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# Prevalence of *A2143G* and *A2144G* Point Mutations Responsible for Clarithromycin Resistance among *Helicobacter pylori* Strains in Bushehr, Iran

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## Abstract

**Background:** Resistance to clarithromycin in *Helicobacter pylori* has become one of the most important reasons for failure of antibiotic eradication therapies. This resistance is predominantly caused by point mutations in the peptidyl transferase region of 23S rRNA.
**Objectives:** The aim of this study was to determine the prevalence of the A2143G, A2144G, and A2143C point mutations among *H. pylori* strains from gastric biopsy specimens in Bushehr, in the southwest of Iran.
**Patients and Methods:** Gastric biopsy specimens were obtained from patients with upper gastrointestinal symptoms during endoscopy. Fluorescent in situ hybridization (FISH) using oligonucleotide probes was applied to detect the point mutations responsible for clarithromycin resistance in *H. pylori*.
**Results:** Of the 135 *H. pylori*-positive specimens, two harbored strains with the A2143G mutation and nine contained strains with the A2144G mutation. Thus, the prevalences of the A2143G and A2144G point mutations were 1.5% and 6.7%, respectively. The A2143C point mutation was not found.
**Conclusions:** The prevalences of the point mutations A2143G and A2144G were low in our geographic area. Based on the findings of this study, clarithromycin still seems to be a useful antibiotic for initial treatment regimens in Bushehr, Iran.

*Keywords:* Clarithromycin Resistance, Point Mutation, *Helicobacter pylori*

## 1. Background

*Helicobacter pylori* is a highly motile, curved or spiral-shaped Gram-negative bacterium that has been isolated from the human stomach in all parts of the world. *H. pylori* colonization is associated with various upper gastrointestinal diseases, including gastritis, peptic ulcers, gastric carcinoma, and gastric lymphoma (1). These diseases may regress or be completely cured after therapy with antibiotics to eradicate *H. pylori*. Clarithromycin, amoxicillin, metronidazole, tetracycline, and levofloxacin are among the antibiotics utilized for the eradication of *H. pylori* infections (2, 3). However, resistance to antibiotics is a major problem in effective treatment (2). Clarithromycin is the most effective antimicrobial used for the treatment of *H. pylori* infection, and resistance to this drug is the major cause of treatment failure (3-5). The prevalence of clarithromycin resistance may vary in different geographical regions, and continuous monitoring of the clarithromycin resistance rate in each region is important for designing the most effective therapies (6, 7).

Clarithromycin attaches to the peptidyl transferase region of 23S rRNA and inhibits protein synthesis (5). Resistance to clarithromycin in *H. pylori* is caused predominantly by point mutations in the peptidyl transferase center of 23S rRNA (5, 8, 9). These mutations prevent the binding of clarithromycin to the 50S subunit of the ribosome (6). It has been proven that fluorescent in situ hybridization (FISH) by the use of rRNA-targeted oligonucleotide probes is an accurate and convenient molecular technique for the detection of three 23S rRNA point mutations responsible for clarithromycin resistance, in which the adenine residues at positions 2143 and 2144 are replaced by guanine (A2143G and A2144G) or cytosine (A2143C) (8, 10, 11). The FISH technique was used in this study.

## 2. Objectives

The aim of this study was to determine the prevalence of the A2143G, A2144G, and A2143C point mutations among *H. pylori* strains from gastric biopsy specimens in Bushehr, southwest Iran.

## 3. Patients and Methods

### 3.1. Collection and Preparation of Specimens

This study was approved by the ethics committee of Bushehr University of Medical Sciences. The specimens used in this study have been previously investigated to determine the prevalence of *H. pylori* infection (12). Between May 2011 and June 2012, gastric biopsy specimens were collected from 310 patients with various upper gastrointestinal symptoms, who were referred for endoscopy to Bentolhoda hospital, a major university hospital in the city of Bushehr, southwest Iran. The investigation of the point mutations was done on 135 *H. pylori*-positive samples from patients, of whom 62 (45.9%) were male and 73 (54.1%) were female. The mean ± standard deviation for age was 46.59 ± 17.14 years. Patients previously treated for eradication of *H. pylori* infection (2), who had active gastrointestinal bleeding (13), who had a history of gastric surgery (7), or who declined to participate were excluded from the study. The patients who agreed to participate signed consent forms.

The gastric biopsy samples were obtained from the antrum and corpus of the stomach during routine endoscopy and placed into 10% formalin for fixation. Thereafter, the specimens were embedded in paraffin, cut into sections, and placed on glass slides, then incubated overnight at 55°C. The tissue sections were subsequently deparaffinized with hexane (Merck, Darmstadt, Germany) and absolute ethanol (Merck, Darmstadt, Germany) as previously described (14, 15). The slides were then ready for investigation by FISH.

### 3.2. Probes

The oligonucleotide probes used in this work were synthesized and 5'-labeled with fluorochromes Fluo or Cy3 (Metabion, Martinsried, Germany). These probes have been formerly described and evaluated by Trebesius et al. (8). Probe Hpy-1 (5'-CAC ACC TGA CTG ACT ATC CCG-3') specifically hybridizes a 16S rRNA region of the species *H. pylori* and detects this organism. Hpy-1 was 5'-labeled with Fluo, which exhibits a green color. Probes ClaR1 (5'-CGG GGT CTT CCC GTC TT-3'), ClaR2 (5'-CGG GGT CTC TCC GTC TT-3'), and ClaR3 (5'-CGG GGT CTT GCC GTC TT-3') detect the 23S rRNA point mutations responsible for clarithromycin resistance, and were used for the detection of clarithromycin-resistant strains of *H. pylori* in this study. Probes ClaR1, ClaR2, and ClaR3 detect the A2143G, A2144G, and A2143C point mutations, respectively. The 5' ends of these three probes were labeled with fluorochrome Cy3, which emits a red fluorescent signal. Probe ClaWT (5'-CGG GGT CTT TCC GTC TT-3'), which targets the 23S rRNA of wild-type *H. pylori*, was unlabeled (8).

### 3.3. FISH Protocol

FISH was carried out according to a protocol described elsewhere (8, 11, 15). For the first step, hybridization of all of the samples was performed using a mixture of five probes: Hpy-1, ClaR1, ClaR2, ClaR3, and ClaWT. Hybridization was done at 46°C for 90 minutes with a hybridization buffer (8), and then stringent washing was performed using a washing buffer (8). Thereafter, DNA was stained with 4′, 6-diamidine-2′-phenylindole dihydrochloride (DAPI; Roche, Mannheim, Germany). The slides were visualized and analyzed with an epifluorescence microscope (Nikon 80i, Tokyo, Japan) equipped with a filter set. Microscopy was performed in a blinded manner by two persons. Each examination was done twice (16).

### 3.4. Identifying Point Mutations by FISH

Next, the specimens that had shown a positive result with the mixture of the probes were also examined separately with each probe ClaR1, ClaR2, or ClaR3, accompanied by probe Hpy-1, to discriminate between single point mutations. The FISH procedure was performed as described above.

### 3.5. Statistical Analysis

To analyze the data for the frequency, mean, and standard deviations, the Chi-square test (to check the association between sex and clarithromycin resistance), and the Mann-Whitney U-test (to compare the mean age of the patients infected with mutant strains and of the remaining infected patients) were used. A $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS version 13 (SPSS Inc., Chicago, IL, USA).

## 4. Results

The results of the examination of the gastric biopsy specimens for the detection of the 23S rRNA point mutations responsible for clarithromycin resistance in *H. pylori* are summarized in Table 1.

The specimens were taken from 310 patients suffering from various upper gastrointestinal symptoms. One hundred thirty-five of these gastric specimens were *H. pylori*-positive. The strains in 124 of the 135 *H. pylori*-containing

**Table 1.** Results of the Examination of Gastric Biopsy Specimens via FISH for the Identification of Point Mutations in *H. pylori*

| Number of specimens | Hpy-1 | Hybridization With Probe/Detected Point Mutation | | |
|---|---|---|---|---|
| | | ClaR1/A2143G | ClaR2/A2144G | ClaR3/A2143C |
| 175 | - | - | - | - |
| 124 | + | - | - | - |
| 2 | + | + | - | - |
| 9 | + | - | + | - |

biopsy specimens did not hybridize to any of the ClaR probes, and therefore did not have the point mutations studied in our investigation. Of the 135 *H. pylori*-positive specimens, 11 were infected with mutant (resistant) strains, of which two harbored strains with the A2143G point mutation and nine contained strains with the A2144G point mutation. Therefore, the prevalences of the A2143G and A2144G point mutations among *H. pylori* strains from gastric biopsy specimens in Bushehr were 1.5% and 6.7%, respectively. No strain with the A2143C point mutation was observed among these specimens.

Out of the 11 patients infected with mutant strains, eight were female and three were male; however, no significant association was found between sex and clarithromycin resistance (P = 0.23). There was no significant difference between the mean age of the patients infected with mutant strains (46.64 $\pm$ 22.66) and of the remaining infected patients (46.59 $\pm$ 16.68) (P = 0.86).

## 5. Discussion

In our study, the prevalence of the A2143G point mutation was 1.5% (2/135) and the prevalence of A2144G was 6.7% (9/135). In other words, among the mutations found in our investigation, 18.2% were A2143G and 81.8% were A2144G. Various results regarding the prevalence of these point mutations have been reported in other parts of Iran. In a study done by Mohammadi et al. (17), 79% of the clarithromycin-resistant strains from Tehran had A2143G. In another study in Sari (northern Iran), 31 of 147 *H. pylori* strains were detected as clarithromycin-resistant; the mutations were A2143G in 30 strains and A2144G in one strain. Therefore, in contrast to our results, the prevalence of the A2143G mutation was higher than of the A2144G mutation (7). An investigation in Ilam (western Iran) revealed that 16% of *H. pylori* strains were resistant to clarithromycin, and all of the resistant isolates had the mutation A2143G (18). In Kerman (southeast Iran), three out of 63 *H. pylori* isolates harbored the A2143G point mutation (19). Also, various results have been reported in other countries. In a study in Malaysia, the prevalence of the point mutation A2143G among 105 *H. pylori* strains was 1.9% (2/105), which is close to its prevalence in our work (6). In a study from Turkey, of 37 *H. pylori*-positive specimens, four (10.8%) had the A2143G mutation and 11 (29.7%) had the A2144G mutation. Thus, similar to our results, the rate of the A2144G mutation was higher than that of A2143G, but the rates of both mentioned mutations were higher than their corresponding rates in our study (20). Moreover, in an investigation among Tunisian patients, 37 of 273 *H. pylori* isolates harbored the A2143G mutation, which was more prevalent than in our study (21). It is understood from the foregoing articles that the prevalence of the point mutations vary widely from region to region. The reasons for these different results are difficult to explain, but may be due to differences in the prescription and administration of clarithromycin (6, 21). In fact, the primary risk factor for resistance to clarithromycin is the previous use of clarithromycin or other macrolides (20). It should be emphasized that cross-resistance to different types of macrolides can be developed (5, 20).

It should also be mentioned that the point mutations explored in our study are not the only mutations associated with clarithromycin resistance, and there may be some others related to such resistance (19). For instance, the A2142G point mutation has been investigated in many studies (4-6, 17, 19, 21, 22). Moreover the A2142C point mutation has been studied by some researches (17, 19).

A limitation of our study was that we did not have oligonucleotide probes for the detection of the A2142G and A2142C point mutations. However, it is noteworthy that the prevalence of these mutations at position 2142 is much lower than that of A2143G. The global average mutation frequency for the A2143G and A2142G mutations are 69.8% and 11.7%, respectively (23). In many studies, the point mutations A2142G (13, 18, 23, 24) and/or A2142C (4, 13, 21, 24) were not found, and numerous investigations reported that A2143G was the only detected mutation or the most prevalent (4, 5, 13, 17, 18, 21-25). On the other hand, it has been found that the presence of the A2143G point mutation, but not of A2142G or A2142C, significantly lowered the *H. pylori* eradication rate (26, 27). De Francesco et al.

(26) reported that clarithromycin resistance related to the A2142G point mutation seemed to be clinically meaningless because the eradication rates were higher than 90% in both the mutated and the wild strains. The different therapeutic outcomes observed among various point mutations may be due to diverse three-dimensional changes on the binding site, leading to different losses of affinity for clarithromycin. It should be kept in mind that, firstly, the A2143G mutation is associated with a low eradication rate of *H. pylori* infections, and secondly, although this mutation is reported as the most common one in many articles, it has a low prevalence in Bushehr. This supports the continuation using of clarithromycin in initial treatment regimens in this region.

In the present study, of the 11 patients infected with mutant strains, eight were female and three were male; however, no significant association was found between sex and clarithromycin resistance, which might be due to the small number of patients infected with mutant strains. Controversial results concerning an association between clarithromycin resistance and sex have been published. Onder and colleagues found no association between clarithromycin resistance and sex (28). In contrast, Toracchio and Marzio (29), as well as Meyer et al. (30), reported a significant association between female sex and resistance to clarithromycin; however, it was not clear why females were more likely to be infected with clarithromycin-resistant strains (30).

In our study, there was no significant difference between the mean age of the patients infected with mutant strains and of the remaining infected patients. In some studies, clarithromycin resistance was not statistically associated with age (28, 29). However, in a study by Meyer et al. (30), clarithromycin resistance was significantly associated with older age; the authors argued that this was because elderly patients are more likely to suffer respiratory tract infections, for which macrolides are most often used. In another study, the rate of clarithromycin resistance was significantly higher in children than in adults, and these authors similarly mentioned that children have had more exposure to macrolides because of treatment for respiratory infections (5).

In conclusion, the point mutation A2143C was not found at all in this study, and the prevalences of the point mutations A2143G and A2144G were low in our geographic area. This finding supports the continuation using of clarithromycin in initial treatment regimens in Bushehr.

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## Footnotes

**Authors' Contribution:** Study concept and design: Saeed Tajbakhsh; analysis and interpretation of data: Saeed Tajbakhsh, Niloofar Motamed, and Abbas Bahador; drafting of the manuscript: Saeed Tajbakhsh; critical revision of the manuscript for important intellectual content: Saeed Tajbakhsh, Niloofar Motamed, and Abbas Bahador; specimen collection: Seyed Masoud Tabib; carrying out of laboratory tests: Saeed Tajbakhsh, Jamal Falahi, and Somayyeh Gharibi; acquisition of data: Saeed Tajbakhsh, Jamal Falahi, Seyed Masoud Tabib, and Somayyeh Gharibi; statistical analysis: Niloofar Motamed.

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