Antimicrobial Susceptibility Testing of Less Commonly Isolated
Haemophilus Species Using Haemophilus Test Medium

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Haemophilus test medium (HTM) was developed recently for dilution and disk diffusion antimicrobial agent susceptibility testing of Haemophilus influenzae. The application of HTM to the testing of other, less frequently encountered Haemophilus species recovered from humans was evaluated in this study by using commercially prepared HTM (BBL Microbiology Systems, Cockeysville, Md.) in broth microdilution and agar disk diffusion susceptibility tests with 18 antimicrobial agents. A total of 93.3% of 90 isolates belonging to six Haemophilus species provided acceptable growth in HTM agar disk diffusion tests, while only 63.3% (57 of 90) provided acceptable growth in the broth microdilution tests. However, HTM agar dilution testing provided an alternative method for those strains (primarily H. haemolyticus) which failed to grow adequately in broth. Based on the latest National Committee for Clinical Laboratory Standards guidelines (standard M2-T4) for interpretation of HTM disk tests of H. influenzae, the overall very major, major, and minor errors for all 18 drugs and six species tested were 0.2, 0.7, and 3.4%, respectively. Thus, the use of HTM in agar or broth susceptibility tests can be recommended for testing the less commonly encountered Haemophilus species by using the same test conditions and interpretive guidelines developed for H. influenzae.

Recently, a new medium, Haemophilus test medium (HTM) (6), has been recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for dilution and disk diffusion testing of Haemophilus influenzae (9, 10). The MIC and zone size interpretive criteria advocated by the NCCLS for this new medium apply specifically to H. influenzae. A number of other Haemophilus species are prominent members of the human oral microflora but also cause occasional cases of infective endocarditis or osteomyelitis or infections of other normally sterile body cavities (1-4, 7, 11, 12). The purpose of this study was to determine whether HTM can be used for antimicrobial agent susceptibility testing of clinically significant isolates of these less frequently encountered Haemophilus species and whether the NCCLS interpretive criteria (9, 10) can be uniformly applied to the results of such tests.

MATERIALS AND METHODS

Test strains. The strains of Haemophilus species which we used were either clinical isolates from our laboratory or isolates kindly provided to us for this study by the following individuals: Carolyn Baker and Clyde Thornsberry, Centers for Disease Control, Atlanta, Ga.; Hugh Gerlach, St. Francis Medical Center, Wichita, Kans.; Cynthia Knapp and John Washington, Cleveland Clinic, Cleveland, Ohio; Patrick Murray, Washington University, St. Louis, Mo.; Bert Woolfrey, St. Paul-Ramsey Medical Center, St. Paul, Minn.; and Karla Tomfohrde and Linda Van Pelt, Microscan Division, Baxter Healthcare Corp., Sacramento, Calif.

HTM microdilution tests. Broth microdilution tests were performed by using commercially prepared HTM (BBL Microbiology Systems, Cockeysville, Md.) formulated as originally described (6). Twofold concentration increments of antimicrobial agents prepared in HTM were dispensed (100 μl per well) into plastic 96-well microdilution trays. For inoculum preparation, a suspension of colonies from a chocolate agar plate which had been incubated for 20 to 24 h at 35°C in 5% CO₂ was adjusted photometrically to the turbidity of a no. 0.5 McFarland standard. This suspension was further diluted to achieve an inoculum of 5 x 10⁵ CFU/ml in the microdilution tray wells. Following incubation at 35°C for 20 to 24 h in ambient air, MIC endpoints were determined and recorded.

HTM agar dilution tests. HTM agar was prepared as described previously (6) by using Mueller-Hinton agar base (BBL) supplemented with 15 μg of bovine hematin (Sigma Chemical Co., St. Louis, Mo.) per ml, 15 μg of β-NAD (Sigma) per ml, and 5 mg of yeast extract (BBL) per ml. A 30-ml portion of hematin stock solution and 5 g of yeast extract were added to 1 liter of dissolved Mueller-Hinton agar base before autoclaving. A fresh hematin stock solution was prepared by dissolving 50 mg of bovine hematin (Sigma) in 100 ml of 0.01 N NaOH with gentle heat and stirring for 20 to 30 min or until the powder was completely dissolved. After autoclaving and cooling, 3 ml of NAD stock solution was added aseptically. The NAD stock solution was made by dissolving 50 mg of NAD in 10 ml of distilled water, followed by filter sterilization using a membrane filter (pore size, 0.22 μm). Twofold concentration increments of the various antimicrobial agents were incorporated into the molten HTM agar, and the preparations were dispensed into plastic petri plates (diameter, 100 mm). The inoculum suspensions were adjusted to the density of a no. 0.5 McFarland standard as described above and then diluted so that the final inoculum was 10⁴ CFU per spot; this inoculum was delivered with a Steers replicator. Following incubation at 35°C in 5% CO₂ for 16 to 20 h, MICs were interpreted.

HTM agar disk diffusion tests. Commercially prepared HTM agar (BBL) in plastic petri plates (diameter, 150 mm) was used for this study. Inocula for disk diffusion tests were prepared by suspending colonies from chocolate agar plates incubated at 35°C in 5% CO₂ for 22 to 24 h to achieve the density of a no. 0.5 McFarland standard as described above. The photometrically adjusted suspensions were used to swab the surfaces of the HTM agar plates in the usual manner; this was followed by application of up to nine
TABLE 1. Production of acceptable growth in HTM by various Haemophilus species

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates tested</th>
<th>No. (%) of isolates producing acceptable growth in HTM:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Broth and agar</td>
</tr>
<tr>
<td>H. parainfluenzae</td>
<td>35</td>
<td>26 (74.3)</td>
</tr>
<tr>
<td>H. haemolyticus</td>
<td>21</td>
<td>5 (23.8)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>H. aphrophilus</td>
<td>14</td>
<td>10 (71.4)</td>
</tr>
<tr>
<td>H. parahaemolyticus</td>
<td>13</td>
<td>11 (84.6)</td>
</tr>
<tr>
<td>H. parapneumonia</td>
<td>6</td>
<td>4 (66.7)</td>
</tr>
<tr>
<td>H. segnis</td>
<td>1</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>57 (63.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Only 2 of 90 (2.2%) strains failed to produce acceptable growth in either broth or agar tests.  
<sup>b</sup> Four strains produced adequate growth in the agar dilution test but not for the disk diffusion test.

antimicrobial agent disks per plate. The plates were incubated for 16 to 18 h at 35°C in 5% CO₂. Zones of inhibition were then measured and recorded.

β-Lactamase tests. All isolates were examined for β-lactamase production by using nitrocefin-impregnated paper disks (Cefinase; BBL.).

CAT tests. Strains demonstrating elevated chloramphenicol MICs (>2 µg/ml) were tested for the presence of chloramphenicol acetyltransferase (CAT) by using a commercial paper disk method (Remel, Lenexa, Kans.). Modifications of the method of the manufacturer were made as described by Matthews et al. (8) for testing of pneumococci when it was noted that, unlike tests with H. influenzae, CAT tests of the unusual Haemophilus species were falsely negative without induction of the enzyme. Specifically, each strain was grown overnight on a chocolate agar plate having a 30-µg chloramphenicol disk on the agar surface (for enzyme induction). Growth was taken from around the zone margin and suspended in sterile 0.9% saline to match the turbidity of a no. 1 McFarland standard. A CAT test disk and 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 color in the test disk tube was more intense than the color in the control disk tube.

RESULTS

A total of 90 human isolates of unusual Haemophilus species were examined for their ability to grow in HTM broth or on HTM agar and for appropriate antimicrobial agent susceptibility test results when HTM was used; 11 of 13 H. parahaemolyticus strains, 26 of 35 H. parainfluenzae strains, 10 of 14 H. aphrophilus strains, 4 of 6 H. parapneumonia strains, and the single strain of H. segnis tested produced acceptable growth in both HTM broth microdilution and agar disk diffusion susceptibility tests (Table 1). However, only 5 of 21 (23.8%) H. haemolyticus strains grew acceptably in both HTM tests. Of the 33 (36.6%) Haemophilus species isolates which failed to grow in HTM broth microdilution tests, 31 (94%) were retested successfully by using HTM in an agar dilution test. Moreover, 84 of 90 (93.3%) isolates provided acceptable growth characteristics for interpretation of HTM disk diffusion tests. Only two isolates, one each of H. aphrophilus and H. parainfluenzae, failed to grow adequately for reliable testing in both broth and agar formulations of HTM.

The HTM-derived MICs (whether determined by broth- or agar-based tests) for all six species tested were plotted versus their disk diffusion zone diameters for the 18 antimicrobial agents included in the study. Figure 1 shows selected scattergrams for data from all six species combined. The current NCCLS interpretive breakpoints (indicated on Fig. 1) allowed appropriate classification of susceptibility and resistance in the majority of strains. Table 2 shows the very major, major, and minor errors for each of the drugs tested. No interpretive errors occurred with ampicillin-sulbactam, amoxycillin-clavulanate, aztreonam, cefonicid, cefotaxime, cefotaxime, ceftiraxone, chloramphenicol, ciprofloxacin, imipenem, or trimethoprim-sulfamethoxazole. The only very major errors occurred with ceftazidime (3.6% of all tests) (Fig. 1D). Likewise, the highest single category of major errors occurred with ceftazidime (8.3%). Very major and major errors incurred with the other drugs were quite low. Overall, there were 0.2% very major, 0.7% major, and 3.4% minor errors for the 18 drugs and six Haemophilus species tested. All ampicillin-resistant (β-lactamase-producing), chloramphenicol-resistant (CAT-producing), and tetracycline-resistant strains were correctly classified by the HTM-based tests.

DISCUSSION

Haemophilus species (other than H. influenzae) are occasionally the causes of endocarditis, bacteremia, osteomyelitis, and other infectious processes arising from an oropharyngeal source (1-4, 7, 11, 12). Clinical microbiology laboratories may find it necessary to perform antimicrobial agent susceptibility tests on such isolates when their clinical significance appears likely. Relatively little guidance is available from sources such as the NCCLS regarding the appropriate methodology for susceptibility testing of unusual, fastidious, gram-negative bacteria.

HTM has been recommended recently by the NCCLS for either dilution or disk diffusion susceptibility testing of H. influenzae (9, 10). MIC and zone size interpretive criteria for H. influenzae are now available for 18 different antimicrobial agents when they are tested by using HTM. An H. influenzae quality control strain (ATCC 49247) has also been selected, and control ranges have been developed specifically for HTM (NCCLS documents M2-A4 and M7-A2, in press). It is possible that the enhanced nutritive properties of HTM might allow it to be used for testing of other fastidious bacteria. We have shown recently that HTM supports the growth of pneumococci and that susceptibility results determined in HTM agree closely with results of pneumococcal tests performed by using lysed horse blood-supplemented Mueller-Hinton broth (J. H. Jorgensen, L. A. Maher, and A. W. Howell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, C-73, p. 405). In this study we attempted to document the usefulness of HTM for testing infrequently isolated Haemophilus species.

The six Haemophilus species included in this study could be grown and tested successfully by using either HTM broth or HTM agar-based test formats with 97.8% of the isolates. Those isolates (primarily H. haemolyticus) which did not demonstrate turbidity adequate to determine broth microdilution MIC endpoints could be tested satisfactorily by using the HTM agar disk diffusion or the HTM agar dilution
method. When the approved NCCLS interpretive guidelines for HTM tests of *H. influenzae* (9, 10) were applied to the results of MIC and disk tests of the other *Haemophilus* species included in this study, only 0.2% very major, 0.7% major, and 3.4% minor errors were noted overall. The largest number of very major and major errors occurred with ceftazidime. However, the MIC susceptibility breakpoints of ≤2 µg/ml and ≥26 mm have been established only tentatively, since no *H. influenzae* strains with documented resistance to this agent have been encountered thus far (5). For eight isolates of other *Haemophilus* species included in this study, ceftazidime MICs were ≥4 µg/ml; for five of the isolates, MICs were either 8 or 16 µg/ml (Fig. 1D). These isolates were uniquely less susceptible to ceftazidime than to cefotaxime, ceftriaxome, or cefixime (MICs ≤0.5 µg/ml for the latter three drugs for all eight isolates [Fig. 1E]). These isolates included three *H. paraphrophilus* strains, two

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**FIG. 1.** HTM MICs and disk diffusion zone sizes for 84 strains. The dashed lines indicate the current NCCLS interpretive breakpoints for *H. influenzae*.

*H. aphrophilus* strains, and one strain each of *H. parahaemolyticus*, *H. parainfluenzae*, and *H. segnis*. The reason for this difference in ceftazidime activity against these unusual *Haemophilus* species cannot be determined from our data.

Strains resistant to ampicillin (as determined by β-lactamase production), to chloramphenicol (as determined by

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**TABLE 2.** Interpretive errors of HTM disk diffusion zones compared with HTM MICs based on NCCLS guidelines for *H. influenzae*

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Very major</th>
<th>Major</th>
<th>Minor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0</td>
<td>0</td>
<td>14 (16.7)</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>0</td>
<td>0</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>0</td>
<td>0</td>
<td>4 (4.8)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>3 (3.6)</td>
<td>7 (8.3)</td>
<td>NA*</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>0</td>
<td>0</td>
<td>3 (3.6)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0</td>
<td>2 (2.4)</td>
<td>10 (11.9)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0</td>
<td>2 (2.4)</td>
<td>20 (23.8)</td>
</tr>
</tbody>
</table>

* MICS were determined by the HTM broth microdilution test for 56 of 90 isolates; the remainder of the MICs were determined by the HTM agar dilution test.

* No interpretive errors occurred with ampicillin-sulbactam, amoxicillin-clavulanate, aztreonam, cefonicid, cefotaxime, ceftriaxome, cefixime, chloramphenicol, ciprofloxacin, imipenem, or trimethoprim-sulfamethoxazole.

* NA, Not applicable.
CAT production), to tetracycline, and to trimethoprim-sulfamethoxazole were correctly classified by the HTM MIC and disk diffusion tests performed in this study. Thus, the overall favorable results of this study suggest that the medium now recommended by the NCCLS for susceptibility testing of *H. influenzae* (HTM) can also be used to test other, less commonly encountered *Haemophilus* species of human origin.

ACKNOWLEDGMENTS

BBL Microbiology Systems kindly provided the HTM broth and HTM agar plates used in this study. We also thank Carolyn Baker, Hugh Gerlach, Cynthia Knapp, Patrick Murray, Clyde Thornsberry, Karla Tomfohrde, Linda Van Pelt, John Washington, and Bert Woolfrey for providing some of the isolates used in the study.

LITERATURE CITED