

PCR-RFLP detection of point mutations in 23s RNA gene responsible for clarithromycin resistance in *Helicobacter Pylori* isolates of Ahvaz, Iran

Abstract

Helicobacter pylori is the most common human pathogen and is the main cause of chronic gastritis, gastric ulcer and gastric malignancies. Clarithromycin is the key antibiotic in the treatment of *Helicobacter pylori* infections. Clarithromycin resistance is due to point mutations in the variable region of 23s rRNA peptidyl transferase, and the most important of which is the displacement of A-G at position 2143, 2142 (A2143G, A 2142G). The aim of this study was to determine the resistance of *Helicobacter pylori* isolated strains to Clarithromycin and subsequently to detect Clarithromycin resistant point mutations. 224 gastric biopsies were collected by endoscopy during 2016. Samples were homogenized and immediately cultivated on Colombia agar containing sheep blood and various antibiotics at 37°C under microaerophilic conditions. By using biochemical methods and identifying the glmM gene, *Helicobacter pylori* strains were confirmed. Standard strain ATCC26695 was used as control. The prevalence of resistance to clarithromycin was determined by E-test method based on CLSI standard. The MIC \geq 1 µg/ml was considered as resistant. Existing point mutations were identified by the PCR-RFLP method followed by sequencing of 224 endoscopic specimens, 93 strains of *H. pylori* were identified. 49 strains (52.7%) were resistant to clarithromycin. Prevalence of A2143G, A2142G, A2142C was 77.5%, 4.1% and 4.1%, respectively. 7 samples (14.2%) contained 2 types of mutations. The high prevalence of *Helicobacter pylori* resistance to clarithromycin reported in this study is a serious concern. This necessitates performing of genotypic and phenotypic Antibiotic susceptibility methods. In this study, the relationship between the resistance of *Helicobacter pylori* strains to clarithromycin and point mutations in 23srRNA was fully demonstrated.

Keywords: *Helicobacter pylori*, clarithromycin, point mutation, PCR-RFLP

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Introduction

Helicobacter pylori is a spiral-shaped gram-negative bacterium that infects half of the world's population and is the main cause of chronic gastritis, gastric ulcer and gastric malignancies including gastric adenocarcinoma and mucus-associated lymphoid tissue (MALT) lymphoma.^{1,2} *Helicobacter pylori* is the most common human pathogen and a successful pathogen and has been specifically transmitted to humans in early childhood and through the family and can persist for decades in the gastric mucosa despite inherent and acquired immune responses.^{3,4} The prevalence of bacteria in developing countries is between 70%-90%, and in developed countries it is 35-40%.⁵ During a *Helicobacter pylori* infection, persistent inflammation and abnormal epithelial proliferation might be the major causes of *Helicobacter pylori* associated gastric disorders.^{6,7} Successful bacterial eradication is an effective method not only for the improvement of peptic ulcer, but also for gastric cancer. Successful bacterial elimination reduces the inflammation and atrophy of the gastric mucosa and can be effective in reducing the incidence of peptic ulcer and gastric cancers associated with *Helicobacter pylori* infection.⁸ *Helicobacter pylori* eradication therapy is usually a combination of one proton pump inhibitor with several antibiotics such as clarithromycin, metronidazole, amoxicillin and tetracycline.⁹⁻¹¹ Expecting an increase in the resistance of *Helicobacter pylori* to these antibiotics is due to an increase in the number of treated patients, incomplete contribution of patients during treatment and an increase in the use of antibiotics in recent years.^{9,10} The prevalence of resistance to mentioned drugs in different

geographical areas is very different. Therefore, prior to administration of any drug for the treatment of *Helicobacter pylori* infections, it is necessary to determine the regional susceptibility pattern for each geographical region.^{9,12,13} Several factors have contributed to reducing the efficacy of current treatments on *Helicobacter pylori* infections, but antibiotic resistance is the first factor of this decline in the world.¹⁴

Helicobacter pylori rapidly becomes resistant to many antibiotics after treatment, which leads to incomplete elimination of bacteria and rapid return of disease.^{10,12} Clarithromycin is the key antibiotic in the treatment of *Helicobacter pylori* infection, which is due to the bactericidal effect of the drug on the bacteria.^{11,15,16} Resistance to Clarithromycin is due to the lack of binding of the drug to the 23s rRNA of the bacterial ribosome, which is caused by the occurrence of point mutations in the variable domains of the peptidyl transferase of 23s rRNA. Several point mutations have been reported in about clarithromycin resistance, but the most important of which is the transition of A-G at position 2143 and 2142 (A2143G, A2142G).^{11,15-18} Although these mutations are responsible for most of the primary resistance to clarithromycin, each of them is specifically associated with different MIC values. The effect of the A2143G mutation on the outcome of the treatment is much greater than other mutations and is associated with a lower eradication rate.^{19,20} The resistance of *Helicobacter pylori* strains to antibiotics is determined in clinical specimens by standard methods such as Agar dilution, Microbroth dilution, Disk diffusion and E-test. These phenotypic methods are highly effective in isolating susceptible and resistant strains, but are

time consuming and unable to detect resistance-inducing mutations. Among these methods, the E.Test is a simple, and easy, but expensive method to determine the MIC for most antibiotics. Genotypic methods such as Real Time PCR, Sequencing, RFLP require lesser time than phenotypic methods, and are capable of identifying a variety of resistance-causing mutations. Genotypic methods have limited efficiency because of insufficient data about the mechanism of bacterial resistance to some antibiotics.^{11,16,17} Among genotypic methods, PCR-RFLP is highly efficient in detection of 23s rRNA gene mutations and its application to find resistance to clarithromycin leads to more effective and better management of *Helicobacter pylori* infections.¹⁶

Aim of study

The aim of this study was to determine the resistance of *Helicobacter pylori* isolates to clarithromycin and subsequently to identify the mutations responsible for clarithromycin resistance.

Materials and methods

Sample collection, isolation of strains and storage in a freezer

224 gastric biopsy samples taken from patients referring to the Endoscopy Division Unit of Imam and Mohr hospitals in Ahvaz city (southwest of Iran) during 2016 were collected in sterile tubes containing BHI broth (Merck-Germany) and 5% non Activated fetal calf serum (Baharafshan-Iran) and transferred on ice to the Department of Microbiology, Faculty of Medicine. The samples were fragmented and homogenized on a sterile glass slide by a bistoury blade and were cultivated on Colombia agar (Merck-Germany) containing 7% fresh sheep blood, 5% inactivated fetal calf serum (Baharafshan-Iran) And several antibiotics including Vancomycin (10mg/l-Sigma-

USA), Amphotericin B (4mg/L-Sigma-USA), Trimethoprim (5mg/L-Sigma-USA) and Polymyxin (10mg/L-Sigma-USA). The inoculated plates were incubated for 3 to 5 days at 37°C under microaerophilic conditions (7% CO₂, 5% oxygen, saturated humidity) produced by Anaerocult C system (Merck-Germany).²¹ The isolates were recognized as *H. pylori* by urease, catalase, oxidase positive and gram negative staining tests. Afterward a bacterial suspension was prepared in the BHI broth (Merck-Germany), containing 30% sterile glycerol and 10% inactivated fetal calf serum and were stored in -80°C. Before the endoscopy, a questionnaire was filled out by the patients. Also, patients who took clarithromycin for two weeks before endoscopy were excluded from the study.²¹

DNA extraction

DNA was extracted from all 93 *H. pylori* grown isolates using DNA extraction kit (Roche-Germany) according to the manufacturer's instruction. To prevent the possible error in the diagnosis of bacterium, amplification of glmM gene was done by PCR method. Extracted DNA stored in -20°C until the test was performed.^{11,22} First, to confirm the isolation of *Helicobacter pylori*, glmM (ureC) gene was amplified by PCR, resulting in a 294bp fragment. This gene is a house keeping gene which found in all strains of *Helicobacter pylori*. PCR conditions included denaturation at 94°C for 60 seconds, annealing at 57°C for 90 seconds, and extension at 72°C for 120 seconds in 40 cycles. The final extension at 72°C for 7 minutes was done to complete the PCR process. Master mix (Sinaclon-Iran) of reaction contains 800 μmol of dNTPs, 2.5 μg mgCl₂, 50 μm buffer, 2.5 μg of Taq enzyme, 1.8 μl of each of the primers (metabion-Germany) and 0.7 μl template DNA in total volume of 20 μl. Obtained PCR products were electrophoresed on 2% agarose gel with ethidium bromide.²³ The name and sequence of the primers, the size of PCR product and the name of the gene have been shown in (Table 1).

Table 1 Product size and primers used for amplification of GLMM gene

Gene name	Primer sequence	Product size(bp)	Reference
glmM	F- AAGCTTTTAGGGGTGTTAGGGGTTT	294	24
	R- AAGCTTACTTCTAACACTAACGC		

Evaluation of strains resistance to clarithromycin using E. test

For *H. Pylori* isolated strains, the antibiogram test was performed using the E. test method with clarithromycin strips (Lioflichem-Italy) on muller-Hinton agar (Merck-Germany) enriched with 7% sheep blood and MIC of Clarithromycin is determined for all strains. At first, a suspension of pure *H.Pylori* colonies in sterile physiologic saline with turbidity equals to 3 McFarland tube (9x10⁸ CFU/ml) was prepared, and inoculated on enriched Muller-Hinton agar with a cotton swab immersed in bacterial suspension. After drying the surface of the medium, the clarithromycin strips were placed on the inoculated medium and were incubated at 37°C for 72 hours under microaerophilic conditions. If the MIC values were greater than or equal to one microgram per liter, the strains were considered resistant to Clarithromycin.²¹

Table 2 Product size and primers used for amplification of peptidyl transferase gene

Gene name	Primer sequence	Product size(bp)	Reference
Peptidyl transferase of V domain of 23srRNA	F-CCACAGCGATGTGGTCTCAG	425	26
	R-CTCCATAAGAGCCAAAGCCCC		

Amplification of variable domain of peptidyl transferase gene

The amplification of variable domain of peptidyl transferase gene by PCR method involves the initial denaturation for 4 minutes at 94°C and 32 cycles of denaturation at 94°C for 40 seconds, annealing at 61.5°C for one minute, and extension at 72°C for one minute. At the end of the reaction, a final extension process takes place for 7 minutes at 72°C. The master mix of reaction (Sinaclon-Iran) contains 16.5 μl of distilled water, 3.3 μl buffer, 2.5 μl dNTPs, 0.2 μl of Taq enzyme, 1.8 μl of each of the primers (metabion-Germany) and 0.7 μl of the template DNA in a total volume of 25 μl. PCR products were electrophoresed on 1.5% agarose gel containing Safe Stain (Sinaclon-Iran).²⁵ The name and sequence of the primers, the size of PCR products and the name of the gene have been shown in Table 2.

Determination of point mutations in Clarithromycin resistant strains by PCR-RFLP

Among the strains that were resistant to clarithromycin, PCR-RFLP was performed to identify point mutations. First, a fragment of 425bp length from the variable domain of 23s rRNA peptidyl transferase was amplified by PCR. Afterward PCR products were digested using BSAI and BBSI (Thermo scientific-Lithuania) restriction enzymes and obtained fragments were detected by electrophoresis on 1.5% agarose gel. Using the size and number of fragments obtained, the point mutations responsible for the resistance to clarithromycin in strains were defined resistant in E-test has been identified.^{9,16,25,26} Also, if there are other types of point mutations, these mutations will be identified by the Sequencing method using ABI system (Abott-USA). For enzymatic digestion of PCR products gained from peptidyl transferase variable region amplification, mixed 2µl of PCR product with 8µl of distilled water and 2µl of digestive enzymes, for the BsaI enzyme, the mixture was incubated for 30 minutes at 37°C and for BBSI, 24hours at 37°C. Then digested products were electrophoresed in 1.5% agarose.^{9,15,16}

Results & discussion

In this cross-sectional study, 224 patients with gastric problems referring to the endoscopy division unit of Imam and Mehr hospitals of Ahwaz city were examined. Of 224 endoscopic samples, 93 strains (41.5%) of *Helicobacter pylori* were isolated by culture (Figure 1). Of these, 51(55%) were isolated from men and 42 (45%) of female. Patients age for men were 25-82years old and women were 24-79years old. The isolates were recognized as *H. Pylori* by urease, catalase, oxidase positive and gram negative staining tests. To confirm *Helicobacter pylori* strains, a PCR test was conducted to amplify the UREC gene (glmM) (Figure 2). The clinical diagnosis given by endoscopy includes gastritis, peptic ulcer, duodenal ulcer and gastric cancer. Results showed that 49 isolates (52.7%) of 93 strains of *H. Pylori* were detected resistant to clarithromycin by E.Test. Resistant strains have a MIC of 1 to 96µg/ml and all susceptible strains have MIC <1µg/ml. In the next step, a 425bp fragment from the variable region of 23srRNA was amplified by the PCR method. The resultant PCR product was affected by the endonuclease enzymes of BSAL and BBSL. The PCR product from strains containing A2143G mutation when affected by the BSAL enzyme produce two fragments of 304bp and 101bp (Figure 3), and strains contains A2142G mutation when affected by the BBSL enzyme produce two fragments of 332bp and 93bp (Figure 4). All Clarithromycin resistant strains produced 425bp fragment in the PCR. In this study, among 49 clarithromycin resistant isolates were affected by enzymes, 38 strains (77.5%) had A2143G mutation and 2 strains (4.1%) had A2142G mutation. Seven strains (14.2%) had both A2143G and A2142G mutations. Sequencing was performed for two strains lacking both mutations, but were resistant to Clarithromycin in the E.Test method, sequences of these strains were analyzed using MEGA7 software: molecular evolutionary genetics analysis version 7 for bigger datasets (2016), and Both strains had A2142C mutations. *Helicobacter pylori* standard strain 26695 was used as control strain in the study.

Helicobacter pylori resistance to antibiotics is expanding in most parts of the world, including Iran. Clarithromycin is one of the most important components used in the treatment regimens of *Helicobacter pylori* infections, and expanded resistance to this drug has become

a serious concern for the eradication of this bacterium.²⁷ The use of culture-based methods to determine the sensitivity of the drug to *H. pylori* is very difficult, because the bacteria are fastidious and require a long time to grow on selective culture media.^{28,29} *Helicobacter pylori* resistance to clarithromycin varies in different parts of the world, for example, in southern Europe 30% and in the United States, between 15% and 30%,¹⁸ While in the Asian countries such as Vietnam and Iran 42.4% & 31.7% respectively.^{27,30} In this study, isolated *Helicobacter pylori* strains displayed resistance to clarithromycin in 49/93 cases (52.7%) using the E.Test method, which is consistent with some studies in different parts of the world and incompatible with some others. For example, Fan et al. In a study in Vietnam in 2015, reported the resistance rate of *Helicobacter pylori* strains to clarithromycin 42.4%,³⁰ which is close to the results of this study. Also, in a study by Rashid et al.³¹ In Pakistan in 2014, resistance to clarithromycin was 47.8%,³¹ which is largely similar to the current study. Asaka & colleagues in Japan in 2010 also reported the resistance rate to clarithromycin of 27.7%.³²

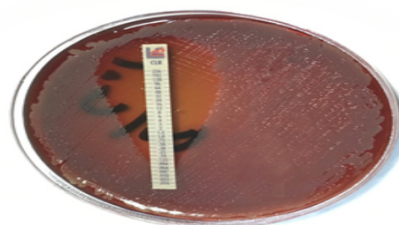


Figure 1 *H.Pylori* sensitive strain to clarithromycin (E.Test).

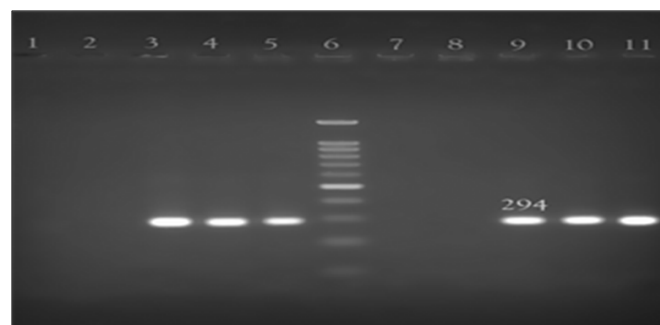


Figure 2 Gel electrophoresis of PCR products of 23srRNA Gene. Lane 6, 100bp DNA ladder, lanes 3, 4, 5, 9, 10, 11 *H. Pylori* isolates. Lane 1 negative control, lanes 2, 7, 8 Non *H.Pylori* bacteria.

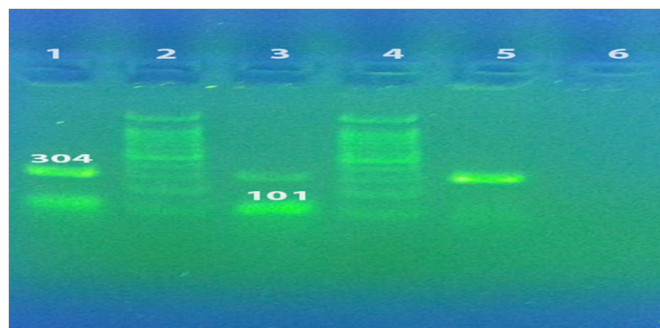


Figure 3 RFLP analysis of 23S r RNA to detect the A2143G mutation using BsaI restriction enzyme. Lanes 1, 3 and 5: Digestion products of two clarithromycin resistant strains with BsaI. Lane 2 & 4 100bp DNA ladder, lane 6 negative control.

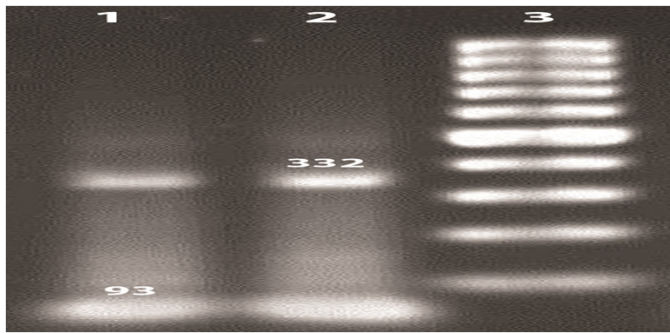


Figure 4 RFLP analysis of 23S r RNA to detect the A2142G mutation using BbsI restriction enzyme. Lanes 1 and 2: Digestion products of two clarithromycin resistant strains with BbsI, lane 3 100bp DNA ladder.

In different regions of Iran, *Helicobacter pylori* resistance to clarithromycin was different, for example, in Tehran in 2005, this rate was reported by Mohammadi et al.,³³ 20% while reported rate by Abdullahi et al.²⁷ was 31.7%.²⁷ According to the high level of *H. pylori* resistance to clarithromycin in this study it seems to be due to the high consumption of macrolides in the treatment of respiratory diseases at this point in Iran because of severe respiratory disorders and also the high use of clarithromycin in the treatment of *Helicobacter pylori* infections because of high incidence of gastric disorders in residents of the studied area. Versalovich et al. were the first researchers to show that *Helicobacter pylori* resistance to clarithromycin is associated with point mutations in the variable region 23srRNA. They also discovered that point mutations that cause the A to G transition in the 23srRNA sequence occur at 2143 and 2142 positions.²⁷ In this study, using PCR-RFLP method, two common mutations of A2143G and A2142G were detected in *Helicobacter pylori* clarithromycin resistant strains and in cases where these mutations were not found, other mutations were identified by sequencing method. In all resistant strains, at least one mutation from point mutations exists and therefore, complete association between the resistance of *Helicobacter pylori* strains to clarithromycin and the presence of point mutations in this study was confirmed. The prevalence of A2143G and A2142G mutations in this study was 77.5% and 4.1% respectively, which was compatible with the results of Nishizawa et al.³² in Japan, Agudo et al.¹⁵ In Spain and Doroud et al.²⁶ in Iran,^{15,26,32} but differs from the results of the studies by Kelzwick et al. in Poland and De Francesco et al.¹³ In Italy.^{13,16} This indicates the existence of a geographical difference in the level of resistance to clarithromycin and this fact highlights the importance of determining the resistance of regional strains of *Helicobacter pylori* to the selection of appropriate therapeutic methods. It should be noted that the mutations reported in this study are not the only point mutations known in the resistance of *Helicobacter pylori* to clarithromycin, and several other point mutations have been reported elsewhere in the world. In addition, a small part of the *Helicobacter pylori* resistance to clarithromycin is due to the Efflux system, which makes bacteria send out the macrolide.

Conclusion

The high prevalence of *Helicobacter pylori* resistance to clarithromycin reported in this study is a serious concern that should be considered by physicians in determining the patient's drug regimen. This necessitates performing of genotypic and phenotypic Antibiotic susceptibility methods. In this study, the relationship between the resistance of *Helicobacter pylori* strains to clarithromycin and point mutations in 23srRNA was fully demonstrated. Also, the results of

this study showed that the resistance of *Helicobacter pylori* strains to clarithromycin in Iran has been rising and this has reduced the effectiveness of this drug.

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Ethical approval

This study was approved by ethics committee of the Jundishapur University of Medical Science in Ahvaz-southwest of Iran (IR.AJUMS.REC.1395.581).

Conflict of interest

Author declares that there is no conflict of interest.

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