The treatment of helicobacter pylori infection and its sequelae with emphasis on nitroimidazole resistance
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Chapter 3

Subpopulations of *Helicobacter pylori* are responsible for discrepancies in the outcome of nitroimidazole susceptibility testing.

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Abstract.

Susceptibility testing for metronidazole by E-test was compared to disk diffusion in 263 and to breakpoint agar dilution in 90 *H. pylori* isolates. In 5% and 6% of cases, results were discrepant. In 52 clinical isolates an E-test was performed on 10 separate colonies. Subpopulations of resistant and susceptible bacteria were found in 5 cases. In 3 isolates, each colony was subcultured and tested up to ten times. All but one of 292 tests showed the same result. We conclude that the E-test is reliable and that subpopulations are responsible for discordant results.
**Introduction.**

Nitroimidazoles are frequently used to treat *Helicobacter pylori* (*H. pylori*) infection (1). The relevance of *in vitro* nitroimidazole resistance (NIR) of *H. pylori* for treatment efficacy is still debated (2-6). Furthermore, data on the evolution of NIR prevalence are conflicting (7-11). As nitroimidazole susceptibility testing is not standardized, methodology may be, at least partly, responsible for these controversies (4). In addition, susceptibility testing may also be influenced by the fact that patients can be infected with both susceptible and resistant bacteria (12). In this study the relevance of this phenomenon for the E-test results was evaluated.

**Methods.**

Antral biopsy specimens were rubbed on two selective agar plates, one with Belo-horizonte medium (BHM-medium) and one with Campylobacter selective medium. The plates were incubated at 36°C under micro-aerophilic conditions and were examined after 2-3 days. Colonies were confirmed to be *H. pylori* by Gram staining and enzyme activity. For susceptibility testing, Columbia agar plates, supplemented with 7% horseblood but without antibiotics (*H. pylori* susceptibility testing medium, HPS-medium) were inoculated with a suspension of multiple colonies. Metronidazole susceptibility was tested by E-test, disk diffusion, and breakpoint agar dilution. The plates were read after 2-3 days. For the E-test (AB-Biodisk, Solna, Sweden) strains were considered resistant if the minimal inhibitory concentration (MIC) of metronidazole was above 8 µg per ml. For disk diffusion a 5 µg metronidazole disk (Mast Laboratories, Liverpool, UK) was used. Strains with an inhibition zone of less then 10 millimeter were regarded as resistant (13). For breakpoint agar dilution two plates were used, one HPS-medium supplemented with metronidazole (8 µg per ml) and one such plate without antibiotics as a control. If *H. pylori* grew on both plates, the strain was considered resistant.

In the first part of the study the E-test was compared with disk diffusion in 263 consecutive *H. pylori* isolates and with breakpoint agar dilution in another 90 isolates. All isolates were obtained from different patients. In the second part 52 isolates, each obtained from a single antral biopsy specimen from one of 52 patients, were investigated. From each isolate 10-11 separate colonies were subcultured and an E-test was performed on each subculture. Results were compared with the E-test results using
multiple colonies. In three of these 52 isolates (two with MIC ≥256 µg per ml and one with MIC 0.5 µg per ml by E-test on multiple colonies) the 10-11 colonies were also subcultured on BHM-medium. Bacteria were harvested after 2-3 days and resuspended on HPS-medium for the E-test and on BHM-medium for further subculture. After 2-3 days bacteria were again harvested from the BHM-medium and the same procedure was repeated. In this way, 32 colonies were tested up to 10 times.

**Results.**

When the E-test results were compared to the disk diffusion and breakpoint agar dilution results, discordance was found in 5% (95% CI: 3-9%) and 6% (95% CI: 2-12%) of cases, respectively (E-test vs disk diffusion: 166 strains susceptible (S) by both tests, 83 strains resistant (R) by both tests, 8 strains R by E-test but S by disk diffusion, 6 strains S by E-test but R by disk diffusion; E-test vs breakpoint agar dilution: 60 strains S by both tests, 25 strains R by both tests, 1 strain R by E-test but S by agar dilution, 4 strains S by E-test but R by agar dilution). In the discordant cases differences between E-test and disk diffusion were mostly not around the cut-off, but bacteria were tested completely resistant by one test and fully susceptible by the other test (table 1). When 10-11 different colonies of an isolate were studied, 34 biopsy specimens harbored only susceptible bacteria and in 13 specimens only resistant bacteria were found. In all of these cases the results were congruent with the outcome of routine susceptibility testing using multiple colonies. In 5 biopsy specimens (10%; 95% CI:3-21%) both resistant and susceptible bacteria were found. When multiple colonies were used, 3 of these 5 isolates were tested resistant and the other two were tested susceptible (table 2). In our last study, repeating the E-test up to 10 times on 32 single colonies obtained from three isolates, variations in MIC were frequently seen. In one strain for example, MIC varied between 0.032 and 2 µg per ml. However, only one strain initially designated as resistant (MICs varying between 24 and ≥256 µg per ml) was reclassified as susceptible (MIC of 8 µg per ml) (1/292 tests (0.34%, 95% CI: 0.01-1.84%).

**Discussion.**

It has been generally recognized that, in comparison with susceptibility testing of *H. pylori* for other antibiotics, testing nitroimidazole susceptibility is problematic (4,14-17). In our study we also found disagreement between the tests in approximately 5% of
Table 1. MICs and inhibition zones of the 14 strains with discordant results in the study comparing E-test to disk diffusion.

<table>
<thead>
<tr>
<th></th>
<th>E-test MIC (ug/ml)</th>
<th>Disk diffusion inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.125</td>
<td>no zone</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>no zone</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>no zone</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>no zone</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>no zone</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>no zone</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>8</td>
<td>&gt;32</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td>&gt;32</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>&gt;32</td>
<td>16</td>
</tr>
<tr>
<td>11</td>
<td>&gt;32</td>
<td>17</td>
</tr>
<tr>
<td>12</td>
<td>&gt;32</td>
<td>22</td>
</tr>
<tr>
<td>13</td>
<td>&gt;32</td>
<td>26</td>
</tr>
<tr>
<td>14</td>
<td>&gt;32</td>
<td>26</td>
</tr>
</tbody>
</table>

cases. Although differences in methodology may cause variations in test results (4), we think that this does not account for the discrepancies found in our study, namely that isolates were classified as highly resistant by one test and fully susceptible by another test. As the reproducibility of the E-test in the offspring of a single bacterium was almost perfect, the most likely explanation for the discordant results is a coinfection with both resistant and susceptible bacteria. In our population such a coinfection could be demonstrated in approximately 10% of the cases, when only one biopsy specimen was examined. This is not unexpected as it is known from DNA fingerprinting that patients can be infected with two or more different strains (18-20). Moreover, even within the same strain different susceptibility patterns to metronidazole have been found (20,21). As a study in gnotobiotic piglets has shown a microclonal mode of growth with limited migration of bacteria between different sites.
Table 2. Results of the E-test performed on 10-11 separate colonies as compared to the E-test performed on multiple colonies in the 5 biopsy specimens containing both resistant and susceptible bacteria.

<table>
<thead>
<tr>
<th></th>
<th>multiple colonies</th>
<th>single colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>≥256</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>≥256</td>
<td>0.19</td>
</tr>
<tr>
<td>3</td>
<td>≥256</td>
<td>≥256</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>192</td>
</tr>
<tr>
<td>5</td>
<td>≥256</td>
<td>≥256</td>
</tr>
</tbody>
</table>

--- represents a missing test result due to strain death, very slow growth, or fungal overgrowth.

of the stomach (22), it is likely that the more gastric biopsy specimens are examined, the more different subpopulations will be encountered (20). It is remarkable that coinfections with both resistant and susceptible bacteria have not been described for other antibiotics (21). The special position of nitroimidazoles in this respect may be explained by the genetic basis of NIR (15). NIR is related to null mutations in the \(rdxA\) gene that encodes for a nitroreductase. This enzyme is not essential for bacterial survival as no decrease in metabolic or growth capacity is observed in the absence of functional enzyme (23). When a nitroimidazole containing therapy fails to eradicate \(H. pylori\), resistant mutants will become the major population in the stomach. After the antibiotic pressure has been removed, the wild type may reappear, but will have no significant survival advantage. This results in a stable coinfection with both resistant and susceptible bacteria. In contrast, clarithromycin resistance is associated with a point mutation in rRNA genes (24). Inefficient protein synthesis, due to the mutation, is likely to diminish the chances of survival in the
absence of clarithromycin. Therefore, if clarithromycin susceptible bacteria are present they will eventually constitute the majority of the population.

In conclusion, our study shows that the E-test is reliable for nitroimidazole susceptibility testing of \textit{H. pylori}. A co-infection with both resistant and susceptible bacteria, however, is common and may lead to discordant results between different tests.

References.


