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Rates of clarithromycin resistance in *Helicobacter pylori* sampled from healthy subjects in Cheonan, Korea

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RESEARCH

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ABSTRACT

Background

The increasing use of the standard triple therapy for eradication of *Helicobacter pylori* (*H. pylori*) has led to an increase in the prevalence of strains resistant to existing drugs, thereby lowering the success rate of eradication therapies.

Aims

This study aimed at promoting effective eradication therapy by investigating the *H. pylori* infection rate, incidence of clarithromycin resistance, and types of mutations.

Methods

Using a PCR kit that amplifies a gene site known to be resistant to antibiotics in *H. pylori*, the resistance gene retention rate is determined and analyzed using various methods. The rapid urease test (RUT) was performed on patients undergoing routine health exams in Cheonan, and residual specimens were analyzed through DPO-based multiplex PCR to examine point mutations of the 23S rRNA gene, a gene responsible for clarithromycin resistance in *H. pylori*.

Results

The statistical program R was used for data analysis. Data are presented as medians and ranges. A chi-square test was used to analyze the categorical data. A p-value <0.05 was considered statistically significant. RUT and DPO-based multiplex PCR were 95.9 per cent in agreement with regard to the *H. pylori* infection rate, and the prevalence of the A2142G and A2143G mutations—point mutations for clarithromycin resistance—was 3.9 per cent and 22.8 per cent, respectively.

Conclusion

This data will serve as a basis for research on drug resistance in *H. pylori*, reflecting regional differences in Korea.

Key Words

Helicobacter pylori, clarithromycin resistance, drug resistance, rapid urease test, sequencing

What this study adds:

1. What is known about this subject?

H. pylori infection is a known cause of chronic gastritis, ulcer, and gastric cancer. *H. pylori* resistance has risen recently, frequently leading to treatment failure.

2. What new information is offered in this study?

This study is the first to investigate the prevalence of clarithromycin resistance in the Cheonan region using DPO-based multiplex PCR.

3. What are the implications for research, policy, or practice?

Information on regional antibiotic resistance rates is required to establish effective *H. pylori* eradication therapies, and this study will form the basis for future investigation.

Background

Helicobacter pylori (*H. pylori*) has a high infection rate (approximately 50 per cent worldwide),[1] and approximately 50 per cent of the Korean population is known to be infected with *H. pylori.*[2] In recent years, the first-line therapy against *H. pylori* has been the standard triple therapy, which combines a proton pump inhibitor (PPI) and two types of antibiotics (AMX (amoxicillin) and CAM (clarithromycin) or AMX (amoxicillin) and MNZ (metronidazole)).[3] Although the eradication rate of the triple therapy varies depending on whether the patient is infected with sensitive (81–95 per cent) or resistant strains (0–48 per cent),[4] the prevalence of *H. pylori* resistance to these drugs has risen recently due to the growing use of these antibiotics, frequently leading to treatment failure. Further, the decreasing success rate of the triple therapy due to increasing clarithromycin resistance has become a problem worldwide.[5]

Clarithromycin resistance in *H. pylori* is caused by a point mutation in the V domain of the 23S rRNA gene, most commonly A2143G, A2142G, and A2142C.[6] Previous studies have reported that 23S rRNA mutations at base pairs 2057–2611 impact erythromycin efficacy by reducing the affinity of erythromycin for ribosomes.[7]

In Korea, clarithromycin resistance is on the rise, from below 10 per cent in 2001 to 21.6 per cent in 2008.[8] Clarithromycin resistance has also increased in Japan, from 18.9 per cent in 2002 to 27.7 per cent in 2004,[9] and in Iran, from 1.4 per cent in 1997 to 26.5 per cent in 2013.[10] Thus, clarithromycin resistance is on the rise worldwide, albeit with regional variations. The rise in the resistance rate has also lowered the treatment success rate, calling for appropriate actions by health authorities worldwide. Studies on antibiotic resistance of *H. pylori* in Korea have focused on a few regions, including Seoul, and no studies have been conducted on clarithromycin resistance and the underlying mutations in the Cheonan region. Therefore, we performed the rapid urease test (RUT) on patients undergoing routine health examinations in the Cheonan region, as well as DPO-based multiplex PCR with residual specimens to analyze the prevalence of clarithromycin resistance and 23S rRNA point mutations (A2143G, A2142G). This study will contribute to understanding the regional differences and importance of Korea's *H. pylori* antibiotic resistance strains.

Method

This study was approved by the institutional review board Dankook University (IRB-2015-12-001). This study was a retrospective study using data obtained from medical records of previously isolated *H. pylori* strains. Data were collected for one month in November 2015. RUT was using CLO kit (campylobacter-like organism; Kimberly-Clark, Choongju, Korea) performed with samples obtained after endoscopy on patients undergoing routine health examinations at Dankook University Hospital in Cheonan. After randomly selecting patients with positive or negative results (413 positive samples and 56 negative samples), DPO-based multiplex PCR was performed; the ATCC strain was also included in the analysis.

DNA extraction

DNA to be used for clarithromycin resistance gene testing was extracted from residual specimens after performing RUT. Nucleic acid extraction was performed using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with the QIAcube system (Qiagen), which is an automated system for DNA extraction.

DPO-based multiplex PCR

DPO-based multiplex PCR was performed using residual samples collected during gastric endoscopy on patients undergoing a routine health examination.

The primers and probes were designed using the portion of the *H. pylori* genome that codes for the 5'-untranslated region. Specimens collected for DNA extraction underwent extraction on the same day or were stored at 4°C for nucleic acid extraction on the following day. For PCR specimens, nucleic acid extraction was performed using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol, with the QIAcube system (Qiagen). *H. pylori* PCR was performed using 40μL of filtrate that had been passed through a QIAamp Spin column (Qiagen). The extracted DNA was stored at −80°C until use for DPO-based multiplex PCR. The stored specimens were assayed for *H. pylori* using a Seeplex® *H. pylori*-ClaR ACE Detection kit (Seegene, Seoul, Korea) based on a 5' non-coding region (NCR) of a highly conserved portion in the *H. pylori* genome, according to the manufacturer's protocol.

Clarithromycin resistance gene extraction and sequencing

A diagnostic kit that can detect *H. pylori* infection with specificity for two types of point mutations (A2142G and A2143G in 23S rRNA) that induce clarithromycin resistance (Seegene) was used for PCR. Test reagents included in the kit were 5× ClaR-HP PM, 8-MOP solution, and 2× Multiplex Master. Completely dissolved test agents and 0.2mL and 1.5mL tubes were prepared. The 1.5mL tube was used to

mix the test agents. In the 1.5mL tube, 5× ClaR-HP PM (4μL×total number of samples), 8-MOP solution (3μL×total number of samples), 2× Multiplex Master (10μL×total number of samples) were mixed. In the 0.2mL tubes, 3μL of each DNA sample and 17μL of the mixed agents from the 1.5mL tube were mixed slowly, to prevent bubble formation. Three 0.2mL tubes (NC, PC1, PC2) were prepared for quality control, and 3μL of DW was added to NC while 3μL of PC1 and PC2 agents were added to PC1 and PC2, respectively. The following PCR protocol was used: denaturing at 94°C for 15 minutes, 40 cycles of amplification consisting of 30 seconds at 94°C, 30 seconds at 65°C, and 1 minute at 72°C, followed by a final elongation for 5 minutes at 72°C.

After gene extraction, sequencing was performed using a BigDye Terminator v.3.1 sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol on an ABI3730XL instrument (Thermo Fisher Scientific).

Data analysis

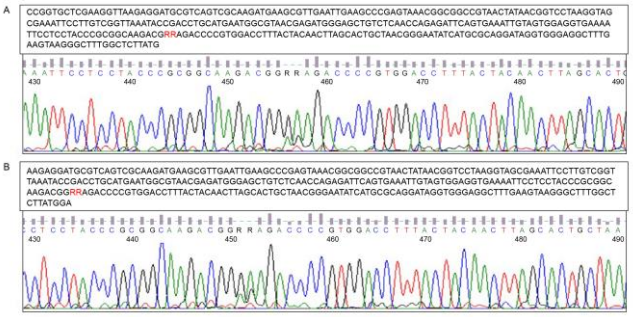
The statistical program R (Comprehensive R Archive Network; http://www.r-projects.org) was used for data analysis. Data are presented as medians and ranges. A chi-square test was used to analyze the categorical data. A p-value <0.05 was considered statistically significant.

Results

We performed DPO-based multiplex PCR on 469 randomly selected specimens (413 positive, 56 negative). A total of 89.1 per cent of the samples were positive according to DPO-based multiplex PCR and 95.9 per cent (450 of 469) agreement with the RUT results (Table 1). The remaining 19 cases that showed disparity between the two tests were analyzed. Seven specimens (1.5 per cent) were positive according to RUT but negative according to DPO-based multiplex PCR, whereas 12 specimens (2.6 per cent) were negative according to RUT but positive according to DPO-based multiplex PCR.

The A2142G mutation was detected in 16 specimens (3.4 per cent) and the A2143G mutation was detected in 94 specimens (20.0 per cent). Multi-mutation was detected in two specimens (0.4 per cent) (Table 1). *H. pylori*-specific sequencing was performed on the two specimens with both mutations. Both A and G peaks were detected at the mutation target site that causes drug resistance (Figure 1).

Figure 1: Sequence analysis of two specimens (A, B) in which both A2142G and A2143G were detected



Discussion

H. pylori infection is a known cause of chronic gastritis, gastric marginal zone B lymphoma, digestive ulcer, and gastric cancer.[11] A meta-analysis conducted outside Korea showed that, compared to *H. pylori*-negative individuals, *H. pylori*-positive individuals have a 3.0-fold higher risk of gastric cancer; Korean studies have also highlighted the need for rapid eradication due to the high risk posed by *H. pylori* infection.[2] Antibiotic resistance is an important factor that may affect *H. pylori* eradication.[12] Determining resistance prior to treatment is very important, as a high resistance rate is associated with a high possibility of treatment failure. Further, doing so helps in selecting the appropriate antibiotics, decreasing the unnecessary use of antibiotics.

In this study, we investigated the effectiveness of DPO-based multiplex PCR using residual specimens from RUT for detecting clarithromycin resistance and determined the prevalence of clarithromycin resistance in the Cheonan area.

A total of 89.1 per cent of the 469 *H. pylori* specimens analyzed with DPO-based multiplex PCR were positive, which was in 95.4 per cent agreement with the RUT results (88.1 per cent positive on RUT). This result is similar to that of a previous study that employed DPO-based multiplex PCR using residual specimens from a RUT kit; 74.3 per cent of the specimens were positive for *H. pylori* by RUT whereas 97.1 per cent of the specimens were positive for the DPO-based multiplex PCR, suggesting that the latter test has a higher sensitivity.[13]

We checked for A2142G and A2143G mutations related to clarithromycin resistance through DPO-based multiplex PCR. The results showed that 23.0 per cent (108/469) contained a mutation for clarithromycin resistance, similar to a previous finding that in Korea the rate of primary

clarithromycin resistance is approximately 20 per cent (17.2–23.7 per cent, p=0.323).[14] Notably, we observed a high clarithromycin resistance even though our study population was the general public undergoing routine health examination, not patients. Although reports that the acquisition of *H. pylori* resistance has risen due to the use of clarithromycin to treat respiratory infections[15] and that previous eradication therapy is related to clarithromycin resistance[14] suggest that these factors have increased resistance, it is difficult to definitively confirm the direct causes of the rising prevalence of resistance because the use of specimens from individuals undergoing health examinations who are apparently asymptomatic prevents us from confirming their previous and current drug regimens.

The A2142G mutation was detected in 16 specimens (3.4 per cent), whereas the A2143G mutation was detected in 94 specimens (20.0 per cent), indicating that the latter mutation is more common. Our results are in line with previous reports that the A2143G mutation has a greater impact on primary clarithromycin resistance than the A2142G mutation,[16] and that the A2143G point mutation is dominant over the A2142G point mutation.[17] In terms of regional distribution, clarithromycin resistance is highly prevalent in East Asian regions, such as Japan,[18] and least prevalent in Southeast Asian regions,[19] suggesting the possibility that cultural, regional, and climatic differences play a role in the varying mutation rates.

Interestingly, both the A2143G and A2142G mutations were detected in two specimens (0.4 per cent). Previous reports have speculated that such specimens may be infected by hetero-resistant strains, where both the wild-type and mutated strain coexist, or that they may be infected by both susceptible and resistant bacterial strains. *H. pylori* infections are more common in Korea than in more developed countries, with a high risk for multiple infections.[20] A prior study that performed genotyping for clarithromycin resistance via DPO-based multiplex PCR identified cases in which the A2142G and A2143G mutations were both present. Such cases, however, also showed disagreement between genotype and phenotype, with a sensitivity for phenotypes, and the authors questioned whether the 23S rRNA mutation leads to absolute resistance,[20] calling for additional studies to shed light on this matter.

This study has a few limitations. First, although the subjects were individuals undergoing routine health examinations, it is not certain that all subjects were healthy, due to the lack of data on underlying diseases or antibiotic exposure. Hence, these data on clarithromycin resistance should be interpreted carefully. Second, despite the fact that the specimens for RUT and DPO-based multiplex PCR were obtained from the same area of the same subject, the time interval between the two tests leaves the possibility that specimen management and experimental mismatch.

Conclusion

This study is the first to investigate the prevalence of clarithromycin resistance and types of mutations in the Cheonan area using DPO-based multiplex PCR. Information on national and regional antibiotic resistance rates is required to establish effective *H. pylori* eradication therapies, and findings in these studies will form the basis for future investigation. Currently in Korea, not all university hospitals report antibiotic resistance rates for *H. pylori*. However, resistance rates vary heavily across antibiotics, and no clear evidence has been presented that elucidates the cause of the variation. Therefore, it is important to establish a standardized reference system to integrate studies conducted by multiple institutions and a central system to effectively manage the *H. pylori* antibiotics resistant data, to form the basis for additional studies.

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PEER REVIEW

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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ETHICS COMMITTEE APPROVAL

This study was approved by the institutional review board Dankook University (IRB-2015-12-001). This study was a retrospective study using data obtained from medical records of previously isolated *H. pylori* strains.

Table 1: Prevalence of clarithromycin resistance gene in 469 persons

	H. pylori PCR			
Rapid urease test		Negative	Positive	Total
	Negative	44 (9.4%)	12 (2.6%)	56 (11.9%)
	Positive	7 (1.5%)	406 (86.6%)	413 (88.1%)
	Total	51 (10.9%)	418 (89.1%)	469 (100.0%)
Gene mutation	A2142G	453 (96.6%)	16 (3.4%)	
	A2143G	375 (80.0%)	94 (20.0%)	
	Both	467 (99.6%)	2 (0.4%)	