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Impact of High-Inoculum *Staphylococcus aureus* on the Activities of Nafcillin, Vancomycin, Linezolid, and Daptomycin, Alone and in Combination with Gentamicin, in an In Vitro Pharmacodynamic Model

Kerry L. LaPlante[1]† and Michael J. Rybak[1,2]*

Anti-Infective Research Laboratory, Eugene Applebaum College of Pharmacy and Health Sciences,[1] and School of Medicine,[2] Wayne State University, Detroit, Michigan

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We evaluated the impact of high (9.5 $\log_{10}$ CFU/g) and moderate (5.5 $\log_{10}$ CFU/g) inocula of methicillin-susceptible and -resistant *Staphylococcus aureus* (MSSA and MRSA, respectively) on the activities of nafcillin, linezolid, vancomycin, and daptomycin, alone and in combination with gentamicin in an in vitro pharmacodynamic model with simulated endocardial vegetations over 72 h. Human therapeutic dosing regimens for nafcillin, daptomycin, vancomycin, linezolid, and gentamicin were simulated. At a moderate inoculum, nafcillin (MSSA only), vancomycin, and daptomycin demonstrated equivalent and significant ($P < 0.01$) bactericidal (99.9% kill) activities (decreases of 3.34 ± 1.1, 3.28 ± 0.4, and 3.34 ± 0.8 $\log_{10}$ CFU/g, respectively). Bactericidal activity was demonstrated at 4 h for nafcillin and daptomycin and at 32 h for vancomycin. Linezolid demonstrated bacteriostatic activity over the course of the study period. At a high inoculum, daptomycin exhibited bactericidal activity against both MSSA and MRSA by 24 h (decrease of 5.51 to 6.31 ± 0.10 $\log_{10}$ CFU/g). Nafcillin (versus MSSA), vancomycin, and linezolid (MSSA and MRSA) did not achieve bactericidal activity throughout the 72-h experiment. The addition of gentamicin increased the rate of 99.9% kill to 8 h for daptomycin ($P < 0.01$) and 48 h for nafcillin (MSSA only) ($P = 0.01$). The addition of gentamicin did not improve the activity of vancomycin or linezolid for either isolate for the 72-h period. Overall, high-inoculum *Staphylococcus aureus* had a significant impact on the activities of nafcillin and vancomycin. In contrast, daptomycin was affected minimally and linezolid was not affected by inoculum.

Sequestered high-bacterial-density infections such as those observed with endocarditis and osteomyelitis are often difficult to eradicate (4, 53). Antibiotic failure secondary to problems with antibiotic penetration, inoculum effect, increased protein binding, and stationary-phase organisms make these infections difficult to treat.

Staphylococcus aureus is a common pathogen in sequestered infections, a major cause of infective endocarditis (IE), and has been shown to be an independent predictor of mortality (12, 40). Vancomycin is currently the drug of choice in treating methicillin-resistant *S. aureus* (MRSA) infections, which are currently reported to be as high as 51.3% nationwide in intensive care units (14). Several reports have questioned the efficacy of vancomycin based upon the slow clinical response that often is seen with its use in sequestered infections (34, 39, 51) Additional studies observed that vancomycin is less rapidly bactericidal against *S. aureus*, especially at higher inoculums (approximately 10^7 CFU) (51). Also, studies have demonstrated that patients with MRSA infections treated with vancomycin have higher mortality rates than those with methicillin-susceptible *S. aureus* (MSSA) infections who received nafcillin (55 versus 28%) (27).

* Corresponding author. Mailing address: Anti-Infective Research Laboratory, Pharmacy Practice, 4148, Eugene Applebaum College of Pharmacy & Health Sciences, Wayne State University, 259 Mack Ave., Detroit, MI 48201. Phone: (313) 577-4376. Fax: (313) 577-8915. E-mail: m.rybak@wayne.edu.

† Present address: University of Rhode Island, Department of Pharmacy Practice, Kingston, RI 02881.

Rapid clearance of a bacterial infection may be necessary to achieve a better prognosis for the patient and may be preferable to use bactericidal agents when treating severe infections such as sepsis, meningitis, and endocarditis (42, 47). Although limited data exist regarding combination with aminoglycosides, recommendations have been made that when treating *S. aureus* IE, adding gentamicin for synergy may allow for more rapid sterilization of the cardiac vegetations (56).

Recently daptomycin, a new lipopeptide antibiotic, was approved for use in complicated skin and soft tissue infections and has demonstrated efficacy against multidrug-resistant gram-positive isolates (7, 9, 57; R. D. Arbeit and M. F. DeBruin, Abstr. 41st Intersci. Conf. Antimicrob. Agents and Chemother., abstr. L-1482, 2001). This compound currently is in phase 3 clinical trials evaluating its efficacy in patients with endocarditis or bacteremia at a dose of 6 mg/kg every 24 h (q24h) (55).

We used a previously described in vitro pharmacodynamic model to evaluate several antibiotic regimens using *S. aureus* at a high inoculum representing the organism density often associated with sequestered infections such as endocarditis and a moderate inoculum incorporated into simulated endocardial vegetations (SEVs) (2, 31). To date, there has been no direct comparison between the use of nafcillin, daptomycin, linezolid, and vancomycin alone and in combination with gentamicin in the treatment of sequestered high-inoculum infections such as infective endocarditis.

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MATERIALS AND METHODS

Bacterial strains. The clinical isolates tested in this investigation were MSSA 1199 and MRSA 494. Both isolates were obtained from the bloodstream of patients with bacterial endocarditis and were provided by Glenn W. Kaatz, John D. Dingell VA Medical Center, Detroit, Mich.

Antimicrobials. Vancomycin, nafcillin, and gentamicin were purchased from Sigma Chemical Company, St. Louis, Mo. Daptomycin (lot no. CM2:282A) was provided by Cubist Pharmaceuticals, Inc., Lexington, Mass. Linezolid (Pharmacia and Upjohn) was purchased commercially. Stock solutions of each antibiotic were freshly prepared at the beginning of each week and kept frozen at −4°C.

Media. Mueller-Hinton broth (Difco, Detroit, Mich.) supplemented with 25-μg/ml calcium and 12.5-μg/ml magnesium (SMHB) was used for all in vitro pharmacodynamic models simulating nafcillin, vancomycin, and linezolid alone and in combination with gentamicin. Mueller-Hinton broth supplemented with 12.5-μg/ml magnesium and physiological ionized calcium concentrations (1.02 ± 0.38 mmol of ionized calcium liter) to meet the National Committee for Clinical Laboratory Standards guidelines was used for daptomycin simulations due to its dependency on calcium for antimicrobial activity (9, 57). Colony counts were determined by plating serial dilutions on tryptic soy agar (TSA; Difco) plates.

In vitro susceptibility testing. MICs of study antimicrobial agents were determined by broth microdilution with SMHB as described according to National Committee for Clinical Laboratory Standards guidelines (43). Additionally, MICs were determined with a higher inoculum (5×10^9 CFU/ml) to simulate bacterial densities in the high-inoculum models. After 24 h of incubation at 37°C, observation of growth at high inoculum was facilitated by addition of 20 μl of a 5-μg/ml methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to each of the wells and incubated for 20 min. This change in color allowed for the examination of the wells containing live bacteria at a high inoculum. MTT is reduced by mitochondrial dehydrogenase in living cells to produce insoluble purple MTT formazan crystals that results in a color change from light yellow to a bright blue (30).

MICs and minimal bactericidal concentrations were also determined in the presence of human albumin (American Red Cross, Detroit, Mich.) at 4 g/dl. All samples were incubated at 35°C for 24 h prior to interpretation of results.

SEVs. Organism stocks were prepared by inoculating 5-ml test tubes of Mueller-Hinton broth with colonies harvested from fresh overnight growth on TSA. Cultures were incubated for 24 h, the organisms were recovered by centrifugation, and the pellet was resuspended to achieve a concentration of approximately 10^{10} CFU/ml. SEVs containing 10^9 or 10^6 CFU/g were prepared by combining 0.1-ml dilutions of the organism suspension with 0.5 ml of human cryoprecipitate AHF from volunteer donors (American Red Cross) and 0.025 ml of platelet suspension (platelets mixed with normal saline at 250,000 to 500,000 platelets per clot in 1.5 ml of siliconized Eppendorf tubes). Bovine thrombin (5,000 U/ml, 50 μl) was added to each tube after insertion of a sterile monofilament line into the mixture. The resultant SEVs were removed from the Eppendorf tubes with a sterile 21-gauge needle and introduced into the model. This methodology results in SEVs containing approximately 3 to 3.5 g of albumin per dl and 6.8 to 7.4 g of total protein per dl (15).

In vitro pharmacodynamic infection model. An in vitro infection model consisting of a 250-ml one-compartment glass apparatus with ports where the SEVs were suspended was utilized for all simulations. The apparatus was prefilled with medium, and antibiotics were administered as boluses over a 72-h period into the central compartment via an injection port. The models were placed in a 37°C water bath throughout the procedure with magnetic stir bar for thorough mixing of the drug in the model. Fresh medium was continuously supplied and removed from the model via a peristaltic pump (Masterflex; Cole-Parmer Instrument Company, Chicago, Ill.) set to simulate the half-lives of the antibiotics. The pH was monitored throughout all experiments. SEVs were removed at 0, 4, 8, 24, 28, 32, 48, and 72 h. SEVs were homogenized, diluted, plated onto TSA, and incubated at 35°C for 24 h followed by colony count enumeration. This method results in a lower limit of detection of 2.0 $\log_{10}$ CFU/ml. Antimicrobial carryover was minimized by serial dilution (10 to 10,000) of plated samples in conjunction with vacuum filtration where samples were washed through a 0.22-μm-pore filter with normal saline. These filters were plated onto TSA and incubated at 35°C for 24 h. Colonies were counted on filter paper containing a grid; the limit of detection is 2.0 $\log_{10}$ CFU/ml.

Daptomycin was administered to simulate a 6-mg/kg (peak, 80 μg/ml) dose every 24 h with the pump rate set to achieve a half-life of 8 h. Vancomycin was administered to simulate 1 g q12h with the pump rate set to achieve a half-life of 6 h and a peak concentration of 30 to 35 and a trough concentration of 5 to 10 μg/ml. Nafcillin and linezolid were administered to simulate 2 g q4h (peak, 40 μg/ml) and 600 mg q12h (peak, 18 μg/ml), respectively, with the pump rate set to achieve half-lives of 1 and 5 h, respectively (26). Gentamicin was administered to simulate a dose of 1.3 mg/kg q12h (approximate peak, 6 μg/ml; trough, 0.4 μg/ml), and the pump rate was set to achieve a half-life of 3 h.

For combination regimen experiments, the elimination rate was set for the drug with the shortest half-life, the drug with the longer half-life was supplemented (11). All model experiments were performed in triplicate to ensure reproducibility. In addition, simulations in the absence of antibiotics were performed to ensure adequate growth of the organisms in the model.

Pharmacodynamic analysis. Two SEVs were removed from each model, for six simulated vegetations at 0, 4, 8, 24, 32, 48, and 72 h. The SEVs were homogenized and diluted in cold saline and plated onto TSA plates. The pH was monitored throughout all experiments with daptomycin due to the possible effects on its activity (37). Plates were incubated at 35°C for 24 h, at which time colony counts were performed. Reductions in $\log_{10}$ CFU per gram over 72 h were determined by plotting time-kill curves. Bactericidal activity (99.9% kill) was defined as a ≥3-$\log_{10}$-CFU/ml reduction in colony count from the initial inoculum. Bacteriostatic activity was defined as a <3-$\log_{10}$-CFU/ml reduction in colony count from the initial inoculum, while inactivity was defined as no observed reductions in initial inoculums. The time to achieve 99.9% kill was determined by nonlinear regression (using a minimum of 4 data points) if $r^2 \geq 0.95$, or by visual inspection. Enhancement of activity was defined as an increase in kill of ≥2-$\log_{10}$ CFU/ml by a combination of antimicrobials versus the most active single agent of that combination. Improvement was defined as a 1- to 2-$\log_{10}$-CFU/ml increase in kill in comparison to the most active single agent, while combinations that resulted in ≥1-$\log_{10}$ bacterial growth in comparison to the least active single agent were considered to represent antagonism (1). The terms "improvement" and "enhancement" were used because our simulations used therapeutically obtained serum drug concentration, and this did not permit the mathematical modeling necessary to consider the standard terms "additivity" and "synergy" (3). Indifference was defined as no differences. Reductions in colony counts were determined over a 72-h period and will be compared between regimens.

Pharmacokinetic analysis. Samples for pharmacokinetic analyses were obtained through the injection port at 0.5, 1, 2, 4, 8, 24, 32, 48, 56, and 72 h for verification of target antibiotic concentrations. In addition, antimicrobial concentrations in SEVs were determined and compared to model concentrations to determine percent penetration over time. All samples were stored at −70°C until ready for analysis. Vancomycin and gentamicin concentrations were determined by fluorescence polarization immunoassay (Abbott Diagnostics TDx). The assay for vancomycin and gentamicin has limits of detection of 2.0 and 1.13 μg/ml, respectively, and between-day sample precision levels (percent coefficient of variation [CV%]) of ≤4.8 and ≤3%, respectively. Linezolid concentrations were determined by a previously described validated high-pressure liquid chromatography assay (3). Linezolid concentrations were determined at the Division of Infectious Diseases at the National Jewish Medical and Research Center (Denver, Colo.) using a validated high-performance liquid chromatography assay that conforms to the guidelines set forth by the College of American Pathologists. The standard curve for linezolid in plasma ranged from 2.06 to 19.51 μg/ml. The between-day sample precision CV% was ≤5.3%. Concentrations of daptomycin and nafcillin were determined by microbioassay utilizing *Micrococcus luteus* ATCC 9341. Blank 1/4-mm disks were spotted with 20 μl of the standards or samples. Each standard was tested in triplicate by placing the disk on Mueller-Hinton agar plates that were preswabbed with a 0.5 McFarland suspension of the test organism. Plates were incubated for 18 to 24 h at 37°C, after which time the zone sizes were measured. Concentrations of 150, 50, 10, and 5 and 8, 4, 2, and 1 μg/ml were used as standards for daptomycin and nafcillin, respectively. The nafcillin samples were inoculated onto disks at a 1:10 dilution. The daptomycin and nafcillin bioassay has lower limits of detection of 2.5 and 1 μg/ml, respectively, and between-day sample CV%s of ≤11.1 and ≤7.8%, respectively (1). The half-lives and area under the curve (AUC), maximum concentration (C_{max}), and minimum concentration (C_{min}) of the antibiotics were determined by the trapezoidal method, utilizing PK Analyst software (version 1.10; MicroMath Scientific Software, Salt Lake City, Utah).

Resistance. Development of resistance was evaluated for each combination and monotherapy model at 24, 48, and 72 h. One hundred-microliter samples from said time points were plated on TSA plates containing four- and eightfold the MIC of the respective antibiotic to assess the development of resistance. Plates were examined for growth after 48 h of incubation at 35°C.

Statistical analysis. Changes in CFU per gram at 24, 48, and 72 h and time to 99.9% kill were compared by two-way analysis of variance with Tukey's post hoc test. A P value of ≤0.05 was considered significant. All statistical analyses were performed with SPSS Statistical Software (release 11.1; SPSS, Inc., Chicago, Ill.).

TABLE 1. Susceptibility testing results using standard and high inocula

Antimicrobial[a]	MIC (μg/ml) for[b]:	
	MSSA 1199	MRSA 494
Daptomycin	0.25 (8)	0.25 (8)
Daptomycin with albumin	0.5 (8)	1 (16)
Nafcillin	0.5 (2)	>32
Nafcillin with albumin	2 (16)	>32
Linezolid	2 (2)	2 (2)
Linezolid with albumin	2 (2)	2 (2)
Vancomycin	1 (4)	0.5 (2)
Vancomycin with albumin	4 (8)	2 (4)
Gentamicin	0.5 (0.5)	0.5 (2)
Gentamicin with albumin	0.5 (1)	2 (4)

[a] For antimicrobials with albumin, albumin was added to broth at 4 g/dl.
[b] The standard inoculum was 5×10^5 CFU/g, and the high inoculum was 5×10^9 CFU/g. Data for the high inoculum are presented parenthetically.

RESULTS

Susceptibility testing. All susceptibility results are reported in Table 1. At a high inoculum, MICs for both MSSA and MRSA strains increased 32-fold for daptomycin, 4-fold for vancomycin, and 4-fold for nafcillin against the MSSA strain. With respect to the MSSA test strain, the presence of albumin resulted in fourfold MIC increases for nafcillin and vancomycin and a twofold increase for daptomycin. In the presence of a high inoculum and albumin, the daptomycin, vancomycin, and nafcillin MICs increased 32-, 8-, and 128-fold, respectively. MICs of linezolid were unchanged in the presence of physiological concentrations of albumin and/or by increased inoclum.

Pharmacokinetics. Pharmacokinetic parameters (± standard deviation) for the tested agents are shown in Table 2. Observed kinetics and peak concentrations were within 8 to 10% of the targeted value. The concentrations in the SEVs were obtained and compared to concentrations in the broth at 24 h. The C_{max} percent drug concentration (micrograms per gram) within the first 24 h in the homogenized SEVs for nafcillin, vancomycin, linezolid, daptomycin, and gentamicin ranged from 22 to 61%.

Pharmacodynamics. Results of 72-h pharmacodynamic models for moderate- and high-inoculum MSSA and MRSA SEVs are shown in Fig. 1 and 2. Quantitative changes in the bacterial population (change in log CFU per gram over 72 h) with each drug exposure are portrayed in Table 2. For all model simulations, pH ranged between 6.98 and 7.06 and the temperature was maintained at 37°C throughout the 72-h experiment.

Moderate inoculum. At a moderate inoculum (5×10^5 $\log_{10}$ CFU/g), (Fig. 1A and B), nafcillin and daptomycin were equally effective against MSSA and MRSA (not nafcillin), as demonstrated by achieving bactericidal activity as early as 4 h, and remained bactericidal throughout the 72-h experiment. The time above the MIC in the presence of albumin, $T > MIC_{(albumin)}$, was 100% for nafcillin throughout the study, and the AUC/$MIC_{(albumin)}$ ratio for daptomycin was 1,511.

Vancomycin demonstrated bactericidal activity at 48 h against both isolates and remained bactericidal to the 72-h endpoint. The achieved AUC/$MIC_{(albumin)}$ ratios were 149 for the MSSA isolate and 298 for the MRSA isolate. Linezolid demonstrated bacteriostatic activity throughout the 72-h study, the AUC/$MIC_{(albumin)}$ ratio was 133. Both vancomycin and linezolid concentrations demonstrated 100% $T > MIC_{(albumin)}$.

High inoculum. Changes in bacterial density ($\log_{10}$ CFU per milliliter) over 72 h are in Table 3, and the achieved pharmacodynamic parameters are shown in Table 4. The high-$inoculum_{(albumin)}$ MIC of nafcillin was 16, and therefore the $T > MIC_{(albumin)}$ for nafcillin was less than 38%. At a high inoculum, nafcillin alone did not demonstrate significant activity throughout the 72-h experiment, however, bactericidal activity was noted by 48 h when gentamicin was used in combination with nafcillin ($P = < 0.01$).

Against MSSA and MRSA isolates at a high inoculum (5×10^9 $\log_{10}$ CFU/g) (Fig. 2A and B), daptomycin achieved bactericidal activity by 24 h and maintained this for the duration of the experiment ($P = <0.001$). Nonlinear regression extrapolated 99.9% kill to occur at 11.8 and 13.2 h ($R^2 = \geq 0.94$) for MSSA and MRSA, respectively. The high-inoculum AUC/$MIC_{(albumin)}$ ratios for daptomycin against MSSA and MRSA were 94 and 47, respectively. Although not statistically significant, gentamicin increased the rate of daptomycin's bactericidal activity to 8 h for both MSSA and MRSA, thus increasing the time to 99.9% kill by approximately 3.8 to 5.2 h.

Vancomycin achieved bactericidal activity at 72 h, and no differences were noted when gentamicin (simulated doses targeting a peak of 6 μg/ml every 12 h) was added to vancomycin. Vancomycin's $T > MIC$ was 100% for each simulation. At a high inoculum, the achieved AUC/$MIC_{(albumin)}$ ratios were 75 against the MSSA isolate and 149 against the MRSA isolate. This apparent discrepancy was the result of baseline differences in MICs between the two organisms.

Linezolid demonstrated bacteriostatic activity similar to what was observed at the moderate inoculum. Our results illustrate that for linezolid the time above the MIC ($T > MIC$) was 100% throughout the experiments. The AUC/MIC ratio for linezolid was 133. Bacteriostatic activity was noted at 24 h and remained bacteriostatic throughout the 72-h experimental period. There was no additional benefit when gentamicin was added to the regimen.

TABLE 2. Values of pharmacokinetic parameters obtained with infection models

Regimen	Peak concn (μg/ml)	Trough concn (μg/ml)	Half-life (h)	AUC_{0-24} (μg · h/ml)	C_{max} SEV concn (μg/g) within 24 h
Daptomycin, 6 mg/kg	79.62 ± 0.51	8.70 ± 0.10	7.3 ± 0.17	755.54 ± 59	48 ± 2.83
Nafcillin, 4 g q4h	42.88 ± 1.69	4.99 ± 1.98	1.45 ± 0.22	671.91 ± 78	9.11 ± 1.25
Linezolid, 600 mg q12h	19.07 ± 0.95	3.58 ± 0.07	5.05 ± 1.01	266.61 ± 28	10.98 ± 1.55
Vancomycin, 1 g q12h	42.15 ± 1.33	9.52 ± 0.11	5.56 ± 0.49	597.65 ± 42	23.14 ± 0.11
Gentamicin, 1.5 mg/kg q12h	6.95 ± 0.16	0.375 ± 0.54	2.91 ± 0.02	59.50 ± 22	2.83 ± 0.18

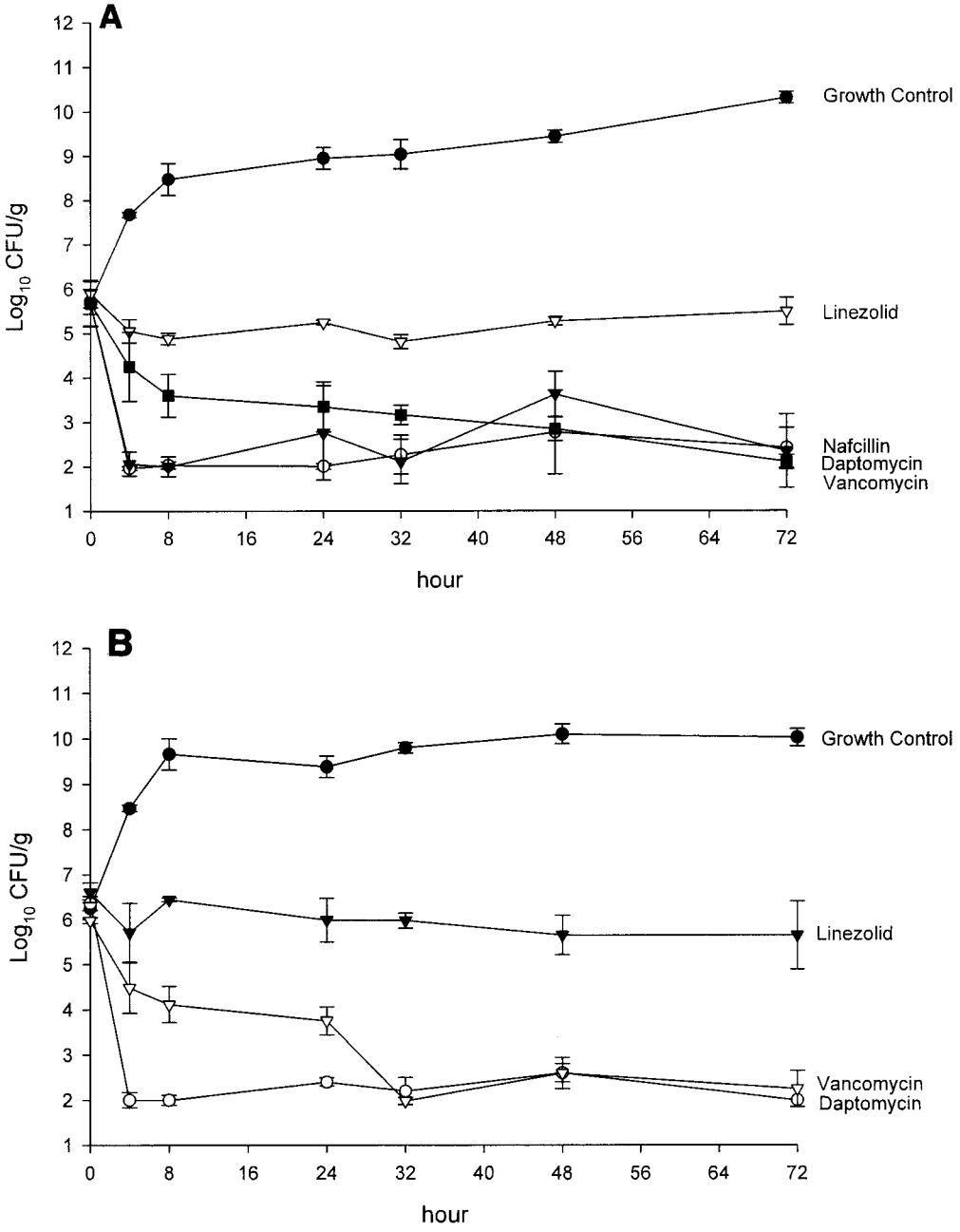

FIG. 1. Activities of tested antimicrobials (alone and in combination) at a moderate inoculum versus MSSA (A) and MRSA (B).

Detection of resistance. Resistance was detected in the gentamicin monotherapy model. There was a four- to sixfold increase in MIC at 48, 56, and 72 h against both the MSSA and the MRSA isolates. There was no resistance noted at any time point in the nafcillin, daptomycin, vancomycin, or linezolid models with monotherapy or combination regimens.

DISCUSSION

IE is a sequestered infection that often yields a high bacterial density (10^8 to 10^{10} organisms per g of tissue). As these infections progress, rates of metabolism and cell division appear to be reduced and biofilm production can occur, perhaps as a result of nutrient limitations (4). Clinical cure is often achieved, but prolonged administration of relatively high doses of a bactericidal cell wall-active antibacterial agent is generally required for sterilization of the vegetation (44). Additionally, these types of infections are commonly seen in immunocompromised patients who possess several risk factors for harboring multidrug-resistant organisms (4, 59).

Over 60 years ago, Eagle described an in vitro phenomenon called the "inoculum effect." This effect has been observed in gram-positive bacteria exposed to β-lactam antibiotics and glycopeptides such as vancomycin (52, 53). This is believed to occur as a result of the cell wall-active agents' mechanism of

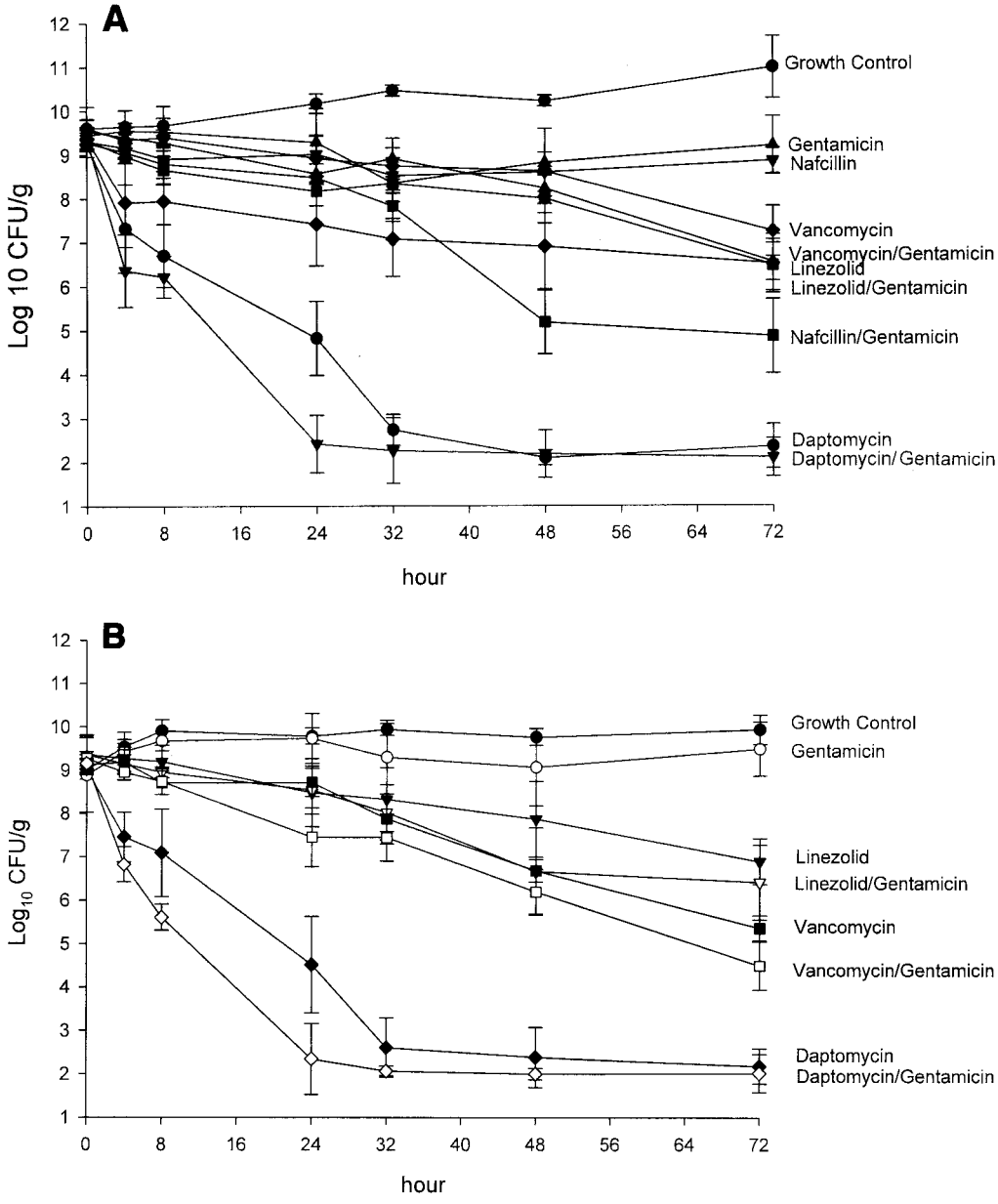

FIG. 2. Activities of tested antimicrobials (alone and in combination) at a high inoculum versus MSSA (A) and MRSA (B).

action. For example, vancomycin, penicillin, and other β-lactams interact and inhibit synthesis of the cell wall in the bacterial cytoplasmic membrane. This binding results in a series of interactions that lead to inhibition of growth and eventual death of the organism. When there is an overwhelming number of bacteria and lack of nutrients at the core of the infection (and therefore organisms in stationary phase), these agents are unable to reach their target site on a growing cell wall (24).

Controversy exists regarding the true impact of an in vitro inoculum effect in clinical settings; however, several studies have demonstrated this effect in vitro (23, 25, 33, 37, 45, 54).

Stevens et al. demonstrated that during the stationary phase of bacterial growth, penicillin binding proteins 1 and 4 were undetectable in a high inoculum (10^8); therefore, primary target proteins for penicillin were nonexistent (52, 53).

In our study, nafcillin at high inoculum (9.5 $\log_{10}$ CFU/g) did not demonstrate any significant reduction in bacterial densities throughout the 72-h study. However, when the study was repeated at a moderate inoculum of 5.5 $\log_{10}$ CFU/g, nafcillin elicited a rapid decline in bacterial densities as early as 4 h and throughout the entire 72-h experiment.

Nafcillin's efficacy has been pharmacodynamically correlated to $T >$ MIC, which is the cumulative percentage of time over a 24-h period that the drug concentration exceeds the MIC for the organism (8). At the moderate inoculum, the $T >$ $MIC_{(albumin)}$ was 100% throughout the 72-h experiment; however, the $T >$ $MIC_{(albumin)}$ at a high inoculum was 38%; this

TABLE 3. Inoculum change over 72 obtained in the SEV model[a]

Antimicrobial	Change in bacterial density ($\log_{10}$ CFU/ml) over 72 h			
	MSSA 1199 (5×10^5)	MRSA 494 (5×10^5)	MSSA 1199 (5×10^9)	MRSA 494 (5×10^9)
Growth control	+4.59	+3.78	+1.37	+0.93
Daptomycin	−3.34[b]	−4.36[b]	−6.89[b]	−6.85[b]
Nafcillin	−3.34[b]		−0.47[b]	
Linezolid	−0.75[b]	−0.96[b]	−1.36[b]	−2.45[b]
Vancomycin	−3.28[b]	−3.67[b]	−2.38[b]	−3.76[b]
Gentamicin			+0.25	−0.62[b]
Daptomycin + gentamicin			−7.28[b]	−5.45[b]
Nafcillin + gentamicin			−4.44[b,c]	
Linezolid + gentamicin			−2.87[b]	−2.93[b]
Vancomycin + gentamicin			−3.00[b]	−4.77[b,d]

[a] Note that positive values indicate regrowth.
[b] $P \leq 0.01$ for significance versus growth control.
[c] $P \leq 0.001$ for significance of monotherapy versus gentamicin.
[d] $P \leq 0.05$ for significance of monotherapy versus gentamicin.

may account for the observed difference in kill between the moderate and high inocula.

We also observed significant and improved activity when nafcillin was combined with gentamicin at a high inoculum. These results are consistent with the clinical findings of Chambers et al., who observed a more rapid cure of right-sided endocarditis when using nafcillin plus an aminoglycoside and the observation of Korzeniowski et al., who demonstrated that the combination of nafcillin and gentamicin compared to nafcillin alone demonstrated earlier resolution of bacteremia (2.8 versus 4.0 days; $P \leq 0.05$) associated in patents with IE (16, 36).

Vancomycin has been the drug of choice for treating MRSA infections for several decades. This drug has demonstrated efficacy against multidrug-resistant gram-positive bacteria in sequestered infections such as endocarditis and osteomyelitis (39, 51). There have been several studies which demonstrate vancomycin's kill to be significantly encumbered during stationary phases of bacterial growth, under anaerobic conditions, and at an increased inoculum (29, 37, 38). In addition, patients with *S. aureus* IE have demonstrated positive blood cultures after 7 days of therapy with vancomycin and have a slower response and longer duration of bacteremia than patients treated with β-lactams (27, 39, 40). One in vivo study evaluated the efficacy of β-lactam antibiotics versus vancomycin in the treatment of *S. aureus* infections. Investigators observed that β-lactam antibiotics were more effective at the 3- and 7-day time points than vancomycin (58). We demonstrated a delay in killing when a high inoculum (9.5 $\log_{10}$ CFU/g) was compared to a moderate inoculum (5.5 $\log_{10}$ CFU/g): 48 versus 72 h for both the MSSA and MRSA organisms.

A number of studies have evaluated the potential use of synergy with gentamicin combined with vancomycin; they demonstrate mixed results. This combination is not considered a more effective option for the treatment of MRSA infections due to the increased risk of nephrotoxicity (as much as four to five times baseline) but has been used frequently due to slow response to monotherapy with vancomycin and lack of suitable alternatives (28, 49, 50). In our investigation of a high-inoculum sequestered infection, we noted slow bactericidal activity of vancomycin, which was obtained at 72 h and showed no improvement with the addition of gentamicin. Furthermore, we noted an eightfold increase in MICs when vancomycin was tested at a high inoculum in the presence of physiological concentrations of albumin in humans.

The therapeutic efficacy of vancomycin has been pharmacodynamically correlated with both $T >$ MIC and AUC over 24 h divided by the MIC (AUC/MIC) (20, 38). We evaluated both parameters at moderate- and high-inoculum MICs in the presence of albumin (41). The $T >$ MICs were 100 and 80% for all for simulations with vancomycin against both isolates at moderate and high inocula, respectively. Despite achieving maximal target attainment for this parameter, we observed a significant reduction in overall kill and time to achieve bactericidal activity at high inocula. This may be related to the fact that the $AUC/MIC_{(albumin)}$ ratio was reduced by 50% for both the MSSA and MRSA isolates when vancomycin was evaluated at high inoculum.

There have been mixed results demonstrating an inoculum effect with daptomycin. Although we observed a 16-fold increase in the MIC under a high bacterial inoculum, this only translated to a delay in bactericidal kill activity of about 4 h, by extrapolation of nonlinear regression. However, the overall extent of killing at high inocula was equal to that observed at

TABLE 4. Pharmacodynamic properties of antimicrobials tested against MSSA and MRSA at both moderate and high inocula

Regimen	$T > MIC_{(albumin)}$ (%)		$C_{max}/MIC_{(albumin)}$		$AUC/MIC_{(albumin)}$	
	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA
Moderate inoculum						
Daptomycin, 6 mg/kg			159	159	1,511	1,511
Nafcillin, 4 g q4h	100	100				
Linezolid, 600 mg q12h	100	100			133	133
Vancomycin, 1 g q12h	100	100			149	299
Gentamicin, 1.5 mg/kg q12h			12.8	3.2	119	30
High inoculum						
Daptomycin, 6 mg/kg			9.95	9.95	94	47
Nafcillin, 4 g q4h	0					
Linezolid, 600 mg q12h	100	100			133	133
Vancomycin, 1 g q12h	100	100			75	149
Gentamicin, 1.5 mg/kg q12h			6.4	1.6	59	15

the moderate inoculum. Earlier studies noted that an increase in the bacterial inoculum from 10^3 to 10^6 CFU/ml had little effect on the activity of daptomycin (6). Another study reported that inoculum size had minimal effect on daptomycin in time-kill studies (19). In our high-inoculum model, we identified a delay in, but not a significant impact on, the overall activity of daptomycin in the presence of a high bacterial load.

We also observed in our high-inoculum sequestered infection model, that daptomycin demonstrated rapid bactericidal kill of both MSSA and MRSA test strains by 24 h. Several in vitro studies have addressed the effect of combination therapy containing daptomycin; however, a limited number of clinical studies have examined this effect (1, 18). Most commonly, these studies evaluate the effect of daptomycin in combination against *Enterococcus* spp. (2). One study demonstrated that daptomycin in combination with an aminoglycoside resulted in synergistic activity at 48 h against glycopeptide-intermediate *S. aureus* isolates (1). We observed no additional additivity at the 72-h endpoint when gentamicin was added to daptomycin. However, we did demonstrate an increase in the rate of bactericidal activity at 8 h when gentamicin combination therapy was used, while daptomycin alone demonstrated bactericidal activity at 24 h.

Daptomycin efficacy has also been correlated with AUC/MIC (1, 22, 36). Our study resulted in an $AUC/MIC_{(albumin)}$ ratio of 1,511 for both MSSA and MRSA at moderate inoculum. This ratio is consistent with previous studies testing the same isolates at a moderate inoculum and falls within the range of AUC/MIC_{free} of 171 to 442 providing maximum kill (15, 22, 41; M. J. Rybak, P. S. McKinnon, R. Cha, and B. H. Dvorchik, 41st Intersci. Conf. Antimicrob. Agents and Chemother., abstr. A-2195, 2001). At high inoculum, the AUC/MIC_{free} ratios dropped to 94.4 and 47.2 for MSSA and MRSA, respectively, which translated into a delay of bactericidal kill.

Linezolid is an oxazolidinone antibiotic that exerts its antibacterial effect by interfering with protein synthesis. In our study, linezolid did not demonstrate an inoculum effect in moderate- or high-bacterial-density experiments. Our data are consistent to observations reported in similar studies.

Recently, there have been published case reports and animal studies that found mixed results in treating *S. aureus* IE with linezolid (21, 32). One study reported that vancomycin alone was more effective than either the combination of linezolid and vancomycin or linezolid alone for the treatment of MRSA endocarditis (18). Additionally, there have been several clinical reports of treatment failures (10, 46, 48). Our results are consistent with several published in vivo and in vitro studies suggesting that linezolid is bacteriostatic and may not be effective in patients with complicated sequestered infections such as infective endocarditis. Synergy or additivity is rarely demonstrated in combination therapy with linezolid, which was what we observed in our infection model (3).

Linezolid efficacy have been pharmacodynamically correlated with both $T >$ MIC and AUC/MIC. In our investigation, linezolid concentrations exceeded the $T >$ MIC for 100% of the dosing interval and the AUC/MIC parameter was 133, a known ratio to achieve success against staphylococci (5). Although this parameter was maximized, we did not find any significant decrease in bacterial density with linezolid at moderate and high inocula against MSSA and MRSA. This may be related to the fact that linezolid acts on the ribosomes, whereas daptomycin, nafcillin, and vancomycin target the cell wall.

S. aureus is capable of producing biofilm especially at a higher inoculum and stationary phase (37, 52, 53, 59). Once a biofilm is present, antibiotics may have difficulty penetrating to the site at which the organism resides (4, 59). However, more recent evidence suggests that it may not be the antibiotic's inability to penetrate but rather inactivity of the antibiotic in the biofilm (4, 13, 35, 59). We did not directly investigate the presence of a biofilm in our in vitro model. It is feasible that such production may have been a contributory factor to the lack of efficacy demonstrated by nafcillin and vancomycin at a high inoculum.

Conclusion. Although monotherapy and combination regimens were investigated in our in vitro models, a limitation of this study is the use of single MSSA and MRSA isolates. In addition, we cannot conclude with certainty that our results will hold true with treatment durations longer than 72 h. Two- and 4-week courses of therapy with nafcillin and vancomycin have demonstrated clinical efficacy in patients with IE (16, 17). The majority of our monotherapy and combination regimens have been consistent with previous clinical studies and other in vitro and animal models (1, 15, 17, 27). However, our results should be applied to clinical practice with caution. The inoculum appears to influence the activity of nafcillin > vancomycin > daptomycin > linezolid. Nonetheless, confirmation of our results with further clinical studies would be beneficial before these regimens can be adopted for use in the care of patients.

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