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Original Article

A retrospective study of the antibiotic-resistant phenotypes and genotypes of *Helicobacter pylori* strains in China

Zishao Zhong[1,2,3,9*], Zhenyu Zhang[4*], Jing Wang[6*], Yunlian Hu[7*], Yang Mi[5*], Bangshun He[4], Yushu Zhang[4], Ximei Zhang[4], Xingzhou Xia[5], Huang Huang[5], Yuexing Lai[6], Min Lin[7], Chengxia Su[7], Zhiyi Zhang[8], Zhengqi Wu[8], Linzhi Lu[8], Beiping Zhang[9], Suiping Huang[9], Cailing Zhong[9], Xiaoming Zeng[10], Yun Peng[10], Guangxia Chen[11], Haihan Zhang[11], Guangqing Zhou[11], Shiyu Liu[11], Changqing Yang[1,2], Lijuan Yan[3], Aojun Chen[3], Guiying Zhang[10], Ping Xu[6], Shukui Wang[4], Pengyuan Zheng[5], Shuchang Xu[1,2], Hengjun Gao[1,2,3]

[1]Tongji Hospital, School of Medicine, Tongji University, Shanghai, China; [2]Institute of Digestive Disease, School of Medicine, Tongji University, Shanghai, China; [3]China Center for Helicobacter Pylori Molecular Medicine, Shanghai, China; [4]Nanjing First Hospital, Nanjing Medical University, Nanjing, China; [5]The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou, China; [6]Songjiang District Central Hospital, Shanghai, China; [7]Hubei Provincial Hospital of Traditional Chinese Medicine, Wuhan, China; [8]Gansu Wuwei Tumour Hospital, Wuwei, China; [9]The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China; [10]Xiangya Changde Hospital, Chengde, China; [11]Xuzhou First People's Hospital, Xuzhou, China. [*]Equal contributors and co-first authors.

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Abstract: *Helicobacter pylori* antibiotic resistance is a serious concern in China, where it severely influences treatment for *H. pylori* infection. To overcome this, it is essential to apply personalized therapies based on local or individual data on antibiotic-resistant phenotypes or genotypes. We conducted a large-scale multi-center study with a retrospective cross-sectional observational design to investigate the antibiotic-resistant phenotypes and genotypes of *H. pylori* in China. Strains were isolated from the gastric biopsy samples of *H. pylori*-infected patients from five different regions in China. The strains were tested for antibiotic-resistant phenotypes and genotypes, and the agreement between the two was assessed. In total, 4242 *H. pylori* strains were isolated and cultured, with an 84.43% success rate. The primary and secondary antibiotic resistance rates of *H. pylori* were 37.00% and 76.93% for clarithromycin, 34.21% and 61.58% for levofloxacin, 2.20% and 6.12% for amoxicillin, 1.61% and 3.11% for furazolidone, 1.18% and 3.31% for tetracycline, and 87.87% and 93.48% for metronidazole, respectively. The dual-resistance patterns for metronidazole/clarithromycin, metronidazole/levofloxacin, and clarithromycin/levofloxacin were 43.6%, 38.4%, and 26.1%, respectively. Clarithromycin- and levofloxacin-resistant *H. pylori* phenotypes and genotypes showed satisfactory agreement. Based on these findings, clarithromycin- and levofloxacin-resistant genotype testing could partially replace traditional antibiotic susceptibility testing in China. Continuous monitoring and personalized treatments based on individual and local *H. pylori* antibiotic-resistance data remain necessary.

Keywords: *Helicobacter pylori*, antibiotics, phenotypic resistance, genotypic resistance

Introduction

Helicobacter pylori is a common pathogen that colonizes the stomach in more than 4.4 billion people worldwide. In China, more than half of the population is infected with *H. pylori* [1]. *H. pylori* has been identified as a group 1 carcinogen for non-cardia gastric cancer by the International Agency for Research on Cancer and is strongly associated with the incidence of chronic gastritis, peptic ulcers, and MALT lymphoma [2, 3]. Globally, experts have emphasized the eradication of *H. pylori* infection to reduce the incidence of intestinal gastric cancer, peptic ulcers, and *H. pylori*-related dyspepsia.

However, *H. pylori* antibiotic resistance has become increasingly severe in China, resulting in a decline in eradication rates [4]. A meta-analysis showed that from 2006 to 2016, *H. pylori* resistance rates in China for clarithromy-

cin, metronidazole, and levofloxacin were 37%, 77%, and 33%, respectively [5]. Another multicenter study conducted by Chinese scholars showed that the primary resistance rates of *H. pylori* to metronidazole, clarithromycin, levofloxacin, amoxicillin, and tetracycline from 2010 to 2016 were 78.2%, 22.1%, 19.2%, 3.4%, and 1.9%, respectively [6]. These high resistance rates to clarithromycin, metronidazole, and levofloxacin in China have rendered the standard triple therapy ineffective [7], making treatment regimens increasingly complex. Therefore, monitoring of *H. pylori* antibiotic resistance for personalized treatment has become necessary; however, there are insufficient large-scale and multi-regional studies on *H. pylori* antibiotic resistance in China.

To address the failure of the eradication of *H. pylori* infection due to antibiotic resistance, personalized treatments based on antibiotic susceptibility represent a novel therapeutic option [8]. Traditional antibiotic susceptibility testing by culture is microaerobic, time-consuming, and complex with a limited possibility of scaling-up testing. Alternatively, polymerase chain reaction (PCR) and DNA sequencing have been used to detect the susceptibility of *H. pylori* to antibiotics. Although 23S rRNA and gyrA mutations have been determined as critical for susceptibility to clarithromycin and levofloxacin, resulting in the development of validated and commercial kits, there are no large-scale validation studies for other antibiotic resistance-related mutation loci [9-12].

In view of the extent of *H. pylori* antibiotic resistance in China, the China Center for *H. pylori* Molecular Medicine (CCHpMM), headed by the late gastroenterologist, Professor Shudong Xiao, was established in 2016 for monitoring *H. pylori* antibiotic resistance and developing personalized treatments. In addition, because there are no large-scale multi-regional studies on *H. pylori* antibiotic-resistant phenotypes and genotypes in China currently, we conducted a nationwide multicenter large-sample study on *H. pylori* antibiotic-resistant phenotypes and genotypes and assessed the agreement between them.

Methods

Study design and patients

A retrospective cross-sectional observational study investigating the phenotypic and genotypic resistance of *H. pylori* strains to antibiotics was conducted from January 2018 to June 2020 across different regions of China, including Jiangsu (Nanjing First Hospital), Shanghai (Songjiang District Central Hospital), Hubei (Hubei Provincial Hospital of Traditional Chinese Medicine), Henan (The Fifth Affiliated Hospital of Zhengzhou University), Gansu (Gansu Wuwei Tumour Hospital), Guangdong (Guangdong Provincial Hospital of Chinese Medicine), Hunan (Xiangya Changde Hospital), Anhui (Huangshan Shoukang Hospital), and Beijing (Beijing Tonghui Clinic of G. I. Health Care).

All enrolled patients had been diagnosed with *H. pylori* infection, which was confirmed by the urea breath, rapid urease, or fecal antigen tests, and had undergone *H. pylori*-strain phenotypic and genotypic antibiotic resistance testing. Patients who received antibiotics, bismuth, or other herbal drugs with antibacterial effects 1 month before the testing were excluded, along with those who received proton-pump inhibitors or H2 receptor blockers 2 weeks before the testing. The baseline characteristics of the patients (age, sex, and history of *H. pylori* infection treatment) were recorded by the physician who prescribed the test. The study design is illustrated in **Figure 1**.

This study was approved by the Ethics Committee of Shanghai Outdo Biotech Company, which is the headquarters of the China Center for *Helicobacter pylori* Molecular Medicine (approval number YB M-05-01). The need for informed consent was waived owing to the patients being lost to follow-up.

H. pylori strains

H. pylori strains were isolated from gastric-biopsy specimens. After conducting the gastroscopic biopsy, the gastric specimens were immediately transferred to the liquid transport medium (Sigma Transwab®, Medical Wire & Equipment, Wilts, United Kingdom) and transported to the CCHpMM (Shanghai Outdo Biotech Company, Shanghai, China) at 0-4°C.

H. pylori strains were inoculated in Columbia agar plates containing 5% sheep blood; treated with vancomycin (1 mg/mL), polymyxin B (0.5 mg/mL), and amphotericin B (0.5 mg/mL) to avoid undesired bacterial growth; and cultured under microaerobic conditions (10% CO_2, 5% O_2) at 37°C for 96-120 h. *H. pylori* strains were

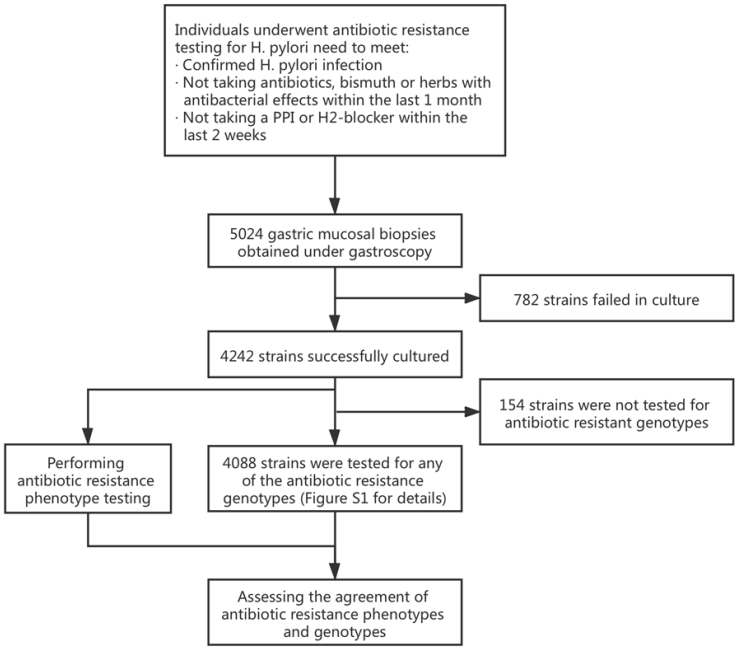

Figure 1. Flowchart depicting the study design. PPI, proton pump inhibitor.

confirmed by microscopy using Gram staining, catalase test, and urease test.

Phenotypic antibiotic-resistance testing

Phenotypic resistance to antibiotics was determined using the Kirby-Bauer disk diffusion method. Suspensions of the *H. pylori* strains were prepared to a final turbidity of 0.5 McFarland standard, passaged onto a new culture plate, and cultured for 48 h. Antibiotic disks were applied as follows: clarithromycin (15 μg), levofloxacin (5 μg), amoxicillin (10 μg), furazolidone (100 μg), tetracycline (30 μg), and metronidazole (5 μg). The antibiotic disks were pressed onto the agar surface of each culture plate, followed by incubation for 48 h. The diameter of the inhibition ring was measured, and strains were identified as sensitive, intermediate, or resistant according to the criteria listed in Supplementary Table 1.

Genotypic antibiotic-resistance testing

H. pylori DNA was extracted from the specimens in the liquid transport medium using a HiPure bacterial DNA kit (Magen Biotech, Guangzhou, China). PCR was performed to amplify the following genes: 23S rRNA, gyrA, PBP1A, porD, oorD, 16S rRNA, and rdxA, which have all been reported to be associated with antibiotic resistance [13-24]. The PCR conditions were set as follows: predenaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 50 s and final extension at 72°C for 5 min. The PCR products were sequenced using Sanger sequencing. Several point mutations and amino-acid mutations that have been previously reported to be associated with antibiotic resistance were interpreted using Chromas 2.6.5 software [13-24]. The PCR primers used and mutation loci obtained are shown in Supplementary Table 2.

Statistical analysis

Data were analyzed using R version 4.0.1. Continuous measures are reported as mean and standard deviation, while categorical measures are reported as percentages and counts. Proportional differences were evaluated using Fisher's exact test or the Chi-square test. The agreement between antibiotic-resistant phenotypes and genotypes was evaluated using accordance rates and the kappa consistency test. A *P*-value <0.05 was considered to denote statistical significance.

Results

In total, 4242 *H. pylori* strains successfully isolated from 5024 subjects who underwent phenotypic and genotypic testing for antibiotic resistance were included in this study. The success rate of strain isolation and culture was 84.43%. **Table 1** shows the baseline information of the included subjects: 2098 (49.5%) men and 2144 (50.5%) women, with a mean (standard deviation) age of 47.0 (12.9) years. Approximately a quarter (23.5%) of the subjects had received *H. pylori* eradication therapy and 71.7% had not, while 203 (4.8%) subjects were unaware of the history of *H. pylori* infection treatment. Not all strains were tested for all antibiotic-resistant genotypes. The types of testing performed on each *H. pylori* strain are shown with UpSet plots in Supplementary Figure 1.

Table 2 shows the prevalence of *H. pylori* phenotypic resistance to antibiotics in each region

Table 1. Baseline characteristics of study cohort

	Overall (N=4242)	Eastern China (N=3020)	Central China (N=776)	Northwest China (N=214)	South China (N=173)	North China (N=59)
Gender, n (%)						
Male	2098 (49.5)	1531 (50.7)	364 (46.9)	108 (50.5)	71 (41.0)	24 (40.7)
Female	2144 (50.5)	1489 (49.3)	412 (53.1)	106 (49.5)	102 (59.0)	35 (59.3)
Age[a], mean (SD)	47.0 (12.9)	47.1 (13.1)	44.6 (12.2)	51.2 (9.29)	50.6 (13.8)	46.9 (11.0)
Detect year, n (%)						
2018	384 (9.1)	253 (8.4)	119 (15.3)	0 (0)	1 (0.6)	11 (18.6)
2019	2688 (63.4)	1910 (63.2)	479 (61.7)	121 (56.5)	132 (76.3)	46 (78.0)
2020	1170 (27.6)	857 (28.4)	178 (22.9)	93 (43.5)	40 (23.1)	2 (3.4)
Therapy history, n (%)						
No	3043 (71.7)	2539 (84.1)	320 (41.2)	161 (75.2)	7 (4.0)	16 (27.1)
Yes	997 (23.5)	448 (14.8)	407 (52.4)	40 (18.7)	59 (34.1)	43 (72.9)
Unknown	202 (4.8)	33 (1.1)	49 (6.3)	13 (6.1)	107 (61.8)	0 (0)

a, Age data were missing for 6 individual subjects.

of China. The overall resistance rate for clarithromycin was 47.24%, while the secondary resistance rate was higher than the primary resistance rate (76.93% vs. 37.00%). When the resistance rate is higher than 15%, a region is generally considered to have high resistance to clarithromycin [3]; in all regions of China, the primary resistance rate exceeded 15%, with the highest rate reaching 48.45% in northwest China. The resistance rate for levofloxacin in China, with a primary resistance rate of 34.21%, was also noteworthy; the secondary resistance rate increased to 61.58% in patients with a treatment history, and the overall resistance rate was 41.40%. The overall resistance rates for amoxicillin, furazolidone, and tetracycline were relatively low in China at 3.23%, 2.05%, and 1.65%, respectively. However, in northwest China, *H. pylori* had significantly higher resistance rates, both primary and secondary, for these three antibiotics than those in other regions of China. The primary resistance rates of *H. pylori* strains to amoxicillin, furazolidone, and tetracycline in the northwest region were 12.42%, 20.50%, and 19.25%, respectively, whereas the secondary resistance rates were 30.00%, 25.00%, and 25.00%, respectively, which were noteworthy. For metronidazole, both primary and secondary resistance rates were very high in China (>87% in all regions).

Figure 2 demonstrates the dual and triple antibiotic resistance of *H. pylori* in China. The rates of dual resistance to metronidazole + clarithromycin, metronidazole + levofloxacin, and clarithromycin + levofloxacin were quite high, reaching 43.6%, 38.4%, and 28.1%, respectively. The rate of triple resistance to clarithromycin + levofloxacin + metronidazole was high, reaching 26.5%. Dual-resistance rates for other antibiotic combinations were low (<5%).

The correlation between the types of mutations in antibiotic-resistance genes and phenotypic resistance in *H. pylori* is presented in **Table 3**. The most common mutation locus for clarithromycin resistance was A2143G, with 91.4% of the resistant strains carrying mutation at this locus, while the prevalence of the A2142G and A2144G mutations was lower (0.9% and 0.1%, respectively). The amino-acid substitution types in gyrA at positions 87 and 91 for levofloxacin resistance included Asn87Ile, Asn87Lys, Asn87Thr, Asn87Tyr, Asp91Asn, Asp91Gly, and Asp91Tyr. All but Asn87Thr were significantly distinct between the levofloxacin-sensitive and levofloxacin-resistant strains. Amino-acid substitutions Val374Leu, Asn562Tyr, and Thr593Ala in PBP1A were most frequent in amoxicillin-resistant strains and differed significantly from sensitive strains. The mutations G353A, A356G, and C357T in porD and A41G and A335G in oorD were significantly different between the furazolidone-resistant and furazolidone-sensitive strains, but the prevalence of these mutations was not low in the sensitive strains. The 16S rRNA AGA926-928 mutation, which was considered to be related to tetracycline resistance, revealed AGC, CGA, GGA, TGC, and TTC mutation types. However, the inci-

Table 2. Prevalence of *Helicobacter pylori* phenotypic resistance

	Overall (N=4242)	Eastern China (N=3020)	Central China (N=776)	Northwest China (N=214)	South China (N=173)	North China (N=59)
CLA, n (%)						
Primary	1126 (37.00)	917 (36.12)	121 (37.81)	78 (48.45)	2 (-)	8 (-)
Secondary	767 (76.93)	368 (82.14)	311 (76.41)	21 (52.50)	26 (44.07)	41 (95.35)
Unknown	111 (54.95)	16 (48.48)	26 (53.06)	5 (38.46)	64 (59.81)	-
All	2004 (47.24)	1301 (43.08)	458 (59.02)	104 (48.6)	92 (53.18)	49 (83.05)
LEV, n (%)						
Primary	1041 (34.21)	838 (33.01)	112 (35.00)	85 (52.80)	3 (-)	3 (-)
Secondary	614 (61.58)	280 (62.50)	257 (63.14)	25 (62.50)	24 (40.68)	28 (65.12)
Unknown	101 (50.00)	17 (51.52)	25 (51.02)	7 (53.85)	52 (48.60)	-
All	1756 (41.40)	1135 (37.58)	394 (50.77)	117 (54.67)	79 (45.66)	31 (52.54)
AMX, n (%)						
Primary	67 (2.20)	23 (0.91)	20 (6.25)	20 (12.42)	1 (-)	3 (-)
Secondary	61 (6.12)	12 (2.68)	28 (6.88)	12 (30.00)	1 (1.69)	8 (18.60)
Unknown	9 (4.46)	0 (0)	4 (8.16)	2 (15.38)	3 (2.80)	-
All	137 (3.23)	35 (1.16)	52 (6.70)	34 (15.89)	5 (2.89)	11 (18.64)
FZD, n (%)						
Primary	49 (1.61)	12 (0.47)	1 (0.31)	33 (20.50)	0 (-)	3 (-)
Secondary	31 (3.11)	3 (0.67)	17 (4.18)	10 (25.00)	0 (0)	1 (2.33)
Unknown	7 (3.47)	0 (0)	3 (6.12)	2 (15.38)	2 (1.87)	-
All	87 (2.05)	15 (0.50)	21 (2.71)	45 (21.03)	2 (1.16)	4 (6.78)
TET, n (%)						
Primary	36 (1.18)	4 (0.16)	1 (0.31)	31 (19.25)	0 (-)	0 (-)
Secondary	33 (3.31)	2 (0.45)	18 (4.42)	10 (25.00)	1 (1.69)	2 (4.65)
Unknown	1 (0.50)	0 (0)	1 (2.04)	0 (0)	0 (0)	-
All	70 (1.65)	6 (0.20)	20 (2.58)	41 (19.16)	1 (0.58)	2 (3.39)
MET, n (%)						
Primary	2674 (87.87)	2213 (87.16)	281 (87.81)	159 (98.76)	7 (-)	14 (-)
Secondary	932 (93.48)	425 (94.87)	373 (91.65)	39 (97.50)	52 (88.14)	43 (100.00)
Unknown	188 (93.07)	30 (90.91)	45 (91.84)	13 (100.00)	100 (93.46)	-
All	3794 (89.44)	2668 (88.34)	699 (90.08)	211 (98.60)	159 (91.91)	57 (96.61)

Resistance rates were hidden for subgroups with sample sizes less than 20. CLA: clarithromycin, LEV: levofloxacin, AMX: amoxicillin, FZD: furazolidone, TET: tetracycline, MET: metronidazole.

dence of the AGA926-928GGA mutation, which is a single-base mutation, was different between the tetracycline-resistant and tetracycline-sensitive strains (16.3% vs. 3.7%, P<0.01). The rdxA mutation loci, which have been reported previously [24], were not found to differ between the metronidazole-resistant and metronidazole-sensitive strains.

Table 4 presents the agreement between *H. pylori* phenotypic and genotypic antibiotic resistance. Phenotypic and genotypic resistance to clarithromycin and levofloxacin were in good agreement, and the resistance patterns of 23S rRNA and gyrA genotypes performed well in discriminating strain susceptibility to clarithromycin and levofloxacin (kappa coefficient =0.810 and 0.782, respectively). However, the agreement between the phenotypic and genotypic resistance to amoxicillin, furazolidone, tetracycline, and metronidazole was not satisfactory.

Discussion

This nationwide, multicenter, large-sample study was organized by the CCHpMM, which was initiated by Prof. Shudong Xiao, to investigate antibiotic-resistant phenotypes, multi-antibiotic resistance, and antibiotic resistance-conferring genotypes in *H. pylori* and to assess

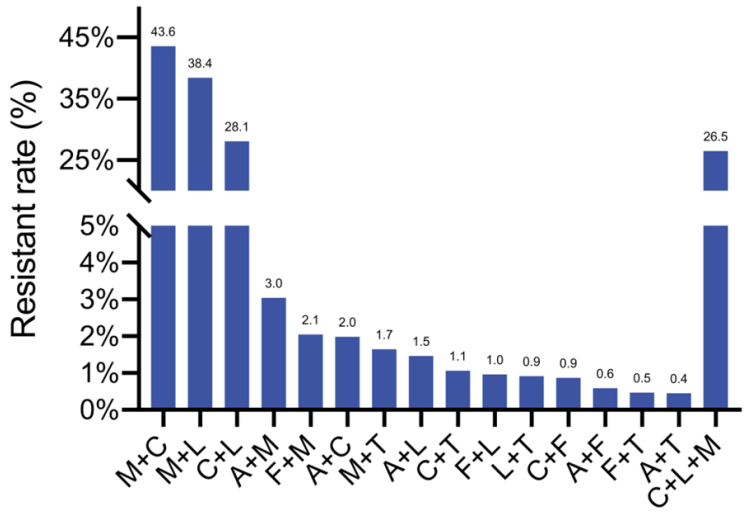

Figure 2. Dual and triple antibiotic resistance of *Helicobacter pylori* in China. M, metronidazole; C, clarithromycin; L, levofloxacin; A, amoxicillin; F, furazolidone; T, tetracycline.

the agreement between resistance phenotypes and genotypes in China. This is the most widespread and large-scale study of *H. pylori* antibiotic-resistant phenotypes and genotypes conducted in China thus far, including strains from five regions (nine provinces). Our results indicate that the state of antibiotic resistance of *H. pylori* in China is severe. The resistance rates for clarithromycin, levofloxacin, and metronidazole were higher than those reported in the past decade [6, 25]. Clarithromycin-resistance rates are well above 15%, and clarithromycin-containing empirical triple therapy is no longer appropriate. Levofloxacin is no longer recommended as a first-line drug and is instead recommended mainly as a second-line empirical drug by expert consensus in China [7]. The current primary and secondary levofloxacin-resistance rates are so alarming that the appropriateness of second-line empiric administration without antibiotic susceptibility testing should be reconsidered.

H. pylori resistance rates for amoxicillin, furazolidone, and tetracycline remained low in China. However, we noted that the primary resistance rates of *H. pylori* for amoxicillin, furazolidone, and tetracycline in northwest China (Gansu Province) reached 12.42%, 20.5%, and 19.25%, respectively, which were much higher than those in other regions. Whether this is related to the clinical or agricultural overuse of these antibiotics in the region requires further investigation. Since the antibiotic combinations recommended by the consensus of Chinese experts contain one or two of these three antibiotics [7], the efficacy of the expert-recommended regimen in this region should be studied further.

This study also investigated multidrug-resistant *H. pylori* strains in China. Various antibiotic combinations, including metronidazole + clarithromycin, metronidazole + levofloxacin, clarithromycin + levofloxacin, and clarithromycin + levofloxacin + metronidazole had co-antibiotic resistance rates above 25%, and these antibiotic combinations may no longer be suitable for use in empiric therapy in China.

Pertaining to the antibiotic-resistance genotypes of *H. pylori*, this study found that the major genotypic alterations for clarithromycin resistance were A2143G and A2142G mutations in 23S rRNA, and those for levofloxacin resistance were mutations resulting in substitutions in amino acids 87 and 91 of gyrA. These loci were significantly different between the resistant and sensitive strains, with good concordance between genotypic and phenotypic resistance (kappa >0.75). These results are generally consistent with those of previous studies [9-12]. Thus, detecting resistance to clarithromycin and levofloxacin by the resistance genotype is an applicable alternative method.

Mutations in bases 926-928 of 16S rRNA are the main cause of tetracycline resistance in *H. pylori*. Single- or double-base mutations in these three bases may lead to low levels of tetracycline resistance in *H. pylori*, whereas simultaneous mutations in the three bases may lead to high levels of resistance [22, 23, 26, 27]. However, in the present large-scale study in China, mutations did not differ significantly between the tetracycline-resistant and tetracycline-sensitive strains, except for AGA926-928GGA; therefore, there was a poor agreement between tetracycline-resistance phenotypes and genotypes. This finding was inconsistent with those of previous studies [22, 23]. Hence, further research on the mechanism of tetracycline resistance is required, in order to

Table 3. Correlation of mutation types of antibiotic resistance genes and phenotypic resistance in *Helicobacter pylori*

Mutation type	Resistant phenotype		χ^2	*P* value
	Resistant n or n (%)	Sensitive n or n (%)		
CLA (23S rRNA)	1739	1951		
A2142G	16 (0.9)	3 (0.2)	-	0.002
A2143G	1589 (91.4)	214 (11.0)	2375.75	<0.001
A2144G	1 (0.1)	1 (0.1)	-	1
LEV (GyrA)	927	957		
Asn87Ile	63 (6.8)	3 (0.3)	-	<0.001
Asn87Lys	448 (48.3)	45 (4.7)	461.602	<0.001
Asn87Thr	2 (0.2)	1 (0.1)	-	0.619
Asn87Tyr	11 (1.2)	0 (0)	-	<0.001
Asp91Asn	141 (15.2)	23 (2.4)	95.579	<0.001
Asp91Gly	109 (11.8)	14 (1.5)	80.111	<0.001
Asp91Tyr	52 (5.6)	7 (0.7)	35.345	<0.001
AMX (PBP1A)	83	2120		
Ala320Val	2 (2.4)	2 (0.1)	-	0.008
Phe366Leu	0 (0)	10 (0.5)	-	1.000
Ala369Thr	1 (1.2)	12 (0.6)	-	0.394
Val374Leu	9 (10.8)	34 (1.6)	30.965	<0.001
Ser414Arg	3 (3.6)	9 (0.4)	-	0.009
Leu423Phe	0 (0)	1 (0)	-	1.000
Thr556Ser	3 (3.6)	21 (1.0)	-	0.059
Asn562Tyr	20 (24.1)	83 (3.9)	68.533	<0.001
Thr593Ala	5 (6.0)	8 (0.4)	34.322	<0.001
Thr593 deletion	1 (1.2)	0 (0)	-	0.038
Gly595Ser	1 (1.2)	0 (0)	-	0.038
FZD (porD)	65	1835		
G353A	29 (44.6)	571 (31.1)	4.687	0.030
A356G	30 (46.2)	569 (31.0)	5.988	0.014
C357T	30 (46.2)	571 (31.1)	5.886	0.015
FZD (oorD)	65	1835		
A41G	25 (38.5)	468 (25.5)	4.832	0.028
A122G	34 (52.3)	758 (41.3)	2.689	0.101
A335G	26 (40.0)	471 (25.7)	5.955	0.015
TET (16s rRNA)	49	1734		
AGA926-928AGC	12 (24.5)	275 (15.9)	2.028	0.154
AGA926-928CGA	3 (6.1)	73 (4.2)	-	0.463
AGA926-928GGA	8 (16.3)	64 (3.7)	16.509	<0.001
AGA926-928TGC	7 (14.3)	142 (8.2)	1.585	0.208
AGA926-928TTC	0 (0)	1 (0.1)	-	1.000
MET (rdxA)	1572	102		
A61G	189 (12.0)	18 (17.6)	2.301	0.129
T62C	188 (12.0)	18 (17.6)	2.369	0.124
A91G	1537 (97.8)	102 (100)	-	0.268
C92A	1531 (97.4)	102 (100)	-	0.173
C92G	6 (0.4)	0 (0)	-	1.000

reveal other mutation loci that may contribute to tetracycline resistance.

The PBP1A mutation has been reported to be the major cause of *H. pylori* resistance to amoxicillin [18, 28-30]. Our results revealed that the occurrence of amino-acid substitutions or deletions at positions 320, 374, 414, 562, 593, and 595 in PBP1A was significantly higher in amoxicillin-resistant strains than in the amoxicillin-sensitive strains, and the resistance phenotype of more than 90% of the strains was consistent with the resistance genotype. However, there existed a subset of phenotypically resistant strains not harboring mutations at these loci, thereby resulting in a lack of consistency in determining amoxicillin-resistant phenotypes using genotypes. It is possible that there are other mechanisms (such as expression of efflux-pump genes) that contribute to the incidence of amoxicillin resistance [31], and the determination of the sensitivity of amoxicillin susceptibility by genotype requires further study.

This study also investigated the mutation loci in furazolidone resistance-related genes reported previously [21], and the occurrence of the G353A, A356G, and C357T mutations in *porD* and the A41G and A335G mutations in *oorD* was found to be more frequent in furazolidone-resistant strains than in furazolidone-sensitive strains. However, a number of these mutation loci were also found in furazolidone-sensitive strains; therefore, the susceptibility of *H. pylori* to furazolidone cannot be determined simply by identifying these mutation loci.

Metronidazole resistance has been demonstrated to be associated with loss of rdxA function due to genetic mutations. This study investigated several metronidazole resistance-related mutations reported previously [24], including one identified by natural transformation studies [32], and significant differences between metronidazole-sensitive and metronidazole-

G392A	1524 (96.9)	101 (99.0)	-	0.362
A610G	1550 (98.6)	102 (100)	-	0.64
A614C	5 (0.3)	0 (0)	-	1.000

CLA: clarithromycin, LEV: levofloxacin, AMX: amoxicillin, FZD: furazolidone, TET: tetracycline, MET: metronidazole.

resistant strains were found. Zhang et al. indicated that some of the rdxA mutations are phylogenetically related, but do not offer metronidazole resistance [33], which may explain the lack of significant differences between metronidazole-resistant and metronidazole-sensitive strains in rdxA at positions 91, 92, 392, and 610 in this study. There may be some other unknown mutation loci that contribute to H. pylori resistance to metronidazole and therefore metronidazole-resistant H. pylori genotypes require further in-depth analysis.

This large-sample, multicenter study provided insights into the status of antibiotic resistance in *H. pylori* in China and identified high antibiotic-resistance rates in some regions as well as issues of multi-antibiotic resistance, with important implications for the clinical use of antibiotics. This study identified a strong concordance between phenotypes and genotypes of clarithromycin-resistant and levofloxacin-resistant *H. pylori* strains through a large-scale survey. The present study also revealed a weak concordance between the resistance phenotypes and the tetracycline resistance-conferring 16S rRNA926-928 mutations that have been defined well previously, as well as genotypes conferring resistance to amoxicillin, furazolidone, tetracycline, and metronidazole. Determining antibiotic resistance may require assays targeting multiple genetic loci to improve accuracy; therefore, we are conducting a multilocus antibiotic-resistance genotype assay for *H. pylori* using next-generation sequencing technology, with the expectation of improving the accuracy of genotype-based methods in determining antibiotic resistance in *H. pylori*. A shortcoming of this study is that the strains were mainly isolated from patients from big cities; the study lacks antibiotic resistance data for *H. pylori* strains originating in small cities, villages, and towns. In the future, we will establish more branches of the CCHpMM in such places, which were not included in this study, to monitor *H. pylori* antibiotic resistance more comprehensively.

In conclusion, the status of *H. pylori* resistance to antibiotics in China is critical. Before working on the availability of novel antimicrobial drugs and vaccines, antibiotic stewardship should be strengthened. Antibiotics for eradication therapy should be selected based on antibiotic-susceptibility testing or regional resistance status in order to reduce the occurrence of *H. pylori* antibiotic resistance. Genotypic resistance testing has several advantages over traditional phenotypic resistance testing. The traditional testing method of *H. pylori* resistance to clarithromycin and levofloxacin, which is based on phenotypic studies, can be replaced with genotypic testing in China.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Hengjun Gao and Shuchang Xu, Tongji Hospital, School of Medicine, Tongji University, Shanghai, China; Institute of Digestive Disease, School of Medicine, Tongji University, Shanghai, China. Tel: +86-13816802848; E-mail: hengjun_gao@tongji.edu.cn (HJG); Tel: +86-13601999711; E-mail: xschang@163.com (SCX); Dr. Pengyuan Zheng, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou, China. Tel: +86-371-66965783; E-mail: medp7123@126.com; Dr. Shukui Wang, Nanjing First Hospital, Nanjing Medical University, Nanjing, China. Tel: +86-25-52271000; E-mail: sk_wang@njmu.edu.cn; Dr. Ping Xu, Songjiang District Central Hospital, Shanghai, China. Tel: +86-21-67720001; E-mail: sjzxxp@yeah.net; Dr. Guiying Zhang, Xiangya Changde Hospital,

Table 4. Agreement between phenotypic and genotypic resistance

Antibiotics	Genotypic resistance	Phenotypic resistance		Accordance rate	Kappa coefficient (95% CI)	*P* value
		Resistant	Sensitive			
CLA	Resistant	1606	218	90.49%	0.81 (0.791-0.829)	<0.001
	Sensitive	133	1733			
LEV	Resistant	814	92	89.12%	0.782 (0.754-0.81)	<0.001
	Sensitive	113	865			
AMX	Resistant	45	180	90.10%	0.251 (0.184-0.318)	<0.001
	Sensitive	38	1940			
FZD[a]	Resistant	29	569	68.16%	0.027 (0.002-0.053)	0.02
	Sensitive	36	1266			
TET	Resistant	30	555	67.81%	0.046 (0.021-0.071)	<0.001
	Sensitive	19	1179			
MET	Resistant	1571	102	93.85%	-0.001 (-0.003-0.001)	0.799
	Sensitive	1	0			

a, Both *porD* and *oorD* mutations are considered to be resistance mutations in furazolidone. CLA: clarithromycin, LEV: levofloxacin, AMX: amoxicillin, FZD: furazolidone, TET: tetracycline, MET: metronidazole.

Chengde, China. Tel: +86-736-2120213; E-mail: guiyingzhang@hotmail.com

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Supplementary Table 1. Criteria for interpreting phenotypic resistance based on diameter of inhibition ring

Antibiotics	Types of interpretation		
	Resistant	Intermediate	Sensitive
CLA	≤13 mm	14-17 mm	≥18 mm
LEV	<13 mm	13-17 mm	>17 mm
AMX	<14 mm	14-17 mm	>17 mm
FZD	≤14 mm	15-16 mm	≥17 mm
TET	≤14 mm	15-18 mm	≥19 mm
MET	<16 mm	16-21 mm	>21 mm

CLA: Clarithromycin; LEV: Levofloxacin; AMX: Amoxicillin; FZD: Furazolidone; TET: Tetracycline; MET: Metronidazole.

Supplementary Table 2. PCR primers and mutation loci interpreted in H. pylori genotypic resistance testing

Genes	Primers	Loci of interpretation	Annotation
23S rRNA	Forward: 5'-GAATTGAAGCCCGAGTAAACG-3' Reverse: 3'-TCAAGCAGAGACGAAAGTCGG-5'	A2142, A2143, A2144 [1-3]	Related to charithromycin resistance
GyrA	Forward: 5'-GGCGTATTTTGTATGCGATGC-3' Reverse: 3'-GAAAGTGCGGGCCAAAGTG-5'	Asn87, Asp91 [2, 4-6]	Related to levofloxacin resistance
PBP1A	Forward 1: 5'-AGGCGGTTGTATTCTTTAGGC-3' Reverse 1: 3'-CACTGACCAACAAAACGATGTCAA-5' Forward 2: 5'-TACGGCACCATGCTCAAACC-3' Reverse 2: 3'-ATAGGGACTTTCACATCGCTG-5'	Ala320, Phe366, Ala369, Val374, Ser414, Leu423, Thr556, Asn562, Thr593 [7, 8]	Related to amoxicillin resistance
porD	Forward: 5'-CCATTACACCGAGCAAAGCTA-3' Reverse: 3'-GCCTATCCTATCACCCCATCA-5'	G353, A356, C357 [9]	Related to furazolizone resistance
oorD	Forward: 5'-CATGCTTTCAGCGCGACTTAT-3' Reverse: 3'-GCGTATCTTTAGGGGCAAGC-5'	A41, A122, A335 [9]	
16S rRNA	Forward: 5'-GCGACCTGCTGGAACATTAC-3' Reverse: 3'-TCGTGTCGTGAGATGTTGGG-5'	AGA926-928 [10, 11]	Related to tetracycline resistance
rdxA	Forward: 5'-CATGGGTTGCTGATTGTGGTT-3' Reverse: 3'-GGTGTTTTCAAGCGTTTCATTAAG-5'	A61, T62, A91, C92, G392, A610, A614 [12]	Related to metronidazole resistance

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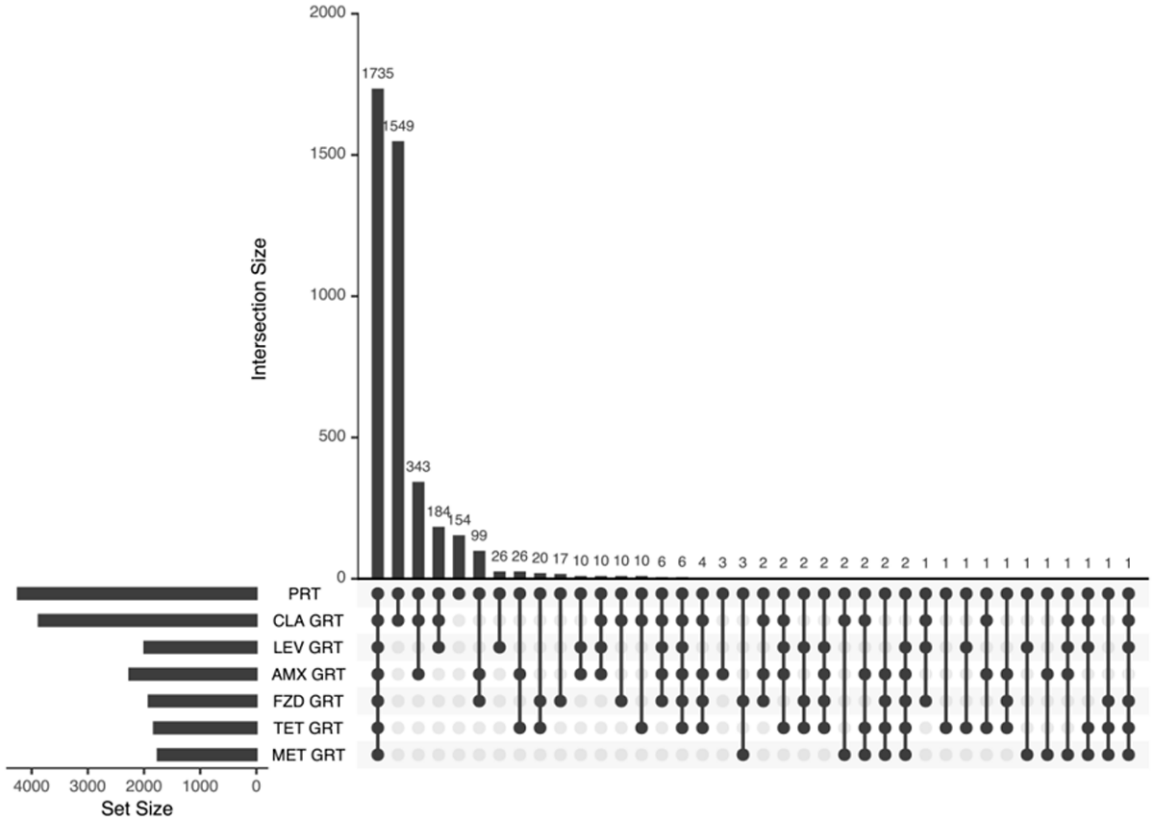

Supplementary Figure 1. Upset plots of testing type performed for each *H. pylori* strain. PRT: Phenotypic resistance testing; GRT: Genotypic resistance testing; CLA: Clarithromycin; LEV: Levofloxacin; AMX: Amoxicillin; FZD: Furazolidone; TET: Tetracycline; MET: Metronidazole. One strain was not underwent metronidazole susceptibility test.