

Antimicrobial susceptibility of *Helicobacter pylori* strains isolated from patients in Shiraz, Southern Iran

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Abstract

AIM: To improve our understanding of Iranian regional variation in *Helicobacter pylori* (*H. pylori*) antibiotic resistance rates to find the best antibiotic therapy for eradication of *H. pylori* infections.

METHODS: A total of 266 patients undergoing endoscopy in Shiraz, Southern Iran, were included in this study. *H. pylori* strains were isolated from antral biopsies by culture and confirmed by the rapid urease-test and gram staining. Antibiotic susceptibility of *H. pylori* isolates was determined by E-test.

RESULTS: A total of 121 *H. pylori* strains were isolated, 50 from male and 71 from female patients. Data showed that 44% ($n = 53$), 20% ($n = 24$), 5% ($n = 6$), and 3% ($n = 4$) of all strains were resistant to the antibiotics metronidazole, amoxicillin, clarithromycin, and tetracycline, respectively. When the antibiotics were considered together we found 11 sensitivity patterns for the strains. Resistance to metronidazole was significantly higher in female than in male patients ($P < 0.05$). In about 71% of the metronidazole-resistant isolates, the minimum inhibitory concentrations (MICs) exceeded 256 $\mu\text{g/mL}$.

CONCLUSION: We found a moderate rate of primary resistance to metronidazole. However, a high MIC ($> 256 \text{ mg/L}$) which was found in 71% of the isolates is considerable. In the case of amoxicillin, an increased resistance rate of 20% is worrying. Resistance to clarithromycin and tetracycline is also emerging among the *H. pylori* strains in our region.

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Key words: Gastric disorders; *Helicobacter pylori*; Iran; Sensitivity; Treatment

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is involved in the pathogenesis

of a number of gastrointestinal diseases, including acute and chronic gastritis, peptic ulceration, gastric carcinoma and gastric lymphoma^[1]. Eradication treatment of *H. pylori* infection usually consists of various combinations of drugs. Most commonly, an acid suppressor (usually a proton pump inhibitor) or an H₂-receptor antagonist (e.g. ranitidine) is prescribed in combination with two antibiotics usually amoxicillin, metronidazole or clarithromycin^[1]. The combination of two antibiotics can increase the success of eradication therapy and decrease the possibility of secondary antibiotic resistance^[2]. Antibiotic resistance in *H. pylori* is the major cause of eradication failure. Growing resistance often parallels the patterns of antibiotic consumption, and may vary within patient groups according to the geographic region, patient age and sex, type of disease, birthplace, other infections and other factors. The geographic map and the process of primary *H. pylori* resistance are clinically important, and should be considered when choosing eradication regimens, as is constant monitoring at both national and global level in an attempt to reach the recently recommended goal of eradicating more than 95% of resistant cases^[3]. The prevalence of clarithromycin, metronidazole and amoxicillin resistance varies between countries and is highest for metronidazole^[4,5]. Resistance to tetracycline and ciprofloxacin has been reported but appears uncommon^[6-8].

There are several problems with antimicrobial susceptibility testing of *H. pylori*^[9,10]. Agar or broth dilution methods are difficult to perform routinely^[11], thus, disk-diffusion testing is often used because it is simple, easy to perform, and economical^[12]. However, the E-test has proved to be an accurate method for assaying the susceptibility of fastidious organisms, including *H. pylori*, to antibiotics. The E-test has a more stable pattern of antibiotic release and has been found to tolerate prolonged incubation^[13]. This is the main reason why the E-test rather than the disk diffusion method, has been recommended for *H. pylori*.

Pre-treatment resistance rates in *H. pylori* vary markedly between countries and regions. In Europe, mean resistance rates of 27% for metronidazole and 10% for clarithromycin are typical^[14,15]. There is no systematic surveillance of primary antibiotic resistance rates in Shiraz, and widely divergent rates have been reported in Iran, depending on the local population.

The present study aimed to improve our understanding of the Iranian regional variation in *H. pylori* antibiotic resistance rates in relation to gender, and to find the best antibiotic therapy for the eradication of *H. pylori* infections.

MATERIALS AND METHODS

The patient groups and sample collection

In this study, 266 patients attending the endoscopy ward of Motahhary Clinic of Shiraz University of Medical Sciences during the period between October 2008 and October 2009 were enrolled. Exclusion criteria for patient recruitment to the study were: previous attempts to eradicate *H. pylori*, use of antibiotics or proton pump inhibitors within the last 2 wk prior to endoscopy, and previous gastric surgery. The diagnosis of *H. pylori* infec-

tion and confirmation of gastric disease by histology were established by a central study pathologist. Antral biopsies taken from each patient were transferred to the lab in an appropriate transfer medium (brain heart infusion broth, supplemented with 20% glucose) for *H. pylori* isolation and identification.

Isolation and identification of H. pylori

Biopsy samples were gently homogenized and cultured on rapid urease-test media and colombia agar base (Merck, Germany), supplemented with 10% lysed horse blood and 7% fetal calf serum and the antibiotics amphotericin B (5 µg/L), trimethoprim (5 µg/L) and vancomycin (10 µg/L). The cultures were kept in a microaerophilic atmosphere (7% O₂, 7.1% CO₂, 7.1% H₂, 79.8% N₂), provided by Anoxomate (Mark II, Mart Microbiology BV, Netherlands) at 37°C for 2-4 d. The isolates were then confirmed as *H. pylori* by positive oxides, catalase and rapid urease-tests. The samples were also evaluated for the presence of *H. pylori* by the modified gram staining and rapid urease-tests. If any of the two tests were positive simultaneously, the sample was considered *H. pylori* positive.

Antibiotic susceptibility test

For *in vitro* susceptibility testing of the *H. pylori* strains, a suspension equal to the McFarland tube no. 3 was prepared for each isolate. We used only one colony from each patient for the analysis. Brain heart infusion broth (Merck, Germany) plates, supplemented with fetal calf serum (Gibco, USA) were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions. The E-test strips (Biomérieux, France) for the antibiotics amoxicillin, metronidazole, tetracycline, and clarithromycin, were aseptically placed onto the dried surface of inoculated agar plates. The plates were then incubated at 37°C under microaerophilic conditions. The minimum inhibitory concentrations (MICs) were read after 48-72 h of incubation on the basis of the intersection of the elliptical zone of growth inhibition using the MIC scale on the E-test strip, as per the manufacturer's instructions^[16]. Susceptibility results were recorded as resistant according to the following interpretive criteria; for metronidazole, clarithromycin, tetracycline and amoxicillin, MIC break-points of ≥ 8 mg/L, ≥ 1 mg/L, ≥ 4 mg/L and ≥ 0.5 mg/L, respectively^[17-19].

Statistical analysis

Fisher's exact test and *P* values were determined. A *P* value of < 0.05 was considered significant.

RESULTS

A total of 121 *H. pylori* strains were isolated from the patients under study, 50 from males and 71 from females. The antimicrobial susceptibility results of the *H. pylori* strains are presented in Table 1. According to the data, 44% (*n* = 53), 20% (*n* = 24), 5% (*n* = 6), and 3% (*n* = 4) of the strains were resistant to metronidazole, amoxicillin, clarithromycin, and tetracycline, respectively. Fifty isolates were

Table 1 Rates of antibiotic resistance in *Helicobacter pylori* isolates in relation to patient gender

Sex No.	No. of isolates (% of resistance)			
	MTZ	AMX	CLA	TET
Male (n = 50)	17 (34)	9 (18)	1 (2)	1 (2)
Female (n = 71)	36 (50)	15 (21)	5 (7)	3 (4)
Total (n = 121)	53 (43)	24 (19)	6 (5)	4 (3)

MTZ: Metronidazole; AMX: Amoxicillin; CLA: Clarithromycin; TET: Tetracycline.

Table 2 Antibiotic resistance patterns of the *Helicobacter pylori* strains

Antibiotic resistance patterns	n	Male	Female
MTZ	44	15	29
AMX	14	7	7
CLA	1	0	1
TET	1	1	0
MTZ-AMX	3	1	2
MTZ-TET	1	0	1
AMX-TET	1	0	1
AMX-CLA	1	0	1
MTZ-AMX-TET	1	0	1
MTZ-AMX-CLA	4	1	3
Sensitive	50	25	25
Total	121	50	71

MTZ: Metronidazole; AMX: Amoxicillin; CLA: Clarithromycin; TET: Tetracycline.

sensitive to all the tested antibiotics. When the antibiotics were considered together, we found 11 sensitivity patterns for drug sensitivity among the strains (Table 2). When the data were analyzed on the basis of patient gender (Table 1), 32% (n = 16), and 51% (n = 36) of the strains isolated from males and females, respectively, were resistant to metronidazole. Statistical analysis showed that resistance to metronidazole was significantly higher in female than in male patients (P < 0.05). With regard to clarithromycin and amoxicillin, the percentage of resistance in female patients was 7% and 20%, respectively and in male patients was 2% and 20%, respectively. The differences in resistance to these two antibiotics among the strains isolated from both genders were not significant (P > 0.05). We found that 3 (4%) and 1 (2%) strains isolated from female and male patients were resistant to tetracycline, respectively.

In about 71% of the metronidazole-resistant isolates, the MICs exceeded 256 µg/mL. The MIC ranges for the antibiotics tested in both genders are shown in Table 3.

DISCUSSION

Resistance to antimicrobials is of particular concern to practitioners in this field, and is a major cause of the failure to eradicate *H. pylori* infections^[20,21]. It has also been shown that resistance to different antibiotics develops in *H. pylori* strains by acquiring chromosomal mutations at the site where the drug acts^[22]. However, many reports have

Table 3 Range of minimum inhibitory concentrations for antibiotics tested against *Helicobacter pylori* strains in relation to patient gender

Sex	Range of MICs for antibiotics (mg/L)			
	MTZ	AMX	CLA	TET
Male	0.064 to > 256	< 0.016 to > 256	< 0.016 to 64	0.016 to 8
Female	0.047 to > 256	< 0.016 to > 256	< 0.016 to > 256	0.016 to 8

MIC: Minimum inhibitory concentration; MTZ: Metronidazole; AMX: Amoxicillin; CLA: Clarithromycin; TET: Tetracycline.

indicated that the prevalence of resistance varies geographically and that there is a broad range of resistance variability depending on the drug used^[23]. The special nutritional and atmospheric conditions required by these organisms make susceptibility testing relatively difficult; however, the E-test technique developed to determine the minimum inhibitory concentration (MIC) has remained valid^[24]. Accordingly, in the present study we evaluated the sensitivity of *H. pylori* strains isolated from patients with gastric disorders to 4 antibiotics using the E-test to find the resistance pattern in these strains in our region.

It was observed that 44% of the isolates in this study were resistant to metronidazole with a MIC range of 0.064 to > 256 µg/mL. This resistance rate was consistent with reports from some developed countries, where it has been reported that 15.8%-40% of *H. pylori* strains were resistant to metronidazole^[25-27]. However, most reports from developing countries describe a high level of resistance to metronidazole, which varies from 66.2% to 100%^[28,29]. Resistance rates to metronidazole may also vary within a country. For example, in India, the resistance rate to metronidazole was high in Lucknow, Chennai and Hyderabad (68%, 88.2% and 100%, respectively), whereas a moderate rate was observed in Delhi (37.5%) and Chandigarh (38.2%)^[30]. Similarly, the resistance rate was high (78%) in Tehran, Iran in one study carried out by Falsafi *et al*, while in another study it was reported to be 34%^[31,32]. In Europe, according to studies conducted between 1989-2001 and 1990-2002, respectively, the resistance rate varied between 16.0% and 43% in pediatric patients and between 14.9% and 40.3% in adult patients^[22]. It seems that primary resistance to nitro-imidazole has been attributed to frequent use of the drug, which is commonly prescribed for other diseases, especially parasitic conditions, and periodontal or gynecological infections. The higher resistance rate to metronidazole in females reported in this and other studies could be due to the treatment of gynecological infections using this drug which is also used in the treatment of bacterial vaginosis. Moreover, the use or abuse of this inexpensive drug may contribute to the increased metronidazole resistance seen in developing countries^[33]. For this reason, metronidazole has been excluded from first-line empirical therapy plans in some countries^[23]. However, it has been reported that the results of *in vitro* resistance to this drug are also poorly correlated with the outcome of therapy, and consequently, susceptibility testing is not rou-

tinely indicated^[34]. Therefore, assessment of the drug concentration in blood samples could be recommended to assess the correlation with *in vitro* results. Another important finding from the present study was that in about 71% of the metronidazole-resistant isolates, the MICs exceeded 256 µg/mL, which has rarely been reported^[17].

In contrast to most studies, we found a high resistance (20%) to amoxicillin among *H. pylori* isolates. Most studies have shown that *H. pylori* resistance to this drug is either very rare or non-existent^[25,33,35]. Usually, the MIC of amoxicillin for *H. pylori* is very low (0.03 µg/mL); nevertheless, in our study, we found a few sensitive isolates with reduced susceptibility (MICs: < 0.016 µg/mL)^[33]. However, high resistance rates have been reported in some studies from other parts of the world: 18.5% in South Korea^[36], 19.4% in Indonesia^[37], 32.8% in India^[38], and 38% in Brazil^[39]. In a study conducted in Ile-Ife, southwest of Nigeria, 100% of the 32 isolates were resistant^[28]. It has been shown that resistance to amoxicillin could have emerged by genomic mutation in the *pbp1A* gene^[39]. When comparing different sets of data, it is important to note that variations in rates may arise due to the effects of inter-laboratory reproducibility, caused by the lack of standardized testing protocols or regional prescribing practice. This may be the reason why the resistance rates for amoxicillin in *H. pylori* isolates have been reported to be 1.6% and 27% in different studies conducted in Iran^[31,32]. Moreover, high resistance to amoxicillin observed in the present study reflects the importance of its use in our country.

Clarithromycin is a macrolide used frequently in combination with other antimicrobial agents for the treatment of *H. pylori* infection^[40]. However, resistance to clarithromycin has become one of the major reasons for treatment failure^[41]. The prevalence of *H. pylori* resistance to clarithromycin varies in different countries, and was 12% in Japan, 1.7%-23.4% in Europe and 10.6%-25% in North America^[22]. Resistance in 5% of our isolates resembles data from the Northern regions of Europe^[27]. In contrast, two other studies from Tehran, Iran reported a high rate of resistance to clarithromycin ranging from 16.7% to 21%^[31,32]. Since clarithromycin is not currently used in Iran, emerging resistance to this antibiotic is unexpected. On the other hand, it has been shown that there is cross-reactivity between clarithromycin and other macrolides such as erythromycin, which implies that resistance to one macrolide could cause the emergence of resistance to other macrolides^[42]. Genetic studies have revealed that clarithromycin resistance is often associated with point mutation of the 23S rRNA^[2].

We observed a low resistance (3%) to tetracycline among the isolates, which is consistent with most studies which have reported no resistance or low resistance to this antibiotic in *H. pylori* strains^[2,31,35,43]. In contrast, a high resistance rate (20%) to tetracycline was reported by Falsafi *et al.*^[32]. Similarly, these variations could be due to the effects of inter-laboratory reproducibility caused by the lack of standardized testing protocols or regional prescribing practice. However, resistance to tetracycline, mainly caused by mutations in the 16S rRNA gene, is

emerging and can impair the efficacy of such second-line regimens^[44]. Thus, it seems that molecular methods can help verify the exact rate of resistance to this antibiotic. Recently a novel real-time PCR has been described which is able to detect the strains carrying the mutant genes for tetracycline resistance^[44].

In conclusion, this study showed a moderate rate of primary resistance to metronidazole which is included in the guidelines for the empirical therapy of *H. pylori* infections. However, a high MIC (> 256 mg/L) observed in about 71% of the isolates is considerable. On the other hand, in the case of amoxicillin, there was an increased resistance which is worrying. In particular, it is important to determine whether the increased resistance to amoxicillin is a result of its increased use or due to the ethnic differences of the populations described herein. The data also indicate that resistance to clarithromycin and tetracycline is emerging among the *H. pylori* strains in our region. Therefore, considering the increasing resistance rate in many countries, monitoring of susceptibility of *H. pylori* to these antibiotics appears to be necessary in order to choose effective therapy to eradicate *H. pylori* infections and to optimize the regimen in case of treatment failure. Finally, taking into account the present findings along with other reported findings, continued surveillance of the resistance profiles and the resistance mechanisms present in *H. pylori* strains isolated in Iran is essential, if therapeutic plans are to satisfy the country's needs.

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COMMENTS

Background

Antibiotic resistance in *Helicobacter pylori* (*H. pylori*) is the major cause of eradication failure. Growing resistance often parallels the patterns of antibiotic consumption, and may vary within patient groups according to the geographic region, patient age and sex, type of disease, birthplace, other infections and other factors.

Research frontiers

Geographic mapping and the process of primary *H. pylori* resistance are clinically important, and should be considered when choosing eradication regimens. These should also be constantly monitored both at national and global level in an attempt to reach the recently recommended goal of eradicating the highest rate of resistance. The present study aimed to improve our understanding of Iranian regional variation in *H. pylori* antibiotic resistance rates in relation to gender and to find the best antibiotic therapy for the eradication of *H. pylori* infections.

Innovations and breakthroughs

The E-test has proven to be an accurate method of assaying the susceptibility of fastidious organisms, including *H. pylori*, to antibiotics. Using this method and improved culture conditions, we found a moderate rate of primary resistance to metronidazole which is included in the guidelines for the empirical therapy of *H. pylori* infections. However, a high minimum inhibitory concentration (MIC) (> 256 mg/L) observed in 71% of the isolates is considerable. On the other hand, in the case of amoxicillin, there was an increased resistance (20%) which is worrying. The data also indicate that resistance to clarithromycin and tetracycline is emerging among the *H. pylori* strains in our region.

Applications

Considering the increasing rate of resistance in many countries and based on the varied results from different studies, even in the same regions, the results of this study can improve the monitoring of *H. pylori* susceptibility to antibiotics, which is necessary in order to choose effective therapy to eradicate *H. pylori* infections and to optimize the regimen in case of treatment failure in our region.

Terminology

MIC is minimum inhibitory concentration. E test is the epsilometry test, a test to determine the MIC of antimicrobial agents using strips with epsilometric concentrations of antimicrobials.

Peer review

It is an interesting publication showing the prevalence of antibiotic resistance of *H. pylori* in southern part of Iran (Shiraz).

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