Antibiotic resistance genes rate of *Helicobacter pylori* isolates in gastric biopsies of gastrointestinal patients in Golestan province, Iran

Running Title: Antibiotic resistance of *Helicobacter pylori* and gastrointestinal disorder

Azam Shareh
Department of Microbiology, Faculty of Medical Sciences, Golestan University of Medical Sciences, Gorgan, Iran.

Behnoush Khasheii
Department of Pathobiology, Faculty of Veterinary Sciences, Bu-Ali Sina University, Hamedan, Iran.

Tayebeh Faraji
Department of Microbiology, Faculty of Medical Sciences, Golestan University of Medical Sciences, Gorgan, Iran.

Masoud Khoshnia
Golestan Research Center of Gastroenterology and Hepatology, Golestan University of Medical Sciences, Gorgan, Iran. 0000-0001-7352-8058

Shaghayegh Anvari
Laboratory Sciences Research Center, Golestan University of Medical Sciences, Gorgan, Iran. 0000-0002-2694-5796

Ailar Jamalli
Laboratory Sciences Research Center, Golestan University of Medical Sciences, Gorgan, Iran. 0000-0002-4612-8144

Corresponding Author(s): Ailar jamalli and Shaghayegh Anvari
Email: jamali@goums.ac.ir and shaghayeghanvari@yahoo.com
Address: Laboratory Sciences Research Center, Golestan University of Medical Sciences, Gorgan, Iran.
Tel: +981732421651; Fax: +981732440225

Abstract

Background: Antibiotic resistance in *Helicobacter pylori* is a significant cause of failure in the treatment of this infection. This study aimed to evaluate the antibiotic resistance to metronidazole, clarithromycin, and fluoroquinolone in *H. pylori* strains isolated from biopsy specimens of patients.

Methods: This study was carried out (2016 to 2017) on 80 biopsy specimens from the Golestan province. Resistance to metronidazole (*rdxA*), fluoroquinolones (*gyrA*) was determined by PCR. Mutations at the A2143G and A2142G loci in the 23srRNA gene associated with clarithromycin resistance in strains were performed by PCR-RFLP with *BsaI* and *BbsI* enzymes.

Results: In this study, 25% of *H. pylori* strains were resistant to clarithromycin. A mutation in the A2143G locus (65%) and the A2142G locus (35%) were detected in these strains. Resistance to fluoroquinolones (27.5%) was observed, among which mutations at the 91 amino acid position of aspartate (63.63%) and mutations at the 87 amino acid position of asparagine (36.36%) were observed in the samples. 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.

Conclusions: According to our study, in Iran, the resistance of *H. pylori* to clarithromycin, is increasing, which is a factor in treatment failure. The mechanism of clarithromycin resistance is related to mutations in the A2143G and A2142G positions, and resistance to fluoroquinolones is caused by a mutation in the *gyrA* gene, and more in the 91 amino acid position.

Keywords: *Helicobacter pylori*; Antibiotic resistance; PCR-RFLP

Introduction

Helicobacter 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 bacterium 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 determined to some extent by geographical factors (6). Treatment for *H. pylori* is based on the use of antibiotics. One of the reasons for the failure of the treatment is the colonization of bacteria in the subcutaneous layer and the presence of stomach acid, which reduces the effects of antibiotics (6, 7). A PPI proton pump inhibitor or ranitidine bismuth and two antibiotics, such as amoxicillin and clarithromycin, are often used (8). Although three-drug regimens are usually effective in eradicating *H. pylori* infection, increasing resistance, especially to clarithromycin and metronidazole among clinical strains of *H. pylori*, reduces the success and sometimes failure of treatment (9). Clarithromycin is a macrolide antibiotic that inhibits protein synthesis by binding to the 23SrRNA peptidyl transferase region. Clarithromycin resistance in *H. pylori* is due to distinct point mutations in the peptidyl transferase region of the second V 23SrRNA 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 must be regenerated to be active. There are several nitroreductases in *H. pylori* that 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, and one of the reasons for resistance to metronidazoles is a mutation in this gene (13). Fluoroquinolones inhibit the growth of *H. pylori* in vitro (14). Fluoroquinolones are antibiotics that affect DNA gyrase (15). The resistance mechanism to fluoroquinolones in *H. pylori* is associated with mutations in the regions determining the quinolone resistance of *gyrA* (QRDRs) and resulting from point mutations in the *gyrA* gene encoding gyrase DNA at positions encoding amino acids N87, 88A, 91D And is 97 A (16). This study aimed to determine the antibiotic resistance of *Helicobacter pylori* strains in biopsy specimens and investigate mutations in genes encoding resistance to the antibiotics clarithromycin, metronidazole, and fluoroquinolone by PCR and PCR-RFLP in Golestan province.

Methods

This study was performed on 80 biopsy specimens from 335 specimens collected from patients with gastrointestinal problems and gastric ulcers referred to Sayad Shirazi Hospital in Gorgan for one year (July 2016 to July 2017), with an age range between 14 to 86 years. The gastric biopsy specimen was taken during endoscopy by a gastroenterologist with a sterile endoscope. Code of Ethics (IR.GOUMS.REC.1394.351).

Two gastric biopsies were taken from each patient. One sample was transferred to the urease medium, for rapid urease test (RUT) for *H. pylori* screening, and the second sample was transferred to 1.5 ml micro tubes containing 1 ml of sterile phosphate-buffered saline (PBS) until DNA extraction. For Subsequent steps were kept at -70 °C. Before participating in this study, informed consent was obtained from each patient and approved by Golestan University of Medical Sciences, Iran.

RUT was performed according to the method described by Siavoshi et al (17). Aliquot 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 of *H. pylori* strains was extracted by the Kit / Germany Macherey NagelMn Weber, and the extracted DNAs were stored for further analysis at -20 ° C. All primers for PCR in this study were synthesized by the German Metabion and used.
The primers described by Momtaz *et al* (18), and were used identify the *ureC* gene [Table 1].
PCR amplification was performed to identify the resistance of *H. pylori* isolates to fluoroquinolones (*gyrA*) and metronidazole (*rdxA*). The primers described by Glocker et al and Mahmoudi et al, to identify the *gyrA* and *rdxA* genes (19, 20). [Table 1]
For identify the *gyrA* and *ureC* genes, 2.5 µL of PCR master mix (1×), consisting of Taq DNA polymerase (0.06 U/µL), MgCl2 (1.5 mM), dNTPs (0.2 mM), was admixed with 1 µL of each primer, 5 µL of template DNA, and 13.2 µL of distilled water.
To identify the *rdxA* gene, Touchdown PCR was done, and for this purpose, 1.25 µL of PCR master mix (0.6×), consisting of Taq DNA polymerase (0.04 U/µL), MgCl2 (1.5 mM), dNTPs (0.5 mM), was admixed with 1 µL of each primer, 5 µL of template DNA, and 14.55 µL of distilled water.
PCR reactions were performed in an Eppendorf thermocycler, and PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with SYBR green dye. The results were visualized under a 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.
Finally, the sequencing of genes for each sample was performed using Chromas software (version 2.6.4) and sequenced for the desired genes of Helicobacter pylori 26695 at https://blast.ncbi.nlm.nih.gov/. The sequences were 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 performed by PCR (21). [Table1]
The PCR values in this study are as follows. For this purpose, 2.5 µL of PCR master mix (1×), consisting of Taq DNA polymerase (0.08 U/µL), MgCl2 (1.5 mM), dNTPs (0.2 mM), was admixed with 1 µL of each primer, 5 µL of template DNA, and 13.1 µL of distilled water.
We used *BsaI* and *BbsI* (Thermo) enzymes to detect point mutations, respectively, to detect A2143G and A2142G mutations. The PCR-RFLP protocol for detecting mutations at the A2143G locus using *BsaI* enzyme was as follows: 10 µl of PCR products along with 2 µl of 10X buffer G, 1 µl of *BsaI* enzyme with 16 µl of sterile distilled water were poured into the micro tube and also, the PCR-RFLP protocol for detecting mutations at the A2142G site using the *BbsI* enzyme is as follows: 10 µl of PCR products with 2 µl of 10X buffer G, 1 µl of *BbsI* enzyme with 18µl of sterile distilled water into the micro tube, then spin the micro tubes for a few seconds and incubate at 37 ° C for 3 hours. In order to inactivate the enzyme, inactivation was incubated for 20 minutes at 65 ° C. Finally, the digested PCR and undigested PCR products were examined by UV transilluminator after electrophoresis in 2% agarose gel stained with cyber green.
The data were analyzed by SPSS16 software. P values were calculated using the Chi-square test. $P < 0.05$ was considered as a statistically significant result in all cases.

Results

In total, 335 biopsy specimens were collected from patients during one year from July 2016 to July 2017, and out of 335 specimens (37.9%), 127 urease isolates were positive. 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.

In the present study, 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 are created by enzymatic digestion of 20bp, 101bp, and 304bp. The 20bp fragment is not visible on the gel (Figure 1). The *BbsI* enzyme has a cleavage site created by enzymatic digestion of two fragments of 93 bp and 332 bp. Among the 80 isolates, 25% were resistant to clarithromycin, of which 35% had the A2142G mutation, and 65% had the A2143G mutation.

Prevalence of metronidazole resistance

In our study, Deletion of 200 nucleotides that make metronidazole resistant was not observed among the studied isolates.

Prevalence of fluoroquinolone resistance

Among the 80 isolates studied, 22 isolates (27.5 %%) had resistance to fluoroquinolones, of which the number of mutations at the position of 87 amino acids of asparagine was 8 (36.36%), and the number of mutations at the position of 91 amino acids of aspartate was 14 (63.63%). 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%), and another mutation reported in some studies was 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 studies, these mutations did not induce resistance to fluoroquinolones.

The highest antibiotic resistance among the isolates was 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*. Also, there was no association between antibiotic resistance with age, sex, and patients' symptoms in this study, such as the history of peptic ulcer and gastrointestinal problems, and there was no statistically significant difference.

Discussion

Antibiotic resistance in *H. pylori* is a significant factor influencing current treatment regimens (22). Various antibiotics are used to treat *H. pylori* infection, including metronidazole, clarithromycin, levofloxacin, amoxicillin (23). This study investigated the antibiotic resistance of *Helicobacter pylori* strains and resistance-causing mutations by PCR and PCR-RFLP. Metronidazole resistance is a global concern (24). According to epidemiological studies that examined the antibiotic resistance of *H. pylori* in Iran from 1997 to 2013, the highest antibiotic resistance was reported for metronidazole with 61.6% (25). Abdollahi et al. in 2010 in Iran, conducted a study on 63 isolates of *H. pylori*, of which 35 samples were resistant to metronidazole, and by examining the *rdxA* gene, 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 and tested for the *rdxA* gene. No deletion of 200 nucleotides was

observed among the samples (28). Mahmoudi et al. (2015) conducted a study in Iran on 46 *H. pylori* isolates. The findings of this study indicate that 64.3% of the isolates were resistant to metronidazole. Notably, none of the resistant samples possessed *rdxA* gene deletion (20).

Our study was similar to the studies of Mirzaei, Mahmoudi and in none of the strains we studied, the removal of 200 nucleotides was observed. The diversity of clarithromycin resistance in different regions emphasizes the need to study the level of resistance in each geographical area to guide treatment regimens better (29). Clarithromycin is a macrolide (30) and the most important mutation of them are 2142 and 2143 (10). Clarithromycin resistance varies in different parts of Iran, and the lowest and highest rates are reported in Rasht 5.5% and Kashan 33.7%, respectively (31). A2143G mutation with a frequency of 69.8%, A2142G mutation 11.7%, and A2142C mutation 2.6% cause clarithromycin resistance (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 a 2011, Abdollahi et al. conducted a study in Iran 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 23r RNA gene, 20 (31.7%) out of 63 of the *H. pylori* isolates were resistant to clarithromycin and 3 isolates (15%) had mutations at the A2143G locus, 11 (55%) at the A2142G locus and 6 (30%) at the A2142C locus (36). Also, by 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, and 5 strains were resistant to clarithromycin and had A2143G mutation, and no strains had A2142G mutation (37). Suzuki et al. tested clarithromycin resistance on 488 *H. pylori* in 2013 in Brazil, with 25% A2142G mutations, 58.3% A2143G mutations, and 8.7% mutations at both sites (38). In a 2020 study by Vazirzadeh et al. performed in Iran, 21 (25.3%) out of 83 *H. pylori* strains were resistant to clarithromycin and 19 isolates had mutations, 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).

In our study, the resistance to clarithromycin was 25%. The mutation at the A2143G site was 65%, and the mutation at the A2142G site was 35% and Similar to the Suzuki study and Vazirizadeh. The mechanism of action of fluoroquinolones is the effect on *H.pylori* DNA gyrase. (40). In 2015, Zaki et al. in Egypt tested 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 in the Congo, Ngoyi et al. tested resistance to fluoroquinolones in 36 patient samples, with 18 (50%) isolates *H.pylori* having mutations at positions 87 and 91 (42). In 2020, Kipritci et al. conducted a study in Turkey to evaluate the antibiotic resistance of *H. pylori* strains to clarithromycin and fluoroquinolone on 140 biopsy specimens. The results showed that the resistance to fluoroquinolones in 21 (25.6%) isolates and clarithromycin resistance in 31 (37.9%) isolates and 11 (14.1%) isolates had 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. Also, in our study, similar to the Kipritci study, simultaneous resistance to fluoroquinolone and clarithromycin was observed. Studies show that resistance to fluoroquinolones is due to mutations in the *gyrA* gene and that increased resistance to fluoroquinolones may be associated with enhanced use of these antibiotics in infections. Because this antibiotic has a wide range of therapies and is used for different types of diseases, and is not the only drug of choice for treating *H. pylori*.

Therefore, the resistance rate to this antibiotic in Iran from 1997-2013 increased from 5.3% to 27.5% in the present study.

Conclusion

Antibiotic resistance is increasing among *H. pylori* strains worldwide and is one of the main reasons for treatment failure, resistance to metronidazole, clarithromycin, and fluoroquinolones is becoming an increasing problem. Therefore, other treatment regimens should be used if resistance to clarithromycin and fluoroquinolones. Because the highest drug resistance in the world is metronidazole resistance, it is better to use the phenotypic method to study metronidazole resistance and the PCR method. It is also better to study other genes involved in metronidazole activation, such as *frxA*, *fdxA*, *fdxB*, etc. In order to evaluate the resistance to clarithromycin, in addition to mutations A2143G and A2142G, which are the most common mutations that cause resistance to clarithromycin, other mutations should be examined using sequencing.

Acknowledgements

We thank the staff of Golestan University of Medical Sciences, Naemeh Javid and Masoud Bazori for logistical support. This work was supported by Technology and Research Deputy of Golestan University of Medical Sciences (contract no. 9412183500).

Author contributions

A. SH performed the experiments, analyzed the experimental data, and B. KH wrote the manuscript. M. KH contributed to the concept of the article. A. J and SH. A guided the experiments and critically revised the manuscript.

Funding source

This work was supported by Technology and Research Deputy of Golestan University of Medical Sciences.

Data availability

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

Ethical statement

Written informed consent was obtained from the participants. The original study was approved by the Human Research Ethics Boards at Golestan University of Medical Sciences. Code of Ethics (IR.GOUMS.REC.1394.351).

Consent for publication

Not applicable.

Competing interests

No conflict of interest is declared by the authors.

References

1. Wu W, Yang Y, Sun G. Recent insights into antibiotic resistance in Helicobacter pylori eradication. Gastroenterology research and practice. 2012;2012.
2. Ishaq S, Nunn L. Helicobacter pylori and gastric cancer: a state of the art review. Gastroenterology and hepatology from bed to bench. 2015;8(Suppl1):S6.
3. Dabiri H, Maleknejad P, Yamaoka Y, Feizabadi MM, Jafari F, Rezadehbashi M, et al. Distribution of Helicobacter pylori cagA, cagE, oipA and vacA in different major ethnic groups in Tehran, Iran. Journal of gastroenterology and hepatology. 2009;24(8):1380-6.
4. Jafari F, Shokrzadeh L, Dabiri H, Baghaei K, Yamaoka Y, Zojaji H, et al. vacA genotypes of Helicobacter pylori in relation to cagA status and clinical outcomes in Iranian populations. Japanese journal of infectious diseases. 2008;61(4):290.
5. Chang W-L, Yeh Y-C, Sheu B-S. The impacts of H. pylori virulence factors on the development of gastroduodenal diseases. Journal of biomedical science. 2018;25(1):1-9.
6. De Francesco V, Giorgio F, Hassan C, Manes G, Vannella L, Panella C, et al. Worldwide H. pylori antibiotic resistance: a systematic. J Gastrointestin Liver Dis. 2010;19(4):409-14.
7. Nishizawa T, Suzuki H. Mechanisms of Helicobacter pylori antibiotic resistance and molecular testing. Frontiers in molecular biosciences. 2014;1:19.
8. Vale FF, Rosa MR, Oleastro M. Helicobacter pylori resistance to antibiotics. Science against microbial pathogens: communicating current research and technological advances. 2011:p745-56.
9. Karczewska E, Wojtas-Bonior I, Sito E, Zwolińska-Wcisło M, Budak A. Primary and secondary clarithromycin, metronidazole, amoxicillin and levofloxacin resistance to Helicobacter pylori in southern Poland. Pharmacological Reports. 2011;63(3):799-807.
10. Agudo S, Pérez-Pérez G, Alarcón T, López-Brea M. High prevalence of clarithromycin-resistant Helicobacter pylori strains and risk factors associated with resistance in Madrid, Spain. Journal of clinical microbiology. 2010;48(10):3703-7.
11. Mansour KB, Burucoa C, Zribi M, Masmoudi A, Karoui S, Kallel L, et al. Primary resistance to clarithromycin, metronidazole and amoxicillin of Helicobacter pylori isolated from Tunisian patients with peptic ulcers and gastritis: a prospective multicentre study. Annals of clinical microbiology and antimicrobials. 2010;9(1):1-7.
12. Klesiewicz K, Nowak P, Karczewska E, Skiba-Kurek I, Wojtas-Bonior I, Sito E, et al. PCR-RFLP detection of point mutations A2143G and A2142G in 23S rRNA gene conferring resistance to clarithromycin in Helicobacter pylori strains. Acta Biochimica Polonica. 2014;61(2).
13. Kargar M, Baghernejad M, Doosti A. Role of NADPH-insensitive nitroreductase gene to metronidazole resistance of Helicobacter pylori strains. DARU: Journal of Faculty of Pharmacy, Tehran University of Medical Sciences. 2010;18(2):137.
14. Bauernfeind A. Comparison of the antibacterial activities of the quinolones Bay 12-8039, gatifloxacin (AM 1155), trovafloxacin, clinafloxacin, levofloxacin and ciprofloxacin. The Journal of antimicrobial chemotherapy. 1997;40(5):639-51.
15. Moore RA, Beckthold B, Wong S, Kureishi A, Bryan LE. Nucleotide sequence of the gyrA gene and characterization of ciprofloxacin-resistant mutants of Helicobacter pylori. Antimicrobial agents and chemotherapy. 1995;39(1):107-11.
16. Gerrits MM, van Vliet AH, Kuipers EJ, Kusters JG. Helicobacter pylori and antimicrobial resistance: molecular mechanisms and clinical implications. The Lancet infectious diseases. 2006;6(11):699-709.
17. Siavoshi F, Saniee P, Khalili-Samani S, Hosseini F, Malakutikhah F, Mamivand M, et al. Evaluation of methods for H. pylori detection in PPI consumption using culture, rapid urease test and smear examination. Annals of Translational Medicine. 2015;3(1).
18. Momtaz H, Souod N, Dabiri H, Sarshar M. Study of Helicobacter pylori genotype status in saliva, dental plaques, stool and gastric biopsy samples. World journal of gastroenterology: WJG. 2012;18(17):2105.

19. Glocker E, Stueger H-P, Kist M. Quinolone resistance in Helicobacter pylori isolates in Germany. Antimicrobial agents and chemotherapy. 2007;51(1):346-9.
20. Mahmoudi L, Sharifzadeh F, Mousavi S, Pourabbas B, Niknam R. Susceptibility testing of Helicobacter pylori: Comparison of E-test and disk diffusion for metronidazole and mutations in rdxA gene sequences of Helicobacter pylori strains. Trends in Pharmaceutical Sciences. 2015;1(4):235-42.
21. Agudo S, Pérez-Pérez G, Alarcón T, López-Brea M. Rapid detection of clarithromycin resistant Helicobacter pylori strains in Spanish patients by polymerase chain reaction-restriction fragment length polymorphism. Revista espanola de quimioterapia: publicacion oficial de la Sociedad Espanola de Quimioterapia. 2011;24(1):32.
22. Vakil N, Megraud F. Eradication therapy for Helicobacter pylori. Gastroenterology. 2007;133(3):985-1001.
23. Ghotaslou R, Leylabadlo HE, Asl YM. Prevalence of antibiotic resistance in Helicobacter pylori: A recent literature review. World journal of methodology. 2015;5(3):164.
24. Debets-Ossenkopp YJ, Pot RG, Van Westerloo DJ, Goodwin A, Vandenbroucke-Grauls CM, Berg DE, et al. Insertion of mini-IS 605 and deletion of adjacent sequences in the nitroreductase (rdxA) gene cause metronidazole resistance in Helicobacter pylori NCTC11637. Antimicrobial agents and chemotherapy. 1999;43(11):2657-62.
25. Khademi F, Poursina F, Hosseini E, Akbari M, Safaei HG. Helicobacter pylori in Iran: A systematic review on the antibiotic resistance. Iranian journal of basic medical sciences. 2015;18(1):2.
26. Abdollahi H, Savari M, Zahedi MJ, DARVISH MS, HAYATBAKHSH AM. A study of rdxA gene deletion in metronidazole resistant and sensitive Helicobacter pylori isolates in Kerman, Iran. 2011.
27. Ramzy I, Elgarem H, Hamza I, Ghaith D, Elbaz T, Elhosary W, et al. Genetic mutations affecting the first line eradication therapy of Helicobacter pylori-infected Egyptian patients. Revista do Instituto de Medicina Tropical de São Paulo. 2016;58.
28. Mirzaei N, Poursina F, Moghim S, Rahimi E, Safaei HG. The mutation of the rdxA gene in metronidazole-resistant Helicobacter pylori clinical isolates. Advanced biomedical research. 2014;3.
29. Thung I, Aramin H, Vavinskaya V, Gupta S, Park J, Crowe S, et al. the global emergence of Helicobacter pylori antibiotic resistance. Alimentary pharmacology & therapeutics. 2016;43(4):514-33.
30. Zhao L-J, Huang Y-Q, Chen B-P, Mo X-Q, Huang Z-S, Huang X-F, et al. Helicobacter pylori isolates from ethnic minority patients in Guangxi: Resistance rates, mechanisms, and genotype. World Journal of Gastroenterology: WJG. 2014;20(16):4761.
31. Khademi F, Sahebkar AH, Vaez H, Arzanlou M, Peeridogaheh H. Characterization of clarithromycin-resistant Helicobacter pylori strains in Iran: A systematic review and meta-analysis. Journal of global antimicrobial resistance. 2017;10:171-8.
32. Karczewska E, Wojtas I, Budak A. Prevalence of Helicobacter pylori primary resistance to antimicrobial agents in Poland and around the world. POSTEPY MIKROBIOLOGII. 2009;48(1):31-41.
33. Magraud F. Helicobacter pylori antibiotic resistance. Prevalence, importance and advance in testing. Gut. 2004;53:1374-84.
34. Francavilla R, Lionetti E, Castellaneta S, Margiotta M, Piscitelli D, Lorenzo L, et al. Clarithromycin-resistant genotypes and eradication of Helicobacter pylori. The Journal of pediatrics. 2010;157(2):228-32.
35. Sadeghifard N, Seidnazari T, Ghafourian S, Soleimani M, Maleki A, Qomi MA, et al. Survey in Iran of clarithromycin resistance in Helicobacter pylori isolates by PCR-RFLP. Southeast Asian Journal of Tropical Medicine & Public Health. 2013;44(1):89-95.
36. Abdollahi H, Savari M, Zahedi MJ, Moghadam SD, Abasi MH. Detection of A2142C, A2142G, and A2143G mutations in 23s rRNA gene conferring resistance to clarithromycin among Helicobacter pylori isolates in Kerman, Iran. Iranian journal of medical sciences. 2011;36(2):104.
37. Eghbali Z, Mojtahedi A, Ansar MM, Asl SF, Aminian K. Detection of 23SrRNA mutations strongly related to clarithromycin resistance in Helicobacter pylori strains isolated from patients in the north of Iran. Jundishapur journal of microbiology. 2016;9(2).

38. Suzuki RB, Lopes RAB, da Câmara Lopes GA, Ho TH, Sperança MA. Low Helicobacter pylori primary resistance to clarithromycin in gastric biopsy specimens from dyspeptic patients of a city in the interior of São Paulo, Brazil. BMC gastroenterology. 2013;13(1):1-7.
39. Vazirzadeh J, Falahi J, Moghim S, Narimani T, Rafiei R, Karbasizadeh V. Molecular assessment of resistance to clarithromycin in Helicobacter pylori strains isolated from patients with dyspepsia by fluorescent in situ hybridization in the center of Iran. BioMed Research International. 2020;2020.
40. Trespalacios-Rangél AA, Otero W, Arévalo-Galvis A, Poutou-Piñales RA, Rimbara E, Graham DY. Surveillance of levofloxacin resistance in Helicobacter pylori isolates in Bogotá-Colombia (2009-2014). PLoS One. 2016;11(7):e0160007.
41. Zaki M, Othman W, Ali MA, Shehta A. Fluoroquinolone-resistant Helicobacter pylori strains isolated from one Egyptian University Hospital: molecular aspects. J Microbiol Antimicrobial Agents. 2016;2:26-31.
42. Ontsira Ngoyi EN, Atipo Ibara BI, Moyen R, Ahoui Apendi PC, Ibara JR, Obengui O, et al. Molecular detection of Helicobacter pylori and its antimicrobial resistance in Brazzaville, Congo. Helicobacter. 2015;20(4):316-20.
43. Kipritci Z, Gurol Y, Celik G. Antibiotic Resistance Results of Helicobacter pylori in a University Hospital: Comparison of the Hybridization Test and Real-Time Polymerase Chain Reaction. International Journal of Microbiology. 2020;2020.

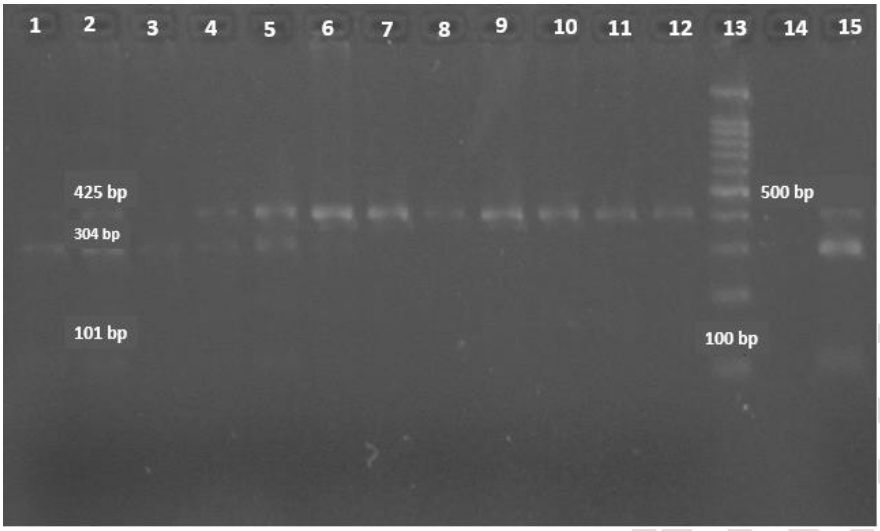

Figure 1. PCR-RFLP analysis of 425bp fragment 23srRNA gene; No 1 to 6 and 15 clarithromycin-resistant strains using *BsaI* enzyme with A2143G mutation; No 7 to 12: Clarithromycin-sensitive strains; No 13: DNA marker 100bp; No 14: without sample

Table 1. Primer pairs for PCR amplification of *ureC*, *gyrA*, *rdxA*, *23r RNA* genes in *Helicobacter 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	