

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*^r 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 10^3 CFU was spotted onto four areas of the agar plates (16). The pretreatment strains showed susceptibility to all antibiotics tested. For post-treatment *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 *Mbo*II and *Bsa*I 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*^r *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*^r *H. pylori* was digested with restriction enzyme *Hind*III or *Sau*3A and ligated into *E. coli* vector pUC18, and transformation of *E. coli* DH5 α was carried out

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

Patient isolate ^a	Antibiotic treatment ^b	Antibiotic susceptibility (MIC [μ g/ml]) ^c					23S rRNA ^d	Restriction enzyme assay result ^e	
		Tet	Cla	Cla (agar dilution)	Azi	Ery		<i>Bsa</i> I	<i>Mbo</i> II
1									
a		0.25	<0.016	<0.015	0.064	0.094	Wild type	-	-
b	Cla	0.25	32	64	>256	>256	A2143G	-	+
2									
a		0.25	<0.016	<0.015	0.125	0.19	Wild type	-	-
b	Cla	0.5	12	16	>256	>256	A2144G	+	-
3									
a		0.8	<0.016	<0.015	0.094	0.19	Wild type	-	-
b	Cla	0.75	4	64	>256	>256	A2143G	-	+
4									
a		1.5	0.019	<0.015	0.19	0.25	Wild type	-	-
b	Cla	1.5	>256	16	>256	>256	A2144G	+	-
5									
a		1.5	<0.016	<0.015	0.094	0.38	Wild type	-	-
b	Cla + Tet	0.75	>256	128	>256	>256	A2143G	-	+
6									
a		0.5	<0.016	<0.015	0.19	0.25	Wild type	-	-
b	Cla + Tet	1.0	8	64	>256	>256	A2143G	-	+
7									
a		0.75	<0.016	<0.015	0.064	0.064	Wild type	-	-
b	Cla + Tet	0.5	4	64	>256	>256	A2116G, A2142G	+	+

^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 *Bsa*I, *Mbo*II, or both enzymes resulted in smaller fragments (+) or no digestion (-).

(11). Selection was performed on Isosensitest agar plates (Oxoid Unipath Ltd., Basingstoke, United Kingdom) with ampicillin (50 μ g/ml) and erythromycin (2 μ g/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).

Cla^r *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 *Mbo*II into 700-bp fragments. The other *Mbo*II site and the restriction site for *Bsa*I 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,

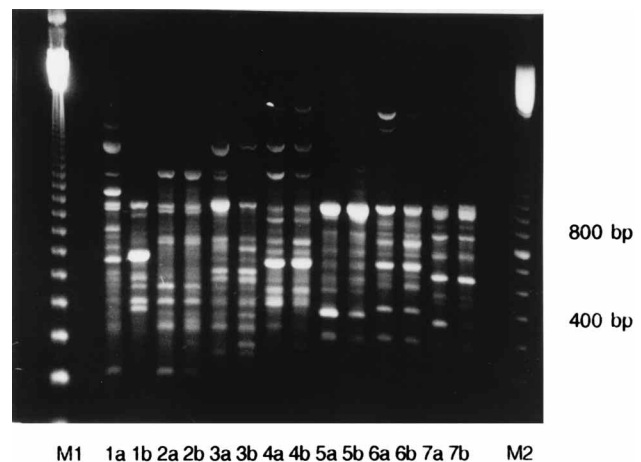


FIG. 1. Genomic fingerprinting of pretreatment (a) and posttreatment (b) *H. pylori* strains by AP-PCR. M1, 100-bp DNA ladder (Pharmacia Biotech, Sollentuna, Sweden); M2, 123-bp DNA ladder (Gibco BRL, Life Technologies, Täby, Sweden).

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

Patient no.	MIC ($\mu\text{g/ml}$) ^a			
	Clarithromycin	Clarithromycin (agar dilution [20 passages])	Erythromycin	Azithromycin
1	32	16	>256	>256
2	12	16	>256	>256
3	4	64	>256	>256
4	>256	16	>256	>256
5	>256	128	>256	>256
6	4	16	>256	>256
7	8	64	>256	>256

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

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^r 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 de-

tect 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

- Akopyanz, N., N. O. Bukanov, T. U. Westblom, and D. E. Berg. 1992. PCR-based RFLP analysis of DNA sequence diversity in the gastric pathogen *Helicobacter pylori*. *Nucleic Acids Res.* **20**:6221–6225.
- Andremont, A., G. Gerbaud, and P. Courvalin. 1986. Plasmid-mediated high-level resistance to erythromycin in *Escherichia coli*. *Antimicrob. Agents Chemother.* **29**:515–518.
- Arthur, M., C. Molinas, C. Mabilat, and P. Courvalin. 1990. Detection of erythromycin resistance by polymerase chain reaction using primers in conserved regions of *erm* rRNA methylase genes. *Antimicrob. Agents Chemother.* **34**:2024–2026.
- Berryman, D. I., and J. I. Rood. 1989. Cloning and hybridization analysis of *ermP*, a macrolide-lincosamide-streptogramin B resistance determinant from *Clostridium perfringens*. *Antimicrob. Agents Chemother.* **33**:1346–1353.
- Debets-Ossenkopp, Y. J., M. Sparrius, J. G. Kusters, J. J. Kolkman, and C. M. J. E. Vandenbroucke-Grauls. 1996. Mechanism of clarithromycin resistance in clinical isolates of *Helicobacter pylori*. *FEMS Microbiol. Lett.* **142**:37–42.
- DeKoster, E., A. Cozzoli, C. Jonas, R. Ntounda, J. P. Butzler, and M. Deltenre. 1996. Six years resistance of *Helicobacter pylori* to macrolides and imidazoles. *Gut* **39**(Suppl. 2):5. (Abstract 1A:04.)
- Hultén, K., B. H. Jaup, B. Stenquist, and L. Engstrand. Combination treatment with ranitidine is highly efficient against *Helicobacter pylori* despite negative impact of macrolide resistance. *Helicobacter*, in press.
- Jaup, B. H., and A. Norrby. 1995. Low dose short term triple therapy for cure of *Helicobacter pylori* infection and healing of peptic ulcers. *Am. J. Gastroenterol.* **6**:943–945.
- Lind, T., S. Veldhuyzen van Zanten, P. Unge, R. Spiller, E. Bayerdörffer, C. O'Morain, K. D. Bardhan, M. Bradette, N. Chiba, M. Wrangstad, C. Cederberg, and J. P. Idström. 1996. Eradication of *Helicobacter pylori* using one week triple therapies combining omeprazole with two antimicrobials: the Mach 1 study. *Helicobacter* **1**:138–144.
- Lucier, T. S., K. Heitzman, S.-K. Liu, and P.-C. Hu. 1995. Transition mutations in the 23S rRNA of erythromycin-resistant isolates of *Mycoplasma pneumoniae*. *Antimicrob. Agents Chemother.* **39**:2770–2773.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Meier, A., L. Heifets, R. J. Wallace, Jr., Y. Zhang, B. A. Brown, P. Sander, and E. C. Böttger. 1996. Molecular mechanism of clarithromycin resistance in *Mycobacterium avium*: observation of multiple 23S rDNA mutations in a clonal population. *J. Infect. Dis.* **174**:354–360.
- Monod, M., S. Mohan, and D. Dubnau. 1987. Cloning and analysis of *ermG*, a new macrolide-lincosamide-streptogramin B resistance element from *Bacillus sphaericus*. *J. Bacteriol.* **169**:340–350.
- Murphy, E. 1985. Nucleotide sequence of *ermA*, a macrolide-lincosamide-streptogramin B determinant in *Staphylococcus aureus*. *J. Bacteriol.* **162**:633–640.
- Murphy, E., and S. Löfdahl. 1984. Transposition of Tn 544 does not generate a target duplication. *Nature* **307**:292–294.

16. **National Committee for Clinical Laboratory Standards.** 1993. Methods for dilution. Antimicrobial susceptibility tests for bacteria that grow aerobically, 3rd ed. Approved strategies. NCCLS document M7-A3. National Committee for Clinical Laboratory Standards. Villanova, Pa.
- 16a. **Österlund, A., and L. Engstrand.** 1995. DNA fingerprinting of *Streptococcus pyogenes* from patients with recurrent pharyngotonsillitis by means of random amplified polymorphic DNA analysis. *Scand. J. Infect. Dis.* **27**:119–121.
17. **Rinkel, L. A., and D. C. Savage.** 1990. Characterization of plasmids and plasmid-borne macrolide resistance from *Lactobacillus* sp. strain 100-33. *Plasmid* **23**:119–125.
18. **Sanger, F., and A. R. Coulson.** 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* **94**:441–448.
19. **Stone, G. G., D. Shortridge, R. K. Flamm, D. Staimler, J. Versalovic, J. Beyer, A. T. Ghoneim, D. Y. Graham, and S. K. Tanaka.** 1996. Clarithromycin-resistant *Helicobacter pylori*: prevalence of 23S rRNA gene mutations in strains from European and Argentinian clinical trials. *Gut* **39**(Suppl. 2):8. (Abstract 1A:16.)
20. **Versalovic, J., D. Shortridge, K. Kibler, M. V. Griffy, J. Beyer, R. K. Flamm, S. K. Tanaka, D. Y. Graham, and M. F. Go.** 1996. Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **40**:477–480.
21. **Weisblum, B.** 1995. Erythromycin resistance by ribosome modification. *Antimicrob. Agents Chemother.* **39**:577–585.
22. **Xia, H. X., C. T. Keane, and C. A. O'Morain.** 1994. Clarithromycin resistance in *Helicobacter pylori*: prevalence, stability, and clinical implications, p. 165. *In* European Study Group on Pathogenicity and Immunology in *Helicobacter* Infections. Meeting at LO-skolen, Elsinore, Denmark, 21 to 24 June 1994.
23. **Xia, H. X., C. T. Keane, and C. A. O'Morain.** 1996. A 5-year survey of metronidazole and clarithromycin resistance in clinical isolates of *Helicobacter pylori*. *Gut* **39**(Suppl. 2):6. (Abstract 1A:09.)
24. **Yan, W., and D. E. Taylor.** 1991. Characterization of erythromycin resistance in *Campylobacter jejuni* and *Campylobacter coli*. *Antimicrob. Agents Chemother.* **35**:1989–1996.