Quantitative Antimicrobial Susceptibility Testing of *Haemophilus influenzae* and *Streptococcus pneumoniae* by Using the E-Test

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Received 9 August 1990/Accepted 18 October 1990

The E-test (PDM Epsilometer; AB Biodisk, Solna, Sweden) is a new product for quantitative (MIC) antimicrobial susceptibility testing. The E-test consists of a plastic strip with a predefined antimicrobial agent concentration gradient immobilized on one side and a continuous MIC scale covering 15 twofold dilutions on the opposite side. To determine an MIC with the E-test, the surface of an agar plate is swab inoculated with an adjusted bacterial suspension in the same manner as a disk diffusion test. One or more E-test strips for the antimicrobial agents to be tested are then placed on the inoculated agar surface. After overnight incubation, the interaction of the antimicrobial agent gradient and the test bacterial inoculum gives rise to elliptical inhibitory zones. The intersection of the growth ellipse margin with the E-test strip gradient indicates the MIC of the drug for the organism.

Increasing resistance by various mechanisms to several classes of antimicrobial agents among clinical isolates of *Haemophilus influenzae* (3, 9, 11) and *Streptococcus pneumoniae* (7, 8) has focused new attention on reliable methods for in vitro susceptibility testing of these species and on guidelines for interpretation of such tests (2, 4-6, 10). The National Committee for Clinical Laboratory Standards (NCCLS) has recently endorsed new media, quality control guidelines, and interpretive criteria for *Haemophilus* testing (12, 13) and is exploring potential new standards for pneumococcal testing. The purpose of the present study was to evaluate the E-test for determining the susceptibilities of *H. influenzae* and *S. pneumoniae* to antimicrobial agents of clinical relevance for those species.

**MATERIALS AND METHODS**

Antimicrobial agents. E-test strips containing ampicillin, penicillin, cefaclor, cefuroxime, cefotaxime, chloramphenicol, erythromycin, doxycycline, tetracycline, and trimethoprim-sulfamethoxazole were provided by the manufacturer for the purpose of this study. Reagent grade powders of the same antimicrobial agents were used for performance of reference agar and broth dilution MIC tests.

**Test strains.** One hundred strains of *H. influenzae* and 50 strains of *S. pneumoniae* which demonstrated various resistance mechanisms and levels of antimicrobial susceptibility (Table 1) were utilized for this evaluation.

**Control strains.** The control strains employed in this study included *H. influenzae* ATCC 49247 and ATCC 10211, *Escherichia coli* ATCC 25922, and *Streptococcus faecalis* (Enterococcus faecalis) ATCC 29212.

**E-tests.** E-tests of *H. influenzae* were performed on both *Haemophilus* test medium (HTM) agar (BD Microbiology Systems [BDMS], Cockeysville, Md.) and PDM ASM II agar (Biodisk). The latter medium was supplemented with 1% hemoglobin (BDMS) and 1% IsoViteX (BDMS) and is hereafter referred to as ASM II chocolate agar. For *S. pneumoniae*, E-tests were performed on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood (BDMS). Inocula were prepared by direct suspension in Mueller-Hinton broth of colonies grown overnight on enriched chocolate agar (*H. influenzae*) or sheep blood agar (pneumococci) to achieve turbidity equivalent to a 0.5 McFarland opacity standard. The 150-mm-diameter agar plates were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions. With both species, four or five E-test strips were placed in an equidistant radial fashion on the surface of the plates. After application of the E-test strips, *H. influenzae* plates were incubated at 35°C in
TABLE 1. Resistance properties\(^a\) of 100 *H. influenzae* and 50 *S. pneumoniae* test strains

<table>
<thead>
<tr>
<th>Species and resistance property</th>
<th>No. of strains with resistance property</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. influenzae</em></td>
<td></td>
</tr>
<tr>
<td>β-Lactamase positive</td>
<td>53</td>
</tr>
<tr>
<td>β-Lactamase negative, ampicillin resistant</td>
<td>5</td>
</tr>
<tr>
<td>(MIC ≥ 4 μg/ml)</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol resistant (CAT positive)(^b)</td>
<td>10</td>
</tr>
<tr>
<td>Tetracycline resistant (MIC ≥ 16 μg/ml)</td>
<td>14</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole resistant (MIC ≥ 4 μg/ml)</td>
<td>5</td>
</tr>
<tr>
<td>Fully susceptible</td>
<td>37</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>Penicillin resistant (MIC ≥ 2 μg/ml)</td>
<td>4</td>
</tr>
<tr>
<td>Penicillin relative resistance (MIC = 0.12 to 1 μg/ml)</td>
<td>18</td>
</tr>
<tr>
<td>Chloramphenicol resistant (CAT positive)(^b)</td>
<td>5</td>
</tr>
<tr>
<td>Erythromycin resistant (MIC ≥ 8 μg/ml)</td>
<td>2</td>
</tr>
<tr>
<td>Tetracycline resistant (MIC ≥ 16 μg/ml)</td>
<td>14</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole resistant (MIC ≥ 4 μg/ml)</td>
<td>19</td>
</tr>
<tr>
<td>Fully susceptible</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\) Some strains were multiply resistant.
\(^b\) Produced chloramphenicol acetyltransferase (CAT).

5% CO\(_2\) for 16 to 18 h, while pneumococcal test plates were incubated in ambient air at 35°C for 20 to 22 h. The longer incubation period for pneumococcal tests was chosen on the basis of preliminary studies that indicated better definition of growth ellipses after 20 to 22 h than after only 16 to 18 h of incubation. The use of CO\(_2\) for E-tests of *H. influenzae* was based on prior experience, which showed that some strains do not grow well on HTM agar if incubated in ambient air (5). E-test MICs were interpreted by noting the point of intersection of the growth ellipse margin with the MIC scale on the E-test strip when viewed from the upper agar surface with the plate lids removed (see Fig. 1 and 2).

**Reference broth microdilution susceptibility tests.** Broth microdilution MIC tests were performed in the manner recommended by the NCCLS (12) by using HTM broth (BDMS) for *H. influenzae* and cation adjusted (25 mg of Ca\(^{2+}\) per liter and 12.5 mg of Mg\(^{2+}\) per liter) Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with 3% lysed horse blood for *S. pneumoniae* (12). With both species, the final inoculum density was ca. 5 × 10\(^5\) CFU/ml, and incubation was carried out at 35°C in ambient air for 20 to 24 h.

**Agar dilution tests.** Agar dilution MIC tests were also performed with *H. influenzae* by using twofold concentration increments of the antimicrobial agents incorporated in molten ASM II chocolate agar in the manner suggested by the NCCLS for nonfastidious bacteria (12). Inoculum sus-

FIG. 1. E-tests with a β-lactamase-producing *H. influenzae* strain performed with HTM agar. Antibiotic abbreviations on the E-test strips and MIC interpretations (indexed to base 1) are AM (ampicillin; 8 μg/ml), DC (doxycycline; 4 μg/ml), XM (cefoxime; 1 μg/ml), CF (cefaclor; 2 μg/ml), and CT (ceftaxime; 0.015 μg/ml).
pensions were prepared as described above and then diluted in Mueller-Hinton broth and delivered to the surface of the agar plates with a Steers replicator, which resulted in a final inoculum of ca. 10^4 CFU per spot. Agar dilution plates were incubated at 35°C in 5% CO_2 for 16 to 20 h before interpretation of the MICs in the usual manner (12).

**RESULTS**

One hundred *H. influenzae* and 50 *S. pneumoniae* strains were examined by E-tests performed on the agar media currently recommended for disk diffusion testing of those species by the NCCLS (HTM and Mueller-Hinton sheep blood agars, respectively), and those results were compared with the results of broth microdilution MIC tests performed with the media currently recommended by the NCCLS (HTM and Mueller-Hinton lysed horse blood broth, respectively). E-tests were also performed on a subset of 50 *Haemophilus* strains (including predominantly those strains with known resistance mechanisms) with the medium (PDM ASM II chocolate agar) recommended by the manufacturer of the E-test and by agar dilution with the same medium. E-test MICs were easily interpreted for most of the drugs with either of the agar media with *H. influenzae* and with Mueller-Hinton sheep blood agar with *S. pneumoniae*. The E-test inhibition ellipses were generally clearly demarcated, and the points of intersection of the zone edge with the strips were generally easily interpreted (Fig. 1). However, trimethoprim-sulfamethoxazole with both species and ampicillin with *H. influenzae* were exceptions. Some β-lactamase-producing *H. influenzae* yielded growth of numerous small colonies within the inhibition ellipse with ampicillin (Fig. 2). E-test MICs with such strains could be interpreted in several different ways (see Discussion). Inhibition ellipse margins with the trimethoprim-sulfamethoxazole combination were sometimes diffuse and poorly defined with *H. influenzae* on ASM II chocolate agar and with pneumococcal tests on Mueller-Hinton sheep blood agar. The problem was less pronounced with *H. influenzae* tested with HTM agar.

Despite the difficulty in interpreting the E-test MICs for the two drugs mentioned above, the overall agreement between E-test MICs and MICs determined by the conventional dilution methods was generally good. The overall agreement between E-test MICs for *H. influenzae* measured on HTM agar and microdilution MICs measured in HTM broth was 89.8% (Table 2). The highest agreement occurred with erythromycin tests (99.0%), and the lowest agreement values were seen with trimethoprim-sulfamethoxazole (67.7%) and ampicillin (76.5%) tests. The overall agreement between E-test MICs determined with ASM II chocolate agar and microdilution MICs determined with HTM broth was lower, i.e., 81.4% (Table 3), principally because of an even lower correlation between trimethoprim-sulfamethox-

![FIG. 2. Ampicillin E-test of a β-lactamase-producing *H. influenzae* strain performed with ASM II chocolate agar. The MIC was interpreted as >256 μg/ml because of the in-growth colonies.](http://jcm.asm.org/)
azole results (55.3%) and a poor correlation of doxycycline results (56.0%). With the exception of low agreement between trimethoprim-sulfamethoxazole results (67.4%), the most favorable correlation (91.8%) was seen between E-tests performed on ASM II chocolate agar and agar dilution MICs determined with the same medium (data not depicted). The agreement between E-test MICs with H. influenzae determined on the two different agars (HTM and ASM II chocolate) was 90.8% (Table 4).

The overall agreement between E-test and NCCLS reference broth microdilution MICs with S. pneumoniae was 80.4% (Table 5). The highest agreement occurred with chloramphenicol (98.0%), and the lowest agreement was seen with trimethoprim-sulfamethoxazole (42.0%). If the trimethoprim-sulfamethoxazole values were excluded, the overall agreement between E-test and broth microdilution MICs with pneumococci increased to 90%.

Interpretive category errors resulting from E-tests of both species were generally low (Table 6). Overall, only 0.7% of the E-tests had very major errors and none had major errors in the case of H. influenzae E-tests performed on HTM agar compared with the interpretive guidelines for the NCCLS reference broth microdilution test (12). Thus, the E-test MICs accurately categorized the Haemophilus strains which were resistant to the various study drugs. Likewise, the overall error rates of E-tests with pneumococci were relatively low, i.e., 0.8% of the tests had very major and 2.4% had major errors with a variety of interpretive criteria for the reference MICs (Table 6). No very major or major errors occurred with penicillin E-tests of pneumococci in comparison with the NCCLS criteria (11). Two very major errors (4%) occurred with tetracycline E-tests of pneumococci if an MIC of ≥16 μg/ml was regarded as indicative of tetracycline resistance.

A few other drugs yielded lower than average agreement between the E-test and conventional methods. Cefaclor and cefuroxime tests with H. influenzae resulted in 2% of the tests having very major errors and, in the case of cefaclor, 11% of the tests having minor errors (Table 6). E-tests of the quality control strains included in this study yielded MICs within the acceptable ranges for all of the study drugs.

### DISCUSSION

The E-test represents a new and innovative approach to the determination of antimicrobial susceptibility which is potentially applicable to a wide array of drugs and microorganisms. In a manner reminiscent of the agar disk diffusion method, the E-test provides quantitative MICs simply and reproducibly. The E-test approach may be well suited to the testing of certain fastidious bacteria (e.g., H. influenzae and pneumococci) or bacteria that are difficult to test (e.g., anaerobes [1]). The present study has demonstrated the potential of the E-test for use with H. influenzae and S. pneumoniae with the media most often used to test these two species in the United States, i.e., HTM agar for H. influ-

### TABLE 3. Comparison of E-test MICs determined on ASM II agar with HTM broth microdilution MICs

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of strains²</th>
<th>No. of E-test MICs within indicated concn (log₂) of HTM broth dilution MICs</th>
<th>% Agreement within 1 log₂ concn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt;-2</td>
<td>-2</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>50</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>50</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>50</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>50</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>50</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>50</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>50</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole⁶</td>
<td>47</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

Overall agreement 81.4

² Indicates number of strains in which both tests resulted in a finite MIC; off-scale values were excluded from comparison.

⁶ Trimethoprim-sulfamethoxazole ratio, 1:19.

### TABLE 4. Comparison of H. influenzae E-test MICs determined on HTM agar with E-test MICs determined on ASM II chocolate agar

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of strains²</th>
<th>No. of E-test MICs within indicated concn (log₂) of ASM II agar E-test MICs</th>
<th>% Agreement within 1 log₂ concn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt;-2</td>
<td>-2</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>50</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>50</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>50</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>50</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>50</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>50</td>
<td>5</td>
<td>43</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>50</td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole⁶</td>
<td>47</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

Overall agreement 90.4

² Indicates number of strains in which both tests resulted in a finite MIC; off-scale values were excluded from comparison.

⁶ Trimethoprim-sulfamethoxazole ratio, 1:19.
enae and Mueller-Hinton sheep blood agar for S. pneu-
niae. ASM II chocolate agar was also used for E-tests of H. in-
fluenzae, since it is the medium recommended by the
manufacturer of the E-test strips and is sold for that purpose
in Europe. However, the correlation between E-tests per-
formed on that medium and HTM microdilution susceptibil-
ity tests was less favorable, and only slightly better overall
agreement was noted between E-test and conventional agar
dilution MICs determined by using the ASM II agar in
contrast to the HTM agar recommended by the NCCLS.

E-test MICs were generally easily interpreted with both
Haemophilus and pneumococcus isolates. Since E-test re-
results are interpreted from the upper surface of the agar with
the plate lid removed, there is no particular disadvantage in
using opaque media such as blood- or hemoglobin-supple-
mented agars. The major exceptions were the diffuse growth
ellipses encountered with the two blood- or hemoglobin-
containing agars and trimethoprim-sulfamethoxazole E-
tests. The indistinct endpoints with this antimicrobial com-
bination resulted in a high rate of disagreement with the
conventional dilution test results. The likely presence of
fолate metabolism antagonists in the hemoglobin supplement
added to the ASM II agar and the sheep blood added to the
Mueller-Hinton agar may account for the poorly delineated
growth ellipses which made it difficult to interpret accurately
the MIC endpoints. A further confounding factor was the
preparation and labeling of the E-test strips for trimeth-
prim-sulfamethoxazole with the arithmetic total drug con-
tent, rather than only the trimethoprim component, indexed
to the base 1, as is more common in the United States. Thus,
when the 1:19 trimethoprim-to-sulfamethoxazole test ratio
was considered, the trimethoprim-sulfamethoxazole MIC
results did not coincide exactly. For example, the reference
dilution MICs contained trimethoprim and sulfamethoxazole
concentrations such as 4 and 76, 2 and 38, and 1 and 19
µg/ml, while the nearest E-test concentrations were 3.2 and
60.8 µg/ml (labeled 64 on the test strip), 1.6 and 30.4 µg/ml
(labeled 32), and 0.8 and 15.2 µg/ml (labeled 16). Thus, any
discordance between the E-test and reference methods was
no doubt worsened by the inequitable concentration incre-
ments which were used for comparison. Despite the diffi-
culties, there were no very major or major interpretive errors
encountered with H. influenzae. However, the folate an-
tagonists apparently present in the sheep blood resulted in
substantial (12%) major errors in pneumococcal tests.

The problem of in-growth colonies which occurred with
ampicillin and β-lactamase-producing H. influenzae meant
that E-test MICs could be read where the most obvious
ellipse intersected the test strip (ignoring the in-growth
colones) or where the in-growth colonies diminished appreci-
ably (the approach used in this study), or the MIC could be
recorded as equal to or greater than the highest concentra-
tion on the test strip if some colonies occurred throughout
most of the ellipse. The last option is recommended by the
manufacturer of the E-test. That strategy reduces the possi-
bility of false susceptibility (1% of tests had very major
errors in this study) but does not improve agreement with the
exact ampicillin MICs obtained by either conventional broth
or agar dilution methods.

E-test MICs compared favorably with those of most of the
other drugs tested by conventional methods against H. in-
fluenzae and with all but penicillin and trimethoprim-
sulfamethoxazole against S. pneumoniae. It is not immedi-
ately clear why the penicillin MICs for pneumococci did not
agree more closely. There was a trend toward somewhat
higher (1 to 2 log; units) penicillin MICs determined by the

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TABLE 5. Comparison of S. pneumoniae E-test MICs with broth microdilution MICs

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of strains</th>
<th>No. of E-test MICs within indicated concn (log2) of broth microdilution MICs</th>
<th>% Agreement within 1 log2 concn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>50</td>
<td>&gt;2  50  40  30  20  10  0  -1</td>
<td>-2 -1 Same +1 +2 &gt;+2</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>50</td>
<td>&gt;2  50  40  30  20  10  0  -1</td>
<td>-2 -1 Same +1 +2 &gt;+2</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>50</td>
<td>&gt;2  50  40  30  20  10  0  -1</td>
<td>-2 -1 Same +1 +2 &gt;+2</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>50</td>
<td>&gt;2  50  40  30  20  10  0  -1</td>
<td>-2 -1 Same +1 +2 &gt;+2</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazolea</td>
<td>50</td>
<td>&gt;2  50  40  30  20  10  0  -1</td>
<td>-2 -1 Same +1 +2 &gt;+2</td>
</tr>
</tbody>
</table>

Overall agreement: 80.4%

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* E-tests performed on HTM agar with H. influenzae and Mueller-Hinton sheep blood agar with S. pneumoniae.
* Based on interpretive criteria of NCCLS standard M7-A2 (12).
* Trimethoprim-sulfamethoxazole ratio, 1:19.
* Based on resistance at MIC of ≥8 µg/ml (10).
* NA, Not applicable.
* Based on resistance at MIC of ≥16 µg/ml.
* Based on resistance at MIC of ≥8 µg/ml.
* Based on resistance at MIC of ≥4 µg/ml.
Mueller-Hinton lysed horse blood broth microdilution method compared with the E-test MICs determined on Mueller-Hinton sheep blood agar. Another study has shown recently that penicillin MICs for pneumococci were slightly higher in lysed horse blood broth than in a second microdilution test medium, HTM (5). The slightly lower penicillin E-test values did not result in any very major errors in this study; however, there were strains with reference penicillin MICs in the resistant range (i.e., ≥0.06 μg/ml) which were considered relatively resistant (MIC, 0.12 to 1 μg/ml) by E-tests, and likewise strains which were relatively resistant to penicillin by the reference method but susceptible (MIC, ≤0.06 μg/ml) by the E-test. While these differences are strictly classified as minor errors, they would lead to important differences in the categorization of pneumococcal penicillin resistance rates, and thus they seriously question the utility of the E-test for penicillin determinations with S. pneumoniae.

The E-test method represents a potential alternative for convenient performance of quantitative (MIC) susceptibility tests of H. influenzae and S. pneumoniae. With a few important exceptions noted above, E-test MICs correlated well with conventional MICs and with existing interpretive categories. The E-test method may be a convenient means for testing fastidious or anaerobic bacteria against a limited array of drugs. Since no more than six E-test strips can be placed on the surface of the 150-mm-diameter round petri plates most often used for susceptibility testing in the United States, the E-test will probably not represent an economical method for testing a large number of drugs on each isolate. The cost effectiveness of this new method has not been addressed in this article, since the final market price of the strips in the United States has not yet been decided by the manufacturer. Further studies are needed to fully explore the potential of this new method for antimicrobial susceptibility testing.

REFERENCES