Distribution of gyrA mutations in fluoroquinolone-resistant Helicobacter pylori strains

Li-Hui Wang, Hong Cheng, Fu-Lian Hu, Jiang Li

Abstract

AIM: To investigate the resistance of Helicobacter pylori (H. pylori) to ciprofloxacin (CIP), levofloxacin (LVX) and moxifloxacin (MOX) in the Beijing area and to elucidate the resistance mechanisms.

METHODS: Seventy-nine H. pylori clinical strains, isolated from patients who had undergone upper gastrointestinal endoscopy in Peking University First Hospital from 2007 to 2009, were tested for their susceptibility to CIP, LVX and MOX using the E-test method. H. pylori strain 26695 was included in the susceptibility testing as a control strain. According to the minimal inhibitory concentration (MIC) values, a strain was classified as resistant to CIP, LVX or MOX when the MIC was > 1 µg/mL. We amplified by polymerase chain reaction (PCR) and sequenced the quinolone resistance-determining regions of the gyrA and gyrB genes from 29 quinolone-resistant and 16 quinolone-susceptible H. pylori strains selected at random.

RESULTS: In this study, the resistance rates of H. pylori to CIP, LVX or MOX were 55.7% (44/79), and the primary resistance rates were 26.6% (21/79). Patients with secondary resistance had received LVX in previous eradication treatments, but not MOX or CIP. Forty-five strains, including 29 CIP, LVX or MOX-resistant strains (MIC: 1.5-32 µg/mL) and 16 susceptible strains, were selected randomly from the 79 strains and used in PCR analysis. Among these 45 strains, 27 resistant strains had mutations in the gyrA gene, including 11 strains with mutations corresponding to Asp-91 (MIC: 2-32 µg/mL), one of which also had a mutation corresponding to Val-150, and 16 strains had mutations at Asn-87 (MIC: 4-32 µg/mL), three of which also had mutations corresponding to Arg-140 or Val-150. In addition, Arg-140, Val-150 or Ala-97 mutations were separately detected in three susceptible strains. Analysis of the gyrB gene showed that one strain of low resistance had a mutation corresponding to Ser-457 that coexisted with an Asp-91 mutation. There was a significant difference in the occurrence of mutations in the gyrA gene between CIP, LVX and MOX-resistant and -susceptible strains (P < 0.05), but 2 resistant strains were found to possess no quinolone resistance-determining region mutations.

CONCLUSION: Resistance is primarily mediated through point mutations in gyrA. Whether other mechanisms are responsible for resistance in strains without mutations in the QRDR should be detected.

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Key words: Helicobacter pylori; Antibiotic resistance; Quinolones; Mutation; gyrA

Peer reviewers: Dr. György M Buzás, Department of Gastroenterology, Ferencváros Health Center, IX. District Policlinic, Mester u 45, 1095 Budapest, Hungary; Can Gonen, MD, Department of Gastroenterology, Kutahya State Hospital, 43100 Kutahya, Turkey; Phil Sutton, Associate Professor, Centre for Animal Biotechnology, School of Veterinary Science, University of Melbourne, Melbourne, VIC 3010, Australia
INTRODUCTION

*Helicobacter pylori* (H. pylori) infection, which affects half of the world’s population, is responsible for gastritis [1], peptic ulcers [2,3] and gastric mucosa-associated lymphoid tissue lymphoma [4], and is a major risk factor for the development of gastric adenocarcinoma [5]. Eradication therapy plays an important role in the treatment of *H. pylori* infection. According to the Maastricht III and Chinese Consensus Report, triple therapy using a proton-pump inhibitor (PPI) combined with clarithromycin and amoxicillin or metronidazole, is recommended as the first choice treatment [6,7]. With the increasing frequency of clarithromycin-resistance among *H. pylori* strains, there is rising concern about the potential decline in the eradication rate of this infection [8-10]. There is therefore an urgent need to introduce other treatment options. Fluoroquinolones, such as levofloxacin (LVX) and moxifloxacin (MOX), have been evaluated as an alternative to standard antibiotics against *H. pylori* [6,7].

Some studies have shown good results when using fluoroquinolone-based triple therapies for *H. pylori* eradication. In a German study, a 7-d course including LVX in patients with persistent *H. pylori* infection, resulted in eradication rates of greater than 85% [10]. In an Italian study, *H. pylori* eradication was achieved in 90% of patients treated with MOX, clarithromycin and lansoprazole [11]. However, the widespread use of fluoroquinolones for the treatment of *H. pylori* infection has led to an increase in its resistance rate in some areas, leading to unacceptably low eradication rates [12]. Several studies have shown that LVX-based therapies are not superior to traditional quadruple therapy or Maastricht triple therapy in the treatment of *H. pylori* infection, especially in the case of resistant *H. pylori* strains [13,14]. A Turkish study speculated that the low eradication rate with MOX-containing treatment regimens may be due to the development of resistance to this quinolone [15]. The findings from all of these studies indicate that a regimen that is effective in one area may not be effective in another area, as antibiotic-resistant rates for *H. pylori* may be different in different areas.

The fluoroquinolone resistance rates of *H. pylori* have been reported for several regions, including China (Mainland), Hong Kong, Taiwan, France, Japan and Korea [12,13,26-28], and range from 3% to about 20%. It was reported that LVX resistance was associated with prior fluoroquinolone use over the previous 10 years, and with the total number of fluoroquinolone courses prescribed [21]. A study from Korea speculated that resistance to MOX might be acquired through prior use of fluoroquinolones due to the development of cross-resistance to other fluoroquinolones like ciprofloxacin (CIP) and LVX [12]. Bogaerts et al [22] reported that mutations in the *gyrA* gene elevated the minimal inhibitory concentrations (MICs) to LVX, CIP and MOX.

The mechanism of action of fluoroquinolones is via inhibition of DNA gyrase and topoisomerase, which control and modify the topological state of DNA in cells. Fluoroquinolone then interferes with bacterial DNA replication. Both DNA gyrase and topoisomerase are composed of two A and two B subunits, encoded by the *gyrA* and *gyrB* genes for DNA gyrase and the *parC* and *parE* genes for topoisomerase IV. The mechanism of fluoroquinolone resistance in *H. pylori* has been found to be linked to mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene. Mutations in the *gyrB* gene have also been reported in LVX-resistant strains isolated in Japan, but these often occurred along with *gyrA* mutations [23]. The resistance of *H. pylori* strains to fluoroquinolones in the Beijing area has not yet been reported. The aim of this study was to assess the prevalence of fluoroquinolone resistance against CIP, LVX and MOX in *H. pylori* strains isolated from patients over the past 3 years in Beijing and to compare the susceptibility of these strains with CIP and two newer fluoroquinolones in vitro. Mutations in the QRDRs of *gyrA* and *gyrB* genes were also evaluated in these strains.

MATERIALS AND METHODS

Patients and bacterial strains

Seventy-nine clinical *H. pylori* strains were collected from adult patients, who were randomly selected and had undergone a gastroendoscopy at Peking University First Hospital between January 2007 and July 2009. Some patients had received *H. pylori* eradication therapy before, but none of the patients had received MOX or CIP-based therapy. The biopsy specimens were cultured on Columbia blood agar (BBL Microbiology Systems, Cockeysville, MD, USA), supplemented with 30% glycerol. Brain-heart infusion broth (BHI, Difco Laboratory, Detroit, MI, USA) supplemented with 8% defibrinated sheep blood, and incubated for 5-7 d under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂). Clinical isolates were identified as *H. pylori* based on positive tests for urease, oxidase and catalase. All cultures were stored at -80°C in brain-heart infusion broth (BHI, Difco Laboratory, Detroit, MI, USA) supplemented with 30% glycerol.

MIC determination

The MICs of LVX, CIP and MOX were determined by E-test strips (AB Biodisk, Solna, Sweden) according to the recommendations of the Clinical and Laboratory Standards Institute (Pennsylvania, USA) and the manufacturer’s instructions. *H. pylori* 26695 was used as the quality control strain. The resistance breakpoints of fluoroquinolones were defined as > 1 µg/mL, as previously suggested [19,20].

Polymerase chain reaction amplification and nucleotide sequence analysis

*H. pylori* genomic DNA was extracted by the High Pure
polymerase chain reaction (PCR) Template Preparation kit (Tiangen, Beijing China). Oligonucleotide primers gyr APF (5′-AGCTTATTTCCATGACCGTGAG-3′) and gyr APR (5′-TCAGGGCTTTTGAACAAAATT-3′), gyrBPF (5′-CCCTAACGAGGCACCAATTCA-3′) and gyr BPR (5′-GGGGCGAAATACGATGAAA-3′) were designed to amplify a 582-bp and 465-bp region of the \( H. \) pylori gyr-A and gyr-B genes respectively. Primers were synthesized by Shenggong (Shanghai, China). PCR was performed in a 25 μL reaction volume containing 2 pmol of the oligonucleotide primer, 200 μmol/L (each) of dATP, dCTP, dGTP and dTTP (GE Healthcare, Little Chalfont, UK), 1.5 mmol/L of reaction buffer (GE Healthcare), 1 μL of template DNA, and 2.5 U of Taq polymerase (GE Healthcare). Thermocycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 30 s, with a final extension step of 72°C for 10 min. The reaction products were visualized by running 5 μL of the reaction mixture on 1.5% agarose gels. Sequencing of the amplified DNA was performed on an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were then compared with the published sequence of the \( H. \) pylori gyr-A and gyr-B gene (GenBank accession number NC_000915.1).

Statistical analysis
The association between MIC levels and the occurrence of \( \text{gyrA/B} \) mutations relating to quinolone susceptibility was determined using Fisher’s exact probability test, a \( P \)-value < 0.05 was considered significant.

RESULTS

Antimicrobial susceptibility
The MICs of CIP, LVX and MOX were determined for the 79 \( H. \) pylori strains isolated between 2007 and 2009 from gastric biopsies. The resistance rate to either CIP, LVX or MOX was 55.7% (44/79). Primary CIP, LVX and MOX resistance was detected in 21 (26.6%) isolates. Based on the breakpoints, we found that the strains susceptible to CIP (MIC ≤ 1.0 μg/mL) were also susceptible to LVX and MOX, whereas the isolates resistant to CIP (MIC > 1.0 μg/mL) were also resistant to LVX and MOX (Tables 1 and 2).

| Strains | MIC (μg/mL) | Mutations of GyrA | Mutations of GyrB |
|---------|-------------|-------------------|-------------------|
| LVX | MOX | CIP |
| 1R | 1.5 | 2 | 32 | Asp91Gly and Val150Ala | 0 |
| 2R | 2 | 2 | 32 | Asp91Asn | 0 |
| 3R | 2 | 2 | 32 | Asp91Gly | 0 |
| 4R | 3 | 1.5 | 1.5 | Asp91Asn | Ser457Ala |
| 5R | 4 | 3 | 4 | Asp91Tyr | 0 |
| 6R | 4 | 3 | 32 | Asp91Asn | 0 |
| 7R | 6 | 4 | 32 | Asp91Asn | 0 |
| 8R | 6 | 6 | 12 | Asn87Lys and Arg140Lys | 0 |
| 9R | 8 | 4 | 12 | Asn87Tyr | 0 |
| 10R | 12 | 8 | 32 | Asp91Gly | 0 |
| 11R | 12 | 12 | 12 | Asn87Lys and Arg140Lys | 0 |
| 12R | 12 | 12 | 32 | Asp91Asn | 0 |
| 13R | 32 | 12 | 32 | Asp91Tyr | 0 |
| 14R | 32 | 16 | 32 | Asp91Asn | 0 |
| 15R | 32 | 32 | 32 | Asp91Asn | 0 |
| 16R | 32 | 32 | 32 | Asp87Lys | 0 |
| 17R | 32 | 32 | 32 | Asn87Lys and Val150Ala | 0 |
| 18R | 32 | 32 | 32 | Asn87Lys | 0 |
| 19R | 32 | 32 | 32 | Asn87Lys | 0 |
| 20R | 32 | 32 | 32 | Asn87Lys | 0 |
| 21R | 32 | 32 | 32 | Asn87Lys | 0 |
| 22R | 32 | 32 | 32 | Asn87Lys | 0 |
| 23R | 32 | 16 | 8 | Asn87Lys | 0 |
| 24R | 24 | 32 | 32 | Asn87Lys | 0 |
| 25R | 12 | 12 | 32 | Asn87Lys | 0 |
| 26R | 6 | 6 | 32 | Asn87Lys | 0 |
| 27R | 3 | 6 | 12 | Asn87Lys | 0 |
| 15 | 0.02 | 0.02 | 0.016 | Val150Ala | 0 |
| 26 | 0.016 | 0.016 | 0.032 | Ala97Tyr | 0 |
| 35 | 0.064 | 0.094 | 0.064 | Arg140Lys | 0 |
| 45 | 0.064 | 0.02 | 0.023 | 0 | Val451Gly |

1Primary resistance. R: Resistant strains; S: Susceptible strains; 0: No mutation; MIC: Minimal inhibitory concentration; LVX: Levofloxacin; MOX: Moxifloxacin; CIP: Ciprofloxacin; \( H. \) pylori: Helicobacter pylori.

Table 2 Mic and amino acid changes in quinolone-resistant and susceptible strains of \( H. \) pylori

| Strains | MIC (μg/mL) | Mutations of GyrA | Mutations of GyrB |
|---------|-------------|-------------------|-------------------|
| LVX | MOX | CIP |
| 28R | 24 | 16 | 32 | 0 | 0 |
| 29R | 16 | 32 | 32 | 0 | 0 |

1Primary resistance. QRDR: Quinolone resistance-determining region.

DISCUSSION
In this study, we investigated the resistance of \( H. \) pylori strains isolated from patients at Peking University First Hospital during 2007-2009 to three quinolone antibiotics. Of the 79 \( H. \) pylori strains isolated, 44 (55.7%) strains fell into the following categories: (1) substitution of the amino acid at position 91 in 10 of the 27 isolates; (2) substitution of the amino acid at position 87 in 13 isolates; and (3) two mutations leading to substitution of amino acids in four isolates (Table 1). We also found 2 resistant strains with no QRDR mutations; in these strains the MICs to fluoroquinolones were > 12 μg/mL (Table 2).
were found to be resistant to quinolones and 21 (26.6%) showed primary resistance to quinolones. This is the first report of primary H. pylori resistance to quinolones in the Beijing area. In a previous study carried out in Hong Kong, the prevalence of LVX resistance in H. pylori was reported to be 11.5%\(^\text{[10]}\). An increase in fluoroquinolone resistance has also been reported in other areas, for example, the resistance rate to either CIP or LVX increased from 2.8% (1998-2003) to 11.8% (2004-2007) in southern Taiwan\(^\text{[13]}\). In other parts of the world, geographical differences and differences in treatment regimens result in a range of resistance rates, for example, rates of more than 20% in Korea\(^\text{[12]}\), 15% in Japan\(^\text{[9]}\) and 14% in Italy\(^\text{[14]}\) have been reported.

According to research from Japan\(^\text{[19]}\), caution is needed when interpreting these results because no criteria have been published for establishing the breakpoint of fluoroquinolones against H. pylori. According to the Maastricht III Consensus Report\(^\text{[3]}\), the threshold of clarithromycin resistance at which the empirical use of this antibiotic should be abandoned, or pretreatment clarithromycin susceptibility testing performed, is 15%-20%. Although some studies have reported good eradication rates using fluoroquinolones in some areas of China\(^\text{[25,26]}\), quinolone treatment regimens in Beijing should be based on the findings of local antimicrobial susceptibility tests as the resistance rate is higher than 20% in this area.

In China, quinolone antibiotics have been widely used in hospitals to treat various infections and in the poultry industry as a supplement in feed. This has resulted in drug resistance becoming a serious problem\(^\text{[15,26-28]}\). In our study and a previous study from Korea\(^\text{[12]}\), cross-resistance of the H. pylori strains to MOX, CIP and LVX was observed in patients who had not received MOX before the other eradication regimens.

Fluoroquinolone resistance is attributed to specific mutations in the genes encoding DNA gyrase and/or topoisomerase IV. In Neisseria gonorrhoeae\(^\text{[29]}\), for example, mutations in the QRDRs of the gyrA and parC genes may be responsible for fluoroquinolone resistance. In H. pylori, only mutations in the DNA gyrase gene have been considered responsible for fluoroquinolone resistance because neither parC nor parE have been detected in the genome sequence and the drug efflux system is not considered important in this organism\(^\text{[30,31]}\). Consequently, DNA gyrase is the unique target for quinolones in H. pylori. Fluoroquinolone resistance of H. pylori is considered to depend on point mutations in the QRDR of the gyrA/B gene. GyrA mutations at Asn-87 and Asp-91 have been reported previously\(^\text{[9]}\). In our study, 11 resistant strains (37.93%) possessed mutations at Asp-91, 16 resistant strains (55.17%) possessed mutations at Asn-87, but mutations at both Asp-91 and Asn-87 were not found to coexist in any strain. Mutations at position Asn-87 were more frequent than at Asp-91 in LVX-resistant strains in Japan, according to a previous report\(^\text{[9]}\). We also identified more mutations at Asn-87 than at Asp-91 in this study. In Escherichia coli, Nakamura et al\(^\text{[32]}\) found that mutations in the gyrB gene also lead to low-level quinolone resistance. Yoshida et al\(^\text{[33]}\) identified two possible mutations in the GyrB protein of Escherichia coli: Asp426→Asn and Lys447→Glu. We also identified mutations in the gyrB gene in this study, but there was no statistically significant difference between the genotype of this gene and quinolone resistance. The role of GyrB in fluoroquinolone resistance therefore remains to be determined. In H. pylori, one mutation within the QRDR of the gyrA gene was associated with low level fluoroquinolone resistance, and two or more mutations may relate to high level of fluoroquinolone resistance\(^\text{[19]}\). One previous study reported that in Campylobacter spp.\(^\text{[34]}\), a single mutation in the gyrA gene can lead to a high level of resistance not only to nalidixic acid but also to CIP, but it requires a second mutation to become MOX-resistant. In our study, two strains with two mutations in the QRDR of the gyrA gene had different levels of resistance to quinolones. Consistent with the findings of the Japanese study, we were also unable to identify any significant association between gyrA mutation patterns and the MICs of three fluoroquinolones\(^\text{[10]}\). In our study, we found that two resistant strains had no mutations in the QRDR of the gyrA and gyrB gene, and in these strains the MICs of three fluoroquinolone antibiotics reached 32 μg/mL. We also found that the same mutation can exist in resistant and susceptible strains. We therefore speculate that low-level resistance is most likely mediated through a point mutation in the gyrA gene. There may be other mutations in non-QRDRs of the gyrA and gyrB genes or other mechanisms, such as efflux systems, that lead to high level resistance.

A previous Japanese study reported that differences in amino acid substitutions associated with fluoroquinolone resistance correlate with geographical differences\(^\text{[35]}\), with the occurrence of the Asn-87 GyrA mutation being more frequent than the Asp-91 GyrA mutation in LVX-resistant strains. Different from the findings of our study, it has previously been reported that in Hong Kong the most frequent mutation site in the GyrA protein was position 91\(^\text{[14]}\).

In conclusion, the prevalence of resistance of H. pylori to fluoroquinolones is of concern in the Beijing area. Our results suggest that the susceptibility of H. pylori to fluoroquinolones should be tested before administration of a therapy, especially in the Beijing area. Resistance is most likely mediated through point mutations in the gyrA gene. Future studies should investigate whether other mechanisms are responsible for resistance in strains in which no mutations in the QRDR were detected.

**Comments**

**Background**

Resistance to antibiotics is one of the most important reasons behind Helicobacter pylori H. pylori eradication failure. H. pylori resistance to metronidazole and clarithromycin is becoming an increasingly serious problem and as a result first-line treatment programs based on these drugs are declining. As an alternative to these standard regimens, quinolones can be recommended for the first-line treatment of H. pylori infection.

**Research frontiers**

Fluoroquinolone-based regimens for H. pylori eradication are widely employed...
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9 Chinese Helicobacter pylori Research Group, Chinese Society of Gastroenterology. Prevalence of Helicobacter pylori in some areas of China, but studies investigating fluoroquinolone resistance are limited. Innovations and breakthroughs

Traditional E-tests and modern genetic mutation analysis of 45 H. pylori isolates showed a high rate of resistance to fluoroquinolones in Beijing. Resistance was found to be most likely mediated through point mutations in the gyrA gene, but two resistant strains were found with no quinolone resistance-determining region mutations. The possibility that other mechanisms are responsible for fluorquinolone resistance in these strains remains to be determined. Applications

The results of this study show a high prevalence of H. pylori resistance to fluoroquinolones in Beijing. The authors recommend that the susceptibility of H. pylori to fluoroquinolones is tested before the administration of a therapy. Terminology

gyrA: The gene encoding DNA gyrase (type II topoisomerase), subunit A; gyrB: The gene encoding DNA gyrase (type II topoisomerase), subunit B. Peer review

The manuscript investigates the prevalence and mechanisms of resistance of H. pylori to fluoroquinolones in Beijing. The methodology of resistance determination consists of a traditional E-test and modern genetic testing of gyrA mutation. The results show a primary resistance of 26% and secondary resistance of 55%, which is higher than in European countries. The article is good, using a sound methodology and correct conclusions.
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S- Editor Wang JL  L- Editor O’Neill M  E- Editor Lin YP