

# THE PRACTICAL APPLICATION OF OZONE GAS AS AN ANTIMICROBIAL AGENT

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Bacterial infections continue to pose a threat to health in many Institutional and communal settings, including hospitals, health-care Institutes, hotels, cruise liners, and damaged buildings, and epidemics are frequently reported. There is a desperate need for a simple, effective, and safe way to remove infectious organisms.

We evaluated the efficacy of a portable ozone-generating machine, equipped with a catalytic converter to reconvert the ozone to oxygen after use, and an accessory humidifier, to inactivate 15 different species of medically important bacteria.

Quantitative assays were used to measure the number of bacterial colonies inactivated by the ozone treatment.

We found that, with an ozone dosage of 20-30 ppm, for 20-30 minutes, and a short burst of humidity in excess of 80% relative humidity, we were able to inactivate more than 3 log<sub>10</sub> cfu of most of the bacteria, and in many cases complete eradication was achieved. Dried and wet samples were vulnerable to the ozone.

The catalytic converter removed excess ozone within 15 minutes, so that it was possible to re-enter the room within an hour of the start of the treatment cycle.

We conclude that the ozone generator would be a valuable decontamination tool in these settings.

## **Introduction**

The prevention and control of nosocomial infections – those contracted during health care – are not new subjects of concern. For many years, health professionals, particularly infection prevention and control nurses, have been devoting time and energy to this subject in health network institutions, where the fight against these infections starts and where the first responsibility for their prevention and control lies

Health care acquired infections are a significant public health problem and patient safety issue. In Canada an estimated 220,000 infections acquired in health care facilities and 8,000 deaths attributable to these infections occur annually. <sup>1</sup>

The use of disinfectants is standard practice in a variety of common clinical situations. Many hospitals use formaldehyde vaporization, peracetic acid or chlorhexidine for this purpose,<sup>2</sup> although the extent to which either contributes to a reduction in transmission of infection in hospitals is usually unknown. Such methods have inherent drawbacks including cost, labor, and inhalation of the disinfectant vapors by the hospital staff, the formation of dirty flecks on glass surfaces, and the retention of unpleasant disinfectant odor after decontamination.

Ozone has well documented bactericidal properties<sup>3</sup>, is cheap to generate and although toxic, rapidly dissociates to oxygen. As a decontamination agent, gaseous ozone therefore offers potential advantages over chlorine releasing agents and other disinfectants. Ozone has been reported to be useful in the decontamination of water<sup>4</sup> and contact lenses.<sup>5</sup> One of the major advantages is that the release of ozone can be controlled from outside the room.

This report presents data on the use of ozone gas, provided by a proprietary portable ozone generator, against various bacteria causing infections in hospitals and health-care facilities

## **Materials and Methods**

### **Equipment.**

1. The laboratory test chamber was a molded polycarbonate chamber with a transparent plastic front window that could be lifted to allow access to samples. Within the test chamber was the TAS (Treated Air Systems) ozone generator, fitted with a control dial that could be pre-set to determine the ozone dosage in ppm, an ozone sampler tube connected to the exterior ozone measuring system, and the probe of a hygrometer for measuring relative humidity and temperature..

2. 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.. In addition a portable commercial humidifier (Humidifirst Inc, Florida) was used to provide a burst of water vapor when required. All the components were controlled remotely from outside the test room. Ozone concentration was monitored continuously by means of an 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.

### **Test chamber/rooms and protocol**

The tests were carried out either in the test chamber with the TAS (Treated Air Systems) ozone generator or in the office, volume 34 m<sup>3</sup>, containing normal office furniture, adjoining the laboratory, with the prototype Viroforce ozone generator.

The standard protocol was as follows (based upon preliminary laboratory tests):

Bacterial 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 within the test chamber/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 vents, windows, and doors were sealed with tape, 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.

Alternatively, following the 20 min ozone exposure in the polycarbonate test chamber, the window was lifted briefly to allow a mist of water from a spray bottle. This resulted in a rapid increase in relative humidity to 90-99 %.

The samples were reconstituted in PBS (phosphate buffered saline), and serial 10-fold dilutions were made in PBS. Aliquots of 2.5µL were spotted and spread out with plastic inoculating loops onto blood agar or other agar plates . Control untreated samples were kept in the biosafety cabinet during the entire operation. Agar plates were incubated at 35°C for a minimum of 24 h, after which bacterial colonies were counted.

### **Materials.**

The lids of sterile polystyrene tissue culture trays were used as plastic surfaces. Samples of fabrics and cotton (typical of those used in hospital 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.

Serum and PBS were obtained from Invitrogen (Gibco; Ontario). Sterile plastic 24 well plates and other supplies were BD-Falcon brand obtained from VWR Scientific (Ontario). Sheep blood agar, chocolate agar, charcoal agar and Middlebrook agar plates were obtained from PML Microbiologicals, Willsonville, Oregon

### **Bacterial strains:**

The following bacteria were all American Type Culture Collection (ATCC) strains

1. *Acinetobacter baumannii*
2. *Bacillus cereus* (spores and cells)
3. *Bacillus subtilis*
4. *Clostridium difficile* (spores and cells)
5. *Enterococcus faecalis*
6. *Escherichia coli*
7. *Hemophilus influenzae*
8. *Klebsiella pneumoniae*
9. *Legionella pneumophila*
10. MRSA (methicillin-resistant *Staphylococcus aureus*)
11. MSSA (methicillin-sensitive *Staphylococcus aureus*)
12. *Mycobacterium smegmatis*
13. *Pseudomonas aeruginosa*
14. *Propionibacterium acne*
15. *Streptococcus pyogenes*

### **Growth of bacteria and Preparation of spores**

Preparation of spore suspensions was done according to the ethanol method of Wullt et al 2003<sup>6</sup>

### **Bacterial Assay**

Using bacterial isolates in pure culture, a 2.0 McFarland standard ( $6 \times 10^8$  colony-forming-units [cfu] per mL) was prepared and viable bacteria counts were performed by serial dilutions on blood agar plates. Quantitative tests were carried out; each consisting of 100  $\mu$ L drops of suspension, in duplicate, dried onto plastic trays (usually the underside of the lid of a micro well plate), under standard conditions of ozone

exposure (20-30 ppm ozone for 20 min followed by 15 min exposure to 90 – 99.9% relative humidity).

The 15 bacteria were grown and assayed on blood agar plates (*C.difficile* and *P.acne* in anaerobic chambers), except for *Legionella pneumophila*, on Charcoal agar plates, *Hemophilus influenzae*, on chocolate agar plates, and *M.smegmatis*, on Middlebrook agar plates. The blood/agar plates were incubated in a conventional incubator at 35°C for a minimum of 24 h and *L.pneumophilla* , *H.influenzae* and *M.smegmatis* were maintained in a 5% CO<sub>2</sub> \_95% air atmosphere at 36° after which the plates were removed and the colony-forming units were manually counted.

## Results

All operations, except the ozone exposure, were carried out within a certified class 2- bio-safety cabinet.

### **Bactericidal properties of ozone**

The activity of ozone was tested against 15 bacteria including *C.difficile* spores suspension ( $6 \times 10^8$  colony-forming-units [cfu] per mL) with and without organic contamination. The results are shown in the table 1.

Replicate samples of bacteria each consisting of 100  $\mu$ L drops of suspension (with and without 10%FBS), dried onto plastic trays, and were placed in different locations in the office. Samples were subsequently assayed after ozone exposure. The bacteria were grown on appropriate agar plates (*C.difficile* and *P.acne* in anaerobic chambers), and incubated at 35°C for a minimum of 24 h, *L.pneumophilla*, *H.influenzae* and *M.smegmatis* were maintained in a 5% CO<sub>2</sub> \_95% air atmosphere at 36° after which the plates were removed and the colony counts were performed.

Comparison of colony counts between control and treated samples at different dilutions permitted calculation of the log<sub>10</sub> decrease in titer of the treated samples to measure the number of surviving organisms

The results of this study demonstrate that ozone at 20-30ppm could inactivate more than 3 log cfu of most of the bacteria (Fig.1), and in many cases complete eradication was achieved (Table 1.). Both dried and wet samples were vulnerable to the ozone

### **Bacteria on Soft Surfaces:**

In additional tests, conducted in the office, replicate samples of bacteria both gram positive and gram-negative were dried onto plastic trays, as usual, and also on to samples of fabric, cotton, filter paper and card board. These were placed at various locations within the office to mimic possible contamination sites in the hospital. The standard ozone exposure protocol was used, and subsequently samples were assayed for bacteria survival. All samples showed similar sensitivity to ozone, regardless of their location or the surface on which they were dried.

## Discussion

Nosocomial infections are estimated to more than double the mortality and morbidity risks of any admitted patient. It is estimated that one in ten patients admitted to hospital will acquire an infection after admission, resulting in substantial morbidity and economic cost to health care system. Patients with hospital-acquired infection (HAIS) stay longer, require additional diagnostic and therapeutic procedures and are at increased risk of other medical complications.<sup>7-9</sup> Approximately one-third of HAIS are preventable with an effective infection control program.

Many methods have been used for the decontamination of rooms in the hospitals, such as peracetic acid and chlorine dioxide based disinfectants.<sup>2</sup> Disinfectants currently in use are inadequate in many respects, being unreliable for rapid use, toxic, corrosive, unstable or expensive depending on the choice of disinfectant used.<sup>10</sup> The most frequent complaints regarding these procedures are inhalation of disinfectants by the hospital staff, inconvenience and long retention of the unpleasant odor of the disinfectants.

Ozone decontamination has been shown to have substantial advantages. 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. It can be switched on and off from the outside after the room has been made airtight. Ozone is known to have antibacterial activity, is cheap to generate and although toxic, rapidly dissociates to oxygen with a half-life of about 20 min. As a decontamination agent, gaseous ozone therefore offers potential advantages over chlorine releasing agents and other disinfectants.

Most studies into antibacterial effects of ozone have been performed in aqueous solution.<sup>11</sup> Activity has also been demonstrated against bacterial spore<sup>12</sup> and viruses.<sup>13</sup> Less is known about the bactericidal effects of gaseous ozone although there is evidence that it is enhanced by humidity. It probably acts through oxidation of cell-wall targets such as fatty acids and peptides.

Our studies have demonstrated that our prototype ozone generator produced bactericidal concentration of ozone (in the order of 20-30ppm). The potent biocidal activity of the ozone generator after 20 min exposure time and 90% RH was demonstrated across a range of gram positive and gram-negative bacteria (micro-organisms) including spores and a Mycobacterium species with 10% organic contamination. For our experiment we chose to suspend the organism in PBS rather than nutrient broth media because saline maintains the bacteria in a stasis in which they neither multiply nor die. Evidence of the static situation was the relatively stable cfu/mL in the controls. Inactivation of bacterial samples dried onto soft surfaces, such as fabric, cotton, and filter paper, were comparable to that observed for samples on plastic. Thus confirming that ozone gas can be bactericidal to samples on curtains, linen, furniture and walls in the health care facilities. While wiping with liquid disinfectant for general decontamination requires a great deal of

work and is unsuitable for curtains, walls and ceilings, decontamination with gas is easier than wipe down decontamination

To conclude, the results of this study demonstrate that ozone at 20-30ppm and RH 90% is bactericidal ( $>3\text{-log}_{10}$  reduction in bacterial cfu/mL) to strains of bacteria that commonly cause nosocomial infection and the bactericidal effect was accomplished with a short exposure of 20min. Prototype O<sub>3</sub> generator may merit consideration an alternative to liquid disinfectants. Thanks to a very efficient scrubber system built in to the generator, that speeds up the removal of the gas. Since it is used in rooms that are sealed off for the duration of the treatment, there is no danger of toxicity caused by high concentration of ozone. Ozone decontamination is much superior to other disinfectants with regard to convenience, ready expulsion after use and insignificant inhalation of the disinfectant by the hospital staff. It can assist Infection control programs in preventing transmission of infection to health care staff and promote a climate of safety.

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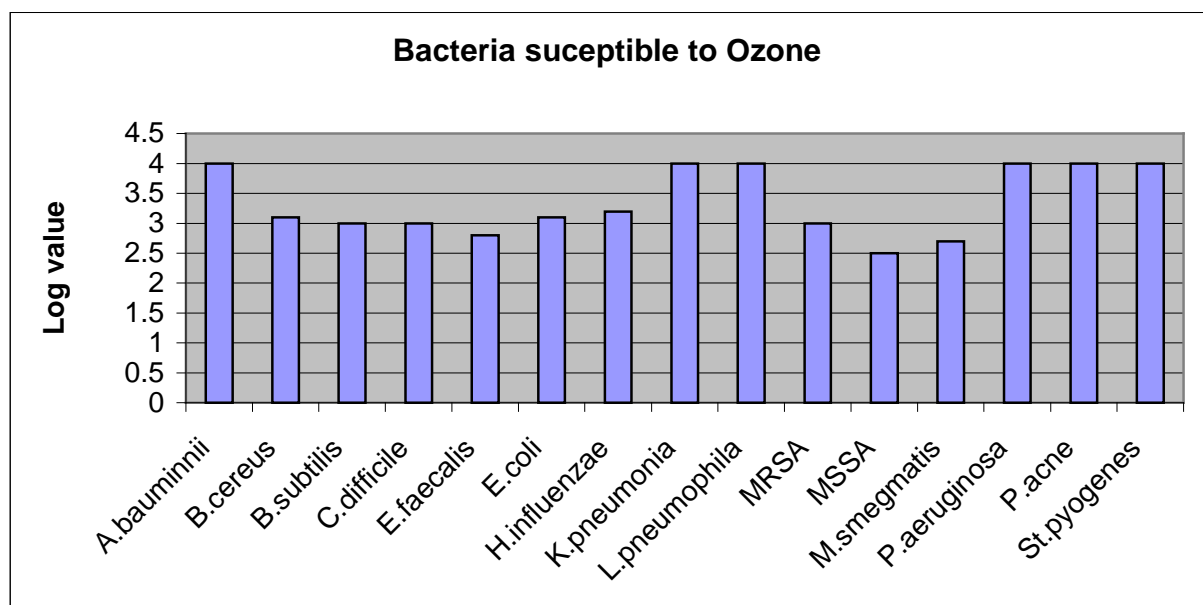
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Fig. 1.



**Table 1. Bacterial Susceptible to ozone gas**

<b>Bacteria</b>	<b>Bacteria reduction (Log<sub>10</sub> value)</b>
<i>Acinetobacter baumannii</i>	≥4
<i>Bacillus cereus</i> (spores and cells)	>3.1
<i>Bacillus subtilis</i>	>3.0
<i>Clostridium difficile</i> (spores and cells)	>3.0
<i>Enterococcus faecalis</i>	>2.8
<i>Escherichia coli</i>	>3.1
<i>Hemophilus influenzae</i>	>3.2
<i>Klebsiella pneumoniae</i>	≥4
<i>Legionella pneumophila</i>	≥4
MRSA (methicillin-resistant <i>Staphylococcus aureus</i> )	>3.0
MSSA (methicillin-sensitive <i>Staphylococcus aureus</i> )	>2.5
<i>Mycobacterium smegmatis</i>	>2.7
<i>Pseudomonas aeruginosa</i>	≥4
<i>Propionibacterium acne</i>	≥4
<i>Streptococcus pyogenes</i>	≥4

