

Increase in antibiotic resistant *Helicobacter pylori* in a University Hospital in Japan

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Background: Eradication effectively prevents *Helicobacter pylori*-associated diseases; however, *H. pylori* antibiotic resistance has increased throughout Japan and worldwide. This study aimed to assess rates of resistance to antibiotics; amoxicillin, clarithromycin and metronidazole in a University Hospital in Japan.

Materials and methods: *H. pylori* (208 strains) were isolated from patients at the Okayama University Hospital in Japan. The minimum inhibitory concentrations (MIC) were determined using the mean values of the E-test to determine the antimicrobial susceptibilities of the strains. Sequencing and gene analysis were performed to analyze resistance genes to clarithromycin and amoxicillin.

Results: Rates of amoxicillin, clarithromycin, and metronidazole resistance were 13%, 48%, and 49%, respectively. Genetic analysis indicated that the A2143G point mutation in 23S rDNA is closely associated with the MIC of clarithromycin. The MIC in amoxicillin-resistant strains increased with an increase in the number of PBP1A amino acids mutations.

Conclusion: Genetic analysis for resistant strains is not clinically effective in cases of amoxicillin resistance. Numerous bacteria with already high antibiotic resistance rates have been isolated in large hospitals such as a University Hospital. For effective eradication therapy, MIC measurement should be considered via several methods.

Keywords: *Helicobacter pylori*, resistance, clarithromycin, amoxicillin, University Hospital, genotype

Introduction

Helicobacter pylori is a Gram-negative spiral bacterium usually found in the gastric mucosa. *H. pylori* is associated, not only with gut diseases such as, peptic ulcers, gastric mucosa-associated lymphoid tissue (gastric-MALT) lymphoma, and gastric cancer,¹⁻⁴ but also with systemic diseases.^{5,6} Eradication therapy is effective to prevent diseases and is widely practiced nationwide in hospitals and clinics within Japan.^{7,8} In particular, eradication therapy for *H. pylori* has evidently reduced the incidence of gastric cancer. Antibiotic resistance in *H. pylori* has recently increased.^{9,10} There are many reports that antibiotic resistance in *H. pylori* has increased alarmingly worldwide, thereby greatly affecting therapeutic efficacy.¹¹⁻¹⁵ Many difficult-to-diagnose patients or those wherein eradication therapy failed at other common hospitals are admitted at Okayama University hospital and are carefully treated because the hospital administers third-line treatment. Therefore, it is possible that more resistant bacteria may be isolated at Okayama University Hospital than at other general hospitals.

The resistant strain surveillance committee in the Japanese Society for *Helicobacter* research reported that the prevalence rates of clarithromycin and metronidazole resistance are 38.5% and 3.0%, respectively, among patients requiring first-line triple therapy with a proton pump inhibitor, amoxicillin, and clarithromycin.¹⁶ However, clarithromycin and metronidazole resistance rates, after unsuccessful eradication by first- or second-line therapy, increased up to 90.5% and 52.4%, respectively. Furthermore, the committee has been admonished owing to the drastic increase in clarithromycin-resistant strains in Japan. We performed antibiotic susceptibility tests to amoxicillin, clarithromycin, and metronidazole in isolated *H. pylori* strains in the Okayama University Hospital. SNPs involving substitution of adenine to guanine residues at positions 2142 and 2143 of the 23S rRNA of *H. pylori* (A2142G and A2143G) are reportedly associated with clarithromycin resistance.¹⁷ Amoxicillin resistance depends on mutations in the amino acid sequence of PBP1A.¹⁸

Therefore, we assessed antibiotic resistance rates in *H. pylori* strains to amoxicillin, clarithromycin, and metronidazole at the Okayama University Hospital. In addition, we performed a genotype analysis of clarithromycin resistance and mutations in PBP1A in amoxicillin-resistant *H. pylori* because these antibiotics are being used for not only first-line treatment but also for all treatments.

Materials and methods

Patients and materials

Gastric biopsy specimens were obtained with written informed consent from patients who underwent endoscopy at Okayama University Hospital from January 2011 to February 2016. Ethical approval to carry out the study was obtained a priori from the Okayama University Ethics Committee. Gastric biopsy specimens were obtained from the antrum and body for *H. pylori* culture. Biopsy samples were homogenized and spread onto both non-selective brain heart infusion (BHI) agar supplemented with 7% (v/v) sterile defibrinated horse blood and selective BHI horse blood agar containing trimethoprim (50 mg mL⁻¹), vancomycin (100 mg mL⁻¹), and polymyxin B (25,000 IU), and incubated at 37°C under micro-aerobic conditions for 7 days. *H. pylori* growth was monitored by assessing colony morphology and positive biochemical reactions, such as those with urease. *H. pylori* (208 strains) were isolated from 131 culture-positive patients. Cultured *H. pylori* strains were maintained in -80°C until use.

Antibiotic susceptibility tests

H. pylori susceptibility to clarithromycin and amoxicillin was quantified using the E-test.^{19,20} After initial bacterial isolation from biopsy samples, colonies were harvested from pure *H. pylori* cultures. The pure-cultured strain was sub-cultured for 7 days and suspended in 1 mL sterile saline at McFarland 1.0. The suspension was spread on brain heart infusion (BHI) agar supplemented with 7% (v/v) horse blood, with sterile cotton swabs. E-test strips were placed on the plates in accordance with the manufacturer's instructions. These plates were incubated at 37°C under micro-aerobic conditions for 7 days. Strains were considered resistant to amoxicillin, clarithromycin, and metronidazole if the MIC was ≥ 0.1 $\mu\text{g/mL}$, ≥ 2 $\mu\text{g/mL}$, and ≥ 8 $\mu\text{g/mL}$, respectively.

PCR amplification of the clarithromycin resistance gene

Bacterial DNA was extracted and purified using the QIAamp DNA Micro Kit (QIAGEN, Venlo, Netherlands). The most common point mutations (A2142G and A2143G) in 23S rDNA, conferring resistance to clarithromycin in *H. pylori* strains, were detected via PCR, using primers 23 S-F and 23 S-R (Table 1). The reaction mixture comprised 5 μL of 10 \times Ex Taq Buffer, 4 μL of dNTP Mixture, 1 μL of each primer, 1 μL of DNA template, 0.5 μL of TaKaRa Ex Taq (5-unit μL^{-1}) and 38 μL of sterile distilled water. Cycling conditions were as follows: 98°C for 5 minutes, 35 cycles of denaturation at 98°C for 15 seconds, annealing at 60.5°C for 20 seconds, extension at 72°C for 1 minute, and final extension step at 72°C for 10 minutes. Five microliters of the PCR product were electrophoresed on a 2% agarose gel for 30 minutes and stained with ethidium bromide for 20 minutes, and bands were visualized using Dolphin-View (Dolphin-View, KURABO, Japan), an electrophoresis gel photographing apparatus.

PCR amplification of the amoxicillin resistance gene

Bacterial DNA was extracted and purified using QIAamp DNA Micro Kit (QIAGEN, Venlo, Netherlands). Mutations in PBP1A, conferring amoxicillin resistance in *H. pylori* strains, were also detected via PCR, using primers PBP1-F and PBP1-R (Table 1). The reaction mixture comprised 5 μL of 10 \times Ex Taq Buffer, 4 μL of dNTP Mixture, 1 μL of each primer, 1 μL of DNA template, 0.5 μL of TaKaRa Ex Taq (5-unit μL^{-1}) and 38 μL of sterile distilled water. Cycling conditions were as follows: 35 cycles of denaturation at 94°C for 1 minute,

Table 1 Primers for PCR

Primer	Primer sequence	Amplicon size (bp)
23 S-F	5-TCGAAGGTTAAGAGGATGCGTCAGTC-3	320
23 S-R	5-GACTCCATAAGAGCCAAAGCCCTTAC-3	
PBPI-F	5-GCATGATCGTTACAGACACG-3	
PBPI-R	5-ATCCACGATTTCTTTACGC-3	
		905

annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. PCR products were assessed via electrophoresis on a 2% agarose gel, which was photographed using Dolphin-View (Dolphin-View, KURABO, Japan).

Sequencing of PCR products

PCR-amplified DNA samples were purified via ethanol precipitation. The samples were sequenced using forward and reverse primers, using a DNA sequencer (PRISM3130 Genetic Analyzer, Applied Biosystems) at Hokkaido System Science Co., Ltd. (Sapporo, Japan). Sequences were analyzed using Genetyx software (<https://genetyx.software.informer.com>).

Statistical analysis

Statistical analysis of mutations in the clarithromycin resistance gene among resistant strains and susceptible strains was performed using the chi-squared test. Mutations in the amoxicillin resistance gene were analyzed using Bartlett's test. $P < 0.05$ was considered statistically significant.

Results

Resistance rate of *H. pylori* at the Okayama University Hospital

From January 2011 to February 2016, 208 strains of *H. pylori* were isolated from 131 culture-positive patients who underwent gastric biopsy at the Okayama University Hospital. These strains were tested for amoxicillin, clarithromycin, and metronidazole susceptibility via the E-test. The amoxicillin-resistant strains accounted for 26 of the 207 (13%) strains isolated; 100 of 208 (48%) were clarithromycin-resistant strains; and 101 of the 189 (49%) strains. Cumulative curves of MICs of these three antibiotics are shown in Figure 1.

Clarithromycin resistance gene and resistance value

Mutations in the clarithromycin resistance gene (the most common point mutations being A2142G and A2143G in 23S rDNA) were examined via sequencing of 86 of the 208

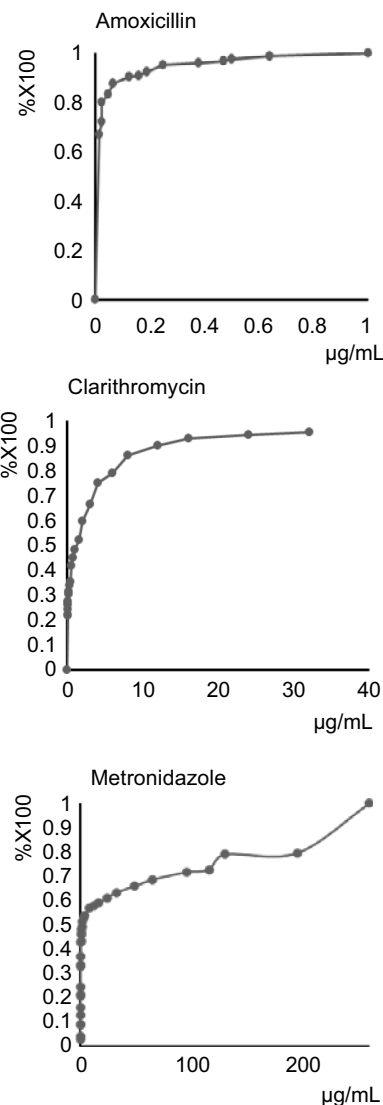


Figure 1 Cumulative curves for the minimum inhibitory concentrations of amoxicillin, clarithromycin, and metronidazole.

(41%) strains. Thirty-seven of these isolates were resistant to clarithromycin ($MIC \geq 2$ mg/L), 27 displayed intermediate resistance ($0.1 \leq MIC < 2$ mg/L), and 22 were sensitive ($MIC < 0.1$ mg/L). Only two isolates harbored the A2142G mutation, which was not associated with clarithromycin resistance. The A2143G mutation was present in 81% of

clarithromycin-intermediate-resistant isolates and in 95% of clarithromycin-resistant isolates (Table 2). The A2143G mutation is closely associated with the corresponding MIC values.

Amoxicillin resistance gene and resistance value

After sequencing of PBP1A from each isolated strain, amino acid mutations were detected at 14 points in the amoxicillin-resistant (Amx^R) strains and 19 points in amoxicillin-sensitive (Amx^S) strains. All mutations were not significantly associated with amoxicillin resistance (Table 3). These strains were categorized into 3 groups based on the MICs of amoxicillin: $\text{MIC} < 0.016$, $0.016 < \text{MIC} < 0.1$, and $0.1 < \text{MIC}$. The amino acid mutation numbers were compared in each group. In the

group with the lowest MICs ($\text{MIC} < 0.016$, $n=29$), the average value for the mutation number was 2.14; intermediate MICs ($0.016 < \text{MIC} < 0.1$, $n=20$), 2.35; highest MICs ($0.1 < \text{MIC}$, $n=8$), 3.38. There was significant ($P < 0.05$) difference between the lowest MIC and highest MIC groups (Figure 2). The number of mutations contributes to a higher MIC in Amx^R strains.

Discussion

Antibiotic resistance of *H. pylori* is increasing; however, the resistance rate is still low during first-line therapy in Japan.^{21,22} Clarithromycin- and metronidazole-resistant strains have different geographical distributions worldwide and are divided into four groups. The US, Austria, and Japan constitute one group wherein clarithromycin resistance is high; however, metronidazole resistance is low.²³ The prevalence rate of amoxicillin-resistant *H. pylori* is less than 1% in many areas.²⁴⁻²⁶ However, in the present study performed at Okayama University Hospital, the resistance rate of clarithromycin and metronidazole were high. In addition, the amoxicillin resistance rate was also high. In the near future, we believe that not only the clarithromycin and metronidazole resistance rates, but also the amoxicillin resistance rate will increase in Japan.

Clarithromycin resistance is associated with a mutation in 23S rDNA in the 50S ribosome. Mutations at both locations, ie, 2142 and 2143, involve an adenine-to-guanine transition, the latter leading to an adenine-to-cytosine trans-version at position 2142.^{27,28} The predominant mutations among resistant strains in Europe were A2143G (44.1%) and A2142G (32.6%). A recent study from India reported that the dominant point mutation was at the A2142G site, while the A2143G

Table 2 Correlation between the minimum inhibitory concentration of clarithromycin (mg/L) and the type of mutation in 23S rDNA of clarithromycin-resistant *Helicobacter pylori* strains

Mutation	No. (%) of strains			
	MIC < 0.1 mg/L (n=22)	$0.1 \leq \text{MIC} < 2$ mg/L (n=27)	MIC ≥ 2 mg/L (n=37)	Total (n=86)
A2142G	0 (0)	1 (4)	1 (3)	2 (2)
A2143G ^a	1 (5)	22 (81)	35 (95)	58 (67)
No mutation ^a	21 (95)	4 (15)	1 (3)	26 (30)

Notes: ^a $P < 0.01$ by chi-squared test.

Abbreviation: MIC, minimum inhibitory concentrations.

Table 3 Amino acid substitutions in PBP1A in amoxicillin-sensitive and amoxicillin-resistant *Helicobacter pylori* strains

Amino acid position in PBP1A	Change of amino acid in PBP1A	Amx^S group (n=49)	Amx^R group (n=8)
374	V374L	1	1
400	N400D	4	0
406	E406K	6	0
414	S414R	3	1
461	N461K	1	0
474	A474T	5	0
480	A480V	1	2
509	V508I	7	2
511	T511I	1	1
515	I515M	9	2
562	N562Y	0	1
	N562D	2	0
563	I563V	3	0
589	S589V	1	0
593	T593A	6	1
	T593G	4	2
595	G595S	10	3
604	Y604H	2	0

Abbreviation: Amx, amoxicillin.

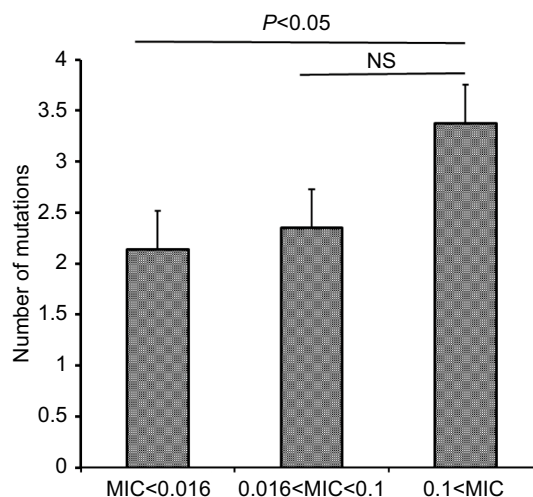


Figure 2 Correlation between the minimum inhibitory concentration of amoxicillin (mg/L) and mutation number in penicillin-binding protein 1A.

Abbreviations: MIC, minimum inhibitory concentration; NS, not significant.

mutation was not detected.²⁹ The prevalence of the A2143G as a primary mutation is 10–14% among the Chinese population; however, administration of clarithromycin to obliterate an *H. pylori* infection increased the prevalence (32%) of the A2143G mutation in Chinese patients.³⁰ These reports indicate that the A2143G mutation in 23S mRNA is most important for clarithromycin resistance. In the present study, A2143G was associated with clarithromycin resistance. Other Japanese studies have also reported that A2143G was the primary mutation in clarithromycin-resistant strains.^{31–33} These mutations in 23S rDNA associated with clarithromycin resistance differed among different countries; therefore, genetic tests consistent with the strains prevalent in each country are required.

Amoxicillin resistance is caused by a mutation in PBP1A. Previous Japanese studies have reported that an amino acid substitution of Asn562→Tyr (N562Y) was similarly observed in all amoxicillin-resistant strains.³⁴ Other substitutions including Ser414→Arg (S414R), Thr593→Ala (T593A), Gly595→Ser (G595S), and Ala599→Thr (A599T) were detected in amoxicillin-resistant strains in studies reported in Korea.³⁵ The substitution of Ser414→Arg (S414R) has also been reported from Netherlands.³⁶ Another study from Korea reported that the T593 substitution particularly contributes to amoxicillin resistance.¹² However, in the present study, these substitutions were not detected or were detected in both resistant and sensitive strains. Similarly, no amino acid substitutions were detected in all eight amoxicillin-resistant strains. All detected amino acid substitutions were not specific for amoxicillin-resistant strains. The present results indicate that amoxicillin resistance in *H. pylori* is associated with multiple amino acid substitutions in PBP1A and tends to increase with an increase in the number of amino acid substitutions in PBP1A. Hence, it is better to avoid determining amoxicillin resistance exclusively on the basis of amino acid substitutions at a specific position. Currently, the detailed mechanism underlying amoxicillin resistance in *H. pylori* is unclear. Amoxicillin-resistant *H. pylori* in Japan are expected to increase in the future, and further studies on the acquisition of amoxicillin resistance are necessary.

Conclusion

Many bacteria with high antibiotic resistance have been isolated in large hospitals such as the Okayama University Hospital in Japan. To detect antibiotic resistance in *H. pylori*, culture-based assessment and SNP analysis are useful to detect clarithromycin-resistant bacteria. However, genetic analysis of PBP1A is not applicable for detecting amoxicillin resistance owing to the need to analyze amino acid

substitution mutations. The method for detecting antibiotic resistance should be selected appropriately in accordance with the type of antibiotic.

Abbreviations

Amx, amoxicillin; BHI, brain heart infusion; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; SNP, Single Nucleotide Polymorphism.

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Disclosure

The authors report no conflicts of interest in this work.

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