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Antibiotic resistance genes in *helicobacter pylori* isolates from gastric biopsies of patients with gastrointestinal diseases in Golestan province, Iran

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Abstract

Background: Antibiotic resistance in *Helicobacter pylori* infections can lead to treatment failure. This study aims to evaluate the antibiotic resistance to metronidazole, clarithromycin, and fluoroquinolone in *H. pylori* strains isolated from gastric biopsy specimens.

Methods: This study was conducted between 2016 and 2017 on 80 biopsy specimens obtained from Golestan province. Resistance to metronidazole (*rdxA*) and fluoroquinolones (*gyrA*) was determined using PCR. Mutations in the loci of the 23S rRNA gene associated with clarithromycin resistance were analyzed using PCR-RFLP with the BsaI and BbsI enzymes.

Results: In this study, 25% of *H. pylori* strains showed resistance to clarithromycin. Mutations in the A2143G locus (65%) and the A2142G locus (35%) were detected in these strains. Resistance to fluoroquinolones (27.5%) was observed, with the most common mutations being at the 91 amino acid position of aspartate (63.63%) and the 87 amino acid position of asparagine (36.36%). Resistance to metronidazole was not observed in any of the strains of this study, and concomitant resistance to clarithromycin and fluoroquinolones was observed in 13.75% of *H. pylori* strains.

Conclusion: According to our study, in Iran, the resistance of *H. pylori* to clarithromycin is increasing, which may lead to treatment failure. The mechanism of clarithromycin resistance is related to mutations in the A2143G and A2142G positions, and a mutation in the gyrA gene causes resistance to fluoroquinolones, which often occurs in the 91 amino acid position.

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Introduction

Helicobacter pylori (H. pylori) is a gram-negative, flagellated, microaerophilic bacterium (1). *H. pylori* infection is a major cause of gastrointestinal diseases, including chronic gastritis, peptic ulcer, and gastric cancer (2). The prevalence of this infection in Iran is 60 to 90%, which indicates that Iran is a high-risk area for *H. pylori* infection (3,4). Virulence factors in the pathogenesis of *H. pylori* include urease, flagellum, binding, cytotoxin-associated gene A (cagA), and vacuolating cytotoxin A (vacA) (5). The prevalence of *H. pylori* antibiotic resistance varies from country to country and may be related to geographical factors (6).

Treatment for H. pylori often involves the use of antibiotics. A common reason for treatment failure is the bacteria's ability to colonize the subcutaneous layer, and when it is combined with stomach acid, it can reduce the effectiveness of these antibiotics (6,7). A PPI (Proton pump inhibitor) or ranitidine, bismuth, and two antibiotics, such as amoxicillin and clarithromycin, are often used for its treatment (8). While three-drug regimens typically succeed in eradicating H. pylori infections, rising resistance-particularly to clarithromycin and metronidazole-among clinical strains can lead to treatment failures (9). Clarithromycin is a macrolide antibiotic that inhibits protein synthesis by binding to the 23S rRNA peptidyl transferase region. Clarithromycin resistance in H. pylori results from specific point mutations in the peptidyl transferase region of the second V23S rRNA gene (10). The most common mutation is the replacement of A to G at positions 2143 (A2143G) and 2142 (A2142G) (11,12). Metronidazole is a prodrug that requires regeneration for activation.

Several nitroreductases in *H. pylori* regenerate nitroimidazoles. Reduction of the nitro group produces imidazole mediators and toxic radicals that are toxic to DNA. One of the essential nitroreductases is oxygen-insensitive NAD(P)H, which is encoded by the rdxA gene; a mutation in this gene can lead to resistance to metronidazole (13). Fluoroquinolones, which inhibit the growth of *H. pylori* in vitro (14), can affect DNA gyrase (15). The resistance mechanism to fluoroquinolones in *H. pylori* is related to mutations in the regions determining the quinolone resistance of *gyrA* (QRDRs) and is caused by point mutations in the *gyrA* gene encoding gyrase DNA at positions encoding amino acids N87, 88A, 91D, and 97A (16). This study aimed to determine the antibiotic resistance of Helicobacter pylori strains in biopsy specimens and to investigate mutations in genes that confer resistance to clarithromycin, metronidazole, and fluoroquinolones using PCR and PCR-RFLP methods in specimens collected from Golestan Province, Iran.

Methods

In this study, 80 biopsy specimens from 335 patients referred to Sayad Shirazi Hospital in Gorgan for gastrointestinal issues and gastric ulcers were analyzed. The specimens were collected over a period of one year, from July 2016 to July 2017, and the patients' ages ranged from 14 to 86 years. The gastric biopsy specimens were taken during endoscopy by a gastroenterologist with a sterile endoscope.

Two gastric biopsies were taken from each patient. One sample was transferred to the urease medium for a rapid urease test (RUT) for *H. pylori* screening, and a second sample was transferred to 1.5 mL

microtubes containing 1 mL of sterile phosphate-buffered saline (PBS) until DNA extraction. For Subsequent steps, the samples were kept at -70 °C. Informed consent was obtained from the patient before participating in this study. The study was approved by Golestan University of Medical Sciences, Iran. Ethical approval was granted under the Code of Ethics (IR.GOUMS.REC.1394.351).

The RUT was conducted following the method outlined by Siavoshi et al (17). Aliquots were collected in sterilized microtubules with a 700-1000 microliter volume. One of the biopsy specimens was placed in a urease medium for 1-2 hours at room temperature, and the change in color from yellow to pink indicated a positive test. Samples of patients whose urease test was positive were included in the study and kept at -70. According to the kit manufacturer's protocol, the total genomic DNA of 80 H. pylori strains was extracted using the Macherey-Nagel Kit (Germany). The extracted DNAs were stored for further analysis at -20° C. All primers for PCR in this study were synthesized by the German Metabion.

The primers described by Momtaz *et al* (18) were used to identify the *ureC* gene (Table 1). PCR amplification was performed to investigate the resistance of *H. pylori* isolates to fluoroquinolones (*gyrA*) and metronidazole (*rdxA*). The primers developed by Glocker et al. and Mahmoudi et al. were used to identify the *gyrA* and *rdxA* genes (19,20) (Table 1). To identify the *gyrA* and *ureC* genes, a PCR master mix was prepared by combining 2.5 µL of 1× PCR master mix, which contained Taq DNA polymerase (0.06 U/µL), MgCl2 (1.5 mM), and dNTPs (0.2 mM). This was mixed with 1 µL of each primer, 5 µL of template DNA, and 13.2 µL of distilled water.

Table 1. Primer pairs for the PCR amplification of ureC, gyrA, rdxA, and 23S rRNA genes in *H. pylori isolates*

Primer pair	Nucleotide sequence (5'-3')	Amplicon size (bp)
ureC-F	GGATAAGCTTTTAGGGGTGTTAGGGG	296
ureC-R	GCTTACTTTCTAACACTAACGCGC	
gyrA-F	ATATTGTAGAAGTGGGGATTGAT	569
gyrA-R	ATGCACTAAAGCGTCTATGATT	
rdxA-F	AATTTGAGCATGGGGCGA	850
rdxA-R	AAACGCTTGAAAACACCCT	
23S rRNA-F	CCACACAGCGATGTGGTCTCAG	425
23S rRNA-R	CTCCATAAGAGCCAAAGCCC	

To identify the rdxA gene, touchdown PCR was performed. For this process, we prepared a PCR master mix of 1.25 µL at a concentration of 0.6×. This mix included Taq DNA polymerase at 0.04 U/µL, MgCl2 at 1.5 mM, and dNTPs at 0.5 mM. Additionally, we added 1 µL of each primer, 5 µL of template DNA, and 14.55 µL of distilled water. PCR reactions were performed using an Eppendorf thermocycler, and the PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with SYBR Green dye. The results were visualized under UV light using a gel documentation system. Amplified products of the *gyrA* gene from PCR in the samples were sent to the Macrogen Company of South Korea for sequencing.

Gene sequencing for each sample was performed using Chromas software (Version 2.6.4) for the genes of *H. pylori* 26695 at https://blast.ncbi.nlm.nih.gov/. The sequences were then compared and analyzed using BLAST. Detection of point mutations in the second V of 23S rRNA gene related to clarithromycin resistance of *H. pylori* was performed by the PCR-RLFP method. Amplification was carried out using the polymerase chain reaction (PCR) technique (Table 1) (21).

The PCR procedure details in this study were as follows. To prepare the PCR reaction, 2.5 µL of PCR master mix (1×) was combined with 1 µL of each primer, 5 µL of template DNA, and 13.1 µL of distilled water. The master mix contained Taq DNA polymerase (0.08 U/µL), MgCl2 (1.5 mM), and dNTPs (0.2 mM). We used BsaI and BbsI (Thermo) enzymes to detect A2143G and A2142G point mutations, respectively.

The PCR-RFLP protocol for detecting mutations at the A2143G locus using the BsaI enzyme was as follows. 10 µL of PCR products, along with 2 µL of 10X buffer G, 1 µL of BsaI enzyme, and 16 µL of sterile distilled water, were added to microtubes. The PCR-RFLP protocol for detecting mutations at the A2142G site using the BbsI enzyme was as follows. 10 µL of PCR products with 2 µl of 10X buffer G, 1 µl of BbsI enzyme, and 18 µL of sterile distilled water were mixed in a microtube. The microtubules were then spun for a few seconds and then incubated at 37 °C for three hours. To inactivate the enzyme, the sample was incubated for 20 minutes at 65 °C. Ultimately, the digested PCR and undigested PCR products were examined by UV transilluminator after electrophoresis in 2% agarose gel stained with ethidium bromide.

The data were analyzed using SPSS 16 software. P values were obtained through the Chi-square test, with P values < 0.05 considered statistically significant in all cases.

Results

In total, 335 biopsy specimens were collected from patients over the course of one year, from July 2016 to July 2017. Out of all 335 specimens, 37.9% (127) were positive for urease isolates. Among 127 urease-positive isolates (81.88%), 80 had a conserved *ureC* gene, including 54 females (67.5%) and 26 males (32.5%).

Prevalence of clarithromycin resistance

After amplifying the target fragment by PCR using specific primers for the *23S rRNA* gene and electrophoresis of PCR products, a 425 bp band was created. We examined clarithromycin resistance and mutations at the A2142G and A2143G loci using BbsI and BsaI enzymes, respectively. The BsaI enzyme has two cleavage sites on the PCR product, 425 bp. Three fragments were created by enzymatic digestion of DNA with lengths of 20 bp, 101 bp, and 304 bp. The 20bp fragment is not visible on the gel (Figure 1). The BbsI enzyme created a cleavage site through the enzymatic digestion of two pieces, 93 bp and 332 bp. Among the 80 isolates, 25% were resistant to clarithromycin; 35% had the A2142G mutation, and 65% had the A2143G mutation.

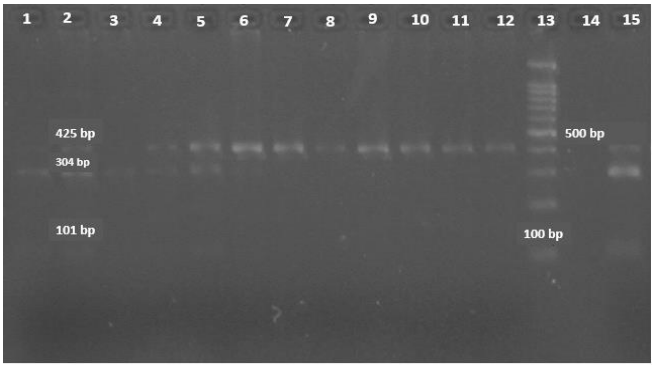

Figure 1. PCR-RFLP analysis of a 425 bp fragment of the 23S rRNA gene; Samples No. 1 to 6 and 15 are clarithromycin-resistant strains using the BsaI enzyme with A2143G mutation; Samples No. 7 to 12 are clarithromycin-sensitive strains; Sample No. 13 is a 100 bp DNA marker; Sample No. 14 is without a sample.

Prevalence of metronidazole resistance

Our study found no deletion of 200 nucleotides in the studied isolates that confer metronidazole resistance.

Prevalence of fluoroquinolone resistance

Out of the 80 isolates studied, 22 isolates (27.5%) exhibited resistance to fluoroquinolones. Among these resistant isolates, there were 8 mutations (36.36%) at the 87th amino acid position, where asparagine is located, and 14 mutations (63.63%) at the 91st amino acid position, where aspartate is found. At position 87, the highest mutation leading to resistance to fluoroquinolones was due to a change in the amino acid asparagine to lysine (31.82%). Another mutation reported in some studies is a change in the amino acid asparagine to isoleucine (4.54%), which was observed in one of the isolates. At position 91, changes in the amino acid aspartate to glycine (27.27%), aspartate to asparagine (22.72%), and aspartate to tyrosine (13.63%) were observed among the isolates in this study, and two isolates (2.06%) had a D91A mutation. Among the specimens studies, these mutations did not lead to resistance to fluoroquinolones.

The highest antibiotic resistance among the isolates was against fluoroquinolone (27.5%), and metronidazole resistance was the lowest. None of the 80 samples had 200 nucleotide deletions. Concomitant resistance to clarithromycin and fluoroquinolone was observed in 11 samples (13.75%) of *H. pylori*. Additionally, no statistically significant association was found between antibiotic resistance and age, sex, or patients' symptoms, including a history of peptic ulcer and gastrointestinal problems.

Discussion

Antibiotic resistance in *H. pylori* is a crucial factor influencing treatment regimens (22). Various antibiotics are used to treat *H. pylori* infection, including metronidazole, clarithromycin, levofloxacin, and amoxicillin (23). This study investigated the antibiotic resistance of *H. pylori* strains and resistance-causing mutations by PCR and PCR-RFLP.

Metronidazole resistance is a global concern (24). From 1997 to 2013, epidemiological studies in Iran reported the highest antibiotic resistance of *H. pylori* to metronidazole, with a rate of 61.6% (25). Abdollahi et al. (2010) in Iran conducted a study on 63 isolates of *H. pylori*, of which 35 samples were resistant to metronidazole. Examining the *rdxA* gene demonstrated that eight isolates (22.9%) had 200 nucleotide deletions (26). Ramzy et al. examined the *rdxA* gene in Egypt in 2013 and observed the removal of 200 nucleotides from 70 samples in 44 strains (62.9%) (27). In a 2013 study by Mirzaei et al. in Iran, 27 of 48 patients had metronidazole resistance. They were tested for the *rdxA* gene, and no deletion of 200 nucleotides was observed (28). Mahmoudi et al. (2015) conducted a study in Iran on 46 *H. pylori* isolates. The findings of this study indicated that 64.3% of the isolates were resistant to metronidazole. Notably, none of the resistant samples demonstrated deletion of the *rdxA* gene (20).

Our study confirmed the findings of Mirzaei and Mahmoudi; none of the strains we studied exhibited the removal of 200 nucleotides. The diversity of clarithromycin resistance in different regions highlights the need to study the level of resistance in each geographical area to inform more effective treatment regimens (29). Clarithromycin is a macrolide (30), and the most important related mutations are 2142 and 2143 (10). Clarithromycin resistance rates vary across different regions in Iran, with the lowest rate observed in Rasht at 5.5% and the highest rate in Kashan at 33.7% (31). Clarithromycin resistance can arise from the A2143G mutation, which occurs with a frequency of 69.8%, the A2142G mutation with a frequency of 11.7%, and the A2142C mutation with a frequency of 2.6% (32-34). Sadeghifard et al. conducted a study in 2010 on 50 patients to evaluate clarithromycin resistance in Iran. Eight patients (16%) had clarithromycin resistance at the A2143G locus, and no mutation was observed at the A2142G locus (35). In 2011, Abdollahi et al. conducted a study in the same country on 63 H. pylori isolates obtained from gastric biopsy specimens to investigate the resistance and sensitivity of the isolates to clarithromycin and to investigate mutations in the 23S RRNA gene. They found that 20 out of 63 *H. pylori* isolates (31.7%) were resistant to clarithromycin. Among these resistant isolates, 3 (15%) exhibited mutations at the A2143G locus, 11 (55%) at the A2142G locus, and 6 (30%) at the A2142C locus (36). Also, Eghbali et al. in 2016 in Iran conducted a study on 89 gastric biopsies of patients to investigate the resistance of *H. pylori* strains to clarithromycin; 5 strains were resistant to clarithromycin and had A2143G mutation, and no strains had A2142G mutation (37). Suzuki et al. investigated clarithromycin resistance in 488 *H. pylori* cases in 2013 in Brazil and observed 25% A2142G mutations, 58.3% A2143G mutations, and 8.7% mutations at both sites (38). In a 2020 study by Vazirzadeh et al. in Iran, 21 (25.3%) out of 83 *H. pylori* strains were resistant to clarithromycin, and 19 isolates had mutations. Among them, 13 strains (68.4%) had A2143G mutation and 6 strains (31.5%) had A2144G mutation; the mutation A2143C was not detected in any of the isolated (39).

Our findings indicated that the resistance to clarithromycin was 25%. The mutation at the A2143G site was 65%, and the mutation at the A2142G site was 35%, similar to the Suzuki and Vazirizadeh studies. The mechanism of action of fluoroquinolones is through their effect on *H.pylori* DNA gyrase (40). In 2015, Zaki et al. in Egypt investigated mutations in the *gyrA* gene at positions 87 and 91 on 82 samples. Nineteen strains had mutations in *gyrA*, of which 12 isolates (63.2%) had mutations in aspartate 91, and 7 isolates (36.8%) had mutations in asparagine 87 (41).

In 2014, Ngoyi et al. conducted a study in the Congo on the resistance of 36 patient samples to fluoroquinolones. They found that 18 (50%) of the *H. pylori* isolates had mutations at positions 87 and 91 (42). In 2020, Kipritci et al. conducted a study on 140 biopsy specimens in Turkey to investigate the antibiotic resistance of *H. pylori* strains to clarithromycin and fluoroquinolones. The results indicated that 21 isolates (25.6%) showed resistance to fluoroquinolones, while 31 isolates (37.9%) exhibited resistance to clarithromycin. Additionally, 11 isolates (14.1%) demonstrated simultaneous resistance to fluoroquinolones and clarithromycin (43). In this study, the resistance to fluoroquinolones was 27.5%, which was 63% in the 91-amino acid position of aspartate and 36% in the 87-amino acid position of asparagine. Our study was similar to the Zaki study. Additionally, identical to the Kipritci study, our study demonstrated simultaneous resistance to fluoroquinolones and clarithromycin. Resistance to fluoroquinolones seems to be due to mutations in the *gyrA* gene. Increased resistance to fluoroquinolones may be associated with excessive use of these antibiotics. Since this antibiotic has a wide range of therapeutic applications and is used against various types of infections, it is not recommended for the treatment of *H. pylori*. The resistance rate to fluoroquinolones in Iran increased from 5.3% in 1997 to 27.5% in 2013.

Conclusion

Antibiotic resistance among *H. pylori* strains is rising globally, leading to treatment failures. Since resistance to metronidazole, clarithromycin, and fluoroquinolones has become a grave concern, alternative treatment regimens should be considered if resistance to clarithromycin and fluoroquinolones is suspected. Considering high levels of metronidazole resistance worldwide, metronidazole resistance should be further investigated using phenotypic methods and PCR techniques. It is also recommended to study other genes involved in metronidazole activation, such as *frxA*, *fdxA*, *fdxB*. To investigate clarithromycin resistance, research should not be limited to A2143G and A2142G mutations, which are the most common mutations associated with resistance, and other mutations should also be considered.

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Ethical statement

Informed consent was obtained from all participants. The study received approval from the Human Research Ethics Boards at Golestan University of Medical Sciences under the Code of Ethics (IR.GOUMS.REC.1394.351).

Conflicts of interest

The authors declare that there are no conflicts of interest.

Author contributions

A.SH conducted the experiments and analyzed the data. B. KH wrote the manuscript and contributed to the article's concept. A. J and SH guided the experiments and critically revised the manuscript.

Data availability statement

All data generated and analyzed during this study are included in our manuscript.

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