Comparative Evaluation of the E Test, Agar Dilution, and Broth Microdilution for Testing Susceptibilities of Helicobacter pylori Strains to 20 Antimicrobial Agents

RAFFAELE PICCOLOMINI,1* GIOVANNI DI BONAVENTURA,1 GIOVANNI CATAMO,1 FLAVIA CARBONE,2 and MATTEO NERI2

Clinical Microbiology Laboratory, Department of Biomedical Sciences,1 and Department of Medicine and Ageing Sciences,2 “G. D’Annunzio” University, I-66100 Chieti, Italy

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The purpose of this study was to assess the reliability of methods for determining the in vitro susceptibility of Helicobacter pylori. Seventy-one H. pylori strains isolated from patients with duodenal ulcers were tested against 20 antimicrobial agents. The E test and the agar dilution method were carried out on Mueller-Hinton agar; the broth microdilution method was performed with Mueller-Hinton broth. The E-test results showed excellent correlation with the agar dilution results, with 91.3% and 98.8% agreement within 1 and 2 dilution steps, respectively, in a total of 1,350 tests. The correlation between the E-test results and the broth microdilution results was slightly higher, with 91.6% and 99.1% agreement within 1 and 2 dilution steps, respectively, in a total of 1,317 tests. There were six major errors and two very major errors by the metronidazole E test compared to the results obtained by reference methods. Excellent agreement between E-test, agar dilution, and broth microdilution results was found for resistance to erythromycin (8%), clarithromycin (6%), and tetracycline (6%). Our results confirm that the E test is comparable to standardized methods for susceptibility testing. Therefore, the E test is a reliable and alternative method for testing H. pylori susceptibility to a wide range of antimicrobial agents in clinical practice.

Helicobacter pylori is now accepted as a major cause of chronic type B gastritis (9, 14, 16), and there is a strong association with peptic ulceration (16, 19, 30) and gastric cancer (10, 11, 23), two of the most important diseases in the upper gastrointestinal tract. Treatment with antimicrobial agents and/or bismuth salts successfully eradicates H. pylori from the gastric mucosa, producing favorable clinical responses (1), but relapses frequently occur after therapy (12, 18). In fact, resistance of H. pylori to metronidazole and other 5-nitroimidazoles has emerged worldwide and now constitutes a major problem in therapy (1, 3, 12, 25). Therefore, the treatment of infections caused by H. pylori requires that special attention be given toward reliable methods for determining the in vitro susceptibility of this microorganism. Susceptibility testing of H. pylori is not yet either standardized or routinely performed in most laboratories. Several investigators have reported the susceptibilities of H. pylori to antimicrobial agents using various MIC methods (7, 12, 15, 18, 20), but no standard methods exist because of the slow growth of the bacterium combined with its requirement for numerous additives in the growth medium and a microaerophilic atmosphere. The Epsilometer tests (E test; AB Biodisk, Solna, Sweden), a modification of the disk diffusion method were carried out on Mueller-Hinton agar; the broth microdilution method was performed with Mueller-Hinton broth. The E-test results showed excellent correlation with the agar dilution results, with 91.3% and 98.8% agreement within 1 and 2 dilution steps, respectively, in a total of 1,350 tests. The correlation between the E-test results and the broth microdilution results was slightly higher, with 91.6% and 99.1% agreement within 1 and 2 dilution steps, respectively, in a total of 1,317 tests. There were six major errors and two very major errors by the metronidazole E test compared to the results obtained by reference methods. Excellent agreement between E-test, agar dilution, and broth microdilution results was found for resistance to erythromycin (8%), clarithromycin (6%), and tetracycline (6%). Our results confirm that the E test is comparable to standardized methods for susceptibility testing. Therefore, the E test is a reliable and alternative method for testing H. pylori susceptibility to a wide range of antimicrobial agents in clinical practice.

MATERIALS AND METHODS

Bacterial strains. Seventy-one consecutive nonduplicate clinical strains of H. pylori isolated from patients with duodenal ulcer or gastritis were tested. The strains were identified by Gram staining and oxidase, catalase, and urease reactions. After identification, the bacteria were stored at −80°C in aliquots of 1 ml of dehydrated sheep blood (Bioline Italana S.r.l., Milan, Italy) supplemented with 10% (vol/vol) glycerol (Sigma Chemical Co., Milan, Italy) (27) until they were ready for use. Before they were used, the bacteria were subcultured twice on Mueller-Hinton agar (Unipath S.p.A., Garbagnate Milanese, Milan, Italy) supplemented with 5% dehydrated sheep blood (Bioline) at 37°C in a microaerophilic atmosphere (5% O2, 10% CO2, and 85% N2; CampyGen; Unipath) for 72 h.

Control strains. H. pylori NCTC 11637 and NCTC 11638 and Esherichia coli ATCC 25922 were included as control organisms with each run of each method. To compare the antimicrobial susceptibility test methods, we also used 35 selected strains of H. pylori with known resistance patterns. These strains were isolated from patients subjected to several treatment trials: 15 strains were resistant to metronidazole (breakpoint MIC, >32 μg/ml), 8 strains were resistant to clarithromycin (breakpoint MIC, >8 μg/ml), 9 strains were resistant to tetracycline (breakpoint MIC, >16 μg/ml), and 3 strains were resistant to both metronidazole (breakpoint MIC, >32 μg/ml) and ciprofloxacin (breakpoint MIC, >4 μg/ml).

Antimicrobial agents. The antimicrobial agents tested against H. pylori included amoxicillin, amoxicillin-clavulanate (tested at a 2:1 ratio); ampicillin, azithromycin, aztreonam, cefadroxil, cefotetan, cefezime, ciprofloxacin, clarithromycin, erythromycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, pefloxacin, roxithromycin, tetracycline, ticarcillin, and tobramycin. The E-test strips of each antibiotic were purchased from AB Biodisk. Antibiotic powders of known potency for the agar dilution and broth microdilution MIC tests were purchased from Sigma except as follows: tobramycin was from Eli Lilly Italy S.p.A., Sesto Fiorentino, Florence, Italy; cefradine was from Farmacia Carlo Erba, Milan, Italy; aztreonam was from Menarini, Florence, Italy; pefloxacin-
TABLE 1. Comparison of antimicrobial susceptibility test results by the E test and the agar dilution and broth microdilution methods for 71 isolates of H. pylori

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>E test</th>
<th>AD</th>
<th>MD</th>
<th>% Resistant</th>
<th>MIC (µg/ml) (geometric mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC90 (µg/ml)</td>
<td>E test</td>
<td>AD</td>
<td>MD</td>
<td>E test</td>
</tr>
<tr>
<td>Amoxicillin-clavulanate</td>
<td>0.032</td>
<td>0.064</td>
<td>0.064</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.032</td>
<td>0.064</td>
<td>0.064</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>1.5</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ceftriaxime</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.094</td>
<td>0.25</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.75</td>
<td>1</td>
<td>0.5</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>30</td>
<td>32</td>
</tr>
<tr>
<td>Nitrofurantin</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0.19</td>
<td>0.25</td>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pefloxacin</td>
<td>0.19</td>
<td>0.25</td>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>0.094</td>
<td>0.125</td>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.125</td>
<td>0.25</td>
<td>0.25</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>0.19</td>
<td>0.25</td>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>1.5</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Strains were classified as resistant when the MIC was greater than the breakpoint concentration.
* AD, agar dilution method.
* MD, broth microdilution method.
* Six major errors (resistant by the E test and susceptible by the agar dilution and broth microdilution methods) and two very major errors (susceptible by the E test and resistant by the agar dilution and broth microdilution methods).

Evaluation criteria. Because the twofold dilution scheme for the agar dilution and broth microdilution methods was different from that for the E test, those MICs determined by the E test with one-half an increment were rounded up to the next higher dilution (e.g., 0.75 µg/ml was rounded up to 1 µg/ml), and these values were used in the comparison of the results between the E test and the conventional methods. Agreement between two of the test methods evaluated was defined as MICs that differed by 1 log₂ dilution or less. Discrepancies in MICs were characterized as very major (reference method result was resistant and the E-test result was susceptible) or major (reference method result was susceptible and the E-test result was resistant) errors. Calculations of very major and major errors have been based only on the number of resistant strains tested; likewise, major errors have been calculated only on the basis of the number of susceptible strains tested (21).

Statistical analysis. The significance of the differences between MICs obtained by using two methods was determined by the χ² test. A P value of less than 0.05 was considered to represent a statistically significant difference between the results of the two methods compared. Microsoft Excel, version 6.0, was used to perform statistical analysis. The mode, geometric mean, MIC at which 50% of isolates are inhibited (MIC₅₀), and MIC₉₀ were also calculated.

RESULTS

Antimicrobial susceptibility test results are presented in Table 1. The most active compounds in vitro were amoxicillin-clavulanate and ampicillin (MIC₅₀, 0.032 µg/ml for the E test and 0.064 µg/ml for the reference methods). The highest MIC₉₀ was observed for metronidazole (>32 µg/ml by all three methods). The E test yielded greater numbers of results indicating resistance than did the reference methods when metronidazole (P > 0.05) was tested: 23 (32%) H. pylori isolates were resistant to metronidazole by the reference methods, and 27 (38%) were resistant to metronidazole by the E test. All three methods detected the following resistance rates: 8% (6 of 71) to erythromycin and 6% (4 of 71) to both clarithromycin and tetracycline.

For amoxicillin-clavulanate, the MIC (geometric mean) by the E-test method was 0.018 µg/ml, compared with 0.021 µg/ml by the agar dilution method and 0.022 µg/ml by the broth
TABLE 2. Distribution of differences in MICs of 20 antimicrobial agents for 71 isolates of H. pylori: E test versus agar dilution method

<table>
<thead>
<tr>
<th>Drug (no. of strains)</th>
<th>&gt; +2</th>
<th>+1</th>
<th>0</th>
<th>−1</th>
<th>−2</th>
<th>No. (%) of E-test MICs within indicated no. of log2 dilution steps of agar dilution MICs</th>
<th>% Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin-clavulanate (71)</td>
<td>1 (1.4)</td>
<td>3 (4.2)</td>
<td>22 (31)</td>
<td>37 (52.1)</td>
<td>8 (11.3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amoxicillin (65)</td>
<td>0</td>
<td>8 (12.3)</td>
<td>27 (41.5)</td>
<td>24 (36.9)</td>
<td>4 (6.2)</td>
<td>2 (3.1)</td>
<td>0</td>
</tr>
<tr>
<td>Ampicillin (54)</td>
<td>0</td>
<td>0</td>
<td>14 (25.9)</td>
<td>31 (57.4)</td>
<td>9 (16.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Azithromycin (70)</td>
<td>1 (1.4)</td>
<td>4 (5.7)</td>
<td>23 (32.9)</td>
<td>26 (37.1)</td>
<td>16 (22.9)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aztreonam (70)</td>
<td>0</td>
<td>1 (1.4)</td>
<td>16 (22.9)</td>
<td>28 (40)</td>
<td>22 (31.4)</td>
<td>3 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td>Cefaclor (71)</td>
<td>0</td>
<td>1 (1.4)</td>
<td>29 (40.8)</td>
<td>22 (31)</td>
<td>18 (25.4)</td>
<td>1 (1.4)</td>
<td>0</td>
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<tr>
<td>Cefotetan (66)</td>
<td>1 (1.5)</td>
<td>5 (7.6)</td>
<td>23 (34.8)</td>
<td>18 (27.3)</td>
<td>17 (25.8)</td>
<td>2 (3)</td>
<td>0</td>
</tr>
<tr>
<td>Ceftriaxone (70)</td>
<td>0</td>
<td>0</td>
<td>18 (25.7)</td>
<td>34 (48.6)</td>
<td>18 (25.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin (60)</td>
<td>1 (1.7)</td>
<td>6 (10)</td>
<td>14 (23.3)</td>
<td>28 (46.7)</td>
<td>11 (18.3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clarithromycin (68)</td>
<td>2 (2.9)</td>
<td>2 (2.9)</td>
<td>15 (22.1)</td>
<td>44 (64.7)</td>
<td>5 (7.4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Erythromycin (69)</td>
<td>1 (1.4)</td>
<td>4 (5.8)</td>
<td>20 (29)</td>
<td>33 (47.8)</td>
<td>8 (11.6)</td>
<td>1 (1.4)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Gentamicin (70)</td>
<td>0</td>
<td>7 (10)</td>
<td>18 (25.7)</td>
<td>21 (30)</td>
<td>18 (25.7)</td>
<td>6 (8.6)</td>
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<tr>
<td>Metronidazole (69)</td>
<td>2 (2.9)</td>
<td>3 (4.3)</td>
<td>1 (1.4)</td>
<td>49 (71)</td>
<td>12 (17.4)</td>
<td>1 (1.4)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Nitrofurantoin (68)</td>
<td>0</td>
<td>4 (5.9)</td>
<td>13 (19.1)</td>
<td>30 (44.1)</td>
<td>21 (30.9)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin (69)</td>
<td>2 (2.9)</td>
<td>15 (21.7)</td>
<td>19 (27.5)</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Pefloxacin (71)</td>
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<td>5 (7)</td>
<td>15 (21.1)</td>
<td>45 (63.4)</td>
<td>6 (8.5)</td>
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<tr>
<td>Roxithromycin (70)</td>
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<td>4 (5.7)</td>
<td>34 (48.6)</td>
<td>24 (33.5)</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Tetracycline (64)</td>
<td>0</td>
<td>6 (9.4)</td>
<td>21 (32.8)</td>
<td>30 (46.9)</td>
<td>6 (9.4)</td>
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<tr>
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<td>2 (3.1)</td>
<td>27 (42.2)</td>
<td>18 (28.1)</td>
<td>12 (18.8)</td>
<td>3 (4.7)</td>
<td>2 (3.1)</td>
</tr>
<tr>
<td>Tobramycin (71)</td>
<td>0</td>
<td>0</td>
<td>10 (14.1)</td>
<td>26 (36.6)</td>
<td>34 (47.9)</td>
<td>1 (1.4)</td>
<td>0</td>
</tr>
<tr>
<td>All agents (1,350)</td>
<td>11 (0.8)</td>
<td>80 (5.9)</td>
<td>379 (28.1)</td>
<td>597 (44.2)</td>
<td>257 (19)</td>
<td>20 (1.5)</td>
<td>6 (0.4)</td>
</tr>
</tbody>
</table>

* Number of strains for which MICs were within the concentration range of the E test.
* A dilution of 0 indicates number (percent) of isolates for which MICs are identical; −1 and +1 indicate ± log2 dilution difference, etc.
* Percentage of isolates within the accuracy limits of the test (±1 log2 dilution).

**DISCUSSION**

The efficacy of the treatment of gastric infection caused by H. pylori can be reduced by the occurrence of primary or acquired resistance to various drugs, especially to metronidazole (1). This has made susceptibility testing of H. pylori increasingly important for the search for efficient antimicrobial combinations that allow for the eradication of this bacterium from the stomach. However, up to now there are no standard methods for in vitro antimicrobial susceptibility testing for this fastidious organism. Agar or broth dilution methods have been used in most studies (2, 13, 27), but they are difficult to perform routinely. Moreover, this approach is economically impractical for clinical laboratory use when testing individual isolates. The disk diffusion method is inappropriate for microorganisms like H. pylori, requiring a microaerophilic atmosphere, a prolonged incubation time, and numerous additives in the growth medium.

The accuracy of the E-test MIC results for H. pylori that we found is in agreement with the findings of previous studies encompassing a variety of other bacteria and fungi (2, 5, 6, 8, 26).

Other investigators have reported an excellent correlation of the E-test results with those obtained by standard methods for H. pylori. Glupczynski et al. (13), who compared the E test with the agar dilution method to assess the in vitro activities of 12 antimicrobial agents against H. pylori, found that 86 and 99.5% of the results correlated within 1 and 2 log2 dilution steps, respectively. Van Horn et al. (28), who compared the E test and the reference agar dilution method to evaluate the activities of five antimicrobial agents against H. pylori, found a correlation of 86%. Cederbrant et al. (7), who determined the susceptibilities of 20 isolates of H. pylori to six antimicrobial agents, found that 81% of the E-test-determined MICs were within 1 twofold dilution and 95% were within 2 twofold dilu-
In the present study, excellent agreement between the 
E test and the agar dilution method (91.1%)
was found. This may be due in part to the common batch of 
Mueller-Hinton agar and in part to the common inoculum. 
However, Cederbrant et al. (7) showed that, in contrast to 
the standard broth or agar dilution method, the results 
of the 
E test were not significantly affected by the inoculum density. 
The results of the 
E test also 
yielded excellent agreement compared with those of the broth 
microdilution method (89.7%).

In general, the 
MICs obtained by the 
E test tended to be 
lower than those obtained by the reference methods. This is most apparent for 
roxithromycin and norfloxacin (E test versus the agar dilution method; Table 2) and for 
clarithromycin, 
zarotetanam, and roxithromycin (E test versus the broth mi-
crodilution method; Table 3). The underestimate of 
MICs by the 
E test has been described in previous studies (2, 7, 29). The 
reason for this observation in the present study is not known, 
since all three susceptibility tests were performed from the 
same inoculum.

With regard to the antimicrobial agents tested in the present 
study, the 
E test produced results comparable to those 
obtained by the agar dilution and broth microdilution methods: 
the 
E test had greater than 80% agreement with the reference 
methods except for tests with tetracycline (70.8%) by the broth 
microdilution. The most active compounds in vitro were 
amoxicillin-clavulanate and ampicillin.

When evaluating new methods for susceptibility testing, it is 
important to test an adequate number of resistant strains to 
verify the ability of the new test to detect resistance. Jorgensen 
(17) proposed that very major errors determined for a large 
sample (n ≥ 35) of known resistant isolates should be ≤3%.

In our study, we have found an excellent correlation (100% within 
1 log₂ dilution step) for the 35 collected strains known to be 
resistant; furthermore, no major or very major errors were 
found between the 
E-test, agar dilution, and broth microdilution 
MICs of metronidazole, clarithromycin, and tetracycline. Among the 71 clinical isolates of H. pylori tested, only two very 
major errors and six major errors were detected. We have no 
explanation other than chance to explain why these errors were 
observed only with metronidazole.

In our experience, the 
E test is much less labor-intensive and 
is easier to perform than the agar and broth dilution methods.
Also, the 
E test requires the material and principles of the wide-
ly used Kirby-Bauer disk diffusion susceptibility method, 
which allows the 
E test to be quickly and economically adapted 
into the laboratory work flow. We conclude that the 
E test appears to represent an excellent alternative, reproducible 
method for determining the antimicrobial susceptibilities of 
H. pylori strains to a wide array of antimicrobial agents.

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