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Multicenter Evaluation of the Use of *Haemophilus* Test Medium for Broth Microdilution Antimicrobial Susceptibility Testing of *Streptococcus pneumoniae* and Development of Quality Control Limits

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A five-laboratory collaborative study was undertaken to determine the precision and accuracy of broth microdilution susceptibility tests of *Streptococcus pneumoniae* isolates performed with *Haemophilus* test medium (HTM) compared with tests performed with lysed horse blood-supplemented Mueller-Hinton broth (LHB). The intra- and interlaboratory reproducibilities of MICs of 10 antimicrobial agents determined with the two media were found to be quite similar and highly reproducible in both media. On the basis of favorable performance in this study, *S. pneumoniae* ATCC 49619 is recommended as a quality control strain to assess the performance of HTM when this medium is used for testing of pneumococci. Testing of 293 unique clinical isolates of *S. pneumoniae* with both media in the respective participant laboratories allowed a direct comparison of MIC results and a calculation of interpretive error rates. Although there were some slight differences between MICs determined with HTM and MICs determined with LHB, few very major or major errors resulted from testing the clinical isolates against the 10 antimicrobial agents. However, MIC-interpretive criteria specific for *S. pneumoniae* should be developed and promulgated through a national consensus mechanism.

Resistance to several antimicrobial agents commonly used for therapy of both serious and localized infections due to *Streptococcus pneumoniae* has been recently documented (1–5, 9, 10, 12, 17, 18, 20, 22). The increased possibility of encountering resistant strains has underscored the need for a simple and accurate means of carrying out routine susceptibility testing of pneumococcal clinical isolates. The present study has compared the use of *Haemophilus* test medium (HTM) and lysed horse blood-supplemented Mueller-Hinton broth (LHB), the medium recommended for broth microdilution testing of streptococci by the National Committee for Clinical Laboratory Standards (NCCLS) (14). The two media were evaluated during a collaborative study involving five laboratories. Studies were designed to determine the intra- and interlaboratory reproducibilities of MICs determined with each medium and to develop quality control limits for use with HTM. In addition, a group of unique clinical isolates recovered by each laboratory was tested in parallel with the two media to determine the comparability of MIC results.

MATERIALS AND METHODS

Participant laboratories. These studies were conducted in five collaborating laboratories. They included the Centers for Disease Control (Atlanta, Ga.), the Cleveland Clinic Foundation (Cleveland, Ohio), Massachusetts General Hospital (Boston), the University of Massachusetts Medical Center (Worcester), and the University of Texas Health Science Center (San Antonio).

Quality control strains. A candidate *S. pneumoniae* quality control strain (now designated ATCC 49619) with relative resistance to penicillin (MIC = 0.25 μg/ml) and *Staphylococcus aureus* ATCC 29213 were tested in each laboratory on multiple occasions during reproducibility studies as outlined in NCCLS document M23-T (13).

Test strains. Four *S. pneumoniae* strains for which the study drugs had previously determined MICs were tested initially in each laboratory to ascertain interlaboratory agreement and to assure appropriate performance of reagents in each laboratory. In addition, each of the collaborating laboratories tested approximately 60 unique clinical isolates of pneumococci from its own facility.

Antimicrobial agents. Penicillin, cefaclor, cefotaxime, ceftriaxone, cefuroxime, chloramphenicol, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin were tested with each isolate.

Broth microdilution panels. All microdilution test panels were prepared by the coordinating laboratory (University of Texas Health Science Center) with common and unique test lots of media. These included a common lot of LHB (Mueller-Hinton broth [Difco, Detroit, Mich.] supplemented with 3% lysed horse blood) (12), a common lot of HTM broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.), and five unique test lots of panels prepared with HTM from two different manufacturers (Becton Dickinson Microbiology Systems and Remel, Lenexa, Kans.), as well as an

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TABLE 1. Interlaboratory reproducibility of MICs for *S. aureus* 29213 determined in five laboratories with common and unique test lots of media

Antimicrobial agent[a]	HTM test lots[b,c]		HTM common lot[b]		LHB common lot[b]	
	Modal MIC (μg/ml)	% of tests within ±1 dilution of mode[d]	Modal MIC (μg/ml)	% of tests within ±1 dilution of mode[d]	Modal MIC (μg/ml)	% of tests within ±1 dilution of mode[d]
Penicillin (0.25–1)	1	60[e]	1	80	4	80
Cefaclor (1–4)	2	100	1	100	2	100
Cefotaxime (1–4)	1	100	1	100	1	100
Ceftriaxone (1–8)	2	100	2	100	2	100
Cefuroxime (0.5–2)	1	100	1	100	1	100
Chloramphenicol (2–8)	8–16	100	16	100	8	100
Erythromycin (0.12–0.5)	1	100	1	100	0.5	100
Tetracycline (0.25–1)	0.5	100	0.5	100	0.25	96
Trimethoprim-sulfamethoxazole[f] (≤0.5)	0.25	100	0.25	100	0.06	96
Vancomycin (0.25–2)	1	100	1	100	1	100

[a] Values in parentheses are NCCLS MIC quality control ranges (in micrograms per milliliter) for tests performed in unsupplemented Mueller-Hinton broth.
[b] Total of 25 tests.
[c] Unique to each laboratory (total, five lots).
[d] One laboratory reported that 39 of 40 MIC determinations were 2 $\log_2$ dilutions below the mode. On the basis of data from four laboratories, 100% of the MICs were within ±1 $\log_2$ dilution of the mode.
[e] One laboratory reported that all five MIC determinations were ≥2 $\log_2$ dilutions of the mode. On the basis of data from four laboratories, 100% of the MICs were within ±1 $\log_2$ of the mode.
[f] Based on the trimethoprim component, tested at a 1:19 trimethoprim-sulfamethoxazole ratio.

HTM lot prepared from basic ingredients (8) by the coordinating laboratory. Broth microdilution panels were prepared to contain twofold concentration increments of the 10 antimicrobial agents in the various common and unique test lots of media (100 μl of medium per well). Panels were frozen at −70°C until needed for testing.

Broth microdilution tests. The final inoculum density used for all susceptibility tests was approximately 5×10^5 CFU/ml, which was derived by suspending overnight growth (obtained on blood agar incubated at 35°C in 5% CO_2) of pneumococcal strains in 0.9% NaCl to a specified turbidity. Each laboratory verified that the desired final inoculum density was achieved by performing colony counts from the microdilution trays on the first day of testing and once daily thereafter with *S. pneumoniae* ATCC 49619. Microdilution trays were incubated for 20 to 24 h in ambient air prior to interpretation of MICs.

Agar dilution tests. A group of 48 *S. pneumoniae* strains was tested in one of our laboratories (University of Texas Health Science Center) with penicillin by the broth microdilution method with both of the media described above and by agar dilution. The agar dilution tests incorporated the use of Mueller-Hinton agar (Difco) supplemented with 5% defibrinated sheep blood (MHSB). The final inoculum density was ca. 10^4 CFU per spot, and the inoculum was applied to the surface of the plates with a Steers replicator. The plates were incubated for 20 to 24 h in ambient air prior to interpretation of MICs.

RESULTS

Replicate tests were performed with *S. pneumoniae* ATCC 49619 and *S. aureus* ATCC 29213 on 10 separate days in each laboratory with common lots of HTM and LHB and the HTM lot unique to each laboratory. Intralaboratory reproducibility (MICs within a 3-$\log_2$-dilution range, i.e., mode ± 1 $\log_2$ dilution) for the 10 antimicrobial agents with *S. pneumoniae* ATCC 49619 was 100% for 500 MICs determined with HTM broth and 100% for 100 MICs determined with LHB. The intralaboratory reproducibility of tests with *S. aureus* ATCC 29213 was 100% for four of five laboratories (93.8% for the fifth laboratory) when HTM was used and 100% for tests performed with LHB for all laboratories, on the basis of 100 tests performed in each medium. Interlaboratory reproducibility (within 1 $\log_2$ dilution of the modal MIC values of each agent, as determined in the five laboratories) with *S. aureus* ATCC 29213 ranged from 60 to 100% with HTM and 80 to 100% with LHB (Table 1). It can also be noted from Table 1 that the majority of MICs for *S. aureus* ATCC 29213 were within the approved NCCLS control ranges (12) for 9 of 10 antimicrobial agents tested with LHB and for 8 of 10 tested with HTM. Interlaboratory reproducibility of MICs for *S. pneumoniae* ATCC 49619 ranged from 80.5 to 100% with HTM and 84 to 100% with LHB (data not shown). The interlaboratory testing comparisons for *S.*

TABLE 2. Proposed acceptable MIC quality control ranges for *S. pneumoniae* ATCC 49619 tested by the HTM broth microdilution method

Antimicrobial agent	MIC (μg/ml)		% of MICs within proposed range[a]
	Mode[b]	Proposed range[c]	
Penicillin	0.25	0.125–0.5	100
Cefaclor	1	0.5–2	100
Cefotaxime	0.06	0.03–0.125	96.4
Ceftriaxone	0.06	0.03–0.125	96.4
Cefuroxime	0.25	0.125–0.5	98.0
Chloramphenicol	2	1–4	100
Erythromycin	0.06	0.03–0.125	96.4
Tetracycline	0.25	0.125–0.5	84.4[d]
Trimethoprim-sulfamethoxazole[e]	1	0.5–2	100
Vancomycin	0.25	0.125–0.5	100

[a] Determined by using 200 tests performed with HTM test lots and 50 tests performed with the HTM common lot.
[b] Based upon 200 tests performed in five laboratories with HTM test lots.
[c] Mode ±1 $\log_2$ dilution of MICs determined with HTM test lots.
[d] One laboratory reported that 39 of 40 test lot MICs were 2 $\log_2$ dilutions below the mode. On the basis of data from the other four laboratories, 100% of the MICs were within the proposed range.
[e] Based on the trimethoprim component, tested at a 1:19 trimethoprim-sulfamethoxazole ratio.

TABLE 3. Comparison of MICs determined in five laboratories with HTM versus LHB with 293 unique clinical isolates of *S. pneumoniae*

Antimicrobial agent (no. of tests)	No. of HTM MICs within the following $\log_2$ dilution of LHB MICs[a]:							% within ±1 $\log_2$ dilution
	>−2	−2	−1	0[b]	+1	+2	>+2	
Penicillin (293)		6	106	169	11	1		97.6
Cefaclor (275)	1	20	116	122	15	1		90.9
Cefotaxime (281)		3	124	144	9	0	1	98.6
Ceftriaxone (279)		7	127	138	6	1		97.1
Cefuroxime (127)	1	3	52	65	5	1		96.1
Chloramphenicol (290)	1	3	130	148	8			98.6
Erythromycin (276)		5	67	162	42			98.2
Tetracycline (241)		2	34	90	101	14		93.4
Trimethoprim-sulfamethoxazole (272)				2	52	156	62	19.9
Vancomycin (293)		1	40	217	35			99.7

[a] Only on-scale MICs were used for comparisons.
[b] That is, MICs were the same.

pneumoniae ATCC 49619 can be used to develop proposed acceptable MIC quality control ranges for the 10 antimicrobial agents tested in HTM (Table 2). All MIC determinations yielded a single modal value for each of the various drugs among the five laboratories, which allows a proposed quality control range of only 3 $\log_2$ dilutions (i.e., mode ± 1 dilution) for each antimicrobial agent.

In general, MICs determined for the 10 antimicrobial agents with HTM compared well with those determined with LHB, with 293 unique pneumococcal clinical isolates tested individually in the five laboratories (Table 3). Overall, 89% of MICs determined with the two media agreed within 1 $\log_2$ dilution (96.7% if trimethoprim-sulfamethoxazole comparisons were excluded). However, penicillin, cefaclor, cefotaxime, ceftriaxone, cefuroxime, and chloramphenicol MICs were 1 $\log_2$ dilution lower with HTM for approximately one-half of the test isolates (Table 3). Conversely, tetracycline MICs tended to be 1 dilution higher and trimethoprim-sulfamethoxazole MICs were often 2 $\log_2$ dilutions higher with HTM than with LHB (Table 3).

Table 4 compares penicillin MICs determined by broth microdilution with HTM and LHB and by MHSB agar dilution for a group of 48 selected strains. It is apparent that the MICs determined with HTM were often 1 $\log_2$ dilution lower than those determined in LHB, as seen in the larger group of strains described above. However, it appears that the penicillin MICs determined with HTM broth agreed more closely with MICs determined with MHSB agar. Similarly, the penicillin MICs determined with LHB tended to be 1 $\log_2$ dilution higher than those determined by MHSB agar dilution.

TABLE 4. Comparison of penicillin MICs for 48 *S. pneumoniae* strains determined by MHSB, LHB, and HTM dilution methods

Medium	No. of MICs within the indicated $\log_2$ concn of:										
	MHSB agar dilution MICs						LHB MICs				
	−2	−1	0[a]	+1	+2	>+2	−2	−1	0[a]	+1	+2 >+2
LHB		3	23	20	1	1					
HTM	1	12	25	8	2			19	26	3	

[a] That is, MICs were the same.

TABLE 5. Interpretive category errors resulting from MIC determinations with HTM compared with MIC determinations with LHB for 293 unique clinical isolates

Antimicrobial agent and reference category (intepretive breakpoint [μg/ml])[a]	No. of strains	No. (%) of interpretive category errors[b]		
		Very major	Major	Minor
Penicillin				
R (≥2)[c]	21	0		
S (≤0.06)[c]	272		0	
Total				18 (6.1)
Cefaclor				
R (≥32)[d]	14	0		
S (≤8)[d]	279		0	
Total				6 (2.0)
Cefotaxime				
R	5	3 (60)		
S (≤2)[d]	288		0	
Total				NA
Ceftriaxone				
R	4	3 (75)		
S (≤2)[d]	289		0	
Total				NA
Cefuroxime				
R (≥16)[d]	0	NC		
S (≤4)[d]	293		0	
Total				5 (1.7)
Chloramphenicol				
R (≥8)[d]	21	0		
S (≤2)[d]	272		0	
Total				133 (45.4)
Erythromycin				
R (≥8)[e]	20	0		
S (≤0.5)[e]	273		0	
Total				2 (0.7)
Tetracycline				
R (≥8)[d]	47	0		
S (≤2)[d]	246		1 (0.4)	
Total				8 (3.3)
Trimethoprim-sulfamethoxazole				
R (≥4)[d,f]	31	0		
S (≤0.5)[d,f]	262		3 (1.1)	
Total				194 (66.2)
Vancomycin				
R (≥32)[e]	0	NC		
S (≤4)[e]	293		0	
Total				0

[a] Susceptible category includes strains with susceptible or intermediate reference MICs. S, susceptible; R, resistant.
[b] NA, not applicable since the NCCLS has not defined an intermediate category; NC, not calculated since resistant strains were not encountered in this study.
[c] Interpretive breakpoint stipulated for *S. pneumoniae* in NCCLS M7-A2 (14).
[d] Interpretive breakpoint used for *H. influenzae* in NCCLS M7-A2 (14).
[e] Interpretive breakpoint used for nonfastidious organisms in NCCLS M7-A2 (14).
[f] Based on the trimethoprim component, tested at a 1:19 trimethoprim-sulfamethoxazole ratio.

Despite some systematic differences in MICs noted above when the clinical isolates were tested with the two media, few very major or major interpretive category errors were observed in the results of the study with HTM compared with results obtained with LHB. Table 5 indicates the interpretive error calculations and the criteria invoked for the purposes of this study to define susceptibility of pneumococci to the 10 agents. No very major or major errors

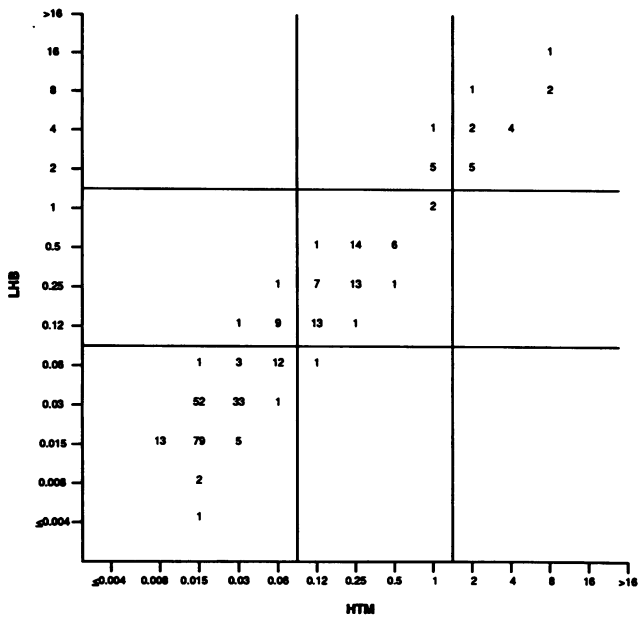

FIG. 1. Scattergram of penicillin MICs determined with LHB versus MICs determined with HTM for 293 clinical isolates of *S. pneumoniae*. The correlation coefficient (*r*) was 0.97. The solid lines indicate the NCCLS (12) interpretive criteria for penicillin when pneumococci are tested.

were detected with penicillin when the clinical isolates were tested. However, there were some minor errors (Table 5) because penicillin MICs determined with HTM were sometimes 1 $\log_2$ dilution lower and because of clustering of such strains near the two interpretive breakpoints (Fig. 1). Other apparent interpretive errors which deserve comment include those observed with cefotaxime, ceftriaxone, chloramphenicol, and trimethoprim-sulfamethoxazole. In the case of the two cephalosporins, the definition of susceptibility (MIC $\leq$ 2 μg/ml) advocated by the NCCLS for *Haemophilus* spp. (14) was employed here for *S. pneumoniae*. The very major interpretive errors recorded in Table 4 were attributed to four to five penicillin-resistant strains for which cefotaxime and ceftriaxone MICs were 2 μg/ml (1 μg/ml for one strain) when the organisms were tested with HTM and 4 to 8 μg/ml when the organisms were tested with LHB. Numerous minor errors occurred with chloramphenicol because of a number of strains for which MICs were 2 μg/ml (susceptible) when the organisms were tested with HTM and 4 μg/ml (intermediate) with LHB (no additional data shown). Lastly, the systematically higher trimethoprim-sulfamethoxazole MICs observed with HTM gave rise to numerous minor errors when the *Haemophilus* breakpoints were applied to the data. In particular, MICs for a number of strains were 0.12 to 0.5 μg/ml with LHB, while MICs for those strains were 1 μg/ml with HTM.

DISCUSSION

This study has confirmed and extended the findings of a prior study (7), which showed that HTM represents an alternative to LHB for broth microdilution susceptibility testing of *S. pneumoniae*. The complexity of preparation of LHB and its lack of commercial availability have been cited previously as reasons for the development of HTM for susceptibility testing of *Haemophilus influenzae* (8). The NCCLS has endorsed the use of HTM for *Haemophilus* testing, and more recently, HTM has been approved as an alternative to LHB for testing of pneumococci (15), largely on the basis of the findings of the present study. This study has shown that intra- and interlaboratory reproducibilities of pneumococcal susceptibility tests are similar for the two media. In addition, no growth failures were encountered with either medium when 293 unique clinical isolates were examined in this study.

Quality control ranges for broth microdilution MIC tests performed with HTM are herein proposed for *S. pneumoniae* ATCC 49619. This strain was selected as a candidate pneumococcal control strain in part because it demonstrates relative resistance to penicillin (modal MIC = 0.25 μg/ml), which might assist in providing on-scale values in antimicrobial test panels intended for routine clinical laboratory use. This study has also documented the reproducibility of MICs of 10 antimicrobial agents with this strain during testing in five laboratories. One obvious question is whether it is necessary to have a pneumococcal strain for quality control of HTM or whether *H. influenzae* ATCC 49247 (14) can be relied upon for an adequate assessment of HTM. The NCCLS has recently advocated the addition of *H. influenzae* ATCC 10211 for more critical assessment of the growth-promoting properties of HTM and *H. influenzae* ATCC 49766 for use with several cephalosporins (15). Since the inception of this study, it was discovered in one of our laboratories (University of Texas Health Science Center) that certain commercial Mueller-Hinton broth dehydrate preparations which have been preadjusted to contain the currently recommended levels of calcium and magnesium cations (14) may not support growth of pneumococci when used to prepare HTM, although growth of *H. influenzae* strains appears unaffected by such media. When the cations were added to Mueller-Hinton broth in the course of HTM preparation after autoclaving, there was no detrimental effect on the growth of pneumococci (our unpublished observation). Thus, if HTM is to be used for testing of pneumococci as well as *Haemophilus* spp., quality control testing should be performed with a pneumococcal control strain to verify the adequacy of the medium for growth of pneumococci.

The only antibiotic for which the NCCLS has specifically defined MIC interpretive breakpoints for *S. pneumoniae* is penicillin (14). For the purposes of this report, the NCCLS MIC-interpretive breakpoints for nonfastidious bacteria (14) have been utilized for interpreting the erythromycin and vancomycin results, and the *Haemophilus* breakpoints have been used for the remaining agents (Table 4). The selection of the latter breakpoints for interpretation of pneumococcal susceptibility test results may be justified on the basis that pneumococci and *H. influenzae* often cause similar life-threatening infections (e.g., meningitis, bacteremia, and pneumonia) and are involved in similar localized infections (e.g., otitis media, maxillary sinusitis, and acute exacerbations of chronic bronchitis).

Use of the interpretive breakpoints outlined above for analysis of the data gathered in this study resulted in few very major or major interpretive errors. However, error rates probably could be lowered further by adjustment of certain breakpoints to better fit the data generated with HTM as the test medium. For example, penicillin resistance might be defined as MICs of $\geq$1 μg/ml and penicillin susceptibility as MICs of $\leq$0.03 μg/ml. This change in breakpoints would provide a somewhat more conservative delineation of susceptible strains with either HTM or LHB, although it would

not lower substantially the rate of minor errors when HTM is used (i.e., 5.1 versus 6.1%). The fact that trimethoprim-sulfamethoxazole MICs are often twofold higher with HTM than with LHB might argue for raising the susceptibility breakpoint from ≤0.5 μg/ml (as applied to *Haemophilus* spp.) to ≤1 μg/ml (for pneumococci), while retaining a resistance breakpoint of ≥4 μg/ml. Such a change would reduce the minor errors from 66 to 14.7% without creating any very major errors.

Since pneumococci for which chloramphenicol MICs are 8 to 16 μg/ml have been shown to produce the inactivating enzyme chloramphenicol acetyltransferase in prior studies (5, 11), the *Haemophilus* interpretive breakpoints (Table 4) are probably appropriate for use with pneumococci. Therefore, it may not be possible to reduce the incidence of minor interpretive errors between testing with LHB and HTM without creating very major or major errors. The very major errors observed with both cefotaxime and ceftriaxone relate in part to use of the conservative *Haemophilus* susceptibility breakpoint of ≤2 μg/ml. This breakpoint was adopted because no resistant strains of *H. influenzae* have been identified to date, and susceptible strains have been found to yield MICs no higher than 2 μg/ml (6, 14). Use of cefotaxime or ceftriaxone has been recommended for therapy of relatively penicillin-resistant strains on the basis of favorable, although limited, experience in treating serious infections due to such strains (16, 21). However, the cases of two patients who failed therapy with cefotaxime for meningitis caused by relatively penicillin-resistant strains have recently been reported (19). The cefotaxime MICs for those strains were 8 and 32 μg/ml. The most appropriate breakpoints for *S. pneumoniae* with cefotaxime and ceftriaxone have not yet been determined.

In summary, HTM represents a reasonable alternative to LHB for performance of broth microdilution MIC tests of *S. pneumoniae*. The precision of MIC results and the ability to support growth of clinical isolates of pneumococci appear to be similar for both media. However, there are some antimicrobial agents which may yield slightly lower or higher MICs when tested with HTM than when tested with LHB. Thus, it will be important that interpretive breakpoints specific for pneumococci be determined through a national consensus process such as that provided by the NCCLS. Lastly, *S. pneumoniae* ATCC 49619 is recommended for routine quality control testing of HTM if this medium is to be used for susceptibility testing of pneumococci.

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