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Corresponding author
Hwoon-Yong Jung, MD, PhD
Department of Gastroenterology,
Asan Medical Center,
University of Ulsan College of Medicine,
88 Olympic-ro 43-gil, Songpa-gu,
Seoul 05505, Korea
E-mail: hyjung@amc.seoul.kr

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The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

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Helicobacter pylori Isolation and Antibiotic Susceptibility Testing Using Rapidly Frozen Biopsy Samples

Kee Don Choi[1]*, Jung Mogg Kim[2]*, Gwang Ho Baik[3], Jun Chul Park[4], Hye-Kyung Jung[5], Han Seung Ryu[6], Soo-Jeong Cho[7], Cheol Min Shin[8], Hwoon-Yong Jung[1]; and Korean College of *Helicobacter* and Upper Gastrointestinal Research

[1]Department of Gastroenterology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea
[2]Department of Microbiology, Hanyang University College of Medicine, Seoul, Korea
[3]Department of Internal Medicine, Hallym University College of Medicine, Chuncheon, Korea
[4]Department of Internal Medicine, Severance Hospital, Yonsei University College of Medicine, Seoul, Korea
[5]Department of Internal Medicine, Ewha Womans University School of Medicine, Seoul, Korea
[6]Department of Internal Medicine, Wonkwang University School of Medicine, Iksan, Korea
[7]Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul, Korea
[8]Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, Korea

Objectives: To involve institutions without the ability to perform susceptibility testing, long-term storage of tissue sample is critical to isolate the bacteria in a central laboratory. The aim of the study was to investigate the feasibility of *H. pylori* isolation and antibiotic susceptibility testing using rapidly frozen biopsy specimens collected from various institutions. **Methods:** Eight institutions located in various regions of Korea participated in the study. Patients requiring upper endoscopy and *H. pylori* testing were screened. Two biopsy samples were taken from the stomach. One was placed in a sterile Eppendorf tube and then immediately placed in a vacuum bottle containing dry ice, which was stored at -80°C. The other was used in a rapid urease test. Collected samples were delivered to a central laboratory. The bacteria were isolated from the frozen samples under microaerophilic conditions. The agar dilution method was used to determine the minimum inhibitory concentration (MIC) of amoxicillin, clarithromycin, metronidazole, tetracycline, ciprofloxacin, and levofloxacin for each *H. pylori* isolate. **Results:** Patients with a positive rapid urease test result (n=113) were enrolled. The mean age was 56.6±12.3 years. The male:female ratio was 64:49. The overall culture success rate was 77.0% (87/113). MIC values were determined using isolated 87 *H. pylori* strains. Rates of resistance to amoxicillin, clarithromycin, metronidazole, tetracycline, levofloxacin, and ciprofloxacin were 23.0%, 25.3%, 28.7%, 1.1%, 33.3%, and 34.5%, respectively. **Conclusions:** It is feasible to perform *H. pylori* isolation and antimicrobial susceptibility testing using rapidly frozen and transported biopsy specimens.

Keywords *Helicobacter pylori*; Culture; Biopsy; Freezing.

INTRODUCTION

Since 1998, when regimens for *Helicobacter pylori* eradication were first recommended in Korea, triple therapy—consisting of a proton pump inhibitor, clarithromycin, and amoxicillin—has been the primary treatment choice.[1-3] However, the

eradication rate with standard triple therapy has proven suboptimal.[4] A meta-analysis of first-line triple therapy in Korea indicated a significant decline in eradication rates.[5] Several factors contribute to this reduced efficacy, with the most critical being the increased resistance of *H. pylori* to antibiotics, especially clarithromycin. Studies assessing *H. pylori* resistance have typically been conducted in a limited number of metropolitan centers in Korea,[6,7] suggesting potential regional differences in antibiotic resistance and corresponding eradication rates.[8,9] Therefore, it is important to employ standardized susceptibility testing methods to gather nationwide data on the antibiotic resistance of *H. pylori* clinical isolates.

Because most centers lack a microbiology laboratory capable of performing *H. pylori* isolation and antimicrobial susceptibility tests, transporting gastric biopsy specimens to a central laboratory is essential. However, transport time and temperature significantly impact the viability of *H. pylori*.[10] Several freezing methods have been reported for the long-term storage of *H. pylori*.[11-14] A previous study showed that the recovery rate of bacteria from biopsy specimens stored at -70°C for 2 to 8 weeks was 97%.[12] Long-term preservation of *H. pylori* can be achieved through cryopreservation. In one study, *H. pylori* was successfully isolated from 72.4% of patients whose gastric biopsy specimens had been stored at -70°C for over 10 years.[13] However, standardized methods for freezing and transporting *H. pylori* for long-term storage have not been established. Additionally, most studies in this area have been conducted in a single laboratory.

This study aimed to assess the feasibility of *H. pylori* isolation and antibiotic susceptibility testing by applying a standardized protocol for the rapid freezing of gastric biopsy specimens and their transport from various institutions to a central laboratory.

METHODS

Patients

Eight institutions across various regions of Korea—including Seoul, Gyeonggi, Gangwon, Busan, and Jeolla—participated in the study. From January to August 2016, patients requiring upper endoscopy and *H. pylori* testing were screened. Informed consent was obtained from all participants, and the study received approval from the Institutional Review Boards of all participating institutions.

Isolation of *H. pylori* strains

Two biopsy samples were collected from the greater curvature of the stomach. One sample was placed in a sterile Eppendorf tube and immediately stored in a vacuum bottle with dry ice, while the other was used for the rapid urease test. The Eppendorf tubes, stored in the vacuum bottle, were then placed in a -80°C deep freezer. Only patients with positive rapid urease results were enrolled in the study. Frozen samples from these enrolled patients were delivered to the Asan Institute for Life Sciences in Seoul, Korea.

Frozen biopsy specimens were thawed at room temperature and inoculated onto *H. pylori* isolation medium (Brucella broth agar supplemented with 7% sheep blood, vancomycin [10 mg/L], trimethoprim [5 mg/L], amphotericin B [5 mg/L], and polymyxin B [2.5 IU]). *H. pylori* strains were cultured under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2). After initial isolation, a single colony from each biopsy specimen was subcultured and identified as *H. pylori* based on colony morphology, Gram staining, and urease reaction. The strains were preserved at -70°C in Brucella broth with 15% glycerol, thawed, and subcultured before use in subsequent experiments.[15,16]

Determination of the minimum inhibitory concentration

H. pylori isolates were examined using the serial 2-fold agar dilution method, as previously described.[16] Briefly, bacteria were subcultured for 48 h on Mueller–Hinton agar supplemented with 5% defibrinated sheep blood. The bacterial suspension was adjusted to 1×10^7 colony-forming units and inoculated directly onto antibiotic-containing agar dilution plates. After 72 hours, the minimum inhibitory concentration (MIC) of each antibiotic was determined. The standard strain, *H. pylori* ATCC 43504, served as a control. Resistance breakpoints for amoxicillin, clarithromycin, metronidazole, tetracycline, levofloxacin, and ciprofloxacin were set at ≥0.5, >1, ≥8, ≥4, ≥1, and ≥1 μg/mL, respectively.[7,17]

Statistical analysis

Categorical variables, expressed as numbers and percentages, were compared using the chi-square test. All statistical analyses were conducted with SPSS software (version 21.0; IBM Corp., Armonk, NY, USA). A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Baseline characteristics

From January to August 2016, 137 patients from eight institutions were screened, and 113 patients with positive rapid urease test results were enrolled. The baseline characteristics of these patients are summarized in Table 1.

Culture success rate

The median duration of tissue storage at -80°C was 79 days (range, 8–186 days). The overall culture success rate was 77.0% (87/113). Fig. 1 shows the culture success rates by institution, with institution 5 showing the lowest success rate at 42.9%. The culture success rate for patients without a history of *H. pylori* eradication was 79.4% (77/97), compared to 69.2% (9/13) for those with a history of eradication.

Table 1. Baseline characteristics of the study patients

Characteristics	Value (n=113)
Age (yr)	56.6±12.3
Male:female	64:49
Underlying disease	
Benign gastric ulcer	17 (15.0)
Duodenal ulcer	10 (8.8)
Gastric cancer or adenoma	26 (23.0)
Gastric marginal zone lymphoma of MALT	6 (5.3)
Chronic gastritis	41 (36.3)
Others	13 (11.5)
Residence	
Seoul	28 (24.8)
Incheon, Gyeonggi	28 (24.8)
Gangwon	20 (17.7)
Chungcheong	5 (4.4)
Busan, Gyeongsang	14 (12.4)
Gwangju, Jeolla	18 (15.9)
Residence area	
Urban	84 (74.3)
Rural	29 (25.7)
History of *H. pylori* eradication	
Yes	13 (11.5)
No	97 (85.8)
Unknown	3 (2.7)

Values are presented as mean±standard deviation, n (%), or numbers only.
MALT, mucosa-associated lymphoid tissue.

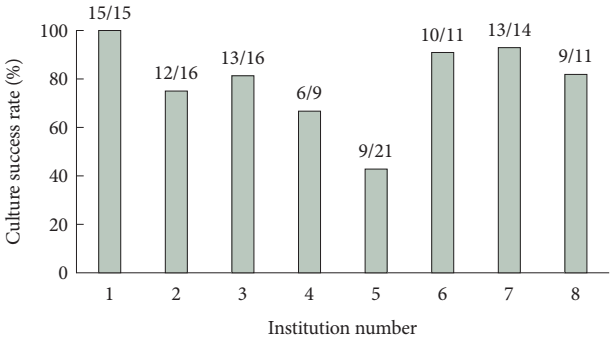

Fig. 1. Culture success rates according to institution. Institution 1, 2, and 3 from Seoul; 4 and 8 from Gyeonggi; 5 from Gangwon; 6 from Busan; and 7 from Jeolla.

Prevalence of antibiotic resistance

MIC values were determined for 87 isolated *H. pylori* strains. The distribution of antibiotic MIC values is shown in Table 2. Resistance breakpoints for amoxicillin, clarithromycin, metronidazole, tetracycline, levofloxacin, and ciprofloxacin were set at ≥0.5, >1, ≥8, ≥4, ≥1, and ≥1 μg/mL, respectively.[7,17] The rates of resistance to amoxicillin, clarithromycin, metronidazole, tetracycline, levofloxacin, and ciprofloxacin were 23.0%, 25.3%, 28.7%, 1.1%, 33.3%, and 34.5%, respectively.

DISCUSSION

The present study demonstrated that the overall *H. pylori* recovery rate from rapidly frozen biopsy specimens across various institutions in the country was 77.0%. This result is promising, considering the relatively low sensitivity of the culture methods employed.[18-20]

Previous studies have reported good *H. pylori* recovery from frozen biopsy specimens.[11,12,14] Han et al.[11] achieved *H. pylori* isolation in 14 patients (87.5%) after 5 to 6 years of storage at -70°C; however, the recovery rate dropped to 57% after 12 weeks at -20°C. Heep et al.[12] reported a recovery rate of 97% when samples were stored in glycerol broth at -70°C for 2 to 8 weeks. The present study differs from these previous studies in that no transport medium was used. Previous studies utilized Stuart's transport medium, Portagerm pylori, or cysteine-albumin broth supplemented with 20% glycerol for rapid freezing.[11,12] In this study, biopsy specimens were immediately placed in sterile Eppendorf tubes without medium and then rapidly frozen. This approach resulted in a high recovery rate without the need for a transport medium.

Different institutions reported varying culture success rates. The recovery rate at Institution 1 was 100% (15/15), while at Institution 5, it was 42.9% (9/21). Therefore, we reviewed the sample processing procedures at Institution 5. Eppendorf tubes containing biopsy tissue were stored in a standard refrigerator (4°C) for several hours before being transferred to a vacuum bottle with dry ice. This deviation from the standard protocol raises particular concerns, as the biopsy samples were not immersed in a protective transport medium during the interim storage period. The lack of such a medium, combined with the relatively warm temperature of 4°C, likely resulted in cellular degradation and metabolic changes. Consequently, it can be assumed that improper initial handling contributed significantly to the low culture success rates. A study showed that when the transfer time exceeded 4 h, isolating *H. pylori* from biopsy specimens became difficult, even when the temperature was maintained at 4°C.[21] Recently, a large-scale multicenter study involving 66452 patients from 26 hospitals in China

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Table 2. Distributions of antibiotic MIC for *H. pylori* isolates

Agents	MIC (μg/mL)												
	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128
Amoxicillin	25	11	19	12	11	4	0	4	1	0	0	0	0
Clarithromycin	45	15	3	0	1	1	1	0	0	10	7	3	1
Metronidazole	0	0	0	0	2	9	28	23	10	5	2	5	3
Tetracycline	0	11	31	26	14	3	1	1	0	0	0	0	0
Levofloxacin	0	3	7	25	23	0	1	10	11	6	1	0	0
Ciprofloxacin	0	0	12	35	10	0	2	5	18	5	0	0	0

MIC, minimum inhibitory concentration.

was conducted. Gastric mucosal biopsy specimens were transferred to a central laboratory at ambient temperatures, with transport times ranging from 5 to 72 h and temperatures varying between 4.7°C and 29.1°C. The specimens were categorized into three groups based on transport time: 5 h, 24 h, and 48 h. The culture rate for the 24-h transport group was 32.8%, and for the 48-h group, it was 26.3% (p<0.001).[22] Thus, optimal recovery of *H. pylori* requires that biopsy specimens be cultured as soon as possible. However, transferring all biopsy specimens to a laboratory within 24 h can be challenging. To ensure optimal bacterial recovery, we believe that rapidly freezing biopsy specimens before transfer to the central laboratory is preferable.

After *H. pylori* eradication, the culture success rate in post-treatment biopsy samples tended to decrease in the present study. This reduction in culture positivity could be attributed to several factors, including the effect of antibiotics used in eradication therapy, which may suppress the bacterial load to undetectable levels. Further studies are needed to understand this mechanism better.

Understanding local patterns of antibiotic resistance is essential for selecting the most appropriate first-line treatment. Several centers in Korea, primarily single centers in Seoul and the Gyeonggi area, have documented antibiotic resistance rates.[6,7,9,16,23,24] However, resistance patterns may differ in other regions. For example, a previous study in Japan found that the prevalence of metronidazole resistance was significantly higher in Kyoto (23.8%) than in Sapporo (8.1%).[8] Similarly, the prevalence of clarithromycin resistance in *H. pylori* strains isolated from Korean patients in the Gyeonggi area between 2009 and 2012 was 37%,[6] whereas another study from Seoul reported lower resistance rates (8.5%), leading to higher eradication rates (84.9%) with clarithromycin-based triple therapy.[9] These results suggest the need for *H. pylori* resistance analysis at both nationwide and regional levels using standardized susceptibility testing. The agar dilution method is recommended for measuring the MIC of antibiotics against *H. pylori*. However, few centers have laboratories equipped to isolate and test *H. pylori* using the agar dilution method. Therefore, effective transport methods are essential to obtaining meaningful nationwide antibiotic susceptibility data.

This study had several limitations. First, we did not observe regional differences in antibiotic susceptibility, as only a small number of patients from each region were included. However, the purpose of this study was not to identify regional differences in antibiotic susceptibility but to confirm the feasibility of antibiotic susceptibility testing using rapidly frozen gastric biopsy specimens. A nationwide multicenter study with a larger patient cohort is currently underway to investigate regional differences in antibiotic susceptibility. Another possible limitation is the variation in culture success rates reported across institutions. Although this study was conducted prospectively with a standardized protocol, we identified protocol deviations that could explain the differing culture success rates (ranging from 42.9% to 100%). Despite these limitations, this was the first prospective study to evaluate the feasibility of *H. pylori* isolation and antibiotic susceptibility testing using rapidly frozen gastric biopsy specimens collected from multiple institutions.

In conclusion, the isolation and antimicrobial susceptibility testing of *H. pylori* using frozen and transported biopsy specimens is both feasible and effective. This method can facilitate the collection of nationwide antibiotic resistance data, which can then inform the development of empirical regimens for *H. pylori* eradication.

Authors' Contribution

Conceptualization: Kee Don Choi, Jung Mogg Kim, Hwoon-Yong Jung. Data curation: Kee Don Choi, Gwang Ho Baik, Jun Chul Park, Hye-Kyung Jung, Han Seung Ryu, Soo-Jeong Cho, Cheol Min Shin, Hwoon-Yong Jung. Formal analysis: Kee Don Choi. Funding acquisition: Jung Mogg Kim, Hwoon-Yong Jung. Investigation: Kee Don Choi, Jung Mogg Kim. Methodology: Jung Mogg Kim. Project administration: Hwoon-Yong Jung. Resources: Jung Mogg Kim, Hwoon-Yong Jung. Software: Kee Don Choi. Supervision: Jung Mogg Kim, Hwoon-Yong Jung. Validation: Jung Mogg Kim, Hwoon-Yong Jung. Visualization: Kee Don Choi. Writing—original draft: Kee Don Choi. Writing—review & editing: Jung Mogg Kim, Hwoon-Yong Jung. Approval of final manuscript: all authors.

ORCID iDs

Kee Don Choi https://orcid.org/0000-0002-2517-4109
Jung Mogg Kim https://orcid.org/0000-0002-6506-7519
Gwang Ho Baik https://orcid.org/0000-0003-1419-7484
Jun Chul Park https://orcid.org/0000-0001-8018-0010
Hye-Kyung Jung https://orcid.org/0000-0002-6653-5214
Han Seung Ryu https://orcid.org/0000-0002-9359-0075
Soo-Jeong Cho https://orcid.org/0000-0001-7144-0589
Cheol Min Shin https://orcid.org/0000-0003-2265-9845
Hwoon-Yong Jung https://orcid.org/0000-0003-1281-5859

REFERENCES

1. Korean *H. pylori* Study Group. Diagnosis and treatment of *Helicobacter pylori* infection in Korea. Korean J Gastroenterol 1998;32:275-289.
2. Kim SG, Jung HK, Lee HL, et al. Guidelines for the diagnosis and treatment of *Helicobacter pylori* infection in Korea, 2013 revised edition. J Gastroenterol Hepatol 2014;29:1371-1386.
3. Jung HK, Kang SJ, Lee YC, et al. Evidence-based guidelines for the treatment of *Helicobacter pylori* infection in Korea: 2020 revised edition. Korean J Helicobacter Up Gastrointest Res 2020;20:261-287.
4. Kim Y, Ahn JY, Jung HY, et al. Comparison of *Helicobacter pylori* eradication rates using standard triple therapy and sequential therapy. Korean J Helicobacter Up Gastrointest Res 2023;23:277-282.
5. Gong EJ, Yun SC, Jung HY, et al. Meta-analysis of first-line triple therapy for *helicobacter pylori* eradication in Korea: is it time to change? J Korean Med Sci 2014;29:704-713.
6. Lee JW, Kim N, Kim JM, et al. Prevalence of primary and secondary antimicrobial resistance of *Helicobacter pylori* in Korea from 2003 through 2012. Helicobacter 2013;18:206-214.
7. Kim JM, Kim JS, Jung HC, Kim N, Kim YJ, Song IS. Distribution of antibiotic MICs for *Helicobacter pylori* strains over a 16-year period in patients from Seoul, South Korea. Antimicrob Agents Chemother 2004;48:4843-4847.
8. Kato M, Yamaoka Y, Kim JJ, et al. Regional differences in metronidazole resistance and increasing clarithromycin resistance among *Helicobacter pylori* isolates from Japan. Antimicrob Agents Chemother 2000;44:2214-2216.
9. Yoon KH, Park SW, Lee SW, Kim BJ, Kim JG. Clarithromycin-based standard triple therapy can still be effective for *Helicobacter pylori* eradication in some parts of the Korea. J Korean Med Sci 2014;29:1240-1246.
10. Morton D, Bardhan KD. Effect of transport medium and transportation time on culture of *Helicobacter pylori* from gastric biopsy specimens. J Clin Pathol 1995;48:91.
11. Han SW, Flamm R, Hachem CY, et al. Transport and storage of *Helicobacter pylori* from gastric mucosal biopsies and clinical isolates. Eur J Clin Microbiol Infect Dis 1995;14:349-352.
12. Heep M, Scheibl K, Degrell A, Lehn N. Transport and storage of fresh and frozen gastric biopsy specimens for optimal recovery of *Helicobacter pylori*. J Clin Microbiol 1999;37:3764-3766.
13. Seo JH, Youn HS, Goo MJ, et al. Recovery of *Helicobacter pylori* from gastric tissue cryopreserved for more than 10 years. Helicobacter 2013;18:167-168.
14. Tompkins DS, Rotowa NA. Isolation of *Helicobacter pylori* from frozen gastric biopsy specimens. Lancet 1991;338:253.
15. Choi KD, Kim N, Lee DH, et al. Analysis of the 3' variable region of the cagA gene of *Helicobacter pylori* isolated in Koreans. Dig Dis Sci 2007;52:960-966.
16. Chung JW, Lee GH, Jeong JY, et al. Resistance of *Helicobacter pylori* strains to antibiotics in Korea with a focus on fluoroquinolone resistance. J Gastroenterol Hepatol 2012;27:493-497.
17. Kim JM, Kim JS, Jung HC, Kim N, Song IS. Antibiotic resistance of *Helicobacter pylori* isolated from Korean patients in 2003. Korean J Gastroenterol 2004;44:126-135.
18. Loffeld RJ, Stobberingh E, Flendrig JA, Arends JW. *Helicobacter pylori* in gastric biopsy specimens. Comparison of culture, modified giemsa stain, and immunohistochemistry. A retrospective study. J Pathol 1991;165:69-73.
19. Grove DI, Koutsouridis G, Cummins AG. Comparison of culture, histopathology and urease testing for the diagnosis of *Helicobacter pylori* gastritis and susceptibility to amoxycillin, clarithromycin, metronidazole and tetracycline. Pathology 1998;30:183-187.
20. Hirschl AM, Makristathis A. Methods to detect *Helicobacter pylori*: from culture to molecular biology. Helicobacter 2007;12(Suppl 2):6-11.
21. Soltesz V, Zeeberg B, Wadström T. Optimal survival of *Helicobacter pylori* under various transport conditions. J Clin Microbiol 1992;30:1453-1456.
22. Gong YN, Li YM, Yang NM, et al. Centralized isolation of *Helicobacter pylori* from multiple centers and transport condition influences. World J Gastroenterol 2015;21:944-952.
23. Kim JY, Kim N, Park HK, et al. Primary antibiotic resistance of *Helicobacter pylori* strains and eradication rate according to gastroduodenal disease in Korea. Korean J Gastroenterol 2011;58:74-81.
24. An B, Moon BS, Kim H, et al. Antibiotic resistance in *Helicobacter pylori* strains and its effect on *H. pylori* eradication rates in a single center in Korea. Ann Lab Med 2013;33:415-419.