Comparison of Agar Dilution, Broth Dilution, and Disk Diffusion Testing of Ampicillin against *Haemophilus* Species by Using In-House and Commercially Prepared Media

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An evaluation to determine the optimal method for the in vitro suscepti-

bility testing of *Haemophilus* strains to ampicillin was undertaken. In our hands, in-house-prepared *Haemophilus* Test Medium used by either the broth macrodilution or agar dilution method produced the most consistent results, especially for \( \beta \)-lactamase-negative, ampicillin-resistant *H. influenzae* strains.

In 1985, the National Committee for Clinical Laboratory Standards (NCCLS) published disk diffusion (DD) and dilution susceptibility testing criteria for the in vitro susceptibility testing of *Haemophilus influenzae* (M2-A3 [12] and M7-A [13], respectively). These standards applied to the testing of ampicillin, amoxicillin-clavulanate, ampicillin-sulbactam, and chloramphenicol when *H. influenzae* was tested with chocolatized Mueller-Hinton agar for DD testing and Mueller-Hinton broth supplemented with lysed horse blood for dilution susceptibility testing.

In January 1990, NCCLS published revised guidelines for *H. influenzae* DD and dilution susceptibility testing with Haemophilus Test Medium (HTM) (M2-A4 [14] and M7-A2 [15], respectively). HTM was adopted for DD testing because it had certain advantages over chocolatized Mueller-Hinton agar, including transparency and the ability to support DD testing of trimethoprim-sulfamethoxazole (chocolatized Mueller-Hinton agar contained large quantities of thymidine and thymidine analogs). HTM was also advantageous for both DD and broth dilution testing because of its low cost and its ability to adequately support the growth of *H. influenzae* (2, 9). Nevertheless, problems with HTM have been reported, including its inability to support the growth of *H. influenzae* and provide consistent results in susceptibility testing results (11). Inter- and intramanufacturer variabilities with batches of HTM (1, 3, 6, 7) as well as lot-to-lot and within-lot variabilities of HTM have also been reported (1, 3, 7).

In June 1992, NCCLS realized that the HTM DD method could falsely categorize \( \beta \)-lactamase-negative, ampicillin-susceptible (BLNAS) *H. influenzae* strains as \( \beta \)-lactamase-negative, ampicillin-resistant (BLNAR) strains. New interpretive criteria for zone diameters for DD testing were developed to alleviate this problem (2).

We undertook a study to determine the optimal method for the in vitro susceptibility testing of *H. influenzae* strains to ampicillin. Specifically, we were interested in comparing the performance characteristics of agar dilution (AD) methods with HTM in comparison with those of our standard Mueller-Hinton agar supplemented with 1% hemoglobin and 1% IsoViteX (SMH). Furthermore, we wished to evaluate the broth dilution method using HTM and the DD method using HTM from two commercial sources (Remel and BBL). Finally, although current NCCLS guidelines do not specifically address other *Haemophilus* species, we included BLNAS *Haemophilus parainfluenzae* strains in our evaluation. These strains were previously characterized as ampicillin susceptible by our conventional SMH agar dilution technique. We wished to evaluate these *H. parainfluenzae* strains to ensure that the methods with HTM did not falsely categorize these strains as BLNAR and, for that matter, to ensure that the SMH agar dilution method did not falsely categorize these strains as BLNAS. The frequency that BLNAR *H. parainfluenzae* strains are recognized by our technique is unknown.

**Methods.** Thirty-eight *H. influenzae* strains, including 9 \( \beta \)-lactamase positive, 10 BLNAR, and 19 BLNAS strains, were evaluated for their susceptibilities to ampicillin according to NCCLS guidelines published in 1990 (14, 15). Six of 10 BLNAR *H. influenzae* strains were kindly provided by Ronald N. Jones (University of Iowa Hospital and Clinics, Iowa City). Three additional BLNAR strains were kindly provided by James Jorgensen (University of Texas Health Science Center, San Antonio). The last BLNAR strain evaluated was ATCC 49247. Although NCCLS guidelines do not specifically address other *Haemophilus* species, 17 *H. parainfluenzae* strains were also evaluated for their susceptibilities to ampicillin by using the same guidelines provided for *H. influenzae*. All \( \beta \)-lactamase-positive and BLNAS *H. influenzae* strains and all *H. parainfluenzae* strains were isolated from clinical specimens at the Mayo Clinic. All *H. influenzae* and *H. parainfluenzae* strains evaluated in the study were confirmed as such by screening for X- and V-factor requirements. \( \beta \)-Lactamase determinations were done by the Cefinase disk method (Becton Dickinson, Cockeysville, Md.).

Table 1 summarizes the susceptibility testing methods that we used, which included the AD, broth macrodilution (BMD), and DD tests. Also summarized are the test media that we used, including whether the media were prepared in-house or were commercially prepared, inocula, incubation parameters, and interpretive guidelines. For the latter, both the 1990 (14) and the 1992 (2) revised standards for disk diffusion interpretation are displayed.

**Results.** Tables 1, 2, and 3 summarize the results of the study. Table 2 displays the interpretive discrepancies for the susceptibility testing methods and media that we used. In general, there were no major differences in the ability of any
media to support the growth of Haemophilus species, although the commercially prepared HTM media (Remel and BBL) resulted in less exuberant growth. For the latter, colonies were smaller, resulting in films of growth which were difficult to discern at times. Very major interpretive errors (a resistant strain misinterpreted as a susceptible strain) occurred only by AD testing with SMH. Major interpretive errors (a susceptible strain misinterpreted as a resistant strain) were infrequent. Minor interpretive errors (susceptible or resistant strains misinterpreted as intermediate) were frequent, especially by the AD method with SMH and the DD method with HTM from either commercial supplier (BBL or Remel). Of interest, fewer minor interpretive errors occurred by the DD method with HTM from BBL when 1992 NCCLS zone diameter guidelines (2) rather than 1990 NCCLS zone diameter guidelines (14) were used. However, the opposite occurred with HTM from Remel; more minor interpretive discrepancies occurred when 1992 NCCLS zone diameter guidelines (2) rather than 1990 NCCLS zone diameter guidelines (14) were used.

**Discussion.** In 1988, Doern and colleagues reviewed the prevalence of antimicrobial resistance among 2,811 clinical isolates of H. influenzae obtained from 30 medical centers in the United States (4). Ampicillin resistance related to the TEM type 1 β-lactamase occurred in 20.0% of isolates. Ampicillin resistance not related to β-lactamase occurred in 0.1% of isolates. This latter type of resistance has been referred to as BLNAR. Additional studies have implicated these BLNAR strains as being resistant or relatively resistant to amoxicillin-clavulanate, ampicillin-sulbactam, and the relatively β-lactamase-labile cephalosporins, including cefaclor, cefuroxime, cefonicid, cefamandole, ceprozil, and the carbacephem loracarbef (8, 10). If the frequency of BLNAR strains remains low (i.e., about 1 in 1,000, as described by Doern et al. [4]), one may argue that the routine screening of all H. influenzae isolates for BLNAR, which serves as a surrogate for resistance to cephalosporins and carbacephems, may be overly excessive. This is particularly true since detection of BLNAR resistance may be unreliable if one follows current NCCLS HTM-based methods and interpretive criteria (7).

In the current study, however, we found that the use of the broth dilution method with HTM or the AD method with HTM produced the best results. No very major interpretive errors occurred by either of these methods, major and minor interpretive errors were infrequent, and both methods produced identical results for BLNAR strains. In contrast, SMH agar performed poorly, especially when BLNAR strains were evaluated.

DD methods with HTM produced different results when 1992 NCCLS guidelines (2) rather than 1990 NCCLS guidelines (14) were followed. Fewer susceptible (BLNAS) isolates were falsely classified as intermediate or resistant (minor and major interpretive errors were less frequent) when HTM supplied by BBL was used, but the opposite results occurred when HTM supplied by Remel was used (minor interpretive errors were more frequent). By following the 1992 NCCLS guidelines (2), more BLNAR isolates were classified as intermediate by using HTM from either BBL or Remel. Inhibitory zones by the DD method with HTM from BBL were more

**TABLE 1. Materials and methods used in the study**

<table>
<thead>
<tr>
<th>Test method</th>
<th>Test medium (manufacturer)</th>
<th>Ampicillin concn</th>
<th>Incubation conditions</th>
<th>Interpretation criteria&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NCCLS, 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td>AD</td>
<td>HTM prepared in-house and</td>
<td>8-0.12 µg/ml&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16-20 h at 35°C in CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>≤1 µg/ml</td>
</tr>
<tr>
<td>MBD</td>
<td>HTM prepared in-house</td>
<td>8-0.12 µg/ml&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20-24 h at 35°C in CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>≤1 µg/ml</td>
</tr>
<tr>
<td>DD</td>
<td>HTM from BBL and Remel</td>
<td>10-µg disk</td>
<td>16-18 h at 35°C in CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>≥25 mm</td>
</tr>
</tbody>
</table>

<sup>a</sup>The same direct inoculum was used for all methods. The inoculum was equivalent to that of a 0.5 McFarland standard.

<sup>b</sup>The 1990 NCCLS criteria are from references 14 and 15; the 1992 NCCLS criteria are from reference 2.

<sup>c</sup>The test organism was H. influenzae ATCC 49247; NCCLS 1990 (14, 15) and 1992 (2) guidelines were used.

<sup>d</sup>Twofold dilutions.

**TABLE 2. Interpretive discrepancies by the various test methods**

<table>
<thead>
<tr>
<th>Error</th>
<th>BMD method with HTM</th>
<th>AD method with:</th>
<th>DD method with HTM from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SMH</td>
<td>HTM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BBL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Remel</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>NCCLS 1990 guideline</td>
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<tr>
<td></td>
<td></td>
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<td>NCCLS 1990 guideline</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>NCCLS 1992 guideline</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Very major&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Major</th>
<th>Minor</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD with HTM</td>
<td>0</td>
<td>0</td>
<td>2 (1/1)</td>
</tr>
<tr>
<td>AD with SMH</td>
<td>4 (4/0)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0</td>
<td>1 (1/0)</td>
</tr>
<tr>
<td>AD with HTM</td>
<td>0</td>
<td>1 (1/0)</td>
<td></td>
</tr>
<tr>
<td>DD with BBL</td>
<td>0</td>
<td>1 (1/0)</td>
<td></td>
</tr>
<tr>
<td>DD with Remel</td>
<td>16 (8/8)</td>
<td>5 (4/1)</td>
<td>4 (3/1)</td>
</tr>
</tbody>
</table>

<sup>e</sup>The reference or “gold standard” susceptibility for each organism to which all results in our study were compared was established by a consensus among a majority of testing methods in the current study. Of note, all of the previously characterized BLNAR H. influenzae strains and the BLNAR H. influenzae ATCC 49247 were also BLNAR by consensus among a majority of test methods in the current study.

<sup>f</sup>Resistant strains called susceptible.

<sup>f</sup>Numbers in parentheses indicate number of H. influenzae/number of H. parainfluenzae.
clear-cut and consistently smaller than those obtained by the DD method with HTM from Remel. A review with each manufacturer indicated that the formulation of the HTM of each manufacturer was similar; however, the medium from Remel differed from that from BBL in hematin or X factor concentration. This difference in formulation, as well as the stabilities of the constituents, may have accounted for the differences described above.

In summary, in our hands, HTM medium prepared in-house by the AD or BMD method following the 1990 guidelines of NCCLS (15) produced the most consistent results. The AD method with in-house-prepared SMH performed poorly. The results of DD testing were inconsistent and varied according to the commercial source of HTM and the NCCLS guidelines (1990 [14] versus 1992 [2]) that were followed. These inconsistencies are likely related to the stabilities of the HTM constituents. Although we did not study this, DD testing of Haemophilus species with in-house-prepared HTM may have resulted in more consistent results. Zone diameter criteria for ampicillin DD tests with HTM may need to be reevaluated by NCCLS, especially if the incidence of BLNAR H. influenzae strains increases.

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REFERENCES


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**TABLE 3. BLNAR H. influenzae strains**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>BMD method with HTM</th>
<th>AD method with SMH</th>
<th>HTM</th>
<th>DD method with HTM from Remel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (µg/ml)</td>
<td>Resistance</td>
<td>MIC (µg/ml)</td>
<td>Resistance</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>R</td>
<td>2</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>R</td>
<td>2</td>
<td>I</td>
</tr>
<tr>
<td>3</td>
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<td>R</td>
<td>2</td>
<td>I</td>
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<tr>
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<td>1</td>
<td>S</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>R</td>
<td>2</td>
<td>I</td>
</tr>
<tr>
<td>7</td>
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<tr>
<td>8</td>
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<td>R</td>
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</tr>
<tr>
<td>9</td>
<td>8</td>
<td>R</td>
<td>0.5</td>
<td>S</td>
</tr>
</tbody>
</table>

* S, susceptible; I, intermediate; R, resistant.