

Comparison of various antimicrobial agents as catheter lock solutions: preference for ethanol in eradication of coagulase-negative staphylococcal biofilms

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Coagulase-negative staphylococci (CoNS) are the main causative agents of bacteraemia in infants managed in neonatal intensive care units (NICUs). Intraluminal colonization of long-term central venous catheters by these bacteria and subsequent biofilm formation are the prerequisites of the bloodstream infections acquired in NICUs. The catheter lock technique has been used to treat catheter colonization; however, the optimum choice of antimicrobial agents and their corresponding concentrations and exposure times have not been determined. The effectiveness of catheter lock solutions (CLSs) was assessed by determining the minimal biofilm eradication concentration of antimicrobial agents against CoNS biofilms. Five conventional antibiotics (oxacillin, gentamicin, vancomycin, ciprofloxacin and rifampicin) alone or in combination, as well as ethanol, were evaluated. Ethanol was found to be superior to all of these conventional antibiotics when used as a CLS. A time–kill study and confocal laser scanning microscopy revealed that exposure to 40% ethanol for 1 h was sufficient to kill CoNS biofilm cells. To our knowledge, this is the first *in vitro* study to provide solid evidence to support the rationale of using ethanol at low concentrations for a short time as a CLS, instead of using conventional antibiotics at high concentrations for a long period to treat catheter-related bloodstream infections.

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INTRODUCTION

Coagulase-negative staphylococci (CoNS), predominantly *Staphylococcus epidermidis*, are the most common causative agents of bloodstream infections in neonatal intensive care units (NICUs) (Stoll *et al.*, 2002; Villari *et al.*, 2000). These bacteria are relatively avirulent compared with *Staphylococcus aureus* and other organisms that also cause bloodstream infections. The pathogenesis of CoNS infection depends mainly on their ability to form biofilms on the surfaces of various polymers (Klingenberg *et al.*, 2005; von Eiff *et al.*, 2002). Long-term catheters are used in both ambulant and hospitalized patients in areas such as intensive care and oncology to provide central venous access for various therapies (Ackoundou-N'guessan *et al.*, 2006; Onland *et al.*, 2006; Opilla *et al.*, 2007; van de Wetering & van Woensel, 2007). Major adverse effects of

long-term catheterization are catheter-related bloodstream infections (CRBSIs), due to intraluminal colonization of catheters by CoNS, and subsequent biofilm formation on the surface of the catheter lumen.

Systemic antibiotics have been widely used to treat CRBSIs, but failures have been reported frequently (Allon, 2004; Benjamin *et al.*, 2001; Berrington & Gould, 2001; Gagnon *et al.*, 1993) due to the inability of most conventional antibiotic therapies to eradicate biofilm-grown bacteria (Donlan, 2000; Klingenberg *et al.*, 2005) or to poor access of antibiotics to the surface of the catheter lumen (Bastani *et al.*, 2000). These deficits can be overcome by the catheter lock technique (CLT), which involves filling the lumen of the catheter with an antimicrobial agent at high concentration (100–1000 times higher than is used systemically) and allowing the compounds to dwell for a period of time while the catheter is not in use (Messing *et al.*, 1988).

The effectiveness of various antimicrobial agents used as catheter lock solutions (CLSs) to prevent or treat CRBSIs has been compared in numerous studies. Previous studies have shown that the effectiveness of antibiotics is

Abbreviations: CLS, catheter lock solution; CLSM, confocal laser-scanning microscopy; CLT, catheter lock technique; CoNS, coagulase-negative staphylococci; CRBSI, catheter-related bloodstream infection; MBEC, minimal biofilm eradication concentration; NICU, neonatal intensive care unit; PI, propidium iodide.

suboptimal (Allon, 2003; Curtin *et al.*, 2003; Fernandez-Hidalgo *et al.*, 2006; Kite *et al.*, 2004). In addition, the application of antibiotics may be associated with undesirable effects, such as the development of resistance, allergic reactions and toxicity. In three recent cohort studies, ethanol was found to be extremely effective in the treatment of CRBSIs when applied as a CLS (Metcalf *et al.*, 2004; Onland *et al.*, 2006; Opilla *et al.*, 2007). However, there is still a lack of scientific data on the effectiveness of ethanol in eradication of biofilm-grown bacteria.

Besides the paucity of data on the most effective antimicrobial agents used as CLSs, their optimum concentrations and exposure times also need to be clarified further (Bailey *et al.*, 2002; Berrington & Gould, 2001). Most investigators have chosen concentrations and exposure time arbitrarily, and hence there is much variation in the effectiveness reported in different studies (Berrington & Gould, 2001; Lee *et al.*, 2006; Onland *et al.*, 2006; Opilla *et al.*, 2007).

METHODS

Bacterial isolates and growth conditions. Eight CoNS isolates were used in this study, including two reference strains (RP62a, a biofilm-positive *S. epidermidis* strain, and SP2, a biofilm-negative *Staphylococcus hominis* strain) and six invasive clinical CoNS isolates from newborns with confirmed CRBSI. These isolates were obtained from blood cultures from infants at the Royal Women's Hospital NICU, Victoria, Australia. Biofilm production of these isolates was examined quantitatively in a previous study by staining the biofilms with Hucker's crystal violet and measuring the A_{600} (Bradford *et al.*, 2006). The isolates were: a biofilm-positive *S. epidermidis* (isolate no. 3), a biofilm-weak *S. epidermidis* (no. 4), another biofilm-positive *S. epidermidis* (no. 5) and three *Staphylococcus capitis* isolates, which only produce biofilms under stress induced by sodium chloride or ethanol (nos 6, 8 and 9). The *icaA* gene was found in all of the biofilm-positive *S. epidermidis* and *S. capitis* isolates, except for the biofilm-weak isolate *S. epidermidis* no. 4 (Bradford *et al.*, 2006).

Biofilms were developed in tryptone soy broth (TSB; Oxoid) in 96-well microplates as described by Deighton *et al.* (2001). As *S. capitis* only produces biofilms when stressed with salt or ethanol, TSB with 4% (w/v) sodium chloride was used for biofilm production of *S. capitis* nos 6, 8 and 9.

Antimicrobial agents. Representatives of five different classes of antibiotic and one disinfectant were chosen for the catheter lock experiments: oxacillin, gentamicin, vancomycin, ciprofloxacin, rifampicin (Sigma-Aldrich) and ethanol (Merck). Antibiotic stock solutions were prepared according to the manufacturer's instructions. To compare the effectiveness of single antimicrobial agents in catheter lock experiments, dilutions were prepared with sterile saline, starting from very high concentrations used in the CLT as reported previously (Lee *et al.*, 2006; Mermel *et al.*, 2001; Onland *et al.*, 2006; Opilla *et al.*, 2007; Sherertz *et al.*, 2006). The selected high concentrations were also consistent with published guidelines for the management of CRBSIs (Mermel *et al.*, 2001). The starting concentrations were 10 000 $\mu\text{g ml}^{-1}$ for gentamicin and rifampicin, 5000 $\mu\text{g ml}^{-1}$ for oxacillin, vancomycin and ciprofloxacin and 80% (v/v) for ethanol. These concentrations are defined as pharmacological concentrations at which no apparent precipitation of antibiotic powder or evaporation of solutions occurs.

The double or triple combinations of conventional antibiotics were chosen based on their reported effectiveness against CoNS biofilms (Saginur *et al.*, 2006). Therapeutic concentrations were used for individual antibiotics in combinations that have been defined as the Clinical and Laboratory Standards Institute breakpoints for determining resistance (Table 1).

In vitro model to predict the efficacy of CLSs. The efficacy of single antibiotics, antibiotic combinations and ethanol against CoNS biofilms was examined. Preformed 24 h biofilms were exposed to different concentrations and combinations of antimicrobial agents in saline for 24 h as follow. Two hundred microlitres of single agents at concentrations ranging from 4 $\mu\text{g ml}^{-1}$ to 5000 or 10 000 $\mu\text{g ml}^{-1}$, double or triple antibiotic combinations at therapeutic concentrations (Table 1) or ethanol (10–80%) were added to the biofilms. After 24 h of exposure, the biofilms were washed three times with saline to remove any residual antimicrobial agents. Sterile resin beads (0.2 g per well, Amberlite XAD-16; Rohm and Haas) were then added to minimize the chance of antibiotic carryover. TSB (200 μl) was then added to each well and microplates were incubated at 37 °C for a further 48 h. A microplate shaker (speed 2, Titretrek; Flow Laboratories) was used to facilitate the multiplication and release of any living cells remaining in the biofilms. After 48 h, 150 μl of the contents in each well of the microplate was transferred to a U-bottomed microplate and examined visually for turbidity. The lowest concentration of antimicrobial agent corresponding to clear wells was defined as the minimal biofilm eradication concentration (MBEC) for successful use in the CLT. This concentration is generally higher than the MIC for planktonic cultures and also higher than the minimum biofilm inhibitory concentration for biofilms, and targets complete killing of biofilm-embedded cells. This method was defined as the broth recovery method, in contrast to the plate recovery method, which employs agar plates to recover the remaining living cells after treatment.

Optimum concentration and duration of ethanol exposure for catheter lock applications. Two hundred microlitres of ethanol at concentrations ranging from 10 to 80% was added to 24 h CoNS biofilms and allowed to remain in contact for 1 min, 15 min, 1 h, 4 h, 8 h or 24 h. After the specified contact time, the MBEC of ethanol was determined as described above, but without adding resin beads to the wells. The ethanol-treated biofilms of RP62a and SP2 in the original microplate were then collected with sterile cotton-tipped

Table 1. Antibiotic combinations and the test concentrations of individual agents

Combination*	Therapeutic concentrations ($\mu\text{g ml}^{-1}$)†
G:R	16:4
O:R	0.5:4
V:R	32:4
C:R	4:4
G:O:R	16:0.5:4
G:V:R	16:32:4
G:C:R	16:4:4
O:V:R	0.5:32:4
O:C:R	0.5:4:4
V:C:R	32:4:4

*G, Gentamicin; O, oxacillin; V, vancomycin; C, ciprofloxacin; R, rifampicin.

†G:R ratio, for example, of 16:4 represents 16 $\mu\text{g ml}^{-1}$ gentamicin plus 4 $\mu\text{g ml}^{-1}$ rifampicin.

swabs and cultured on nutrient agar plates to detect any viable cells embedded in the biofilms that could not be detected by the broth recovery method.

Confocal laser-scanning microscopy (CLSM). Sterile polystyrene pieces ($\sim 0.5 \times 0.5$ cm) were aseptically cut from the bottom of a 24-well microplate with extreme caution to avoid any scratch to the tissue culture treated surface. The polystyrene pieces were then transferred to a microwell containing an *S. epidermidis* RP62a bacterial suspension of $\sim 10^7$ c.f.u. ml^{-1} and incubated overnight at 37 °C. The pieces, with biofilm, were then rinsed three times with 0.9% saline to remove planktonic bacteria. One of the following treatments was applied to the *S. epidermidis* RP62a biofilms grown on the polystyrene pieces: (i) 0.9% saline for 24 h, as an untreated biofilm control; (ii) 5000 μg oxacillin ml^{-1} for 24 h; (iii) 5000 μg vancomycin ml^{-1} for 24 h; (iv) 256 μg ciprofloxacin ml^{-1} for 24 h; (v) 40% ethanol for 1 h; or (vi) 40% ethanol for 4 h. The treated biofilms were then stained with a LIVE/DEAD BacLight viability kit, containing 3.35 μM SYTO-9 and 20 μM propidium iodide (PI), at 22 °C for 15 min in the dark. The fluorescently stained polystyrene pieces were then washed twice with 0.9% saline. The viability of biofilm cells was examined immediately using a Zeiss LSM 510 Pa confocal laser-scanning microscope with Zeiss Axioplan upright microscopes (Leica Microsystems). To minimize artefacts associated with simultaneous dual wavelength excitation, all samples were sequentially scanned, frame-by-frame, first at 488 nm and then at 561 nm. A $\times 63$ oil objective was used in all imaging experiments.

Data analysis. All experiments were run in duplicate and repeated at least three times. A fourth duplicate was performed if the results were inconsistent.

RESULTS AND DISCUSSION

Comparison of the effectiveness of antibiotics and ethanol as a CLS

Ethanol was found to be the most efficient of the antimicrobial agents examined for eradication of the CoNS biofilms after 24 h exposure. A low concentration of 20% ethanol killed all cells embedded in the biofilms after overnight exposure. Gentamicin, oxacillin and vancomycin, even at very high pharmacological concentrations (10 000, 5000 and 5000 μg ml^{-1} , respectively), failed

to eradicate the biofilm cells of most CoNS isolates. The only exceptions were the biofilms formed by *S. epidermidis* nos 3 and 4, which responded to gentamicin. In addition, ciprofloxacin and rifampicin achieved biofilm killing of all isolates when exposure lasted for 24 h, but only at relatively high concentrations (32–128 and 256–512 μg ml^{-1} , respectively) (Table 2).

The inability of conventional antibiotics commonly used in NICUs (gentamicin, oxacillin and vancomycin) at very high concentrations to kill all biofilm cells indicates their unsuitability as a CLS. This *in vitro* finding is supported by frequent reports of failure of CRSBIs to respond to oxacillin, gentamicin, vancomycin or teicoplanin in the absence of catheter removal (Allon, 2004; Castagnola *et al.*, 2006; Fernandez-Hidalgo *et al.*, 2006; Guedon *et al.*, 2002). Although vancomycin and teicoplanin lock solutions have been found to be effective in the prevention of catheter-related infections (van de Wetering & van Woensel, 2007), their effectiveness on established CoNS biofilms was found to be poor in this study. Ciprofloxacin and rifampicin were able to completely kill biofilm-embedded cells. However, the value of ciprofloxacin and rifampicin in biofilm killing is limited by the high doses and related expense and drug toxicity, and the ease with which rifampicin induces resistance in bacterial populations (Dunne *et al.*, 1993). Our findings were consistent with a recent study by Lee *et al.* (2006), which found that ciprofloxacin (1000–5000 μg ml^{-1}) and rifampicin (5000 μg ml^{-1}) completely eradicated staphylococcal biofilms after a short-term exposure, in contrast to vancomycin (5000 μg ml^{-1}) and gentamicin (10 000 μg ml^{-1}), which were much less effective, even after long-term exposure (Lee *et al.*, 2006). Differences in the MBEC values of single antibiotics reported in our study and the study by Lee *et al.* (2006) could be due to the different diluents used for preparing the CLS or the different ages of the biofilms. Sterile saline was used in our study as the test medium to mimic the clinical situation, whilst TSB, a rich medium for bacterial growth, was used in the study by Lee *et al.* (2006). We examined the effect of antimicrobial agents on 24 h

Table 2. MBECs of single antimicrobial agents against 24 h CoNS biofilms

Values are geometric means of at least three duplicates. Variation between three duplicates was less than fourfold in all instances.

Strain identity	Gentamicin (μg ml^{-1})	Oxacillin (μg ml^{-1})	Vancomycin (μg ml^{-1})	Ciprofloxacin (μg ml^{-1})	Rifampicin (μg ml^{-1})	Ethanol (%)
<i>S. epidermidis</i> RP62a	>10 000	>5000	>5000	64	512	20
<i>S. hominis</i> SP2	>10 000	5000	>5000	32	512	20
<i>S. epidermidis</i> no. 3	128	>5000	>5000	64	512	20
<i>S. epidermidis</i> no. 4	8	>5000	>5000	128	256	20
<i>S. epidermidis</i> no. 5	>10 000	>5000	>5000	128	512	20
<i>S. capitis</i> no. 6	>10 000	>5000	5000	64	512	20
<i>S. capitis</i> no. 8	>10 000	>5000	5000	128	512	20
<i>S. capitis</i> no. 9	>10 000	>5000	5000	64	512	20

biofilms, whilst Lee *et al.* (2006) used 5-day biofilms, which could be expected to behave differently.

Interestingly, there were no significant differences found in the present study in the MBECs of single antimicrobial agents for strains with different biofilm-forming abilities. The concentrations of these agents required to eradicate the adherent cells of the biofilm-negative *S. hominis* SP2 were the same as those required for *S. epidermidis* RP62a biofilms, although the latter formed much thicker biofilms than the former (Table 2). Although classified as a biofilm-negative strain, SP2 does form an adherent monolayer on various biomaterials (Christensen *et al.*, 1985). It is therefore likely that the biofilm mode of growth, rather than the ability to form dense biofilm, leads to its resistance to antimicrobial agents. A parallel study by us comparing the antimicrobial susceptibilities of biofilms, adherent monolayers and planktonic cells at similar densities reported the same conclusion when targeting 99.99 % biofilm bacterial killing (unpublished data). Similar results were also found in a recent study, in which a biofilm-negative mutant strain, M187sn3, had the same or an even higher resistance level to most antibiotic locks as its biofilm-positive ancestor (Edmiston *et al.*, 2006).

At therapeutic concentrations, most combinations failed to kill the biofilm cells, indicating the ineffectiveness of biofilm killing of antibiotic combinations at achievable serum concentrations (Table 3). Combinations containing gentamicin were effective against biofilms of *S. epidermidis* no. 4, unless they contained oxacillin (Table 3). Vancomycin combined with rifampicin has been suggested for the treatment of *S. epidermidis* biofilm-associated infections (Monzon *et al.*, 2001; Shama *et al.*, 2002), but our study found no advantage of this combination over rifampicin alone in biofilm eradication. Vancomycin may, however, minimize the risk of rifampicin resistance developing (Zimmerli *et al.*, 1994). Besides the suboptimal effectiveness of conventional antibiotics, the risk of developing resistance, the high cost of antibiotic usage and the possibility of the accidental infusion of highly concentrated solutions into the circulation during the lock procedure and related toxicity cannot be ignored.

Optimum concentration of ethanol and duration of treatment for catheter locks

Biofilm cells of all isolates were killed within 1 min of exposure to 60–80 % ethanol and in 1 h of exposure to 40 % ethanol. Exposure for >1 h was required only for 20 % ethanol and no further advantage was achieved by increasing the dwell time beyond 4 h (Fig. 1).

In agreement with Sherertz *et al.* (2006), we showed that ethanol eradicates biofilm bacteria much more effectively than commonly used antibiotics if used as a CLS. Moreover, the use of ethanol for the CLT is simpler and easier than previously recommended antibiotic regimens. We also extended these findings further by showing that exposure of CoNS biofilms to 40 % ethanol for 1 h was sufficient to eradicate the biofilm bacteria. In addition, low concentrations of ethanol (25–60 %) do not cause catheter occlusion (Laird *et al.*, 2005) or degradation of the catheter biomaterial (Opilla *et al.*, 2007), and ethanol is compatible with heparin and EDTA, the anticoagulant agents commonly used in the CLT (Ackoundou-N'guessan *et al.*, 2006; Raad *et al.*, 2007). A shorter dwell time also reduces the risk of spillage of ethanol into the circulation and its associated toxicity (Ackoundou-N'guessan *et al.*, 2006). We therefore suggest that this concentration and exposure time is optimum for clinical use.

The superiority of ethanol over conventional antibiotics can be explained by its hydrophilic nature and small size, which enable it to obtain access to deep biofilm cells, as the biofilm matrix is highly hydrated. This is in contrast to other antimicrobial agents that may have restricted diffusion gradients through the biofilm matrix. Although it has been suggested that 1–2 mm thick biofilms may have a diffusion delay for ethanol to pass through the dense biofilm structure, no solid evidence has been presented (Sissons *et al.*, 1996). In addition, as ethanol has extremely high bactericidal activity against planktonic cells, it could also be effective in killing the 'less active' cells in biofilms, i.e. the cells presenting biofilm-specific genes or the more tolerant 'persister' cells.

Our CLSM study revealed that exposure to 40 % ethanol for 1–4 h, or 256 µg ciprofloxacin ml⁻¹ for 24 h, worked

Table 3. Regrowth of biofilm bacteria after exposure to double and triple combinations of antibiotics at therapeutic concentrations

For abbreviations and therapeutic concentrations of individual antibiotics, see Table 1. +, Regrowth; –, no regrowth.

Strain identity	R:G	R:V	R:O	R:C	R:G:O	R:G:V	R:G:C	R:O:V	R:O:C	R:V:C
<i>S. epidermidis</i> RP62a	+	+	+	+	+	+	+	+	+	+
<i>S. hominis</i> Sp2	+	+	+	+	+	+	+	+	+	+
<i>S. epidermidis</i> no. 3	+	+	+	+	+	+	+	+	+	+
<i>S. epidermidis</i> no. 4	–	+	+	+	+	–	–	+	+	+
<i>S. epidermidis</i> no. 5	+	+	+	+	+	+	+	+	+	+
<i>S. capitis</i> no. 6	+	+	+	+	+	+	+	+	+	+
<i>S. capitis</i> no. 8	+	+	+	+	+	+	+	+	+	+
<i>S. capitis</i> no. 9	+	+	+	+	+	+	+	+	+	+

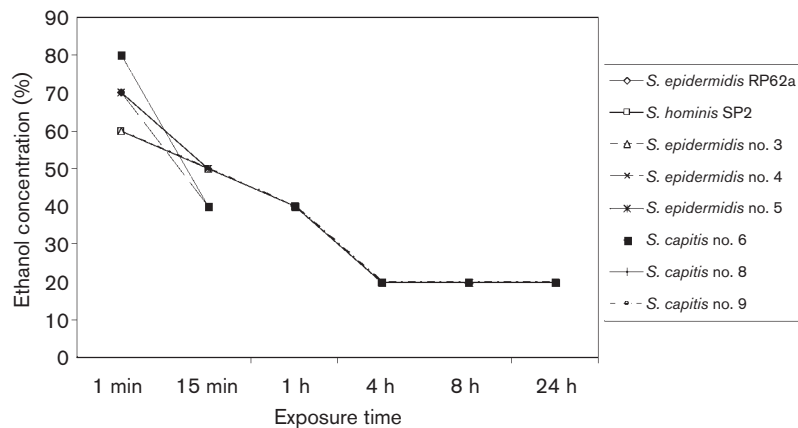


Fig. 1. Concentrations and exposure times of ethanol required to kill all CoNS residing in biofilms. Experiments were repeated in duplicate three times and the data were identical.

most efficiently in biofilm cell killing (Fig. 2d–f). Exposure to first-line conventional antibiotics, such as oxacillin and vancomycin, at very high concentrations only partially killed the biofilm cells (Fig. 2b, c).

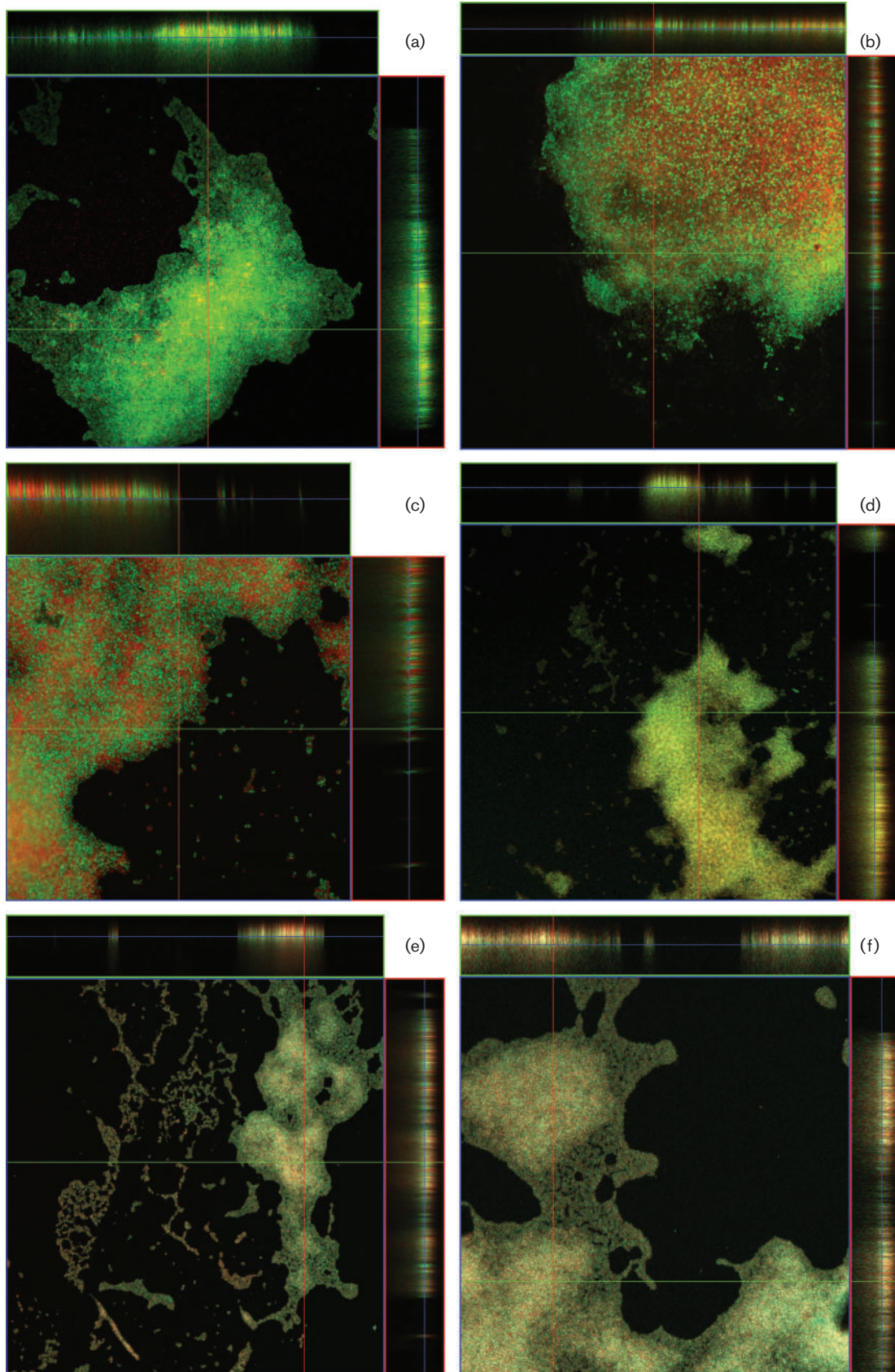
CLSM in combination with a LIVE/DEAD BacLight viability kit has been used successfully to examine the efficacy of antimicrobial agents on *S. epidermidis* biofilm eradication (Qin *et al.*, 2007a, b). The LIVE/DEAD BacLight viability kit is a fluorescence-based stain for determining bacterial cell viability and contains two components. SYTO-9 is a membrane-permeant nucleic acid-labelling dye that labels all cells and fluoresces green. The second dye, PI, is another nucleic acid-labelling dye that only enters cells with compromised or damaged cytoplasmic membranes. PI has been reported to have a stronger affinity for nucleic acids, competing with and quenching the SYTO-9 and then fluorescing red (Stocks, 2004). According to the manufacturer, once stained with the LIVE/DEAD BacLight viability kit, the dead cells in a bacterial population present as a bright red colour, whilst live cells are a bright green colour, leaving the background non-fluorescent. This theory is consistent with our results when cell-wall-attacking antibiotics, such as oxacillin and vancomycin, were tested (Fig. 2b, c). However, when antimicrobial agents with intracellular targets or more complex killing mechanisms were tested in our study, the dead cells presented as a reduced green colour or displayed an orange colour, indicating that only partial quenching of SYTO-9 by PI or simultaneous staining of cells by PI and SYTO-9 had occurred. Similar results were obtained in a previous study by Berney *et al.* (2007). These authors found that *Escherichia coli* with an intact outer membrane showed strong green fluorescence intensity and those cells with cytoplasmic membrane damage displayed a decreased green fluorescence intensity. An incomplete replacement

and quenching of SYTO-9 fluorescence by PI has been proposed to explain this phenomenon (Berney *et al.*, 2007). Based on the report by Berney *et al.* (2007) as well as the current study, reduction of green fluorescence density, but not the absolute absence of green signal, should be used as indicators of bacterial killing when the BacLight kit is used to examine viability. This theory is more significant when antimicrobial agents with intracellular activity rather than cell-wall-active agents are tested, i.e. ciprofloxacin and ethanol. The incomplete replacement could be explained by the poor effect of these antimicrobial agents on membrane integrity in *Staphylococcus* spp. (O'Neill *et al.*, 2004), the much lower fluorescent intensity of PI compared with SYTO9 (8:300) when excited (Stocks, 2004) or the presence of viable but non-culturable cells in the biofilms after antimicrobial exposure (Alam *et al.*, 2007).

Concerning the methodology, the definition of successful catheter lock application used in the present study was the complete absence of recoverable living cells following *in vitro* exposure. Successful treatment using the catheter lock technique has been defined as negative blood cultures and resolution of fever a few days after completion of a 3-week treatment course (Allon, 2004). However, this definition has not been validated by *in vitro* studies. Unless complete eradication of biofilm cells is achieved, the remaining living cells will rebuild the biofilm once antimicrobial stress diminishes, as host immune mechanisms can neither reach the lumen of catheters nor eradicate any remaining living cells in biofilms (Lewis, 2005; Spoering & Lewis, 2001).

Currently, there is no universally accepted *in vitro* model of catheter-related biofilm infection. The model of biofilm formation and antimicrobial treatment used in our study was intended to replicate the clinical situation as closely as possible. Biofilm development and treatment were carried

Fig. 2. Biofilms of *S. epidermidis* RP62A treated with antimicrobial agents and then stained with SYTO-9 (bright green for live cells) and PI (red or orange, or loss of bright green for dead cells). (a) Untreated biofilm control; (b) biofilm treated with 5000 μg oxacillin ml^{-1} for 24 h; (c) biofilm treated with 5000 μg vancomycin ml^{-1} for 24 h; (d) biofilm treated with 256 μg ciprofloxacin ml^{-1} for 24 h; (e) biofilm treated with 40% ethanol for 1 h; and (f) biofilm treated with 40% ethanol for 4 h.



out under static conditions and antimicrobial agents were prepared in 0.9 % saline. However, our biofilm model does differ from clinical biofilms. We used polystyrene as the biofilm platform, whereas catheters are mostly manufactured from polyurethane and Teflon. In addition, we prepared biofilms with inocula of 10^7 c.f.u. ml⁻¹, in contrast to the clinical situation, where catheters are generally seeded with bacteria at densities of 50–600 c.f.u. ml⁻¹ (Shah *et al.*, 2002). We chose polystyrene because it is commonly available in microbiology laboratories and has been widely used for biofilm studies. The initial inoculum of 10^7 c.f.u. ml⁻¹ was adopted for our study as this density has been accepted as a standard inoculum for *in vitro* biofilm formation (Deighton *et al.*, 2001).

As we anticipated that agent carryover could be a problem in this study, which used antimicrobial agents at high concentrations, resin beads (Amberlite XAD-16) were used to remove any antimicrobial agents that remained after treatment and washing of biofilms. Although there are limited data about the efficacy of Amberlite XAD-16 in removal of antibiotics, the structurally similar but functionally weaker Amberlite XAD-4 efficiently reduces carryover of aminoglycosides, penicillin, macrolides, ciprofloxacin and vancomycin from bacterial cultures (Hyatt *et al.*, 1994; Zabinski *et al.*, 1993). Moreover, these resin beads have no adverse effect on bacterial growth (Hyatt *et al.*, 1994; Zabinski *et al.*, 1993).

Two bacterial recovery methods have been widely used by biofilm researchers (Raad *et al.*, 2007; Zimmerli *et al.*, 1994). One method consists of dissociating the biofilms by scraping, vortexing or/and sonication, followed by plating the bacteria on recovery agar plates (dissociating/plating method). This quantitative method has low sensitivity in qualitatively detecting the remaining living biofilm cells (Raad *et al.*, 2007). This could be due to the inability of the scraping or sonication method to completely dissociate biofilm cells or the failure to recover shocked cells by direct plating (Raad *et al.*, 2007; Zimmerli *et al.*, 1994). In biofilm studies, sonication is more commonly used to dissociate the biofilms grown at the air–liquid interface of plastic pegs or inserts, such as those formed by the Gram-negative bacillus *Pseudomonas aeruginosa* (Ceri *et al.*, 1999). For the bottom-growing CoNS biofilms used in our study, there was poor efficiency and a large variation in cell release when sonication was used (unpublished data). Moreover, cross-contamination between wells frequently occurred when sonication was employed. In another method, the treated biofilms were incubated for an additional 24 h in fresh broth prior to dissociating and plating (broth incubation/dissociating/plating). Whilst this method is labour-intensive, it has been reported to have greater accuracy in qualitative determination of viable cells (Raad *et al.*, 2007). The method that we used to recover the living cells in biofilms after antimicrobial treatment is a variation of the broth incubation/dissociating/plating method. An extended incubation period of 48 h was used instead of direct plating. We showed that our recovery method had

the same accuracy as the broth incubation/dissociating/plating method, but was less labour-intensive when dealing with ethanol.

In summary, in the present study, we have described the first *in vitro* model to compare the efficacy of ethanol and antibiotics as a CLS. This study showed that ethanol has a higher efficacy than most of the commonly used antibiotics. We also suggest that using 40 % ethanol for 1 h is ideal as a CLS targeting CoNS biofilms. Additional *in vivo* studies are required to confirm the clinical utility of our findings. The efficacy of ethanol locks could also be confirmed on catheters with CoNS biofilms removed from patients with clinical infections.

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