ISSN: 2538-5887; Pathobiology Research. 2022;26(2): 69-74

High clarithromycin resistance of *Helicobacter pylori* strains isolated from naive patients

ARTICLE INFO

Article Type
Original Research

Authors

Shiva Rezaei[1]

Amin Talebi Bezmin Abadi[1]

Ashraf Mohabatti Mobarez[1]

1- Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

***Corresponding author:**

A.Talebi Bezmin Abadi

Tarbiat Modares University, Department of Bacteriology, P. O. Box 14115-111, Tehran, Iran

Amin.talebi@modares.ac.ir

ABSTRACT

Introduction This study investigates the susceptibility and resistance of *H. pylori* isolates recovered from gastroduodenal patients naive to clarithromycin.

Materials and Methods To that end, *H. pylori* strains were isolated from antral biopsies of pretreatment patients, and antral biopsy specimens were subsequently cultured. Presumptive *H. pylori* colonies were also confirmed on enriched Brucella agar by biochemical tests, including catalase, oxidase, rapid urease, and the standard polymerase chain reaction (PCR) method. The antimicrobial susceptibility testing was performed by standard disk diffusion methods according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Then, the Epsilometer test (E-test) was used to determine the minimal inhibition concentration (MIC).

Results Of 180 samples, 80 (44%) were positive for urease and were included for further analysis. 65 were also positive in culture base method. The sensitivity test indicated a 23% resistance rate to clarithromycin among the six clarithromycin-resistant strains: four have a common form of the A2143G mutation, and two have A2142G mutation.

Conclusion The PCR indicated that the level of resistance to clarithromycin was very similar to the resistance level in Iran.

Keywords: Helicobacter pylori; resistance; clarithromycin, susceptibility test.

INTRODUCTION

Helicobacter pylori (H. pylori) is a microaerophilic bacterium that colonizes the human gastric mucosa and stays lifelong if untreated. This infectious agent is the most common cause of chronic gastritis. Also, it has been classified by the International Agency for Research on Cancer (IARC) as the cause of gastric cancer since 1994 [1]. Various guidelines introduce antibiotic-oriented treatment strategies for H. pylori infection cases. The Maastricht V/Florence Consensus Report and the Toronto Consensus Statements recommend clarithromycin (CLR) as the main antibiotic in triple therapy for H. pylori eradication [2, 3]. Similarly, many studies have shown the beneficial effects of this therapeutical procedure, although universal high efficacy achievement is diminished. The effective elimination of the bacteria can be helpful for patients with gastric cancer and peptic ulcer. The prevalence of H. pylori resistance to CLR is increasing worldwide, specifically in Iran. This prevalence has led to a significant increase in treatment failures [3, 4]. Clarithromycin resistance associated with a 23s ribosomal RNA (rRNA) point mutation is the most crucial factor in the failure of H. pylori eradication [5]. The European guideline states that clarithromycin-containing triple therapy without susceptibility testing should be abandoned when the clarithromycin resistance rate in the region is higher than 15% [6]. Hence, an updated profile of prescribed antibiotics against H. pylori is needed. Disk diffusion and

Epsilometer methods were utilized to achieve our central goals. It was detected in 1996 that point mutation in domain V of the 23S rRNA gene is responsible for the CLR-resistant status of H. pylori strains [7]. Apart from the abovementioned mechanisms of resistance developed by H. pylori strains to the prime antibiotics used in the treatment of infection, other factors such as the virulence genotype status of bacteria have been reported to affect drug resistance [8]. Specific mutations have been recently nominated as chief factors leading to x resistance in H. pylori, such as A2143G, A2142C/G, and A2143C/G. The above resistant strains were examined to find point mutations in 23s rRNA genes. This study aimed to determine the prevalence of clarithromycin antibiotic resistance in clinically isolated H. pylori strains collected from June 2016 to December 2017. Also, another objective of this study was to evaluate the association between genotype V of the 23S rRNA region and CLR resistance phenotypic.

MATERIALS AND METHODS

Between June 2016 and December 2017, gastric biopsy specimens were taken from patients with gastric disorders who indicated endoscopy before receiving routine anti-H.pylori therapy at the Gastrointestinal Endoscopy Center, Mehrad Hospital (Tehran, Iran). Patients were excluded if they had received H. pylori eradication therapy within 1-year, antiplatelet drugs during the past week, and surgery for gastric cancer. The written informed consent to participate was obtained from all participants, and study activities were approved and overseen by the Ethical Committee of Tarbiat Modares University, Iran. The ethics code is IR.TMU.REC.1395.514. Other exclusion criteria were: surgery for malignant tumors other than gastric cancer, a severe systemic disorder such as end-stage renal disease or liver cirrhosis, patients under hemodialysis, autoimmune diseases, pregnancy, and lactation. The biopsies were transported to the laboratory in a transport media (thioglycolate broth) and plated on the brucella agar (Merck, Germany). Then, they were supplemented with: 5% defibrinated sheep blood (Bahar Azma, Iran), 10% of fetal bovine serum (Sigma, USA), selective campylobacter supplement (Merck, Germany), and 5 mg/l of amphotericin B (Merck, Germany). The culture was performed under microaerophilic conditions in anaerobic jars at 37°C for maximally two weeks and checked for growth every 48 hours. Macroscopic identification of H. pylori was confirmed by modified Gram staining, catalase, oxidase, and rapid urease reactions. The characterized isolates were kept at -80 °C in BHI agar containing 16% glycerol. The modified disk diffusion method (MDDM) was utilized to assess the sensitivity of H. pylori strains to clarithromycin (15 mg) (HIMEDIA, India). Suspensions of bacteria were prepared in the sterile normal saline (2 ml) equivalent to standard 3 McFarland. The suspension was spread on Brucella agar supplemented with 5% sheep blood (Bahar Afshan, Iran). When plates dried, antibiotic disks were placed and incubated in a microaerophilic atmosphere at 37°C for 3-5 days. Susceptibility results were recorded as resistant following interpretive criteria of CLSI guideline; for clarithromycin, no zone of growth inhibition [9].

The E-test was applied in the antibiogram test for *H. pylori* strains using strips of clarithromycin (E-test, Biomerieux, France) on Mueller-Hinton agar (MHA; Merck, Germany) which was enriched with a 5% of sheep blood (Bahar Azma, Iran). In all strains, the MICs of clarithromycin were measured. A suspension containing pure *H. pylori* colonies was first prepared using sterile physiological saline (9×10^8 CFU/mL; turbidity, 3 McFarland). Then it got inoculated on MHA. After drying the medium surface, the strips were placed on the medium; then incubated under microaerophilic conditions for 72 hours at 37°C. MICs≥ 1 were considered resistant to clarithromycin. Also, the reference strain was cultured and was *H. pylori* 26695 in this study [10].

DNA was extracted from fresh resistant *H. pylori* isolates and reference strain 26695 before storage at -80°C using a DNA extraction kit (Sinaclon CO, Iran), according to the manufacturer's instructions. Then, DNA concentration was assessed by Nanodrop. The isolated DNA was eluted in 200 µl of 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and stored at -20°C until use. All extracted DNAs were amplified for *glmM* by PCR using an

automatic Thermos cycler (Eppendorf Personal 5332, Germany). Phosphoglucosamine mutase (*glmM*) is a "housekeeping" gene to confirm the genetic identity of *H. pylori* existence in clinical samples [Table 1].

Table 2 : Primer sequence and PCR condition

	Primer sequence and PCR condition			
genes	Primer sequence (5' _3')	PCR product	amplification conditions	**Ref**
glmM	AAGCTTACTT TCTAACACTA AAGCTTTTAG GGGTGTT	294	94°C, 5 mins 94°C, 45 s, 55°C, 1 min, 72°C, 30 s (30 cycles)	[11]
23srR NA	CCACAGCGAT GTGGTCTCAG CAAA ATGACTCCAT AAGAGCCAAA GCCCT	429	95°C, 5 mins 95°C, 30 s, 54°C, 30 S, 72°C, 30 s (35 cycles)	[11]

PCR reactions were carried out in a volume of 25 µl for detection of 23s rRNA gene, 1 µl of extracted DNA from culture (approximately 200 ng), 0,1 µM of primer 23SF2, and 0,14 µM of 23Ssc. The amplification was performed after a denaturation step (95°C for 15s) for 50 cycles, annealing (55°C for 30 s), and extension (72°C for 20 s).

The PCR products were separated on 1.5% agarose gels (Cinna gen, Iran) in TAE 1X (Tris/Acetate/EDTA) buffer. The bands were visualized under UV gel documentation and photographed. Ethidium bromide stain (Merck, Germany) was added to the agarose gel during preparation to give a 0.2 µl/ml concentration. The 23s rRNA domain V gene was sequenced (Pishgam Biotech co) using primers 23srRNA in clarithromycin-resistant strains and compared with the sequences from clarithromycin-sensitive isolates. ATCC 26695 (AE000511.1), J99 (AE001439.1) SPSS version 16 (SPSS, Inc.) was used for data analysis. P-values of £0.05 were considered statistically significant.

RESULTS

Sixty-five *H. pylori* isolates without a history of anti-*H. pylori* therapy were identified among 180 consecutive patients admitted to the endoscopy unit at Mehrad Hospital in Tehran, Iran. The prevalence rate of *H. pylori* infection was 36%. However, 65 patients (21 male (32%) and 44 female (68%)), aging from 40 to 50 and with disease statuses, were included in this assay. The significant level of less than 0.05 (P>0.05) in the Binomial test (SPSS software) suggests that patient sex is associated with the prevalence of *H. pylori*, with females displaying a higher prevalence rate. The significant level of less than 0.05 (P>0.05) in the k2 test (SPSS) suggests that the 40–50 age range is directly related to the prevalence of *H. pylori*. The MICs of all isolates were tested by E-test. Among 47 *H. pylori* isolates, 11 (23%) were resistant to clarithromycin (MIC1-2 µg = 5, MIC ≥2 µg= 6). As expected, the 26695 strain was sensitive to clarithromycin. In MIC1-2 µg, two out of 5 reported gastritis; one had a peptic ulcer, and two had a duodenal ulcer. In MIC ≥2 µg, in six cases, 2 had gastritis, 3 had a peptic ulcer, and one had gastric cancer.

Complementary sequencing analysis for mutations of the 23s rRNA gene was performed only in clarithromycin-resistant strains with MIC ≥64. Mutation at A2143G was found in 67% of *H. pylori* strains and mutation at A2142G in 33.3%. GenBank accession number: (KJ194496–KJ194498 and KJ488950, KJ488951 and KM035756). Histological analysis of each biopsy specimen revealed that of the 65 infected individuals, 39 were patients with gastritis (60 %), 8 with gastric ulcers (11 %), 10 with duodenal ulcers (14%), and 3 with gastric cancer (5%). No significant association was found between diseases outcome and antibiotic resistance among the *H. pylori* isolates (*P* >0.05). Among patients with a positive culture (65 cases), 51 (78%) were with no previous history of treatment, 8 (12%) were with complete treatment, 3 (5%) were with incomplete treatment, and 2 (4%) did not have any information about their history of treatment. According to the questionnaire on the history of treatment, 8 (12%) had regular consumption of Omeprazole in the past two weeks, 4 (6%) had irregular consumption, and 53 (81%) had no

history of taking it. Also, 2 (3%) had regular consumption of ranitidine in the past two weeks, and 63 (96%) had no history of taking it. Further, 11 (17) had regular consumption of Aspirin in the last two weeks, and 54 (83%) had no history of taking it. 2 (3%) had regular consumption of Plavix in the past two weeks, and 63 (96%) had no history of taking it.

DISCUSSION

The eradication of *H. pylori* infection has been decreasing progressively, mainly due to increased resistance to antimicrobial agents, especially in developing countries [12]. Maastricht V recommends performing susceptibility testing before therapy in regions with high clarithromycin resistance rates [13]. In Iran, nearly 40–90% of the adult population is infected with *H. pylori*, acquired early in childhood. PCR, RUT, and histopathological tests indicated that the incidence of *H. pylori* was independent of gender. Also, the prevalence of *H. pylori* infection in the adult population of Iran is about 80% [14]. Therefore, we carried out this study to assess the phenotypic and molecular characteristics of *H. pylori* antibiotic resistance. The MICs of all isolates were tested by E-test. Of 47 *H. pylori* isolates, 11 (23%) were resistant to clarithromycin. The resistance of *H. pylori* to clarithromycin varies worldwide. This resistance is the leading cause of the failure of *H. pylori* infection eradication. Further increasing antibiotic resistance in recent years has reduced the efficacy of treatment. Resistance to clarithromycin has been reported to be 30% in Southern Europe and 15%–30% in the United States [15]. Among Asian countries, in Pakistan, resistance was estimated to be 48% [16]. The prevalence of resistance to clarithromycin in Iran (Tehran, Jahrom, and Kerman) was 22%, 24%, and 32%, respectively [17]. The estimated value in the present study (23%) approximated the observed prevalence rate. It has been claimed that the three most frequently reported mutations, including A2143G, A2142G, and A2142C, are responsible for more than 90% of the cases of primary resistance to clarithromycin [18]. Moreover, some other mutations are associated with clarithromycin resistance, although their precise role is not yet clear. In the isolates of this study, only two mutations, including A2142G and A2143G, are found. The most common ones are A2142G and A2143G, and according to our research as well as some studies, the most abundant mutation was A2143G [19, 20]. In the most recent study in Shahrekord, Southwest Iran, the clarithromycin resistance rate was 57%, and the A2142G mutation was recognized as the most common mutation [21]. In another study, the prevalence of A2143G and A2144G mutations was 68% (13/19) and 31% (6/19), respectively. A2143C mutation was not tracked in any isolate [22]. In addition, we failed to identify additional mutations like T2183C and A2223G, which are frequently reported to be the cause of clarithromycin resistance in Eastern countries rather than in Western countries [23]. ASP-PCR of 23s rRNA showed four strains had A2143G mutation, six strains had A2142G mutation, and one strain had a Wt+A2143G mutation [24]. Additionally, no point mutation was identified in the sequence of the 23SrRNA gene in five clarithromycin-resistant strains. For those isolates, we can speculate that other resistance mechanisms, such as the presence of an efflux pump, may be implicated in developing resistance to clarithromycin [23]. During this study, we understood that antimicrobial susceptibility assessment in *H. pylori* by culture-based phenotypic tests could be challenging due to the pathogen's fastidious growth requirements. Moreover, it was not always possible to determine an explicit MIC of a drug by E-Test because of the small and transparent colonies that *H. pylori* forms. Phenotypic tests may also be challenged when a mixed population of resistant and susceptible *H. pylori* strains is present in the same patient [25]. A study in Turkey highlights that there is a high rate of resistance to clarithromycin in Turkish children with *H. pylori* infection. Hence, it is essential to note other classes of antibiotics except clarithromycin for effectively treating the *H. pylori* infection in children younger than 18 years in the study region [26]. It is shown in another study that clarithromycin resistance is the most common in that population, followed by amoxicillin and tetracycline. Excessive use of these antibiotics and self-prescription should be analyzed in future studies.

Also, public-awareness programs might be required.

Limitations of the study

The sample size was relatively small. Our study was only performed in Tehran, and it would be a good idea if we could collect some samples from other regions in Iran. The cloning method can be utilized to confirm the mutations and ensure that single polymerase enzyme errors did not cause the mutations. The mutations found in our study are among the most mutations reported for this kind of resistance. Nevertheless, other mutations may not have been identified due to the short length of the selected region. We can also use the amplification-refractory mutation system (ARMS), a simple method for detecting any mutation involving single base changes or small deletions. ARMS is based on using sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. Thus, we could use it for mutations occurring in 23sr RNA related to clarithromycin resistance.

ACKNOWLEDGMENTS

This study was supported by Tarbiat Modares University, Tehran, Iran. We are thankful to the physicians and staff of the endoscopy department of Mehrad hospital.

FUNDING

This work was supported by Tarbiat Modares University, Tehran, Iran.

ABBREVIATIONS

PCR	*Polymerase chain reaction*
CLSI	Clinical and laboratory standards institute
E-test	Epsilometer test
MIC	Minimal inhibition concentration
IARC	**International Agency for Research on Cancer**
CLR	Clarithromycin
ARMS	Amplification-refractory mutation system

Availability of data and materials

All data generated or analyzed during this study was done, and data reports are available in the department of Bacteriology, Tarbiat Modares University.

Ethics Approval and Consent to participate

The written informed consent to participate was obtained from all participants, and study activities were approved and overseen by the Ethical Committee of Tarbiat Modares University, Iran. The ethics code is IR.TMU.REC.1395.514.

DECLARATION

The authors declare they have no competing interests.

CONSENT FOR PUBLICATION

Not applicable.

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