Influence of delivery system on the efficacy of low concentrations of H_2O_2 in the disinfection of common healthcare associated infection pathogens

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RUNNING TITLE: Impact of delivery system of the efficacy of H_2O_2

Abstract

INTRODUCTION: The ability of healthcare associated infection pathogens to survive on environmental surfaces is well known. Disinfection is employed to reduce or remove these pathogens however disinfection failures still occur. One method with the potential to improve disinfection efficacy is whole room disinfection with H_2O_2.

AIM: To determine the influence of delivery system on the efficacy of low concentration H_2O_2 on common healthcare associated infection pathogens.

METHODS: We compared SanoStatic (electrostatic spray) with SanoFog (fogging) in terms of performance for delivery of 5% H_2O_2 and trace silver ions for disinfection. The bacterial test challenges were VRE, ESBL- producing *K. pneumoniae* (ESBLK), CPE, MRSA, *Clostridium difficile* spores, *Bacillus atropheus* and *Geobacillus stearothermophilus* commercial spore strips.

FINDINGS: SanoFog and SanoStatic were effective when tested under the conditions of experimentation reported here. For VRE, ESBLK, CPE and MRSA, SanoFog and SanoStatic were comparable in performance. For *C. difficile* we concluded the following: SanoFog was most effective for disinfection of *C. difficile* spores when compared to SanoStatic.

CONCLUSION: We concluded that while SanoFog and SanoStatic were effective against bacterial cells, the current practice of using SanoFog and SanoStatic together would be effective for disinfection of *C. difficile* spores based on investigations under the conditions of experimentation reported here. For the spore strips we found results that were not comparable to the results for either the vegetation cells (VRE, ESBLK, CPE, and MRSA) or *C. difficile* spores.

Keywords

Disinfectant testing, Hydrogen peroxide, Healthcare Associated Infection, Infection control, VRE, ESBL, CPE, MRSA, *C. difficile*.

Introduction

The ability of healthcare associated infection (HAI) pathogens to survive on environmental surfaces is well known [1][2]. Meticillin Resistant *Staphylococcus aureus (S. aureus)* (MRSA), Vancomycin Resistant Enterococci (VRE), Extended Spectrum Beta-lactamase producers (ESBLs), Carbapenemase Producing Enterobacteriaceae (CPE) and *Clostridioides difficile* (*C. difficile*) amongst others are capable of surviving for days to weeks on dry inanimate surfaces. Multiple studies in adult settings have demonstrated that surfaces in the rooms of patients who themselves are colonised or infected with important healthcare-associated pathogens are frequently contaminated. The proportion of hospital surfaces contaminated with MRSA has varied in published reports from 1 to 27% in patient rooms in general hospital wards and from a few percent to 64% of burns units [3][4]. As long as these bacteria are contained within the room and removed by cleaning at the end of the patient's stay then they present no risk of cross infection. However, studies have frequently reported on bacterial contamination of the healthcare environment [1][3][5][6] even though procedures for regular cleaning and disinfection are in place.

One method of reducing environmental contamination with potential pathogens is whole room treatments or methods of targeted treatment with compounds such as H_2O_2. Here we report on a comparison of SanoStatic (electrostatic spray) with SanoFog (fogging) in terms of performance for delivery of 5% H_2O_2 and trace silver ions for disinfection. The Sanofog system is the traditional way of deploying H_2O_2 in an enclosed room for even provision of disinfectants on surfaces. As it is a touchless means of disinfection, the risk of cross infection associated with cleaning and disinfection using wipes is minimized. It has however limitations of being less effective for air handling units or in situations where multiple objects in a room make complete coverage difficult and in large atrium or reception areas. The Electrostatic spray delivering H_2O_2 can overcome these issues as the equipment employs a sprayer nozzle that confers an electrostatic negative charge on the disinfecting mixture. This allows the spray to surround and attach strongly to surfaces and with the wand, one can work through an entire room to ensure that all surfaces are treated. The

presence of silver trace ions in the mixture stabilizes H_2O_2 enhancing its biocidal activity [7]. In this work the impact of the two delivery systems on efficacy of 5% v/v preparation of H_2O_2 and trace levels of silver ions on some important human pathogens was compared.

The bacterial test challenges were VRE, ESBL- producing *K. pneumoniae* (ESBLK), CPE, MRSA, *C. difficile* spores, *Bacillus atropheus* and *Geobacillus stearothermophilus* commercial spore strips.

Methods

H_2O_2 disinfectant and delivery systems

The H_2O_2 preparation used was a 5% v/v preparation of H_2O_2 and trace levels of silver ions. SanoFog is a H_2O_2 fogging system that delivers 5% H_2O_2 as a fog. SanoSpray is a H_2O_2 electrostatic spray method applied via a handheld device that allows all surface contact points to be moved or exposed and allows access into confined spaces (Sanondaf UK).

Bacterial cultures and growth conditions

Enterococcus faecalis (*E. faecalis* [VRE]) was a clinical isolate supplied by the Department of Microbiology, University Hospitals of Morecambe Bay NHS Foundation trust. The following were provided from recognised culture collections: *K. pneumoniae* (ESBL-producing) ATCC 700603 (American Type Culture Collection [Manassa, USA]), *K. pneumoniae* (CPE) NCTC 13438 (National collection of type cultures, Public Health England [Porton Down, UK]), *S. aureus* (MRSA) NCTC 12493. *Clostridioides difficile* (*C. difficile*) atoxigenic strain was prepared by Blutest (Glasgow UK) and spore strips (*Bacillus atropheus* [10^5 CFU] and *G. stearothermophilus* [10^6 CFU]) were provided by Crosstex (New York, USA) as pre-prepared products.

All media were prepared following instructions provided by the manufacturer. Cell stocks (apart from *C. difficile*) were maintained on cysteine lactose electrolyte deficient plates (ThermoFisher Scientific, Waltham, USA) incubated at 37°C for 18 hours in a HERAtherm incubator (ThermoFisher Scientific, Waltham, USA). Cultured plates containing the bacteria were stored at 4°C until

required and used within two weeks. Overnight liquid cultures of bacteria were prepared using Luria broth (ThermoFisher Scientific, Waltham, USA) except for *E. faecalis* which was prepared using tryptic soy broth (TSB) (Sigma Aldrich, Dorset, UK). All overnight cultures were incubated for 18 hours at 37°C and 150rpm (Incubating orbital shaker, VWR International, Lutterworth, UK). A sterility control was also incubated (without inoculation). The overnight cultures were only used if the negative control was clear of growth.

Preparation of the bacterial master stocks

The bacterial master stocks were prepared from the overnight liquid cultures. Two 1mL volumes of each culture were transferred to sterile 1.5mL microcentrifuge tubes and centrifuged at 6,000 x g for five minutes in a Micro Centaur centrifuge (MSE UK Ltd, London, UK), washed twice and resuspended in Phosphate Buffered Saline (PBS) (ThermoFisher Scientific, Waltham, USA). The bacterial master stocks were standardised by absorbance to 10^9 colony forming units (CFU)/ml (OD_{570} of 0.2 ± 0.02) using an infinite f200 pro plate reader (Tecan, Grödig, Austria) and confirmed by plate culture.

The *C. difficile* spore master stock was prepared by Blutest (Glasgow, UK). The final titre of the *C. difficile* spore stock was $5.15x10^7$ cultivable spores/ml in PBS and was stored at 4°C until required.

Determination of the minimum inhibitory concentration

The MIC for the test isolates were determined by a modification [8] of the microdilution method reported by Andrews [9]. One hundred microLitres of the required growth media was dispensed into 8 wells of a non-tissue culture treated round bottom 96 well plate (ThermoFisher Scientific, Waltham, USA). TSB (Sigma Aldrich, Dorset, UK) was used as the growth media for all but *C. difficile* (brain heart infusion broth [Sigma Aldrich, Dorset, UK] used instead). Into the first well of each column, 100µL of 5% H_2O_2 (Sanondaf UK, Coatbridge, UK) was added into the broth and gently mixed with a micropipette. One hundred microLitres was then removed from the mixture into the next well and by doubling dilution the concentration of the antimicrobial agent was diluted in decreasing order to the last well from which 100µL was discarded. Ten

microLitres of the mixture was removed from all the wells and replaced with 10µL of bacterial suspension (10^9 CFU/mL). This was prepared in triplicate for each test isolate. Three wells containing 100% growth media and another three containing growth media and bacteria were used as negative and positive controls respectively. Three wells containing 100% antimicrobial agent and bacteria were also prepared to ascertain the efficacy of the antimicrobial agent in the absence of the growth media. The plates were incubated for 24h at 37˚C in air (except for *C. difficile* which was incubated for 48h at 37˚C under anaerobic conditions) and the MIC was read as the lowest concentration of disinfectant that prevented visible growth of bacteria (based on visual turbidity).

Experimental contamination of the test surfaces

On the day before experimentation, sterile 24 well microtitre plates (ThermoFisher Scientific, Waltham, USA) were challenged with 100 µL of each bacterium in triplicate wells (approx. 10^8 CFU). Bacterial challenges were dried overnight in a class I biosafety cabinet (MDH Ltd, Andover, UK) with the lids slanted to aid drying. The positive control consisted of a bacterial challenge that was not exposed to H_2O_2 treatment (either SanoFog or SanoStatic). The negative control consisted of a microtitre well exposed to H_2O_2 without bacterial challenge.

On the morning of experimentation, the microtitre plates were verified for compete drying, removed from the biosafety cabinet and sealed with Parafilm (Bemis Company Inc., Neenah, USA).

The laboratory was prepared as follows. All incubators containing biological agents (shaking and static) were sealed to protect against possible H_2O_2 exposure. Two windows in the laboratory were opened at either end to allow for air flow through the laboratory to reduce the risk of H_2O_2 exposure. The laboratory was then closed to non-essential personnel for the period of the H_2O_2 experimentation. All personnel involved in the testing wore suitable personal protective clothing including gloves, safety glasses and FP3 masks. The application of the H_2O_2 (5% H_2O_2 [Sanondaf UK, Coatbridge, UK]) fog (SanoFog) and electrostatic spray (SanoStatic) was undertaken by trained

Sanondaf UK personnel according to standard operating procedures. Before application of the H_2O_2 treatment the test plates containing replicates (n=3) of the bacterial challenge were placed in the class I biosafety cabinet, parafilm and lids removed. The plates containing the positive controls (sealed with parafilm) were left on the bench in the same laboratory as the class I biosafety cabinet. For the fogging the front of the class I biosafety cabinet was modified with a replacement panel containing a door port that fitted with the nozzle of the fogging unit (Figure 1, supplementary material). For electrostatic spraying of H_2O_2 the upper panel of the front was raised to allow for access by the person applying the electrostatic spray (Figure 2, supplementary material). The size of the droplets in SanoFog and SanoStatic which was obtained from the Sanondaf UK was between 10 and 15µm. H_2O_2 indicator strips were placed in the cabinet and around the lab. When the H_2O_2 treatment was completed the fogging unit was left *in situ* and when spraying was completed the class I biosafety cabinet was closed and sealed with the microtitre plates containing the bacterial challenges left in place for a defined period (called the dwell time).

Following a suitable dwell time (time between end of H_2O_2 treatment and processing of the samples) the microtitre plates were processed for bacterial survival (CFU estimation). Each well containing bacteria, positive and negative control were processed in an identical manner. One hundred microLitres of sterile PBS was added to each well. Plates were sonicated for 5 minutes (37 kHz) in an Elmasonic S30 sonicating waterbath (Elma, Singen, Germany) placed within a class II biosafety cabinet. The surface of each well was scraped with the tip of a sterile plastic pipette tip to resuspend cells from the surfaces. The resultant bacterial suspensions were transferred to sterile microcentrifuge tubes and assessed for CFU/mL using the modified Miles and Misra plate counting technique [10]

Data analysis

Data generated following processing of the samples were analysed using Jamovi (version 1.0.0).

The lower limit of detection for the Miles and Misra assay was estimated to be 50 CFU (1.699 Log_{10} CFU). Where the Log_{10} value was 'zero' the Log_{10} density could not be calculated. To calculate the Log_{10} reduction we substituted an artificial count of 0.5 CFU (assuming a normal distribution with a range between 0 and 1) for the observed zero on one Miles and Misra 20 microLitre spot at the first counted dilution (KSA-SM-07: The Log reduction (LR) measure of disinfectant efficacy [http://www.biofilm.montana.edu/documents/KSA-SM-07.pdf [accessed May 2019]). Details of the equations used to calculate the Log_{10} and % reduction values is supplied in the supplementary materials.

Results

The minimum inhibitory concentration of H_2O_2 varied by bacteria

All bacteria were inhibited by concentrations of H_2O_2 less than the working concentration (5%) but there were variations in the MIC values observed with *E. faecalis* being the most sensitive (MIC of less than 0.0049%). MRSA was inhibited by 0.0195% H_2O_2, ESBLK by 0.0781% and the CPE strain (*K. pneumoniae*) by 0.0391% H_2O_2. The highest MIC was observed for *C. difficile* (1.25% H_2O_2).

Length of dwell time affected disinfection efficacy

Two H_2O_2 (5%) delivery systems were investigated: SanoFog (fogging) and SanoStatic (electrostatic spray). SanoFog and SanoStatic disinfection efficacy was generally comparable.

H_2O_2 (5% fogging) with the SanoFog delivery system

Treatment times were 1.5 and 5 minutes and dwell times were 30 and 90 minutes (Table I). The most effective combination of H_2O_2 exposure was 5 minutes treatment time and 90 minutes dwell time with all bacteria unrecoverable following exposure. Log_{10} reductions for this most effective exposure ranged from 4.166 to 6.204 (Table I).

H_2O_2 (5% spray) with the SanoStatic delivery system

The impact of treatment time and dwell time on the disinfection efficacy of SanoStatic (electrostatic spraying with 5% H_2O_2) was assessed. Treatment times were 3 and 6 minutes and dwell times were 30 and 90 minutes (Table II). The most effective combination of H_2O_2 treatment and dwell times was 6 minutes and 90 minutes with all bacteria unrecoverable following exposure. Log_{10} reductions for this most effective exposure ranged from 4.166 to 6.204 (Table II).

In the second of the experiments on the impact of dwell time on disinfection efficacy of the SanoStatic delivery system work concentrated on the two *K. pneumoniae* bacterial isolates as they showed the greatest resilience to shorter treatment and dwell times. The treatment time was standardised at 6 minutes, and the dwell times were 30, 60 and 90 minutes. Results demonstrated a clear relation between Log_{10} reduction and dwell time (Table 1, supplemental materials). The most effective combination of H_2O_2 treatment and dwell times was 6 minutes and 90 minutes with all bacteria unrecoverable following exposure. The Log_{10} reductions for this most effective exposure were 4.166 (ESBLK) and 4.271 (CPE).

Disinfection of C. difficile using 5% H_2O_2 was successful but varied by method of delivery (SanoFog / SanoStatic)

The SanoFog and SanoStatic H_2O_2 delivery systems were tested for their efficacy in disinfection of *C. difficile* spores using the optimum treatment and dwell times identified in the prior experiments on VRE, ESBLK, CPE and MRSA (Tables I & II).

For treatment with SanoFog, the percentage reduction in CFU was 99.999 and the Log_{10} reduction was 5.368 (Table III). For SanoStatic, the percentage reduction in CFU was 94.38 and the Log_{10} reduction was 1.265. This experiment was repeated with similar results (Table 2, supplemental materials). Following on from these initial experiments, extended treatment and dwell times of 10 minutes and 2 hours respectively were investigated. The results were

similar to those achieved with the shorter treatment and dwell times (Table 3, supplemental materials).

Spore strip survival patterns do not concur with results for multi-drug resistant pathogens or *C. difficile*

H_2O_2 fogging (SanoFog) and spray (SanoStatic) were assessed for their efficacy against commercially available spore strips containing *B. atropheus* and *G. stearothermophilus*. The interpretation of the results for these spore strips was confined to visual evidence for the presence or absence of growth. One aspect that required prior consideration was whether to expose the spore strips within their primary packaging to H_2O_2 ("closed") or to remove them from the primary packaging first ("open"). Both approaches have been observed in practice.

The format in which the spores were exposed to H_2O_2 (either in their primary packaging ["closed"] or not ["open"]) influenced the results of the disinfection test with "open" spore strips more readily disinfected. Both methods of H_2O_2 delivery (SanoFog and SanoStatic) were generally comparable in terms of performance. Disinfection with SanoFog resulted in no recoverable growth of *G. stearothermophilus* when spore strips were "open", but all three "closed" spore strips did show signs of growth. *B. atropheus* spore strips were incubated for 7 days before reading the results (1 day results are shown for information only). SanoStatic performed more effectively than SanoFog in terms of disinfection. "Open" spore strips were more sensitive to disinfection than "closed" for SanoFog but not for SanoStatic treatment (Table 4, Supplemental materials).

Discussion

For *E. faecalis* (VRE – clinical isolate), *K. pneumoniae* (ESBL – ATCC 700603), *K. pneumoniae* (CRE – NCTC 13438), *S. aureus* (MRSA – NCTC 12493), the two delivery systems of 5%H_2O_2 which were SanoFog and SanoStatic reduced the bacteria to undetectable levels using the most effective combinations of

treatment time and dwell time of 5 minutes/90 minutes and 6 minutes/90 minutes respectively. For *C. difficile* SanoFog resulted in the same outcome yet SanoSpray treatment did not. However, in cases of *C. difficile* disinfection SanoSpray is used in conjunction with SanoFog for efficacy to be achieved.

Piskin and colleagues reported a >4 Log_{10} reduction for MRSA after treatment with a 5% dry mist H_2O_2 preparation used in accordance with the manufacturer's instructions (details of treatment time and dwell time were not provided) [11]. A study by Ali and colleagues comparing the disinfection efficacy of two H_2O_2 vapour systems (Bioquell Q10, Bioquell, Andover, UK [30% H_2O_2] and Deprox, Hygiene solutions, Kings Lynn, UK [5% H_2O_2]) found comparable results to those reported here for *C. difficile* (approximately 5.1 Log_{10} reduction), MRSA and *K. pneumoniae* (approximately 6.3 Log_{10} reduction) [12]. In their comprehensive study Otter and French reported complete eradication of multiple strains of MRSA, *C. difficile*, *K. pneumoniae*, *E. faecalis* and VRE following 90 minute treatment with a Clarus R suite (Bioquell UK Ltd, Andover, Hampshire, UK) reporting approximately 6 Log_{10} reductions [13]. It is clear from the available literature that H_2O_2 is an effective disinfectant against these human pathogens and the results we report here for SanoFog and SanoStatic are in general agreement with prior literature. However, more studies are required investigating the influence of different H_2O_2 concentrations, treatment, and dwell time combinations on disinfection efficacy.

Our results for *C. difficile* 5% H_2O_2 treatment were mixed. The bacterial spore form is considered one of the most difficult to disinfect possibly because they were not as clean as the purified spore strips. In practice where *C. difficile* associated disease is an issue and disinfection over and above standard cleaning and disinfection is required then SanoFog and SanoStatic are used together. Such procedures are likely to be effective based on our experimental observations. Little information on what levels of *C. difficile* contamination are likely to be present in healthcare settings has been published. Prior literature suggests levels far below those used in this study. For example, Shapey and colleagues [14] in their study sampled 10 high risk elderly care rooms (Nottingham University Hospital, UK) using culture methods and found a total

of 138 CFU (203 samples). The sampling methods used resulted in a surface area of 400 cm^2 (20×20 cm) or 100 cm^2 (10×10 cm and 5×20 cm). Taking the higher surface area value, the levels of *C. difficile* contamination can be estimated as $1.38x10^2$ CFU/8.12 m^2. For comparative purposes the *C. difficile* CFU used in the current study was $2.1x10^{11}$ CFU/8.12 m^2 (based on a test surface area of 1.96 cm^2 and average test challenge of $5.06x10^6$ CFU *C. difficile*). Such high levels of test challenge are used in disinfection efficacy tests to allow for the reporting of the Log_{10} reduction (and % reduction). These high titre test challenges bear little resemblance to the levels of bacteria likely to be encountered in the clinical setting. Lawley and colleagues considered 17 to 29 spores/cm^2 to be “heavily contaminated” in their murine model of *C. difficile* contamination and disinfection [15]. We therefore consider, based on our observations, that the combination treatment of SanoFog and SanoStatic is likely to be an effective measure in reducing the levels of *C. difficile* in the healthcare environment and therefore reducing the risk of infection transmission via this route.

It is worth reflecting on a comparison of the spore strip results with those for both the vegetative bacteria where challenge with the same treatment / dwell times led to no recovery of cultivable cells and the spore form of *C. difficile* where quite different results were observed when SanoFog and SanoStatic were compared. This brings into question the utility of using spore strip preparations to estimate disinfection efficacy for H_2O_2. Since the spore strips provide a yes/no answer the interpretation of the results is much less graduated than the methods used in this report for the other test bacteria where Log_{10} reduction and percentage reduction values were reported. A single spore that survived could have led in theory to a positive result (growth) and this would be interpreted as a disinfection failure. Whereas Log_{10} reduction and percentage reduction data coupled with knowledge from the published literature on the likely contamination levels in healthcare settings allows for a more evidence-based nuanced assessment by infection control professionals of the likely outcome of disinfection in practice. The current expectations stated in EN 13697 [16] are that a disinfectant should demonstrate a 4 Log_{10} reduction in vegetative bacterial counts. Specific guidance for spores is currently lacking.

However spore preparations with Log_{10} 5 or 6 cultivable spores are at concentrations where negative results (an estimated 5 or 6 Log reduction) are higher than those expected by EN 13697 and very much higher than levels of contamination found in practice [13]. We advise that spore strips are calibrated against other test organisms for ease and accuracy in results interpretation.

There has yet to be a robust economic analysis of H_2O_2 whole room disinfection published. Regarding the Sanondaf H_2O_2 delivery systems a single occupancy room (or equivalent) can be disinfected for less than £170.00. In the most recent version of the Health Protection Scotland Literature review on airborne H_2O_2 [17] the authors stated that a 'comprehensive cost-effectiveness evaluation would be timely' – it has yet to appear.

Conclusions

We compared the efficacy of SanoFog and SanoStatic for the disinfection of VRE, ESBLK, CPE, MRSA and *C. difficile*. For the vegetative bacteria (VRE, ESBLK, CPE and MRSA) both H_2O_2 delivery systems were effective and comparable when subjected to investigations under the conditions of experimentation reported here. For *C. difficile* and the commercially available spore strips we observed the following: SanoFog was most effective for disinfection of *C. difficile* spores when compared to SanoStatic. However, in practice both H_2O_2 delivery systems are used together. Therefore, we concluded that this process was effective at disinfection of *C. difficile* spores when subject to investigations under the conditions of experimentation reported here. For the spore strips we found results that were not comparable to the results for either the vegetation cells (VRE, ESBLK, CPE, MRSA) or *C. difficile* spores and questioned their use for the assessment of H_2O_2 treatment in practice.

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Table I: Impact of treatment and dwell time on the disinfection efficacy of SanoFog (5% H_2O_2)

Bacterial challenge	Time profile	Log10 CFU not treated	Log10 CFU treated	% reduction	Maximum detectable log10 reduction	Log10 CFU reduction	Post treatment survival score
VRE (*E. faecalis* clinical isolate)	T=1.5, D=30	7.176	3.821	99.95	5.778	3.355	1.000
	T=5, D=30	7.091	1.398	100.00	5.693	5.693	0.000
	T=5, D=90	7.602	1.398	100.00	6.204	6.204	0.000
ESBL (*K. pneumoniae* ATCC 700603)	T=1.5, D=30	6.954	5.979	88.39	5.556	0.974	1.000
	T=5, D=30	6.684	6.289	52.07	5.286	0.395	1.000
	T=5, D=90	5.564	1.398	99.99	4.166	4.166	0.000
CPE (*K. pneumoniae* NCTC 13438)	T=1.5, D=30	5.426	4.668	79.59	4.028	0.758	1.000
	T=5, D=30	6.985	5.445	90.93	5.587	1.540	1.000
	T=5, D=90	5.778	1.398	99.99	4.271	4.271	0.000
MRSA (*S. aureus* NCTC 12493)	T=1.5, D=30	6.778	3.063	99.97	5.380	3.715	1.000
	T=5, D=30	5.301	2.508	91.10	3.903	2.793	0.333
	T=5, D=90	6.620	1.398	100.00	5.222	5.222	0.000

Key: Bacterial challenge: the identity of the bacteria used as the challenge. Time profile: The time (in minutes) of H_2O_2 treatment (T) and minimum dwell time (D) (the time between end of treatment and processing for CFU analysis by Miles and Misra plate counting). Log_{10} CFU not treated: the CFU of the test sample without H_2O_2 treatment expressed in Log_{10} units. Log_{10} CFU treated: the mean CFU of the treated test samples (n=3) following treatment with H_2O_2 expressed in Log_{10} units. % reduction: the reduction in CFU post treatment with H_2O_2 expressed as a percentage of the CFU for the untreated test sample. Maximum detectable Log_{10} reduction: the maximum detectable Log_{10} reduction based on the titre of the test challenge. Log_{10} CFU reduction: the reduction in CFU post treatment with H_2O_2 compared to the CFU of the untreated test sample expressed as Log_{10} reduction. Post treatment survival: A score based on the presence or absence of bacterial growth following treatment. One point was awarded for growth and 0 points awarded for no growth. Based on the 3 replicates the possible scores were 1 – all

three replicates exhibited growth; 0.66 – two of the three replicates exhibited growth; 0.33 – one of the three replicates exhibited growth; and 0 – none of the three replicates exhibited growth.

Table II: Impact of treatment and dwell time on the disinfection efficacy of SanoStatic (5% H_2O_2)

Bacterial challenge	Time profile	Log10 CFU not treated	Log10 CFU treated	% reduction	Maximum detectable log10 reduction	Log10 CFU reduction	Post treatment survival
VRE (*E. faecalis* clinical isolate)	T=3 D=30	6.156	2.779	99.96	4.758	3.377	1.000
	T=6 D=90	7.602	1.398	100.00	6.204	6.204	0.000
ESBL (*K. pneumoniae* ATCC 700603)	T=3 D=30	7.885	6.848	90.80	6.487	1.036	1.000
	T=6 D=90	5.564	1.398	99.99	4.166	4.166	0.000
CPE (*K. pneumoniae* NCTC 13438)	T=3 D=30	7.000	5.989	90.22	5.602	1.011	1.000
	T=6 D=90	5.669	1.398	99.99	4.271	4.271	0.000
MRSA (*S. aureus* NCTC 12493)	T=3 D=30	4.885	1.398	99.97	3.487	3.487	0.000
	T=6 D=90	6.620	1.398	100.00	5.222	5.222	0.000

Key: Bacterial challenge: the identity of the bacteria used as the challenge. Time profile: The time (in minutes) of H_2O_2 treatment (T) and minimum dwell time (D) (the time between end of treatment and processing for CFU analysis by Miles and Misra plate counting,). Log_{10} CFU not treated: the CFU of the test sample without H_2O_2 treatment expressed in Log_{10} units. Log_{10} CFU treated: the mean CFU of the treated test samples (n=3) following treatment with H_2O_2 expressed in Log_{10} units. % reduction: the reduction in CFU post treatment with H_2O_2 expressed as a percentage of the CFU for the untreated test sample. Maximum detectable Log_{10} reduction: the maximum detectable Log_{10} reduction based on the titre of the test challenge. Log_{10} CFU reduction: the reduction in CFU post treatment with H_2O_2 compared to the CFU of the untreated test sample expressed as Log_{10} reduction. Post treatment survival: A score based on the presence or absence of bacterial growth following treatment. One point was awarded for growth and 0 points awarded for no growth. Based on the 3 replicates the possible scores were 1 – all three replicates exhibited growth; 0.66 – two of the three replicates exhibited growth; 0.33 – one of the three replicates exhibited growth; and 0 – none of the three replicates exhibited growth.

Table III: Impact of SanoFog and SanoStatic (5% H_2O_2) against *C. difficile* spores

a) SanoFog

Bacterial challenge	Time profile	log10 CFU not treated	Log10 CFU treated	% reduction	Maximum detectable log10 reduction	Log10 CFU reduction	Post treatment survival
C. difficile (atoxigenic)	T=5, D=90	6.766	1.398	99.999	5.368	5.368	0.000

b) Sanostatic

Bacterial challenge	Time profile	log10 CFU not treated	Log10 CFU treated	% reduction	Maximum detectable log10 reduction	Log10 CFU reduction	Post treatment survival
C. difficile (atoxigenic)	T=6, D=90	6.766	5.501	94.38	5.368	1.265	1.000

Key: Bacterial challenge: the identity of the bacteria used as the challenge. Time profile: The time (in minutes) of H_2O_2 treatment (T) and minimum dwell time (D) (the time between end of treatment and processing for CFU analysis by Miles and Misra plate counting. Log_{10} CFU not treated: the CFU of the test sample without H_2O_2 treatment expressed in Log_{10} units. Log_{10} CFU treated: the mean CFU of the treated test samples (n=3) following treatment with H_2O_2 expressed in Log_{10} units. % reduction: the reduction in CFU post treatment with H_2O_2 expressed as a percentage of the CFU for the untreated test sample. Maximum detectable Log_{10} reduction: the maximum detectable Log_{10} reduction based on the titre of the test challenge. Log_{10} CFU reduction: the reduction in CFU post treatment with H_2O_2 compared to the CFU of the untreated test sample expressed as Log_{10} reduction. Post treatment survival: A score based on the presence or absence of bacterial growth following treatment. One point was awarded for growth and 0 points awarded for no growth. Based on the 3 replicates the possible scores were 1 – all three replicates exhibited growth; 0.66 – two of the three replicates exhibited growth; 0.33 – one of the three replicates exhibited growth; and 0 – none of the three replicates exhibited growth.

Influence of delivery system on the efficacy of low concentrations of H_2O_2 in the disinfection of common healthcare associated infection pathogens

Supplementary materials

Figure 1: Operational setup used for the H_2O_2 fogging (SanoFog) experiments

Key: The removable door of the front of the class I biosafety cabinet (MDH Ltd, Andover, UK) was replaced by a purpose-built replacement with a door port made to fit the nozzle of the fogging machine exactly. The H_2O_2 fogging machine was operated as normal and in accordance with standard operating procedures. Front view (left) and side view (right).

Figure 2: Operational setup used for the H_2O_2 electrostatic spraying (SanoStatic) experiments

Key: The front panel of the class I biosafety cabinet (MDH Ltd, Andover, UK) was lifted and secured in place using heavy duty masking tape. The H_2O_2 electrostatic spray machine was operated as normal and in accordance with standard operating procedures.

Analysis of the data

The Log_{10} reduction of bacteria was assessed based on the difference in Log_{10} CFU counts reported for the H_2O_2-treated bacterial challenges compared to the untreated bacterial challenge.

$$\text{Log reduction (LR)} = \log_{10}(A) - \log_{10}(B)$$

Where A = CFU of the untreated control and B = the CFU of the bacteria after H_2O_2 treatment

Percent reduction was calculated as follows:

$$P=(1-10^{-L})*100$$

Where P is the percent reduction, and where L is the log10 reduction.

(https://microchemlab.com/information/log-and-percent-reductions-microbiology-and-antimicrobial-testing [accessed May 2019]).

In each experimental run representative colonies for each bacteria were assessed for colony morphology and Gram stained to assess purity and identity.

Table 1: Impact of dwell time on the disinfection efficacy of SanoStatic (5% H_2O_2) against multi-drug resistant *K. pneumoniae* strains

Bacterial challenge	Time profile	Log10 CFU not treated	Log10 CFU treated	% reduction	Maximum detectable log10 reduction	Log10 CFU reduction	Post treatment survival
ESBL (*K. pneumoniae* ATCC 700603)	T=6, D=30	5.564	3.874	97.64	4.166	1.690	1.000
	T=6, D=60	5.564	1.815	99.98	4.166	3.749	0.667
	T=6, D=90	5.564	1.398	99.99	4.166	4.166	0.000
CPE (*K. pneumoniae* NCTC 13438)	T=6, D=30	5.669	1.398	100.00	4.271	4.271	0.000
	T=6, D=60	5.669	1.540	99.99	4.271	4.129	0.333
	T=6, D=90	5.669	1.398	100.00	4.271	4.271	0.000

Key: Bacterial challenge: the identity of the bacteria used as the challenge. Time profile: The time (in minutes) of H_2O_2 treatment (T) and minimum dwell time (D) (the time between end of treatment and processing for CFU analysis by Miles and Misra plate counting). Log_{10} CFU not treated: the CFU of the test sample without H_2O_2 treatment expressed in Log_{10} units. Log_{10} CFU treated: the mean CFU of the treated test samples (n=3) following treatment with H_2O_2 expressed in Log_{10} units. % reduction: the reduction in CFU post treatment with H_2O_2 expressed as a percentage of the CFU for the untreated test sample. Maximum detectable Log_{10} reduction: the maximum detectable Log_{10} reduction based on the titre of the test challenge. Log_{10} CFU reduction: the reduction in CFU post treatment with H_2O_2 compared to the CFU of the untreated test sample expressed as Log_{10} reduction. Post treatment survival: A score based on the presence or absence of bacterial growth following treatment. One point was awarded for growth and 0 points awarded for no growth. Based on the 3 replicates the possible scores were 1 – all three replicates exhibited growth; 0.66 – two of the three replicates exhibited growth; 0.33 – one of the three replicates exhibited growth; and 0 – none of the three replicates exhibited growth.

Table 2: Experimental repeat of impact of SanoStatic (5% H_2O_2) against *C. difficile* spores

Bacterial challenge	Time profile	log10 CFU not treated	Log10 CFU treated	% reduction	Maximum detectable log10 reduction	Log10 CFU reduction	Post treatment survival
C. difficile (atoxigenic)	T=6, D=90	6.790	4.853	98.78	5.392	1.937	1.000

Key: Bacterial challenge: the identity of the bacteria used as the challenge. Time profile: The time (in minutes) of H_2O_2 treatment and minimum dwell time (the time between end of treatment and processing for CFU analysis by Miles and Misra plate counting. Log_{10} CFU not treated: the CFU of the test sample without H_2O_2 treatment expressed in Log_{10} units. Log_{10} CFU treated: the mean CFU of the treated test samples (n=3) following treatment with H_2O_2 expressed in Log_{10} units. % reduction: the reduction in CFU post treatment with H_2O_2 expressed as a percentage of the CFU for the untreated test sample. Maximum detectable Log_{10} reduction: the maximum detectable Log_{10} reduction based on the titre of the test challenge. Log_{10} CFU reduction: the reduction in CFU post treatment with H_2O_2 compared to the CFU of the untreated test sample expressed as Log_{10} reduction. Post treatment survival: A score based on the presence or absence of bacterial growth following treatment. One point was awarded for growth and 0 points awarded for no growth. Based on the 3 replicates the possible scores were 1 – all three replicates exhibited growth; 0.66 – two of the three replicates exhibited growth; 0.33 – one of the three replicates exhibited growth; and 0 – none of the three replicates exhibited growth.

Table 3: Impact of extended treatment and dwell times of SanoStatic (5% H_2O_2) against *C. difficile* spores

Bacterial challenge	Time profile	log10 CFU not treated	Log10 CFU treated	% reduction	Maximum detectable log10 reduction	Log10 CFU reduction	Post treatment survival
C. difficile (atoxigenic)	T=10, D=90	6.501	5.654	85.61	5.103	0.847	1.000
	T=10, D=120	6.501	5.799	80.00	5.103	0.701	1.000

Key: Bacterial challenge: the identity of the bacteria used as the challenge. Time profile: The time (in minutes) of H_2O_2 treatment and minimum dwell time (the time between end of treatment and processing for CFU analysis by Miles and Misra plate counting. Log_{10} CFU not treated: the CFU of the test sample without H_2O_2 treatment expressed in Log_{10} units. Log_{10} CFU treated:

the mean CFU of the treated test samples (n=3) following treatment with H_2O_2 expressed in Log_{10} units. % reduction: the reduction in CFU post treatment with H_2O_2 expressed as a percentage of the CFU for the untreated test sample. Maximum detectable Log_{10} reduction: the maximum detectable Log_{10} reduction based on the titre of the test challenge. Log_{10} CFU reduction: the reduction in CFU post treatment with H_2O_2 compared to the CFU of the untreated test sample expressed as Log_{10} reduction. Post treatment survival: A score based on the presence or absence of bacterial growth following treatment. One point was awarded for growth and 0 points awarded for no growth. Based on the 3 replicates the possible scores were 1 – all three replicates exhibited growth; 0.66 – two of the three replicates exhibited growth; 0.33 – one of the three replicates exhibited growth; and 0 – none of the three replicates exhibited growth.

Table 4: Disinfection efficacy of SanoFog and SanoStatic against commercially available spore strips

Bacterial challenge	Disinfection method	Incubation time	Spore strip status	Post treatment survival
G. sterothermophilus	Fogging	24	Closed	1.000
			Open	0.000
		168	Closed	1.000
			Open	1.000
	Spray	24	Closed	0.000
			Open	0.000
		168	Closed	0.333
			Open	0.000
B. atropheus	Fogging	24	Closed	0.333
			Open	0.000
		168	Closed	0.667
			Open	0.667
	Spray	24	Closed	0.000
			Open	0.000
		168	Closed	0.333
			Open	0.000

Key: Bacterial challenge: the identity of the bacteria used as the challenge. Disinfection method: SanoFog (treatment time = 5 minutes, dwell time = 90 minutes) or SanoStatic (treatment time = 6 minutes, dwell time = 90 minutes). Incubation time: the time in hours that the tubes containing the spore strips were incubated for. Spore strip status: Spore strips were either 'closed' in their primary packaging or 'open' – aseptically removed from their primary packaging. Post treatment survival: A score based on the presence or absence of bacterial growth following treatment. One point was awarded for growth and 0 points awarded for no growth. Based on the 3 replicates the possible scores were 1 – all three replicates exhibited growth; 0.66 – two of the three replicates exhibited growth; 0.33 – one of the three replicates exhibited growth; and 0 – none of the three replicates exhibited growth.