

Antimicrobial efficacy of copper surfaces against spores and vegetative cells of *Clostridium difficile*: the germination theory

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Objectives: Persistent contamination of surfaces by spores of *Clostridium difficile* is a major factor influencing the spread of *C. difficile*-associated diarrhoea (CDAD) in the clinical setting. In recent years, the antimicrobial efficacy of metal surfaces has been investigated against microorganisms including methicillin-resistant *Staphylococcus aureus*. This study compared the survival of *C. difficile* on stainless steel, a metal contact surface widely used in hospitals, and copper surfaces.

Methods: Antimicrobial efficacy was assessed using a carrier test method against dormant spores, germinating spores and vegetative cells of *C. difficile* (NCTC 11204 and ribotype 027) over a 3 h period in the presence and absence of organic matter.

Results: Copper metal eliminated all vegetative cells of *C. difficile* within 30 min, compared with stainless steel which demonstrated no antimicrobial activity ($P < 0.05$). Copper significantly reduced the viability of spores of *C. difficile* exposed to the germinant (sodium taurocholate) in aerobic conditions within 60 min ($P < 0.05$) while achieving a ≥ 2.5 log reduction (99.8% reduction) at 3 h. Organic material did not reduce the antimicrobial efficacy of the copper surface ($P > 0.05$).

Conclusions: The use of copper surfaces within the clinical environment and application of a germination solution in infection control procedures may offer a novel way forward in eliminating *C. difficile* from contaminated surfaces and reducing CDAD.

Keywords: nosocomial infection, susceptibility, time–kill, germinant

Introduction

Clostridium difficile-associated diarrhoea is currently the leading healthcare-acquired infection in the UK.¹ Excretion of large numbers of vegetative cells and spores of *C. difficile* by infected patients contaminates inanimate surfaces that serve as potential reservoirs of infection.² Spores of *C. difficile* can persist in the environment for months and are resistant to commonly used surface disinfectants, for example 70% industrial methylated spirits. Sporicidal agents such as hypochlorite and peracetic acid are required to eliminate the spores from the clinical setting.³

Recently, research into the antimicrobial efficacy of metal surfaces including stainless steel, zeolite (silver/zinc) and, in particular, copper has escalated.^{4,5} The only report of activity of copper against *C. difficile* spores is by Weaver *et al.*⁶ in 2008, in which complete death of *C. difficile* spores was observed in 24–48 h when exposed to various copper alloys. However, it is well known that when bacterial spores are exposed to a

suitable germinant, they are significantly more susceptible to antimicrobials.⁷ In this investigation, the ability of copper metal and stainless steel to reduce the viable load of vegetative cells and dormant and germinating spores of two strains of *C. difficile* (NCTC 11204 and ribotype 027) was investigated.

Materials and methods

Microorganisms

C. difficile NCTC 11204 and *C. difficile* 027 R20291 (Anaerobe Reference Laboratory, Cardiff, UK) were stored on Microbank[®] beads (Pro-Lab Diagnostics, Cheshire, UK) and kept at -70°C until required.

Preparation of vegetative cell and spore suspensions of *C. difficile*

An overnight culture of *C. difficile* grown anaerobically in Wilkins Chalgren broth (WCB) was used to produce vegetative

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cell suspensions. Spore suspensions of *C. difficile* were produced following the method described by Shetty *et al.*⁸ and stored at 4°C until needed. To determine the concentration of spores in each suspension, both a viable count and a total count were performed.

Preparation of a soil load

The soil load used comprised both protein and carbohydrate to simulate a contaminated clinical environment and was adapted from Perez *et al.*⁹

Preparation of copper and stainless steel coupons

Coupons (1 cm²) of copper (UNS C19700, Copper Development Association, USA) and stainless steel (GTSS Engineering Supplies, Coventry, UK) were prepared according to the method of Noyce *et al.*¹⁰

Assessment of copper neutralization and non-toxicity of D/E neutralizer

Fifty microlitres of a suspension of *C. difficile* NCTC 11204 vegetative cells in WCB, containing 10⁶ cfu/mL, was mixed with 50 µL of D/E neutralizer¹¹ and inoculated onto the surface of a copper coupon inside a sterile bottle. After 1 h of exposure, viable counts were compared with those of a similar volume mixed with sterilized distilled water. To ensure non-toxicity of D/E neutralizer, 900 µL of neutralizer was mixed thoroughly with 100 µL of *C. difficile* cell suspension and incubated for 1 h before comparing viable counts with those from controls (with sterilized distilled water).

Assessment of antimicrobial efficacy of copper metal and stainless steel against vegetative cells and dormant spores of *C. difficile*

Coupons of copper and stainless steel were inoculated with 10 µL of each test strain of *C. difficile* (10⁶ cfu/mL) and placed in sterile glass bottles. Tests involving vegetative cells of *C. difficile* were undertaken at 37°C in anaerobic conditions, Whereas those involving spore suspensions were carried out in air at room temperature. At 30 min intervals over a 3 h period, 990 µL of D/E neutralizer and 5–10 glass beads (3 mm diameter, Merck, UK) were added to the appropriate bottles, vortex mixed for 1 min and left to stand at room temperature (for spores) or 37°C in anaerobic conditions (for vegetative cells) for 30 min (with the lids on all of the bottles). This time period allowed for adequate neutralization of copper. Each suspension was diluted in sterile distilled water if appropriate and mixed with 15 mL of molten Fastidious Anaerobe Agar (Lab M, UK) supplemented with 0.1% (w/v) sodium taurocholate (≥95% sodium taurocholate, Sigma Aldrich, UK). For tests involving vegetative cells, samples were inoculated onto Wilkins Chalgren agar. All plates were incubated at 37°C in anaerobic conditions for 48 h.

Assessment of antimicrobial efficacy of copper metal and stainless steel against spores of *C. difficile* exposed to 1% (w/v) sodium taurocholate

Ten microlitres of each spore suspension was mixed with 10 µL of 2% (w/v) sodium taurocholate in double strength thioglycollate medium and inoculated onto the surface of copper and stainless steel coupons in sterile bijoux bottles. At the sampling times

described previously, 980 µL of D/E neutralizer and 5–10 glass beads were added to each bottle which was then vortex mixed for 1 min and allowed to stand at room temperature for 30 min. All spore suspensions were then diluted and cultured, as described previously.

Statistical analysis

The statistical analysis of data was performed using an unpaired *t*-test (two-tailed), using the InStat[®] package (GraphPad Software Inc., version 3.06) and repeated measures analysis of variance (ANOVA), with Fisher's LSD *post hoc* test, using the Statistica package (StatSoft Inc., version 6.0).

Results

Efficacy and non-toxicity of D/E neutralizer

Preliminary studies showed that D/E neutralizer nullified the antimicrobial activity of copper and was non-toxic to *C. difficile* over 1 h of exposure (results not shown).

Efficacy of copper and stainless steel against the dormant and germinating spores and vegetative cells of *C. difficile*

Stainless steel did not demonstrate any antimicrobial activity against the vegetative cells of *C. difficile* after 30 min of exposure. In contrast, copper demonstrated significant ($P < 0.05$) antimicrobial activity against vegetative *C. difficile*, achieving a ≥6 log reduction in cfu/mL in 30 min (Table 1). Stainless steel did not demonstrate antimicrobial activity against either dormant or germinating spores of *C. difficile* NCTC 11204 and 027, as no reduction in viability was observed following 3 h of exposure ($P > 0.05$). Furthermore, there was no reduction in viability of dormant spores of either strain of *C. difficile* on copper metal within the 3 h study period. However, the viability of spores of both *C. difficile* strains exposed to the germination solution on copper was significantly reduced ($P < 0.05$) within 60 min, compared with the viability of spores with the germinant solution on stainless steel. At 3 h, a 2.67 (99.79% reduction) and 2.96 (99.87% reduction) log reduction of germinating *C. difficile* NCTC 11204 and 027 spores, respectively, was achieved on copper metal. The antimicrobial activity of copper and stainless

Table 1. Antimicrobial efficacy of copper metal and stainless steel against the vegetative cells of *C. difficile*

Surface/exposure time	Mean <i>C. difficile</i> cfu/mL remaining (range)	
	NCTC 11204	027
Stainless steel		
0 min	7.6×10^6 (6.8–8.3)	5.6×10^6 (5.5–5.6)
30 min	7.3×10^6 (6.8–7.8)	3.2×10^6 (2.4–4.0)
Copper		
0 min	1.2×10^6 (1.0–1.4)	4.6×10^6 (3.6–5.6)
30 min	0*	0*

* $P < 0.05$.

Antimicrobial efficacy of copper against *Clostridium difficile*

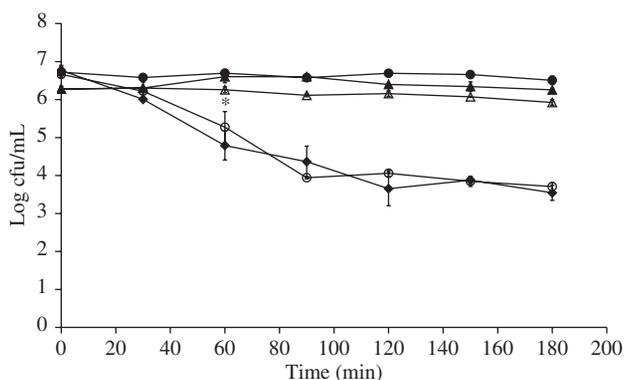


Figure 1. Antimicrobial effects of copper metal and steel on dormant and germinating spores of *C. difficile* ribotype 027. Filled triangles, spores on steel; filled circles, spores on copper; open triangles, spores and germinant on steel; open circles, spores and germinant on copper; filled diamonds, spores, germinant and a soil load on copper. * $P < 0.05$.

steel against *C. difficile* 027 is shown in Figure 1. Data for the strain NCTC 11204 are not shown.

Effect of a soil load on the antimicrobial activity of copper

In both strains of *C. difficile* tested, there was a significant decrease in cfu/mL over time after incubation of spores with the germinant solution and a soil load on the surface of copper ($P < 0.05$) within the first 60 min of exposure. A log reduction of 2.71 and 3.22 was achieved for NCTC 11204 and 027 strains, respectively, after 3 h of exposure to copper (Figure 1; data not shown for the strain NCTC 11204). This reduction in cfu/mL was not significantly different from the reduction achieved in the absence of a soil load in either strain tested ($P > 0.05$).

Discussion

In line with other recent studies that have evaluated the efficacy of copper against nosocomial pathogens including methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*,⁵ copper metal, in this study, exhibited rapid antimicrobial activity against the vegetative cells of *C. difficile* compared with stainless steel, which remained inert.

In the 3 h study period, both copper and stainless steel surfaces were ineffective at reducing the numbers of viable dormant *C. difficile* spores, and no log reduction was observed. However, in the recent study by Weaver *et al.*⁶ it was shown that a >5 log reduction of dormant spores of *C. difficile* was achieved following a 24–48 h of exposure to pure copper.⁶ This finding clearly indicates that copper metal is effective at eliminating dormant spores of *C. difficile*, but an extended exposure period is required. In the current investigation, a 3 h time period was chosen in order to assess the value of copper metal for application as an antimicrobial surface in the clinical setting, within a realistic time frame. The introduction of a specific germinant (1% sodium taurocholate in thioglycollate medium) significantly enhanced the kill of *C. difficile* spores in aerobic conditions on copper metal, with a 1.47 log reduction of NCTC 11204 spores and a 1.39 log reduction of 027 spores within 60 min in the absence of a soil load. Furthermore, at 3 h, there

was a log reduction of 2.67 (99.79%) and 2.96 (99.87%) in the NCTC 11204 and 027 strains, respectively. Germinating spores of *C. difficile* on stainless steel were not eliminated in 3 h, thus highlighting the antimicrobial efficacy of copper metal. The application of specific spore germinants to eliminate spores in the presence of antimicrobials has been adopted in a recent study by Hornstra *et al.*¹² with *Bacillus cereus* spores. Spores were germinated on stainless steel coupons prior to the introduction of a cleaning agent; this treatment resulted in a reduction of over 3 decimal log units in the number of surviving spores, whereas there was no effect on dormant spores in the presence of the cleaning agent alone. The incorporation of a germination step may be of particular importance with *C. difficile* as it has been found that the use of subinhibitory levels of certain cleaning agents can induce spore formation in *C. difficile*.¹³

The mode of action of copper is not completely understood as yet, but it has been suggested that it exerts its cytotoxic effects by degrading proteins, damaging the bacterial membrane and binding DNA.¹⁴

The incorporation of a soil load into the *C. difficile* spore suspension (in order to simulate environmental contamination) did not demonstrate any inhibitory effect on the antimicrobial activity of copper metal. There was no significant difference in either the rate or extent of reduction in cfu/mL of *C. difficile* spores on exposure to copper and a germinant in the presence of a soil load compared with that in the absence of a soil load ($P > 0.05$). These findings are important as they demonstrate that even in the presence of a soil load, copper metal is still effective at rapidly reducing the number of viable spores of *C. difficile* in the presence of a specific germinant. However, in a recent study by Airey and Verran,¹⁵ it was shown that a build-up of bacterial cells (*S. aureus*) and organic matter (bovine serum albumin) on copper surfaces occurs after several cycles of soiling and cleaning. As this current investigation assessed the antimicrobial effects of copper alone (without the incorporation of a cleaning agent), further *in vitro* studies may be warranted to assess the effect of multiple cleaning and soiling cycles on the antimicrobial activity of copper.

The incorporation of copper metal surfaces into the clinical environment is currently being widely considered as one approach to ‘design out’ healthcare-acquired infection in modern medicine. The novel approach of introducing a specific germinant to significantly enhance the susceptibility of *C. difficile* spores to copper metal should be considered if copper surfaces are adopted in clinical practice.

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Transparency declarations

None to declare.

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