Evaluation of new *in vitro* efficacy test for antimicrobial surface activity reflecting UK hospital conditions

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A R T I C L E   I N F O

S U M M A R Y

Background: Antimicrobial surfaces aim to reduce microbial bioburden and improve hygiene. The current antimicrobial surface efficacy test (ISO22196) is an initial screening test but its conditions, high temperature (37 °C) and relative humidity (RH) (100%) bear little relationship to in-use conditions.
Aim: To develop an antimicrobial surface efficacy test providing a realistic second-tier test, simulating in-use conditions.
Methods: Surface relative humidity, temperature and soiling were measured over one year at a UK hospital, enabling realistic parameters to be set for our surface efficacy test. A nebulizer, connected to a cascade impactor, aerosolized and uniformly deposited a *Staphylococcus aureus* suspension over test copper alloys and control stainless steel surfaces. Bacteria were enumerated following nebulization, and after a range of contact times, under [20 °C, 50% RH] and [20 °C, 40% RH] parameters reflecting in-use conditions; [37 °C, 100% RH] was employed to reflect conditions used in ISO22196.
Findings: All copper alloys produced a $>4 \log_{10}$ reduction after 24 h under all conditions tested. Copper alloys were more effective at [37 °C, 100% RH] showing a $>4 \log_{10}$ reduction after 30 min than at in-use conditions [20 °C, 50% RH and 20 °C, 40% RH], for which 60 min was required to achieve the same level of kill, for most but not all alloys.
Conclusion: The use of the nebulizer to deposit bacterial inocula on surfaces showed little variability in results. Our method was more discriminatory than the ISO22196 enabling distinction between the bactericidal surface activity, which allows for a more rigorous selection of antimicrobial surfaces for potential use in healthcare settings.
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Introduction

It is estimated that healthcare-associated infections (HCAIs) cost the National Health Service around £1 billion annually.[1]

The hospital environment has been identified as an ideal 'reservoir' for micro-organisms.[2] A link between microbial surface contamination and infection was first identified in the 1960s and the role of surfaces in cross-contamination has now been well documented.[2–4] There is an interest in the potential role surfaces with antimicrobial properties could play to help reduce further the rate of HCAIs.[5] In terms of surface fittings in hospitals, stainless steel is the material of choice due to its durability and appearance. However, stainless steel does not

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possess any antimicrobial activity and studies have shown that meticillin-resistant *Staphylococcus aureus* (MRSA) can persist on dry inanimate surfaces for up to seven months, and that *C. difficile* spores can survive up to five months.[6] One of the major obstacles for the use of antimicrobial surfaces is the lack of evidence of their performance based on appropriate efficacy test protocols. Currently the test protocol of choice for testing surfaces with an antimicrobial claim is the Japanese Industry Standard, JIS Z 2801, recently also published as ISO22196.[7] Control and test surfaces are assessed against a liquid bacterial suspension, covered with a plastic film and incubated for 24 h at high temperature and under 100% humidity. Viable bacteria are enumerated at time 0 h from control surfaces and compared with counts after 24 h on both control and test surfaces. Many manufacturers utilize the test, which aims to maximize the opportunity for biocide diffusion from the material, on their products to confirm antimicrobial activity before making them available commercially. However, this test has been described as inappropriate since the parameters used (temperature, relative humidity, and presentation of the inocula) do not reflect conditions found in practice.

The aim of this study was to develop a new antimicrobial surface test to replace the JIS Z 2801 that evaluates the activity of antimicrobial surfaces under parameters reflective of conditions in healthcare settings.

Methods

Sample copper surfaces

Copper alloy surfaces; CuSn5, CuDHP, CuZn30 and CuNi10-Fe1Mn (see Table I for compositions) were kindly provided by the Copper Development Association (CDA, Hemel Hempstead, UK). These four alloys are currently registered by the US Environmental Protection Agency (EPA) as public health antimicrobial products. Surfaces measured 22 mm × 22 mm. Surfaces were not treated when received from the CDA. After testing surfaces were disinfected by immersion in 70% 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 not contaminated post disinfection.

Table I
JIS Z 2801 results: $\log_{10}$ reduction in *S. aureus* on copper alloy surfaces after 24 h at [37 °C, 100% relative humidity] relative to viable bacteria on stainless steel surfaces at 0 h ($N = 3$)

Surface	Surface composition	Log reductions (CFU/mL)
Stainless steel	C 0.08%, Mn 2%, P 0.045%, S 0.03%, Si 0.75%, Cr 18–20%, Ni 8–12%, N 0.1%, Fe balance	$0.31^{a} \pm 0.07$
CuSn5	Cu 95%, Sn 5%	$>5.11 \pm 0.00$
CuDHP	99.90% minimum, P 0.015–0.040%	$>4.91 \pm 0.35$
CuZn30	Cu 70%, Zn 30%	$>5.11 \pm 0.00$
CuNi10Fe1Mn	Cu 86–89.7%, Ni 9–11%, Fe 1–2%, Mn 0.3–1%	$>4.85 \pm 0.00$

[a] Denotes an increase in number rather than a $\log_{10}$ reduction.

Hospital sampling

A number of factors, including surface relative humidity, temperature and soiling level, can affect the efficacy of microbicides; these factors need to be taken into account while performing efficacy tests. In order to gain information about these parameters, ward sampling of surfaces took place regularly at the University Hospital of Wales (UHW), Cardiff, UK over a one-year period.[8] The Protimeter MMS (GE Sensing, Taunton, UK) was used to measure surface relative humidity and surface temperature according to the manufacturer's guidelines. The 'hygrometer' mode of the device was utilized to measure surface relative humidity and its 'condensator' mode was used for surface temperature readings. The Protimeter also provided the user with dew temperature information and an indication of the risk of condensation on surfaces. The measurement of ATP using a SystemSURE Plus ATP Hygiene Monitoring System (Hygiena, Watford, UK) was used to estimate how clean the surfaces sampled were. The procedure was carried out according to the manufacturer's guidelines. The swabs used, Ultrasnap (Hygiena), contain a liquid-stable luciferase/luciferin agent that emits light in direct proportion to the amount of ATP collected on the swab. The manufacturer indicates the following 'pass, caution or fail' outcomes depending on the reading: <10 relative light units (RLU) indicates that the surface is clean (pass), 11–29 RLU (caution), and >30 RLU suggests that a surface is dirty (fail). Wards selected for sampling were gastroenterology and adult critical care (ACC). In addition, surfaces in one of the hospital's theatre rooms were sampled although it was not possible to sample the same theatre room on each occasion and we were unable to obtain results during October 2011. Surfaces sampled included door handles ($N = 2$), door push plates ($N = 2$), computer keyboard ($N = 1$), computer mouse ($N = 1$), trolleys ($N = 2$), light switches ($N = 1$), wall panels ($N = 2$), taps ($N = 1$), waste bin ($N = 1$), bed rails ($N = 3$), chairs ($N = 3$) and tables ($N = 2$). Operating machinery was additionally subject to sampling in theatre ($N = 1$). Sampling took place every two months over a one-year period to take into account seasonal variations.

JIS Z 2801: test for antimicrobial activity and efficacy of antimicrobial products

A modified JIS Z 2801 protocol was followed. Briefly, a tryptone soya agar (TSA) slope of *Staphylococcus aureus* (*S. aureus*) NCIMB 9518 was prepared and stored for a maximum of one month at 4 °C. A loopful of culture was transferred to a fresh slope and incubated at 37 °C for 24 h to make the first subculture. From this a further sub-culture was prepared to produce the working culture. On the day of testing, 9 mL maximum recovery diluent (MRD) (1 g/L peptone, 8.5 g/L sodium chloride) and 5 g, 3 mm glass beads were added to the slope to recover the working culture, which was adjusted to $\sim 10^{8}$ cfu/mL following OD_{600} measurements. The four copper alloy surfaces were tested together with stainless steel (Grade 2B finish; Goodfellow Cambridge, Huntingdon, UK) as a control. All surfaces were tested in triplicate. Each surface was inoculated with 50 µL of $\sim 10^{7}$ cfu/mL bacterial suspension and placed in a sterile Petri dish. To maintain high humidity the surfaces were covered with a plastic film. Test and control surfaces were incubated for 24 h at 37 °C and 100% relative humidity in a

plastic box containing a saturated solution of zinc sulphate to further maintain high humidity. The inoculum level at 0 h on the control surface and at 24 h on both the test and control surfaces was determined. Surfaces were transferred to a stomacher bag to which 9 mL MRD and 1 mL neutralizer [3 g/L lecithin, 30 ml/L Tween 80, 5 g/L sodium thiosulphate, 1 g/L L-histidine, 10 mL/L phosphate diluent (34 g/L K_2HPO_4), 30 g/L saponin all dissolved in 1 L deionized water; all from Oxoid, Basingstoke, UK] were added. The stomacher bag was massaged for 30 s by hand to recover the inoculum. One mL of solution was serially diluted to 10^{-4} in MRD and bacterial counts enumerated via the pour plate method. Plates were incubated for 24 h at 37 °C and the number of colony-forming units (CFU) determined. Log_{10} reductions were calculated by subtracting the 24 h log_{10} count on control and test surfaces from the 0 h log_{10} count of the control.

The efficacy of the neutralizer to quench the activity of Cu^{2+} was tested using a protocol described by Wheeldon et al.[9] The neutralization test was performed in triplicate.

Development of a new antimicrobial surface test based on surface exposure to bacterial aerosols

Test arrangement

A new antimicrobial surface test was developed based on surface exposure to bacterial aerosols. A Porta-neb Vent-Stream nebulizer (Philips Respironics, Best, The Netherlands) was used to generate the aerosol. The nebulizer was connected to an Andersen cascade impactor (Westech Instrument Services Ltd, Henlow, UK) of which only Stage 0 was used (Figure 1). Test copper alloy surfaces and control stainless steel discs were aligned around the edge of a large stainless steel collecting plate, which was placed over Stage F of the impactor. A vacuum pump (Fisherbrand, Loughborough, UK) was connected to the cascade impactor. The procedure was carried out in a class 2 microbiological safety cabinet. The test arrangement delivered an average flow rate of 4.84 L/min, measured by a DFM2000 flow meter (Copley Scientific, Nottingham, UK).

Microbial aerosol testing

Staphylococcus aureus NCIMB 9518 was grown in 10 mL tryptone soya broth for 24 h at 37 °C. The culture was centrifuged at 2500 g for 15 min, re-suspended and OD_{600} adjusted in MRD to give a bacterial concentration of 10^8 CFU/mL. MRD contains 1 g/L peptone which contributes to soiling on surfaces, particularly as this solution subsequently dries on the surface and becomes more concentrated. This concentration of peptone mixed with the bacterial inoculum corresponded to an ATP reading of 3910 RLU when dried on a stainless steel surface, although the variability in the measurement was high (data not shown).

The deposition of the microbial inocula on the stainless steel disc following nebulization was investigated and validated by viable count in a preliminary experiment that assessed the optimum volume and time of nebulization to ensure uniformity in the bacterial suspension deposited on the disc surface (data not shown). Following this validation process the following conditions were employed: 10 mL of the suspension was placed in the nebulizer and nebulized for 30 min at a flow rate of 4.84 L/min. After 30 min nebulization (labelled as 0 h), test and control surfaces were aseptically transferred individually to a bottle containing 9 mL MRD, 1 mL neutralizer and 5 g 3 mm glass beads. Bottles were shaken for 1 min at 150 rpm on a Grant Bio POS 300 rotating platform (Patterson Scientific, Luton, UK), then left to stand for 5 min. Viable bacteria were enumerated by serial dilution in MRD to 10^{-4}, plated via the spread plate method and incubated for 24 h at 37 °C. The remaining inoculated surfaces were placed in individual Petri dishes and incubated at various combinations of temperature, relative humidity (RH) and contact times. Surfaces were exposed to the following conditions: [37 °C, 100% RH], [20 °C, 50% RH] and [20 °C, 40% RH] for 30 min, 60 min and 24 h. Surfaces were incubated at [37 °C, 100% RH] for comparison to the JIS Z 2801. [20 °C, 50% RH] and [20 °C, 40% RH] were selected as additional incubation parameters to reflect conditions observed in practice at UHW.

For the JIS Z 2801 test, high humidity [37 °C, 100% RH] was maintained by covering the test surface with a plastic film. PROsorb™ silica gel cassettes (Conservation by Design Ltd, Bedford, UK) were used to stabilize relative humidity at 40% and 50%; no film was placed over the surfaces. Following the required contact time, viable bacteria were recovered and enumerated as described above. Each surface was tested in triplicate for each contact time, relative humidity and temperature. Log_{10} reductions were calculated by subtracting the 30 min, 60 min and 24 h count from the 0 h count of each
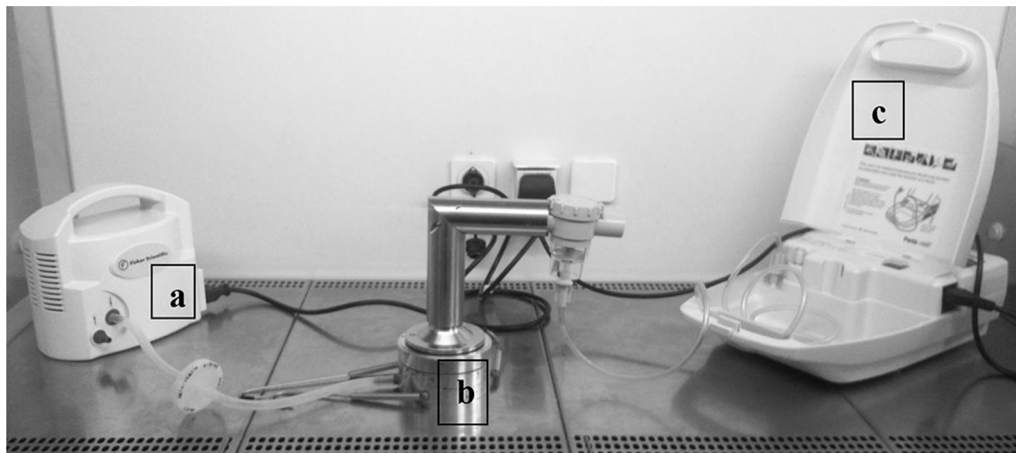

Figure 1. Arrangement of new antimicrobial surface test. (a) Vacuum pump, (b) Andersen cascade impactor, (c) Philips nebulizer.
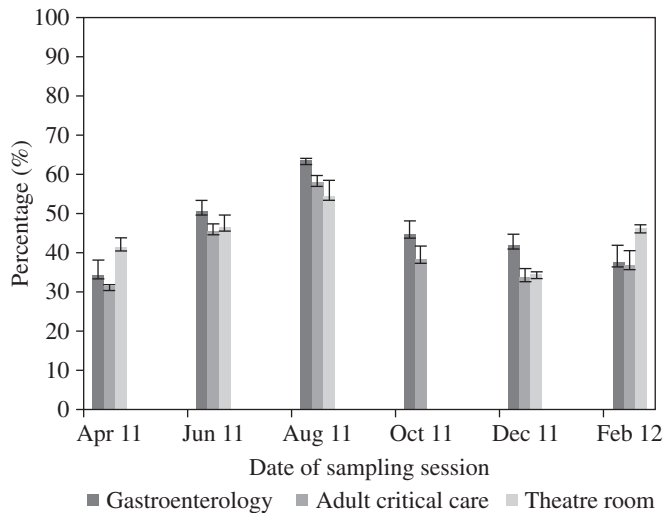
Figure 2. Surface relative humidity.

Figure 4. Surface soiling.

surface. The test was also performed under 'dirty' conditions where 3 g/L bovine serum albumin (BSA; final concentration) was added to the bacterial suspension and aerosolized.

Statistical analysis

In addition to the validation process mentioned above, the experiment was conducted on 36 separate occasions in clean conditions and on 24 separate occasions in dirty conditions. All data are expressed as mean and standard deviation; for some data such as the protimeter data the standard deviation represents the standard deviation of the readings from all surfaces sampled from each ward on each occasion. General linear models were carried out to compare grouped data using R version 3.0.1 software.
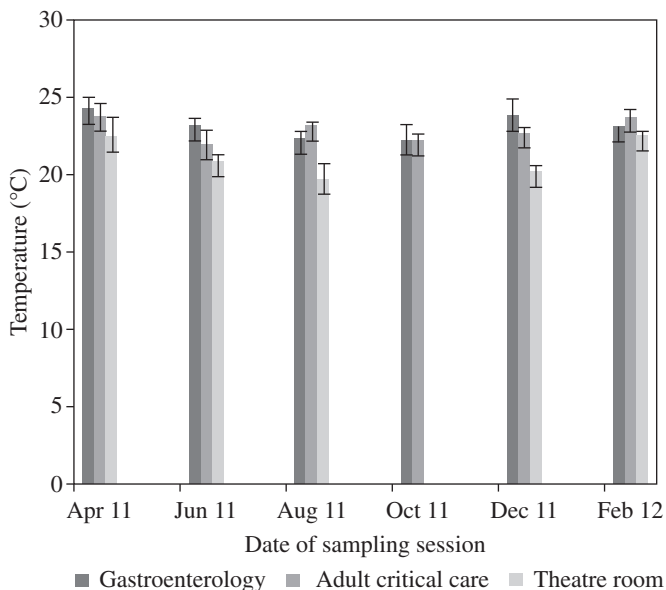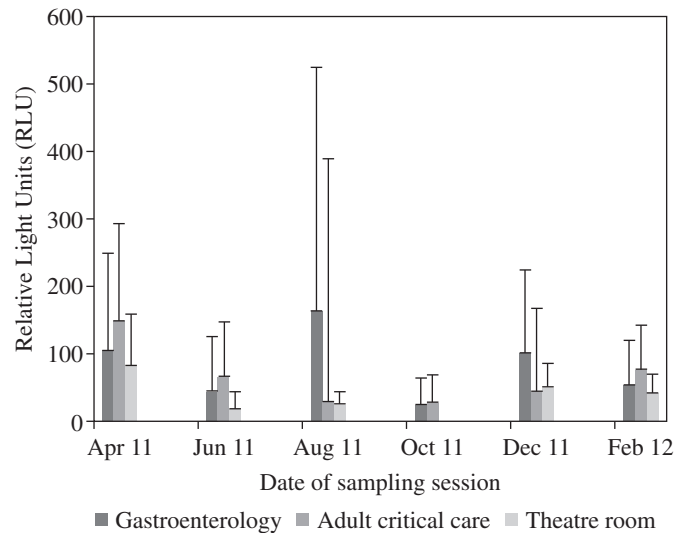

Figure 3. Surface temperature.

Results

Hospital sampling

The six sampling sessions provided a comprehensive data set regarding surface relative humidity, surface temperature conditions and surface cleanliness in a range of wards. The data are represented in three graphs (Figures 2—4). Relative humidity ranged from $31.4 \pm 0.5\%$ (ACC, April 2011) to $63.6 \pm 0.4\%$ (gastroenterology, August 2011). Surface temperature ranged from $19.74 \pm 1.0\,°C$ (theatre room, August 2011) to $24.3 \pm 1.3\,°C$ (gastroenterology, April 2011). Temperature difference data — how many degrees a surface is above or below the dew point temperature (not shown) — confirmed that all surfaces sampled were not at risk of condensation as all surface temperature differences exceeded their dew point by $>3\,°C$. ATP measurements varied greatly on each sampling occasion, as shown by the large error bars (Figure 4). The lowest ATP reading observed on several occasions was 0 RLU and the highest was 1465 RLU. Average ward RLU readings ranged from 19.43 ± 24.61 (theatre, June 2011) to 164.86 ± 358.98 (gastroenterology, August 2011).

JIS Z 2801 results

All copper alloy surfaces were bactericidal, producing a >4 $\log_{10}$ reduction in viable bacterial count (Table I) when incubated for 24 h at 100% humidity. The stainless steel control surfaces showed very little change in count after 24 h incubation, showing a mean $\log_{10}$ increase of 0.31 (Table I).

Antimicrobial surface test

The bactericidal efficacy of the test surfaces against *S. aureus* NCIMB 9518 is presented in Figures 5—9. After 30 min nebulization the amount of bacterial aerosols deposited on stainless steel discs was between 6.56 ± 0.05 and 6.71 ± 0.14 $\log_{10}$ CFU/cm^2. In contrast, the amount recovered from copper alloys at time 0 h was between 5.01 ± 0.38 and 5.96 ± 0.42 $\log_{10}$ CFU/cm^2. These results suggest that some antimicrobial
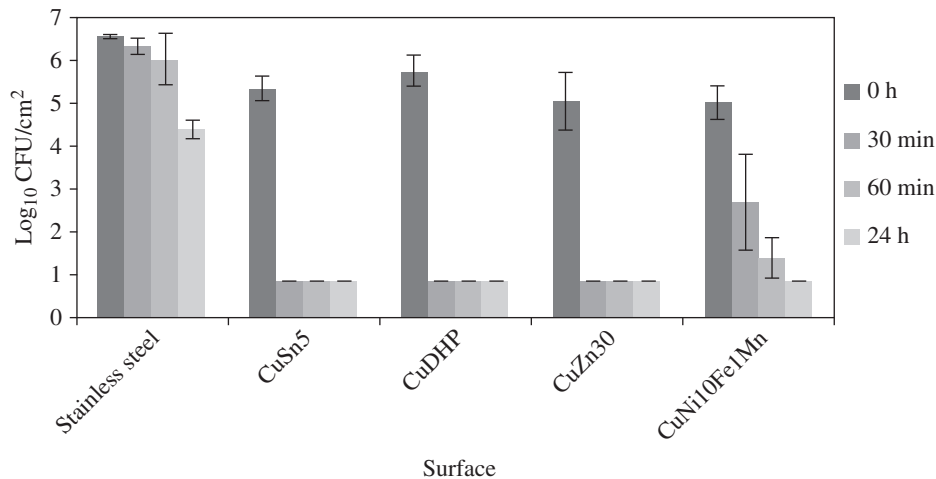
Figure 5. Recovery of *Staphylococcus aureus* from deposited aerosols at 0 h and $\log_{10}$ reduction after 30 min, 60 min and 24 h incubation at [37 °C, 100% relative humidity] ($N = 3$).

activity by the surfaces occurred during nebulization. The nebulization time of 30 min was adopted following test development and validation (data not shown).

All copper alloy surfaces showed a >4 $\log_{10}$ reduction in viable bacteria at all temperature and relative humidity conditions after 24 h. At [37 °C, 100% RH] a >4 $\log_{10}$ reduction was observed for CuSn5, CuDHP and CuZn30 after 30 min incubation (Figure 5). At [20 °C, 50% RH] a >4 $\log_{10}$ reduction was only observed after 60 min by CuSn5, CuDHP and CuZn30 (Figure 6). At [20 °C, 40% RH] CuDHP and CuZn30 produced >4 $\log_{10}$ reduction following 60 min incubation (Figure 7). CuNi10Fe1Mn displayed the slowest bactericidal activity at [20 °C, 50% RH] and at [20 °C, 40% RH]. At [20 °C, 40% RH] CuSn5 similarly did not exhibit >4 $\log_{10}$ reduction until after 24 h incubation. The stainless steel control did not show any bactericidal activity after 30 and 60 min at all conditions, although, after 24 h a 2.17 ± 0.21 $\log_{10}$ reduction was observed at [37 °C, 100% RH]. Statistical analysis of data for the copper surfaces showed statistically significant differences between [37 °C, 100% RH]

and [20 °C, 50% RH] ($P < 0.001$) and between [37 °C, 100% RH] and [20 °C, 40% RH] ($P < 0.001$). There was no significant difference between [20 °C, 50% RH] and [20 °C, 40% RH] conditions ($P = 0.27$). As expected all copper alloy surfaces showed significantly reduced counts to the stainless steel control ($P < 0.001$). There was no significant difference in activity with our protocol between the copper alloy surfaces tested, except between CuZn30 and CuNi10FeMn ($P = 0.04$), suggesting that a copper concentration as low as 70% provided the same lethality as a concentration of 95%. In terms of contact time, a significant difference was observed between all contact time ($P < 0.001$) when looking at all three test conditions combined.

No significant difference in counts was observed by the addition of 3 g/L BSA at [37 °C, 100% RH] ($P = 0.653$) but a difference was observed at [20 °C, 40% RH] ($P < 0.001$) (Figures 8, 9). At [37 °C, 100% RH], similar to the absence of BSA, three of the four copper alloys displayed >4 $\log_{10}$ reduction after 30 min. CuNi10Fe1Mn was more bactericidal in the presence of organic load presenting >4 $\log_{10}$ reduction, compared
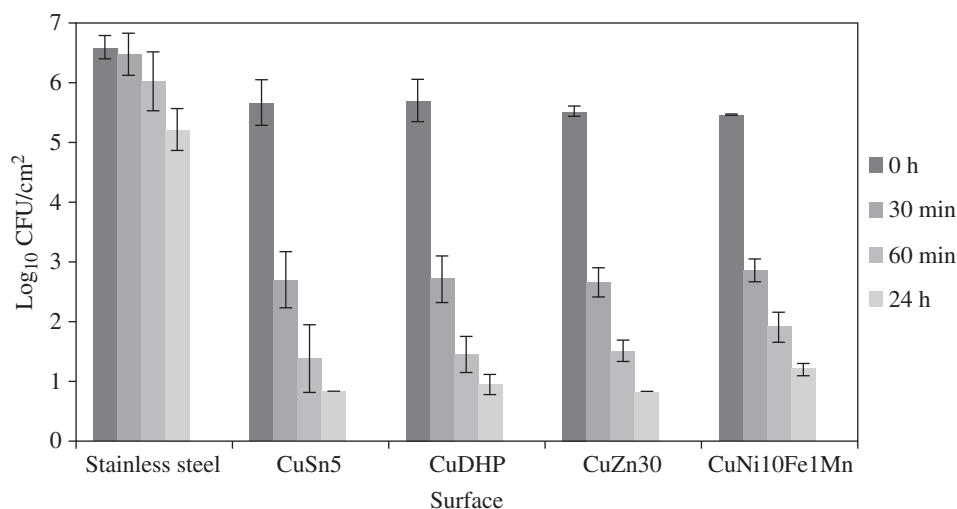

Figure 6. Recovery of *Staphylococcus aureus* from deposited aerosols at 0 h and $\log_{10}$ reduction after 30 min, 60 min and 24 h incubation at [20 °C, 50% relative humidity] ($N = 3$).
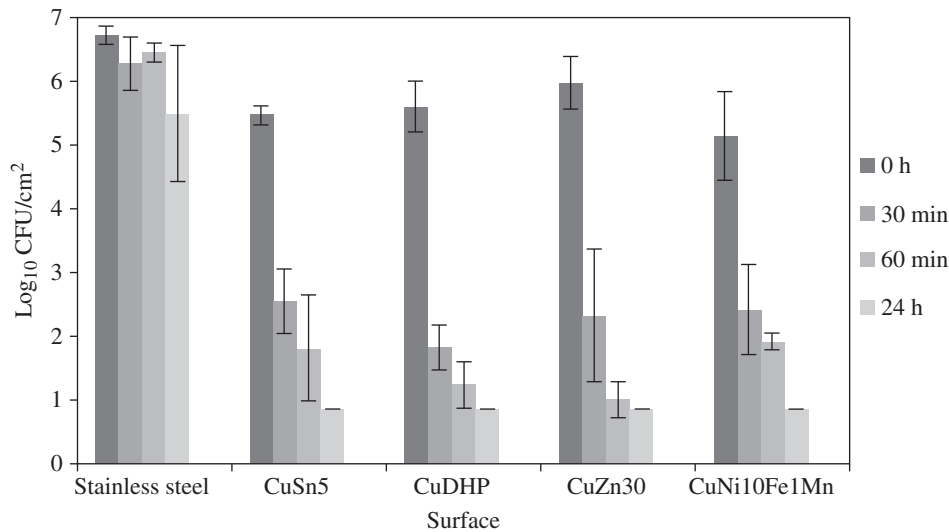
Figure 7. Recovery of *Staphylococcus aureus* from deposited aerosols at 0 h and $\log_{10}$ reduction after 30 min, 60 min and 24 h incubation at [20 °C, 40% relative humidity] ($N = 3$).

to only 2.34 ± 1.12 $\log_{10}$ reduction in the absence of BSA. After 60 min at [37 °C, 100% RH] all copper alloys showed >4 $\log_{10}$ reduction. Under dirty conditions at [20 °C, 40% RH] CuSn5 produced >4 $\log_{10}$ reduction after 30 min; in the absence of BSA this high reduction was only apparent after 24 h. Three of the four alloys showed >4 $\log_{10}$ reduction after 60 min and all displayed this high reduction after 24 h.

Discussion

Both the JIS Z 2801 test and our newly developed antimicrobial test are simple, straightforward tests for determining the antimicrobial efficacy of surfaces with antimicrobial claims. The data obtained from the JIS Z 2801 showed that all four copper alloy surfaces tested were bactericidal at [37 °C, 100% RH]. Results from our new antimicrobial surface test at 24 h [37 °C, 100% RH] were comparable to those of the JIS Z 2801. The key requirement for the efficacy of antimicrobial

surface, and notably metallic surfaces, is usually the availability of the active antimicrobial agent. When a liquid is introduced to the surface, the liquid facilitates the diffusion of the agent and its interaction with the micro-organisms when present. Hospital sampling data obtained has been useful for setting parameters in our new antimicrobial surface test. The parameters used ([20 °C, 50% RH] and [20 °C, 40% RH]) reflecting in-use conditions allowed greater discrimination in the identification of efficacious antimicrobial surfaces, which in turn will allow for a more rigorous selection of surfaces for antimicrobial application. Sampling provided an insight into changes in environmental conditions in hospital wards over a one-year period; it was apparent that there were seasonal variations in RH, peaking in the summer and at its lowest in winter. There were no obvious seasonal variations in surface temperature over the sampling period.

It could be argued that overall our protocol was more discriminatory than the JIS Z 2801 test, enabling the distinction
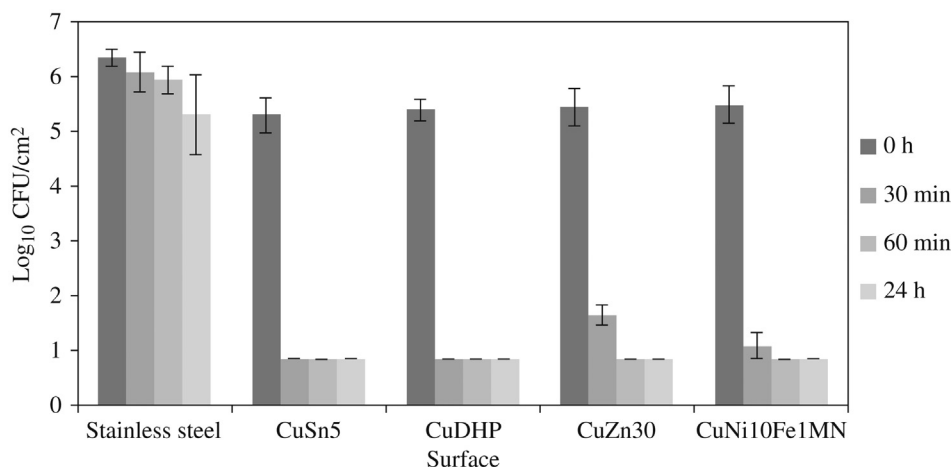

Figure 8. Recovery of *Staphylococcus aureus* from deposited aerosols at 0 h and $\log_{10}$ reduction after 30 min, 60 min and 24 h incubation at [37 °C, 100% relative humidity] in the presence of organic load ($N = 3$).
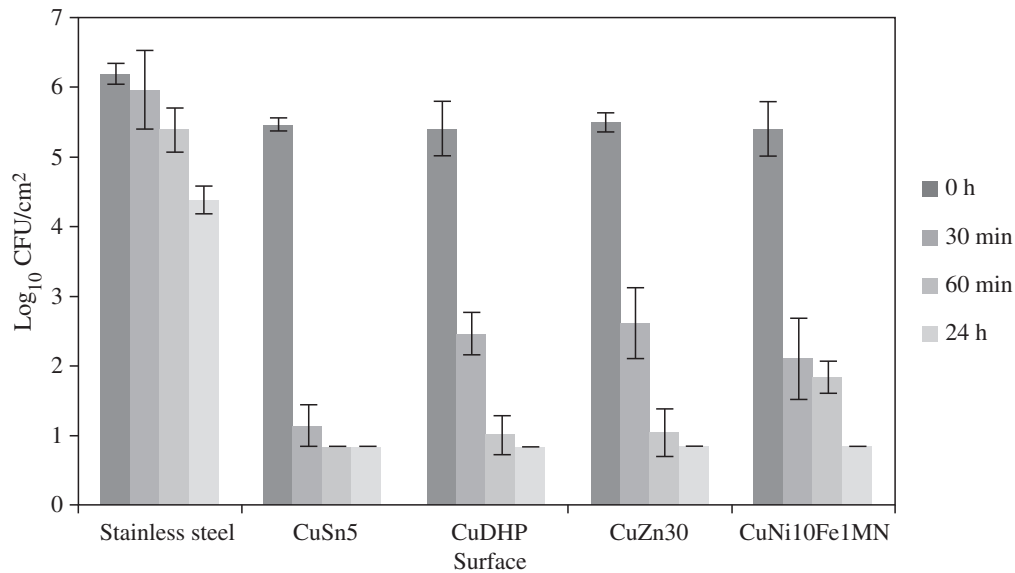
Figure 9. Recovery of *Staphylococcus aureus* from deposited aerosols at 0 h and $\log_{10}$ reduction after 30 min, 60 min and 24 h incubation at [20 °C, 40% relative humidity] in the presence of organic load ($N = 3$).

of efficacy between different copper alloys. We and others[10] suggest that the JIS Z 2801 is not a suitable antimicrobial surface efficacy test for predicting the in-use antimicrobial efficacy for surfaces to be used in clinical settings. This is primarily because the incubation conditions surfaces are exposed to high relative humidity and high temperature that are not a true reflection of conditions *in situ*. Surfaces may present antimicrobial activity under conditions of the JIS Z 2801 but not at lower humidity and temperatures reflecting indoor conditions, as found by Michels *et al.*[10] This could potentially lead to false-positive results and mislead healthcare professionals if such surfaces were to be introduced in hospitals based on the predictions of the JIS Z 2801 test alone. Random ATP measurements on a wide range of surfaces, undertaken regardless of when cleaning regimes took place, might account for the high variability of the results observed. The use of MRD, which contains 1 g/L peptone, added some organic soil to the test, especially upon drying, although such a level did not reflect the type of soiling, e.g. blood, faeces, sometimes found on healthcare surfaces. The ATP Hygiena device measured ATP levels from all organic matter on surfaces; it is not exclusive to microbial bioburden and does not measure levels of proteins. The addition of BSA, as used with CEN efficacy tests to mimic dirty conditions, showed surprising results; some surfaces presented faster antimicrobial activity when exposed to aerosols containing BSA. In general, organic matter acts to protect micro-organisms from the action of microbicidal agents, thus further investigation is required to explain these particular results.

In a hospital environment aerosols can be generated by talking, sneezing, coughing and vomiting.[11,12] Other sources of airborne pathogens include fabrics, textiles, dry skin, hair, floors, furniture, nebulizers, ventilation and air-conditioning systems.[13] Sneeze particles can range from 0.01 to 500 μm in size, and in infected patients from 0.05 to 500 μm.[14] Bacterial aerosols are a particular concern in dental surgeries as they are easily generated from instruments used in routine procedures, and are able to form biofilms once the aerosols have settled.[15]

Currently there are very few studies regarding bacterial aerosol deposition on surfaces and the effect of antimicrobial surfaces on bacterial aerosol viability. A study previously published found *Enterococcus faecalis* aerosol viability decreased on stainless steel surfaces with increasing relative humidity. Bacterial aerosols were completely inactivated on copper surfaces after 24 h at 100% relative humidity but survived at zero relative humidity.[16] Our test showed that the quickest kill by copper alloys was at [37 °C, 100% RH] suggesting that they work rapidly in the presence of moisture. The use of aerosolized bacteria might reflect better the deposition of bacteria following sneezing, coughing and splashing on surfaces.

Copper is a well-known antimicrobial agent and its potential as an antimicrobial surface in clinical settings is being explored.[17,18] The use of copper-containing surfaces was first suggested in 1981; brass doorknobs were recommended by Kuhn to replace stainless steel doorknobs to impede bacterial growth on the surfaces.[19] The clinical efficacy of copper alloy antimicrobial surfaces was tested at Selly Oak Hospital, Birmingham, UK. Copper alloy fittings were installed on toilet seats, tap handles and door push plates and their microbial load compared to that on standard fittings. The copper alloy surfaces presented a 90–100% reduction in median numbers of micro-organisms in comparison to control surfaces over the 10-week sampling period. In addition, MRSA, *C. difficile*, meticillin-sensitive *S. aureus*, vancomycin resistant enterococci and *Escherichia coli* were not detected on the copper surfaces.[20]

For materials that have been shown to be antimicrobial in the JIS Z 2801 screening test, our new protocol provides an effective second-tier test method to predict the antimicrobial material's in-use efficacy under conditions of relative humidity and temperature representative of UK hospital environments. Studies have demonstrated that small volumes of bacterial suspension require >30 min to dry on metal surfaces; in our study microbial aerosols dried within 60 min under in-use conditions.[21] The greatest majority of antimicrobial activity occurred within 60 min, suggesting that water is critical for the antimicrobial activity of copper. Despite the greater

relevance of the conditions applied in our test is it is still essentially a 'wet' test. The use of dried microbial inoculum would be ideal to allow simulation of the antimicrobial performance of test surfaces when contaminated under dry conditions.

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