Multiple Combination Bactericidal Testing of Staphylococcal Biofilms from Implant-Associated Infections

Raphael Saginur,^{1,2*} Melissa St. Denis,^{2,3} Wendy Ferris,^{2,3} Shawn D. Aaron,^{1,2} Francis Chan,^{2,3}
Craig Lee,^{1,2} and Karam Ramotar^{1,2}

The Ottawa Hospital,¹ University of Ottawa,² and Children's Hospital of Eastern Ontario,³ Ontario, Canada

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Standardized susceptibility testing fails to predict in vivo resistance of device-related infections to antimicrobials. We assessed agents and combinations of antimicrobials against clinical isolates of *Staphylococcus epidermidis* and *S. aureus* (methicillin-resistant *S. aureus* and methicillin-sensitive *S. aureus*) retrieved from device-associated infections. Isolates were grown planktonically and as biofilms. Biofilm cultures of the organisms were found to be much more resistant to inhibitory and bactericidal effects of single and combination antibiotics than planktonic cultures ($P < 0.001$). Rifampin was the most common constituent of antibiotic combinations active against staphylococcal biofilms. Other frequently effective antimicrobials were vancomycin and fusidic acid. Susceptibility testing involving biofilm-associated bacteria suggests new options for combination antibiotic therapy.

For many patients, surgical implantation of bioengineered medical devices such as valvular prostheses, vascular prostheses, ventricular assist devices, or ventricular shunts can be life saving. However, implantation of these foreign bodies carries risk of infection. Although the risk of infection of these devices is in general only between 1 and 7%, the impact of implant-associated infection is major (3). Implant-associated infections are associated with considerable morbidity, repeated surgeries, and prolonged antibiotic therapy. Mortality of prosthetic valve endocarditis ranges up to 30%, and mortality rates associated with an infected aortic graft approach 40% (3).

Key to the pathogenesis of device infections is bacterial adherence to the prosthetic surface and formation of a bacterial biofilm. A biofilm has been defined as “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface” (6). Biofilms are characterized by high concentrations of organisms with little turnover. Bacteria within the biofilm communicate with each other through the elaboration and recognition of small molecules, a process called “quorum sensing” (13).

Antimicrobial therapy is guided by the results obtained from conventional antimicrobial testing of planktonic (free-growing) bacteria. However, this may not necessarily reflect the susceptibilities of these same bacteria when they are grown as biofilms. Biofilm associated bacteria are 100 to 1,000 times less susceptible to antibiotics than are planktonic bacteria (7), and agents active against planktonic bacteria, but not against biofilms, fail to cure patients with infected prostheses.

The mechanism of biofilm-associated antibiotic resistance is uncertain and likely multifactorial. A number of factors have been postulated, including binding of antibiotic to the slime, poor penetration of antibiotic into the biofilm, slow growth rate of organisms in the biofilm, high bacterial density, and

changes in gene expression in biofilm bacteria (7, 12). Bacteria released from biofilms retain susceptibility to antibiotics characteristic of free-growing bacteria rather than biofilms, implying that the mechanism of resistance is not genetic change. The consequence of this resistance is frequently the need for surgery to explant and replace the device in addition to antibiotic therapy, vastly increasing cost of therapy and debility attributable to infection.

Staphylococcus epidermidis and *Staphylococcus aureus* are the most common pathogens associated with infections of surgical implants and other prosthetic devices (10, 14, 15). These organisms have been shown to form in vivo biofilms on implanted devices. The objective of this study was to compare susceptibilities of planktonic and biofilm-grown bacteria to single antibiotics and to combinations of antibiotics in order to identify antibiotic combinations that were effective against staphylococci retrieved from implant-associated infections. It was hypothesized that biofilm-grown bacteria would demonstrate greater resistance to single and combination antibiotics compared to the same bacteria grown under planktonic conditions.

MATERIALS AND METHODS

Bacterial strains. Staphylococci associated with device infections were obtained from the clinical microbiology laboratories of The Ottawa Hospital (Ottawa, Ontario, Canada) and Mount Sinai Hospital (Toronto, Ontario, Canada). Preference was given to isolates recovered from the local site of infection or the device itself. Blood culture isolates from patients with clinically defined line sepsis were also used. Staphylococci were identified by colonial morphology, Gram stain, and coagulase testing. Methicillin-resistant *S. aureus* (MRSA) was further confirmed by tube coagulase testing, growth on oxacillin salt agar screen plate, and PBP 2a testing (MRSA-Screen; Denka Seiken Co. Ltd., Tokyo, Japan). In total, 17 isolates of *S. epidermidis*, 11 isolates of methicillin-sensitive *S. aureus* (MSSA), and 12 isolates of methicillin-resistant *S. aureus* (MRSA) were obtained from the sites of device infections and were studied.

Antibiotics. Antibiotics tested were azithromycin (Pfizer, Montréal, Canada), cefazolin (Sigma, Oakville, Canada), ciprofloxacin (Bayer, Toronto, Canada), fusidic acid (Sigma), gentamicin (Sigma), linezolid (Pharmacia, Toronto, Canada), oxacillin (Sigma), rifampin (Aventis Pharma, Laval, Canada), and vancomycin (Sigma). Interpretation criteria for susceptibility testing were based on NCCLS, now CLSI (11), or British Society for Antimicrobial Chemotherapy (BSAC) (4) guidelines (Table 1).

* Corresponding author. Mailing address: Department of Medicine, CPC 470, The Ottawa Hospital, Civic Campus, 1053 Carling Avenue, Ottawa, ON K1Y 4E9. Phone: (613) 798-5555, ext. 14155. Fax: (613) 761-5260. E-mail: rsaginur@ohri.ca.

TABLE 1. Antibiotic break points

Antibiotic	Break point ($\mu\text{g/ml}$)			Reference
	Sensitive	Intermediate	Resistant ^a	
Linezolid	4		8	BSAC
Rifampin	1	2	4	NCCLS
Cefazolin	8	16	32	NCCLS
Oxacillin	2		4	NCCLS
Vancomycin	4	8–16	32	NCCLS
Gentamicin	4	8	16	NCCLS
Azithromycin	2	4	8	NCCLS
Ciprofloxacin	1	2	4	NCCLS
Fusidic acid	1		2	BSAC

^a Concentration used in MCBT testing.

Planktonic MIC and MBC. Microtiter MIC and minimal bactericidal concentration (MBC) tests were done according to standard techniques (11). Briefly, serial twofold dilutions of antibiotic were performed in Mueller-Hinton broth with cations (MHB II; Becton Dickinson, Oakville, Ontario, Canada) and containing 2% NaCl. A suspension of the organism was added to wells at a concentration of 5×10^5 CFU/ml, and the microtiter plates were incubated aerobically at 35°C. The MIC was defined as the lowest concentration of antibiotic in which there was no visible growth after overnight incubation. In wells where there was no visible growth, 10 μl was subcultured to Columbia agar plates with 5% defibrinated sheep blood (PML Microbiologics, Mississauga, Ontario, Canada), and the agar plates were incubated aerobically at 35°C for colony count. MBC was defined as the highest dilution showing $\geq 99.9\%$ kill after 24 h of incubation.

Biofilm MIC and MBC. All isolates were grown as biofilms using a modified version of the Calgary Biofilm Device shown previously to be both reliable and reproducible for growing *Pseudomonas aeruginosa* organisms as biofilms (5). Briefly, each isolate was resuspended in MHB II containing 2% NaCl to a 0.5 McFarland standard, and 100 μl was added to wells of a 96-well round-bottomed microtiter plate (NUNC, Roskilde, Denmark). A transferable solid-phase (TSP) pin lid (NUNC, Roskilde, Denmark) was placed into the microtiter plate and incubated overnight at 35°C on a rocking table (Bellco Glass Inc., Vineland, NJ) to produce a shear force. The TSP pin lid was then removed and placed into a new microtiter plate containing the twofold dilutions of antibiotics, similar to the MIC and MBC methods for planktonic bacteria. This plate was incubated for 24 h at 35°C on the rocker platform. The MIC was read as the last well in which there was no visible growth. The TSP pin lid was then transferred to a 96-well microtiter plate containing sterile MHB II with 2% NaCl and sonicated for 5 min. The TSP pin lid was discarded and replaced by a sterile microtiter lid, and the plate was incubated overnight at 35°C. The MBC was determined as the last well showing no turbidity after incubation.

Planktonic multiple-combination bactericidal testing (MCBT). Combination antibiotic susceptibility testing was performed using the MCBT technique as previously described for *Pseudomonas aeruginosa* and *Burkholderia cepacia* (1, 2, 9). Minor changes were made to accommodate multiple combination bactericidal testing for staphylococci. In addition to supplementation of MHB II with 2% NaCl, different antibiotics known to be active against staphylococcal bacteria were used. There were 94 combinations of two or three antibiotics, as shown in the MCBT template (Table 2). Frozen stock solutions of each antibiotic were stored in a concentration 10 times the concentration used in testing. Combinations of the nine antibiotics were added to each well of a 96-well microtiter plate in aliquots of 10 μl . In wells containing two antibiotics, MHB II with 2% NaCl was added to bring volumes of all wells to 30 μl . The inoculum was 70 μl of a 100-fold dilution of a 0.5 McFarland turbidity suspension of organism prepared from a culture in MHB II with 2% NaCl in the growth phase, resulting in a final inoculum concentration of 5×10^5 CFU/ml and the desired concentration of antimicrobials in each well. Plates were incubated at 35°C. At 24 h, inhibition of growth was read; bacterial killing was confirmed by subculturing the TSP lid to another microtiter plate containing 100 μl of MHB II with 2% NaCl per well, with demonstration of no growth after 24 h of incubation. The final concentrations of antibiotics tested corresponded to the criteria for resistance (Table 1).

Biofilm MCBT. Biofilms were cultured using a modification of the Calgary Biofilm Device (5). Isolates were grown as biofilms using the NUNC microtiter plate and NUNC TSP pin lid system as described in the MIC and MBC procedures. After a biofilm was grown on the pegs, the pin lid was then transferred to a 96-well microtiter plate containing the same antibiotic combinations used for the planktonically grown staphylococcus (Table 2). This plate with the pin lid was

TABLE 2. Template of antibiotic combinations used in MCBT testing^a

Row	Drugs in column:											
	1	2	3	4	5	6	7	8	9	10	11	12
A	LZD, RIF	LZD, CFZ	LZD, OXA	LZD, VAN	LZD, GEN	LZD, AZM	LZD, CIP	LZD, FA	LZD, RIF, CFZ	LZD, RIF, OXA	LZD, RIF, VAN	LZD, RIF, GEN
B	LZD, RIF, AZM	LZD, RIF, CIP	LZD, RIF, FA	LZD, CFZ, GEN	LZD, CFZ, AZM	LZD, CFZ, CIP	LZD, CFZ, FA	LZD, OXA, GEN	LZD, OXA, AZM	LZD, OXA, CIP	LZD, OXA, FA	LZD, VAN, GEN
C	LZD, VAN, AZM	LZD, VAN, CIP	LZD, VAN, FA	LZD, GEN, AZM	LZD, GEN, CIP	LZD, GEN, FA	LZD, AZM, CIP	LZD, AZM, FA	LZD, CIP, FA	RIF, CFZ	RIF, OXA	RIF, VAN
D	RIF, GEN	RIF, AZM	RIF, CIP	RIF, FA	RIF, CFZ, GEN	RIF, CFZ, AZM	RIF, CFZ, CIP	RIF, CFZ, FA	RIF, OXA, GEN	RIF, OXA, AZM	RIF, OXA, CIP	RIF, OXA, FA
E	RIF, VAN, GEN	RIF, VAN, AZM	RIF, VAN, CIP	RIF, VAN, FA	RIF, GEN, AZM	RIF, GEN, CIP	RIF, GEN, FA	RIF, AZM, CIP	RIF, AZM, FA	RIF, CIP, FA	CFZ, GEN	CFZ, AZM
F	CFZ, CIP	CFZ, FA	CFZ, GEN, AZM	CFZ, GEN, CIP	CFZ, GEN, FA	CFZ, AZM, CIP	CFZ, AZM, FA	CFZ, CIP, FA	OXA, GEN	OXA, AZM	OXA, CIP	OXA, FA
G	OXA, GEN, AZM	OXA, GEN, CIP	OXA, GEN, FA	OXA, AZM, CIP	OXA, AZM, FA	OXA, CIP, FA	VAN, GEN	VAN, AZM	VAN, CIP	VAN, FA	VAN, GEN, AZM	VAN, GEN, CIP
H	VAN, GEN, FA	VAN, AZM, CIP	VAN, AZM, FA	VAN, CIP, FA	GEN, AZM	GEN, CIP	GEN, FA	AZM, CIP	AZM, FA	AZM, CIP, FA	Growth control	Sterility control

^a AZM, azithromycin; GEN, gentamicin; CFZ, cefazolin; LZD, linezolid; OXA, oxacillin; RIF, rifampin; VAN, vancomycin; CIP, ciprofloxacin; FA, fusidic acid.

TABLE 3. Single antibiotic susceptibilities: planktonic MIC and MBC values (in micrograms/milliliter)

Isolate ^a and isolate no.	Linezolid		Rifampin		Cefazolin		Oxacillin		Vancomycin		Gentamicin		Azithromycin		Ciprofloxacin		Fusidic acid	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1 SE	2.0	>64	0.06	0.06	32	64	>32	>32	2.0	4.0	>128	>128	>64	>64	8.0	8.0	0.12	8.0
2 SE	1.0	>64	0.06	0.06	128	256	>32	>32	2.0	>128	32	>128	>64	>64	>32	>32	0.12	4.0
3 SE	0.5	>64	0.06	0.5	64	>256	>32	>32	1.0	128	128	>128	>64	>64	>32	>32	0.12	>64
4 SE	1.0	64	0.06	0.5	64	128	>32	>32	2.0	>128	2.0	8.0	0.5	>64	>32	>32	0.12	>64
5 SE	1.0	32	0.06	0.06	2.0	8.0	8.0	>32	2.0	16	2.0	2.0	>64	>64	16	>32	0.12	64
6 SE	1.0	>64	0.06	4.0	64	>256	>32	>32	2.0	128	128	>128	>64	>64	>32	>32	0.25	>64
7 SE	2.0	64	0.06	2.0	128	256	>32	>32	2.0	64	2.0	128	1.0	64	>32	>32	0.12	64
8 SE	2.0	>64	0.06	1.0	256	>256	>32	>32	2.0	2.0	128	128	>64	>64	>32	>32	0.12	>64
9 SE	1.0	>64	>32	>32	2.0	16	4.0	>32	2.0	>128	128	128	>64	>64	>32	>32	0.12	>64
10 SE	1.0	>64	0.06	16	8.0	16	>32	>32	2.0	4.0	128	>128	>64	>64	>32	>32	0.5	>64
11 SE	1.0	>64	0.06	2.0	32	64	>32	>32	2.0	32	1.0	2.0	>64	>64	>32	>32	0.12	>64
12 SE	1.0	>64	0.06	8.0	2.0	32	4.0	>32	2.0	4.0	128	>128	>64	>64	>32	>32	8.0	>64
13 SE	1.0	>64	0.06	0.25	4.0	32	>32	>32	2.0	64	128	>128	>64	>64	>32	>32	0.12	16
14 SE	2.0	>64	0.06	4.0	4.0	32	8.0	>32	2.0	2.0	4.0	4.0	>64	>64	16	>32	0.12	>64
15 SE	2.0	>64	0.06	4.0	4.0	32	16	>32	2.0	2.0	>128	>128	>64	>64	>32	>32	16	>64
16 SE	1.0	>64	0.06	8.0	8.0	64	>32	>32	4.0	4.0	>128	>128	>64	>64	>32	>32	0.12	64
17 SE	2.0	>64	0.06	0.06	2.0	128	4.0	>32	>128	64	>128	>128	>64	>64	>32	>32	0.12	>64
1 MSSA	2.0	>64	0.06	0.06	0.5	0.5	0.12	0.25	0.5	0.5	2.0	>128	0.5	64	0.25	0.5	0.12	0.5
2 MSSA	2.0	>64	0.06	0.06	0.5	4.0	0.12	2.0	1.0	128	1.0	2.0	0.5	64	0.5	16	0.12	2.0
3 MSSA	2.0	>64	0.06	0.06	0.5	64	0.25	16	0.5	32	8.0	8.0	0.5	64	0.5	64	0.12	>64
4 MSSA	1.0	>64	0.06	4.0	0.5	32	0.12	32	0.5	32	16	16	0.25	>64	0.25	4.0	0.12	>64
5 MSSA	1.0	>64	0.06	0.5	2.0	8.0	0.5	>32	2.0	32	2.0	16	>64	>64	>32	32	0.12	>64
6 MSSA	2.0	>64	0.06	16	0.5	256	0.12	>32	1.0	>128	8.0	8.0	>64	>64	>32	>32	0.12	>64
7 MSSA	2.0	>64	0.06	16	0.5	128	0.25	>32	1.0	128	32	32	0.5	>64	>64	>32	0.12	>64
8 MSSA	2.0	>64	0.06	8.0	0.5	128	0.25	>32	0.5	32	4.0	64	>64	>64	>32	>32	0.12	>64
9 MSSA	4.0	>64	0.06	16	1.0	16	0.5	>32	0.5	32	16	16	2.0	>64	>64	1.0	0.12	>64
10 MSSA	4.0	>64	0.06	32	1.0	128	0.5	>32	0.5	128	16	16	2.0	>64	>64	1.0	0.12	>64
11 MSSA	2.0	>64	0.06	>32	0.5	128	0.5	>32	0.5	>128	16	16	0.5	>64	0.25	32	0.12	>64
1 MSSA	4.0	>64	0.06	16	8.0	>256	32	>32	0.5	>128	2.0	>128	>64	>64	0.5	16	0.12	>64
2 MSSA	2.0	>64	0.06	4.0	>256	>256	>32	>32	0.5	8.0	2.0	2.0	>64	>64	>32	>32	0.12	64
3 MSSA	2.0	>64	0.06	32	64	128	16	>32	1.0	16	4.0	8.0	0.5	>64	>32	>32	0.12	>64
4 MSSA	2.0	>64	0.06	8.0	>256	>256	>32	>32	1.0	128	16	16	>64	>64	>32	>32	4.0	>64
5 MSSA	2.0	>64	0.06	4.0	256	>256	>32	>32	0.5	1.0	16	32	>64	>64	>32	>32	2.0	>64
6 MSSA	2.0	>64	0.06	>32	128	>256	>32	>32	0.5	>128	4.0	64	>64	>64	>32	>32	0.25	>64
7 MSSA	2.0	>64	0.06	32	>256	>256	>32	>32	1.0	128	4.0	16	>64	>64	>32	>32	0.12	>64
8 MSSA	2.0	>64	>32	>32	>256	>256	>32	>32	1.0	>128	>128	>128	>64	>64	>32	>32	4.0	>64
9 MSSA	4.0	>64	0.06	16	>256	>256	>32	>32	0.5	>128	>128	>128	>64	>64	>32	>32	0.12	>64
10 MSSA	4.0	>64	0.06	16	>256	>256	>32	>32	0.5	64	>128	>128	>64	>64	>32	>32	0.12	>64
11 MSSA	2.0	>64	8.0	>32	128	256	>32	>32	2.0	>128	>128	>128	>64	>64	0.25	0.25	0.12	>64
12 MSSA	2.0	>64	0.06	16	>256	>256	>32	>32	1.0	>128	8.0	16	>64	>64	>32	>32	0.12	>64

^a SE, *S. epidermidis*.

TABLE 4. Single antibiotic susceptibilities; biofilm MIC and MBC values (in micrograms/milliliter)

Isolate ^a and no.	Linezolid		Rifampin		Cefazolin		Oxacillin		Vancomycin		Gentamicin		Azithromycin		Ciprofloxacin		Fusidic acid	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1 SE	>64	>64	>32	8.0	>256	>256	>32	>32	>128	64	>128	>128	>64	>64	>32	>32	>64	64
2 SE	>64	32	0.5	0.06	>256	>256	>32	>32	>128	>8.0	>128	>128	>64	>64	>32	>32	>64	>64
3 SE	>64	64	>32	0.06	256	>256	>32	>32	64	128	>128	>128	>64	>64	>32	>32	>64	>64
4 SE	>64	32	>32	1.0	>256	>256	>32	>32	>128	128	>128	128	>64	>64	>32	>32	>64	>64
5 SE	>64	32	>32	2.0	>256	256	>32	>32	>128	128	>128	128	>64	>64	>32	>32	>64	64
6 SE	>64	>64	>32	0.5	>256	>256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
7 SE	>64	64	>32	32	256	>256	>32	>32	>128	128	>128	128	>64	>64	>32	>32	>64	>64
8 SE	>64	>64	>32	32	>256	>256	>32	>32	>128	2.0	>128	>128	>64	>64	>32	>32	>64	>64
9 SE	>64	64	>32	>32	16	>256	>32	>32	4.0	8.0	>128	>128	>64	>64	>32	0.5	>64	2.0
10 SE	>64	64	32	0.12	128	256	>32	>32	32	128	>128	>128	>64	>64	>32	>32	>64	8.0
11 SE	>64	>64	>32	4.0	>256	>256	>32	>32	>128	64	>128	64	>64	>64	>32	>32	>64	64
12 SE	>64	64	>32	0.25	256	256	>32	>32	>128	128	>128	>128	>64	>64	>32	>32	>64	0.12
13 SE	>64	>64	>32	0.12	>256	>256	>32	>32	>128	64	>128	>128	>64	>64	>32	>32	>64	64
14 SE	>64	>64	>32	8.0	>256	256	>32	>32	>128	128	>128	128	>64	>64	>32	>32	>64	>64
15 SE	>64	64	>32	1.0	128	256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
16 SE	>64	64	>32	2.0	>256	>256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
17 SE	>64	64	>32	32	256	256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
1 MSSA	>64	>64	>32	>32	>256	64	>32	>32	>128	128	>128	>128	>64	>64	>32	>32	>64	>64
2 MSSA	>64	>64	>32	16	>256	256	>32	>32	>128	128	>128	>128	>64	>64	>32	>32	>64	>64
3 MSSA	>64	>64	>32	0.5	>256	256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
4 MSSA	>64	>64	>32	2.0	>256	256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
5 MSSA	>64	>64	>32	2.0	>256	256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
6 MSSA	>64	64	>32	32	>256	256	>32	>32	>128	128	>128	>128	>64	>64	>32	>32	>64	>64
7 MSSA	>64	>64	>32	>32	>256	256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
8 MSSA	>64	>64	>32	0.25	>256	256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
9 MSSA	>64	>64	16	2.0	4.0	256	>32	>32	>128	1.0	>128	>128	>64	>64	>32	>32	16	32
10 MSSA	>64	>64	>32	2.0	>256	256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
11 MSSA	64	>64	>32	32	256	256	>32	>32	>128	128	>128	>128	>64	>64	>32	>32	>64	4.0
1 MRSA	>64	>64	>32	>32	>256	>256	>32	>32	>128	128	>128	>128	>64	>64	>32	>32	>64	>64
2 MRSA	>64	>64	>32	0.5	>256	>256	>32	>32	>128	64	>128	>128	>64	>64	>32	>32	>64	>64
3 MRSA	>64	>64	>32	1.0	>256	>256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
4 MRSA	>64	>64	32	1.0	>256	>256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	>64
5 MRSA	16	>64	>32	16	>256	>256	>32	>32	16	128	>128	>128	>64	>64	>32	>32	>64	>64
6 MRSA	>64	64	>32	16	>256	>256	>32	>32	>128	128	>128	>128	>64	>64	>32	>32	>64	64
7 MRSA	>64	>64	>32	0.5	>256	>256	>32	>32	>128	2.0	>128	>128	>64	>64	>32	>32	>64	64
8 MRSA	>64	64	>32	>32	>256	>256	>32	>32	>128	2.0	>128	>128	>64	>64	>32	>32	>64	64
9 MRSA	>64	>64	>32	32	128	>256	>32	>32	16	128	>128	>128	>64	>64	>32	>32	>64	8.0
10 MRSA	>64	>64	>32	>32	>256	>256	>32	>32	>128	>128	>128	>128	>64	>64	>32	>32	>64	16
11 MRSA	4.0	32	32	>32	>256	>256	>32	>32	>128	128	>128	>128	>64	>64	>32	>32	>64	1.0
12 MRSA	>64	>64	>32	0.12	256	>256	>32	>32	>128	128	>128	>128	>64	>64	>32	>32	>64	4.0

^a SE, *S. epidermidis*.

TABLE 5. Number of staphylococcus isolates susceptible to single antibiotics

Drug ^a	<i>S. epidermidis</i> (n = 17)				MSSA (n = 11)				MRSA (n = 12)			
	Planktonic		Biofilm		Planktonic		Biofilm		Planktonic		Biofilm	
	Inhibit	Kill	Inhibit	Kill	Inhibit	Kill	Inhibit	Kill	Inhibit	Kill	Inhibit	Kill
LZD	17	0	0	0	11	0	0	0	12	0	1	0
RIF	16	8	1	8	11	3	1	2	10	0	3	5
CFZ	9	1	0	0	11	3	3	0	1	0	0	0
OXA	0	0	0	0	11	2	1	0	0	0	0	0
VAN	17	7	2	0	11	1	2	0	12	1	4	0
GEN	5	4	0	0	4	2	0	0	4	1	0	0
AZM	4	0	0	0	8	0	0	0	1	0	0	0
CIP	0	0	0	0	7	4	1	0	2	1	0	0
FA	14	0	1	1	11	1	0	0	9	0	3	1

^a See the footnote to Table 2 for drug abbreviations.

incubated for 24 h at 35°C on the rocking platform. After incubation, the TSP pin lid was transferred to a new microtiter plate containing sterile MHB II with 2% NaCl and sonicated for 5 min. The TSP pin lid was discarded and replaced with a microtiter plate cover, and the plate was incubated overnight at 35°C. Using a plate reader, the wells with no visible growth were determined as bactericidal antibiotic combinations.

RESULTS

MIC and MBC results are shown in Tables 3, 4, and 5. There was uniform susceptibility of planktonic staphylococci to vancomycin and linezolid by MIC testing. MSSA was also uniformly susceptible to rifampin, cefazolin, oxacillin, and fusidic acid. Ninety-four percent (16 out of 17) of *S. epidermidis* strains were sensitive to rifampin, and 82% (14 out of 17) were sensitive to fusidic acid by MIC testing ("Inhibit"). By MBC testing, 8 out of 17 (47%) *S. epidermidis* strains were killed by rifampin. Ciprofloxacin was bactericidal against 4 out of 11 (36%) *S. aureus* strains, rifampin and cefazolin was bactericidal against 3 out of 11 (27%) strains, and oxacillin and gentamicin was bactericidal against 2 out of 11 (18%) strains.

Biofilm cultures of all three groups of organisms were found to be much more resistant to inhibitory effects of the antibiotics compared to planktonic cultures (Table 5; $P = 0.004$, $P < 0.001$, and $P = 0.008$ by paired t tests for *S. epidermidis*, MSSA, and MRSA, respectively; no antibiotic was very cidal against biofilms in our assay). The most active antimicrobial against biofilms was rifampin, which was bactericidal against 2 of 11 MSSA (18%), 5 of 12 MRSA (42%), and 8 of 17 *S. epidermidis* (47%) strains. Unexpectedly, rifampin exhibited more bactericidal than inhibitory activity.

In order to explicate rifampin's apparently greater bactericidal than bacteriostatic activity, we estimated bacterial release from biofilms coating the pegs by measuring bacterial counts in

the wells immediately after sonication. We compared rifampin to oxacillin and vancomycin. Four of six isolates of *S. epidermidis*, three of five MSSA isolates, and three of four MRSA isolates tested showed evidence of release of adherent organisms from the peg after overnight exposure to rifampin. This was not seen in two strains of each of *S. epidermidis*, MSSA, and MRSA after exposure to oxacillin or vancomycin.

MCBT results are shown in Table 6. A total of 94 separate antibiotic combinations were evaluated. When grown planktonically, the *S. epidermidis* isolates were susceptible to a mean of 82.9 combinations of antimicrobials; however, when grown as biofilms the same isolates were susceptible to a mean of 47.8 antibiotic combinations ($P < 0.001$). Similar results were observed for MSSA and MRSA, demonstrating far fewer bactericidal antibiotic combinations when the organisms were grown as biofilms compared to when they were grown planktonically ($P < 0.001$).

Table 7 lists the combinations of antimicrobials that were bactericidal against at least 90% of the biofilm-grown organisms. A total of 11 antibiotic combinations were bactericidal against $\geq 90\%$ of MSSA, and 9 antibiotic combinations were similarly found to be highly effective against *S. epidermidis*. Rifampin was the antibiotic most frequently included in these active combinations: 9 out of 9 combinations active against *S. epidermidis*, 2 out of 2 combinations active against MRSA, and 4 out of 11 combinations active against MSSA. Fusidic acid was part of both com-

TABLE 7. Antimicrobial combinations active against $\geq 90\%$ of biofilms of isolates^a

MSSA (n = 11)	MRSA (n = 12)	<i>S. epidermidis</i> (n = 17)
OXA, AZM, FA	RIF, VAN, FA	RIF, VAN, GEN
OXA, GEN, FA	RIF, CIP, FA	RIF, VAN, CIP
RIF, CFZ		RIF, CIP, FA
RIF, VAN, GEN		LZD, RIF, VAN
CFZ, AZM, FA		RIF, VAN
CFZ, CIP, FA		RIF, OXA, CIP
VAN, FA		RIF, OXA, FA
VAN, AZM, FA		LZD, RIF, AZM
GEN, FA		RIF, VAN, FA
AZM, CIP, FA		
RIF, VAN		

^a AZM, azithromycin; FA, fusidic acid; GEN, gentamicin; CFZ, cefazolin; LZD, linezolid; OXA, oxacillin; RIF, rifampin; VAN, vancomycin; CIP, ciprofloxacin.

TABLE 6. Number of bactericidal antibiotic combinations effective against planktonically and biofilm-grown isolates

Isolate	Mean no. of active combinations (maximum = 94)		P value
	Planktonic	Biofilm	
<i>S. epidermidis</i>	82.9 $\pm$ 6.6	47.8 $\pm$ 13.0	<0.001
MSSA	93.2 $\pm$ 1.3	42.7 $\pm$ 15.2	<0.001
MRSA	80.6 $\pm$ 7.8	44.7 $\pm$ 16.8	<0.001

TABLE 8. Summary of combinations and number of combinations active against planktonic and biofilm forms of isolates

Row	Drug combination and no. active against planktonic and biofilm (in parentheses) forms for column no. ^a :											
	1	2	3	4	5	6	7	8	9	10	11	12
A	LZD, RIF	LZD, CFZ	LZD, OXA	LZD, VAN	LZD, GEN	LZD, AZM	LZD, CIP	LZD, FA	LZD, RIF, CFZ	LZD, RIF, OXA	LZD, RIF, VAN	LZD, RIF, GEN
	11 (3)	11 (0)	11 (0)	11 (0)	10 (0)	11 (0)	11 (0)	10 (7)	12 (9)	11 (8)	11 (9)	11 (5)
	<i>11 (9)</i>	<i>12 (0)</i>	<i>10 (0)</i>	<i>11 (1)</i>	<i>11 (0)</i>	<i>11 (0)</i>	<i>11 (1)</i>	<i>11 (7)</i>	<i>12 (8)</i>	<i>11 (8)</i>	<i>11 (10)</i>	<i>11 (5)</i>
B	LZD, RIF, AZM	LZD, RIF, CIP	LZD, RIF, FA	LZD, CFZ, GEN	LZD, CFZ, AZM	LZD, CFZ, CIP	LZD, CFZ, FA	LZD, OXA, GEN	LZD, OXA, AZM	LZD, OXA, CIP	LZD, OXA, FA	LZD, VAN, GEN
	11 (6)	11 (4)	11 (7)	11 (0)	11 (1)	11 (1)	11 (6)	10 (2)	11 (1)	11 (0)	11 (9)	10 (8)
	<i>11 (8)</i>	<i>11 (9)</i>	<i>11 (9)</i>	<i>10 (3)</i>	<i>12 (1)</i>	<i>12 (1)</i>	<i>12 (4)</i>	<i>11 (3)</i>	<i>11 (2)</i>	<i>11 (2)</i>	<i>11 (7)</i>	<i>11 (7)</i>
C	LZD, VAN, AZM	LZD, VAN, CIP	LZD, VAN, FA	LZD, GEN, AZM	LZD, GEN, CIP	LZD, GEN, FA	LZD, AZM, CIP	LZD, AZM, FA	LZD, CIP, FA	RIF, CFZ	RIF, OXA	RIF, VAN
	11 (0)	11 (0)	11 (9)	11 (0)	11 (1)	11 (5)	11 (3)	11 (8)	11 (9)	11 (11)	11 (8)	11 (10)
	<i>11 (2)</i>	<i>12 (1)</i>	<i>12 (10)</i>	<i>10 (2)</i>	<i>10 (0)</i>	<i>10 (7)</i>	<i>10 (0)</i>	<i>11 (7)</i>	<i>11 (8)</i>	<i>11 (9)</i>	<i>10 (9)</i>	<i>12 (10)</i>
D	RIF, GEN	RIF, AZM	RIF, CIP	RIF, FA	RIF, CFZ, GEN	RIF, CFZ, AZM	RIF, CFZ, CIP	RIF, CFZ, FA	RIF, OXA, GEN	RIF, OXA, AZM	RIF, OXA, CIP	RIF, OXA, FA
	11 (4)	10 (4)	11 (4)	10 (2)	11 (8)	11 (7)	10 (8)	11 (9)	11 (9)	11 (8)	11 (9)	11 (7)
	<i>9 (8)</i>	<i>16 (13)</i>	<i>10 (10)</i>	<i>11 (8)</i>	<i>10 (6)</i>	<i>11 (6)</i>	<i>11 (7)</i>	<i>10 (6)</i>	<i>9 (8)</i>	<i>11 (10)</i>	<i>11 (10)</i>	<i>11 (10)</i>
E	RIF, VAN, GEN	RIF, VAN, AZM	RIF, VAN, CIP	RIF, VAN, FA	RIF, GEN, AZM	RIF, GEN, CIP	RIF, GEN, FA	RIF, AZM, CIP	RIF, AZM, FA	RIF, CIP, FA	CFZ, GEN	CFZ, AZM
	11 (10)	11 (8)	11 (8)	11 (9)	11 (5)	11 (8)	11 (9)	11 (8)	11 (9)	11 (9)	11 (7)	11 (1)
	<i>12 (11)</i>	<i>12 (10)</i>	<i>11 (10)</i>	<i>12 (11)</i>	<i>7 (6)</i>	<i>9 (9)</i>	<i>10 (7)</i>	<i>9 (8)</i>	<i>10 (10)</i>	<i>10 (11)</i>	<i>8 (1)</i>	<i>2 (0)</i>
F	CFZ, CIP	CFZ, FA	CFZ, GEN, AZM	CFZ, GEN, CIP	CFZ, GEN, FA	CFZ, AZM, CIP	CFZ, AZM, FA	CFZ, CIP, FA	OXA, GEN	OXA, AZM	OXA, CIP	OXA, FA
	11 (0)	11 (4)	11 (1)	11 (0)	11 (7)	11 (0)	11 (10)	11 (10)	11 (6)	11 (0)	11 (0)	11 (6)
	<i>2 (0)</i>	<i>12 (7)</i>	<i>7 (1)</i>	<i>9 (1)</i>	<i>11 (8)</i>	<i>3 (1)</i>	<i>11 (6)</i>	<i>12 (8)</i>	<i>7 (3)</i>	<i>1 (0)</i>	<i>2 (1)</i>	<i>11 (7)</i>
G	OXA, GEN, AZM	OXA, GEN, CIP	OXA, GEN, FA	OXA, AZM, CIP	OXA, AZM, FA	OXA, CIP, FA	VAN, GEN	VAN, AZM	VAN, CIP	VAN, FA	VAN, GEN, AZM	VAN, GEN, CIP
	10 (0)	11 (0)	11 (11)	11 (0)	11 (11)	11 (8)	11 (5)	11 (2)	11 (1)	11 (10)	11 (5)	11 (1)
	<i>5 (2)</i>	<i>8 (2)</i>	<i>10 (8)</i>	<i>3 (1)</i>	<i>12 (9)</i>	<i>12 (8)</i>	<i>11 (6)</i>	<i>11 (5)</i>	<i>12 (5)</i>	<i>12 (10)</i>	<i>11 (3)</i>	<i>12 (2)</i>
H	VAN, GEN, FA	VAN, AZM, CIP	VAN, AZM, FA	VAN, CIP, FA	GEN, AZM	GEN, CIP	GEN, FA	AZM, CIP	AZM, FA	AZM, CIP, FA	Growth control	Sterility control
	10 (9)	11 (1)	11 (10)	11 (8)	11 (1)	11 (3)	11 (10)	11 (0)	11 (9)	11 (10)	11 (10)	11 (9)
	<i>11 (8)</i>	<i>11 (2)</i>	<i>12 (10)</i>	<i>12 (8)</i>	<i>5 (1)</i>	<i>7 (1)</i>	<i>11 (10)</i>	<i>3 (1)</i>	<i>11 (8)</i>	<i>11 (9)</i>	<i>11 (9)</i>	<i>11 (9)</i>
	17 (13)	17 (5)	17 (14)	17 (12)	6 (1)	4 (0)	13 (12)	3 (5)	13 (13)	14 (13)		

^a MSSA, *n* = 11; MRSA, *n* = 12; *S. epidermidis*, *n* = 17. Results in italics are for MRSA; those in boldface are for *S. epidermidis*; those in regular font are for MSSA. See the footnote to Table 2 for drug abbreviations.

binations active against MRSA, 3 of 9 active against *S. epidermidis*, and 8 of 11 active against MSSA. Vancomycin was in five combinations active against *S. epidermidis*, one combination active against MRSA, and four combinations active against MSSA. Only two antibiotic combinations (containing rifampin and fusidic acid, plus either ciprofloxacin or vancomycin) were consistently bactericidal against MRSA biofilms. Table 8 summarizes all the results, indicating the activity of each combination against planktonic forms and biofilms of each isolate.

DISCUSSION

In this study, we evaluated antimicrobial susceptibility of pathogenic strains of staphylococci. We confirmed increased resistance of biofilm-associated staphylococci, and we demonstrated that bacteria in biofilms were susceptible to a smaller number of antimicrobial combinations than were planktonic forms of the same strains of staphylococci. Rifampin, vancomycin, and fusidic acid were the antibiotics most commonly included in combinations active against staphylococcal biofilms. Rifampin was a constituent of all the combinations active against *S. epidermidis* and MRSA and should be part of any antibiotic therapy directed against biofilms due to these organisms. Fusidic acid was implicated in 10 of 13 most active combinations against *S. aureus* and is a useful constituent of presumptive therapy against *S. aureus* biofilms.

Rifampin was the single most active agent against biofilms. Paradoxically, it was better as a bactericidal agent than as an inhibitory one. Our data suggest that rifampin acts to reduce adherence of the biofilm organisms to the surfaces of the pins causing the release of increasing numbers of bacteria off the biofilm into the broth, thereby resulting in an apparent lack of inhibitory activity against the organism.

Although biofilms were generally insensitive to individual antimicrobials, they were frequently susceptible to combinations. Almost half of the combinations tested were bactericidal against staphylococcal biofilms, whereas individual antibiotics with the exception of rifampin were less active. This finding is consistent with accepted clinical practice where combinations of two or more antimicrobials are used to treat implant-associated infections (10, 15). Combinations that are frequently active are ones that should be considered for presumptive therapy of staphylococcal foreign body infections.

MCBT sacrifices multiple dilutions of a given antibiotic for a single breakpoint in order to test multiple combinations. The test is suitable for the clinical laboratory in that it can screen a large number of antibiotic combinations against a pathogenic strain of bacterium to attempt to predict efficacy. We plan to continue work to assess the usefulness of biofilm MCBT testing through clinical trials to determine whether antibiotic therapy

guided by results of MCBT biofilm cultures results in improved clinical outcomes for patients with biofilm-associated infections.

Preliminary clinical data support the use of agents active against biofilms in device-related infection (8, 16). The development of our understanding of biofilms as a primary element in the pathogenesis of infection, and the development of new in vitro techniques for testing of biofilm antibiotic susceptibilities, offers the potential for novel uses of old antibiotic agents as well as novel possible targets for antimicrobial chemotherapy.

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