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ORIGINAL ARTICLE

Antibiotic resistance of *Helicobacter pylori* isolated from children in Chongqing, China

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Abstract

The resistance of Helicobacter pylori (*H. pylori*) to antibiotics has been increasing worldwide and varies across different geographic areas and times. Limited studies reported the prevalence of antibiotic resistance and its related gene mutations in children in Chongqing, a city located in southwest China. We collected 112 *H. pylori* strains isolated from gastric biopsies of 156 children at Children's Hospital of Chongqing Medical University and calculated resistance rates of these strains to six antibiotics. The A2143G and A2142G mutations in 23S rRNA gene, which are related to clarithromycin resistance, and Asn87 and Asp91 mutations in gyrA gene, which are related to levofloxacin resistance, were investigated in 102 strains. The resistance rates to clarithromycin, metronidazole, and levofloxacin were 47.3% (53/112), 88.4% (99/112), and 18.8% (21/112), respectively. No resistance to amoxicillin, tetracycline, and furazolidone was observed. Dual and triple resistance percentages were 37.5% (42/112) and 10.7% (12/112), respectively. The detection rate of A2143G mutation in 23S rRNA gene was 83.3% (40/48). The detection rates of mutations of Asn87 and Asp91 in gyrA gene were 52.6% (10/19) and 36.8% (7/19), respectively.

Conclusion: The prevalence of *H. pylori* resistance to clarithromycin, metronidazole, and levofloxacin was high in children in Chongqing, China. The A2143G mutation was detected in most clarithromycin-resistant strains, and Asn87 and Asp91 of gyrA mutation points were common in levofloxacin-resistant strains. In clinical practice, anti-*H. pylori* therapy should be individualized based on a susceptibility test.

What is Known:
- *The resistance of H. pylori to antibiotics changes with the geographic areas and that in Asia the resistance rate is high.*
- *Mutation plays a vital role in antibiotics resistance of H. pylori.*

What is New:
- *High resistance rates to single and multiple antibiotics in children of Chongqing, a city located in southwest China, were observed.*
- *Molecular assays showed good conformance with susceptibility test results to direct antibiotic resistance of H. pylori.*

Keywords Helicobacter pylori (*H. pylori*) · Antibiotic resistance · Children

Abbreviations

AMO Amoxicillin
CLA Clarithromycin

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FUR Furazolidone
LEV Levofloxacin
MTZ Metronidazole
NUD Non-ulcer disease
PUD Peptic ulcer diseases
PPI Proton pump inhibitor
TET Tetracycline

Introduction

Helicobacter pylori (*H. pylori*) infection is one of the most common chronic bacterial infections in humans, infecting about 50% of the population [1]. *H. pylori* infection can lead to gastrointestinal diseases such as chronic gastritis, peptic ulcer diseases (PUD), gastric cancer, and gastric mucosa–associated lymphoid tissue lymphoma. It may also be associated with non-digestive diseases such as halitosis, short stature, malnutrition, and refractory iron deficiency anemia in children [2]. Eradication of *H. pylori* could help to treat chronic gastritis, PUD, and other related diseases, as well as reduce the risk of developing gastric cancer [3].

As the first-line regimen for *H. pylori*, standard triple therapy consists of one proton pump inhibitor (PPI) and two antibiotics: clarithromycin and amoxicillin, (amoxicillin can be substituted with metronidazole if allergic to penicillin). When the therapy was first introduced, it achieved a high eradication rate (> 90%). However, in recent years the efficacy of the regimen has been decreasing in many regions of the world, with eradication rates of < 80%, making it no longer suitable for clinical practice [4]. The causes of the low eradication rates may involve inappropriate therapies, poor patient compliance, smoking, genetic polymorphisms in *CYP2C19*, and high bacterial load in the stomach [4]. Resistance to antibiotics in *H. pylori* is generally thought to be the major reason for eradication failure [4]. The prevalence of antibiotic resistance has been increasing with the excessive consumption of antibiotics. A recent review on the global emergence of *H. pylori* antibiotic resistance comprising 178 studies from 65 countries reported severe antibiotic resistance worldwide, especially resistance to clarithromycin and metronidazole [5]. According to Maastricht V/Florence Consensus report, when the clarithromycin resistance rate is more than 15% in the region, empiric triple therapy containing clarithromycin should not be used unconditionally without prior susceptibility test [1]. The ESPGHAN/NASPGHAN guidelines also emphasize the importance of susceptibility test before eradication therapy [2]. When prescribing anti-*H. pylori* therapies, clinicians should consider the local antibiotic resistance rates and choose an appropriate regimen to improve eradication.

Mutation plays an important role in antibiotic resistance of *H. pylori*. As one of the most commonly used macrolides, clarithromycin inhibits protein synthesis through binding to the peptidyl transferase loop of domain V of the bacterial 23S rRNA molecule in 50S ribosome subunit reversibly. Point mutations in the domain V of 23S rRNA gene have been reported to be correlated with clarithromycin resistance [6]. Transition mutations of A2142G and A2143G are the most prevalent mutations [7]. Fluoroquinolones interfere with the synthesis of DNA in bacterium by inhibiting the activity of the enzyme DNA gyrase. The resistance to fluoroquinolones in *H. pylori* is related to point mutations in the quinolone resistance–determining region of the gyrA gene. The most frequent mutations are found at the codons for amino acids 87 (Asn to Lys, Tyr) and 91 (Asp to Gly, Asn, Ala, or Tyr) of gyrA gene [7].

Studies from Chinese children have revealed that the resistance of *H. pylori* was alarming [8–11]. However, there is a lack of study on antibiotic resistance and relevant mutations in 23S rRNA and gyrA gene in children in Chongqing, a city located in southwest China. Therefore, in this study we aimed to assess the resistance to six antibiotics and related mutations of *H. pylori* isolated from children in Chongqing.

Patients and methods

Isolation of *H. pylori* strains

Gastric biopsy was performed in 156 children who suspected of having a *H. pylori* infection at gastroenterology outpatient department of Children's Hospital of Chongqing Medical University from March to July 2020. All children did not take any H2 receptor antagonists, PPIs, bismuth, and antibiotics within the last 2 months before the investigation. Gastric mucosa biopsy specimens were collected at the antrum via upper gastrointestinal endoscopy and were reserved in the brain–heart infusion broth (Oxoid) with 5% glycerin at − 80 °C and then transported to the laboratory of Hangzh -ou Zhiyuan Medical Inspection Institute with dry ice. Suspensions of gastric biopsy specimens were inoculated onto Columbia agar (Oxoid) plates with 5% defibrinated sheep blood and cultivated at 37 °C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) for 3 to 5 days. The suspected transparent colonies were identified by Gram stain and urease, catalase, oxidase activity test. Strain which was Gram-negative and -positive for all three tests was identified as *H. pylori*.

Antibiotic susceptibility testing

The antibiotic resistance of *H. pylori* strains to six antibiotics (clarithromycin, metronidazole, levofloxacin, amoxicillin, tetracycline, and furazolidone) was tested via agar dilution method. Suspensions of *H. pylori* were plated onto Mueller–Hinton agar (Oxoid) plates containing 5% sheep blood and a single antibiotic under a critical concentration, then incubated at 37 °C for 3 to 5 days under microaerophilic conditions. The resistance breakpoints of antibiotics were set at clarithromycin ≥ 1.0 μg/mL, metronidazole ≥ 8.0 μg/mL, levofloxacin ≥ 2.0 μg/mL, amoxicillin ≥ 2.0 μg/mL, tetracycline ≥ 2.0 μg/mL, furazolidone ≥ 2.0 μg/mL [11–13]. ACTC11637 was used as the control strain.

Mutations analysis of 23S rRNA and gyrA gene

The DNA of isolated strains was extracted by using PureLink™ tissue extraction kit (PL, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Primers were designed to amplify 23S rRNA and gyrA genes: 23S rRNA FP (5′-ATGAATGGCGTAACGAGATG-3′), 23S rRNA (5′-ACACTCA ACTTGCGATTTCC-3′), gyrA FP (5′-GATCATAGGGCGCGCTTTACC-3′), gyrA RP (5′-AAGTCGCCATCCCTACAGCGA-3′). The fragments were 360 bp of 23S rRNA and 458 bp of gyrA gene. PCR was performed using the BIO-GENER PCR amplifier as follows: initially 3 min pre-denaturation at 98 °C, then denaturation at 98 °C for 30 s, annealing at 58 °C for 10 s and 72 °C for 10 s by 30 cycles, finally extension of 5 min at 72 °C. The PCR products was identified by running it in 2% agarose and performed Sanger sequencing to detect the mutations at the 2143, 2142 positions in 23S rRNA, 87 and 91 codons in gyrA gene.

Statistical analysis

Data were analyzed by SPSS 26.0 (SPSS Inc., Chicago, IL, USA) statistical software. The prevalence of resistance among different gender, age groups, and endoscopic findings was accessed with chi-square test (χ^2 test). A probability (p) value ≤ 0.05 was regarded as statistically significant.

Results

A total of 112/156 (71.8%) *H. pylori* strains were isolated. There were 56 boys and 56 girls; the mean age of all children was 9.6 ± 3.0 years, ranged from 3.0 to 17.9 years. The most frequent symptom was abdominal pain (77.7%) and the main endoscopic finding was *H. pylori*-ralated gastritis (86.6%) among 112 patients, only 15 presented with duodenal ulcer. The baseline characteristics of the 112 children are summarized in Table 1.

Table 1 Baseline characteristics of included children (n=112)

Characteristics	
Boys, no.(%)	56 (50.0)
Age,years	9.6±3.0
Boys age, years	9.5±3.0
Girls age, years	9.7±3.1
Endoscopic findings	
H. pylori-related gastritis, no. (%)	97 (86.6)
Duodenal ulcer, no. (%)	15 (13.4)
Symptoms	
Abdominal pain, no. (%)	87 (77.7)
Vomiting, no. (%)	7 (6.3)
Anorexia, no. (%)	13 (11.6)
Halitosis, no. (%)	7 (6.3)
Gastrointestinal hemorrhage, no. (%)	6 (5.4)
Asymptomatic, no. (%)	7 (6.3)

Antibiotic susceptibility test results

Among the 112 isolated *H. pylori* strains, 5 (4.5%) was sensitive to all of six antibiotics and 107 (95.5%) were found resistance to one or more antibiotics. No resistance

Table 2 Antibiotics resistance results of 112 *H. pylori* strains isolated

Resistance patterns	No. of strains	Resistance rate (%)
Total resistance		
AMO	0	0
TET	0	0
FUR	0	0
CLA	53	47.3
MTZ	99	88.4
LEV	21	18.8
Single resistance		
CLA	5	4.5
MTZ	47	42.0
LEV	1	0.9
Double resistance		
CLA+MTZ	34	30.4
CLA+LEV	02	1.8
MTZ+LEV	06	5.4
Triple resistance		
CLA+MTZ+LEV	12	10.7

AMO amoxicillin, TET tetracycline, FUR furazolidone, CLA clarithromycin, MTZ metronidazole, LEV levofloxacin

Table 3 MIC of 112 isolated strains, single and multiple resistant strains (μg/mL)

Antimicrobial	Range (n=112)	Single (mean)	Double (mean)	Triple (mean)
CLA	≤0.016~4	c2.82	2.44	2.58
MTZ	≤0.5~64	38.64	42.82	36
LEV	0.0625~8	4	3.50	5.33

CLA clarithromycin, MTZ metronidazole, LEV levofloxacin

to tetracycline, amoxicillin, and furazolidone was observed of all strains. Table 2 showed the resistance rates of isolated *H. pylori* strains. We found that 53 (47.3%) were single resistance to clarithromycin, metronidazole, or levofloxacin. Multiple resistance rate accounted for 48.2% (54/112), and four types of multiple resistance were found. The dual drug resistance rate was 37.5% (42/112), the dual resistance rate to metronidazole and clarithromycin was 30.4% (34/112) as the predominant resistance pattern. Triple resistance rate of clarithromycin, metronidazole, and levofloxacin was 10.7% (12/112) and no quadruple resistance was found. The distributions of minimal inhibitory concentration (MIC) values in isolated *H. pylori* strains were demonstrated in Table 3. Strains resistant to clarithromycin showed a low level of MIC in the range of 1–4 μg/mL, similarly in levofloxacin-resistant strains, the range of MIC was 2–8 μg/mL. Among 99 metronidazole-resistant strains, 42.4% (44/99) had high level resistance with MIC ≥ 64 μg/mL. There was no significant difference between single and multiple resistance strains on average values of MIC ($p > 0.05$).

Mutations of 23S rRNA and gyrA gene

Of the 112 samples, 102 strains were detected the mutations of 23S rRNA and gyrA gene (Table 4). The mutation rate of A2143G was 48.0% (49/102); we did not found any A2142G mutation. The mutation rate of gyrA gene was 16.7% (17/102), among the 17 strains, the mutation rate corresponding Asn87 and Asp91 was 9.8% (10/102) and 6.9% (7/102) respectively. We also found that 11 strains had both mutations of 23S rRNA and gyrA gene simultaneously. No significant difference of MIC values between Asn87 and Asp91 mutations was observed ($p > 0.05$).

Analysis of the genotype and phenotype of clarithromycin and levofloxacin resistance

Results of resistance to two antibiotics on genotype by detection the mutation and phenotype on MIC in 102 strains were compared. There were 19 strains showed inconsistencies in the phenotype and genotype resistance (17 of clarithromycin and 2 of flevofloxacin). Comparison with susceptibility test, the concordant rate of genotypes to clarithromycin and levofloxacin was 83.3% (85/102), 98.0% (100/102), respectively. Of 53 strains which were resistant to clarithromycin by phenotypes, 5 lack of genotype results and only 40 strains showed A2143G mutations, so the detection rate of A2143G in clarithromycin-resistant strains was 83.3% (40/48). The 21 strains which were resistant to levofloxacin by phenotypes, 2 without genotype results and 17 strains were found related gyrA gene mutations, so the detection rate of Asn87, Asp91 mutations were 52.6% (10/19) and 36.8% (7/19) in levofloxacin-resistant strains.

Influence of different factors on resistance

The prevalence of clarithromycin in different age groups was 56.5% (0–6-year-group), 49.3% (7–12-year-group), 27.8% (13–18-year-group), respectively, which showed an age-dependent decrease but without statistical difference ($p > 0.05$). The resistant rate to metronidazole in 13–18-year-group (100.0%) was higher than other two age groups (87.0% in 0–6-year-group, 85.9% in 7–12-year-group). No significant difference was found in resistance of the whole isolated *H. pylori* strains to clarithromycin, metronidazole, and levofloxacin among different genders, age groups, endoscopic findings, and histopathological appearance of antral mucosa based on severity of inflammation (Table 5).

Discussion

Clarithromycin resistance in children has been reported in many countries, with its prevalence ranging from 16.0 to 50.9% [14–19]. In our study, the resistance rate to clarithromycin was 47.3%, which was higher than those in previous reports in other parts of China (16.4–31.8%) [9, 10], but

Table 4 MIC and mutation rate of 23S rRNA and gyrA gene in 102 Strains

Mutation point	No.	Mutation rate (%)	Mean of MIC (μg/mL)	Range of MIC (μg/mL)
23S rRNA of clarithromycin				
A2143G	49	48.0	2.43	1~4
gyrA of levofloxacin				
Asn87	10	9.8	5.43	2~8
Asp91	7	6.9	4.20	2~8

Table 5 Alalysis of factors affecting antibiotic resistance rate

Characteristics	Resistance rate (%)		
	CLA	LEV	MTZ
Genda			
Boy	44.6	16.1	82.1
Girl	50.0	21.4	94.6
Age(y)			
0-6	56.5	13.0	87.0
7-12	49.3	22.5	85.9
13-18	27.8	11.1	100.0
Endoscopic findings			
PUD	50.5	20.6	87.6
NUD	26.7	6.7	93.3
Histopathological			
Mild	44.4	7.4	88.9
Medorate	48.0	22.7	86.7
Severe	50.0	20.0	100.0

CLA clarithromycin, MTZ metronidazole; LEV levofloxacin, PUD peptic ulcer disease, NUD non-ulcer disease

lower than those in Beijing (84.9–96.6%) [8, 11]. A multicenter study in Europe analyzed the association between antibiotic use and resistance to antibiotics for the treatment of *H. pylori* infection in outpatient communities, and showed that use of long-acting macrolides was correlated with the resistance of *H. pylori* to clarithromycin [20]. Strong cross-resistance was also observed among macrolides. Thus, the high resistance of *H. pylori* to clarithromycin in Chinese children may attribute to frequent use of macrolides in respiratory and other diseases. Regulating and restricting the administration of macrolides in clinical practice may be beneficial to reduce the high resistance.

In our study, metronidazole resistance rate was 88.4%, higher than that in other regions of China and other countries (7.4–75.2%) [8–11, 14–18], but lower than that in Iran (95.0%) [19]. A meta-analysis published in 2017 demonstrated that the resistance to metronidazole increased from 36% before 2000 to 45% in 2015 in the Asia–Pacific region [21]. Several studies from European countries also observed an increasing trend in resistance to metronidazole in recent years [17, 22]. This may likely attribute to the massive use of metronidazole in the treatment of gynecological, dental infections, and parasite-related diseases. However, metronidazole resistance has less impact on eradication rate of *H. pylori* compared to clarithromycin resistance, because the latter can be partially overcome by increasing the doses and prolonging the duration of *H. pylori* eradication treatment regimens [23, 24].

Fluoroquinolones were contraindicated in children for the suspected side effects of causing cartilage malformation. This could explain the lack of *H. pylori* resistance to fluoroquinolones in children. The resistance to levofloxacin was 18.8% in our study, higher than that in other areas, for instance, in Beijing (13.7%) [8] and Hangzhou (9.0%) [10] of China, in South Korea (15.2%) [14] and in Europe countries (2.5%) [20], but lower than that in Chongqing adults (34.9%) [25]. The resistance of *H. pylori* isolated from children to fluoroquinolones may be due to infections of fluoroquinolones-resistant strains, intake of foodstuffs from antibiotic-treated animals or intrafamilial spread of fluoroquinolones-resistant strains. A study in China indicated that the resistance rate of *H. pylori* to levofloxacin increased from 2.0 to 10.6% from 2009 to 2015 in pediatric population [9]. No resistance to tetracycline, amoxicillin, and furazolidone was observed in our study. Amoxicillin was widely used to treat respiratory tract, skin, and other infections, yet low and stable resistance rates (< 5%) were found in most regions of the world [4]. Tetracycline was contraindicated in children under 8 years of age because of the risk of enamel staining and dysplasia, so there was limited data about resistance to tetracycline in children. The resistance to furazolidone was rare because furazolidone was banned in many countries due to the uncertain genotoxicity and carcinogenic effects. Several published studies from areas where furazolidone was licensed for use demonstrated that furazolidone was effective in the treatment of *H. pylori* infection [26, 27]. Although previous studies based in Chinese population demonstrated that *H. pylori* strains had a quite low resistance to furazolidone (< 0.1%) [11, 28], the side effects limit its use in children.

High resistance rate of multiple antibiotics was observed in our study, especially the metronidazole and clarithromycin dual resistance (30.4%), which was higher than those reported in children from Vietnam (28.8%), France (7.9%), Germany (7.7%), Japan (6.6%), but lower than Beijing (52.3%) and Iran (42.0%) [11, 15–19]. According to published consensus, antimicrobial susceptibility test should be obtained before eradication therapy to guide treatment regimens in areas with high prevalence of *H. pylori* resistant strains [1]. A meta-analysis in 2015 included 12 randomized controlled clinical trials and compared cure rates of susceptibility-guided therapy versus empirical therapy. The results suggested that the susceptibility test–guided therapy was more efficacious than empiric therapy [29]. Cosme et al. performed a study to evaluate the efficacy of antimicrobial therapy guided by *E*-test in patients with dual antibiotic resistance (clarithromycin, metronidazole or levofloxacin) to *H. pylori* strains and found that antimicrobial susceptibility–guided triple regimens resulted in an eradication rate > 90% in patients with dual antibiotic resistance [30].

Molecular assays have been recommended for detection of clarithromycin and fluoroquinolones resistance based on the gastric biopsy samples, and seem to be more cost-effective

and rapid than antimicrobial test to predict the antibiotic resistance of *H. pylori* [4]. Few studies have revealed that tailored treatment based on molecular methods improved the eradication rate [31]. Papastergiou et al. showed that a 7-day triple therapy based on molecular assay to detect the resistance of *H. pylori* strains in Greek achieved an eradication rate of > 90% [32]. In this study, molecular methods to detect gene mutations showed good concordance (83.3% versus 98.0%) with susceptibility test results, similar to previous studies [8, 33]. However, there were some strains showed discordance in results of phenotypic and genotypic methods. The phenotypes by MIC of 8 isolated strains were resistant to clarithromycin but no mutation of A2143G or A2142G was detected. One possible explanation is that mutations in other genes such as *rpl22* and *infB* connected to clarithromycin resistance were not detected [7]. In the study, we only detected the most frequent mutations. Besides, outer membrane proteins, efflux pumps mechanism have also been shown to be related to clarithromycin resistance [7]. Nine strains were susceptible by phenotype to clarithromycin but A2143G mutation was detected. On the one hand, perhaps the infected strains were a mixture of sensitive and resistant strains [34]. On the other hand, *H. pylori* has two 23S rRNA operons, when mutations are only found in one copy of 23S rRNA maybe be not enough to confer resistance. Two strains were resistant by phenotype but susceptible by genotype to levofloxacin, this could be due to the existence of mutations in other genes connected to quinolone resistance, such as gyrB gene [7].

Several studies have found that A2142G point mutation was associated with higher clarithromycin MIC values compared with A2143G mutation [35, 36]. That could explain the low MIC values (1–4 μg/mL) in clarithromycin-resistant strains in our study. Human with 2143G mutation seemed to suffer higher eradication failure risk compared with other clarithromycin resistance mutations [37]. Detecting the A2143G mutation could be helpful to predict eradication results. However, we lack related data about A2142G mutation strains and eradication treatment outcomes to support the above hypotheses. Future studies are needed to explore the relationship of gene mutation and MIC, eradication results.

To improve the accuracy of molecular methods, different techniques have been designed, including hybridization, fluorescence in situ hybridization (FISH), and whole-genome sequencing [38]. Furthermore, a new molecular method based on stool samples without the invasive procedure of gastroscopy has been suggested, making it convenient to detect the resistance of *H. pylori* strains [39, 40]. More accurate and convenient methods for detecting resistance of *H. pylori* are needed to guide the therapy in the future.

Authors' contributions Tian Geng and Zhong-Su Yu conceived and designed the experiments, collected materials, analyzed and interpreted the data, drafted the manuscript. Xi-Xi Zhou, Hui-Hua Zhang, Bo Liu conceived and designed the experiments, collected materials. Zhong-Yue Li conceived and designed the experiments, analyzed and interpreted the data, drafted and revised the manuscript, and approved the final version.

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Data availability The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Declarations

Ethics approval This study was approved by the Ethics Committee of Children's Hospital of Chongqing Medical University (File No. 2017–36-1) and informed consent of the clinical trial was signed from legal guardians of the whole subjects.

Consent to participate All participants gave their written consent to participate.

Conflict of interest The authors declare no competing interests.

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