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Quantitative Antimicrobial Susceptibility Test for *Streptococcus pneumoniae* Using Inoculum Supplemented with Whole Defibrinated Sheep Blood

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The National Committee for Clinical Laboratory Standards recommends the use of lysed horse blood-supplemented Mueller-Hinton broth for determining the quantitative antimicrobial susceptibility of *Streptococcus pneumoniae*. This procedure may be difficult for laboratories using previously prepared or commercial MIC systems. Therefore, a study was undertaken to determine whether previously prepared microdilution trays containing Mueller-Hinton broth without blood could be used for determining the antimicrobial susceptibility of *S. pneumoniae* by adding whole defibrinated sheep blood to the bacterial suspension used to inoculate the trays. The presence of alpha-hemolysis was used as an indicator of bacterial growth. One hundred isolates of *S. pneumoniae* selected to represent a distribution of susceptibility patterns were tested by the National Committee for Clinical Laboratory Standards method and the sheep blood-supplemented-inoculum method. Greater than 94% agreement between the two methods was achieved. The sheep-blood-supplemented-inoculum procedure was highly reproducible and easy to perform and provides an acceptable alternative for determining the MICs for *S. pneumoniae* for laboratories using previously prepared or commercial microdilution systems.

The isolation of *Streptococcus pneumoniae* with relative resistance (MIC, 0.12 to 1 μg/ml) and complete resistance (MIC, >1 μg/ml) to penicillin has been reported with increasing frequency (1–11, 13, 14, 16). Screening for penicillin resistance is easily accomplished by the disk diffusion method with 1-μg oxacillin disks (12, 18). If quantitative values are desired, the National Committee for Clinical Laboratory Standards (NCCLS) dilution standard M7-A (15) recommends the use of lysed horse blood, which may be difficult for laboratories using previously prepared or commercial microdilution broth systems. Tarpay et al. (17) described a microdilution broth technique which uses Mueller-Hinton broth (MHB) supplemented with 5% defibrinated whole sheep blood. Hemolysis of erythrocytes indicated bacterial growth, and the MIC endpoint was defined as the lowest concentration of the antimicrobial agent demonstrating no hemolysis. This method can be used by those laboratories preparing their own microdilution trays but precludes the use of commercial microdilution broth systems or other plates previously prepared without blood. Therefore, we undertook a study to determine whether previously prepared microdilution trays containing MHB without blood could be used to determine the antimicrobial susceptibility of *S. pneumoniae* by adding whole defibrinated sheep blood to the bacterial suspension used to inoculate the trays.

MATERIALS AND METHODS

Antimicrobial susceptibility tests. (i) Reference method. Microdilution broth trays containing 0.1 ml of cation-supplemented MHB (Difco Laboratories, Detroit, Mich.) per well also supplemented with 5% lysed and centrifuged horse blood were prepared in accordance with NCCLS M7-A recommendations (15) at the Centers for Disease Control (CDC). The antimicrobial agents and the concentrations tested are given in Table 1. After preparation, all trays were stored at −70°C. Test organisms were streaked onto 5% sheep blood agar plates and incubated overnight at 35°C. A sufficient number of organisms to equal a 0.75 McFarland turbidity standard were suspended in sterile distilled water. This standard was chosen because plate counts indicated that the inoculator used in this phase of the study delivered the proper inoculum when a 0.75 McFarland turbidity standard was used to make the initial bacterial suspension. A 1:5 dilution of the suspension was made with sterile distilled water, and approximately 0.001 ml (1×10^5 to 5×10^5 CFU/ml) was delivered to each well of the microdilution tray with a Dynatech MIC 2000 inoculator (Dynatech Laboratories, Inc., Alexandria, Va.). The inoculated trays were incubated at 35°C for 18 h. Endpoints were read as the lowest concentration of antimicrobial agent that inhibited visible growth.

(ii) Whole defibrinated sheep blood-supplemented-inoculum method. Microdilution broth trays containing 0.05 ml of double-strength cation-supplemented MHB (Difco) and double-strength antimicrobial agent were prepared as directed in NCCLS M7-A (15) for testing at The Catholic Medical Center (CMC) and Analytab Products (API). The antimicrobial agents and concentrations tested were identical to those tested at CDC (Table 1). After preparation, all plates were stored at −70°C. For inoculum preparation, the test organisms were first streaked onto 5% sheep blood agar plates and incubated overnight at 35°C. A sufficient number of organisms to equal a 0.5 McFarland turbidity standard were

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TABLE 1. Distribution of antimicrobial susceptibility of *S. pneumoniae* isolates[a]

Antimicrobial agent	MIC (μg/ml) for category[b]			No. of strains in category			Concn (μg/ml) tested
	S	MS	R	S	MS	R	
Cefazolin	≤8	16	≥32	94	6	0	0.06–32
Cefotaxime	≤8	16–32	≥64	100	0	0	0.008–1
Ceftazidime	≤8	16–32	≥64	84	12	4	0.12–16
Ceftriaxone	≤8	16–32	≥64	100	0	0	0.008–1
Cefuroxime	≤8	16	≥32	100	0	0	0.015–2
Chloramphenicol	≤8	16	≥32	81	14	5	0.5–32
Erythromycin	≤0.5	1–4	≥ 8	85	2	13	0.12–16
Penicillin	≤0.06	0.12–1	≥ 2	36	48	16	0.015–8
Tetracycline	≤4	8	≥16	63	2	35	0.25–16
Vancomycin	≤4	8–16	≥32	100	0	0	0.12–16

[a] A total of 100 isolates were tested.
[b] From NCCLS standard M7-A. S, Susceptible; MS, moderately susceptible (relatively resistant, for penicillin); R, resistant.

suspended in 0.85% sterile saline. The inoculum was diluted to 1×10^5 to 5×10^5 CFU/ml, as determined by plate count, by using 0.85% saline containing 10% whole defibrinated sheep blood. Each well of the microdilution trays received 0.05 ml of inoculum. The final concentration of sheep blood in each well was 5%.

The inoculated trays were incubated at 35°C for 18 h. Endpoints were read as the lowest concentration of antimicrobial agent demonstrating no hemolysis (no greening of the blood).

Bacteria. One hundred isolates of *S. pneumoniae* (19 blind duplicates) selected from the CDC collection to represent a distribution of susceptibility patterns (Table 1) were blindly coded and distributed to CMC and API. All bacteria were stored at −70°C until used. All isolates were tested at CDC by the reference method, and 97 and 98 were tested at CMC and API, respectively, by the whole defibrinated sheep blood-supplemented-inoculum method.

Quality control. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Streptococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* CDC 78-008101, and *S. pneumoniae* CDC 78-008102 were included in each test run to assess the performance of both methods.

RESULTS

Each result obtained at CDC with the reference method was compared with the results obtained with the whole defibrinated sheep blood-supplemented-inoculum method at CMC and API. The use of alpha-hemolysis as an indicator of bacterial growth resulted in readily discernible MIC endpoints. A comparison of the on- and off-scale results from CMC and CDC is presented in Table 2. The overall agreement (correlation) between the two methods (±1 dilution) was 94.3%, with 60.5% of the results identical and 99.3% within ±2 dilutions. The correlation was ≥91.8% for all drugs evaluated except cefotaxime (89.7%). All isolates determined to be penicillin resistant or relatively resistant by the reference method were resistant or relatively resistant by the whole defibrinated sheep blood-supplemented-inoculum method.

The correlation between the reference method and the whole defibrinated sheep blood-supplemented-inoculum method performed at API is presented in Table 3. The overall agreement, including on- and off-scale results, between the two methods was 95.0%, with 61.8% of the results identical and 99.0% within ±2 dilutions. The correlation was ≥92.9% for all antimicrobial agents tested except cefazolin and cefotaxime, which gave 87.8 and 88.8% correlations, respectively. All penicillin-resistant and relatively resistant isolates were detected.

For CMC and API data (using the same method) (Table 4), 97.1% were within ±1 dilution, 71.2% were the same, and 99.5% were within ±2 dilutions. The percentage of agreement for all antimicrobial agents evaluated was ≥93.7%. All penicillin-resistant and relatively resistant isolates of *S. pneumoniae* were detected at both laboratories.

TABLE 2. Agreement of results from CDC obtained with MHB plus lysed horse blood and those from CMC obtained with the inoculum supplementation method[a]

Antimicrobial agent	No. of tests varying by the following $\log_2$ dilutions							% Agreement[d]
	>−2	−2	−1[b]	0	+1[c]	+2	>+2	
Cefazolin	0	4	35	51	7	0	0	95.9
Cefotaxime	1	9	34	43	10	0	0	89.7
Ceftazidime	0	7	21	55	13	0	1	91.8
Ceftriaxone	0	2	15	46	31	3	0	94.8
Cefuroxime	1	2	10	56	27	1	0	95.9
Chloramphenicol	0	3	37	50	7	0	0	96.9
Erythromycin	2	3	1	90	0	1	0	93.8
Penicillin	0	2	11	51	30	3	0	94.8
Tetracycline	1	2	34	56	3	1	0	95.9
Vancomycin	0	4	11	56	24	2	0	93.8
Total or avg	5	38	209	554	152	11	1	94.3

[a] Includes off-scale MICs; 97 isolates were tested.
[b] CMC values lower than CDC values.
[c] CMC values higher than CDC values.
[d] Percentage of MICs within ±1 dilution.

TABLE 3. Agreement of results from CDC obtained with MHB plus lysed horse blood and those from API obtained with the inoculum supplementation method[a]

Antimicrobial agent	No. of tests varying by the following $\log_2$ dilutions							% Agreement[d]
	>−2	−2	−1[b]	0	+1[c]	+2	>+2	
Cefazolin	1	11	23	57	6	0	0	87.8
Cefotaxime	5	6	44	41	2	0	0	88.8
Ceftazidime	1	6	24	64	3	0	0	92.9
Ceftriaxone	1	0	15	60	22	0	0	99.0
Cefuroxime	0	1	12	54	31	0	0	99.0
Chloramphenicol	0	4	38	56	0	0	0	95.9
Erythromycin	1	2	2	92	0	0	1	95.9
Penicillin	0	2	7	75	14	0	0	98.0
Tetracycline	0	4	36	55	3	0	0	95.9
Vancomycin	0	3	7	52	36	0	0	96.9
Total or avg	9	39	208	606	117	0	1	95.0

[a] Includes off-scale MICs; 98 isolates were tested.
[b] API values lower than CDC values.
[c] API values higher than CDC values.
[d] Percentage of MICs within ±1 dilution.

The overall agreement between the two methods, separating on-scale results from on- plus off-scale results, is presented in Table 5. On-scale results were almost identical to on- and off-scale results for CDC and CMC, CDC and API, and CMC and API.

Using the results of the testing of the 19 blind duplicates, we found that the intralaboratory reproducibility for both methods, including on- and off-scale results, was greater than 98%.

The results of the reference method and the whole defibrinated sheep blood-supplemented-inoculum method were compared by examining the interpretive category changes. A total of 21 minor and 2 very major errors (1 with tetracycline and 1 with erythromycin) were noted when the CMC results were compared with the CDC results. A comparison of the API results with those of the reference method revealed 18 minor, 1 major (erythromycin), and 1 very major (tetracycline) error.

The quality control results were within acceptable ranges for all drug-organism combinations except for penicillin and *S. aureus* ATCC 29213. There was a tendency toward slightly higher MICs with this strain (range, 1 to 8 μg/ml) in all three laboratories than those given by NCCLS (0.25 to 1.0 μg/ml) (15).

DISCUSSION

The increasing frequency of isolation of *S. pneumoniae* with relative or complete resistance to penicillin (1–11, 13, 14, 16) necessitates routine antimicrobial susceptibility testing of all isolates from central nervous system infections and, some believe, from all body sites. Although the mechanism of resistance has not been fully elucidated, it is most likely due to altered penicillin-binding proteins in resistant strains (19). Although isolates can readily be screened for penicillin resistance with the oxacillin (1 μg) disk diffusion test (12, 18), many microbiologists prefer to perform quantitative antimicrobial susceptibility tests on all isolates or those with oxacillin zones of inhibition ≤19 mm. However, for determining MICs, NCCLS standard M7-A (15) recommends the use of MHB supplemented with lysed horse blood, which many laboratories using previously prepared or commercial MIC systems may find difficult.

The results of this study clearly indicate that preprepared microdilution trays without blood can be used to determine the susceptibility of *S. pneumoniae* to a variety of antimicrobial agents by supplementing the bacterial suspension used to inoculate the trays with whole defibrinated sheep blood. The MIC endpoints were easy to read, and no ambiguous results were encountered. The overall agreement

TABLE 4. Agreement of the inoculum supplementation method results from CMC and API[a]

Antimicrobial agent	No. of tests varying by the following $\log_2$ dilutions							% Agreement[d]
	>−2	−2	−1[b]	0	+1[c]	+2	>+2	
Cefazolin	0	1	14	72	7	1	0	97.9
Cefotaxime	0	4	20	63	7	1	0	94.7
Ceftazidime	1	4	19	60	10	1	0	93.7
Ceftriaxone	0	1	20	66	7	1	0	97.9
Cefuroxime	0	0	14	65	14	2	0	97.9
Chloramphenicol	0	0	16	71	8	0	0	100
Erythromycin	0	0	1	90	2	0	2	97.9
Penicillin	0	3	27	51	13	1	0	95.8
Tetracycline	1	1	12	70	10	0	1	96.8
Vancomycin	0	0	10	68	15	2	0	97.9
Total or avg	2	14	153	676	93	9	3	97.1

[a] Includes off-scale MICs; 95 isolates were tested.
[b] API values lower than CMC values.
[c] API values higher than CMC values.
[d] Percentage of MICs within ±1 dilution.

TABLE 5. Overall agreement of test methods

Antimicrobial agent	% Agreement[a]					
	CDC/CMC[b]		CDC/API[c]		CMC/API[d]	
	On[e]	All[f]	On	All	On	All
Cefazolin	96.7	95.9	88.4	98.0	97.7	97.9
Cefotaxime	91.4	89.7	88.5	88.8	94.3	94.7
Ceftazidime	94.6	91.8	95.0	92.9	94.2	93.7
Ceftriaxone	96.1	94.8	100	99.0	98.6	97.9
Cefuroxime	96.5	95.9	98.4	99.0	98.5	97.9
Chloramphenicol	97.9	96.9	96.9	95.9	100	100
Erythromycin	—[g]	93.8	—	98.0	—	97.5
Penicillin	97.4	94.8	100	95.9	97.4	95.8
Tetracycline	95.2	95.9	88.5	96.9	95.5	96.8
Vancomycin	98.9	93.8	100	87.8	100	97.9
Avg	96.3	94.3	95.6	95.0	97.7	97.0

[a] Percentage of MICs within ±1 dilution.
[b] CDC results compared with CMC results.
[c] CDC results compared with API results.
[d] CMC results compared with API results.
[e] On-scale values only (no values ≤ or >).
[f] On- and off-scale values.
[g] —, Insufficient number of MICs.

between the reference method and the whole defibrinated sheep blood-supplemented-inoculum method was ≥94%, and the method was highly reproducible and easy to perform. An analysis of individual antimicrobial agents also indicated excellent correlation between the methods. It should be noted that there was a tendency toward slightly lower MICs with the inoculum supplementation method.

This collaborative evaluation demonstrates that the supplementation of the bacterial suspension used to inoculate previously prepared or commercial microdilution broth trays with whole defibrinated sheep blood is an acceptable method for quantitative antimicrobial susceptibility testing of pneumococci.

LITERATURE CITED

1. **Applebaum, P. C., A. Bhamjee, J. N. Scragg, A. F. Hallett, A. J. Bowen, and R. C. Copper.** 1977. *Streptococcus pneumoniae* resistant to penicillin and chloramphenicol. Lancet **ii**:995–997.
2. **Cates, K. L., J. M. Gerrard, G. S. Giebink, M. E. Lund, E. Z. Bleeker, S. Lau, M. C. O'Leary, W. Krivit, and P. G. Quie.** 1978. A penicillin-resistant pneumococcus. J. Pediatr. **93**:624–626.
3. **Centers for Disease Control.** 1985. Isolation of multiply antibiotic resistant pneumococci—New York. Morbid. Mortal. Weekly Rep. **34**:545–546.
4. **Dajani, A. S.** 1982. Antibiotic-resistant pneumococci. Pediatr. Infect. Dis. **1**:143–144.
5. **Dixon, J. M. S.** 1974. Pneumococcus with increased resistance to penicillin. Lancet **ii**:474.
6. **Feldman, C., J. M. Kallenbach, S. D. Miller, J. R. Thorburn, and H. J. Koornhof.** 1985. Community-acquired pneumonia due to penicillin-resistant pneumococci. N. Engl. J. Med. **313**:615–617.
7. **Hansman, D., and M. M. Bullen.** 1967. A resistant pneumococcus. Lancet **ii**:264–265.
8. **Hansman, D., H. Glasgow, J. Sturt, H. L. Devitt, and R. Douglass.** 1971. Increased resistance to penicillin of pneumococci isolated from man. N. Engl. J. Med. **284**:175–177.
9. **Istre, G. R., J. T. Humphreys, K. D. Albrecht, C. Thornsberry, J. M. Swenson, and R. S. Hopkins.** 1983. Chloramphenicol and penicillin resistance in pneumococci isolated from blood and cerebrospinal fluid: a prevalence study in metropolitan Denver. J. Clin. Microbiol. **17**:472–475.
10. **Jackson, M. A., S. Shelton, J. D. Nelson, and G. H. McCracken.** 1984. Relatively penicillin-resistant pneumococcal infections in pediatric patients. Pediatr. Infect. Dis. **3**:129–132.
11. **Jacobs, M. R., H. J. Koornhof, R. M. Robins-Browne, C. M. Stevenson, Z. A. Vermaak, I. Freiman, G. B. Miller, M. A. Whitcomb, M. Isaacson, J. I. Ward, and R. Austrian.** 1978. Emergence of multiply resistant pneumococci. N. Engl. J. Med. **299**:735–740.
12. **Jacobs, M. R., M. N. Robins-Browne, and H. J. Koornhof.** 1980. Antimicrobial susceptibility testing of pneumococci. 2. Determination of optimal disc diffusion test for detection of penicillin G resistance. J. Antimicrob. Chemother. **6**:53–64.
13. **Krause, K. L., C. Stager, and L. O. Gentry.** 1982. Prevalence of penicillin-resistant pneumococci in Houston, Texas. Am. J. Clin. Pathol. **77**:210–213.
14. **Liñares, J., J. Garau, C. Domínguez, and J. L. Pérez.** 1983. Antibiotic resistance and serotypes of *Streptococcus pneumoniae* from patients with community-acquired pneumococcal disease. Antimicrob. Agents Chemother. **23**:545–547.
15. **National Committee for Clinical Laboratory Standards.** 1985. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M7-A. National Committee for Clinical Laboratory Standards, Villanova, Pa.
16. **Saah, A. J., J. P. Mallonee, M. M. Tarpay, C. Thornsberry, M. A. Roberts, and E. R. Rhoades.** 1980. Relative resistance to penicillin in the pneumococcus. A prevalence and case-control study. J. Am. Med. Assoc. **243**:1824–1827.
17. **Tarpay, M. M., D. F. Welch, and M. I. Marks.** 1980. Antimicrobial susceptibility testing of *Streptococcus pneumoniae* by micro-broth dilution. Antimicrob. Agents Chemother. **18**:579–581.
18. **Thornsberry, C., and J. M. Swenson.** 1980. Antimicrobial susceptibility tests for *Streptococcus pneumoniae*. Lab. Med. **11**: 83–86.
19. **Zighelboim, S., and A. Tomasz.** 1980. Penicillin-binding proteins of multiply antibiotic-resistant South Africa strains of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **17**:434–442.