ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1997, p. 712–714 Vol. 41, No. 3
0066-4804/97/$04.00+0

A PCR-Oligonucleotide Ligation Assay To Determine the Prevalence of 23S rRNA Gene Mutations in Clarithromycin-Resistant *Helicobacter pylori*

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Received 25 July 1996/Returned for modification 25 October 1996/Accepted 10 December 1996

We have developed a rapid PCR-oligonucleotide ligation assay that can discriminate single base substitutions that are associated with clarithromycin resistance in *Helicobacter pylori*. Susceptible isolates were wild type at positions 2143 and 2144 (cognate to 2058 and 2059 in *Escherichia coli*), while 93% of the resistant isolates contained A-to-G mutations at either position and 7% of the isolates contained A-to-C mutations at position 2143. In addition, the MIC for 86% of the resistant isolates with an A2143 mutation was ≥64 μg per ml, and that for 89% of the resistant isolates with an A2144 mutation was ≤32 μg per ml.

Clarithromycin inhibits protein synthesis by binding to the peptidyltransferase loop of 23S rRNA (4). In *Escherichia coli*, ribosomal binding sites for clarithromycin and other macrolides have been shown at residues A2058 and A2059 of the 23S rRNA gene (8). Mutations in the 23S rRNA gene in *Helicobacter pylori* appear to be associated with clarithromycin resistance. Versalovic et al. (16) have shown that A-to-G mutations in domain V of the 23S rRNA gene in *H. pylori*, cognate to residues 2058 and 2059 in *E. coli*, are associated with resistance to clarithromycin. We have chosen to numerate the residues 2143 and 2144 according to the transcriptional start site from the known sequence of the 23S rRNA gene (GenBank accession number U27270). However, Debets-Ossenkopp et al. (2) have reported another numbering system for the same residues, specifically, 2514 and 2515.

We developed a rapid assay based on PCR followed by the oligonucleotide ligation assay (OLA) for the rapid detection of these point mutations within the 23S rRNA gene of *H. pylori*, which is associated with resistance to macrolides. The PCR-OLA has been shown to be a rapid method that can discriminate single base pair mutations in DNA (10). A unique aspect of the PCR-OLA is that it can discriminate sequence variations (transitions, transversions, and deletions). This can be done by constructing probes to identify the variation of interest. This is different than using restriction enzymes to identify point mutations, because restriction enzymes are limited to sequence specificity. For instance, a restriction enzyme may be able to discriminate an A-to-G mutation by the creation of a restriction site. But if an A-to-C mutation occurs, the restriction enzyme may not restrict the DNA at that site; therefore, the sequence appears to be wild type.

In the present study, we used the PCR-OLA method to (i) analyze the association between macrolide resistance and the presence of mutations at 2143 and 2144 in the 23S rRNA gene of *H. pylori*, (ii) analyze the prevalence of both mutations in *H. pylori*, and (iii) correlate the MIC with both mutations.

Clinical isolates of *H. pylori* were isolated from gastric biopsy samples from patients entered into clarithromycin clinical trials sponsored by Abbott Laboratories. Genomic DNA was extracted from *H. pylori* isolates as described previously (3). Oligonucleotides (Table 1) were constructed by standard phosphoramidite chemistry on an Applied Biosystems model 380A DNA synthesizer. Oligonucleotides used as PCR primers were derived from the known sequence of the 23S rRNA gene (GenBank accession number U27270) and resulted in a 163-bp amplified product. Oligonucleotides that served as capture probes were modified with a 5′ biotin group by incorporation of biotin-labeled phosphoramidite during synthesis. Oligonucleotides that served as reporter probes were modified by phosphorylating the 5′ ends with 5′ phosphate-ON (Clontech, Palo Alto, Calif.) according to the manufacturer's directions. The phosphorylated reporter probes were labeled with digoxigenin-11-ddUTP on the 3′ end by using the Genius 5 oligonucleotide labeling system (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's directions.

Amplification reaction mixtures (50 μl) contained 50 mM Tris-Cl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, Md.), and 2 μl of the DNA preparation. The DNA was denatured for 45 s at 94°C prior to thermocycling in a model 9600 thermocycler (Perkin-Elmer, Norwalk, Conn.). Cycling conditions were 40 cycles of 94°C for 15 s, 43°C for 45 s, and 72°C for 45 s, followed by a final extension step at 72°C for 2 min. Ligation reactions (20 μl) and detection of ligated products were performed as described previously (12), with the exception that reactions were conducted in a model 9600 DNA thermocycler (Perkin-Elmer) for 10 cycles of 94°C for 15 s and 68°C for 5 min.

A total of 72 isolates of *H. pylori* were examined by PCR-OLA for point mutations at either position 2143 or position 2144. Of the 72 isolates, 32 were susceptible to clarithromycin and 40 were resistant to clarithromycin. *H. pylori* isolates from patients prior to treatment and after treatment were shown to be identical strains by PCR-restriction fragment length polymorphism of the *ureC* gene (14) and ribotyping (13), suggesting that selection of clarithromycin-resistant mutants arose

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TABLE 1. Oligonucleotides used as PCR primers and OLA probes in this study[a]

Ligation probe[b]		Mutation detected
Capture probe	Reporter probe	
B-CCTACCCGCGGCAAGACGGA	pAAGACCCCGTGGACCTTTAC-D	Wild-type 2143
B-CCTACCCGCGGCAAGACGGG		A2143G
B-CCTACCCGCGGCAAGACGGC		A2143C
B-CTACCCGCGGCAAGACGGAA	pAGACCCCGTGGACCTTTACT-D	Wild-type 2144
B-CTACCCGCGGCAAGACGGAG		A2144G
B-CTACCCGCGGCAAGACGGAC		A2144C

[a] Amplification primers used were 5′-TCAACCAGAGATTCAGT and 5′-TCCATAAGAGCCAAAGC.
[b] B, biotin; p, phosphate; D, digoxigenin.

from a single clone of *H. pylori* resident in the gastric mucosa during treatment with clarithromycin. All 32 clarithromycin-susceptible isolates possessed wild-type sequence at positions 2143 and 2144, while 37 of 40 (93%) clarithromycin-resistant isolates possessed A-to-G mutations at either position 2143 or position 2144 as determined by PCR-OLA. In addition, three isolates (7%) possessed A-to-C mutations at position 2143. Mutations were evenly distributed, with 22 (55%) at position 2143 and 18 (45%) at position 2144. This is in agreement with other reports that mutations at these residues or cognate residues are associated with resistance to macrolides (6, 7, 9, 15).

We found that the MIC for the majority of the isolates with mutations at position 2143 was higher than that for mutations at position 2144 (Fig. 1). Of the 22 isolates with the A2143G(C) mutation, 19 (86%) were associated with an MIC of ≥64 μg per ml. Of the 18 isolates with the A2144G mutation, 16 (89%) were associated with an MIC of ≤32 μg per ml. A statistically significant difference between mutations at positions 2143 and 2144 with respect to the MIC was observed with a one-way analysis of variance ($P < 0.05$). Nash and Inderlied (9) have suggested that the affinity of binding of macrolides to ribosomes is drastically reduced when the A2274 (cognate to 2058 in *E. coli*) residue of *Mycobacterium avium* is mutated. In *H. pylori*, mutations at residue A2143 may cause a greater conformational change than those at A2144, resulting in differences in MICs; however, further research is needed to determine if conformational changes result in differences in MICs.

H. pylori has been reported to possess two copies of the rRNA operon (1, 5). In studies with *E. coli*, the presence of multicopy plasmids carrying the mutant 23S rRNA gene has shown that only 50% of the gene copies need to be mutated at position 2058 to confer resistance to macrolides (11, 17). There is evidence to suggest that a heterozygous condition exists in *H. pylori* that is resistant to clarithromycin, where one 23S rRNA allele is of the wild-type genotype and the other copy is of the mutant genotype (16). We found two isolates of *H. pylori* that possessed an A-to-G heterozygous characteristic, one isolate at position 2143 and one at position 2144. The MIC for the 2143 heterozygote was 32 μg per ml, and the MIC for the 2144 heterozygote was 16 μg per ml.

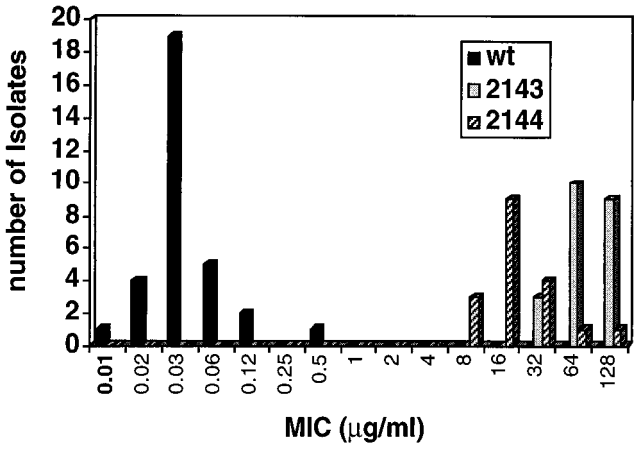

FIG. 1. Frequency of 23S rRNA gene mutations of *H. pylori* in relation to the MIC of clarithromycin. wt, wild type.

The application of the PCR-OLA for detection of macrolide resistance markers in *H. pylori* has the potential to benefit both treatment and diagnosis of an infection. The PCR-OLA can potentially be performed directly on biopsy material rapidly to detect the presence of the macrolide resistance marker. The presence, or absence, of the resistance marker would then help direct the treatment regimen for the patient.

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