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In-vitro bactericidal activity of quinupristin/dalfopristin alone and in combination against resistant strains of *Enterococcus* species and *Staphylococcus aureus*

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In-vitro activities of quinupristin/dalfopristin, a semisynthetic injectable streptogramin, and vancomycin were compared against multidrug-resistant *Enterococcus faecium* and *Enterococcus faecalis*, methicillin-susceptible *Staphylococcus aureus* ATCC 25923 and a methicillin-resistant *S. aureus* (MRSA). Combinations of quinupristin/dalfopristin and/or vancomycin with ofloxacin or gentamicin were evaluated using the chequerboard technique. The only synergy observed was that between quinupristin/dalfopristin plus vancomycin and quinupristin/dalfopristin plus gentamicin against *E. faecium* (FIC index <0.5). Time–kill curves were performed over 24 h with an inoculum of 1 x 10^7 cfu/mL and clinically achievable concentrations of quinupristin/dalfopristin, vancomycin, ofloxacin and gentamicin (6, 30, 5 and 5 mg/L, respectively). In time–kill studies, combinations of quinupristin/dalfopristin plus vancomycin and quinupristin/dalfopristin plus gentamicin were additive, not synergic, against *E. faecium* and achieved 99.9% killing in 21.2 h and 19.6 h, respectively. None of the combination regimens suppressed the regrowth of *E. faecalis*. Quinupristin/dalfopristin combined with vancomycin demonstrated consistent synergy against ATCC 25923 and the MRSA, achieving 99.9% killing in 12.1 h and 11.9 h, respectively. Overall, quinupristin/dalfopristin alone demonstrated inhibitory activity against *E. faecium*, but not against *E. faecalis*, and bactericidal activity was achieved only with quinupristin/dalfopristin in combination with vancomycin or gentamicin against *E. faecium*. Quinupristin/dalfopristin plus vancomycin was the most potent and reliable combination against both strains of *S. aureus* in time–kill studies.

Introduction

Over the past decade we have observed the emergence and escalation of antimicrobial resistance in the clinically important Gram-positive cocci, including enterococci and staphylococci.[1,2] For many years, enterococci have been recognized as being resistant to many commonly used antimicrobial agents. However, the increasing incidence of vancomycin-resistant enterococci is causing great concern among clinicians and microbiologists. In the USA, the National Nosocomial Infections Surveillance System reported a 20-fold increase in the percentage of nosocomial enterococci that were resistant to vancomycin between 1 January 1989 and 31 March 1993.[3] Recent reports describing large outbreaks of vancomycin-resistant enterococci continue to appear.[4–6]

Similarly, staphylococci remain one of the major nosocomial pathogens. In the USA, the overall incidence of methicillin resistance in *Staphylococcus aureus* is approximately 18%, and it is much higher, 70–80%, among the coagulase-negative staphylococci.[2] A survey of *S. aureus* isolates from ten western European countries indicated that 12.8% were methicillin-resistant.[7]

Quinupristin/dalfopristin is a semisynthetic antibiotic consisting of two water-soluble, naturally occurring components: quinupristin is a derivative of pristinamycin I_A and dalfopristin is a derivative of pristinamycin II_A. Quinupristin/dalfopristin binds irreversibly to bacterial ribo-

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somes, thereby inhibiting protein synthesis.[8] This 30:70 preparation demonstrates synergic activity *in vitro* against a wide range of Gram-positive organisms including methicillin-susceptible and methicillin-resistant *S. aureus* and enterococci.[9–13] Collins *et al.*[11] and Freeman *et al.*[14] have also reported bacteriostatic activity against glycopeptide-resistant enterococci.

In this study, we evaluated the in-vitro activity of quinupristin/dalfopristin alone and in various combinations to identify the most potent regimen against multidrug-resistant strains of enterococci and staphylococci.

Materials and methods

Bacterial strains

Clinical isolates of *Enterococcus faecium* (strain 7242) and *Enterococcus faecalis* (strain 6375) were obtained from the blood of patients at William Beaumont Hospital, a tertiary teaching hospital in Royal Oak, MI, USA. Both strains had the VanA phenotype, i.e. high-level resistance to vancomycin (MIC $\geqslant$ 64 mg/L) and teicoplanin (MIC $\geqslant$ 16 mg/L). A reference strain of a methicillin-susceptible *S. aureus* (ATCC 25923) and a clinical isolate of a methicillin-resistant *S. aureus* (MRSA 67) were also tested.

Antimicrobial agents

Quinupristin/dalfopristin susceptibility-grade powder (batch 1030) and ofloxacin susceptibility-grade powder (lot KN-777) were supplied by Rhône-Poulenc Rorer, Collegeville, PA, USA and R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ, USA, respectively. Susceptibility-grade powders of vancomycin (lot 112H075025) and gentamicin (lot 121171) were purchased from Sigma Chemical Company, St Louis, MO, USA. Each agent was dissolved in sterile distilled water, and aliquots of 1 mL were stored at −80°C until use.

MIC/MBC and FIC index determinations

MICs and MBCs of quinupristin/dalfopristin, vancomycin, ofloxacin and gentamicin were determined by the microdilution method with an inoculum of 5×10^5 cfu/mL following National Committee for Clinical Laboratory Standards guidelines.[15] After preliminary tests, some stock solution concentrations were adjusted to accommodate the more susceptible and more resistant strains. Studies of synergic activities of combinations of quinupristin/dalfopristin and/or vancomycin with gentamicin or ofloxacin at an inoculum of 5×10^5 cfu/mL were performed by the chequerboard dilution technique[16] to obtain a fractional inhibitory concentration (FIC) index. The concentrations of antibiotics used in the FIC index determinations were based on the MIC results. Using the MIC as the midpoint concentration in the chequerboard dilution set up, a minimum of three dilutions below and above the MIC were tested to determine the concentrations needed to observe synergy and antagonism. Synergy was defined as an FIC index of <0.5, antagonism as an FIC index of >4.0, and indifference as an FIC index between these values.

Time–kill curves

The bactericidal activities of antimicrobial agents over time were characterized by time–kill experiments. Each agent was tested alone and in various combinations. Overnight cultures diluted to give an inoculum of $>10^7$ cfu/mL, were chosen to represent a bacterial load of a serious infection. A higher inoculum of between 10^8 and 10^9 was chosen for staphylococci, allowing brief bacterial growth before reaching stationary phase, similar to that obtained with a lower inoculum enterococci. Clinically achievable serum concentrations of quinupristin/dalfopristin, vancomycin, ofloxacin and gentamicin (6, 30, 5 and 5 mg/L, respectively)[17] in Mueller–Hinton broth (SMHB; Difco Laboratories) supplemented with calcium and magnesium (25 and 12.5 mg/L, respectively) were used. All experiments were performed, in duplicate, over 24 h. Samples (100 μL) were removed at 0, 2, 4, 6 and 24 h, and after each dilution with cold 0.9% sodium chloride, 20 μL was plated on tryptic soy agar (TSA; Difco) in triplicate. The plates were then incubated at 37°C for 24 h, and the colonies were counted thereafter. Antibiotic carryover experiments were conducted to identify the drug concentrations that might affect the colony counts. Samples (100 μL) were placed in 10 mL 0.9% sodium chloride, and filtered through 0.45 μm pore-size Millipore filters. Filters were then placed on TSA plates, and the plates were incubated for 24 h. The reliable limit of detection in our laboratory was 100 cfu/mL.[18,19]

Time–kill curves over 24 h were plotted graphically as $\log_{10}$ cfu/mL against time to observe the changes in $\log_{10}$ cfu/mL over 24 h. The time required to achieve a 99.9% reduction in bacterial population for each antimicrobial agent alone and in various combinations was determined by linear regression. Synergy was defined as a reduction in cfu by the combination at the end of 24 h of at least 2 logs greater than that produced by the more active single drug.

Results

Susceptibility testing in vitro

MIC and MBC results are summarized in Table I. The enterococcal strains were highly resistant to several antibiotics, including vancomycin. Overall, *E. faecalis* 6375 appeared to be more resistant than *E. faecium* 7242. The staphylococcal strains were highly susceptible to all of the antimicrobial agents tested.

Table I. MIC/MBC of four antimicrobial agents against Gram-positive cocci

Strain	MIC/MBC (mg/L) quinupristin/ dalfopristin	vancomycin	ofloxacin	gentamicin
E. faecium 7242	0.19/0.78	312.50/>1250	3.9/15.6	9.77/9.77
E. faecalis 6375	12.5/>50	78.10/>1250	>31.2/>31.2	>1250/>1250
Methicillin-susceptible *S. aureus* ATCC 25923	<0.19/0.78	3.13/6.25	0.24/0.49	<0.06/0.24
Methicillin-resistant *S. aureus* 67	<0.19/0.39	0.78/0.78	0.24/0.98	0.12/0.24

Table II. FIC indices of various combinations against Gram-positive cocci

Combinations	*E. faecium* 7242	*E. faecalis* 6375	*S. aureus* ATCC 25923	*S. aureus* MRSA 67
Quinupristin/dalfopristin + vancomycin	0.5	1.5	1.9	2.5
Quinupristin/dalfopristin + ofloxacin	1.6	1.6	2.0	2.0
Quinopristin/dalfopristin + gentamicin	0.5	1.5	2.0	2.0
Vancomycin + ofloxacin	1.0	1.5	1.3	1.6
Vancomycin + gentamicin	2.5	1.5	2.0	1.3

Synergy studies

The FIC indices of the combinations of quinupristin/dalfopristin plus vancomycin, ofloxacin or gentamicin, and vancomycin plus ofloxacin or gentamicin for *E. faecium* 7242 and *E. faecalis* 6375 are shown in Table II. Although the combinations of quinupristin/dalfopristin with either vancomycin or gentamicin were synergic against *E. faecium* 7242, all the combinations demonstrated indifference against *E. faecalis* 6375. FIC indices for the combinations against *S. aureus* ATCC 25923 and MRSA 67 again indicated indifference.

Time–kill curves

Time–kill curves of monotherapy and combination regimens against enterococci are depicted in Figure 1. The starting inoculum was approximately 10^7 cfu/mL for the experiment using enterococci. There was regrowth of *E. faecium* 7242 with all of the monotherapy regimens except quinupristin/dalfopristin (Figure 1a). This was expected since the MBCs of vancomycin, ofloxacin and gentamicin were much higher than the antibiotic concentrations applied in the experiments. Regimens of quinupristin/dalfopristin combined with either vancomycin or gentamicin demonstrated additive effects, achieving a 99.9% killing in 21.2 h and 19.6 h, respectively (Figure 1b). The addition of ofloxacin to quinupristin/dalfopristin was slightly antagonistic. Against *E. faecalis* 6375, the more resistant strain of the two, regrowth occurred with monotherapy and all of the combination regimens (Figure 1c and d).

Gentamicin was the most effective monotherapy against *S. aureus* ATCC 25923, achieving a 99.9% killing in 7.3 h (Figure 2a). Although the rate of bactericidal activity of ofloxacin was greater than those of quinupristin/dalfopristin and vancomycin, the final bacterial counts were comparable at the end of 24 h. Regimens consisting of quinupristin/dalfopristin plus vancomycin and vancomycin plus gentamicin were the most effective combination regimens (Figure 2b). However, the time to achieve a 99.9% killing was much faster with vancomycin plus gentamicin than the quinupristin/dalfopristin plus vancomycin regimen (6.3 h versus 12.1 h). Activities of quinupristin/dalfopristin and vancomycin against MRSA 67 *in vitro* were very similar to those against *S. aureus* ATCC 25923 (Figure 2c). When these agents were combined, they were the most effective and the only regimen to achieve a 99.9% killing activity in 11.9 h (Figure 2d). There was regrowth of MRSA 67 with gentamicin alone and in combination with vancomycin, unlike the activities seen against *S. aureus* ATCC 25923.

Discussion

In recent years, Gram-positive cocci have emerged as important pathogens in nosocomial infections. Currently, the therapeutic options for infections caused by resistant Gram-positive cocci are limited. In enterococci, resistance to vancomycin is due to the expression of an inducible vancomycin-resistance gene. The plasmid on which this gene is carried could possibly be transferred to other Gram-positive organisms including staphylococci. Noble

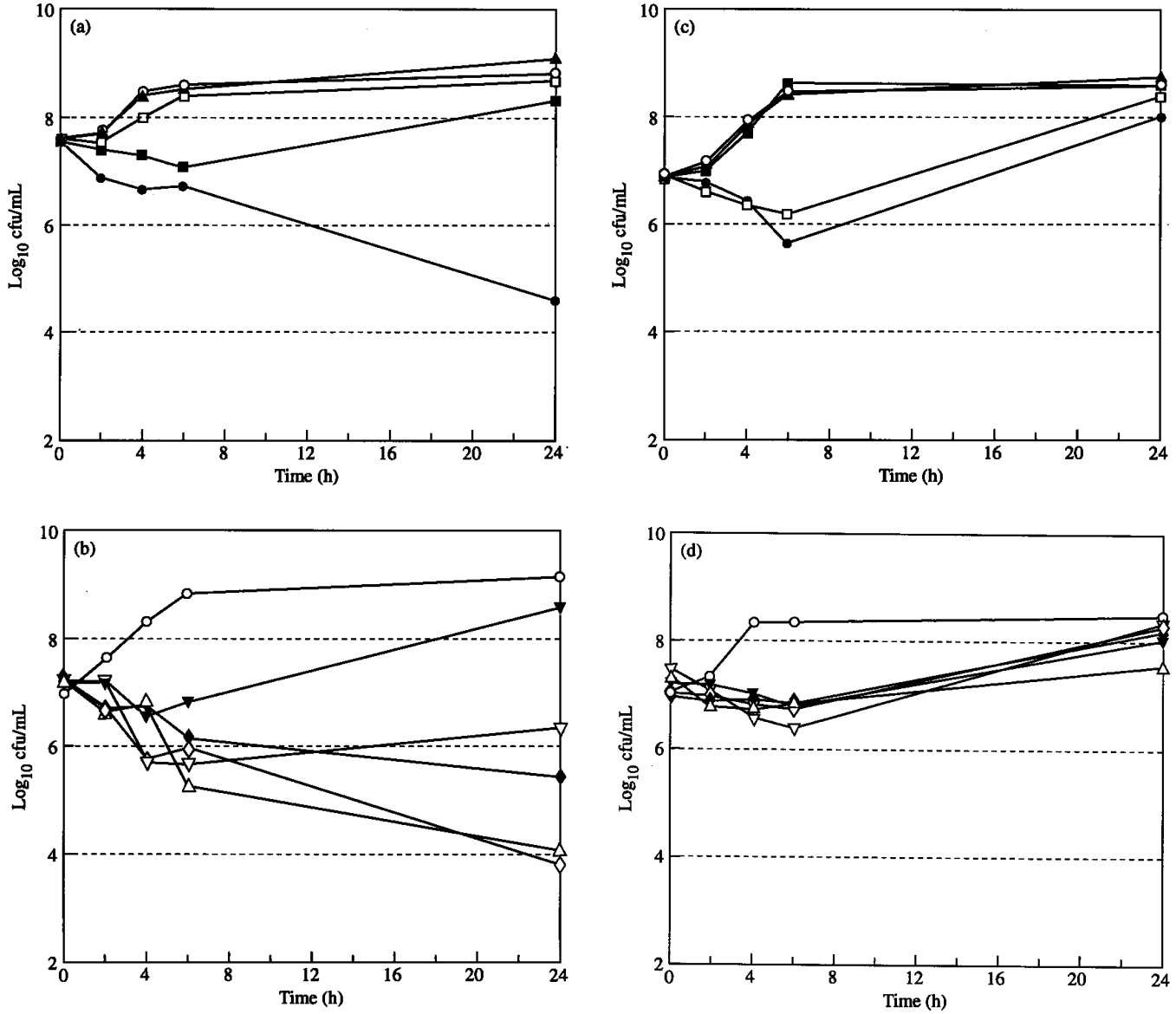

Figure 1. Killing curves of quinupristin/dalfopristin (QD) alone and in combination against *E. faecium* 7242 (a, b) and *E. faecalis* 6375 (c, d). ○, control; ●, QD; □, vancomycin; ■, ofloxacin; ▲, gentamicin; △, QD plus vancomycin; ♦, QD plus ofloxacin; ◇, QD plus gentamicin; ▽, vancomycin plus ofloxacin; ▼, vancomycin plus gentamicin.

et al.[20–22] have reported the experimental transfer of the resistant gene from enterococci to *S. aureus*, resulting in a vancomycin-resistant *S. aureus.* Although clinical strains of vancomycin-resistant *S. aureus* have not yet been reported, this could occur in the future. To date, 37 isolates of staphylococci with elevated MICs of vancomycin have been reported.[23] This is of concern since no effective alternative agents are commercially available at present.

Quinupristin/dalfopristin is active against both glycopeptide-susceptible and glycopeptide-resistant enterococci as well as MRSA.[11,13,14,24] Unfortunately, the agent achieves only limited bactericidal activity against enterococci.[25] This may limit its usefulness in the treatment of serious infections such as enterococcal endocarditis. However, the results of a compassionate-use programme indicate that quinupristin/dalfopristin may be effective against these organisms in the clinical setting.[26,27]

We analysed the activity of various antimicrobial agents combined with quinupristin/dalfopristin to identify the most effective regimen. The isolates had MICs of quinupristin/dalfopristin of <1 mg/L, except for *E. faecalis* 6375. As observed in previous studies, quinupristin/dalfopristin possessed greater activity against *E. faecium* than against *E. faecalis*.[9,11,24] At a concentration of 6 mg/L, a concentration readily achievable in humans following a 7.5 mg/kg dose,[17] quinupristin/dalfopristin killed almost 99.9% of *E. faecium* by the end of 24 h. However, there was regrowth of *E. faecalis* at sub-MIC concentration of

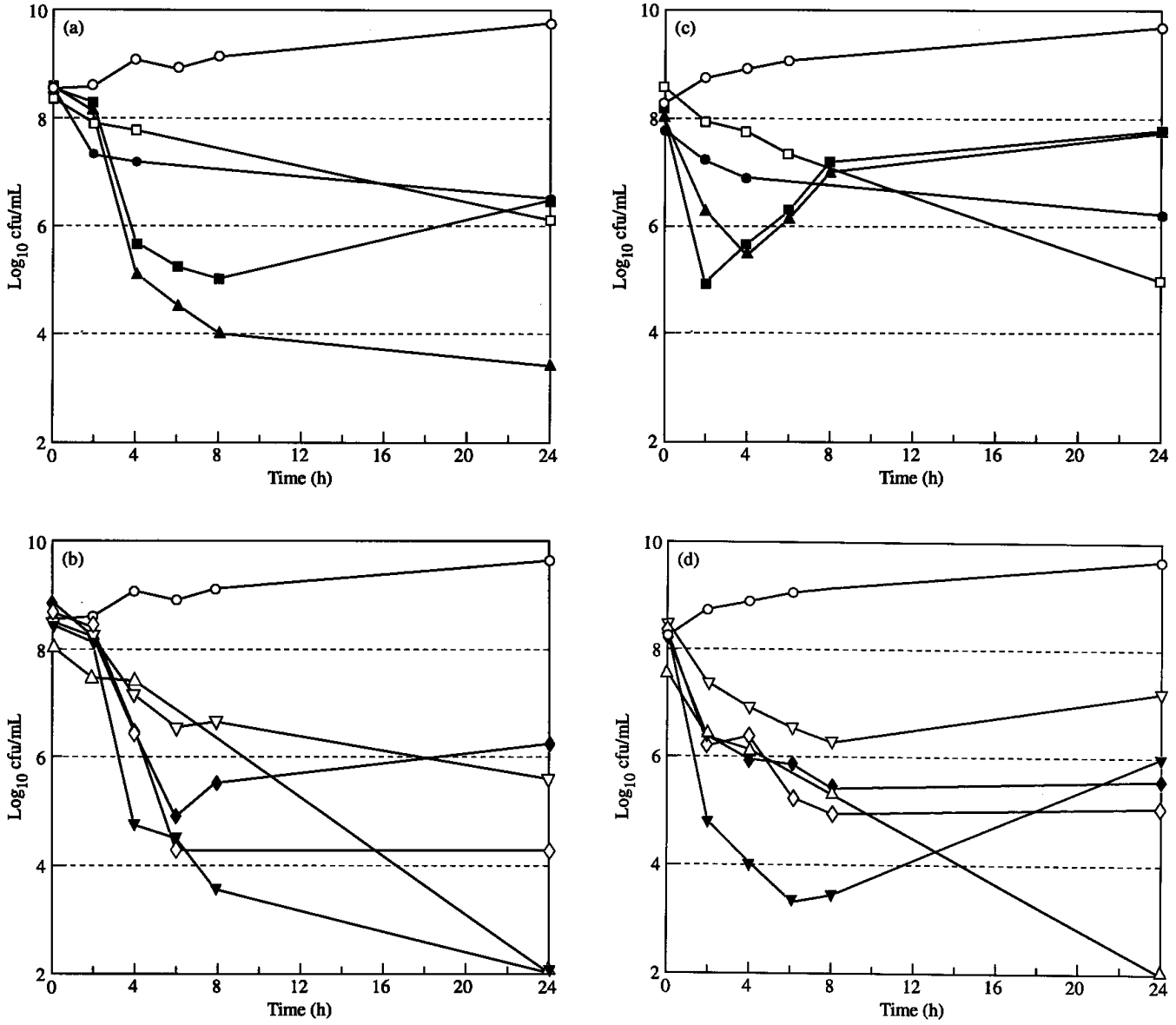

Figure 2. Killing curves of quinupristin/dalfopristin (QD) alone and in combination against methicillin-susceptible *S. aureus*, ATCC 25923 (a, b) and methicillin-resistant *S. aureus*, MRSA 67 (c, d). ○, control; ●, QD; □, vancomycin; ■, ofloxacin; ▲, gentamicin; △, QD plus vancomycin; ♦, QD plus ofloxacin; ◇, QD plus gentamicin; ▽, vancomycin plus ofloxacin; ▼, vancomycin plus gentamicin.

the drug after the initial killing of the susceptible subpopulation.

Notably, three out of the four isolates tested had raised MBCs, a minimum of eight-fold higher than the MICs. Some investigators have reported that raised MBCs may affect the post-antibiotic effect.[28] Boswell *et al.*[29] compared the killing activity of quinupristin/dalfopristin at a concentration of 2 mg/L against *S. aureus* with or without a raised MBC. Initially, the killing activities against strains with raised MBCs and a normal MBC were very similar (1.4 $\log_{10}$ cfu/mL and 1.6 $\log_{10}$ cfu/ml, respectively at 4 h). However, the reduction in viability at 24 h was significantly higher for *S. aureus* with the normal MBC. This may explain the slower rate of killing observed with quinupristin/dalfopristin than with gentamicin against *S. aureus* ATCC 25923 in our experiment.

Overall, the regimen consisting of quinupristin/dalfopristin plus vancomycin was the most potent and reliable combination against both *S. aureus* ATCC 25923 and MRSA 67. This may be due to the differences in the sites and the mechanisms of action. However, the rate of killing by quinupristin/dalfopristin during the initial period was not influenced significantly by the addition of either vancomycin or gentamicin. Regrowth of MRSA 67 was observed with the combination of vancomycin and gentamicin. The pattern of killing kinetics of this combi-

nation appears to follow that of gentamicin for both *S. aureus* ATCC 25923 and MRSA 67. This observation needs to be verified with further studies on different isolates.

Many investigators have analysed the killing activities of various combinations against vancomycin-resistant enterococci including quinupristin/dalfopristin, vancomycin, teicoplanin, aminoglycosides, daptomycin, novobiocin, fluoroquinolones, rifampicin and fusidic acid.[24, 30–33] When Nicolau *et al.*[33] examined the efficacy of vancomycin and teicoplanin, alone and in combination with streptomycin, using an in-vivo model of experimental endocarditis caused by VanB-type *E. faecalis*, both combinations significantly reduced the bacterial density. However, a teicoplanin and aminoglycoside combination was not so successful in experimental endocarditis caused by a highly glycopeptide-resistant *E. faecium*.[30] A high-dose regimen of teicoplanin (40 mg/kg every 12 h) and gentamicin (6 mg/kg every 12 h) marginally reduced the bacterial load in the vegetation. A high-dose daptomycin regimen (12 mg/kg every 8 h) combined with gentamicin was the most effective regimen in reducing the vegetative bacterial concentration as well as sterilizing the vegetation. However, the investigators did not recommend either regimen for the treatment of endocarditis because of concerns regarding their tolerance in humans. Novobiocin, alone or in combination with a fluoroquinolone, appears to be very potent against vancomycin-resistant *E. faecium*.[31] However, the toxicity of novobiocin has reduced its clinical use.

Since quinupristin/dalfopristin demonstrates inhibitory activity against vancomycin-resistant enterococci, some investigators have evaluated its activity in combination with other agents. Pasculle *et al.*[32] reported antagonism with the combination of quinupristin/dalfopristin and ciprofloxacin against VanA-type *E. faecium*. We replaced ciprofloxacin with ofloxacin in our study and the combination resulted in indifference. In time–kill studies, Johnson *et al.*[24] observed no synergic activity against several strains of vancomycin-resistant *E. faecium* between quinupristin/dalfopristin (at the MIC, i.e. 0.78 mg/L) and gentamicin (3 mg/L). Whereas they reported indifference for these combinations, we have observed an additive effect. However, we used higher concentrations of both quinupristin/dalfopristin (6 mg/L) and gentamicin (5 mg/L).

Quinupristin/dalfopristin generally possesses bacteriostatic, but usually not bactericidal activity against enterococcal strains (especially against *E. faecalis*). Therefore, it is optimal to combine this agent with another to achieve bactericidal activity. In our study, combinations of quinupristin/dalfopristin with vancomycin or gentamicin appeared to be bactericidal against vancomycin-resistant *E. faecium*. However, further studies of various two- and perhaps three-drug combinations are needed to find the most effective combination against both vancomycin-resistant *E. faecium* and *E. faecalis.*

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