PCR detection of clarithromycin-susceptible and -resistant *Helicobacter pylori* from formalin-fixed, paraffin-embedded gastric biopsies

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Antimicrobial resistance to clarithromycin is a growing concern in the treatment of *Helicobacter pylori* and is associated with three major point mutations of the 23S rRNA, A2142C, A2142G, and A2143G. The use of traditional culture-based methods for determination of clarithromycin resistance in *H. pylori* are time consuming and lack sensitivity. We implemented a real-time PCR with melt curve analysis to detect and characterize *H. pylori* in formalin-fixed, paraffin-embedded gastric biopsy specimens to assess the frequency of clarithromycin resistance mutations in our study population. One hundred and fifty-three formalin-fixed, paraffin-embedded gastric biopsies were chosen on the basis of positive immunohistochemical staining for *H. pylori* and an accompanying histopathological diagnosis of Helicobacter-associated gastritis. New adjacent sections were taken for immunohistochemical staining and DNA extraction with subsequent testing by PCR assay and melt curve analysis using a primer and probe combination first described by Oleastro *et al.*¹² One hundred and forty-six samples demonstrated adequate amplification of a human DNA control target. Of these, there were 122 *H. pylori* immunohistochemistry-positive samples. In all, 103 out of 122 (84%) immunohistochemistry-positive samples demonstrated amplifiable *H. pylori* 23S rRNA gene target and 19 (16%) demonstrated no amplification of *H. pylori*. Twenty-two samples were negative for *H. pylori* by immunohistochemistry and PCR. Two were negative for *H. pylori* by immunohistochemistry, but were positive for *H. pylori* by PCR. In all, 52 out of 105 (50%) PCR-positive samples demonstrated resistance mutations, and it was determined that a heterogeneous population of mutated and unmutated organisms was present in 11 out of 52 samples. The use of PCR assays allows for a timely assessment of clarithromycin resistance status without the disadvantages of culture-based methods, and may lead to a decrease in treatment failure rates.

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Helicobacter pylori is a Gram-negative bacillus associated with gastroesophageal reflux disease, gastric and duodenal ulcers, gastric cancer, and MALT lymphoma. *H. pylori* has a worldwide distribution, with a higher incidence of infection among the population in developing countries.¹ It is estimated that up to 30–40% of the population of the United States harbors *H. pylori* within the upper gastrointestinal tract;² however, the majority of

people infected with *H. pylori* are asymptomatic. According to the American College of Gastroenterology, treatment is indicated for active peptic ulcer disease, a confirmed history of peptic ulcer disease that has not been previously treated for *H. pylori*, low-grade gastric MALT lymphoma, and following endoscopic resection of early gastric cancer. In addition, a ‘test and treat’ strategy has been recommended for uninvestigated dyspepsia in patients under 55 years of age who do not demonstrate specific ‘alarm’ features, such as anemia, vomiting, or family history of gastrointestinal malignancy.³

The ‘triple therapy’ regimen is the mainstay of pharmacological treatment for *H. pylori* infection in the United States, and typically consists of either amoxicillin or metronidazole used together with a proton pump inhibitor and clarithromycin.³

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Although increased antimicrobial resistance has been seen with each of the agents used in treatment of *H. pylori*, the increase in treatment failure can be attributed in large part to resistance to clarithromycin.^{4–6} It has been previously demonstrated that point mutations in domain V of the 23S rRNA gene are responsible for the resistance of *H. pylori* to macrolides,⁷ with the three major point mutations being A2142G, A2143G, and A2142C.⁷ The most frequent of these are transitions of adenine to guanine at the 2143 or 2142 positions, which account for >80% of clarithromycin resistance seen worldwide and ~90% of resistance seen in developed countries.⁸

A variety of diagnostic methods have been developed for *H. pylori*, including culture and histopathology, rapid urease tests, stool antigen testing, the urea breath test, serology, and more recently molecular assays.^{3,9} Of these, culture is the only widely available method that also allows for antimicrobial susceptibility testing. In addition to requiring tissue obtained through endoscopy, culture has many inherent disadvantages, which include requirements of special transportation conditions with restrictive timelines to ensure optimal viability of organisms,¹⁰ the use of special media and environments for maintenance of cultures, and lengthy incubation time.¹¹

Owing to the limitations of current laboratory methods available for the assessment of clarithromycin resistance in *H. pylori*, we set out to implement a method that would rapidly detect clarithromycin resistance and susceptible *H. pylori* without the constraints of traditional culture methods. In this report, we describe the use of real-time PCR based on methods previously described by Oleastro *et al*¹² to detect *H. pylori* in formalin-fixed, paraffin-embedded gastric biopsy specimens, and to assess the frequency of clarithromycin resistance within our study population.

Materials and methods

Bacterial Culture and Sample Processing

Seven *H. pylori* reference strains with known mutations in the 23s rRNA gene (two A2142G and five A2143G strains) and one unmutated strain were obtained from the Centers for Disease Control and Prevention, National *Campylobacter* and *Helicobacter* Reference Laboratory. Samples were grown on Brucella agar w/5% sheep blood, Hemin, and Vitamin K (Remel, Lenexa, KS, USA) under a microaerophilic environment at 37 °C for up to 7 days. Phenotypic clarithromycin susceptibilities of the seven *H. pylori* strains with 23S mutations and the unmutated sample were determined using the E-test method (AB Biodisk, Solna, Sweden) with previously established guidelines of MIC $\geq 1 \mu\text{g/ml}$ indicating resistance.¹³

DNA was extracted by placing a heavy inoculum of bacteria into 250 μl of TritonX buffer solution

(stock: 10 ml 2% Triton X-100, 5 ml TWEEN 20, 20 ml 0.5 M EDTA, 1 ml 1M Tris HCL (pH 8.0) and 964 ml molecular grade water) followed by placement in a heat block at 99 °C for 10 min.

Preparation of Fresh and Formalin-Fixed, Paraffin-Embedded Dilution Series

The limit of detection of the assay was evaluated using fresh isolates via a tenfold dilution series of extracted DNA from 10^7 to 10^1 genome equivalents per a final 20 μl PCR reaction volume. Genome equivalents were calculated on the basis of an *H. pylori* genome size of 1.64 megabases, resulting in 5.655×10^5 copies/ng purified DNA. A spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, Wilmington, DE, USA) was used to determine the concentration of DNA per μl of initial sample.

For determination of the limit of detection using formalin-fixed, paraffin-embedded specimens, mock biopsy blocks were created to serve as quantitative standards: a 0.5 McFarland dilution of the cultured strains of *H. pylori* was prepared and mixed with collagen at tenfold dilutions of 10^6 – 10^2 organisms per μl , to a final volume of 50 μl . The resulting collagen plug was formalin-fixed and paraffin-embedded according to our institutional protocol for gastric biopsy specimens. 10- μm -thick sections were prepared and mounted to glass slides, and the DNA was extracted from the sections using Pinpoint (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions.

Gastric Biopsy Selection and Processing

One hundred and fifty-three formalin-fixed, paraffin-embedded gastric biopsy specimens were obtained from the archives of three institutions within our hospital system with IRB approval. The gastric biopsies were each retrospectively reviewed and evaluated, and specimens were chosen on the basis of positive immunohistochemical staining for *H. pylori* and an accompanying histopathologic diagnosis of *Helicobacter*-associated gastritis. 10- μm -thick unstained sections were prepared from the gastric biopsies, and adjacent sections were submitted for immunohistochemical staining. The immunohistochemically stained glass slides were reviewed for the presence of *H. pylori* organisms with subsequent performance of DNA extraction from the corresponding unstained sections using Pinpoint (Zymo Research), according to the manufacturer's instructions.

Detection of Point Mutations of the 23 rRNA Gene of *H. pylori* by Real-Time PCR

A 267-bp fragment of the 23S rRNA gene of *H. pylori* was amplified using primers HPYS and HPYA, which have been previously described.¹⁴ Amplification was detected using a 5' LC-Red

640-labeled sensor probe with a 3' phosphorylated end (5'-GGCAAGACGGAAAGACC-3'; nucleotides 2504–2520), and a 3' fluorescein-labeled anchor probe (5'-TGTA GTGGAGGTGAAAATTCCTCCTAC CC-3'; nucleotides 2473–2501). The sensor probe hybridized to the mutation site containing DNA region whereas the anchor probe hybridized three bases upstream, as previously described.¹² The LightCycler 1.5 thermocycler (Roche Diagnostics, Indianapolis, IN, USA) was used for the PCR reactions. The glass capillaries contained a total volume of 20 μ l (2 μ l of DNA template, 4.0 μ l of MgCl₂ (25 mM), 0.5 μ l of forward and reverse primers (20 μ M each), 0.2 μ l of sensor and anchor probes (20 μ M each), 2.0 μ l of FastStart DNA Master Hybridization Probes (Roche Diagnostics)). The PCR conditions consisted of an initial denaturation cycle of 95 °C for 10 min, followed by 50 amplification cycles with a temperature transition rate of 20 °C/s, with denaturation at 95 °C for 15 s, an annealing step at 57 °C for 30 s, and extension step at 72 °C for 30 s. Amplification was immediately followed by a melting program, which consisted of heating to 95 °C for 15 s, and cooling to 45 °C for 30 s with a temperature elevation rate of 20 °C/s, and an increase in the temperature to 75 °C at a rate of 0.2 °C/s with step acquisition of decreasing fluorescence. Successful amplification of a 231-bp human DNA target (p53) was used to assess the nucleic-acid quality of specimens. DNA extracted from the known samples was used in each assay run as a positive control. Melting temperature (T_m) value s.d. were determined by analysis of control samples on three separate runs. Analyses were performed on two different LightCycler instruments.

Results

Confirmation of Clarithromycin Susceptibility of Reference Strains

Each of the seven reference strains demonstrated MIC values $\geq 1 \mu\text{g/ml}$ when using the E-test, and were therefore phenotypically resistant to clarithromycin by current CLSI guidelines.¹³ The unmutated strain demonstrated an MIC value of $\leq 0.25 \mu\text{g/ml}$, and was determined to be phenotypically susceptible to clarithromycin.

Detection/Confirmation of Point Mutations Conferring Resistance to Clarithromycin in the Control Strains

Melt curve analysis of DNA from the cultured reference strains produced three melt curves, with T_m of ~ 61.2 , 51.8 , and 52.2 °C for the wild-type strain and mutant strains A2142G and A2143G, respectively (data not shown). As expected, real-time PCR with melt curve analysis accurately differentiated each of the *H. pylori* reference strains.

The significant difference in T_m allowed for unmutated strains to be easily distinguished from the mutant strains included in the study. However, the smaller differences observed in T_m between the various mutant strains compounded by run-to-run temperature variations precluded reliable determination of specific mutations using this approach.

Detection of Point Mutations Conferring Resistance to Clarithromycin in Formalin-Fixed, Paraffin-Embedded Specimens

PCR/melt curve analysis successfully differentiated mutated and unmutated *H. pylori* 23S gene targets, as shown in Figure 1. T_m values of samples prepared from formalin-fixed, paraffin-embedded reference strains and from formalin-fixed, paraffin-embedded gastric biopsies demonstrated a small shift toward higher temperatures compared with fresh samples, likely because of the use of differing DNA extraction methods. Non-mutated *H. pylori* samples showed a T_m value of ~ 62.3 °C ± 1.0 °C, whereas mutated *H. pylori* samples demonstrated T_m values of ~ 53.8 °C ± 1.0 °C. Mutant strains A2142G and A2143G were considered indistinguishable in formalin-fixed, paraffin-embedded specimens owing to the small differences in T_m s and the possibility of significant overlap in T_m ranges.

Limits of Detection

When using fresh isolates, the limit of detection was determined to be 10 genome equivalents of *H. pylori*. Using 10- μm -thick sections of the formalin-fixed, paraffin-embedded collagen plugs to simulate formalin-fixed, paraffin-embedded biopsy tissue, detection was good but somewhat less sensitive, with a limit of detection of <100 genome equivalents reproducibly detected (data not shown).

Assay Performance

Of the 153 total gastric biopsies studied, 7 failed to show adequate amplification of the human DNA control target and were excluded from the remainder of the study. Of the remaining 146 amplifiable samples, 122 demonstrated immunohistochemical positivity. In all, 103 out of 122 (84%) demonstrated amplifiable *H. pylori* 23S rRNA gene target and 19 out of 122 (16%) demonstrated no amplification of *H. pylori* using the PCR assay. This was likely because of formalin overfixation, which has been demonstrated to decrease the sensitivity of PCR.¹⁵ The failure of amplification in these cases may also be because of the lack of adequate numbers of organism in the sections used for PCR analysis. Alternatively, immunohistochemical methods are known to cross react with other *Helicobacter* species,^{16,17} and these findings may represent infection with *Helicobacter* organisms other than

Figure 1 Typical melt curve analysis, with wild-type (unmutated DNA sample) and A to G mutation controls. Formalin-fixed, paraffin-embedded wild-type (unmutated) and a mutated (A to G) samples are demonstrated. A mixed formalin-fixed, paraffin-embedded sample is also demonstrated.

Table 1 Summary of detection of *H. pylori* using PCR and immunohistochemistry in 146 formalin-fixed, paraffin-embedded biopsies that passed human DNA control amplification

Total samples (n = 146)	Immunohistochemistry		Mutation	
	Positive/ %	Negative/ %	Present/ %	Absent/ %
PCR positive	103/84%	2/8%	52/50%	53/51%
PCR negative	19/16%	22/92%		

The presence of mutations associated with clarithromycin resistance by melt curve analysis is also shown.

H. pylori. The primer and probe combinations used in this study have been previously shown to be specific for *H. pylori*,¹² which raises the possibility of false-positive immunohistochemistry results due to cross reactivity with an expected lack of detection by PCR.

Twenty-two samples were negative for *H. pylori* by immunohistochemistry and PCR; however two were negative for *H. pylori* by immunohistochemistry, but were positive by the PCR assay. This could have been due to either greater sensitivity of the PCR assay compared with immunohistochemical staining or due to differences in the number of organisms present in the sections used for PCR versus immunohistochemical staining.

Detection of Mutations

Of the total 105 samples demonstrating PCR positivity for *H. pylori*, 52 (50%) were positive for mutations associated with resistance (A2142G or A2143G) (Table 1). In addition, the PCR assay was able to demonstrate a mixture of mutated and unmutated wild-type organisms in 11 of the 52 samples.

Discussion

Several previous reports have described the identification of clarithromycin-resistant *H. pylori* by PCR from clinical specimens, using cultures grown from gastric biopsies^{12,18,19} and more recently from formalin-fixed, paraffin-embedded gastric biopsy tissue.²⁰

The use of a PCR assay to detect clarithromycin-resistant *H. pylori* in formalin-fixed, paraffin-embedded tissues offers several potential advantages over culture-based methods. As testing can be performed from paraffin-embedded tissue, the special conditions and time constraints for transport can be avoided. The use of formalin-fixed, paraffin-embedded tissues also allows for testing decisions to be made following microscopic interpretation by a pathologist. The most appropriate sections may then be taken for PCR testing, minimizing sampling error that can occur with culture-based methods. Prior microscopic examination of tissues may also reveal that changes characteristic of *H. pylori* infection are not present, avoiding the need for further testing altogether. The presence of *H. pylori* in gastric biopsies and clarithromycin resistance status may be assessed simultaneously, guiding correct treatment at the time of diagnosis. Lastly, PCR may be used retrospectively on previously procured specimens to assess whether clarithromycin resistance may have been a factor in treatment failure.

This study suggests that the sensitivity of PCR may be less than that of immunohistochemistry-based methods for the detection of *H. pylori* in formalin-fixed, paraffin-embedded gastric biopsy specimens, detecting 84% of cases identified by immunohistochemistry. Careful selection of paraffin sections submitted for PCR testing as well as optimal control of formalin fixation times should serve to further mitigate any differences in sensitivity. Immunohistochemistry offers a suitable method for

an initial presumed diagnosis of *H. pylori* infection, particularly in cases where sparse numbers of bacteria are present; however, a subsequent positive PCR assay can be used for confirmation of *H. pylori* infection, in addition to providing valuable information regarding clarithromycin susceptibility. Also of note, is the fact that the PCR assay was able to detect *H. pylori* DNA in 2 (of 24) cases demonstrating negative immunohistochemical staining. These findings suggest that utilization of a PCR assay may be helpful in some cases where suspicion for *H. pylori* infection remains despite negative immunohistochemical staining.

The most recent data obtained from the *Helicobacter pylori* Antimicrobial Resistance Monitoring Program (HARP) demonstrated an overall clarithromycin resistance rate of 12.9% for the population of the United States.²¹ In total, nearly half of the patients in our study (50%) were positive for mutations associated with clarithromycin resistance. Although this high percentage is striking in comparison with the HARP study, it is important to note that patients in our study population typically present for biopsy following presumed failure of initial therapy. It is known that the prevalence of antimicrobial resistant *H. pylori* varies significantly by region worldwide, including different regions of the United States, and has been shown to have a strong correlation to regional antimicrobial practices.^{22,23} Future studies including correlation with clinical history will be helpful in elucidating the percentage of patients presenting for biopsy, following failure of therapy that includes a clarithromycin component.

In this study, the melting curves were indistinguishable between the two mutations seen (data not shown). Previous studies have suggested that the presence of the A2143G mutation confers a significantly decreased *H. pylori* eradication potential when using clarithromycin-containing regimens, more so than the presence of the A2142C or A2142G mutations.²⁴ At this time, the clinical manifestations and current therapies for each are identical and there is no utility for differentiation between the strains in the clinical laboratory setting. However, differentiation of mutations may have importance in future epidemiological studies or as recommendations for antimicrobial therapies change.

The number of known positive *H. pylori* strains included in this study is limited, and because this was a retrospective study utilizing formalin-fixed tissues, correlation with bacterial culture was unable to be conducted using patient samples. Phenotypic findings of clarithromycin resistance using the E-test did demonstrate 100% concordance with PCR using the supplied *H. pylori* strains. Although we suspect that our results would be largely concordant, a prospective clinical validation would be helpful using bacterial cultures obtained from fresh endoscopy-obtained biopsy tissue, such

as that obtained for rapid urease testing. Comparison with simultaneously obtained formalin-fixed, paraffin-embedded biopsy tissue would be advantageous in discerning the true sensitivity and specificity of the assay in a clinical setting.

It should be noted that no A2142C mutations were seen within the study population. Although our reference strains did not include a sample with the A2142C mutation, the melt curve has been previously shown to be readily distinguishable from unmutated strains and from either of the A to G mutations.¹² This result is not surprising as A2142C mutations are significantly more rare and could likely have been absent from this particular study population.²⁵

In summary, we have described the use of a real-time PCR assay based on the LightCycler platform for the rapid detection of *H. pylori*, and assessment of clarithromycin susceptibility status directly from formalin-fixed, paraffin-embedded gastric biopsy specimens utilizing a probe and primer combination first reported by Oleastro *et al.*¹² in 2003. Failure to amplify the 23S gene was minimally problematic, and likely due to formalin overfixation or sampling error in detecting sparse numbers of organisms. Utilization of PCR primers amplifying a smaller DNA target, or the use of unfixed specimens, may yield a greater number of samples with interpretable results.

The implementation of this method into the clinical laboratory will allow for the identification of *H. pylori* and for assessment of clarithromycin resistance in previously fixed gastric biopsy specimens in <4-h analytic time. This method could substitute for the time-intensive culture and antimicrobial susceptibility testing process. It would also permit evaluation of formalin-fixed specimens in cases where cultures were not performed, either because they are not routinely performed by the microbiology laboratory or unsuspected *H. pylori*-associated gastritis is detected on histopathological examination. A rapid means of detecting clarithromycin-resistant *H. pylori* directly from patient specimens could potentially have a major impact on clinical management of *H. pylori*-associated gastritis, allowing for the timely assessment of clarithromycin resistance status leading to a decrease in treatment failure rates.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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