







ORIGINAL ARTICLE

Establishment of a reference panel of *Helicobacter pylori* strains for antimicrobial susceptibility testing

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Abstract

Background: Eradication treatment for *Helicobacter pylori* gastritis is covered by national health insurance since 2013 in Japan. However, eradication failure due to the increase of antimicrobial resistance has become a serious problem. The present study aims to establish a reference panel of Japanese *H. pylori* strains for antimicrobial susceptibility testing.

Method: A total of 28 strains were collected from 4 medical facilities in Japan. Antimicrobial susceptibility tests (ASTs) to clarithromycin (CLR), amoxicillin (AMX), and metronidazole (MNZ), were used to select standard reference strains. Complete genome sequences were also determined.

Results: Three *H. pylori* strains (JSHR3, JSHR6 and JSHR31) were selected as standard reference strains by the Japanese Society for Helicobacter Research (JSHR). The minimum inhibitory concentrations (MICs) of the antibiotics against these 3 strains by agar dilution method with Brucella-based horse-serum-containing agar medium were as follows: JSHR3 (CLR 16 µg/ml, AMX 0.032 µg/ml and MNZ 4 µg/ml), JSHR6 (CLR

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0.016 µg/ml, AMX 0.032 µg/ml and MNZ 4 µg/ml), and JSHR31 (CLR 16 µg/ml, AMX 1 µg/ml and MNZ 64 µg/ml).

Conclusions: A reference panel of *H. pylori* JSHR strains was established. The panel consisted of JSHR6, which was antibiotic-susceptible, JSHR3, which was CLR-resistant, and JSHR31, which was multi-resistant. This reference panel will be essential for standardized ASTs before the optimal drugs are selected for eradication treatment.

KEYWORDS

antibiotic resistance, antimicrobial susceptibility tests, genome information, *Helicobacter pylori*, reference panel

1 | INTRODUCTION

Helicobacter pylori is a Gram-negative, spiral bacterium, and a causative agent of chronic gastritis, peptic ulcers, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer.

In Japan, eradication therapy for gastritis caused by *H. pylori* infection has been covered by insurance since 2013,¹ and it was the first country in the world to do this. Since this time, the number of patients with gastric and duodenal ulcers has decreased to about a quarter of the peak prevalence and the number of gastric cancer deaths, which have been around 50,000 per year for many years, is gradually decreasing.^{2,3}

The first-line regimen for eradication therapy in Japan consists of twice-daily dosing (b.i.d.) with a proton pump inhibitor (PPI) or potassium-competitive acid blocker (P-CAB), amoxicillin (AMX), and clarithromycin (CLR) for 1 week.⁴ The second-line therapy is replacement of CLR with metronidazole (MNZ). However, antibiotic resistance of *H. pylori* is now increasing and often causes eradication failure. Savoldi et al⁵ indicated that resistance of *H. pylori* to antibiotics has reached alarming levels worldwide, which has a great effect on the efficacy of treatment. The eradication rate with Japanese standard first-line triple therapy was initially reported as just over 85% but it has gradually decreased due to increased prevalence of CLR-resistant *H. pylori*.⁶ In February 2015, P-CAB (vonoprazan) was launched. The eradication rate of 7-day triple therapy including CLR and AMX with P-CAB has been reported to be significantly higher than those with PPIs.⁷⁻¹⁰ Furthermore, the eradication rates in major studies conducted in Japan using secondary eradication therapy with omeprazole, lansoprazole, rabeprazole, esomeprazole, and P-CAB were around 90%.^{7,9,11}

Antimicrobial susceptibility tests (ASTs) can provide useful information for eradication therapy. If antibiotic resistance is found before eradication, there is no need to use ineffective antimicrobial agent(s). In the Japanese Society for *Helicobacter* Research (JSHR) Guidelines 2016,⁴ it was recommended that antimicrobials for eradication therapy should be chosen on the basis of ASTs and used in a combination expected to achieve the highest eradication rate.

However, in Japan, ASTs have not been standardized, and each clinical laboratory may perform different standards of testing. The

present study aims to establish a reference panel of Japanese *H. pylori* strains for ASTs to AMX, CLR, and MNZ.

2 | MATERIALS AND METHODS

2.1 | Collected strains and culture

This study was carried out with approval from the Ethics Committees of the Japanese Society of *Helicobacter* Research and Kyorin University. In total 28 strains were collected from 4 medical facilities in Japan.

2.2 | Susceptibility tests

The -80°C freeze-stocked *H. pylori* strains were cultured in Brucella medium (Nippon Becton Dickinson and Co.) containing 7% horse serum and 1.5% agar at 37°C under microaerobic conditions (Mitsubishi Gas Chemical Company, Inc.) and used in ASTs.¹² The strains were sub-cultured one or two times before ASTs.

Twofold dilutions of CLR (Tokyo Chemical Industry Co., Ltd.), AMX (Tokyo Chemical Industry Co.), MNZ (Fujifilm Wako Chemical), and sitafloxacin hydrate (STX) (Med Cheme Express) were prepared in Brucella agar with 5% horse serum (BR-HS), Brain heart infusion agar (Oxoid) with 5% horse serum (BHI-HS), and Mueller-Hinton agar (Nippon Becton Dickinson Co.) with 5% horse serum (MH-HS). *H. pylori* strains were cultured in broth medium for 18–24 h under microaerobic conditions at 37°C with shaking (130 rpm), and 1 µl of the culture (OD > 1.0:OD₆₀₀) was inoculated onto each agar plate with a multi-point inoculator (MIT-F; Sakuma). Following incubation at 37°C for 72 h under microaerobic conditions, the minimum inhibitory concentration (MIC) value was determined as the lowest concentration that inhibited bacterial growth. MICs of CLR and MNZ against *H. pylori* strains were measured with a range of 0.008–256 µg/ml, and MICs of AMX against *H. pylori* strains were measured with a range of 0.008–16 µg/ml.

The resistance breakpoints for CLR, AMX, and MNZ were >0.5, >0.125, and >8 µg/ml, respectively, according to EUCAST.¹³ The tentative resistance cutoff value for STX was defined as more than 0.12 µg/ml.¹⁴

2.3 | E-test

H. pylori was cultured on Brucella medium containing 7% horse serum and 1.5% agar under microaerobic conditions at 37°C unless otherwise stated. *H. pylori* strains were subjected to ASTs with 3 antibiotics (CLR, AMX and MNZ) using E-test (bioMérieux) to obtain MICs according to the manufacturer's instructions. Briefly, the strains grown were suspended in Mueller-Hinton broth containing 5% horse serum at McFarland equivalent 3 and spread on Mueller-Hinton agar plates containing 5% sheep blood (≥ 2 weeks old). For the AST of MNZ, the agar medium was preincubated under anaerobic condition for 24 h before inoculation of *H. pylori*. E-test strips were placed at the edge of the plates, and the plates were cultured for 3 days. Based on MIC values, antibiotic resistance was determined by the resistance breakpoints described above.

2.4 | Detection of AMX-resistance mutations in PBP1A, macrolide-resistance mutations in 23S rRNA gene and fluoroquinolone-resistance mutations in GyrA

Resistance mutations were detected by direct sequencing of PCR products. DNA was extracted using QIAamp DNA Micro Kit (QIAGEN). Sequences of primers and polymerase chain reaction (PCR) conditions are shown in Table 1. The *pbp1* gene encoding penicillin-binding protein 1A (PBP1A) conferring AMX resistance in *H. pylori* strains was amplified by PCR using primers PBP1-F and PBP1-R.¹⁵ A part of the 23S rRNA gene conferring CLR resistance was amplified by PCR using primers Hp23S 1835F and Hp23S 2327R.¹⁶ Amplification of the quinolone resistance determining region (QRDR) of *gyrA* was done using PCR primers *gyrA* F1 and *gyrA* R1.¹⁷

The PCR reaction mixture consisted of 5 μ l of 10 \times Ex Taq Buffer, 4 μ l of dNTP Mixture, 1 μ l of each primer, 1 μ l of DNA template, 0.5 μ l of TaKaRa Ex Taq (5 unit/ μ l), and 38 μ l of sterile distilled water.

2.5 | Sequence of PCR products

The PCR products were purified by the ethanol precipitation method. The nucleotide sequence was determined by DNA sequencer (PRISM3130 Genetic Analyzer; Life Technologies Japan Ltd.) using forward and reverse primers at the facilities of Hokkaido System Science Co., Ltd. Sequence and amino acid mutations were analyzed by Genetyx software (v.15, Genetyx Corporation).

2.6 | Detection of *H. pylori* phages

We previously reported complete genome sequences of two *H. pylori* phages (KHP30 and KHP40) in clinical isolates from Japan.¹⁸

Using the database, PCR was performed to determine whether phages infected the *H. pylori* strains. Briefly, total genomic DNA extracted (QIAamp DNA mini kit, Qiagen K.K.) was used in PCR with 3 primer sets specific to KHP30 ORFs (13, 14 and 15) and to KHP40 ORFs (14, 15 and 16). The PCR reaction mixture (20 μ l) contained 2 μ l of 10 \times Blend Taq Buffer (Toyobo Osaka), 1.6 μ l of dNTPs, 0.5 μ l of each primer, 1 μ l of genomic DNA template, 0.2 μ l of Blend Taq, and 14.2 μ l of sterile distilled water. The PCR conditions and primers used are shown in Table 1.

2.7 | Genomic sequencing and comparative genomic analysis

Genomic DNA from *H. pylori* strains was extracted using Genomic-tips 100/G and buffers (Qiagen K.K.). One μ g of genomic DNA was treated with Short read eliminator XS (Circulomics Inc.). After treatment, 600 ng of genomic DNA was prepared using a native barcoding expansion kit (EXP-NBD104; barcodes 1 to 12; Oxford Nanopore) and ligation sequencing kit (SQK-LSK108; Oxford Nanopore). The library DNA was prepared using Ligation Sequencing Kit and sequenced on the GridION platform (Oxford Nanopore) using FLO-MIN106 R9.4.1revD. For the short-read sequencing, 400 ng of DNA was prepared using MGIEasy FS PCR Free DNA Library Prep Set (MGI Tech Co., Ltd.) and MGI Easy PF Adapters-16Kit. The library DNA was sequenced on DNBSEQ G400RS (MGI). For complete genome analysis, after quality trimming (average Phred quality value of 10.0, short reads of 1000 bp, and adaptor sequences) using NanoFilt v.2.7.1 software,¹⁹ obtained Nanopore and MGI reads were hybrid-assembled de novo using Unicycler v.0.4.8.²⁰ The genome sequences of the 3 strains have been deposited at GenBank/EMBL/DDBJ under BioProject number PRJDB10083. DDBJ FAST Annotation (DFAST: <https://dfast.nig.ac.jp/>) was used for gene annotation.

3 | RESULTS

3.1 | Determination of *H. pylori* standard strains for reference panel

We performed ASTs with 28 strains collected from 4 medical facilities in Japan. Based on the results, 3 strains (JSHR3, JSHR6 and JSHR31) were selected as standard strains by JSHR for practice in laboratory medicine. The MIC values of each antibiotics against the 3 strains using the agar dilution method with BR-HS medium were as follows: JSHR3 (CLR 16 μ g/ml, AMX 0.032 μ g/ml and MNZ 4 μ g/ml), JSHR6 (CLR 0.016 μ g/ml, AMX 0.032 μ g/ml and MNZ 4 μ g/ml), and JSHR31 (CLR 16 μ g/ml, AMX 1 μ g/ml and MNZ 64 μ g/ml) (Table 2). The results of E-test for the 3 strains are also shown in Table 2. JSHR6 is susceptible to all 3 antibiotics. JSHR3 is CLR-resistant and susceptible to the other two antibiotics. JSHR31 is resistant to all 3 antibiotics.

TABLE 1 List of PCR conditions and primers used in this study

Target gene	Primer	Sequence (5'-3')	Condition	Cycle	Amplicon (bp)	Reference
<i>pbp1A</i>	PBP1-F PBP1-R	5'-GCATGATCGTTACAGACACG-3' 5'-ATCCACGATTTCTTTACGC-3'	Pre-heating: 96°C, 2 min Denature: 94°C, 1 min Annealing: 60°C, 1 min Extension: 72°C, 1 min	35	905	15
23S rRNA	Hp23S 1835F Hp23S 2327R	5'-GGTCTCAGCAAAGAGTCCCT-3' 5'-CCCACCAAGCATTGTCCT-3'	Pre-heating: 96°C, 2 min Denature: 96°C, 30 s Annealing: 57°C, 30 s Extension: 72°C, 1 min	35	493	16
<i>gyrA</i>	gyrA F1 gyrA R1	5'-AAAGCCCGTGCATAGGC-3' 5'-TCCCATTAGCCCCATTGA-3'	Pre-heating: 96°C, 2 min Denature: 96°C, 30 s Annealing: 52°C, 30 s Extension: 72°C, 1 min	35	398	17
KHP30 ORF13/KHP40 ORF14	orf13-F orf13-R	5'-GAAACYTTTAAARCGWGARATCGCGCAA-3' ^a 5'-ACRCCRTTRCCTAACGCTTCTTAGG-3' ^a	Pre-heating: 96°C, 2 min Denature: 96°C, 30 s Annealing: 60°C, 30 s Extension: 72°C, 30 s	40	482	18
KHP30 ORF14/KHP40 ORF15	orf14-F orf14-R	5'-ATTTAGARATYTRAGYCAAACGATCTACCCG -3' ^a 5'-ACGGTTTCATCAATRARAAYCTYGTTCCTTT -3' ^a	Pre-heating: 96°C, 2 min Denature: 96°C, 30 s Annealing: 50°C, 30 s Extension: 72°C, 30 s	40	751	18
KHP30 ORF15/KHP40 ORF16	orf15-F orf15-R	5'-CGRGAAGTSGTRAAGGTGAGYTTAGAA-3' ^a 5'-ACGCTBGYAGAAAARTAAACACTCT-3' ^a	Pre-heating: 96°C, 2 min Denature: 96°C, 30 s Annealing: 50°C, 30 s Extension: 72°C, 30 s	40	269	18

^aB = C + G + T, H = A + C + T, R = A + G, S = C + G, W = A + T, Y = C + T.

TABLE 2 MIC for each antibiotic against each of the 3 strains measured by antimicrobial sensitivity test

Strain	MICs ($\mu\text{g/ml}$)						Features for eradication therapy
	Agar dilution method ^a			E-test ^b			
	CLR	AMX	MNZ	CLR	AMX	MNZ ^c	
JSHR6	0.016	0.032	4	0.016	<0.016	0.5	Susceptible strain
JSHR3	16	0.032	4	256	<0.016	0.023	CLR-resistant strain
JSHR31	16	1	64	16	0.25	96	Multi-resistant strain

^aMICs were measured by using Brucella agar containing 5% horse serum.

^bMICs were measured by using Mueller-Hinton agar containing 5% sheep blood.

^cThe medium was incubated under anaerobic conditions for 24 h before inoculation of *H. pylori*.

All 3 standard strains were defined as negative for phage-specific PCR by using all 3 primer sets, which showed no *H. pylori* phage infection.

3.2 | AMX susceptibilities and PBP1A mutations

We selected #5, #13, #18, #30, and #31 (JSHR31) as AMX-resistant strains using the MIC determined by agar dilution methods. The *pbp1* gene PCR product of each strain was sequenced directly and replaced with amino acids and compared with the reference gene of *H. pylori* ATCC 43504. Number of amino acid substitution mutations in PBP1A of AMX-resistant (MIC range 1–4 $\mu\text{g/ml}$, $n = 5$) and susceptible (MIC ≤ 0.125 $\mu\text{g/ml}$, $n = 21$) *H. pylori* strains were shown in Figure 1. The number of mutations in PBP1A in the resistant strains was significantly higher than that in the susceptible strains. Of these,

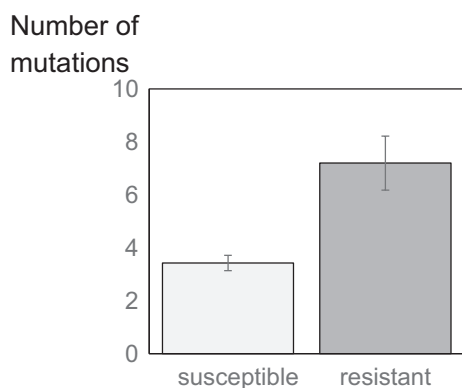


FIGURE 1 Number of amino acid substitution mutations in PBP1A of AMX-resistant (MIC range 1–4 $\mu\text{g/ml}$) ($n = 5$) and susceptible (MIC ≤ 0.125 $\mu\text{g/ml}$) ($n = 21$) *Helicobacter pylori* strains

TABLE 3 Genome information of *H. pylori* JSHR6, JSHR3, and JSHR31 strains

Strain	Total genome size (BP)	GC (%)	CDS	tRNA	rRNA
JSHR6	1,551,197	38.9	1495	36	4
JSHR3	1,624,837	38.8	1571	37	4
JSHR31 (chromosome)	1,610,599	38.7	1526	36	4
(Plasmid)	9122	33.6	8	0	0

it was shown that JSHR 31 had the largest number (10 amino acids) of mutations, among the AMX-resistant strains (Figure 1).

Comparing the sequences of PBPs with JSHR3 and JSHR6, JSHR31 was found to have mutations at position 562, 593, and 595 in PBP1A, a mutation at position 444 in PBP2 and mutations at position 541 and 519 in FtsI. The prediction of the structures of *H. pylori* PBPs revealed that all 3 mutations in PBP1A of JSHR31 are located close to the penicillin-binding pocket, whereas the PBP2 and FtsI mutations are not located close to the penicillin-binding pocket (Figure S1).

3.3 | CLR susceptibilities and 23S rRNA gene mutations

Clarithromycin resistance of *H. pylori* is linked to a single nucleotide polymorphism (SNP) in the 23S rRNA gene. All the 11 CLR-resistant strains had an A2142G (3 strains) or A2143G (8 strains) mutation. JSHR 3 had A2143G and JSHR31 had A2142G mutation. *H. pylori* has two 23S rRNA genes, and the complete genome sequences of *H. pylori* JSHR3, and JSHR31 strains (shown below) confirmed the same mutations in both genes.

3.4 | MNZ and fluoroquinolone susceptibilities

A total of 8 *H. pylori* strains were resistant to MNZ in this study. The higher MIC (64–128 $\mu\text{g/ml}$) of MNZ was shown in 4 *H. pylori* strains, and the middle range MIC (16–32 $\mu\text{g/ml}$) was shown in 4 *H. pylori* strains by agar dilution methods using BR-HS and BHI-HS. The MICs of MNZ against these middle range strains measured using MH-HS medium were 8 $\mu\text{g/ml}$, which was lower than those using BR-HS and

BHI-HS. The MIC of MNZ against *H. pylori* JSHR31 strain was 64–128 µg/ml using all 3 media.

JSHR3 had a resistance mutation from Asn to Tyr at position 87 and JSHR31 had a resistance mutation from Asp to Tyr at position 91 in *GyrA*, and MICs for STX were 1 and 0.125 µg/ml, respectively. JSHR6 is susceptible to STX (MIC 0.06 µg/ml) and the resistant mutations in *gyrA* gene were not shown in JSHR6 genome.

3.5 | Genomic structure

Genome sequences of *H. pylori* JSHR 3, JSHR6, and JSHR31 strains were determined by short- and long-read sequencing. The genome sequence was assembled using de novo sequence assembler software and complete genomes of 3 strains were determined. Only the JSHR31 strain has a plasmid. Comparative genome information of the 3 chromosomes of the *H. pylori* strains was shown in Table 3 and Figure S2. In the neighbor-joining tree of CagA protein sequences from Kumar reports,²¹ JSHR 3 and 6 belong to ABD type I and JSHR 31 belongs to ABD type II (data not shown).

The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers, JSHR6; AP023347, JSHR3; AP023344, JSHR31; AP023345, JSHR31-PL; AP023346.

4 | DISCUSSION

Standard bacterial strains are important to quality control antimicrobial susceptibility testing. We aimed to establish a reference panel of control strains that can be used easily in Japan to standardize antimicrobial susceptibility testing for *H. pylori*. *H. pylori* ATCC 43504 strain has been used as a control strain in several previous studies and CLSI M100-S18²² for ASTs and pathogenicity studies as the complete genome sequences are available.²³

Japanese clinical testing companies perform various methodologies for antimicrobial susceptibility testing for *H. pylori*. The agar dilution method, liquid medium dilution method using a microtiter plate, dry plate method (distributed by Eiken Chemical Co. Ltd.), and E-test are common in Japan. The microtiter plate liquid medium dilution methods and E-test are simple and easy-to-handle compared with agar dilution methods. There are some problems with ASTs in *H. pylori*, such as that the organism is microaerophilic and poor growth of *H. pylori* strains may lead to false susceptibility results. Judging the MIC results also requires a good deal of discernment, and sometimes a microscope is required for microtiter plates. Therefore, for the establishment of reference strains for susceptibility testing, two methods were applied for accuracy: agar dilution method and E-test.

The discrepancy between the E-test method and the agar dilution method has been pointed out in prior studies.^{24–27} For the E-test, ambient temperature, humidity, depth of medium, media type, and supplemental additives may affect the diffusion efficiency of the drug on the medium and therefore the results.²⁶ Furthermore, MNZ

is also affected by oxygen concentration. Since the hemoglobin in blood is bound to oxygen, aged blood and anaerobic pre-incubated blood agar is recommended.²⁵ On the other hand, in the agar dilution method, it is important to measure the drug accurately, dissolve it completely, and dilute it exactly.

Brucella and BHI media are used commonly for culture of *H. pylori* as basal media, but Mueller-Hinton medium is the standard medium for the AST. Brucella and BHI media supplemented with horse serum are also commonly used for liquid culture of *H. pylori*. The CLSI recommends an agar dilution method with Mueller-Hinton agar supplemented with 5% aged sheep blood for MIC interpretation for *H. pylori*.²¹ Mueller-Hinton agar is used to control the diffusion of the antibiotics on the medium. As mentioned above, the medium is important for the diffusion efficiency of the antibiotics and as such, Mueller-Hinton agar with 5% aged sheep blood is recommended for E-test.

For the agar dilution method, we recommend BR-HS that is usually used for sub-culture of *H. pylori*. We generally use medium containing 5%–10% serum for sub-culture of *H. pylori* and the lowest concentration of 5% serum was used for AST to avoid too much growth of *H. pylori*. As it is difficult to stock sheep blood all the time, we eventually often used sheep blood with different lot numbers. The horse serum is easily obtained and can be frozen for stock. *H. pylori* clinical isolates grow better on BR-HS medium compared to MH-HS medium. Thus, bacterial adaptation to the media for AST (ie, MH-HS medium) is necessary in Japan whenever we perform, leading to time consuming.

The mechanism of resistance to CLR is well known. Mutations in the 23S rRNA gene of *H. pylori* reduces the affinity to CLR and causes the MIC to markedly elevate.²⁸ In this study, CLR-resistant strains JSHR3 and JSHR31 demonstrated A2143G and A2142G mutations, respectively. A2142G has been shown to confer higher resistance to CLR than A2143G, but the MICs of CLR were the same for JSHR3 and JSHR31 strains.²⁹

The mechanism of resistance to AMX in *H. pylori* has been shown to be related to the number of mutations in PBPA1 and increasing mutations correlate with MIC¹⁵ AMX-resistant strain JSHR31 possessed several mutations structurally close to the penicillin-binding pocket, and these mutations could have altered the affinity of AMX to PBPs resulting in AMX resistance (Figure S1).

The mechanism of resistance to MNZ is complex. MNZ is a pro-drug, which is reduced by intracellular reductases and converted into active nitroso radicals that disrupt DNA. The major reductases for MNZ in *H. pylori* are RdxA, oxygen-insensitive nitroreductase, and FrxA, flavin reductase, as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases.³⁰ Goodwin et al³¹ demonstrated that a mechanism of MNZ resistance in *H. pylori* is due to null mutations in the *rdxA* gene, which encodes an oxygen-insensitive nitroreductase. Kwon et al³² reported the role of mutations in an additional gene *frxA*, which encodes NADPH flavin oxidoreductase, in MNZ-resistant *H. pylori*. The significant mutations previously reported in these studies were not demonstrated in the *rdxA* and *frxA* genes of MNZ-resistant *H. pylori* JSHR31 strain in this study. In previous

reports, mutations in *rdxA* and *frxA* alone are unable to explain MNZ resistance in *H. pylori* isolates.³⁰ Further studies are needed to elucidate the mechanism.

Helicobacter pylori possesses a high genetic diversity and is thought to produce a variety of clones, which leads to persistent infection in the stomach. During persistent infection, within-host evolution of *H. pylori* shaped by niche-specific adaptation and intra-gastric migration increases diversity of *H. pylori* strains colonized.³³ Furthermore, *H. pylori* bacteriophages (phages) contribute to evolution and promote the development of a flexible *H. pylori* community with variable characteristics.³⁴ Thus, phage infection should be investigated in strains to predict the characteristic diversity during in vitro culture for sustainable standard strains in a medical laboratory. In this study, we did not find evidence of phage infection.

As mentioned earlier, AMX and CLR or MNZ are used as the first- and second-line eradication therapy for *H. pylori* infection in Japan. Fluoroquinolones are valuable drugs for alternative therapy. However, the isolation rate of levofloxacin-resistant *H. pylori* was found to be around 30% in Japan in a recent study.³⁵ Patients with eradication failure are treated with STX as 3rd line therapy, as it has a higher antimicrobial activity than other fluoroquinolones.^{14,36} Susceptibility to STX was also tested in this study. Mutations that cause fluoroquinolone resistance have been found in the QRDR with the major mutations being at position 87 and 91 in the QRDR of GyrA.^{37,38} Mutation from Asn to Lys at position 87 of GyrA confers higher resistance to levofloxacin and gatifloxacin than mutation from Asp to Asn at position 91.¹⁷ JSHR3 has a mutation from Asn to Tyr at position 87 of GyrA and JSHR31 has a mutation from Asp to Tyr at position 91 in GyrA and showed MICs of STX of 1 and 0.125 µg/ml, respectively. It was reported that the relative risk of the eradication failure is significantly lower in GyrA mutation at D91 compared with GyrA mutation at N87. It was also reported that the MICs of double-mutated strains were extremely higher than those of single-mutated strains.³⁹ In this study, none of strains showed STX MICs greater than 1 µg/ml and double-mutated strain was not found (data not shown).

In conclusion, in this study, we have established a reference panel of *H. pylori* JSHR strains for ASTs. The panel consisted of JSHR6 which was antibiotic-susceptible, JSHR3 which was CLR-resistant, and JSHR31 which was multi-resistant.

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CONFLICT OF INTEREST

All other authors declare no conflicts of interest.

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REFERENCES

1. Tsuda M, Asaka M, Kato M, et al. Effect on *Helicobacter pylori* eradication therapy against gastric cancer in Japan. *Helicobacter*. 2017;22(5):e12415.
2. Uno Y. Prevention of gastric cancer by *Helicobacter pylori* eradication: a review from Japan. *Cancer Med*. 2019;8(8):3992-4000.
3. Hiroi S, Sugano K, Tanaka S, Kawakami K. Impact of health insurance coverage for *Helicobacter pylori* gastritis on the trends in eradication therapy in Japan: retrospective observational study and simulation study based on real-world data. *BMJ Open*. 2017;7(7):e015855.
4. Kato M, Ota H, Okuda M, et al. Guidelines for the management of *Helicobacter pylori* infection in Japan: 2016 Revised Edition. *Helicobacter*. 2019;24(4):e12597.
5. Savoldi A, Carrara E, Graham DY, Conti M, Tacconelli E. Prevalence of antibiotic resistance in *Helicobacter pylori*: a systematic review and meta-analysis in World Health Organization Regions. *Gastroenterology*. 2018;155(5):1372-1382.e17.
6. Kawai T, Takahashi S, Suzuki H, et al. Changes in the first line *Helicobacter pylori* eradication rates using the triple therapy—a multicenter study in the Tokyo metropolitan area (Tokyo *Helicobacter pylori* study group). *J Gastroenterol Hepatol*. 2014;29(Suppl 4):29-32.
7. Murakami K, Sakurai Y, Shiino M, Funao N, Nishimura A, Asaka M. Vonoprazan, a novel potassium-competitive acid blocker, as a component of first-line and second-line triple therapy for *Helicobacter pylori* eradication: a phase III, randomised, double-blind study. *Gut*. 2016;65(9):1439-1446.
8. Suzuki S, Gotoda T, Kusano C, Iwatsuka K, Moriyama M. The efficacy and tolerability of a triple therapy containing a potassium-competitive acid blocker compared with a 7-day PPI-based low-dose clarithromycin triple therapy. *Am J Gastroenterol*. 2016;111(7):949-956.
9. Mori H, Suzuki H, Omata F, et al. Current status of first- and second-line *Helicobacter pylori* eradication therapy in the metropolitan area: a multicenter study with a large number of patients. *Therap Adv Gastroenterol*. 2019;12:1756284819858511.
10. Deguchi H, Uda A, Murakami K. Current status of *Helicobacter pylori* diagnosis and eradication therapy in Japan using a nationwide database. *Digestion*. 2020;101(4):441-449.
11. Asaoka D, Nagahara A, Matsuhisa T, et al. Trends of second-line eradication therapy for *Helicobacter pylori* in Japan: a multicenter study in the Tokyo metropolitan area. *Helicobacter*. 2013;18(6):468-472.
12. Osaki T, Mabe K, Zaman C, et al. Usefulness of detection of clarithromycin-resistant *Helicobacter pylori* from fecal specimens for young adults treated with eradication therapy. *Helicobacter*. 2017;22(5):e12396.
13. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_11.0_Breakpoint_Tables.pdf
14. Matsuzaki J, Suzuki H, Nishizawa T, et al. Efficacy of sitafloxacin-based rescue therapy for *Helicobacter pylori* after failures of first- and second-line therapies. *Antimicrob Agents Chemother*. 2012;56(3):1643-1645.
15. Kageyama C, Sato M, Sakae H, et al. Increase in antibiotic resistant *Helicobacter pylori* in a University Hospital in Japan. *Infect Drug Resist*. 2019;12:597-602.

16. Noguchi N, Rimbara E, Kato A, et al. Detection of mixed clarithromycin-resistant and -susceptible *Helicobacter pylori* using nested PCR and direct sequencing of DNA extracted from faeces. *J Med Microbiol*. 2007;56(Pt 9):1174-1180.
17. Rimbara E, Noguchi N, Kawai T, Sasatsu M. Fluoroquinolone resistance in *Helicobacter pylori*: role of mutations at position 87 and 91 of GyrA on the level of resistance and identification of a resistance conferring mutation in GyrB. *Helicobacter*. 2012;17(1):36-42.
18. Uchiyama J, Takeuchi H, Kato S, et al. Complete genome sequences of two *Helicobacter pylori* bacteriophages isolated from Japanese patients. *J Virol*. 2012;86(20):11400-11401.
19. De Coster W, D'Hert S, Schultz DT, Cruys M, Van Broeckhoven C. NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics*. 2018;34(15):2666-2669.
20. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol*. 2017;13(6):e1005595.
21. Pachathundikandi SK, Gutiérrez-Escobar AJ, Tegtmeyer N. Tailor-made detection of individual phosphorylated and non-phosphorylated EPIYA-motifs of *Helicobacter pylori* oncoprotein CagA. *Cancers*. 2019;11(8):1163.
22. Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standard; eighteenth informational supplement, M100-S18, 28(1), 2008.
23. Kinoshita-Daitoku R, Ogura Y, Kiga K, et al. Complete genome sequence of *Helicobacter pylori* strain ATCC 43504, a type strain that can infect gerbils. *Microbiol Resour Announc*. 2020;9(18):e00105-20.
24. Cederbrant G, Kahlmeter G, Ljungh A. Proposed mechanism for metronidazole resistance in *Helicobacter pylori*. *J Antimicrob Chemother*. 1992;29(2):115-120.
25. Chida-Sakata N, Baba M, Inagawa H, et al. Significance of anaerobic preincubation of *Helicobacter pylori* for measuring metronidazole susceptibility by the Etest. *Microbiol Immunol*. 1999;43(5):397-401.
26. Citron DM, Ostovari MI, Karlsson A, Goldstein EJ. Evaluation of the E test for susceptibility testing of anaerobic bacteria. *J Clin Microbiol*. 1991;29(10):2197-2203.
27. Miftahussurur M, Fauzia KA, Nusi IA, et al. E-test versus agar dilution for antibiotic susceptibility testing of *Helicobacter pylori*: a comparison study. *BMC Res Notes*. 2020;13(1):22.
28. Alarcón T, Domingo D, Prieto N, López-Brea M. Clarithromycin resistance stability in *Helicobacter pylori*: influence of the MIC and type of mutation in the 23S rRNA. *J Antimicrob Chemother*. 2000;46(4):613-616.
29. Boyanova L, Markovska R, Yordanov D, Gergova G, Mitov I. Clarithromycin resistance mutations in *Helicobacter pylori* in association with virulence factors and antibiotic susceptibility of the strains. *Microb Drug Resist*. 2016;22(3):227-232.
30. Marais A, Bilardi C, Cantet F, Mendz GL, Mégraud F. Characterization of the genes *rdxA* and *frxA* involved in metronidazole resistance in *Helicobacter pylori*. *Res Microbiol*. 2003;154(2):137-144.
31. Goodwin A, Kersulyte D, Sisson G, Veldhuyzen van Zanten SJ, Berg DE, Hoffman PS. Metronidazole resistance in *Helicobacter pylori* is due to null mutations in a gene (*rdxA*) that encodes an oxygen-insensitive NADPH nitroreductase. *Mol Microbiol*. 1998;28(2):383-393.
32. Kwon DH, Kato M, El-Zaatari FA, Osato MS, Graham DY. Frame-shift mutations in NAD(P)H flavin oxidoreductase encoding gene (*frxA*) from metronidazole resistant *Helicobacter pylori* ATCC43504 and its involvement in metronidazole resistance. *FEMS Microbiol Lett*. 2000;188(2):197-202.
33. Ailloud F, Didelot X, Woltemate S, et al. Within-host evolution of *Helicobacter pylori* shaped by niche-specific adaptation, intragastric migrations and selective sweeps. *Nat Commun*. 2019;10(1):2273.
34. Takeuchi H, Kira M, Konishi S, Uchiyama J, Matsuzaki S, Matsumura Y. Polymorphisms in the *Helicobacter pylori* NY43 strain and its prophage-cured derivatives. *Microbiology*. 2018;164(6):877-882.
35. Kuo YT, Liou JM, El-Omar EM, et al. Primary antibiotic resistance in *Helicobacter pylori* in the Asia-Pacific region: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol*. 2017;2(10):707-715.
36. Murakami K, Okimoto T, Kodama M, et al. Sitafloxacin activity against *Helicobacter pylori* isolates, including those with *gyrA* mutations. *Antimicrob Agents Chemother*. 2009;53(7):3097-3099.
37. Miyachi H, Miki I, Aoyama N, et al. Primary levofloxacin resistance and *gyrA/B* mutations among *Helicobacter pylori* in Japan. *Helicobacter*. 2006;11(4):243-249.
38. Tankovic J, Lascols C, Sculo Q, Petit JC, Soussy CJ. Single and double mutations in *gyrA* but not in *gyrB* are associated with low- and high-level fluoroquinolone resistance in *Helicobacter pylori*. *Antimicrob Agents Chemother*. 2003;47(12):3942-3944.
39. Mori H, Suzuki H, Matsuzaki J, Masaoka T, Kanai T. Acquisition of double mutation in *gyrA* caused high resistance to sitafloxacin in *Helicobacter pylori* after unsuccessful eradication with sitafloxacin-containing regimens. *United European Gastroenterol J*. 2018;6(3):391-397.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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