

Helicobacter pylori antibiotic resistance in Iran

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OBJECTIVE

AIM: To examine the frequency of antibiotic resistance in Iranian *Helicobacter pylori* (*H. pylori*) strains isolated from two major hospitals in Tehran.

METHODS: Examination of antibiotic resistance was performed on 120 strains by modified disc diffusion test and PCR-RFLP methods. In addition, in order to identify the possible causes of the therapeutic failure in Iran, we also determined the resistance of these strains to the most commonly used antibiotics (metronidazole, amoxicillin, and tetracycline) by modified disc diffusion test.

RESULTS: According to modified disc diffusion test, 1.6% of the studied strains were resistant to amoxicillin, 16.7% to clarithromycin, 57.5% to metronidazole, and there was no resistance to tetracycline. Of the clarithromycin resistant strains, 73.68% had the A2143G mutation in the 23S *rRNA* gene, 21.05% A2142C, and 5.26% A2142G. None of the sensitive strains were positive for any of the three point mutations. Of the metronidazole resistant strains, deletion in *rdxA* gene was studied and detected in only 6 (5%) of the antibiogram-based resistant strains. None of the metronidazole sensitive strains possessed *rdxA* gene deletion.

CONCLUSION: These data show that despite the fact that clarithromycin has not yet been introduced to the Iranian drug market as a generic drug, nearly 20% rate of resistance alerts toward the frequency of macrolide resistance strains, which may be due to the widespread prescription of erythromycin in Iran. *rdxA* gene inactivation, if present in Iranian *H. pylori* strains, may be due to other genetic defects rather than gene deletion.

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Key words: *H. pylori*; Clarithromycin; Metronidazole; Resistance; Iran

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infects the majority of the adult population in developing countries including Iran. The rate of infection in Iranian adults according to serology data is up to 80%^[1]. The outcomes include gastritis, peptic ulcers and gastric adenocarcinomas, which are highly prevalent in Iran^[2]. Patient compliance with the prescribed medications, presence of pre-existing resistance to the key anti-*H. pylori* antibiotics (clarithromycin and metronidazole), final duration of therapy and prescribed dose of antibiotics are all the factors which influence the effectiveness of therapy^[3]. Host genetic polymorphisms, i.e. IL-1-511 and CYP450 and CYP2C19, may affect the efficiency of the therapy as well^[4]. Antibiotic resistance varies geographically and there is a great need for local studies.

Clarithromycin is recommended as a key component in anti-*H. pylori* eradication^[5]. In different geographic locations, resistance to clarithromycin differs. In countries with a higher use of macrolides, the rate of resistance to clarithromycin is reported to be proportionally higher^[6]. The basis of the resistance is the presence of defined mutations in the 23S *rRNA* gene, which results in a decrease in the antibiotic binding to the bacterial ribosome^[7-9]. Mutations in the form of A-G transition at nucleotide positions 2143, 2144, and A-C at nucleotide position 2142 have been found to confer clarithromycin resistance in *H. pylori* strains^[10]. Recently in *H. pylori* isolates from northeast China, three new mutation points (G2224A, C2245T, and T2289C) were found to be related to the clarithromycin resistance^[11]. Several different PCR-based methods have been developed for the detection of the above-mentioned mutations in order to amplify part of the V domain of 23S *rRNA* gene^[12]. The amplified fragments are digested by restriction enzymes, indicating mutations in the positions 2143 and 2144 which yield a high level clarithromycin resistance^[10,13]. A2142C is reported to be less frequent and is detectable by PCR using 3'-mismatched specific primers^[14,15].

Though most countries include clarithromycin in the prescribed anti-*H. pylori* treatment regimens, this drug is infrequently prescribed in Iran due to its high cost.

As regards to metronidazole, the basis of resistance in *H. pylori* has been partly associated with inactivation of *rdxA*, the gene encoding an oxygen-insensitive NADPH nitroreductase may be enhanced by mutations in *fixA* gene

encoding a NAD(P)H-flavin oxidoreductase^[16].

This study presents the first documented report from Iran on the *rdxA* gene deletion. Frameshift mutations in *frxA* gene were not studied as their frequent occurrence has questioned its reliability as a resistance marker^[17].

We have used modified disc diffusion test for primary analysis followed by the described molecular assays to examine resistance among Iranian *H. pylori* strains with particular emphasis on clarithromycin and metronidazole.

RESULTS

Bacterial strains and growth conditions

Gastric antral biopsies were taken from 120 non-*H. pylori* pretreated patients (52 males and 68 females, aged 42±20 years) with epigastralgia referring to gastric endoscopy (during 2001-2002) followed by rapid urease test. These patients did not undergo any prior treatment. Urease positive samples were cultured on brucella agar (Merck) supplemented with 5% sheep blood, (6 mg/L) vancomycin (Fluka BioChemika), (5 mg/L) trimethoprim (Fluka BioChemika) and (2 mg/L) amphotericin-B (Gibco) under microaerophilic conditions (85% N₂, 100 mL/L CO₂, 5% O₂) at 37 °C for 3-5 d. Single colonies of *H. pylori* were then isolated and confirmed for identity according to colony morphology, wet mount, microscopic observation after Gram staining and biochemical analysis (urease and catalase tests).

Disc diffusion susceptibility test

Bacterial resistance to clarithromycin^[18], metronidazole^[19], amoxicillin, and tetracycline^[20] was determined using modified Kirby-Bauer procedure as previously described^[21,22]. Suspensions of 4-d-old cultures were prepared in sterile saline to opacity of No. 4 (108 CFU/mL) McFarland standard. Muller-Hinton agar plates supplemented with 5% sheep blood were then inoculated with a swab from the prepared suspension. House-made discs containing 2 µg clarithromycin, 5 µg metronidazole, 10 µg amoxicillin, and 30 µg tetracycline were placed separately on the culture plates for each strain. Plates were then incubated at 37 °C for 72 h under microaerophilic conditions generated by Gas Pak jars. The disc diffusion tests were made thrice for each strain. The inhibition zone diameters were measured in millimeters with a ruler. If similar results were observed in two experiments out of three, the isolates were considered susceptible or resistant but as mixed populations. A *H. pylori* control strain susceptible to metronidazole (Sydney strain, ss1) was used. The breakpoints with the Mueller-Hinton agar for the inhibition diameters are described in Table 3, briefly: 20 mm for tetracycline, 11 mm for amoxicillin, for metronidazole zones areas; <16 mm resistant, 16-21 mm intermediate and >21 mm susceptible (but in this study isolates in the intermediate and resistant zone were both considered as resistant), for clarithromycin development of any size zone is considered resistant^[23].

DNA extraction and PCR assays

Genomic DNA was extracted as previously described^[10]. PCR primers used for this study were chosen from published reports with the implementation of the same PCR cycles^[10]

(Table 1). Primer sets 1 and 2 were designed to detect point mutations in the 23S *rRNA* gene responsible for clarithromycin resistance. Set 1 was used to amplify a 1 400-bp PCR fragment following digestion with *Bsa*I produced three fragments of 700, 400, and 300 bp, if the A2143G mutation was present. *Mbo*II digestion of the 1 400-bp fragment produced two 700-bp fragments, if A2142G mutation was present. A 750 and a 850 bp were produced, if A2144G mutations existed^[10]. Set 2 was used in 3'-mismatched PCR to obtain a 700-bp-amplified fragment, an indication of the A2141C mutation^[15].

For detection of *rdxA* gene deletion, set 3 was used. In this case, the native gene yielded a 850-bp PCR product and its deletion resulted in a 650-bp fragment^[24].

Table 1 Primer sets used in this study

Gene	Primers	Expected fragment (bp)	References
23S <i>rRNA</i>	CLA 18: 5'-AGTCGGGAC CTAAGGCGAG-3'	1 400	10
(set 1)	CLA 21: 5'-TTCCCGCTTA GATGCTTTCAG-3'		
23S <i>rRNA</i>	CLA 18: 5'-AGTCGGGACC TAAGGCGAG-3'	700	15
(set 2)	CLA 3: 5'-AGGTCCACGGG GTCTTG-3'		
<i>RdxA</i>	RdxA1: 5'-AATTGAGCATG GGGCAGA-3'	850	24
(set 3)	RdxA2: 5'-GAAACGCTTGAA AACACCCCT-3'		

Statistical analysis

Data were analyzed by SPSS version 11.0. The Pearson χ^2 test and Fisher's exact test were used to assess the relationships between the results of disc diffusion and PCR/RFLP methods. Standard error of mean for each frequency was determined accordingly.

RESULTS

Primary antibiotic resistance by disc diffusion

According to the disc diffusion test, all 120 strains were sensitive to tetracycline. Of these, 2 strains (1.6%) were resistant to amoxicillin, 20 (16.7%) to clarithromycin and 69 (57.5%) to metronidazole. Five of the one hundred and twenty (4.2% of the strains) showed dual resistance to metronidazole and clarithromycin. Table 2 presents the frequency of single and multiple resistances to the tested antibiotics.

Detection of 23S *rRNA* mutations via PCR-RFLP

All the DNA samples were positive by the PCR assay for the amplification of the 1 400 bp of 23S *rRNA* gene. RFLP analysis pattern showed that 19 out of 120 (15.8%) strains were mutated (Table 2). In this group, 73.68% had the A2143G mutation and 5.26% the A2142G mutations. Moreover, none of the clarithromycin resistant samples showed the A2144G mutation, 21.05% of the samples were positive for the 3'-mismatched PCR, revealing the presence

of the A2142C mutation. Among the 20 clarithromycin resistant strains, only one did not show any of these mutations. None of the sensitive strains were positive for any of the mentioned mutations.

PCR detection of *rdxA* gene deletion

Among the 69 metronidazole resistant strains (based on antibiograms), only 6 (5% of total) demonstrated 200 bp deletion in the *rdxA* gene (Table 2).

According to the data analysis by the SPSS program, there were no relations between the 23S *rRNA* mutations, *rdxA* deletion and antibiotic resistance with age, gender, and *cagA* status.

Table 2 Frequency of single and multi-drug resistance based on listed methods of detection

Antibiotics	Method of detection					
	Disc diffusion		23S <i>rRNA</i> PCR-RFLP		<i>rdxA</i> gene deletion	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Amoxicillin only	1/120	0.8				
Amoxicillin and metronidazole	1/120	0.8				
Clarithromycin only	15/120	12.5	19/120	15.8		
Clarithromycin and metronidazole	5/120	4.2				
Metronidazole only	63/120	52.5			6/120	5
Metronidazole and amoxicillin	1/120	0.8				
Metronidazole and clarithromycin	5/120	4.2				
Tetracycline	0/120	0				

Table 3 Susceptibility rates of 120 isolates to selected antibiotics by modified disk diffusion method

Antibiotics	Disc content (μg)	Zone size breakpoint (resistance) (mm)	Resistant isolates (%)
Metronidazole	5	16	34.2 resistant 23.3 intermediate
Clarithromycin	2	Any zone	16.7
Amoxicillin	10	11	1.6
Tetracycline	30	20	0

DISCUSSION

Several studies comparing the different susceptibility techniques for *H. pylori* have been published, but the results are controversial, as *H. pylori* is a very slow growing bacterium with particular growth needs^[23,25-28]. The oxygen-dependent metabolism of metronidazole in *H. pylori* via several nitroreductases possibly results in great difference in MIC when isolates are tested repeatedly. Nevertheless, strains continue to be classified as resistant or susceptible^[25].

As regards to culture-based antimicrobial susceptibility testing (especially to metronidazole), routine methods of agar dilution, disc diffusion, and *E*-test seem to be poorly standardized^[29]. Though time consuming agar dilution is accepted as the gold standard and is reported to be highly reproducible in several studies^[23,30,31], nowadays, *E*-test is

used most frequently as a substitute as it is easier to perform and relatively reproducible^[32]. Finally, a cheap and easy way to perform susceptibility testing is via disc diffusion, but no MIC value can be obtained. Recently in UK, susceptibility testing to metronidazole, tetracycline, macrolide, and amoxicillin is performed by the modified disc diffusion method and the results are adequate to determine the resistance rates in a large cohort of patients from a single clinical center whom resistance patterns have been evaluated over time^[33]. Then again in a study in France, reproducibility study on randomly selected strains declared that disc diffusion is more reproducible than *E*-test for both clarithromycin and erythromycin^[34]. Due to these observations and high cost of *E*-test, modified disc diffusion test was chosen for this study.

Worldwide antibiotic resistance has been carefully reviewed by Megraud^[35], and our resistance rate to clarithromycin and metronidazole is comparable to the resistance rate of 50% to metronidazole and 8% to clarithromycin in China^[36].

Current regimens for the eradication of *H. pylori* in Iran consist of a proton pump inhibitor (omeprazole) or an H₂ receptor blocker (ranitidine), a bismuth salt plus two antibacterial agents, such as amoxicillin, furazolidone/metronidazole or recently clarithromycin^[37]. Treatment regimens of 4 or 7 d are unacceptable for *H. pylori* infection in Iran, even in the presence of a favorable sensitivity profile^[38]. Although the presented data do not support the association with the failure of eradication in our country, according to our results, such rates of resistance to metronidazole and clarithromycin could be the major cause of eradication failure in Iran. For developing countries, the standard triple therapy remains as the best option for the eradication regimen because of its low cost. However, in Iran bismuth triple therapy in the presence of high prevalence of metronidazole resistance has a poor efficacy unless higher doses of metronidazole are prescribed to increase the cure rate of therapy^[39].

Our results indicate that making clarithromycin available may not be an effective strategy to improve the eradication rates in Iran, as the level of resistance is already significant. There is a worldwide need for simple and cost effective anti-*H. pylori* therapies. Since the effectiveness relates to the level of antibiotic resistance, knowledge of the resistance patterns is essential for choosing empiric therapy. Metronidazole is widely used in Iran because of the prescription in most periodontal and parasitic diseases. Resistance to metronidazole among Iranian patients, as most of the developing countries, is relatively high and this is the major cause of eradication failure in these countries. Thus, the need for a suitable alternative for this drug is highly perceptible and crucial to plan a more effective therapeutic strategy. (OABC) Omeprazole, Amoxicillin, Bismuth, Clarithromycin and (OABF) Omeprazole, Amoxicillin, Bismuth, Furazolidone are both effective in eradicating *H. pylori* in countries where metronidazole resistance is a problem. OABF is a good alternative in the face of growing resistance to clarithromycin in developed countries, and is attractive for developing countries where clarithromycin is not readily available and is particularly recommended for Iran^[37].

In recent years, *rdxA* gene analysis of the fresh samples showed that the metronidazole resistance is mainly attributed to the mutations in this gene including gene deletion^[24]. AntibioGram analysis of our strains demonstrated 57% metronidazole resistance; however, the majority of the metronidazole resistant strains did not show the 200 bp deletion in the *rdxA* gene. One obvious explanation is that the deletion in this gene is not the only defect causing gene inactivation and is not an informative indication of metronidazole resistance in Iranian *H. pylori* strains (with ~60% metronidazole resistance). The data are supported by data from Germany^[40] and France^[16] (with 40-50% metronidazole resistance) and contradicted by data from China^[41] and Taiwan^[42] (with 50-60% resistance). Due to the reported disparity, it seems that *rdxA* gene mutation is not a suitable marker for molecular prediction of metronidazole resistance. Although *rdxA* gene deletion is not a good resistance indicator, it is only detected in resistant strains and all of the strains with *rdxA* deletion clusterized in specific rappedemes (analyzed by RAPD-PCR), suggesting that they are genetically similar (data not shown). Unlike clarithromycin, metronidazole resistance *in vitro* does not reflect *in vivo* resistance. Other existing genetic defects causing metronidazole resistant phenotypes and the high incurring cost of molecular diagnostic assays lead us to the conclusion that standard culture-based techniques (simple and inexpensive disc diffusion method) remain the recommended method of choice in Iran.

In conclusion, the discovery of such a high rate of resistance to clarithromycin, which is most likely due to the vast consumption of erythromycin in cases of upper respiratory infections, calls for an effective eradication program and disqualifies clarithromycin as an alternative to metronidazole. These results further confirm the essence of continuous monitoring of antibiotic resistance patterns in order to reduce the rate of eradication failure in Iran or any other target population.

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