RAPID DETECTION OF POINT MUTATIONS CONFERRING RESISTANCE TO FLUOROQUINOLONE IN THE GYRA OF HELICOBACTER PYLORI BY ALLELE-SPECIFIC POLYMERASE CHAIN REACTION

Toshihiro Nishizawa¹, Hidekazu Suzuki¹, Ayako Umezawa², Hiroe Muraoka³, Eisuke Iwasaki¹, Tatsuhiro Masaoka⁴, Intetsu Kobayashi³, Toshifumi Hibi¹

Department of Internal Medicine¹ & Center for Integrated Medical Research², Keio University School of Medicine, Tokyo; Mitsubishi Kagaku Bio-Clinical Laboratories³, Department of Gastroenterology, Eiju Hospital⁴, Japan

Short Title: Allele-specific PCR for *H. pylori* gyrA mutations

Key Word: *Helicobacter pylori*, allele-specific PCR, *gyrA* mutation

Address for Correspondence:
Hidekazu Suzuki, M.D., Ph.D.
Department of Internal Medicine,
Keio University School of Medicine
35 Shinanomachi, Shinjuku-ku, Tokyo
160-8582, JAPAN
Phone: 81-3-5363-3914
Fax: 81-3-5363-3967
E-mail: hsuzuki@sc.itc.keio.ac.jp
ABSTRACT

Background/Aims: Helicobacter pylori (H. pylori) with reduced susceptibility to fluoroquinolones has a mutation at either codon 87 Asn or 91 Asp of the gyrA gene. A rapid test based on an allele-specific polymerase chain reaction (AS-PCR) was designed to detect the gyrA mutations. Material and Methods: Clinical H. pylori isolates were obtained from the stomach of 51 patients with H. pylori infection showing treatment failure. The minimal inhibitory concentrations (MICs) of gatifloxacin (GAT) were determined by the agar dilution method. Identical genotyping results were obtained with AS-PCR as with conventional PCR. Results: The gyrA mutations of H. pylori showing reduced susceptibility to fluoroquinolones could be successfully detected by this method. Significant association was observed between the presence of mutations as detected by AS-PCR and the resistance of the strains to GAT. Moreover, the genotyping by AS-PCR took less than 3-4 h. Conclusion: The AS-PCR method for the detection of gyrA mutations in H. pylori is useful for easy identification of fluoroquinolone-resistant strains of H. pylori.
INTRODUCTION

Failure of proton pump inhibitor-based triple therapies for the eradication of *Helicobacter pylori* (*H. pylori*) infection has been shown to be frequently due to resistance to either clarithromycin or metronidazole. Furthermore, concomitant resistance to both drugs is not rare either, particularly in cases showing failure of first-line therapy (4). Alternative regimens need to be developed for such cases showing concomitant resistance to both drugs.

Recently, Sharara et al. reported a 7-day regimen of gatifloxacin (8-methoxy fluoroquinolone, GAT), amoxicillin and rabeprazole as an effective and safe secondary eradication treatment regimen for *H. pylori*, with an eradication rate of 84.4 % (20). Therefore, GAT-based triple therapy might be a promising alternative treatment option for *H. pylori* infection. However, we recently reported a high resistance rate (47.9%) to GAT of *Helicobacter pylori* (*H. pylori*) strains isolated from Japanese patients after unsuccessful eradication therapy (17).

The resistance of *H. pylori* to fluoroquinolones, which exert their antimicrobial action by affecting the A-subunit of the DNA gyrase of *H. pylori*, has been reported to be caused by point mutations in the so-called quinolone resistance-determining region (QRDR) of the *gyrA* gene of *H. pylori* (9, 12, 17, 19, 22, 23), mainly at amino acid 87 (Asn to Lys) or 91 (Asp to Gly, Asp to Asn, Asp to Tyr) (17, 22). *H. pylori* does not possess a gene encoding topoisomerase IV, an important fluoroquinolone target in other bacteria. Therefore, bacterial resistance to fluoroquinolone can be tested by a genetic test of *gyrA*. Development of an inexpensive and reliable high-throughput method for the detection of *gyrA* mutations is expected to be highly useful for *H. pylori* eradication therapy with regimens containing fluoroquinolones.

Allele-specific polymerase chain reaction (AS-PCR) is one of the techniques used to determine point mutations with a high degree of specificity and sensitivity in genomic samples. Point mutations can be identified easily within a short period of time by PCR amplification alone, without direct sequencing or digestion with restriction enzymes. In
the AS-PCR analysis, PCR amplification is performed using a specific primer in which the second nucleotide from the 3’ end is designed to match the site of the point mutation and the third nucleotide is designed to produce a mismatch in order to yield allele-specific PCR amplification (8). The point mutations can be identified by determining whether or not the PCR amplicons corresponding to the specific primers can be observed.

The aim of this study was to develop a rapid genotyping method based on AS-PCR for detecting \textit{gyrA} mutations in \textit{H. pylori}.

\textbf{Materials and Methods}

\textbf{Patients and isolation of} \textit{H. pylori}

A total of 51 patients (33 males and 18 females; age, 56.7±12.7 years, mean±SD) with \textit{H. pylori} infection showing treatment failure were enrolled in this study. Of the total, 43 patients had one treatment failure, 6 patients had two treatment failures, and 2 patients had three treatment failures. (first-line treatment used: triple therapy with CLR, amoxicillin (AMX) and proton pump inhibitor (PPI) for 7 days; second-line treatment used: triple therapy with MNZ, AMX and PPI for 7 days; third-line treatment used: triple therapy with LVX, AMX and PPI) Informed consent was obtained from all the patients prior to their participation in the study. All the patients underwent upper gastrointestinal endoscopy; biopsy specimens obtained from the greater curvature of the upper corpus were then used to isolate the \textit{H. pylori}. The clinical isolates of \textit{H. pylori} used in the present study were previously examined and reported only in terms of \textit{gyrA} mutation and the minimal inhibitory concentrations (MICs) for GAT (17). In addition to previous 48 isolates (17), isolates with known GAT MICs were examined for \textit{gyrA} mutation (KS0203, KS0205, KS0193, KS0195). However, KS0166 (GAT MIC: 0.5 µg/ml) was excluded in the present study, because the sequencing of \textit{gyrA} gene revealed that it was mixed strain of main wild type and trivial mutant (A272G).

\textbf{Microaerobic bacterial culture and determination of the MICs}
Primary culture was performed using the Columbia HP agar (Becton Dickinson, Cockeysville, MD, USA) under a 10% CO$_2$ and 5% O$_2$ atmosphere at 35°C for 4-7 days. The colonies were harvested and subcultured on Sheep Blood Agar (Becton Dickinson) under a 10% CO$_2$ and 5% O$_2$ atmosphere at 35°C for 3 days.

The susceptibility of the *H. pylori* isolates to GAT (Kyorin Pharmaceutical Co., Ltd, Tokyo, Japan) was determined by the agar dilution method according to the guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS) (13). A saline suspension equivalent to a 2.0 McFarland standard (containing $1 \times 10^7$ to $1 \times 10^8$ CFU/ml) was prepared from a 72-hour subculture from a blood agar plate. The inoculum (1 to 3 µl per spot) was plated directly on the antimicrobial agent-containing agar dilution plates. All the plates were incubated in a 10% CO$_2$ and 5% O$_2$ atmosphere at 35°C for 3 days. The MIC value was defined as the lowest concentration of antibiotic that completely inhibited the growth of the inoculum. Isolates were considered resistant to GAT if the MIC of the drug was $\geq 1$ µg/ml (2, 11, 14, 17).

**DNA preparation and PCR assay**

Total DNA was extracted from *H. pylori* isolates using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). Primers complementary to regions flanking the 428-bp coding sequence of the quinolone resistance-determining region of *gyrA* (from codon 38 to 154) were used. The PCR mixture (50 µl final volume) contained HotStar Taq Master Mix (QIAGEN) and 0.5 µM each of primer *gyrA.f* (5’-TTRGCTTATTCMATGAGCGT, forward) and *gyrA.r* (5’-GCAGACGGCTTGGTARAATA, reverse) for the *gyrA* gene. PCR was performed in a gene Amp PCR System 9700 (Applied Biosystems, Foster City, USA) under the following amplification conditions: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 30 s), annealing at 55°C for 30 s, and extension at 72°C for 1min, and a final extension at 72°C for 10 min. The products of amplification were purified using the
Sequencing

Amplicons of gyrA were sequenced using gyrA.f /gyrA.r and the ABI Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Norwalk, CT, USA). The sequenced PCR products were analyzed in an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). The sequences obtained were determined using a Sequencher (Lasergene, DNASTar, Madison, WI, USA), and compared with the published sequences of the H. pylori gyrA gene (GenBank accession no. L29481) (2).

Allele-specific PCR with mismatch primers

Analysis of alleles with point mutations – gyrA (C261A, C261G, G271A, G271T, A272G) – was performed with standard thin-walled PCR tubes using the KOD-Plus (Toyobo, Osaka, Japan) on the gene Amp PCR System 9700. Details concerning the designing of the allele-specific primers used in this study are given in Table 1. The following mixtures were prepared for the AS-PCR : 7.9 μl of distilled water, 2.5 μl of 10X reaction buffer, 2.5μl of 2mM dNTPs, 0.5 μl of KOD-Plus DNA polymerase, 1 μl of 25mM MgSO₄, 9.6 μl of primer mixture (10 pmoles of gyrA R primer, 5 pmoles of F261A1 primer, 2.5 pmoles of F261G1 primer, 5 pmoles of F271A5 primer, 16 pmoles of F271A9 primer, 5.5 pmoles of F271T9 primer, 1.25 pmoles of F272G1 primer and 1 pmoles of F272G9 primer) and 1 μl of genomic DNA. The amplification was conducted under the following conditions: an initial denaturing step at 95°C for 5 min, followed by 35 cycles at 95°C for 15 s, 62.5°C for 30 s, and 68°C for 30 s, and a final elongation step at 68°C for 2 min. The PCR products were analyzed by electrophoresis on 2% agarose gels, followed by ethidium bromide staining and inspection under ultraviolet light.
Statistical Analysis

Fisher’s exact test was used to test the association between the presence of mutations in the genotype as determined by AS-PCR and resistance of the strains to GAT. A P value of <0.05 was considered to denote statistical significance.

RESULTS

Point mutations in the gyrA gene at codon 87 Asn or 91 Asp were detected in 24 of the 25 (96 %) GAT-resistant strains isolated in this study. On the other hand, only one of the 26 (3.8 %) susceptible strains had the mutation, with a substitution at amino acid 87. At base triplet 87, we found two wild-type variants, an AAC triplet and an AAT triplet (C261T mutation, both coding for Asn).

Of all the strains, 26 were the wild-type of H. pylori strains (AAC triplet; 17 isolates, AAT triplet (C261T); 9 isolates), 8 strains had the C261A mutation (Asn 87 Lys), 3 strains had the C261G mutation (Asn 87 Lys), 9 strains had the C271A mutation (Asp 91 Asn), 2 strains had the C271T mutation (Asp 91 Tyr), and 3 strains had the A272G mutation (Asp 91 Gly). We did not see any significant association between the type of the gyrA mutation and the MICs of GAT.

In the 51 samples, the AS-PCR clearly differentiated the wild-type from the mutant of gyrA of H. pylori (Fig. 1). Significant association was observed between the presence of mutations in the genotype as determined by AS-PCR and the resistance of the strains to GAT (p<0.001). Moreover, the AS-PCR for the genotyping of the strains took less than 3-4 h.

DISCUSSION

Various assay techniques have been developed to investigate polymorphisms,
including PCR restriction fragment length polymorphism (RFLP), AS-PCR, multiplex PCR, single-strand confirmation polymorphism, oligonucleotide ligation assay, and real-time PCR (3, 5, 6, 10, 15, 16, 18). AS-PCR is an excellent genotyping method, although it frequently requires extensive optimization and background amplification is often high. It has been reported that the configuration of mismatched base pairs at the template primer 3’ terminal and the penultimate nucleotide are involved in the extension efficiency of PCR (7). In this study, PCR amplification was performed by allele-specific primers in which the second nucleotide from the 3’ end was designed to match the site of the point mutation; furthermore, the third, fourth or fifth nucleotide from the 3’ end was designed to produce a 1-base pair mismatch in order to attain high specificity in the AS-PCR of mutant alleles with point mutations. AS-PCR does not require restriction enzyme cleavage, purification of PCR products, or a real-time PCR machine.

In the present study, we applied the AS-PCR method to detect mutations in the gyrA gene of H. pylori. The method was useful for easily identifying whether an H. pylori strain was sensitive or resistant to GAT. To the best of our knowledge, this is the first report of successful simultaneous detection of five kinds of point mutations by AS-PCR requiring only two PCR tubes.

Because the traditional culture test for bacterial susceptibility to antibiotics is costly and requires 10-14 days, this test has not been feasible in routine clinical practice and MIC-based individualized H. pylori eradication therapy has not been prevalent among general practitioners. However, the AS-PCR method can be used to determine the bacterial susceptibility to fluoroquinolones within only several hours, which we suggest would make individualized treatment really feasible in daily clinical practice. This tailored- to- each-individual therapeutic strategy may be expected to increase the eradication rates of H. pylori achieved by fluoroquinolone-based triple therapy as an alternative treatment regimen.

In conclusion, we developed a reliable AS-PCR technique to detect GAT-resistant H. pylori strains with the aim of identifying potential responders to a
fluoroquinolone-based eradication regimen. The method was developed on DNA extracts from *H. pylori* isolates, however, it may be performed directly on gastric specimens(1). Because of the genetic heterogeneity of *H. pylori* (21) and other new mutants, the assay may fail for strains isolated outside Japan, however, it could be adapted to genetic gyrA variants found in different geographical regions.

**ACKNOWLEDGEMENTS**

Supported by a Grant-in Aid for Scientific Research C from JSPS (No. 15590686, to H.S.), and a grant from Keio University and Keio Health Counseling Center.
FIGURE LEGENDS

Figure 1

Representative electrophoresis patterns of gyrA QRDR polymerase chain reaction (PCR) products and allele specific PCR products with mixed primers for determination of C261A, C261G, G271A, G271T and A272G genotypes of gyrA of H. pylori. The theoretical fragment sizes according to the gyrA sequence are mentioned in the text.
Table 1. Oligonucleotide sequences of primers used in the allele specific primer polymerase chain reaction (AS-PCR) method to determine gyrA mutation of *H. pylori* at the position of 261, 271 and 272. The penultimate nucleotide to distinguish between sequences of wild type and mutant type are lowercase. The 1-base pair mismatches at another nucleotide are underlined.

<table>
<thead>
<tr>
<th>Position Name</th>
<th>Primer sequence PCR product size (bp)</th>
<th>Primer sequence</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C 261 A</td>
<td>F261A1</td>
<td>CCCCATGGCGAGAAaG</td>
<td>262</td>
</tr>
<tr>
<td>C 261 G</td>
<td>F261G1</td>
<td>CCCCATGGCGAGAaG</td>
<td>262</td>
</tr>
<tr>
<td>G 271 A</td>
<td>F271A5</td>
<td>GCGATAACGCGGTTTAGaA</td>
<td>254</td>
</tr>
<tr>
<td></td>
<td>F271A9</td>
<td>GCGATAATGC GGTTTAGaA</td>
<td>254</td>
</tr>
<tr>
<td>G 271 T</td>
<td>F271T9</td>
<td>GGCGATAATGCGGTTATATa</td>
<td>255</td>
</tr>
<tr>
<td>A 272 G</td>
<td>F272G1</td>
<td>GCGATAACGCGGTTTAGGgT</td>
<td>254</td>
</tr>
<tr>
<td></td>
<td>F272G9</td>
<td>GCGATAATGCGGTTAGGgT</td>
<td>254</td>
</tr>
<tr>
<td><strong>Reverse primer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gyrA R</td>
<td>GTTAGGCAGACGGCTTGTTAATA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES


Fig. 1

 gyrA QRDR PCR

AS-PCR

Negative control
Wild type
Wild type (C 261 T)
87 mutation (C 261 A)
87 mutation (C 261 G)
91 mutation (G 271 A)
91 mutation (G 271 T)
91 mutation (A 272 G)

Marker

254 bp

428 bp