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ORIGINAL RESEARCH

Mutations in the Antibiotic Target Genes Related to Clarithromycin, Metronidazole and Levofloxacin Resistance in *Helicobacter pylori* Strains from Children in China

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Purpose: This study aimed to characterize common mutations of antibiotic-resistant gene of clarithromycin, metronidazole and levofloxacin in *Helicobacter pylori* (*H. pylori*) and determine their association with antibiotic resistance of *H. pylori* for providing a strategy for eradication therapy of *H. pylori* infection in children.

Patients and Methods: The antibiotic resistance to clarithromycin, metronidazole and levofloxacin for *H. pylori* strains isolated from children was determined by E-test. The mutation of domain V of 23S rRNA, *rdxA* and *frxA genes, gyrA* and *gyrB* genes was performed by PCR-based sequencing of DNA fragments.

Results: Out of the 79 *H. pylori* strains examined, 66 (83.5%) were resistant to at least one of the tested antibiotics and 13 (16.5%) were fully sensitive. A total of 29 (36.7%) strains were resistant to clarithromycin. Analysis of the 23S rRNA gene showed that most mutations occurred at the A2143G and T2182C sites, showing a frequency of 82.8% (24/29) and 89.7% (26/29) respectively. In the 11 sensitive strains to clarithromycin, the frequency of A2143G mutation was only 45.5%, which was significantly lower than that in resistant strains ($P<0.05$). There were 54 strains (68.4%) resistant to metronidazole, with most mutations occurring at G47A and T184G in the *rdxA* gene. T184G mutation was recognized in metronidazole-sensitive strains, but no G47A mutation was identified. Twelve strains (15.2%) were resistant to levofloxacin. Position 91 mutation of the *gyrA* gene occurred only in resistant strains, whereas position 87 mutations were detected in both sensitive and resistant strains to levofloxacin.

Conclusion: In *H. pylori* resistant strains isolated from children in China, most mutations occurred at A2143G of the 23S rRNA gene for clarithromycin; G47A mutation of *rdxA* gene for metronidazole; and at 91 mutation of *gyrA* gene for levofloxacin. It is suggested that susceptibility testing together with screening the mutation of antimicrobial-resistant gene prior to treatment is important for the eradication of *H. pylori* in children.

Keywords: *Helicobacter pylori*, Metronidazole, Clarithromycin, Levofloxacin, antibiotic resistant, gene

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Introduction

Helicobacter pylori (*H. pylori*) is a gram-positive, microaerophilic bacterium that was first identified from human gastric mucosa by Australia scientists Barry Marshall and Robin Warren in 1982.[1] *H. pylori* plays a significant role in the pathogenesis of gastrointestinal diseases, including chronic gastritis, peptic ulcer,

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gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma.[2] In 1994, the International Association for Cancer Research listed *H. pylori* as a class I carcinogen. *H. pylori* is one of the most common human bacterial infections in the world, with a global infection rate as high as 50%.[3,4] The prevalence of *H. pylori* infection in developing countries is higher than in developed countries, and studies showed that the prevalence of *H. pylori* infection in China is about 58.07%.[5] In the past few decades, standard therapy regimen consisting of a proton pump inhibitor (PPI) in combination with two antibiotics such as amoxicillin, clarithromycin, metronidazole, or levofloxacin has been recommended as the first-line treatment regimen for *H. pylori* infection. However, with the increase of *H. pylori* resistant strains, this traditional treatment regimen is being replaced by quadruple therapy or sequential therapy.[6]

The increasing prevalence of antibiotic resistance in *H. pylori* is a cause for concern as this is one of the most important causes of therapy failure. The resistance rate of *H. pylori* in developing countries is significantly higher than that in developed countries.[7] We also found that the total resistance rates of *H. pylori* to clarithromycin, metronidazole, and levofloxacin in children were 20.6%, 68.8%, and 9.0%, respectively, and the *H. pylori* resistance rate increased significantly from 2012 to 2014.[8] Generally, *H. pylori* acquires antibiotic resistance by chromosomal mutations, not by acquiring plasmids.[9] Although drug efflux proteins can contribute to the natural insensitivity to antibiotics and emerging antibiotic resistance, the main mechanism that contributes to *H. pylori* resistance is vertically transmitted point mutations in the DNA.[10] Clarithromycin interacts with the peptidyl transferase region of the domain V of 23S rRNA subunit, an interaction that suppresses bacterial ribosome activity and inhibits bacterial protein synthesis.[10] Point mutations in the 23S rRNA gene have been shown to lead to a modification in ribosome conformation, which consequently reduces clarithromycin affinity and leads to bacterial resistance to the drug.[10] The most common mutations are A2143G, A2142G and A2142C. In addition, mutations A2115G, G2141A, C2147G, T2190C, C2195T, A2223G and C2694A have also been reported, but their role in resistance to clarithromycin is not yet clear.[11] Metronidazole, a nitroimidazole, acts as a biocidal agent by its interaction with a nitroreductase homolog, *rdxA*. Mutations in *rdxA* were shown to be the cause of *H. pylori* resistance to metronidazole, while mutations in another gene, *frxA*, encoding to NADH flavin oxidoreductase was also implicated in *H. pylori* metronidazole resistance.[12,13] Levofloxacin, a fluoroquinolone, generally target chromosome replication and in particular, DNA gyrase, which allows DNA unraveling before replication. This resistance has been associated with point mutations occurring at positions Asn87 and Asp91 of the quinolone resistance determining region (QRDR) within *gyrA* gene.[14] Amino acid substitutions at positions 91 (D91G, N, A, Y or H) and 87 (N87L, I, A or K) of *gyrA* were most frequently associated with levofloxacin resistance, while *gyrB* frequently occurred alongside *gyrA* mutations.[15,16] Despite the studies on the resistance-related genes, resistant isolates with other mutations seem to be emerging, and the mutations of resistance genes of *H. pylori* in children are poorly understood.

H. pylori antimicrobial resistance can be investigated in the laboratory by phenotypic and genotypic methods. Bacterial culture and determination of the minimum inhibitory concentration (MIC) of the antibiotic are characteristics of the phenotypic method. In this study, PCR amplification and DNA sequencing were performed to detect genetic mutations in drug-resistance and sensitive *H. pylori* strains from children, and we also determined the mutations of *H. pylori* strains resistant to clarithromycin, metronidazole and fluoroquinolone. We analyzed the correlation between drug resistance and gene mutations by molecular biology software.

Materials and Methods

Isolation and Culture of *H. pylori*

Gastric mucosa samples were collected from children (aged from 2 to 16 years old) with gastrointestinal diseases such as chronic gastritis or ulcer during endoscopy from 2012 to 2014 in Children's Hospital, Zhejiang University School of Medicine. Isolation, culture and identification of *H. pylori* were performed at the laboratory of the Hangzhou Zhiyuan Medical Inspection Institute as described in previous studies.[17] The antibiotic susceptibility testing was performed by E-test and agar dilution methods according to the protocols of the Clinical and Laboratory Standards Institute (Wayne, PA, USA).[18] Written informed consents were obtained from the parents of the children involved in our study. The study protocol was approved by the Medical Ethics Committee in the Children's Hospital, Zhejiang University School of Medicine (2018-IRB-003) and was conducted in accordance with the declaration of Helsinki.

Exclusion criteria included history of treatment by use of antibiotic, antacid, H_2 receptor antagonist, PPI, bismuth-containing compounds, or non-steroidal anti-inflammatory drugs (NSAID) in the last 4 weeks.

Antibiotic Susceptibility Tests

Susceptibility of *H. pylori* to five antibiotics (clarithromycin, amoxicillin, tetracycline, metronidazole, and levofloxacin) was tested via agar dilution method using reference standards obtained from the National Institutes for Food and Drug Control. Tenmicroliter suspensions (10^8 CFU/mL) of each isolate from a mixture of colonies in brain-heart infusion broth (Oxoid) were inoculated onto Mueller-Hinton agar plates (Oxoid) that included 5% sheep blood and a single antibiotic and incubated at 37°C for 3 days under microaerophilic conditions. The resistance break points to clarithromycin, amoxicillin, tetracycline, metronidazole, and levofloxacin were set at ≥1 μg/mL, ≥2 μg/mL, ≥2 μg/mL, ≥8 μg/mL, and ≥2 μg/mL, respectively. ATCC43504 (NCTC11637) was used as the control strain.

PCR and DNA Sequencing

H. pylori Strain Culturing and DNA Extraction

H. pylori strains were recovered and cultured under microaerophilic conditions. DNA extraction was performed using the Takara LA Taq Kit according to the manufacturer's instructions, and the isolated genomic DNA was stored at −20°C.

Detection of DNA Purity and Concentration

DNA purity was examined using UV spectroscopy. DNA extracted from each strain was diluted in 5 μL water, and measured with light absorbance at 260 nm and 280 nm. The concentration and purity of DNA were determined. Purified DNA should have an A260/A280 ratio within 1.7–1.9.

PCR Amplification

PCR was performed for amplification regions, including following genes: 16S rRNA (139bp), 23S rRNA (280bp),[19] *rdxA* (749bp),[20] *frxA* (913bp),[20] *gyrA* (582bp),[21] *gyrB* (465bp).[21] Based on the sequence of reference strain 26695 available on the GenBank website, the following primers were designed to detect point mutations (Table 1). Primers were diluted to 10 μM before use. The 50 μL PCR system contained Takara LA Taq (5 U/μL) 0.5 μL, 10×LA PCR Buffer II (Mg^{2+} Plus) 5 μL, dNTP Mixture (2.5 mM each) 4 μL, template DNA 5 μL, primer F (10 μM) 1 μL, primer R (10 μM) 1 μL, and dH_2O 33.5 μL. The reaction condition was set as: 95°C predenaturation 5 min, 94°C 30 s, 58°C 30 s, 72°C 30 s, 30 cycles, and 72°C final elongation 10 min, followed by preservation at 4°C. After that, PCR products (9 μL) were added with 10×loading Buffer (1 μL), and electrophoresed through 1% agarose gel with 0.5 mg/L ethidium bromide at 100 mV for 30 min. For validation, electrophoresis strips were observed under a gel imager.

DNA Sequencing

PCR products were first recognized by electrophoresis, and determined with DNA purity as well as concentration by measuring the A260/A280 ratio. DNA sequencing was accomplished by Qingke Zixi Biotechnology Co., Ltd (Hangzhou, China). Each sequence was compared to the sequences of *H. pylori* 26695 reference strains, available on the GenBank website. With sequences from NCBI for reference, mutations were identified from each sensitive or resistant strain using the MEGA 5 software.

Results

Clinical Data

A total of 79 *H. pylori* strains be isolated, identified and preserved from the patients because of abdominal pain, vomiting, and gastrointestinal bleeding. The mean age was 9.7± 2.8 years old. All of them were diagnosed as *H. pylori*-related gastritis by gastroendoscopy, histopathological examination, and *H.pylori* culture of gastric mucosa. Among them, 21 cases were diagnosed as duodenal ulcer, 1 case was gastric ulcer, 1 case was concomitant gastric and duodenal ulcer. Out of the 79 *H. pylori* strains examined, 66 (83.5%) were

Table 1 Primer Sequences for Molecular Detection of *H. pylori*

Genes	Forward Primer	Reverse Primer
16S rRNA	CTCATTGCGAAGGCGACCT	TCTAATCCTGTTTGCTCCCCA
23S rRNA	GTAACTATAACGGTCCTAAG	GAAACATCAAGGGTGGTATC
rdxA	GCCACTCCTTGAACTTTAATTTAGG	CGTTAGGGATTTTATTGTATGCTAC
frxA	CGAATTGGATATGGCAGCCG	TATGTGCATATCCCCTGTAGG
gyrA	AGCTTATTCCATGAGCGTGA	TCAGGCCCTTTGACAAATTC
gyrB	CCCTAACGAAGCCAAAATCA	GGGCGCAAATAACGATAGAA

resistant to at least one of the tested antibiotics and 13 (16.5%) were fully sensitive. Among these strains, 66 were resistant strains, and most of them were resistant to multiple antibiotics. 29 (36.7%) were resistant to clarithromycin, 54 (68.4%) to metronidazole, and 12 (15.2%) to levofloxacin. No resistance of *H. pylori* strains to amoxicillin and to tetracycline was observed in our study.

rRNA Gene Mutations in Clarithromycin-Resistant Strains

Among 29 *H. pylori* resistant strains to clarithromycin, 22 were multi-drug resistant. A 280-bp segment on the 23S rRNA gene was successfully amplified by PCR and sequenced from both 11 sensitive strains and 29 resistant strains. We found most mutations in resistant strains occurred at A2143G and T2182C, with a frequency of 82.8% (24/29) and 89.7% (26/29), respectively, but mutations at A2142G and C2131T were detected only in one case, respectively. As for those 11 sensitive strains, the frequency of A2143G mutation was 45.5% (5/11), which was significantly lower than that in resistant strains (X^2=6.714, P<0.05); the frequency of T2182C was 90.9% (10/11), which was not significantly different from that in resistant strains (89.7%, 26/29); No mutations were detected at A2142G or C2131T in sensitive strains as shown in Table 2. The figure of sequencing fluorograms of 23S rRNA gene amplification products in resistant and sensitive strains of *H. pylori* was shown in Supplementary Figure S1.

rdxA and *frxA* Mutations in Metronidazole-Resistant Strains

The resistant rate of metronidazole to *H. pylori* was 68.4% (54/79), and 28 out of them had multiple drug resistance.

rdxA Mutations

A 749-bp segment of *rdxA* gene was successfully amplified by PCR and sequenced from both 6 sensitive strains and 54 resistant strains. The frequency of G47A mutation in resistant strains was 20.4% (11/54), and T184G mutation 70.4% (38/54), other mutations including G60A mutation in 3 resistant strains, C273T mutation in 2 resistant strains, and C49T mutation in 1 resistant strain. However, a mutation rate of T184G in sensitive strains was 83.3% (5/6), similar to the resistant strains. The above other 4-point mutations were not detected in any sensitive strains. In addition, deletion of A at position 329,330/Frameshift, a stop codon at position 111 and deletion of G at position 570/Frameshift at position 191 of *rdxA* gene were observed both in resistance strains and in sensitive strains of *H. pylori* as shown in Table 3. The figure of sequencing fluorograms of *rdxA* gene amplification products in resistant and sensitive strains of *H. pylori* was shown in Supplementary Figure S2.

frxA Mutations

A 913-bp segment of *frxA* gene was successfully amplified by PCR and sequenced from both 6 sensitive strains and 43 resistant strains. The frequency of A48G mutations in resistant strains was 70% (30/43). Other 13 resistant strains did not have these point mutations. Among 30 resistant strains had the A48G mutations, 1 strain had a G441A mutation, 1 strain had a deletion of G at position 208. Besides, A48G mutation was also identified from all the 6 sensitive strains, and 1 strain had a C13A mutation in sensitive strain as shown in Table 4. The figure of sequencing fluorograms of *frxA* amplification products in resistant and sensitive strains of *H. pylori* was shown in Supplementary Figure S3.

gyrA and *gyrB* Mutations in Levofloxacin-Resistant Strains

The resistant rate of levofloxacin to *H. pylori* was 15.2% (12/79), and 11 out of them had multiple drug resistance. A 582-bp segment of *gyrA* was successfully amplified by PCR and sequenced from both 12 sensitive and 12 resistant strains. Three resistant strains of them had a mutation at ASP 91, and one mutated from Asp to Tyr, and it was resistant to both metronidazole and levofloxacin; the other two resistant strains mutated from Asp to Asn. In addition, there were N87K (Asn87Lys) mutations for both sensitive strains (6/12) and resistant strains (10/12). Other mutation including R140K (Arg140Lys) and V150A (Val 150Ala) were identified in one resistant strain and one sensitive strain, respectively. No D91Y or D91N mutation was found in sensitive strains as shown in Table 5. This study did not identify any mutations for *gyrB* gene in both resistant strains and sensitive strains. The figure of sequencing fluorograms of *gyrA* gene amplification products in resistant and sensitive strains of *H. pylori* was shown in Supplementary Figure S4.

Discussion

H. pylori infection has become a global problem. Nearly half of the world population are infected with the pathogen, and the infectious rate among children is estimated to be 10–80% with different age, with more than a half being infected before the age of 10 years old.[15,16] In fact, many adult gastric

Table 2 Mutations Within 23S rRNA Gene for Clarithromycin-Resistant and Sensitive Strains of *H. pylori*

No. of Strain	Site of Mutation				Resistance to Antibiotics		
	T2182C	A2143G	A2142G	C2131T	Clarithromycin	Metronidazole	Levofloxacin
002	T2182C	A2143G	N	N	+	+	−
004	T2182C	A2143G	N	N	+	+	−
165	T2182C	A2143G	N	N	+	+	−
223	T2182C	A2143G	N	N	+	+	−
250	N	N	N	N	+	+	−
272	N	A2143G	N	C2131T	+	+	−
281	T2182C	A2143G	N	N	+	−	−
318	T2182C	A2143G	N	N	+	−	−
371	T2182C	N	A2142G	N	+	+	−
372	T2182C	A2143G	N	N	+	−	−
404	T2182C	A2143G	N	N	+	+	+
409	T2182C	A2143G	N	N	+	+	−
453	T2182C	A2143G	N	N	+	+	−
457	T2182C	A2143G	N	N	+	+	−
461	T2182C	A2143G	N	N	+	+	−
466	T2182C	A2143G	N	N	+	+	−
484	T2182C	A2143G	N	N	+	−	−
580	T2182C	A2143G	N	N	+	+	+
592	T2182C	A2143G	N	N	+	+	−
636	T2182C	A2143G	N	N	+	+	−
701	T2182C	A2143G	N	N	+	+	−
724	T2182C	N	N	N	+	−	−
729	N	N	N	N	+	+	−
798	T2182C	A2143G	N	N	+	+	−
820	T2182C	A2143G	N	N	+	+	−
846	T2182C	A2143G	N	N	+	+	−
878	T2182C	A2143G	N	N	+	−	−
951	T2182C	A2143G	N	N	+	−	−
984	T2182C	N	N	N	+	+	+
111	T2182C	N	N	N	−	−	−
113	T2182C	A2143G	N	N	−	−	−
159	T2182C	N	N	N	−	−	−
282	T2182C	N	N	N	−	−	−
433	T2182C	N	N	N	−	−	−
476	T2182C	N	N	N	−	−	−
480	T2182C	A2143G	N	N	−	−	−
527	T2182C	A2143G	N	N	−	−	−
746	T2182C	A2143G	N	N	−	−	−
750	N	N	N	N	−	−	−
751	T2182C	A2143G	N	N	−	−	−

Abbreviations: N, no mutation; +, resistant; −, sensitive.

H. pylori infections are suffered from childhood. *H. pylori* eradication is the key to the treatment of *H. pylori* related diseases, but it is controversial in children with *H. pylori* infection. Therapy failure is inherent and can be due to multiple factors (human and bacterial), including improper drug dose, short treatment duration, early treatment discontinuation, drug activity associated with the use of other substances, quick reinfection of successfully treated patients, and the presence of antibiotic-resistant strains.[11] Many reports have indicated that antibiotic resistance has become a predominant factor for therapy failure in which containing clarithromycin and metronidazole. No consensus of indications and therapy of *H. pylori* eradication in children have yet been reached. It is very important to use sensitive antibiotics

Table 3 Mutations Within *rdxA* for Metronidazole-Resistant and Sensitive Strains of *H. pylori*

No. of Strain	Site of Mutation			Resistance to Antibiotics		
	G47A	T184G	Other	Clarithromycin	Metronidazole	Levofloxacin
002	N	T184G		+	+	–
003	N	T184G		–	+	+
004	G47A	N		+	+	–
044	N	T184G		–	+	–
112	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	+	–
144	N	N		–	+	–
160	G47A	N	G60A	–	+	–
165	N	T184G	C273T	+	+	–
184	N	T184G		–	+	+
208	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	+	–
223	N	T184G		+	+	–
250	N	T184G		+	+	–
251	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	+	+
256	G47A	N		–	+	–
267	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	+	+
272	N	T184G	Deletion of G at position 570/ Frameshift at position 191	+	+	–
276	N	N		–	+	–
277	N	T184G		–	+	–
371	G47A	N		+	+	–
404	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111, Deletion of G at position 570/ Frameshift at position 191	+	+	+
409	N	T184G		+	+	–
436	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111, Deletion of G at position 570/ Frameshift at position 191	–	+	–
437	N	T184G		–	+	–
455	G47A	T184G	G60A, Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	+	+	–
457	N	T184G	C273T	+	+	–
461	N	T184G		+	+	–

(*Continued*)

Table 3 (Continued).

No. of Strain	Site of Mutation			Resistance to Antibiotics		
	G47A	T184G	Other	Clarithromycin	Metronidazole	Levofloxacin
466	N	N	C49T	+	+	–
481	N	N		–	+	–
538	N	T184G		–	+	–
580	N	N		+	+	+
592	N	T184G		+	+	–
594	G47A	T184G		–	+	–
634	N	T184G		–	+	–
636	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	+	+	–
637	N	T184G		–	+	–
701	N	N		+	+	–
720	G47A	T184G		–	+	+
725	N	T184G		–	+	–
729	G47A	T184G		+	+	–
731	N	N		–	+	–
752	N	N		–	+	–
766	N	T184G		–	+	–
767	N	T184G		–	+	+
796	N	T184G		–	+	–
798	N	N		+	+	–
818	N	T184G		–	+	–
820	N	N		+	+	–
832	G47A	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	+	–
833	G47A	T184G	G60A, Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	+	+
846	N	N		+	+	–
873	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	+	–
904	G47A	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	+	–
940	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	+	–
952	N	N		–	+	–

(*Continued*)

Table 3 (Continued).

No. of Strain	Site of Mutation			Resistance to Antibiotics		
	G47A	T184G	Other	Clarithromycin	Metronidazole	Levofloxacin
113	N	T184G		–	–	–
282	N	T184G		–	–	–
746	N	T184G	Deletion of A at position 329,330/ Frameshift, a stop codon at position 111	–	–	–
751	N	N		–	–	–
281	N	T184G		+	–	–
318	N	T184G	G576A, Deletion of G at position 570/ Frameshift at position 191	+	–	–

Abbreviations: N, no mutation; +, resistant; –, sensitive.

to treat *H. pylori* infection. Unfortunately, most of them have not performed any sensitivity tests including the E-test before *H.pylori* eradication therapy in the clinics. Point mutations detection by PCR or sequencing of the amplified fragment has higher sensitivity and specificity than that of MIC determination, which could be served as a new treatment regimen for antibiotics selection in the *H. pylori* eradication, especially in children.

In this study, the resistant rate of clarithromycin of *H. pylori* was 36.7% (29/79). A2143G and T2182C were the major mutations among the 29 strains with clarithromycin resistance. The frequency of A2143G in sensitive strains was significantly lower than that in resistant strains (45.5 vs 82.8%), while the frequency of T2182C had not reached significant difference between in sensitive strains and in resistant strains (90.9 vs 89.7%), indicating that clarithromycin resistance might be associated with A2143G mutation in children. A study in adults from the same district in Zhejiang Province showed that all of phenotype-resistant strains (n=6) presented mutation A to G at position 2143 of the 23S rRNA, while 2 mutant-type of 23S rRNA gene at position 2143 (A>G) was also detected in sic phenotype-susceptible strains.[22] Another study in adults from the same area showed that 90.4% gene mutations of the 23S rRNA were the A2143G mutation.[23] *H. pylori* strains were found to carry A2143G, A2142G, A2147G and A2146G mutations that can lead to clarithromycin-resistance in Africa, Asia, South America, Europe and North America.[24–30] The T2183C and A2223G transformations have been frequently found to be the cause of observed clarithromycin resistance in Asian countries than those in Europe and North America,[31] but not be observed in our study. In addition to these mutations, other mutations in different positions have been found in other parts of the world to confer clarithromycin resistance in *H. pylori* strains (T2182C, T2190C, C2195T, A2223G, G2141A, C2694A, G2224A, C2245T, T2289C).[30] The T2182C was detected in this study both in sensitive strains and in resistant strains, which were identified with low resistance level in other studies, and were detected in most of the strains in China for geographical and genetic factors.[22,32,33]

The resistant rate of metronidazole to *H. pylori* was 68.4% (54/79), and 28 out of resistant strains had multiple drug resistance. In recent years, many studies believe that mutations in NADPH *rdxA* and *frxA* genes are the main causes of metronidazole resistance. In this study, the mutation frequency of *rdxA* gene G47A mutation in resistant strains was 20.4% (11/54), and T184G mutation 70.4% (38/54). However, a mutation rate of T184G in sensitive strains was 83.3% (5/6), but no G47A mutation was identified. Luo et al reported that 87.2% of the resistant strains identified in the Guiyang Medical College contained 4 fixed-site mutations in their *rdxA* gene, and that might be associated with *H. pylori* resistance to metronidazole.[34] Mirzaei et al suggested that the W(209)R mutation within *rdxA* might be related to metronidazole resistance.[35] Tanih et al identified 13 amino acid alterations at 9 sites, and truncations at 14 sites within the *rdxA* gene of metronidazole-resistant strains.[36] The mutation frequency of *frxA* gene A48G mutations in resistant strains was 70% (30/43), while A48G mutation was also identified from all 6 sensitive strains. With these different mutations, more studies are needed to verify which mutations are related to drug resistance.

Table 4 Mutations Within *frxA* for Metronidazole-Resistant and Sensitive Strains of *H. pylori*

No. of Strain	Site of Mutation		Resistance to Antibiotics		
	A48G	Other	Clarithromycin	Metronidazole	Levofloxacin
002	A48G		+	+	–
003	A48G	Deletion of G at position 208	–	+	+
044	A48G		–	+	–
112	N		–	+	–
144	N		–	+	–
165	A48G		+	+	–
184	A48G		–	+	+
208	A48G		–	+	–
250	A48G		+	+	–
251	A48G		–	+	+
256	A48G	G441A	–	+	–
263	A48G		–	+	–
267	N		–	+	+
272	A48G		+	+	–
277	A48G		–	+	–
371	N		+	+	–
404	A48G		+	+	+
409	A48G		+	+	–
436	A48G		–	+	–
461	N		+	+	–
481	A48G		–	+	–
538	A48G		–	+	–
580	A48G		+	+	+
592	N		+	+	–
594	N		–	+	–
634	A48G		–	+	–
636	A48G		+	+	–
701	N		+	+	–
719	A48G		–	+	+
725	N		–	+	–
729	N		+	+	–
731	N		–	+	–
752	A48G		–	+	–
796	A48G		–	+	–
798	A48G		+	+	–
820	A48G		+	+	–
832	A48G		–	+	–
833	A48G		–	+	+
846	A48G		+	+	–
873	N		–	+	–
904	N		–	+	–
940	A48G		–	+	–
952	A48G		–	+	–
113	A48G		–	–	–
282	A48G		–	–	–
746	A48G		–	–	–
751	A48G		–	–	–
281	A48G	C13A	+	–	–
318	A48G		+	–	–

Abbreviations: N, no mutation; +, resistant; –, sensitive.

Table 5 Mutations Within *gyrA* for Levofloxacin-Resistant and Sensitive Strains of *H. pylori*

No. of Strain	Site of Mutation			Resistance to Antibiotics		
	Asp91	Asn87	Other	Clarithromycin	Metronidazole	Levofloxacin
003	D91Y(Asp91Tyr)	N87K(Asn87Lys)		−	+	+
109	D91N(Asp91Asn)	N87K(Asn87Lys)		−	−	+
184	N	N87K(Asn87Lys)		−	+	+
251	N	N		−	+	+
267	D91N(Asp91Asn)	N87K(Asn87Lys)		−	+	+
403	N	N87K(Asn87Lys)		−	+	+
404	N	N87K(Asn87Lys)	R140K(Arg140Lys)	+	+	+
580	N	N87K(Asn87Lys)		+	+	+
719	N	N87K(Asn87Lys)		−	+	+
767	N	N87K(Asn87Lys)		−	+	+
833	N	N		+	+	+
984	N	N87K(Asn87Lys)		+	+	+
111	N	N		−	−	−
113	N	N87K(Asn87Lys)		−	−	−
159	N	N87K(Asn87Lys)		−	−	−
282	N	N		−	−	−
433	N	N87K(Asn87Lys)		−	−	−
476	N	N		−	−	−
480	N	N		−	−	−
524	N	N		−	−	−
527	N	N87K(Asn87Lys)	V150A(Val 150Ala)	−	−	−
746	N	N87K(Asn87Lys)		−	−	−
750	N	N87K(Asn87Lys)		−	−	−
751	N	N		−	−	−

Abbreviations: N, no mutation; +, resistant; −, sensitive.

Point mutations at Asp-91 and Asn-87 in the quinolone-resistance determining region of gyrase are the key to drug resistance.[37–39] In this study, 3 out of the 12 levofloxacin-resistant strains carried mutations at Asp-91, and another 10 had mutations at Asn-87, but 6 out of the 12 sensitive strains also had Asn-87 mutations but not Asp-91 mutations. Thus, we speculate that Asp-91 mutation might be a key point to the resistance of *H. pylori*. One study in adults from the same district in Zhejiang Province showed that the N87K mutation was the predominant mutation in the 14 levofloxacin-resistant isolates with *gyrA* mutations, while only 2 of the 29 isolates with *gyrA* mutations contained the D91G mutation, and these 2 isolates were susceptible to levofloxacin.[23] The results were not consistent with our results in levofloxacin-resistant gene mutations. Binkowska et al reported that the most common mutation presented in 40% of *H. pylori* strains resistant to levofloxacin was a change at position 91 of *gyrA* in children.[25] De Palma et al reported that resistance of levofloxacin was due to substitution at position 87 (K,I) and 91 (G,N,A) of *gyrA* QRDR in the 92.8% of the resistant isolates.[40] Miftahussurur et al reported that mutations at Asn-87 and/or Asp-91 were predominantly in levofloxacin-resistant strains, and the *gyrB* mutation had a steady relationship with *gyrA* 87–91 mutations.[41] None of the substitutions found in *gyrB* proved conferring resistance in this study. These findings revealed that the mutations of genes related to *H. pylori* resistance are diversified, especially in children.

In conclusion, in *H. pylori* resistant strains isolated from children in China, most mutations occurred at A2143G of the 23S rRNA gene for clarithromycin; G47A mutation of *rdxA* gene for metronidazole; and at 91 mutation of *gyrA* gene for levofloxacin. It is suggested that susceptibility testing together with screening the mutation of antimicrobial-resistant gene prior to treatment is important for future clinical practice. Further studies of *H. pylori* resistant gene mutation and its relationship to *H. pylori* resistance to antibiotics should be highlighted in the eradication of *H. pylori* in children.

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Disclosure

The authors report no conflicts of interest in this work.

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