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.
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 solution was added to each test well. The initial concentrations of the antibiotics and antiseptics were tested against biofilms formed over the bacterial strains used. 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 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 iodine (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). Guide-lines on breakpoints to the antiseptics included in this testing are not available.

#### Inactivation of mature *S. aureus* biofilms by antisepic and antibiotic treatment

IVC infections progress due to bacterial biofilm accumulation over a number of days. To reflect this, antibi-otics 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 1  *S. aureus* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH1000</td>
<td>MSSA reference strain, functional <em>rsbU</em> derivative</td>
<td>33</td>
</tr>
<tr>
<td>BH48 (04)</td>
<td>MSSA clinical isolate, serotype 8, clonal complex 8</td>
<td>34</td>
</tr>
<tr>
<td>BH1CC</td>
<td>MRSA clinical isolate, SCC mec type II, serotype 8,</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>clonal complex 8</td>
<td></td>
</tr>
<tr>
<td>USA300 J2E</td>
<td>MRSA strain USA300 derivative lacking plasmids P01</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>and P03, J2E LAC serotype 8, clonal complex 8</td>
<td></td>
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</tbody>
</table>

### TABLE 2 Concentration of agents used for biofilm susceptibility testing

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concen used for biofilm testing</th>
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</thead>
<tbody>
<tr>
<td><em>Antibiotics</em></td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>10 mg/ml</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>40 mg/ml</td>
</tr>
<tr>
<td>Linezolid</td>
<td>4 mg/ml</td>
</tr>
<tr>
<td>Rifampin</td>
<td>10 mg/ml</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td><em>Antiseptics</em></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>30% (vol/vol)</td>
</tr>
<tr>
<td>EDTA</td>
<td>32 mg/ml</td>
</tr>
<tr>
<td>Duralock-C</td>
<td>46.7% (wt/vol) sodium citrate</td>
</tr>
<tr>
<td>Taurolock</td>
<td>4% sodium citrate, 1.35% tauroidine</td>
</tr>
</tbody>
</table>
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 daptomycin, 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

<table>
<thead>
<tr>
<th>TABLE 3 MICs of antibiotics and antiseptics against <em>S. aureus</em></th>
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<tr>
<td><strong>Agent</strong></td>
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<tr>
<td><strong>Antibiotics</strong></td>
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<tr>
<td>Vancomycin</td>
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<td>Tigecycline</td>
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<td>Daptomycin</td>
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<td>Rifampin</td>
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<td>Fosfomycin</td>
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<tr>
<td><strong>Antiseptics</strong></td>
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<tr>
<td>Ethanol</td>
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<tr>
<td>Sodium citrate</td>
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<tr>
<td>EDTA</td>
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<tr>
<td>Taurolidine</td>
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</tbody>
</table>

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 ± SD. Statistically significant results are indicated (two-tailed Student t tests, *, *P* < 0.05).
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 $128 \mu g/ml$ 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 $\mu g/ml$, respectively (Fig. 2).

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, vii), reducing the number of live cells to less than 2% (Fig. 5B). Further analysis revealed that both daptomycin and tigecycline at concentrations of 128 $\mu g/ml$ inactivated biofilm, while vancomycin and rifampin were effective at 8 and 4 mg/ml, respectively (see Fig. S1 in the supplemental

![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 ± SD.](downloaded from http://aac.asm.org on October 25, 2017 by guest)
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 fibronecin, 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 material.

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 ± SD. Statistically significant results are indicated (two-tailed Student t tests; *, P ≤ 0.05).
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.
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. Su 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.
ACKNOWLEDGMENTS

This research was funded by a major grant award from the Healthcare Infection Society and a health research award from the Irish Health Research Board to E.O., J.P.O., and H.H.

FUNDING INFORMATION

This work, including the efforts of Hilary Humphreys, James P. O‘Gara, and Eoghan O’Neill, was funded by Health Research Board (HRB) (HRA-POR-2012-52). This work, including the efforts of Eoghan O’Neill, was funded by Healthcare Infection Society (HIS) (Major Grant Award).

The funders had no role in the design, execution, or interpretation of this study and were not involved in the decision to submit the work for publication.

REFERENCES


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 ± SD. Assays were performed in triplicate, and representative images are shown.
ALT of Catheter-Related Infections Due to S. aureus


