Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates

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The hospital environment is particularly susceptible to contamination by bacterial pathogens that grow on surfaces in biofilms. The effects of hospital biocides on two nosocomial pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*, growing as free-floating (planktonic) and adherent biofilm populations (sessile) were examined. Clinical isolates of MRSA and *P. aeruginosa* were grown as biofilms on discs of materials found in the hospital environment (stainless steel, glass, polyethylene and Teflon) and treated with three commonly used hospital biocides containing benzalkonium chloride (1 % w/v), chlorhexidine gluconate (4 % w/v) and triclosan (1 % w/v). Cell viability following biocide treatment was determined using an XTT assay and the LIVE/DEAD BacLight Bacterial Viability kit. The minimum bactericidal concentration (MBC) of all biocides for planktonic populations of both organisms was considerably less than the concentration recommended for use by the manufacturer. However, when isolates were grown as biofilms, the biocides were ineffective at killing bacteria at the concentrations recommended for use. Following biocide treatment, 0–11 % of cells in MRSA biofilms survived, and up to 80 % of cells in *P. aeruginosa* biofilms survived. This study suggests that although biocides may be effective against planktonic populations of bacteria, some biocides currently used in hospitals are ineffective against nosocomial pathogens growing as biofilms attached to surfaces and fail to control this reservoir for hospital-acquired infection.

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INTRODUCTION

Hospital-acquired infections are responsible for significant morbidity and mortality in today's healthcare environment. Recent estimates indicate that 9 % of in-patients in England and Wales suffer from a hospital-acquired infection at any one time, resulting in 5000 deaths and costing the National Health Service over £1 billion per year (National Audit Office, 2004). During the last decade, there has been an alarming rise in hospital-acquired infections by multi-drug-resistant micro-organisms (Livermore, 2000; Reacher *et al.*, 2000).

Two organisms of particular concern within the healthcare environment are methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant isolates of *Pseudomonas aeruginosa*. *S. aureus* is a leading cause of nosocomial bacteraemia, with 30–50 % of invasive isolates in the UK resistant to methicillin (Diekema *et al.*, 2001; Fluit *et al.*, 2001; EARSS, 2003). MRSA infections range from those of the skin and surgical sites, to infections relating to catheters

and prosthetic implants, to pneumonia (Diekema *et al.*, 2001). *P. aeruginosa* is a ubiquitous environmental bacterium that has become a major opportunistic pathogen among immuno-compromised patients in intensive care units or patients in neonatal or burns units, and is the principal cause of chronic infection, leading to lung damage, in cystic fibrosis (CF) sufferers (Govan & Deretic, 1996; Cunha, 2001; Gales *et al.*, 2001; Lawrence, 2002; Rossolini & Mantengoli, 2005).

Current procedures for infection control in hospitals have not been successful in curbing the rise in infections by multi-drug-resistant pathogens. MRSA and *P. aeruginosa* have been isolated from hospital surfaces including stethoscopes, catheters, and even disinfectant soap dispensers (Brooks *et al.*, 2002; Guinto *et al.*, 2002; Stickler, 2002). Bacteria grow in the natural environment attached to surfaces in biofilms (Costerton *et al.*, 1994). A biofilm comprises a functional consortium of cells enveloped within a matrix of extracellular polysaccharides. Surface association allows the organisms to persist in a favourable environment, while the biofilm structure protects cells from dehydration and other environmental pressures (Donlan & Costerton, 2002). Biofilms can form on almost any biological or abiotic surface and generally have susceptibilities towards antibiotics and biocides that are

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Abbreviations: CF, cystic fibrosis; HA-MRSA, hospital-acquired MRSA; MBC, minimum bactericidal concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; SEM, scanning electron microscopy.

100–1000-fold less than equivalent populations of planktonic bacteria (Gander, 1996; Gilbert *et al.*, 2002; O'Toole, 2002). Biofilms have an enormous impact on healthcare, and are estimated to be associated with 65 % of nosocomial infections (Potera, 1999). There is a desperate need to limit contamination of surfaces, equipment and implanted medical devices, and to find suitable agents for infection control that will contribute to strategies to eradicate this reservoir for infection.

In this study, the efficacy of commonly used hospital biocides was tested with biofilms of clinical isolates of hospital-acquired MRSA (HA-MRSA) and multi-drug-resistant *P. aeruginosa* grown on a range of surfaces found in the hospital environment. A colorimetric XTT assay was used to determine the percentage of viable cells in biofilms following biocide treatment, and the LIVE/DEAD BacLight Bacterial Viability kit was used to view the effect of biocides on biofilm viability by microscopy.

METHODS

Clinical isolates of nosocomial bacterial pathogens. Eight clinical isolates of HA-MRSA, including EMRSA-15 and EMRSA-16 strains and sporadic Scottish and European clones, were collected from the Scottish MRSA Reference Laboratory (Stobhill Hospital, Glasgow, UK) from a large library of strains. Eight clinical isolates of *P. aeruginosa* were collected from children with CF at the Royal Hospital for Sick Children (Yorkhill, Glasgow, UK). MRSA isolates were maintained on tryptone soy agar (Oxoid) and *P. aeruginosa* isolates were maintained on nutrient agar (Oxoid). Isolates were cultured from patients on blood agar (Oxoid) and stored in 80 % (v/v) glycerol at -70°C . Isolates were freshly subcultured before each experiment.

Biocides. Biocides were obtained in commercial preparations. These were: Anticide Bac-50, containing the quaternary ammonium compound benzalkonium chloride (50 %, w/v) (THOR); the cationic biocide MediHex-4, containing 4 % (w/v) chlorhexidine gluconate (Medichem International); and Mediscrub, containing 1 % (w/v) triclosan (Medichem International).

Minimum bactericidal concentrations of biocides with planktonic cells. The minimum bactericidal concentrations (MBC) of Anticide Bac-50, MediHex-4 and Mediscrub were determined by serial twofold dilution of the biocide in Mueller–Hinton broth (MHB) (Oxoid). For all experiments with *S. aureus*, the medium was supplemented with 2 % (w/v) NaCl (CLSI, 2002). Each dilution was inoculated with 2×10^3 c.f.u. of overnight culture in fresh MHB. The final volume of each culture was 1 ml. The cultures were incubated at 37°C for 24 h at 200 r.p.m., then 100 μl of each dilution was plated on Mueller–Hinton agar (MHA). The plates were incubated at 37°C for 24 h and colony counts were determined. Each dilution series was performed in triplicate, the entire MBC experiment was replicated twice, and the data from these experiments were pooled.

Culture of bacteria as biofilms. Discs (1 cm in diameter and 1 mm depth) of polished medical-grade stainless steel (304) and plastics (Teflon and polyethylene) were sterilized in 70 % (v/v) ethanol for 10 min (Sopwith *et al.*, 2002). One colony of each isolate of HA-MRSA and *P. aeruginosa* was used to inoculate 5 ml MHB, and the cultures were incubated at 37°C for 24 h at 250 r.p.m. The sterile discs were placed in a Petri dish and covered with 19 ml MHB.

Overnight culture of each strain (1 ml) was then added to the Petri dish containing discs of the three materials. Triplicate discs were used for each strain for each biocide treatment, together with biocide-free controls. Discs were incubated in the cultures for 24 h at 37°C .

Bacterial growth in biofilms on hospital-associated materials treated with biocides. Triplicate discs coated in biofilms of HA-MRSA and *P. aeruginosa* that had been grown for 24 h were removed from culture and rinsed three times with 5 ml sterile $1 \times$ PBS to remove planktonic cells. These were then transferred to 7 ml bijoux tubes and completely covered with 200 μl biocide. Anticide Bac-50 was used at a final concentration of 1 % (w/v) benzalkonium chloride; MediHex-4 and Mediscrub were used in the original commercial preparations, which have concentrations of 4 % (w/v) chlorhexidine gluconate and 1 % (w/v) triclosan, respectively (dilutions were made in MHB). These concentrations were used to mimic the concentrations recommended for use in the hospital environment by the biocide manufacturers. Biofilm-coated discs were incubated with biocides for 24 h at 37°C . Each biocide was tested on triplicate biofilms of each strain and experiments were repeated once.

Quantification of viable cells in the biofilm by XTT assay. The principle of the XTT assay is that viable cells will reduce the tetrazolium salt XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5 carboxanilide; Sigma] to an orange-coloured water-soluble product (Tunney *et al.*, 2004). The XTT salt was prepared as a saturated solution (1 mg ml^{-1} in $1 \times$ PBS), filter-sterilized and stored at -70°C . Menadione (Aldrich), the electron mediator for the reaction, was prepared (50 mM in acetone) and stored at -70°C . Prior to each assay, aliquots of XTT and menadione were thawed and diluted in filter-sterilized $1 \times$ PBS to final concentrations of 0.5 mg ml^{-1} and 50 μM , respectively.

There was a linear relationship between cell number and reduction of XTT. This was determined by preparing a dilution series of 18 h cultures of HA-MRSA isolate 2 and *P. aeruginosa* isolate 2 (1×10^3 – 1×10^9 c.f.u. ml^{-1}) in $1 \times$ PBS. Cells were collected by centrifugation, resuspended in 150 μl XTT/menadione solution, and incubated in the dark for 2 h. Cells were pelleted and the supernatant from each tube was transferred to a 96-well flat-bottom plate (Nunc) and the reduced XTT was measured at 492 nm using a microtitre plate reader (iEMS Reader, Thermo Labsystems). The cells were plated on MHA, incubated for 18 h at 37°C , and colonies were counted. There was a correlation coefficient (R^2) of 0.908 and 0.953, respectively, between the number of HA-MRSA and *P. aeruginosa* c.f.u. and the reduction of XTT.

Following exposure to biocides, discs were rinsed three times with 5 ml $1 \times$ PBS and 300 μl XTT/menadione solution was added to each disc in a 7 ml bijoux tube. The discs were incubated at 37°C in the dark for 2 h. Biocide-free biofilms and discs without biofilms were used as positive and negative controls, respectively. The liquid from each tube was then transferred to a 96-well plate and the reduced XTT was measured at 492 nm. The percentage of viable cells in biocide-treated biofilms was calculated by comparing the reduction of XTT to that of biocide-free control biofilms.

Enumeration of viable cells by fluorescent confocal microscopy. Biofilms were grown from HA-MRSA isolate 2 and *P. aeruginosa* isolate 1 on triplicate poly-lysine-coated glass coverslips (64 $\times$ 21 mm no. 1) for 24 h and treated with biocides, as described previously for biofilms grown on stainless steel and plastic discs. These isolates were chosen because of their innate ability to form substantial biofilms. After incubation, coverslips were rinsed three times with 5 ml sterile $1 \times$ PBS, and stained using the LIVE/DEAD BacLight Bacterial Viability kit (L7007, Molecular Probes, Invitrogen). A solution composed of 1.5 μl Component A with

1.5 µl Component B in 997 µl sterile distilled water was made up, and 300 µl was added to each coverslip; these were incubated in darkness for 15 min at room temperature. Each coverslip was inverted, mounted on a microscope slide and viewed using a Nikon Eclipse TE2000 S inverted confocal microscope using fluorescein and Texas red band-pass filters to visualize SYTO 9 and propidium iodide, respectively. Images were captured with an integrated Hamamatsu digital camera (C4742-95; Nikon UK) from six areas of each of the triplicate biofilms. IPLab Analytical Imaging Software (BD Biosciences) was used to calculate the mean number of green and red pixels in each area.

Statistical analysis of data. The data from all experiments were analysed using the Prism software package (GraphPad Software). Standard deviations between replicate samples for all experiments were calculated and an unpaired two-tailed *t*-test was performed to determine whether there was a statistically significant difference between growth as biofilms on the three hospital surfaces.

RESULTS

MBCs of hospital biocides for planktonic cultures

The MBCs of Anticide Bac-50, MediHex-4 and Mediscrub were determined by broth micro-dilution for eight HA-MRSA isolates and eight *P. aeruginosa* isolates grown in planktonic culture. The MBC of a biocide is the concentration which kills 99.9% of the bacterial inoculum (Gilbert & McBain, 2003). The concentration of benzalkonium chloride recommended for use by the manufacturer of Anticide Bac-50 is 1% (w/v), the concentration of chlorhexidine gluconate recommended for use by the manufacturer of MediHex-4 is 4% (w/v) and the concentration of triclosan recommended for use by the manufacturer of Mediscrub is 1%. For HA-MRSA isolates, the MBC of Anticide Bac-50 and MediHex-4 ranged from 0.0004 to 0.001% (w/v) and the MBC of Mediscrub ranged from 0.0005 to 0.001% (w/v). MBCs for *P. aeruginosa* were higher, ranging from 0.01 to 0.1% (w/v) for Anticide Bac-50, 0.4 to 2% (w/v) for MediHex-4, and 0.1% (w/v) with Mediscrub.

Growth of biofilms on hospital surfaces

The eight HA-MRSA and eight *P. aeruginosa* isolates formed biofilms on stainless steel, Teflon and polyethylene. Following incubation for 24 h, growth of HA-MRSA in biofilms was less vigorous than that of *P. aeruginosa*. Biofilm formation by HA-MRSA isolates depended on the surface, with the greatest growth exhibited by all isolates on stainless steel discs. This increased colonization of stainless steel was statistically significant ($P < 0.05$) for five out of the eight isolates tested compared to growth on the plastics (Fig. 1a). *P. aeruginosa* isolates grew equally on all surfaces, but the level of biofilm growth varied between isolates. Biofilms formed after 24 h by isolates 5 and 6 displayed significantly less growth ($P < 0.05$) than all other *Pseudomonas* isolates (Fig. 1b).

Fig. 1. Growth of eight HA-MRSA (a) and eight *P. aeruginosa* biofilms (b) on polyethylene (black bars), Teflon (white bars) and stainless steel (grey bars) as estimated by reduction of XTT (*= $P < 0.05$). Error bars represent the standard deviation between triplicate samples.

Quantification of cell viability in biofilms following biocide treatment

HA-MRSA biofilms retained 0–11% cell viability following treatment with Anticide Bac-50 (1% w/v benzalkonium chloride: Fig. 2a), MediHex-4 (containing 4% w/v chlorhexidine gluconate: Fig. 3a), and Mediscrub (1% w/v triclosan: Fig. 4a). HA-MRSA biofilms grown on stainless steel discs retained the highest proportion of live cells following treatment with Anticide Bac-50 and MediHex-4. From initial growth experiments, the biofilms formed by five out of eight HA-MRSA isolates on stainless steel displayed significantly ($P < 0.05$) more growth than the biofilms on plastic surfaces (Fig. 1a). This may explain why biofilms grown on stainless steel discs had a significantly higher proportion of live cells following treatment with Anticide Bac-50 and MediHex-4 ($P < 0.01$ and $P < 0.001$, respectively).

P. aeruginosa biofilms treated with Anticide Bac-50 retained 4–80% cell viability (Fig. 2b), MediHex-4 treatment resulted in 30–80% cell viability (Fig. 3b), and biofilms treated with Mediscrub displayed 20–80% cell viability (Fig. 4b), in comparison to biocide-free controls. There was no statistically significant difference in cell viability between the three surfaces ($P = 0.9$, $P = 0.3$ and $P = 0.9$ for Anticide Bac-50, MediHex-4 and Mediscrub, respectively).

Fig. 2. Cell viability (%) in biofilms of HA-MRSA (a) and *P. aeruginosa* (b) following treatment with Anticide Bac-50 (1%, w/v, benzalkonium chloride). Error bars represent the standard deviation between triplicate samples; this is too small to visualize for many of the samples.

Enumeration of viable cells by confocal microscopy using the LIVE/DEAD stain

The LIVE/DEAD BacLight Bacterial Viability kit, containing SYTO 9 and propidium iodide, was used to stain biofilms of HA-MRSA isolate 2 and *P. aeruginosa* isolate 1 grown on poly-lysine-coated glass coverslips following biocide treatment, to visualize live and dead cells. Following 24 h incubation, the HA-MRSA isolate adhered to the surface in dense monolayers rather than forming a

mature three-dimensional biofilm structure (Fig. 5). This may have been a result of growth in the limited nutrients of the MHB or a consequence of the microbes growing on glass. The majority of cells in the HA-MRSA monolayer treated with Anticide Bac-50 stained with propidium iodide (Fig. 5b). Analysis of the image concluded that the mean percentage of live cells in this monolayer was 1% ($\pm 0.59\%$) (mean $\pm$ SD), suggesting that 99% of cells were

Fig. 3. Cell viability (%) in biofilms of HA-MRSA (a) and *P. aeruginosa* (b) following treatment with MediHex-4 (4%, w/v, chlorhexidine gluconate). Error bars represent the standard deviation between triplicate samples; this is too small to visualize for many of the samples. See Fig. 2 for key to bars.

Fig. 4. Cell viability (%) in biofilms of HA-MRSA (a) and *P. aeruginosa* (b) following treatment with Mediscrub (1%, w/v, triclosan). Error bars represent the standard deviation between triplicate samples; this is too small to visualize for many of the samples. See Fig. 2 for key to bars.

Fig. 5. Effect of biocides on an HA-MRSA biofilm (isolate 2). (a) Biocide-free control; (b) biofilm treated with Anticide Bac-50; (c) biofilm treated with MediHex-4; (d) biofilm treated with Mediscrub. Bacteria stained fluorescent green (SYTO 9) have intact membranes, whereas bacteria stained red (propidium iodide) have damaged membranes, allowing propidium iodide to enter the cell.

killed by Anticide Bac-50 in comparison to the biocide-free control (Fig. 5a). Following treatment with MediHex-4, 12 % (± 1.6 %) of cells survived (Fig. 5c). Mediscrub treatment resulted in the monolayer retaining 11 % (± 1.8 %) cell viability (Fig. 5d).

Following Anticide Bac-50 treatment, 30 % of the *P. aeruginosa* biofilm stained green (Fig. 6b), indicating that this biocide failed to kill 30 % (± 0.73 %) of cells in comparison to the biocide-free control (Fig. 6a). MediHex-4 had similar efficacy against the *P. aeruginosa* biofilm, killing 75 % (± 1.25 %) of cells (Fig. 6c), and Mediscrub killed 84 % (± 1.5 %) cells (Fig. 6d).

DISCUSSION

Bacterial biofilms are thought to be responsible for 65 % of nosocomial infections (Potera, 1999). In the hospital environment, micro-organisms colonize virtually all surfaces as biofilms, creating a reservoir for infection for hospitalized individuals (Stickler, 2002; Vincent, 2003). In this environment, it is imperative that suitable biocides and infection control procedures are enforced to limit this risk of infection. In this study, the efficacy of commonly used hospital biocides, Anticide Bac-50, MediHex-4 and Mediscrub (containing the active agents benzalkonium chloride, chlorhexidine gluconate and triclosan,

respectively), was determined using clinical isolates of HA-MRSA and *P. aeruginosa* grown in liquid culture and as biofilms. Biofilms were grown on materials found universally in the hospital environment: stainless steel, widely used for surfaces and equipment, and polyethylene and Teflon, which are used in the manufacture of endoscopes, protective splash aprons, catheters and prosthetic joints (Neely & Maley, 2000; Donlan & Costerton, 2002).

The MBCs of hospital biocides were tested initially by broth micro-dilution using planktonic cultures of clinical isolates of HA-MRSA and *P. aeruginosa* (CLSI, 2002). The concentration recommended by the manufacturer for use of Anticide Bac-50 is 1 % (w/v) benzalkonium chloride, for MediHex-4 is 4 % (w/v) chlorhexidine gluconate, and for Mediscrub is 1 % (w/v) triclosan. MBC of all biocides for all HA-MRSA isolates in liquid culture were 100–1000-fold lower than that recommended by the manufacturer. MBCs with *P. aeruginosa* isolates were 10–100-fold lower than the recommended concentrations of these biocides. This might indicate that when these biocides are used in the hospital environment they effectively kill over 99.9 % of bacteria. However, in the hospital environment, bacteria grow attached to surfaces in biofilms, and the susceptibility of bacteria to antimicrobials in this mode of growth has been reported previously to be lower than the same isolates

Fig. 6. Effect of biocides on a *P. aeruginosa* biofilm (isolate 1). (a) Biocide-free control; (b) biofilm treated with Anticide Bac-50; (c) biofilm treated with MediHex-4; (d) biofilm treated with Mediscrub. Bacteria stained fluorescent green (SYTO 9) have intact membranes, whereas bacteria stained red (propidium iodide) have damaged membranes, allowing propidium iodide to enter the cell.

grown in planktonic culture (Lewis, 2001; Mah & O'Toole, 2001; Gilbert *et al.*, 2002).

In this study, hospital biocides were used at concentrations recommended by the manufacturer, to mirror the conditions of use in the hospital environment. Using the XTT assay and the LIVE/DEAD staining kit, the MBCs of all three biocides with clinical isolates of HA-MRSA were found to be 100–1000-fold higher than the same isolates grown in planktonic culture. *P. aeruginosa* isolates had MBCs for all three biocides that were 10–100-fold higher than the same isolates grown in planktonic culture. When challenged with the three hospital biocides at the recommended concentrations, *P. aeruginosa* biofilms displayed up to 80 % cell viability and HA-MRSA biofilms exhibited 0–11 % cell viability. The proportion of live cells varied between isolates and depended on the surface on which the biofilm was grown. *P. aeruginosa* biofilms displayed a higher percentage of viable cells in comparison to HA-MRSA biofilms for all hospital biocides tested, and none of the biocides killed 100 % of *P. aeruginosa* cells in biofilms formed by the eight clinical isolates. These results highlight the importance of testing biocide efficacy with bacterial biofilms rather than using traditional Clinical and Laboratory Standards Institute (CLSI) methodology, when developing recommendations for the use of these products in the hospital environment (Donlan & Costerton, 2002; CLSI, 2002).

The effect of biocides on bacterial biofilms has been determined by others, by treating the biofilm with an antimicrobial, removing the cells by sonication and estimating the number of viable cells by colony counts. This method can result in the incomplete removal of cells from the surface, non-dispersal of bacterial aggregates and rupture of some cells by sonication that combine to produce inaccuracy in viable cell counts. In this study, two robust methods were used to measure cell viability without disrupting or removing the intact biofilm; the LIVE/DEAD BacLight Bacterial Viability kit gave a visual representation of live and dead cells by green or red staining on the basis of membrane integrity, and the XTT assay gave an estimation of metabolically active cells. This assay has been used successfully to test the effect of antimicrobials on a range of bacterial and fungal pathogens (Ramage *et al.*, 2001; Tunney *et al.*, 2004; Cerca *et al.*, 2005). Together these assays allow the viability of cells within the biofilm to be more accurately determined.

When viability of cells within the biofilms of HA-MRSA isolate 2 and *P. aeruginosa* isolate 1 treated with biocides were determined by XTT assay and the LIVE/DEAD stain, there was no systematic difference in results by either method. The viability of the HA-MRSA biofilm following treatment with Anticide Bac-50, MediHex-4 and Mediscrub was detected by the XTT assay as 0.8–3.5 %, 3–11 % and 3–8 %, respectively, depending on the surface material (Figs 2a, 3a and 4a). With the LIVE/DEAD stain, the HA-MRSA biofilm had 1 % (± 0.59 %), 12 % (± 1.6 %)

and 11 % (± 1.8 %) cell viability. For the *P. aeruginosa* biofilm, the XTT assay showed 12–38 %, 35–82 % and 18–22 % cell viability following exposure to Anticide Bac-50, MediHex-4 and Mediscrub respectively (Figs 2b, 3b and 4b). The LIVE/DEAD stain displayed 30 % (± 0.73 %), 25 % (± 1.25 %) and 16 % (± 1.5 %) cell viability following exposure to Anticide Bac-50, MediHex-4 and Mediscrub, respectively. The XTT assay and LIVE/DEAD stain are two highly useful methods which can be used for the detection of viable cells within intact biofilms. They provide an alternative tool to the traditional sonication-based methods for the quantification of cell viabilities in biofilms.

All clinical isolates of HA-MRSA and *P. aeruginosa* included in this study formed biofilms on stainless steel and plastics found in the hospital environment. However, these two organisms had different potentials for forming biofilms. *P. aeruginosa* isolates formed thicker biofilms compared to HA-MRSA isolates, following 24 h of incubation at 37 °C. This may be due to the enhanced intrinsic ability of *P. aeruginosa* to form recalcitrant biofilms, as displayed in the CF lung, or that the HA-MRSA isolates selected for this study may not have been the most adherent (Govan & Deretic, 1996). To test this hypothesis, a larger number of HA-MRSA strains would have to be screened for the ability to form biofilms. In this study, biofilm formation by HA-MRSA isolates was significantly enhanced on stainless steel. There have been a number of reports that support the enhanced binding of bacteria, including *S. aureus*, to stainless steel over other metals in orthopaedic implants and surfaces used in the food production industry, due to the hydrophilic nature of this material (Hyde *et al.*, 1997; Chmielewski & Frank, 2003; Sheehan *et al.*, 2004). Although the surface of the stainless steel was polished and appeared smooth, when the three materials were examined by scanning electron microscopy (SEM), the stainless steel was marked by pronounced striations (data not shown). These irregularities increase the surface area of the material and may be the reason why the *S. aureus* isolates formed more robust biofilms on the stainless steel discs, in comparison to the plastics. Both plastic surfaces appeared smooth when inspected by SEM (data not shown). From these results it can be concluded that in the case of *S. aureus*, the surface topography of the material that the cells are attached to has a major effect on biofilm development.

P. aeruginosa isolates formed biofilms on all surfaces, but two isolates formed diminished biofilm structures compared to the other isolates. These isolates were taken from CF patients with primary *P. aeruginosa* infections and had a non-mucoid phenotype, whereas all other isolates had a mucoid phenotype and overproduced alginate. However, this is not a necessary requisite as it has been suggested that alginate is not required for biofilm formation by the non-mucoid *P. aeruginosa* strains PAO1 and PA14 (Wozniak *et al.*, 2003). This study has shown that non-mucoid clinical isolates are still able to form biofilms, but they do not establish the enhanced level of growth of mucoid strains.

Anticide Bac-50, at a concentration of 1 % benzalkonium chloride (w/v), had the greatest bactericidal activity against HA-MRSA and *P. aeruginosa* biofilms, with the majority of biofilms displaying less than 8 % and 60 % cell viability following treatment, respectively. The efficacy of biocides varied between strains and, to an extent, between surfaces. Biocides were more effective against HA-MRSA biofilms than *P. aeruginosa* biofilms, and none of the *P. aeruginosa* biofilms were rendered completely non-viable by the hospital biocides. The results showed clearly that up to 11 % of cells in HA-MRSA biofilms and up to 80 % of cells in *P. aeruginosa* biofilms survived exposure to the biocides. This survivor population was confirmed by LIVE/DEAD staining. These cells may have survived attack due to their spatial arrangement and physical protection in the biofilm or they may possess a more favourable phenotype which allowed them to survive the effects of the antimicrobial. Such cells have the ability to resurrect the biofilm and sustain a recalcitrant infection or source of environmental contamination (Lewis, 2001; Spoering & Lewis, 2001).

This study has highlighted the varying efficacy of common hospital biocides on biofilms formed by clinical isolates of two important nosocomial pathogens. Each biocide was used at the concentration recommended by the manufacturer, to emulate the concentration that would be used in the hospital environment. None of the biocides killed 100 % of cells in the biofilms formed by multi-drug-resistant isolates of MRSA and *P. aeruginosa*. This suggests that when these biocides are used in hospitals, they fail to eradicate bacterial biofilms, leaving a survivor population to provide a reservoir for the spread and preservation of the infectious agent. In the hospital setting, the application of biocides is not sufficient to eliminate live bacteria growing in biofilms and should be used in conjunction with a rigorous manual cleansing regime to disrupt surface contamination, and a vigilant attitude to cleanliness and infection control.

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REFERENCES

Brooks, S. E., Walczak, M. A., Hameed, R. & Coonan, P. (2002). Chlorhexidine resistance in antibiotic-resistant bacteria isolated from the surfaces of dispensers of soap containing chlorhexidine. *Infect Control Hosp Epidemiol* **23**, 692–695.

Cerca, N., Martins, S., Cerca, F., Jefferson, K. K., Pier, G. B., Oliveira, R. & Azeredo, J. (2005). Comparative assessment of antibiotic susceptibility of coagulase negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. *J Antimicrob Chemother* **56**, 331–336.

Chmielewski, R. A. N. & Frank, J. F. (2003). Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety* **2**, 22–32.

CLSI (2002). M7–A5. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Second Edition*: Approved Standard, 5th edn. Villanova, PA, USA: Clinical and Laboratory Standards Institute.

Costerton, J. W., Lewandowski, Z., De Beer, D., Caldwell, D., Korker, D. & Jones, G. (1994). Biofilms, the customized microniche. *J Bacteriol* **176**, 2137–2142.

Cunha, B. A. (2001). Nosocomial pneumonia. Diagnostic and therapeutic considerations. *Med Clin North Am* **85**, 79–114.

Diekema, D. J., Pfaller, M. A., Schmitz, F. J., Smayevsky, J., Bell, J., Jones, R. N., Beach, M. & SENTRY Participants Group (2001). Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. *Clin Infect Dis* **32** (Suppl. 2), S114–S132.

Donlan, R. M. & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**, 167–193.

EARSS (2003). On-going surveillance of *S. pneumoniae*, *S. aureus*, *E. coli*, *E. faecium*, *E. faecalis*. Bilthoven, the Netherlands: RIVM.

Fluit, A. C., Wielders, C. L., Verhoef, J. & Schmitz, F. J. (2001). Epidemiology and susceptibility of 3,051 *Staphylococcus aureus* isolates from 25 university hospitals participating in the European SENTRY study. *J Clin Microbiol* **39**, 3727–3732.

Gales, A. C., Jones, R. N., Turnidge, J., Rennie, R. & Ramphal, R. (2001). Characterization of *Pseudomonas aeruginosa* isolates: occurrence rates, antimicrobial susceptibility patterns, and molecular typing in the Global SENTRY Antimicrobial Surveillance Program, 1997–1999. *Clin Infect Dis* **32**, S146–S155.

Gander, S. (1996). Bacterial biofilms: resistance to antimicrobial agents. *J Antimicrob Chemother* **37**, 1047–1050.

Gilbert, P. & McBain, A. J. (2003). Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clin Microbiol Rev* **16**, 189–208.

Gilbert, P., Allison, D. G. & McBain, A. J. (2002). Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *J Appl Microbiol* **92**, 98S–110S.

Govan, J. R. W. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60**, 539–574.

Guinto, C. H., Bottone, E. J., Raffalli, J. T., Montecalvo, M. A. & Wormser, G. P. (2002). Evaluation of dedicated stethoscopes as a potential source of nosocomial pathogens. *Am J Infect Control* **30**, 499–502.

Hyde, F. W., Alberg, M. & Smith, K. (1997). Comparison of fluorinated polymers against stainless steel, glass and polypropylene in microbial biofilm adherence and removal. *J Ind Microbiol Biotechnol* **19**, 142–149.

Lawrence, D. (2002). Clues to mechanism of *Pseudomonas* resistance in cystic fibrosis. *Lancet* **359**, 1410.

Lewis, K. (2001). The riddle of biofilm resistance. *Antimicrob Agents Chemother* **45**, 999–1007.

Livermore, D. M. (2000). Antibiotic resistance in staphylococci. *Int J Antimicrob Agents* **16**, S3–S10.

Mah, T. F. & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**, 34–39.

- National Audit Office (2004).** Improving Patient Care by Reducing the Risk of Hospital Acquired Infection: a Progress Report. London: Stationery Office.
- Neely, A. N. & Maley, M. P. (2000).** Survival of enterococci and staphylococci on hospital fabrics and plastic. *J Clin Microbiol* **38**, 724–726.
- O'Toole, G. A. (2002).** A resistance switch. *Nature* **416**, 695–696.
- Potera, C. (1999).** Forging a link between biofilms and disease. *Science* **283**, 1837–1838.
- Ramage, G., Vande Walle, K., Wickes, B. L. & López-Ribot, J. L. (2001).** Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* **45**, 2475–2479.
- Reacher, M. H., Shah, A., Livermore, D. M., Wale, M. C. J., Graham, C., Johnson, A. P., Heine, H., Monnickendam, M. A., Barker, K. F. & other authors (2000).** Bacteraemia and antibiotic resistance of its pathogens reported in England and Wales between 1990 and 1998: trend analysis. *BMJ* **320**, 213–216.
- Rossolini, G. M. & Mantengoli, E. (2005).** Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. *Clin Microbiol Infect* **11**, 17–32.
- Sheehan, E., McKenna, J., Mulhall, K. J., Marks, P. & McCormack, D. (2004).** Adhesion of *Staphylococcus* to orthopaedic metals, an *in vivo* study. *J Orthop Res* **22**, 39–43.
- Sopwith, W., Hart, T. & Garner, P. (2002).** Preventing infection from reusable medical equipment: a systematic review. *BMC Infect Dis* **2**, 4.
- Spoering, A. L. & Lewis, K. (2001).** Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* **183**, 6746–6751.
- Stickler, D. J. (2002).** Susceptibility of antibiotic-resistant Gram-positive bacteria to biocides: a perspective from the study of catheter biofilms. *Symp Ser Soc Appl Microbiol* **31**, 163S–170S.
- Tunney, M. M., Ramage, G., Field, T. R., Moriarty, T. F. & Storey, D. G. (2004).** Rapid colorimetric assay for antimicrobial susceptibility testing of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **48**, 1879–1881.
- Vincent, J.-L. (2003).** Nosocomial infections in adult intensive-care units. *Lancet* **361**, 2068–2077.
- Wozniak, D. J., Wyckoff, T. J. O., Starkey, M., Keyser, R., Azadi, P., O'Toole, G. A. & Parsek, M. R. (2003).** Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A* **100**, 7907–7912.