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Changing pattern of antimicrobial resistance of *Helicobacter pylori* in Korean patients with peptic ulcer diseases

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Background. Antibiotic resistance of *Helicobacter pylori* is problematic because it reduces the efficacy of eradication therapy. It has been suggested that the incidence of resistance is rising. In Korea, information on the antimicrobial resistance of *H. pylori* is rare. The aim of this study was to assess the prevalence of *H. pylori* antibiotic resistance at a single center in Korea, and the changes in its antimicrobial resistance, and to detect the mutation foci of clarithromycin-resistant strains. ***Methods.*** *H. pylori* isolates obtained from 224 patients with peptic ulcer disease in Korea between June 1996 and March 2000 were tested for antimicrobial resistance. The minimum inhibitory concentration (MIC) for metronidazole and clarithromycin was determined by the broth microdilution method. Isolates were considered resistant when the MIC was more than 8μg/ml for metronidazole and more than 1μg/ml for clarithromycin. To detect *H. pylori* 23S rRNA mutations, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed. Sequencing was performed on the two strands of the nonrestricted amplicons. ***Results.*** Overall, resistance to metronidazole and clarithromycin was detected in 41.9% and 5.4% of patients, respectively. There was no significant difference in metronidazole and clarithromycin resistance according to age group and sex. Six strains were resistant to both metronidazole and clarithromycin. Six of nine clarithromycin-resistant isolates possessed the A2144G mutation in the gene encoding 23S rRNA. Sequencing of the three non-restricted clarithromycin-resistant strains revealed a T-to-C mutation at position 2182. ***Conclusions.*** In Korea, there was no significant increase in the prevalence of metronidazole resistance, but clarithromycin-resistant *H. pylori* strains had increased relatively over the 5-year period. There was an increasing tendency for the emergence of strains with dual resistance to metronidazole and clarithromycin. Many of the clarithromycin-resistant strains possessed the A2144G mutation.

Key words: *Helicobacter pylori*, antimicrobial resistance, clarithromycin, metronidazole

Introduction

Helicobacter pylori infection is a major cause of chronic active gastritis and peptic ulcer disease, and has been implicated in the development of gastric adenocarcinoma and lymphoma.[1] Eradication of *H. pylori* infection is essential for patients with *H. pylori*-associated peptic ulcer diseases.[2] The proton pump inhibitor (PPI)-based triple therapies are currently the most popular first-line treatment, followed by bismuth or ranitidine-bismuth-citrate-based triple therapies.[3]

Recently, antimicrobial resistance has become a growing problem in *H. pylori* treatment because it is an important cause of eradication failure of *H. pylori* infection.[4] The prevalence of *H. pylori* resistance to metronidazole ranges from about 20% to more than 50% in the United States,[4,5] from 10% to 50% in Europe,[6] and from 26.8% to 49.4% in East Asia.[7,8] Recently, it has been suggested that the prevalence of resistance to clarithromycin has significantly increased in many countries. The prevalence of *H. pylori* resistance to clarithromycin varies from 7% to 14% in the United States[4,5] and from 0% to 15% in Europe,[6] and it is around 10% in East Asia.[7,8]

In Korea, the prevalence of *H. pylori* infection is about 60%–70% in adults, but information on the antimicrobial resistance of *H. pylori* is difficult to obtain; especially, resistance to clarithromycin.

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In this study, we evaluated the prevalence of the primary resistance of *H. pylori* to metronidazole and clarithromycin at a single center in Korea, and the changes in its antimicrobial resistance, and we also determined the mutation foci in clarithromycin-resistant strains.

Subjects and methods

Patients

Between June 1996 and March 2000, 224 consecutive newly diagnosed peptic ulcer disease patients whose *H. pylori* strains were isolated at the Gastroenterology Unit of Hanyang University Kuri Hospital were enrolled in the study.

Patients who received bismuth compounds, antisecretory drugs, or antimicrobial agents in the 4 weeks before endoscopy were excluded. Other exclusion criteria included previous gastric surgery, prior treatment for *H. pylori* infection, and any of several concomitant medical illnesses, including cardiac, respiratory, renal, and liver diseases.

Strains and preparations used for antimicrobial susceptibility testing

Biopsy specimens for culture were obtained during endoscopy from the antrum. The specimens were immediately transported in 20% glucose solution to the microbiology laboratory. *H. pylori* was cultured from gastric biopsy specimens on blood agar plates with 5% sheep blood under microaerophilic conditions for 3–6 days. *H. pylori* was identified by Gram staining and by positive urease, oxidase, and catalase tests. Suspensions of *H. pylori* were stored at −80°C in brain heart infusion (BHI) broth with 20% glycerol.

H. pylori was subcultured on BHI agar with 7% horse blood and incubated at 37°C under microaerophilic conditions. With *H. pylori* isolates, antimicrobial susceptibility tests were performed by the broth microdilution method. Suspensions of 2.0 McFarland standard in BHI were used as the inoculum for the broth microdilution method.

Broth microdilution method

Metronidazole (Sigma, Seoul, Korea) and clarithromycin (Abbott, Seoul, Korea) were dissolved in distilled water and ethanol, respectively, and were subsequently diluted in distilled water. Ninety-six-well microplates, with 160 μl of BHI broth containing metronidazole in concentrations ranging from 0.25–128 μg/ml, or clarithromycin in concentrations ranging from 0.125 to 64 μg/ml, were prepared. A 20-μl suspension of each isolate was inoculated into the antimicrobial-containing plates. Plates were incubated for 72 h at 37°C under microaerophilic conditions.

Definition of susceptibility

After 72-h incubation under microaerophilic conditions, the minimal inhibitory concentrations (MICs) were determined. The MIC was determined as the lowest concentration producing complete growth inhibition. Isolates were considered resistant when the MIC was more than 8 μg/ml for metronidazole and more than 1 μg/ml for clarithromycin.[9]

Detection of mutation foci in clarithromycin-resistant strains

Strains initially identified as clarithromycin-resistant were subcultured. Genomic DNA was prepared from confluent plate cultures of clarithromycin-resistant isolates with InstaGene Matrix (BioRad Laboratories, Hercules, CA, USA).

The peptidyltransferase region of the *23S rRNA* gene, which contains the known sites of mutation associated with clarithromycin resistance, was amplified. A forward primer from positions 2191 to 2210 and a reverse primer from positions 2596 to 2615 were used to amplify a 425-bp fragment.

Polymerase chain reaction (PCR) amplification of DNA was performed using 1 μg of genomic DNA, 1 μM of primer, 2 units of Taq DNA polymerase, and a 0.2 mM concentration of deoxynucleoside triphosphate mixture with standard PCR buffer in a volume of 50 μl, for 35 cycles, with the following cycling conditions: denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s, in a Thermal Cycler 9600 (Perkin-Elmer, Norwalk, CT, USA). The PCR amplified product was then purified.

PCR restriction fragment length polymorphism (RFLP) was performed to detect point mutations associated with clarithromycin resistance. Ten μl of purified PCR product was incubated with the restriction enzymes, *Bsa*I and *Bbs*I (New England Biolabs, Beverly, MA, USA), in order to detect the restriction site occurring when the mutation was A-to-G at position 2144 and at position 2143, respectively. Products were then electrophoresed in 2% agarose gels.

Sequencing was performed on the two strands of the non-restricted amplicons, using the ABI PRISM 377XL DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Results

Prevalence of and changes in antibiotic resistance of H. pylori

This study included *H. pylori* isolates from 224 patients (168 males, mean age 43.5 years; 56 females, mean age 47.7 years) who satisfied the inclusion and exclusion criteria.

Overall, resistance to metronidazole and clarithromycin was detected in 41.9% and 5.4% of patients, respectively. Resistance to metronidazole was detected in 41.8% (23/55), 44.4% (28/63), 37.5% (3/8), 44.9% (22/49), and 35.7% (15/42) of the patients in each year from 1996 to 2000, respectively. There was no significant increase in the prevalence of metronidazole resistance over the 5-year period in Korea.

However, resistance to clarithromycin was detected in 1.6% (1/62), 3.2% (2/63), 12.5% (1/8), 12.2% (6/49), and 4.8% (2/42) of the patients in each year from 1996 to 2000, respectively (Table 1). The prevalence of clarithromycin resistance has increased relatively over the 5-year period in Korea. Six of 12 strains resistant to clarithromycin were also resistant to metronidazole. The prevalence of strains with dual resistance has increased over the period.

Distribution of the MICs of metronidazole against *H. pylori* strains showed a continuous spectrum, while the distribution of the MICs of clarithromycin showed relatively distinct separation between sensitive and resistant strains (Figs. 1, 2).

There was no significant difference in resistance to metronidazole or clarithromycin, or both, according to age group or sex.

Mutations in the 23S rRNA gene in clarithromycin-resistant strains

PCR-RFLP was performed to detect point mutations in the *23S rRNA* gene associated with clarithromycin resistance in 9 of the 12 clarithromycin-resistant strains. Among the 9 clarithromycin-resistant strains examined, 6 strains were restricted with *Bsa*I, which means that there was an A2144G point mutation in the *23S rRNA* gene (Fig. 3). The amplicons of the other 3 strains were not restricted with either the *Bsa*I or the *Bbs*I enzyme, which means that there was no mutation at positions 2144 and 2143 (Fig. 4). Sequencing of these non-

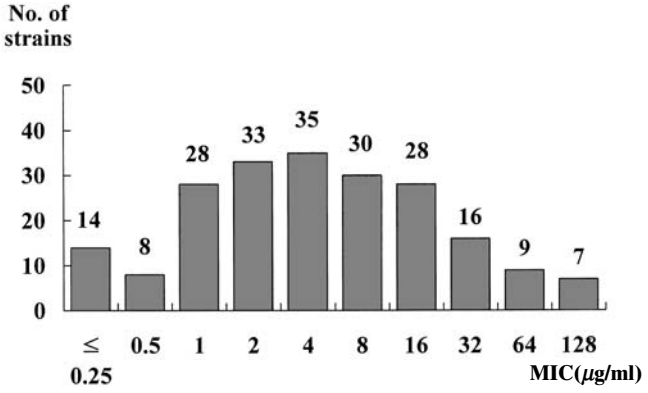

Fig. 1. Distribution of minimum inhibitory concentrations (*MICs*) among the 224 *H. pylori* isolates for metronidazole showed a continuous spectrum

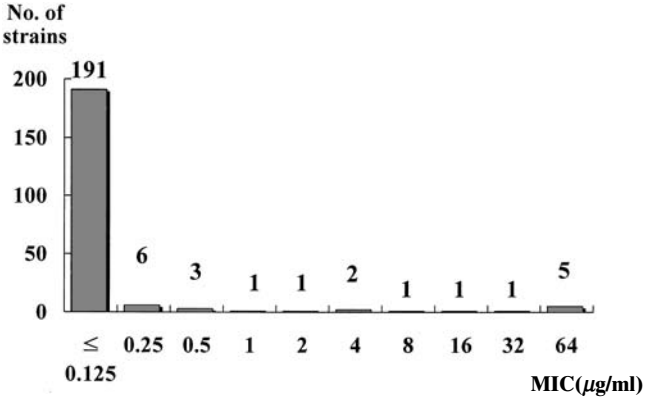

Fig. 2. Distribution of minimum inhibitory concentrations (*MICs*) among the 224 *H. pylori* isolates for clarithromycin showed distinct separation between sensitive and resistant strains

Table 1. Changes in the prevalence of metronidazole and clarithromycin resistance in *Helicobacter pylori* over the 5-year period 1996–2000

Year of isolation	MTZ resistance		CLA resistance	
	Number of isolates	MTZ-R (%)	Number of isolates	CLA-R (%)
1996	55	41.8	62	1.6
1997	63	44.4	63	3.2
1998	8	37.5	8	12.5
1999	49	44.9	49	12.2
2000	42	35.7	42	4.8

MTZ-R; Proportion of *H. pylori* isolates resistant to metronidazole; CLA-R, proportion of *H. pylori* isolates resistant to clarithromycin

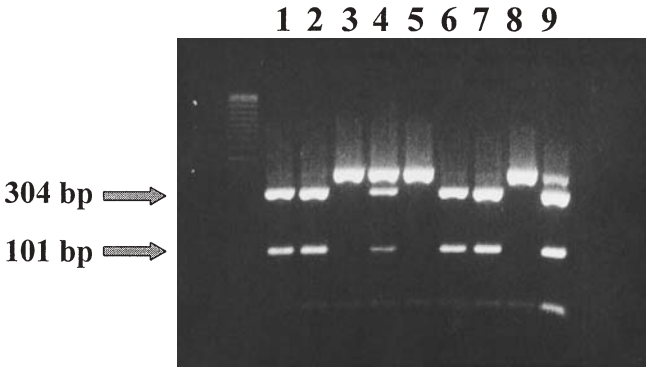

Fig. 3. Detection of the point mutation at 2144 in the *23S rRNA* gene by *Bsa*I digestion of polymerase chain reaction (PCR) products. Strains 1, 2, 4, 6, 7, and 9 were restricted with *Bsa*I, which means that there was an A-to-G mutation at position 2144

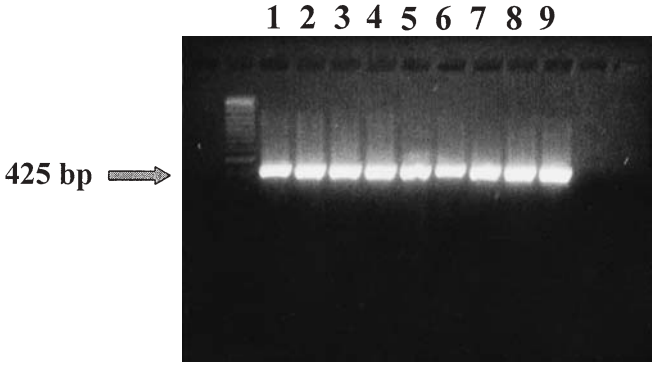

Fig. 4. Detection of the point mutation at 2143 in the *23S rRNA* gene by *Bbs*I digestion of 425-bp PCR products. There was no restriction of PCR products by the enzyme *Bbs*I, which means that there was no point mutation at position 2143

restricted 425-bp PCR products from 3 clarithromycin-resistant strains revealed a T-to-C mutation at position 2182 (Fig. 5).

The MICs of most of the A2144G mutant strains were relatively low, ranging from 1 to 8 μg/ml. In contrast, the MICs of the T2182C mutants were relatively high, ranging from 16 to over 64 μg/ml (Table 2).

Discussion

The eradication of *H. pylori* facilitates the healing and prevents the recurrence of peptic ulcer. There are many treatment options to cure *H. pylori* infection. Currently, the most popular treatment strategy for *H. pylori* infection is a combination of a PPI and two antibiotics.[3]

In Korea, the overall prevalence of *H. pylori* infection is about 46.6%, and the prevalence increases with age, at 17.2% in children and 66.9% in adults.[10] Eradication rates with a 7-day schedule of a PPI-based triple regimen have been reported to be about 80.6% (range, 53.2%–93.1%) by intention-to-treat analysis and 90.0% (range, 85.2%–93.1%) by per-protocol analysis in

Table 2. Minimum inhibitory concentrations (MICs) and mutation profiles of nine clarithromycin-resistant strains

Strains	Clarithromycin MIC (μg/ml)	Mutation site
1	≥64	A2144G
2	8	A2144G
3	≥64	T2182C
4	2	A2144G
5	≥64	T2182C
6	4	A2144G
7	1	A2144G
8	16	T2182C
9	8	A2144G

Fig. 5. DNA sequence of a clarithromycin-resistant strain that was not restricted with either *Bsa*I or *Bbs*I revealed a T-to-C mutation at position 2182

Korea.[11] A combination of clarithromycin, amoxicillin, and a PPI is the most popular treatment regimen, due to the high prevalence of metronidazole-resistant *H. pylori* strains in Korea.

Antibiotic resistance is a growing problem in *H. pylori* treatment. The increasing prevalence of strains resistant to commonly used antimicrobial agents is an important cause of failure to eradicate *H. pylori* infection.[4]

There is little information on the antimicrobial resistance of *H. pylori* in Korea. The prevalence of metronidazole-resistant *H. pylori* is known to be as high as 40%–50%. Recently, an increasing prevalence of resistance to clarithromycin has been reported from a few studies, ranging from 5.9% to 20.3%.[12,13] The present study showed that primary resistance of *H. pylori* to metronidazole and clarithromycin was found in 41.9% and 5.4% of patients, respectively, in Korea.

Metronidazole resistance is generally high in most Asian countries; for example, it is 49.4% in Hong Kong.[7] The metronidazole resistance rate, 41.9%, in our study is comparable with data reported from other Asian countries.[7,14,15] Metronidazole has been widely used for the treatment of anaerobic infections, amebic infections, and gynecological infections in Korea. There was no significant increase in the prevalence of metronidazole-resistance over the past 5 years in our study, and no significant difference in the prevalence of metronidazole resistance was found between men and women in this study.

The present study showed that the prevalence of clarithromycin resistance has relatively increased over the past 5-year period in Korea. Although the overall prevalence of primary resistance to clarithromycin, 5.4%, was still low, the increase in prevalence in this short period is problematic. Recently, an increasing prevalence of resistance to clarithromycin has been reported in many countries.[5,16–18] The use of clarithromycin has been increased not only for *H. pylori* eradication treatment but also for respiratory infections in Korea. Therefore, clarithromycin resistance may continue to increase in the future.

In this study, there was no significant difference in the prevalence of clarithromycin resistance according to age group or sex. In some studies, age and sex of the patient had a significant effect on the resistance rates, with the older individuals and females having higher rates.[5,19] Although the reason for our result being different from these studies is not clear, regional differences, in the preference and indications for antibiotics may have had an effect.

In general, metronidazole-resistant *H. pylori* shows a continuous spectrum of MICs, while clarithromycin resistance presents as a bimodal distribution, with isolates being characterized as sensitive or resistant.[4] In our study, the distribution of MICs for metronidazole and clarithromycin supported this pattern. We considered the isolates resistant to metronidazole when the MIC of the isolates was more than 8μg/ml, in agreement with other studies,[9] but the MICs of the *H. pylori* isolates examined were distributed to a considerable extent around the breakpoint, 8μg/ml. So, it may be reasonable to increase the threshold of the MIC for metronidazole or to have an intermediate zone between the sensitive and resistant strains.

We have determined the prevalence of *H. pylori* resistance to amoxicillin over the past 2 years (data not shown). In that study, amoxicillin-resistant *H. pylori* strains were not found. In Korea, strains resistant to amoxicillin are considered to be absent or very rare. This is consistent with other countries.[4,9]

Clarithromycin resistance is thought to involve point mutations in 23S rRNA at sites critical for macrolide interaction. Most of the mutations have been found in the two gene positions 2143 and 2144.[16] Mutations in positions in 2116 and 2142 have also been reported.[20] In the present study, six of the nine clarithromycin-resistant strains examined possessed the A2144G mutation in 23S rRNA. The MICs of most of the A2144G mutant strains were relatively low, ranging from 1 to 8μg/ml. According to our results, the A2144G (formerly A2059G) mutation is probably related to low clarithromycin MICs. Our findings of these low MICs are similar to previously reported data.[21,22] The other three strains revealed a T-to-C mutation at position 2182 on sequencing. Our study is the first to confirm the T2182C mutation in three strains, and the strains with the T2182C mutation showed a high MIC. However, to elucidate the significance of this mutation, further study is needed.

In our study, 6 of the 12 clarithromycin-resistant strains were also resistant to metronidazole. It has been suggested that the prevalence of strains with dual resistance to both metronidazole and clarithromycin has increased in recent years.[7,23] Particularly in areas of high prevalence of primary metronidazole resistance, such as our country, metronidazole resistance could reduce the efficacy of combination therapy including clarithromycin and lead to secondary clarithromycin resistance. Also, these strains with dual resistance are difficult to eradicate.[24] In the future, dual resistance to metronidazole and clarithromycin might become a major problem in *H. pylori* eradication. In countries where metronidazole resistance is common, it may be considered not to use the combination of metronidazole and clarithromycin as the first-line treatment, to avoid the emergence of more dual-resistant strains.

In the future, close monitoring of the strains with dual resistance will be of great importance. If antibiotic resistance continues to increase, a pretreatment antibiotic susceptibility test may become necessary in many re-

gions. In areas with high primary clarithromycin resistance (i.e., more than 15%–20%), new alternative first-line treatment strategies, such as quadruple therapy, should be considered and tested.

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