Study on the value of antibiotic-resistant gene detection in Helicobacter pylori in China

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Abstract. The aim of the present study was to explore the value of detecting antibiotic-resistant genes in Helicobacter pylori (H. pylori) and the association between genotype and antibiotic resistance. Two gastric mucosa samples from each H. pylori-positive patient were collected. Each patient’s H. pylori sample was cultured in vitro, and the agar plate dilution method was conducted. In addition, all patient samples were analyzed for the detection of antibiotic resistance-related mutant genes and VacA gene genotypes. The association between VacA genotypes and antibiotic resistance was also determined and the value of mutant gene detection in predicting H. pylori resistance to antibiotics was evaluated. In total, 133 H. pylori-positive patients were enrolled. A total of 22 strains of H. pylori failed to grow in vitro culture and 25 strains were negative in a H. pylori gene test. Among 108 strains detected by PCR, a total of 39 VacA s1m1 strains, 69 VacA s1m2 strains and no VacA s2 strain were identified. There was no significant association between VacA genotypes and antibiotic resistance. The mutation rates of G616A in the rdxA gene, T87A, G91A, A91G and G91T in the gyrA gene and A2143G and A2142G in the 23S rRNA were 32.3, 22.6, 12.9, 6.5, 81.8 and 0.0%, respectively. Among these mutant sites, the mutation coincidence rates were as follows:

| Gene   | Coincidence Rate (%) |
|--------|----------------------|
| gyrA   | 12.6                 |
| rdxA   | 8.7                  |
| 23S rRNA | 7.1              |

There was no significant association between mutant genes and antibiotic resistance. The mutation rates of G616A in the gyrA gene, T87A, G91A, A91G and G91T in the gyrA gene and A2143G and A2142G in the 23S rRNA gene were 32.1, 32.3, 22.6, 12.9, 6.5, 81.8 and 0.0%, respectively. Among these mutant sites, the mutation coincidence rates were as follows, according to the agar plate dilution method: rdxA G616A (81.8%), gyrA G91T (65.7%), gyrA G91A (54.5%), 23S rRNA A2143G (49.1%), gyrA T87A (45.5%), gyrA A91G (33.3%), penicillin-binding protein 1 (PBP1) C556G (0.0%), PBP1 A562T (0.0%), PBP1 A562G (0.0%) and 16S rRNA 926-927 (AT-GT) (0.0%). VacA subtypes were not associated with H. pylori antibiotic resistance. In conclusion, the present findings suggested that the detection of related mutant genes had a clinical application value in predicting the antibiotic resistance of H. pylori, particularly resistance to clarithromycin and levofloxacin.

Introduction

Helicobacter pylori (H. pylori) is a spiral-shaped, microaerophilic and Gram-negative bacillus that was isolated by Marshall and Warrenin 1983 (1). H. pylori infection has been considered as an independent risk factor for gastric cancer for 27 years (2). Both laboratory and clinical studies have confirmed that H. pylori is a key factor in the occurrence of gastric cancer. The Kyoto consensus published in 2015 reported that H. pylori infection is an infectious disease. The earlier H. pylori is eradicated, the lower the incidence of gastric cancer will be. The eradication program should contain the most effective antibiotics available (3). It has been reported that eradication is more successful when antibiotics are selected based on individual susceptibility, community antibiotic sensitivity testing or antibiotic use and clinical prognosis data (3). However, the increased antibiotic resistance rates have become a severe challenge for the clinical eradication of H. pylori. The resistance rate to tetracycline (TET), amoxicillin (AMX) and furazolidone (FUR) in the majority of countries and regions in the world is relatively low (<10%) (4-10). However, it has been reported that the abuse of antibiotics in Iran has led to a high antibiotic resistance rate of H. pylori to TET (38.5%), AMX (27.1%) and FUR (23.9%) (11). As of June 2017, in all World Health Organization regions, the primary and secondary resistance rates of clarithromycin (CLA), metronidazole (MNZ) and levofloxacin (LEFX) were ≥15%, except for the primary and secondary resistance rates of CLA in America (10%; 95% CI, 4-16%) and Southeast Asia (10%; 95% CI, 5-16%) and LEFX in Europe (11%; 95% CI, 9-13%), while the global double resistance rate of CLA and MNZ was 19% (12). It is generally accepted that the main detection method of the antibiotic resistance of H. pylori is a drug sensitivity test based on the in vitro culture of H. pylori (13). Since H. pylori is a microaerophilic bacterium, its in vitro culture is challenging and time-consuming. In addition, numerous hospitals do not dispose the facilities and equipment to carry out this test. A previous study has indicated that there are several resistance mechanisms of H. pylori to antibiotics, including enhanced oxidative free radical scavenging activity, increased DNA repair enzyme activity, drug efflux pump expression and activation,
as well as resistant gene expression, which is the focus of the present study (8). The Vacuolating cytotoxic gene A (VacA) gene exists in almost all H. pylori strains. However, only ~50% of the strains can express VacA, an important virulence factor in H. pylori production that causes vacuolation (13). The VacA gene is divided into middle region types ml1 and ml2, intermediate region i1, i2 and i3 and signal sequence types slA, slB, slC and s2 (14). The association between VacA subtypes and H. pylori resistance remains controversial (15,16). The detection technology of drug-resistant genes is simpler and faster than the drug sensitivity in vitro culture-based test, and it can detect the H. pylori genotype at the same time (17). Therefore, the present study explored the value of antibiotic-resistant gene detection in H. pylori and the association between genotype and antibiotic resistance.

Patients and methods

Patients. Patients with H. pylori infection who were subjected to gastroscopy were enrolled between December 2017 and January 2020 in The First Affiliated Hospital of Zhejiang Chinese Medical University (Zhejiang, China). A total of 133 H. pylori-positive patients were enrolled in the present study, with a mean age of 45.1 years (age range, 18-71 years). The inclusion criteria were as follows: i) Age, 18-70 years old; ii) pathological findings of H. pylori infection; and iii) positive in 14C-urea and 14C-breath tests. The exclusion criteria were as follows: i) Gastroscopy contraindications; ii) diagnosis of severe cardiovascular diseases, immune disorders, poorly controlled diabetes, hypertension or recently planned subtotal gastrectomy; iii) inability to obtain biopsies due to poor coagulation function or anticoagulant drugs; iv) inability to complete gastroscopy due to lack of cooperation; and v) refusal to participate in a clinical trial.

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang Traditional Chinese Medical University (approval no. 2017-KL-054-02; Hangzhou, China). All patients provided written informed consent and general information, such as sex and age. Gastric mucosa samples were obtained from all patients (three samples per patient; each sample was 0.4-0.6 mm²). One sample was sent for routine pathological examination, one was cultured in vitro and subjected to a drug sensitivity test (agar plate dilution method), and one was sent for antibiotic resistance gene detection (PCR reverse dot blot; a chip with mutation sites presented in Table 1). Finally, the sensitivity of H. pylori detection was compared between the two methods. The mutation sites tested in the present study were as follows: C556G, A562T and A562G in PBPI for AMX; T87A, A91G, G91T and G91A in gyrA for LEFX; 926-928 (AGA to TTC) and 926-927 (AG to GT) in 16SrRNA for TET; A2142G and A2143G in 23rRNA for CLA; and G616A in rdxA for MNZ (Table 1). The mutation rate of the related gene sites was calculated in different antibiotic-resistant strains and the antibiotic resistance rate was calculated in gene mutant strains. Finally, the drug resistance rate was compared among different VacA genotypes.

\[ H. pylori \text{ culture and drug sensitivity test} \]

\[ H. pylori \text{ culture. Gastric mucosa tissue immersed in Bacterial Preservation Fluid (Hangzhou ZhiYuan Medical Laboratory)} \]

was aspirated using a sterile pipette. A total of 0.5 ml RIPA Lysis Buffer (Hangzhou ZhiYuan Medical Laboratory) was to form tissue homogenate. The tissue homogenate was then added to the culture medium of Columbia blood agar (500 g; Thermo Fisher Scientific Inc.). The medium was incubated in a microaerobic environment (5% O₂; 10% CO₂; 85% N₂; 37°C) for 2-3 days. The suspected colonies were selected for hematoxylin-eosin staining (6.0% hematoxylin for 5 min and 0.5% eosin 2 min at room temperature) and were subjected to three enzyme identification tests [urease (Suzhou Haibo Biotechnology Co., Ltd., 37°C, 180 min), oxidase (Beijing Luqiao Technology Co., Ltd., room temperature 5 min) and catalase (Suzhou Haibo Biotechnology Co., Ltd., room temperature 5 min; data not shown]. If the cultured bacteria were identified as H. pylori, the result was positive; otherwise, it was negative.

\[ \text{Drugsensitivity test of H. pylori. The H. pylori colonies were selected in sterile normal saline, shaken and mixed well. Next, the bacterial suspension containing 0.5 MCF (150 million bacteria/millilitre) was prepared. A total of 3 µl bacterial suspension was collected using a pipette gun and added to the blood agar medium, with and without the following antibiotics: CLA (100 mg x 10 tablets; National Medical Products Administration), LEFX (100 mg x 10 capsules; China Academy of Food and Drug Control), MNZ (100 mg x 10 tablets; China Academy of Food and Drug control), AMX (0.25 g x 30 capsules; Dr. Ehrenstorfer GmbH). Three gas incubators were purchased from Thermo Fisher Scientific, Inc. (cat. no. 3427). The antibiotic concentration was the drug-sensitive minimum inhibitory concentration of the antibiotic: 1 clarithromycin, 2 levofloxacin, 2 amoxicillin, 2 furazolidone, 2 tetracyclins and 8 µg/ml metronidazole. The medium was incubated in a microaerobic environment (5% O₂; 10% CO₂; 85% N₂; 37°C) for 2 days. The growth state of the strain was first observed in the culture medium without antibiotics. If it grew normally, the drug sensitivity test result could be interpreted; otherwise, the test needed to be repeated. Next, the colony growth state was observed in the culture medium containing antibiotics. If the colony growth was inhibited, H. pylori was considered to be sensitive to the antibiotic; otherwise, H. pylori was considered to be resistant to the antibiotic.} \]

\[ \text{PCR reverse dot blot} \]

\[ \text{Preservation of samples and sample processing. Gastric mucosa samples were preserved at -80°C. For genotyping and drug resistance gene mutation testing of H. pylori, a nucleic acid extraction reagent was used [Nucleic acid extraction kit, Yaneng Biosciences (Shenzhen) Co., Ltd., cat. no. Yuehen Xiebei no. 20150098; model no. pathogen DNA (centrifugal column type); specification, 25 copies/box]. The concentration and purity of the template DNA prior to PCR was determined using a nucleic acid quantitative analyzer (Multi-mode Readers BioTek).} \]

\[ \text{Preparation of amplification reaction solution. The sequences of the gene primers used are listed in Table II. The number of reaction solutions required for each test was n+2, where n=number of samples to be tested, and 2 equals one H. pylori positive quality control sample and one H. pylori negative} \]
quality control material. Reaction solutions I, II and III were removed from the reagent box, melted and mixed. The amplification reaction solution was prepared according to the manufacturer's instructions. After the mixture was prepared, it was evenly mixed and centrifuged at 447.2 x g for 10 sec at 4˚C.

**Sample addition.** The sample DNA was tested (three times per sample; 4 µl each) and added into three different amplification reaction solutions. Quality control samples (DNA of known dose) were also included (three times per sample; 4 µl each) and added into three different amplification reaction solutions for PCR amplification.

**PCR amplification.** PCR was performed using the *H. pylori* Genotypes and drug resistance mutations detecting kit (PCR-reverse dot blot; Hangzhou Qianji Biosciences Co., Ltd. China). The PCR parameters are presented in Table III. The temperature rise and fall rate was 3.0˚C/sec.

| Table I. A total of 12 gene mutation types associated with antibiotic resistance. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Mutation type Gene | Amoxicillin | Levofloxacin | Tetracycline | Clarithromycin | Metronidazole |
| Mutant site | PBP1 | gyrA | 16S rRNA | 23S rRNA | rdxA |
| 556 | 87 | 2142 | 1616 | |
| 562 | 91 | 2143 | | |
| T87A | | 2142 | | |
| A91G | | 2143 | | |
| G91T | | | | |
| G91A | | | | |

| Table II. Sequences of the forward primers. |
|-------------------------------------------|
| A, Wild-type |
| Primer (site) | Number | Sequence, 5’→3’ |
| 16S rRNA (926–928) | 16S W | CGAAGATACACGGAAGAAC |
| 23S rRNA (2142/2143) | 23S W | ACGGAAAGACCCCGTGT |
| gyrA (87) | 87 W | GGCAGTATACGGCTTT |
| gyrA (91) | 91 W | TTTATGATGCACTAGTGAG |
| PBP1 (556) | 556 W | AACCCGGGACTTTCAATA |
| PBP1 (562) | 562W | AATGGTGATTGCTTTGTTCA |
| rdxA (565) | 565W | ATTTGGTAAGGGT |
| rdxA (616) | 616W | AAGTTGATGCAATTACCTT |

| B, Mutant |
| Primer (site) | Number | Sequence, 5’→3’ |
| 16S rRNA (926–928) | 16S TTC | TCGATTCTACACGGAAGAA |
| 23S rRNA (2142) | A2142C | GACGGCAAGACCC |
| gyrA (87) | 87K | ATGGTGCGTATAGTGAG |
| gyrA (91) | 91Y | TTATGATGCACTAGTGAG |
| PBP1 (556) | 556S | GTAAAAGCGGRACTTCTT |
| PBP1 (562) | 562Y | AAAAACTATATGATGCTTT |
| rdxA (565) | 565T | GCTTTAGTAAGGGT |
| rdxA (616) | 616A | CAAAATGTAACAGATCTT |
| β-globin | IC | CCTCTATCTTCCACCCAC |
Table III. Parameters for PCR amplification.

| Step no. | Step name                          | Temperature, °C | Time   | Cycle number |
|----------|------------------------------------|-----------------|--------|--------------|
| 1        | Uracil-DNA glycosylase enzyme reaction | 50              | 10 min | 1            |
| 2        | Initial denaturation                | 95              | 10 min | 1            |
| 3        | Denaturation                        | 95              | 30 sec | 45           |
| 4        | Annealing                           | 56              | 30 sec |              |
| 5        | Extension                           | 72              | 30 sec |              |
| 6        | Final extension                     | 72              | 5 min  | 1            |

Hybridization. Film strips marked with the sample number were placed in a plastic centrifuge tube (15 ml), and 7 ml liquid A was added. Next, three PCR products corresponding to the sample number were added, and the tube cover was tightened. Next, the centrifuge tube was heated in a boiling water bath for 10 min. It was then removed, the cover was tightened, and the tube was placed into the hybridization instrument (Combi-H12 FINEPCR) at 48°C for 1.5 h. A total of 45 ml liquid B was added to a 50 ml plastic centrifuge tube, which was then preheated at 48°C in the hybridization instrument.

Membrane washing. The membrane strips were removed and transferred to a 50 ml tube containing preheated liquid B, followed by gentle washing at 48°C for 15 min.

Color rendering. The formula of the incubation solution was as follows: Solution A: Peroxidase=2,000:1. The membrane was washed twice with liquid A at room temperature for 5 min each time. It was then washed with liquid C at room temperature for 1 min, and the color developing solution was prepared. The membrane strip was then immersed in the color developing solution at room temperature for 10 min and rinsed with pure water twice to observe the results. Subsequently, mutation points turned blue (Fig. 1).

Statistical analysis. Fisher's exact test was used to compare two groups of classification data. The \( \chi^2 \) test was used for comparison of multiple sample rates. SPSS v17.0 (SPSS, Inc.) statistical software package was used for statistical analysis. \( P<0.05 \) was considered to indicate a statistically significant difference.

Results

General patient information. The male:female ratio of the enrolled patients was 1.17:1 (P>0.05). A total of 111 strains were successfully cultured in vitro, 109 strains were identified by pathologists (Fig. 2), while 108 strains were detected using PCR reverse dot blot. No significant difference in the sensitivity of \( H. pylori \) detection was identified between the three methods (agar plate dilution method, 83.5%; PCR, 81.2%; pathology, 82.0%; \( P=0.89 \)). A total of 96 antibiotic-resistant strains were confirmed by both the agar plate dilution method and PCR reverse dot blot. According to genotyping, all strains were VacA-positive and type s1, including 44 cases that were type s1m1 and 69 that were type s1m2. The agar plate dilution method revealed that the resistance rate of MNZ was 90.1% (100/111), followed by 35.1% (39/111) for LEFX and 32.4% (36/111) for CLA. No strains were resistant to AMX, FUR and TET. A total of 34 strains were resistant to both CLA and MNZ, 37 strains were resistant to both LEFX and MNZ, and 23 strains were resistant to CLA, LEFX and MNZ.

Association analysis between mutations of different gene loci and drug resistance. The results of the agar plate dilution method and PCR reverse dot blot were not entirely consistent; 32.1% of strains that, according to the agar plate dilution method, were MNZ-resistant exhibited an rdxA G616A mutation. In addition, numerous LEFX-related resistance gene sites were identified in gyrA. Among them, the T87A mutation accounted for 32.3%, the G91A mutation for 22.6%, the A91G mutation rate for 12.9% and the G91T mutation for 6.5%. The main mutation in CLA-resistant strains was 23S rRNA A2143G, accounting for 81.8%, with the 23S rRNA A2142G mutation accounting for 0.0%. Neither AMX-resistant nor TET-resistant strains were identified in vitro (Table IV).

Among all the gene sites studied, rdxA G616A had the highest accuracy in predicting \( H. pylori \) resistance (81.8%), followed by gyrA G91T (66.7%), gyrA G91A (54.5%), 23S rRNA A2143G (49.5%), gyrA T87A (45.5%), gyrA A91G (33.3%) and 23S rRNA A2142G (0.0%), while no drug resistance was identified in the strains with AMX (PBP1 C556G, PBP1 A562T and PBP1 A562G) and TET-resistance-related gene mutations [926-927 (AG to GT) and 926-928 (AGA-TCG) in 16S rRNA], according to the \( \chi^2 \) test (Table V).

Among the genes, 81.8% of strains with a rdxA mutation were revealed to be resistant to MNZ, while 47.8% of strains with a gyrA mutation and 49.1% strains with a 23S rRNA mutation were indicated to be resistant to LEFX and CLA, respectively (Table VI).

Drug resistance analysis of different VacA genotypes. All 108 patients with positive \( H. pylori \) genes presented the VacA s1 type; there were no VacA s2 type patients. Out of those, 39 (36.1%) had VacA s1m1 strains and 69 (63.9%) VacA s1m2 strains. As indicated using Fisher's exact test and \( \chi^2 \) analysis, there was no significant association between genotypes and overall drug resistance rate, multidrug resistance rate, CLA, LEFX or MNZ resistance rate (\( P>0.05 \); Tables VII-XI).
Discussion

There are several mechanisms of *H. pylori* antibiotic resistance, and they vary among different antibiotics (8). The mechanism underlying CLA resistance involves reverse binding to the domain V of the 23S rRNA gene in the ribosomal subunit 50S in bacteria, thereby interfering with protein synthesis and promoting the early release of peptide tRNA from the recipient site, thus preventing the synthesis of the *H. pylori* peptide chain extension protein (18). Therefore, a mutation in domain V of the 23S rRNA gene may decrease the affinity between the ribosome and drug, leading to the lack of binding between CLA and 50S ribosome subunit. If the mutation cannot affect protein synthesis, *H. pylori* resistance occurs. In addition, if the mutation cannot entirely block the affinity between ribosome and antibiotics, sensitivity to antibiotics can still occur in vitro. Among the point mutations, certain are clinically more important than others. Seo et al (19) reported that 19.5, 0.9, 0.2 and 0.7% patients presented A2143G, A2142G, A2142C and both A2143G and A2142G mutations, respectively. The CLA-resistant group was treated with proton-pump inhibitor (PPI) twice a day, AMX (1,000 mg) twice a day and MNZ (500 mg) three times a day for 7 days (PAM). Groups with other mutations were treated with PPI twice a day, AMX (1,000 mg) twice a day and CLA (500 mg) twice a day for 7 days (PAC). As revealed by ITT (Intention-To-Treat (ITT) and PP(Per-Protocol) analysis, there was a significant difference between the two groups [PAM vs. PAC, 55.4 vs. 74.3%, P=0.001 (ITT); 66.2 vs. 88.4%, P=0.0001] (16). Similarly, Ong et al (20) treated patients infected with *H. pylori* with

![Figure 1. Membrane strips.](image1)

- (A) S1/M2/UreA/CagA; sensitive to all the antibiotics tested.
- (B) S1/M2/UreA/CagA; resistant to clarithromycin (23S rRNA A2143G) and metronidazole (rdxA G616A).
- (C) *Helicobacter pylori* negative.

![Figure 2. Pathological results.](image2)

- (A) *Helicobacter pylori* negative (magnification, x200).
- (B) *Helicobacter pylori* positive (magnification, x100).
Table IV. Proportion of gene mutations in drug-resistant heliobacter pylori strains shown by agar plate dilution.

| Antibiotic | Resistant-strain number | Mutant sites | Mutant strain number | Mutant rate, % |
|------------|-------------------------|--------------|----------------------|----------------|
| MNZ        | 81                      | G616A        | 26                   | 32.1           |
| LEFX       | 31                      | T87A         | 10                   | 32.3           |
|            |                         | A91G         | 4                    | 12.9           |
|            |                         | G91T         | 2                    | 6.5            |
|            |                         | G91A         | 7                    | 22.6           |
| CLA        | 33                      | A2143G       | 27                   | 81.8           |
|            |                         | A2142G       | 0                    | 0.0            |
| AMX        | 0                       | C556G        | 0                    | -              |
|            |                         | A562T        | 0                    | -              |
|            |                         | A562G        | 0                    | -              |
| TET        | 0                       | 926–928 (AGA-TTC) | 0 | - |
|            |                         | 926–927 (AG-GT) | 0 | - |

MNZ, metronidazole; LEFX, levofloxacin; CLA, clarithromycin; AMX, amoxicillin; TET, tetracylin.

Table V. Proportion of resistant strains *in vitro* in strains with single gene loci mutation.

| Mutant site | Mutant-strain number | Resistant-strain number | Resistance rate, % |
|-------------|----------------------|-------------------------|--------------------|
| G616A       | 33                   | 27                      | 81.8               |
| T87A        | 22                   | 10                      | 45.5               |
| A91G        | 12                   | 4                       | 33.3               |
| G91T        | 3                    | 2                       | 66.7               |
| G91A        | 11                   | 6                       | 54.5               |
| A2143G      | 53                   | 26                      | 49.1               |
| A2142G      | 1                    | 0                       | 0                  |
| C556G       | 10                   | 0                       | 0                  |
| A562T       | 1                    | 0                       | -                  |
| A562G       | 0                    | 0                       | -                  |
| 926–928 (AGA-TTC) | 0 | 0 | - |
| 926–927 (AG-GT) | 0 | 0 | - |

Table VI. Drug resistance ratio in different resistance gene mutant strains.

| Mutant gene | Mutant-strain number | Resistant-strain number | Positive rate, % |
|-------------|----------------------|-------------------------|-----------------|
| rdxA        | 33                   | 27                      | 81.8            |
| gyrA        | 46                   | 22                      | 47.8            |
| 23S rRNA    | 53                   | 26                      | 49.1            |
| PBP1        | 13                   | 0                       | 0               |
| 16S rRNA    | 0                    | 0                       | -               |

Table VII. Association between Vac A genotype and antibiotic resistance.

| Genotype | Resistant strains | Sensitive strains | Total |
|----------|------------------|-------------------|-------|
| s1m1     | 32               | 2                 | 34    |
| s1m2     | 53               | 5                 | 58    |
| Total    | 85               | 7                 | 92    |

no CLA mutation points in 23S rRNA with concomitant (1,000 mg AMX; 500 mg CLA; 500 mg MNZ and 30 mg lansoprazole twice a day for 14 days) or tailored (1,000 mg AMX; 500 mg CLA and 30 mg lansoprazole twice a day for 14 days) therapy. There was no significant difference in the eradication rates between the groups in both point mutation-negative subjects (91.7 vs. 87.3%; P=0.154). These studies demonstrated that the detection of CLA mutation
points in 23S rRNA could be efficient for screening of CLA resistant strains in regions where the CLA resistance rate is >15%. In the present study, the CLA resistance rate was as high as 32.4%, and the mutation rate of A2143G and A2142G in 23S rRNA was 81.8 and 0% (Table IV), respectively. The sensitivity of A2142G and A2143G mutation in 23S rRNA was 81.8%, rendering them ideal mutations for the detection of CLA resistance in *H. pylori* in China. Adding bismuth to standard triple therapy with CLA may eradicate 77.6% resistant strains with A2143G or A2142G mutation (21).

LEFX interferes with bacterial DNA synthesis by inhibiting DNA gyrase and topoisomerase IV (8). The gyrA gene encodes DNA gyrase, so its mutations can lead to the resistance of *H. pylori* to LEFX (22). Liou et al (23) used a modified sequential therapy containing LEFX and high-dose esomeprazole (40 mg esomeprazole and 1,000 mg AMX for the first 5 days, followed by 40 mg esomeprazole, 250 mg LEFX and 500 mg MNZ for another 5 days; all administered twice daily) in patients who did not respond to first-line treatment. The eradication rates were 84.6% (11/13) and 95.1% (58/61) in patients with and without a gyrA mutation, respectively (P=0.210), which indicated little value of gyrA mutation testing. However, Papastergiou et al (24) used a 7-day, genotypic resistance-guided triple *H. pylori* eradication therapy comprising esomeprazole, AMX and either CLA (wild-type 23S rRNA), LEFX (23S rRNA mutated/wild-type gyrA) or rifabutin (mutated 23S rRNA/gyrA). The mutation sites of CLA in Papastergiou et al (24) were A2146G, A2146C and A2147G in the 23S rRNA gene, while the mutation sites of LEFX were N87K, D91N, D91G and D91Y in the gyrA gene. For patients who received CLA-, LEFX- and rifabutin-containing triple therapy, the respective eradication rates were 24/27, 20/20 and 2/4 by ITT and 24/24, 19/19 and 2/3 by PP analysis. The overall eradication rates exhibited no significant difference between treatment-naïve and -experienced patients (ITT, 87.5 vs. 94.7%, P=0.64; PP, 96.4 vs. 100%, P=1.00). Therefore, the unsatisfactory results of the previous trial may be linked to the high resistance rate of MNZ. The present results indicated that 74.2% of LEFX-resistant strains had gene mutations. The mutation rates of T87A, G91A, A91G and G91T in gyrA were 32.3, 22.6, 12.9 and 6.5%, respectively. The resistance rate of loci 91 (36.1%) was similar to that in the study by Palmitessa et al (25). The coincidence rates of mutation sites, as determined by *in vitro* drug sensitivity test, were as follows: rdxA G616A (81.8%), gyrA G91T (66.7%), gyrA G91A (54.5%), 23 S rRNA A2143G (49.5%), gyrA T87A (45.5%), gyrA A91G (33.3%), and 23 S rRNA A2142G (0.0%). Since the resistance rate of gyrB is low (26), it was not detected in the present study, based on the economic benefit ratio. Meta analysis showed that second-line anti-*H. pylori* levofloxacin/amoxicillin-based triple (10-day regimen) therapy was significantly more effective than standard quadruple therapy [PP=bismuth+two antibiotics; odds ratio, 5.05; 95% CI, 2.74-9.31; P<0.001; heterogeneity

| Genotype | Single-antibiotic resistant strains | Double-antibiotic resistant strains | Triple-antibiotic resistant strains | Multi-antibiotic resistant strains | Total |
|----------|------------------------------------|------------------------------------|------------------------------------|------------------------------------|-------|
| s1m1     | 16                                 | 7                                  | 9                                  | 16                                 | 32    |
| s1m2     | 29                                 | 13                                 | 11                                 | 24                                 | 53    |
| Total    | 45                                 | 20                                 | 20                                 | 40                                 | 85    |

*Single-vs. double-vs. triple-antibiotic resistant strains, P=0.74; x²=0.60. *Single-vs. multi-antibiotic resistant strains, P=0.82.*

| Genotype | CLA-resistant strains | CLA-sensitive strains | Total |
|----------|----------------------|----------------------|-------|
| s1m1     | 14                   | 20                   | 34    |
| s1m2     | 19                   | 39                   | 58    |
| total    | 33                   | 59                   | 92    |

CLA, clarithromycin.

| Genotype | MNZ-resistant strains | MNZ-sensitive strains | Total |
|----------|----------------------|----------------------|-------|
| s1m1     | 30                   | 7                    | 37    |
| s1m2     | 51                   | 11                   | 62    |
| total    | 81                   | 18                   | 99    |

MNZ, metronidazole.

| Genotype | LEFX-resistant strains | LEFX-sensitive strains | Total |
|----------|-----------------------|-----------------------|-------|
| s1m1     | 13                    | 21                    | 34    |
| s1m2     | 18                    | 40                    | 58    |
| total    | 31                    | 61                    | 92    |

LEFX, levofloxacin.

### Table VIII. Association between Vac A genotype and multi-antibiotic resistance.

| Genotype | Single-antibiotic resistant strains | Double-antibiotic resistant strains | Triple-antibiotic resistant strains | Multi-antibiotic resistant strains | Total |
|----------|------------------------------------|------------------------------------|------------------------------------|------------------------------------|-------|
| s1m1     | 16                                 | 7                                  | 9                                  | 16                                 | 32    |
| s1m2     | 29                                 | 13                                 | 11                                 | 24                                 | 53    |
| Total    | 45                                 | 20                                 | 20                                 | 40                                 | 85    |

*Single-vs. double-vs. triple-antibiotic resistant strains, P=0.74; x²=0.60. *Single-vs. multi-antibiotic resistant strains, P=0.82.*

### Table IX. Association between Vac A genotype and CLA resistance.

| Genotype | CLA-resistant strains | CLA-sensitive strains | Total |
|----------|----------------------|----------------------|-------|
| s1m1     | 14                   | 20                   | 34    |
| s1m2     | 19                   | 39                   | 58    |
| total    | 33                   | 59                   | 92    |

### Table X. Association between Vac A genotype and MNZ resistance.

| Genotype | MNZ-resistant strains | MNZ-sensitive strains | Total |
|----------|----------------------|----------------------|-------|
| s1m1     | 30                   | 7                    | 37    |
| s1m2     | 51                   | 11                   | 62    |
| total    | 81                   | 18                   | 99    |

### Table XI. Association between Vac A genotype and LEFX resistance.

| Genotype | LEFX-resistant strains | LEFX-sensitive strains | Total |
|----------|-----------------------|-----------------------|-------|
| s1m1     | 13                    | 21                    | 34    |
| s1m2     | 18                    | 40                    | 58    |
| total    | 31                    | 61                    | 92    |

CL, clarithromycin; AMX, amoxicillin; LEFX, levofloxacin; MNZ, metronidazole; PPI, proton pump inhibitors; CI, confidence interval.
index (I) = 75% (27). Simple molecular susceptibility testing for CLA and LEFX may achieve a high H. pylori eradication rate. If CLA or LEFX resistance does exist, administration of probiotic microorganisms along with triple therapy would be another treatment possibility (28).

In the present study, no case showed 926-928 and 926-927 loci mutations in 16S rRNA, a TET-related resistance gene and 556 and 562 loci mutations in an AMX-related drug-resistant gene, PBP1, occurred in 10 cases (C556G) and 3 case (A562T), respectively. However, TET- and AMX-resistant strains were not revealed to be among these mutant strains via the in vitro drug sensitivity test. At the same time, AMX-resistant strains detected in the in vitro drug sensitivity test identified no gene mutation. No FUR-resistant strains were cultured in the present study. In a review, Zamani et al (29) analyzed studies on the resistance of H. pylori to FUR published worldwide, suggesting that the resistance rate of H. pylori to FUR in most regions was <5%, which may be associated with A041G, A122G, C349A or G A78G, A112G, A335G, C156T and C165T mutations in the oorD gene and G353A, A356G, C357T, C347T, C347G and C346A mutations in the porD gene. The specificity and sensitivity of several mutation sites mentioned in the review by Zamani et al (29) requires further investigation, as they were not detected in the present study. Generally, the drug resistance rate of H. pylori to AMX, TET and FUR is low, and thus no drug resistance test is required prior to eradication if any of these three antibiotics are used (30) However, it should be noted that TET has been out of production for many years in China.

In a study by Lee et al (31), which enrolled 53 H. pylori-positive patients treated with MNZ-containing quadruple therapy between 2011 and 2015, rdxA and frxA mutations in patients with H. pylori were analyzed via DNA sequencing. The results indicated that rdxA mutations served a critical role in MNZ resistance and the outcomes of eradication therapy. Apart from rdxA, frxA and frxB are among the most frequently studied mutations; however, previous studies have suggested that mutations in these two genes have a synergistic effect with rdxA mutations in causing MNZ resistance, but cannot cause it alone (32-34). In theory, if the mutation rate of rdxA in resistant strains is high enough, tailored therapy could achieve a high eradication rate of H. pylori, according to the rdxA gene mutation detection results. However, only 32.1% of the MNZ-resistant strains had a rdxA mutation in G616A in the present study, which was slightly higher than the rate observed in a study in Pakistan (32.1 vs. 23.5%) (35). In addition, all strains (20/20) that were resistant to both CLA and LEFX were also resistant to MNZ in the present study. According to a study by Zhang et al (36), the rdxA mutation was identified in 90.7% (49/54) of Chinese children. Therefore, rdxA mutation detection in G616A may not be a good strategy to improve the eradication rate of H. pylori among adults in high MNZ-resistant areas, while it may benefit Chinese children.

As aforementioned, VacA is an important virulence factor in H. pylori. The association between VacA subtypes and H. pylori resistance remains controversial. Karabiber et al (15) demonstrated that VacA s1c-positive H. pylori was more likely to lead to CLA resistance, while in MNZ-resistant strains VacA s1 and s1c were more likely to lead to resistance. Wang et al (37) reported that the resistance rate of MNZ in the VacA s1m1/m2 strains was lower than that in the VacA s1m2 strain. However, Bachir et al (38) and Xu and Xie (39) demonstrated that the VacA genotype was not linked to H. pylori resistance. The present results indicated that there was no significant difference in H. pylori multidrug CLA, MNZ and LEFX resistance between VacA m1 and VacA m2 strains. The present results were consistent with studies by Bachir et al (38) and Xu and Xie (39). However, the s2 strain was not detected and the s1 subtype was not classified into s1a, s1b and s1c in the current study. Intermediate region i1, i2 and i3 were not detected.

Antibiotic resistance is divided into primary and secondary resistance (40). The antibiotic resistance rate increases in association with the number of therapy failures (38). The majority of patients included in the present study has received treatment before; however, due to the unknown antibiotic history of the patient, it is not possible to accurately calculate the primary and secondary drug resistance rates of H. pylori in Zhejiang, China, thus further research is required. The average time required to culture H. pylori and perform an in vitro drug sensitivity test is 1 week. However, it only takes 1 day to detect drug-resistant genes, which can save valuable time. In addition, Giorgio et al (41) demonstrated that reverse transcription-quantitative PCR on feces, which is non-invasive compared with the use of gastric mucosa, can be a useful tool for the detection of DNA sequence and antibiotic resistance-related gene mutations in H. pylori. A previous study used gene chip technology to detect the drug resistance of H. pylori in children, and the main mutation sites were similar to those identified in the present study (17). Studies focused on adults have only been used for the detection of single antibiotic resistance (42,43). The chip used in the present study covers five commonly used antibiotics. The results of gene detection were not identical to drug sensitivity test in vitro. At present, there is a lack of relevant clinical data to compare the accuracy of the two methods. However, a relevant clinical trial is in progress. There are numerous non-conventional rapid methods for antimicrobial resistance diagnostics, including whole genome sequencing (WGS), matrix-assisted laser desorption/ionization time-of-flight spectrometry, Fourier transform infrared spectroscopy and microfluidics technology. WGS is being expanded in China, while other methods require high purchase and maintenance costs and large space for equipment. Therefore, none of them can be widely carried out in the clinic (44).

In conclusion, VacA m subtypes were not linked to H. pylori antibiotic resistance. The detection of associated mutant genes C556G, A562T and A562G in PBP1 for AMX; T87A, A91G, G91T and G91A in gyrA for LEFX; 926-928 (AGA to TTC) and 926-927 (AG to GT) in 16S rRNA for TET; A2142G and A2143G in 23S rRNA for CLA; and G616A in rdxA for MNZ has a clinical application value in predicting the antibiotic resistance of H. pylori, particularly the resistance to CLA and LEFX.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
BL designed the study. JZ analyzed and interpreted data. JD performed data and wrote the manuscript. YH and LM confirmed the authenticity of all the raw data.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang Traditional Chinese Medical University (approval no. 2017-KL-054-02; Hangzhou, China). All patients provided written informed consent.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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