Journal of Hospital Infection (2008) ■, 1–7

Available online at www.sciencedirect.com

ScienceDirect

www.elsevierhealth.com/journals/jhin

Survival of *Clostridium difficile* on copper and steel: Futuristic options for hospital hygiene

L. Weaver [a,*], H.T. Michels [b], C.W. Keevil [a]

[a] *Environmental Healthcare Unit, School of Biological Sciences, University of Southampton, Southampton, UK*
[b] *Copper Development Association Inc., New York, New York, USA*

Received 31 October 2007; accepted 16 November 2007

KEYWORDS
Clostridium difficile;
Touch surfaces;
Copper; Hygiene;
Infection

Summary *Clostridium difficile* is rapidly becoming a major cause of hospital-acquired infections worldwide, due in part to transmission of the faecal pathogen between contaminated hands and contact surfaces. Accordingly, this study evaluated survival of *C. difficile* vegetative cells and spores on the contact surface commonly found in healthcare settings, stainless steel, compared to five copper alloys (65–100% copper content). *C. difficile* requires prolonged incubation to grow and therefore the total number and number of viable cells was estimated using a fluorescence dual-staining technique. For viability assessment the redox dye 5-cyano-2,3-ditolyl tetrazolium (CTC) was used to measure metabolic activity. Results demonstrated that copper alloys with a copper content >70% provide a significant reduction in survival of *C. difficile* vegetative cells and spores on copper alloys compared with stainless steel. Complete death of spores was observed after 24–48 h on copper alloys whereas no significant death rate was observed on stainless steel even after 168 h. The use of CTC gave comparable results to culture and offers a more rapid viability analysis (8 h) than culture. The results suggest that using copper alloys in hospitals and other healthcare facilities could offer the potential to reduce spread of *C. difficile* from contaminated surfaces.

Introduction

The anaerobic spore-forming bacterium, *C. difficile*, is a major cause of nosocomial diarrhoeal

* Corresponding author. Address: Environmental Healthcare Unit, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK. Tel.: +44 23 80592034; fax: +44 23 80594459.
E-mail address: lw14@soton.ac.uk

0195-6701/$ - see front matter © 2007 The Hospital Infection Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.jhin.2007.11.011

Please cite this article in press as: Weaver L et al., Survival of *Clostridium difficile* on copper and steel: Futuristic options for hospital hygiene, J Hosp Infect (2008), doi:10.1016/j.jhin.2007.11.011

infections worldwide.[1–4] *C. difficile* produces exotoxins that are pathogenic to humans, resulting in disease symptoms ranging from mild diarrhoea to fulminant colitis and death. As well as severe health implications, especially for those aged >65 years, *C. difficile* infections cause a large financial burden on hospitals.[5] Although there are few data on the number of deaths caused by *C. difficile*, there was an increase in the number of death certificates mentioning *C. difficile* from 1999 to 2005 in England and Wales.[6] A study in the USA reported that 4.8% of deaths were associated with *C. difficile* in 2003, increasing to 9.9% in 2004.[2]

The use of culture analysis for enumeration and detection of viable bacteria has been shown to be lacking as pathogenic bacteria are often found in a viable but non-culturable state (VBNC) in the environment.[7,8] The use of viability dyes has been demonstrated as a useful alternative to culture analysis in vegetative cells.[9–12] In particular the use of tetrazolium redox dyes for direct visualisation of metabolic activity in bacterial cells has gained much interest. Tetrazolium redox dyes scavenge electrons from oxidation/reduction reactions and are reduced intracellularly to brightly chromogenic or fluorescent formazan precipitates by the electron transport system components or dehydrogenases in metabolically active cells.[12,13] The precipitates formed can be detected within cells by direct microscopy techniques.

5-Cyano-2,3-ditolyl tetrazolium (CTC) is reduced from colourless to a red/orange fluorescent formazan in metabolically active cells. Although CTC has been used previously in environmental and ecological studies, there has been little research into the use of CTC for detecting metabolic activity in anaerobic bacteria. Research by Bhupathiraju *et al.* demonstrated that it was possible to use CTC for metabolic activity investigations in a range of anaerobic bacteria using various substrates.[12,13]

The present study investigated the potential usefulness of copper and copper-based alloys as contact surfaces in reducing the survival of *C. difficile* cells and spores compared with stainless steel. To establish counts, including VBNC cells, both culture analysis and viability dyes were applied.

Methods

Culture preparation

Clostridium difficile (ATCC 9689) was maintained on glycerol-protected beads (Fisher Scientific, UK) at −80 °C. For each experiment a culture of *C. difficile* was prepared by aseptically inoculating 15 mL of brain heart infusion broth (Oxoid, UK) with one bead of *C. difficile* and incubating anaerobically at 37 °C for 3–5 days.

Spore production

Grown cultures were subsequently incubated overnight at 5 °C to initiate spore production. Ethanol (95% vol/vol) was added (1:1 ratio) to the cultures and mixed by vortexing to kill vegetative cells. Cultures were left at room temperature for 30 min and then centrifuged at 3000*g* for 20 min. To purify spores the method of Gombas and Gomez was used.[14] Briefly, low-speed centrifugation of the spore suspension (1475*g*) was conducted. After centrifugation the upper layer, containing vegetative cells, was removed by gently rocking the pellet in 5 mL filter-sterilised deionised water, leaving pelleted spores. Further centrifugations of the upper layer were performed. Pellets were combined and resuspended in 2 mL filter-sterilised deionised water. Cultures of spores were stored at 5 °C until used.

Coupon preparation

Table I lists the compositions of the alloys tested in this research. Sheets (3 mm thick) of each metal alloy (supplied by the Copper Development Association, New York, NY, USA) were cut into coupons (10 mm by 10 mm). Coupons were degreased and cleaned by vortexing in acetone containing 2 mm diameter glass beads for 30 s and then immersed in absolute ethanol until used. Prior to use, excess ethanol was removed from coupons by flaming in a Bunsen burner.

Analysis

Aliquots (20 μL) of total cells (vegetative cells and spores) or purified spore culture were pipetted

Table I Composition of alloys tested

UNS no.	% Composition					
	Cu	Zn	Sn	Ni	Fe	Cr
C11000	100					
C26000	70	30				
C51000	95		5			
C70600	89			10	1	
C75200	65	17		18		
S30400				8	74	18

UNS, Unified Numbering System.

Please cite this article in press as: Weaver L et al., Survival of *Clostridium difficile* on copper and steel: Futuristic options for hospital hygiene, J Hosp Infect (2008), doi:10.1016/j.jhin.2007.11.011

onto the surface of the coupons. Coupons were incubated at room temperature (22 ± 2 °C) for various time periods. For each experiment triplicate coupons were analysed by culture or direct staining.

Culture analysis

Coupons were aseptically transferred to 5 mL phosphate-buffered saline (PBS) containing 20–30 glass beads (2 mm diameter) and vortexed for 30 s. Immediately thereafter, 0.1 mL and 1 mL aliquots were spread over *C. difficile* agar plates (CDA) supplemented with 5% (vol/vol) horse blood (Oxoid, Basingstoke, UK) in triplicate. Plates were allowed to dry before inverting and incubating anaerobically at 37 °C for 5 days. Colonies on plates were counted by eye and the concentration per coupon was calculated. The effect of copper release into the PBS on the viability of recovered organisms was investigated by the addition of ethylenediaminetetra-acetic acid (EDTA, 20 mM) which readily complexes free copper. No significant difference was seen (data not shown) between samples recovered into PBS or PBS with EDTA. Thorough analysis of coupons by episcopic differential interference contrast (EDIC) microscopy revealed no attached organisms after washing.

Direct staining protocol

After exposure, coupons were air-dried by placing in a flow of air (Class II cabinet).

SYTO9

Three microlitres of 1.67 mM SYTO9 (L7007; Invitrogen, Paisley, UK) was pipetted into 1 mL filter-sterilised deionised water; 20 μL of this working solution was pipetted onto coupons and incubated at room temperature for 1 h in the dark.

5-Cyano-2,3-ditolyl tetrazolium chloride (CTC)

Twenty microlitres of CTC (Sigma–Aldrich, Gillingham, UK) at a final concentration of 5 mM was pipetted onto the surface of the coupon. Coupons were placed in a humid chamber and incubated in the dark at 37 °C for 8 h.

Microscopy

Coupons were scanned directly using EDIC and epifluorescent microscopy (Nikon Eclipse Model ME600; Best Scientific, Wroughton, UK).[15] A minimum of 10 fields of view were photographed using a digital camera (Model Coolsnap CF; Roper Industries, Bury St Edmunds, UK) connected to a computer with digital image analysis software (Image-Pro Plus, version 4.5.1.22; Media Cybernetics, Marlow, UK). Total cells or spores (SYTO9-stained) and metabolically active cells or spores (CTC-stained) were enumerated.

Statistical analysis

Data are expressed as mean ± SEM. Differences between duplicate samples were assessed using Mann–Whitney rank *U*-test. Group comparisons were analysed using Mann–Whitney *U*-test where statistical significance was expressed as $P < 0.05$. Statistical analyses were performed using Sigma Stat version 3.5 and graphical representations were performed using Sigma Plot version 10 (Systat Software Inc., Hounslow, UK).

Results

Using culture methods and viability staining, survival of *C. difficile* on copper and copper alloy surfaces was compared with that on stainless steel. As no significant difference ($P \geq 0.05$) was found between duplicate coupons these results were pooled. All surfaces (copper and copper alloys and stainless steel) showed a significant reduction ($P < 0.05$) in numbers of both total cells (vegetative cells and spores) and purified spores of *C. difficile* obtained by culture methods (Figures 1 and 2). Although all surfaces showed a significant reduction in culturable *C. difficile* after 48 h, most of the cell death occurred within 6 h of exposure. Time for complete death (no culturable *C. difficile*) was fastest both in total cells and purified spores on pure copper (C11000) which occurred within 24 h. Alloy C51000 was the next most effective, taking 48 h to obtain no culturable *C. difficile* either as total cells or purified spores, followed by alloy C70600 which took 72 h. This appears to follow the concentration of copper (Cu) contained in the alloys: C11000, 100% Cu; C51000, 95% Cu; and C70600, 90% Cu. Alloys C26000 and C70600 did not show a significant difference compared with stainless steel S30400.

The number of viable cells on all copper alloys over a period of 48 h, estimated by CTC reduction, were observed by microscopy (Figures 3 and 4). Stainless steel resulted in no significant reduction in CTC-positive cells over an exposure period of 72 h. Moreover, when this exposure time was increased to 168 h (1 week), still no significant reduction was observed (data not shown). The rate of reduction in viability for the total counts (vegetative cells and spores) of *C. difficile* was fastest

Please cite this article in press as: Weaver L et al., Survival of *Clostridium difficile* on copper and steel: Futuristic options for hospital hygiene, J Hosp Infect (2008), doi:10.1016/j.jhin.2007.11.011

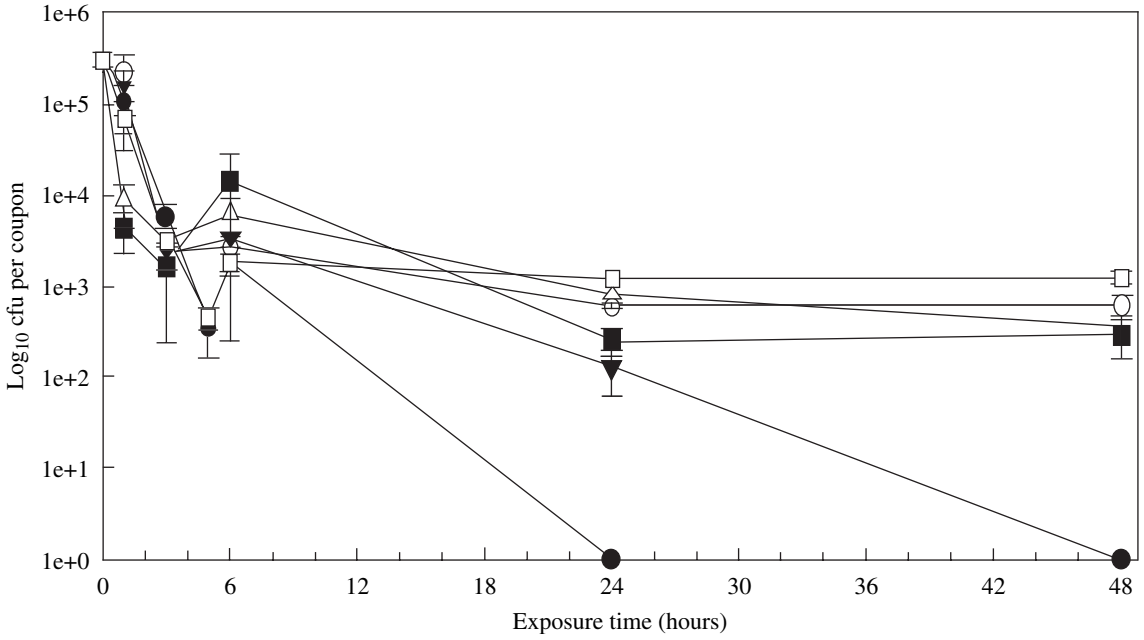

Figure 1 Effect of *C. difficile* (total vegetative cells and spores) viability after exposure to C11000 (●), C26000 (○), C51000 (▼), C70600 (△), C75200 (■) or S30400 (□) at 22 °C, using plate count method. Data points represent mean ($N = 6$) ± SEM.

on C51000, taking 24 h; followed by C11000, 48 h; and C26000, 72 h. The differences between the counts on C51000 and C11000 were very small (0 cells per coupon on C51000 and a mean of 1.2 cells per coupon on C11000, $N = 6$) and were not significant ($P \geq 0.05$). For purified spores, the fastest reduction in CTC activity was observed on C11000, 6 h; followed by C51000, 24 h; and C26000, 72 h.

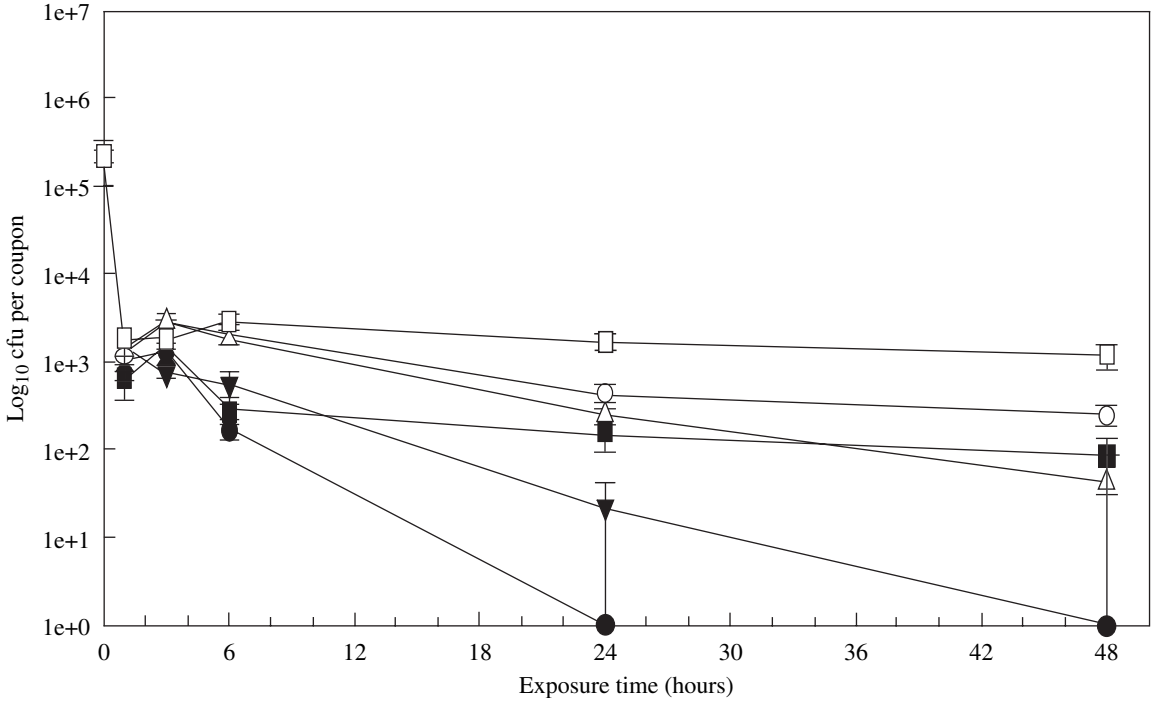

Figure 2 Effect of *C. difficile* (purified spores) viability after exposure to C11000 (●), C26000 (○), C51000 (▼), C70600 (△), C75200 (■) or S30400 (□) at 22 °C, using SYTO9 and CTC staining. Data points represent mean ($N = 6$) ± SEM.

Please cite this article in press as: Weaver L et al., Survival of *Clostridium difficile* on copper and steel: Futuristic options for hospital hygiene, J Hosp Infect (2008), doi:10.1016/j.jhin.2007.11.011

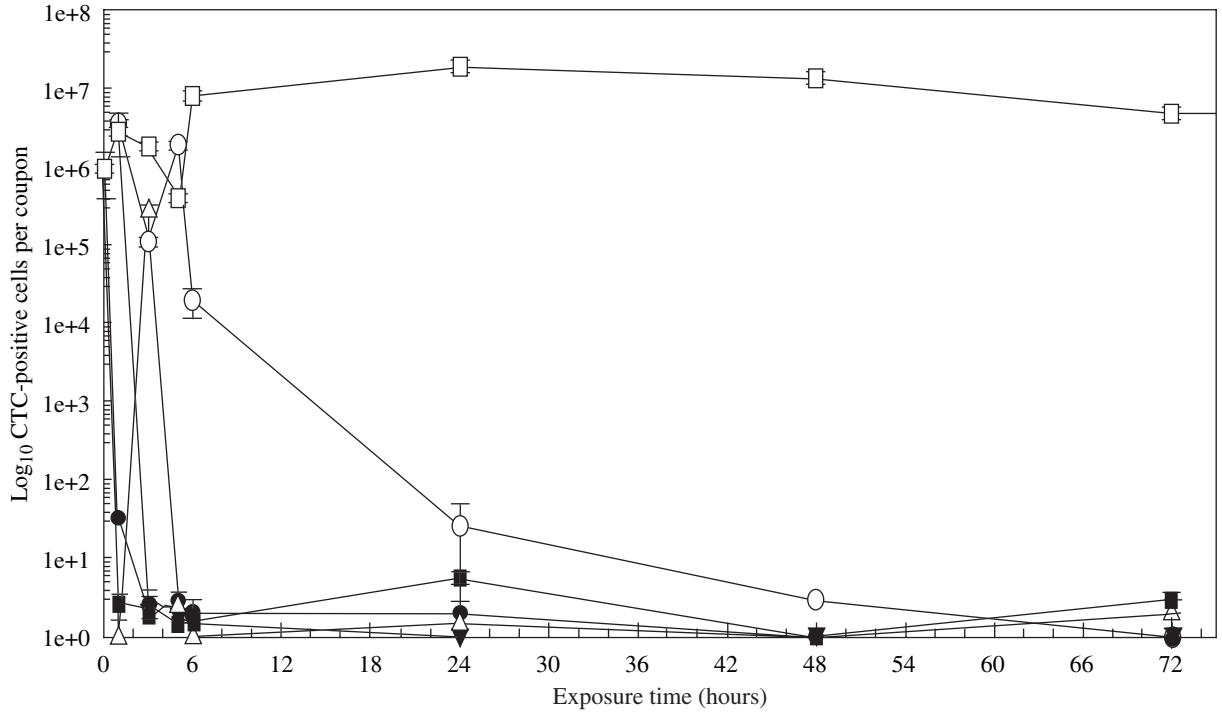

Figure 3 Effect of *C. difficile* (total vegetative cells and spores) viability after exposure to C11000 (●), C26000 (○), C51000 (▼), C70600 (△), C75200 (■) or S30400 (□) at 22 °C, using SYTO9 and CTC staining. Data points represent mean ($N = 6$) ± SEM.

The reduction of CTC observed for total *C. difficile* exposed to C70600 and C75200 was variable and although there was initially a significant decrease (by a factor of 10^4 total cells and spores) CTC-positive cells were detected up to 72 h (one to three CTC positive cells on C70600 and two to six CTC positive cells on C75200).

The number of cultured *C. difficile* was higher than the number of CTC-positive cells on all copper alloys but lower on stainless steel. Using

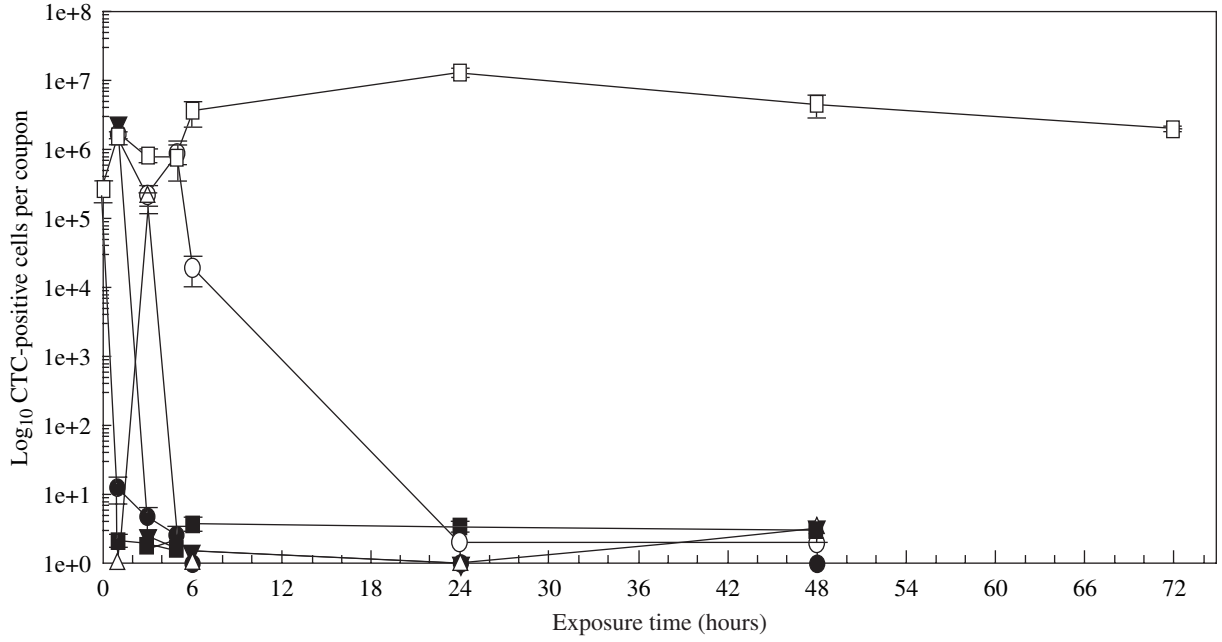

Figure 4 Effect of *C. difficile* (spores) viability after exposure to C11000 (●), C26000 (○), C51000 (▼), C70600 (△), C75200 (■) or S30400 (□) at 22 °C, using SYTO9 and CTC staining. Data points represent mean ($N = 6$) ± SEM.

Please cite this article in press as: Weaver L et al., Survival of *Clostridium difficile* on copper and steel: Futuristic options for hospital hygiene, J Hosp Infect (2008), doi:10.1016/j.jhin.2007.11.011

Mann–Whitney rank *U*-test, the differences in counts were only significant ($P \leq 0.05$) on C26000, C75200 and S30400. The rate of reduction in CTC activity was comparable with culture analysis except on C51000 where CTC reduction ceased before culture results.

Discussion

Hospital infections by *C. difficile* are increasing and can be serious in some patients, especially the elderly. Current cleaning regimens appear unable to remove spores which can then remain viable for long periods of time. These spores are resistant to a range of disinfectants, including the alcohol hand rubs recommended to control MRSA transmission. Therefore, it is essential to consider alternative strategies to control transmission between contaminated hands and contact surfaces; for example, by considering the use of surfaces with inherent antibacterial properties. Stainless steel is widely used for surfaces in hospitals (e.g. door plates, door handles and handwashing faucets) but despite being easy to clean it offers no antimicrobial effect. Previous studies have indicated that stainless steel is not a suitable material due to its poor performance as an antimicrobial agent.[16–19] As spores of *C. difficile* are resistant to cleaning regimens the installation of materials which offer antimicrobial effect could be of significant advantage in the hospital environment.

The data presented in this research demonstrate that the viability of *C. difficile* total cells and spores persists for long periods of time (>72 h) on stainless steel surfaces. Although the number of viable *C. difficile* cultured after exposure to stainless steel was significantly reduced, this was only true of the initial 3 h. A large decrease in the number of viable culture-positive and CTC-positive *C. difficile* was observed in the first 3–6 h on all surfaces tested. This is most likely due to cell death caused by exposure to aerobic atmosphere, as *C. difficile* is an anaerobic organism. In control experiments where *C. difficile* cultures were not exposed to copper alloys or stainless steel but left at room temperature on glass slides, similar results were observed in the first 3 h (data not shown).

The use of pure copper has not been exploited as a contact surface for use in healthcare environments due to its relative softness, high tarnishing rate and low corrosion resistance compared with other alloys.[19] An early study on the effectiveness of using brass, a copper alloy, for door knobs in hospitals recommended its use to aid prevention of nosocomial infections.[20] In the present study, alloys of high copper content, C51000 (95% Cu) and C70600 (70% Cu) along with pure copper (C11000), gave the highest cell death rates of *C. difficile*. These increased cell death rates have been demonstrated in previous research on aerobic bacteria relating to food preparation surfaces.[16,18] The extended time for copper alloys to kill *C. difficile* is probably due to the fact that the pathogen forms spores that are more resilient than non – spore forming cells of *Escherichia coli* and *Listeria monocytogenes*.

The use of CTC viability dye coupled with SYTO9 to establish total cell counts offers a rapid and convenient method to estimate cell death of *C. difficile* on surfaces. The numbers of CTC-positive cells were lower than with culture methods on copper alloys, but, with the exception of C51000, gave statistically similar results with culture analysis. The lower numbers may be due to the action of copper on bacterial cells. Copper has been shown to cause damage to bacterial cells by producing hydroperoxide radicals and possibly through damage to the cell membrane.[21,22] In this study, no indication of damage to cell membranes was observed using the BacLight (SYTO9 plus Propidium iodide) dye exclusion methodology (data not shown). CTC is reduced as an alternative electron acceptor within a cell's electron transfer chain and previous research has demonstrated the capability of assessing metabolic activity in anaerobic cells using CTC.[12] *C. difficile* ferments leucine via an oxidation reduction pathway.[23,24] One possibility is that CTC may replace acyl-CoA dehydrogenase or NADH within the pathway, thereby being reduced to the insoluble formazan. Copper ions could interfere with this electron transport pathway, thus preventing metabolism within the cell. This would also mean that CTC would not be reduced as an electron acceptor in the electron transport pathway.

The use of copper alloys in hospital environments may offer the potential to reduce the spread of *C. difficile* infections in healthcare establishments, particularly if coupled with optimal cleaning procedures. A significant increase in cell death rate can be seen on copper alloy surfaces (those with >70% copper) when compared with stainless steel. Using the redox dye, CTC offers a rapid method (8 h) for assessing viability of *C. difficile* instead of the more prolonged conventional culture analysis (5 days). In-situ staining also offers the advantage of whole sample analysis where low numbers are present, whereas culture analysis has a detection limit of 50 cells. As the infective dose may be low, it is

Please cite this article in press as: Weaver L et al., Survival of *Clostridium difficile* on copper and steel: Futuristic options for hospital hygiene, J Hosp Infect (2008), doi:10.1016/j.jhin.2007.11.011

imperative to be able to detect small numbers of *C. difficile* present on contaminated surfaces; the methodology developed in the current study may be useful in this regard.

Conflict of interest statement
None declared.

Funding sources
This research was supported by the Copper Development Association, New York, with assistance from the International Copper Association, New York.

References

1. Frost F, Craun GF, Calderon RL. Increasing hospitalisation and death possibly due to *Clostridium difficile* diarrhoeal disease. *Emerg Infect Dis* 1998;**4**:619–625.
2. Lafaro PE, Nahass RG. Increased number of deaths with concomitant diagnosis of *Clostridium difficile* in a community hospital. *Am J Infect Control* 2006;**34**:E151–E152.
3. McDonald LC, Owings M, Jernigan DB. *Clostridium difficile* infection in patients discharged from US short-stay hospitals, 1996–2003. *Emerg Infect Dis* 2006;**12**:409–415.
4. Wilcox MH, Fawley WN. Hospital disinfectants and spore formation by *Clostridium difficile*. *Lancet* 2000;**356**(9238): 1324.
5. Wilcox MH, Cunniffe JG, Trundle C, Redpath C. Financial burden of hospital-acquired *Clostridium difficile* infection. *J Hosp Infect* 1996;**34**:23–30.
6. Office of National Statistics, UK. *Clostridium difficile* deaths increase in 2005. London: Office for National Statistics; 2007.
7. Chaiyanan S, Chaiyanan S, Huq A, Maugel T, Colwell RR. Viability of the non-culturable *Vibrio cholerae* O1 and O139. *Syst Appl Microbiol* 2001;**24**:331–341.
8. Cools I, D'Haese E, Uyttendaele M, Storms E, Nelis JH, Debevere J. Solid phase cytometry as a tool to detect viable but non-culturable cells of *Campylobacter jejuni*. *J Microbiol Methods* 2005;**63**:107–114.
9. Hoefel D, Grooby WL, Monis PT, Andrews S, Saint CP. Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. *J Microbiol Methods* 2003;**55**:585–597.
10. Creach V, Baudoux A-C, Bertru G, Le Rouzic B. Direct estimate of active bacteria: CTC use and limitations. *J Microbiol Methods* 2003;**52**:19–28.
11. Laflamme C, Lavigne S, Ho J, Duchaine C. Assessment of bacterial endospore viability with fluorescent dyes. *J Appl Microbiol* 2004;**96**:684–692.
12. Bhupathiraju VK, Hernandez M, Landfear D, Alvarez-Cohen L. Application of a tetrazolium dye as an indicator of viability in anaerobic bacteria. *J Microbiol Methods* 1999;**37**:231–243.
13. Bhupathiraju VK, Hernandez M, Landfear D, Alvarez-Cohen L. A new direct microscopy based method for evaluating *in-situ* bioremediation. *J Hazard Mater* 1999; **67**:299–312.
14. Gombas DE, Gomez RF. Sensitization of *Clostridium perfringens* spores to heat by gamma radiation. *Appl Environ Microbiol* 1978;**36**:403–407.
15. Keevil CW. Rapid detection of biofilms and adherent pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy. *Water Sci Technol* 2003;**47**:105–116.
16. Noyce JO, Michels H, Keevil CW. Use of copper cast alloys to control *Escherichia coli* O157 cross-contamination during food processing. *Appl Environ Microbiol* 2006;**72**:4239–4244.
17. Robine E, Boulange-Petermann L, Derangere D. Assessing bactericidal properties of materials: the case of metallic surfaces in contact with air. *J Microbiol Methods* 2002;**49**: 225–234.
18. Wilks SA, Michels HT, Keevil CW. Survival of *Listeria monocytogenes* Scott A on metal surfaces: implications for cross contamination. *Int J Food Microbiol* 2006;**111**:93–98.
19. Wilks SA, Michels HT, Keevil CW. The survival of *Escherichia coli* O157 on a range of metal surfaces. *Int J Food Microbiol* 2005;**105**:445–454.
20. Kuhn PJ. Doorknobs: a source of nosocomial infection? *Diag Med* 1983 (Nov/Dec).
21. Rodriguez-Montelongo L, de la Cruz-Rodriguez LC, Farias RN, Massa EM. Membrane-associated redox cycling of copper mediates hydroperoxide toxicity in *Escherichia coli*. *Biochim Biophys Acta* 1993;**1144**:77–84.
22. Suwalsky M, Ungerer B, Quevedo L, Aguilar F, Sotomayor CP. Cu^{2+} ions interact with cell membranes. *J Inorg Biochem* 1998;**70**:233–238.
23. Kim J, Darley D, Buckel W. 2-Hydroxyisocaproyl-CoA dehydratase and its activator from *Clostridium difficile*. *FEBS J* 2004;**272**:550–561.
24. Kim J, Hetzel M, Boiangiu CD, Buckel W. Dehydration of (*R*)-2-hydroxyacyl-CoA to enoyl-CoA in the fermentation of α-amino acids by anaerobic bacteria. *FEMS Microbiol Rev* 2004;**28**:455–468.

Please cite this article in press as: Weaver L et al., Survival of *Clostridium difficile* on copper and steel: Futuristic options for hospital hygiene, J Hosp Infect (2008), doi:10.1016/j.jhin.2007.11.011