Analysis of gene mutations associated with antibiotic resistance in *Helicobacter pylori* isolated from Korean patients

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Analysis of gene mutations associated with antibiotic resistance in *Helicobacter pylori* isolated from Korean patients

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ABBREVIATIONS

ATCC	:	American Type Culture Collection
bid	:	twice a day
CLSI	:	Clinical and Laboratory Standards Institute
DEIA	:	DNA enzyme immunoassay
dNTPs	:	deoxynucleotide triphosphates
fdxA	:	ferredoxin
fdxB	:	ferredoxin-like protein
fldA	:	flavodoxin
FISH	:	fluorescence in situ hybridization
frxA	:	NAD(P)H flavin nitroreductase
LipA	:	line probe assay
MALT	:	mucosa associated lymphoid tissue
MIC	:	minimum inhibitory concentration
OLA	:	oligonucleotide ligation assay
oorD	:	2-oxoglutarate oxidoreductase
PCR	:	polymerase chain reaction
PBPs	:	penicillin-binding proteins
PHFA	:	preferential homoduplex formation assay
PPI	:	proton pump inhibitor
porD	:	pyruvate:ferredoxin oxidoreductase
qid	:	four times a day
QRDR	:	quinolone resistance-determining region

rdxA	:	oxygen-insensitive NAD(P)H nitroreductase
RFLP	:	restriction fragment length polymorphism
RND	:	Resistance-Nodulation-cell Division
tid	:	three times a day
3M-PCR	:	3’-mismatched reverse primer PCR

ABSTRACT

Helicobacter pylori is an etiologic agent of acute or chronic gastritis and can be a predisposing condition for peptic ulcer diseases, gastric cancer, and mucosa associated lymphoid tissue (MALT) lymphoma. Eradication of *H. pylori* is expected to cure gastritis and peptic ulcer disease and prevent atrophic gastritis and gastric cancer. To treat *H. pylori* infections, clarithromycin, amoxicillin, metronidazole, tetracycline, or fluoroquinolone are usually prescribed along with the proton pump inhibitor (PPI). Recently, however, the resistance to these antibiotic agents has been increased, which is regarded as the main cause of therapy failure. For this reason, it is necessary to choose proper antibiotics based on the antimicrobial susceptibility test results. But the antimicrobial susceptibility test is not widely used due to low *H. pylori* culture rate and lack of standard methods. In this study, minimum inhibitory concentration (MIC) on 5 antibiotics against 82 strains isolated at Yongin Severance Hospital in Korea was analyzed and then relationship between the resistance gene

mutations and curative effects was investigated. The aims of this study are to identify the domestic pattern of resistance gene mutations and to ascertain whether the molecular methods are suitable ways to predict the antibiotics resistance or not. In the case of clarithromycin, resistant strain groups and susceptible strain groups were clearly manifested. In 23S rRNA gene in 5 strains resisting to clarithromycin, A2143G was observed to have a point mutation. Also, the bacteriostatic treatment in 2 patients ended in failure, which was resulted from clarithromycin resistance and amoxicillin sensitivity. In 4 strains (MIC 1-2 μg/mL) resisting to amoxicillin, S414 and N562 in penicillin-binding proteins (PBPs) gene was observed not to have mutations. However, a substitution occurred at N562Y in a strain (MIC 2 μg/mL). Full-length nucleotide sequence of *rdxA* in 28 strains resisting to metronidazole was analyzed. Truncations was observed in 8 strains (28.5%), which was a result of the mutation caused by nucleotide deletion, insertion or stop codon. In MIC there was no strain resisting to tetracycline. 16S rRNA genes,

including $AGA_{926\text{-}928}$ of strains between 0.5 and 2 μg/mL of tetracyclin were analyzed, but no point mutations were observed. Eleven (57.8%) out of 19 strains resisting to levofloxacin showed amino acid substitutions in GyrA: N87K in 8 strains, N87I, A88V and D91N. In short, resistance gene mutation tests on 5 antibiotics were performed. Clarithromycin resistance gene observed at A2143G in 23S rRNA and levofloxacin resistance gene observed at N87, A88 and D91 have high correlation with MIC results. In the case of amoxicillin and tetracycline resistance, patterns of mutations were not diverse owing to a small number of resistance cases, but the mutation regions related to resistance could be detected. It is considered that multiple factors are leading to resistance to metronidazole. In conclusion, resistance mutation patterns related to antibiotics resistance in *H. pylori* strains isolated from Koreans are expected to be a basic data necessary in the development of the molecular-based method for detection of antibiotics resistance.

Key words: *Helicobacter pylori*, clarithromycin, amoxicillin, metronidazole, tetracycline, levofloxacin, 23S rRNA, PBPs, *rdxA*, 16S rRNA, GyrA, mutation

I. INTRODUCTION

Helicobacter pylori is a gram-negative rod with a spiral or slightly curved shape. The bacterium is relatively small (2.5-3.5 μm long and 0.5-1.0 μm wide) and contains 4-6 unipolar-sheathed flagellae[1]. Its genome is smaller (1.64-1.67 Mb) than that of *Escherichia coli* (4.60 Mb) and has virulence factors that are suitable to survive in the mucosa of the gastric anthrum. It has been reported that about half of the world's population and 59-67% of the South Korean population are colonized by *H. pylori*[2, 3]. *H. pylori* is an etiologic agent of acute or chronic gastritis and can be developed into peptic ulcer disease, gastric cancer, and mucosa associated lymphoid tissue (MALT) lymphoma[4-8].

H. pylori eradication from colonized stomach leads to healing of gastritis and peptic ulcer disease, probably also has beneficial effect on regression of atrophic gastritis and prevention of distal gastric cancer[9, 10]. *In vitro*, *H. pylori* is susceptible to the majority of antibiotics, but *in vivo* only a few antibiotics have been used successfully for eradication, i.e. clarithromycin,

amoxicillin, metronidazole, tetracycline, or fluoroquinolones (ciprofloxacin, levofloxacin and moxifloxacin)[11]. A proton pump inhibitor (PPI) is included in all treatment regimens for *H. pylori* infection because it primarily increases the intragastric pH level, enhances the activity of antibiotics, and reduces the side effects of any given regimen, consequently increasing the cure rate[12, 13]. For 7-10 days, triple treatments consisting of two of the previous mentioned antibiotics and a PPI or ranitidine bismuth citrate are now mostly recommended[14-16]. There seems to be preference for combination treatment that includes amoxicillin and clarithromycin[16].

In spite of the good results in many clinical trails, these treatments are not always successful in each clinical practice. Failure of first-line treatment is usually related to insufficient patient compliance and/or development of antibiotic resistance. For retreatment, 10 to 14 day treatment courses with higher doses of the antibiotics and/or bismuth component is often advised with inclusion of previously unused antibiotics[17]. Most patients who

still remain *H. pylori*-positive after two consecutive courses of eradication treatment have been infected with an *H. pylori* strain resisting to one or more of the previously used antibiotics[18, 19]. To select an appropriate third-line treatment, endoscopy followed by bacterial culture and antibiotic susceptibility testing is advisable.

Since antibiotic resistance in *H. pylori* seems to be the main reason in therapy failure, detection of antibiotic resistance is very crucial. Antibiotic susceptibilities of *H. pylori* are usually assessed by culture-based methods such as E-test, agar dilution and disc diffusion. While these methods offer the opportunity to determine the minimum inhibitory concentration (MICs) of antibiotics, they are time-consuming and their results show low reproducibility[20, 21]. Factors such as cell viability, inoculum size, incubation condition, and growth media may affect their outcome[22].

Molecular-based methods for antibiotic resistance are independent of these factors, and thus they offer attractive alternatives, compared to conventional antimicrobial

susceptibility tests. These tests give reproducible results and are easily standardized. Moreover, they are faster than the conventional culture-based assay and data can be obtained at the day of endoscopy.

Recently numerous molecular-based methods are now available in assessing clarithromycin, tetracycline, and ciprofloxacin resistance in *H. pylori*, but the assessment for metronidazole and amoxicillin resistance is more needed. For rapid detection of clarithromycin resistance in *H. pylori*, PCR-based method is used in PCR-restriction fragment length polymorphism (RFLP)[23-26], PCR-oligonucleotide ligation assay (OLA)[27], PCR-DNA enzyme immunoassay (DEIA)[28], PCR-line probe assay (LipA)[29], PCR-preferential homoduplex formation assay (PHFA)[30], 3'-mismatched reverse primer PCR (3M-PCR)[31]. Recently, real-time PCR hybridization assays[32,33] and fluorescence in situ hybridization (FISH)[34] without PCR are practiced. Molecular-based tests for metronidazole resistance in *H. pylori* are not easy to perform because the resistance is

associated with various unrelated mutations within the *rdxA* gene and other reductase-encoding genes. Despite these barriers, there is new evidence that a system could be developed based on detection of the RdxA protein (immunoblotting with specific antibodies)[35]. PCR-RFLP[36] and real-time PCR hybridization assay[37] was developed to detect tetracycline resistance in *H. pylori*. In accordance with the same principle, real-time PCR assay has been developed for ciprofloxacin resistance[38].

For these useful molecular-based methods, the mechanism of the resistance to the major antibiotics and the information of resistant patterns in strains isolated from local areas should be understood.

Currently clarithromycin remains the most powerful antibiotic available against *H. pylori* with MIC being the lowest as compared to the other molecules. The MIC values as low as 0.016-0.5 μg/mL are generally reported, antibiotic resistance being recognized with MIC values ≥ 1 μg/mL (range: 2-256 μg/mL)[39, 40]. Local studies have shown that the primary resistance

rate varied from 12 to 26% among regions[41, 42]. If *H. pylori* becomes resistant, its eradication rate drops considerably up to 0-50%. *H. pylori* resistance is due to the prevention of clarithromycin from binding to domain of 23S rRNA because of the point mutation in the 23S rRNA gene and due to the activity of the efflux pump system.

The Resistance-Nodulation-cell Division (RND family) is responsible for macrolide intrinsic resistance in several gram-negative bacteria and it has been recently proposed also for *H. pylori*. Four RND gene clusters in the efflux pump system play a role in promoting multi-drug resistance in *H. pylori*[43]. Clarithromycin binds preferentially to the ribosomal subunits rather than to the efflux pumps. The bacteriostatic activity of clarithromycin depends on its capacity to inhibit the protein synthesis by binding to the 50S bacterial ribosomal subunit.

PCR-based tools have demonstrated that point mutations in the peptidyl transferase region encoded in domain V of 23S rRNA are responsible for the bacterial resistance to macrolides[44].

These mutations are able to inhibit the binding between clarithromycin and the ribosomal subunit dedicated to the specific antibiotics related protein synthesis[44,45]. Resistance to clarithromycin in *H. pylori* is caused by point mutations in three adjacent 23S rRNA nucleotides, namely at position 2142, 2143 and 2144. It can be induced by an adenine (A) to guanine (G) substitution at one of these positions[46] or an adenine (A) to cytosine (C) substitution solely at position 2142. These mutational events are responsible for more than 90% of clarithromycin resistance in developing countries[47]. In detail, the mutation at position 2143 seems to be associated with different resistant level, with MIC values widely ranging from 0.016 to 256 μg/mL. Conversely, the mutations at position 2142 are associated with more restricted MIC values, close to 64 μg/mL[48].

Despite the high resistance rate of local strains in Korea, the data on the mutation patterns of epidemic strains at the molecular level relating to *H. pylori* resistance and eradication are generally insufficient. A large volume of data from the

surveillance is required for the development of molecular detection methods that will be available for a given geographic region.

Amoxicillin is a bactericidal antibiotic that belongs to a group of penicillins. It binds to penicillin-binding proteins (PBPs), and thus interferes with bacterial cell wall synthesis, resulting in the lyses of replicating bacteria. MIC values ranging from 0.06 to 0.25 μg/mL are generally reported in susceptible strains. Cut-off value of antibiotic resistance is recognized as MIC value ≥ 1 μg/mL (range: 1-8 μg/mL)[39, 49].

This drug has been used as the first line antibiotics in *H. pylori* therapy because of a presumed absence of resistance. According to a recent local report, however, the amoxicillin resistance rate varied from 0 to 14% among regions[41, 42], and resistance was one of the major reasons for the high failure rate of the eradication even though it has the susceptibility to clarithromycin.

The major causes of *H. pylori* resistance are the point mutation in *pbp1*, *hopB* and *hopC* genes that encode porin, and multi-drug efflux pumps. The PBPs is currently believed to have the greatest influence on *H. pylori* resistance. PBPs are enzymes involving in the synthesis of the peptidoglycan layer of the bacterial wall with a glycosyl transferase-acyl transpeptidase activity. This enzymatic activity is located in 3 distinct motifs ($SKN_{368-370}$, $SNN_{433-435}$ and $KTG_{555-557}$) of PBPs within the C-terminal region

Several investigations indicate that multiple point mutations in *pbp1* gene are the major mechanism of amoxicillin resistance, leading to loss of affinity between amoxicillin and PBP-transpeptidase[49, 50]. It has been observed that the Ser414 to Arg substitution, adjacent to the SKN motif in PBP1, is responsible for amoxicillin resistance with a significantly increased MIC (0.5-1 μg/mL)[51]. Another study reported that the Asn_{562} amino acid substituted to a Tyr residue in near KTG motif of PBP1[52]. Such a point mutation is able to confer high resistance to all

strains *in vitro* and is considered as the main mutation conferring resistance.

In gram-negative bacteria, resistance to β-lactam antibiotics is mostly due to the production of β-lactamase, either chromosome, or plasmid encoded[53]. In *H. pylori*, however, there are no indications that amoxicillin resistance is caused by the acquisition or expression of β-lactamase. As the *H. pylori* resistance rate is increasing in Korea with the use of amoxicillin as a first-line treatment increased. Therefore, the influence of the point mutation of the *pbp1* on the eradication rate must be investigated.

Metronidazole is administered as a prodrug that needs to be activated within the target cell through one or two electron reduction processes. This reduction leads to the formation of nitro-anion radicals and metronidazole intermediates that cause lethal damage to subcellular structures and DNA[54]. Theoretically, any protein that possesses a low redox potential can accept electrons from metronidazole and thus activate the drug. In *H.*

pylori several putative electron acceptors including ferredoxin (*fdxA*), ferredoxin-like protein (*fdxB*), flavodoxin (*fldA*), NAD(P)H flavin nitroreductase (*frxA*), 2-oxoglutarate oxidoreductase (*oorD*), pyruvate:ferredoxin oxidoreductase (*porD*), and oxygen-insensitive NAD(P)H nitroreductase (*rdxA*)[55] have been identified.

In *H. pylori*, the levels of metronidazole resistance are very diverse, with MICs ranging from 8 to ≥ 256 μg/mL. Previous studies have reported that the metronidazole resistance rate varied from 26 to 66%[56, 57], and recent studies have reported that it varied from 10 to 27%[41, 42], with high differences in prevalence among regions and periods. Despite the high antibiotic resistance the eradication rate does not show any difference compared with that of clarithromycin. This indicates that the mechanism of resistance and the *in vivo* activity of *H. pylori* involve various pathways.

Potential mechanisms of metronidazole resistance of *H. pylori* include (i) deficient drug uptake and/or increase drug

efflux[58]; (ii) enhanced activity of DNA repair enzymes[59]; (iii) increased oxygen scavenging capabilities[60]; and (iv) decreased drug activation arising from changes in metronidazole-reducing enzymes[61]. The *rdxA* mutations are recognized as the main mechanism conferring metronidazole resistance in *H. pylori* [62].

Tetracycline is a fundamental antibiotic in quadruple regimens for *H. pylori* eradication. Tetracycline MICs are usually reported with 0.25-2 μg/mL[63], but MIC values ≥ 4 μg/mL is considered with resistance [39].

Although bacterial resistance to tetracycline is rare, it appears to be increasing. It has been reported that the resistance rate was 7-36% in Korea[41, 42]. Tetracycline acts as a bacteriostatic against either gram-positive or gram-negative species by inhibiting codon-anticodon link at the level of 30S ribosomal subunit and blocking the attachment of aminoacyl-tRNA to the acceptor site. In general, 4 tetracycline resistance mechanisms have been identified; (i) deficient drug uptake and/or increased drug efflux; (ii) decreased antibiotic binding by changes in

ribosomal protection proteins, and (iii) mutation 16S rRNA tetracycline binding site; and (iv) enzymatic inactivation of tetracycline[64]. The most reliabe mechanism for tetracycline resistance in *H. pylori* is based upon a nucleotide-base pair substitution in three adjacent 16S rRNA residues, namely $AGA_{926\text{-}928}$ →TTC 3 base mutations.

The use of levofloxacin for *H. pylori* eradication is increasing worldwide because of its role in rescue therapy regimens followed by the failure of clarithromycin-based treatments. Levofloxacin MICs are generally reported with 0.25-0.5 μg/mL, MIC ≥ 1 μg/mL (range: 4-32 μg/mL) is considered to have resistance[65,66]. Fluoroquinolones (i.e. ciprofloxacin, moxifloxacin, trovafloxacin and levofloxacin) are bactericidal antibiotics that exert their antibiotics activity by inhibiting the enzyme DNA gyrase. This enzyme is a tetramer consisting of two A subunits and B subunits, encoded by the *gyrA* and *gyrB*, respectively [67]. The main function of this enzyme is to catalyze the negative supercoiling of DNA.

In *H. pylori*, the resistance to quinolone is caused by point mutation in the so-called quinolone resistance-determining region (QRDR, located between amino acid position 67 and 106) of the *gyrA* at amino acids position 87, 88, 91 and 97[67, 68]. The resistance rate has been reported to be 22-26% in Korea.[41, 42]. As the use of antibiotics to eradicate *H. pylori* is increasing, a susceptibility test is required before the use of rescue therapy regimens is practiced and the presence of resistance should be verified with the molecular detection method.

The aims of this study were to identify the resistance in 5 major therapeutic antibiotics (i.e., clarithromycin, amoxicillin, metronidazole, tetracycline, and levofloxacin) through antimicrobial susceptibility test and to identify the relationship between antibiotic resistance and eradication rates of *H. pylori* infection in Korea. Also, it aims to verify whether resistance gene mutation test in molecular-based methods is correlated with antibiotic resistance in MICs.

II. MATERIALS AND METHODS

Bacterial isolates

A total of 493 patients underwent gastric endoscopy at the Yongin Severance Hospital of Yonsei University, Korea from July 2009 to December 2010. When gastric disorders (dyspepsia, acute gastritis, chronic gastritis, gastro-duodenal ulcer, atrophic gastritis, and gastric cancers) were detected via gastric endoscopy, gastric mucosa biopsy was performed. The specimens were placed in 1.5 mL conical tube, transferred to the culture room and inoculated within 10 min. If, immediate inoculation was unfeasible, the specimens were kept into the freezer and then inoculated within the 24 hr. A total of 82 strains were isolated from 493 patients. Thirty two strains out of 82 isolated from 32 patients, which had a history of treatments including a 7 day first-line treatment with PPI (30 mg, bid), amoxicillin (2250 mg, tid), clarithromycin (1000 mg, bid) and a second-line treatment with PPI (30 mg, bid), bismuth (300 mg, bid), metronidazole (2250 mg,

bid), and tetracycline (1000 mg, qid), showed eradication in treatment. The eradication of *H. pylori* is verified in case of being negative in ^{13}C-urea breath test (Isotechnika, Alberta, Canada) after two months of drug administration.

After *H. pylori* DNA had been extracted in gastric tissues from twelve patients who had had failure history in the first-line treatment and whose *H. pylori* culture had failed, 23S rRNA gene went through sequencing to see whether it had clarithromycin resistance.

Culture condition

The medium used in this study was composed of Brucella broth (BBL, Sparks, USA) that containing 1.2% agar and supplements that included 10% bovine serum and selected antibiotics (Oxoid Limited, Hampshire, England) (10 μg/mL of vancomycin, 5 μg/mL of trimethoprim, 5 μg/mL of cefsulodin, and 5 μg/mL of amphotericin B). The biopsy specimens were

fully minced by using homogenizer and then inoculated into the culture media.

The culture media were incubated under 10% CO_2, 5% O_2, 100% humidity, at 37℃ for 3-5 days. Single isolated colorless, pinpoint, convex, and milky colonies were chosen for further characterization. An S-shaped Gram-negative bacilli was observed in Gram stain. For the biochemical confirmation, urease test and oxidase test were performed. For long term storage isolated strains was suspended in Brucella broth containing 10% bovine serum and 20% glycerol, and stored at -70℃ or in liquid nitrogen until use. For subculture, the strains were thawed immediately to 37℃ for optimal growth and were subcultured up to 2 times from liquid nitrogen. The *H. pylori* American Type Culture Collection (ATCC) 43504 and *Helicobacer pylori* strain 51 was cultured using the same methods described above for standard and quality control assessment.

Determination of minimum inhibitory concentration

All 82 strains were examined for antibiotic resistance to clarithromycin (Sigma-Aldrich Co., St. Louis, MO, USA), amoxicillin (Sigma-Aldrich), metronidazole (Sigma-Aldrich), tetracycline (Sigma-Aldrich), and levofloxacin (Sigma-Aldrich) by slightly modified agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI). All of these antibiotics are used for *H. pylori* eradication regimens. Brucella broth base with 1.2% agar was used for MIC tests. Sterilized broth was cooled to 50℃ before antibiotic incorporation. Then each antibiotic was twofold serially diluted in media containing 10% bovine serum.

The strains cultured for 72 hr on Brucella agar medium were used. Ten μL of saline suspension with MacFarland standard 2.0 was inoculated on test medium. The inoculated media was incubated at 37℃ under 10% CO_2, 5% O_2, 100%

humidity condition for 3 days. MIC breakpoint was recognized at the areas which all the growth was completely inhibited and hazes appeared. Clarithromycin resistance was defined according to the CLSI-approved breakpoint (≥ 1 μg/mL) [69]. Isolates were classified as resistant to amoxicillin, metronidazole, tetracycline and levofloxacin when the MICs were ≥ 1, ≥ 8, ≥ 4, and ≥ 1 μg/mL, respectively. Since no criteria for interpretation of levofloxacin MIC against *H. pylori* according to CLSI-approved breakpoints, resistance to this drug was defined for levofloxacin against gram-negative pathogens in general. *H. pylori* ATCC 43504 was used for quality control of the selective medium and antimicrobial susceptibility test. For *H. pylori* ATCC 43504, the MIC range of clarithromycin was 0.016-0.125 μg/mL, amoxicillin 0.016-0.125 μg/mL, metronidazole 64-256 μg/mL, tetracycline 0.125-1 μg/mL, and levofloxacin 0.064-0.5 μg/mL.

DNA extraction

DNA extraction was performed on colonies that were isolated and cultured from the gastric biopsy using AccuPrep® Genomic DNA Extraction Kit (Bioneer Co., Daejeon, Korea). After colony suspension had been mixed with 200 μL of DW, it was mixed with proteinase K afterward, it was incubated at 60℃ for 10 min adding isopropanol afterward. The mixture was transferred to a binding column tube and allowed to fully precipitate. The column was washed twice and finally the genomic DNA was eluted with an elution solution. The control specimens were also tested under the parallel condition.

Detection of *Helicobacter pylori* specific genes

Amplication of a 642 bp fragment of the 16S rRNA gene was performed using the primers HPU185 (5'-CCTACGGGGGAAAGATTTAT-3') and HPU826 (5'-AGCTGCATTACTGGAGAGACT-3')[70]. The template DNA (2 μL) was added to 18 μL aliquots of AccuPower PCR PreMix

(Bioneer Co.) containing 1 U *Top* DNA polymerase, 250 μM deoxynucleotide triphosphates (dNTPs), 10 μM Tris-HCl (pH 9.0), 30 mM KCl and 1.5 μM $MgCl_2$.

PCR was performed with initial denaturation at 94℃ for 5 min, followed by 35 cycles with denaturation at 94℃ for 50 sec, annealing at 52℃ for 50 sec and elongation step at 72℃ for 1 min. Cycling was followed by a final extension at 72℃ for 7 min. The amplification reactions were performed with a thermal cycler (GenePro Thermal Cycler® BIOER, Tokyo, Japan). Amplification products were separated by a 1.5% agarose gel electrophoresis, stained with 0.5 μg/mL ethidium bromide and visualized using a ultraviolet trans-illuminator (Vilber louramat, Mame La Valle, France)

Determination of clarithromycin resistance by PCR sequence analysis

Eleven bacterial stains, 5 of which were resistant to clarithromycin and 6 of which were susceptible to clarithromycin, were tested. Amplication of a 389 bp fragment of the 23S rRNA gene was performed using the primers *rrn*23S-F (5'-ATGAATGGCGTAACGAGATG-3') and *rrn*23S-R (5'-GTCTTACAGTCAGGCTGGCT-3'). The PCR primer designed using the Oligo Program Version 6 (Molecular Biology Insights, Inc., Cascade, CO, USA). Cycling conditions for PCR reaction and was shown in Table 1. DNA sequencing was carried out at Macrogen (Seoul, Korea). The resulting consensus sequences were compared to GenBank (www.ncbi.nlm.nih.gov/GenBank) reference sequence *rrn* 23S of *Helicobacer pylori* strain 51.

Determination of amoxicillin resistance by PCR sequence analysis

Eight bacterial strains, 5 of which were resistant to amoxicillin and 6 of which were susceptible to amoxicillin, were tested. Amplication of an 802 bp fragment of the *pbp1* was performed using the primers *pbp1*-F (5'-CCACGCAAGCCAAACGGCG-3') and *pbp1*-R (5'-CCTTTGGGGACATCAAACTTT-3'). Cycling conditions for PCR reaction and was shown in Table 1. DNA sequencing was carried out at Macrogen (Seoul, Korea).

Determination of metronidazole resistance by PCR sequence analysis

Thirty bacterial strains, 28 of which were resistant to metronidazole and 2 of which were susceptible to metronidazole, were tested. Amplication of the *rdxA* was performed using the two pairs of oligonucleotide primers *rdxA*-F1 (5'-TAGGGATTTTATTGTATGCTACG-3') and *rdx*-R1 (5'-

CCACAGCGATATAGCATTGCT-3'), *rdxA* F2 (5'-GTTAGAGTGATCCCGTCTTTT-3') and *rdxA* R2 (5'-CCTAAAAGAGCGATTAAAACCA-3'). The two overlapping PCR products (489 bp and 407 bp) that constitute a total of 772 bp were designed to contain the entire *rdxA*. Cycling conditions for PCR reaction and was shown in Table 1. DNA sequencing was carried out at Macrogen (Seoul, Korea).

Determination of tetracycline resistance by PCR sequence analysis

Because tetracycline resistance is restricted to the occurrence of specific 16S rRNA gene mutations, PCR-based sequencing in four strains susceptible to tetracycline was performed to detect the triple-base-pair substitution $AGA_{926-928}TTC$.

Amplication of a 314 bp fragment of the 16S rRNA gene was performed using the primers *rrn*16S-F (5'-TGCAGCTAACGCATTAAGCATC-3') and *rrn*16S-R (5'-

GAGGCAGTATCCTTAGAGTTCT-3'). Cycling conditions for PCR reaction and was shown in Table 1. DNA sequencing was carried out at Macrogen (Seoul, Korea).

Determination of levofloxacin resistance by PCR sequence analysis

Twenty-one strains, 19 of which were resistant to levofloxacin and 2 of which were susceptible to levofloxacin, were tested. Amplication of an 802 bp fragment of the *gyrA* was performed using the primers *gyrA*-F (5'-GTGCATAGGCGTATTTTGTATG-3') and *gyrA*-R (5'-CATTCTGGCTTCAGTGTAACG T-3'). Cycling conditions for PCR reaction and was shown in Table 1. DNA sequencing was carried out at Macrogen (Seoul, Korea).

Table 1. Oligonucleotides used in this study

Target gene	Primer	Oligonucleotide sequence (5`-3`)[a]	Position[b]	Anneling Tm	Reference
H. pylori	HPU185	CCTACGGGGGAAAGATTTAT	185 to 204, forward	52℃	Kim *et al.*[70]
	HPU826	AGCTGCATTACTGGAGAGACT	806 to 826, reverse		
rrn 23S (23S rRNA gene)	*rrn*23S-F	ATGAATGGCGTAACGAGATG	2051 to 2070, forward	52℃	This study
	*rrn*23S-R	GTCTTACAGTCAGGCTGGCT	2420 to 2439, reverse		
	*rrn*24S-SF	GAGATGGGAGCTGTCTCA			
pbp1	*pbp1*-F	CCACGCAAGCCAAACGGC	1076 to 1093, forward	58℃	
	pbp1-R	CCTTTGGGGACATCAAACTTT	1857 to 1877, reverse		
	pbp1-SF	ATCGCTTTTGATAATGGCTATT			
rdxA	*rdxA*-F1	TAGGGATTTTATTGTATGCTACG	969932 to 969911, forward	52℃	
	rdxA-R1	CCACAGCGATATAGCATTGCT	969458 to 969435, reverse		
	rdxA-SF1	GTATGCTACGAAAAATTCTAAA			
	rdxA-F2	GTTAGAGTGATCCCGTCTTTT	969543 to 969516, forward	52℃	
	rdxA-R2	CCTAAAAGAGCGATTAAAACCA	969181 to 969161, reverse		
	rdxA-SF2	TGCTTGGCGTGAGATTCAA			
rrn 16S (16S rRNA gene)	*rrn*16S-F	TGCAGCTAACGCATTAAGCATC	818 to 839, forward	54℃	
	*rrn*16S-R	GAGGCAGTATCCTTAGAGTTCT	1110 to 1131, reverse		
	*rrn*16S-SF	AAGCATCCCGCCTGGGG			
gyrA	*gyrA*-F	GTGCATAGGCGTATTTTGTATG	142 to 163, forward	52℃	
	gyrA-R	CATTCTGGCTTCAGTGTAACG	373 to 393, reverse		
	gyrA-SF	GCGTATTTTGTATGCGATGC			

[a] Oligonucleotides used for amplification were based on the published genome sequence of *Helicobacer pylori* strain 51 [GenBank accession CP000012]. [b] Position of oligonucleotides are given to the mutation of antibiotics resistance of *H. pylori*. Cycling conditions for PCR reaction was described in Materials and Methods with the modification of annealing temperature as indicated in Table 1.

III. RESULTS

Bacterial isolates

Out of 493 patients who had undergone endoscopy, 256 (51.9%) were urease test-positive, and 237 (48.1%) were urease test-negative. A total of 87 strains of *H. pylori* were isolated from 87 patients, 82 strains were from urease test-positive specimens, and 5 were from urease test-negative specimens. Out of 87 strains, 5 strains were contaminated during the study and thus, discarded. Eighty-two strains were used for the antimicrobial susceptibility tests, PCR, and sequencing.

Minimum inhibitory concentration for *H. pylori*

antimicrobial susceptibility tests were performed with 5 antibiotics (clarithromycin, amoxicillin, metronidazole, tetracycline, and levofloxacin) using the agar dilution method. The MIC distributions for the 82 clinical isolates are described in Table 2. Although the MIC of clarithromycin was 0.064 μg/mL or less, about 87.8% (72/82) of the isolated strains showed clear

dual peaks in the distribution of MICs due to the strains that had a high MIC and resistance rate was 8.5% (7/82). The 81.7% of strains (67/82) were inhibited at 0.125 μg/mL or less concentration of amoxicillin. There is no clearly established criteria for resistance yet, but ≥ 1 μg/mL was reported as the resistance criterion in recent reports. According to this criterion, the resistance rate of clinical was about 4.8%. The MIC of metronidazole was showing very diverse distributions with a 46.3% resistance rate, though a dual peak appeared at 2 μg/mL and 64 μg/mL without clear boundaries. The MIC of tetracycline was 0.064-2 μg/mL, and there was no strains showing resistance to tetracycline based on the cut-off of ≥ 4 μg/mL. The MIC of levofloxacin varied very widely from 0.032 to 32 μg/mL in the distribution. The peak MIC was 0.5 μg/mL, with a 35.4% resistance rate according to the cut-off ≥ 1 μg/mL.

Table 2. Distribution of MICs of 82 *Helicobacter pylori* strains*

MICs (μg/mL)	Number of strains (%)				
	Clarithromycin	Amoxicillin	Metronidazole	Tetracycline	Levofloxacin
0.008					
0.016	4 (4.9)	14 (17.1)			
0.032	47 (57.3)	13 (15.9)			1 (1.2)
0.064	21 (25.6)	17 (20.7)		12 (14.6)	
0.125		23 (28.0)		14 (17.1)	2 (2.4)
0.25	1 (1.2)	8 (9.8)	1 (1.2)	41 (50.0)	20 (24.4)
0.5	2 (2.4)	3 (3.7)	2 (2.4)	11 (13.4)	30 (36.6)
1		**3 (3.7)**	3 (3.7)	3 (3.7)	**4 (4.9)**
2		**1 (1.2)**	28 (34.1)	1 (1.2)	**3 (3.7)**
4	**1 (1.2)**		10 (12.2)		**2 (2.4)**
8	**1 (1.2)**		**11 (13.4)**		**8 (9.8)**
16	**3 (3.7)**		**5 (6.1)**		**8 (9.8)**
32	**1 (1.2)**		**8 (9.8)**		**4 (4.9)**
64			**10 (12.2)**		
128	**1 (1.2)**		**3 (3.7)**		
256			**1 (1.2)**		

*Cut-off values for resistance were defined as ≥ 1 μg/mL for clarithromycin, ≥ 1 μg/mL for amoxicillin, ≥ 8 μg/mL for metronidazole, ≥ 4 μg/mL for tetracycline, and ≥ 1 μg/mL for levofloxacin. Resistance strains are indicated as bold type letter.

Eradication rate of *H. pylori* infection

Thirty-two patients were monitored for the eradication program (Table 3). The eradication was achieved in 82.1% (23/28) patients who had the strains susceptible to both clarithromycin and amoxicillin. The first-line treatment *H. pylori* of eradication failed in 7 patients (strain Nos. 6, 20, 37, 63, 74, 90, and 93). The second-line treatment of (PPI, bismuth, amoxicillin, metronidazole, and tetracycline) was successful in 3 patients (strain Nos. 6, 37, and 97) and failed in 1 patient (strain No. 63). Treatment records were not found in 3 patients (strain Nos. 74, 90, and 93). Strain No. 37 (CLA; MIC 8 μg/mL) and No. 6 (CLA; MIC 16 μg/mL) were resistant to clarithromycin and susceptible to amoxicillin (Table 4). The resistance gene mutation was observed at A2143G point in the 23S rRNA gene. The first-line treatment was failed in those strains.

Strains No. 97 (AMO; MIC 0.5 μg/mL) and No. 17 (AMO; MIC 1 μg/mL) were susceptible to clarithromycin and resistant to amoxicillin. No mutations were observed at the S414

and the N562 position in *pbp1*. All of them ended in the success of the first-line therapy.

The therapeutically failed cases of other 5 strains (strains No. 20, 63, 74, 90, 93) were susceptible to clarithromycin and amoxicillin. Mutations were not observed at position 2142 and 2143 in 23S rRNA gene and S414 and N562 position in *pbp1*.

Table 3. The effect of resistance of amoxicillin and clarithromycin on the eradication rate of *Helicobacter pylori* after first-line treatment with a proton pump inhibitor, clarithromycin and amoxicillin

CLA	AMO	N. of patients	Eradication rate N. (%)	
			Success	Failure
Susceptible	Susceptible	28	23 (82.1)	5 (17.9)
Susceptible	Resistant	2	2 (100)	
Resistant	Susceptible	2		2 (100)

Abbreviations: CLA, clarithromycin; AMO, amoxicillin; N, number.

Cut-off values of resistance were defined as ≥ 1 μg /mL for CLA and ≥ 1 μg/mL for AMO.

Table 4. The effect of gene mutation on the eradication rate of *Helicobacter pylori* after therapy

Strain Nos.	1st			Gene mutation	2nd		
	CLA	AMO	Eradication		MET	TET	Eradication
7	S	S	Succeeded				
12	S	S	Succeeded				
21	S	S	Succeeded				
24	S	S	Succeeded				
28	S	S	Succeeded				
29	S	S	Succeeded				
38	S	S	Succeeded				
42	S	S	Succeeded				
45	S	S	Succeeded				
46	S	S	Succeeded				
47	S	S	Succeeded				
50	S	S	Succeeded				
53	S	S	Succeeded				
54	S	S	Succeeded				
57	S	S	Succeeded				
58	S	S	Succeeded				
68	S	S	Succeeded				
72	S	S	Succeeded				
77	S	S	Succeeded				
78	S	S	Succeeded				
99	S	S	Succeeded				
106	S	S	Succeeded				
101	S	S	Succeeded				
17	S	R	Succeeded	No mutation			
97	S	R	Succeeded	No mutation			
20	S	S	Failed	No mutation	S	S	Succeeded
63	S	S	Failed	No mutation	S	S	Failed
74	S	S	Failed	No mutation	S	S	No record
90	S	S	Failed	No mutation	S	S	No record
93	S	S	Failed	No mutation	R	S	No record
37	R	S	Failed	23S rRNA A2143G	S	S	Successed
6	R	S	Failed	23S rRNA A2143G	S	S	Successed

Abbreviations: CLA, clarithromycin; AMO, amoxicillin; MET, metronidazole; TET, tetracycline; S, susceptible; R, resistant. Gene mutations regions were targeted at 23S rRNA gene in clarithromycin and at *pbp1* in amoxicillin.

Sequence analysis of the 23S rRNA gene

The base sequence was analyzed for a total of 163 bp (position 2,137-2,299) out of the entire 2,971 bp. This region is where the peptidyl-tRNA that is located in near the 50S ribosome P and E sites undergoes translocation from the acceptor site to the donor site. Clarithromycin is well known to interrupt binding to bases at positions 2142, 2143, and 2144.

Sequence analysis of the 23S rRNA gene was performed on 11 strains. At A2142 and A2144, point mutations did not occur. But in A2143, a point mutation occurred in all 5 resistant strains including strain No. 76 (MIC 4 μg/mL), No. 37 (MIC 8 μg/mL), No. 6 (MIC 16 μg/mL), No. 91 (MIC 16 μg/mL), and No. 44 (MIC 128 μg/mL). Although a mutation of T2182C occurred, it was detected from a susceptible strain with a MIC of 0.06 μg/mL. A mutation of A2223G was also detected on strain No. 99 (MIC 0.03 μg/mL) and No. 91 (MIC 16 μg/mL) (Figure 1).

Sequence analysis of the 23S rRNA gene was performed on 12 biopsy specimens from 12 patients. Target region for

sequencing was total of 163 bp between 2137 and 2299 (Figure 2). In the tissue No. 21, a mutation was detected in a region A2143C known to be the action region of clarithromycin resistance. The mutations diversely occurred in C2197T, C2287T, and T2294C. A2223G mutation was detected on 6 specimens (tissue Nos. 14, 15, 20, 21, 23, and 27).


Strains No.
(MIC μg/mL)
Eradication
2143
2182
2223
GACGGAAAGACCCCGTGGACCTTTACTACAACTTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTAAGG
93 (0.03) Failure
99 (0.03) Succeeded
74 (0.03) Failure
20 (0.03) Failure
63 (0.06) Failure
90 (0.5) Failure
76 (4) No record
37 (8) Failure
6 (16) Failure
91 (16) No record
44 (128) No record
GCTTTGGCTCTTATGGAGCCATCCTTGAGATACCACCCTTGATGTTTCTGTTAGCTAACTGGCCTGTGTTATCC
93 (0.03)
99 (0.03)
74 (0.03)
20 (0.03)
63 (0.06)
90 (0.5)
76 (4)
37 (8)
6 (16)
91 (16)
44 (128)

Figure 1. The correlation of minimum inhibitory concentration of clarithromycin with the presence of *Helicobacter pylori* 23S rRNA gene mutations. MICs of isolates were determined by agar dilution, and the 23S rRNA mutations were assessed by PCR sequence analysis. Gaps in the nucleic acid sequence are marked by substituted sequence and identical nucleic acid is indicated with dots.

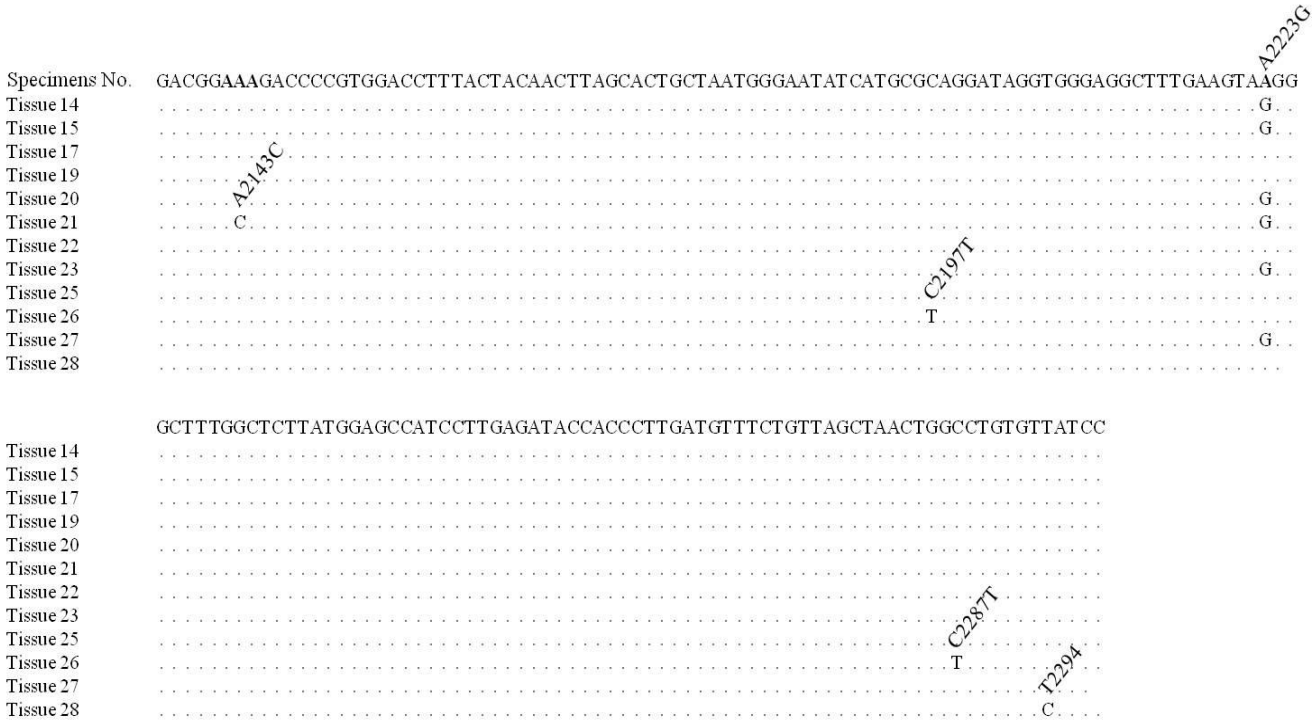
A2223G
Specimens No. GACGGAAAGACCCCGTGGACCTTTACTACAACTTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTAAGG
Tissue 14 . . . . . G . .
Tissue 15 . . . . . G . .
Tissue 17 . . . . . .
Tissue 19 . . . . . .
Tissue 20 . . . . . G . .
Tissue 21 C . . . . . G . .
Tissue 22 . . . . . .
Tissue 23 . . . . . G . .
Tissue 25 . . . . . .
Tissue 26 . . . . . T . . . . . .
Tissue 27 . . . . . G . .
Tissue 28 . . . . . .
A2143C
C2197T
GCTTTGGCTCTTATGGAGCCATCCTTGAGATACCACCCTTGATGTTTCTGTTAGCTAACTGGCCTGTGTTATCC
Tissue 14 . . . . . .
Tissue 15 . . . . . .
Tissue 17 . . . . . .
Tissue 19 . . . . . .
Tissue 20 . . . . . .
Tissue 21 . . . . . .
Tissue 22 . . . . . .
Tissue 23 . . . . . .
Tissue 25 . . . . . .
Tissue 26 . . . . . T . . . . . .
Tissue 27 . . . . . .
Tissue 28 . . . . . C . . .
C2287T
T2294

Figure 2. The distribution of *Helicobacter pylori* 23S rRNA mutations related with clarithromycin resistance from the gastric biopsy specimens. The *H. pylori* 23S rRNA was isolated from the gastric biopsy of the 12 patients from whom no *H. pylori* was cultured. PPI, clarithromycin and amoxicillin treatment were unsuccessful. Gaps in the nucleic acid sequence are marked by substituted sequence and identical nucleic acid is indicated with dots.

Sequence analysis of *bpb1*

The sequences of 5 amoxicillin resistant strains with a resistance cut-off value of ≥ 1 μg/mL or more were analyzed. The gene *bpb1* encodes a protein which containing 660 amino acids, and the base sequence between 390 to 617 which encodes 228 amino acids, where the substitution was occurred are shown in Figure 3. No substitution occurred in the position S414R; a region well-known as amoxicillin-resistant. Substitutions of I515M, K518R, T558S, N562Y, and G594S occurred in strain No. 44 (MIC 2 μg/mL). In the strains No. 97 (MIC 0.5 μg/mL) and No. 17 (MIC 1 μg/mL), N was inserted at position 463, and substitutions of G591R, Y593S, and G594S occurred. In the strain No. 49 (MIC 0.5 μg/mL) T556S and T593A occurred (Figure 3).

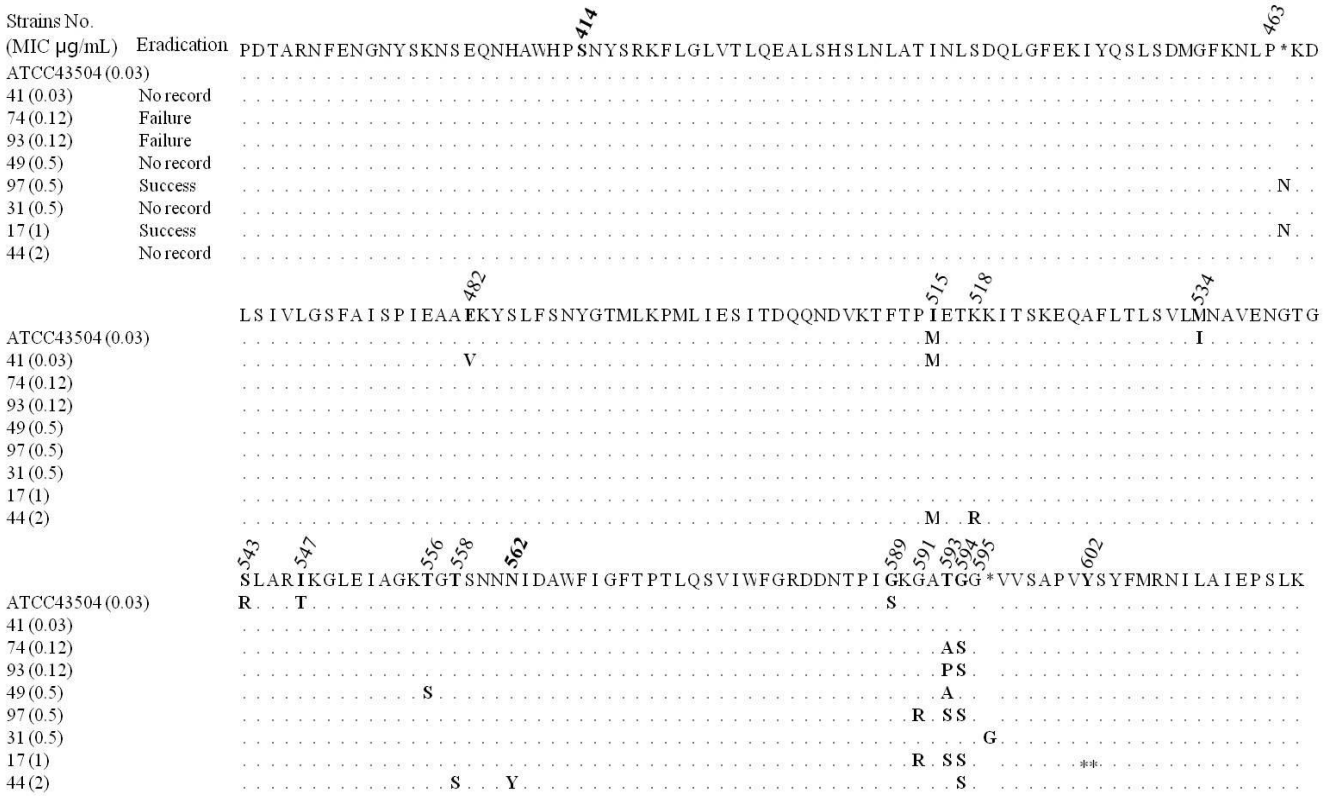
Strains No.
(MIC μg/mL) Eradication
PDTARNFENGNYSKNSEQNHAWHPSNYSRKFLGLVTLQEALSHSLNLATINLSDQLGFEKIYQSLSDMGFKNLP*KD
ATCC43504 (0.03)
41 (0.03) No record
74 (0.12) Failure
93 (0.12) Failure
49 (0.5) No record
97 (0.5) Success
31 (0.5) No record
17 (1) Success
44 (2) No record
LSIVLGSFAISPIEAAEKYSLFSNYGTMLKPMLIESITDQQNDVKTFTPIETKKITSKEQAFLTLSVLMNAVENGTG
ATCC43504 (0.03)
41 (0.03)
74 (0.12)
93 (0.12)
49 (0.5)
97 (0.5)
31 (0.5)
17 (1)
44 (2)
SLARIKGLEIAGKTGTSNNNIDAWFIGFTPTLQSVIWFGRDDNTPIGKGATGG*VVSAPVYSYFMRNILAIEPSLK
ATCC43504 (0.03)
41 (0.03)
74 (0.12)
93 (0.12)
49 (0.5)
97 (0.5)
31 (0.5)
17 (1)
44 (2)

Figure 3. The correlation of minimal inhibitory concentration of amoxicillin with the presence of *Helicobacter pylori* PBP1 mutations. The S414 and N562 amino acid substitutions potentially involved in amoxicillin resistance[66-68]. MICs of isolates were determined by agar dilution and the PBP1 mutations were assessed by PCR sequence analysis. Gaps in the amino acid sequences are marked by substituted sequence and identical amino acid is indicated with dots. Nucleic acid insertion[*] and deletion[**]

Sequence analysis of *rdxA*

Amino acids were identified in the full length of the *rdxA*. Diverse genetic changes occurred in 28 strains that had a cut-off value of ≥ 8 μg/mL. In the resistant strains, no identical mutation changes were observed (Figure 4). In 5 strains, the following substitutions occurred: substitutions position of mutation $CAG_{150-152}$ to TAG in strain No. 31 (MIC 8 μg/mL), $GAA_{399-402}$ to TAA in No. 71 (MIC 32 μg/mL), $CAA_{195-197}$ to TAA in No. 17 (MIC 64 μg/mL), $CAG_{150-152}$ to TAG in No. 100 (MIC 64 μg/mL) and $CAG_{150-152}$ to TAG in No. 75 (MIC 128 μg/mL). Finally, the substitution ended up the stop codon . In the strain No. 36 (MIC 16 μg/mL) the deletion of "TT" from $\underline{TT}AAT_{459-463}$ and in the strain No. 47 (MIC 256 μg/mL) the deletion of "CATGGCA" from $TA\underline{CATGGCA}AAA_{300-311}$ occurred, this resulted in the substitution of an amino acid shown in Table 5.

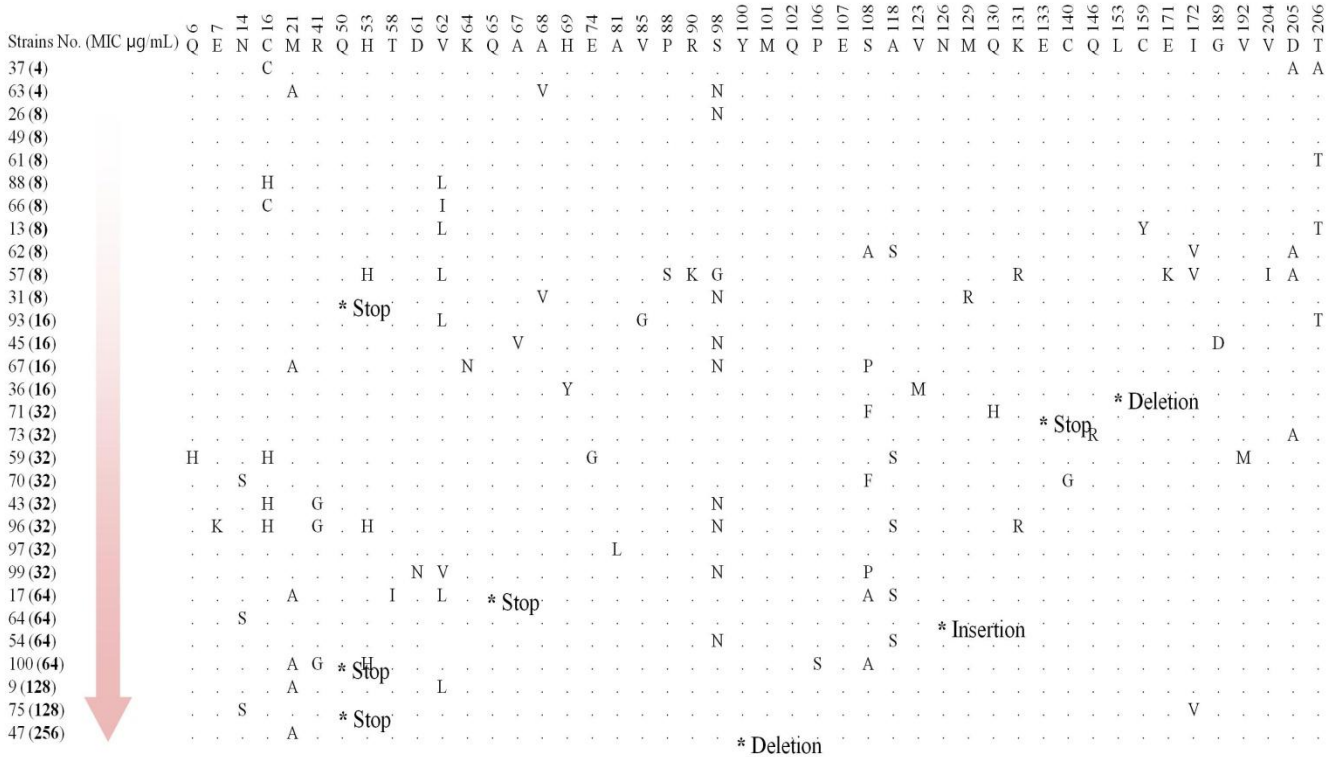
Strains No. (MIC μg/mL)
6 7 14 16 21 41 50 53 58 61 62 64 65 67 68 69 74 81 85 88 90 98 100 101 102 106 107 108 118 123 126 129 130 131 133 140 146 153 159 171 172 189 192 204 205 206
Q E N C M R Q H T D V K Q A A H E A V P R S Y M Q P E S A V N M Q K E C Q L C E I G V V D T
37 (4)
63 (4)
26 (8)
49 (8)
61 (8)
88 (8)
66 (8)
13 (8)
62 (8)
57 (8)
31 (8)
93 (16)
45 (16)
67 (16)
36 (16)
71 (32)
73 (32)
59 (32)
70 (32)
43 (32)
96 (32)
97 (32)
99 (32)
17 (64)
64 (64)
54 (64)
100 (64)
9 (128)
75 (128)
47 (256)
* Stop
* Stop
* Stop
* Stop
* Deletion
* Insertion
* Stop
* Deletion

Figure 4. The correlation of minimum inhibitory concentration of metronidazole with the presence of *Helicobacter pylori* RdxA mutations. MICs of isolates were determined by agar dilution and the RdxA mutations were assessed by PCR sequence analysis. Gaps in the amino acid sequences are marked by substituted sequence and identical amino acid is indicated with dots.

Table 5. Genetic truncation and mutation in *Helicobacter pylori rdxA*

Strain Nos. (MIC, μg/mL)	Substitution	
31 (8)	$CAG_{150\text{-}152}$ → **T**AG	Q 50 Stop
36 (16)	TTA $AT_{459\text{-}463}$ → **A AT	L 153 Frameshift
71 (32)	$GAA_{399\text{-}401}$ → **T**AA	E 133 Stop
17 (64)	$CAA_{195\text{-}197}$ → **T**AA	Q 65 Stop
64 (64)	$AAC_{378\text{-}380}$ → AA(**AA**)C	N 126 Frameshift
100 (64)	$CAG_{150\text{-}152}$ → **T**AG	Q 50 Stop
75 (128)	$CAG_{150\text{-}152}$ → **T**AG	Q 50 Stop
47 (256)	TAC ATG GCA $AAA_{300\text{-}311}$ → TA* *** *** AAA	Y 100 Frameshift

* Deletion and ( ) insertion of 2 nucleotides.

Sequence analysis of the 16S rRNA gene binding to tetracycline

The tetracycline-resistant *H. pylori* strain (MIC value of ≥ 4 μg/mL) was not observed in this study. No substitution was occurred in the region of $AGA_{926\text{-}928}$. The C989T, T1103C, G1121A, and A1122T mutations did not appear to be consistent with the MIC results (Figure 5).

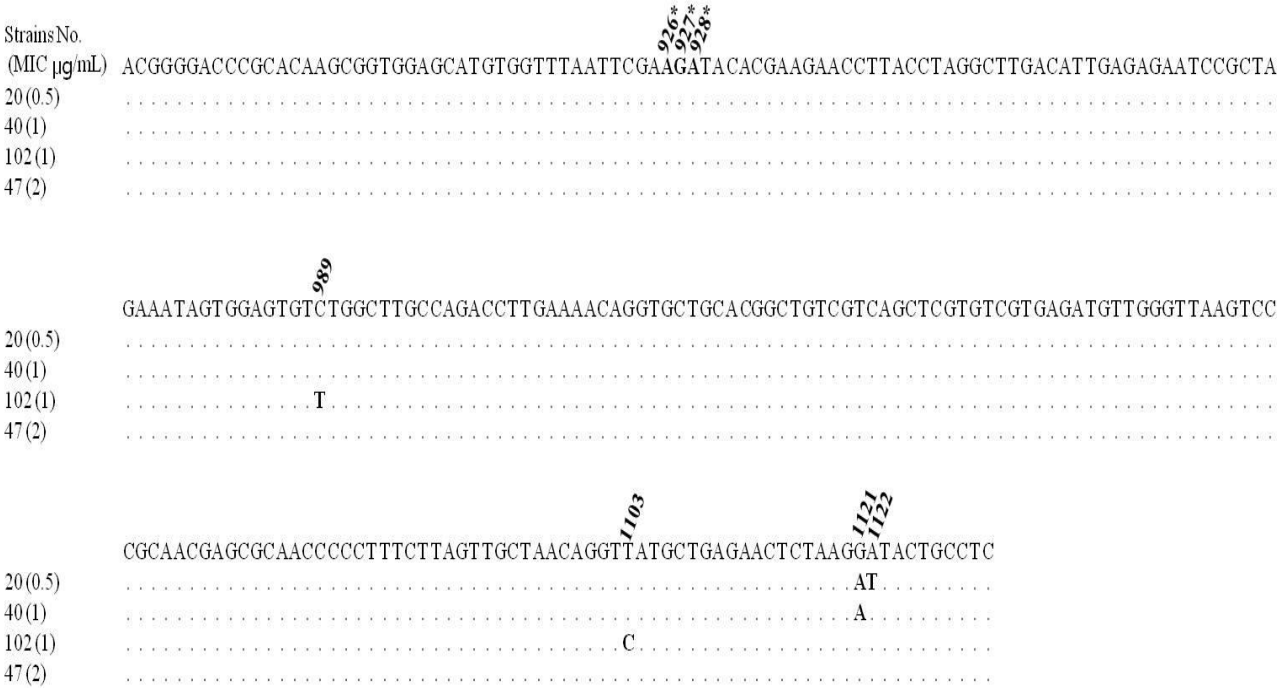
Strains No.
(MIC μg/mL)
926*
927*
928*
ACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGATACACGAAGAACCTTACCTAGGCTTGACATTGAGAGAATCCGCTA
20 (0.5)
40 (1)
102 (1)
47 (2)
989
GAAATAGTGGAGTGTCTGGCTTGCCAGACCTTGAAAACAGGTGCTGCACGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
T
1103
1121
1122
CGCAACGAGCGCAACCCCCTTTCTTAGTTGCTAACAGGTTATGCTGAGAACTCTAAGGATACTGCCTC
AT
A
C

Figure 5. The correlation of minimum inhibitory concentration of tetracycline with the presence of *Helicobacter pylori* 16S rRNA gene mutations. Nucleotide at position 926-928 was located in the front binding site of tetracycline. MICs of isolates were determined by agar dilution and the 16S rRNA gene mutations were assessed by PCR sequence analysis. Gaps in the nucleic acid sequence are marked by substituted sequence and identical nucleic acid is indicated with dots.

Sequence analysis of *gyrA*

Mutations were observed between amino acids 86 and 97 of the *gyrA* gene, which is known to be the major cause of resistance to quinolone antibiotics. The MIC of the levofloxacin-resistant *H. pylori* strains was ≥ 1 μg/mL; and substitution occurred in 11 of 19 resistant strains such as with A88V in strain No. 44 (MIC 2 μg/mL); N87K in strain No. 94 (MIC 2 μg/mL), No. 60 (MIC 8 μg/mL), No. 93 (MIC 8 μg/mL), No. 90 (MIC 16 μg/mL), No. 99 (MIC 16 μg/mL), No. 91 (MIC 16 μg/mL), No. 40 (MIC 32 μg/mL), and No. 55 (MIC 32 μg/mL); N87I in strain No. 101 (MIC 32 μg/mL); and D91N in strain No. 74 (MIC 8 μg/mL) are shown in Figure 6. In 57.8% (11/19) of the antibiotics-resistant strains with a MIC of ≥ 1 μg/mL, amino acid mutations occurred. As MICs are higher, the correlation in gene mutation becomes more consistant. In strain No. 63 (MIC 0.5 μg/mL) and No. 58 (MIC 1 μg/mL), although point mutation (amino acid N87, AAT→AAC) occurred, no change in the amino acid occurred.

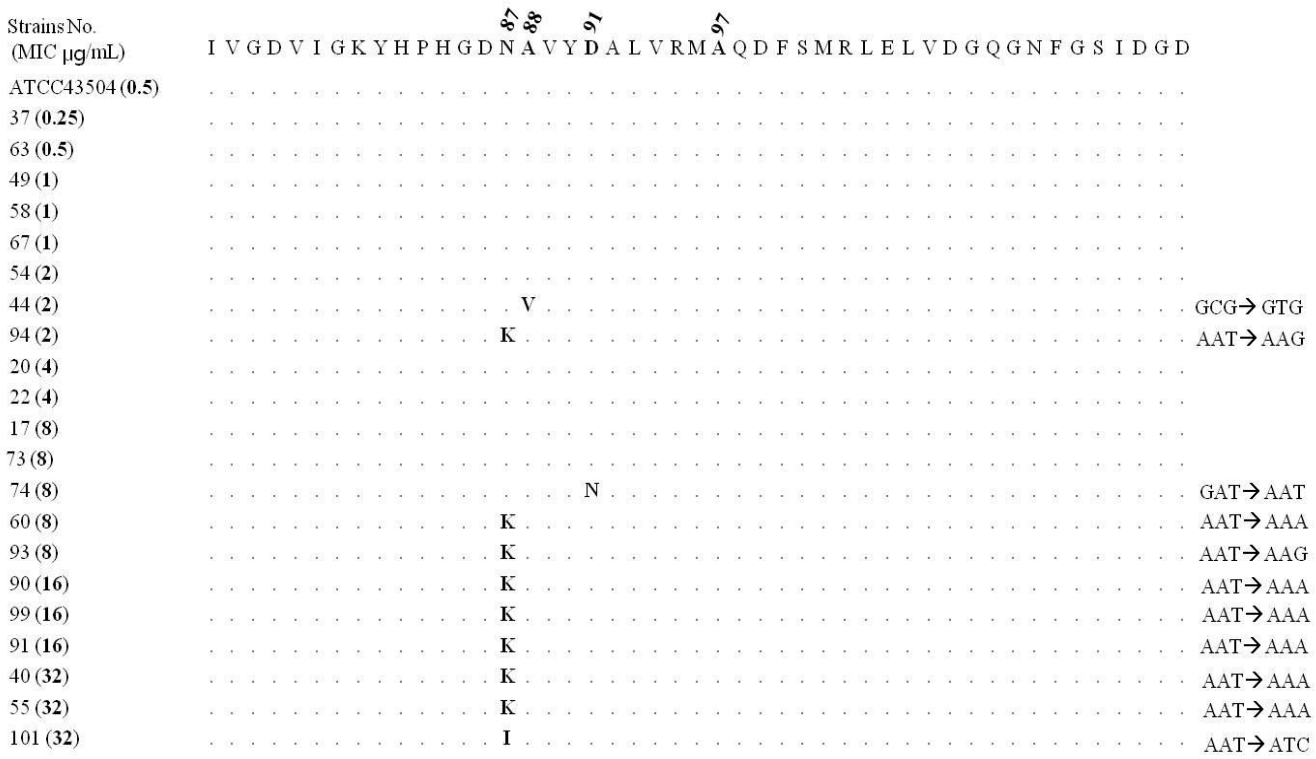
Strains No.
(MIC μg/mL)
87 88 91 97
I V G D V I G K Y H P H G D N A V Y D A L V R M A Q D F S M R L E L V D G Q G N F G S I D G D
ATCC43504 (0.5)
37 (0.25)
63 (0.5)
49 (1)
58 (1)
67 (1)
54 (2)
44 (2) V GCG→GTG
94 (2) K AAT→AAG
20 (4)
22 (4)
17 (8)
73 (8)
74 (8) N GAT→AAT
60 (8) K AAT→AAA
93 (8) K AAT→AAG
90 (16) K AAT→AAA
99 (16) K AAT→AAA
91 (16) K AAT→AAA
40 (32) K AAT→AAA
55 (32) K AAT→AAA
101 (32) I AAT→ATC

Figure 6. The correlation of minimum inhibitory concentration of levofloxacin with the presence of *Helicobacter pylori* GyrA mutations. The isolates were considered to be resistant when the MIC of levofloxacin was ≥ 1 μg/mL. MICs of isolates were determined by agar dilution and the GyrA gene mutations were assessed by PCR sequence analysis. Gaps in the amino acid sequences are marked by substituted sequence and identical amino acid is indicated with dots.

IV. DISCUSSIONS

In patients with peptic ulcer diseases and B-cell MALT lymphoma related to *H. pylori* infection, eradication therapy is performed[9, 10]. Currently, the availability of effective antibiotics for *H. pylori* eradication is limited. The eradication rate is 70-95%, and the major factor of eradication failure is considered to be the antibiotic resistance. The necessity of antimicrobial susceptibility test-based selection of antibiotics has risen compared to using conventional empirical treatment.

Antimicrobial susceptibility test in *H. pylori* is usually assessed with culture-based methods, but these tests are slow as the results are usually obtained only after 6-10 days. Furthermore, they fail in approximately 5-10% of the cases, and are difficult to compare between different institutions due to lack of standardization[71, 72]. In this light, molecular-based methods can offer an attractive alternative. They are independent of cell viability and growth rates of the bacteria and are easily standardized.

In this study, the molecular patterns in relation to *H. pylori* resistance to 5 antibiotics were investigated based on the antimicrobial susceptibility test and the eradication rate. A total of 82 strains of *H. pylori* isolated from the patients who had undergone endoscopy and whose samples had been cultured were tested to have an antimicrobial susceptibility by using the agar dilution on 5 antibiotics (clarithromycin, amoxicillin, metronidazole, tetracycline, and levofloxacin).

The strains resistance to clarithromycin was 8.5% (7/82), and MIC range was 4-128 μg/mL. The MIC range of the clarithromycin-susceptible strains was low (0.016-0.5 μg/mL), with a distinctive difference in the peaks of the two groups. This appeared to indicate that a factor responsible for determining the resistance to clarithromycin was clear. Having macrolides, point mutations in 23S rRNA are known to decrease the affinity of antibiotics to ribosomes[44]. In regions known to be principally responsible for resistance, A2142G/C and A2144G mutations did not occur. In all 5 clarithromycin-resistant strains, A2143G

mutations occurred (100%). In the United States, A2142G mutations were reported to have occurred in 48-53% of *H. pylori* strains, A2142C mutation in 0-7%, and A2143G mutations in 39-45%[27, 48]. In Japan, it was reported that mutations in A2144G were more than 93% while mutations did not occur in A2142C[30]. In Korea, mutations in A2143G were reported to be more than 71.4%[73]. T2182C mutations occurred at most of the MIC ranges, and A2223G mutations were occurred in each one of susceptible and resistant strains. These mutations are regarded as a non-specific mutation which did not affect the antibiotic resistance.

The eradication of *H. pylori* was not achieved in strains No. 37 (MIC 8 μg/mL) and No. 6 (MIC 16 μg/mL) because they had mutations at position A2143G, even though the strains were amoxicillin-susceptible. In contrast, eradication was possible in the strain No. 17 (MIC 1 μg/mL) and No. 97 (MIC 0.5 μg/mL) that were clarithromycin-susceptible, but amoxicillin-resistant. These results were consistent with the outcomes of previous studies which showed that clarithromycin resistance was more

important than the amoxicillin resistance in the clarithromycin and amoxicillin combination therapy[74].

From the gastric tissues of 12 patients whom the *H. pylori* eradication treatment (PPI, clarithromycin, and amoxicillin) failed and mutation were observed on *H. pylori* 23S rRNA. From the 12 samples collected from patients, there was no bacterial growth observed. Mutation in A2143C, a region known to be an action site of clarithromycin resistance, was observed in tissue No. 21. Remarkably, A2223G mutation (Figure 2) occurred in over 50% (6/12). Further studies are required to identify the effects of the mutation related with antibiotic resistance and the eradication rate.

The amoxicillin-resistant strains were 4.8% (4/82) with a MIC range of 0.016-2 μg/mL, which is the lowest MIC among the tested 5 antibiotics. Although resistance was observed at the cut-off value of ≥ 1 μg/mL, and it was observed only in the strain No. 44 (MIC 2 μg/mL) in relation to the resistant mutation of *pbp1* gene, and substitution with N562Y was considered as a

major cause. In another region that is known to be the cause of amoxicillin resistance, substitution with S414R did not occur. It is known that substitutions with S414R near the SKN motif and the N562Y KTG motif would inhibit binding of amoxicillin to PBP1. Substitutions of various amino acids near the motif are expected to influence the MIC values. Although the mutations were observed to have high MIC values, more investigations are required from more cases and diverse types of mutations.

The metronidazole-resistant strains were 46.3% (38/82) with a MIC range of 0.25-256 μg/mL which is the highest among the 5 antibiotics. In the United States and Europe, the metronidazole-resistant strains were 20-40%; and in developing countries, 50-80%. Recent studies in Korea reported that the proportion of the metronidazole-resistant strains was as high as at 10-27%[41, 42]. Despite this resistance rate, the use of metronidazole has steadily increased because of its eradication rate that is comparable with that of clarithromycin.

The treatment success rate of the clarithromycin-resistant strains is from 0 through 50%. Thus, clarithromycin resistance plays a decisive role in the eradication. Resistance in metronidazole has less clinical relevance because it results in a 25% decrease in the treatment success rate, compared with susceptible strains. In this study, a full-length amino acid was analyzed for the *rdxA* of 28 metronidazole-resistant strains. In the *rdxA*, 28.5% (8/28) truncation occurred, displaying a trend of occurring more at a high MIC than at a lower MIC (Figure 6). Five strains (Q50 in strain No. 31 MIC 8 μg/mL, E133 in No. 71 MIC 32 μg/mL, Q65 in No. 17 MIC 64 μg/mL, Q50 in No. 100 MIC 64 μg/mL, and Q50 in No. 75 MIC 128 μg/mL) came to have a stop codon due to point mutation. A frameshift occurred in 3 strains (L153 in strain No. 36 MIC 16 μg/mL, N126 in No. 64 MIC 64 μg/mL, and Y100 in No. 47 MIC 256 μg/mL) due to deletion and insertion (Table 5).

In *H. pylori*, the MIC for the metronidazole resistance was diverse in the range of 8 to ≥ 256 μg/mL and functional mutation

in *rdxA* tended to be more correlative as the MIC was getting higher. Metronidazole is activated within the target cells by one or more electron reductions. Studies have shown that the diversity of the MIC levels was due to the involvement of several electron acceptors and that the resistance to metronidazole disappeared under low oxygen conditions[75, 76]. Taken together, it is meaningful to identify *rdxA* mutation by the molecular detection method for metronidazole resistance, and identification of several genes that are involved in the reductase is also required. In addition, a susceptibility test should be considered under anaerobic conditions.

The most possible mechanism for tetracycline resistance in *H. pylori* is based on triple-bp substitution in three adjacent 16S rRNA residues, namely, $AGA_{926\text{-}928}$→TTC. In this study, no tetracycline resistance was observed in strains with MICs of ≥ 4 μg/mL. Mutation did not occur in the sequence analysis on the $AGA_{926\text{-}928}$ position in the strains with MICs of 0.5, 1, and 2

μg/mL. The C989T, T1103C, G1121A, and A1122T mutations are considered to be inconsistent with the MIC results.

Besides, this triple-bp substitution (i.e., $A_{926}G$, $A_{926}T$, $A_{928}C$, $AG_{926\text{-}927}GT$, and $A_{926}G/A_{928}C$) has been reported to be involved in tetracycline resistance in *H. pylori*[77]. Studies have reported that a deficit in the drug uptake, increased drug efflux, a change in the ribosomal protection proteins, and enzymatic inactivation of tetracycline are also responsible for the tetracycline resistance mechanism. However, the use of the molecular method is considered useful to detect $AGA_{926\text{-}928}$ mutation in a high level of MIC.

The use of fluoroquinolones is increasing worldwide as a rescue therapy regimen due to the failure of clarithromycin-based treatments. Because of this, resistance to fluoroquinolones sharply increased within a short period. A local study reported that the levofloxacin resistance drastically increased from 4.5% in 2003-2005 to 29.5% in 2005-2007[78]. Likewise, this study showed the similar results with 34.5% of resistance.

Fluoroquinolones inhibit the function of DNA gyrase by forming irreversible binding between the DNA and the DNA gyrase in the course of the DNA recombination caused by the DNA gyrase subunit A. The major cause of the resistance is known to be point mutation in amino acids 87, 88, 91, and 97 of the *gyrA*[67, 68]. The MIC of the levofloxacin-resistant *H. pylori* strains was ≥ 1 μg/mL; and in 11 of the 19 resistant strains, substitution occurred. N87K substitution occurred most commonly (8 strains) and substitution in N87I, A88V, and D91N occurred. In 27.8% of the resistant strains, amino acid mutation was observed, and the higher the MIC values was, the more distinctive the mutation was. For levofloxacin, the MIC level and the *gyrA* mutation showed a positive correlation.

In conclusion, the MIC values and the gene sequences that were responsible for the antibiotics resistance for 5 antibiotics were compared. The A2143G mutation of 23S rRNA gene was completely consistent with the MIC in the clarithromycin-resistant strains. N562Y mutation of the *pbp1* gene in the

amoxicillin-resistant strains was consistent with the MIC and *rdxA* truncation was observed in 28% of the metronidazole-resistant strains.

Analysis on $AGA_{926\text{-}928}$ in 16S rRNA genes did not show any point mutation because there was no tetracycline-resistant strain in MIC. The mutations in the QRDR regions of the *gyrA* were observed in 57.8% of the levofloxacin-resistant strains.

In this study, patterns of resistance mutation were identified in *H. pylori* strains isolated from Koreans. The results from this study would be useful for the development of molecular-based for detection of antibiotics resistance.

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국문요약

***Helicobacter pylori*의 항생제 내성에 관련된 유전자 변이 분석**

*H. pylori*는 급성 또는 만성위염의 원인이 되며, peptic ulcer disease, gastric cancer, mucosa associated lymphoid tissue (MALT) lymphoma로 진행할 수 있는 주요한 역할을 한다. *H. pylori* 제균은 위염과 소화성궤양에서 치유를 유도하며 위축성 위염과 위암으로 진행되는 것을 막는데 효과가 있는 것으로 알려져 있다. *H. pylori* 감염의 치료는 proton pump inhibitor (PPI)를 기본으로 하여 주로 clarithromycin, amoxicillin, metronidazole, tetracycline, 혹은 levofloxacin의 복합 요법이 이용된다. 최근 약제에 대한 내성률이 증가하여 제균 실패의 주요원인이 되며, 항균제 감수성검사를 기초로 적합한 항생제 선택이 필요하다. 하지만 낮은 배양률과 표준화된 방법이 없어 검사가 보편화되지 못하고 있다. 본 연구에서는 용인세브란스병원에 분리한 82 주의 *H. pylori*를 대상으로 5 종류의 치료제에 대하여 항균제 감수성검사를 시행하였고, 내성과 관련된 유전자 변이 분석과 치료효과 사이에 관련성을 알아 보았으며 내성유전자와 관련된 돌연변이 pattern을 확인하여 분자진단방법으로 항생제 내성을 예측 할 수 있는지 알아 보았다. Clarithromycin은 내성과 감수성 균주 사이에 뚜렷한 dual peaks를 보이고, 내성을 보이는 5 균주 모두에서 23S rRNA gene의 A2143G 돌연변이를 확인하였다. 또한 clarithromycin 내성, amoxicillin 감수성을 보이는 균주를 가진 2명의 환자에서 제균에 실패하였다. Amoxicillin에 내성을 보이는 균주 중에서, MIC 1-2 ㎍/mL인 4 균주에서는 PBP1의 S414와 N562 위치에 돌연변이는 없었으며 MIC 2 ㎍/mL인 한 균주에서 N562Y를 발견하였다. Metronidazole 내성을 보이는 28 균주에 대

하여 *rdxA* gene 전체 염기서열분석을 하였다. 8 균주 (28.5%)에서 nucleotide가 삽입, 삭제 또는 정지염기서열로 돌연변이 되어 기능적으로 결손이 되었다. Tetracycline 내성을 보이는 균주는 없었으며 MIC 0.5-2 μg/mL 범위의 균주에서 16S rRNA gene의 $AGA_{926-927}$ 돌연변이는 일어나지 않았다. Levofloxacin에 내성을 보이는 19 균주 가운데 11 균주 (57.8%)에서 GyrA에서 N87K (8 균주), N87I, A88V, D91N 돌연변이가 일어났다. 결론적으로 clarithromychin 내성과 관련된 23S rRNA gene A2143과 levofloxacin 내성과 관련된 GyrA의 N87, A88, D91 부위의 돌연변이는 고도의 내성을 보이는 균주에서 일치도가 높았으며, amoxicillin과 tetracycline에 내성을 나타내는 균수는 수가 적어 다양한 pattern을 볼 수는 없었으나 돌연변이부위를 분자진단방법으로 확인할 수 있었다. Metronidazole 내성을 결정하는 요인은 복합적인 요소가 작용하는 것으로 보인다. 이상의 결과로부터 한국에서 분리한 *H. pylori* 균주에 대하여 내성돌연변이 pattern을 확인할 수 있었으며, 분자적인 진단방법을 개발하는데 기초자료로 활용할 수 있을 것으로 사료된다.

핵심이 되는 말: *Helicobacter pylori*, clarithromycin, amoxicillin, metronidazole, tetracycline, levofloxacin, 23S rRNA, PBP1, *rdxA*, 16S rRNA, GyrA, mutation