

Primary Antibiotic Resistance of *Helicobacter pylori* Isolated from Beijing Children

Guodong Liu,^{*,†} Xiwei Xu,[‡] Lihua He,^{*,†} Zhaolu Ding,[‡] Yixin Gu,^{*,†} Jianzhong Zhang^{*,†} and Liya Zhou[§]

^{*}National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, [†]State Key Laboratory for Infectious Disease Prevention and Control, Beijing, [‡]Department of Internal Medicine, Beijing Children's Hospital Affiliated to Capital Medical University, Beijing, [§]Department of Gastroenterology, Peking University Third Hospital, Beijing, China

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Reprint requests to: Jianzhong Zhang, State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 155 Changbai Road, Changping, Beijing 102206, China. E-mail: zhangjianzhong@icdc.cn and Liya Zhou, Department of Gastroenterology, Peking University Third Hospital, 49 North Garden Rd., Haidian District, Beijing 100191, China. E-mail: liyazhou@medmail.com.cn

The authors, Guodong Liu, Xiwei Xu and Lihua He, contributed equally to this work as the first authors.

Abstract

Background: The antimicrobials resistance of *Helicobacter pylori* (*H. pylori*) was able to sharply decline the eradication rate of *H. pylori* both in adults and children, but there are limited studies about the primary antibiotic resistance and the related gene mutations, specifically in China.

Materials and Methods: The primary resistance to 9 antibiotics of 73 *H. pylori* strains isolated from gastric biopsies of children recruited at Beijing Children's Hospital was assessed, and the mutations in 23S rRNA gene of 65 macrolide-resistant strains and in *gyrA* and *gyrB* of 12 quinolone-resistant strains were investigated.

Results: The resistance rate to clarithromycin, azithromycin, metronidazole, levofloxacin, moxifloxacin, and rifampicin was 84.9%, 87.7%, 61.6%, 13.7%, 15.1%, and 6.8%, respectively. No resistance to amoxicillin, gentamicin, and tetracycline was observed. Dual, triple, and quadruple antibacterial resistant percentage was 46.6% (34/73), 15.1% (11/73), and 2.7% (2/73), respectively. The gene mutation rate of A2142C, A2142G, and A2143G in 23S rRNA gene was 1.5% (1/65), 6.2% (4/65), and 84.6% (55/65), respectively. The detection rate of mutations of Asn87, Asp91, and Met191 in *GyrA* was 41.7% (5/12), 25% (3/12), and 25% (3/12), respectively.

Conclusion: The high prevalence of primary antibiotic resistance was out of expectation in *H. pylori* strains isolated from the children in Beijing. Antibiotic susceptibility should be made clear before the antibiotic was used in the anti-*H. pylori* therapy in this population. The A2143G was the most populated mutation in macrolide-resistant strains, and Asn87 and Asp91 of *GyrA* were the most common mutation points in quinolone resistance strains.

Helicobacter pylori (*H. pylori*) infection can lead to gastric ulcer, gastritis, gastric cancer, and mucosa-associated lymphoid tumors (MALT) [1]. The patients were usually infected with *H. pylori* during their childhood [2], and the *H. pylori* infectious prevalence in children is 7.1–73.3% worldwide [3,4] and 25.6% in Beijing, China [5].

It is believed that eradication therapy is the powerful way to cure the *H. pylori*-related diseases. The triple therapy with two antibiotics combine with one proton-pump inhibitor (PPI) was commended in the Maastricht III and the Chinese Consensus Report [6,7]. Whereas, the *H. pylori* eradication rate is declining because of the increasing antimicrobial resistance [8]. According to the recommendation,

if the clarithromycin resistance rate is up to 15–20% or the metronidazoleand resistance rate reaches 40% in a population, these antibiotics should not be used in anti-*H. pylori* therapy without susceptibility test.

Clarithromycin is a key antibiotic in triple therapy of *H. pylori* eradication; the clarithromycin resistance has been considered as the main reason of eradication failure [9]. Resistance to clarithromycin in *H. pylori* is mediated by the mutations of domain V in the 23S ribosomal RNA (rRNA) gene [10]. A2143G and A2142G are the common prevalent mutations [11].

High resistance (26.6%) to quinolones in *H. pylori* isolated from the adults in Beijing [12] has been reported. The reasons of quinolone resistance in

H. pylori have been reported because of mutations, most of the Asn87 and Asp91, in the quinolone resistance-determining regions (QRDRs) of *gyrA* [12,13].

Antimicrobial resistance levels are different in various countries and regions. Triple therapy was widely used in the children in Beijing, but there were limited studies about the primary antibiotic resistance and the associated mutations in 23S rRNA gene and *gyrA*. Herein, the aim of this study is to assess the primary antibiotic resistance to nine antibiotics of *H. pylori* isolated from Beijing children.

Materials and Methods

Strains Collection

Seventy-three *H. pylori* strains were isolated from consecutive gastric biopsies of 120 children (77 boys and 43 girls) recruited at Beijing Children's Hospital from Jan 1, 2009 to Jun 30, 2010. The age range of these children is from 3 to 16 years old, and the average age is 10.0 ± 5.8 . Children were divided into three groups by age (0–6, 7–12, and 13–16-year-old group). All children underwent gastroscopy examination and did not receive any anti-*H. pylori* therapy before the investigation. This study was permitted by the Ethics Committee of China CDC, and a written consent for the endoscopy was obtained from their legal guardians.

Gastric mucosa biopsy samples were collected and stored in the brain–heart infusion broth (Oxoid, Dardilly, France) with 5% glycerin at $-80\text{ }^{\circ}\text{C}$ and then transported to the laboratory in the National Institute for Communicable Disease Control and Prevention, China CDC with dry ice. The frozen samples thawed at room temperature for 30 minutes then were grinded and cultivated on a Columbia Agar (Oxoid) plate supplemented with 5% defibrinated sheep blood, 3 $\mu\text{g}/\text{mL}$ synergist, 2.5 $\mu\text{g}/\text{mL}$ vancomycin, 2 $\mu\text{g}/\text{mL}$ amphotericin B, and 2 $\mu\text{g}/\text{mL}$ bacillosporin B under microaerophilic conditions (5% O_2 , 10% CO_2 , 85% N_2). Translucent colonies about 0.5–2 mm from selective agar plates were selected to gram stain and tested for urease, catalase, and oxidase activity test. Isolates with curved gram-negative rods resembling *Helicobacter* together with positive in all the three enzyme activity tests mentioned earlier were identified as *H. pylori*.

Antibiotic Susceptibility Testing

The antibiotic resistance of *H. pylori* to nine kinds of antibiotics was tested by E-test method using Mueller–Hinton agar (Oxoid) supplemented with 5% sheep blood and incubated in microaerophilic conditions for 2 days.

Experiments were performed according to the protocols of the Clinical and Laboratory Standards Institute (Wayne, PA, USA) [14]. The resistance breakpoints of antibiotics were defined for amoxicillin $\geq 0.5\text{ }\mu\text{g}/\text{mL}$, clarithromycin, azithromycin, levofloxacin and moxifloxacin $\geq 1.0\text{ }\mu\text{g}/\text{mL}$, tetracycline and rifampicin $\geq 4\text{ }\mu\text{g}/\text{mL}$, metronidazole and gentamicin $\geq 8\text{ }\mu\text{g}/\text{mL}$ [14,15]. Susceptibility test of amoxicillin was tested again after the resistance strains have been stored for 2 weeks at $-80\text{ }^{\circ}\text{C}$.

Mutations Analysis of 23S rRNA gene, GyrA, and GyrB

The 23S rRNA gene of all the 65 macrolide-strains in our study was sequenced and compared with that of *H. pylori* 26695 strain. The protein sequences of GyrA and GyrB of 12 quinolone-resistant strains were also analyzed.

Total DNA of *H. pylori* was extracted by using QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. PCRs were carried out to amplify 23S rRNA, *gyrA*, and *gyrB* genes using primers: Hp23S PF1820–1839 (5'-CCA CAG CGA TGT GGT CTC AG-3') and Hp23S PR 2244–2225 (5'-CTC CAT AAG AGC CAA AGC CC-3') [16], *gyrA* PF (5'-AGC TTA TTC CAT GAG CGT GA-3') and *gyrA* PR (5'-TCA GGC CCT TTG ACA AAT TC-3'), *gyrB* PF (5'-CCC TAA CGA AGC CAA AAT CA-3') and *gyrB* PR (5'-GGG CGC AAA TAA CGA TAG AA-3') [12]. The products were 425, 582, and 465 bp, respectively. The PCR products were purified using gel extraction kits (QIAGEN) according to the manufacturer's protocol and then sequenced using ABI 3730xl sequencer (Applied Biosystems, Foster City, USA). The sequences were analyzed using Vector NTI suite 7.1 software (Invitrogen, CA, USA). The mutations in domain V of the 23S rDNA, GyrA, and GyrB were compared with the related gene of *H. pylori* 26695.

Statistical Analysis

The prevalence of resistance in different age groups and gender was accessed with chi-square test (χ^2 test); difference of MICs between single and multi-resistance strains was accessed with paired/matched *t*-test; the association between ages and multi-resistance was accessed with linear regression test. All tests performed with $p < .05$ considered significant.

Results

Antibiotic Susceptibility Test Results

Susceptibility tests were investigated in 73 strains and listed in Table 1. Two resistant strains of amoxicillin

Table 1 Prevalence of resistance to clarithromycin, azithromycin, metronidazole, levofloxacin, moxifloxacin, rifampicin, gentamicin, tetracycline, amoxicillin of 73 *Helicobacter pylori* isolated from children in Beijing

Antimicrobial	Number	Resistance %	Mean of MICs (µg/mL)	Range of MICs (µg/mL)
Clarithromycin	62	84.9	24.4	0.016–512
Azithromycin	64	87.7	111.3	0.032–512
Metronidazole	45	61.6	14.3	0.125–512
Levofloxacin	10	13.7	0.3	0.064–64
Moxifloxacin	11	15.1	0.4	0.032–64
Rifampicin	50	6.8	0.3	0.008–512
Amoxicillin	0	0	0.1	0.008–1.5
Gentamicin	0	0	0.5	0.125–2
Tetracycline	0	0	0.1	0.023–512

with minimal inhibitory concentration (MIC) at 0.5 and 1.5 µg/mL restored the sensitivity to amoxicillin after they were kept for 2 weeks at –80 °C, indicating they were tolerant to amoxicillin. Cross-resistance was observed between clarithromycin and azithromycin in 60 strains, as well as between levofloxacin and moxifloxacin in ten strains. The percentage of dual, triple, and quadruple antibacterial resistance was 46.6% (34/73), 15.1% (11/73), and 2.7% (2/73), respectively (Table 2). Multiple drug-resistant strains had higher MIC than single drug-resistant strains (Table 3). The average age of single, dual, triple, and quadruple

Table 3 Average level of MIC for single and multiple resistant strains (µg/mL)

Antibiotics	Single	Dual	Triple	Quadruple
Amoxicillin	0.057	0.047	0.092	0.077
Gentamicin	0.393	0.506	0.475	1.225
Rifampicin	0.247	0.231	0.685	16
Levofloxacin	0.174	0.288	2.239	4.899
Moxifloxacin	0.169	0.301	2.881	4.899
Azithromycin	163.066	196.537	68.31	16
Clarithromycin	30.931	37.066	44.334	1.732
Metronidazole	0.98	56.458	84.129	362.039
Tetracycline	0.07	0.097	0.14	0.218

Multi-resistance strains have higher MICs.

antibacterial resistant strains was 10, 10, 8, and 12, respectively, and no significant difference in age was observed between single and multiple resistant strains ($p > .05$).

Resistance in Different Gender and Ages

The resistance rates of metronidazole and rifampin in girls were higher than in boys (70.4% vs 56.5% and 14.8% vs 2.2%, $p < .05$). No significant difference of other antibiotics was found in different genders.

The quinolones-resistant incidence of *H. pylori* isolated from gastric mucosa biopsies of 0–6-year-old group (30.8%) was higher than that of the other two

Table 2 Multi-resistance of 73 *Helicobacter pylori* strains isolated from children in Beijing

Multi-resistance pattern ^a	Number of multiresistant strains	Percentage	Total percentage
AZM + MTR	2	2.7	Dual resistance
CLR + MTR	1	1.4	
(CLR AZM) ^b + MFX	1	1.4	46.6 (34/73)
(CLR AZM) ^b + MTR	27	37.0	
(CLR AZM) ^b + (LVX MFX) ^c	3	4.1	
(CLR AZM) ^b + MFX + RIF	1	1.4	
(CLR AZM) ^b + MFX + MTR	1	1.4	
(LVX MFX) ^c + MTR + AZM	2	2.7	
CLR + MTR + RIF	1	1.4	
(CLR AZM) ^b + MTR + RIF	1	1.4	
(CLR AZM) ^b + MTR + LVX	1	1.4	
(CLR AZM) ^b + (LVX MFX) ^c + MTR	4	5.8	
(LVX MFX) ^c + AZM + MTR + RIF	1	1.4	Triple resistance
(CLR AZM) ^b + (LVX MFX) ^c + MTR + RIF	1	1.4	
Total		64.4% (47/73)	15.1 (11/73)
			Quadruple resistance
			2.7 (2/73)

^aCLR, clarithromycin; AZM, azithromycin; MTR, metronidazole; LVX, levofloxacin; MFX, moxifloxacin; AMX, amoxicillin; RIF, rifampicin; TCY, tetracycline.

^bBoth CLA and AMZ resistant.

^cBoth LVX and MFX resistant.

groups (12.2% in 7–12 and 15.8% in 13–16-year-old group, respectively). The incidence of resistance to macrolide is 84.6% in 0–6-year-old group, 92.7% in 7–12-year-old group, and 68.4% in 13–16-year-old group. No difference of the resistances to other antibiotics was observed among all three groups.

Mutations of 23S rRNA Gene in Macrolide-resistant Strains

Mutations of 23S rRNA gene were shown in Table 4. The mutation rate of A2142C, A2142G, and A2143G was 1.5% (1/65), 6.2% (4/65), and 84.6% (55/65), respectively. The mutation of T2182C was detected with the rate of 72.3% (47/65) and mostly accompanied the mutation of A2143G and A2142G. No mutation was

Table 4 Mutations of 23S rDNA in 65 macrolide-resistant *Helicobacter pylori* strains

Mutations in 23S rRNA	No.	Percentage in resistant strains	AZM	CLA
			MIC µg/mL	MIC µg/mL
A2142C	1	1.5	0.06	>256
A2142G	1	1.5	>256	>256
A2142G + T2182C	2	3.1	>256	>256
A2142G + G2196A	1	1.5	256	256
A2143G	14 ^a	21.5	>256	108.5
A2143G + T2182C	41 ^b	63.1	>256	61.6
T2182C	4 ^c	6.2	2.9	0.2

CLR, clarithromycin; AZM, azithromycin.

^aOne with G1940A and one with C2127T.

^bOne with A1948G, One with G1952A, One with C2129T and One with C2195T.

^cCompanied by A1948G, G1952A, C2129T, C2195T respectively.

detected in a low-resistant strain (MIC of clarithromycin at 1.5 µg/mL). The average level of MIC of clarithromycin and azithromycin resistant strains with A2143G mutation was 70.5 and 256 µg/mL, respectively, and both were 256 µg/mL to the strains with A2142G mutation.

Mutations of GyrA and GyrB in Quinolone-resistant Strains

Mutations in GyrA and GyrB of the 12 quinolone-resistant strains were showed in Table 5. The mutation rate of Asn87 and Asp91 in GyrA was 41.7% (5/12) and 25% (3/12). Eleven of the 12 strains had Met191Ile mutation.

Discussion

Antibiotic resistance in *H. pylori* is a global problem, which is the hinder to *H. pylori* eradication therapy. For pediatrics, the situation may be more serious than in adults [17]. High percentages of antibiotic resistance were observed in this study.

For macrolides, resistance rates of azithromycin and clarithromycin were 87.7% and 84.9%. The resistance rate of clarithromycin in our study was much higher than those reported in previous publications, a percentage of 18.2% in children in Zhejiang province, and 37.2% in adults in Beijing, China [18,19], and in other areas or countries (from 4% to 49.2%) [20–27]. Clarithromycin was recommended as a first-line antibiotic, and the resistance to it was considered as the main reason of triple treatment failure [28]. According to the recommendation in Maastricht III, the clarithromycin should be used when the resistance rate is below

Table 5 Mutations of GyrA and GyrB in 12 *Helicobacter pylori* strains isolated from children in Beijing

Mutations in GyrA	Mutations in GyrB	No.	LEV	MFX
			MIC µg/mL	MIC µg/mL
Asn87Lys + Met191Ile	–	1	1	1
Asn87Lys + Met191Ile	–	2	32	32
Asn87Lys + Met191Ile + Asp145Gly	–	1	8	>32
Asn87Lys + Met191Ile	Ser397Gly	1	32	32
Asp91Gly + Met191Ile	–	1	8	32
Asp91Tyr + Met191Ile	–	1	>32	>32
Asp91Asn + Met191Ile + Ile194Thr	–	1	>32	>32
Arg140Lys	–	1	>32	0.75
Met191Ile	–	1	32	32
Met191Ile	–	2	0.25	32

LVX, levofloxacin; MFX, moxifloxacin.

15–20%, otherwise, it should not be used or the drug susceptibility test should be performed. In our study, the resistance of clarithromycin was much higher than the threshold. The results of this study indicated that macrolide should not be used as a first-line antibiotic in anti-*H. pylori* therapy for Beijing children without a susceptibility test.

Strong cross-resistance of azithromycin and clarithromycin was also observed. There were 60 strains resistant to the two macrolide at the same time. Our result corresponds to the previous reports that strains resistant to one macrolide become resistant to all others [29,30]. In China, macrolides were widely used for respiratory diseases, especially in children. Resistance of macrolides in pneumoniae-associated mycoplasma was also high in Beijing [31]. The consumption of macrolide was much more than other antibiotics included in our study in the last 2 years in Beijing Children's Hospital, and the consumption of azithromycin in the year 2009 and 2010 in this hospital was 14 times more than clarithromycin. The high consumption may be the important reason of high resistance level of *H. pylori* to macrolides although we had not got the defined daily doses per 1000 inhabitants per day (DIDs) of macrolide in children in Beijing. A study in Bulgaria revealed that resistance in clarithromycin increased with the DIDs [21]. Another reported from France reported that resistance in clarithromycin had maintained stability since a policy of restriction of antimicrobial, mainly for upper respiratory tract infections, was performed in 2002 [15].

Metronidazole resistance rates of *H. pylori* are from 50% to 100% in developing countries. The rate from our study was 61.6% (45/73), which was higher than that in Zhejiang province, China [18] and some certain areas and countries [20,21,25], but lower than that in Iran (95%) [22] and Egypt (100%) [27]. And it was approximate to the result in Beijing adults (63.9%) [19]. Recently, another study in adults in Shanghai of China revealed that the resistant rate of *H. pylori* to metronidazole had been remained stable at 40–50% from year 2000 to 2009 [32]. A study in Europe found the similar rate of the metronidazole resistance both in children and adults [26]. In China, as in other countries, metronidazole was widely used. In Maastricht III, the threshold for metronidazole resistance rate is 40%. Consider the high resistance in our study, metronidazole should not be used as a first-line antibiotic in *H. pylori* therapy in Beijing children.

The mechanism of amoxicillin resistance is complex and multiplex. The "amoxicillin-resistant" strain often changed to sensitive by freezing at -80°C [33–35]. Considering the reason mentioned above, we repeated the susceptibility test on the two strains that appeared

"amoxicillin resistant" after having been frozen at -80°C for 2 weeks. Both of the strains became sensitive, which indicated that they were tolerant to amoxicillin. This indicated that the amoxicillin susceptibility test should be performed as soon as possible before the strain having been frozen. Amoxicillin is a key antimicrobial in the triple anti-*H. pylori* regime for children, it becomes more important because of the high resistance of other antibiotics.

For fluoroquinolones, the primary resistance rate (levofloxacin, 13.7%; moxifloxacin, 15.1%,) was lower than that in Beijing adults (50.3–61.9%) and Iran (35%, ciprofloxacin) [19,29], but higher than that in Bulgarian (2.7% ciprofloxacin) and other European countries (3.0–5.8%) [21,36]. There were few reports about the resistance of *H. pylori* to quinolones in children, because this antimicrobial was forbidden to be used in children. The reason of high resistance to this antibiotic may because of the infection by quinolones-resistant strains and might be an indication of adults-children transmission of drug-resistant strains.

For rifampicin, the resistance rate was 6.8%. There were few reports about resistance of *H. pylori* to rifampicin, especially in children in China. The percentage in our result is higher than that in adults of Germany (1.4%) in 2007 [37]. Rifampicin is a common anti-*Mycobacterium tuberculosis* drug. In the past years, tuberculosis has been become a sticky issue around the world because of the multi-drug-resistant *M. tuberculosis* infections. According to the recently report, the primary and secondary resistance of rifampicin in *M. tuberculosis* in China was 2.7% and 10.4% [38]. In our study, *H. pylori* strains were isolated from girls with higher resistance rate than those from boys, this confirms to the recently report that the resistance of *M. tuberculosis* to rifampicin in women was higher than in men [38].

There was no gentamicin resistant strain in our study. While, the using of this antibiotic to eradicate *H. pylori* has been reported in the past two decades in adults [39,40].

By the conjoint data analysis of the six antibiotics-related *H. pylori* drug resistance in our study, high rate of multi-drug-resistance strains was observed. Dual, triple, and quadruple antibiotics resistant rate was 46.6% (34/73), 15.1% (11/73), and 2.7% (2/73), respectively. Multi-drug resistances in *H. pylori* strains isolated from adults have been reported in many areas or countries [41–44]. There were a few reports in untreated children, such as China (6.8%) [18], Mexico (4%) [44], and Bulgarian (1%) [41]. Quadruple resistance in children, however, has not been reported before. It is known that multi-drug resistance can markedly reduce the *H. pylori* eradication rate [45]. In

this study, we confirmed that strains resistant to multi-drug usually have higher MIC than single resistant strains, which was consistent with previous report [41]. There was no statistics significance between multi-resistance and ages. Although the reasons of resistance to different antibiotic are various, there seems as exist synergistic actions among these antimicrobials [46]. High rate of multi-resistance (triple and quadruple) *H. pylori* strains isolated from untreated children highlights the severe circumstance of *H. pylori* eradication in pediatric in Beijing.

The circumstance of mutations in 23S rDNA and GyrA in *H. pylori* isolated from Beijing children was not reported before. The detection rate of A2142C, A2142G, and A2143G was 1.5% (1/65), 6.2% (4/65), and 84.6% (55/65), respectively, in macrolide-resistant strains. It indicated that A2143G is the most popular mutation in 23S RNA gene of *H. pylori* in Beijing. This conformed to the most of previous reports in other countries [15,20,24,33,47]. High rate of T2182C coexisting with the main mutation, such as A2143G, corresponded to the report in Korea in secondary resistance in adults [47]. Among quinolone-resistant strains, mutations of Asn87 and Asp91 were the most popular. This corresponded to the previous report in adults in Beijing area [12].

In summary, primary antimicrobial resistances of *H. pylori* strains isolated from children in Beijing were observed. High resistance rates to macrolide, metronidazole, quinolone, and rifampicin were detected. High multi-resistances levels were found. A2143G was the most common mutation in 23S rDNA in macrolide-resistant *H. pylori*. Changes of Asn87 and Asp91 in GyrA related to the resistance of quinolone. According to the threshold of Maastricht III and Chinese Consensus Report, macrolide and meronidazole should be used according to the susceptibility test of antimicrobial resistance in children in Beijing area.

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Conflict of interests

There is no conflict of interests.

Author contributions

Guodong Liu sequenced DNA, analyzed data, and drafted manuscript; Xiwei Xu collected samples and analyzed data; Lihua He and Yixin Gu performed antimicrobials susceptibility

tests; Zhaolu Ding collected samples; Jianzhong Zhang and Liya Zhou designed the experiments, analyzed data, and revised manuscript.

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