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, meticillin-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.

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 meticillin-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 meticillin (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 prostatic 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
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 × 10^8 cfu 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. 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 × PBS to remove planktonic cells. These were then transferred to 7 ml bijou 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 × 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 × 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 × 10^7 c.f.u. ml^-1) in 1 × 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 × PBS and 300 μl XTT/menadione solution was added to each disc in a 7 ml bijou 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-lysin-coated glass coverslips (64 × 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 × 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 benzalk-
onium 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).

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 chlor-
hexidine 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. 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).
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% (±0.59%) (mean ± SD), suggesting that 99% of cells were dead.
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.
The HA-MRSA biofilm had 1% (3–11%) and 3–8% respectively, depending on the surface. Mediscrub was detected by the XTT assay as 0.8–3.5%, treatment with Anticide Bac-50, MediHex-4 and Mediscrub, respectively. The viability of the HA-MRSA biofilm following 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., 2007; 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; Spoerling & 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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