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Ampicillin Disk Diffusion Susceptibility Testing of *Haemophilus influenzae*

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A total of 114 strains of *Haemophilus influenzae* were characterized with respect to beta-lactamase production and ampicillin MIC. Of this total, 41 strains produced a TEM-type beta-lactamase, and ampicillin MICs for these strains were ≥ 2.0 $\mu\text{g/ml}$. It was found that 54 strains lacked TEM-type beta-lactamase activity, and ampicillin MICs for them were ≤ 0.5 $\mu\text{g/ml}$. The remaining 19 strains were beta-lactamase negative, but ampicillin MICs were ≥ 2.0 $\mu\text{g/ml}$. Disk diffusion susceptibility tests were performed with two media, i.e., Mueller-Hinton agar containing 1.0% hemoglobin and 1.0% IsoVitaleX supplement (CHOC-MHA) and enriched chocolate agar (CHOC), by using disks containing 10 and 2 μg of ampicillin. If strains of *H. influenzae* for which ampicillin MICs were ≥ 2.0 $\mu\text{g/ml}$ were considered resistant, while strains for which MICs were ≤ 0.5 $\mu\text{g/ml}$ were considered susceptible, the following zone diameter interpretive criteria were identified as indicating ampicillin susceptibility: CHOC-MHA (10- μg disks), ≥ 20 mm; CHOC-MHA (2- μg disks), ≥ 17 mm; CHOC (10- μg disks), ≥ 25 mm; and CHOC (2- μg disks), ≥ 20 mm. In all cases, zones of inhibition less than those listed above would be interpreted as indicating resistance. Each of these four combinations was found to be essentially equivalent in identifying susceptible and resistant strains of *H. influenzae*, irrespective of beta-lactamase production.

It is now recognized that plasmid-mediated production of a TEM-type beta-lactamase is not the only mechanism of ampicillin resistance among clinical isolates of *Haemophilus influenzae* (1, 3, 9, 11). Possible alternative mechanisms of ampicillin resistance include production of a beta-lactamase different from the TEM type, altered penicillin-binding proteins, and diminished permeability of beta-lactam antimicrobial agents through the cell wall outer membrane (4, 6). Irrespective of the mechanism of ampicillin resistance among such strains, conventional beta-lactamase assays, such as those which readily detect the TEM-type beta-lactamase of *H. influenzae*, yield negative results. As a result, the detection of ampicillin resistance among strains of *H. influenzae* which lack the TEM-type beta-lactamase requires some direct measure of ampicillin activity.

The most recent approved standards of the National Committee for Clinical Laboratory Standards (NCCLS) provide guidelines for ampicillin disk diffusion susceptibility testing of *H. influenzae* (7). Use of Mueller-Hinton agar supplemented with 1% hemoglobin or 5% horse blood and V factor enrichments is advocated. Strains with zones of inhibition of ≤ 19 mm surrounding 10- μg ampicillin disks are considered resistant; strains with zone diameters of ≥ 20 mm are considered susceptible.

In a recent investigation of disk diffusion susceptibility testing of *H. influenzae*, use of the NCCLS method resulted in the false-susceptible classification of 8 of 18 (44%) ampicillin-resistant strains which lacked the TEM-type beta-lactamase (5). In contrast, when disks which contained 2 μg of ampicillin were used, all ampicillin-resistant strains of *H. influenzae* were properly classified. The previous study, however, used brain heart infusion-based media. Furthermore, a relatively small total number of isolates of *H. influenzae* (41 isolates) were used to assess the disk diffusion procedure.

The intent of the present investigation was to reexamine the issue of ampicillin disk diffusion susceptibility testing of *H. influenzae* by using a large number of test strains (114 strains). Two media were evaluated: Mueller-Hinton agar containing 1.0% bovine hemoglobin and 1.0% IsoVitaleX supplement (CHOC-MHA), as recommended for use by the NCCLS, and enriched chocolate agar (CHOC). In addition, disks containing either 10 or 2 μg of ampicillin were used. The results of disk diffusion susceptibility tests were compared with ampicillin MICs determined by using a macrotube broth dilution procedure with Mueller-Hinton broth supplemented with 3% lysed horse blood and 15 μg of NAD per ml. The final inoculum concentration was 1×10^5 to 2×10^5 CFU/ml.

MATERIALS AND METHODS

Strains of *H. influenzae*. A total of 114 strains of *H. influenzae* were examined in this investigation. Of these strains, 64 were obtained from patients in central Massachusetts with a variety of *H. influenzae* infections during the 5-year period 1980 to 1985. The remaining 50 strains were provided by James H. Jorgensen, University of Texas Health Science Center at San Antonio, and represented clinical isolates of *H. influenzae* obtained from several different locations in the United States as well as various Western European countries. Organisms were maintained at -70°C in 2% skim milk. Prior to testing, stock cultures were thawed, inoculated onto enriched chocolate agar plates (GC agar base supplemented with 1% bovine hemoglobin and 1% IsoVitaleX), and incubated at 35°C in 5 to 7% CO_2 for 18 to 24 h. Isolated colonies from the initial subculture were transferred to a second enriched chocolate agar plate, which was incubated under identical conditions. Growth from this plate was used to prepare inocula for all subsequent tests.

Ampicillin MIC determinations. Ampicillin MICs were determined by using a macrotube broth dilution procedure in cation-supplemented Mueller-Hinton broth containing 3%

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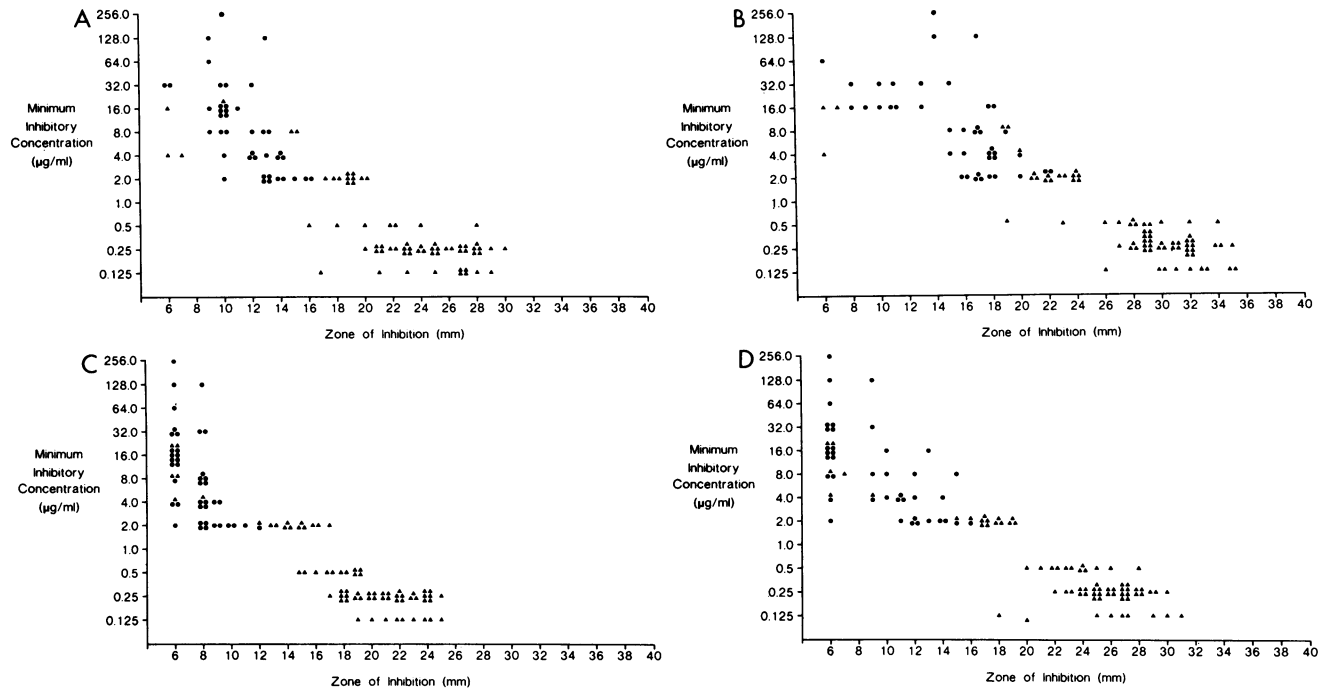


FIG. 1. Comparison between ampicillin MICs and zones of inhibition obtained with 114 strains of *H. influenzae* by using two media and disks containing 10 and 2 µg of ampicillin. ▲, Beta-lactamase-negative strains; ●, beta-lactamase-positive strains. (A) CHOC-MHA and 10-µg ampicillin disk. (B) CHOC and 10-µg ampicillin disk. (C) CHOC-MHA and 2-µg ampicillin disk. (D) CHOC and 2-µg ampicillin disk.

lysed horse blood and 15 µg of NAD (Sigma Chemical Co., St. Louis, Mo.) per ml (pH 7.2; final volume, 2.0 ml). Ampicillin, obtained as reagent-grade powder from Bristol Laboratories, Syracuse, N.Y., was tested at twofold concentration increments ranging from 0.008 to 512 µg/ml. The final concentration of test organism, confirmed by viable cell counts, was 1×10^5 to 2×10^5 CFU/ml. Tubes were incubated for 24 h at 35°C in ambient air and examined macroscopically for evidence of turbidity. The MIC was defined as the lowest concentration of ampicillin tested which yielded no macroscopic evidence of turbidity. All MIC determinations were performed in duplicate, and the results were averaged to obtain an estimate of the MIC for a given organism-antimicrobial agent combination. In cases in which duplicate MICs varied by only one twofold concentration, the lower value was assigned as the ampicillin MIC. Among the 114 study isolates, in 96 cases (84.2%), the results of duplicate MIC determinations were the same. In 16 cases (14.0%), duplicate MICs varied by one twofold concentration. In the remaining two cases (1.8%), duplicate MICs yielded a fourfold difference.

Beta-lactamase determination. All study strains were examined for beta-lactamase activity with the conventional tube chromogenic cephalosporin assay with nitrocefin as a substrate (8).

Disk diffusion susceptibility studies. Disk diffusion susceptibility tests were performed by the method described by the NCCLS (7). CHOC-MHA and CHOC plates were both obtained preprepared as 100-mm plastic petri dishes from Scott Laboratories, Fiskeville, R.I. Paper disks contained 10 and 2 µg of ampicillin (BBL Microbiology Systems, Cockeysville, Md.). After incubation of plates for 18 h at 35°C in 5 to 7% CO₂, zones of inhibition were measured with a caliper to the nearest millimeter. All determinations were made in duplicate.

Linear regression analysis. Comparison of zones of inhibition obtained by the disk diffusion procedure with MICs (log₂) obtained by the macrotube broth dilution technique was accomplished by linear regression analysis by using the method of least squares. Test strains with no measurable zone of inhibition were excluded from linear regression analyses.

RESULTS

Among the 114 strains of *H. influenzae* examined in this study, 41 produced beta-lactamase (Fig. 1). For all beta-lactamase-producing strains, ampicillin MICs were ≥ 2.0 µg/ml. The remaining 73 strains failed to produce beta-lactamase detectable by the conventional nitrocefin assay and could be categorized into two groups based on ampicillin MIC. For 54 of these 73 beta-lactamase-negative strains, ampicillin MICs were ≤ 0.5 µg/ml; for the other 19 strains, ampicillin MICs were ≤ 2.0 µg/ml. In the latter group of 19 beta-lactamase-negative strains, the ampicillin MIC for 13 isolates was 2.0, and ampicillin MICs of 4.0, 8.0, and 16 µg/ml were determined for the remaining 6 isolates (2 isolates for each MIC).

The results of duplicate determinations of ampicillin zones of inhibition on CHOC-MHA and CHOC by using both 10- and 2-µg ampicillin disks are presented in Table 1. The degree of variability was greatest on CHOC. With CHOC-MHA, duplicate determinations of zone diameters varied by no more than 2 mm for 113 of the 114 (99.9%) test strains irrespective of the disk content. In contrast, when CHOC was used with disks containing 10 µg of ampicillin, 25.4% of duplicate determinations of zone sizes varied by more than 2 mm. Similarly, with disks containing 2 µg of ampicillin tested on CHOC, 19.3% of duplicate determinations varied by more than 2 mm.

TABLE 1. Reproducibility of disk diffusion susceptibility test results with 114 strains of *H. influenzae* examined by using two media and disks containing 10 and 2 μg of ampicillin

Medium	Ampicillin content of disk (μg)	No. of strains (cumulative %) for which duplicate determinations of ampicillin zone diam varied by mm:							
		0	1	2	3	4	5	6	7
CHOC-MHA	10	58 (50.9)	48 (93.0)	7 (99.1)					1 (100)
CHOC	10	20 (17.5)	43 (55.3)	22 (74.6)	17 (89.5)	5 (93.9)	4 (97.4)	2 (99.1)	1 (100)
CHOC-MHA	2	74 (64.9)	33 (93.9)	6 (99.1)	1 (100)				
CHOC	2	44 (38.6)	29 (64.0)	19 (80.7)	14 (93.0)	4 (96.5)	2 (98.3)	1 (99.1)	1 (100)

Disk diffusion tests were performed with plates incubated in an atmosphere of 5 to 7% CO_2 . This was necessary insofar as preliminary experiments revealed that 29 of the 114 (25.4%) test strains of *H. influenzae* examined in this investigation grew poorly on both CHOC-MHA and CHOC when plates were incubated in ambient air. The extent of growth of these strains on CHOC-MHA and CHOC in ambient air was insufficient to permit reliable measurements of zones of inhibition.

Plots comparing MICs with zones of inhibition obtained on CHOC-MHA and CHOC with 10- and 2- μg ampicillin disks are shown in Fig. 1. In each case, the mean of duplicate zone diameter determinations was used for comparisons. As seen in Fig. 1, a distinct bimodal distribution of organisms existed with respect to ampicillin MIC; i.e., ampicillin MICs for study isolates were either ≥ 2.0 or ≤ 0.5 $\mu\text{g}/\text{ml}$. Furthermore, composition of the test medium and the disk content had a pronounced influence on the size of zones of inhibition. Specifically, zone diameters were larger on CHOC than on CHOC-MHA and with disks containing 10 μg of ampicillin versus those containing 2 μg of ampicillin. Correlation coefficients for the four comparisons were as follows: CHOC-MHA (10- μg disks), 0.888; CHOC (10- μg disks), 0.920; CHOC-MHA (2- μg disks), 0.868; and CHOC (2- μg disks), 0.898.

In general, the extent of separation according to zone diameters of strains for which the ampicillin MICs were ≥ 2.0 $\mu\text{g}/\text{ml}$ versus those for which ampicillin MICs were ≤ 0.5 $\mu\text{g}/\text{ml}$ was greater on CHOC than on CHOC-MHA. Disk content, however, appeared to have little effect on the extent of separation according to zone sizes of the two organism groups. Finally, it should be noted that among strains for which ampicillin MICs were 2.0 $\mu\text{g}/\text{ml}$, beta-lactamase-negative organisms tended to have considerably larger zone diameters than did beta-lactamase-producing organisms. This pattern was observed on both media with both ampicillin disks.

DISCUSSION

Selection of a particular method for performing disk diffusion susceptibility testing may be predicated on a number of different factors. These include the precision of the method as evidenced by the reproducibility of zone diameters when individual organisms are tested repetitively, test accuracy as judged by the ability of a particular method to discriminate susceptible organisms from those that are resistant, and the degree of correlation between zones of inhibition and MICs as assessed by linear regression analysis.

In the present investigation of *H. influenzae* disk diffusion susceptibility tests with ampicillin, greater precision was achieved with CHOC-MHA than with CHOC. The ampicillin content of disks, however, was not found to influence test

precision. Furthermore, the degree of correlation between zones of inhibition and MICs was similar irrespective of the medium used or the content of the disk (range of correlation coefficients, 0.868 to 0.920).

These correlation coefficients were derived from comparisons between zone diameters and MICs, with MICs determined by a macrotube broth dilution procedure at a final inoculum of ca. 10^5 CFU/ml. Recently, Mendelman et al. (5) reported that the degree of correlation between ampicillin zones of inhibition and MICs is greatest with *H. influenzae* when inocula of 10^3 or 10^4 CFU are used for MIC determinations, irrespective of disk content and the media used to perform disk diffusion tests. With these small inoculum sizes, they observed correlation coefficients of 0.893 to 0.935 for disks containing 2 or 10 μg of ampicillin tested with CHOC-MHA or CHOC. In contrast, when comparisons were based on MIC determinations at an inoculum size of 10^5 CFU, correlation coefficients varied from 0.719 to 0.899.

The difference between our results and those of Mendelman et al. (5) might be explained by the methods used to determine MICs. We used a macrotube broth dilution procedure with a relatively large volume (2.0 ml) of a medium that has been widely used for broth dilution susceptibility testing of *H. influenzae* (Mueller-Hinton broth supplemented with lysed horse blood and NAD). Mendelman et al. determined MICs with an agar dilution method which used brain heart infusion agar supplemented with hemin, L-histidine, and NAD. Perhaps the differences between our results and those of Mendelman et al. could be explained by the different methods and media used to determine MICs. In any case, we believe that the method we used to determine MICs affords a meaningful basis for assessing disk diffusion results insofar as this method used a medium that is widely accepted for testing *H. influenzae* and an inoculum size (ca. 10^5 CFU/ml) that is broadly advocated for broth dilution susceptibility tests.

Estimates of test accuracy are dependent on the ability of a test to clearly define strains as being resistant or susceptible. With respect to *H. influenzae*, the results of the present investigation suggest that strains for which ampicillin MICs are ≥ 2.0 $\mu\text{g}/\text{ml}$ be considered resistant, while those for which ampicillin MICs are ≤ 0.5 $\mu\text{g}/\text{ml}$ be considered susceptible. This interpretation is based on the observation that when viewed according to MICs a clear-cut bimodal distribution of organisms was observed when MICs were determined by a conventional, accepted method. Ampicillin MICs for the strains were either ≥ 2.0 or ≤ 0.5 $\mu\text{g}/\text{ml}$. Furthermore, the range of ampicillin MICs for beta-lactamase-producing strains started at 2.0 $\mu\text{g}/\text{ml}$ and went higher. There were no beta-lactamase-positive organisms for which ampicillin MICs were < 2.0 $\mu\text{g}/\text{ml}$. In other words, among strains of *H. influenzae* known to be resistant to ampicillin, that is, beta-lactamase-producing organisms, ≥ 2.0 $\mu\text{g}/\text{ml}$ appeared to be the best MIC breakpoint for defining resistance. These

results are consistent with the observations of others (2, 10, 12).

If these definitions of susceptible and resistant, which are predicated on MICs, are accepted, then there exists a basis for assessing the accuracy of disk diffusion tests performed by using the four combinations of media and disks examined in this investigation. With CHOC-MHA and 10- μ g ampicillin disks, the smallest number of interpretive errors was observed by using cutoffs of ≥ 20 and ≥ 21 mm to define susceptible strains. In both cases, five interpretive errors were noted among the 114 test organisms, i.e., three major errors and two very major errors at ≤ 20 mm and five major errors at ≥ 21 mm. When CHOC was used with 10- μ g ampicillin disks, a criterion of ≥ 25 mm yielded the smallest number of interpretive errors, i.e., two major errors. With CHOC-MHA and disks containing 2 μ g of ampicillin, the smallest number of interpretive errors was obtained when ≥ 17 mm was used to define the susceptible category, i.e., three major and one very major error. At a cutoff of ≥ 18 mm, no very major errors were noted but six major errors were observed. Finally, when CHOC was used in combination with 2- μ g ampicillin disks, the smallest number of interpretive errors was observed when ≥ 20 mm was used to define susceptible strains, i.e., one major error. These results suggest that the greatest test accuracy could be achieved with CHOC.

The question arises: what is the optimum approach to performing ampicillin disk diffusion susceptibility tests with *H. influenzae*? If this decision were predicated solely on test accuracy, the use of CHOC with 2- μ g ampicillin disks would be the method of choice. As stated above, however, zones of inhibition obtained on CHOC, irrespective of the ampicillin content of disks, were conspicuously less reproducible than those obtained on CHOC-MHA. It is difficult, therefore, to recommend a single method.

In the absence of a preferred method, we believe the results of the current investigation define four different and equally acceptable approaches to disk diffusion susceptibility testing of *H. influenzae*. When CHOC-MHA is used with 10- μ g ampicillin disks, the current guideline of the NCCLS should be used, i.e., ≥ 20 mm indicates susceptibility (7). With 2- μ g disks and CHOC-MHA, strains with zone diameters of ≥ 17 mm should be considered susceptible. If CHOC is used in combination with 10- μ g ampicillin disks, ≥ 25 mm best defines susceptible strains. Finally, with 2- μ g disks and CHOC, organisms with zone diameters of ≥ 20 mm should be considered susceptible. In each case, strains with zones of inhibition less than those used to define the susceptible category would be considered resistant. In all cases, ampi-

cillin MIC correlates are: ≥ 2.0 μ g/ml for resistance and ≤ 0.5 μ g/ml for susceptibility.

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