In Vitro Approach for Identification of the Most Effective Agents for Antimicrobial Lock Therapy in the Treatment of Intravascular Catheter-Related Infections Caused by *Staphylococcus aureus*

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Infection of intravascular catheters by *Staphylococcus aureus* is a significant risk factor within the health care setting. To treat these infections and attempt salvage of an intravascular catheter, antimicrobial lock solutions (ALSs) are being increasingly used. However, the most effective ALSs against these biofilm-mediated infections have yet to be determined, and clinical practice varies greatly. The purpose of this study was to evaluate and compare the efficacies of antibiotics and antiseptics in current clinical use against biofilms produced by reference and clinical isolates of *S. aureus*. Static and flow biofilm assays were developed using newly described *in vivo*-relevant conditions to examine the effect of each agent on *S. aureus* within the biofilm matrix. The antibiotics daptomycin, tigecycline, and rifampin and the antiseptics ethanol and Taurolock inactivated established *S. aureus* biofilms, while other commonly used antistaphylococcal antibiotics and antiseptic agents were less effective. These findings were confirmed by live/dead staining of *S. aureus* biofilms formed and treated within a flow cell model. The results from this study demonstrate the most effective clinically used agents and their concentrations which should be used within an ALS to treat *S. aureus*-mediated intravascular catheter-related infections.

The use of intravascular catheters (IVCs) in modern health care has increased over the last decades. Infection of these devices by surface-adhering bacteria, resulting in catheter-related bloodstream infection (CRBSI), is associated with significant patient morbidity and mortality, prolonged hospitalization, and excess hospital-related costs. The Centers for Disease Control and Prevention (CDC) attributes 12 to 25% mortality among critically ill patients alone to CRBSI (1).

Biofilms formed by staphylococci, in particular *Staphylococcus epidermidis* and *Staphylococcus aureus*, have for many years been recognized as the most frequent cause of CRBSI (2, 3). These biofilms are highly resistant both to the action of the innate and adaptive immune defense systems and to the action of antimicrobial agents, resulting in persistent infections and treatment failure.

The majority of guidelines recommend catheter removal and systemic antimicrobial treatment on suspicion or confirmation of a CRBSI (4). However, clinical circumstances, for example, lack of alternative venous access, bleeding disorders, or comorbid conditions, often preclude device removal. Alternative strategies, such as the use of antimicrobial lock therapy (ALT), to treat these biofilm-related IVC infections, have generated considerable interest in recent years. Antimicrobial lock solutions (ALSs) have been used with variable success to fill the lumen of the IVC in order to eradicate biofilms (5). This technique provides very high concentrations of antimicrobial agents at the site of infection. However, concerns around selection of resistant organisms, toxicity, and treatment failure have thus far limited their widespread application in the treatment of CRBSIs. The Infectious Diseases Society of America (IDSA) has issued guidelines on the management of CRBSIs, recommending the use of ALT for the salvage of an IVC associated with CRBSI (4). However, the choice of which antibiotic (or antiseptic) to be used in the ALS is often based on the *in vitro* susceptibilities determined using conventional susceptibility testing, which may not necessarily indicate that the antibiotic is

active against the same organisms at higher density and embedded within the biofilm matrix (6).

A range of antibiotics, including vancomycin, daptomycin, gentamicin, rifampin, and linezolid, have been used in studies investigating their effectiveness as ALSs in patients with CRBSI due to *S. aureus* (7–9). Similarly, various antiseptic agents, including ethanol, EDTA, and sodium citrate derivatives, have been studied, with significant variations in their effectiveness reported (10–12). Many *in vitro* studies investigating the effectiveness of ALT have been limited in that they do not mimic features such as physiologically relevant shear (e.g., host blood flow), the deposition of serum proteins on IVC surfaces, and the nutrient-limited environment found in the host. Furthermore, studies often involve only a small number of reference strains using a static 96-well polystyrene plate biofilm assay. Therefore, there remain insufficient insight and knowledge about which agent, or combination of agents, is likely to be most efficacious in ALT against biofilm-forming staphylococci under *in vivo* conditions (13). The aim of this study was to use a biofilm model of IVC infection, more representative of the clinical environment, to investigate the bio-

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TABLE 1 *S. aureus* strains used in this study

Strain	Characteristics	Reference
SH1000	MSSA reference strain, functional <i>rsbU</i> derivative of 8325-4 <i>rsbU</i> ⁺ , serotype 8, clonal complex 8	33
BH48 (04)	MSSA clinical isolate, serotype 8, clonal complex 8	34
BH1CC	MRSA clinical isolate, SCCmec type II, serotype 8, clonal complex 8	34
USA300 JE2	MRSA strain USA300 derivative lacking plasmids P01 and P03, JE2 LAC serotype 8, clonal complex 8	35

film eradication effectiveness of ALSs against a number of methicillin-susceptible and -resistant *S. aureus* reference strains and clinical isolates.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The main bacterial strains used in this study are described in Table 1. *S. aureus* strains were grown with aeration at 200 rpm at 37°C in RPMI 1640 (Gibco) medium.

MIC testing. MICs were established using a modification of the British Society for Antimicrobial Chemotherapy (BSAC) guidelines. To determine the MICs of each bacterium to vancomycin, gentamicin, and linezolid, Etest strips (bioMérieux) were used in accordance with manufacturer's instructions. MICs of the remaining agents were determined via serial broth dilution by preparing a range of concentrations of each test agent, and to this 100 µl of a 0.5 McFarland suspension of each bacterial strain was added. Solutions were incubated for 24 h at 37°C before results were examined to determine the minimum concentration required to inhibit growth.

Microtiter plate biofilm assay. Microtiter plate biofilm assays were carried out as described previously (14). Briefly, blood (approximately 40 ml) was drawn from a healthy volunteer using the S-Monovette system (Sarstedt) containing heparin or EDTA (15). Blood was transferred into 15-ml tubes and centrifuged at 150 × *g* for 10 min. Plasma (upper layer) was collected and either used immediately or frozen at −20°C. The plasma was then diluted to a 20% (vol/vol) concentration in carbonate buffer (pH 9.6) to promote immobilization of the host matrix proteins to the plate surface (16). Then, 100 µl of the plasma was added to the individual wells of a 96-well plate (Nunc, Denmark), incubated at 37°C for 2 h, and then withdrawn from the wells. An overnight culture of the test organism in RPMI 1640 was diluted 1:2 in prewarmed fresh medium (RPMI 1640). From this suspension, 100 µl was inoculated into the microtiter plate wells prior to incubation at 37°C for 24 h, 3 days, or 5 days, as indicated. RPMI 1640 medium was removed and replaced daily for three- and five-day biofilms.

Treatment of biofilm. Biofilms were prepared as described above. Following the initial incubation and washing, 100 µl of the test antimicrobial (Table 2) was added to each test well and incubated for a further 24 h at 37°C. Following this, test wells were washed twice with sterile distilled water. The antimicrobial solutions used were prepared in accordance with the manufacturer's guidelines. To investigate the effectiveness of antimicrobial solutions within our biofilm, the initial concentrations of the antimicrobials chosen for testing were concentrations which were the upper limit of those used routinely in clinical practice, or they were taken from previous studies investigating ALT (10, 17–19); following identification of the most effective agents, concentration gradients of these agents were performed.

Resazurin conversion assay. One hundred microliters of the redox indicator dye alamarBlue (Invitrogen, United Kingdom) (20% [vol/vol] alamarBlue in RPMI 1640 broth) or resazurin (88 µM resazurin in water) was added to each well. Plates were incubated for a further 60 min at 37°C in the dark to determine biofilm viability after antimicrobial treatment. Biofilm viability was determined using a fluorimeter with excitation at 544

TABLE 2 Concentration of agents used for biofilm susceptibility testing

Agent	Concn used for biofilm testing
Antibiotics	
Vancomycin	50 mg/ml
Tigecycline	10 mg/ml
Daptomycin	50 mg/ml
Gentamicin	40 mg/ml
Linezolid	4 mg/ml
Rifampin	10 mg/ml
Fosfomycin	50 mg/ml
Antiseptics	
Ethanol	30% (vol/vol)
EDTA	32 mg/ml
Duralock-C	46.7% (wt/vol) sodium citrate
Taurolack	4% sodium citrate, 1.35% taurolidine

nm and an emission value of 590 nm. The amount of fluorescence produced was proportional to the number of living cells present; i.e., the higher the fluorescent unit, the higher the level of actively metabolizing live cells present. Each experiment was performed in triplicate on three occasions, and the results represent mean fluorescence density ± standard deviation (SD).

Measurement of antibiofilm activity of antimicrobial agents under flow. A flow cell and pump were used to create a microfluidic model (Kima; Cellix Ltd., Ireland). Bacteria were grown overnight on solid medium. One colony was taken from this and used to inoculate 5 ml of RPMI 1640, which was then incubated at 37°C at 200 rpm for 6 h. The flow chambers were subsequently inoculated with an optical density at 600 nm (OD₆₀₀) of *S. aureus* grown in RPMI 1640 up to exponential phase. Each chamber in a chip was coated with 100% plasma and left for 2 h at 37°C, which was then flushed through with sterile phosphate-buffered saline (PBS) before bacteria were injected into each chamber and allowed to attach for 1 h on a heated platform (set at 37°C) connected to an inverted microscope. An infusion rate of 200 µl/min of RPMI 1640 was set to maintain constant flow through the chip for 24 h. Antimicrobial test agents were then injected into each chamber of the chip and allowed to treat the biofilm statically for 24 h. Chambers were analyzed using bright-field and confocal microscopy.

Confocal microscopy. Biofilm structure and treatment efficacy were analyzed using an inverted confocal microscope (Zeiss LSM 510 Meta) and image capture software LSM510 (Zeiss). Bacterial cells within the biofilm were visualized using Syto 9 green (3.34 mM) and propidium iodide (20 mM). Optimized lasers, argon (488 nm) and HeNe (632.8 nm), were used to excite the dyes and capture the fluorescence emitted from the cells, under a magnification of ×40. ImageJ software was used to calculate the fluorescence intensity for each chamber.

RESULTS

Planktonic susceptibility testing. The *in vitro* susceptibilities of four *S. aureus* isolates to a range of clinically relevant antibiotics and antimicrobials were determined. The MICs for each isolate fell below the EUCAST clinical breakpoints for susceptibility, deeming them sensitive to all antibiotics tested (Table 3). Guidelines on breakpoints to the antiseptics included in this testing are not available.

Inactivation of mature *S. aureus* biofilms by antiseptic and antibiotic treatment. IVC infections progress due to bacterial biofilm accumulation over a number of days. To reflect this, antibiotics and antiseptics were tested against biofilms formed over 1, 3, and 5 days by the four *S. aureus* strains SH1000, BH1CC, BH48, and JE2. Increased resistance to treatment has been ob-

TABLE 3 MICs of antibiotics and antiseptics against *S. aureus*

Agent	MIC against strain:			
	SH1000	BH48	BH1CC	USA300 JE2
Antibiotics				
Vancomycin	1 µg/ml	0.25 µg/ml	1 µg/ml	1 µg/ml
Tigecycline	0.125 µg/ml	<0.016 µg/ml	0.094 µg/ml	0.125 µg/ml
Daptomycin	0.25 µg/ml	0.25 µg/ml	0.25 µg/ml	0.125 µg/ml
Gentamicin	1 µg/ml	0.5 µg/ml	1 µg/ml	1 µg/ml
Linezolid	1 µg/ml	0.5 µg/ml	1 µg/ml	1 µg/ml
Rifampin	0.125 µg/ml	0.06 µg/ml	0.06 µg/ml	0.125 µg/ml
Fosfomycin	2 µg/ml	1 µg/ml	1 µg/ml	2 µg/ml
Antiseptics				
Ethanol	30% (vol/vol)	30% (vol/vol)	30% (vol/vol)	30% (vol/vol)
Sodium citrate	25% (wt/vol)	25% (wt/vol)	25% (wt/vol)	25% (wt/vol)
EDTA	1.87 mg/ml	1.87 mg/ml	1.87 mg/ml	1.87 mg/ml
Taurolidine	>250 µg/ml	>250 µg/ml	>250 µg/ml	>250 µg/ml

served previously in other bacterial species, such as *Escherichia coli* and *Klebsiella pneumoniae* (20, 21). One-, three-, and five-day-old biofilms formed by these four *S. aureus* isolates (Table 1) were treated with antibiotics for 24 h (Fig. 1). The antibiotics dapto-

mycin, vancomycin, tigecycline, and rifampin resulted in an almost complete inactivation of 24-h biofilms. However, while vancomycin was not observed to be significantly effective against 3- and 5-day-old biofilms, daptomycin, tigecycline, and

FIG 1 Susceptibility of mature *S. aureus* biofilms to antibiotics. Methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) biofilms were grown in RPMI 1640 for 1, 3, or 5 days and treated with antibiotics at 37°C for 24 h. Viability of biofilms was measured using the resazurin conversion assay. Assays were performed in triplicate, and the data represent mean fluorescence intensity units $\pm$ SD. Statistically significant results are indicated (two-tailed Student *t* tests; *, $P \geq 0.05$).

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FIG 2 Susceptibility of *S. aureus* biofilms to daptomycin and tigecycline. MRSA and MSSA biofilms were grown in RPMI 1640 for 1, 3, or 5 days and treated with daptomycin or tigecycline, at the indicated concentrations, at 37°C for 24 h. Viability of biofilms was measured using the resazurin conversion assay. Assays were performed in triplicate, and the data represent mean fluorescence intensity units $\pm$ SD.

rifampin were significantly ($P \leq 0.05$) effective against all biofilms tested (Fig. 1).

To further investigate the concentrations of antibiotics required to inactivate mature biofilm, concentration gradients of daptomycin and tigecycline were examined. We previously reported that vancomycin and rifampin concentrations as low as 1 mg/ml inactivated biofilms formed in this assay; however, vancomycin was less effective versus three- and five-day-old biofilm, and rifampin required increasing concentrations of 4 to 8 mg/ml to inactivate mature biofilm (14). Tigecycline concentrations of ≥ 128 µg/ml inactivated biofilm formed over 24 h, while 4 mg/ml proved effective against more mature biofilms formed over 3 and 5 days (Fig. 2). The concentrations of daptomycin required to inactivate *S. aureus* biofilms formed over 1, 3, and 5 days were 32, 256, and 1,024 µg/ml, respectively (Fig. 2).

The antiseptics ethanol and Taurolock were shown to be most effective at inactivating biofilm; this was evident against all stages of biofilm investigated (Fig. 3). EDTA and Duralock-C were effective only against 24-hour biofilms and did not result in a significant reduction in biofilm viability on more mature biofilms formed over 3 and 5 days (Fig. 3).

Further analysis showed that 15% (vol/vol) ethanol was effective

against biofilms formed after 3 and 5 days, while 7.5% (vol/vol) was sufficient to inactivate 24-hour biofilms (Fig. 4). Taurolock at commercially available concentrations inactivated *S. aureus* biofilms at all time points investigated; lower concentrations were not effective at significantly reducing biofilm viability (Fig. 4).

Impact of physiological shear on biofilm susceptibility to antibiotic and antiseptic treatment. To extend these studies to an experimental model that mimics a clinical infection, biofilms were grown under venous shear in microfluidic flow cells precoated with human blood plasma. Live/dead staining followed by microscopic analysis was used to assess biofilm susceptibility to antibiotic and antiseptic treatment. The concentrations of the antimicrobial agents used are outlined in Table 2. Initial experiments performed on SH1000 biofilms revealed that daptomycin, vancomycin, tigecycline, and rifampin exhibited the most potent inactivating activity (Fig. 5A, panels iv, v, vi, viii), reducing the number of live cells to less than 2% (Fig. 5B). Further analysis revealed that both daptomycin and tigecycline at concentrations of 128 µg/ml inactivated biofilm, while vancomycin and rifampin were effective at 8 and 4 mg/ml, respectively (see Fig. S1 in the supplemental

FIG 3 Susceptibility of mature *S. aureus* biofilms to antiseptics. MRSA and MSSA biofilms were grown in RPMI 1640 for 1, 3, or 5 days and treated with antiseptics at 37°C for 24 h. Viability of biofilms was measured using the resazurin conversion assay. Assays were performed in triplicate, and the data represent mean fluorescence intensity units $\pm$ SD. Statistically significant results are indicated (two-tailed Student *t* tests; *, $P \geq 0.05$).

material). Similar susceptibility patterns were observed for strains BH48, BH1CC, and JE2 (data not shown).

Ethanol was the most effective antiseptic against biofilms formed under flow conditions on plasma-coated surfaces (Fig. 6A, panel iv). As in the static biofilm assays, Taurolock also resulted in a substantial reduction in biofilm viability (Fig. 6A, panel iii). EDTA and Duralock-C did not reduce the number of live cells within the biofilm; both agents showed a ratio of live to dead cells similar to that of the untreated control (Fig. 6A, panels ii and v, and B). In addition, ethanol at concentrations of 7.5% (vol/vol) and above inactivated biofilm (see Fig. S2 in the supplemental material). In general terms, the patterns of susceptibility of biofilms formed under static or flow conditions to the antibiotics and antiseptics were similar.

DISCUSSION

The treatment of IVC infections due to biofilm-producing staphylococci is an extensively studied area, and significant progress has been made over the last years in our understanding of the complexity of the various stages involved in staphylococcal biofilm formation and potential treatment strategies (22). The use of ALT,

in addition to systemic antibiotics, to fill the lumen of the IVC to eradicate biofilms has been one such important treatment option for the salvage of an IVC in patients with a CRBSI (4). However, the experimental evidence for the selection of effective antimicrobial agents used as ALSs has not highlighted an optimum specific agent or approach, resulting in widely varied clinical practice. Furthermore, efforts to investigate effective therapeutics for biofilm-associated infections have been underpinned by studies investigating the mechanisms of staphylococcal biofilm formation on polystyrene surfaces, in nutrient-rich bacteriological broths, and using mainly reference strains of staphylococci. However, when the environmental growth conditions in the host are considered, such as nutrient availability *in vivo* and the rapid conditioning of implanted device surfaces by the extracellular proteins fibronectin, fibrinogen, and collagen, the relevance of biofilm mechanisms already elucidated under *in vitro* conditions must come into question, as they may not be as important as once thought. To address this concern, we employed our recently described *in vitro* biofilm assay, which more closely mimics the physiological environment that is proposed to exist during the pathogenesis of an *S. aureus* catheter-related infection involving biofilm mediated by the

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FIG 4 Susceptibility of *S. aureus* biofilms to ethanol and Taurolock. MRSA and MSSA biofilms were grown in RPMI 1640 for 1, 3, or 5 days and treated with ethanol and Taurolock, at the indicated concentrations, at 37°C for 24 h. Viability of biofilms was measured using the resazurin conversion assay. Assays were performed in triplicate, and the data represent mean fluorescence intensity units $\pm$ SD.

enzyme coagulase (14). Using this more appropriate assay facilitated a more clinically relevant investigation that examined the effects of a range of commonly used antibiotics and antiseptic agents against a number of reference and clinical staphylococcal isolates (14).

While some previous *in vitro* studies have shown the effectiveness of antibiotics, such as vancomycin, daptomycin, and rifampin, as ALTs (7, 8, 19, 23), others have demonstrated the opposite effect, as well as the ineffectiveness of other antibiotics, such as linezolid, gentamicin, and oxacillin (12, 24, 25). In our study, the antibiotics daptomycin, vancomycin, tigecycline, and rifampin resulted in a significant reduction in the viability of *S. aureus* biofilms formed over a 24-h incubation period. Heparin is often used in combination with antimicrobials within an ALS; in this study, the effect of the addition of the anticoagulant heparin was measured, and no effect of heparin, at the concentrations applied as anticoagulants within an ALS, on the thickness or viability of *S. aureus* biofilms was found (data not shown). Under conditions of flow, confocal

microscopy revealed a reduction in viability of up to 95% after treatment. Importantly, vancomycin was not effective in eradicating older biofilms, and the presence of mature biofilm is more likely to reflect the “real-life” clinical scenario where ALT is initiated following the diagnosis of catheter-related infection caused by *S. aureus*. Daptomycin, tigecycline, and rifampin had a more sustained effect, remaining active against more mature biofilms of three and five days old. The extended efficacy of daptomycin may, in part, be related to the structure of the drug. The C₁₀ fatty acid, which forms part of daptomycin, may aid in the penetration of this antibiotic into mature biofilms (26). Findings from concentration gradients described in this study indicate minimum antibiotic concentrations which should be used as ALT in patients with an IVC infection due to *S. aureus*. Successful patient outcomes with ALT can occur only by using high enough concentrations of antibiotics that are based on concentrations required to inhibit mature *S. aureus* biofilm. Furthermore, the concentrations of antibiotics required to kill biofilms formed under conditions of flow were higher than those seen during testing against biofilms

FIG 5 Effect of antibiotics on biofilm formed under shear. (A) SH1000 biofilm was formed under conditions of flow in RPMI 1640 and treated statically with medium (panel i) or the antibiotic linezolid (panel ii), gentamicin (panel iii), daptomycin (panel iv), vancomycin (panel v), tigecycline (panel vi), fosfomycin (panel vii), or rifampin (panel viii) for 24 h at 37°C. Live/dead staining was carried out and visualized using a confocal microscope. (B) Image J software was used to calculate the percentage of live (green) versus dead (red) pixels; the data represent mean percentage $\pm$ SD. Assays were performed in triplicate, and representative images are shown.

formed under static growth conditions. This is consistent with previous studies that have indicated that biofilms formed under conditions of shear are more resistant to treatment (27).

The use of nonantibiotic antimicrobial locks has also been studied extensively, as there are concerns that their use in catheters may also lead to the development of antibiotic-resistant microorganisms (28). The use of ethanol as a ALS has been widely reported. Qu et al. (25) found that treatment with ethanol, at a concentration of 40%, for 1 h was superior to that with conventional antibiotics used in ALT. Sodium citrate is a potent antimicrobial and anticoagulant agent that has been used in previous studies (29). It is the main component of two commercially available ALSs, namely, Duralock-C and Taurolock. Duralock-C is a 46.7% solution of sodium citrate, while Taurolock comprises 4% sodium citrate and 1.35% taurolidine (a modified amino acid with antimicrobial properties). The use of EDTA as a potential ALS has also been investigated, with various results reported (10, 30).

In our study, all four antiseptics investigated resulted in significant reductions in viability against 1-day-old biofilms for all strains tested under static or flow conditions. However, only ethanol and Taurolock remained effective against more mature biofilms (≥ 3 days old). Under conditions of flow, ethanol, followed by Taurolock, was the most effective agent at inactivating biofilm. As with antibiotic testing, the concentrations required to kill biofilms formed under conditions of flow were higher than those seen in static testing. These findings are in contrast those in to previous publications, where the use of Duralock-C and EDTA has been recommended in the treatment of device-related infections involving biofilms (30–32).

Although this study represents findings from an *in vitro* study, we have attempted to replicate *in vivo*-like conditions by using iron-deficient media and human blood. When medical devices, such as IVCs, are implanted, the staphylococcal biofilm accumulates within the lumen of the catheter and is exposed to human blood, and therefore we believe that our conditions represent the *in vivo* situation in the best possible way and thus identify the most effective of the agents in routine use to treat staphylococcal biofilms within an ALS. Clinical studies would therefore be required to replicate these findings and provide conclusive recommendations. Furthermore, the effectiveness of these agents and concentrations described using this model of infection should also be investigated versus other microbial causes of IVC infection.

In conclusion, although the optimal treatment for patients with CRBSI is removal of the IVC, this is not always possible due to the underlying diagnosis or the lack of alternative vascular access; therefore, an alternative treatment such as combination of an ALS and systemic treatment is often required. While many antimicrobial agents kill bacteria in a planktonic state, only a limited number of agents are effective in treating device-related staphylococcal infections involving biofilms. The results from this study, using a novel *in vivo*-relevant model of catheter-related infection mediated by *S. aureus*, give definitive evidence that the antibiotics daptomycin, tigecycline, and rifampin, as well as the antiseptics ethanol and Taurolock, when used at an appropriate dose are likely to be the most highly effective ALSs in the salvage of catheters in vulnerable patients where device removal is not an option.

FIG 6 Effect of antiseptics on biofilm formed under shear. (A) SH1000 biofilm was formed under conditions of flow in RPMI 1640 and treated statically with medium (panel i) or the antiseptic Duralock-C (panel ii), Taurolock (panel iii), ethanol (panel iv), or EDTA (panel v) for 24 h at 37°C. Live/dead staining was carried out and visualized using a confocal microscope. (B) Image J software was used to calculate the percentage of live (green) versus dead (red) pixels; the data represent mean percentage $\pm$ SD. Assays were performed in triplicate, and representative images are shown.

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