A Pilot Study of *Helicobacter pylori* Eradication Using a Polymerase Chain Reaction-based Test for Clarithromycin Resistance

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**Background/Aims:** Clarithromycin resistance is one of the main predictors of eradication treatment failures in *Helicobacter pylori* infections. The aim of this study was to investigate the ideal eradication rate of more than 90% of tailored therapies using a polymerase chain reaction (PCR)-based test for clarithromycin resistance in patients with peptic ulcer disease. In addition, we evaluated the possibility of sequential therapies for infections due to clarithromycin-resistant strains.

**Materials and Methods:** We prospectively enrolled patients referred to the gastroenterology unit for the evaluation and management of peptic ulcer from January 2012 to January 2014. Histology, a rapid urease test, and a dual-priming oligonucleotide-based multiplex (DPO)-PCR test were performed on gastric biopsy specimens. In the absence of 23S rRNA point mutations in *H. pylori*, the patients were treated with standard triple therapy, while in the presence of 23S rRNA point mutations, they were treated with sequential therapies.

**Results:** A total of 93 patients had peptic ulcer disease that was associated with *H. pylori* infections. These patients received eradication therapies, and 78 patients completed the therapies. The total eradication rate was 91% per protocol analysis, whereas it was 78.3% in patients treated with sequential therapies.

**Conclusions:** The eradication rate of *H. pylori* with tailored therapies using the DPO-PCR test was acceptable. However, sequential therapies were not effective in patients who did not respond to clarithromycin. *(Korean J Helicobacter Up Gastrointest Res 2017;17:200-207)*

**Key Words:** Clarithromycin; *Helicobacter pylori*; Polymerase chain reaction

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**INTRODUCTION**

*Helicobacter pylori* is a gram-negative bacterium associated with gastritis, peptic ulcer disease (PUD), gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer. Accurate diagnosis and success of *H. pylori* eradication are crucial in preventing related diseases. Current international guidelines recommend *H. pylori* eradication in patients infected with PUD.¹ Proper diagnosis of *H. pylori* is essential for treatment. Several invasive and non-invasive diagnostic tests including molecular methods are available for the detection of *H. pylori* in PUD patients. However, the efficacy of the most commonly used rapid urease test (RUT) can underestimate the prevalence of *H. pylori* and has low sensitivity.² In recent years, there has been an effort to improve the accuracy of the testing, and polymerase chain reaction (PCR) has been used and is producing good results.

Increasing antibiotic resistance rate, including clarithromycin resistance, that affects *H. pylori* eradication treatment is a challenge for clinicians. Antibiotic resistance is the main reason for *H. pylori* eradication failure; therefore, the standard triple regimen is no longer suitable as first-line treatment in most regions.³ Compared to a triple therapy regimen, higher eradication rates were recently reported using sequential treatment, but present data are still not conclusive.⁴ There is growing interest in personalized, care for patients. Increased eradication rates of tailored therapy based on antibiotic susceptibility have been reported using nucleic acid-based techniques or culture-based techniques for clarithromycin resistance with a focus on the first-line eradication therapy of *H. pylori* infection.⁵

An ideal antibiotic regimen for *Helicobacter pylori* should achieve eradication rates of approximately 90%. The purpose of our study was to investigate the efficacy...
of tailored therapy by diagnosing *H. pylori* using dual-priming oligonucleotide-based multiplex (DPO)-PCR test with the tissue sample from the RUT kit in PUD patients. In addition, we aimed to evaluate the possibility of sequential therapy for clarithromycin resistant strain infection.

**MATERIALS AND METHODS**

1. **Subjects**
   
The clinical trial was carried out between January 2012 and January 2014. We prospectively enrolled patients referred to the gastroenterology unit for the evaluation and management of PUD at St. Vincent’s Hospital, College of Medicine, The Catholic University of Korea. Patients were eligible for recruitment if they were older than 18 years of age and had gastric ulcer and/or duodenal ulcer. An ulcer is diagnosed with endoscopy as the loss of mucosal surface of 5 mm or greater in diameter. Patients with any one of the following criteria were excluded from the study: previous eradication of *H. pylori*, previous gastric surgery; antibiotic consumption in the two months preceding the study.

An estimated sample size gave an 80% power to detect a difference of 15% in the successful *H. pylori* eradication rate compared to the other regimens (assumed to be a successful eradication rate of 80% with previous regimens), with a two-sided alpha of 0.05. If a 10% drop-out rate was expected, at least 92 patients needed to be recruited.

\[
 n = \frac{(z_α \sqrt{p(1-p)} + z_β \sqrt{p_1(1-p_1)})^2}{δ^2}
\]

Written informed consent was obtained from all patients, and the study protocol was approved by the Institutional Review Board of the Catholic Medical Center (IRB no. VC11EISI0200).

2. **Sampling**
   
   During the upper endoscopy, four gastric biopsies were taken from the greater curvature of both the mid-antrum and mid-body of the stomach. Two biopsies were used for Warthin-Starry silver staining and two were used for RUT (CLO<sup>®</sup> test; Kimberly-Clark, UT, USA). On the day of endoscopy, 5 mL blood was taken from patients. A serological assay for immunoglobulin (Ig)G antibodies against *H. pylori* was performed with a commercial *H. pylori* IgG ELISA kit (IBL, Hamburg, Germany) according to the manufacturer’s instructions. The diagnosis of *H. pylori* infection was based on positive results of two out of three tests. After the first diagnostic test, proton pump inhibitor (PPI) therapy for 4 weeks was prescribed to all patients with PUD. If *H. pylori* status was negative, we obtained an additional biopsy specimen through endoscopy 6~8 weeks after initial examination with no PPI therapy for at least 2 weeks. The CLO<sup>®</sup> test was interpreted at 1 and 9 hours in the endoscopy room. If the CLO<sup>®</sup> test was positive, the specimens were immediately placed at −15°C to −20°C. If the CLO<sup>®</sup> test was negative, it was re-interpreted 24 hours later in ambient air. After 24 hours interpretation, all specimens obtained with the CLO<sup>®</sup> test kits were frozen regardless of the results.

3. **DNA extraction for PCR and gene mutation test: DPO-based multiplex PCR**
   
   DNA was extracted from the specimens in the CLO<sup>®</sup> test kit using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol and used for PCR. DNA was stored at −20°C until use. DPO-PCR (Seeplex<sup>®</sup> ClaR-*H. pylori* ACE Detection; Seegene Institute of Life Science, Seoul, Korea) was performed for identifying point mutation-containing gene fragments according to the manufacturer’s recommendations. After an initial incubation at 94°C for 15 minutes, 40 amplification cycles were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) using the following amplification parameters: 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. The final extension was performed at 72°C for 10 minutes. The amplified DNA products (5 μL of PCR products and 5 μL of ClaR-HP marker) were identified using an ultraviolet trans-illuminator following electrophoresis using a 2% agarose gel containing ethidium bromide. The presence of a single 621-bp DNA product was considered as belonging to wild-type *H. pylori*. The
4. Tailored therapy for *H. pylori* eradication

The patients who had documented *H. pylori* infection received therapy for the eradication. In the absence of 23S rRNA point mutations, the patients were treated with standard triple therapy (amoxicillin 1 g, clarithromycin 500 mg, and lansoprazole 30 mg twice daily for 14 days). In the presence of 23S rRNA point mutations, the patients were treated with sequential therapy (lansoprazole 30 mg and amoxicillin 1 g for the first 5 days, followed by lansoprazole 30 mg, clarithromycin 500 mg, and metronidazole 500 mg for another 5 days, with all drugs given twice daily). The standard 13C-urea breath test was performed 4~6 weeks after the completion of treatment to evaluate the efficacy of eradication.

5. Statistical analysis

The eradication and detection rates were compared using the chi-square test and Student t-test. The mean and standard deviation were calculated for quantitative variables. The percentage and 95% confidence interval (95% CI) were calculated for qualitative variables. PASW Statistics ver. 18.0 (IBM Co., Armonk, NY, USA) was used for all analyses. Significance was defined as *P* < 0.05.

**RESULTS**

1. Characteristics of the enrolled patients

A total of 164 patients with PUD were screened, and positive results using the DPO-PCR test were detected for 106 patients. Three patients had false positive results. A total of 103 patients were diagnosed with *H. pylori*-associated PUD, and 6 patients were diagnosed with *H. pylori*-associated gastric cancer by histologic examination and excluded. In addition, 4 patients were excluded because of previous operation history of duodenal ulcer perforation (1) and refusal of eradication therapy (3). Finally, 93 patients (69 males and 24 females) treated with *H. pylori* eradication

| Characteristic | Initial treatment (n=93) | Completion of the protocol (n=78) | *P* value |
|----------------|--------------------------|----------------------------------|----------|
| Age (yr)       | 56.83±14.99              | 56.92±15.70                      | 0.97     |
| Sex (male:female) | 69:24                   | 55:23                            | 0.59     |
| Smoke          | 51                       | 41                               | 0.77     |
| Alcohol        | 50                       | 39                               | 0.62     |
| Concomitant drug* | 30                      | 15                               | 0.05*    |
| Disease pattern| 66                       | 65                               | 0.98     |
| GU             | 56                       | 46                               |          |
| DU             | 26                       | 22                               |          |
| GU+DU          | 11                       | 10                               |          |

GU, gastric ulcer; DU, duodenal ulcer.

*Nonsteroidal anti-inflammatory drugs, anti-platelet and antithrombotic agents, *statistically significant.

**Table 2. Diagnosis of *Helicobacter pylori* in the Patients Enrolled in This Study**

| Diagnosis | *H. pylori* positivity | Eradication therapy |
|-----------|------------------------|---------------------|
| Silver stain+RUT (CLO* test) + positive serologic test (two or three) | 103 | 93 |
| DPO-PCR test | 106 | \ |
| False positive | 3 | 93 |
| 23S rRNA mutations (CLA-resistance) | 26 | 25 |
| *A2143G mutation* | 13 | 13 |
| *A2142G mutation* | 8 | 7 |
| Both | 5 | 5 |

RUT, rapid urease test; DPO-PCR, dual-priming oligonucleotide-based multiplex polymerase chain reaction; CLA, *Campylobacter*-like organism test.
therapy were enrolled. The mean age of the enrolled subjects was 56.83±14.99 years (54.96±13.79 males and 62.21±16.95 female, P=0.04). Patients with gastric ulcer (56), duodenal ulcer (26), or both (11) were enrolled, and 78 patients completed the *H. pylori* eradication therapy (Table 1).

### 2. Resistance to clarithromycin and eradication therapy

Twenty-six patients (25.2%, 26/103) had 23S rRNA point mutations associated with clarithromycin resistance. The mutation subtypes included mutation of A2143G (13), A2142G (8), and the presence of both (5) (Table 2). Successful eradication was achieved in 71 patients, and the eradication rate was 91.0% in per protocol (PP) and 73.2% in the intention-to-treat (ITT) analysis. In 25 patients with 23S rRNA point mutations, eradication therapy with the sequential regimen was performed. Twenty-three patients completed the therapy, and successful eradication was achieved in 18 patients. The eradication rate with the sequential therapy was 78.3% in PP analysis and 72.0% in the ITT analysis.

A total of 15 patients (13 patients without 23S rRNA point mutations and 2 patients with 23S rRNA point mutations) had poor compliance (<80% of prescribed medication). In most of the patients (10), a large amount of co-medication because of underlying chronic illness affected the completion for therapy. Bitter taste (1), diarrhea (1) and loss of follow-up (3) were reasons of poor compliance (Fig. 2).

**DISCUSSION**

The Kyoto global consensus report classified *H. pylori*-induced gastritis into the category of infectious disease. In most infectious diseases, empiric antibiotics are used initially, and antibiotics are re-administered based on subsequent antibiotic susceptibility testing results. However, the majority of *H. pylori* eradication depends on empirical antibiotic therapy because it is difficult to cultivate the bacteria, and the criteria for judging antibiotic resistance vary according to the test method used.
Currently, the most widely used empirical *H. pylori* eradication therapy is the clarithromycin therapy-based standard triple therapy, which adds two antibiotics (amoxicillin and clarithromycin) to the PPI. However, the existing standard triple therapy reported unsatisfactory eradication success rate of sterilization, and several alternatives therapies have been proposed and studied.

The worldwide success rate of *H. pylori* eradication has been reduced to less than 80% and in some countries it has been reduced to less than 70%. The main cause of eradication failure is the increased antibiotic resistance of *H. pylori*, especially to clarithromycin. The eradication rate of triple therapy is 81~95% in the clarithromycin susceptible strains. On the other hand, resistant strains have an eradication rate of 0~48%. Unfortunately, due to the excessive use of antibiotics, clarithromycin resistance has recently increased throughout the world. In Japan, the clarithromycin resistance rate is approximately 20% or higher, and prevalence of clarithromycin-resistant strains of *H. pylori* in Japan is higher than 30%. In Korea, the clarithromycin resistance rate is below 5%, although, this rate has been steadily increasing. Several years ago, the resistance rate for clarithromycin was 17.2% based on bacterial culture (MIC of >1.0 μg/mL). Furthermore, based on the 23S rRNA point mutations, the resistance rate for clarithromycin increased drastically up to 32%. There has been a gradual increase in resistance rate of clarithromycin in Korea from 22.9% in 2003~2005 to 25.5% in 2006~2008 and 37.0% in 2007~2009. In the present study, 23.9% (26/109) of patients had 23S rRNA point mutations associated with clarithromycin resistance.

In order to overcome the limitations of the existing standard triple therapy, antimicrobial therapy has been used in combination with various regimens such as bismuth-containing quadruple, sequential, and concomitant therapy. It is also important to ascertain the susceptibility to antibiotics. Recently, pretreatment susceptibility testing was performed by several studies to avoid antibiotic resistance. In tailored therapy, suitable medications are selected according to individual’s specific conditions to achieve optimal drug responses and to pursue satisfactory therapeutic outcome.

*H. pylori* has been commercialized using PCR to detect a partial resistance to clarithromycin in a relatively simple manner. PCR is the most sensitive and specific method for detecting *H. pylori* in gastric biopsy specimens. DPO has been developed to detect single–nucleotide polymorphisms using 1-step PCR assay. DPO-PCR is a method that blocks nonspecific binding sites, suppresses the incomplete primer loosening, and increases the sensitivity and specificity. DPO-PCR detects more *H. pylori* positive biopsies than culture alone. Woo et al. showed 94.1% concordance rate between DPO-PCR and culture. Cho and Lee compared DPO-PCR to culture and histology, and showed that combination of histology and PCR gave a high detection rate of *H. pylori* infection.

In terms of ease of diagnosis and economic efficiency, a recent study demonstrated that the DPO-PCR test using tissue samples processed by the CLO test was appropriate for detecting *H. pylori*. We investigated the diagnostic yield of the DPO-PCR test using the tissue sample from the RUT (CLO test) kit to diagnose *H. pylori* infection in patients with peptic ulcer bleeding. On the basis of the 13C-urea breath test, *H. pylori* detection by DPO-PCR had a sensitivity of 87.5%, a specificity of 91.3%, a positive predictive value of 84.0%, a negative predictive value of 93.3%, and an accuracy of 90.0%.

In addition, DPO-PCR has a revolutionary peculiarity in providing information about clarithromycin resistance, a main predictor of failure of eradication treatment and has excellent correlation with E-test susceptibility. DPO-PCR for the detection of *H. pylori* 23S rDNA mutations, involved in macrolide resistance was previously evaluated in several studies. Clarithromycin acts inhibiting protein synthesis by binding to 23S rRNA. When point mutations occur in 23S rRNA, clarithromycin cannot bind to rRNA, resulting in resistance. Three mutations at A2143G (69.8%), A2142G (11.7%), and A2142C (2.6%) are associated with clarithromycin resistant *H. pylori* strains. These point mutations can be confirmed by PCR, which is the most sensitive and specific method to detect *H. pylori*. DPO-PCR is rapid and accurate for *H. pylori* diagnosis and determination of clarithromycin susceptibility through confirmation of the presence of A2142G and A2143G mutations of the 23S rDNA.
In regions with a high prevalence of primary clarithromycin resistance, the DPO-PCR test may alleviate the social and economic costs of medical treatment.\textsuperscript{21,22} This test may detect clarithromycin resistance prior to eradication therapy and help in the selection of the appropriate regimen. Therefore, this test may become a key therapeutic tool in the future, especially in populations with high prevalence of \textit{H. pylori} and resistance to antibiotics including clarithromycin.

Recently, there has been a downward adjustment in the Maastricht V/Florence Consensus to perform culture and standard antibiotic susceptibility testing before prescribing first-line treatment in population areas where clarithromycin resistance rate is above 15%.

In countries with high resistance to clarithromycin, efforts should be made to conduct antibiotic susceptibility testing more effectively at the beginning of treatment, DPO-PCR should be considered as an alternative for appropriate testing.

The effective eradication rate of the first-line therapy should be 90\% or greater in PP analysis, and 80\% or greater in ITT analysis. Our results are close to the ideal eradication rate (91.0\% in PP and 73.2\% in ITT analysis), a finding that is in contrast with the eradication rate in clarithromycin resistant \textit{H. pylori} (78.3\% in PP analysis). Clarithromycin-resistant \textit{H. pylori} may be sensitive to the metronidazole-containing regimen, and the possibility of multidrug resistant strains should be considered.

To overcome clarithromycin resistance, a 10-day sequential treatment regimen, including PPI and amoxicillin for 5 days, followed by clarithromycin and imidazole derivatives with a PPI for further 5 days has been investigated.\textsuperscript{27-29} According to studies conducted in Italy, ITT analysis showed a high rate of eradication (98\%), and complications occurred at 6\%.\textsuperscript{30} Several studies have reported higher eradication rates of \textit{H. pylori} than standard triple therapy.\textsuperscript{31-33} However, some reports in areas such as France and Spain showed 85\% of unsatisfactory rates of eradication, while Thailand (96\%) and Taiwan (92\%) showed satisfactory rates.\textsuperscript{34,35} A study showed that sequential therapy was significantly more effective in children infected with clarithromycin resistant strains (81 vs 13\%).\textsuperscript{36}

Several meta-analyses compared the efficacy of sequential therapy, and the results were slightly different.\textsuperscript{37-39} A meta-analysis suggested that sequential therapy appears to be superior to standard triple therapy even in patients infected with clarithromycin resistant strains.\textsuperscript{39} Another meta-analysis showed that, although sequential treatment offered an advantage when compared to standard triple therapy, it cannot be considered a valid alternative.\textsuperscript{38} Our study also revealed that sequential therapy did not offer an advantage over standard triple therapy in the eradication of clarithromycin-resistant \textit{H. pylori}.

When this study was designed, the Maastricht IV/Florence recommended the sequential treatment as an alternative to bismuth-containing quadruple therapy as a first-line treatment in regions with high clarithromycin resistance (\(>15 \sim 20\%\)) and in the Asia. Because of lack of the research data in Asia, it was not generally applicable and adopted. As a thought to be the mechanism of treatment for sequential therapy, the combination of early amoxicillin therapy reduces the bacterial density and increases the effectiveness of eradication therapy, amoxicillin weakens bacterial cell walls, and the development of clarithromycin efflux channels and inhibited the expression of clarithromycin resistance. However, the results of recent studies show a possibility of other mechanism of action. The effect of sequential therapy is known to be caused by the addition of metronidazole rather than overcoming clarithromycin resistance. In Taiwanese studies, the effect of sequential therapy was better than standard triple therapy, only when metronidazole resistance was less than 40\%.\textsuperscript{40} Revised Maastricht V/Florence does not recommend sequential therapy. The sequential treatment has lower eradication rate than concomitant therapy in the case of clarithromycin resistance and metronidazole susceptibility, and in the clarithromycin susceptible and metronidazole-resistant group, sequential treatment has lower eradication rate than clarithromycin-based triple therapy.\textsuperscript{41} Our study is a pre-revision consensus study, and the results of unsatisfactory sequential treatment are likely to support recent trends. However, further studies are needed to confirm the efficacy of sequential therapy.

Several methodological weaknesses may limit the validity of our findings. Another major limitation of this
study is the single center collection of data, raising concern about the generalizability of the results. And this study is a single arm study. Therefore, there are some limitations to compare eradication rate with other regimens. In our study, the ITT and PP analysis of eradication rates were 91% and 73.2%, respectively. The difference between the two analyses was relatively large. These results are probably due to that the number of patients who lost follow-up during the study was as high as 16%. When comparing the protocol to the full analysis group, it was statistically significant difference in co-medication administration. It is considered that the compliance is lowered because it is difficult to take eradication therapy for a certain period in patients who take co-medication. In addition, the CYP2C19 genotypes were not considered as a potential influence on the outcome of eradication therapy. The principal enzyme involved in the metabolism of the PPI is CYP2C19, and there are inter-individual differences in the activity of this enzyme. Large-scale prospective studies are needed to evaluate what regimen is the most effective in eradicating clarithromycin-resistant Helicobacter pylori.

We believe that DPO-PCR may be a useful diagnostic tool in clinical practice, especially in tailored therapy of Helicobacter pylori eradication and provide great benefits for the Helicobacter pylori eradication rate. DPO-PCR is useful in optimal treatment as well as in the diagnosis of Helicobacter pylori, and its role will expand in the future.

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