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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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-*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 *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 *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 *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 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 *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 (Bahar-Azma, Tehran, Iran), 10% fetal bovine serum (Sigma, St Louis, MO, USA), *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 *H. pylori*. Following the incubation period, the *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 *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 (Bahar-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 *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 (Bahar-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 *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	AAGCTTACTTTCTAACACTA AAGCTTTTAGGGGTGTT	294	94°C, 5 min,94°C,45s, 57°C,1min, 72°C, 30s (35 cycles)	[6]
rdxA	GCAACTATCCAATCCCATCAAG GCCAGACTATCGCCAAGC	360	94°C, 5 min 94°C,45s, 55°C,1min, 72°C, 30s (35 cycles)	[6]
rdxA	AATTTGAGCATGGGGCAGA GAAACGCTTGAAAACACCCCT	850	94°C, 5 min 94°C,45s, 58°C,1min, 72°C, 30s (35 cycles)	[6]
23srRNA	CCACAGCGATGTGGTCTCAGCAAA ATGACTCCATAAGAGCCAAAGCCCT	429	95°C, 5 min 95°C,30s, 54°C,30S, 72°C, 30s (35 cycles)	[6]

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

Antibiotic susceptibility	MTZ					CLR				
	R		S		p value	R		S		p value
Number of isolates, n (%)	33 (45.3)		40 (54.7)		>0.05	17 (23.3)		56 (76.7)		>0.05
Gender	18 (F)	15 (M)	18 (F)	22 (M)	>0.05	8 (F)	9 (M)	24 (F)	32 (M)	>0.05
Age group (25-35 years)	4	3	6	5	>0.05	2	3	6	6	>0.05
Age group (36-45 years)	2	5	5	1	>0.05	1	0	2	6	>0.05
Age group (46-55 years)	5	2	0	3	>0.05	0	0	4	3	>0.05
Age group (56-65 years)	0	2	2	4	>0.05	3	2	2	6	>0.05
Age group (66-75 years)	3	3	4	4	>0.05	2	1	4	6	>0.05
Age group (>75 years)	4	0	1	5	>0.05	0	3	6	5	>0.05

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

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

Diseases	Resistance status			
	CLR-resistant	MTZ-resistant	Multidrug resistant isolates	p value
G (n = 44)	8	19	6	>0.05
DU (n = 10)	5	4	2	>0.05
GU (n = 16)	4	9	2	>0.05
GC (n = 3)	0	1	0	>0.05
Total (n = 73)	(n = 17)	(n = 33)	(n = 10)	

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

others, we think that the small size of our population was the main reason for this determined rate. We have sequenced the *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 *rdxA* gene was necessary to induce the resistance to metronidazole among the *H. pylori* NCTC11637; however, we have found metronidazole-resistant strains without *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 *rdxA* gene [16]. In contrast, Abdollahi et al., found that 22% of resistant strains exhibited the *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 *H. pylori* caused by various fungal contaminations. On the other hand, as our findings have shown no mutation in the *rdxA* gene attributed to the resistance phenotype, we assume that other genes or different mechanisms might influence the emergence of metronidazole resistance in *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 *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 *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 *H. pylori* in individuals with severe gastroduodenal disorders. The detection of *H. pylori* isolates with no responsible mutation in the *rdxA* gene shows the need for new studies to check other possible molecular mechanisms conferring the metronidazole resistance phenotype on *H. pylori*.

Conflicts of interest

None declared.

Acknowledgements

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