Metronidazole-resistant *Helicobacter pylori* isolates without *rdxA* mutations obtained from Iranian dyspeptic patients

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**Abstract**

Antibiotic resistance is now accepted as an inevitable factor in *Helicobacter pylori* treatment failure, so a survey on the antibiotic susceptibility profile of *H. pylori* is welcomed. In addition, the main molecular mechanism of antibiotic resistance in *H. pylori* is not fully determined, particularly for metronidazole. Our single-centre study was designed to evaluate the local antibiotic resistance profile of *H. pylori* strains recovered from individuals with dyspepsia. Gastric biopsy specimens from 200 individuals underwent bacterial culture for *H. pylori*, and bacterial identification was confirmed by positive reports from biochemical and genotypic universal protocols. Antibiotic susceptibility tests were performed on the 73 isolates obtained, by both disc diffusion and E-test methods. DNA extraction was carried out on single colonies of *H. pylori* confirmed by biochemical tests, then PCR was used to amplify the *rdxA* and 23srRNA genes. Metronidazole and clarithromycin resistance phenotypes were checked to detect possible mutations at *rdxA* and 23srRNA genes. Successful bacterial culture was reported for 73 of the 200 patients (27 male (36%) and 46 female (63%) with an age range from 25 to 80 years (mean 54 years)). None of the patients reported pre-treatment. Among the 73 biochemically and genotypically confirmed *H. pylori* isolates in this analysis, antibiotic resistance rates were 45% (33/73) for metronidazole and 23% (17/73) for clarithromycin. Additionally, ten *H. pylori* isolates were multidrug resistant (13%). According to the antibiogram analysis, 13/17 (76%) had the A2142G mutation, although 3/17 (17%) samples also showed A2143G. None of the resistant isolates were carrying the A2142C and A2144G mutations. Moreover, none of the metronidazole-resistant strains showed any of the point mutations. Identification of *H. pylori* isolates without the *rdxA* mutation reveals the need for an urgent investigation to select an effective antibiotic before drug prescription by gastroenterologists.

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**Keywords:** Antibiotic resistance, clarithromycin, E-test, *Helicobacter pylori*, metronidazole, mutation

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**Introduction**

The discovery of the association between *Helicobacter pylori* and a wide range of gastroduodenal diseases caused a renaissance in gastroenterology in the twentieth century [1]. This spiral but rapidly motile bacterium can remain long term in the human gastric mucosa if effective antibiotics are not prescribed [2]. It is more than 30 years since we acknowledged the essential role of *H. pylori* as a causative agent of chronic gastritis, as well as being a major risk factor in the development of gastric cancer [3,4]. The designation of an effective therapy providing the successful eradication of *H. pylori* is a key point in the management of these digestive diseases [5,6]. To date, the generally accepted anti-*H. pylori* therapy has been classic proton-pump inhibitors in combination with two antibiotics from amoxicillin, clarithromycin and metronidazole [2]. This therapeutic regimen, termed standard triple therapy, may be under question if the rate of clarithromycin resistance reaches 15% [7,8]. Given an acceptable antibacterial effect of metronidazole on *H. pylori*, even during *in vivo* experiments, it is now considered a permanent member of antibacterial regimens used in clinical approaches by gastroenterologists [9,10]. Clarithromycin is also,
currently, an inevitable drug in all anti-\textit{H. pylori} treatment formulations [11]; however, rising resistance rates hamper many of the recommended therapies for eradication of this persistent bacterium [12,13]. To determine the susceptibility profile, there are different but consistent methods available for microbiologists. Disc diffusion as the easiest and most cost-effective approach was primarily applied to screen the \textit{H. pylori} antibiotic-resistant isolates [14]. The E-test is also a quantitative variant of the disc diffusion method and exhibits a good correlation with other methods [14]. An updated meta-analysis proved that successful eradication of \textit{H. pylori} is clearly bound with reduced occurrence of gastric cancer and such severe gastroduodenal diseases [15]. The clinical reasons mentioned here highlight the importance of having up-to-date data on the antibiotic resistance of this bacterium. Unfortunately, there is currently no active surveillance system by Iranian health authorities to track those resistant strains [16]. We aim to determine the prevalence of antibiotic resistance against both clarithromycin and metronidazole among \textit{H. pylori} isolates recovered from antral biopsies of individuals admitted to the Imam-Khomeini Hospital at Tehran, Iran, during 2015–2018.

Materials and methods

Patients

A total number of 200 antral biopsy specimens were collected from individuals with various gastroduodenal disorders who were admitted to the gastroenterology unit of Imam Khomeini Hospital (Tehran, Iran) during 2015–2018. Exclusion criteria were as follows: report of severe systemic disease (e.g. abdominal surgery) in last 6 months, age <18 years, consumption of antibiotics in last 2 months before the endoscopy, consumption of bismuth salts in last month before the endoscopy, and anti-platelet drugs within 1 week of admission. The \textit{H. pylori} strains were isolated from antral biopsies of individuals with dyspepsia who had undergone endoscopy after the first visit because of the gastroduodenal problems registered by expert clinicians. In this survey, written informed consent was taken from each participating patient, and our study was approved by the ethics committee of Tarbiat Modares University following a rigorous peer-review process (ethics code: IR.TMU.REC.1395.514).

Antral biopsy collection and \textit{H. pylori} culture

Two biopsy specimens were taken, the first was sent to the pathology department for histopathological examination, the second was shipped within 2–4 hours to the laboratory in Eppendorf tubes containing thioglycollate broth medium in a cold-box [14]. Bacterial culture was conducted briefly, as follows: the biopsy specimen was gently vortexed, then the homogenate was inoculated into Brucella agar (Merck, Darmstadt, Germany) plates supplemented with 5% defibrinated sheep blood (Bahir-Azma, Tehran, Iran), 10% fetal bovine serum (Sigma, St Louis, MO, USA), \textit{Campylobacter} selective supplement (Merck), and 5 mg/L of amphotericin B (Merck) [14]. Incubation period was 7–14 days, and the plates were checked for suspected colonies after 5 days. Microaerophilic conditions (10% CO$_2$, 5% O$_2$ and 85% N$_2$) were used during the incubation period to provide optimum growth conditions for \textit{H. pylori}. Following the incubation period, the \textit{H. pylori} cultures were investigated using Gram-staining, translucent colonies, as determined with the naked eye, and three common biochemical tests (catalase, oxidase and urease) [14]. The confirmed isolates were chosen for second bacterial culture to achieve a single colony (to avoid mixed infections). The selected isolates were the subject of susceptibility tests and PCR.

Antibiotic susceptibility test

A modified disc diffusion method was used to investigate the susceptibility of \textit{H. pylori} isolates to clarithromycin (15 mg) and metronidazole (5 mg) (HIMEDIA, Mumbai, India). For this purpose, bacterial suspensions were prepared in the sterile saline (2 mL) equivalent to 3 McFarland standard (~9.0 × 10$^8$ CFU/mL). The suspensions were streaked onto Müller–Hinton agar supplemented with 5% sheep blood (Bahir-Afshan, Tehran, Iran). After 10 min of inoculation, antibiotic discs were placed and incubated in a microaerophilic atmosphere at 37°C for 5–8 days. Susceptibility testing and interpretive criteria were interpreted according to CLSI guideline; inhibition zone for metronidazole was <16 mm and no inhibition zone for clarithromycin.

Determination of MIC

In our study, MICs were determined by the E-test (E-test, Biomérieux, Marcy l’Etoile, France). A bacterial suspension equal to the concentration equivalent to 3 McFarland standard (~9.0 × 10$^8$ CFU/mL) was used to identify MIC for the selected \textit{H. pylori} strains. To perform E-test (AB Biodisk, Solna, Sweden), suspensions from primary plates were prepared in sterile saline solution at a concentration equivalent to 3 McFarland and streaked onto Müller–Hinton agar medium (Merck) plates supplemented with sheep blood 5% vol/vol (Bahir-Azma). The clean E-test strips were placed on the dried surface of inoculated agar plates. The plates were incubated in microaerophilic conditions at 37°C for 72 hours or maximally for 2 more days until a visible inhibition ellipse was disclosed. As suggested in the European Committee on Antimicrobial Susceptibility Testing recommendations (EUCAST, 2013), MIC values of 0.5 and 8 mg/L are the cut-offs above which \textit{H. pylori} is deemed...
resistant to clarithromycin and metronidazole, respectively. In our survey, the MIC was measured and reported at the lowest antibiotic concentration where *H. pylori* growth was inhibited. The *H. pylori* 26695 reference strain was used as quality control to ensure the accuracy of these findings.

**Polymerase chain reactions**

DNA was extracted from all resistant *H. pylori* isolates and reference strain. Bacterial DNA isolation from *H. pylori* single colonies was performed by using ‘Yekta-Tajhiz-Azma’ as described by the manufacturer’s instructions with minor modifications. DNA samples were the subject of glmM (as the specific species gene for *H. pylori*) PCR to confirm biochemical tests. The remaining DNA was subsequently stored at −20°C until further use. Resistant isolates were selected to evaluate the mutation profile of the rdxA and 23srRNA genes. Using an automatic Thermocycler (Eppendorf Personal 5332; Eppendorf, Hamburg, Germany), PCR was carried out based on the published protocols with minor changes. http://jjmicrobiol.com/en/articles/80156.html Table 1 provides details of the primer sequences and PCR conditions.

**Statistical analysis**

To report any significant association between presence of mutations and gastroduodenal diseases, age and gender, we used Fisher’s exact test; *p* values <0.05 were considered statistically significant. Data analysis was performed using software SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

**Results**

Over the 3 years (2015–2018), 200 individuals who underwent upper gastroscopy in Imam Khomeini hospital, Tehran, Iran were included in our analysis. A total of 73 *H. pylori* isolates were obtained and confirmed as single colonies according to the bacterial culture and glmM-specific PCR method. Overall, 73 individuals (27 male (36%) and 46 female (63%)) were included with a wide age range from 25 to 80 years (mean age 54 years). Endoscopic investigation and histological analysis of the 73 *H. pylori*-positive biopsy specimens revealed that 44 were gastritis (60%), 16 were gastric ulcer (22%), 10 were duodenal ulcer (13.6%), and 3 were gastric cancer (4%). Table 2 shows the antibiotic susceptibility profiles of different patients according to age range and gender. Table 3 presents various diseases groups found for patients carrying the antibiotic-susceptible and antibiotic-resistant *H. pylori* isolates. However, no significant association was detected between clinical outcome, age, gender and antibiotic resistance among the *H. pylori* isolates (Tables 2 and 3) (*p* > 0.05). Overall antibiotic resistance rates were 45% (33/73) for metronidazole and 23% (17/73) for clarithromycin. Ten isolates (13.6%) were multidrug resistant (Table 3). None of the metronidazole-resistant isolates were positive for any rdxA mutations. According to the antibiogram analysis, 13/17 (76%) had the A2142G mutation, but 3/17 (17%) samples also showed A2143G. Additionally, none of our resistant isolates were carrying the A2142C and A2144G mutations.

**Discussion**

The success of the triple therapy against *H. pylori* has been endangered by rapidly rising antibiotic resistance. Many recent meta-analyses confirmed the alarming increase in antibiotic resistance among *H. pylori* strains; so urgent input, especially by microbiologists and gastroenterologists, is required [17,18]. According to national and international records, as *H. pylori* treatment becomes more difficult, updated guidelines with better modifications seem to be the clinical solution [19,20]. Resistance to clarithromycin is alarming as it has reached >20% in many regions [2,21,22], a similar finding to what we found in our study (23%). In Iran, the prevalence of clarithromycin-resistant *H. pylori* ranges from 15% to 45%, which is in line with the results of the current report (23%) [19,23,24]. The prevalence of metronidazole-resistant *H. pylori* varies from 20% to 40% among western countries [25,26]; however, this rate is at least twofold in developing countries, includes Iran [19,27,28]. Although the rate of metronidazole resistance among the *H. pylori* strains in our survey was not higher than

**TABLE 1. Primer sequences and PCR conditions**

| Genes | Primer sequence (5' → 3') | PCR product (bp) | PCR conditions | References |
|-------|--------------------------|----------------|----------------|-----------|
| glmM  | AAGCTTACTCTTCATAACCTA    | 294           | 94°C, 5 min, 94°C, 45s, 57°C, 1min, 72°C, 30s (35 cycles) | [6]       |
|        | AAGCTTTAAGTTGGTATTG      | 360           | 94°C, 5 min, 94°C, 45s, 55°C, 1min, 72°C, 30s (35 cycles) | [6]       |
|        | GCAACTATCCTAACCATCAG     |               |                |           |
| rdxA   | GCTAAGCAGTAGCGCAAGGCA    | 850           | 94°C, 5 min, 94°C, 45s, 58°C, 1min, 72°C, 30s (35 cycles) | [6]       |
|        | GAAAGCCTTAAAAAGCCCCCT    |               |                |           |
|        | CCAAGCGAGTTGCTCAGAGGCAA | 429           | 95°C, 5 min, 95°C, 30s, 54°C, 30s, 72°C, 30s (35 cycles) | [6]       |
| 23srRNA| ATACCTCATAAGGCAAGCCCCCT  |               |                |           |
others, we think that the small size of our population was the main reason for this determined rate. We have sequenced the \textit{rdxA} fragment and understood that no predicted mutation was observed, a novel finding that had never been reported, at least among the Iranian population. Although the prevalence of clarithromycin in this study was also within the expected range for developing countries, it is recommended that alternative regimens should be chosen if the clarithromycin resistance rate is >15%. Interestingly, Deyi et al. showed that the 200-bp deletion in the \textit{rdxA} gene was necessary to induce the resistance to metronidazole among the \textit{H. pylori} NCTC11637; however, we have found metronidazole-resistant strains without \textit{rdxA} mutations [26]. Close to our findings, Mohammadi et al. reported that only 5% of metronidazole-resistant strains carried at least a mutation in the \textit{rdxA} gene [16]. In contrast, Abdollahi et al., found that 22% of resistant strains exhibited the \textit{rdxA} deletion mutation [29]. However, these two recent Iranian studies had controversial results in comparison with our experiment. The main limitation in this research was the relatively small sample size. Indeed, the successful bacterial culture rate of 36.5% for registered participants is a relatively low rate that clearly indicates the difficulties in culturing \textit{H. pylori} caused by various fungal contaminations. On the other hand, as our findings have shown no mutation in the \textit{rdxA} gene attributed to the resistance phenotype, we assume that other genes or different mechanisms might influence the emergence of metronidazole resistance in \textit{H. pylori}. Chisholm et al. evaluated more than 46 clinical isolates to find any mutations contributing to the metronidazole-resistance phenotype, but no mutations were reported [30]. Based on our result, the use of \textit{rdxA} as a marker gene for the detection of the mutation responsible for the metronidazole resistance genotype may not be useful in clinical settings.

**Conclusion**

Our results highlight the critical role of \textit{H. pylori} molecular susceptibility approaches for gastroenterologists to define an improved therapeutic regimen against this persistent bacterium. Our findings will be helpful in designing more effective and logical antibiotic therapy to successfully eradicate the \textit{H. pylori} in individuals with severe gastroduodenal disorders. The detection of \textit{H. pylori} isolates with no responsible mutation in the \textit{rdxA} gene shows the need for new studies to check other possible molecular mechanisms conferring the metronidazole resistance phenotype on \textit{H. pylori}.

**Conflicts of interest**

None declared.

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**TABLE 2.** Antibiotic resistance profile of various patients according to the age range and gender

| Antibiotic susceptibility | MTZ | CLR |
|---------------------------|-----|-----|
| Number of isolates, n (%) | 33 (45.3) | 40 (54.7) | 17 (23.3) | 56 (76.7) |
| Gender | 18 (F) | 15 (M) | 8 (F) | 9 (M) |
| Age group (25-35 years) | 4 | 3 | 2 | 3 |
| Age group (36-45 years) | 5 | 2 | 0 | 3 |
| Age group (46-55 years) | 0 | 2 | 3 | 2 |
| Age group (56-65 years) | 3 | 3 | 2 | 6 |
| Age group (>75 years) | 4 | 0 | 1 | 5 |

Abbreviations: MTZ: Metronidazole, CLR: Clarithromycin, R: Resistance, S: Susceptible, F: Female, M: Male.

**TABLE 3.** Disease distribution of 73 patients infected with \textit{Helicobacter pylori} versus resistance status

| Diseases | MTZ-resistant | CLR-resistant | Multidrug resistant isolates | p value |
|-----------|---------------|---------------|-----------------------------|---------|
| \(G (n = 44)\) | 19 | 6 | >0.05 |
| \(DU (n = 10)\) | 4 | 2 | >0.05 |
| \(GU (n = 16)\) | 9 | 2 | >0.05 |
| \(GC (n = 3)\) | 1 | 0 | >0.05 |
| Total (\(n = 73\)) | (\(n = 33\)) | (\(n = 17\)) | (\(n = 10\)) |

Abbreviations: GC, gastric cancer; G, gastritis; DU, duodenal ulcer; GU, gastric ulcer; MTZ, metronidazole; CLR, clarithromycin.
References

[1] Mégraud F. A humble bacterium sweeps this year’s Nobel Prize. Cell 2005;123:975–6.
[2] Fallone CA, Moss SF, Malfertheiner P. Reconciliation of recent Helicobacter pylori treatment guidelines in a time of increasing resistance to antibiotics. Gastroenterology 2019;157:44–53.
[3] Suerbaum S, Michetti P. Helicobacter pylori infection. N Engl J Med 2002;347:1175–86.
[4] Lee Y-C, Chiang T-H, Liou J-M, Chen H-H, Wu M-S, Graham DY. Mass eradication of Helicobacter pylori to prevent gastric cancer: theoretical and practical considerations. Gut Liver 2016;10:12.
[5] Malfertheiner P, Mégraud F, O’Morain C, Gizbert J, Kuipers E, Axon A, et al. Management of Helicobacter pylori infection—the Maastricht VI Florence consensus report. Gut 2017;66:6–30.
[6] Mégraud F. Time to change approaches to Helicobacter pylori management. Lancet Gastroenterol Hepatol 2017;2:692–3.
[7] Phan TN, Santona A, Tran VH, Tran TNH, Cappuccinelli P, Rubino S, et al. High rate of levofloxacin resistance in a background of clarithromycin and metronidazole-resistant Helicobacter pylori in Vietnam. Int J Antimicrob Agents 2015;45:244–8.
[8] Hu Y, Zhang M, Lu B, Dai J. Helicobacter pylori and antibiotic resistance, a continuing and intractable problem. Helicobacter 2016;21:349–63.
[9] O’Connor A, Lamarque D, Gizbert JP, O’Morain C. Treatment of Helicobacter pylori infection 2017. Helicobacter 2017;22:e12410.
[10] Boyanova L, Erastiev I, Yordanov D, Markovska R, Mitov I. Three unsuccessful treatments of Helicobacter pylori infection by a highly virulent strain with quadruple antibiotic resistance. Folia Microbiol 2016;61:307–10.
[11] Shokri-Shirvani J, Zamani V, Zamami M. Global emergence of Helicobacter pylori antibiotic resistance—unanswered questions. Aliment Pharmacol Ther 2016;43:1249.
[12] Park JY, Dunbar KB, Mitui M, Arnold CA, Lam-Himlin DM, Valseck MA, et al. Helicobacter pylori clarithromycin resistance and treatment failure are common. Dig Dis Sci 2016;61:2373–80.
[13] Thung I, Aramin H, Vavinskaya V, Gupta S, Park J, Crowe S, et al. The global emergence of Helicobacter pylori antibiotic resistance. Aliment Pharmacol Ther 2016;43:514–33.
[14] Mégraud F, Lehours P. Helicobacter pylori detection and antimicrobial susceptibility testing. Clin Microbiol Rev 2007;20:280–322.
[15] Lee Y-C, Chang T-H, Chou C-K, Tu Y-K, Liao W-C, Wu M-S, et al. Association between Helicobacter pylori eradication and gastric cancer incidence: a systematic review and meta-analysis. Gastroenterology 2016;150:1113–1124 e5.
[16] Mohammadi M, Goroud D, Mohajerani N, Massarrat S. Helicobacter pylori antibiotic resistance in Iran. World J Gastroenterol 2005;11:6009.
[17] Savoldi A, Carrara E, Graham DY, Conti M, Tacconelli E. Prevalence of antibiotic resistance in Helicobacter pylori: a systematic review and meta-analysis in World Health Organization regions. Gastroenterology 2018;155:1372–1382.e17.
[18] Muñoz N, Sánchez-Delgado J, Baylina M, Puig I, López-Gongora S, Suarez D, et al. Systematic review, meta-analysis, and meta-regression: successful second-line treatment for Helicobacter pylori. Helicobacter 2018;23:e12488.
[19] Yousefi-Avarvand A, Vaez H, Tafaghodi M, Sahebkar AH, Arzanlou M, Khademi F. Antibiotic resistance of Helicobacter pylori in Iranian children: a systematic review and meta-analysis. Microb Drug Resist 2018;24:980–86.
[20] Zamani M, Ebrahimi Tabar F, Zamani V, Miller W, Alizadeh-Navaei R, Shokri-Shirvani J, et al. Systematic review with meta-analysis: the worldwide prevalence of Helicobacter pylori infection. Aliment Pharmacol Ther 2018;47:868–76.
[21] Siddique O, Ovalle A, Siddique AS, Moss SF. Helicobacter pylori infection: an update for the internist in the age of increasing global antibiotic resistance. Am J Med 2018;131:473–9.
[22] Kageyama C, Sato M, Sakae H, Obayashi Y, Kawahara Y, Mimura T, et al. Increase in antibiotic resistant Helicobacter pylori in a University Hospital in Japan. Infection and drug resistance. Infect Drug Resist 2019:12:597–602.
[23] Haghighi MB, Dara N, Mansour Ghaeie R, Azimi L, Hosseini A, Tajalli S, et al. Evaluation of clarithromycin and metronidazole resistance of Helicobacter pylori infection in symptomatic Iranian children. Int J Pediatr 2019;7:8925–33.
[24] Amin M, Shayesteh AA, Serajian A, Goodarzi H. Assessment of metronidazole and clarithromycin resistance among Helicobacter pylori isolates of Ahvaz (southwest of Iran) during 2015–2016 by phenotypic and molecular methods. Jundishapur J Microbiol 2019;12:e80156.
[25] Marques B, Donato MM, Cardoso O, Luxo C, Martinho A, Almeida N. Study of rdxA and frxA genes mutations in metronidazole-resistant and -susceptible Helicobacter pylori clinical isolates from the central region of Portugal. J Glob Antimicrob Resist 2019;17:300–4.
[26] Deyi VYM, Burette A, Ntounda R, Elkilic O, Cadranel S, Bontems P, et al. Update of primary Helicobacter pylori resistance to antimicrobials in Brussels, Diagn Microbiol Infect Dis 2019:11:4875.
[27] Pourakbari B, Mahmoudi S, Parhiz J, Sadeghi R, Monajemzadeh M, Maniashi S. High frequency of metronidazole and clarithromycin-resistant Helicobacter pylori in formalin-fixed, paraffin-embedded gastric biopsies. Br J Biomed Sci 2018;75:61–5.
[28] Saniee P, Hossein F, Kadkhodaei S, Siavoshi F, Khalili-Samani S. Helicobacter pylori multidrug resistance due to misuse of antibiotics in Iran. Arch Iran Med 2018;21:7.
[29] 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. Jundishapur J Microbiol 2011, epub ahead of print.
[30] Chisholm SA, Owen RJ. Mutations in Helicobacter pylori rdxA gene sequences may not contribute to metronidazole resistance. J Antimicrob Chemother 2003;51:995–9.