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Evaluation of a Proprietary Broth Medium for Microdilution Susceptibility Testing of Nutritionally Fastidious Bacteria

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For microdilution susceptibility tests with nutritionally fastidious microorganisms, a new clear broth medium developed at Micro-Media Systems, Inc., Potomac, Md., was evaluated in a three-laboratory collaborative study. Replicate tests were performed with 80 isolates (51 *Streptococcus* spp., 27 *Haemophilus influenzae* isolates, and 2 *Neisseria meningitidis* isolates) against 15 antimicrobial agents. In standard 100-μl volumes, results of tests in the new broth medium were comparable to those in the reference medium (Mueller-Hinton broth with 2 to 3% lysed horse blood), but MICs were somewhat easier to read in the new broth medium. Results of similar tests in smaller panels, containing 40 μl in each well, were less satisfactory; i.e., growth failures and poorly defined endpoints were more commonly encountered. With drugs other than erythromycin or clindamycin, the 40-μl panels provided MICs which compared favorably with those obtained by standard reference methods.

Modification of standard broth media is necessary to perform antimicrobial susceptibility tests with nutritionally fastidious microorganisms such as *Haemophilus* spp., most nonenterococcal streptococci, and meningococci. The National Committee for Clinical Laboratory Standards (2) recommends the addition of 2 to 3% lysed horse blood to cation-supplemented Mueller-Hinton broth when nonenterococcal streptococci or *Listeria* spp. are being tested. For testing *Haemophilus* spp., 10 μg of NAD per ml should be added, in addition to lysed horse blood.

Micro-Media Systems, Inc., Potomac, Md., has recently developed a proprietary medium for testing nutritionally fastidious microorganisms (M. J. Lawrie and R. B. Carey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, C297, p. 286; J. M. McCarty and J. C. Craft, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, C223, p. 273). This medium is a modification of Schaedler broth with defined supplements and is referred to as fastidious broth. It is a clear medium that supports growth of most streptococci, meningococci, *Haemophilus* spp., and *Listeria* spp.

This study was undertaken to determine whether results of microdilution susceptibility tests in the new broth medium are comparable to those obtained by a standard reference procedure (2). The two media were tested in standard 80-well microdilution panels with 100 μl in each well. Smaller, 118-well panels containing 40 μl of the new broth medium per well were also evaluated.

MATERIALS AND METHODS

Microdilution test panels. Three types of microdilution test panels were prepared by Micro-Media Systems and distributed to the participating laboratories. Each test system contained doubling dilutions of 15 antimicrobial agents (Table 1). Test panels with 118 separate wells, each well containing 40 μl of fastidious broth, were evaluated, and standard 80-well test panels containing 100 μl of fastidious broth were also tested. Two separate panels were required to match all of the concentrations included in the 40-μl test panels. Two additional 80-well panels were prepared with 100 μl of cation-supplemented Mueller-Hinton broth and antimicrobial agents at twice the desired concentration. To inoculate these panels, lysed horse blood in cation-supplemented Mueller-Hinton broth was inoculated, and 100 μl was then added to each well, resulting in a total volume of 200 μl per well. The lysed horse blood was prepared to obtain a final concentration of 2 to 3%. During testing of *Haemophilus* spp., NAD (10 μg/ml) was added to the lysed horse blood.

Test procedure. Isolated colonies were selected from an overnight agar plate and suspended in saline. The suspension was then adjusted to match a 0.5 McFarland turbidity standard. Each suspension was then used to inoculate all three test systems, after appropriate dilution. The final inoculum density in all three systems was approximately 1×10^5 to 5×10^5 CFU/ml. Each laboratory tested each isolate on 3 separate days, by using colonies from overnight cultures. The inoculated panels were incubated for 18 to 20 h at 35°C in ambient air (without increased CO_2).

Microorganisms. A collection of 20 isolates was tested in triplicate by all three laboratories. This collection included seven *Haemophilus influenzae* isolates (four β-lactamase-producing strains), five *Streptococcus pneumoniae* isolates, two *Streptococcus pyogenes* isolates, two *Streptococcus sanguis* isolates, two *Streptococcus salivarius* isolates, and two *Neisseria meningitidis* isolates. In addition, two laboratories each performed triplicate tests with 30 different recent clinical isolates, representing 14 streptococci of the viridans group, 13 *S. pneumoniae* isolates, 13 β-hemolytic streptococci, and 20 *H. influenzae* isolates (10 β-lactamase producing strains).

Control strains. All three laboratories performed triplicate tests, using three methods and five different control strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Streptococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, and *H. influenzae* ATCC 43163). The *H. influenzae* strain was recommended by Micro-Media Systems to be a control for their test panels;

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TABLE 1. Comparison of microdilution MICs in fastidious broth compared to 200-μl tests in Mueller-Hinton broth with lysed horse blood[a]

Antimicrobial agent and test system[b]	No. with both MICs off scale[c]	Differences between modal MICs[d] as 2× dilutions								
		−4	−3	−2	−1	0[c]	+1	+2	+3	+4
Ampicillin										
100 μl FB vs LHB	51				1	59	17	3		
40 μl FB vs LHB	52				3	65	11	1		
Penicillin										
100 μl FB vs LHB	26			2	5	52	17	3	1	
40 μl FB vs LHB	20				4	44	19	12	1	
Oxacillin										
100 μl FB vs LHB	34			2	1	45	28	3	1	
40 μl FB vs LHB	38			2	5	47	22	3	1	
Cefazolin										
100 μl FB vs LHB	47		1	1	7	60	10	1		
40 μl FB vs LHB	47		1	14	10	54	1			
Cefuroxime										
100 μl FB vs LHB	73				1	75	4			
40 μl FB vs LHB	75				2	76	2			
Cefotaxime										
100 μl FB vs LHB	80					80				
40 μl FB vs LHB	80					80				
Ceftizoxime										
100 μl FB vs LHB	76			1		78	1			
40 μl FB vs LHB	76			1		77	2			
Ceftriaxone										
100 μl FB vs LHB	79					79	1			
40 μl FB vs LHB	79					79	1			
Ceftazidime										
100 μl FB vs LHB	68				2	70	7			1
40 μl FB vs LHB	69				3	71	4	1		1
Moxalactam										
100 μl FB vs LHB	32		1		10	55	11	3		
40 μl FB vs LHB	34		1	2	12	51	12	2		
Tetracycline										
100 μl FB vs LHB	44	1		3	7	58	11			
40 μl FB vs LHB	39			2	7	50	21			
Chloramphenicol										
100 μl FB vs LHB	2			2	13	64	1			
40 μl FB vs LHB	2		2	2	9	58	9			
Vancomycin										
100 μl FB vs LHB	58			1	7	71	1			
40 μl FB vs LHB	53			1	15	63	1			
Clindamycin										
100 μl FB vs LHB	53			3	13	63	1			
40 μl FB vs LHB	51	1	12	10	6	51				
Erythromycin										
100 μl FB vs LHB	48		2	2	13	61	2			
40 μl FB vs LHB	48		7	14	10	48	1			
All drugs (% of total)										
100 μl FB vs LHB	64.2	0.1	0.3	1.4	6.7	80.8	9.3	1.1	0.2	0.1
40 μl FB vs LHB	63.6	0.1	1.9	4.0	7.1	76.2	8.8	1.6	0.2	0.1

[a] A total of 51 *Streptococcus* spp. isolates, 27 *H. influenzae* isolates, and 2 *N. meningitidis* isolates were tested.
[b] Tests in 100- or 40-μl volumes of fastidious broth (FB) compared with tests in 200-μl volumes of Mueller-Hinton broth with lysed horse blood (LHB).
[c] Number of strains with both modal MICs off scale; these values are included in the table as 0 differences between modes.
[d] Mode of three values (40 *Streptococcus* spp. isolates and 20 *H. influenzae* isolates) or mode of six to nine values (11 *Streptococcus* spp. isolates, 7 *H. influenzae* isolates, and 2 *N. meningitidis* isolates) were compared.

the other strains are recommended by the National Committee for Clinical Laboratory Standards (2) for quality control of microdilution tests.

RESULTS AND DISCUSSION

Control strains. All MICs for the standard control strains were within the expected ranges (2). However, in both broth media, *Streptococcus faecalis* ATCC 29212 appeared to be unusually susceptible to cefotaxime, ceftizoxime, ceftriaxone, and cefuroxime (modal MICs, 1.0 to 8.0 μg/ml). Sahm et al. (3) have previously documented the effect of blood or blood products on the in vitro activity of such structurally related cephalosporins against *S. faecalis*. If the *S. faecalis* control strain is to be used for monitoring the performance of any one of the systems that we studied, appropriate control limits for the cephalosporins will need to be established. The fastidious broth should not be used for routine susceptibility testing of enterococci.

Replicate tests with the four nonfastidious control strains

TABLE 2. Erythromycin and clindamycin MICs in three microdilution test systems[a] with 27 isolates of *H. influenzae*

MIC (μg/ml)	No. of times each MIC recorded for each test method[b]					
	Erythromycin			Clindamycin		
	200 μl LHB	100 μl FB	40 μl FB	200 μl LHB	100 μl FB	40 μl FB
>8.0				31	16	2
8.0	3	3		46	21	2
4.0	74	34	1	30	43	10
2.0	29	52	13	7	27	25
1.0	11	18	54	6	9	45
0.5	4	8	28	2	3	17
0.25	1	4	6			5
≤0.12			5			1
No growth	1	4	16	1	4	16

[a] Volumes (200 μl) of Mueller-Hinton broth with NAD and lysed horse blood (LHB) and fastidious broth (FB) in 100- or 40-μl volumes.
[b] A total of 7 isolates were tested nine times and 20 isolates were tested three times; 21 growth failures were reported.

demonstrated comparable results among the three systems; i.e., modal MICs were all within a range of ±1 doubling dilution. Among the two standard 80-well panels with different broth media, the *H. influenzae* control strain also provided modal MICs that differed by no more than 1 doubling dilution. However, in the 40-μl volume test system clindamycin, erythromycin, and cefazolin MICs with the *H. influenzae* control strain were two- to eightfold lower than those recorded for the 100- or 200-μl test systems.

Comparability of MICs. Results obtained with the two test systems in which the new broth medium was used were compared with those of the reference tests with lysed horse blood. Table 1 compares modal MICs for each of the 80 isolates included in this study. The two broth media that were tested in standard 80-well trays were in agreement (±1 doubling dilution) with 96.8% of all tests with the 15 antimicrobial agents. However, 64% of the pairs of modal MICs were both off scale (≤ lowest concentration tested or > greatest concentration tested). Because differences cannot be detected when both values are off scale, such data were omitted for this type of analysis. If such off-scale data were omitted, the two systems were in agreement with 91.1% of the 429 data pairs that were compared.

When the 40-μl volume tests were compared with the reference method, 92.1% of the modal values were in agreement (±1 doubling dilution). If tests with two off-scale modal values were excluded, 437 comparisons could be made, and only 78.5% of those values were in agreement. The majority of discrepancies involved tests with clindamycin and erythromycin against *H. influenzae*. Table 2 describes the spread of MICs recorded for replicate tests with 27 *H. influenzae* strains. A total of 123 determinations was made, but results of 21 tests were recorded as no growth. Both erythromycin and clindamycin MICs were significantly lower in the 40-μl volume system than in either of the other two systems. In the 100-μl volume fastidious broth, MICs for both drugs were approximately 1 doubling dilution lower than those obtained by the reference method. Such differences were not observed with other antimicrobial agents. We could find no obvious explanation for the discrepancy with these two drugs, both of which are notoriously susceptible to slight changes in pH.

A similar analysis of data with *H. influenzae* was carried out to determine whether ampicillin or penicillin MICs would distinguish between β-lactamase-producing and -nonproducing strains. Table 3 summarizes the results of tests with ampicillin. With two exceptions, all β-lactamase-producing strains provided ampicillin MICs of ≥4.0 μg/ml, and all β-lactamase-negative strains were inhibited by ≤1.0 μg/ml. The two exceptions represent tests with the reference method. Benzyl penicillin also separated the two types of *H. influenzae*. In all three systems, β-lactamase-producing strains were all resistant to 2.0 μg of penicillin per ml (the greatest concentration tested), and all β-lactamase-negative strains were inhibited by ≤2.0 μg/ml (reference method) or ≤1.0 μg/ml (new broth medium). We conclude that all three systems satisfactorily separate β-lactamase-producing strains of *H. influenzae* from β-lactamase-negative strains

TABLE 3. Ampicillin MICs in three systems[a] with 27 isolates of *H. influenzae*

Ampicillin MIC (μg/ml)	No. of β-lactamase-positive or -negative isolates[b]					
	200 μl LHB		100 μl FB		40 μl FB	
	Positive	Negative	Positive	Negative	Positive	Negative
>8.0	39		41		36	
8.0	8		11		9	
4.0	8		4		5	
2.0	1					
1.0	1	1		7		
0.5		10		48		32
0.25		46		8		24
≤0.12		8				1
No growth	0	1	1	3	7	9

[a] Volumes (200 μl) of Mueller-Hinton broth with NAD and lysed horse blood (LHB) and fastidious broth (FB) in 100- or 40-μl volumes.
[b] Number of times each MIC was recorded with 13 β-lactamase-positive strains or with 14 β-lactamase-negative strains (three to nine MICs were recorded for each isolate).

TABLE 4. Reproducibility of three microdilution test systems[a]

Test system[b]	No. of MICs recorded[c]	% Recorded MICs		
		At mode	Mode ± 1 dilution	Mode ± 2 dilutions
200 μl LHB	2,670	88.9	97.8	99.2
100 μl FB	2,625	88.8	98.7	99.4
40 μl FB	1,995	90.2	98.3	99.5

[a] A total of 20 isolates were tested in triplicate in each of three independent laboratories.

[b] Volumes (200 μl) of Mueller-Hinton broth with lysed horse blood (LHB) and fastidious broth (FB) tested in 100- or 40-μl volumes.

[c] A total of 2,700 MICs should have been recorded for each system. One laboratory reported no growth in 54 of 180 tests performed (47 with 40 μl of FB, 5 with 100 μl of FB, and 2 with 100 μl of LHB). The other two laboratories repeated growth failures until all 900 MICs were recorded by each laboratory.

but the two systems that use the new broth medium provide somewhat better separation than the reference method.

Reproducibility. Data obtained with the 20 isolates that were tested in all three laboratories were examined to document combined inter- and intralaboratory reproducibility (Table 4). Approximately 89 to 90% of the repeated tests provided identical MICs when triplicate tests were performed in each of three laboratories, and approximately 98% of all tests varied by no more than 1 doubling dilution. In that respect there were no important differences among the three testing procedures that were evaluated. However, one participating laboratory reported an inordinate number of growth failures, especially with the 40-μl volume test system. The two other participating laboratories repeated the tests until all desired data points were available. Growth failures were relatively uncommon in the two systems in which standard 80-well trays were used with 100 or 200 μl per well. An unsatisfactory number of growth failures occurred in the panels containing 40-μl volumes, and when growth did occur, the endpoints were difficult to read. In 100-μl volumes, the new broth medium provided the best growth and was judged to be the easiest system to read, but there is still room for improvement in the clarity of endpoint definitions. Use of the new broth medium should reduce, but not eliminate, the subjective element that influences reproducibility of all microdilution tests (1). Subjectivity is a particularly important source of variability when reading tests with *H. influenzae* in broth supplemented with lysed horse blood.

LITERATURE CITED

1. **Barry, A. L., and L. E. Braun.** 1981. Reader error in determining minimal inhibitory concentrations with microdilution susceptibility test panels. J. Clin. Microbiol. **13**:228–230.
2. **National Committee for Clinical Laboratory Standards.** 1985. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard. National Committee for Clinical Laboratory Standards publication M7-A. Villanova, Pa.
3. **Sahm, D. F., C. N. Baker, R. N. Jones, and C. Thornsberry.** 1984. Influence of growth medium on the in vitro activities of second- and third-generation cephalosporins against *Streptococcus faecalis*. J. Clin. Microbiol. **20**:561–567.