Ozone Treatment for Elimination of Bacteria in Medical Environments

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Abstract
Pathogenic bacteria in medical environments can lead to treatment complications and hospital acquired infections (HAIs), and current cleaning protocols do not address hard-to-access areas or that may be beyond line-of-sight treatment such as with ultraviolet radiation. Here, we tested the efficacy of Sanisport ozone as a means to treat hospital equipment and surfaces for killing bacteria. We observed a rapid killing of medically-relevant and environmental bacteria (*Escherichia coli*, *Enterococcus faecalis*, *Bacillus subtlis*, and *Deinococcus radiodurans*) across four surfaces (blankets, catheter, remotes, and syringes) within 30 minutes, and up to a 99% reduction in viable bacteria at the end of 2-hour treatment cycles. These results show the strong promise of ozone treatment for reducing risk of infection and HAIs.

Introduction
The World Health Organization (WHO) has recently stated that we are entering a "post-antibiotic era" due to decades of overuse of antibiotics for therapeutic and agricultural reasons[1]. Antimicrobial resistance (AMR) has been increasing at an alarming rate, whereby prevalent nosocomial infections such as pneumonia, tuberculosis, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Clostridium difficile* infection are becoming difficult to treat with conventional methods, due to multidrug resistance (MDR) and AMR. In the United States alone, hospital acquired infections (HAIs) kill an average of 63,000 patients yearly, with many showing AMR. In order to combat this crisis, resources have been allocated to developing modified

versions of existing antibiotics or discovery of new ones[3]. However, resistance to new and existing drugs continues to persist due to the strong selective pressure of antibiotics, with successful bacteria acquiring antimicrobial resistance genes in a continual 'arms race' between antibiotic development and antibiotic resistance[,3].

As such, there is a need of new methods for effective disinfection tools that control drug resistant pathogens and reduce antibiotic consumption. In the past, ozone has been utilized to safely sanitize a number of products in various industries, such as sewage treatment to kill harmful bacteria[4]. The strong electronegative properties of ozone encourages disruption of proteins, peptidoglycans, and lipids in the cell wall and cell membrane, and interferes with the activity of enzymes and nucleic acids. It has been demonstrated that ozone is up to 3,000 times faster acting and 150 times stronger than chlorine for killing bacteria, fungi, and other pathogens under some conditions[5]. Also, ozone has been used at highly toxic concentrations to sanitize hospital rooms prior to patient occupation[6,7]. However, ozone is inherently unstable as it degrades to diatomic oxygen if its source of production is exhausted[5]. Thus, a method for continually-generating ozone may be an ideal tool for sanitizing various hospital surfaces or equipment, as an alternative to the autoclave or ultraviolet light, but such a device (to our knowledge) has not been tested on medical equipment. Here, we report the impact of an ozone-generating machine on medically-relevant surfaces and materials from a hospital, to gauge the impact on bacterial growth (colony forming units, or CFUs) and response (percent of bacteria killed).

Results

We tested four distinct bacterial species commonly found in the hospital environment (*Escherichia coli* strain K12, *Enterococcus faecalis*, *Bacillus subtlis*, and *Deinococcus radiodurans*) for their susceptibility to ozone. These bacteria were grown and tested in triplicate on four high-traffic surfaces from the hospital equipment: catheters, blankets, hospital remote controls, and syringes, with positive and negative controls also included for comparison. All samples were treated with the same doze of ozone, at increasing lengths of time (30, 60, and 120 minutes), and CFU counts were compared between ozone-treated and controls. Control experiments consisted of allowing bacteria to grow without ozone treatment and then collecting samples as outlined in the **Methods** for each of the time points. For each surface and species tested colony forming units (CFUs) were counted, CFU/mL calculated, and the triplicates were averaged with standard error of the mean calculated to obtain ozone kill curves (**Figure 1**). These results showed as much as a three-log-fold changes in CFUs, with the majority of the impact observed within the first 30 minutes of treatment. These data showed that the ozone treatment causes significant reductions in all bacterial species tested across the various surfaces tested ($p<0.001$, Fisher's Exact Test), with increasing degrees of efficacy as a function of time.

Moreover, both the different surfaces and different species showed distinct rates of reduction and response to the treatment. For example, *E. coli* showed showest the greatest sensitivity to killing in the plate, but less so on the remote controls. Also, the surface with the greatest impact (the most species killed) was the syringe, specifically at the 2-hour time point (>99.3%), vs. the greater variance on the hospital blankets. Also, the 2-hour time points for 12 well plate experiments showed a wide range of bacterial reduction across the different species, from 89.75% to 99.70%. The syringe experiments were the most consistent, with a range of bacterial reduction from 94.24% to 99.57%; conversely, the other surfaces showed a wider range of

bacterial reduction, from 83.59% to 96.00% for the catheter, 86.10% to 99.31% for the remote control, and 82.99% to 98.36% for the hospital blankets. In all cases, these reductions were highlight significant ($p<10^{-5}$) compared to the negative controls (0.0-0.4%).

Discussion

Antibiotic resistance is a huge financial burden on the U.S. health care system, often associated with common infections in hospitals which are caused by the sharing of rooms of infected patients, transmission by hospital workers, overgrowth of pathogens in the patient's own microbiome, and interactions with surfaces and equipment that harbor these pathogenic bacteria. Fortunately, alternatives to antibiotics are being developed to take on this looming challenge.

Our findings provide support that ozone treatment is an effective sterilization method to combat HAIs in medical environments. We report rapid killing of the majority of medically-relevant bacteria within 30 minutes, and up to a 99% reduction in viable bacteria at the end of 2 hour treatment cycles, with as much as a 3-4-fold log kill range. Here, we used the Sanisport Supreme Dupliskate Ozone generator, but without any modifications or changes to the instrument. As such, changes in the flow rate, pressure, or temperature could increase the efficacy of this method, and possibly reduce bacterial species on common hospital surfaces in less time (~10-15 minutes).

It is interesting to note that different surfaces led to variation in the reduction of the same bacterial species. For example, the syringe and catheter experiments killed off bacteria at a slower rate than remote control and 12 well plate experiments. A likely explanation for this is that bacteria were inoculated directly onto the surfaces of 12 well plates and remote controls and so ozone could interact with more bacteria without any obstruction. Regardless, ozone still caused bacterial reductions even when inoculated inside tube-like structures such as catheters and syringes. Furthermore, ozone may not damage sensitive equipment (vs. bleach), which can ensure continued use of sterilized equipment in medical environments. Further work could explore the mechanisms by which bacteria respond to this treatment. Ozone is thought to disrupt membrane integrity, and so monitoring viability in this context can provide for a more conservative methodology[5]. While utilizing CFUs as a method to assess bacterial reduction have been the gold standard in many kill curve experiments, this method is limited in being able to discriminate viable but not cultivable cells (VBNC), and other methods such as PMA-qPCR or Flow Cytometry could also be used[8]. In the context of further testing more pathogenic bacteria known to enter VBNC states it would be worthwhile to use these methods in conjunction with colony forming unit counts.

It would also be interesting to utilize RNA-seq to study stress response in various HAI bacterial species in response to ozone. While our methods here demonstrate that 20 ppm of ozone can severely reduce several bacterial populations, there is little known about the stress response genes activated in response to ozone. The utilization of these stress responses are typically mediated by global regulatory mechanisms which affect biochemical pathways leading to physiological changes that confer survival. We hypothesize that regulatory networks such as heat shock, membrane integrity, and DNA damage may be activated. Particularly, it is interesting to note that ozone kills *D. radiodurans*, as this species has been known to have the unique ability to reconstruct its fragmented genome in response to ionizing radiation. There are a number of

theories of how *D. radiodurans* is able to survive such extreme stressors one of them being an unusual capacity to avoid radiation induced protein oxidation, thus it would be interesting to study the stress response pathways in the context of Sanisport ozone mediated treatment[10-12]. Understanding these mechanisms can be valuable for discovering novel genes for a multitude of bacterial species to decipher phenotypic characteristics, virulence regulation, and survivability, which may impact other medical environments like ambulances[13] and broader urban environments around the world[14].

Methods

We used four common HAI-related, bacterial species to treat with ozone: *Escherichia coli* strain K12 (substrain MG1655/ATCC 700926), *Enterococcus faecalis* (OG1RF ATCC 47077), *Bacillus subtlis* (subtilis strain 168 ATCC 23857), and one isolated strain *Deinococcus radiodurans* (R1). Single, isolated colonies were cultured and treated with ozone (20ppm) inside the continually-generated ozone instrument (Sanisport Supreme Dupliskate) at 0, 30, 60, and 120 minutes, which represent common intervals for cleaning in medical settings. We inoculated various surfaces that are common in hospital settings and produced ozone kill curves for each surface and bacterial strain. After swabbing each surface in triplicate, we isolated the bacteria from the swabs and cultured them overnight on media agar plates from which we assessed bacterial death from counting colony forming units (CFUs). Plates were blinded during counting and annotated afterward.

From fresh grown stock solutions, 100ul of cultured bacteria were obtained and placed in 900ul of appropriate broth (tryptic soy broth or nutrient broth with 1% glucose) in 1.5ml eppendorf tubes. From this 1:10 dilution the bacteria were serially diluted to 1:100, 1:1000, 1:10,000, 1:100,000, 1:1,000,000, and 1:10,000,000. 100ul from each dilution was then plated on corresponding tryptic soy agar plates and nutrient broth with 1% glucose agar plates using Zymo glass shaker beads to randomly distribute colonies across the plate. Plates were then grown at 37°C overnight for 12 hrs. Plates that had about 300 CFUs were chosen for the Sanisport ozone experiment to be able to distinguish between colonies.

Using the correct dilution, 1 ml of each single strain of bacteria was placed in triplicates in a 12-well plate, so as to have enough surface area exposed to ozone treatment. 3 samples were used for each time point of 0, 30, 60, and 120 minutes (min). Ozone treatment was done on separate days for each strain to reduce chance of cross contamination. The 12-well plate was placed uncovered inside of Sanisport and for the 0 min time point, 100ul was taken from the 1 ml sample and placed in a labeled eppendorf tube to be plated at the end of the experiment. The remaining 900ul was placed in a cryotube with 70ul of DMSO added and then placed on dry ice before moving into -80°C freezer.

Sanisport doors were closed and the “cycle” button was pushed. Each 16-minute cycle consists of a 10-minute phase where purified air is blown inside the machine from fans and followed by a 6-minute phase of 20ppm ozone treatment according to manufacturer’s instructions. Ozone is generated each cleaning cycle inside the machine by reacting oxygen available in the air with ultraviolet light. At the end of the 5th cycle 100ul of sample was taken from the three corresponding 30min wells and saved for plating at the end of the experiment while 900ul is

frozen down as described above. This is done for 5 more cycles for the 1 hour (h) time point and then 10 more cycles for the 2h time point. After the 2h time point the saved 100ul samples are plated on appropriate agar plates and labeled. CFUs are then counted the next day after a 12h incubation at 37°C.

The same methods are followed for the different surfaces tested (syringe, catheter, hospital blanket, etc.) with minor modifications. 100ul of bacteria sample were added to each surface via pipette in triplicates and then for each time point 0, 30, 60, and 120 minutes, the surface was swabbed for 3 mins using Isohelix buccal swabs. Bacteria were pipetted directly onto flat surfaces such as remote controls and hospital blankets while bacteria were pipette onto the inside surface of tube like structures such as catheters and 5cc syringes. For dry surfaces, swabs were wetted with respective media and then used for swabbing. Swabs were then placed in 1.5ml Eppendorf tubes and suspended in 100ul of corresponding media. After the 2h time point each Eppendorf tube containing swab and media was vortexed for 10 seconds at max speed to shake bacteria off of swab and into surrounding media at bottom of tube. The tubes were then centrifuged at 6000 rpm for 10 mins at room temperature to collect bacteria at bottom of tube. Swabs were removed using sterile tweezers and media was pipetted up and down 10 times to resuspend bacteria at the bottom of the tube. 100ul of media was then plated onto respective agar plates, labeled, and incubated at 37°C overnight for 12h. CFUs were counted the following day.

Declarations:

Ethics Approval
None required for this study

Contributions and Consent for Publication
CW, SR, DD, NBO, RO, DB, EA, CEM wrote and reviewed the text and data. CW did the culturing with SR. DD and EA supervised the protocols. RO, NBO, and CEM reviewed thed data. All authors reviewed and approved of the manuscript.

Availability of Data and Material
All raw data and CFU counts are included in the manuscript.

Competing interests
NO, RO, and CM hold shares in a company (Biotia) that builds technology to surveil hospital environments and screen patients to identify pathogens, however that company's technology is not used in this study.

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Figure Legends

Figure 1. Ozone Kill Curves indicate ozone treatment reduces bacterial load. 1ml of bacteria for taxa *B. substilis* (a), *E. coli K12* (b), *D. radiodurans* (c), and *E. faecalis* (d) were pipetted into wells of 12 well plates and 100ul was collected at each time point for plating. 100ul of bacteria were pipetted inside 5cc syringes, inside catheters, onto remotes, and onto hospital blankets in triplicates and swabbed for 3 minutes at each time point for plating. Colony forming units were then counted following plating and 12hr incubation. CFUs are converted into CFUs/ml (y-axis). Error bars represent standard error of the mean. Controls are indicated as black lines. Ozone treatment (x-axis) is at 20ppm.

Figure 2. Percent Bacterial Reduction. Bacterial percent reductions for *B. substilis* (red), *E. coli K12* (green), *D. radiodurans* (blue), and *E. faecalis* (purple) from ozone treatment (20ppm). Error bars represent standard error of the mean from triplicate experiments on a variety of surfaces: a) 12 well plates b) syringes c) catheters d) remote controls e) hospital blankets. All differences compared to control are statistically significant ($p<0.001$, Fisher's Exact Test).

Figure 1

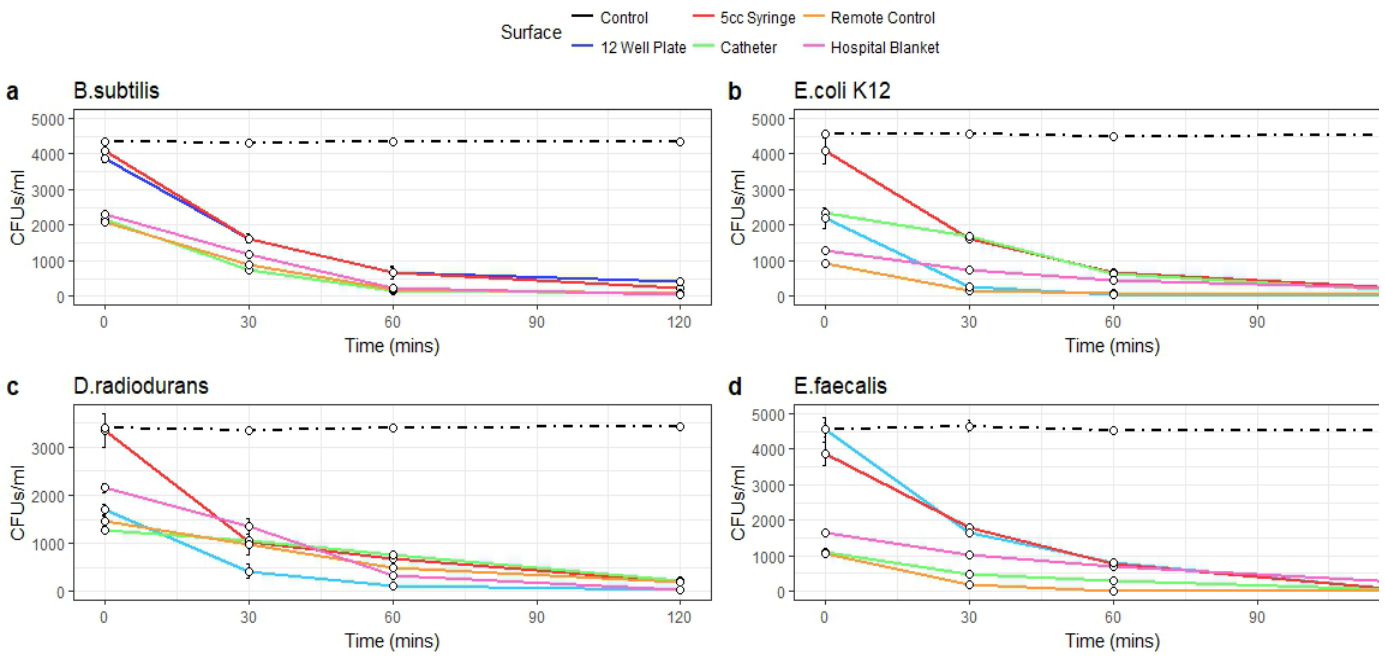

Figure 2