Rapid detection of clarithromycin resistance in *Helicobacter pylori* using a PCR-based denaturing HPLC assay

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**Objectives:** We evaluated a new approach for the rapid detection of clarithromycin resistance in *Helicobacter pylori*, based on PCR and denaturing HPLC (DHPLC).

**Methods:** A 180 bp fragment of the 23S rRNA gene was amplified using DNA from 81 clinical *H. pylori* isolates (51 isolates were shown to be resistant to clarithromycin by Etest), and, directly, from 101 gastric biopsies from patients with digestive diseases, who were infected with *H. pylori* as assessed by a 13C-urea breath test, histology and/or culture. DHPLC was used to detect mutations in all the PCR products.

**Results:** DHPLC profiles for the 30 susceptible isolates all showed homoduplex peaks; the resistant isolates consistently generated heteroduplex peaks that were easily distinguishable from the wild-type *H. pylori* reference strain. Sequencing revealed point mutations in all the resistant isolates. Overall, five different mutations were detected. Four of these mutations (A2142G, A2142C, A2143G and T2182C) are known to be associated with clarithromycin resistance; the remaining mutation (C2195T) has not been previously described. This novel single-base substitution was found in combination with the common mutation A2143G. Of the biopsies tested, 25 specimens generated heteroduplexes due to sequence alterations (mutation A2142G, A2142C or A2143G). In one of these specimens, A2143G was found together with the novel mutation T2221C; in another, a mixture of wild-type and mutant (A2143G) sequences was detected. For 20 culture-positive out of the 25 biopsies DHPLC results confirmed the presence of clarithromycin resistance.

**Conclusions:** Our results suggest that the PCR–DHPLC assay is a valid tool for rapid assessment of clarithromycin resistance in *H. pylori* and that in the future it could be used directly on biopsy specimens, avoiding the need for culture-based methods.

Keywords: 23S rRNA gene, mutations, Etest

**Introduction**

*Helicobacter pylori*, a human-specific colonizer of gastric mucosal epithelium, is associated with a variety of gastrointestinal diseases, including gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphatic tissue lymphoma.1,2 *H. pylori* infection is difficult to eradicate. According to the Maastricht 2-2000 Consensus Report,3 the recommended first-line eradication therapy, especially for patients with peptic ulcer disease, should be based on the combination of a proton pump inhibitor, clarithromycin and amoxicillin or metronidazole.

In recent years, however, resistance to clarithromycin and metronidazole has become increasingly common. It is current opinion that such resistance is the main cause of the failure of *H. pylori* eradication therapy,4 although additional factors, such as poor patient compliance and inappropriate treatment are also...
implicated. The failure of a clarithromycin-based regimen can lead to the development of secondary clarithromycin resistance. The prevalence of resistance ranges from <2% in The Netherlands to 29% in Japan. Resistance to clarithromycin is due to point mutations in the peptidyltransferase-encoding region of 23S rRNA which affect the binding of macrolides to the bacterial ribosome. Three major point mutations in domain V of the 23S rRNA gene have been linked to macrolide resistance: A2142G, A2143G and, less frequently, A2142C. Numerous PCR-based techniques have been developed to detect such mutations. These include the analysis of PCR products by restriction fragment length polymorphism (RFLP), DNA enzyme immunoassay, oligonucleotide ligation assay (OLA), reverse hybridization line probe assay and, more recently, fluorogenic-labelled probes. These molecular-based detection methods offer rapid and sensitive detection of antimicrobial resistance and play a critical role in understanding the mechanisms of resistance. Recently, denaturing HPLC (DHPLC) has emerged as a versatile technology for the analysis of genetic variants, such as single nucleotide polymorphisms, insertions and deletions. Novel polymer chemistry for separation has greatly improved the accuracy, sensitivity and throughput of DNA and RNA analysis by DHPLC. The high DHPLC sensitivity in the heteroduplex analysis has facilitated the development of applications beyond the scope of traditional genotyping, e.g. analysis of genes involved in antimicrobial resistance in bacteria such as Salmonella enterica, Staphylococcus aureus, Bacillus anthracis, Yersinia pestis and Mycobacterium tuberculosis.

In the work reported in this paper, we applied DHPLC technology together with confirmatory sequencing to rapidly and accurately characterize 23S rRNA gene mutations from 81 clinical H. pylori isolates and 101 gastric biopsy specimens from H. pylori-infected patients. To our knowledge, this is the first application of a PCR–DHPLC assay to detect clarithromycin resistance in H. pylori.

Materials and methods

Bacterial isolates and biopsy specimens

Eighty-one H. pylori isolates were recovered from antral gastric biopsies of patients attending our gastroenterology unit. Fifty-one clarithromycin-resistant isolates came from patients participating in a trial to assess the efficacy of a third-line, culture-guided regimen for H. pylori eradication. All of these patients displayed persistent H. pylori infection after two attempts at eradication. The 30 remaining clarithromycin-susceptible isolates served as negative controls. All isolates were sub-cultured from their respective 20% glycerol stocks, as described elsewhere. Positive controls included the H. pylori ATCC 43504 reference strain (with the wild-type 23S rRNA genotype) and clinical strains HPSC68 and HPSC73 harbouring the well-known 23S rRNA mutations, A2142G and A2143G.

In addition, we studied 101 gastric biopsies from dyspeptic patients taken during upper endoscopy in the period January 2004–March 2005. All of these patients had tested positive for H. pylori infection in at least two out of three tests (histology, culture and 14C-urea breath test). Of these patients, 35 had previously received antibiotic treatment for H. pylori infection. In 96 cases we took two biopsy specimens. One was formalin-fixed for histology, the other was immediately transported in Portagerm Pylori Transport Medium (bioMérieux, Marcy l’Etoile, France) to the microbiology laboratory where it was homogenized in 1 mL of 0.9% NaCl. An aliquot of the homogenate was directly cultured and isolates identified, as described elsewhere. A second aliquot was removed for subsequent DNA isolation (see below). In the five remaining patients we took a single biopsy specimen which was used for histology. The corresponding formalin-fixed paraffin-embedded tissues were used for DNA isolation (see below). Twenty gastric biopsies obtained from patients with negative diagnostic tests for H. pylori served as negative controls.

Clarithromycin susceptibility testing

A clarithromycin susceptibility testing assay was performed using the Etest method (AB Biodisk, Solna, Sweden), according to the manufacturer’s instructions. Briefly, 100 µL of a 3 McFarland bacterial suspension was spread on Mueller–Hinton agar plates (bioMérieux) supplemented with yeast extract (5 g/L), horse serum (10%) and nicotinamide adenine dinucleotide (25 mg/L). Plates were incubated at 35°C under microaerobic conditions for 3–5 days. Isolates were considered resistant to clarithromycin when the MIC was ≥1.0 mg/L.

DNA isolation

Genomic DNA was extracted from bacterial isolates and gastric biopsies using a QIAamp DNA Mini Kit (Qiagen, Milan, Italy), according to the manufacturer’s instructions. DNA was eluted in 200 µL of elution buffer and 5 µL of each DNA solution was used in the PCR. Formalin-fixed paraffin-embedded tissue blocks of gastric biopsies were processed as follows: 10 µm thick sections were de-paraffinized with xylene and re-hydrated in 95% ethanol; the DNA was subsequently extracted using the DNeasy Tissue kit (Qiagen), according to the supplier’s instructions. The DNA was eluted in 200 µL of elution buffer and 20 µL was used directly in the PCR.

PCR amplification of the H. pylori 23S rRNA gene

Previous studies have indicated that the ideal size of PCR fragments for use in DHPLC analysis is between 150 and 700 bp and that short fragments are associated with better sensitivity. We therefore used NTI Vector Advance software, version 9.0 (Invitrogen, Inc., Milan, Italy), to design two primers, Hp-F (5’-GGGAGCTGTCTCAACCAGAGA-3‘; positions 2207–2226, GenBank accession no. U27270) and Hp-R (5’-GGATGCTCCATAAGAGCCA-3‘; positions 2230–2250, GenBank accession no. U27270), able to generate a 180 bp amplified product from the peptidyltransferase-encoding region of domain V of the H. pylori 23S rRNA gene. A BLAST search was performed to check the specificity of the primer sequences. The Hp-F primer showed 100% similarity with H. pylori, Helicobacter cetonur, Helicobacter acinonychis and Arcobacter cryaerophilus; the Hp-R primer showed 100% similarity with H. pylori and H. cetonur only. PCRs were performed in a total volume of 50 µL containing 5–20 µL DNA solution (see above), 1.5 mmol MgCl2, 0.5 µmol each primer, 200 µmol dATP, dGTP, dCTP and dTTP, and 1.25 U OPTIMASE Polymerase (Transgenomic, Inc., Omaha, NE, USA). Cycling conditions were as follows: initial denaturation (95°C for 1 min), 35 cycles of denaturation, annealing and extension (95°C for 15 s, 43°C for 30 s, and 72°C for 30 s) and a final elongation step (72°C for 2 min). The amplicons were stored at 4°C until required.

To evaluate primer sensitivity, DNA extracted from the H. pylori ATCC 43504 was quantified by UV spectrophotometry at 260 nm and was used to make 10-fold serial dilutions to be used in PCR.

To evaluate primer specificity, we performed the PCR with DNA from other microorganisms: Helicobacter helimannii (formerly Gastrospirillum hominis) ATCC 49286, Helicobacter felis ATCC 49179, Helicobacter mustelae ATCC 43774, Campylobacter jejuni (clinical isolate), Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Enterococcus faecalis ATCC 29212, Posteroaro et al.
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*Mycobacterium tuberculosis* H37Rv, *Candida albicans* ATCC 90028, *Aspergillus fumigatus* H11-20 (kindly provided by David Perlin) and *Trichomonas vaginalis* (clinical isolate). PCR products were analysed by reverse blot hybridization using a *H. pylori* oligonucleotide probe (5′-ACTACAACCTTAGCAGCTGCTA-3′; positions 2161–2181, GenBank accession no. U27270), specifically designed to detect the amplified 23S rRNA gene fragment, and a protocol previously described.\(^{30}\)

**Verification of PCR product integrity**

Prior to mutation detection analysis all PCR products were analysed for size, yield and specificity determination using non-denaturing analysis at a column temperature of 50°C (indicative of a helical state). This ensured the generation of single, clean chromatographic peaks. Fragment size was determined by comparison with the phiX174/HaeIII DNA standard. Only amplicons that generated a single uniform peak of correct size were used in subsequent experiments.

**DHPLC analysis**

DHPLC analysis was performed using the WAVE DNA Fragment Analysis System (Transgenomic). The WAVE system is a novel commercially available automated technology for detecting variations in DNA sequences. PCR products can be processed in a 96-well format and directly loaded onto the column for analysis, without any initial purification. Gene variations are associated with changes in retention time and peak profile and can be characterized by sequencing of the PCR products. WAVE uses a method based on ion-pair reversed-phase HPLC and temperature-modulated heteroduplex analysis. The WAVE-DNA-Sep cartridge contains an electrostatically neutral hydrophobic polystyrene–divinylbenzene particle matrix that binds DNA in the presence of the ion-pair reagent 0.1 M triethylammonium acetate, pH 7 (buffer A). Bound DNA is eluted with 0.1 M triethylammonium acetate in 25% acetonitrile (buffer B). Elution of the DNA molecules is monitored spectrophotometrically by UV absorption at 260 nm.

Before DHPLC analysis, the wild-type reference PCR product (prepared from *H. pylori* strain ATCC 43504) was used for individual hybridization with each of the test samples. To optimize heteroduplex formation, the ratio of the area of each individual PCR product at 50°C (see above) was used to calculate the required sample volume. Each reaction mix, containing equal molar amounts of test and reference DNA molecules, was denatured at 95°C for 5 min and then slowly re-annealed by cooling to 25°C over 45 min (i.e. using a thermocycler) to form the hetero- and homoduplexes. Cooled PCR products (5–10 μL) were automatically loaded onto the column. Hetero- and homodimer analysis was carried out using an acetonitrile gradient formed by mixing buffers A and B (WAVE Optimized; Transgenomic). The optimal DHPLC conditions for elution of the PCR products were calculated by the WAVEmaker software (version 4.1; Transgenomic), which uses the sequence of the PCR products to predict gradient buffer concentrations and temperature. The optimal temperature for resolving heteroduplexes was determined by titration analysis, 1–3°C above and below the mean melting temperature predicted by the WAVEmaker software. Each run was carried out at a flow rate of 0.9 mL/min and lasted 7.0 min, including the column washing and equilibration steps. Column stability was assessed systematically by directly injecting a high and low range mutation standard (Transgenomic) from the WAVE system autosampler. Each test sample run used controls consisting either of the wild-type PCR product (from *H. pylori* strain ATCC 43504) or of the wild-type product mixed with mutant PCR products (from *H. pylori* strain HPSC68 or HPSC73). Reproducibility of the chromatographic patterns was monitored by analysing the test samples at least twice (on two successive days) using two diverse PCR products from each template.

The chromatogram elution profiles generated by the test samples were compared with the wild-type control profile to determine the presence of sequence variations. Test samples that gave single-peak profiles (homoduplex peak) were defined as wild-type *H. pylori* strains while samples that gave separate (double or multiple) peaks or shoulders on the leading edge of homoduplex peaks (heteroduplex peaks) were defined as mutant *H. pylori* strains. All differences in elution profile were confirmed by overlay of the profiles using WAVEmaker software.

**Sequencing**

PCR products were purified by the rapid PCR purification system (Marligen Biosciences, Ijamsville, MD, USA) and directly sequenced in both directions (ABI Prism 377, Applied Biosystems, Foster City, CA, USA.) using the Hp-F and Hp-R primers. The sequences were analysed for the presence of mutations of interest by sequence alignment against wild-type *H. pylori*, using WinDNASYS software version 2.1 (Hitachi Software Genetic Systems, San Francisco, CA, USA).

**PCR amplification of the ureC (glmM) gene in biopsy specimens**

All biopsy specimens included in the study were subjected to confirmatory tests for *H. pylori* infection by PCR amplification of the ureC (glmM) gene, using primers and conditions as described elsewhere.\(^{31}\)

**Results**

**Evaluation of the assay**

First, the sensitivity of the PCR assay was evaluated with a series of 10-fold dilutions of *H. pylori* DNA ranging from 10 ng to 1 fg. A positive PCR amplification result was obtained with DNA down to 10 fg, which corresponds to approximately five organisms (data not shown).

The specificity of the primer sequences was evaluated by alignment with bacterial 23S rRNA gene sequences deposited in the GenBank database. Primer sequence identity with gastric helicobacters, e.g. *H. felis* and *H. mustelae*, was of particular interest. With the exception of *H. cetorum* no sequence identity was found for any of the species considered in the analysis. No amplified products were generated by DNA from *H. mustelae*, *H. felis* or other microorganisms (e.g. *C. jejuni*, *S. aureus*, *E. coli*, *E. faecalis*, *M. tuberculosis*, *C. albicans*, *A. fumigatus* and *T. vaginalis*) or from human gastric biopsies from healthy individuals. DNA from *H. helicobacter* (the other human gastric pathogen), for which the 23S rRNA gene sequence is not available, also tested negative. By contrast, the 23S rRNA assay gave positive results for all the *H. pylori* isolates studied which included 81 clinical isolates, two well-characterized mutant isolates HPSC68 and HPSC73, and the wild-type reference strain, and for the 101 *H. pylori*-positive patient gastric biopsy specimens (see below). PCR amplification of the ureC (glmM) gene confirmed that *H. pylori* was present in all the biopsy specimens (data not shown). A reverse blot hybridization assay with a *H. pylori*-specific probe produced a positive hybridization signal for all 23S rRNA PCR products, confirming the specificity of the 23S rRNA gene assay for *H. pylori* detection (data not shown).

The specificity, yield and purity of the 23S rRNA PCR products were tested using non-denaturing analysis at a column temperature of 50°C. All samples generated single chromatographic peaks with
identical retention times; comparison with the phiX174 DNA ladder suggested a size of ~180 bp.

We went on to analyse duplex formation between PCR products from the test samples and those from wild-type and mutant H. pylori isolates. Tests were carried out under partially denaturing conditions, using three temperatures and gradients of elution buffer, as recommended by the analytical software. Each sample was tested first at the temperature predicted by the software (T_P) and then at the next two temperature settings where we observed a significant reduction in retention time, corresponding to a DNA denaturation point of 75%. DHPLC conditions were defined as optimal when mixtures of mutant and wild-type PCR products gave elution profiles with heteroduplex peaks, which could be easily distinguished from the single homoduplex peak for wild-type reference DNA. The reproducibility of the elution profiles was assessed by testing samples in duplicate at the optimal analytical column temperature (59.8°C). Similar chromatograms were obtained in a series of independent experiments.

**Detection of 23S rRNA mutations**

Crude PCR products from the 81 clinical H. pylori isolates were analysed by DHPLC. In comparisons with the wild-type reference strain, homoduplex peaks were observed for 30 of the 81 isolates; heteroduplexes, with separate peaks or shoulders on the leading edge of homoduplex peaks, were observed for 51 of the 81 isolates. All 51 DHPLC profiles display clear differences with respect to the normal elution profile, indicating at least one sequence variation within the DNA test fragment. DHPLC detected seven different profiles including the wild-type (Figure 1). All samples containing the same substitutions had identical profiles (shown by overlaying them using the WAVEmaker software).

All PCR products from amplicons with altered profiles were sequenced to assess the validity of the DHPLC results and to characterize sequence variation. Sequence analysis revealed the presence of point mutations in all amplicons with heteroduplex profiles (Table 1). Of the 51 isolates containing sequence variations, 25 (49%) contained the A2143G mutation, 15 (29%) contained the A2142G mutation and 7 (14%) displayed an A→C transition at position 2142. Two point mutations were also found at additional sites in the 23S rRNA gene: three isolates contained the T2182C mutation, which has recently been shown to be associated with clarithromycin resistance;32 one isolate contained C2195T. In two out of three cases, T2182C was found in combination with A2142G. The two isolates harbouring the combined mutations had profiles that were different from their compositive single mutations (Figure 1, profile f versus profiles c and g). The remaining one isolate harboured T2182C alone, leading us to hypothesize that this mutation was involved in the clarithromycin resistance, although much caution is required in interpreting such results.33 C2195T, which has not been previously described, was detected alongside A2143G. As expected, no sequence alterations were observed for the 30 isolates with homoduplex peak DHPLC profiles (Table 1).

To further evaluate the validity of our DHPLC assay, we examined PCR products generated by amplification of DNA from the gastric biopsies of 101 patients with H. pylori infection (see the Materials and methods section). Heteroduplex peaks were observed in 25 of the biopsy specimens. Subsequent sequencing revealed the presence of the A2143G, A2142G and A2142C mutations, respectively in 15, 7 and 3 specimens. One of these specimens contained the A2143G mutation together with a T→C transition at position 2221, a variation that has not been previously described. This specimen had a DHPLC profile identical to that exhibited from specimens harbouring the A2143G mutation.
TABLE 1. Results of the PCR–DHPLC assay for H. pylori culture isolates and gastric biopsies of patients with H. pylori infection

<table>
<thead>
<tr>
<th>Mutation in 23S rRNA domain Vb</th>
<th>No. of samples</th>
<th>Presence of heteroduplex in DHPLC profile</th>
<th>MIC range (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolates (n = 81)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2143G</td>
<td>25</td>
<td>Yes</td>
<td>8–256</td>
</tr>
<tr>
<td>A2142G</td>
<td>15</td>
<td>Yes</td>
<td>16–256</td>
</tr>
<tr>
<td>A2142C</td>
<td>7</td>
<td>Yes</td>
<td>1–32</td>
</tr>
<tr>
<td>T2182C</td>
<td>1</td>
<td>Yes</td>
<td>8</td>
</tr>
<tr>
<td>A2143G + C2195Td</td>
<td>1</td>
<td>Yes</td>
<td>256</td>
</tr>
<tr>
<td>A2142G + T2182C</td>
<td>2</td>
<td>Yes</td>
<td>256</td>
</tr>
<tr>
<td>wild-type</td>
<td>30</td>
<td>No</td>
<td>0.016–0.125</td>
</tr>
<tr>
<td>Culture-positive biopsies (n = 96)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2143G</td>
<td>10</td>
<td>Yes</td>
<td>32–256</td>
</tr>
<tr>
<td>A2142G</td>
<td>5</td>
<td>Yes</td>
<td>16–64</td>
</tr>
<tr>
<td>A2142C</td>
<td>3</td>
<td>Yes</td>
<td>8–32</td>
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<tr>
<td>A2143G + T2221Cd</td>
<td>1</td>
<td>Yes</td>
<td>256</td>
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<td>A2143G + wild-type</td>
<td>1</td>
<td>Yes</td>
<td>32</td>
</tr>
<tr>
<td>wild-type</td>
<td>76</td>
<td>No</td>
<td>0.016–0.06</td>
</tr>
<tr>
<td>Culture-negative biopsies (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2143G</td>
<td>3</td>
<td>Yes</td>
<td>NC</td>
</tr>
<tr>
<td>A2142G</td>
<td>2</td>
<td>Yes</td>
<td>NC</td>
</tr>
</tbody>
</table>

aBiopsies from patients not infected by H. pylori were also examined and were PCR negative.
bAs identified by direct sequencing of PCR products.

cFor all isolates MICs were determined by the Etest method. Isolates with MIC ≥ 1 mg/L were considered clarithromycin-resistant in accordance with the NCCLS M100-S12 document.28

dNever observed.

Not carried out. These specimens were formalin-fixed paraffin-embedded gastric biopsies for which culture was not performed.

Comparison of clarithromycin susceptibility testing by the Etest and PCR–DHPLC assay

Isolates were tested for clarithromycin susceptibility using the Etest phenotypic method, and the results compared with those from PCR–DHPLC. The two methods gave the same results for all 81 (51 resistant and 30 susceptible) isolates (Table 1).

For 96 culture-positive biopsies out of the 101 tested, the PCR–DHPLC assay results correlated perfectly with the clarithromycin susceptibility profile determined by the Etest (Table 1). All 76 specimens with a clarithromycin-susceptible isolate were confirmed by PCR–DHPLC. All of the 20 specimens with a clarithromycin-resistant isolate generated DHPLC profiles showing sequence variations (Table 1). The same variations were observed when PCR products of the 20 corresponding resistant isolates were directly sequenced (data not shown). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) values for clarithromycin susceptibility testing by PCR–DHPLC were all 100%. Five paraffin-embedded biopsy specimens could not be cultured, making evaluation impossible. In each of these specimens, the PCR–DHPLC assay revealed the presence of the resistant genotype.

MICs of clarithromycin-susceptible and -resistant isolates were 0.016–0.125 mg/L and 1–256 mg/L, respectively. The level of clarithromycin resistance differed between resistant isolates, with MICs of 1 mg/L (2 isolates), 2 mg/L (3 isolates), 4 mg/L (3 isolates), 8 mg/L (5 isolates), 16 mg/L (10 isolates), 32 mg/L (9 isolates), 64 mg/L (5 isolates), 128 mg/L (9 isolates) and 256 mg/L (25 isolates).

In isolates where phenotypic susceptibility was associated with 23S rDNA mutations, we observed similar MICs for isolates containing the A2142G mutation and those containing the A2143G mutation. MIC values for these isolates were higher than those for isolates harbouring the A2142C mutation.

Discussion

Given that the prevalence of macrolide-resistant strains of H. pylori is increasing worldwide and that resistance to clarithromycin is an important predictor of the failure of H. pylori eradication therapy the rapid and accurate identification of resistant isolates is an important goal for clinical microbiology. Molecular-based methods provide a rapid and alternative approach to macrolide susceptibility testing. These methods are less time-consuming than conventional methods such as disc diffusion, Etest or microbroth dilution.9 They can also be used with gastric biopsy specimens, omitting the necessity for in vitro culture.

Macrolide resistance in H. pylori strains has been associated with at least eight different mutations, including A2115G, G2141A, A2142G, A2142C, A2142T, A2143G, A2143C and T2182C.11–13,15,32,34 The most frequent of these mutations are A2142G, A2143G and A2142C. A large multicentre study by van Doorn et al.35 confirms the strong association between specific 23S rDNA mutations and macrolide resistance (although A2115G, G2141A, A2142T and A2143C were not observed). Several recent DNA-based methods selectively detect the most common mutations in the H. pylori 23S rRNA gene.10,16,18,30–38 In this context, Stone et al.18 proposed a PCR–OLA assay in which four individual oligonucleotide probes were used to determine the prevalence of 23S rRNA gene mutations in 40 clarithromycin-resistant H. pylori isolates. The mutations were evenly distributed between the positions 2143 (equivalent to 2142) and 2144 (equivalent to 2143), whereas two isolates exhibited an A-to-G heterozygous condition (one isolate at position 2143 and one at position 2144). Alarcón et al.10 studied 25 clarithromycin-resistant H. pylori strains to detect A2142G and A2143G mutations in the 23S rRNA gene by a PCR–RFLP method and an A2142C mutation by PCR using a 3′-mismatched specific primer. The last method, also known as 3M-PCR, was therefore used by Pan et al.37 to detect all the three prevalent types of 23S rRNA gene mutations (A2142G, A2142C and A2143G) associated with clarithromycin resistance among H. pylori isolates from 96 Chinese patients, although only five of these isolates were found to have a point mutation (A2143G). Even though PCR–RFLP has been widely used for the detection of clarithromycin resistance this assay failed to detect most mixed infections since it is able to detect only the dominant genotype when several genotypes coexist, as it was shown recently.39 In addition, the method is technically complex and may not be suitable for clinical application. To overcome these limits, Maeda et al.38 described the application of the preferential homoduplex formation
assay (PHFA), originally used for determination of HLA types, to detect the mutations in the 23S rRNA gene directly from gastric juice specimens. The PCR–PHFA assay was performed on streptavidin-coated microtitre plates with biotin- and dinitrophenyl-labelled amplicons and using distinct probes for the wild-type and each mutant. This assay, which approaches to that described here, can detect each genotype separately, allowing the detection of mixed infections. Finally, Oleastro et al.16 developed a single-step, real-time, PCR-based method for use with gastric biopsies. This method rapidly and accurately detects the three most common mutations (A2142G, A2142C and A2143G) that confer clarithromycin resistance and the wild-type genotype, even though this assay is a probe mismatch assay, it would also detect other mutations in the amplimer sequence complementary to the probe (e.g. G2141A, A2142T and A2143C), but not those outlying the probe (e.g. A2115G, T2182C and T2717C). Yet, despite an observed correlation of 96.4% between these genotypes and the susceptibility profile of tested isolates, three of the clarithromycin-resistant biopsy specimens studied by these authors contained a wild-type genotype. This suggests that other undetected 23S rDNA mutations may be involved in resistance. In a recent evaluation conducted by Elvis et al.35,36 the performances of 3M-PCR, PCR–RFLP and real-time PCR assays were compared for detecting clarithromycin resistance associated with the A2142G, A2142C and A2143G mutations. Though the 3M-PCR and real-time PCR assays gave identical results for 92.4% of the isolates studied, however, the real-time PCR failed to detect A–G mutations in 10 isolates when they were present together with the wild-type allele. Overall, 3M-PCR appeared to be the most specific and sensitive assay, although four individual PCRs for each sample were needed.

In this paper, we have described the successful use of a new PCR-based diagnostic method. In conjunction with DHPLC analysis, the new method offers rapid detection of several different mutations in the H. pylori 23S rRNA gene. It can detect not only single-base substitutions but also a broad range of other sequence variations, including small deletions and insertions. With respect to the existing assays discussed above, that are limited to detecting known sequence variants, we feel that the main advantage of our approach is the possibility to detect a wider range of point mutations than A2142G, A2142C and A2143G, including novel or multiple mutations, although the contribution of many of these other mutations to resistance is still to be established.

In the experiments reported here, DHPLC revealed the existence of sequence variations in DNA obtained from all 51 H. pylori clarithromycin-resistant isolates studied. Sequencing of the DNA products clarified that these variations corresponded to single-base substitutions. The majority (49%) of isolates contained the A2143G mutation. This result confirms the findings of previous studies35,39 but contrasts with studies by Ribeiro et al.40 and others3,18 in which the most commonly detected mutation was A2142G. Since DHPLC, through the formation of mismatched heteroduplexes during reannealing of wild-type and mutant DNA, can simply reveal unknown sequence variations that may not be associated with resistance we examined a large number (30) of clarithromycin-susceptible H. pylori strains to establish whether polymorphisms occurred within the amplified 23S rRNA gene fragment. Nevertheless, no sequence variants were detected in all of these strains allowing the recognition of additional mutations by this DHPLC assay to gain a great deal of importance.

As H. pylori genome contains two copies of 23S rRNA gene,41 it has been reported that H. pylori sometimes exhibits a heterozygous condition in which one of the genes is mutated and the other remains normal. This genotype confers a clarithromycin-resistant phenotype.13,18 This finding is supported by studies using E. coli isolates containing multicyclic plasmids with a mutant 23S rRNA gene. These investigations show that mutations in 50% of gene copies (at position 2058, equivalent to 2142 in H. pylori) are enough to confer macrolide resistance.42 In our own work we detected T2182C in conjunction with A2142G and C2195T in conjunction with A2143. Given that reproduction within colonies of bacteria is clonal and that the three double mutant isolates in question were purified from a single colony of isolate our findings support the hypothesis16 that a single strain of H. pylori may contain multiple variant alleles of the 23S rRNA gene.

In 23 out of 25 gastric biopsies showing an altered DHPLC profile we detected a single mutation (A2142G, A2142C, or A2143G). Of the two remaining specimens, one contained a mixture of wild-type and mutant (A2143G) sequences while the other contained the A2143G mutation together with the novel T2221C mutation.

As recently illustrated by Hao et al.,43 the discovery of new mutations is not unusual. In this case, however, the mutations were found in conjunction with mutations known to be associated with macrolide resistance. Further investigation is therefore required to fully elucidate the role of these two novel H. pylori mutations (C2195T and T2221C) in clarithromycin resistance.

To summarize, the DHPLC assay correctly identified 100% of H. pylori clarithromycin-resistant isolates. Sequence analysis confirmed the existence of mutations in all isolates. Though we cannot exclude the possibility that other 23S rRNA domains may also play a role,44 this finding raises the possibility that domain V of the 23S rRNA gene encompasses almost all mutations conferring clarithromycin resistance in H. pylori.

Our assay is rapid (the temperature-dependent, ion pair chromatography required only 7 min per sample), easy to perform and relatively cost-effective, allowing high throughput with minimal operator time. It can be easily adapted to allow the simultaneous analysis of numerous clinical specimens (96 samples per run). By considering the additional time needed to perform the pre-analytical steps of the DHPLC assay (PCR, quantification of PCR product and heteroduplex formation), our approach is less time-saving than other existing methods, such as LightCycler-based assays or primer-mismatch PCR. However, the DHPLC analysis, by requiring one assay for each sample, remains a valid alternative to real-time PCR (multiple oligonucleotide assays on each sample are frequently required) for detecting previously documented mutations and novel mutations at the same time. Remarkably, since the WAVE system can operate in three different modes, depending on the temperature at which chromatographic separation is performed, the DHPLC system should be utilized in a molecular core facility to enhance services in the areas of mutation detection and rapid genotyping, enabling users to analyse samples from a variety of organisms on the same instrument. Additional cost savings may be achieved by using DHPLC system for automated analysis, isolation and purification of PCR products for cloning to replace standard agarose gel electrophoresis.

Like other existing molecular assays,14,16,17,38 the DHPLC assay can be used directly with biopsy specimens, thereby avoiding the requirement for time-consuming culture-based methods. This is particularly important for patients in whom a first eradication attempt has failed. Current H. pylori patient management strategies call for at least two courses of treatment before resorting
to culture-guided assessment of resistance.\textsuperscript{22} Although we need to test our assay with larger numbers of biopsy specimens we are hopeful that it will prove a reliable method for the rapid detection of clarithromycin resistance in clinical specimens, and that the availability of the new assay will lead to a reassessment of the costs and benefits of current eradication strategies.

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Transparency declarations

None to declare.

References


