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Evaluation of daptomycin combinations with cephalosporins or gentamicin against *Streptococcus mitis* group strains in an *in vitro* model of simulated endocardial vegetations (SEVs)

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Objectives: Among viridans group streptococcal infective endocarditis (IE), the *Streptococcus mitis* group is the most common aetiological organism. Treatment of IE caused by the *S. mitis* group is challenging due to the high frequency of β-lactam resistance, drug allergy and intolerability of mainstay antimicrobial agents such as vancomycin or gentamicin. Daptomycin has been suggested as an alternative therapeutic option in these scenarios based on its excellent susceptibility profile against *S. mitis* group strains. However, the propensity of many *S. mitis* group strains to rapidly evolve stable, high-level daptomycin resistance potentially limits this approach.

Methods: We evaluated the activity of 6 mg/kg/day daptomycin alone or in combination with gentamicin, ceftriaxone or ceftaroline against two daptomycin-susceptible *S. mitis* group strains over 96 h in a pharmacokinetic/pharmacodynamic model of simulated endocardial vegetations.

Results: Daptomycin alone was not bactericidal and high-level daptomycin resistance evolved at 96 h in both organisms. Combinations of daptomycin + ceftriaxone and daptomycin + ceftaroline demonstrated enhanced killing activity compared with each antibiotic alone and prevented emergence of daptomycin resistance at 96 h. Use of gentamicin as an adjunctive agent neither improved the efficacy of daptomycin nor prevented the development of daptomycin resistance.

Conclusions: Addition of ceftriaxone or ceftaroline to daptomycin improves the bactericidal activity against *S. mitis* group strains and prevents daptomycin resistance emergence. Further investigation with combinations of daptomycin and β-lactams in a large number of strains is warranted to fully elucidate the clinical implications of such combinations for treatment of *S. mitis* group IE.

Introduction

The *Streptococcus mitis* group belongs to the viridans group streptococci (VGS) and comprises oral commensal microorganisms. The *S. mitis* group is the most common cause of infective endocarditis (IE) among the VGS, as well as a leading cause of severe septic syndromes in neutropenic hosts (e.g. the 'toxic strep syndrome').[1] Management of *S. mitis* group infections is challenging due to the relative frequency of resistance to β-lactam antibiotics, including penicillins and multiple cephalosporins, which are the first-line antibiotics.[2–4] Penicillin resistance has been reported to be as high as 56% of 352 VGS bloodstream isolates from US medical institutions.[3–6] Although most *S. mitis* group strains are susceptible *in vitro* to vancomycin, this organism frequently exhibits tolerance to the killing caused by this agent.[7,8] In addition, patient characteristics such as drug allergy or intolerance of mainstay antimicrobials make treatment of *S. mitis* group IE more challenging, even in IE caused by penicillin-susceptible strains. Prevalence of penicillin allergy is not minimal, limiting use of penicillin in 8%–12% of

patients.[9] The prolonged use of current alternative agents such as vancomycin and gentamicin is often associated with nephrotoxicity.[10] All these clinical challenges have raised the need for a novel therapeutic approach based on better-tolerated agents.[11,12]

Daptomycin is a cyclic lipopeptide antibiotic that has been extensively evaluated for treatment of Gram-positive infections, including *Staphylococcus aureus* and both vancomycin-susceptible enterococci and vancomycin-resistant enterococci (VRE). Although limited clinical data are available, daptomycin's potent *in vitro* activity demonstrates therapeutic potential for treatment of endocarditis caused by *S. mitis* group strains resistant to front-line antibiotics or in those who are intolerant to first-line antimicrobial agents.[13,14] However, several studies have confirmed the propensity of more than 25% of such daptomycin-susceptible strains to rapidly develop high-level and durable daptomycin resistance during *in vitro* passage or following daptomycin therapy in experimental IE models.[6] We hypothesized that use of an adjunctive antibiotic may not only potentiate the bactericidal activity of daptomycin, but could also prevent emergence of daptomycin resistance in treatment of *S. mitis* group IE.

Ceftaroline fosamil is a novel cephalosporin with excellent activity against *S. aureus* (including MRSA) and VGS.[15] For example, in a recent surveillance study using clinical VGS isolates from the USA, ceftaroline demonstrated *S. mitis* group $MIC_{50/90}$ of $\leq$0.015 and $\leq$0.06 mg/L.[16] It should be noted that the *in vitro* synergistic activity of daptomycin plus ceftaroline has been documented against *S. aureus*, *Enterococcus faecalis* and *Enterococcus faecium*,[17–20] but not against the *S. mitis* group. Ceftriaxone and gentamicin have served as the backbone of antibiotic regimens for treatment of VGS IE in combination with penicillin G or vancomycin.[7] Although daptomycin plus ceftriaxone and daptomycin plus gentamicin are usually synergistic against *S. aureus* strains and many enterococci,[17,21–25] the activities of these combinations against *S. mitis* group strains are unknown. The aim of this study was to evaluate the efficacy of daptomycin alone and in combination with cephalosporins, either ceftriaxone or ceftaroline, or gentamicin, using a pharmacokinetic (PK)/pharmacodynamic (PD) model of simulated endocardial vegetations (SEVs) both in terms of killing of two daptomycin-susceptible *S. mitis* group strains and preventing the emergence of daptomycin resistance.

Materials and methods

Bacterial isolates and growth conditions

Two daptomycin-susceptible *S. mitis* group clinical isolates (penicillin-resistant *S. mitis/oralis* 351 and penicillin-susceptible *S. mitis* SF100) were evaluated in the experiment. *S. mitis/oralis* 351 was identified as an *S. mitis* strain, based on standard biotyping and 16S RNA sequencing.[6] Recently, we have obtained genome-sequenced results and discovered that this strain is more likely to be a member of the closely related species *S. oralis*, based on average nucleotide identity analysis of the whole genome sequence. The strain has therefore been renamed *S. mitis/oralis* 351 and is listed thus in GenBank. These isolates were clinically derived and were previously shown to cause experimental IE.[6,26] Daptomycin, ceftriaxone and gentamicin were purchased commercially (Merck, Kenilworth, NJ, USA; Sandoz, Princeton, NJ, USA; and Sigma–Aldrich, St Louis, MO, USA), while ceftaroline was provided by its manufacturer (Allergan, Parsippany, NJ, USA). For *in vitro* susceptibility testing, cation-adjusted Mueller–Hinton broth supplemented with 5% lysed horse blood was used. Brain heart infusion broth (Difco, Detroit, MI, USA) was used for PK/PD models of SEVs. For all experiments including daptomycin, calcium chloride supplementation was performed to provide 50 mg/L. Tryptic soy agar supplemented with 5% sheep's blood (Difco) was used for colony growth and enumeration upon subculture from the SEV models.

In vitro susceptibility testing

The MICs of daptomycin, ceftriaxone, ceftaroline and gentamicin were determined by broth microdilution in duplicate. Following determination of the MIC values for each isolate, daptomycin MICs were determined again in the presence of ceftriaxone, ceftaroline or gentamicin at 0.5× the MIC, to determine the potential for synergy, evidenced by the 'daptomycin MIC lowering effect' of the cephalosporins and gentamicin.[17]

PK/PD SEV model

Both *S. mitis/oralis* 351 and *S. mitis* SF100 were evaluated in a PK/PD model of SEVs over 96 h as previously described.[19,22] In brief, SEVs were prepared by combining human cryoprecipitate antihaemolytic factor (American Red Cross, Detroit, MI, USA), aprotinin (Sigma–Aldrich), human platelet suspensions (American Red Cross) and a suspension of either strain with the mixture, then solidified by addition of bovine thrombin (Pfizer, New York City, NY, USA). SEVs consisting of approximately 3–3.5 g/dL albumin and 6.8–7.4 g/dL total protein[27] were suspended in the 250 mL *in vitro* glass model by monofilaments (16 SEVs per chamber). Antimicrobials were administered as a bolus at a predefined frequency. Fresh medium was continuously infused and then removed along with the drug via a peristaltic pump (Masterflex, Cole-Parmer Instrument Company, Chicago, IL, USA) at a rate that simulates the human-equivalent half-life ($t_{1/2}$) of each agent. In the case of daptomycin combinations with ceftaroline and gentamicin the models were supplemented with daptomycin to compensate for excessive drug loss when simulating the more rapid $t_{1/2}$ of the two other agents as previously described.[28]

The models were placed in a warm water bath at 37°C. All experiments were performed in duplicate to ensure reproducibility. The following antibiotic regimens were simulated using total drug concentrations: (i) 6 mg/kg daptomycin every 24 h (C_{max} 93.9 mg/L, $t_{1/2}$ 8 h)[29]; (ii) 2 g ceftriaxone every 24 h (C_{max} 257 mg/L, $t_{1/2}$ 8 h)[30]; (iii) 600 mg ceftaroline every 8 h (C_{max} 21 mg/L, $t_{1/2}$ 2.66 h)[31]; (iv) 3 mg/kg gentamicin every 24 h (C_{max} 8.53 mg/L, $t_{1/2}$ 2.5 h)[32,33]; (v) daptomycin + ceftriaxone; (vi) daptomycin + ceftaroline; (vii) daptomycin + gentamicin; and (viii) drug-free growth controls.

For PD evaluations, two SEVs were aseptically removed from each model at 0, 4, 8, 24, 32, 48, 72 and 96 h timepoints. After weighing, each SEV was homogenized with trypsin, serially diluted with normal saline and quantitatively cultured. To minimize antibiotic carryover, those SEV samples where it was anticipated that the drug concentration was within 1 tube dilution of the MIC were diluted appropriately before plating. Plates were incubated in an anaerobic chamber for 18–24 h at 37°C before performing colony counts. Bacterial counts (cfu/g) remaining in the SEVs were plotted against time over 96 h to evaluate the bactericidal activity of the single and combination drug regimens. Bactericidal and bacteriostatic activity were defined as a $\geq$3 and $<$3 $\log_{10}$ cfu/g decrease in colony counts from the initial inoculum, respectively. The effects of daptomycin combinations were interpreted as enhanced if a combination reduced bacterial counts by $\geq$2 $\log_{10}$ cfu/g as compared with the most effective single agent in the combination. Outcomes featuring reductions in bacterial counts by 1–2 $\log_{10}$ cfu/g compared with the most active single agent were deemed 'indifferent'. Changes in cfu/g at 24, 48, 72 and 96 h were compared by one-way analysis of variance with Tukey's *post-hoc* test. A P value of $\leq$0.05 was considered significant. All statistical analyses were performed on SPSS statistical software (version 23; SPSS, Inc., Chicago, IL, USA).

For PK analysis, chamber medium samples were obtained through the injection port at 0, 2, 4, 8 and 24 h to verify the attainment of target antibiotic concentrations. All samples were stored at −80°C until ready for PK assay. Daptomycin concentrations were measured using a

Table 1. MIC data for selected strains

Strain	CPT MIC (mg/L)	PEN MIC (mg/L)	CRO MIC (mg/L)	GEN MIC (mg/L)	DAP MIC (mg/L)	DAP MIC (mg/L) in the presence of CPT (DAP MIC reduction)	DAP MIC (mg/L) in the presence of CRO (DAP MIC reduction)	DAP MIC (mg/L) in the presence of GEN (DAP MIC reduction)
351	0.5	8	8	8	0.5	<0.063 (>8-fold)	0.25 (2-fold)	0.25 (2-fold)
SF100	<0.063	0.125	0.125	4	0.5	<0.063 (>8-fold)	0.25 (2-fold)	0.5 (no change)

CPT, ceftaroline; CRO, ceftriaxone; DAP, daptomycin; GEN, gentamicin; PEN, penicillin.

validated HPLC assay.[22] Gentamicin and ceftriaxone concentrations were determined by bioassay using *Escherichia coli* ATCC 25922 as reference organism and ceftaroline concentration by bioassay using *Kocuria rhizophila* ATCC 9341 as reference organism.[19,22,34] In brief, holes were aseptically punched or sterile blank 0.25 inch paper discs were placed on the agar (Antibiotic Medium 1 for ceftriaxone, Antibiotic Medium 11 for ceftaroline and gentamicin; Difco, Detroit, MI) that were pre-swabbed with 0.5 McFarland bacterial suspension of reference strains. Each hole or blank paper was filled with either a sample or a standard concentration at a fixed volume. Plates were incubated for 18–24 h at 37°C, before the diameter of each inhibition zone was measured using an automatic colony counter (Scan 1200, Interscience Woburn, MA). A standard curve was created using inhibition zone size versus known concentrations, and the inhibition zone size at each sample timepoint was plotted against this curve to obtain sample concentrations. These concentrations allowed calculation of PK parameters such as the half-life and peak concentrations of each antibiotic agent by the trapezoidal method using PK Analyst Software (version 1.10; MicroMath Scientific Software, Salt Lake City, UT, USA).

Emergence of daptomycin resistance over 96 h daptomycin exposures within the SEV model was evaluated by determining daptomycin MICs of isolates recovered from SEVs plated on daptomycin drug plates at 3× the MIC at 96 h.

Results

In vitro susceptibility testing

The MIC values for each antimicrobial agent alone, as well as the daptomycin MIC in the presence of an adjunctive antimicrobial agent at 0.5× MIC, are summarized in Table 1. It should be noted that the daptomycin MICs were reduced by >8-fold in both *S. mitis/oralis* 351 and *S. mitis* SF100 strains in the presence of ceftaroline at 0.5× its MIC. Similarly, ceftriaxone decreased daptomycin MICs by 2-fold in both *S. mitis/oralis* 351 and *S. mitis* SF100. In contrast, gentamicin reduced the daptomycin MIC in strain *S. mitis/oralis* strain 351 by 2-fold, but failed to impact the daptomycin MIC of *S. mitis* SF100.

In vitro PK/PD models

The average C_{max} for daptomycin was 98.79±0.10 mg/L (target C_{max}, 93.9 mg/L), the area under the concentration–time curve over 24 h (AUC_{0-24}) was 1139±14.13 mg·h/L and the average $t_{1/2}$ was 8.62±0.14 h (target $t_{1/2}$, 8 h). For ceftaroline, the average C_{max} observed was 19.74±0.12 mg/L (target C_{max}, 21 mg/L) and the average $t_{1/2}$ was 3.32±0.04 h (target $t_{1/2}$, 2.66 h). For ceftriaxone, the average C_{max} of 263.33±9.22 mg/L (target C_{max}, 257 mg/L) and the average $t_{1/2}$ of 8.46±0.58 h (target $t_{1/2}$, 8 h) were achieved. For gentamicin, the observed C_{max} and C_{min} were 8.39±1.37 mg/L and 0.001±0.0004 mg/L (target C_{max}, 8.53 mg/L) and the average $t_{1/2}$ was 1.9±0.1 h (target $t_{1/2}$, 2.5 h)

Relevant PD responses to simulated human-equivalent antimicrobial regimens are depicted in Figure 1. In SEVs infected with *S. mitis/oralis* 351, daptomycin alone was not bactericidal ($-\Delta 2.03\ \log_{10}$ cfu/g between 0 and 96 h exposure). It is noteworthy that daptomycin resistance emerged at 96 h (MIC increased from 0.5 to 64 mg/L) and was stable to 5 days of passage on antibiotic-free medium. Ceftaroline alone was bactericidal at 96 h ($-\Delta 4.26\ \log_{10}$ cfu/g) and was significantly superior to daptomycin alone ($P < 0.001$). The combination of daptomycin + ceftaroline was highly bactericidal at 96 h ($-\Delta 6.79\ \log_{10}$ cfu/g). This combination was also statistically superior to daptomycin alone by 24 h ($P < 0.001$) and to ceftaroline alone at 48, 72 and 96 h ($P < 0.001$). Daptomycin + ceftaroline also prevented the emergence of daptomycin resistance at 96 h. Ceftriaxone alone was bactericidal as soon as 24 h ($-\Delta 3.7\ \log_{10}$ cfu/g) and statistically superior to any other single agent at 96 h ($-\Delta 5.86\ \log_{10}$ cfu/g; $P < 0.001$). The combination of ceftriaxone with daptomycin demonstrated enhanced bactericidal activity at 8 and 24 h, achieving bacterial killing to the detection limit within the first 8 h ($-\Delta 5.87\ \log_{10}$ cfu/g) and prevented daptomycin resistance emergence at 96 h. Gentamicin alone demonstrated little activity against *S. mitis/oralis* 351 at 96 h ($-\Delta 0.49\ \log_{10}$ cfu/g) and the addition of gentamicin to daptomycin neither improved bactericidal activity compared with daptomycin alone nor prevented evolution of daptomycin resistance.

As was seen with *S. mitis/oralis* 351, daptomycin alone was not bactericidal at 96 h in SEVs ($-\Delta 0.07\ \log_{10}$ cfu/g) against *S. mitis* SF100, and the strain developed daptomycin resistance at 96 h (MIC increased from 0.5 to 16 mg/L) and was stable to 5 days of passage on antibiotic-free medium. Ceftriaxone and ceftaroline alone were statistically superior to any other single antibiotic against SF100 at 96 h ($P < 0.001$). Ceftaroline alone and the daptomycin + ceftaroline combination were bactericidal at 96 h ($-\Delta 5.57$ and $-\Delta 5.65\ \log_{10}$ cfu/g; $P < 0.001$ for both regimens versus daptomycin alone). Ceftaroline alone was bactericidal at 8 h, while the combination of daptomycin + ceftaroline was bactericidal at 4 h. Both ceftaroline and daptomycin + ceftaroline reduced SEV SF100 bacterial load to the detection limits by 24 h and by 4 h. Daptomycin + ceftaroline also prevented daptomycin resistance emergence. Ceftriaxone alone was bactericidal at 24 h

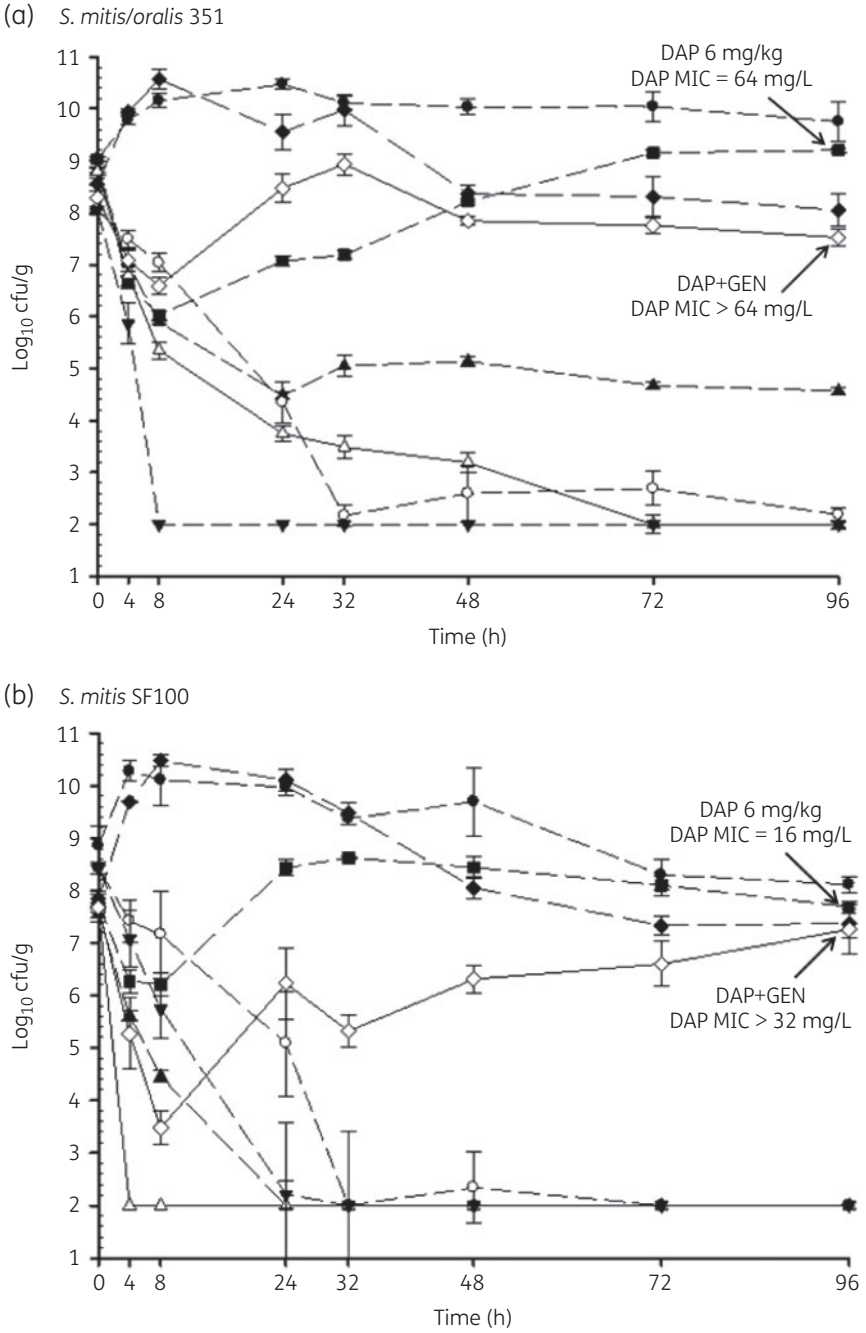

Figure 1. PD of simulated antibiotic regimens against two *S. mitis* group strains in a PK/PD model of SEVs. Broken line with black circles, growth control; broken line with black squares, 6 mg/kg/day daptomycin; broken line with black upward triangles, 600 mg of ceftaroline every 8 h; broken line with black diamonds, 3 mg/kg/day gentamicin; broken line with white circles, 2 g of ceftriaxone every 24 h; continuous line with white upward triangles, daptomycin + ceftaroline; continuous line with white diamonds, daptomycin + gentamicin; broken line with black downward triangles, daptomycin + ceftriaxone. DAP, daptomycin; GEN, gentamicin.

($-\Delta 3.34\ \log_{10}$ cfu/g) and maintained its bactericidal activity for 96 h. Ceftriaxone + daptomycin demonstrated enhanced bacterial killing compared with ceftriaxone alone at 8 and 24 h ($P < 0.001$) and maintained bacterial colony count at the detection limit from 32 to 96 h ($-\Delta 6.43\ \log_{10}$ cfu/g). Addition of ceftriaxone also prevented daptomycin resistance emergence at 96 h. Gentamicin alone failed to reduce SEV bacterial counts compared with untreated controls. Daptomycin + gentamicin neither substantially improved killing compared with daptomycin alone nor prevented emergence of daptomycin resistance. No resistance to ceftriaxone, ceftaroline or gentamicin was detected during monotherapy or combination therapy over the 96 h experiments.

Discussion

Since its FDA approval in 2003, daptomycin has found major clinical usage, especially against resistant Gram-positive bacteria such as MRSA and VRE. This agent represents a viable treatment option for VGS infections including the *S. mitis* group, based on its *in vitro* susceptibility profile against such strains.[6,35,36] However, clinical experience with daptomycin against VGS infections is limited to date. Importantly, two factors have promoted daptomycin as a viable alternative treatment agent for invasive VGS infections, especially in the *S. mitis* group: (i) the relatively high frequency of β-lactam resistance (to both penicillins and cephalosporins); and (ii) the inconsistent clinical outcomes with vancomycin, linked to vancomycin 'tolerance'.[37] In the case of drug allergy or intolerance associated with β-lactam antibiotics and vancomycin, daptomycin has been considered as a reasonable alternative. However, the recent recognition that more than 25% of *S. mitis* group strains develop rapid, high-level and durable daptomycin resistance during both *in vitro* and *in vivo* exposures to daptomycin make therapy with daptomycin alone problematic.[6] In addition, the potential for the development of cross-resistance as demonstrated with *S. aureus* increases the need to evaluate potential therapeutic options against these pathogens.[38]

The current study was designed to investigate the temporal evolution of daptomycin resistance in an *in vitro* model of SEV caused by two prototypic *S. mitis* group strains in the presence of standard-dose daptomycin exposures, either alone or in combination with two cephalosporins, ceftriaxone and ceftaroline, or gentamicin and to evaluate the therapeutic potential of these combinations in the treatment of *S. mitis* group IE. The *in vitro* model of SEV has been validated against rabbit IE models and implemented for decades for assessment of antibiotic potential.[39] As previously demonstrated by García-de-la-Mària *et al*.,[6] 6 mg/kg/day daptomycin was not sufficient for bactericidal activity against either strain in our study and resulted in daptomycin resistance at 96 h. Interestingly, in their experiments with aortic valve IE with *S. mitis/oralis* 351 in rabbits, adding 1 mg/kg gentamicin every 8 h to 6 mg/kg/day daptomycin not only significantly increased the number of vegetations sterilized (10 out of 11 endocardial vegetations) after 48 h of treatment, as compared with daptomycin alone (1 out of 11 endocardial vegetations), but it also decreased the number of recovered isolates with daptomycin MIC $\geq$256 mg/L from 7 out of 11 to 1 out of 11. In contrast, we encountered no such beneficial effect of the addition of gentamicin in our SEV model. It is possible that the steady-state gentamicin dosing regimen employed in our *in vitro* study (3 mg/kg/day), compared with the 1 mg/kg every 8 h regimen used in experimental IE, is the source of this difference. However, previous *in vitro* data have proven inconclusive with regard to the role of gentamicin synergy dosing, as some studies have demonstrated equivalence between the two dosing strategies, while others have shown superiority of thrice-daily dosing.[33,40–42] Regardless, the data available on the efficacy of gentamicin in combination with daptomycin against *S. mitis* group strains are conflicting and further study is warranted.

In contrast, both ceftriaxone and ceftaroline improved the activity of daptomycin against both *S. mitis* group strains and prevented daptomycin resistance emergence. Synergy between these agents has been extensively documented against other Gram-positive bacteria; however, our study is the first to show this positive effect against *S. mitis* group strains. Previous studies in *S. aureus* and enterococci have demonstrated that the addition of a β-lactam antibiotic may enhance daptomycin binding to the bacterial membrane by altering surface charge.[20,43] It is possible that a similar synergistic mechanism exists within *S. mitis* group strains, although our efforts did not include daptomycin binding studies to examine this potential effect.

Our study does have limitations. We evaluated only two strains of the *S. mitis* group, which may constrain the generalizability of our findings. Regimens were evaluated for 96 h which is obviously much shorter than the duration of therapy employed for IE in the clinical setting. The initial starting inoculum varied slightly for some of the experiments which may have affected the overall comparisons. In addition, the achieved half-life (3.32 h) for ceftaroline was slightly higher than targeted (2.66 h) which may have impacted the activity of ceftaroline. Regarding daptomycin, it is possible that a much higher dosage of daptomycin (i.e. 8–12 mg/kg/day), although not tested here, may have suppressed the emergence of resistance, although our prior *in vitro* passage data and our current *ex vivo* passage data would suggest that this is not the case.[44] That being said, the extent of bactericidal activity and resistance suppression observed with the daptomycin + ceftriaxone and daptomycin + ceftaroline combinations would suggest that these combinations are likely to be effective beyond this period. Also, it is worth noting that ceftriaxone and ceftaroline alone were bactericidal, perhaps suggesting that single-drug efficacy will occur without the presence of daptomycin. Although it is unknown what the clinical impact of the more rapid and sustained bactericidal activity observed against both strains with the combinations of ceftriaxone + daptomycin or ceftaroline + daptomycin would be, it was clear that these combinations were able to suppress the emergence of resistance to daptomycin which would be an advantage over daptomycin monotherapy. In this regard, additional studies that include more *S. mitis* group strains will be of value.

In summary, our data demonstrate that addition of ceftriaxone or ceftaroline to daptomycin improves the activity against *S. mitis* group strains *in vitro* and prevents the emergence of daptomycin resistance. Most importantly, these combinations prevented daptomycin resistance in two strains that have demonstrated a high propensity to develop daptomycin resistance on standard daptomycin therapy *in vitro*. The improved activities between daptomycin and these two cephalosporins were superior to any effects observed when daptomycin was combined with gentamicin, an agent used traditionally for combination therapy against VGS IE. Based on the data obtained from this investigation, along with previous reports of daptomycin + β-lactam efficacy against other resistant Gram-positive pathogens, it appears that ceftriaxone + daptomycin and ceftaroline + daptomycin may represent useful combination therapies for endovascular infections due to *S. mitis* group strains.

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