Investigation of Ampicillin-Intermediate Strains of *Haemophilus influenzae* by Using the Disk Diffusion Procedure and Current National Committee for Clinical Laboratory Standards Guidelines

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It was noted in our laboratory that certain strains of *Haemophilus influenzae* yielded zone sizes interpreted as resistant to the ampicillin (AMP) disk on chocolate–Mueller-Hinton agar (CMH) but showed no evidence of β-lactamase (β-Lac) activity. Although it is known that a second mechanism of AMP resistance exists, strains with this mechanism are uncommon. To investigate this apparent discrepancy, a study of 100 consecutive clinical isolates of *H. influenzae* collected over a 6-month period was performed. Isolates were simultaneously tested against five antibiotics (AMP, chloramphenicol, cefotaxime, ciprofloxacin, and AMP-sulbactam) on CMH and on two brands of *Haemophilus* test medium (HTM) by using the disk diffusion procedure and National Committee for Clinical Laboratory Standards (NCCLS) standards. By using CMH and NCCLS standard M2-A3-S2, strains of *H. influenzae* showing zone sizes of ≥20 mm with AMP were considered sensitive. By using HTM and NCCLS standard M2-A4, strains showing zone sizes of ≥25 mm to AMP on HTM were considered sensitive. Intermediate strains had zone sizes of 22 to 24 mm. The majority of isolates (68%) were sensitive to all antibiotics. Two percent of the isolates were resistant to chloramphenicol. Seventeen percent of the isolates were AMP-resistant, β-Lac-producing strains of *H. influenzae*. Thirteen percent of the isolates gave at least one intermediate or resistant zone for AMP but were β-Lac negative. MIC determinations with NCCLS standard M7-A2 were performed with resistant and intermediate strains. MICs for β-Lac-producing strains of *H. influenzae* were ≥8.0 μg/ml. MICs for β-Lac-negative strains were ≤1.0 μg/ml and were highly reproducible. If one uses the current NCCLS zone diameter interpretive criteria, results should be viewed with caution. Further investigation of zone size interpretive criteria is warranted. It is suggested that in the case of serious infections with *H. influenzae*, β-Lac-negative, AMP-resistant or -intermediate strains be confirmed by the MIC procedure.

*Haemophilus influenzae* is now recognized as an important cause of acute otitis media, maxillary sinusitis, and childhood meningitis as well as many other pathologic conditions (4). The increase in resistance of *H. influenzae* to ampicillin (AMP) and other antimicrobial agents necessitates a reliable method for susceptibility testing of this organism. Although rapid tests for β-lactamase (β-Lac) are available, reliable growth-based susceptibility tests are necessary to detect resistance to AMP by alternative mechanisms and resistance to other antimicrobial agents. The fastidious nature of *H. influenzae* necessitates the use of complex growth media, which requires the use of blood products. The opacity of media such as chocolate–Mueller-Hinton agar (CMH) often makes interpretation difficult (7). A new medium called *Haemophilus* test medium (HTM), consisting of Mueller-Hinton agar supplemented with yeast extract, hematin (X factor), and NAD (V factor) (11), supports the growth of *H. influenzae* and has the advantage of clarity, permitting easier measurement of zone diameters. This medium is recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (standards M2-A4) for disk susceptibility testing of *H. influenzae*.

This study was undertaken to investigate the frequent isolation in our laboratory of *H. influenzae* strains that were resistant on CMH but were β-Lac negative. AMP resistance in *H. influenzae* is most often due to the plasmid-mediated production of TEM β-Lac; however, AMP-resistant non-β-Lac-producing strains of *H. influenzae* have been reported. Possible alternative mechanisms of AMP resistance include altered penicillin-binding proteins and diminished permeation of beta-lactam antimicrobial agents through the cell wall outer membrane (5, 12). Isolates with this mechanism are considered rare (6, 18). In the present article, we report the results of disk diffusion studies on CMH and HTM for five antimicrobial agents and the MICs of any strain testing in the resistant or intermediate range for AMP.

**MATERIALS AND METHODS**

Clinical isolates. One hundred strains of *H. influenzae*, mostly serotype non-b, were isolated from adult patients in a 300-bed teaching hospital. Of these strains, 90 were isolated from respiratory sites, 10 were from eye cultures, and 1 was a blood isolate. This isolate was a β-Lac-producing strain of *H. influenzae* serotype b. These organisms were grown on chocolate II agar (BBL, Cockeysville, Md.) at 35°C for 18 to 24 h before testing.

Antimicrobial susceptibility studies. (i) Disk diffusion procedure. Several well-isolated colonies were inoculated into Mueller-Hinton broth and adjusted with an Abbott A-just meter to a turbidity equivalent to a 0.5 McFarland standard.

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Colony counts yielding $1 \times 10^8$ to $4 \times 10^8$ CFU/ml were done on a weekly basis on bacterial suspensions of *H. influenzae* ATCC 49247 to ensure the proper concentration of organisms. Duplicate plates of CMH (BBL) and HTM (BBL and Remel, Lenexa, Kans.) were streaked according to procedures described in the NCCLS standards M2-A3-S2 and M2-A4, respectively, (13, 14). All disks were the BBL Sensi-disc brand, manufactured by Becton Dickinson Microbiology Systems. *H. influenzae* ATCC 49247 was included with each batch of tests.

(ii) MICs. MICs were determined by using dried commercially prepared microdilution panels (Sensititre Microbiology Systems, Westlake, Ohio) and NCCLS standard M7-A2 (15). After the organisms were suspended in Mueller-Hinton broth as described above, 50 μl of each suspension was inoculated into 10 ml of HTM broth (BBL and Remel).

Each well of the Sensititre panel was inoculated with 100 μl of each suspension by using an autoinoculator (Sensititre). Antimicrobial concentration ranges for AMP, chloramphenicol, cefotaxime, ciprofloxacin, and AMP-sulbactam (SAM) were 1 to 64, 0.5 to 64, 0.5 to 64, 0.5 to 4, and 1/0.5 to 32/16, respectively. Plates were incubated at 35°C for 20 to 24 h. MICs were determined according to standard practice relative to the final concentration of antibiotic in each well.

(iii) Enzyme studies. β-Lac production was determined by using nitrocefin-impregnated paper disks (2). Positive and negative control strains were tested along with patient isolates.

### RESULTS

Zone size interpretive criteria for CMH and HTM were taken from NCCLS standards M2-A3-S2 and M2-A4, respectively. MIC Interpretive criteria were taken from NCCLS standard M7-A2 (Table 1). MICs for the recommended *H. influenzae* control strain, ATCC 49247, fell within acceptable ranges. Reproducibility of zone sizes for this strain for both within-run duplicate tests and day-to-day testing was acceptable, with values differing by no more than 1 mm. However, an occasional slightly higher zone size of 22 mm for AMP (acceptable range of 13 to 21 mm) was noted. Of 34 tests with Remel HTM agar, 32% (11 of 34) showed zone sizes of 22 mm, while BBL HTM agar showed 15% (5 of 34). The range for all 34 control tests was 20 to 22 mm. All isolates were sensitive to ciprofloxacin and cefotaxime on all media tested.

Among the 100 strains studied, 17% produced β-Lac. Zone sizes for AMP for these isolates were interpreted as resistant, ranging from 6 to 17 mm on CMH, from 6 to 19 mm on BBL HTM, and from 6 to 20 mm on Remel HTM, with mean zone sizes of 10.5, 11.4, and 12.3 mm, respectively. The distribution of AMP zone sizes on HTM media is shown in Fig. 1. One strain of *H. influenzae* did not grow during repeated testing on Remel HTM agar. MICs for the β-Lac-producing strains of *H. influenzae* were all in the resistant range (from 8 to 64 μg/ml with 84% [27 of 32] of the results being ≥64 μg/ml). One strain of *H. influenzae* did not grow upon repeated testing. Agreement between results with BBL HTM broth and Remel HTM broth occurred in 14 of 17 cases (82%). In only one case did the MIC differ by more than 1 dilution. Of β-Lac-producers, 94% (15 of 16 isolates) were sensitive to SAM; MICs for these isolates were between 0.5/0.25 and 2/1 μg/ml.

All disk diffusion tests were run in duplicate. Thirteen percent of our isolates gave one or both AMP zones in the intermediate (22- to 24-mm) or resistant (≥21-mm) zone size range on BBL and/or Remel HTM, but did not produce β-Lac, and had MICs that were interpreted as sensitive (15). The distribution of these zone diameters is shown in Fig. 2. The total number of results in this range was 24 for BBL HTM, with 20 intermediate and 4 resistant zones (average zone size was 22.5 mm), and 10 for Remel HTM, with 8 intermediate and 2 resistant zones (average zone diameter was 23.0 mm).

Table 2 shows the results of duplicate tests and compares the occurrence of discrepant results between AMP-interme-
TABLE 2. Frequency of discrepant* AMP results on HTM agar

<table>
<thead>
<tr>
<th>Result on HTM from:</th>
<th>BBL</th>
<th>Remel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 1</td>
<td>Test 2</td>
<td>Test 1</td>
</tr>
<tr>
<td>I or R</td>
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<td>I or R</td>
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<td>5</td>
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<tr>
<td>I or R</td>
<td>2</td>
<td></td>
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</tbody>
</table>

* AMP-intermediate (I) or AMP-resistant (R) zone diameters and susceptible MICs.

**Duplicate plates.

diate or -resistant zone sizes with BBL and Remel HTM agar and susceptible MICs. Of the 13 isolates, 6 showed discrepant results on both brands of HTM; 7 strains showed discrepant results on BBL HTM only. Of the four strains showing discrepant results in all HTM tests, 2 strains also gave discrepant results on CMH. No other isolates gave discrepant results on CMH when NCCLS standard M2-A3-S2 (13) was used. When BBL HTM and Remel HTM broth were compared, we found excellent agreement for all antibiotics, with no two values differing by more than 1 dilution in the MIC determination procedure.

**DISCUSSION**

Antimicrobial resistance among clinical isolates of H. influenzae has increased over the last few decades (3, 10). In the past, AMP was generally the drug of choice for serious H. influenzae infections since there were few reports of therapeutic failures when AMP was used and H. influenzae was considered to be uniformly susceptible to it (8). Resistance was first noted in 1972, and by 1988, the overall rate of β-Lac-mediated resistance of H. influenzae was reported to be as high as 20% (9). Although other mechanisms of AMP resistance have been recognized, these isolates are extremely uncommon in the United States (6, 16, 17).

Several methods for in vitro susceptibility testing of H. influenzae, including the disk diffusion and broth dilution procedures, exist. Many bacteria, including β-Lac-producing strains of H. influenzae, exhibit an inoculum effect when tested for antimicrobial susceptibility (16). It is important, therefore, to carefully adjust the inoculum, preferably with a mechanical device, to consistently ensure accurate results (1).

The fastidious nature of H. influenzae prohibits the use of Mueller-Hinton agar, the medium generally recommended for the Bauer-Kirby disk diffusion procedure. Until recently, CMH was recommended for H. influenzae. However, complex constituents of this medium have been shown to antagonize certain antimicrobial agents, and its opaque nature makes interpretation of zone sizes difficult (11). Also, no strain of H. influenzae has been recommended by NCCLS to ensure quality control of this medium.

Jorgensen et al. (11) described a new, simplified medium, HTM, which avoids many of these problems. The medium is transparent, which allows measurement of zone sizes from the back of the plate as is done with nonfastidious organisms. It is stable, reproducible from lot to lot, and commercially available. This medium is recommended by the current NCCLS standards (15) for disk diffusion susceptibility testing of H. influenzae.

In 1987, Jorgensen et al. described new zone size interpretive criteria (11). On the basis of MIC studies involving a large number of H. influenzae isolates, it was proposed that strains having zone sizes of ≤21 mm be considered susceptible, while those with zone diameters of ≥22 mm be considered resistant. In 1990, NCCLS-approved standards that recommended HTM as the medium of choice for in vitro susceptibility testing of H. influenzae were published. However, only strains of H. influenzae having zone diameters of ≥25 mm are considered susceptible. H. influenzae ATCC 49247 was introduced as a quality control strain to be tested routinely on HTM agar (14). Table 1 compares zone size interpretive criteria for CMH and HTM with corresponding MICs.

Between the early 1970s, when β-Lac-mediated resistance was recognized, and the late 1980s, the prevalence of AMP-resistant H. influenzae has continued to increase. Most laboratories reported that less than 5% of their isolates produced β-Lac prior to 1978. The incidence in Great Britain in 1981 had increased to 14% (16).

The prevalence of β-Lac-mediated AMP resistance in this study (17%) with 0% AMP resistance due to other mechanisms parallels the findings of Doern et al. (6), who in 1988 reported an overall rate of 20% for β-Lac-producing strains of H. influenzae. They also reported that high-level AMP resistance among H. influenzae strains lacking the TEM-β-Lac was extremely uncommon in the United States. Since the prevalence of β-Lac-producing strains of H. influenzae has increased so dramatically, the use of empiric therapy with AMP alone for serious H. influenzae infections is not advisable.

AMP MICs were <64 µg/ml for only three isolates among the 17 β-Lac-producing strains. For 16 of these isolates (one β-Lac-producing isolate failed to grow), all zone sizes as well as MICs fell within the resistant range (13–15). Zones were slightly larger with Remel HTM. The use of the β-Lac inhibitor sulbactam with AMP (SAM) resulted in MICs in the sensitive range for 15 of 16 isolates. The isolate remaining resistant (for which the SAM MIC was 4/2 µg/ml) had AMP zone sizes of 6 mm on all media tested and AMP MICs of ≥64 µg/ml.

Although reproducibility of results with the control strain, H. influenzae ATCC 49247, was acceptable, one questions the fact that it is an AMP-resistant, non-β-Lac-producing strain, which is known to be uncommon. It is also of some concern that the allowable zone diameter range (14 to 22 mm) for SAM includes both sensitive and resistant interpretations (Table 1). Our results therefore varied from resistant (at a zone size of 19 mm) to sensitive (when the zone size was 20 to 21 mm). Many of our AMP zone diameters were slightly larger (22 mm) on both brands of HTM than the acceptable range of 13 to 21 mm; our range for all tests was 20 to 22 mm. We therefore suggest that the zone diameter interpretative criteria for this control strain be reevaluated for use of commercially available HTM.

Thirteen non-β-Lac-producing isolates gave at least one AMP zone diameter in either the intermediate (22- to 24-mm) or resistant (≥21-mm) range on HTM. All of these isolates were shown to be susceptible to AMP by MIC testing. MIC tests were consistently reproducible, and most isolates were extremely susceptible to AMP; MICs for these isolates were ≤0.5 µg/ml. Only one of these strains had all six tests score in the intermediate or resistant range for all media tested, including CMH as well as HTM, when NCCLS interpretive guidelines M2-A3-S2 and M2-A4, respectively (13, 14), were used. Of the remaining 12 isolates, a range of 21 to 24 mm was measured on both BBL and Remel HTM. The repro-
ducibility of the disk diffusion procedure appears to be acceptable; duplicate results of all strains varied by ≤2 mm. Given the somewhat subjective nature of the disk diffusion procedure, it is our feeling that a difference of 1 to 2 mm may be inherent in the procedure, especially when fastidious bacteria that show light growth are tested. Since zone sizes on Remel HTM are in general slightly larger than those on BBL HTM, fewer false intermediate or resistant results were obtained. However, fastidious \textit{H. influenzae} strains grew better on BBL HTM. Four of our isolates failed to grow well with Remel HTM during repeated testing. One of these strains produced β-Lac.

Of the 70 AMP-susceptible (on all media) isolates, 30 had at least one AMP zone diameter of 25 or 26 mm. It is quite possible that with repeat testing, some or all of these isolates would appear intermediate. In order to avoid misclassifying many AMP-susceptible strains of \textit{H. influenzae}, we suggest that the recommended zone diameter interpretive criteria for AMP be reevaluated. Further investigation of correlation of zone sizes for AMP with susceptibility or resistance of \textit{H. influenzae}, particularly with AMP-resistant, non-β-Lac-producing strains, is warranted. This study suggests that criteria for AMP recommended in 1987 by Jorgenson et al. (11) might be more suitable in classifying susceptibility of \textit{H. influenzae} to AMP. If strains of \textit{H. influenzae} with zone sizes of ≥22 mm to AMP were considered sensitive, all β-Lac-producing strains in this study would be correctly classified as resistant. Also, the false intermediate or resistant results would be reduced by 82% (from 34 to 6).

It is important to gather additional data on a large number of isolates to support such a change in classification.

Although rapid tests for β-Lac to predict susceptibility of \textit{H. influenzae} to AMP are available, reliable susceptibility tests are still needed to detect resistance to AMP by other mechanisms and resistance to other potentially useful antimicrobial agents. HTM appears to represent an improvement over previous medium formulations used for testing \textit{H. influenzae} since it is relatively simple to prepare, it is inexpensive, and its transparency allows for easier reading of results (11).

When non-β-Lac-producing strains of \textit{H. influenzae} which are AMP resistant or AMP intermediate by disk diffusion susceptibility testing are isolated or when serious life-threatening infections with \textit{H. influenzae} are being treated, MIC systems should be available for further study.

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**REFERENCES**