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ORIGINAL ARTICLE

The status of antimicrobial resistance of *Helicobacter pylori* in Eastern Azerbaijan, Iran: comparative study according to demographics

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Abstract *Helicobacter pylori*-associated infections are extremely common in Iran, but few data about antibiotic sensitivity of *H. pylori* are available for this region. The purpose of this study was to investigate the prevalence of resistance in isolates against commonly used antibiotics in Eastern Azerbaijan, Iran, and the dependence of prevalence on the sex and age of patients. *H. pylori* isolates were collected by culture from gastric biopsies. Antibiotic susceptibility of isolates was determined by use of the disk agar diffusion test, and the minimum inhibitory concentration of clarithromycin was established by use of the Etest. A total 395 of biopsy specimens were studied; 112 samples of *H. pylori* were isolated (28.3 %), 55 (49 %) from males and 57 (51 %) from females. The prevalence of resistance to clarithromycin, metronidazole, erythromycin, amoxicillin, ciprofloxacin, rifampin, nitrofurantoin, and tetracycline were 16 (14.3 %), 86 (76.8 %), 29 (26.0 %), 32 (28.6 %), 37 (33.0 %), 32 (28.6 %), 13 (11.6 %), and 21 (18.7 %), respectively. Antimicrobial resistance was not statistically significantly associated with sex or age. Furthermore, the prevalence of resistance to metronidazole was high and that to clarithromycin was reasonable, consistent with reported low success in *H. pylori* treatment in this area. Therefore, continuous surveillance of antibiotic resistance of *H. pylori* is essential.

Keywords Antimicrobial resistance · Clarithromycin · *H. pylori* · Metronidazole

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Introduction

Helicobacter pylori is a curved Gram-negative bacillus associated with a variety of digestive diseases, for example peptic ulcer, gastritis, mucosa-associated tissue lymphoma, and gastric cancer [1]. *H. pylori* infects approximately 80 % of the population in many developing countries, for example Iran [2, 3]. Management of *H. pylori* infections is difficult, and relapse frequently occurs after apparently successful eradication of the organism with a variety of antimicrobial agents [3]. In general, *H. pylori* strains are susceptible to many antibiotics in in-vitro conditions, but only a few antibiotics, e.g. amoxicillin, clarithromycin, metronidazole, and tetracycline can be successfully used for eradication of *H. pylori* in vivo. Because none of these drugs effectively eradicates *H. pylori* in monotherapy, successful treatment of *H. pylori* infection requires combination therapy, consisting of one or two antibiotics with an anti-secretory agent [4]. Factors involved in management failure include lack of antibiotic penetration into submucosa, inactivation of antibiotics by pH, lack of correlation between in-vitro susceptibility tests and in-vivo efficacy, and the presence of *H. pylori* strains resistant to antibiotics [5]. Resistance to the antibiotics used is the main reason for treatment failure [2].

The purposes of this study were to assess susceptibility of *H. pylori* isolates to commonly used antibiotics, and to

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evaluate minimum inhibitory concentrations (MICs) of clarithromycin, by use of the Etest, for two groups of children and adults.

Methods

Bacterial strains

One hundred and twelve *H. pylori* isolates were recovered from gastric biopsies of patients with gastritis, duodenal ulcer, peptic ulcer, and gastroesophageal reflux disease undergoing endoscopy at Emam Reza and Pediatric Hospitals from December 2010 to November 2011 in Eastern Azerbaijan, Iran. At least 1 week before endoscopy the patients had not received antimicrobial agents. This research was approved by the regional Medical Research Ethics Committee of Tabriz University of Medical Sciences on 18 Nov 2010 (no. 5/4/9591). The specimens were placed in sterile tubes containing 10 ml Stuart medium (Merck, Germany) and transported to the microbiology laboratory within 4 h. For bacterial culture, gastric biopsy samples were homogenized and cultured in Brucella agar (Pronadisa, Spain) containing 5 % sheep blood and antibiotics supplement (vancomycin 6 μg/ml, amphotericin B 2.5 μg/ml, and trimethoprim 20 μg/ml). The plates were incubated under microaerophilic conditions (Anoxomat; Mart, Lichtenvoorde, The Netherlands; O_2 5 %, CO_2 10 %), at 37 °C and high humidity. After incubation for 5–7 days the plates were examined for growth and small rounded colonies subcultured to obtain a pure culture. Organisms were identified as *H. pylori* on the basis of colony morphology, Gram staining, and positive oxidase, catalase, and rapid urease tests [6].

Antibiotic susceptibility tests

Antimicrobial susceptibility of *H. pylori* isolates and the reference strain (*H. pylori* ATCC 26695) were determined by the conventional disk agar diffusion test. Suspensions of 3-day-old cultures were prepared in sterile saline to an opacity of McFarland no. 4 standard [6]. Muller–Hinton agar plates were supplemented with 5 % sheep blood then inoculated with a swab from the prepared suspension. Disks of different antibiotics (Mast, UK) including nitrofurantoin (300 μg), tetracycline (30 μg), clarithromycin (15 μg), ciprofloxacin (5 μg), erythromycin (15 μg), amoxicillin (25 μg), rifampin (5 μg), and metronidazole (5 μg) were placed on the plates and the plates were incubated for 72 h and examined for inhibition zones, which were measured in millimeters. The zones of inhibition were interpreted as reported elsewhere [3, 7, 8].

Clarithromycin MIC determination

The MIC for clarithromycin was determined by use of the Etest (Epsilometer test; Biomeriux, Solna, Sweden). Plates containing Muller–Hinton agar with 5 % sheep blood were used for the test. According to the manufacture's recommendations, clarithromycin was tested at concentrations ranging from 0.016 to 256 μg/ml. The agar plates were inoculated by confluent swabbing of the surface with adjusted inoculum suspensions (McFarland no. 4). The Etest strip was aseptically placed on to the dried surfaces of each inoculated agar plate and incubated under the conditions described above. The MICs were read after 72 h of incubation on the basis of the zone of growth inhibition with the MIC scale. The *H. pylori* resistance breakpoint for clarithromycin (≥1 μg/ml) was adopted by the Clinical and Laboratory Standards Institute (CLSI) [9, 10].

Results

From the total of 395 biopsies studied, 112 samples of *H. pylori* were isolated (28.3 %), which 55 (49 %) were from males and 57 (51 %) from females (sex ratio M/F: 0.96). The mean age of the patients was 35 ± 19 (mean ± SD) years. Of the 112 isolates of *H. pylori*, 91 were from adults (mean age 46) and 21 were from children (age 3–14 years; mean age 5 years).

Antibiotic susceptibility patterns of the 112 *H. pylori* strains, determined by use of the disk-diffusion method, are presented in Table 1. When the clarithromycin MICs for the 112 isolates were measured by use of the Etest, 19 (17 %) of the isolates were found to be resistant. Figure 1 shows the bimodal distribution of clarithromycin MICs for *H. pylori* isolates. However, when tested by disk diffusion, 16 strains were found to be resistant to clarithromycin. Ten of 112 strains (9 %) were resistant to four antibiotics and 4 of the 112 strains (3.6 %) were resistant to more than 4 antibiotics. The incidence of multiple resistance of *H. pylori* isolates to antimicrobial agents is listed in Table 2.

Overall resistance to metronidazole was higher for isolates from females (42 %) than for those from males (32.4 %), whereas more isolates resistant to rifampin, amoxicillin, and nitrofurantoin were recovered from males. Antimicrobial resistance was not statistically significantly associated with sex (*P*v 0.1).

In this study, metronidazole resistance was slightly more frequent in children than in adults (81 vs. 76 %, *P*v 0.7) whereas clarithromycin resistance was slightly more prevalent in adults than in children (17.6 vs. 14.3 %, *P*v 0.4). There were also differences in the prevalence of resistance to the five antibiotics tested (tetracycline, nitrofurantoin,

Table 1 Pattern of resistance of 112 *H. pylori* isolates to different antibiotics by disk-diffusion method according to age of patients

Age groups	CLM[a] (%)	MTZ[b] (%)	ERY[c] (%)	AMX[d] (%)	CIP[e] (%)	RIF[f] (%)	NIT[g] (%)	TET[h] (%)
Children (21 cases)	2 (9.5)	17 (81.0)	3 (14.3)	5 (23.8)	6 (28.6)	6 (28.6)	0 (0)	1 (4.8)
Adults (91 cases)	14 (15.4)	69 (75.8)	26 (28.6)	27 (27.5)	31 (34.0)	26 (28.6)	13 (14.3)	20 (22.0)
Total (112 cases)	16 (14.3)	86 (76.8)	29 (26.0)	32 (28.6)	37 (33.0)	32 (28.6)	13 (11.5)	21 (18.7)

[a] Clarithromycin
[b] Metronidazole
[c] Erythromycin
[d] Amoxicillin
[e] Ciprofloxacin
[f] Rifampin
[g] Nitrofurantoin
[h] Tetracycline

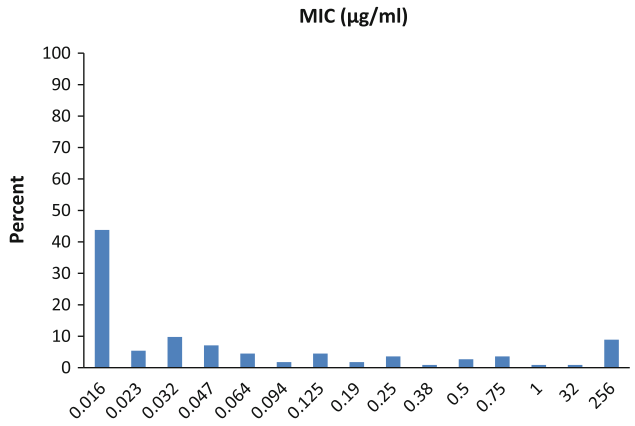

Fig. 1 Distribution of clarithromycin MICs (µg/ml) of *H. pylori* isolates

ciprofloxacin, amoxicillin, and erythromycin) when we compared adults with children (Table 1), but these difference were not statistically significant.

Discussions

H. pylori infection has important implications for health [11], and remains a major healthcare burden, with persistently high prevalence, especially in less-developed countries [12]. Current treatment is primarily based on triple therapy with combinations of a proton-pump inhibitor, amoxicillin, metronidazole, and clarithromycin for 7–14 days [13]. However *H. pylori* resistance to antibiotics is the major factor affecting the efficacy of current therapeutic regimens [14].

In this study, the prevalence of resistance to metronidazole was high (76.8 %). This high prevalence of *H. pylori* resistance to metronidazole has been reported from Iran and some other Asian countries [11, 14–20], and may be related to the greater use of this antibiotic in these countries [16]. Levels of resistance differ from country to country. Overall, metronidazole resistance is uncommon in developed countries, for example Europe and the USA, and relatively uncommon in Japan [11]. Our results revealed that metronidazole resistance was more the prevalent in females than in males. This result has also been reported previously [21, 22]. O'Connor et al. [11], reported that metronidazole resistance is more prevalent in females of reproductive age than in older females and males, which may be because of the use of metronidazole for gynecologic infections. Additionally, no statistically significant association of clarithromycin resistance with sex has been reported [22].

In 2004, the CDC reported 13 % clarithromycin resistance and warned of the potential for eradication failure [23]. In this research, the prevalence of clarithromycin resistance was relatively low. This finding is consistent with previous unrelated studies conducted in Iran, in which clarithromycin resistance was reported as 25, 30, and 9.4 % [19, 20, 24]. However, in North America the prevalence of resistance to clarithromycin was in the range 2.5–12.2 % [25]. Among the 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 %). In Asian countries, high prevalence of clarithromycin resistance was also detected in Japan (40.7 %) whereas prevalence was lowest in Malaysia (2.1 %) [14].

This study showed that double resistance to clarithromycin and metronidazole was relatively high (14.3 %). The findings of a study conducted in Iran revealed 23.5 % resistance to these two agents, 3.7 % to three drugs, and 0.9 % to four antibiotics [24]. Resistance to these two drugs in America, Europe, Africa, and Asia, has been reported to be 29.3, 44.1, 92.4, and 18.9 %, respectively [25]. Low prevalence of clarithromycin/metronidazole double resistance has been observed in Sweden (0.6 %) and Ireland (2.2 %) [14].

Table 2 Incidence of multiple resistance of *H. pylori* isolates to antimicrobial agents

Antibiotics	Incidence (%)
CLM[a] + MTZ[b]	16 (14.3)
CLM[a] + TET[c]	9 (8.0)
CLM[a] + NIT[d]	6 (5.4)
CLM[a] + AMX[e]	11 (9.8)
MTZ[b] + CIP[f]	31 (27.7)
MTZ[b] + TET[c]	19 (17.0)
MTZ[b] + AMX[e]	28 (25.0)
CLM[a] + MTZ[b] + TET[c]	8 (7.1)
CLM[a] + MET[b] + AMX[e]	10 (9.0)

[a] Clarithromycin

[b] Metronidazole

[c] Tetracycline

[d] Nitrofurantoin

[e] Amoxicillin

[f] Ciprofloxacin

The prevalence of *H. pylori* resistance to metronidazole was found to be high, but the prevalence of resistance against nitrofurantoin, tetracycline, erythromycin, and clarithromycin was low, particularly for children. Although, metronidazole resistance results differ among published studies [11, 26], all these studies generally report high prevalence of metronidazole resistance in adults. Our results show that clarithromycin resistance is particularly common in adults. This is, perhaps, a consequence of the extensive use of clarithromycin products to treat adult respiratory infections. Our results are relatively consistent with the results of Torres et al. [26]; resistance to clarithromycin for adults and children were 25 and 21.6 %, respectively.

Finally, results on amoxicillin resistance are highly contradictory. Indeed, amoxicillin resistance is virtually absent in several countries and as high as 85 % in Cameroon [14]. In two previous studies conducted in Iran, researchers reported 6.8 and 20.8 % amoxicillin resistance [20, 24]. In our research, amoxicillin resistance in children and adults was 23.8 and 27.5 %, respectively.

Tetracycline resistance remains very low (<3 %) in all countries, with no substantial increase compared with results from studies published before 2000 [25]. The prevalence of *H. pylori* resistance to tetracycline did not differ significantly between Europe (2.1 %), Asia (2.4 %), and America (2.7 %), although it was significantly higher in Africa (43.9 %) [14]. The prevalence of tetracycline resistance of *H. pylori* isolates in Iran has previously been reported as 9 and 4.7 % [20, 24]. In this area, tetracycline is not routinely used in *H. pylori* therapy regimens, so finding such low resistance is not unexpected. Another major finding is that low resistance to tetracycline (18.7 %) may indicate the importance of this drug for eradication of adult *H. pylori* infection.

Ciprofloxacin is not the drug of choice for *H. pylori* infection therapy but 0–20 % resistance has been reported in a variety of countries [18, 24, 27]. We found 33 % ciprofloxacin resistance among our *H. pylori* isolates. The most interesting finding in our study was the absence of resistance to nitrofurantoin among children. In this region, nitrofurantoin is not used in *H. pylori* therapy regimens, so this finding is not surprising. Quinolone-based second-line triple-therapy seems to be effective and well tolerated [28]. In this research, resistance to both tetracycline and ciprofloxacin was 27.7 %; this finding has been reported but seems uncommon [8]. The results of this study showed that the prevalence of resistance to ciprofloxacin and tetracycline in this region has increased in percentage terms between 2007 and 2011 [16], but not sufficiently to reach statistical significance (*P*v 0.07).

Correlation between disk-diffusion method and Etest results was excellent, in agreement with Mishra et al. [8]. In this research, the reproducibility of the Etest and disk-diffusion methods was good. In a study of reproducibility in France, using randomly selected strains, it was found that disk diffusion is more reproducible than the Etest for both clarithromycin and erythromycin [29]. The disk-diffusion test is less expensive than the Etest, is easy to perform, and is a reliable technique for testing *H. pylori* susceptibility to antimicrobial agents in clinical microbiology laboratories.

In conclusion, the prevalence of antibiotic resistance is relatively high, consistent with the low success of *H. pylori* treatment in this area. These findings suggested that monitoring antibiotic resistance is important for effective *H. pylori* infection management in medical practice.

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Ethical standards This paper has been approved by the regional Medical Research Ethics Committee of Tabriz University of Medical Sciences, number: 5/4/9591.

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