Novel Method for Rapid Determination of Clarithromycin Sensitivity in Helicobacter pylori

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A novel PCR-hybridization assay, performed in single closed capillaries, was developed to detect clarithromycin resistance-associated gene mutations in Helicobacter pylori. Mutations were detected by thermal analysis in 33 of 34 (97%) resistant isolates but not in 66 isolates determined to be sensitive by conventional antibiotic assays. The method was rapid and reproducible, and it reduced PCR product contamination risk.

Helicobacter pylori colonizes the human stomach, where it induces chronic gastritis and has a role in the etiology of gastric and duodenal ulceration (4). Eradication of the bacterium cures peptic ulcer disease, and the macrolide drug clarithromycin is often a key component of combination therapies (1a). However, macrolide resistance occurs in H. pylori and is an important cause of treatment failure. Clarithromycin resistance in this organism is associated with single base mutations within the peptidyltransferase-encoding region of the 23S rRNA gene (5, 7–9). Three mutations have been described in which the adenine residues at positions 2143 and 2144 (equivalent to Escherichia coli coordinates 2058 and 2059) are replaced with guanine (A2143G and A2144G) or cytosine (A2143C).

We have developed a rapid assay for the detection of these point mutations with the LightCycler (Bio/Gene Ltd., Kimbolton, England) (10). In this assay, a fragment of the 23S rRNA gene surrounding bases 2143 and 2144 is amplified in a glass capillary tube with a PCR mixture that incorporates the DNA double-strand-specific fluorophore SYBR Green 1 (10). The LightCycler illuminates the contents of the capillary at the excitation wavelength of SYBR Green 1 and monitors the levels of amplicon by measuring the fluorescent signal with an integrated fluorimeter. Following amplification, the mutations are detected with a probe labelled at the 5' end with a second fluor dye, Cy5. To avoid primer activity, the 3’ end of the probe is blocked with biotin. Hybridization of the probe to the target sequence leads to an increase in fluorescence from the Cy5 fluorophore as a result of fluorescent resonance energy transfer between SYBR Green 1 and Cy5 (1). The peak emission wavelength of Cy5 is different from that of SYBR Green 1 and is monitored separately by the LightCycler. Following probe hybridization, the temperature within the capillary is increased and the level of Cy5 fluorescence is monitored. The temperature at which the maximum rate of decrease in fluorescence occurs is characteristic for a particular probe and target sequence under defined conditions. Where there are mismatched bases between the probe and target, the melting peak occurs at a temperature lower than that of a perfectly matched hybrid. Here we designed a probe that was complementary to the gene sequence of the clarithromycin-sensitive phenotype (8).

In this study we examined 100 strains of H. pylori from 63 patients. These included 55 strains from 18 patients (from antrum and/or corpus biopsy specimens taken before and after treatment). The strains were stored, cultured, and tested for antibiotic sensitivity, and then DNA was extracted with cetyltrimethylammonium bromide as previously described (2). Thirty-four strains from 25 patients were resistant to clarithromycin as indicated by an MIC of >2 mg/liter and/or the absence of a zone of growth inhibition around a 2-μg clarithromycin disc.

A 96-bp PCR fragment was amplified from chromosomal DNA with primers 23F (bases 2080 to 2099 [5'-CAA CCA GAG ATT CAG TGA AA-3']) and 23R (bases 2175 to 2155 [5'-GTG CTA AGT TGT AGT AAA GGT-3']). The amplification and hybridization reaction mixtures (10 μl) contained 10 ng of template DNA, a 200 μM concentration of each deoxynucleoside triphosphate (Gibco BRL, Paisley, Scotland), 1 pmol of forward primer (23F), 5 pmol of reverse primer (23R), 0.4 U of Platinum Taq (Gibco BRL), SYBR Green 1 (Bio/Gene Ltd.) diluted 1/20,000, and 5 pmol of the probe (23Pr; bases 2133 to 2150 [Cy5-GGC AAG ACG GAA AGA AAG CCG-biotin]) in Idaho Technology Rapid Cycler buffer (Bio/Gene) supplemented with MgCl₂ to yield a final MgCl₂ concentration of 3 mM. The primers (unpurified and desalted) and the probe (purified by reverse-phase high-performance liquid chromatography) were synthesized by MWG-Biotech UK Ltd., Milton Keynes, England. Forward and reverse prim-

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**TABLE 1. Amplification and melting cycles**

<table>
<thead>
<tr>
<th>Step and target temp (°C)</th>
<th>Increment time (s)</th>
<th>Temp (°C)</th>
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<tr>
<td>Initial denaturation (1 cycle), 94</td>
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<td>Amplification (50 cycles)</td>
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<td>74</td>
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<tr>
<td>Melting (1 cycle)</td>
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¹ Instrument settings: fluorimeter gains for both channel 1 and channel 2 were set at 8; the light-emitting diode was set at 100.

* Time held at the target temperature.
were added in unequal concentrations in order to favor the amplification of the strand to which the probe bound. The PCR cycling and probe melting conditions are given in Table 1.

All 66 strains sensitive to clarithromycin in the standard antibiotic assays had a probe melting peak at approximately 68°C (Fig. 1). In contrast, 31 of the 34 clarithromycin-resistant strains had melting peaks at approximately 58°C (Fig. 1). Of the remaining three resistant strains, two (strains A and B), isolated from posttreatment antral and corpus biopsy specimens taken from a single patient, had a probe melting peak at approximately 63°C. No mutation was detected in a third resistant strain (strain C). Controls were incorporated into each melt because the observed melting peak temperature in different runs varied up to 3°C with the number of samples analyzed. However, the differences in temperature between the peaks remained constant and the overall shift probably resulted from variations in temperature profile created by the LightCycler.

A PCR-restriction fragment length polymorphism assay for the detection of the A→G mutations in positions 2143 and 2144 was also carried out on all strains. For this, the primers described by Occhialini et al. (5) were used to obtain a 425-bp product spanning bases 1820 to 2244, which was then digested with MboII, to detect A2143G, and BsaI, to detect A2144G. There are no restriction enzymes reported for the detection of the rarer A2143C transversion, which also confers clarithromycin resistance. Mutations that confer clarithromycin resistance were detected in the 31 strains that had probe melting peaks at 58°C in the LightCycler assay. Seventeen strains had the A2143G mutation and 14 had the A2144G base substitution. Mutations were not detected in the 66 clarithromycin-sensitive strains or in 3 of the 34 clarithromycin-resistant strains. Two of those three (strains X and Y) had a LightCycler probe melting temperature indicative of a mismatch (63°C), although this was higher than that observed for the majority of resistant strains (58°C). The third strain (strain Z) had a probe melting temperature of 68°C, indicating homology with the sequence conferring the sensitive phenotype.

Sequencing (ABI PRISM Dye Terminator Cycle sequencing carried out according to the manufacturer’s instructions [Perkin Elmer, Warrington, England], using the four primers described in this paper) was performed on the 385-bp region within the peptidyltransferase-encoding region of the 23S rRNA gene of two strains (X and Z) that were negative for mutations in PCR-restriction fragment length polymorphism analysis. Sequence data showed that strain X contained the A2143C transversion, evident on LightCycler analysis, but the bases of strain Z matched those of a fully sensitive strain, confirming that resistance was not attributable to a mutation in the target region. Our results for strain Z indicated that, on rare occasions, the clarithromycin-resistant phenotype may arise through a different mutation in the 23S RNA gene or through an alternative mechanism.

A PCR-based assay has been developed that enables clarithromycin sensitivity of H. pylori to be determined within 1 h, excluding time for template preparation. The method has advantages over previously described hybridization assays used to detect the mutations (3, 6, 7) in that it involves the preparation of a single combined amplification and probe reaction mixture. In addition, one probe is used to detect all three mutations and the assay is performed in a closed capillary tube, which reduces the risk of PCR product contamination. Studies to assess the applicability of the assay to DNA extracted directly from biopsy material without the need for primary culture are currently under way.

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REFERENCES


