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Analysis of Gene Mutations Associated with Antibiotic Resistance in *Helicobacter pylori* Strains Isolated from Korean Patients

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Background/Aims: This study aims to identify the gene mutation pattern associated with antibiotic resistance for mainly used antibiotics in *Helicobacter pylori* strains isolated from Koreans.
Materials and Methods: Seventy-one *H. pylori* strains were isolated from gastric mucosal biopsy specimens. The specimens were cultivated and the resistance to 5 antibiotics were assessed by using agar gel dilution method. DNA sequencing was carried out to detect the resistance-related gene mutations.
Results: A point mutation at A2143G of 23S rRNA was observed in all of the clarithromycin resistant strains, but tetracycline resistant strains were not found. Substitution N562Y in penicillin binding protein 1 were observed in an amoxicillin resistant strain (minimum inhibitory concentration [MIC] 2.0 μg/mL). Eleven (57.8%) out of 19 levofloxacin resistant strains showed amino acid substitution at N87K (8 strains), N87I, A88V and D91N in GyrA. The truncation in *rdxA* was detected in 8 (25.0%) out of 32 metronidazole resistant strains. Two out of the 7 patients who failed in first-line treatment of clarithromycin and amoxicillin showed A2143G mutation.
Conclusions: 23S rRNA mutation is closely related to the failure of eradication, however, the fact that five people who have no gene mutation failed eradication implies that other factors are related. As MIC levels in clarithromycin and levofloxacin resistance strains are getting higher, their appropriate gene mutation is more correlated. **(Korean J *Helicobacter* Up Gastrointest Res 2014;14:95-102)**

Key Words: *Helicobacter pylori*; Antibiotic resistance; 23S rRNA; Penicillin binding protein 1; GyrA

INTRODUCTION

Helicobacter pylori eradication from colonized stomach leads to healing of gastritis and peptic ulcer disease and probably also has beneficial effect on regression of atrophic gastritis and prevention of distal gastric cancer.[1] Failure of first-line treatment is usually related to insufficient patient compliance and/or development of antibiotic resistance. Most of the 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. To select an appropriate third-line treatment, endoscopy followed by bacterial culture and antimicrobial susceptibility testing is advisable. Culture-based methods offer the opportunity to determine the minimum inhibitory concentration (MIC) of antibiotics, they are time-consuming and their results show low reproducibility. Factors such as cell viability, inoculums size, incubation condition, and growth media may affect their outcome.[2] Molecular-based methods for antibiotic resistance are independent of these factors, and thus they have reproducible results and are easily standardized. 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 (CLA) remains the most powerful antibiotic available against *H. pylori* with MIC being the lowest as compared to the other molecules. Resistance to CLA in *H. pylori* is caused by point mutations in three adjacent 23S rRNA nucleotides, namely at position 2142, 2143, and 2144.[3] The reliable mechanism for tetra-

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cycline (TET) resistance in *H. pylori* is based upon a nucleotide-base pair substitution in three adjacent 16S rRNA residues, namely $AGA_{926-928}$ → TTC 3 base mutations.[4] It has been observed that the Ser414 to Arg substitution, adjacent to the SKN motif in PBP1, is responsible for amoxicillin (AMX) resistance with a significantly increased MIC.[5]

Another study reported that the Asn562 amino acid substituted to a Tyr residue in near KTG motif of PBP1.[6]

In *H. pylori*, the resistance to levofloxacin (LEV) 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.[7] Metronidazole (MET) is administered as a prodrug that needs to be activated within the target cell through one or two electron reduction processes. There are electron acceptors in *H. pylori* such as NAD(P)H flavin nitroreductase (*frxA*), oxygen-insensitive NAD(P)H nitroreductase (*rdxA*). And truncation in *rdxA* is more shown than in *frxA* as MIC level is higher in MET resistance.[8] Although resistant rate on *H. pylori* is continuously reported, data on gene mutation that influences on its treatment are not enough. This study aims to identify the gene mutation pattern associated with antibiotic resistance on mainly used antibiotics in *H. pylori* isolated from Koreans whose gene mutation data were not fully analyzed.

MATERIALS AND METHODS

1. Bacterial isolates

Antimicrobial susceptibility was tested against a total of 71 strains isolated from 71 patients underwent gastric endoscopy at the Yongin Severance Hospital of Yonsei University, Korea, from July 2009 to December 2010. Afterwards, their antibiotic resistance associated with gene mutation was identified. Thirty two out of 71 patients, who had a history of treatments including a 7 day first-line treatment with a proton pump inhibitor (PPI, 30 mg, twice a day), AMX (2,250 mg, three times a day), CLA (1,000 mg, twice a day) and a second-line treatment with PPI (30 mg, twice a day), bismuth (300 mg, twice a day), MET (2,250 mg, twice a day), and TET (1,000 mg, four times a day), were included in an eradication treatment. The eradication of *H. pylori* is verified in case of being negative in ^{13}C-urea breath test (Isotechnika, Edmonton, AB, Canada) after two months of drug administration.

This study was approved by the institutional review board of Yonsei University College of Medicine (No. 4-2011-0508).

2. Culture conditions

The medium used in this study was composed of Brucella broth (BBL, Sparks, MD, USA) containing 1.2% agar, 10% bovine serum and selected antibiotics (Oxoid Limited, Hampshire, UK) (10 μg/mL of vancomycin, 5 μg/mL of trimethoprim, 5 μg/mL of cefsulodin, and 5 μg/mL of amphotericin B). The fully minced biopsy specimens were incubated under 10% CO_2, 5% O_2 and 100% humidity at 37°C for 3~5 days. The *H. pylori* American Type Culture Collection (ATCC) 43504 and *H. pylori* strain 51 were cultured using the same methods described above for standard and quality control assessment.

3. Determination of MICs

Determination of MICs were examined for CLA (Sigma-Aldrich Co., St. Louis, MO, USA), TET (Sigma-Aldrich Co.), AMX (Sigma-Aldrich Co.), LEV (Sigma-Aldrich Co.), and MET (Sigma-Aldrich Co.) by slightly modified (Brucella broth base with 1.2% agar) agar dilution method recommended by the Clinical and Laboratory Standards Institute. CLA resistance was defined according to the Clinical and Laboratory Standards Institute approved breakpoint (≥1 μg/mL).[9] Isolates were classified as resistant to TET, AMX, LEV, and MET when the MICs were ≥4, ≥1, ≥1 and ≥8 μg/mL respectively. *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 CLA was 0.016~0.125 μg/mL, TET 0.125~1 μg/mL, AMX 0.016~0.125 μg/mL, LEV 0.064~0.5 μg/mL, and MET 64~256 μg/mL.

4. PCR and sequencing analysis

DNA extraction was performed on colonies that were isolated from the gastric biopsy using AccuPrep Genomic

Table 1. Oligonucleotides Used in This Study

Target gene	Primer	Oligonucleotides sequence (5'-3')[a]	Position[b]	Annealing temperature	Reference
Helicobacter pylori 16S rRNA	HPU185	CCTACGGGGGAAAGATTTAT	185 to 204, forward	52°C	10
	HPU826	AGCTGCATTACTGGAGAGACT	806 to 826, reverse		
rrn 23S (23S rRNA gene)	*rrn*23S-F	ATGAATGGCGTAACGAGATG	2051 to 2070, forward	52°C	This study
	*rrn*23S-R	GTCTTACAGTCAGGCTGGCT	2420 to 2439, reverse		
	*rrn*24S-SF	GAGATGGGAGCTGTCTCA			
rrn 16S (16S rRNA gene)	*rrn*16S-F	TGCAGCTAACGCATTAAGCATC	818 to 839, forward	54°C	This study
	*rrn*16S-R	GAGGCAGTATCCTTAGAGTTCT	1110 to 1131, reverse		
	*rrn*16S-SF	AAGCATCCCGCCTGGGG			
pbp1	*pbp1*-F	CCACGCAAGCCAAACGGC	1076 to 1093, forward	58°C	This study
	pbp1-R	CCTTTGGGGACATCAAACTTT	1857 to 1877, reverse		
	pbp1-SF	ATCGCTTTTGATAATGGCTATT			
gyrA	*gyrA*-F	GTGCATAGGCGTATTTTGTATG	142 to 163, forward	52°C	This study
	gyrA-R	CATTCTGGCTTCAGTGTAACG	373 to 393, reverse		
	gyrA-SF	GCGTATTTTGTATGCGATGC			
rdxA	*rdxA*-F1	TAGGGATTTTATTGTATGCTACG	969932 to 969911, forward	52°C	This study
	rdxA-R1	CCACAGCGATATAGCATTGCT	969458 to 969435, reverse		
	rdxA-SF1	GTATGCTACGAAAAATTCTAAA			
	rdxA-F2	GTTAGAGTGATCCCGTCTTTT	969543 to 969516, forward	52°C	This study
	rdxA-R2	CCTAAAAGAGCGATTAAAACCA	969181 to 969161, reverse		
	rdxA-SF2	TGCTTGGCGTGAGATTCAA			

[a]Oligonucleotides used for amplification were based on the published genome sequence of *Helicobacter pylori* strain 51 (GenBank accession CP000012). [b]Position of oligonucleotides are given to the mutation of antibiotics resistance of *H. pylori*.

Table 2. Distribution of Minimum Inhibitory Concentrations (MICs) for the 71 *Helicobacter pylori* Isolates Tested

MICs (μg/mL)	Number of strains (%)				
	Clarithromycin	Tetracycline	Amoxicillin	Levofloxacin	Metronidazole
0.016	4 (5.6)		14 (19.7)		
0.032	43 (60.6)		11 (15.5)		
0.064	18 (25.4)	12 (16.9)	17 (23.9)		
0.125		13 (18.3)	18 (25.4)	2 (2.8)	
0.25		35 (49.3)	7 (9.9)	20 (28.2)	1 (1.4)
0.5	**1 (1.4)**	8 (11.3)	2 (2.8)	30 (42.3)	2 (2.8)
1		2 (2.8)	**1 (1.4)**	**4 (5.6)**	2 (2.8)
2		1 (1.4)	**1 (1.4)**	**3 (4.2)**	24 (33.8)
4	**1 (1.4)**			**2 (2.8)**	10 (14.1)
8	**1 (1.4)**			**5 (7.0)**	**11 (15.5)**
16	**2 (2.8)**			**2 (2.8)**	**5 (7.0)**
32				**3 (4.2)**	**5 (7.0)**
64					**7 (9.9)**
128	**1 (1.4)**				**3 (4.2)**
256					**1 (1.4)**

Numbers indicated in bold represent resistant strains to respective antibiotics.

DNA Extraction Kit (Bioneer Co., Daejeon, Korea). The PCR primer was designed using the Oligo Program Version 6 (Molecular Biology Insights Inc., Cascade, CO, USA) shown in Table 1.[10] The template DNA (2 μL) was added to 18 μL aliquots of AccuPower PCR PreMix (Bioneer Co.). PCR was performed with initial denaturation at 94°C for 5 min, followed by 35 cycles with denaturation at 94°C for 50 sec, annealing at appropriate temperature (Table 1) for 50 sec and elongation step at 72°C for 1 min. Cycling was followed by a final extension at 72°C 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). 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 of *H. pylori* strain 51.

RESULTS

The MICs distributions for the 71 clinical isolates are described in Table 2. The clinical resistance rate shows CLA at 7.0%, TET at 0%, AMX at 2.8%, LEV at 26.8%, and MET at 45.1%. For the area of 163 bp (position 2137~2299) of 23S rRNA gene, six susceptible strains and five resistance strains are analyzed to identify their base sequences as shown in Fig. 1A. A2143G point mutation was observed in all the five resistant strains including strain No. 76, No. 37, No. 6, No. 91, and No. 44. But the mutation of T2182 and A2223 was observed in both resistant and susceptible groups. The TET-resistant *H. pylori* strain (MIC value of ≥4 μg/mL) was not observed in this study. No substitution was occurred in the region of $AGA_{926-928}$ in 16S rRNA gene. The C989T, T1103C, G1121A, and A1122T mutations did not appear to be consistent with the MIC results as shown in Fig. 1B.

For the area of 228 amino acids of *pbp1* gene, eight susceptible stains and two resistant strains are analyzed to identify their base sequences as shown in Fig. 2A. No substitution occurred in the position S414; a region known as AMX-resistant. Substitutions of I515M, K518R, T558S, N562Y, and G594S occurred in resistant strain No. 44 (MIC 2 μg/mL). In the strains No. 91 (MIC 0.5 μg/mL) and No. 17 (MIC 1 μg/mL), N was inserted at position 463 and substitutions of G591R, T593S and G594S occurred.

QRDR amino acid regions in *gyrA* were identified in 19 strains of LEV-resistant *H. pylori* as shown in Fig. 2B. N87K substitution was found in 8 strains and N87I, A88V and D91N substitutions in 3 strains, respectively. The full length amino acids in *rdxA* were identified in 32 strains of MET-resistant *H. pylori* as shown in Table 3. Genetic truncation appeared in five out of 8 strains, whose mutations were consequently substituted with stop codons. The other three strains, two of which were deleted and one of which was inserted, were finally frameshifted.

Thirty-two patients were monitored for the eradication program as shown in Table 4. The eradication with the

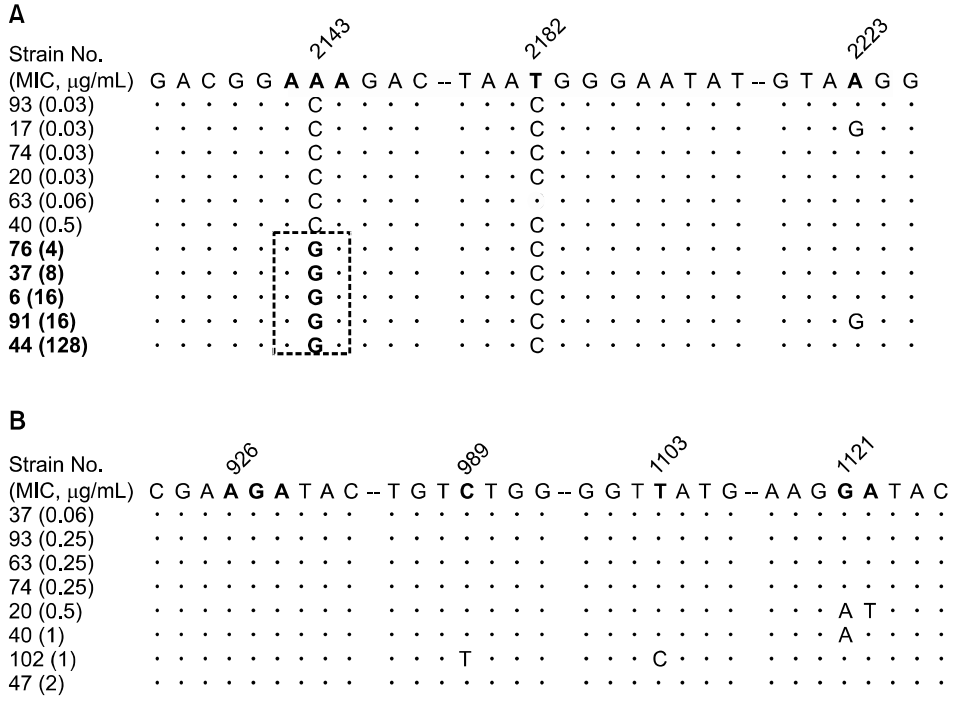

Fig. 1. The correlation of minimum inhibitory concentration against clarithromycin with *Helicobacter pylori* 23S rRNA gene mutations (A) and against tetracycline with *Helicobacter pylori* 16S rRNA gene mutations (B). Genetic sequencer were based on the published genome sequence of *Helicobacter pylori* strain 51 (GenBank accession CP000012). MIC, minimum inhibitory concentration.

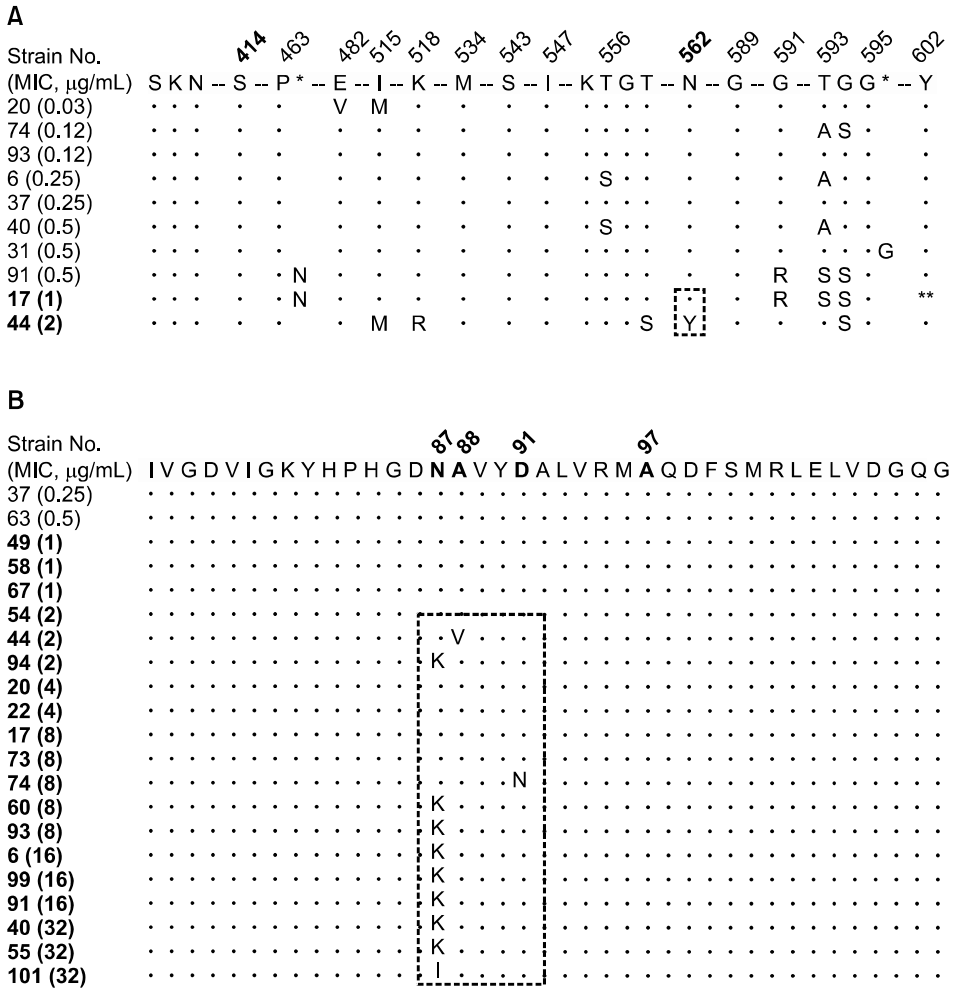

Fig. 2. The correlation of minimum inhibitory concentration against amoxicillin with PBP1 mutations (A) and against levofloxacin with GyrA mutations (B). Genetic sequencer were based on the published genome sequence of *Helicobacter pylori* strain 51 (GenBank accession CP000012). *Amino acid insertion and **deletion. MIC, minimum inhibitory concentration.

Table 3. Genetic Truncation in *rdxA* of *Helicobacter pylori*

Strain No. (MIC, μg/mL)	Substitution	
31 (8)	$CAG_{150\text{-}152}$ → TAG	Q 50 Stop
36 (16)	TTA $AT_{459\text{-}463}$ → **A AT	L 153 Frameshift
71 (32)	$GAA_{399\text{-}401}$ → TAA	E 133 Stop
17 (64)	$CAA_{195\text{-}197}$ → TAA	Q 65 Stop
64 (64)	$AAC_{378\text{-}380}$ → AA(AA)C	N 126 Frameshift
100 (64)	$CAG_{150\text{-}152}$ → TAG	Q 50 Stop
75 (128)	$CAG_{150\text{-}152}$ → TAG	Q 50 Stop
47 (256)	TAC ATG GCA $AAA_{300\text{-}311}$ → TA* *** *** AAA	Y 100 Frameshift

MIC, minimum inhibitory concentration.
*Deletion and ( ) insertion of 2 nucleotides.

first-line therapy was achieved in 82.7% (24/29) in the strains susceptible to both CLA and AMX. The CLA-susceptible and AMX-resistant isolate was successful in eradication, also. However the first-line therapy was failed in five isolates, even though those were susceptible both to CLA and AMX and had no gene mutations. The eradication in 2 CLA-resistant and AMS-susceptible isolates were failed with the first-line therapy, but were successful with the second-line therapy. The mutations found in these strains were A2143G mutation in 23S rRNA.

Table 4. The Effect of Eradication and Gene Mutation of *Helicobacter pylori* Isolated from Treatment Group

1st			2nd			Strain No.	Gene mutation
Clarithromycin	Amoxicillin	Eradication (Number of patients)	Metronidazole	Tetracycline	Eradication		
S	S	Succeeded (24)					No
		Failed (5)	S	S	Succeeded	20	No
			S	S	Failed	63	
			S	S	No record	74	
			S	S	No record	40	
			R	S	No record	93	
R	S	Failed (2)	S	S	Succeeded	6, 37	23S rRNA A2143G
S	R	Succeeded (1)				17	No

Gene mutations regions were targeted at 23S rRNA gene in clarithromycin, at *pbp1* in amoxicillin, at *rdxA* in metronidazole and at 16S rRNA gene in tetracycline. S, susceptible; R, resistant.

DISCUSSION

The eradication rate of *H. pylori* infection is generally around 70~95%, and the antibiotic resistance is considered to be the main factor of eradication failure. Antimicrobial susceptibility test in *H. pylori* is generally performed in a culture-based method, which takes about 6 to 10 days. Moreover, comparing the results with other organizations is not easy due to the lack of standardization. On the contrary, molecular-based methods 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.

The resistant strains to CLA were 7.0% (5/71), and MIC range was 4~128 μg/mL. The MIC range of the CLA-susceptible strains was low (0.016~0.5 μg/mL), with a striking difference in the peaks of the two groups. This indicated that a certain factor is responsible for determining the resistance to CLA. In macrolides, point mutations in 23S rRNA are known to decrease the affinity of antibiotics to ribosomes. Only A2143G mutation occurs in all the five strains, whose base sequences can be identified in CLA-resistant group, and it corresponds to their MIC value.

In this study, A2142G/C and A2144G mutations known to be responsible for resistance did not occur. In the United States, A2143/2144G was reported in 97% of *H. pylori* strains, A2143C in 7%.[11] In Japan, A2143/2144G was reported in 100%.[12] However, T2182 and A2223 are considered as non-specific genetic polymorphism since they occur in both resistant and susceptible groups.

TET resistance of *H. pylori* is based on triple-bp substitution in three adjacent 16S rRNA residues, namely $AGA_{926-928}$→TTC. In this study, TET resistance was not observed in strains that show MICs of ≥4 μg/mL and the strains within MICs of 0.06, 0.25, 0.5, 1, and 2 μg/mL did not show any $AGA_{926-928}$ mutations. However, the C989T, T1103C, G1121A and A1122T mutations are considered to be inconsistent with the MIC results. According to other studies, triple-bp substitutions (i.e., A926G, A926T, A928C, AG926-927 GT, and A926G/A928C) have been reported to be involved in TET resistance in *H. pylori*.[4] Therefore, the use of the molecular method is considered useful to detect $AGA_{926-928}$ mutation in a high level of MIC.

The AMX-resistant strains were 2.8% (2/71) with a MIC range of 1~2 μg/mL and susceptible group showed 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 S414R known as the cause of AMX resistance, substitution did not occur. Substitutions of various amino acids near the motif are considered to influence the MIC

values, so more investigations are required from diverse types of mutations concerning with resistance.

Resistance to fluoroquinolones sharply increased within a short period. A local study reported that the LEV resistance had increased from 4.5% in 2003～2005 to 29.5% in 2005～2007.[13] Likewise, this study showed the similar results within 26.8% of resistance. The mutations in the QRDR regions of the gyrA were observed in 57.8% (11/19) of the LEV-resistant strains. N87K is the most common substitution followed by N87I, A88V, and D91N. The higher the MIC values were, the more distinctive the mutations were, which shows more correlation.

The MET-resistant strains were 45.1% (32/71) with a MIC range of 0.25～256 μg/mL which is the highest among the 5 antibiotics. The MET-resistant strains were 20～40% in the United States and Europe and 50～80% in developing countries. Recent studies in Korea reported that MET-resistant strains were 10～27%.[14] Despite this resistance rate, the use of MET has steadily increased because of its eradication efficacy, which is as effective as CLA. In this study, a full-length amino acid was analyzed in the *rdxA* of MET-resistant strains and *rdxA* truncation was observed in 25.0% (8/32). Five strains came to have a stop codon due to point mutation. A frameshift occurred in 3 strains due to deletion and insertion. These genetic truncations showed a trend that occurs more at a high MIC than at a low MIC. Studies have shown that the diversity of the MIC levels was caused by the involvement of several electron acceptors and that the resistance to MET disappeared under low oxygen conditions.[15] Therefore, it is meaningful to identify *rdxA* mutation by the molecular detection method for MET resistance and it is also required to identify other several genes that are involved in the reductase.

As MIC levels in CLA, AMX, and LEV resistance strains are getting higher, their gene mutation is more correlated. Afterwards, the TET resistance stains are required more case studies to find more information about its gene mutation. In our study, the reason why resistant rate is somewhat low in the same period is that many of the patients have no eradication therapy history of *H. pylori* infection. Also, regional factors are considered to have an influence on it. Though two strains, No. 37 (MIC 8 μg/mL) and No. 6 (MIC 16 μg/mL), from the 7 patients who failed in first-line treatment of CLA and AMX are AMX-susceptible, A2143G mutation of CLA-resistant is considered to effect on eradication failure. However, the other 5 strains failed in eradication, even though they are susceptible and have no gene mutation. We could not find the reason for eradication failure in our study and insufficient patient compliance or heteroresistance strains might be the possible factors, according to the previous paper.[16] On the other hand, eradication was possible in the strain No. 17 (MIC 1 μg/mL), which was CLA-susceptible. In combined treatment, AMX-resistance is less influential than CLA-resistant strains. 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.

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