Rapid Automated Genotyping of CYP2C19 and *Helicobacter pylori* 23S rRNA Gene in Gastric Juice

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**ABSTRACT**

AIM: Genotypes of *Helicobacter pylori* (*H. pylori*) 23S rRNA gene and human CYP2C19 are predictive determinants of efficacy of triple therapy with proton pump inhibitor, clarithromycin, and amoxicillin for eradicating *H. pylori*. We intended to develop a rapid assay system to determine these genotypes.

METHODS: We designed primer sets and specific probes for determining SNPs from adenine to guanine at positions 2142 or 2143 of *H. pylori* 23S rRNA gene and *2 and *3 alleles of CYP2C19*, using the novel full-automated rapid genetic analyzer (GENECUBE®). The results were compared with those acquired by direct nucleotide sequencing of 50 gastric tissue samples. We also determined the presence of SNPs in 132 additional samples of patients’ gastric juices.

RESULTS: The GENECUBE® genotyping results of the 23S rRNA gene and CYP2C19 from gastric tissue samples were in complete agreement with those for direct sequencing. The sensitivity, specificity, and validity of GENECUBE® results were all 100% when compared with clinical data. GENECUBE® genotyping required approximately 30 min for a single test.

CONCLUSIONS: The GENECUBE®-based system can simultaneously measure genotypes of *H. pylori* 23S rRNA gene and human CYP2C19 from gastric tissues and juices rapidly, which can greatly aid the tailored *H. pylori* eradication therapies.

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Key words: *Helicobacter pylori* (*H. pylori*); 23S rRNA; CYP2C19; PCR; SNP


INTRODUCTION

Eradication of *H. pylori* infection is employed as a method of treating disorders of the upper gastrointestinal tract, such as peptic ulcer, gastric mucosa associated lymphoid tissue (MALT) lymphoma, and idiopathic thrombocytopenic purpura, as well as to prevent gastric cancer[1,2]. Eradication therapy includes administration of a proton pump inhibitor (PPI) and two antibiotics. Triple therapy with a PPI (either omeprazole, lansoprazole, rabeprazole, or esomeprazole), clarithromycin, and amoxicillin is now the first-line regimen among modalities currently in use in Japan[3]. The initial eradication rate of this therapy was approximately 90%. This feat attributed to the pathogen’s susceptibility to clarithromycin[4,5]. Thus, as the frequency of clarithromycin-resistant strains of *H. pylori* has increased[6,7], the efficacy of triple therapy regimens has decreased[8], falling to rates as low as 70%[9].

Culture tests are typically employed to test the susceptibility of *H. pylori* strains to antimicrobial agents, such as clarithromycin; however, they require 10-14 days and are of little value in daily clinical practice. Susceptibility of most *H. pylori* strains is caused
Analyses of 23S rRNA gene mutations associated with H. pylori clari

tomycin (CAM). B: When there is a mutation in the H. pylori 23S rRNA gene at nucleotide position 2142 or 2143, the peak is shifted to the left at around 46°C. The strain shown was diagnosed to be resistant to clarithromycin; C: When the sample is negative for H. pylori, there is no signal.

**Table 1** Analyses of 23S rRNA gene mutations associated with susceptibility of H. pylori to clarithromycin.

<table>
<thead>
<tr>
<th>23S rRNA</th>
<th>Direct Sequence</th>
<th>GENECUBE®</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2142G</td>
<td>A A 16</td>
<td>16</td>
</tr>
<tr>
<td>A2143G</td>
<td>A G 33</td>
<td>34</td>
</tr>
</tbody>
</table>

**Table 2** Comparison of results of CYP2C19 genotyping.

<table>
<thead>
<tr>
<th>CYP2C19*2</th>
<th>(681 G→A)</th>
<th>CYP2C19*3</th>
<th>(636 G→A)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>G/A</td>
<td>G/G</td>
<td>G/A</td>
<td>30 s</td>
</tr>
<tr>
<td>25</td>
<td>22</td>
<td>38</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>22</td>
<td>38</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3** Summary of Results of CYP2C19 Genotyping.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.28</td>
</tr>
<tr>
<td>G</td>
<td>0.72</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Study 1 Development of a system that rapidly determines the genotypes of CYP2C19 and H. pylori 23S rRNA gene using the GENECUBE® automated DNA analyzer**

This study enrolled 50 patients with the H. pylori 23S rRNA gene using the GENECUBE® automated DNA analyzer. The subjects were 36 men and 14 women whose median age (range) was 53 (31-78) years. All had endoscopically and histologically proven ulcers or active chronic gastritis and were H. pylori-positive based on the rapid urease test (RUT) (see below). Written informed consent for participation was obtained from all patients before the study, and the human institutional review board of hamamatsu university school of medicine approved the protocol.

Biopsies for the RUT obtained from both the antrum and corpus of the greater curvature by gastroduodenoscopy were analyzed using a commercial kit (HelicocCheck; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). A color change from yellow to pink within 24 hours was judged as a positive result.

The GENECUBE® used in the present study was a novel, fully automated rapid genetic analyzer capable of extracting nucleic acids from biological material, preparing reaction mixtures, and amplifying the target gene all within 30 min. Analysis with this instrument is based on the hybridization of an allele-specific fluorescence-conjugated probe and PCR amplification within 20 min followed by melting point analysis. A total of 16 SNPs analyses can be handled simultaneously. In this study, 4 SNPs, including *2 and *3 mutations of CYP2C19 and A2142G and A2143G mutations of 23S rRNA of H. pylori were analyzed per one sample. Therefore, a total of 4 samples can be handled simultaneously.

We designed primers to amplify the 23S rRNA gene based on the H. pylori sequence deposited in the GenBank: U27270 as follows: forward primer: 5’- GTGGAAGGTGAAAATTCCTCCTACCC-3’, reverse primer 5’- GGCTCCCTCATAAGGCCAAGCCCTTAC-3’), probe for detecting the product (5’- CAAGACGGAAGACC3’ -BODIPYFL). The length of the expected polymerase chain reaction (PCR) product was 141 bp.

PCR was carried out using approximately 0.1 μg of DNA, and TEST BASIC KIT contains KOD mix, PPDmix (TOYOBO, Ltd., Osaka, Japan), primers and probe. PCR conditions were as follows: denaturation at 95°C for 30 s, 50 cycles of denaturation at 97°C for 1 s, annealing at 58°C for 3 s, and extension at 63°C for 5 s. The PCR products were automatically subjected to melting point analysis, which consisted of denaturing at 98°C for 30 s, annealing at 40°C for 30 s, and then heating to 75°C in increments of 0.09°C/s. The display

Figure 1 Analyses of 23S rRNA mutations by GENECUBE®. A: When there is no mutation at nucleotide position 2142 or 2143, the peak is observed around 55°C. The strain shown is to be sensitive to clarithromycin (CAM); B: When there is a mutation in the H. pylori 23S rRNA gene at nucleotide position 2142 or 2143, the peak is shifted to the left at around 46°C. The strain shown was diagnosed to be resistant to clarithromycin; C: When the sample is negative for H. pylori, there is no signal.
of the data on the GENECUBE® monitor is shown in Figure 1. For the measurement of CYP2C19 genotypes, we designed the oligonucleotide primers (forward primer: 5'-ACCAGAGCTTGCCATATTGTATCTATAACC-3' and reverse primer 5' -CCAAATATCATCTTCCATAAAAGCAAG-3') and probe (5'-TTGATTATTCCCCAGAACC-3'-BODIPYFL) for CYP2C19*2 and the oligonucleotide primers (forward primer: 5'-GATGGAAAATTGGAATGAAAATCAGGATTGTA-3' and reverse primer 5'-AAAATGTACCTTGGGCTTGGTATAA-3') and probe (5' -TAAGCACCCCCCTGAAACC-3'-BODIPYFL) for CYP2C19*3 based on CYP2C19 sequence deposited in the GenBank (NG_008384.1). To enable simultaneous measurements of both genotypes, the PCR conditions were the same as described above for detecting 23S rRNA gene mutations. To confirm the GENECUBE® SNP analysis results, we determined the genotypes of CYP2C19 and 23S rRNA gene using a published direct sequencing method[18,19], with minor modifications.

Study 2: Analysis of gastric juice sample
Gastric juice samples were collected by aspiration during gastroduodenoscopy in 132 patients not included in Study 1. These patients suffered from GU (n=31), DU (n=20), or gastritis (n=81). This group included 52 men and 80 women, whose median age (range) was 52 (21-80) years. H. pylori infections in 20 of these patients had been eradicated before the present study. Immediately after the endoscope reached the stomach, 1-10 mL of gastric juice was aspirated through the suction channel and collected in a trap placed in the suction line. When gastric juice was difficult to aspirate due to achlorhydria, 20 mL of water was sprinkled vigorously on the gastric mucosa using a syringe attached to the suction channel and collected by aspiration. A 20-µL aliquot of gastric juice was then added to 180 µL of sample solution buffer (Toyobo) (the final volume was 200 µL) and heated at 95°C for 5 min to extract the DNA. 2-µL aliquot of 200 µL was subjected to GENECUBE® analysis to test whether GENECUBE® system could detect the presence or absence of H. pylori infection using juice samples. The presence of H. pylori infection was also assessed by RUT and 13C-urea breath test, and tests for serum anti-H. pylori IgG antibody. When any one of these tests (RUT, 13C-urea breath test and serum anti-H. pylori IgG antibody test) was positive, the patient was diagnosed with an H. pylori infection. The sensitivity and specificity of the GENECUBE® analysis was evaluated based on the three conventional test.

RESULTS
Validation of GENECUBE® genotyping
Figure 1 shows that GENECUBE® analysis unambiguously demonstrated wild type or mutant sequences in the 23S rRNA gene at nucleotide positions 2142 or 2143. Of note is the fact that the analysis took only 30 min. In contrast, direct sequence analysis of these same 50 samples took approximately 8 hours.

Table 1 presents the results of the analysis of 23S rRNA gene mutations associated with susceptibility to clarithromycin by GENECUBE® and direct sequence analyses. Both methods detected the same mutant sequences in the same 34 samples. Further, sequencing of CYP2C19 by both techniques yielded identical results and were able to detect the CYP2C19*2 and CYP2C19*3 mutations (Table 2).

Analysis of gastric juice samples
In Study 2, 26 of the 132 samples were H. pylori-negative based on analysis of serum anti-H. pylori-IgG, urease and the 13C-urea breath test, and the remaining 106 were H. pylori-positive based on RUT, serum IgG, and 13C-urea breath test findings. The results of GENECUBE® analysis of 23S rRNA gene mutations in subjects based on H. pylori infection status are displayed in table 3. The GENECUBE® could detect H. pylori infection in all patients infected with H. pylori based on analysis of serum anti-H. pylori-IgG, urease and the 13C-urea breath test. Moreover, there were no false positive results. Thus, the sensitivity, specificity and validity of the GENECUBE® assay were all 100% in the present study results. Forty-two of the 132 H. pylori-positive patients harbored clarithromycin-resistant strains of H. pylori based on 23S rRNA gene sequences. CYP2C19 genotyping was successfully performed for each sample (Table 4), and we found that the CYP2C19 allele frequency was almost the same as those reported for samples analyzed in Japan[21].
We report here the development of a rapid (30 min per sample) detection system for determining CYP2C19 genotypes and mutations in the *H. pylori* 23S rRNA gene associated with resistance to clarithromycin. We were able to successfully analyze gastric tissue and fluid samples and validated the assay system by comparing its results to those acquired by direct nucleotide sequencing. The sensitivity of detection of *H. pylori* was equal to that of standard immunological, biochemical, and biological tests. In addition, the time required to complete the assay makes it suitable for use in daily clinical practice.

The samples usually used for the determination of *H. pylori* infection are taken from gastric tissue collected by mucosal biopsy during endoscopy. These samples can be used to detect *H. pylori* by culture tests, RUT, histology, and PCR. Genotyping of human DNA can also be performed using these samples. Unfortunately, not all patients can tolerate this procedure, which is contraindicated in patients with bleeding tendency and those being treated with either or both anti-coagulants or anti-platelet agents, such as warfarin or clopidogrel. In contrast, collection of gastric juice samples during endoscopy can be easily and safely performed in all patients.

In the present study, we analyzed gastric juice samples acquired during endoscopy to determine the genotypes of CYP2C19 and the *H. pylori* 23S rRNA gene, the latter of which is associated with susceptibility to clarithromycin. *H. pylori* and human DNA can be detected by PCR using gastric juice samples, as gastric juice often contains gastric epithelial cells. Therefore, once DNA is extracted from the gastric juice, we can test for human and *H. pylori* DNA. This method of sample collection enabled us to perform simultaneous analyses of CYP2C19 mutations and 23S rRNA mutation of *H. pylori*.

We previously reported that bacterial susceptibility to clarithromycin and the patient’s CYP2C19 genotype were each associated with the success or failure to eradicate *H. pylori* infection and that tailored regimens based on CYP2C19 and 23S rRNA gene mutations yielded higher eradication rates than with the empirical standard therapy. However, standard genotyping methods, such as PCR-RFLP and direct nucleotide sequencing, require at least 5-10 hours, starting with DNA extraction sequence analysis, a length which has rendered such methods virtually useless in daily clinical practice. In the present study, we used the GENECUBE® system, which enabled us to complete simultaneous analysis of CYP2C19 and 23S rRNA gene mutations within 30 min from start to finish. In our hospital, the genotyping of CYP2C19 and 23S rRNA is now completed while patients take a rest for a while after endoscopy. Therefore, the individualized eradication therapy can be given to patients on the same day of the endoscopy.

Our goal here was to develop a GENECUBE®-based system for the simultaneous analyses of SNPs in CYP2C19 and in the *H. pylori* 23S rRNA gene and to compare the results with those acquired by direct DNA sequencing to determine the validity of the new system. This was accomplished successfully, and we were able to show that the sensitivity, specificity and validity of the system correlated perfectly with non-genetic tests.

Determining a patient’s CYP2C19 genotype and clarithromycin sensitivity is useful for devising optimal eradication therapy. In our hospital, we have used this system for advanced treatment in patients infected with *H. pylori*. We confess that performing a genotyping test in advance may seem costly (i.e., around 150 US dollars), but that this cost can be offset by achieving higher eradication rates after the first round of individualized therapy. Given that one of the limitations of the present study is its relatively small sample size, large-scale prospective studies must be performed to assess the full clinical potential of the GENECUBE® system and its economic feasibility.

**CONCLUSION**

The GENECUBE®-based system can simultaneously measure genotypes of *H. pylori* 23S rRNA gene and human CYP2C19 from gastric tissues and juices rapidly, which can greatly aid the tailored *H. pylori* eradication therapies.

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**REFERENCES**


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