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# Clarithromycin Resistance in *Helicobacter Pylori*

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**Objectives:** Eradication rates of *Helicobacter pylori* with standard triple therapy are disappointing in studies from several countries. The main reason for failure was found to be bacterial resistance to one of the most commonly used antibiotics, clarithromycin. Our aim was to determine the prevalence of clarithromycin resistance among *H. pylori* strains isolated from Mongolians and the presence of associated mutations to these antibiotics. **Methods:** All urease-positive samples were cultured according to the standard microbiological procedures. *H. pylori* strains were grown under microaerophilic conditions on *H. pylori* selective agar. *H. pylori* antibiotic sensitivity was examined using the E-test method. The GenoType HelicoDR, which employs reverse hybridization, was used to confirm the presence of *H. pylori,* determine its susceptibility to antimicrobials and detect mutations conferring resistance to clarithromycin. **Results:** Our findings show that 51 (53.7%) of *H. pylori* strains were resistant to clarithromycin. Mutations were observed in 32 (53.7%) strains with A2147G being the most prevalent, A2146G being the least prevalent and A2146C point mutation being undetected. **Conclusion:** In the present study, *H. pylori* strains with moderate-level clarithromycin resistance are more frequently found and clarithromycin is associated with point mutation A2147G in Mongolians.

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

## Introduction

*Helicobacter pylori (H. pylori)* is known to be the major pathologic organism in the development of upper gastrointestinal peptic ulcer disease and is considered the causative agent of chronic gastritis, gastric mucosa-associated lymphoid tissue lymphoma, and the leading cause of gastric cancer worldwide [1, 2]. Current recommendations for the management of *H. pylori* infection were elaborated on by the European Helicobacter Study Group (EHSG) and presented in Maastricht IV/Florence Consensus Report in 2012 [3]. According to these guidelines, an effective treatment requires combined therapy, and it is important to take into consideration *H. pylori* resistance to

clarithromycin in the area the patient is coming from (areas of low (<20%) and high (>20%) prevalence) [3].

In Mongolia antibiotic resistance of *H. pylori* has not been reported. Clarithromycin is found to be one of the most effective antimicrobial agents used in the treatment of *H. pylori* infection. However, it should be remembered that the development of clarithromycin resistance is a major cause of *H. pylori* treatment failure. Clarithromycin resistance in *H. pylori* is due to point mutations in the *rrl* gene encoding the 23S rRNA, with three major mutations described: A2146C, A2146G, and A2147G [4]. Resistance of *H. pylori* to clarithromycin is the prevalence that has been the most-widely studied so far. It ranges from close to 0 to 20-25%. The most common mutation for *H. pylori* resistance to clarithromycin has been A2143G (also known as A2147G in about 70% of the resistant strains, with a range from 53% to 95%), followed by A2142G (also called A2146G, 11.7%) and A2142C (also known as A2146C, 2.6%) [5, 6]. Therefore, the aim of study was to determine the prevalence of clarithromycin resistance among *H. pylori* strains isolated from Mongolians and the presence of associated mutations to these antibiotics.

## Materials and Methods

A total of 95 consecutive patients who visited Shastin Hospital and Songinokhairkhan District Hospital in Ulaanbaatar, Mongolia for upper endoscopy during 2012-2014 were enrolled in the study. All subjects were provided with informed consent, and the study protocol was approved by the Ethics Committee at the Mongolian National University of Medical Sciences. All patients included in the study underwent upper gastrointestinal endoscopy. Two sets of biopsy specimens were obtained from gastric antrum and body and one set was used for the rapid urease test (Becton Dickinson, USA) and the other specimen was used for *H. pylori* culture.

### 1. Bacteria and culture conditions

Biopsy specimens were macerated and homogenized in meat liver dextrose and a 250-µL aliquot was inoculated on *H. pylori* selective agar (bioMérieux, France). Incubation was performed in microaerophilic (5% O2, 15% CO2, 80% N2) conditions at 37°C for a maximum of five days. Colonies were identified as *H. pylori* according to the standard criteria including negative Gram staining, typical cell morphology, and positive reactions to catalase, oxidase, and urease. Positive clinical isolates were stored at −80°C in meat liver dextrose broth until susceptibility tests.

### 2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by the E-test strips (bioMérieux). The bacteria were subcultured on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood under the same microaerophilic atmosphere mentioned above at 37°C for 48-72 hours. Strains were classified into three levels of resistance to clarithromycin depending on MIC as follows: ≤0.25 mg/L = susceptible, equal to 0.5 = intermediate and ≥1 mg/L = resistant [5].

### 3. PCR Method

Amplification of the bacterial DNA was done using hot-start DNA polymerase. Biotinylated primers were used for this study and were provided in the amplification kit. Polymerase chain reaction (PCR) for a single mixture had a final volume of 50 µL containing 35 µL primer/nucleotide mix (PNM), 5 µL of 10x polymerase incubation buffer, 2 µL of 1.5 mM $MgCl_2$, 3 µL of nuclease-free water, 0.2 µL Thermo-Start Taq DNA polymerase (1-2 units were added to each tube), and 5 µL DNA template. The PCR run included 30 cycles for strains. In protocols, the denaturation cycle was 1 cycle at 95°C for 15 minutes, followed by 10 cycles at 95°C for 30 seconds and at 58°C for 2 minutes. Then, 20 cycles had a first step at 95°C for 25 seconds, a second step at 53°C for 40 seconds, and a third step at 70°C for 40 seconds. The PCR ended with eight minutes at 70°C.

### 4. GenoType HelicoDR analysis

Confirmation of isolates as *H. pylori*, antimicrobial susceptibility, and mutational analysis to clarithromycin was performed using the GenoType HelicoDR Kit (Hain Lifescience, Germany). The kit employs the use of reverse-hybridization and was performed using the TwinCubator (Hain Lifescience) at a temperature of 45°C. The denaturation solution was mixed with 20 µL of the amplified sample and submitted to the usual protocol for hybridization [7, 8]. For clarithromycin, one wild type probe (23SWT) and three mutant probes (23SMUT1–23SMUT3) were used for detecting resistance. On the strip, conjugate control (CC), amplification control (AC) and *H. pylori* (HP) were designated. The probes

are listed in Table 1. The presence of a band at CC and AC meant that the conjugate control and amplification control were in the right frame while at HP implied presence of *H. pylori* according to the manufacturer's instruction.

**Table 1.** Probes hybridized on the DNA strip of the GenoType HelicoDR test for detection of mutations in the *rrl* genes

| Probes | Codon | Nucleotides | Associated phenotype |
|---|---|---|---|
| 23S-WT | 2146 and 2147 | AA | CLA-S |
| 23S-MUT1 | 2146 | A2146G | CLA-R |
| 23S-MUT2 | 2146 | A2146C | CLA-R |
| 23S-MUT3 | 2147 | A2147G | CLA-R |

### 5. Statistical analysis

SPSS 22.0 was used for statistical analysis. Fisher's exact tests were applied to our analyses. Null hypotheses of no difference were rejected if the p-values were less than 0.05.

## Results

Of 95 consecutive patients (with both rapid urease test and culture being positive) in this study, 39 out of 95 patients were males (41.1%), and 56 were females (58.9%) ranging in age from 18 to 83 with a mean age of 42.8 years. A total of 44 (46.3%) of the 95 strains were susceptible to clarithromycin while 51 (53.7%) were resistant. Overall, 35.5% of all patients found to have positive cultures for *H. pylori* had isolates fully resistant to clarithromycin. Only one patient had an isolate that demonstrated intermediate resistance to clarithromycin (MIC = 0.50).

Prevalence of clarithromycin resistance in females and males was 58.9% (33/56) and 46.1% (18/39), respectively. In general, there was a higher prevalence of resistant isolates in females compared to male patients. However, this did not reach statistical significance (p = 0.219). When stratified by age, clarithromycin resistance was highest among those 40 years of age and older (40-49 years: 65.0%, 50-59 years: 45%, and ≥60 years: 45.5%), as shown Figure 1. The frequency of clarithromycin resistance was found in patients with gastritis at 42.5%, stomach erosion at 24.0%, gastric ulcer and atrophic gastritis both at 12.9% and nodularity at 7.0%. No statistically significant associations were observed between characteristics like age, sex and dyspeptic disorders and resistance status of *H. pylori* isolates (p >0.05).

**Table 2.** Distribution of 51CLA-R isolated patients with MUT3 based on age and sex

| Characteristic | CLA-R strain A2147G | | p-value |
|---|---|---|---|
| | Positive n (%) | Negative n (%) | |
| Sex | | | |
| Male | 13 (72.2) | 5 (27.8) | 0.301 |
| Female | 19 (57.6) | 14 (42.4) | |
| Age | | | |
| 18-29 | 8 (72.7) | 3 (27.3) | 0.072 |
| 30-39 | 6 (40.0) | 9 (60.0) | |
| 40-49 | 4 (57.1) | 3(42.9) | |
| 50-59 | 11 (91.7) | 1 (8.3) | |
| ≥60 | 3 (50.0) | 3 (50.0) | |
| Total | 32 | 19 | |

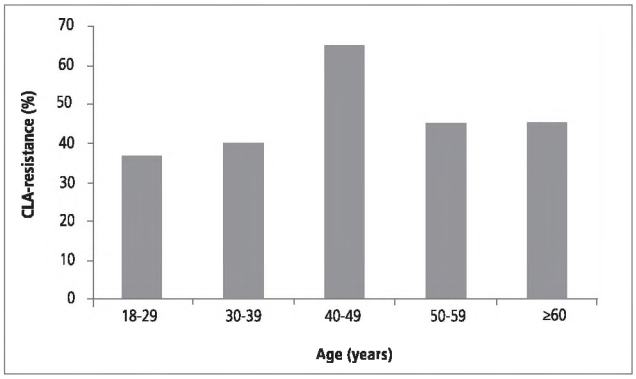


**Figure 1.** Prevalence of clarithromycin resistance in *Helicobacter pylori* in Mongolian patients according to age.

There were 51 results given by GenoType HelicoDR for 23S ribosomal (r-)RNA (rrl) genotyping, as shown in Table 2. All of the 51 Cla-R isolates had at least one of the three common point mutations in the 23s rRNA gene, while none of the Cla-S isolates had such a point mutation. Overall, the most frequent mutation was A2147G (MUT3 profile), observed in 32 strains (62.7% of the mutated alleles), but not in 19 strains, or 37.3% (Table 3). A total of 19 out of 32 (59.3%) of the MUT3 positive strains and 14 out of 19 of the MUT3 negative strains isolated from the female population had this point mutation. A total of 11 out of 32 of the MUT3 positive strains were isolated most frequently in the 50-59 age group (Table 2). There was no significant relation between sex (p = 0.301) and age for this mutation.

Eight percent of the CLA-R isolates (4 out of 51 isolates) had the A2146G point mutation. In our examinations, we did not detect A2146C (MUT2 profile) in all the CLA-S *H. pylori* strains. In general, there was a more frequent detection of MUT 3 in the *H. pylori* susceptible strain compared to resistance strain (p= 0.001).

PCR-based approaches have been developed as alternative tools. These techniques allow assessment of mutations in the peptidyl transferase region encoded in domain V of the *H. pylori* 23S rRNA region that confers clarithromycin resistance [14]. Undeniably, both culture and PCR-based methods have both advantages and limitations [15]. We evaluated a new molecular test that rapidly detects antibiotic resistance in *H. pylori*. The test, based on DNA strip technology, was developed to be applicable to strains, as well as to gastric biopsy specimens, and to be affordable and easy to perform in clinical microbiology laboratories.

**Table 3.** The frequency of clarithromycin susceptibility test for *H. pylori* isolates in both resistant and sensitive isolates in Mongolian

| CLA susceptibility | n (%) | A2146G MUT1 | n (%) | A2146C MUT2 | n (%) | A2147G MUT3 | n (%) |
|---|---|---|---|---|---|---|---|
| Resistance | 51 (53.7) | + | 4 (8) | + | 0 | + | 32 (62.7) |
| | | - | 0 | - | 0 | - | 19 (37.3) |
| Sensitive | 44 (46.3) | + | 0 | + | 0 | + | 0 |
| | | - | 0 | - | 0 | - | 44 (100.0) |
| Total | 95 | | | | | | 95 |

## Discussion

Clarithromycin is one of the core antibiotics of the proton pump inhibitor triple regimen [9, 10]. Many researchers have found that resistance to clarithromycin is detrimental to the effectiveness of *H. pylori* eradication with triple therapy [11]. In European countries, maximum clarithromycin resistance was reported in Spain (49.2%) whereas the lowest was in Sweden (1.5%) and in the Netherlands (0.8%) [12]. In Asian countries, high prevalence of clarithromycin resistance was also detected in Japan (40.7%) while it was the lowest in Malaysia (2.1%) [13]. In the present study, resistance rate to clarithromycin was 35.5% in Mongolia using the E-test method.

The increasing usage of clarithromycin in developing countries, mostly for respiratory tract infections, has caused a large number of clarithromycin-resistant *H. pylori* strains. Our findings show that 51 (53.7%) *H. pylori* strains were resistant to clarithromycin. The guideline for the eradication of *H. pylori* in Mongolia consists of an H2 receptor blocker (such as ranitidine), two antibacterial agents, such as metronidazole and amoxicillin/ clarithromycin plus a bismuth salt. However, based on our results, it has become clear that we need to urgently modify the current regimen to a more effective therapeutic formula for the treatment of this gastric pathogen.

Clarithromycin resistance assessment is currently based on phenotypic detection performed after culture and the agar dilution method or E-test. However, in the past decade, different

The assay used in this study was designed to target the presence of A2147G, A2146G, and A2146C associated with clarithromycin resistant strains [5]. There is a paucity of information in the literature on A2147G, A2146G and A2146C mutation compared to A2142G, and A2143G which are frequently reported [14] to be associated with clarithromycin resistance. We detected that the most-frequent mutation was A2147G (MUT3 profile). This accords with the findings of Cambau et al. who reported a high prevalence of A2147G mutation amongst their strains [5]. In our study, the A2147G mutation was not observed in 37.3% (19/51) of those with the clarithromycin resistance strain (Table 3). It may be associated with another relevant mechanism for macrolide resistance ascribed to the efflux pump system. At least five conserved families of drug efflux mechanisms are associated with bacterial species, including small multidrug resistance, multidrug and toxic compounds extrusion proteins, the major facilitator superfamily, the ATP-binding cassette superfamilies and the resistance-nodulation cell division [16]. The resistance-nodulation-cell division (RND family) is responsible for macrolide intrinsic resistance in several Gram-negative bacteria and it has been recently proposed also for *H. pylori*.

In conclusion, the high rate of clarithromycin resistance in the isolates in the present study is cause for serious alarm, and is in agreement with clinical colleagues' views that many of their patients do not respond to clarithromycin anymore. Point mutations in 23s rRNA are closely related to such a resistance. Therefore, it seems necessary to do antibiotic susceptibility tests for *H. pylori* before therapy begins.

## Conflict of Interest

The authors state no conflict of interest.

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