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Minimum Inhibitory and Bactericidal Concentrations of 44 Antimicrobial Agents Against Three Standard Control Strains in Broth with and Without Human Serum

LARRY G. REIMER,†* CHARLES W. STRATTON,‡ AND L. BARTH RELLER

Division of Infectious Diseases and Clinical Microbiology, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Standard minimum inhibitory and bactericidal concentrations are not established for most antimicrobial agents against strains of bacteria commonly used for quality control in susceptibility testing. The effects of cation and human serum supplementation of broth on the values are also unknown. Therefore, we performed 10 minimum inhibitory and bactericidal concentration determinations for 44 antimicrobial agents against the standard control strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 in Mueller-Hinton broth and in Mueller-Hinton broth supplemented with calcium, magnesium, and 50% pooled human serum. Agreement of replicates was within one twofold dilution 97% of the time. Supplemented Mueller-Hinton broth gave higher minimum inhibitory concentrations for 24 antibiotics against *S. aureus*, for 17 drugs against *E. coli*, and for 12 drugs against *P. aeruginosa*, whereas it gave lower minimum inhibitory concentrations for 1 antibiotic against *S. aureus*, for 5 against *E. coli*, and for 5 against *P. aeruginosa*. Results for minimum bactericidal concentrations were similar. Added serum did not further affect the increased resistance of *P. aeruginosa* to aminoglycosides encountered with cation supplementation of broth. These results provide expected values for the quality control strains when minimum inhibitory and bactericidal concentrations are determined in these two Mueller-Hinton media.

The broth dilution method is frequently used for antimicrobial susceptibility testing in both research and clinical laboratories. The microtiter adaptation is now considered an acceptable alternative (7, 18) to macro methods and to disk diffusion testing. Despite the recognized need for use of standard strains as a quality control measure (7, 12), only one paper has been published with minimum inhibitory concentrations (MICs) for multiple antimicrobial agents tested in Mueller-Hinton broth (6). However, that paper did not include data for *Staphylococcus aureus* ATCC 25923, nor were minimum bactericidal concentrations (MBCs) reported.

The effect of calcium and magnesium supplementation on MIC and MBC values for aminoglycosides against *Pseudomonas aeruginosa* is well known, and the use of Mueller-Hinton broth (MHB) supplemented with calcium and magnesium (MHB-S) has been recommended for broth dilution testing (2, 4, 9, 14). In addition, the use of MHB-S containing 50% pooled human serum (MHB-S/HS) has been proposed as an appropriate method for performing serum dilution tests (15), since protein binding is known to affect antimicrobial activity (13, 16). Therefore, results of MIC and MBC testing of antibiotics against standard control strains in MHB-S and MHB-S/HS also would be of value.

In this study we performed MIC and MBC determinations for control strains of *Escherichia coli*, *S. aureus*, and *P. aeruginosa* against a wide variety of antibiotics in MHB and MHB-S/HS, and for *P. aeruginosa* against aminoglycosides in MHB-S as well. We compared the results obtained in these media, and we reevaluated the reproducibility of microtiter broth dilution testing.

MATERIALS AND METHODS

Microorganisms. We tested the control strains *E. coli* ATCC 25922 (American Type Culture Collection, Rockville, Md.), *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 27853.

Antibiotics. Standard powders or solutions of all antimicrobial agents were kindly provided by their manufacturers. Drugs used are listed in Table 1. Each

† Present address: Division of Infectious Disease, University of West Virginia Medical Center, Morgantown, WV 26506.
‡ Present address: Department of Pathology, Vanderbilt University Medical Center, Nashville, TN 37232.

TABLE 1. *Mean MICs*

Drug	*S. aureus* ATCC 25923		*E. coli* ATCC 25922		*P. aeruginosa* ATCC 27853		
	MHB	MHB-S/HS	MHB	MHB-S/HS	MHB	MHB-S/HS	MHB-S
Penicillins							
Penicillin G	0.1	0.1[a]	76.8	70.4	>512	>512	
Amoxicillin	0.2	0.2	5.2	8.8[a]	>512	>512	
Ampicillin	0.1	0.1	6.0	4.0	>512	>512	
Cloxacillin	0.3	0.9[a]	358	512[a]	>512	>512	
Dicloxacillin	0.2	1.0[a]	>512	≥512	>512	>512	
Methicillin	1.2	1.3	>512	>512	>512	>512	
Nafcillin	0.2	0.8[a]	≥512	>512	≥512	≥512	
Oxacillin	0.2	0.7[a]	333	512[a]	>512	>512	
Carbenicillin	0.9	1.3	16.8	10.0[a]	76.8	32.0[a]	
Piperacillin	0.5	1.0[a]	2.2	1.1[a]	6.0	3.8[a]	
Ticarcillin	0.8	1.6[a]	8.4	11.0	54.4	22.4[a]	
Tetracyclines							
Tetracycline	0.7	1.2[a]	1.1	2.0[a]	18.4	83.2[a]	
Doxycycline	0.1	0.7[a]	0.4	2.4[a]	30.4	>32.0[a]	
Minocycline	0.1	0.3[a]	0.3	1.1[a]	16.0	>64.0[a]	
Chloramphenicol	6.8	8.0	1.9	2.6	>64.0	>64.0	
Cephalosporins and related antibiotics							
Cephalothin	0.1	0.3[a]	16.0	27.2[a]	>512	>512	
Cefaclor	1.3	3.8[a]	6.8	16.8[a]	>512	>512	
Cefamandole	0.2	0.2	1.0	1.0	>512	>512	
Cefazolin	0.2	0.7[a]	1.7	4.4[a]	>512	>512	
Cefoxitin	2.2	3.0	3.6	4.0	>512	>512	
Cefuroxime	0.7	1.6[a]	7.2	6.8	≥512	307[a]	
Cephalexin	2.0	2.0	14.4	16.0	>512	>512	
Cephaloridine	0.1	0.1	6.4	6.4	>512	>512	
Cephapirin	0.3	0.2	16.0	19.2	>512	>512	
Cephradine	2.2	2.2	22.4	16.0	>512	>512	
Moxalactam	4.6	6.0	0.3	0.2	19.2	8.0[a]	
N-Formimidoyl thienamycin (MK0787)	0.1	0.1	0.1	2.2	2.2	2.4	
Aminoglycosides							
Amikacin	0.6	2.9[a]	1.2	2.8[a]	2.0	4.5	6.2[b]
Gentamicin	0.1	0.6[a]	0.4	1.4[a]	0.8	4.0	5.2[b]
Kanamycin	0.4	2.2[a]	1.7	2.1	>128	>128	>128
Netilmicin	0.1	0.1	0.2	0.6[a]	0.7	4.0	4.8[b]
Sisomycin	0.1	0.1	0.2	0.5[a]	0.1	1.2	1.3[b]
Streptomycin	1.1	2.8[a]	2.2	2.0	16.0	20.8	>32.0[c]
Tobramycin	0.1	0.4[a]	0.5	0.5	0.1	0.8	1.3[b]
Peptides							
Colistin	>32.0	>32.0	0.1	0.2[a]	0.1	1.4[a]	
Polymyxin B	20.8	>32.0[a]	0.1	0.2[a]	0.1	1.6[a]	
Miscellaneous							
Clindamycin	0.1	0.1	64.0	>64.0[a]	>64.0	>64.0	
Erythromycin	0.4	0.2[a]	>64.0	24.0[a]	>64.0	>64.0	
Lincomycin	1.2	0.7[a]	>64.0	>64.0	>64.0	>64.0	
Metronidazole	>32.0	>32.0	>32.0	>32.0	>32.0	>32.0	
Nalidixic acid	35.2	>128[a]	1.0	10.4[a]	128	>128[a]	
Nitrofurantoin	12.0	27.2[a]	5.2	13.6[a]	>512	>512	
Rifampin	0.1	0.1[a]	6.4	8.8	32.0	28.8	
Vancomycin	0.8	1.0	≥512	320[a]	>512	≥512	

[a] Means significantly different from MHB value at $P < 0.05$ level.

[b] Mean in broth significantly different from mean in both supplemented media; supplemented media not significantly different from each other.

[c] Mean in broth with serum significantly different from means in broth and broth supplemented with Ca^{2+} and Mg^{2+}.

antimicrobial powder was weighed once and prepared in stock solution by adding distilled water or a recommended diluent to obtain a concentration of 2,560 μg/ml. Ten separate dilutions of the stock solution to an appropriate starting concentration and 10 separate MIC and MBC determinations were performed for a given agent against each bacterial strain. All antimicrobial agents were used fresh on the day prepared.

Broth media. We used commercial MHB (lot 529409; Difco Laboratories, Detroit, Mich.). This

MHB contains 9.8 mg of Ca^{2+} and 2.9 mg of Mg^{2+} per liter of broth (14); MHB-S was prepared by adding 50 mg of Ca^{2+} and 20 mg of Mg^{2+} per liter of broth. MHB-S/HS was prepared by combining MHB-S with pooled human serum (Flow Laboratories, Inglewood, Calif.) in a 1:1 ratio. The pooled human serum was preheated to 56°C for 20 min and had no intrinsic activity against these bacteria when tested by a disk diffusion method.

Broth dilution testing. The MIC for each combination was determined in duplicate by a microtiter modification (17) of the method chosen by the International Collaborative Study (5). Microtiter trays containing 96 U-bottomed wells were used (Cooke Engineering Co., Alexandria, Va.). Each well was filled with 50 μl of MHB or HS, and serial twofold dilutions of the drug were performed on a Cooke automatic diluter. The starting concentration of antibiotic tested was two dilution steps greater than achievable serum concentrations (21). Bacterial strains were suspended in saline 10 separate times after overnight growth on agar medium, diluted in MHB or MHB-S, and added in an equal volume (50 μl) to the plates containing MHB or HS, respectively. The final inoculum size was 10^5 to 10^6 bacteria per ml. For testing *P. aeruginosa* the same procedure was also performed, using MHB-S as both diluent and inoculating broth. MIC endpoints were read as the lowest concentration of antibiotic with no turbidity. If the MIC was less than the lowest concentration tested, 10 new replicates were tested at lower concentrations. The MBC for each strain was determined by subculturing 3 μl from each microtiter well by multipoint inoculator to drug-free Mueller-Hinton agar. MBC endpoints were read as the lowest dilution of drug with no growth (>99.9% killing) after overnight incubation at 35°C.

Statistical analysis. Mean values were calculated as arithmetic means for 10 replicates assumed to have equal value. For combinations with MIC or MBC values greater than or equal to the highest concentration tested, the mean was recorded as greater than or equal to that concentration. The comparison of MHB and MHB-S/HS was analyzed by the Mann-Whitney test statistic performed on a Hewlett-Packard HP/97 programmable printing calculator (Hewlett-Packard Company, Corvallis, Oreg.). Data for all three media in testing *P. aeruginosa* against the aminoglycosides were analyzed by the method of Wilcoxon and Wilcox for more than two variables (20).

RESULTS

Mean MICs are presented in Table 1 for all 44 agents against the three control strains in MHB and MHB-S/HS and for the aminoglycosides against *P. aeruginosa* in MHB-S. Comparisons for which mean values were significantly different in the different media are footnoted. Table 2 presents this comparison grouped by classes of antimicrobial agents. The mean MIC was affected by addition of serum, calcium, and magnesium in testing *S. aureus* for 25 agents, *E. coli* for 22 agents, and *P. aeruginosa* for 17. In general the addition of serum, calcium, and magnesium increased resistance. However, carbenicillin, piperacillin, erythromycin, and vancomycin inhibited *E. coli* at lower concentrations in MHB-S/HS. As expected, MICs for aminoglycosides against *P. aeruginosa* were increased by calcium and magnesium with no further effect upon addition of serum. The only exception was streptomycin, for which serum decreased the MIC significantly.

Mean MBCs appear in Table 3. Comparisons are presented as for MICs. Fewer significant differences were found, in part because more bacteria grew in the highest concentration of antibiotic tested. The trends, however, were the same as with MICs.

Agreement between 10 replicate determinations for each combination of antibiotic and organism was high. Ninety-seven percent of all MICs and 92% of all MBC determinations were within the mean ± one dilution.

DISCUSSION

We determined MICs and MBCs for 44 antibiotics in common usage against the three most

TABLE 2. *Comparison of MICs and MBCs obtained in MHB versus MHB-S/HS*

Determination	Antibiotic group	No. of drugs[a]								
		S. aureus ATCC 25923			*E. coli* ATCC 25922			*P. aeruginosa* ATCC 27853		
		Serum >	Broth >	Same	Serum >	Broth >	Same	Serum >	Broth >	Same
MIC	Penicillins	7	0	4	3	2	6	0	3	8
	Cephalosporins and related antibiotics	4	0	7	3	0	8	0	2	9
	Aminoglycosides	5	0	2	4	0	3	6	0	1
	Others	8	1	6	8	2	5	6	0	9
MBC	Penicillins	6	0	5	0	2	9	0	1	10
	Cephalosporins and related antibiotics	4	0	7	4	2	5	0	1	10
	Aminoglycosides	6	0	1	4	0	3	6	0	1
	Others	2	0	13	7	0	8	5	0	10

[a] Number of drugs for which MIC or MBC is larger in MHB-S/HS (Serum >) or in MHB (Broth >) or is the same in both media (Same).

TABLE 3. *Mean MBCs*

Drug	*S. aureus* ATCC 25923		*E. coli* ATCC 25922		*P. aeruginosa* ATCC 27853		
	MHB	MHB-S/HS	MHB	MHB-S/HS	MHB	MHB-S/HS	MHB-S
Penicillins							
Penicillin G	0.1	0.2[a]	141	70.4[a]	>512	>512	
Amoxicillin	0.3	0.3	9.6	13.6	>512	>512	
Ampicillin	0.1	0.1	8.8	8.4	>512	>512	
Cloxacillin	0.3	1.2[a]	486	≥512	>512	>512	
Dicloxacillin	0.3	1.1[a]	>512	>512	>512	>512	
Methicillin	1.7	1.8	>512	>512	>512	>512	
Nafcillin	0.2	1.2[a]	≥512	>512	>512	>512	
Oxacillin	0.2	0.9[a]	461	≥512	>512	>512	
Carbenicillin	1.0	1.2	35.2	10.8[a]	109	54.4	
Piperacillin	0.7	1.0	2.4	2.0	12.4	7.6	
Ticarcillin	1.1	2.1[a]	14.8	12.6	70.4	41.6[a]	
Tetracyclines							
Tetracycline	>16.0	>16.0	≥32.0	≥32.0	86.4	>256[a]	
Doxycycline	>8.0	>8.0	35.2	≥64.0[a]	≥64.0	>64.0[a]	
Minocycline	>8.0	>8.0	≥32.0	>64.0[a]	41.6	>64.0	
Chloramphenicol	>64.0	>64.0	≥64.0	>64.0	>64.0	>64.0	
Cephalosporins and related antibiotics							
Cephalothin	0.2	0.6[a]	19.2	33.6[a]	>512	>512	
Cefaclor	2.0	6.8[a]	10.4	>64.0[a]	>512	>512	
Cefamandole	0.2	0.2	1.5	1.6	>512	>512	
Cefazolin	0.2	1.2[a]	2.4	7.6[a]	>512	>512	
Cefoxitin	3.2	5.0	6.0	7.6	>512	>512	
Cefuroxime	2.4	3.6	12.8	8.4[a]	>512	>512	
Cephalexin	2.5	3.0	16.0	28.8[a]	>512	>512	
Cephaloridine	0.1	0.1	9.2	10.0	>512	>512	
Cephapirin	0.3	0.3	30.4	27.2	>512	>512	
Cephradine	3.4	3.0	27.2	20.8	>512	>512	
Moxalactam	5.6	8.8[a]	0.3	0.2[a]	30.4	20.8[a]	
N-Formimidoyl thienamycin (MK0787)	0.1	0.1	0.2	0.3	5.6	12.4	
Aminoglycosides							
Amikacin	0.5	2.6[a]	1.5	3.2[a]	3.5	12.0	8.0[b]
Gentamicin	0.1	0.7[a]	0.5	1.6[a]	1.4	7.2	6.0[b]
Kanamycin	1.0	5.6[a]	1.8	2.9	>128	>128	>128
Netilmicin	0.1	0.3[a]	0.2	0.7[a]	1.3	8.0	8.0[b]
Sisomycin	0.1	0.1	0.2	0.5[a]	0.2	2.4	2.3[b]
Streptomycin	1.9	4.0[a]	2.2	2.2	≥32.0	≥32.0	>32.0[b]
Tobramycin	0.1	0.3[a]	0.6	0.7	0.3	1.3	2.1[b]
Peptides							
Colistin	>32.0	>32.0	0.1	0.2[a]	0.1	1.8[a]	
Polymyxin B	>32.0	>32.0	0.1	0.2[a]	0.3	3.2[a]	
Miscellaneous							
Clindamycin	0.2	0.2	≥64.0	>64.0	>64.0	>64.0	
Erythromycin	16.0	24.8	>64.0	≥64.0	>64.0	>64.0	
Lincomycin	>32.0	>32.0	>64.0	>64.0	>64.0	>64.0	
Metronidazole	>32.0	>32.0	>32.0	>32.0	>32.0	>32.0	
Nalidixic acid	64.0	>128[a]	1.6	28.8[a]	>128	>128	
Nitrofurantoin	16.8	32.2[a]	8.4	15.2[a]	>512	>512	
Rifampin	0.5	0.8	8.0	27.2[a]	>32.0	>32.0	
Vancomycin	1.1	1.5	>256	>256	>512	>512	

[a] Means significantly different from MHB value at $P < 0.05$ level.

[b] Mean in broth significantly different from mean in both supplemented media; supplemented media not significantly different from each other.

commonly used bacterial control strains. Since the microtiter broth dilution method is commonly employed with inclusion of these strains for quality control, it is intended that our data will provide guidelines for intra- and interlaboratory control determinations. A major advantage of broth dilution testing is the ability to easily provide MBCs as well as MICs, and we have tabulated values for the control strain MBCs as well.

Several authors have noted the great reproducibility of the microtiter broth methods (1, 3, 8, 10, 11, 19). We found that 97% of MICs and 92% of MBCs were ± one dilution from the mean. No MICs involved more than four dilution steps, and only 1 of 164 interpretable MBCs involved five dilution steps. Past studies have not evaluated the reproducibility of MBCs performed from microtiter broth dilution plates. Despite the increased number of technical steps involved, we found MBCs, as well as MICs, to be reproducible.

The effect of calcium and magnesium ions on MICs for aminoglycosides against *P. aeruginosa* is well known (2, 9, 14). The effect of these cations and pooled human serum on MICs of a wide variety of antibiotics for other bacteria is not as well established. We confirmed the increased resistance of *P. aeruginosa* to aminoglycosides in the presence of increased Ca^{2+} and Mg^{2+} concentrations. This change was not further affected by the addition of pooled human serum. Of 44 antibiotics, 24 were also less effective against *S. aureus*, and 18 were less effective against *E. coli* in MHB-S/HS. We did not examine the effects of human serum and cations individually on altered susceptibility.

The effects of cation and serum supplementation are complex. For example, carbenicillin, piperacillin, and ticarcillin were more effective with supplementation against *P. aeruginosa*, as were carbenicillin and piperacillin against *E. coli*. In contrast, piperacillin and ticarcillin were less effective against *S. aureus* when supplemented. Our results differ from those of D'Amato et al. (4) in testing carbenicillin against *P. aeruginosa*. They found no effect with calcium and magnesium supplementation without serum. Reller et al. (14) did find increased susceptibility of *P. aeruginosa* to carbenicillin at certain concentrations of calcium and magnesium. Finally, aminoglycosides differed from the other antibiotics in that their activity was several-fold less for all three test organisms in MHB-S/HS.

Possible explanations for the variable effects of supplementation include the presence of multiple anions, cations, amino acids, and trace elements in human serum as well as alterations caused by calcium, magnesium, and protein binding. The changes that occur, though statistically significant, are generally of minor consequence since mean MICs were only one dilution step apart by the two methods. However, certain combinations of an antimicrobial agent and an organism have fourfold differences in mean MICs which could be misleading in a clinical setting.

Although the most clinically relevant broth for performing broth dilution testing remains to be established, performance testing with control strains in the same broth is clearly important.

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