Failure to Detect Ampicillin-Resistant, Non-β-Lactamase-Producing 
*Haemophilus influenzae* by Standard Disk Susceptibility Testing

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We questioned whether the apparent rarity of ampicillin-resistant, non-β-lactamase-producing (NBLP) *Haemophilus influenzae* was due to failure of detection. We identified ampicillin-resistant and ampicillin-susceptible *H. influenzae* by the agar dilution technique, using 10²- and 10⁰-CFU inocula. We compared the disk susceptibility of 18 ampicillin-resistant NBLP strains, 13 ampicillin-resistant, β-lactamase-producing strains, and 10 ampicillin-susceptible strains by using standard 10- and 2-μg ampicillin disks on two different media. We also investigated the possibility that disks containing 10 μg of clavulanic acid and 2 μg of ampicillin could be used to distinguish between the two ampicillin-resistant populations. Using the disk containing 2 μg of ampicillin, we correctly differentiated all the ampicillin-resistant isolates from the ampicillin-susceptible isolates on both media (a zone diameter of ≤15 mm was considered resistant). In contrast, the 10-μg ampicillin disk failed to detect 44% (8 of 18) of the ampicillin-resistant NBLP strains (a zone diameter of ≤19 mm was considered resistant). The MIC of ampicillin with the 10³-CFU inoculum correlated better with zone diameters than with the 10²-CFU inoculum. A difference in zone diameters of ≥8 mm between the disk containing 10 μg of clavulanic acid and 2 μg of ampicillin and the disk containing only 2 μg of ampicillin correctly identified all β-lactamase-producing strains. We conclude that the 2-μg ampicillin disk tests more readily identify ampicillin resistance in *H. influenzae* than do the 10-μg ampicillin disk tests. Future investigation should determine whether this in vitro resistance correlates with clinical treatment failures.

Although the most common mechanism of ampicillin resistance (Amp*) in *Haemophilus influenzae* is plasmid-mediated production of TEM β-lactamase (4, 7, 17, 32, 37), pathogenic Amp* type b *H. influenzae* lacking detectable β-lactamase activity has been reported (12, 14, 24, 26). Clinical isolates with this phenotype appear to be even more common among nontypable respiratory tract *H. influenzae* (1, 29, 37). Nontypable *H. influenzae* has been increasingly implicated in community-acquired and nosocomial pneumonia in adults (3, 9, 19, 20, 22, 40, 41) and is associated with exacerbations of chronic bronchitis in patients with chronic obstructive pulmonary disease (35). In addition, nontypable *H. influenzae* plays a major role in certain pediatric diseases, such as otitis media and pneumonia (25, 33).

Failure of an infection to respond to antibiotic administration is an operational correlate of in vitro resistance. Treatment failure with Amp* non-β-lactamase-producing (NBLP) *H. influenzae* has occurred with ampicillin administration (14). Thus, these strains are operationally resistant.

Another way of defining resistance is to examine the distribution of MICs within a specific species. A bimodal distribution suggests two classes of susceptibility (2). When the median concentration for the group with the higher values is fourfold (or greater) than the MIC for the group with the lower values, resistance is inferred (11, 12). This type of analysis allowed accurate definition of penicillin-resistant pneumococci. Those penicillin-resistant strains caused infections which did not respond to penicillin administration (10, 11).

Although the exact incidence of Amp* NBLP strains of *H. influenzae* is unknown, Thornsberry and Kirven reported that 10% (2 of 20) of isolates had this phenotype in the same year (1974) that β-lactamase producers were first identified (37). Many clinical microbiologists and clinicians believe the incidence of Amp* NBLP *H. influenzae* is low. The expectation is that in a diagnostic laboratory Amp* NBLP strains, though detected as ampicillin susceptible by a rapid β-lactamase assay, will be resistant by the standard ampicillin disk susceptibility test. We questioned whether failure of detection by standard disk susceptibility tests played any role in lack of recognition of these isolates. To address this question, we compared the disk susceptibility of 18 Amp* NBLP strains, 13 Amp* β-lactamase-producing (BLP) strains, and 10 Amp* strains by using the standard 10-μg and the commercially available 2-μg ampicillin disks. Furthermore, for validation and comparison of results we used the agar dilution method to determine the MIC of ampicillin for all 41 isolates. The recent availability of clavulanic acid (8), a specific inhibitor of the TEM β-lactamase, allowed us to investigate its use in combination with ampicillin to distinguish Amp* BLP from Amp* NBLP strains.


**MATERIALS AND METHODS**

**Bacterial strains.** All strains required β-NAD⁺ (V factor) and hemin (X factor) for growth when incubated at 37°C in

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room air. Each strain was examined for iridescence on translucent media and tested for agglutination with typing sera (Difco Laboratories, Detroit, Mich.); type b and polyvalent sera were used. Biotyping was performed with the Minitek system (BBL Microbiology Systems, Cockeysville, Md.) as described previously (13). Whole-cell suspensions of each strain were incubated with β-lactamase substrate (chromogenic cephalosporin) at room temperature for 30 min and were inspected visually (23).

**Media.** The medium used for growth and determination of the MIC was brain heart infusion (BHI) agar or broth (Difco) supplemented with 10 μg of hemin (X factor), 10 μg of L-histidine, and 10 μg of β-NAD⁺ (V factor) per ml (sBHI). Chocolate agar plus enrichment (X factor plus V factor) plates and Mueller-Hinton chocolate agar plates were purchased from Prepared Media Laboratories (PML)-Microbiologicals, Tualatin, Oreg. The latter plates fulfill the criteria recommended by the National Committee for Clinical Laboratory Standards (NCCLS [21]). Plate cultures were incubated at 36.5°C, whereas liquid cultures were incubated at 37°C and were shaken at 200 cycles/min.

**Antibiotics and chemicals.** Ampicillin was obtained from Sigma Chemical Co., St. Louis, Mo. Ampicillin susceptibility disks (2 and 10 μg) were obtained from BBL Microbiology Systems. Clavulanic acid powder was a gift of Beecham Laboratories, Bristol, Tenn. The β-lactamase substrate (a chromogenic cephalosporin) was obtained from Calbiochem-Behring, La Jolla, Calif.

**Determination of MIC.** The strains were inoculated onto fresh sBHI agar plates from vials of skim milk stored at −70°C and were incubated overnight. The next day the strains were grown to mid-log phase (A₅₄₀ = 0.60) in liquid media and diluted, and 2 to 5 μl of each strain was plated with a Steers replicator. Colony counts were determined on antibiotic-free media. Inocula tested contained 10⁵, 10⁴, and 10³ CFU/μl. The concentrations of ampicillin in sBHI agar for testing were 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 4.0, 8.0, 10, 16, and 32 μg/ml. The MIC was defined as the lowest concentration of antibiotic which inhibited visible growth of the inoculum in comparison with growth on antibiotic-free media. Plates were examined after 18 to 24 h of incubation in 5% CO₂ at 36.5°C.

**Disk susceptibility testing.** After overnight growth on sBHI agar plates, whole-cell suspensions in phosphate-buffered saline of each strain were adjusted to a density equal to a 0.5 McFarland standard and were swabbed on the surface of 100-mm Mueller-Hinton chocolate agar plates and chocolate agar plus enrichment plates. Disks containing 2 μg of ampicillin, 10 μg of ampicillin, and 2 μg of ampicillin combined with 10 μg of clavulanic acid (added in a 20-μl volume as described by Rippere [31]) were placed on each plate described above. After overnight incubation (~18 h), the zone diameters were determined by at least four readers who recorded the results in a blind fashion. The median values of the four observations were recorded, and the mean value of the median values from separate determinations was calculated for graphic representation. All 41 strains were tested at least twice on both media (four values). False-positive or false-negative determinations were recorded as any median value that misidentified a strain as susceptible or resistant to ampicillin on either medium by using NCCLS criteria for ampicillin (a zone diameter ≤19 mm indicated resistance, and a zone diameter of ≥20 mm indicated susceptibility [21]) in comparison with the agar dilution MIC.

**Statistics.** Median values were compared by the median test, using the statistical package for the social sciences on a CDC-Cyber 174-176 computer. Sensitivities and specificities were calculated as described before (5).

**RESULTS**

**Strains.** All 31 Ampᵢ isolates were collected between the years 1975 and 1982. All 18 Ampᵢ NBLP strains were nontypeable isolates; 15 were isolated from sputum, and 1 each was isolated from the nasopharynx, eye, and blood. These strains were isolated in New Zealand (four strains), England (eight strains) and the United States (six strains) (16, 29). Among these Ampᵢ NBLP strains, biotypes I and II (the two most common biotypes among type b invasive isolates) were observed in equal numbers (seven of each), and one each of biotypes III, IV, V, and VII was observed. The 13 Ampᵢ BLP strains (11 type b and 2 nontypable) were isolated in various U.S. medical centers (4, 17, 18); 10 strains were biotype I, and the others were biotypes II, III, and IV. Six strains were isolates from cerebrospinal fluid, two were from blood, two were from sputum, one was from the nasopharynx, one was from an ear, and one isolate came from an unknown site. Of the 10 Ampᵢ isolates, 9 were nontypeable, and 1 was a type b strain (15, 29). Biotypes for these isolates were I (two strains), II (five strains), III (two strains), and IV (one strain).

**MIC determination.** With an inoculum of 10⁵ CFU, the MIC of ampicillin was similar for all the Ampᵢ isolates and did not differentiate the Ampᵢ NBLP strains from the Ampᵢ BLP strains; both Ampᵢ populations revealed a marked inoculum effect (Fig. 1). However, with an inoculum of 10⁶ CFU, the MIC of ampicillin was clearly higher for the Ampᵢ BLP strains (Fig. 1). The median MIC of 32 μg/ml (range, 8 to >32 μg/ml) contrasted with the median MIC of ampicillin for the Ampᵢ NBLP strains of 8 μg/ml (range, 1.5 to 16 μg/ml; P < 0.001) (Table 1). The MIC of ampicillin for 9 of 10 Ampᵢ isolates with a 10⁵-CFU inoculum was 0.5 μg/ml; the MIC for the other strain was 0.25 μg/ml. One strain showed a marked inoculum effect: an MIC of 32 μg/ml for a 10⁶-CFU inoculum and an MIC of 0.5 μg/ml for a 10⁵-CFU inoculum. This strain was designated Ampᵢ on the basis of the latter value. The data obtained with an inoculum of approximately 10⁴ CFU were either identical to those obtained with an inoculum of 10⁵ CFU or the data varied by a single higher concentration (data not shown).

**Disk susceptibility.** The standard disk containing 10 μg of ampicillin differentiated the 13 Ampᵢ BLP strains from the 10 Ampᵢ isolates (Table 1 and Fig. 2). However, this disk failed to detect 44% (8 of 18) of Ampᵢ NBLP strains (sensitivity, 0.74; specificity, 1.0) on at least one of the four determinations. These strains had a zone diameter of ≥20 mm. An additional 28% (5 of 18) of these isolates had borderline zone sizes of 18 or 19 mm. The apparent discrepancy between these data and those represented in Fig. 2 exists because the histograms represent the mean of the median values, whereas the above results represent any of the median values (taken from four observations), which correlate less...
well with the agar dilution MIC (see Materials and Methods). Using NCCLS guidelines, which recommend the use of Mueller-Hinton chocolate agar plates (Fig. 2), we found that the standard 10-μg ampicillin disk test failed to detect 17% (3 of 18) of the Amp’ NBLP strains (sensitivity, 0.9; specificity, 1.0); an additional 44% (8 of 18) of these isolates had borderline zone diameters of 18 or 19 mm. In contrast, the 2-μg ampicillin disk test (≤15 mm indicated resistance) clearly differentiated all Amp’ strains from Amp’ strains (sensitivity, 1.0; specificity, 1.0) grown on either medium (Table 1 and Fig. 2). Examination of individual median values outside of the standard deviations depicted in Table 1 with the 2-μg ampicillin disk on both media (four values for each strain) revealed the following data. (i) Of 52 observations with the 13 Amp’ BLP strains, only 1 value of 10 mm and 1 value of 8 mm (representing two strains) were observed. (ii) Of 40 observations with the 10 Amp’ isolates, 1 value of 16 mm, 2 values of 17 mm (one was the strain with the 16-mm reading), and 2 values of 18 mm were observed (all of these values represent four strains). In addition, four observations of ≥23 mm among three strains were observed. (iii) Of 72 observations with the 18 Amp’ NBLP strains, 1 value of 15 mm, 4 values of 14 mm (representing two strains), and 7 values of 13 mm (representing four strains) were observed; these 12 values are derived from a total of four strains. Thus, when the 2-μg ampicillin disk with the cutoff of a 15-mm zone diameter as resistant was used, no overlap among the Amp’ and Amp’ isolates was observed.

A similar examination of the individual median values outside the standard deviation with the disk containing 10 μg of ampicillin tested on both media revealed the following data. (i) Of the 52 observations with the 13 Amp’ BLP strains, 5 values (12 mm three times, 15 mm once, and 18 mm once), representing four strains, were observed. (ii) Of the 40 observations with the 10 Amp’ isolates, 1 value of 20 mm and 1 value of 21 mm representing two strains were observed. In addition, 5 values of ≥28 mm were observed for three isolates. (iii) Of the 72 observations with the 18 Amp’ NBLP strains, 12 values from seven strains (22 mm once, 21 mm four times [representing four strains], and ≤14 mm seven times [representing three strains]) fell outside the standard deviation. Thus, when the 10-μg ampicillin disk (recommended cutoff of 19 mm for resistance) was used, an overlap of individual values occurred between the Amp’ NBLP strains and the Amp’ isolates on both media.

Creation of an intermediate zone of 20 to 21 mm for the 10-μg ampicillin disk would have identified two Amp’ isolates and seven Amp’ NBLP strains as intermediate. Furthermore, one Amp’ NBLP strain with a zone diameter of 22 mm (with the 10-μg disk) would have been misidentified as susceptible: that strain had ampicillin MICs of 8 μg/ml with a 10-μg-CFU inoculum and ≥32 μg/ml with a 10-μg-CFU inoculum. If one extended the intermediate zone to 22 mm, an additional two Amp’ isolates (a total of 4 of 10 studied) would be labeled equivocal. Of 243 consecutive invasive H. influenzae strains isolated in our clinical laboratory, 158 were detected as Amp’ with a disk containing 10 μg of ampicillin. Of these 158 Amp’ isolates, 11 had a zone diameter of 20 or 21 mm, and 18 had a zone diameter of 22 mm. Inclusion of these strains in an intermediate category would label 7% (11 of 158) or 18% (29 of 158) of Amp’

<table>
<thead>
<tr>
<th>Phenotype (no. of strains tested)</th>
<th>β-Lactamase activity</th>
<th>MIC (μg/cm² inoculum)</th>
<th>Ampicillin zone diam (mm [mean ± SD])²</th>
<th>Zone diam difference²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp’ (10)</td>
<td>0</td>
<td>0.5 (0.25–0.5)</td>
<td>21 ± 2 (16–27)</td>
<td>1 ± 1 (–4–4)</td>
</tr>
<tr>
<td>Amp’ (13)</td>
<td>+</td>
<td>32 (8–32)</td>
<td>25 ± 3 (20–34)</td>
<td>13 ± 3 (8–20)</td>
</tr>
<tr>
<td>Amp’ (18)</td>
<td>0</td>
<td>8 (1.5–16)</td>
<td>9 ± 3 (7–15)</td>
<td>2 ± 2 (–2–5)</td>
</tr>
</tbody>
</table>

² β-Lactamase activity was detected by chromogenic cephalosporin substrate; +, presence of activity; 0, no activity.
³ MIC of ampicillin with an inoculum of 10⁵ CFU. The median value is indicated, and the range is shown in parentheses.
⁴ Ranges are indicated within parentheses. The criterion for resistance with the 2-μg ampicillin disk is a zone diameter of ≥15 mm (sensitivity, 1.0; specificity, 1.0). The criterion for resistance with the standard 10-μg ampicillin disk is a zone diameter of ≥19 mm (sensitivity, 0.74; specificity, 1.0).
⁵ Zone diameter of a combined disk with 2 μg of ampicillin plus 10 μg of clavulanic acid minus the zone diameter of a 2-μg ampicillin disk; a difference of ≥8 mm correlated with β-lactamase production (sensitivity, 1.0; specificity, 1.0).
isolates, respectively, as uninterpretable. Only one Amp' NBLP strain with the 10-μg ampicillin disk had zone diameters similar to those of the BLP strains, i.e., values of 7, 8, 8, and 9 on the four determinations. This strain was tested further after prolonged incubation with β-lactamase substrate (overnight at room temperature and at 37°C) and after growth on ampicillin-containing media to test for an inducible β-lactamase; no enzyme activity was detected compared to the appropriate control strains.

A difference of ≥8 mm between the zone diameter of the disk containing 2 μg of ampicillin plus 10 μg of clavulanic acid and the zone diameter of the 2-μg ampicillin disk correlated with production of β-lactamase for all 13 strains tested (sensitivity, 1.0; specificity, 1.0; Table 1).

Linear regression analysis consistently revealed that the disk containing 2 μg of ampicillin showed a higher correlation with either medium and with either inoculum (Fig. 3) than did the disk containing 10 μg of ampicillin. This was also observed when the results with each medium were combined and compared by linear regression (Fig. 4). Furthermore, the correlation was always higher with a 10³-CFU inoculum than with a 10⁵-CFU inoculum (Fig. 3 and 4).

**DISCUSSION**

Since 1980, reports of ampicillin-resistant NBLP strains of *H. influenzae* have been increasing (1, 12, 14, 24, 26, 29). Reference laboratories in England and Australia found that 8 and 54%, respectively, of ampicillin-resistant isolates did not have detectable β-lactamase activity (1, 29).

The characterization of the Amp' NBLP strains of *H. influenzae* has been limited: four strains have been studied (16, 26). We characterized three of these and demonstrated that the resistance is chromosomally determined and that the primary mechanism of resistance is an alteration of the penicillin-binding proteins (16). Alterations in the penicillin-binding proteins have also been shown to be the mechanism of resistance in clinical isolates of Pcn' non-penicillinase-producing *Streptococcus pneumoniae* and Pcn' non-penicillinase-producing *Neisseria gonorrhoeae* (6, 43).

Certain problems with disk susceptibility testing with *H. influenzae* have previously been described (34, 38, 39). The current NCCLS guidelines were devised to differentiate Amp' BLP strains of *H. influenzae* from Amp' isolates. With the emergence of a new subset of Amp' strains which do not
produce detectable β-lactamase, the use of standard disks containing 10 μg of ampicillin needs to be reassessed.

Our data with disks containing 2 μg of ampicillin, in contrast to the data for standard 10-μg disks, differentiated all Amp* strains, regardless of the mechanism of resistance, from the Amp* isolates. These data are consistent with another report which examined 10 Amp* NBLP strains with standard disks: 8 of 10 strains were identified as susceptible (27). Those authors recommended the use of a disk containing 10 μg of clavulanic acid, but that approach did not differentiate all the Amp* strains from the Amp* strains tested (27).

Even though antimicrobial therapy is usually not modified until disk susceptibility test results are known, most laboratories perform a commercially available, rapid β-lactamase test to detect Amp* BLP strains. As more Amp* NBLP strains of *H. influenzae* are reported, the use of a screening β-lactamase test may be of limited value for the identification of ampicillin resistance. However, selection of appropriate antimicrobial therapy may be facilitated by determination of the presence of this mechanism of resistance. For example, β-lactamase-stable cephalosporins, including cefuroxime, cefotaxime, and ceftriaxone and augmentin (amoxicillin plus clavulanic acid), are effective for Amp* BLP strains (12, 28, 36, 42). However, augmentin has no effect on the Amp* NBLP strains, and the cephalosporins have reduced potency against Amp* NBLP strains because of the altered targets (penicillin-binding proteins [30]).

Although the rapid β-lactamase test might provide this valuable therapeutic information, it requires another procedure in addition to routine disk susceptibility testing. Placement of a combination disk containing 10 μg of clavulanic acid and 2 μg of ampicillin at the time of initial disk susceptibility testing along with a 2-μg ampicillin disk requires minimal additional labor, is economical, and will provide this useful information.

Amp* NBLP strains of *H. influenzae* have been isolated primarily from the respiratory tract. Patients with chronic pulmonary disease, e.g., cystic fibrosis, chronic bronchitis, or chronic obstructive pulmonary disease, who receive frequent courses of β-lactam antibiotics, appear to be at greatest risk of harboring these strains. Repeated exposure of *H. influenzae* in vivo to β-lactam antibiotics may create a

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**FIG. 3.** Regression line analysis of MICs of ampicillin with *H. influenzae* strains plotted against zone diameters obtained with disks containing 2 μg (△) or 10 μg (●) of ampicillin. Data are presented with inocula of 10⁵ CFU (A and C) and 10⁶ CFU (B and D) and either Mueller-Hinton chocolate agar plates (A and B) or chocolate plus enrichment plates (C and D). Each symbol represents a single isolate unless otherwise noted by either a left-hand subscript to △ or a right-hand superscript to ●.
selective advantage for those strains in which mutational events have altered the targets of these substrates, the penicillin-binding proteins. Whether strains with this in vitro resistance significantly correlate with clinical failure remains to be investigated. Any Amp\(^\beta\) NBLP isolate associated with a treatment failure should have an MIC determination.

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LITERATURE CITED


