Impact of a dry inoculum deposition on the efficacy of copper-based antimicrobial surfaces

McDonald, M. [1], Wesgate, R., [1] Rubiano, M. [1], Holah, J. [2], Denyer, S.P. [3], Jermann C.[4] and Maillard J.-Y. [1*]

[1] Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, Wales, UK

[2] Holchem Laboratories Ltd, UK

[3] University of Brighton, Cockcroft Building, Brighton UK

[4] GNT UK Ltd, Derby, UK

Running Head: Efficacy of copper surface against dried bacterial inocula

*Corresponding author

Prof. Jean-Yves Maillard

Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Redwood Building, King Edward VII avenue, Cardiff CF103NB, Wales, UK

Tel: +44(0)2920979088

Email: maillardj@cardiff.ac.uk

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ABSTRACT

The introduction of antimicrobial surfaces into healthcare environments is believed to impact positively on the rate of healthcare-associated infections by significantly decreasing pathogen presence on surfaces. We report on a novel efficacy test that uses a dry bacterial inoculum to measure the microbicidal efficacy of antimicrobial surfaces. An aerosolised dry inoculum of *S. aureus* or *A. baumannii* was deposited on copper alloy surfaces or a hospital-grade stainless steel surface. Surviving bacteria were enumerated following incubation of the inoculated surfaces at an environmentally relevant temperature and relative humidity (RH). Damage caused to bacteria by the aerosolization process and by the different surfaces was investigated. Dry inoculum testing showed a <2 $\log_{10}$ reduction in *S. aureus* or *A. baumannii* on the copper alloy surfaces tested after 24 h at 20°C and 40% RH. Potential mechanisms of action coper included membrane damage, DNA damage and arrested cellular respiration. The aerosolization process caused some damage to bacterial cells. Once this effect was taken into account, the antimicrobial activity of copper surfaces was evident. Our test provides a realistic deposition of a bacterial inoculum to a surface and as such a realistic protocol to assess the efficacy of dry antimicrobial environmental surfaces *in vitro*.

INTRODUCTION

It has been estimated that 300,000 patients a year acquire a healthcare associated infection (HAI) in England alone [1]. The morbidity and mortality of HAI has been well reported [1,2]. The cost of HAI to the NHS alone has been estimated to be around £1 billion a year [1], although this figure is likely an underestimate as currently there is not a reliable framework for economic evaluation [2]. Antimicrobial surfaces potentially have an important role in infection control in clinical settings by controlling surface bioburden and reducing cross-contamination [3-6]. Microbial contaminants are deposited on surfaces with direct contamination from blood, faeces vomit etc, but also in the form of small droplets which dry readily, for example following sneezing, or in a dry state usually associated with small particles (e.g. dust particles, skin squames). The difference in microbial deposition on surfaces, through contaminated soiling or dry particles, is particularly pertinent whether a biocidal product or an antimicrobial surface is used [7-9].

The standard test used by the majority of manufacturers for determining the activity of an antimicrobial surface is the ISO22196:2010 [10], which derives from the Japanese Industry Standard Z 2801:2010. These tests rely on exposing a surface to an inoculum under 100% humidity for 24 h at 37°C. The performance of antimicrobial surfaces under JIS Z 2801 test conditions does not guarantee efficacy *in situ* where temperature and RH are lower [11]. There are currently no accepted international standard test methods for testing the efficacy of surfaces against a dry microbial inoculum. A number of researchers have attempted to develop a ‘dry’ inoculum surface efficacy tests with reduced inoculum volume to assist faster drying [12-14]. In this context, O’jeil et al. [11] reported the use of a microbial aerosol containing small droplets deposited on surfaces to reduce drying time. These protocols however still involved aqueous suspension and it is accepted that antimicrobial surfaces under wet conditions perform better [15].

The antimicrobial efficacy of copper surfaces has been established *in vitro* and *in situ* and their impact in decreasing microbial bioburden from surfaces in healthcare settings has been observed, with some studies claiming a decrease in HAI [16]. It has also been suggested

that copper surfaces enhance the activity of some disinfectants commonly used in healthcare settings [17]. Unlike antimicrobial coatings, copper surfaces should provide sustained antimicrobial activity, even with surface damage (e.g. scratches abrasions) sustained by repeated surface cleaning and disinfection [18]. The majority of in vitro studies have investigated the effectiveness of copper against a “wet” bacterial inoculum [19-26]. Rarely do studies investigated the efficacy of copper against a dry bacterial inoculum [14]. The aim of this study was to evaluate a new test method for depositing a dry bacterial inoculum on antimicrobial surfaces to better mimic dry surface conditions usually found in healthcare settings.

MATERIALS AND METHODS

Bacterial strains

Staphylococcus aureus NCIMB 9518 and *Acinetobacter baumannii* NCIMB 9214 were grown in tryptone soya broth (TSB) for 24 h at 37°C and 25°C, respectively. Suspensions were centrifuged for 15 min at 2500 *g* at room temperature. The supernatant was discarded, and the remaining pellet were re-suspended in Maximum Recovery Diluent (MRD; 8.5 g/L sodium chloride, 1 g/L peptone) to provide a bacterial concentration of 10^9 cfu/mL.

Antimicrobial surfaces

Stainless steel discs (ø 2 cm) of grade 2B finish (control), were obtained from Goodfellows Cambridge Ltd (Huntington, UK). All discs were immersed in 5 % v/v Decon90 (Decon Laboratories Limited, Hove, UK) in deionised water for 60 min, rinsed, dried then autoclaved before use.

A number of copper alloys (22 mm X 22 mm) containing different concentrations of copper were provided by the Copper Disinfectant Association (Hemel Hempstead, UK) (Table I). After receipt and following testing, surfaces were disinfected by immersion in 70% v/v ethanol, dried, then stored in a sterile Petri dish to prevent contamination. Control

experiments confirmed that disinfection by 70% ethanol was sufficient to ensure that surfaces were fully decontaminated (data not shown).

	Code	Composition					
		% Cu	% Zn	% Sn	% Ni	% Fe	% Mn
Copper	CuDHP	99.99					
Bronze	CuSn5	95		5			
Copper/nickel	CuNi10Fe1Mn	86-89.7			9.0-11.0	1.0-2.0	0.3-1.0
Brass	CuZn30m	70	30				

Table I Composition of copper alloys used in this study

Protocol development and validation of a dry bacterial inoculum aerosolization process

Test set-up

A nebuliser (Philips Respronics, Best, The Netherlands) was used to aerosolise the bacterial suspension as described in [11]. The nebuliser was connected to a 23 cm stainless steel tube wrapped with a 2.4 m heating tape (HT9 Fibre Glass Heating Tape, Electrothermal, Essex, UK) connected at the other end to an Andersen cascade impactor (Westech Instrument Services Ltd, Henlow, UK). Five silica bead sachets were placed in the cascade under the collecting plate and one sachet was placed in the centre of the collecting plate to overcome problems of condensation as a result of the nebulisation. In addition, the cascade impactor was held at 40°C for up to one hour before testing to help prevent further build-up of condensation within the cascade during the dry inoculum deposition period (Figure 1). The heated tape was maintained with a digital temperature controller (MC810B Digital Heating Controller, Electrothermal, Essex, UK) at 70°C during the process to dry the aerosols passing through the stainless steel tube until their deposition on to the sample surfaces

placed on the collecting plate. Temperature and RH conditions on surfaces were determined using a S154TH temperature and RH probe. A vacuum pump (Fisherbrand, Loughborough, UK) was connected to the impactor. A Copley Scientific DFM2000 (Nottingham, UK) flow meter was placed between the cascade impactor and the vacuum pump (Figure 1). The test arrangement delivered an average flow rate of 2.18 ± 0.54 L/min. The procedure was carried out in a class 2 microbiological safety cabinet.

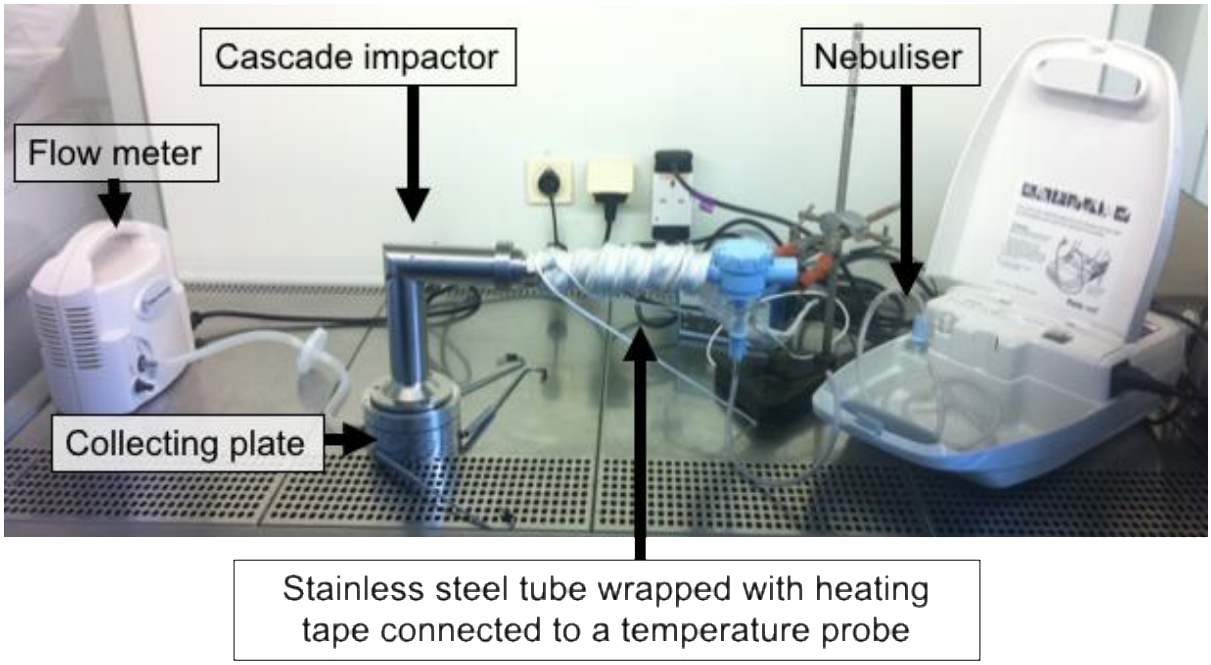

Figure 1 Nebulising test arrangement

Microbial aerosol testing

Ten mL of bacterial culture re-suspended MRD were nebulised over a period of 30 min to deposit over a total of at least one control stainless steel discs and five test copper alloy surfaces randomly placed on the collecting plate. Each test surface was assigned a number and random generator (https://www.random.org/lists/) was used to ascertain a clockwise position on the collecting plate. Test surfaces were placed equidistant to each other on the edge of the collecting plate, away from the centre. Following deposition, inoculated surfaces

were individually transferred to separate 100 mL bottles containing 1 mL neutraliser (3 g/L lecithin, 30 mL/L Tween 80, 5 g/L sodium thiosulphate, 1 g/L L-histidine, 10 mL phosphate diluent (34 g/L K_2HPO_4) and 30 g/L saponin in 1 L deionised water), 9 mL MRD and 5 g of 3mm sterile glass beads. Bottles were then placed on a shaking platform for 1 min then left to stand for 5 min. Viable bacteria were determined by serial dilution and spread plating onto a tryptone soya agar (TSA) plate in triplicate and incubated for 24 h at 37°C.

In subsequent experimental runs, surfaces were incubated for 30 min, 60 min and 24 h at 20°C-40% RH after the deposition of dried inoculum, with timing started immediately after the 30 min nebulisation. Temperature and RH were chosen as the most realistic incubation conditions reflective of an indoor hospital environment [11]. Bacterial viability post-incubation was determined per surface as described above. Each surface and contact time were tested in triplicate.

Both neutraliser toxicity test against the test bacteria and neutraliser efficacy tests were performed. For the neutraliser toxicity test, 1 mL of a bacterial inoculum (approx. 10^9 cfu/mL) was added to 9 mL of neutraliser or water. After 5 min contact time, the suspension was serially diluted and viable bacteria enumerated as described above. For the neutraliser efficacy test, 50 µL of 10^8 cfu/mL of bacterial inoculum and 50 µL of neutraliser were mixed and inoculated on to CuDHP. Water was used as a control in place of the neutraliser for each test. After 30 min exposure surfaces were transferred to a 100 mL bottle containing 5 g glass beads and 10 mL MRD, vortexed and bacterial suspension serially diluted and enumerated as described above.

Efficacy of antimicrobial surfaces against a dried microbial inoculum using a small (1 µl) drop inoculum

A modified method described by Warnes et al. [27] that aimed to simulate dry-touch contamination was carried out with *S. aureus* only. Briefly, *S. aureus* NCIMB 9518 was

prepared as described above but re-suspended in 500 µL MRD (yielding approximately 10^{10} cfu/mL). One µL was inoculated and spread evenly using the end of the pipette tip onto control stainless steel and test copper alloy surfaces. The inoculum was bench dried within 5 sec; no visible moisture was apparent on the surface and surfaces incubated for 30 min, 60 min and 24 h at 20°C and 40% RH. After incubation, surfaces were transferred to a 100 mL bottle containing 9 mL MRD, 1 mL neutraliser and 5 g of 3 mm glass beads. Bottles were placed on a shaking platform for 1 min then left to stand for 5 min. Viable bacteria were determined as described above. Each surface and contact time were tested in triplicate. Temperature and RH conditions on surfaces were determined using a S154TH temperature and RH probe. The probes were set to record temperature and RH at 0 min (before inoculation), at 1.5 min post-inoculation and removed after 5 min. Measurements were recorded every 30 sec, which was the minimum time interval possible.

Impact of combining nebulisation and drying on bacterial health using fluorescence-activated cell sorting (FACS) analysis

FACS analysis was used to determine the impact of combining nebulisation and drying on bacterial health. Propidium iodide (PI) and Bis (1,3-dibarbituric acid) trimethine oxanol (BOX) (both from Sigma-Aldrich, Poole, UK) were used in combination to assess membrane damage and changes in membrane potential. PI stains the DNA of cells with damaged membranes and BOX stains cells with collapsed membrane potentials. PI was prepared in sterile water at 200 µg/mL and used at a working concentration of 5 µg/mL. BOX was prepared in dimethyl sulfoxide (DMSO) at 10 mg/mL and diluted in phosphate buffered saline (PBS) to a working concentration of 10 µg/mL. One hundred µL of ethylenediaminetetraacetic acid (EDTA) at 4 mM was added to 9.9 mL working concentration of BOX to assist with the staining of cells.
5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Sigma-Aldrich, Poole, UK) was used to detect respiring cells. During electron transport respiring cells reduce CTC to insoluble formazan,

which fluoresces. SYTO9 (Invitrogen, UK) was used to assess DNA damage in cells. SYTO9 stains cells with intact DNA. CTC was prepared in sterile water at a stock concentration of 100 mM and used at 5 mM. SYTO9 was prepared at 5 mM in DMSO and diluted in sterile water for use at 5 µM. These two dyes were used in combination.

Following nebulisation of *S. aureus* or *A. baumannii*, bacteria were recovered from surfaces as described above immediately after nebulisation (0 h). The resulting 10 mL mixture was then divided into 2 x 5 mL and transferred to Universal tubes. Tubes were then centrifuged at 2500 *g* for 10 min. One 5 mL test sample was re-suspended in 500 µL phosphate buffered saline (PBS) for PI and BOX staining. The other 5 mL test sample was re-suspended in 500 µL sterile water for CTC and SYTO9 staining. Fifty µL of each sample was added to 1 mL FACS Flow buffer (BD, UK) and the dyes were added at the required concentration.
PI and BOX were added together and left for 5 min in the dark before FACS analysis using a FACS ARIA II (BD, UK). CTC was added first to samples which were incubated for 90 min in the dark at 37°C before SYTO9 was added. Samples were further incubated for 30 min in the dark at room temperature before FACS analysis.
Concurrently, cultures of *S. aureus* and *A. baumannii* were incubated in 10 mL tryptone soya broth for 24 h at 37°C. One mL of each culture was centrifuged for 1 min at 13,000 *g* and re-suspended in 500 µL of 100 % ethanol for 10 min, or in 500 µL of PBS for heat shock treatment at 100°C for 30 min, or in 500 µL of 10 % hydrogen peroxide (H_2O_2) for 30 min. Ethanol, heat shock and H_2O_2 treatment of bacteria were used as positive controls to show membrane damage, arrested cellular respiration or DNA damage.
After each treatment the bacterial suspension was centrifuged for 1 min at 13,000 *g* and the pellet re-suspended in 500 µL PBS for PI and BOX staining or 500 µL sterile water for CTC and SYTO9 staining. Incubation in dyes was conducted as described above. As a negative control, a sample containing just bacterial cells re-suspended in PBS (no treatment) was treated with the dyes.

Cell samples were loaded individually into the FACS Aria II illuminated with a 488 nm laser and data from 10,000 particles were collected. Fluorochromes already calibrated to the FACS Aria II were used. PI fluorescence (red) was collected at an excitation-max 482 nm/emission-max 678 nm and BOX fluorescence (green) was at an excitation-max 494 nm/emission-max 519 nm. CTC fluorescence (green) was collected at an excitation-max 494 nm/emission-max 519 nm and SYTO9 fluorescence (yellow) at an excitation-max 496 nm/emission-max 578 nm. Each experiment was repeated twice on different occasions.

Statistical analysis

All data are expressed as mean and standard deviation. Data were transformed (natural log + 1) and a statistical analysis was carried using SPSS software. A General Linear Model or chi-squared tests were used to analyse data statistically.

RESULTS

Antimicrobial efficacy of surfaces against a dried microbial inoculum using the new test method

The neutraliser used was not toxic against either test bacteria (no difference in bacterial number between the water control and the neutraliser test; p=57 for *S. aureus* and p=0.133 for *A. baumannii*; results not shown). For the neutraliser efficacy test, there was less <1 $\log_{10}$ reduction in bacteria following exposure to CuDHP for 30 min when the neutraliser was used, whilst a >1 $\log_{10}$ reduction was observed when water was used (data not shown).

Dry *S. aureus* bacterial recovery from stainless steel averaged 7.02 ± 0.28 $\log_{10}$ cfu/cm^2 after the 30 min nebulisation (0 h). In contrast, recovery from copper alloy surfaces was 1.61-1.85 $\log_{10}$ cfu/cm^2 lower (Table II). After 30 min contact time at 20°C and 40% RH, there was no difference in bacterial viability between any of the copper alloys and stainless steel (Table II).

After 60 min incubation only CuDHP and CuZn30 produced a >1 log_{10} reduction in viability which was significantly greater ($P<0.001$) than the stainless steel control. After 24 h, all copper alloys except for CuSn5 achieved a >1.5 log_{10} reduction in *S. aureus*. CuSn5 did not performed as well as the other copper alloys ($P<0.001$). None of the copper surfaces achieved 2 log_{10} reduction, discounting the initial bacterial reduction following nebulisation. There was no difference ($P<0.001$) in *S. aureus* recovery from stainless steel disks between the different time points.

Exposure time	Stainless steel	CuDHP	CuSn5	CuNi10Fe1Mn	CuZn30
	Deposition Log_{10} cfu/cm^2				
0 h	7.02 ± 0.28	5.37 ± 0.40	5.20 ± 0.50	5.17 ± 0.21	5.41 ± 0.15
	Log_{10} reduction cfu/cm^2				
30 min	0.45 ± 0.13	0.34 ± 0.20	0.53 ± 0.09	0.33 ± 0.22	0.43 ± 0.40
60 min	0.56 ± 0.19	1.24 ± 0.20	0.99 ± 0.07	0.73 ± 0.45	1.19 ± 0.28
24 h	0.70 ± 0.38	1.67 ± 0.21	1.29 ± 0.11	1.59 ± 0.10	1.80 ± 0.22

Table II. Recovery of viable *S. aureus* after 30 min deposition on surfaces (0 h) and reduction in viability of a dried inoculum deposited on surfaces after 30 min, 60 min and 24 h incubation at 20°C and 40% RH (n = 3)

Recovery of *A. baumannii* dried inocula from stainless steel averaged 5.28 ± 0.25 log_{10} cfu/cm^2 at 0 h. Recovery from all copper alloy surfaces was lower, ranging from 3.38 ± 0.03 to 4.07 ± 0.23 log_{10} cfu/cm^2 at 0 h (Table III). After incubation at 20°C and 40% RH, there was no difference in copper alloys performance. Reductions in bacterial viability were marginal between them. Of note, a 24 h incubation was necessary to achieve at least a 1 log_{10} reduction in cfu/ cm^2, discounting the initial decrease in bacterial viability during nebulisation.

Exposure time	Stainless steel	CuDHP	CuSn5	CuNi10Fe1Mn	CuZn30
	Deposition Log_{10} cfu/cm^2				
0 h	5.28 ± 0.25	3.67 ± 0.67	3.38 ± 0.03	4.07 ± 0.23	3.83 ± 0.39
	Log_{10} reduction cfu/cm^2				
30 min	0.39 ± 0.72	0.75 ± 0.11	0.48 ± 0.34	0.52 ± 0.47	0.90 ± 0.15
60 min	0.59 ± 0.75	1.03 ± 0.44	0.38 ± 0.11	0.64 ± 0.10	0.90 ± 0.25
24 h	0.99 ± 0.93	1.46 ± 0.59	1.01 ± 0.38	1.44 ± 0.25	1.25 ± 0.05

Table III. Recovery of viable *A. baumannii* after 30 min deposition on surfaces (0 h) and reduction in viability of a dried inoculum deposited on surfaces after 30 min, 60 min and 24 h incubation at 20°C and 40% RH (n = 3)

Antimicrobial efficacy of surfaces against a dried microbial inoculum using a small (1 µL) drop inoculum

All copper alloys showed a >4 log_{10} cfu/cm^2 reduction within 30 min incubation at 20°C and 40% RH (Table IV). These results contrasted dramatically with the results observed post-nebulisation. There were no differences ($P<0.001$) in activity between any of the copper alloys. All copper alloys performed significantly better than the stainless steel surface control which only showed a 0.79 ± 0.23 log_{10} cfu/cm^2 reduction after 24 h.

This test relies on the deposition of a wet, albeit small, inoculum on a surface, intending that the inoculum will dry rapidly. The average RH of the surfaces before inoculation was 58 ± 2 %. Within 30 seconds, RH increased to 67 ± 11%. Over the 5 min recording period, the average RH was 75 ± 8%. Surface temperature did not significantly change upon and after deposition of the inoculum on the surface. The recorded surface temperature decreased from 24.6 ± 0.5°C to 24.1 ± 0.5°C during the 5 min recording period.

Exposure time	Stainless steel	CuDHP	CuSn5	CuNi10Fe1Mn	CuZn30
	Deposition Log_{10} cfu/cm^2				
0 h	6.96 ± 0.17	6.60 ± 0.09	6.50 ± 0.31	6.85 ± 0.04	6.14 ± 0.45
	Log_{10} reduction cfu/cm^2				
30 min	0.51 ± 0.06	5.33 ± 0.74	>5.66 ± 0.00	4.41 ± 0.79	4.77 ± 0.92
60 min	0.50 ± 0.17	5.76 ± 0.00	>5.66 ± 0.00	5.30 ± 0.21	4.72 ± 0.50
24 h	0.79 ± 0.23	5.76 ± 0.00	>5.66 ± 0.00	5.28 ± 0.68	5.30 ± 0.00

Table IV. Recovery of viable *S. aureus* after 1 µL deposition and 0 h and log_{10} reduction in viability after 30 min, 60 min and 24 h incubation at 20°C and 40% RH (n = 3)

Impact of combining nebulisation and drying on bacterial health

For each surface and dye combination an average percentage of cells per FACs was calculated. The impact of the protocol combining nebulisation and drying of *S. aureus* for 30 min on stainless steel surface resulted in half the cell population being categorised as healthy (PI^- BOX^-); the remainder of the cells showed either a collapsed membrane potential or membrane damage as indicated by a combination of PI and BOX (Table V). Perhaps not surprisingly, the percentage of healthy cells recovered from copper alloy surfaces after 30 min nebulisation was lower compared to stainless steel surfaces (Table V).

The percentage of cells with intact DNA and with unaffected cellular respiration (CTC^+ $SYTO9^+$) remained constant on the copper alloys over time but increased on the stainless steel surfaces (Table V).

A. baumannii was more significantly affected by combining nebulisation and drying (Table V). Similar to the results observed with *S. aureus*, fewer *A. baumannii* cells suffered from

DNA damage and arrested respiration at the end of the nebulisation process ($CTC^-/SYTO9^-$) compared to membrane damage or collapsed membrane potential (PI^-/BOX^+).

There was a degree of variability in some of the results between the 2 repeats performed, especially with the cells treated with CTC/SYTO9 and as such these results are to be considered indicative only.

Surface	PI^- BOX^- (healthy)	PI^+ BOX^+ (damaged membrane)	PI^- BOX^+ (collapsed membrane potential)	CTC^+ $SYTO9^+$ (healthy)	CTC^- $SYTO9^-$ (damaged respiration or DNA)
S. aureus			% proportion of cells		
SS	50	23	34*	51*	42*
CuSn5	30*	42*	26	63*	35*
CuZn30	35	35	27	68*	32*
A. baumannii			% proportion of cells		
SS	31	17	24*	49*	50*
CuSn5	19*	34*	23	45*	55*
CuZn30	31	31	26	30*	70*

SS: stainless steel

Table V. Summary of PI/BOX and CTC/SYTO results immediately after nebulisation. Average percentages are highlighted with a * when repeats 1 and 2 showed variability; under these circumstances the proportion of cells does not sum to precisely 100%.

DISCUSSION

With our current understanding of the role of surfaces in harbouring and transferring pathogens, antimicrobial surfaces may play a role in reducing acquired infections. To successfully evaluate these however, there is a need for a surface efficacy test representing conditions found on environmental surfaces in healthcare settings. To date tests using a dry bacterial inoculum have not been widely reported. Here, we report on a new method combining nebulisation and drying to deposit a dry bacterial inoculum onto a surface, providing a more accurate way to test for antimicrobial surface efficacy in dry conditions. Using a dry inoculum deposited on various copper alloy surfaces, we observed less than 2 $\log_{10}$ reduction in bacterial viability following a 24 h incubation at 20°C and 40% RH (Tables II & III), not considering the initial bacterial reduction on copper alloys during the 30 min nebulisation. There was no evidence that the copper concentration (ranging from 70 to 99.99% Cu) impacted on the antimicrobial effect against *S. aureus* or *A.* baumannii. These results contrast with data on the efficacy of copper alloys where the methodologies used wet bacterial inocula. O'Jeil and colleagues [11] observed that, using a similar protocol to this study, but instead depositing a wet aerosolised inoculum on surfaces, a 2 $\log_{10}$ reduction in *S aureus* on copper alloy surfaces with 30 min incubation at 20°C and 40% RH and a 4 $\log_{10}$ reduction within 24 h incubation under these same conditions [11]. The efficacy of copper alloys against *A. baumannii* deposited as a wet aerosol was rather better with a 4 $\log_{10}$ reduction observed with 30 min incubation at 20°C and 40% RH [11]. Santo et al. [12] used a wet inoculum deposited on surfaces with a moistened swab and recorded a 9 $\log_{10}$ reduction against *E. coli* within a 1 min contact time at 23°C. Warnes and Keevil [13] used a 1 µL drop inoculum with the purpose of a rapid drying (i.e.<30 seconds) on the surface. They reported a 6 $\log_{10}$ reduction for vancomycin-resistant Enterococci within 10 min contact at 22°C. In our current study, using the 1 µL drop inoculum protocol, we observed a >4 $\log_{10}$ reduction in *S. aureus* within 30 min incubation at 20°C and 40% RH (Table IV). Similarly, Eser et al. [14] reported a > 3 $\log_{10}$ reduction in *S. aureus* on copper surfaces after < 1 h

contact time (20-25°C), using a 10 µL drop inoculum. It is clear that a rapid and extensive bacterial inactivation on copper surfaces will occur when the inoculum is wet even for a short period of time (i.e. < 30 seconds). The presentation of a dry inoculum reduces dramatically the antimicrobial efficacy of these surfaces. Our results are in the same order of magnitude as those obtained from clinical trials, during which copper alloys have been shown to produce a 1-2 $\log_{10}$ reduction in bacterial bioburden over time [28]. These results suggest that in the dry state copper oxide is responsible for antimicrobial efficacy rather than copper ions. Cupric oxide (CuO) which is the common copper oxide formed under wet conditions has been shown to be less antimicrobial against *Enterococcus hirae* than cuprous oxide (Cu_2O) that forms normally under dry, ambient conditions [29]. Santo et al. [12] proposed that the observed antimicrobial activity of copper surfaces following the deposition of a wet *E. coli* inoculum derived from copper oxidation releasing copper ions, which in turn contribute to generating reactive oxidative species, in particular hydroxyl radicals.

The main drawback of our protocol is the time taken to deposit a high concentration of dry inoculum on the test surfaces. The physical conditions inside the cascade impactor during the 30 min nebulisation showed an average of 34.0 ± 1.2 °C and 57 ± 11 % RH. The RH rose slowly with incubation time, reaching 60% after 5 min and 70% after 30 min (data not shown). These conditions could enhance the efficacy of antimicrobial surfaces, and indeed we observed a 1-2 $\log_{10}$ reduction difference in bacterial concentration between stainless steel surfaces and the copper surfaces immediately following deposition (Tables II & III). Although, no condensation, moisture or water droplets were visible after the 30 min nebulisation, we cannot exclude that rising RH contributed to enhancing the activity of copper. Nevertheless, when one compares the dry aerosolised inoculum with the 1 uL drop protocol, the differences in *S. aureus* inactivation on CuDHP (24 h, 20°C and 40% RH) is astounding and such difference in results appear to be attributed to the dryness status of the initial inoculum and possibly the RH during the test (Table VI).

Table VI. Comparison of the 1 μL dried inoculum method with the nebulised dry bacterial aerosol protocol. Both protocols used *S. aureus* NCIMB 9518

	Initial inoculum size	Deposition	Temperature	RH	Inoculum (Log_{10} CFU/mL ± SD) recovered from control surface after deposition[1]	CuDHP efficacy (Log_{10} reduction in viability ± SD)[2]
Nebulised dry aerosol	10^9 cfu/mL	30 min nebulisation	34.0°C	RH increased to 60% after 5 min and to 70% after 30 min	7.02 ± 0.28	1.67 ± 0.21
1 μL dried inoculum	10^{10} cfu/mL	1 μL deposition; drop dried within 5 min	24.6°C	RH increased to 67% after 30 sec and 75% after 5 min	6.96 ± 0.17	5.76 ± 0.00

[1] Control surface: stainless steel coupon

[2] Log_{10} reduction observed on CuDPH after 24 h at 20°C and 40% RH (n=3); data from Tables II and IV

We observed that damaged bacteria following nebulisation showed arrested respiration, collapsed membrane potential and membrane damage (Table V). The percentage of bacteria presenting these damages were generally higher on copper alloy surfaces but did not correlate quantitatively with the viability data. In *A. baumannii*, arrested respiration and DNA damage seemed more prominent than membrane damage. Warnes and Keevil [13] suggested that DNA damage and arrested cell respiration are the initial stages of cell death, followed by membrane damage in Gram-positive bacteria. In Gram-negative bacteria, membrane depolarisation in advance of DNA damage has been reported following exposure to copper [27].

Our study confirms the bactericidal efficacy of copper surfaces and demonstrates the importance of moisture in the development of that action. Our results clearly suggest that the presence of liquid, even as part of a suspending medium and even in small amount and for a brief contact time (< 30 sec), significantly increases the antibacterial efficacy of a copper surface and could compromise the laboratory evaluation of such surfaces when intended for use in the dry state. We offer a novel method for depositing a dry inoculum onto dry surfaces more closely representing environmental surface conditions in healthcare settings. We recognise that, even under these most carefully controlled conditions, bacterial damage can occur immediately following deposition from the nebulisation process onto copper but this can be accurately accounted for. Our study questions the validity of protocols that use a liquid inoculum to determine the activity of antimicrobial surfaces that will be used in a dry environment and offers an alternative approach.

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CONFLICT OF INTEREST

None to declare

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