Macrolide Resistance in *Helicobacter pylori*: Mechanism and Stability in Strains from Clarithromycin-Treated Patients

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*Helicobacter pylori* strains from seven patients treated with clarithromycin were investigated for development, mechanism, and stability of resistance. Genetic relatedness between pre- and posttreatment isolates was shown by arbitrary primed PCR. Clarithromycin resistance was associated with A-to-G transitions at either position 2143 or 2144 or at both positions 2116 and 2142. In four cases, the mutations were homozygous. The Cla' phenotype was stable after 50 subcultivations in vitro. No erythromycin-modifying enzymes or rRNA methylases were found by biological assays, PCR and sequencing, or cloning methods.

Antimicrobial treatment of *Helicobacter pylori* is now widely recommended for patients carrying this bacterium and presenting with peptic ulcers. Antibacterial treatment of *H. pylori* commonly includes a proton pump inhibitor and one or several antibacterial agents, such as clarithromycin, metronidazole, tetracycline, or amoxicillin (8, 9).

The use of clarithromycin for the treatment of *H. pylori* infection has resulted in the development of resistance (6, 23). So far, the only observed mechanism of clarithromycin resistance for *H. pylori* and several other bacteria has been attributable to a single-base mutation in a small region of the 23S rRNA gene (5, 10, 12, 20, 21).

The importance of clarithromycin resistance in treatment of *H. pylori* infection has been questioned. One study suggested that clarithromycin resistance may be of minor consequence based on in vitro results, in which resistant *H. pylori* reverted to susceptible forms after a few subcultivations (22).

In the present study, we investigated the stability and mechanism of resistance for *H. pylori* isolates from seven patients enrolled in a clinical treatment study, who had unsuccessful eradication of *H. pylori* (7). Antral biopsies were acquired before and 3 months after antibiotic treatment. Homogenized biopsies were cultured by standard procedures (7). All isolates were stored at −70°C for later determination of antibiotic susceptibility.

Susceptibility of the strains to tetracycline, azithromycin, clarithromycin, and erythromycin was determined by E-test (Biodisk AB, Solna, Sweden).

Susceptibility to clarithromycin (Abbott Laboratories, Ltd., Queensborough, United Kingdom) was also estimated by an agar dilution method. Approximately 10⁵ CFU was spotted onto four areas of the agar plates (16). The pretreatment strains showed susceptibility to all antibiotics tested. For posttreatment *H. pylori* strains, the MICs of tetracycline were unchanged, while the MICs of the macrolides had increased substantially (Table 1). There were differences in MICs between the E-test and the agar dilution method. However, in most cases, the deviation was only a one- or twofold dilution. The results obtained with both methods were reproducible when the assays were repeated. DNA was prepared from the strains isolated before and after treatment as previously described (1). Genomic fingerprints of the *H. pylori* isolates before and after treatment were studied by an arbitrary primed PCR (AP-PCR) method. The PCR mixtures were prepared as described previously (25), and amplification was carried out in a Perkin-Elmer (Foster City, Calif.) 9600 thermocycler for 40 cycles as follows: 94°C for 30 s, 37°C for 1 min, and 72°C for 1 min. The PCR products were analyzed with a 1.5% agarose gel stained with ethidium bromide. Identical genomic banding patterns were found between the isolates from the same patient in six of seven patients (patients 2 to 7), indicating that the *H. pylori* strains isolated before and after treatment were identical in six of the seven patients (Fig. 1).

Posttreatment strains were subcultivated on agar plates 50 times to examine the stability of the emerged resistance. MICs of azithromycin, clarithromycin, and erythromycin were determined by E-test at 10, 20, and 50 transfers. The clarithromycin MICs were also determined by the agar dilution method at 10 and 20 transfers. Subcultivation of the posttreatment *H. pylori* strains on agar plates did not result in a reversion of the resistance pattern, even though the strains were subcultivated 50 times (Table 2). Part of the 23S rRNA gene was amplified from all strains with primers 18 and 21 (20). The PCR products were digested with *MboII* and *BsaI* to detect the 23S A2143G and the A2144G mutations (19, 20) and were used for DNA sequencing.

Prior to sequencing, PCR products from all strains were purified with the QIAQuick PCR purification kit (Qiagen GmbH, Hilden, Germany). The ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) and primer 19 (20) were used to label the DNA, and the results were analyzed with an ABI 373 sequencer.

Assays for the possible occurrence of an erythromycin-modifying enzyme were performed with the Cla' *H. pylori* isolates as described previously (2). *Staphylococcus aureus* was used as the indicator strain, and *Escherichia coli* BM2571(pIP1527), carrying the *ereA* gene encoding erythromycin esterase, was used as a positive control (24). Fifteen micrograms of erythromycin filter disks (AB Biodisk) was used in the assay.

In order to search for erythromycin resistance genes, purified DNA from Cla' *H. pylori* was digested with restriction enzyme *HindIII* or *Sau3A* and ligated into *E. coli* vector pUC18, and transformation of *E. coli* DH5α was carried out.
Selection was performed on Isosensitest agar plates (Oxoid Unipath Ltd., Basingstoke, United Kingdom) with ampicillin (50 µg/ml) and erythromycin (2 µg/ml). The procedure we used was similar to those used in comparable experiments in which rRNA methylase genes from other species have been successfully cloned in *E. coli* (4, 13, 14).

*H. pylori* phenotypes were also screened for the presence of the *erm* gene(s). A pair of primers corresponding to conserved regions in known Erm methylases were used, and PCR was performed (3). Purified DNA was used as a template, and a negative control was included in each run. Positive controls were *E. coli* (pEM9592) and *E. coli* (pBR328:RV), which carry the *ermA* and *ermC* genes, respectively (15, 17). One of the amplified PCR products was cloned into an M13mp18/19 vector and sequenced (18) in order to look for DNA homology with the *ermA* and *ermC* genes.

Pretreatment strains showed a wild-type genomic sequence, while the posttreatment isolates had an A-to-G transition mutation in the 23S rRNA gene. Four strains were mutated at residue 2143 (cognate to residue 2058 in *E. coli*), and two strains were mutated at residue 2144. One isolate showed mutations at residues 2116 and 2142. Restriction enzymes were used to confirm the sequencing results and to determine whether the mutation occurred in only one or in both of the 23S rRNA copies. The strains with the A2143G and A2144G mutations were digested as expected (Table 1). Isolate 7b, mutated at residues A2116G and A2142G, was digested by both enzymes. The A2142G mutation did not create a new restriction site, but the A2116G mutation, by creating a site, explained the digestion by *MboII* into 700-bp fragments. The other *MboII* site and the restriction site for *BsaI* were not found within the sequenced region of the 23S rRNA gene from isolate 7b.

In four cases, the mutation was homozygous (patients 2, 3, 4, 6). Selection was performed on Isosensitest agar plates (Oxoid Unipath Ltd., Basingstoke, United Kingdom) with ampicillin (50 µg/ml) and erythromycin (2 µg/ml). The procedure we used was similar to those used in comparable experiments in which rRNA methylase genes from other species have been successfully cloned in *E. coli* (4, 13, 14).

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### TABLE 1. MIC test, 23S rRNA sequencing, and restriction enzyme digestion results

<table>
<thead>
<tr>
<th>Patient isolatea</th>
<th>Antibiotic treatmentb</th>
<th>Antibiotic susceptibility (MIC [µg/ml])c</th>
<th>23S rRNA d</th>
<th>Restriction enzyme assay resulte</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Tet</td>
<td>Cla</td>
<td>Cla (agar dilution)</td>
<td>Azi</td>
</tr>
<tr>
<td>1</td>
<td>a</td>
<td>0.25</td>
<td>&lt;0.016</td>
<td>&lt;0.015</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.25</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>a</td>
<td>0.25</td>
<td>&lt;0.016</td>
<td>&lt;0.015</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.5</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>a</td>
<td>0.8</td>
<td>&lt;0.016</td>
<td>&lt;0.015</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.75</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>a</td>
<td>1.5</td>
<td>0.019</td>
<td>&lt;0.015</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>1.5</td>
<td>&gt;256</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>a</td>
<td>1.5</td>
<td>&lt;0.016</td>
<td>&lt;0.015</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.75</td>
<td>&gt;256</td>
<td>128</td>
</tr>
<tr>
<td>6</td>
<td>a</td>
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<td>&lt;0.015</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>1.0</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>7</td>
<td>a</td>
<td>0.75</td>
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<td>&lt;0.015</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.5</td>
<td>&gt;256</td>
<td>64</td>
</tr>
</tbody>
</table>

a Results are presented for each patient before (a) and after (b) treatment.
b Cla, 750 mg of clarithromycin (Cla) plus 300 mg of ranitidine twice a day for 14 days; Cla + Tet, 750 mg of clarithromycin plus 300 mg of tetracycline (Tet) plus 300 mg of ranitidine twice a day for 14 days.
c MICs for all strains before and after treatment were tested by E-test except as noted. Azi, azithromycin; Ery, erythromycin.
d Sequence analysis of the 23S rRNA gene revealed mutations at residues 2116, 2142, 2143, and 2144.
e Treatment of PCR products from the mutated region with either *BsaI*, *MboII*, or both enzymes resulted in smaller fragments (+) or no digestion (–).
and 5); in the remaining cases, the mutations were heterozygous. A mutation in one of the two 23S rRNA copies was enough to cause high resistance. The single-base mutation is thus dominant. No association between therapy with one or two antibiotics and mutation type was observed.

In the assay for erythromycin-modifying enzymes, resistant *H. pylori* strains grew within the zone of inhibition but did not modify erythromycin with a clearly observable arrow-shaped distortion in the zone of inhibition, as did *E. coli* BM2571 (24). This indicates that the resistant *H. pylori* strains do not produce an extracellular enzyme with the capability of modifying erythromycin.

Putative erm genes, mediating macrolide resistance, were sought for by PCR (3). A PCR product of the expected size (about 500 bp) was obtained in five of the macrolide-resistant isolates of *H. pylori*. However, nucleotide sequencing of one of these PCR products did not indicate any homology with the reported *erm* genes. Thus, the erythromycin resistance is unlikely to be mediated by an rRNA methylase. This result is also supported by the failure to clone any erythromycin resistance determinant from the resistant *H. pylori* isolates, despite extensive work with DNA preparations of several isolates with two different restriction enzymes for digestion.

The patients in the present study belonged to a multicenter clinical treatment study in Sweden (7). Of 74 patients who were endoscopically examined before and after treatment, 8 patients were not cured (as determined by urea breath test and culture of *H. pylori*). One posttreatment strain was not culturable after storage at −70°C. The remaining seven posttreatment strains showed high resistance to clarithromycin, as reported here. Thus, the frequency of the acquired resistance in this study was 7%. One of the patients showed different banding patterns before and after treatment and was probably infected with two strains initially. The Cla’ posttreatment strain in this patient could have been resistant prior to treatment or developed resistance during treatment.

All *H. pylori* strains carried point-mutational changes in the small region of the 23S rRNA that has previously been associated with clarithromycin resistance (6, 10, 12, 19, 20). Four of seven resistant *H. pylori* strains carried the 2143 mutation. The posttreatment biopsies with resistant *H. pylori* strains were acquired 3 months after treatment, and 50 subcultivations of isolated bacteria did not change the MICs. We therefore conclude that the resistance conferred by mutations in the 23S rRNA gene was stable.

Several pieces of evidence suggest that the presence of an rRNA methylase is unlikely to be the mechanism of erythromycin resistance in *H. pylori*. First, the bioassays failed to detect any erythromycin-modifying enzymes. Second, the erythromycin resistance determinant from *H. pylori* could not be cloned in *E. coli*. Third, we failed to amplify an rRNA methylase gene in the *H. pylori* isolates by PCR.

The extended use of clarithromycin for the treatment of *H. pylori* may give rise to high frequencies of resistance to macrolides, including cross-resistance within the macrolide group of antibacterial agents. Resistance will affect the cure rates of *H. pylori* infection and cause treatment failures. The suggestion that emerged resistance disappears after treatment is contradicted by this study. Because clarithromycin is effective against *H. pylori* infection, there is an urgent need to find treatment combinations of drugs that prevent development of resistance. The combination with tetracycline in this study did not seem to diminish the selective pressure for macrolide resistance.

From our study, based on 14 strains from seven patients, we conclude that a mutation in one copy of the 23S rRNA is enough to confer a stable and high level of clarithromycin resistance in *H. pylori*.

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**REFERENCES**


**TABLE 2. MICs for posttreatment isolates after 20 or 50 subcultivations estimated by E-test or agar dilution**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Clarithromycin (agar dilution [20 passages])</th>
<th>Erythromycin</th>
<th>Azithromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32:16</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>2</td>
<td>12:16</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>3</td>
<td>4:64</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>4</td>
<td>&gt;256</td>
<td>16:256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>5</td>
<td>&gt;256</td>
<td>128:256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>6</td>
<td>4:16</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>7</td>
<td>8:64</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

* MICs were determined by E-test (50 passages) except as noted. See Table 1 for values before the subcultivations.


