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Rifampicin enhances activity of daptomycin and vancomycin against both a polysaccharide intercellular adhesin (PIA)-dependent and -independent *Staphylococcus epidermidis* biofilm

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Objectives and methods: This study addressed the efficacy of daptomycin, vancomycin, rifampicin, daptomycin/rifampicin and vancomycin/rifampicin against a polysaccharide intercellular adhesin (PIA)-dependent and -independent *Staphylococcus epidermidis* biofilm using flow cell and guinea pig tissue cage models.

Results: The flow cell model of both PIA-dependent and -independent biofilms demonstrated that the viable cell count after treatment with daptomycin/rifampicin was significantly lower ($P < 0.05$) than after treatment with vancomycin, vancomycin/rifampicin, daptomycin or rifampicin alone. To validate these observations, a guinea pig tissue cage model was used. The results demonstrated that the addition of rifampicin to daptomycin or vancomycin sterilized 5/6 tissues cages colonized with *S. epidermidis* 1457 (PIA producing). Similar results were noted with *S. epidermidis* 1457 icaADBC::dhfr (non-PIA producing), where daptomycin/rifampicin and vancomycin/rifampicin sterilized 5/6 and 6/6 tissue cages, respectively. There was no statistical difference in comparison with the no-treatment control when both 1457 and 1457 icaADBC::dhfr were treated with vancomycin and daptomycin alone. Furthermore, treatment with rifampicin alone sterilized 5/6 and 3/6 1457 and 1457 icaADBC::dhfr tissue cages, respectively.

Conclusions: Interpretation of these data suggests that rifampicin is highly active against *S. epidermidis* biofilms and both vancomycin and daptomycin are effective at reducing the subpopulation of bacteria that develop rifampicin resistance.

Keywords: biomaterial infections, guinea pig model, tissue cage, *icaADBC* operon

Introduction

Staphylococcus epidermidis is the most prevalent cause of hospital-acquired infections.[1] A majority of *S. epidermidis* infections are associated with biomaterials. Adherence to these biomaterials and subsequent biofilm synthesis are defined virulence factors as assessed by relevant animal models of infection.[2–5] A major component of *S. epidermidis* biofilm is polysaccharide intercellular adhesin (PIA), which is synthesized by enzymes encoded by the *icaADBC* operon.[6,7] PIA contributes to initial adherence in addition to intercellular accumulation and structural rigidity.[8] Furthermore, PIA protects against major components of the human innate host immune system and was recently shown to be responsible for pro-inflammatory cytokine production.[9,10]

It is clear that, in contrast to *Staphylococcus aureus*, not all *S. epidermidis* isolates encode the *icaADBC* locus.[11–15] Therefore, in these *icaADBC*-negative isolates, biofilm formation and intercellular accumulation are thought to be mediated by extracellular DNA[16,17] and other proteinaceous factors, including accumulation-associated protein (Aap),[18] biofilm-associated protein (Bhp)[19] and the extracellular matrix binding protein (Ebh).[20,21] Accordingly, Rohde *et al.*[14] found that 27% of biofilm-positive *S. epidermidis* isolates obtained from prosthetic joint infections following total hip or knee replacement produced PIA-independent biofilms. Although it is still unclear whether the substructure metabolism is different between PIA-dependent and -independent biofilms, it is well established that bacteria growing in a biofilm state are more recalcitrant to antibiotics. Multiple oxygen and nutrient gradients have been identified within biofilms, allowing unique niche metabolism.[22,23] Further complicating antibiotic treatment of biofilm-associated infections includes the development of persister cells[24] and the

horizontal acquisition of antibiotic resistance determinants, which narrow the available treatment options. Approximately 85% of *S. epidermidis* clinical isolates are resistant to oxacillin; therefore, vancomycin and daptomycin are commonly used treatment modalities against biomaterial-related infections. This study investigated the efficacy of vancomycin and daptomycin alone and in combination with rifampicin in the treatment of an *S. epidermidis* biomaterial-based infection in a guinea pig tissue cage model and an *in vitro* flow cell biofilm model. The contribution of PIA to the recalcitrance of biofilms to antibiotics was addressed by including an isogenic PIA-negative (1457 *icaADBC::dhfr*) *S. epidermidis* strain in both the guinea pig and the flow cell model.

Materials and methods

S. epidermidis strains

The *S. epidermidis* strains used in the study were *S. epidermidis* 1457[7] and the previously constructed isogenic, PIA-negative strain 1457 *icaADBC::dhfr*.[25]

Microscopy

Scanning electron microscopy (EM) was performed using an FEI Quanta 200 (Hillsboro, OR, USA) at the University of Nebraska Medical Center Electron Microscopy Core Laboratory.

Flow cell biofilm model

Plastic convertible Stovall flow cells (24 mm×40 mm×8 mm; Stovall, Greensboro, NC, USA) were used to test an *in vitro* biofilm antibiotic treatment model. Six flow cells were used in each experiment: three control cells (not treated with antibiotics) and three experimental cells (treated with antibiotics). The three control and experimental flow cells were connected to separate 10 L carboys containing tryptic soy broth (TSB; Difco, Detroit, MI, USA). *S. epidermidis* 1457 and 1457 *icaADBC::dhfr* were grown in TSB to an optical density (600 nm) of 0.5 and 100 μL was subsequently added to each flow cell; the bacteria were allowed to adhere to the flow cell for 30 min before initiation of the flow (flow rate=0.5 mL/min). The biofilms were allowed to mature for 48 h, at which time the antibiotic was added to the one carboy supplying TSB to the three experimental flow cells. The viable cell count (cfu/mL) was determined from the control flow cells at 48, 72 and 96 h timepoints by resuspension of the biofilms in 1 mL of TSB and subsequent dilution plating (limit of detection, 10 cfu). The following antibiotic concentrations were added to the carboy supplying antibiotic to the experimental flow cells: vancomycin, 40 mg/L; daptomycin, 50 mg/L; and rifampicin, 12 mg/L. In experiments containing daptomycin, calcium chloride (Sigma, St Louis, MO, USA) was added to a concentration of 50 mg/L. Twenty-four hours after initiation of antibiotic treatment, fresh medium containing antibiotics at the concentrations stated above was added to the flow cell and the experiment was allowed to continue for the remainder of the experiment. At the 96 h timepoint (48 h post-addition of antibiotic), the viable cell count was assessed for the three experimental flow cells as described for the control cells.

Guinea pig tissue cage model

A modification of the tissue cage model developed by Zimmerli *et al.*[26] was used. The most important feature of this model is that it creates a potential space within the tissue cage allowing interaction between host defences and bacteria. In addition, this space allows easy sample aspiration and bacteriological analysis of the tissue cage fluid. The model was performed by first cutting a polypropylene tube (Tygon® S-50-HL Medical/Surgical Tubing 3/8 inch inner diameter×1/2 inch outer diameter×1/16 inch wall) into ∼2.5 cm pieces to construct the tissue cages. Ten glass beads (4 mm) were then added and the tubing was heat-sealed. Finally, nine 1/8 inch holes were drilled into each side of the tissue cage to allow easy access and increased flow into and out of the device. After a single dose of cefazolin (100 mg/kg) prophylaxis, two tissue cages, one on either side, were implanted into the subcutaneous space of the back of Hartley guinea pigs (Charles River Laboratories, Lee's Summit, MO, USA). Following tissue cage insertion, the animals were allowed to heal for 3 days. On the third day, the tissue cages were aspirated and the fluid plated on tryptic soy agar (TSA) to ensure sterility and each tissue cage was inoculated with *S. epidermidis* 1457 or 1457 *icaADBC::dhfr*. Colonization of the tissue cage was confirmed after 5 days by aspiration of the tissue cage and subsequent dilution plating on TSA. This experiment had six experimental arms, with three guinea pigs per arm. Two tissue cages were implanted into each guinea pig for a total of six tissue cages per experimental arm. Treatment was initiated for a duration of 5 days as follows: Group 1: three control animals treated with no anti-infective therapy; Group 2: three animals treated with vancomycin 15 mg/kg intraperitoneally every 12 h; Group 3: three animals treated with daptomycin 5 mg/kg intraperitoneally every 24 h; Group 4: three animals treated with vancomycin 15 mg/kg intraperitoneally every 12 h and rifampicin 25 mg/kg intraperitoneally every 24 h; Group 5: three animals treated with daptomycin 5 mg/kg intraperitoneally every 24 h and rifampicin 25 mg/kg intraperitoneally every 24 h; and Group 6: three animals treated with rifampicin 25 mg/kg intraperitoneally every 24 h. Following 5 days of therapy, the animals were sacrificed. The tissue cages were excised and the tissue cage beads were aseptically removed, vortexed in 1 mL of PBS, serially diluted and plated onto tryptic soy agar plates to reveal cfu/mL (limit of detection, 10 cfu). Statistical analysis was performed using the Mann–Whitney test. P values of <0.05 were considered statistically significant. The University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC) approved these experiments.

Results

In vitro biofilm flow cell antibiotic treatment model

An *in vitro* flow cell biofilm model was developed to determine the antibiotic efficacy of daptomycin, vancomycin, rifampicin, vancomycin/rifampicin and daptomycin/rifampicin against PIA-dependent and -independent biofilms. Flow cells were allowed to mature for 48 h, at which time three of the six flow cells were treated with one of the aforementioned antibiotics or antibiotic combinations. As shown in Figure 1, untreated, PIA-dependent biofilms developed significant three-dimensional structure from 48 to 96 h of growth. 1457 *icaADBC::dhfr* grown in the flow cell setting were devoid of structure formation and appeared as a flat mat (data not shown).[27] However, after treatment with any of the antibiotics (vancomycin, daptomycin or rifampicin) for 48 h, the visible three-dimensional structure did not fully develop (Figure 1) and the viable cell count decreased significantly (Figure 2). After 96 h of growth, the mean control viable cell count for the PIA-dependent biofilm (strain 1457) was 2×10^{10} cfu/mL (from 3×10^{9} cfu/mL after 48 h of growth). Treatment with daptomycin, vancomycin, vancomycin/rifampicin, daptomycin/rifampicin or rifampicin significantly ($P<0.05$) decreased the viable cell count after 48 h of treatment in comparison with the 96 h control (Figure 2). However, the viable cell count after treatment with daptomycin/rifampicin was

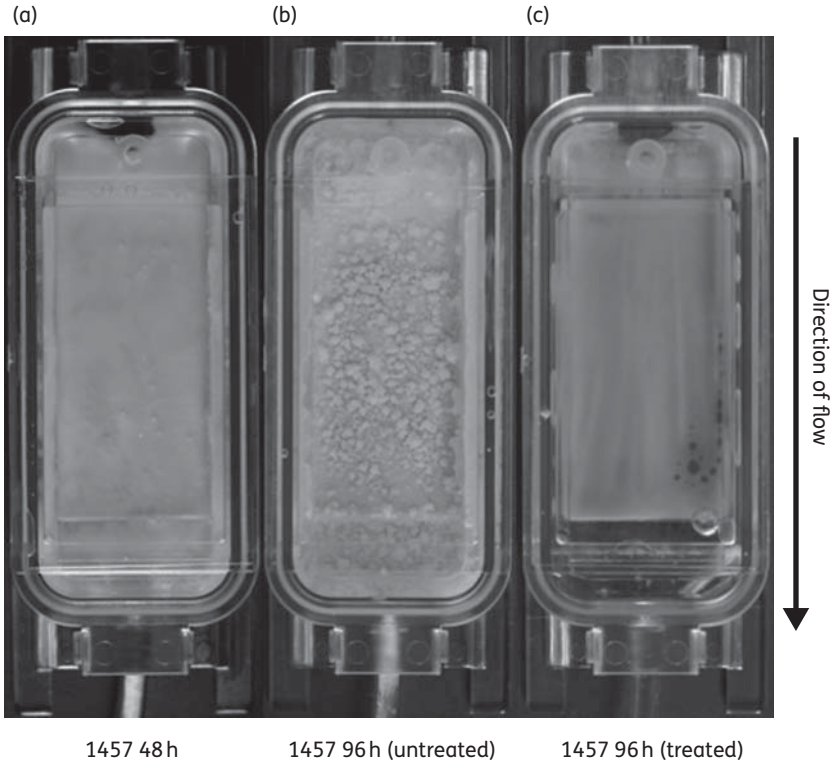

Figure 1. Growth of *S. epidermidis* 1457 in a flow cell chamber. *S. epidermidis* 1457 biofilm at (a) 48 h and (b) 96 h of growth. Note significant three-dimensional structure of the biofilm after 96 h of growth. (c) *S. epidermidis* 1457 after vancomycin/rifampicin treatment for 48 h. Note lack of three-dimensional structure in comparison with (b). Arrow on right-hand side of figure denotes direction of the media flow.

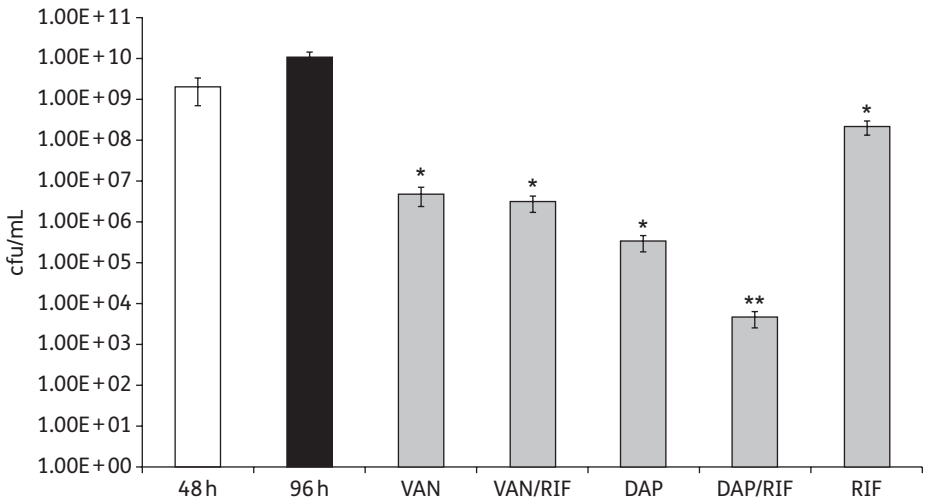

Figure 2. *In vitro* flow cell experiments with *S. epidermidis* 1457. The white bar represents mean cfu/mL after 48 h of growth (time 0) before addition of antibiotic. The black bar represents the mean cfu/mL of the untreated flow cells after an additional 48 h of growth (96 h of growth). The grey bars represent the mean cfu/mL after 48 h of exposure to the indicated antibiotic. *Significance ($P<0.05$) as compared with the 96 h timepoint. **The antibiotic treatment regimen was significantly different from all other treatments tested. VAN, vancomycin; RIF, rifampicin; DAP, daptomycin.

significantly lower ($P<0.05$) than that after treatment with vancomycin, vancomycin/rifampicin, daptomycin or rifampicin. Similar observations were noted using 1457 *icaADBC::dhfr* in the flow cell system. A viable cell count of 2×10^{10} cfu/mL was acquired after 96 h of growth; treatment with all five antibiotics/antibiotic combinations significantly decreased the viable cell count after 48 h of antibiotic treatment (Figure 3). However, as observed with 1457, the viable cell count of 1457 *icaADBC::dhfr* after treatment with daptomycin/rifampicin was significantly lower than after treatment with any other antibiotic.

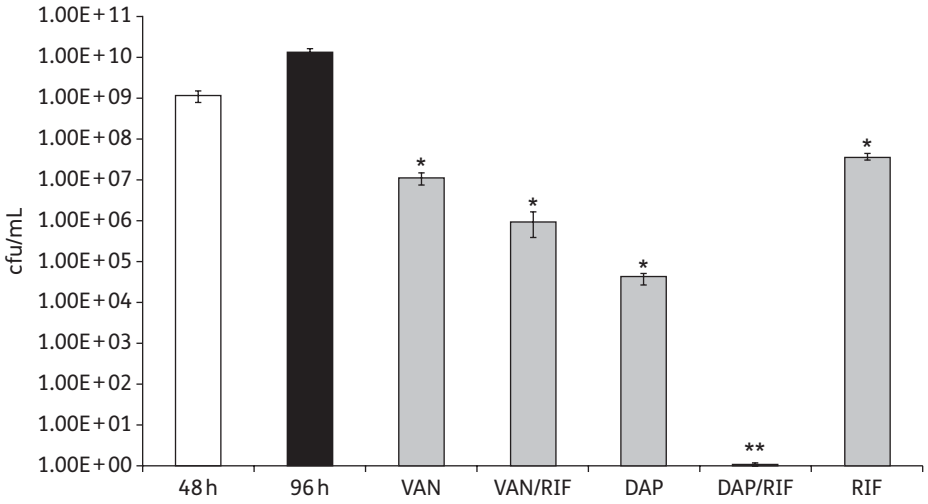

Figure 3. *In vitro* flow cell experiments with *S. epidermidis* 1457 *icaADBC::dhfr*. The white bar represents mean cfu/mL after 48 h of growth (time 0) before addition of antibiotic. The black bar represents the mean cfu/mL of the untreated flow cells after an additional 48 h of growth (96 h of growth). The grey bars represent the mean cfu/mL after 48 h of exposure to the indicated antibiotic. *Significance ($P<0.05$) as compared with the 96 h timepoint. **The antibiotic treatment regimen was significantly different from all other treatments tested. VAN, vancomycin; RIF, rifampicin; DAP, daptomycin.

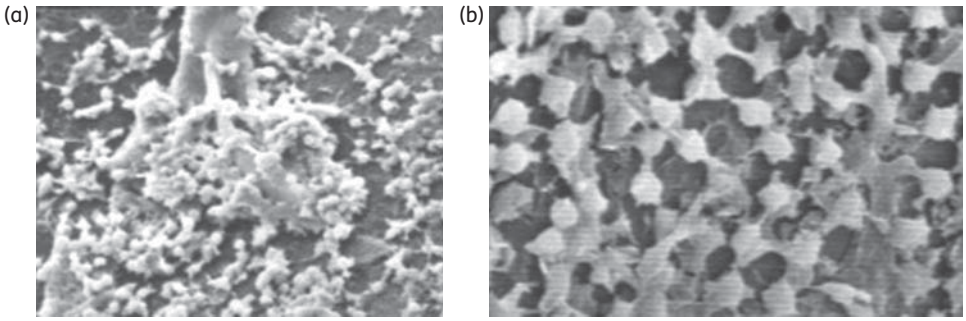

Figure 4. Biofilm formation on tissue cage. Scanning EM of *S. epidermidis* 1457 on Tygon tubing (a) and glass beads (b).

In fact, no colonies were detected (limit of detection, 10 cfu). This experiment was repeated twice ($n=6$ flow cells in total) with identical results. To determine whether rifampicin-resistant mutants were selected during rifampicin treatment, 100 colonies were isolated from both (1457 and 1457 *icaADBC::dhfr*) flow cells treated with rifampicin and colonies were transferred to TSA plates containing 10 mg/L rifampicin. As predicted, all 100 colonies grew on TSA containing rifampicin, suggesting that the failure of rifampicin to eradicate the biofilm was due to the selection of rifampicin-resistant mutants.

Guinea pig tissue cage model

In order to extend our *in vitro* observations and more closely mimic the human infection, a guinea pig tissue cage model was used. Pilot studies were performed to determine the smallest inoculum of both *S. epidermidis* 1457 and 1457 *icaADBC::dhfr* that would colonize each tissue cage. It was determined that an inoculum of 5×10^4 cfu of strain 1457 was reliably able to colonize each guinea pig tissue cage. In addition, we found that the tissue cages remained colonized for at least 28 days ($\sim5\times10^4$ cfu/mL of tissue cage aspirate) and the infection did not metastasize to other organ systems, including the liver, kidney, heart or spleen; therefore, this model mimics a typical indolent *S. epidermidis* biomaterial-related infection seen in humans. Scanning EM verified that both the tissue cages and the tissue cage glass beads were covered with biofilm following colonization with strain 1457 (Figure 4a and b). Following confirmation that the tissue cages were colonized with *S. epidermidis* 1457 (5 days post-inoculation), three animals each were treated with daptomycin, vancomycin, rifampicin, daptomycin/rifampicin and vancomycin/rifampicin for 5 days. Six animals ($n=12$ tissue cages) were used as untreated controls. Following sacrifice of the animals and obtaining the viable count from the glass beads, it was determined that there was no statistical difference between the control group and the animals that were treated with vancomycin and daptomycin alone (Figure 5). The mean cfu/mL in the control group, animals treated with daptomycin and those treated with vancomycin was 3.76×10^4, 1.16×10^4 and 7.88×10^4 cfu/mL, respectively. In contrast, no bacteria were detected following treatment with rifampicin in 5/6 tissue cages. Furthermore, treatment with daptomycin/rifampicin and

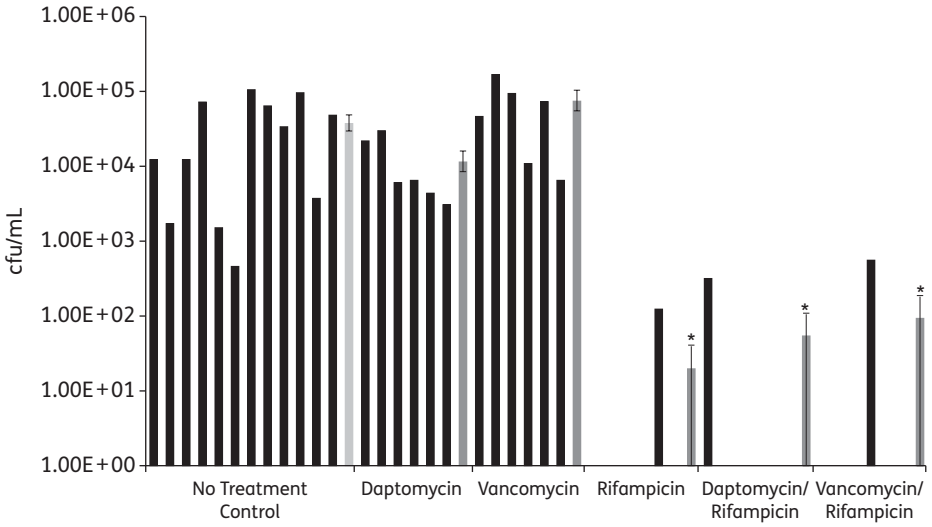

Figure 5. Efficacy of antibiotics against *S. epidermidis* 1457 in a guinea pig tissue cage model. Black bars represent cfu recovered from each individual tissue cage. Grey bars represent the mean and SEM of each dataset. *Significance ($P<0.05$) as compared with the no-treatment control.

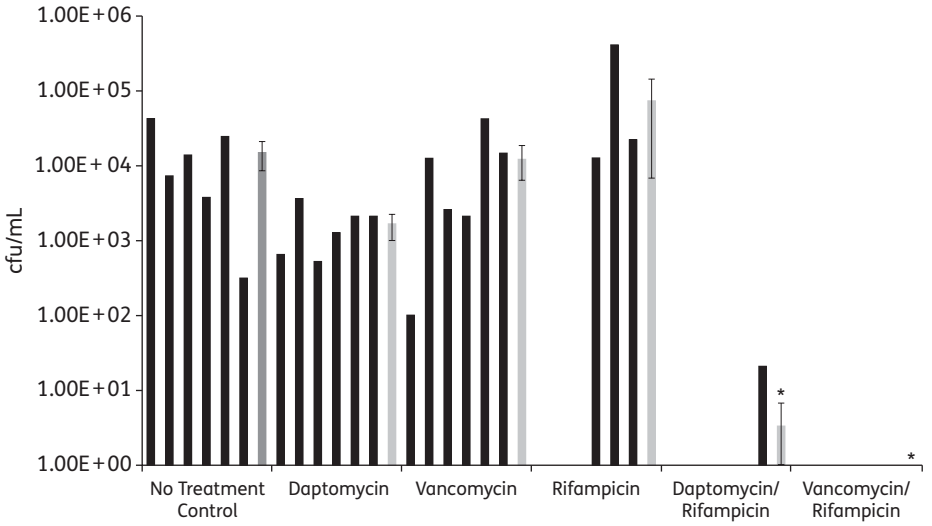

Figure 6. Efficacy of antibiotics against *S. epidermidis* 1457 *icaADBC::dhfr* in a guinea pig tissue cage model. Black bars represent cfu recovered from each individual tissue cage. Grey bars represent the mean and SEM of each dataset. *Significance ($P<0.05$) as compared with the no-treatment control.

vancomycin/rifampicin also sterilized 5/6 tissue cages. To determine whether rifampicin-resistant mutants were selected during therapy with rifampicin, rifampicin/daptomycin and rifampicin/vancomycin, 100 colonies were picked from the three non-sterile tissue cages (one from each treatment arm) and transferred to TSA containing 10 mg/L rifampicin. It was found that, in all three cases, 100/100 colonies grew on media containing rifampicin, suggesting that the selection of rifampicin-resistant mutants during treatment complicated the treatment regimen.

In contrast to strain 1457, pilot studies demonstrated that a higher inoculum of 1457 *icaADBC::dhfr* (5×10^7 cfu) was needed to colonize 100% of the tissue cages. However, a similar level of colonization in strains 1457 and 1457 *icaADBC::dhfr* (3.76×10^4 cfu/mL and 1.45×10^4 cfu/mL, respectively) was observed in terms of cfu/mL extracted from the tissue cage beads following animal sacrifice (Figure 6). Similar to 1457, it was determined that there was no significant difference between the control group of 1457 *icaADBC::dhfr* and the vancomycin treatment group (mean 1.45×10^4 and 1.18×10^4 cfu/mL, respectively). Treatment with daptomycin alone against tissue cages colonized with 1457 *icaADBC::dhfr* resulted in a mean 1.66×10^3 cfu/mL per tissue cage; however, this result did not reach statistical significance ($P=0.06$) (Figure 6). Treatment with rifampicin, daptomycin/rifampicin and vancomycin/rifampicin sterilized 3/6, 5/6 and 6/6 tissue cages, respectively. As observed with strain 1457, all colonies tested ($n=100$ per tissue cage) of 1457 *icaADBC::dhfr* recovered from the non-sterile tissue cages from all three treatment arms containing rifampicin were resistant to rifampicin.

Discussion

Daptomycin is a cyclic lipopeptide antibiotic that is active against Gram-positive bacteria and is especially useful against highly resistant pathogens such as methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium*. Daptomycin is a calcium-dependent antibiotic that causes membrane disruption and depolarization, potassium ion leakage and eventual cell death.[28,29] However, the efficacy of daptomycin in the treatment of *S. epidermidis* biofilms is unclear. Although the effectiveness of daptomycin has been addressed in a catheter lock model[30] and in an experimental model of endocarditis,[31] this is the first study to address the effectiveness of daptomycin and rifampicin against well-defined PIA-dependent and -independent *S. epidermidis* biofilms in *in vitro* and *in vivo* models.

Due to the lack of randomized clinical trials, there are no standard clinical practices regarding the treatment of *S. epidermidis* biofilm-associated infections. Although rifampicin is never used clinically as monotherapy due to rapid development of resistance,[32–34] many studies have demonstrated its effectiveness against staphylococcal biofilms in combination with other anti-staphylococcal antibiotics, including tigecycline,[35,36] trimethoprim/sulfamethoxazole,[36,37] linezolid,[38–40] quinupristin/dalfopristin,[36,39,41] ciprofloxacin[42,43] and levofloxacin.[44] For instance, one blinded, randomized clinical trial for the treatment of orthopaedic implant infections comparing ciprofloxacin against ciprofloxacin plus rifampicin demonstrated that the ciprofloxacin/rifampicin arm had a clinical cure rate of 100% versus 58% in the ciprofloxacin only arm.[43]

This study was designed to address the efficacy of daptomycin and vancomycin in the treatment of both PIA-dependent and -independent biofilms. Because of the aforementioned efficacy against staphylococcal biofilms, rifampicin was added to both vancomycin and daptomycin to determine whether it significantly altered the viability of bacteria growing in a biofilm. First, we found that both vancomycin and daptomycin alone were not effective in the treatment of an *S. epidermidis*-mediated foreign body infection (i.e. tissue cage). Similar results were noted for both 1457 and 1457 *icaADBC::dhfr*. Several studies have demonstrated that daptomycin, vancomycin and rifampicin rapidly penetrate staphylococcal biofilms; thus, their inability to inhibit growth is most likely due to the quiescent state of the bacteria in the biofilm.[45–50] However, both antibiotics in combination with rifampicin were highly efficacious in sterilizing tissue cages colonized by both strains 1457 and 1457 *icaADBC::dhfr*. Surprisingly, no bacteria were detected in 5/6 and 3/6 tissue cages colonized by 1457 and 1457 *icaADBC::dhfr*, respectively, following treatment with rifampicin alone. However, predictably, colonies that were isolated from the non-sterile tissue cages were resistant to rifampicin, further demonstrating that rifampicin should not be used as monotherapy due to rapid development of resistance. In fact, all colonies isolated from any tissue cage treated with rifampicin (either alone or in combination with vancomycin or daptomycin) were resistant to rifampicin (data not shown). Interpretation of these data suggests that rifampicin is highly active against staphylococcal biofilms and that the utility of either vancomycin or daptomycin in combination with rifampicin is to inhibit the outgrowth of rifampicin-resistant mutants that are selected during therapy. Unfortunately, experiments were not performed to determine whether, following successful therapy, the tissue cages would again become colonized by either *S. epidermidis* 1457 or 1457 *icaADBC::dhfr*. Further experimentation will address questions regarding the function of persisters in *S. epidermidis* biomaterial-related infections.

The development of an *in vitro* flow cell model of antibiotic therapy was advantageous for two reasons. First, since the mean number of bacteria detected in the guinea pig tissue model was $\sim 5 \times 10^4$ cfu/mL, we wanted to create a model where the number of bacteria being treated was significantly greater. Second, using a treatment model with a higher inoculum, we hypothesized that subtle differences between the treatment efficacy of vancomycin/rifampicin and daptomycin/rifampicin would be detected. The *in vitro* flow cell model demonstrated that daptomycin/rifampicin was more effective in the treatment of both a PIA-dependent and -independent biofilm. Surprisingly, however, it was determined that daptomycin/rifampicin sterilized flow cells colonized with 1457 *icaADBC::dhfr*. These results may suggest that PIA is protective against the deleterious action of antibiotics or, alternatively, that PIA provides specific niches that allows antibiotic tolerance or persister cells to develop. In addition, it is suggested that rifampicin was not effective at clearing the flow cell biofilm due to the high bacterial burden and subsequent increased chance of selection of rifampicin-resistant mutants.

In conclusion, the *in vivo* guinea pig tissue cage model demonstrated that rifampicin in combination with either vancomycin or daptomycin is highly active against *S. epidermidis* biofilm-mediated infections; this approach deserves greater clinical scrutiny. These results suggest that RNA metabolism is important for biofilm growth and, possibly, that RNA polymerase is an effective target for antibiotic development against bacteria that commonly grow in a biofilm. Furthermore, our *in vitro* flow cell model demonstrated that the combination of daptomycin/rifampicin is superior to vancomycin/rifampicin against both a PIA-dependent and a PIA-independent biofilm. Lastly, our *in vitro* results suggest that PIA protects against the combination of daptomycin and rifampicin, suggesting a function that counteracts, in an unknown manner, the deleterious action of antibiotics against the bacterium.

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Transparency declarations

M. E. R. serves on a Cubist Pharmaceuticals advisory board. All other authors: none to declare.

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