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

Kenji Yokota¹,² | Takako Osaki¹,³ | Shunji Hayashi¹,⁴ | Shin-ichi Yokota¹,⁵ | Hiroaki Takeuchi¹,⁶ | Emiko Rimbara⁷ | Hinako Ojima² | Toyotaka Sato⁵ | Hideo Yonezawa³ | Keigo Shibayama⁸ | Kengo Tokunaga⁹ | Shigeru Kamiya³ | Kazunari Murakami¹⁰ | Mototsugu Kato¹¹ | Toshiro Sugiyama¹²

¹Working Group of the Reference Panel of Helicobacter pylori Strains for Antimicrobial Susceptibility Tests in Japanese Society for Helicobacter Research, Tokyo, Japan
²Graduate School of Health Science, Okayama University, Okayama, Japan
³Department of Infectious Diseases, Kyorin University School of Medicine, Mitaka, Japan
⁴Department of Microbiology, Kitasato University School of Medicine, Sagamihara, Japan
⁵Department of Microbiology, Sapporo Medical University School of Medicine, Sapporo, Japan
⁶Department of Medical Laboratory Sciences, Health Sciences, International University of Health and Welfare Graduate School, Narita, Japan
⁷Department of Bacteriology II, National Institute of Infectious Diseases (NIID), Musashimurayama, Japan
⁸Department of Bacteriology, Nagoya University Graduate School of Medicine, Nagoya, Japan
⁹Department of General Medicine, Kyorin University School of Medicine, Mitaka, Japan
¹⁰Department of Gastroenterology, Faculty of Medicine, Oita University, Oita, Japan
¹¹Department of Gastroenterology, National Hospital Organization National Hakodate Hospital, Hakodate, Japan
¹²Research Division of Molecular Target Therapeutics and Prevention of GI Cancer, Hokkaido University Hospital, Sapporo, Japan

Correspondence
Takako Osaki, Department of Infectious Diseases, Kyorin University School of Medicine, Mitaka, Tokyo, Japan.
Email: osaki@ks.kyorin-u.ac.jp

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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...
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, 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 50,000 per year for many years, is gradually decreasing.

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. 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 al 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*. 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. 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%.

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, 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. 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:OD 600 μl was inoculated onto each agar plate with a multipoint 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. The tentative resistance cutoff value for STX was defined as more than 0.12 μg/ml.
2.3 | E-test

*Helicobacter 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 (bioMerieux) 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 preincubate 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. A part of the 23S rRNA gene conferring CLR resistance was amplified by PCR using primers Hp23S 1835F and Hp23S 2327R. Amplification of the quinolone resistance determining region (QRDR) of gyrA was done using PCR primers gyrA F1 and gyrA R1.

The PCR reaction mixture consisted of 5 µl of 10X 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), and 38 µl 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. 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 µl) contained 2 µl of 10X Blend Taq Buffer (Toyobo Osaka), 1.6 µl of dNTPs, 0.5 µl of each primer, 1 µl of genomic DNA template, 0.2 µl of Blend Taq, and 14.2 µl 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 µg 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 DNBSER 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, obtained Nanopore and MGI reads were hybrid-assembled de novo using Unicycler v.0.4.8. 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-LS medium were as follows: JSHR3 (CLR 16 µg/ml, AMX 0.032 µg/ml and MNZ 4 µg/ml), JSHR6 (CLR 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) (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.
| Target gene | Primer | Sequence (5’-3’) | Condition | Cycle | Amplicon (bp) | Reference |
|-------------|--------|------------------|-----------|-------|---------------|-----------|
| pbp1A       | 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’-CCCACCAAGCATGTCTTCT-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        |
| gyrA        | gyrA F1 gyrA R1 | 5’-AAAGCCCGTGATAGGCG-3’ 5’-TCCCATATGCCATTGA-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’-GAAACYTTTARCRGWCATCGCCAA-3’ 5’-ACRCCTRTCCTAAGGCTTCTTTAGG-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’-ATTAGARATYTTTRGCAAAACGATCTACCAG-3’ a 5’-ACGGTTGTCATCAARTARAAAYCTYGTTTCTTCCG-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’-GCRGAAGTSRAGTGAGGTAGAA-3’ a 5’-ACGCTBGGYAGAAARTAAACACTCTT-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.
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 μg/ml, *n* = 5) and susceptible (MIC ≤ 0.125 μg/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, 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 JHSR6, JHSR31 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 μg/ml) of MNZ was shown in 4 *H. pylori* strains, and the middle range MIC (16–32 μg/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 μg/ml, which was lower than those using BR-HS and BHI-HS.

| Feature for eradication therapy | CLR | AMX | MNZ | CLR | AMX | MNZ |
|---------------------------------|-----|-----|-----|-----|-----|-----|
| Susceptible strain              | 0.016 | 0.032 | 4 | 0.016 | <0.016 | 0.5 |
| CLR-resistant strain            | 16 | 0.032 | 4 | 256 | <0.016 | 0.023 |
| Multi-resistant strain          | 16 | 1 | 64 | 16 | 0.25 | 96 |

* MICs were measured by using Brucella agar containing 5% horse serum.

| 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    |

**TABLE 3** Genome information of *H. pylori* JSHR6, JSHR3, and JSHR31 strains.
were determined by short- and long-read sequencing. The genome panel of control strains that can be used easily in Japan to standard-crobial susceptibility testing. We aimed to establish a reference tations in JSHR31-PL; AP023346. 31 belongs to ABD type II (data not shown).

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, 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 standard-ize 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-S18 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 JHSR31 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 JHSR31 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 prod-rug, 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 intragastric 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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SUPPORTING INFORMATION

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

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