EVALUATION OF GASEOUS OZONE AS AN EFFECTIVE VIRUCIDAL AGENT IN CONDITIONS RELEVANT TO CRUISE LINERS AND HEALTH-CARE FACILITIES [1]

OBJECTIVES:

1. To determine the ability of gaseous ozone to kill human pathogenic viruses, including Norovirus, in defined laboratory experimental conditions, and in situations resembling practical applications in cruise liners, hospitals and health-care facilities.
2. To determine the role of various parameters, including time and dose of ozone exposure, and relative humidity.
3. To develop an efficient, portable, prototype ozone generator capable of killing viruses on contaminated surfaces within a room.

SPECIFIC AIMS:

1. To develop an appropriate and relevant experimental system for testing, under reproducible laboratory conditions, the efficacy of measured ozone doses to inactivate (ie. to kill) known amounts of several important human viruses, including Norovirus and Influenza virus.
2. To compare the virucidal efficacy of ozone gas against viruses known to be important in human health.
3. To examine the effects of different parameters on the virucidal efficacy of ozone gas, including: dose of ozone, time of exposure, relative humidity, composition of drying medium.
4. To validate the use of the RT-PCR technique (real-time polymerase chain reaction) in order to measure the inactivation of caliciviruses and Norovirus in stool samples.
5. To validate the use of feline calicivirus as an indicator of Norovirus inactivation in simulated field conditions.
6. To design and construct a portable ozone generator, based on the results obtained from the laboratory tests, with the capacity to kill viruses on surfaces.
7. To carry out field tests, in different size rooms, to confirm the ability of the prototype ozone generator to kill viruses on contaminated surfaces within the rooms.

[1] Report prepared by Dr. James B. Hudson & Dr. Manju Sharma, Viroforce Systems Inc.

SUMMARY

- We evaluated the use of ozone gas for its ability to inactivate several human pathogenic viruses, namely herpes simplex virus (HSV), influenza virus (Flu), poliovirus (PV), rhinovirus (RV), and the human noroviruses (NVs), as well as the animal surrogates feline calicivirus (FCV) and mouse coronavirus (MCV).
- We found that all of these viruses could be readily inactivated by ozone gas, according to standard virus infectivity tests (except NV, which cannot be measured directly by infectivity tests), at ozone concentrations of less than 100 ppm, in ambient humidity (approximately 40 % RH), although the membrane containing viruses (HSV, Flu, and mouse coronavirus) were more vulnerable.
- However, all the viruses, including those without membranes, were readily inactivated in the presence of a high humidity (approaching 99% RH). In optimum conditions of ozone dose and high humidity, we were able to completely inactivate (eradicate) infectious viruses.
- We established, in our laboratory, the molecular technique of RT-PCR (real- time polymerase chain reaction) to measure quantitatively the amounts of viral RNA for feline calicivirus (the standard surrogate virus for studying inactivation of NV), NV in stool samples, and influenza virus. By means of this approach we were able to correlate FCV and Flu infectivity with RT-PCR measurements, and consequently to relate the results to NV measured by RT-PCR only.
- FCV and several isolates of NV gave similar RT-PCR results. Since under optimum conditions FCV infectivity could be inactivated by ozone in high humidity, we concluded that the infectivity of NV would likewise be inactivated.
- We also concluded that FCV is a suitable surrogate virus for NV in inactivation studies.
- We carried out several field tests in different size rooms, and we demonstrated that our newly developed Viroforce prototype ozone generator could inactivate several viruses, including FCV and NV.

INTRODUCTION

Viruses are ubiquitous, and are responsible for numerous diseases of animals, including humans. There are hundreds of known human viruses, although most of them produce relatively mild infections in healthy individuals (Fields' Virology, 1996). Among these diseases are respiratory diseases, skin infections, blood borne infections, sexually transmitted diseases, and gastro-intestinal (GI) infections (Tyler, 1994; Conner and Ramig, 1996). The latter have been recognized for many decades, and numerous viruses have been isolated from such cases (Kapikian and Estes, 1996). However a significant number of GI infections were until recently associated with an indefinable group of very small viruses which could not be propagated and characterized by conventional laboratory techniques (Sattar, 2004; Steinman, 2004). Many of these are now thought to be due to the Noroviruses (named after the original human virus detected in this family, the Norwalk virus, and which are members of the Calicivirus family, Kapikian and Estes, 1996). The Noroviruses appear to be responsible for numerous outbreaks among populations of people in close proximity, such as exist in hospitals, health-care facilities, and cruise ships. These viruses have resisted attempts to culture them and therefore are currently impossible to measure by standard virus assay techniques.

However, they can now be measured quantitatively by RT-PCR techniques (real – time polymerase chain reaction techniques), although these techniques measure viral RNA, not infectivity. Virus which has been inactivated might still reveal the presence of its RNA. Nevertheless there are several animal caliciviruses, including the feline calicivirus (FCV), which can be conveniently propagated in certain feline cell cultures and assayed by conventional virological methods, such as plaque assays (Bidawed et al. 2003). RT-PCR techniques are also available for FCV, and this allows us to compare the infectivity tests with corresponding RT-PCR measurements (Shin and Sobsey, 2003; Barker et al. 2004; Scansen et al. 2004, 2005). Consequently if FCV and NV are compared under similar conditions, it is possible to make conclusions about the effect of antiviral agents against the two viruses, by measuring NV by RT-PCR and FCV by both techniques (Shin and Sobsey, 2003). 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 tolerated (Steinman, 2004).

Since the advent of NV detection methods by RT-PCR, much has been learned about the epidemiology and economic impact of NV infections (Fankhauser et al.2002;Lopman et al. 2004; Cramer et al. 2006). The usual mode of transmission is through excretion of vomit and feces from infected individuals, from where the virus dries onto surfaces and can be spread by direct contact. Since consecutive transmission to individuals from contaminated surfaces can occur, then the only reliable method for interrupting transmission is proper decontamination of the affected surfaces.

In recent decades virologists have expended considerable effort in attempts to control the spread of virus infections by natural and synthetic agents (Hirsch et al. 1997; Hudson, 1990; Hudson & Towers, 1999), but with little success. Virus infections in the host are notoriously difficult to control because of the normal intracellular location of the viruses. However they are theroretically more accessible on environmental surfaces, although it is advantageous to consider only those antiviral agents that would be relatively safe for humans to use.

The noroviruses are relatively stable in the environment, and consequently decontamination requires efficient disinfection by agents such as bleach , peroxides, or certain organic solvents, although the usefulness of such agents against NV have been questioned (Shin and Sobsey, 2004). In addition these agents are all liquids, and suffer from the limitations of incomplete penetration of contaminated areas within a room. A recent study showed that ozone in solution could act as an effective agent against water-borne Norovirus (Shin and Sobsey, 2004). We therefore decided to evaluate the possibility of using ozone gas as a virucidal agent, with particular emphasis on its efficacy against Norovirus and Influenza virus. We reasoned that, in view of the superior penetrability of gases, it should be feasible to inactivate Norovirus contained anywhere within a contaminated room. We also wanted to confirm the validity of using FCV as a surrogate virus for NV in experimental situations, in which the use of NV itself would not be permitted.

METHODOLOGY.

1. Equipment.

The experimental ozone exposure chamber (reaction vessel) was a simple polycarbonate transparent box (VWR Scientific) with a front hood that could be raised to allow the insertion and withdrawal of samples and equipment probes. The generator was operated inside this chamber, and samples on various test surfaces (plastic trays, glass slides, steel disks, etc) were manually added or withdrawn as required. In later experiments, a humid atmosphere was provided by means of a reservoir of warm water in the chamber.

The ozone generator used in most experiments was a Treated Air Systems (TAS) generator model RA 46-2 (code # 6LK 1006). This machine was able to supply a steady dose of up to 100 – 120 ppm ozone gas within the experimental chamber for at least 30 minutes. The dosage could be regulated by a manually operated dial. The ozone was produced by corona discharge from a stream of ambient air drawn into the machine by a built-in fan. Preliminary experiments used a smaller generator (similar design) which did not produce such high levels of ozone.

The prototype ***Viroforce ozone generator*** was constructed as a portable module containing a flexible number of TAS corona discharge units, a fan to draw room air into the unit and to circulate output ozone gas, plus a highly efficient catalytic converter (scrubber) to reconvert ozone to oxygen at the termination of the ozone exposure period. The unit was controlled by the operator from the outside. In some tests pure oxygen gas (from an oxygen cylinder) was fed into the generator in order to supplement the ozone output. Although this procedure did significantly improve the efficiency of ozone production, concerns were raised about potential safety issues in certain applications. Consequently we abandoned the use of oxygen gas.

In the preliminary experiments we measured ozone concentration in the chamber by means of an Ecosensor portable ozone monitor supplied with a probe which was inserted into the chamber. However we subsequently replaced this device with a more sophisticated and accurate instrument, the American Precision Instruments Inc. model 450 ozone measuring system (from Teledyne), which measures ozone concentration in ppm by frequently withdrawing samples of

the ozonated air and passing them through a UV spectrometer. Input and export tubes were placed in appropriate locations for the duration of the experiment. This monitor measured ozone levels accurately from background (less than 0.1 ppm) to more than 100 ppm.

Relative humidity and temeprature were recorded by a portable hydrometer (VWR Scientific). The probe was periodically inserted into the experimental chamber; or alternatively it was placed adjacent to the chamber for continuous inspection of ambient values. When high humidity was generated inside the chamber, the probe of the hydrometer was taped to the inside for the duration of the experiment.

2. Materials.

Glass slides (clean microscope quality) were sterilized by autoclaving. Sterile commercial polystyrene tissue culture trays (usually the lids) were used as plastic surfaces. Stainless steel disks (produced locally in the form of one cm squares), and samples of wood, fabric and carpet, were all cleaned in detergent, washed, dried, and sterilized by autoclaving.

All media, serum, and other reagents used in cell and virus work were obtained from Invitrogen (Gibco; Ontario). Sterile plastic culture vessels, pipets, tubes, micro-pipettor tips, assay plates, were BD-Falcon brand obtained from VWR Scientific (Ontario). Molecular biology reagents and kits were obtained from Qiagen (Ontario) and PCR primers from Operon (USA).

3. Cell Lines and Viruses

All the cell lines used in this study were obtained from our stocks, which were kept frozen in liquid nitrogen. They were originally acquired from the ATCC (American Type Culture Collection), in the case of Vero cells (monkey kidney cell line), H-1 Cells (H-1 sub clone of human HeLa cells), MDCK cells (Madin-Darby canine kidney cells), or from BC-CDC Virology Laboratory (feline kidney, FK cells), or in the case of the mouse DBT cells, from Dr. Pierre Talbot.

The cells were cultivated in Dulbecco's MEM with 5-10% fetal bovine serum, and transferred once weekly by standard trypsinization procedures. Approximately every 3 months, the lines were replenished from frozen stocks.

The following viruses were used in our studies:
Herpes simplex virus type 1 (HSV-1), was originally obtained from BC-CDC Virology Laboratory. It was passaged and assayed in Vero cells. Titers of stock viruses ranged from 1 to 2 x 10^{8} pfu (plaque-forming units) /mL.
Poliovirus type 1 (vaccine strain, from BC-CDC), was also passaged and assayed in Vero cells. Stock virus was 1 x 10^{8} pfu/mL.
Human Rhinovirus type 14 (RV-14), and RV type 1A, were obtained from ATCC, and passaged and assayed in H-1 cells. Stock viruses ranged from 1 to 10 x 10^{7} pfu/mL.
Murine coronavirus (MCV) was obtained from Dr. Pierre Talbot, and passaged and assayed in DBT cells. Stock virus titers ranged from 2 x 10^{5} to 1 x 10^{6} pfu/mL.

Human Influenza virus (type A, strain H3N2) was originally obtained from BC-CDC, passaged in H-1 or MDCK cells, and assayed in MDCK cells. Stock virus titers ranged from 2 x 10^6 to 2 x 10^7 pfu/mL.
Feline calicivirus (FCV) was originally obtained from BC-CDC, and was passaged and assayed in FK cells. Stock virus was 1 x 10^7 pfu/mL.
Norovirus samples, in the form of virus positive stools (as confirmed by RT-PCR techniques), were obtained from BC-CDC, along with Norovirus-negative stool samples.

4. Virus Assays:

Plaque assays, for the quantitative measurement of infectious virus, were conducted according to standard techniques used for many years in Hudson's laboratory, as well as in other virology laboratories, making use of agarose overlays.

Herpes simplex virus (HSV type 1) and Poliovirus type 1 (PV) were assayed on monolayers of Vero cells; Influenza virus (human type A) on MDCK cells; Coronavirus (MCV, mouse strain JHM) on mouse DBT cells; Rhinovirus type 14 (RV) and RV type 1A on the H-1 sub-clone of HeLa cells, and Feline calicivirus (FCV) on FK cells.

Duplicate test samples, and untreated controls, were serially diluted 10-fold for assays (also in duplicate), on the appropriate cell line grown in 6-well trays. Viruses were allowed to adsorb to the cell monolayers for 60 min. at 37 ^oC (except for RV, which was adsorbed at 34 ^oC), following which the unadsorbed virus was removed by aspiration and replaced by agarose-containing overlay medium. In the case of influenza virus, the inocula and overlay media were supplemented with 10 ug/ mL of trypsin. Following the appropriate incubation time (4 days for HSV, 2 days for FCV, influenza and MCV, 30 – 36 hours for PV, and 5 days for RV at 34 ^oC), infected cell monolayers were fixed in formalin and stained with crystal violet to display individual plaques, which were then manually counted. Results are expressed as arithmetic means of the 4 values for each sample (ie. duplicate samples each assayed in duplicate). Samples were assayed at several dilutions, ranging from 10^{-1} to 10^{-5}, to give suitable numbers of plaques to count. In most cases up to 100 plaques could be counted accurately in each well, although for poliovirus and rhinovirus, the larger sizes of the plaques and their ease of fusion restricted accurate counting to less than 50 per well. In most cases replicate samples gave a variation within +/- 20 %.

In the case of many ozone treated samples, no plaques were observed at the 10^{-1} dilution. Since the starting volume of the dried virus sample was usually 50 – 100 uL, then the lower limit of detectable virus was 100 – 200 pfu/mL.

In some experiments, aliquots of the treated samples were added as undiluted and serial two fold dilutions to monolayers of the appropriate cells, in 96-well trays, without an overlay. This technique, the cpe (cytopathic effect) end-point dilution technique (another standard method in our laboratory, Hudson et al. 2006), was carried out to determine the presence or absence of very small numbers of infectious virus (possibly below the level of detection in the conventional plaque assay, which uses 10-fold serial dilutions). If no viral cpe were seen after 5 days of incubation, the sample was considered completely free of infectious virus. If cpe were present but

only in the more concentrated samples, this was taken to indicate the presence of small numbers of residual infectious viruses.

During the course of the laboratory experiments we investigated the feasibility of exposing more than one virus sample simultaneously to ozone gas, in order to reduce the total number of experiments required, and to permit us to make comparisons between viruses submitted to identical conditions.. To test this we introduced two or more dried virus samples on different glass slides or plastic trays, into the experimental chamber, with or without experimental treatment, and subsequently assayed them for infectious virus on different host cells (by means of the cpe test). In no case did we find evidence for cross-contamination between different viruses. We therefore concluded that it was feasible to carry out experiments and field tests with more than one virus at a time.

5. Quantitative RT-PCR (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. RT-PCR (real time polymerase chain reaction) measurements were made on the Opticon DNA engine (courtesy of Dr. Yossi Av-Gay). Primers were obtained from Operon co. Details for each virus follow.

Influenza virus
Viral RNA was isolated from samples according to the manufacturer's instructions. (QIAamp Viral RNA Kit; Qiagen Canada). Briefly, 140µl of specimen were mixed with 560µl of lysis buffer containing guanidine isothiocynate and carrier RNA (10µg/mL) and incubated at room temperature for 10 minutes . The lysate was then mixed with 560µl of 100% ethanol before it was applied to the silica gel-based extraction spin column, centrifuged, washed once with the buffer containing guanidine hydrochloride and ethanol, and washed once with the second buffer.Viral RNA was eluted in 60µl of RNase-free water containing 0.04% sodium azide and stored at –80ºC until use.

Viral RNA from each sample was assayed in duplicate by use of the one-step RT-PCR system from Qiagen (QuantiTect SYBR Green PCR kit). The RT-PCR amplifications were performed in 50µl reaction mixtures containing 25µl of 2X buffer (containing *Taq* DNA polymerase , SYBR Green I, dNTPs, and 5.0 mM $MgCl_2$), 0.5µl of mixed reverse transcriptases, 10 µM of the forward primer 5' - CAT GGA ATG GCT AAA GAC AAG ACC, 10µM of reverse primer 5'- AAG TGC ACC AGC AGA ATA ACT GAG, and RNAse - free water. Real – t ime RT-PCR amplification was performed on the thermal cycler with an integrated real time optical detection system. Cycling conditions consisted of reverse transcription at 37ºC for 1h, using 10µl of RNA, and a preliminary denaturation step at 95ºC for 5minutes, followed by 45 two step cycles (1min at 92ºC, 1min at 60ºC) and a final elongation step at 72ºC for 7min. Amplification of cDNA was continuously monitored in real time by quantifying the amount of fluorescence emitted at 530nm at each data acquisition step (78ºC). After a post amplification step at 55ºC for 1minute, amplicon melt temperatures were determined by raising the temperature in increments of 0.5ºC from 55ºC to 95ºC. Post-PCR analysis was performed by use of thermal cycler detection software (DNA Engine Opticon System from MJ Research.)

A 10µl portion of RT-PCR product was analyzed by electophoresis on 1.5% agarose gels, which were stained with ethidium bromide and visualized by UV light.

Feline calicivirus

Viral RNA was isolated from virus samples and reverse transcribed and amplified using the same methods described for influenza virus. The RT-PCR amplifications were performed in 50µl reaction mixtures containing 25µl of 2X buffer (containing *Taq* DNA polymerase , SYBR Green I, dNTPs, and 5.0 mM $MgCl_2$), 0.5µl of mixed reverse transcriptases, 10 µM of the forward primer 5' -TGGATGAACTACCCGCCA, 10µM of reverse primer 5'-GCACATCATATGCGGCTC, 10µl of total RNA, and RNase-free water. Cycling conditions consisted of reverse transcription at 50ºC for 3 minutes and a preliminary denaturation step at 95ºC for 15 minutes, followed by 38 cycles of 94ºC for 30 seconds, 53ºC for 30 seconds, and 72ºC for 60 seconds and a data acquisition step at 78ºC for 12 seconds. Post amplification steps were similar to influenza RNA.

Norovirus

Norovirus - positive stool samples were obtained from BCCDC.
50µg of stool sample was added to 500µl of RNase-free water, and mixed with either a disposable wooden stick or disposable plastic pipet, depending on the consistency of the sample. The sample was vortexed for 30 sec. and left at room temperature for 10 min. The sample was then micro centrifuged at 8000 rpm (6000g) for 5 min. 200µl of supernatant above the pellet (avoiding any fatty deposits on surface) were aspirated into a separate Eppendorf tube.
RNA was extracted from the supernatant by means of the Qiagen kit, as described above.
Viral RNA was reverse transcribed and amplified using the one step RT-PCR system described for influenza virus. The RT-PCR amplifications were performed in 50µl reaction mixtures containing 25µl of 2X buffer (containing *Taq* DNA polymerase, SYBR Green I, dNTPs, and 5.0 mM $MgCl_2$), 0.5µl of mixed reverse transcriptases, and 3 µL of each of the following 4 primers (10µM): where
I=Inosine; R=puRine(A/G);Y=pYrimidine(C/T); S=Strong (C/G);

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

RT-PCR amplification was performed, as described above. Cycling conditions consisted of reverse transcription at 42ºC for 10 minutes and a preliminary denaturation step at 95ºC for 5 minute, followed by 40 cycles of 94ºC for 30 seconds, 50ºC for 1.5min, and 60ºC for 30 seconds and final extension at 72ºC for 7min. Post amplification steps were similar to influenza RNA.

6. Construction of the Experimental Laboratory System

After a number of preliminary trials, The following protocol was adopted:

1. Virus of known titer was thawed quickly in a water bath, and immediately placed in the bio-safety cabinet.
2. 50 or 100uL aliquots (unless noted otherwise) were spotted onto the appropriate sterile surface (glass, plastic, steel, wood, fabric), and allowed to dry (about 40 min).
3. The test samples were transferred, in a sterile container, to the polycarbonate treatment chamber (containing the TAS ozone generator) situated within a fume hood.
4. The ozone generator was switched on either before addition of the samples, to reach the desired level (several minutes) or immediately after. Because of the small size of the chamber, and the continuous flow of ozone within the chamber, it was considered unnecessary to use a circulating fan.
5. Ozone measurements were continuously recorded by means of the API model 450 ozone measuring system. Relative humidity and temeperature were recorded by the portable hydrometer.
6. Periodically, treated samples were withdrawn and carried back to the bio-safety cabinet (again inside a sterile container), where they were immediately reconstituted in 1.0 mL medium and placed in the –80 ^{o}C freezer. The standard medium was Dulbecco MEM (the growth medium for all cells and viruses) with or without 1% fetal bovine serum (the presence of serum in the reconstitution medium did not affect results, and therefore it was omitted in later experiments).
7. Control samples, not exposed to ozone, were kept in the bio-safety cabinet for the same time period before reconstituting.
8. Subsequently, all the samples from a given experiment were thawed rapidly at 37^{o}C and assayed for residual virus infectivity by the standard plaque assay technique for that particular virus (as described above). The appropriate cell cultures had been prepared in advance.
9. Virus plaque counts were recorded after the plaques had been fixed and stained. For each test the virus titer was recorded as the mean of 4 values, derived from duplicate assays on duplicate samples.
10. In all experiments, various controls were incorporated. These included measurement of plaque numbers for untreated virus, virus frozen immediately after thawing without treatment, and virus aliquots dried for the same times as test samples but without ozone exposure.
11. In later experiments (laboratory and field trials) samples of Norovirus, FCV, and influenza virus, were also assayed for virus by means of the RT-PCR technique, using the appropriate primer pairs for the specific virus.

7. Field Tests.

Several rooms, of different sizes, located in the Vancouver area, were used in various trials of the ozone generators, including the Viroforce prototype, to determine ozone dosage parameters and to test the efficacy of virus killing under conditions resembling those of practical relevance. Details for each virus test are given in the Results section.

RESULTS:

1. Virus Drying Curves

In order to determine the effect of drying, and the stability of infectious virus in the dried state, viruses were allowed to dry on plastic surfaces, from duplicate 50 uL aliquots, and maintained under ambient conditions of temperature and humidity within a biosafety cabinet, for various periods of time, up to 48 hours. The dry films were reconstituted in 1.0 mL medium and frozen at –80 ^{o}C. Subsequently all samples were assayed for infectious virus (pfu, plaque-forming units per mL).

Data for virus stability are shown in Fig. 1 Each virus showed a different degree of infectivity loss as a result of the drying process itself. Thus poliovirus lost substantial amounts of infectivity (1 to 2 log pfu), FCV lost up to one log pfu, whereas the other viruses showed relatively little loss. Following the initial drying however, the viruses showed more gradual losses during the 48 hour period; but in most cases there was significant infectivity left at this time. Additional experiments (data not shown) indicated that even after a week at ambient temperatre (22 ^{o}C), significant infectious HSV remained (the other viruses were not tested), and the addition of serum (FBS) to 10% of the medium before drying did not affect the outcome. Also similar results were obtained for several combinations of viruses and other surfaces (glass or stainless steel instead of plastic; data not shown). These results indicated that it would be feasible to conduct experiments on dried samples of virus for periods of several hours.

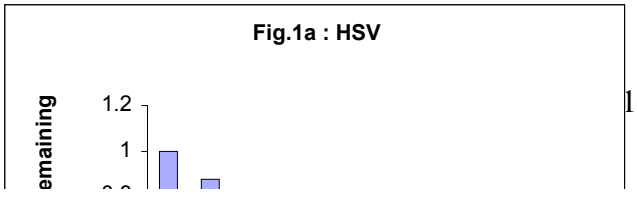
2. Virucidal properties of ozone

We examined the kinetics of the ozone inactivation, under standard conditions, for various virus – surface combinations. Some typical results are shown in Fig. 2 for the three membrane-containing viruses, HSV, influenza and mouse coronavirus. There was no consistent difference between glass, plastic, and stainless steel in these tests. Thus virus dried on any of these surfaces was equally vulnerable to ozone treatment. Fig 3 shows the corresponding results for the non – membranous virus, rhinovirus, which was significantly more resistant to ozone. But this did not depend on the nature of the surface.

All these tests were conducted in ambient humidity and temperature, which varied from 38 % to 48 % RH and 20 – 23 ^{o}C on different occasions. However it was not possible to specify the exact ozone concentrations in these early tests because the probe used to measure ozone had not been adequately calibrated for our conditions, although we did standardize the experimental conditions as far as posible.

In some experiments we dried virus samples onto small pieces of sterile wood, fabric and carpet, followed by ozone exposure and reconstitution. However the complete recovery of virus from these samples was difficult, especially carpet samples, presumably due to adsorption onto or into the surface fibers. Nevertheless it was clear from the limited data obtained that ozone exposure did result in substantial loss in virus infectivity (data not shown).

Figures 1 – 3 appear on succeeding pages



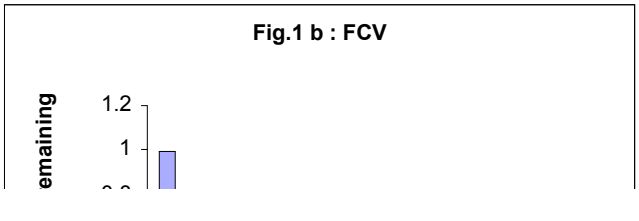
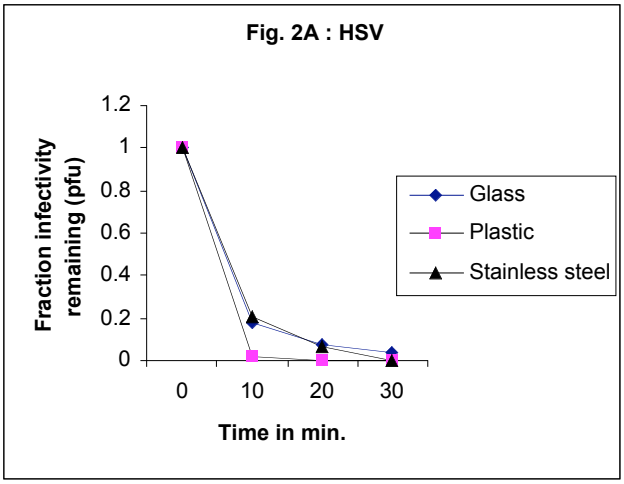
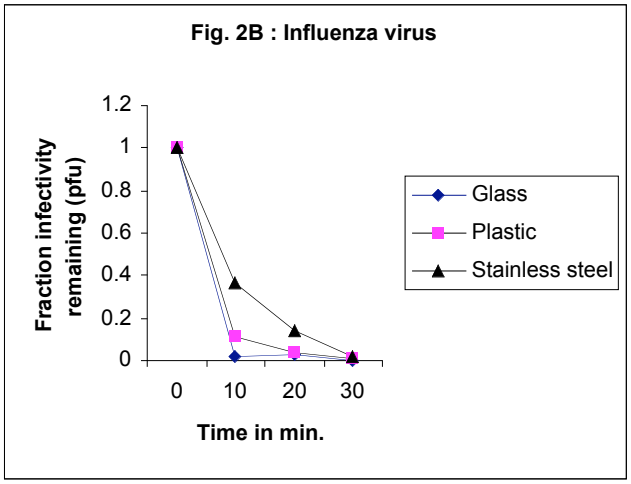


FIG. 1a - 1e. : **Stability of dried viruses**. Fraction of infectious virus remaining (pfu, plaque-forming units). 50µL aliquots of each virus were allowed to dry on glass slides. Immediately after drying, and periodically afterwards, duplicate samples were reconstituted and frozen for subsequent plaque assays as described in methodology section

FIG. 2 A-C. **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, and exposed to ozone gas for the times indicated, followed by reconstitution and freezing. Samples were subsequently thawed out and assayed for pfu. MCV was not tested on stainless steel.





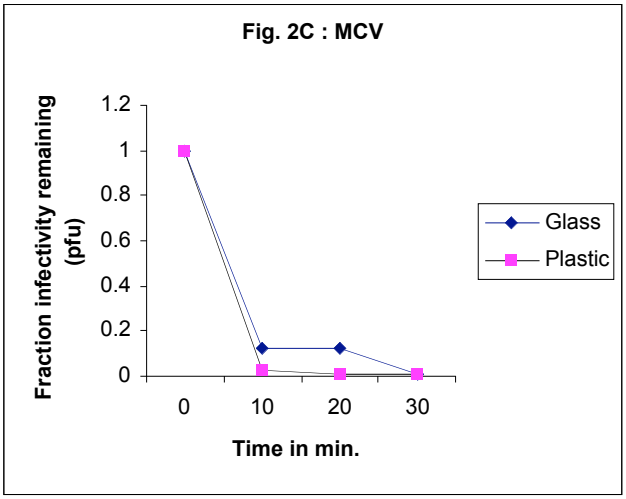
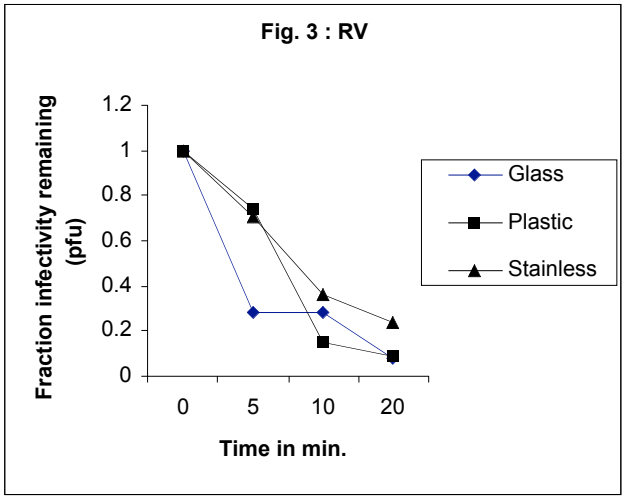
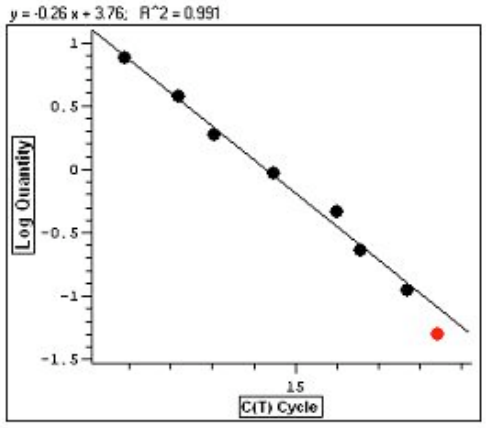


FIG. 3: **Inactivation of Rhinovirus by ozone on different surfaces**. Similar conditions to Fig 2,.



3. The Evaluation of RT-PCR Methodology to Measure Virus Inactivation

We established the RT-PCR method to determine the quantitative reduction in viral RNA genomes by ozone gas, by means of the FCV system. The objective was to validate this approach so that it could be used to measure relative amounts of Norovirus inactivation by ozone treatment. We also utilised influenza virus for an independent assessment, by comparing infectivity and RT-PCR measurements. Details of the techniques are given in the Methodology section.



C(T)	RNA ng	log Quant.
11.40797	7.5	0.875
13.15885	3.75	0.574
13.63019	1.87	0.271
15.30632	0.93	-0.031
16.58299	0.46	-0.337
17.2820	0.23	-0.638
18.45539	0.11	-0.958
19.60129	0.055	-1.25

FIG 4. Standard curve of log Quantity (RNA) vs. C(T), Cycle threshold, for FCV-RT-PCR. The standard graph displays the base-10 logarithm of initial quantity (ng) of FCV RNA in double dilutions versus the C(T) cycle, the cycle number at which the intensity trace intersects the c(t) line. The R^2 value of 0.991 indicates a good fit of the linear standard curve.

Relative amounts of infectious FCV (plaque-forming assay) were found to be proportional to the amounts of viral RNA determined by RT-PCR, as shown in Fig. 4. Similar findings were obtained for influenza virus. This indicated that if ozone could reduce the amount of Norovirus RNA to the same degree as FCV, then the degree of reduction in Norovirus infectivity should correspond to FCV infectivity reduction.

Figures 5, 6, and 7 show typical gel electrophoresis patterns respectively for Influenza virus, FCV, and NV RT-PCR products. The positions of the bands coincide with their expected sizes. Consequently a reduction in the intensity of this band, derived from a treated virus sample, would indicate a reduction in corresponding viral RNA.

FIG. 5 : Identification of 246 bp amplification product of RT-PCR for Influenza virus.
Lanes 1 & 8 (extreme left and right-hand lanes): 100 bp DNA marker ladder.
Lanes 2 – 7 : RT-PCR product was electrophoresed through 2% agarose gel stained with ethedium bromide. Arrow indicates the expected size of product.

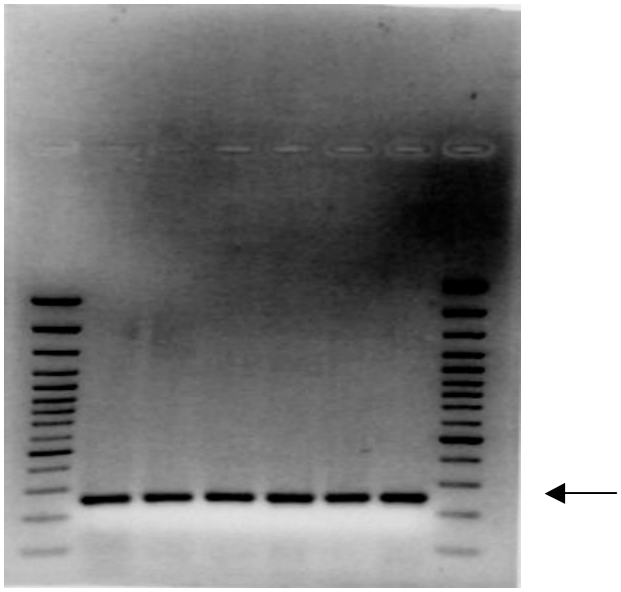


FIG. 6 : Identification of 126 bp amplification product of RT-PCR from treated and untreated FCV.

Lanes 1 & 7 : 100 bp DNA marker ladder.

Lanes 2 & 3 : positive samples of FCV,

Lanes 4 & 5: control FCV (no ozone; ambient humidity, 40 % RH),

Lane 6 : FCV treated with 50 ppm ozone in high humidity (99 % RH).

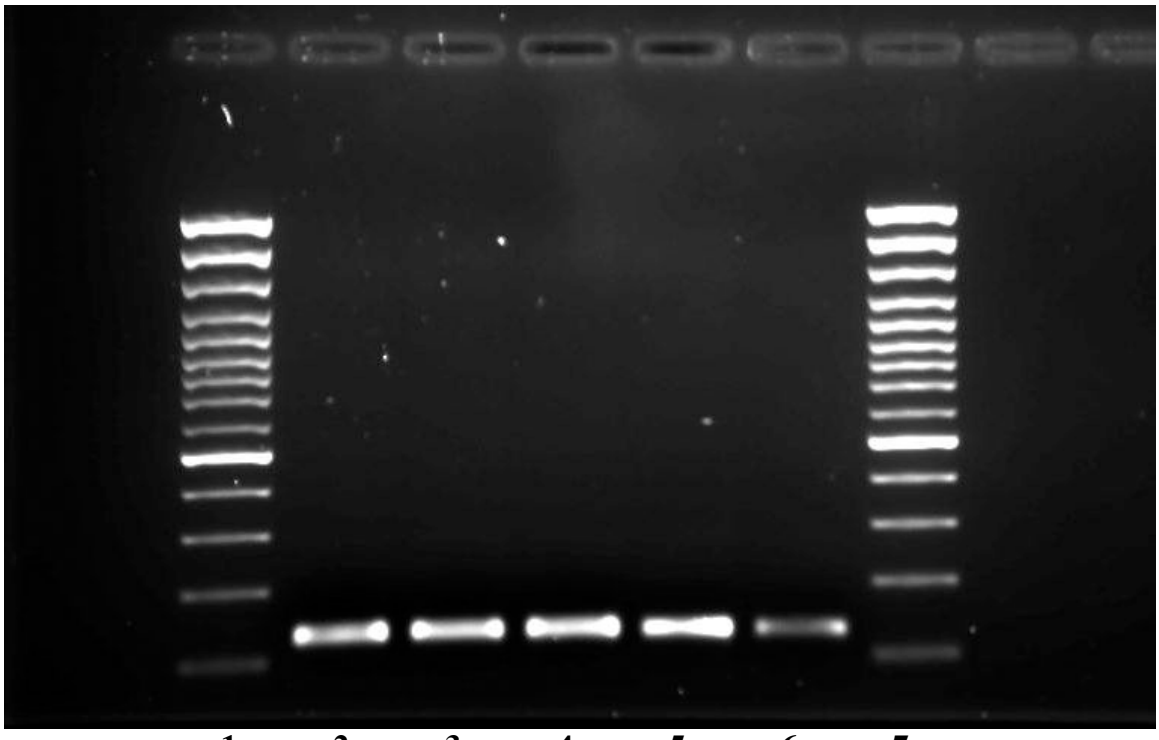


FIG. 7 A : Identification of 213 bp amplification product of RT-PCR from Norwalk virus - positive stool samples .
Lane 1 : 100 bp DNA marked ladder.
Lanes 2-4: NV positive samples.
Lane 5 & 7: NV1/NV2 untreated test samples .
Lane 6 & 8: NV1/NV2 treated with ozone (50 ppm) and high humidity (99 % RH). Arrow indicates the size expected for the amplified NV RNA.

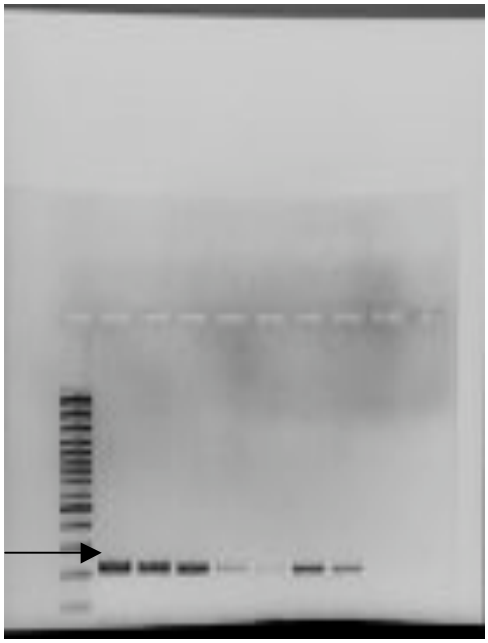
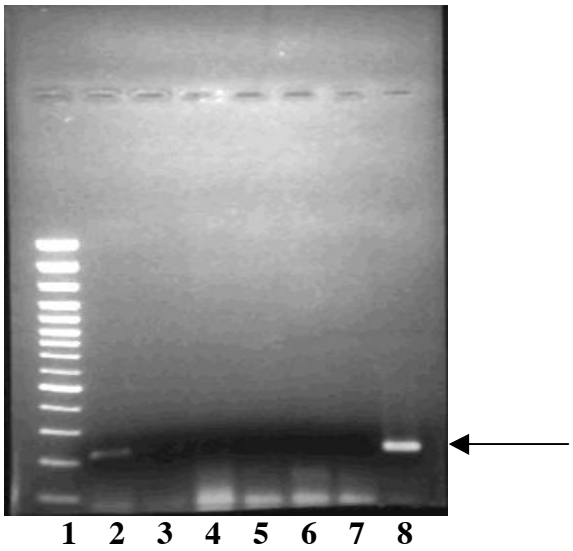


FIG. 7 B : Identification of 213 bp amplification product of RT-PCR from stool samples.
Lane 1 : 100 bp DNA marked ladder.
Lane 2: NV3 control (untreated)
Lane 3: NV3 treated with ozone (50 ppm) and high humidity (99 % RH).
Lanes 4-7 : Stool samples negative for NV.
Lane 8 : NV strongly - positive sample.
Arrow indicates the size expected for the amplified product.



4. The Importance of Humidity

Preliminary experiments had indicated that the virucidal efficacy of ozone, at least in the case of non- membranous viruses, could be enhanced considerably by higher humidity.

Therefore we used feline calicivirus (FCV) in a series of experiments to investigate the role of ozone dosage and relative humidity in the inactivation process. Different exposure times to 50

ppm ozone, from 5 to 30 min. in 99 % RH (relative humidity), all gave more than 99 % inactivation (Fig. 8), and at 30 min there was no detectable infectious virus at all.

These data also showed that high humidity alone inactivates FCV significantly, but the inclusion of ozone dramatically causes more inactivation. Corresponding PCR measurements are also shown. These indicate substantial reduction in PCR product, indicating ozone and humidity – induced damage to the viral RNA (Fig. 8).

In contrast, the membrane - containing influenza virus is not substantially affected by humidity alone, but is completely inactivated by high ozone, in the presence or absence of high RH (Table 1). Therefore the provision of high humidity and a moderate to high dose of ozone is capable of inactivating all of the test viruses.

FIG. 8 A : Ozone inactivation of FCV Infectivity and FCV RNA
Samples of dry FCV, on plastic, were exposed to either high humidity alone (99% RH), or high humidity plus 50 ppm ozone. Duplicate samples, including untreated controls, were reconstituted and measured by plaque assay and RT-PCR (details in Methodology section).
H = 99 % RH. Untreated = ambient humidity (38 % RH) and no ozone.

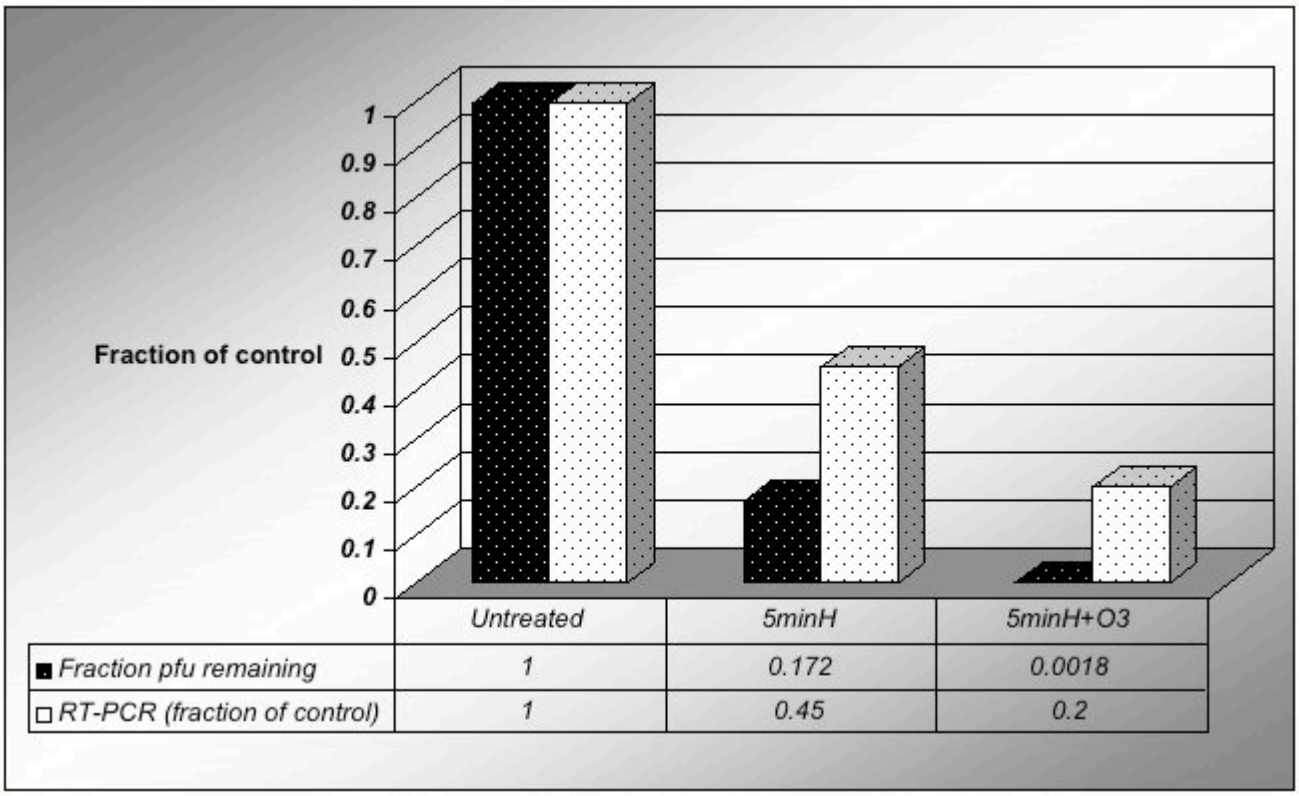
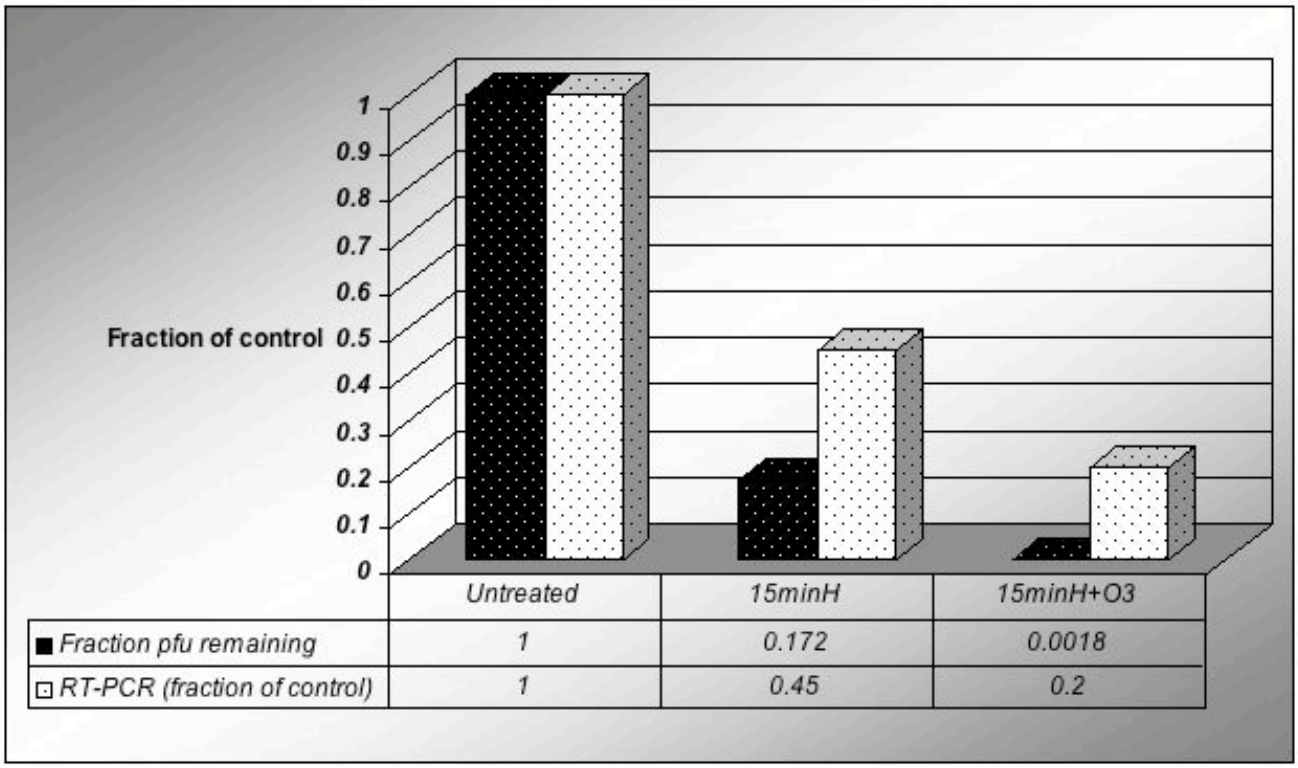
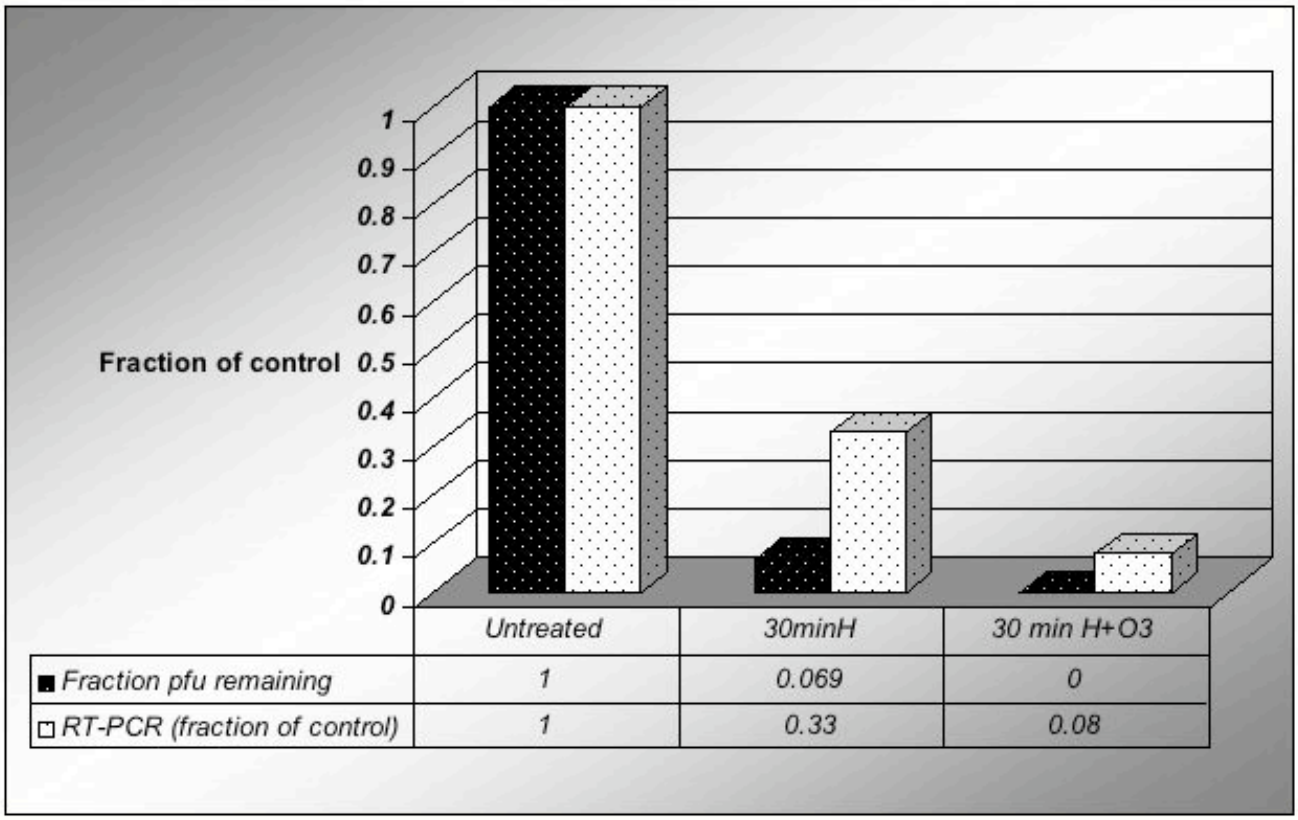


FIG. 8 B : Ozone inactivation of FCV Infectivity and FCV RNA
Samples of dry FCV, on plastic, were exposed to either high humidity alone (99% RH), or high humidity plus 50 ppm ozone. Duplicate samples, including untreated controls, were reconstituted and measured by plaque assay and RT-PCR (details in Methodology section).
H = 99 % RH. Untreated = ambient humidity (38 % RH) and no ozone.



	Untreated	15minH	15minH+O3
■ Fraction pfu remaining	1	0.172	0.0018
□ RT-PCR (fraction of control)	1	0.45	0.2

FIG. 8 C: Ozone inactivation of FCV Infectivity and FCV RNA
Samples of dry FCV, on plastic, were exposed to either high humidity alone (99% RH), or high humidity plus 50 ppm ozone. Duplicate samples, including untreated controls, were reconstituted and measured by plaque assay and RT-PCR (details in Methodology section).
H = 99 % RH. Untreated = ambient humidity (38 % RH) and no ozone.



	Untreated	30minH	30 min H+O3
■ Fraction pfu remaining	1	0.069	0
□ RT-PCR (fraction of control)	1	0.33	0.08

TABLE 1. Comparison of effect of humidity on ozone inactivation of FCV and Influenza virus

	FCV, fraction PFU remaining	Log_{10}	Influenza, fraction PFU remaining	Log_{10}	FCV RT-PCR, fraction of control	Log_{10}
humidity only	< 0.003	< - 2.5	0.867	-0.061	0.54	-0.27
humidity + ozone	< 0.003	< - 2.5	< 0.003	< - 2.5	0.11	-0.95

Samples of FCV and influenza virus were exposed to ozone, 50 ppm, for 20 min. with or without elevated humidity (99% RH), reconstituted and assayed as usual.

TABLE 2. Inactivation of FCV by ozone: no effect of serum

	PFU remaining (Fraction of control)	Log_{10}	RT-PCR (Fraction of control)	Log_{10}
+ Serum	< 0.001	< -3.0	0.08	< -1.0
- Serum	< 0.008	< - 2.1	0.08	< -1.0

Samples of FCV were dried as usual, with the addition of 10 % by volume of fetal bovine serum, or medium. They were exposed to ozone (50 ppm) in 99 % RH.

The addition of serum to the viruses before drying, to simulate the presence of body fluids, did not affect the outcome of the result, as indicated by the data shown in Table 2.

5. Inactivation of Norovirus

In several experiments we showed that Norovirus (NV) RNA could be reduced substantially by treatment of NV-positive stool samples with ozone in high humidity (see Table 3, and the corresponding results of gel electrophoresis, in Fig's. 7A and 7B). Different NV – containing samples contained different amounts of virus, but all were reduced by the ozone treatment.

TABLE 3. Inactivation of different Norovirus samples by ozone in high humidity

Sample #	ng NV RNA(untreated) No	ng NV RNA (treated) Nt	Ratio Nt/No	Log_{10}
1	157.1	81.9	0.52	- 0.28
2	1141.2	671	0.59	- 0.22
3	192.5	116	0.60	- 0.22

Three different NV-positive stool samples were dried and treated with 100 ppm ozone, in 99.9 % RH, for 30 min. RNAs were extracted and measured by RT-PCR.

6. Field Tests

We carried out several experiments in rooms. In these tests samples of dried viruses, on plastic or glass surfaces, were placed in different locations within the rooms, and exposed to ozone generators. Following dissipation or active removal of the ozone, the samples were removed and reconstituted and frozen as soon as possible.

6.1 Tests with individual TAS generators

The first two tests were carried out, with only ambient humidity (approximately 40 % RH), in a spare laboratory of capacity approx 2,300 cu ft.(sealed with duct tape). In these tests three individual TAS generators were used in different parts of the room, together with a fan to aid in the even distribution of ozone and to provide a steady level of ozone.

In the first test (V1, Table 4), we placed three slides, containing duplicate samples of dried HSV, in random locations within the room, and the generators and fan were immediately switched on. The ozone level rose over a period of 30 min to a steady level of 28 ppm, which was maintained for 60 min. followed by a decrease to background level during a further 30 min once the generators had been switched off. Residual levels of virus on the three slides were less than 2.5 %. There was reasonable agreement between the duplicates and between samples at diffent locations, with the exception of sample 2B, which was exceptionally low in residual virus.

In the second experiment (V2, Table 4), under approximately the same conditions (28 ppm ozone, 40 % RH), three different viruses, HSV, poliovirus, and rhinovirus (RV 14), were placed at the same location on different plastic trays (in duplicate spots as usual). These data are shown in Table 4. The three viruses were all substantially reduced, including the non-membrane viruses PV and RV, in spite of only ambient humidity.

TABLE 4. Field tests V1 & V2. Virus inactivation by ozone in ambient humidity

Virus	Sample #	Fraction pfu remaining	Log_{10}
V1, HSV	1A 1B	0.0061 0.0056	- 2.21 - 2.25
V1, HSV	2A 2B	0.021 0.00002	- 1.68 - 4.70
V1, HSV	3A 3B	0.018 0.025	- 1.74 - 1.60
V2, HSV	A B	0.014 0.078	- 1.85 - 1.11
V2, RV	A B	0.007 0.012	- 2.15 - 1.92
V2, PV	A B	<0.01 < 0.01	< - 2.0 < - 2.0

Two separate tests were carried out in a large room (2,300 cu ft), with virus samples dried on glass slides (A and B, duplicate samples). 1, 2, 3, refer to separate locations within room. See text for details.

6.2 Field Tests with *Viroforce prototype ozone generator*

Subsequent tests were conducted in a room (1220 cu ft) containing various items of office furniture. Air spaces were sealed with tape. Humidity was provided by a combination of a household humidifier and steam generated by kettles of water on a hot plate (the average room temperature did not rise above 30 C). The maximum RH attained was 70 %. The ozone gas was generated by the ***Viroforce prototype portable generator*** (containing multiple TAS generators, and equipped with fans), controlled remotely, and was measured continuously by a sampling tube connected to the external API monitor. At the cessation of the ozone exposure period the scrubber was switched on to remove ozone within a period of 10-15 min, at which time the samples were retrieved and reconstituted. In these tests, we calculated total ozone exposure by multiplying minutes of exposure by the ppm reading for each minute, including the rise and fall

periods. Peak ozone levels were always less in the presence of ozone, a phenomenon already documented by the manufacturer of the generators.

In test D1 two consecutive experiments were conducted with the same type of virus preparations, using similar ozone exposures, but in the first experiment ambient humidity was used (38 % RH), and shortly afterwards 70 % RH was attained for experiment 2.
Relatively low levels of virus inactivation were achieved in ambient humidity (as determined by infectivity for FCV, influenza and PV, Table 5), as anticipated, whereas in the presence of moderate RH (70 % RH), dramatic reductions in virus infectivity were obtained, accompanied by substantial reductions in PCR levels for influenza, FCV and NV, as shown in Table 5.

TABLE 5. ***Viroforce prototype generator*****, Field Test D1: Ozone inactivation of Norovirus, FCV, & other viruses.**

Virus	PFU (fraction of control) @ 38% RH	Log_{10}	PFU (fraction of control) @ 70% RH	Log_{10}	RT-PCR (fraction of control) @ 70% RH	Log_{10}
FCV	0.255	- 0.59	< 0.0012	< -2.9	0.56	- 0.25
Norovirus	_		_		0.57	- 0.24
Influenza virus	0.80	-0.097	0.0027	- 2.56	0.25	- 0.60
Poliovirus	1.00	0	< 0.0017	< -2.8	_	

Duplicate samples of the different viruses, on plastic, were placed, side by side, on the surface of a desk contained within the test room. The first experiment was carried out in ambient humidity (38% RH) and a total exposure to ozone of 580 ppm x minutes (peak ozone level 30 ppm). The second experiment, shortly after clearing the room, was carried out with 70 % RH, and a total ozone exposure of 520 ppm x minutes (peak level 20 ppm – ozone levels are always significantly lower in the presence of high humidity), using duplicate preparations of the same viruses. Controls were kept outside the test room for the duration of both experiments. All samples were reconstituted at the termination of the second experiment.

TABLE 6. ***Viroforce prototype generator*****, Field Test D2: Ozone inactivation of Noroviruses and other viruses.**

Virus	PFU (fraction of control)	Log_{10}	RT-PCR (fraction of control)	Log_{10}
FCV	0.026	- 1.58	0.51	- 0.29
Norovirus (NV) sample 1	_		0.52	- 0.28
NV sample 2	_		0.59	- 0.22
NV sample 3	_		0.60	- 0.22
Infuenza virus	< 0.024	< -1.6	0.22	- 0.66
Poliovirus	0.038	- 1.4	_	

Test D2 was carried out in a similar fashion to experiment 2 of test D1, with a RH of 70 %, on a different day. Total ozone exposure was 450 ppm x minutes (peak level 19 ppm).

In test D2, FCV, influenza, and poliovirus, together with three different NV – positive samples, were all placed at the same location in the room, and subjected to doses of ozone and humidity (70 % RH) similar to those in test D1, experiment 2. These results are shown in Table 6. All three viruses measured for infectivity showed substantial reductions in pfu. The corresponding reductions in PCR levels are also shown, along with the values for the three NV samples. Thus influenza RNA was reduced to 0.22 of the control level, whereas FCV was reduced to 0.51, which was similar to each of the NV samples (Table 6). By analogy we can conclude that the real infectivity of the NV samples should be reduced by the same amount as the FCV, ie. approximately 97 %.

Based on these data we can conclude that, in dried virus samples, FCV and NV have similar sensitivity, and therefore the use of ozone in a highly humid environment (close to 99 % RH) should result in considerable inactivation, and possibly complete eradication, as seen for FCV above, and by extension for Norovirus in stool samples.

7. Evaluation of alternative humidifiers

Since humidity plays such an important role in maximizing the ozone virucidal effect, for certain viruses such as caliciviruses, we decided to evaluate the possibility of an alternative approach to humidification, which would not require exposing the entire room to prolonged high humidity. We are curently evaluating devices for acheiving rapid and brief humidification.

CONCLUSIONS

1. Different viruses, of human and animal origin, can be readily inactivated (killed) by gaseous ozone, on several different types of surface and material.

2. The efficiency of ozone inactivation is dependent on ozone dose, as expected, and is considerably enhanced by high humidity, such that complete eradication of several viruses can be achieved (including feline calicivirus, the surrogate virus for Norovirus), both in experimental conditions and in field tests carried out in different rooms.

3. The presence of body fluids, such as serum and fecal components, does not appear to affect the outcome of ozone exposure

4. In general viruses with membranes are more vulnerable to ozone gas than the non-membrane containing viruses, such as caliciviruses, but under optimal conditions, all of them can be eradicated.

5. Based on the RT-PCR comparisons between FCV and NV samples, we can conclude that FCV is probably a valid indicator virus for NV contamination on surfaces, and consequently it should be reasonable to use FCV in field tests in situations, such as cruise liner cabins and hotel rooms, in which the use of real NV samples would be impractical.

REFERENCES:

Barker, J, Vipond IB. Bloomfield, SF. (2004) effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces. J. Hospital Inf. 58: 42-49

Bidawed, S. Malik, N. Adegunrin, O. Sattar, SA. Farber, JM. (2003) A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. J. Virol Methods, 107: 163-167

Conner, ME. Ramig, RF.(1997) Viral enteric diseases, in: Viral Pathogenesis, ed Nathanson, N. et al. Lippincot – Raven pub. 713-743

Cramer, EH. et al. (2006) Epidemiology of gastroenteritis on cruise ships, 2001-2004 Amer. J. Preventive Med. 30: 252-257
Fankhauser, RL. Et al. (2002) Epidemiology and molecular trends of "Norwalk – like virus" associated with outbraks of gastroenteritis in the US. J. Infec. Dis. 186: 1-7

Fields' Virology (1996) 2 volumes. Lippincot - Raven Pub

Hirsch, MS. Kaplan, JC. D'Aquila, RT. (1997) Antiviral agents, in: Viral Pathogenesis, ed Nathanson, N. et al. Lippincot – Raven pub. 401-432.

Hudson, JB (1990) Antiviral Compounds from Plants. CRC Press

Hudson, JB. Towers, GHN (1999) Phytomedicines as Antivirals-Invited Review. Drugs of the Future 24: 295-320

Hudson, JB. et al. (2006) Characterization of anti-viral activities in Echinacea root preparations. Pharmac. Biol. 44:

Kapikian, AZ. Estes, MK. (1994) Norwalk and Related Viruses, in Encyclopedia of Virology, vol 2. ed Webster RG & Granoff, A. Academic Press 925 - 933

Lopman, BA. et al. (2004) Epidemiology and cost of nosocomial gastroenteritis, Avon, England, 2002-2003. Emerg. Inf Dis. 10: 1827-1834

Sattar, SA. (2004) Microbicides and the environmental control of nosocomial viral infections. J. Hosp. Inf. 56: S64-S69

Scansen, BA. et al. (2004) Evaluation of a p30 gene-based real time reverse transcriptase polymerase chain reaction assay for detection of feline caliciviruses J. Vet. Intern. Med 18: 135-138

Scansen, BA. et al. (2005) In vitro comparison of RNA preparation methods for detection of feline calicivirus in urine of cats by use of a revere transcriptase polymerase chain raction assay. Am. J. Vet Res. 66: 915-920

Shin, G-A. Sobsey, MD. (2003) Reduction of Norwalk virus, Poliovirus 1, and Bacteriophage MS2 by ozone disinfection in water. Appl. Environ. Microbiol. 69: 3975-3978

Steinman, J. (2004) Surrogate viruses for testing virucidal efficacy of chemical disinfectants. J. Hosp. Inf. 56: S49-S54

Tyler, KL (1994) Pathogenesis, in Encyclopedia of Virology, vol 3. ed Webster RG & Granoff, A. Academic Press 1077 - 1083