on the Opticon DNA engine. Methods followed those described in Frankhauser *et al.* and Scansen *et al.*[6,8]

Primers for FCV were: forward primer 5'-TGGA TGAACTACCCGCCA; reverse primer 5'-GCACATCAT ATGCGGCTC; and for NV strains the following pairs were used):

MON 431 tgg acI agR ggI ccY aaY ca, RNA sense;
MON 432 tgg acI cgY ggI ccY aaY ca, RNA sense;
MON 433 gaa Yct cat cca Yct gaa cat, DNA sense;
MON 434 gaa Scg cat cca Rcg gaa cat, DNA sense;

where I = Inosine; R = puRine (A/G); Y = pYrimidine(C/T); S = Strong (C/G).

Results

Office tests

Replicate samples of FCV and NV (three different stool samples) were tested in the office. In some samples of FCV, fetal bovine serum or NV-positive stool was added (1:1) to determine the effect of a representative organic load on the treatment. Following the standard ozone protocol, samples were assayed for infectious FCV and for viral RNA by QRT-PCR (Table I).

Substantial inactivation of FCV and NV samples was achieved, with a comparable reduction in RT-PCR values, indicating that infectivity of both viruses would be similarly affected if it were possible to assay for NV infectivity. This was an important finding since it would be necessary to conduct subsequent tests in hotel rooms and cabins with FCV only for practical reasons.

Table I Field test in office

Virus	Pfu (fraction of control)	Log_{10}	RT-PCR (fraction of control)	Log_{10}
FCV	0.012	−1.92	0.029	−1.54
FCV, + FBS (1:1)	0.017	−1.77	0.021	−1.68
FCV, + stool (1:1)	0.015	−1.82	0.020	−1.70
NV sample 1	_		0.070	−1.15
NV sample 2	_		0.055	−1.26
NV sample 3	_		0.046	−1.34

Control values: for FCV infectivity, 5.1×10^4 Pfu/mL; and for PCR, 116 to 218 ng RNA; for NV samples, NV 1 = 58.15 ng RNA, NV 2 = 129.5 ng RNA, NV 3 = 114.1 ng RNA.
Pfu, plaque forming units; FCV, feline calicivirus (FCV); RT-PCR, reverse transcriptase real-time polymerase chain reaction; Norovirus (NV).

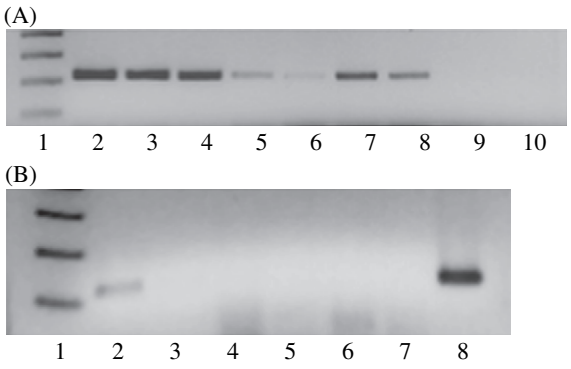

Figure 1 Electrophoresis of PCR-amplified Norovirus (NV) RNA (213 base pair band). (A) Lane 5, NV-1, no ozone; lane 6, NV-1, ozone-treated; lane 7, NV-2, no ozone; lane 8, NV-2, ozone-treated. Other lanes: 1, DNA base pair ladder; 2—4, NV-negative stool samples; 9, 10, NV-negative samples. (B) Lane 1, DNA base pair ladder; lane 2, NV-3, no ozone; lane 2, lane 3, NV-3, ozone-treated. Other lanes: 4—7, NV-negative stool samples; 8, NV-positive stool sample.

In addition the presence of added serum and stool (from an NV-positive sample) did not adversely affect the response of FCV to ozone treatment (Table I). Figure 1 illustrates the gel electrophoresis patterns of amplified RNA from the three NV samples. The bands from all the ozone-treated samples were substantially reduced in intensity, indicating that the treated virus particles had been disrupted, although some residual pieces of viral RNA were still present and capable of being amplified in the PCR reactions.

Hotel room

Replicate samples of FCV were placed in three different locations, one in the bathroom next to the sink, one on top of the bed and the third on top of the table.

Samples were subsequently assayed for virus infectivity and QRT-PCR. The results are shown in Table II. The FCV samples that gave no virus plaques at 1:10 dilution (bathroom and table samples) were reassayed by the CPE end-point dilution test to determine if there were any infectious viruses remaining after treatment. Control and bed samples were also assayed for comparison. Since the bathroom and table samples gave no CPE in these assays, we concluded that for these samples the virus had been eradicated.

Cruise liner cabin

Two tests were carried out with FCV on plastic trays. The first test used the standard protocol, and the second one used a more compact schedule that would be more attractive to cruise liner schedules.

Samples were placed on top of the bed, on the table and in the adjoining bathroom. Results from both tests were essentially the same, indicating that the abbreviated protocol could work with the desired efficiency. Data from test no. 2, the abbreviated schedule, are shown in Table III. In this test, the operation times were reduced to 15 min ozone, followed by 4 min water vapour pulse (RHD), no post-humidity 'incubation' period, and 15 min scrubber.

No residual infectious virus was detected in the CPE tests. Thus the virus had been eradicated in these samples (inactivation by a factor of more than 10^4). The corresponding RT-PCR measurements showed residual amplified viral RNA at a level of 0.03 or less (Table III).

Virus on soft surfaces

For additional tests conducted in the office, replicate samples of both FCV- and NV-positive stools were dried onto plastic trays as usual and also onto samples of fabric, cotton and carpet. These were placed at various locations within the office to mimic possible contamination sites during an NV outbreak. The standard ozone exposure protocol was used and subsequently samples were assayed for FCV infectivity and for viral RNA by QRT-PCR. The results are summarized in Table IV.

Table II Field test in hotel room

Virus	Pfu (fraction of control)	Viral CPE at dilution	Log_{10}	RT-PCR (fraction of control)	Log_{10}
FCV, bathroom	0	None	<-4.0	0.077	-1.11
FCV, bed	<0.0002	1:8	<-3.7	0.077	-1.11
FCV, table	0	None	<-4.0	0.075	-1.12
Control values	8.0×10^4 Pfu/mL	>1:4096		415.5 ng	

Pfu, plaque forming units; CPE, cytopathic effects; RT-PCR, reverse transcriptase real-time polymerase chain reaction; FCV, feline calcivirus.

Table III Cruise liner cabin. Feline calicivirus (FCV) infectivity

Sample	Infectivity (Pfu/mL)	Surviving fraction	RT-PCR surviving fraction
Control	5.37×10^4	1.0	1.0
Treated (bathroom, bed and table)	$<10^1$	<0.0002	0.003—0.03

Pfu, plaque forming units; RT-PCR, reverse transcriptase real-time polymerase chain reaction.

All samples showed similar sensitivity to ozone, regardless of their location or the surface onto which they were dried.

Discussion

We have shown that we can inactivate Norovirus contained in dried stool samples in an office, a hotel room and a cruise liner cabin. In comparison with the feline calicivirus, a generally acceptable surrogate virus for the evaluation of NV titres, it is reasonable to conclude from our data that we were able to achieve inactivation of virus by a factor of more than 10^3, and, under optimal conditions, to eradicate the virus.

The QRT-PCR technique, currently the only convenient method for measuring NV, does not measure virus infectivity per se, but rather a defined sequence of the viral genome, which one would expect to be more resistant to the damaging effects of ozone gas than infectivity. Consequently, this nucleic-acid-based technique probably underestimates the effectiveness of antiviral agents, hence the need for comparison with a related virus that can be assayed for infectivity as well as by QRT-PCR. The finding that our treatment protocol could in many cases eradicate FCV infectivity indicates that under the same conditions NV should also be rendered non-infectious, even though its genome may still be partly intact.

Addition of serum and NV-positive stool to FCV samples did not adversely affect their sensitivity to ozone, an observation that confirmed the validity of FCV as a surrogate for NV in locations where it would be impractical to use live NV samples.

Virus samples dried onto soft furnishings were also vulnerable to ozone. The degree of virus inactivation was comparable to that observed for samples on plastic, confirming that any virus deposited onto curtains, bedding, linen and chair covers, etc. should not present a barrier to inactivation. Recently, it was reported that some liquid disinfectants were not very effective against FCV on fabrics and carpet.[12] We had previously shown in laboratory tests that virus dried onto other hard surfaces such as glass and steel could be inactivated by ozone gas as readily as on plastic (unpublished results).

Ozone gas has several advantages as a practical antiviral agent. It can effectively penetrate every part of a room, including sites that might prove difficult to gain access to with conventional liquids and manual cleaning procedures. For example, in our tests, virus deposited under the table or adsorbed to fabric taped to a window were just as vulnerable to attack as virus placed in more accessible sites. The gas is easy and economical to produce, and is a natural compound which decays quickly back to oxygen with a half-life of about 20 min. The use of a catalytic converter (scrubber) considerably speeds up the removal of the gas. In addition, in the event of possible malfunction during application, the gas is readily detected by smell and hence can be avoided.

Its major disadvantage is its potential toxicity at high concentration, which precludes its use in areas continuously populated by people. In practice this means it can only be used in rooms that can be sealed off or quarantined for the duration of the treatment. Since the standard protocol requires less than an hour to perform, however,

Table IV Norovirus (NV) and surrogate feline calicivirus (FCV) on different surfaces

Sample type	FCV infectivity fraction of control	FCV QRT-PCR fraction of control	NV QRT-PCR fraction of control
Plastic: table top, underside of table and wall	All $\leq 6 \times 10^{-5}$	0.0013—0.0016	0.05—0.069
Fabric: table top, wall, window	All $\leq 3 \times 10^{-4}$	0.0036—0.0048	0.056—0.065
Cotton: table top, different locations	All $\leq 3 \times 10^{-5}$	0.076—0.079	0.030—0.031
Carpet: floor, different locations	All $\leq 4 \times 10^{-5}$	0.0028—0.0032	0.042—0.059
Control values	$2.7—3.6 \times 10^5$ Pfu	18.7—57.3 ng RNA	98.6—132.7 ng RNA

this should not be a barrier to utilization given its potential efficacy.

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