OZONE GAS AS A PRACTICAL ANTIVIRAL AGENT

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SUMMARY

We evaluated the ability of ozone gas, produced by a commercial portable generator, to inactivate Norovirus, and various other viruses in dried and wet samples placed at various locations within a laboratory, a hotel room, a cruise liner cabin, and an office. Norovirus was measured by quantitative reverse transcriptase real-time polymerase chain reaction (QRT-PCR) assays, and other viruses by a combination of QRT-PCR and virus infectivity assays.

Under optimal conditions of ozone exposure, with less than an hour of total operation, we were able to reduce the concentration of infectious viruses by more than 3 $\log_{10}$, and in some cases beyond detection. QRT-PCR assays indicated similar substantial decreases in 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 have shown that Norovirus and other viruses can be inactivated readily and safely by exposure to ozone gas in settings such as hotel rooms, cruise ship cabins, and health-care facilities.

INTRODUCTION:

In addition to the numerous viruses responsible for human infections, the Noroviruses (NV's, calicivirus family) are increasingly recognized as the main cause of gastroenteritis outbreaks in health care facilities, senior citizens' homes and cruise ships. A challenge in developing effective disinfectants against these environmentally resistant agents has been the lack of a sensitive methodology to quantitate 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 titer 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 definitively assess the effect of antiviral 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]

The Noroviruses 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 on contaminated fabrics and carpet.[12] In addition these agents are used in liquid form, and suffer from the limitations of incomplete penetration of contaminated areas within a room, and are labor-intensive to use. Furthermore they are difficult to apply to contaminated soft surfaces such as bedding and curtains where they may have a bleaching effect.

However several studies have shown that ozone in water can be an effective antiviral agent, and a recent study showed that ozone dissolved in water could inactivate water-borne Norovirus.[2] We therefore decided to evaluate the possibility of using ozone gas as a virucidal agent against a variety of human viruses, including Norovirus. We tested the efficacy of ozone gas, provided by 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 heath-care facility, which could be temporarily isolated for treatment with ozone gas.

METHODOLOGY.

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 vapor when required. Preliminary tests in our laboratory had indicated that an ozone level of 25 ppm, at high relative humidity (in excess of 80%), resulted in more than 99.9% inactivation of several viruses (Table 1, Results).

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

Relative humidity and temperature were recorded by a portable hygrometer (VWR Scientific, Ontario). The probe was taped in a convenient location inside the test room. Temperature did not exceed 23^0C.

Preliminary experiments in the laboratory were carried out on samples placed in a polycarbonate chamber containing a Treated Air Systems generator model 1,000. Humidity was provided by spraying sterile water or saline into the chamber when required. Tubes from the ozone monitor and the hygrometer probe were taped inside the the chamber.

Test rooms and protocol

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

The standard protocol was as follows:

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 20-30 ppm within several minutes, and was maintained at this level for 20 minutes. The RHD was then activated to produce a burst of water vapor 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 were retrieved for transport back to the laboratory. There, the samples were reconstituted in 1.0 mL medium and kept frozen at -80^0 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). Sterile plastic culture vessels and other supplies were BD-Falcon brand obtained from VWR Scientific (Ontario). Molecular biology reagents and kits were obtained from Qiagen (Missisauga, Ontario) and PCR primers from Operon (Huntsville, AL).

Cell Lines and Viruses

Cell lines were grown in DMEM with 5% fetal bovine serum, without antibiotics or antimycotics. Most viruses were propagated and assayed in Vero (monkey kidney) cells, except for feline calicivirus (FCV), in FK (feline kidney) cells, murine coronavirus (MCV) in mouse DBT cells, rhinovirus in H1 cells, and influenza virus in MDCK cells. Norovirus specimens, in the form of stools in which Norovirus had been diagnosed by RT-PCR, were obtained from BCCDC, along with Norovirus-negative stool samples.

Virus Assays:

Plaque assays, for the quantitative measurement of infectious FCV, 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 RNAs 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.[8] and Scansen et al.[6]

RESULTS

Laboratory Tests:

Table 1. Viruses Susceptible to ozone gas (> 3 log $_{10}$ inactivation)

Virus	Significance	Membrane (+ or -)	Assay method
HSV (herpes simplex virus)	Herpes virus	yes	infectivity
Influenza virus (human strain H3N2)	Representative human and avian flu	yes	Infectivity QRT-PCR
Murine coronavirus (MCV)	Surrogate for SARS virus	yes	infectivity
Sindbis virus (SINV)	Surrogate for Hepatitis C	yes	infectivity
Yellow fever virus (YFV)	Surrogate for Hepatitis C	yes	infectivity
Vesicular stomatitis virus (VSV)	Rhabdovirus	yes	infectivity
Poliovirus (PV)	Enteric virus	no	Infectivity
Rhinovirus types 1A & 14	Common cold viruses	no	Infectivity QRT-PCR
Norovirus	GI epidemics	no	QRT-PCR
Feline calicivirus (FCV)	Surrogate for Norovirus	no	Infectivity QRT-PCR

Table 1 summarizes the results of numerous laboratory tests on various viruses, under a variety of different conditions. In optimal conditions, all viruses tested were inactivated by at least 99.9%, and in some cases infectious virus could be eradicated completely.

Office tests:

Replicate samples of FCV (feline calicivirus) and NV (Norovirus, 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 2).

TABLE 2. Ozone Gas in Office

Virus	**PFU (fraction of control)**	**Log_{10} reduction**	**RT-PCR (fraction of control)**	**Log_{10} reduction**
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
Norovirus (NV)sample 1	_		0.070	1.15
NV sample 2	_		0.055	1.26
NV sample 3	_		0.046	1.34

Substantial inactivation of FCV and NV samples was achieved, and the reduction in RT-PCR values was similar, 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, for practical reasons, to conduct subsequent tests in hotel rooms and cabins with FCV only.

In addition the presence of added serum and stool (from a NV-positive sample) did not adversely affect the response of FCV to ozone treatment (Table 2). Fig. 1 illustrates the gel electrophoresis patterns of amplified RNA's from the 3 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 amplification in the PCR reactions.

A

1 2 3 4 5 6 7 8 9 10

B

1 2 3 4 5 6 7 8

FIGURE 1 Electrophoresis of PCR-amplified Norovirus RNA's (213 base pair band)
Panel 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-positive stool samples, 9, 10, NV-negative samples.
Panel B: lane 2, NV-3, no ozone; lane 3, NV-3, ozone treated. Other lanes: 4-7, NV-negative stool samples, 8, NV-positive stool sample

Hotel room:
Replicate samples of FCV were placed in 3 different locations, one in the bathroom next to the sink, one on top of the bed, and the third on top of the table.

TABLE 3.

Ozone Gas in Hotel Room:

Virus	PFU (fraction of control)	Viral CPE @ dilution	Log_{10} reduction	RT-PCR (fraction of control)	Log_{10} reduction
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	> 1:4,096		415.5 ng	

Samples were subsequently assayed for virus infectivity and QRT-PCR. The results are shown in Table 3 The FCV samples that gave no virus plaques at 1:10 dilution (bathroom and table samples), were re-assayed by the cpe end-point dilution test, to determine if there were a few remaining infectious viruses left 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. Influenza virus was also tested in the same room, with similar results (data not shown).

Cruise Liner Cabin:

Samples were placed on top of the bed, on the table, and in the adjoining bathroom. Results from two tests were essentially the same, and data from one of them are shown in Table 4. No residual infectious virus was detected in the cpe tests. Thus the virus had been eradicated in these samples (> 4 log_{10} inactivation). The corresponding RT-PCR measurements showed residual amplified viral RNA at a level of 0.03 or less (Table 4).

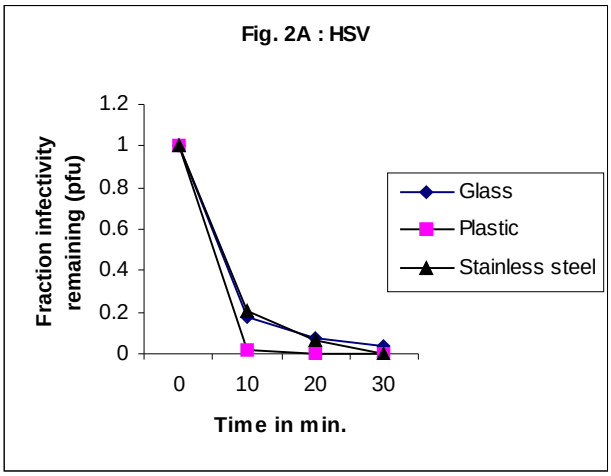
TABLE 4. Ozone Gas in Cruise Liner Cabin.

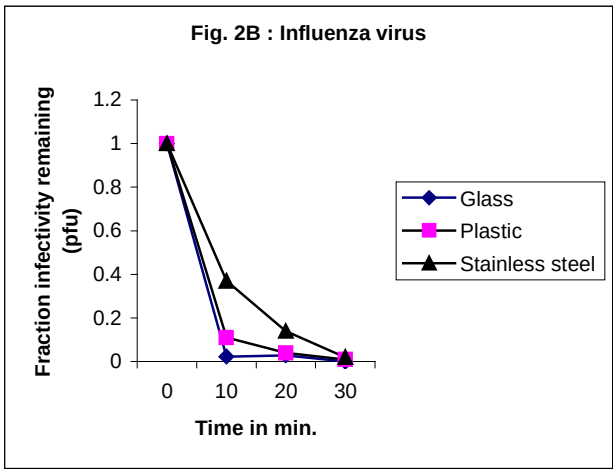
sample	FCV Infectivity pfu/mL	Surviving fraction	FCV RT-PCR surviving fraction
Control	5.37×10^4	1.0	1.0
Treated, (bathroom, bed, and table)	$< 10^1$	< 0.0002	0.003 – 0.03

Virus on Soft Surfaces:

Laboratory tests had shown that viruses, such as HSV and influenza virus (Fig 2), could be inactivated at similar rates on different hard surfaces.

FIG 2. Inactivation of viruses by ozone on different surfaces. 50µL aliquots of viruses were allowed to dry on glass slides, plastic tray lids, or stainless steel disks, in different experiments, and exposed to ozone gas for the times indicated, followed by reconstitution and freezing. Samples were subsequently thawed out and assayed for pfu Zero time controls ranged from: HSV, 2.01 to 3.7×10^6 ; influenza, 1.90 to 2.44×10^4 .





In tests conducted in the office, replicate samples of both FCV and NV-positive stools were dried onto plastic trays, as usual, and also on to samples of fabric, cotton, and carpet. These were placed at various locations within the office to mimic possible contamination sites during a Norovirus outbreak. Samples were assayed for FCV infectivity and for viral RNAs by QRT-PCR. The results are summarized in Table 5. All samples showed similar sensitivity to ozone, regardless of their location or the surface on which they were dried.

TABLE 5. NV & 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

DISCUSSION

We have shown that, in several different rooms, an office, a hotel room, and a cruise liner cabin, we can inactivate Norovirus contained in dried stool samples. By comparison with the feline calicivirus, the generally accepted surrogate virus for the evaluation of

Norovirus titers, it is reasonable to conclude from our data that we were able to achieve more than 3 $\log_{10}$ inactivation of infectious virus, and in optimal conditions to eradicate the virus.

The QRT-PCR technique, currently the only convenient method for measuring Noroviruses, 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 anti-viral agents, hence the need for comparison with a related virus which 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 be still 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, as for example in hotel rooms and public venues.

Virus samples dried onto soft surfaces, such as fabric, cotton, and carpet, were also vulnerable to the 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 anti-viral 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 smelling, 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 are sealed off or quarantined for the duration of the treatment. However since the standard protocol requires less than an hour to perform, thanks to a very efficient scrubber system built in to the generator, this is not generally a barrier to utilization.

In addition to caliciviruses, other viruses that we tested, representing different families, were equally vulnerable to ozone gas in the same conditions. Consequently we believe that the system described could be used to inactivate human and animal viruses in many different applications.

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