Use of *Haemophilus* Test Medium for Broth Microdilution Antimicrobial Susceptibility Testing of *Streptococcus pneumoniae*

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A recently described medium (*Haemophilus* test medium [HTM]) for antimicrobial susceptibility testing of *Haemophilus influenzae* was evaluated in this study for broth microdilution testing of *Streptococcus pneumoniae*. A total of 137 clinical isolates was tested against 11 antimicrobial agents, using Mueller-Hinton broth supplemented with 3% lysed horse blood in parallel with HTM. Inocula of 5 x 10³ CFU/ml and incubation for 20 to 24 h were used with both media. All isolates of *S. pneumoniae* produced acceptable growth in both media, and MICs determined in HTM agreed closely with those determined in lysed horse blood. Drugs which provided a MIC within 1 log₂ concentration difference in both media included penicillin (100%), ampicillin (98.0%), amoxicillin-clavulanate (100%), ampicillin-sulbactam (100%), cephalaxin (98.9%), cefaclor (96.8%), cefuroxime (99.0%), chloramphenicol (96.2%), tetracycline (96.2%), and erythromycin (100%). HTM MICs with trimethoprim-sulfamethoxazole were 1 to 2 log₂, concentration increments higher in 92.0% of isolates than MICs determined in lysed horse blood. Based on the results of this study, HTM appears to represent a promising alternative medium for broth microdilution susceptibility testing of *S. pneumoniae*.

The increasing prevalence of relative penicillin resistance in *Streptococcus pneumoniae* (3, 4, 9, 15), along with recognition of resistance to chloramphenicol, tetracycline, and erythromycin (2, 3, 5, 9, 17), places new emphasis on the need for routine antimicrobial susceptibility testing of this organism when isolated from serious infections. The oxacillin disk diffusion test, which many clinical laboratories now use, cannot differentiate frank penicillin resistance from relative resistance (7, 14). A penicillin MIC test must be performed to distinguish between these two resistance categories. However, false susceptibility to penicillin has been reported when laboratories have attempted to adapt certain commercial microdilution MIC systems for testing of *S. pneumoniae* (7, 16).

The medium currently recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for broth microdilution testing of pneumococci is Mueller-Hinton broth supplemented with 2 to 5% lysed horse blood (LHB) (13). While pneumococci grow reliably in this medium, the horse blood lysate is not available commercially and is difficult for most clinical laboratories to prepare.

The purpose of this investigation was to determine whether an optically clear, relatively simple medium developed recently for testing *Haemophilus* species (*Haemophilus* test medium [HTM]) (8) could be used for broth microdilution testing of *S. pneumoniae*.

**MATERIALS AND METHODS**

**Test strains.** A group of 100 *S. pneumoniae* isolates was selected randomly from a larger collection of 487 clinical isolates recovered during a recent U.S. national surveillance study of antimicrobial resistance in respiratory isolates of *S. pneumoniae* (J. H. Jorgensen, L. A. Maher, A. W. Howell, J. S. Redding, and G. V. Doern, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1286, 1989). Thirty-seven additional clinical isolates from the same collection were selected for testing because they demonstrated resistance or diminished susceptibility to one or more of the study antimicrobial agents. Control strains used with both susceptibility test media included *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *Enterococcus faecalis* ATCC 29212.

**Reference microdilution susceptibility tests.** Broth microdilution susceptibility tests were performed on each isolate by the method recommended by the NCCLS (12), which incorporates use of cation-supplemented Mueller-Hinton broth (Difco, Detroit, Mich.) with 3% LHB. LHB was prepared at 50% by aseptically adding equal volumes of sterile distilled water to defibrinated horse blood (Remel, Lenexa, Kans.) and then performing five freeze-thaw cycles, i.e., completely freezing the 50% blood at −20°C and then completely thawing the blood at room temperature. The lysed blood was then clarified by centrifugation at 12,000 g for 20 min. The supernatant was aseptically decanted (now 50% LHB) and added to cation-supplemented Mueller-Hinton broth to yield a final concentration of 3% LHB. Twofold concentration increments of the antimicrobial agents were dispensed in 100-μl amounts in plastic 96-well microdilution trays. A suspension of colonies from an overnight blood agar plate culture was adjusted to the turbidity of a 0.5 McFarland standard. This suspension was further diluted to achieve a final inoculum of 5 x 10⁶ CFU/ml in the microdilution wells. Following incubation at 35°C for 20 to 24 h in ambient air, MIC endpoints were interpreted in the usual manner.

**HTM microdilution tests.** Broth microdilution tests were also performed with HTM (8), which consisted of Mueller-Hinton broth base (Difco) supplemented with 15 μg of bovine hematin (Sigma Chemical Co., St. Louis, Mo.), 5 mg of yeast extract (Scott Laboratories, Fiskeville, R.I.), and 15 μg of β-NAD (Sigma) per ml. A 30-ml amount of hematin stock solution and 5 g of yeast extract were added to 1 liter of dissolved Mueller-Hinton broth base before autoclaving. A fresh hematin stock solution was prepared on the day of use by dissolving 50 mg of the bovine hematin in 100 ml of 0.01 N NaOH with gentle heat and stirring for 20 to 30 min until the powder was completely dissolved. After autoclaving and cooling, 3 ml of β-NAD stock solution was added aseptically. The NAD stock solution was made by dissolving

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TABLE 1. Agreement between MICs performed with LHB and HTM on S. pneumoniae isolates

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of strains tested</th>
<th>No. of HTM MICs within the given log₂ concentration increments of LHB MICs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>112</td>
<td>45 64 3</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>99</td>
<td>2 8 63</td>
</tr>
<tr>
<td>Amoxicillin-clavulanate</td>
<td>98</td>
<td>43 53 4</td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>98</td>
<td>11 83 4</td>
</tr>
<tr>
<td>Cephalixin</td>
<td>94</td>
<td>1 22 55 16</td>
</tr>
<tr>
<td>Cefalexin</td>
<td>93</td>
<td>3 36 52 2</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>100</td>
<td>22 72 5 1</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>106</td>
<td>22 72 5 1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>104</td>
<td>4 52 47 1</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>94</td>
<td>7 60 27</td>
</tr>
<tr>
<td>Trimethoprin-sulfa-methoxazole</td>
<td>112</td>
<td>4 45 58 5</td>
</tr>
</tbody>
</table>

50 mg of β-NAD in 10 ml of distilled water followed by filter sterilization using a 0.22-µm-pore size membrane filter. Cations (Ca²⁺ and Mg²⁺) were added after chilling the HTM broth. Since trimethoprin-sulfa-methoxazole tests were to be performed, thymidine phosphorylase (Burroughs Wellcome Co., Research Triangle Park, N.C.) was also added aseptically at a final concentration of 0.2 IU/ml to the sterilized and cooled HTM broth. Antimicrobial agent-containing HTM broth was dispensed into plastic 96-well microdilution trays as described above. Inoculum preparation and incubation conditions were as stated above.

CAT tests. Strains demonstrating elevated chloramphenicol MICs were tested for the presence of chloramphenicol acetyltransferase (CAT), using a commercial paper disk method (Remel). Modifications of the manufacturer's method were made as described by Matthews et al. (11). Specifically, strains were grown overnight on a sheep blood agar plate with inclusion of a 30-µg chloramphenicol disk on the agar surface (for induction of the enzyme). Growth was taken from around the inhibition zone margin and suspended in sterile 0.9% saline to match the turbidity of a no. 1 McFarland standard. A CAT test disk and a control disk, placed in separate glass test tubes, were each overlaid with 0.2 ml of the growth suspension. After incubation for 30 min at 35°C, the color of the eluate in the tube containing the test disk was compared with that in the tube containing the control disk. The test was considered positive when the yellow in the test disk tube was more intense than the color in the control disk tube.

RESULTS

All 137 S. pneumoniae clinical isolates tested in this study produced acceptable growth characteristics in both LHB and HTM, although the density of growth generally appeared somewhat heavier in LHB. Interpretation of growth endpoints was quite simple in the optically clear HTM broth. MIC endpoints in the HTM broth were sharp, with either clear growth buttons or no visible turbidity (at the MICs). MICs determined by use of the two media agreed closely with the majority of antimicrobial agents tested (Table 1). MICs were the same or agreed within 1 log₂ concentration increment with penicillin (100%), ampicillin (98.0%), amoxicillin-clavulanate (100%), ampicillin-sulbactam (100%), cephalixin (98.9%), cefaclor (96.8%), cefuroxime (99.0%), chloramphenicol (96.2%), tetracycline (96.2%), and erythromycin (100%). However, MICs determined in HTM tended to be 1 concentration increment lower than when determined in LHB with penicillin, amoxicillin-clavulanate, cefaclor, cefuroxime, and tetracycline (Table 1). This trend combined with the fact that a number of penicillin MICs were clustered near the interpretive breakpoints (Fig. 1) led to 8.9% minor interpretive errors with that drug.

Trimethoprin-sulfa-methoxazole MICs were 1 to 2 log₂ concentration increments higher with 92% of isolates in HTM as compared with the parallel LHB determinations.

FIG. 1. Comparison of penicillin MICs (n = 112) determined in LHB and in HTM. Dotted lines indicate current NCCLS interpretive criteria (14).
FIG. 2. Comparison of trimethoprim-sulfamethoxazole MICs (n = 118) determined in LHB and in HTM. Dotted lines indicate current NCCLS interpretive criteria (14).

(Table 1 and Fig. 2). Similarly, erythromycin MICs tended to be 1 concentration increment higher when determined in HTM.

Chloramphenicol MICs determined in HTM correlated well with MICs determined in LHB and with the production of CAT (Fig. 3). All six CAT-producing strains had chloramphenicol MICs of ≥16 μg/ml when tested in HTM. Induction of the CAT enzyme with a 30-μg chloramphenicol disk was essential for a correct CAT test result, in that all six strains were CAT negative when tested without induction. Tetracycline-resistant strains were also readily detected by MIC testing in HTM (Fig. 4).

MIC testing with HTM provided accurate categorization of susceptible and resistant strains according to current NCCLS interpretive guidelines (Table 2) (13). No very major or major errors of interpretation occurred with penicillin, erythromycin, tetracycline, or trimethoprim-sulfamethoxazole. Error rates were not calculated for ampicillin, the cephalosporins, and β-lactamase inhibitor combinations since specific interpretive guidelines for pneumococci have not been described by the NCCLS for those drugs. Error rates calculated for chloramphenicol used the interpretive breakpoints suggested by Matthews et al. (11). Tests of five separate lots of HTM and five lots of LHB provided MIC results consistently within the NCCLS control ranges (for unsupplemented Mueller-Hinton broth) with all three of the quality control strains.

DISCUSSION

The oxacillin disk diffusion test has been shown to be quite reliable for screening pneumococci for susceptibility to penicillin (7). However, the disk test cannot make the

FIG. 3. Comparison of chloramphenicol MICs (n = 106) determined in LHB and in HTM. Dotted lines indicate interpretive criteria proposed by Matthews et al. (11).
important distinction between frank and relative resistance to penicillin (7, 14). To classify accurately the level of resistance, it is necessary to determine the penicillin MIC by either the broth or the agar dilution method. Pneumococci may also be resistant to chloramphenicol (by CAT production), tetracycline, trimethoprim-sulfamethoxazole, erythromycin, or rifampin (2, 3, 5, 9, 17). Therefore, it may now be important to determine routinely the susceptibility of pneumococcal isolates causing serious infections to several other agents in addition to penicillin.

The NCCLS recommends use of LHB for broth microdilution or sheep blood supplementation of Mueller-Hinton agar for agar dilution tests of streptococci (including pneumococci; 12, 13). However, relatively few laboratories routinely perform agar dilution MICs, and LHB has not been available commercially. The LHB supplement is laborious to prepare, and when not clarified properly, it makes interpretation of microdilution growth endpoints very difficult. Significant very major errors have been reported with certain commercial microdilution or automated susceptibility test methods when they have been used to test pneumococci (7, 16). However, one recent study (1) has suggested that whole defibrinated sheep blood may be used as a growth supplement in commercial microdilution trays, with the growth endpoints detected by the greening of the medium which occurs in the presence of pneumococcal growth.

HTM has been recommended recently by the NCCLS as the medium of choice for susceptibility testing of Haemophilus spp. (13, 14). While HTM contains growth supplements tailored to the specific growth requirements of H. influenzae, this study has shown that it also provides adequate nutritive properties for testing of S. pneumoniae clinical isolates. All of the strains examined in this study grew well in HTM microdilution tests.

MICs determined in HTM were quite comparable to MICs for pneumococci tested in LHB. While there were no very major or major errors in this data set, there were a number of minor errors with penicillin and trimethoprim-sulfamethoxazole based on the current NCCLS breakpoints because of slightly lower or higher MICs (respectively) in HTM. The definition of relative penicillin resistance of pneumococci as a MIC of 0.1 to 1 µg/ml was proposed as a working definition.
which allowed separation of such strains from those that appeared to possess a normal level of susceptibility to penicillin (5, 6). Because penicillin MICs in HTM are sometimes 1 concentration increment lower than those in LHB, relative resistance to penicillin could be defined as MICs of 0.06 to 1 μg/ml for tests performed in HTM. This change would be quite consistent with the recent report that pneumococci with penicillin MICs of ≥0.06 μg/ml possess various alterations of their penicillin-binding proteins (10).

Trimethoprim-sulfamethoxazole MICs for S. pneumoniae are generally 1 to 2 log₂ concentration increments higher in HTM than when determined in LHB. An analogous situation has been seen with trimethoprim-sulfamethoxazole MICs for H. influenzae when tested in these two media (8). Since categorization of resistant isolates is not affected by the shift in MICs for susceptible strains when tested in HTM (Fig. 2), it is probably unnecessary to alter the trimethoprim-sulfamethoxazole breakpoints to adjust for these slight medium differences.

HTM has been shown in this study to represent a promising alternative medium for determining broth microdilution MICs of a variety of antimicrobial agents with S. pneumoniae clinical isolates. Because HTM is simpler to prepare than LHB, and because HTM can be obtained commercially in prepared form, it may represent a practical choice for routine determination of broth dilution MICs for both H. influenzae and S. pneumoniae in clinical laboratories.

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LITERATURE CITED


