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Antimicrobial activity of copper surfaces against carbapenemase-producing contemporary Gram-negative clinical isolates

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Objectives: The antimicrobial activity of copper surfaces against a variety of contemporary carbapenemase-producing Gram-negative bacteria representative of the most problematic nosocomial pathogens worldwide was evaluated.

Methods: Twenty-four clinical isolates, comprising four of *Escherichia coli*, two of *Enterobacter* spp., eight of *Klebsiella pneumoniae* and five each of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* producing either VIM-1 and/or KPC-2 or VIM-2 or OXA-type carbapenemases, were studied. The antimicrobial activity of 99% copper (Cu99%) and a 63% alloy (Cu63%) was evaluated in comparison with that of stainless steel (SS) and polyvinylchloride (PVC) by incubating $\sim 10^6$ cfu/cm^2 of the tested strains on each surface at room temperature.

Results: Copper demonstrated antimicrobial activity against all studied isolates. This effect was observed earlier and was more pronounced for Cu99% than for Cu63%. Cu99% showed a bactericidal effect after <2 h for *A. baumannii*, 3 h for *Enterobacter* spp., 5 h for *K. pneumoniae* and 6 h for *P. aeruginosa* and *E. coli*. No viable colonies were recovered from five (20.8%) isolates after 3 h and from nine (37.5%) isolates after 5 h of incubation on Cu99%.

Conclusions: Copper has significant antimicrobial activity against multidrug-resistant nosocomial Gram-negative pathogens. This supports the hypothesis that replacement of high-contact materials with copper could reduce the high burden of environmental contamination around high-risk patients. However, this strategy should be seen as an adjunctive measure to established cleaning protocols and to good hygiene practices for prevention of hospital-acquired infections.

Keywords: multidrug resistant, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter*, *Enterobacter* spp.

Introduction

The sanitizing properties of copper (Cu) have been known and exploited since ancient times, but they have received renewed attention over recent decades as laboratory studies have shown that various bacteria, yeasts and viruses are rapidly killed on metallic copper surfaces by 'contact killing'.[1]

Living organisms require copper at low concentrations as cofactors for metalloproteins and enzymes. However, at high concentrations, Cu(II) exhibits a toxic effect on most microorganisms. Various species have already been tested, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Campylobacter jejuni*, *Escherichia coli* 0157, *Acinetobacter baumannii*, *Clostridium difficile* and influenza virus as well as *Candida albicans* and a variety of moulds, all with positive results.[1] This led to the registration of 300 different copper alloys as antimicrobial surfaces by the US Environmental Protection Agency in 2008.[2]

The mechanism of contact killing has been the subject of research, and, although it is not yet fully elucidated, a number of contributing factors have been identified. As well as reactive oxygen species, surface-released free copper ions play an important role in contact killing, probably generated by redox cycling between the different copper species, Cu(0), Cu(I) and Cu(II). In the current state of knowledge, it appears that contact killing proceeds by successive membrane damage, copper influx into the cells, oxidative damage, cell death and DNA degradation.[3–5]

In the past decade there has been an alarming emergence of multidrug-resistant (MDR), mainly carbapenem-resistant, Gram-negative bacteria in the hospital environment around the world. These strains have been extremely difficult to contain and they have been very difficult to treat once they have caused clinical infections.[6] The aim of the present study was to evaluate the antimicrobial activity of copper surfaces against a variety of contemporary MDR Gram-negative isolates representative of the most problematic nosocomial pathogens worldwide.

Methods

Bacterial strains

Gram-negative clinical isolates were used in the study if they were MDR, i.e. if they were resistant to at least three groups of relevant antibiotics. These were isolated from various specimens of patients hospitalized in University General Hospital 'Attikon'. Species identification and MIC determinations were performed using an automated system (BD Phoenix automated microbiology system; BD Diagnostic Systems, Sparks, MD, USA). Results were interpreted according to CLSI criteria,[7] with the exception of those for fosfomycin and colistin. For these agents, the breakpoints proposed by EUCAST[8] were used (susceptibility ≤32 mg/L for fosfomycin against Enterobacteriaceae and ≤2 or ≤4 mg/L for colistin against Enterobacteriaceae and *Acinetobacter* spp. or against *P. aeruginosa*, respectively) because relevant breakpoints were not available from the CLSI.

The EDTA–imipenem disc synergy test was performed for the phenotypic detection of metallo-β-lactamase production[9] and the imipenem–boronic acid disc synergy test was used for the dection of production of KPC carbapenemase in Enterobacteriaceae.[10] PCR with specific primers was used for confirmation of the presence of bla_{VIM}, bla_{KPC} or bla_{OXA} genes.[11–13]

Table 1. Susceptibilities of the studied isolates to selected antimicrobial agents

Antimicrobial agent	MIC range (mg/L)	Susceptibility (%)
Amikacin	≤8 to >32	20.8
Gentamicin	≤2 to >8	41.7
Aztreonam[a]	≤2 to >16	5.3
Cefepime[b]	16 to >16	0
Imipenem	≤1 to >8	8.3
Ciprofloxacin	≤0.5 to >2	25
Trimethoprim/sulfamethoxazole[b]	≤1/19 to >2/38	21.4
Fosfomycin[b]	≤16 to 128	78.6
Colistin	≤0.5 to >4	87.5

[a]Only Enterobacteriaceae and *P. aeruginosa* isolates were evaluated.
[b]Only Enterobacteriaceae were evaluated.

Metal coupons

To assess the antimicrobial activity of copper, we used metal coupons (1×1 cm) containing either 99% Cu (Cu99%) or a brass alloy containing 63% Cu and 37% Zn (Cu63%). Similar size coupons of stainless steel (SS) or polyvinylchloride (PVC) were used as controls. Metal coupons were supplied by the Hellenic Copper Development Institute and prior to use they were degreased and cleaned by vortexing in acetone for 30 s, immersion in absolute ethanol and flaming in a Bunsen burner. PVC coupons were only immersed in absolute ethanol and allowed to dry. All coupons were stored in sterile Petri dishes.

Bacterial viability experiments

On the day of the experiment, a bacterial suspension with a turbidity equivalent to that of a 0.5 McFarland standard was made from pure colonies of an overnight culture of each isolate. Twenty microlitres of this suspension (~10^6 cfu) was inoculated on each of the four coupons, which were then incubated at room temperature (20–25°C) for 0, 1, 2, 3, 5, 6 and 24 h. Time 0 was considered the time that the droplet was placed on the coupon. At each time interval, each coupon was aseptically removed and immersed into 10 mL of sterile Dey/Engley (D/E) broth (BD Diagnostic Systems) to neutralize any further antimicrobial activity of copper.[14] Coupons were vortexed for 60 s and 0.1 mL of broth was removed and serially diluted 10-fold in sterile PBS. Twenty-five microlitres of each dilution was plated onto MacConkey agar (BD Diagnostic Systems) plates and incubated at 37°C for 18 h for colony counting. Results were expressed as $\log_{10}$ cfu/cm^2. The lower limit of detection was 2.6 $\log_{10}$ cfu/cm^2.

The survival over time on each of the surfaces was expressed as mean $\log_{10}$ cfu/cm^2 of all isolates of the same species at each timepoint. In cases where no viable colonies were detected, the lower limit of detection was used in calculations. Bacteriostatic activity was defined as a ≥2 to <3 $\log_{10}$ reduction and bactericidal activity as a ≥3 $\log_{10}$ reduction in the cfu from the inoculum at time 0 on that surface.

Statistical analysis

One-way analysis of variance was used to compare $\log_{10}$ cfu/cm^2 at each timepoint among the four studied surfaces and *post hoc* analysis was performed by the Bonferroni method. All differences with $P<0.05$ were considered statistically significant. All statistical analyses were done using IBM SPSS Statistics 20 software.

Ethics

Ethical approval was not required.

Results

The 24 clinical isolates that were used in the experiments comprised four isolates of *E. coli* (three KPC-2 producers and one VIM-1 producer), two isolates of *Enterobacter* spp. (one KPC-2 producer and one KPC-2/VIM-1 producer), eight isolates of *Klebsiella pneumoniae* (seven KPC-2 producers and one VIM-1 producer), five isolates of VIM-2-producing *P. aeruginosa* and five isolates of *A. baumannii* (two OXA-51 and three OXA-51/OXA-58 producers). These isolates were recovered from blood (eight isolates), bronchial secretions (six isolates), faecal flora (seven isolates), urine (two isolates) and pus (one isolate) of hospitalized patients.

All tested isolates were resistant to second- and third-generation cephalosporins and β-lactam/β-lactamase inhibitor combinations. The susceptibilities of the studied isolates to

various antimicrobial agents are presented in Table 1 and in Table S1 (available as Supplementary data at *JAC* Online).

The survival of test organisms on each surface over time was expressed as the difference between the mean starting inoculum and the mean viable cell count at each time interval of all isolates of the same species (Δ $\log_{10}$ cfu/cm^2). A negative sign represented a reduction and a positive sign an increase from the inoculum at time 0. Results are shown in Table 2 and Table S2 (available as Supplementary data at *JAC* Online). Time–kill curves were constructed for each group of isolates of the same species by plotting the mean $\log_{10}$ cfu/cm^2 over time (Figure 1a–e).

For the group of four *E. coli* isolates, Cu99% demonstrated a reduction of initial inoculum by 2 $\log_{10}$ cfu/cm^2 at 3 h of incubation in indoor conditions and a bactericidal effect at 6 h, whereas none of the other surfaces showed bactericidal activity during the first 6 h. A bactericidal effect was shown for Cu63% at 24 h (Table 2 and Figure 1a). For the group of two *Enterobacter* spp., a bactericidal effect was detected at 3 h for Cu99% and at 5 h for Cu63%. Among the control surfaces, SS showed a bactericidal effect after 6 h (Table 2 and Figure 1b). Against the group of eight *K. pneumoniae* isolates, a bactericidal effect was demonstrated by 5 h for Cu99%. Cu63% reduced the initial inoculum by 2 $\log_{10}$ cfu/cm^2 at 5 h. Cu63% and SS showed a bactericidal effect at 24 h (Table 2 and Figure 1c). Cu99% reduced the initial inoculum of *P. aeruginosa* by 2.08 $\log_{10}$ cfu/cm^2 at 3 h, but a bactericidal effect was shown at 6 h. Cu63% showed a bactericidal effect after 24 h (Table 2 and Figure 1d). Against *A. baumannii*, the bactericidal effect of Cu99% was shown in <2 h, whereas that of Cu63% was evident after 6 h. Neither of the control surfaces showed a significant reduction of viable cells even at 24 h (Table 2 and Figure 1e).

Table 2. Difference between mean starting inoculum and mean viable cell count (Δ $\log_{10}$ cfu/cm^2) of the four *E. coli*, the two *Enterobacter* spp., the eight *K. pneumoniae*, the five *P. aeruginosa* and the five *A. baumannii* isolates at each time interval

	Time (h)					
Surface material	1	2	3	5	6	24
	Δ $\log_{10}$ cfu/cm^2 for *E. coli* isolates					
Cu99%	0.21	−0.13	−2.00	−2.81	−4.15	−4.15
Cu63%	0.09	0.15	0.32	−0.81	−2.90	−3.67
SS	0.06	0.07	0.51	−0.01	−0.41	−1.87
PVC	−0.17	0.38	0.47	−0.60	0.67	−2.39
	Δ $\log_{10}$ cfu/cm^2 for *Enterobacter* spp. isolates					
Cu99%	−0.02	−0.13	−4.74*	−4.76*	−4.74	−4.74*
Cu63%	−0.20	0.34	−0.06	−3.36*	−4.60	−4.60*
SS	−0.07	0.05	0.12	−0.69	−3.59	−4.70*
PVC	0.12	0.07	0.63	−0.68	−0.04	−2.35
	Δ $\log_{10}$ cfu/cm^2 for *K. pneumoniae* isolates					
Cu99%	0.03	0.03	0.22	−3.29*	−3.72*	−4.05*
Cu63%	−0.08	0.15	0.32	−2.00	−2.34	−3.96*
SS	0.13	0.12	0.80	−0.65	−2.01	−3.39*
PVC	0.07	0.16	0.68	0.04	−0.34	−2.25
	Δ $\log_{10}$ cfu/cm^2 for *P. aeruginosa* isolates					
Cu99%	NE	−1.61	−2.08	−2.71*	−3.70*	−4.59*
Cu63%	NE	−0.97	−1.52	−1.87*	−1.56	−3.09
SS	NE	−0.40	−0.57	−1.06	−1.18	−1.54
PVC	NE	−0.15	−0.39	−0.82	−0.53	−2.09
	Δ $\log_{10}$ cfu/cm^2 for *A. baumannii* isolates					
Cu99%	NE	−3.74*	−3.12*	−3.31*	−3.76*	−4.56*
Cu63%	NE	−1.98	−1.61*	−2.67*	−2.98	−3.91
SS	NE	−0.64	−0.77	−0.79	−0.87	−1.01
PVC	NE	−0.78	−1.10	−1.11	−1.31	−1.69

NE, values at 1 h were not evaluated; Cu99%, pure copper; Cu63%, copper alloy.
A negative sign represents a reduction and a positive sign an increase from the inoculum at time 0. Asterisks denote differences that were statistically significant in the within-group comparisons.

Comparing the antimicrobial activity of Cu99% with that of copper alloy for the whole collection of studied isolates, we observed an earlier and more pronounced antimicrobial effect of Cu99% versus Cu63% (a median of 3 versus 5 h for a bacteriostatic effect and 5 versus 24 h for a bactericidal effect). No viable colonies were recovered from five (20.8%) isolates (two *Enterobacter* spp. and one each of *E. coli*, *P. aeruginosa* and *A. baumannii*) after 3 h and from nine (37.5%) isolates (six *K. pneumoniae*, two *E. coli* and one *A. baumannii*) after 5 h of incubation on Cu99% in indoor conditions. For all groups of isolates, Cu99% demonstrated a statistically significant effect at 3 h compared with all other surfaces [$F(79.1,209.1) = 12.11$, $P < 0.001$]. This effect persisted throughout the experiment and remained significant in comparison with SS and PVC, but in comparison with the alloy a significant difference was noted at 3 and 5 h only. The effect of the copper alloy was significant from 6 h onwards compared with PVC and from 24 h compared with SS.

Discussion

Carbapenemase-producing Gram-negative organisms have become endemic in hospital settings in several geographical regions and have been reported to cause important nosocomial outbreaks in others. High morbidity and mortality rates have been observed in relation to infections by these strains.[15] The containment of carbapenemase producers has been the focus of guidelines issued by several expert committees, but despite these efforts the prevalence rates of carbapenem-resistant bacteria have been increasing.[6,16] There is definitely a need for new approaches in the field of infection control and antimicrobial copper could provide one such approach to supplement the current measures for prevention of dissemination of MDR strains in hospitals.

Currently, the antimicrobial activities of a variety of copper alloys have been successfully evaluated in the laboratory[1] and in hospital settings against various Gram-negative bacteria, but not against carbapenemase producers.[17,18] The present study was conducted to investigate whether a variety of carbapenemase-producing Gram-negative bacteria representative of the contemporary 'hospital nightmares' were susceptible to the antimicrobial properties of copper surfaces.

We observed a significant effect of metallic copper alloys against all tested species. This effect was exerted earlier and was more pronounced for Cu99% than for the Cu63% alloy. Nevertheless, survival was longer than that observed in studies

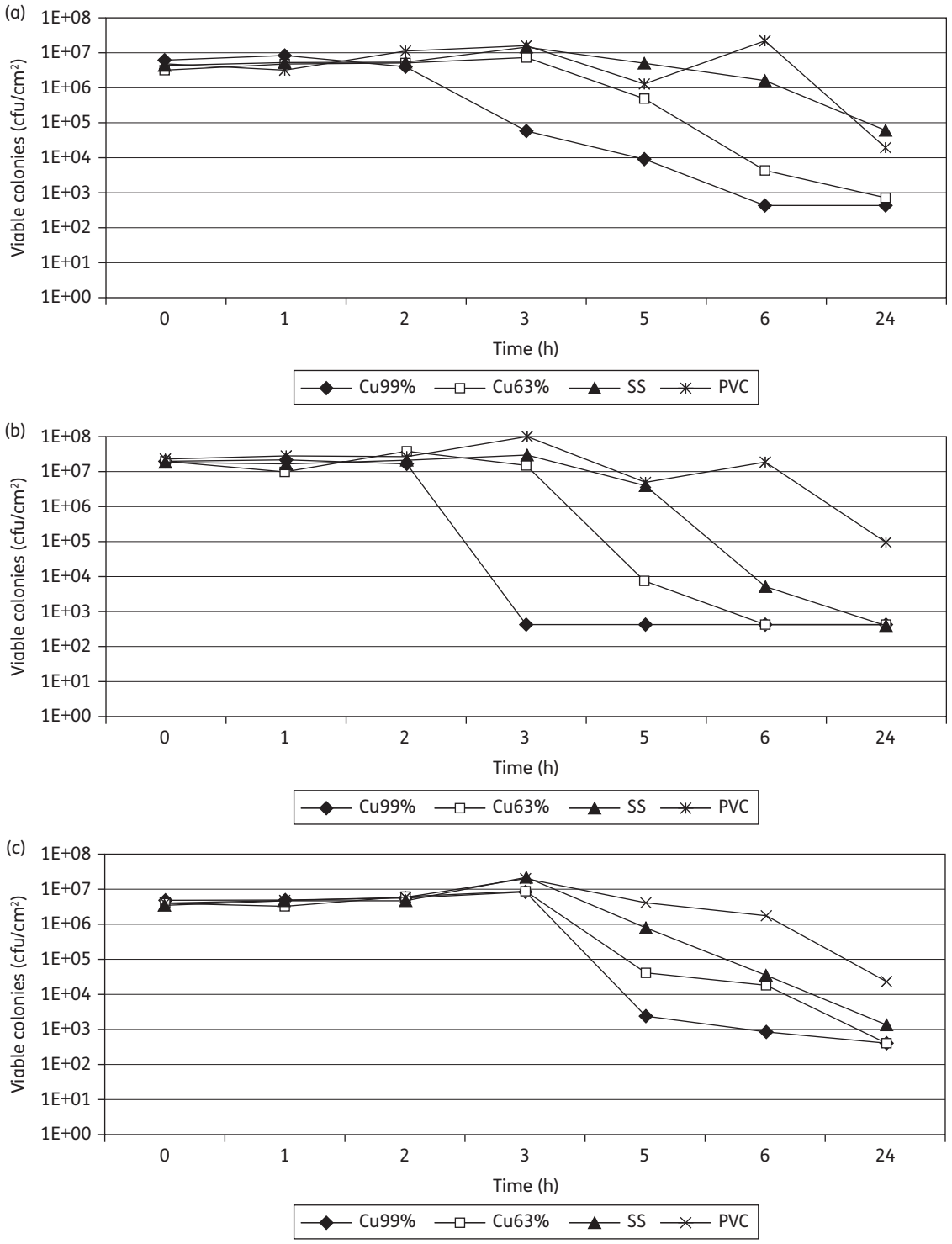

Figure 1. Survival over time of four *E. coli* (a), two *Enterobacter* spp. (b), eight *K. pneumoniae* (c), five *P. aeruginosa* (d) and five *A. baumannii* (e) isolates on copper (Cu99%), copper alloy (Cu63%), SS or PVC. Points represent the mean number of viable colonies for each group of isolates.

evaluating susceptible strains. For example, *E. coli* was inactivated within a few minutes,[19] *P. aeruginosa* after 120 min[20] and *A. baumannii* after 180 min of exposure to copper.[21] Although factors related to the resistance genes cannot be excluded, we believe that factors related to the methodology could probably account for these differences. It has been shown that higher copper content of alloys,[20] higher temperature,[20,22] higher relative humidity[23] and a 'dry' as opposed to a

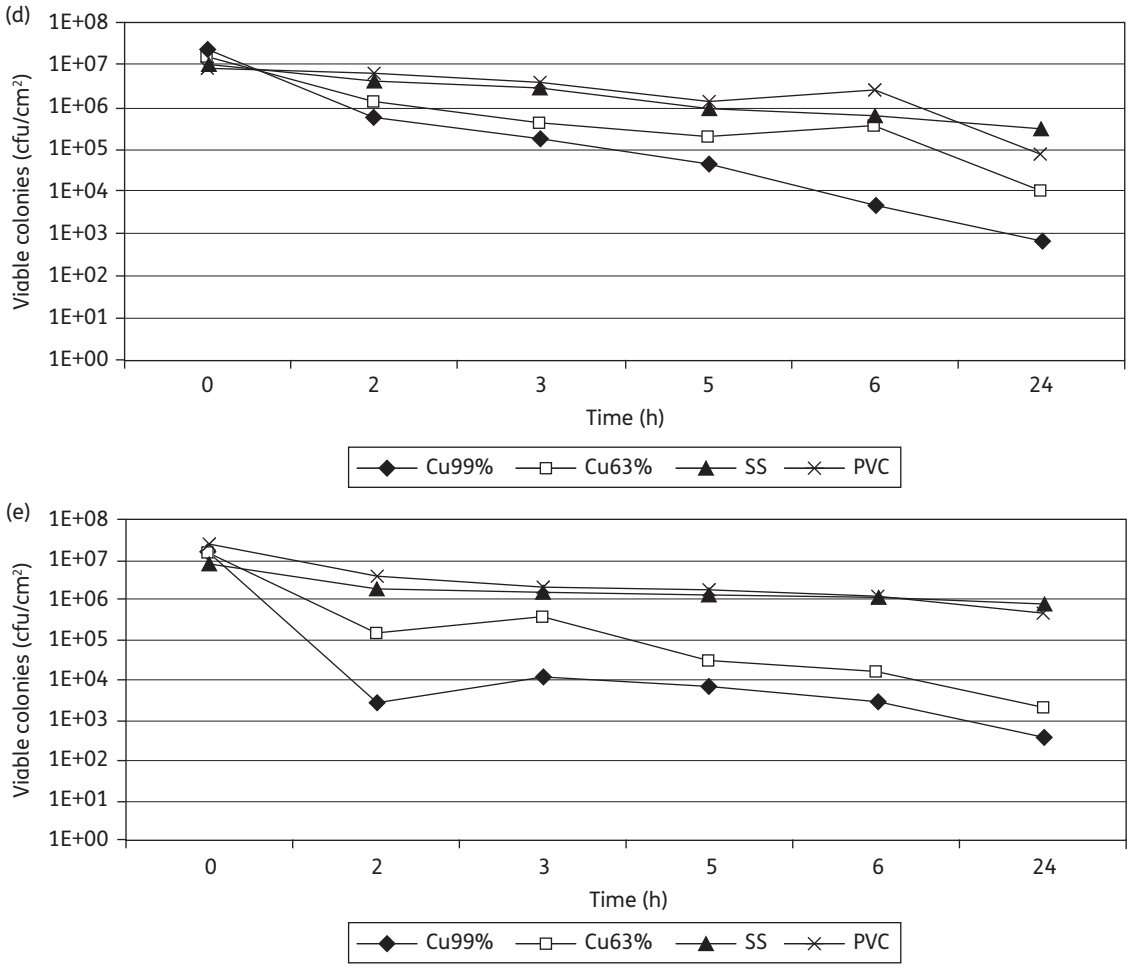

Figure 1. *Continued*

'wet' inoculation technique[4,24] increased the efficacy of contact killing. It was shown by Elguindi *et al.*[20] that the survival of *P. aeruginosa* PAO1 was shortened by 2 h when the inoculum was spread on the copper coupon instead of being left as a droplet, but the results were reproducible only when the 'droplet' technique was used. Furthermore, when a cotton swab was used to spread the inoculum evenly on tested surfaces, the amount of bacteria left on the surface was not standardized. We decided to apply the droplet inoculation technique, which was most often used in related published literature in order to obtain reproducible results and to be able to compare our results with previous studies.

Control SS surfaces exhibited a -3.59 $\log_{10}$ cfu/cm^2 change in initial inoculum of *Enterobacter* spp. at 6 h and a -3.39 $\log_{10}$ cfu/cm^2 change in the inoculum of *K. pneumoniae* at 24 h and control PVC surfaces a -2.09 to -2.39 $\log_{10}$ cfu/cm^2 change in initial inoculum of *E. coli*, *Enterobacter* spp., *K. pneumoniae* and *P. aeruginosa* at 24 h. These reductions represented the inherent incapability of these species of surviving on dry surfaces over time, in contrast to *A. baumannii*. Michels *et al.*[25] showed a similar effect after exposure of *E. coli* on SS for $\geq$24 h.

The killing of carbapenemase-producing MDR Gram-negative bacteria on copper surfaces suggests that the use of copper alloys in high-contact surfaces in the hospital may offer the potential to reduce the spread of 'difficult-to-treat' nosocomial pathogens, if coupled with optimal cleaning procedures and compliance with infection-control practices. Clinical studies showing reduction in cross-transmission as well as reduction in nosocomial infection rates in settings with high prevalences of carbapenemase producers would be welcomed by the medical community.

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Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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