Determination of Penicillin MICs for \textit{Streptococcus pneumoniae} by Using a Two- or Three-Disk Diffusion Procedure

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The potential for the use of the disk diffusion method to accurately predict penicillin MICs for \textit{Streptococcus pneumoniae} was investigated with penicillin (6 \(\mu\)g), methicillin (5 \(\mu\)g), and oxacillin (1 \(\mu\)g) disks. A total of 183 \textit{S. pneumoniae} isolates were tested by three MIC procedures (agar dilution, microdilution, and E-test). Regression analyses of the geometric mean of the three MIC results against (i) the sum of the zone diameters for methicillin, penicillin, and oxacillin disks; (ii) the sum of the zone diameters for methicillin and penicillin disks; and (iii) each of the three individual zone diameters were performed. Calculated MICs were determined from each of these regression analyses and compared to the mean reference MICs. A high level of correlation was obtained with both the two- and the three-disk procedures (\(r = 0.97\)), with essential agreement rates (\(\geq 1\) doubling dilution) between MICs calculated by the three-disk procedure and the two-disk procedure and the mean reference MICs of 98.4 and 99.8\%, respectively. No major or very major errors were obtained with the two- or three-disk procedures. The accuracy of the disks used individually was lower (\(r = 0.84\) to 0.93). However, oxacillin and methicillin disk testing remained excellent for screening strains, with all penicillin-susceptible strains having zones of \(> 21\) and \(> 22\) mm, respectively. The combination disk procedure, which involves the use of three disks (methicillin, oxacillin, and penicillin) or two disks (methicillin and penicillin) for testing \textit{S. pneumoniae}, can provide accurate penicillin MICs and qualitative category results that are comparable to results obtained by the E-test, agar, and microdilution MIC methods.

Clinical microbiology laboratories are faced with the challenge of reliably detecting bacterial resistance to antimicrobial agents, and at the same time, reducing their laboratory costs. As resistance rates increase, additional susceptibility testing is required, which increases laboratory costs. For example, since it was first reported in 1967, \textit{Streptococcus pneumoniae} resistance to penicillin has been recognized worldwide (1, 6, 8, 10, 22). The current recommendation for the determination of penicillin susceptibility is to perform a MIC analysis by an approved method or to first screen for penicillin resistance by disk diffusion with a 1-\(\mu\)g oxacillin disk, followed by MIC determination for strains with zones of 7 to 19 mm, usually having MICs at the upper limit of susceptible strains having zones of \(> 21\) and \(> 22\) mm, respectively. The combination disk procedure, which involves the use of three disks (methicillin, oxacillin, and penicillin) or two disks (methicillin and penicillin) for testing \textit{S. pneumoniae}, can provide accurate penicillin MICs and qualitative category results that are comparable to results obtained by the E-test, agar, and microdilution MIC methods.

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Clinical microbiology laboratories are faced with the challenge of reliably detecting bacterial resistance to antimicrobial agents, and at the same time, reducing their laboratory costs. As resistance rates increase, additional susceptibility testing is required, which increases laboratory costs. For example, since it was first reported in 1967, \textit{Streptococcus pneumoniae} resistance to penicillin has been recognized worldwide (1, 6, 8, 10, 14, 16, 18, 23). In recent studies, penicillin resistance rates as high as 30\% have been reported in some pediatric populations (2, 6).

Based on the current prevalence of resistance, susceptibility testing of pneumococcal strains is important for patient care as well as for tracking epidemiological trends. \textit{S. pneumoniae} isolates are considered susceptible to penicillin if MICs are \(\leq 0.06\) \(\mu\)g/ml, intermediate if MICs are 0.12 to 1 \(\mu\)g/ml, and resistant if MICs are \(\geq 2\) \(\mu\)g/ml (10, 22). The current recommendation for the determination of penicillin susceptibility is to perform a MIC analysis by an approved method or to first screen for penicillin resistance by disk diffusion with a 1-\(\mu\)g oxacillin disk, followed by MIC determination for strains with zone diameters of \(\leq 19\) mm. Penicillin-susceptible strains are also susceptible to other \(\beta\)-lactam antibiotics approved for treating pneumococcal infections, and other \(\beta\)-lactams usually do not need to be tested (1, 22).

The use of disk diffusion for screening strains of \textit{S. pneumoniae} was first described by Dixon et al. in 1977 with 1-\(\mu\)g oxacillin disks, as 6-\(\mu\)g penicillin disks did not reliably detect all resistant strains (5). Methicillin was reported to have similar utility shortly thereafter (10, 11). Since then, other studies have confirmed the utility of oxacillin and methicillin disks (12, 24), and the oxacillin disk is the currently recommended screening procedure (21, 22). Susceptible strains of pneumococci reliably produce oxacillin zones of 20 mm or greater. However, it has been clearly demonstrated that oxacillin zones of \(\leq 19\) mm have been obtained not only with penicillin-resistant and -intermediate strains but also with some penicillin-susceptible strains (6, 12, 17, 24). These strains, which produce oxacillin zones of 7 to 19 mm, usually have MICs at the upper limit of the susceptible category (0.06 \(\mu\)g/ml) and have been shown to have altered penicillin-binding proteins (9).

The present study was conducted in order to evaluate the use of a less costly procedure for obtaining penicillin MIC results for \textit{S. pneumoniae}. Disk diffusion testing with oxacillin, penicillin, and methicillin disks and three different MIC procedures were performed on 183 isolates of \textit{S. pneumoniae}. The results of MIC and disk diffusion tests were compared in order to determine if results from any single disk or a combination of disks could be used to accurately calculate MICs. The use of a combination of disks of varying penicillin content has been demonstrated previously to be an accurate method of determining penicillin MICs for \textit{Neisseria gonorrhoeae} (13).

**MATERIALS AND METHODS**

**Bacterial isolates.** One hundred eighty-three \textit{S. pneumoniae} strains isolated from clinical infections and carriers in the United States, Europe, and South Africa were selected. Strains were identified by typical Gram stain and colonial morphology, alpha-hemolysis, inhibition by optochin, bile solubility, and positive capsular swelling with pneumococcal omnisera (14). Strains were chosen to include approximately one-third penicillin susceptible, one-third penicillin intermediate, and one-third penicillin resistant. Strains were stored at \(-70\)°C and subcultured to blood agar plates at least twice prior to testing.

**Susceptibility testing.** (i) Agar dilution MIC determination. MICs were determined on Mueller-Hinton agar (Difco, Detroit, Mich.) supplemented with 5\%
sheep blood (Cleveland Scientific, Bath, Ohio) (11, 20). Concentrations of penicillin tested were doubling dilutions from 0.008 to 64 μg/ml. Agar dilution plates were incubated in 5% CO₂ at 35°C for 24 h.

(i) Microdilution MIC determination. Microdilution trays were prepared according to the National Committee for Clinical Laboratory Standards (NCCLS) method with cation-adjusted Mueller-Hinton broth (Difco) supplemented with 5% lysed horse blood (Cleveland Scientific) (4, 20). The penicillin concentration in the trays ranged from 0.008 to 16 μg/ml. Organism suspensions were prepared in saline from overnight growth on blood agar plates to the density of a 0.5 McFarland standard, diluted 1:10, and inoculated onto penicillin-containing plates with a Steers replicator with 3-mm pins delivering inocula of 1 million CFU/spot. Plates were incubated in 5% CO₂ at 35°C for 24 h.

(ii) Microdilution MIC determination. Microdilution trays were prepared according to the National Committee for Clinical Laboratory Standards (NCCLS) method with cation-adjusted Mueller-Hinton broth (Difco) supplemented with 5% lysed horse blood (Cleveland Scientific) (4, 20). The penicillin concentration in the trays ranged from 0.008 to 16 μg/ml. Organism suspensions were prepared in saline from overnight growth on blood agar plates to the density of a 0.5 McFarland standard. This organism suspension was then diluted to provide a final inoculum of 10⁵ to 10⁶ CFU/ml. The microdilution trays were incubated in ambient air at 35°C for 24 h.

(iii) E-test MIC determination. E-test penicillin G strips (AB Biodisk, Solna, Sweden) graduated from 0.016 to 256 μg/ml were used (15). Organism suspensions were prepared in saline from overnight growth on blood agar plates to the density of a 0.5 McFarland standard. A swab from this organism suspension was used to inoculate 15-cm-diameter Mueller-Hinton plates with 5% sheep blood (Becton Dickinson, Cockeysville, Md.) with a three-direction inoculation procedure. The penicillin E-test strips were placed on the plates, which were incubated at 35°C in 5% CO₂ for 24 h. MICs were read at the point of intersection between the ellipse edge and the E-test strip. Intermediate E-test MICs were adjusted up to the next-highest doubling-dilution value for comparative purposes (15).

(iv) Disk diffusion testing. Disk diffusion tests were performed according to NCCLS methods with Mueller-Hinton agar supplemented with 5% sheep blood (Becton Dickinson) (21, 22). Disks containing 6 μg of penicillin, 1 μg of oxacillin, and 5 μg of methicillin were applied to the same plates inoculated for the E-test procedure. Plates were incubated at 35°C in 5% CO₂ for 24 h. Zone diameters were measured with calipers to the nearest millimeter.

(v) Quality control. For all methods, NCCLS-recommended quality control strains, including S. pneumoniae (ATCC 49619), were included in each test run (22). Results were accepted only if quality control results were within the NCCLS-specified ranges.

Validation. Sixty selected strains of S. pneumoniae were chosen from recent clinical isolates to include 20 strains in each category (susceptible, intermediate, and resistant) for validation of the disk methods with criteria described by Tenover et al. (25). Strains were tested by broth microdilution with commercially available dried panels (Accumed, Westlake, Ohio) reconstituted with Mueller-Hinton broth with 5% lysed horse blood according to the manufacturer’s directions. This method complies with the requirements of the NCCLS broth microdilution method (20). Disk diffusion was performed as above with different lots of disks and plates than those used for generating the interpretative criteria. Results of combined disk diffusion zones were interpreted according to the criteria developed in this study to evaluate the accuracy of the method.

Statistical methods. Regression analysis of scatterplots was done by the least-squares method, and correlation coefficients (r) were calculated for MICs versus MICs, MICs versus zone diameters, and MICs versus sums of zone diameters (13). MICs were calculated from zone diameters and sums of zone diameters with the BIOMIC system (Giles Scientific Inc., New York, N.Y.) algorithm (3). The method of summing zone diameters from several disks has been used in prior studies and provides greater precision in MIC prediction (13). The ratios of these calculated MICs to the mean MICs by the three methods and the ratios of the MICs by the three methods to each other were determined. When the MICs by the two methods were identical, the ratio was 1; when the calculated MIC was greater than the reference method MIC, the ratio was greater than 1; and when the calculated MIC was less than the reference method MIC, the ratio was less than 1. When a reference MIC was less than or equal to the minimum concentration tested, the minimum value was used for calculating the ratio. There were no values greater than the maximum concentration for the MIC methods. The two methods were considered to be essentially in agreement when the ratios ranged from 0.5 to 2.0, i.e., within 1 doubling dilution.

With NCCLS-recommended interpretive criteria for penicillin (i.e., ≤0.06 μg/ml, susceptible; 0.12 to 1 μg/ml, intermediate; and ≥2 μg/ml, resistant) (22), discrepancy rates were calculated according to minor, major, and very major error criteria (24). A minor error is a one-category difference between the methods (e.g., susceptible versus intermediate or intermediate versus resistant). A major error is indicated when the reference result is susceptible and the comparative result is resistant. A very major error is indicated when the reference result is resistant and the comparative result is susceptible.

RESULTS

The results of the three reference MIC methods are shown as the MICs at which 50% of the isolates are inhibited (MIC<sub>50</sub>) and MIC<sub>90</sub> in Table 1. These values show excellent agreement between the three MIC methods. The percentages of isolates according to penicillin category by the agar dilution method were 32.8% susceptible (Pen<sup>S</sup>), 36.1% intermediate (Pen<sup>I</sup>), and 31.1% resistant (Pen<sup>R</sup>). In comparison, microdilution showed 37.3% to be Pen<sup>S</sup>, 33.9% to be Pen<sup>R</sup>, and 28.4% to be Pen<sup>I</sup>. While the E-test showed 27.9% to be Pen<sup>S</sup>, 42.6% to be Pen<sup>R</sup>, and 29.5% to be Pen<sup>I</sup>. The results of the three MIC methods were compared by regression plots, comparisons of MIC ratios, and categorical error rates. This analysis showed that the E-test and agar dilution results were closest, with a 99.5% essential agreement rate; no major or very major category discrepancies; 7.7% minor discrepancies; and a correlation coefficient (r) of 0.99. The correlation of agar dilution versus microdilution and E-test versus microdilution was slightly lower (95.1% essential agreement, r = 0.98 for both), with
9.9 and 13.3% minor errors, respectively; no major or very major category discrepancies were present. As the three MIC methods were in such close agreement, their geometric mean MICs (referred to as mean reference MICs) were determined and used for correlation with disk diffusion results.

Regression analyses of individual zone diameters and the sum of zone diameters of various combinations of the oxacillin, methicillin, and penicillin disks against mean reference MICs were performed. These scatterplots are depicted in Fig. 1 and 2, and essential agreement rates, correlation coefficients, and error rates are shown in Table 2. The individual disk correlation coefficients were 0.84, 0.92, and 0.93 for oxacillin, methicillin, and penicillin, respectively. Essential agreement scores of MICs calculated from individual oxacillin and methicillin disks were 79% and 87%, respectively. The penicillin disk, however, had the best essential agreement of the single disks (93%), although the MIC ratios for only 54% of the strains were 1. The oxacillin and methicillin disks did not differentiate Penr from Peni strains, and penicillin disks showed considerable overlap between categories.

The sum of the oxacillin, methicillin, and penicillin disks and the sum of the methicillin and penicillin disks produced better agreement with mean reference MICs than did the single disks. The correlation coefficient was 0.97 for both disk combinations, and essential agreement between MICs calculated by the three-disk procedure (oxacillin, methicillin, and penicillin) with the mean reference MICs was 98.4%, and that between MICs calculated by the two-disk procedure (methicillin and penicillin) was 98.9%. In addition, MIC ratios of 1 were obtained for 76% of strains by the three-disk method and for 81% by the two-disk method.

Analysis of discrepancies in Penr, Peni, and Peni categories between calculated MICs and mean reference MICs showed all discrepancies to be minor, with most being within 1 doubling dilution of a breakpoint. The fewest discrepancies occurred with the three-disk, two-disk, and methicillin disk procedures, with values similar to those obtained by the various MIC methods. Oxacillin discrepancies were substantially higher (27.9%), even when discrepancies within 1 dilution of breakpoints were excluded (11.5%).

Further analysis of oxacillin disk and individual MIC method data with the NCCLS oxacillin zone interpretations of ≥20 mm as Penr and ≤19 mm as Peni or Peni showed agreement for 181 of the 183 strains (98.9%) with E-test categories; the MICs for the discrepant strains were 0.06 and 0.12 μg/ml. Oxacillin zones were in agreement with agar dilution categories for 173 strains (94.5%); the MICs for the discrepant strains were 0.06 μg/ml (9 strains) and 0.12 μg/ml (1 strain). Agreement with microdilution category occurred for 164 strains (89.6%); the MICs for the discrepant strains were 0.03 μg/ml (1 strain), 0.06 μg/ml (17 strains), and 0.12 μg/ml (1 strain).

The MICs that can be calculated from the two- and three-

### TABLE 2. Comparison of MIC ratios of MICs, calculated from zone diameter measurements and mean reference MICs, and categorical error rates

<table>
<thead>
<tr>
<th>Drug disk used to calculate MIC</th>
<th>% of tests with MIC ratio of:</th>
<th>% Essential agreement</th>
<th>Correlation coefficient</th>
<th>Minor discrepancy rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25 0.5 1 2 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxacillin, methicillin, and penicillin</td>
<td>1.1  9.8  76.0  12.6  0.6</td>
<td>98.4</td>
<td>0.97</td>
<td>11.5 (0)</td>
</tr>
<tr>
<td>Methicillin and penicillin</td>
<td>1.1  8.2  81.4  9.3  0.0</td>
<td>98.9</td>
<td>0.97</td>
<td>14.2 (0.6)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>11.5 10.4  47.5  21.3  9.3</td>
<td>79.2</td>
<td>0.84</td>
<td>27.9 (11.5)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>8.7  4.9  65.0  16.9  4.4</td>
<td>86.9</td>
<td>0.92</td>
<td>10.4 (1.6)</td>
</tr>
<tr>
<td>Penicillin</td>
<td>3.3  23.5  54.1  15.9  3.3</td>
<td>93.4</td>
<td>0.93</td>
<td>23.5 (5.0)</td>
</tr>
</tbody>
</table>

* No major or very major errors occurred, and therefore only minor errors are shown in this table.

* Within 1 doubling dilution.

* Minor errors, with value excluding errors within 1 doubling dilution in parentheses.
disk combination procedures include a wide range (from 0.004 to 16 μg/ml), assuring that all currently encountered susceptible, intermediate, and resistant strains can be differentiated (Fig. 2). This is not the case with the single-disk oxacillin procedure, which is unable to differentiate strains for which MICs are 1.1 μg/ml from strains for which MICs are higher (Fig. 1). Similarly, the highest MIC predicted by the methicillin disk is 2.5 μg/ml. A wide range of MIC results can be calculated with the penicillin disk (0.012 to 48 μg/ml); however, the intermediate range (from 19 to 35 mm) has a 5% minor error rate, with both susceptible and resistant organisms in this range.

Validation of the data generated by this study with 60 recently isolated strains (20 Pen\textsuperscript{i}, 20 Pen\textsuperscript{r}, and 20 Pen\textsuperscript{ii}) showed essential agreement rates of 95.0% for the three-disk procedure and 96.7% for the two-disk procedure (Table 3). Calculated penicillin MICs were correctly categorized for all Pen\textsuperscript{i} and Pen\textsuperscript{r} strains. Of the 20 Pen\textsuperscript{i} strains, 18 were correctly categorized by the three-disk method and 19 were correctly categorized by the two-disk method; calculated MICs for the misclassified strains were 1.1 to 1.3 μg/ml, while reference MICs for these two strains were 1.0 μg/ml.

**DISCUSSION**

Resistance of *S. pneumoniae* to agents other than β-lactams is usually high level, and strains therefore have bimodal distributions to these agents, with few (if any) intermediate strains (16, 18). β-Lactam resistance, on the other hand, results from multiple changes to one or more penicillin-binding proteins in strains for which penicillin MICs are 0.06 μg/ml to as high as 64 μg/ml, depending on the number of changes and the affinities of the penicillin-binding proteins (9). Disk diffusion has been shown to be an accurate method for obtaining categorical susceptibility results with *S. pneumoniae* for many non-β-lactams, including erythromycin, clindamycin, trimethoprim-sulfamethoxazole, tetracycline, and chloramphenicol (7, 11, 17, 22).

Prior to this study, the use of disk diffusion for β-lactams has been limited to screening for penicillin susceptibility with 1-μg oxacillin disks (14, 21, 24). In addition, the use of the oxacillin disk is limited by the fact that strains for which penicillin MICs are 0.06 μg/ml, the upper limit of the susceptible category, usually have oxacillin zones of ≤19 mm (14, 16–18). This occurs more frequently when MICs are determined by microdilution, which was the case in this study, in which 17 such discrepancies occurred with microdilution MICs, compared to 9 with agar dilution, and 1 with the E-test. Although this limits the value of the oxacillin disk as a screening method, in the current study most of the discrepant Pen\textsuperscript{r} strains had oxacillin zones of 11 to 19 mm, while most Pen\textsuperscript{i} strains had zones of ≤10 mm.

This study has shown that the standard NCCLS disk diffusion method for testing *S. pneumoniae* can be adapted to provide accurate MICs and qualitative category (Pen\textsuperscript{i}, Pen\textsuperscript{r}, and Pen\textsuperscript{ii}) results for penicillin that are comparable to E-test, agar dilution, and microdilution MIC methods by summing zone diameter measurements from either three (penicillin, oxacillin, and methicillin) or two (penicillin and methicillin) disks. Accurate MIC results can be determined from regression plots at a considerably lower cost than that for any MIC method that is currently available. The best results were obtained with the sum of the three zone diameters from standard penicillin, methicillin, and oxacillin disks. The sum of two disks also provided excellent results, with only minimally more minor category discrepancies than with the three disks. In fact, the essential agreement rates of 98.4% for MICs determined by the three-disk procedure and 98.9% for MICs determined by the two-disk procedure were higher than the essential agreement rate of 95.1% for MICs obtained by microdilution versus agar dilution and microdilution versus E-test. MICs can be determined manually from regression plots or generated electronically with the BIOMIC system programmed to perform these conversions (3, 19).

The validation of the two- and three-disk procedures performed as part of this study confirmed the accuracy of these methods for determining penicillin MICs. Essential agreement rates for penicillin (95.0 and 96.7%) were similar to those found with several commercially available systems (91.3 to 100%) which have been approved for use in diagnostic laboratories (25). The few categorical errors found in the validation study were minor, and the frequency of these minor errors was similar to or lower than recently published values (25).

In summary, this study has demonstrated the value of disk diffusion in accurately and economically determining the penicillin susceptibility of *S. pneumoniae* at both a categorical level and a calculated MIC. This approach may have additional applications with other β-lactams.

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**REFERENCES**


