Ozone gas is an effective and practical antibacterial agent

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Background: Bacterial infections continue to pose a threat to health in many institutional and communal settings, and epidemics are frequent. Current control measures are clearly inadequate; thus, there is a need for a simple, effective, and safe way to decontaminate surfaces.

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

Results: An ozone dosage of 25 ppm for 20 minutes, with a short burst of humidity in excess of 90% relative humidity, was able to inactivate more than 3 $\log_{10}$ colony-forming units of most of the bacteria, including *Acinetobacter baumannii, Clostridium difficile*, and methicillin-resistant *Staphylococcus aureus*, in both in a laboratory test system and simulated field conditions. In many cases, complete eradication was achieved. Dried and wet samples were equally vulnerable to the ozone. Inactivation of bacterial samples dried onto soft surfaces (eg, fabric, cotton, filter paper) were comparable with that observed for samples on plastic.

Conclusions: The ozone generator can provide a valuable decontamination tool for the removal of bacteria in many institutional and communal settings, including hospitals and other health care institutions. (Am J Infect Control 2008;36:559-63.)

Bacterial infections continue to pose a threat to health in many institutional and communal settings, including hospitals and other health care institutions, hotels, cruise liners, and damaged buildings, and epidemics are frequently reported. In addition to the increasingly frequent episodes of antibiotic-resistant *Clostridium difficile*–associated diarrhea (CDAD) [1-3] and methicillin-resistant *Staphylococcus aureus* (MRSA),[4] there have been recent epidemics associated with various other genera, including *Acinetobacter* and *Klebsiella*.[5-8] These and many other recent incidents demonstrate the often severe impact of persistent contamination by potentially pathogenic bacteria. There is a desperate need for a simple, effective, and safe way to remove infectious organisms within these settings. Modeling studies have shown that early intervention by appropriate decontamination also could have a substantial economic impact.[9]

The use of disinfectants is standard practice in various common clinical situations. Many hospitals use formaldehyde vaporization, peracetic acid, or chlorhexidine for this purpose;[10] more recently, formulations of hydrogen peroxide have been advocated,[11,12] although the extent to which these contribute to reduced infection transmission in hospitals remains unclear. Such methods have inherent drawbacks, including high cost, labor-intensiveness, and potential for inhalation of disinfectant vapors by hospital staff, formation of dirty flecks on glass surfaces, and retention of unpleasant disinfectant odor after decontamination.

Ozone has well-documented bactericidal properties,[13-16] can be generated cheaply, and, although toxic, rapidly dissociates to oxygen. In addition, the release of ozone can be controlled from outside the room. Thus, as a decontamination agent, gaseous ozone offers potential advantages over chlorine-releasing agents and other disinfectants. A recent case of MRSA contamination was effectively and economically alleviated by ozone gas.[4]

This report presents data on the use of ozone gas, provided by a proprietary portable ozone generator, against various pathogenic bacteria. We recently reported that this system was capable of inactivating norovirus in similar settings.[17]

MATERIALS AND METHODS

Equipment

The laboratory test chamber was a molded polycarbonate box (4.47 ft^3 volume) with a transparent plastic front window that could be lifted to allow access to samples. Within the test chamber was a small ozone generator (Treated Air Systems, Surrey, BC, Canada). Relative humidity and temperature were recorded by a portable hygrometer (cat no. 12777-834; VWR Scientific, Ontario, Canada). Humidity was provided in the form of a deionized sterile water mist using a spray bottle.

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The ozone generator used in all field tests (Viroforce 1000; Viroforce Systems, Kelowna, BC, Canada) was a portable module containing multiple corona discharge units, a circulating fan, and an efficient catalytic converter (ie, scrubber) to reconvert ozone to oxygen at the termination of ozone exposure. In addition, a portable commercial humidifier (model MP-15; Humidifirst, Boynton Beach, FL) was used to provide a burst of water vapor when required. All of the components were remote-controlled from outside the test room. Ozone concentration in the room was monitored continuously using a TAPI model 450 system (Teledyne Advanced Pollution Instrumentation, San Diego, CA). All ozone concentrations reported herein refer to ppm of ozone measured by this monitor. Relative humidity and temperature were recorded by a portable hygrometer (cat no. 12777-834; VWR Scientific). The test room was an office (volume 34 m^3, or 1350 ft^3) containing normal office furniture, located adjacent to the laboratory.

Materials

The lids of sterile polystyrene tissue culture trays were used as plastic surfaces. In a previous study, we evaluated ozone gas as an antiviral agent on glass and stainless steel.[15] These gave the same results as for the plastic surfaces used here. Unfinished wooden surfaces were less suitable, because they gave inconsistent recoveries of test organisms. Samples of fabrics and cotton typical of those used in hospital and hotel rooms were cut into small pieces, cleaned in detergent, washed, dried, and sterilized by autoclaving. Cotton tips and other materials were heated for 2 minutes in a microwave oven.

Fetal bovine serum and phosphate-buffered saline (PBS) were obtained from Invitrogen (Burlington, Ontario, Canada). The sterile plastic 24-well plates and other supplies were BD-Falcon brand (BD, Franklin Lakes, NJ). Sheep's blood agar, chocolate agar, charcoal agar, and Middlebrook agar plates were obtained from PML Microbiologicals (Willsonville, OR).

Bacterial strains

The 15 test bacteria were all American Type Culture Collection (ATCC) strains, obtained from PML Microbiologicals, except for the 2 strains of *S aureus*, which were clinical isolates obtained from the Clinical Microbiology Proficiency Testing Laboratory at the University of British Columbia. All of the test bacteria are all listed in Table 1.

The bacteria were grown and assayed on blood agar plates (*C difficile* and *P acnes* in anaerobic chambers), except for *Legionella pneumophila* (on charcoal agar plates), *Hemophilus influenzae* (on chocolate agar plates), and *Mycobacterium smegmatis* (on Middlebrook agar plates). The plates were incubated in a conventional incubator at 35°C for a minimum of 24 hours, and *L pneumophila*, *H influenzae*, and *M smegmatis* were maintained in a 5% CO_2–95% air atmosphere at 36°C, after which the plates were removed and the colony-forming units (cfu's) were counted manually.

Table 1. Bacterial susceptibility to ozone gas

		Log $_{10}$ reduction in cfu's	
	ATCC #	Wet sample	Dry sample
Gram-positive bacteria			
Bacillus cereus	11778	> 3.1	> 3.1
Bacillus spizizenii	6633	> 3.2	> 3.2
Clostridium difficile	43593	> 4.0	> 4.0
MRSA	Clinical isolates	> 3.0	> 3.0
Methicillin-sensitive *Staphylococcus aureus*	Clinical isolates	> 2.5	> 2.5
Propionibacterium acnes	11827	≥ 4	≥ 4
Streptococcus pyogenes	12384	≥ 4	≥ 4
Gram-negative bacteria			
Acinetobacter baumannii	19606	≥ 4	≥ 4
Enterococcus faecalis	51299	> 3	> 3
Escherichia coli	25922	> 3.1	> 3.1
Haemophilus influenzae	19418	≥ 4	≥ 4
Klebsiella pneumoniae	10031	≥ 4	≥ 4
Legionella pneumophila	33152	≥ 4	≥ 4
Pseudomonas aeruginosa	27853	≥ 4	≥ 4
Acid-fast bacteria			
Mycobacterium smegmatis	14468	> 2.7	> 2.7

Test protocol

Bacterial samples (100 μL), approximately 6×10^8 cfu/mL (2.0 McFarland standard) in PBS, were dried onto sterile plastic or other surfaces, in duplicate, in the Viroforce Laboratory. When dry, the samples were quickly transported to the test site in sterile containers. The samples were placed at various locations within the test chamber or within the test room; in the latter case, the ozone generator and rapid humidifying device (RHD) were placed in a central location. These units were remote-operated from outside the room. All vents and windows were sealed. At the start of the test, the samples were uncovered, the door was closed and sealed with tape, and the generator was switched on. The ozone level reached 25 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 minutes. Both the generator and the RHD were then switched off for another 10 minutes to allow ''incubation'' in the humid atmosphere (with relative humidity usually reaching > 95%). The ozone level decreased gradually to 15 to 20 ppm during this incubation period. The scrubber was then turned on to remove all ozone gas. The ozone level decreased to < 1 ppm

within 15 minutes, at which point the door was opened and the test samples were retrieved. All tests were conducted at ambient temperature (19 to 21°C).

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

The samples were reconstituted in PBS, and then serial 10-fold dilutions were made in PBS. Aliquots of 2.5 μL were spotted and spread out with plastic inoculating loops onto blood agar or other agar plates. Untreated control samples were kept in the biosafety cabinet during the entire operation. Agar plates were incubated at 35°C for a minimum of 24 hours, after which bacterial colonies were counted. All organisms were evaluated, in duplicate, in 2 or more experiments to ensure consistency of results. Because in most cases no residual colonies were found on the plates of treated bacteria, statistical analyses were considered unnecessary. Control samples not exposed to ozone demonstrated a variation of ± 5% between replicates.

Spore suspensions from *C difficile* and *B cereus* were prepared following the ethanol method.[10] These suspensions were treated in the same manner as the bacterial cells.

RESULTS

All 15 bacteria were initially evaluated for susceptibility to standard ozone doses in the laboratory test system. Replicate 100-uL samples of the bacterial suspensions at the 2.0 McFarland standard (6×10^8 cfu/mL in PBS) were dried onto sterile plastic trays in a biosafety cabinet and then exposed to a standard ozone dosage of 25 ppm for 20 minutes. Preliminary tests had demonstrated that the minimum requirements for efficient inactivation of bacteria were an ozone dosage of 20 ppm for 20 minutes and a relative humidity of > 80% (provided by a water vapor mist); consequently, we programmed the Viroforce 1000 generator to produce the standard conditions described in Materials and Methods.

The results are summarized in Table 1. Because of the cfu losses from drying, as well as the dilution factors involved in the assay procedures, we were not always able to determine the exact endpoints of inactivation. Nevertheless, in nearly all cases, decreases > 3 $\log_{10}$ were consistently obtained. For *M smegmatis* and methicillin-sensitive *S aureus*, the corresponding values were > 2.5 $\log_{10}$. Our findings also indicate that wet films of all of the bacteria were equally susceptible.

In some experiments, 10% (by volume) of fetal bovine serum was included in the samples before drying. This did not affect the results, however.

Table 2. Susceptibility of *C difficile* to ozone on different surfaces: $\log_{10}$ cfu reduction

Plastic	Cotton	Fabric	Filter paper	Cardboard
≥ 4	≥ 4	≥ 4	≥ 4	≥ 4

All bacteria except *H influenzae* and *B spizizenii* also were tested in the office. Samples (dry or wet) were prepared as described previously, and the trays were placed at various strategic locations in the room, including windows, walls, and floor. The results were the same as found in the laboratory tests, regardless of the location of samples within the room. Purified spore preparations of *C difficile* and *B cereus*, prepared by the ethanol method described by Wullt et al,[10] also were evaluated and found to lose > 3 log cfu after standard ozone treatment.

In additional tests conducted in the office, replicate samples of bacteria (both gram- positive and gram-negative) were dried onto plastic trays as before, as well as onto samples of fabric, cotton, filter paper, and cardboard. These samples were placed at various locations within the office to mimic possible contamination sites in the hospital. The standard ozone exposure protocol was used, and the samples were assayed for bacterial survival after the recovery of residual bacteria by repeated squeezing and vortexing of the materials in PBS. Controls were treated similarly but without exposure to ozone. All samples demonstrated similar sensitivity to ozone, regardless of their location or the surface on which they were dried. The results for *C difficile* are given in Table 2.

DISCUSSION

Nosocomial infection is considered to more than double the mortality and morbidity risks of any hospitalized patient. An estimated 1 in 10 patients admitted to the hospital will acquire an infection after admission, resulting in substantial morbidity and economic cost to the health care system.[18] Patients with hospital-acquired infections (HAIs) stay longer, require additional diagnostic and therapeutic procedures, and are at increased risk for other medical complications.[18] Approximately 1/3 of HAIs may be preventable through an effective infection control program.

Methods to decontaminate hospital rooms include the use of peracetic acid and chlorine dioxide–based disinfectants and hydrogen peroxide vapor decontamination.[10-12] The disinfectants in current use are inadequate in many respects, being unreliable for rapid use, toxic, corrosive, unstable, and/or expensive. The most frequent complaints are inhalation of disinfectants by the hospital staff, inconvenience, and persistent unpleasant disinfectant odor.

Ozone decontamination has been shown to have substantial advantages. The gas can effectively penetrate every part of a room, including sites for which access is difficult using 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,[13-16] can be generated cheaply, and, although toxic, rapidly dissociates to oxygen, with a half-life of about 20 minutes. The use of a catalytic converter speeds up removal considerably.

Our prototype ozone generator produced a bactericidal concentration of ozone (25 ppm). The potent biocidal activity of the ozone generator after 20 minutes of exposure at 90% RH was demonstrated across a range of gram-positive and gram-negative bacteria, including spores and a *Mycobacterium* species. The inactivation of bacterial samples dried onto soft surfaces (eg, fabrics, cotton, and filter paper) was comparable with that found for samples dried onto plastic, confirming that ozone gas can be bactericidal to samples on curtains, linen, furniture, and walls in health care facilities. Whereas wiping with liquid disinfectant for general decontamination requires much work and is unsuitable for curtains, walls and ceilings, decontamination with gas is quick, easy, and suitable for soft surfaces.

It is interesting that the dry and wet samples demonstrated equivalent sensitivity to ozone. Our earlier antiviral studies demonstrated that certain viruses were less vulnerable in the dry state, and this was the rationale for introducing high RH into the treatment protocol. As a result of those findings, we adopted the use of high humidity as a routine measure for subsequent tests. Incorporation of serum into the samples was evaluated to mimic the possible effects of an organic load on the efficacy of ozone treatment; no differences were found. Because our objective was to determine whether or not bacterial samples could be effectively inactivated in practical situations, we used the optimal conditions defined previously for viruses.[17] There seemed little point in testing the antibacterial effect in low humidity.

This discussion also is relevant to the mechanism of action of the ozone gas. Ozone is a known oxidizing agent in aqueous solutions and in the gas phase,[19] and it is possible that the apparent indiscriminate antiviral and antibacterial activity is a reflection of multiple oxidation effects.[20] However, the optimal requirement for high humidity suggests the possible involvement of additional radicals, such as hydroxyl ion and peroxides, that could be generated in those conditions. It would be interesting to pursue more research in this direction.

CONCLUSION

Our findings demonstrate that ozone at 25 ppm and RH 90% is bactericidal ($> 3 \log_{10}$ reduction in bacterial cfu/mL) to strains of bacteria that commonly cause nosocomial infection, and the bactericidal effect was accomplished with a short exposure (20 minutes). Thanks to the highly efficient scrubber system built into the generator, gas removal after exposure is fast and efficient. Because it is used in rooms that are sealed off for the duration of treatment, there is no danger of toxicity due to high ozone concentrations. Thus, ozone decontamination is superior to other disinfectants in terms of convenience, ready expulsion after use, and insignificant disinfectant inhalation by hospital staff. It can aid infection control programs in preventing transmission of infection to staff and promote a climate of safety.

References

1. Aslam S, Hamill RJ, Musher DM. Treatment of *Clostridium difficile*–associated disease: old therapies and new strategies. Lancet Infect Dis 2005;5:549-57.
2. Bartlett JG. The new epidemic of *Clostridium difficile*–associated enteric disease. Ann Intern Med 2006;145:758-64.
3. Cloud J, Kelly CP. Update on *Clostridium difficile*–associated disease. Curr Opin Gastroenterol 2007;23:4-9.
4. de Boer HEL, van Elzelingen-Dekker CM, van Reeberg-Verberg CMF, Spanjaard L. Use of gaseous ozone for eradication of methicillin-resistant *Staphylococcus aureus* from the home environment of a colonized hospital employee. Infect Control Hosp Epidemiol 2006;27: 1120-2.
5. Robenshtok E, Paul M, Leibovici L, Fraser A, Pitlik S, Ostfeld I, et al. The significance of *Acinetobacter baumannii* bacteraemia compared with *Klebsiella pneumoniae* bacteraemia: risk factors and outcomes. J Hosp Infect 2006;64:282-7.
6. Falagas ME, Bliziotis IA, Siempos II. Attributable mortality of *Acinobacter baumannii* infections in critically ill patients: a systemic review of matched cohort and case-control studies. Crit Care 2006;10: 1-8.
7. Scott P, Deye G, Srinivasan A, Murray C, Moran K, Hultan E, et al. An outbreak of multidrug-resistant *Acinetobacter baumanii–calcoaceticus* complex infection in the US military health care system associated with military operations in Iraq. Clin Infect Dis 2007;44:1577-84.
8. Kerr KG, Beggs CB, Dean SG, Thornton J, Donnelly JK, Todd NJ. Air ionization and colonization/infection with methicillin-resistant *Staphylococcus aureus* and *Acinetobacter* species in an intensive care unit. Intensive Care Med 2006;32:315-7.
9. Lopman BA, Reacher MH, Vipond IB, Hill D, Perry C, Halladay T, et al. Epidemiology and cost of nosocomial gastroenteritis, Avon, England, 2002–2003. Emerg Infect Dis 2004;10:1827-34.
10. Wullt M, Odenholt I, Walder M. Activity of three disinfectants and acidified nitrite against *Clostridium difficile* spores. Infect Control Hosp Epidemiol 2003;24:765-8.
11. Omidbakhsh N, Sattar SA. Broad-spectrum microbicidal activitiy, toxicologic assessment, and materials compatibility of a new generation of accelerated hydrogen peroxide–based environmental surface disinfectant. Am J Infect Control 2006;34:251-7.
12. French GL, Otter JA, Shannon KP, Adams NMT, Watling D, Parks MJ. Tackling contamination of the hospital environment by methicillin-resistant *Staphylococcus aureus* (MRSA): a comparison between conventional terminal cleaning and hydrogen peroxide vapor decontamination. J Hosp Infect 2004;57:31-7.
13. Fan L, Song J, Hildebrand PD, Forney CF. Interaction of ozone and negative air ions to control microorganisms. J Appl Microbiol 2002;93: 144-8.

14. Kowalski WJ, Bahnfleth WP, Striebig BA, Whittam TS. Demonstration of a hermetic airborne ozone disinfection system: studies on *E coli*. AIHA J 2003;64:222-7.
15. Gaunt L, Higgins S, Hughes J. Decontamination of surface-borne bacteria by ionized antimicrobial vapours. J Electrostatics 2005;63:809-14.
16. Fletcher LA, Gaunt LF, Beggs CB, Shepherd SA, Sleigh PA, Noakes CJ, et al. Bactericidal action of positive and negative ions in air. BMC Microbiol 2007;7:1-9.
17. Hudson JB, Sharma M, Petric M. Inactivation of norovirus by ozone gas in conditions relevant to health care. J Hosp Infect 2007;66:40-5.
18. Jarvis WR. Selected aspects of the socioeconomic impact of nosocomial infections. Infect Control Hosp Epidemiol 1996;17:552-7.
19. Berrington AW, Pedler SJ. Investigation of gaseous ozone for MRSA decontamination of hospital side rooms. J Hosp Infect 1998;40:61-5.
20. Li C-S, Wang Y-C. Surface germicidal effects of ozone for microorganisms. AIHA J 2003;64:533-7.