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Original Article

Helicobacter pylori Multidrug Resistance Due to Misuse of Antibiotics in Iran

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Abstract

Background: *Helicobacter pylori* might become highly resistant to antibiotics taken through the life time of patients. This study examined the change in antibiotic resistance of *H. pylori* by time.

Methods: Out of 985 dyspeptic patients who were referred to the endoscopy unit of Shariati hospital during 2010-2017, 218 patients with gastric biopsies positive for rapid urease test (RUT) and *H. pylori* culture were recruited in the study. *H. pylori* isolates were examined for resistance to 8 currently used antibiotics by the disc diffusion method. Results were compared with those from our three previous studies. The frequency of multidrug resistance (MDR) was also assessed.

Results: The highest resistance rate was to metronidazole (MTZ) (79.4%) followed by ofloxacin (OFX) (58.7%), ciprofloxacin (CIP) (46.8%), levofloxacin (LVX) (45%), tetracycline (TET) (38.5%), clarithromycin (CLR) (34.4%), amoxicillin (AMX) (27.1%) and furazolidone (FRZ) (23.9%). No significant difference was found between resistance of *H. pylori* isolates from male and female <40 and >40 years old and patients with gastritis and peptic ulcer. The highest rates of MDR were to MTZ+OFX (4.6%), MTZ+OFX+TET (2.8%), MTZ+OFX+CIP+LVX (6.4%) and MTZ+OFX+TET+ CIP+LVX (5%).

Conclusion: Resistance to MTZ increased from 33%–55.6% in previous studies to 79.4% by time, to CLR increased from 1.4–7.3% to 34.4%, to TET increased from 0–38.1% to 38.5%, to AMX increased from 1.4%–7.3% to 27.1% and to FRZ increased from 0%–4.5% to 23.9%. Resistance to FQs was 45%–58.7%. Increase in *H. pylori* antibiotic resistance indicates antibiotic misuse. In Iran, with a considerable number of *H. pylori*- infected patients, antibiotic therapy should be saved for high risk patients and according to local antibiotic resistance patterns.

Keywords: Antibiotic resistance, *H. pylori*, Misuse

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Introduction

Helicobacter pylori is well- equipped with a genetic depository which enables the bacterium to adapt to conditions of the gastric epithelium and avoid surveillance of the immune system.[1] Another important bacterial strategy is the potential to exhibit resistance to antibiotics, taken throughout the life time of patients, for treatment of different bacterial infections.[2,3] Therapeutic regimens recommended by international guidelines include 2 antibiotics selected from metronidazole (MTZ), clarithromycin (CLR), amoxicillin (AMX) and levofloxacin (LVX) and a proton pump inhibitor for 1–2 weeks[4] although failure occurs due to antibiotic resistance. Resistance to CLR,[5] LVX[6] and MTZ[7] has been found as the main reason for failure in antimicrobial therapy against *H. pylori*.

Sensitive microbes may survive the crisis of antibiotic exposure by becoming resistant through mutations or acquisition of resistance genes carried on mobile genetic elements by horizontal gene transfer (HGT). HGT has been found as the most common and important route for the spread of the resistance phenotype within bacteria and thus is responsible for failure in treatment of infectious diseases.[8] Reports indicate the occurrence of an efficient HGT system and homologous recombination in *H. pylori*. Indeed, *H. pylori* has been recognized as a genetically heterogeneous bacterium which undergoes repeated DNA rearrangements and insertions or deletions of mobile genetic elements.[9-11] These genetic alterations constitute the perfect evolutionary strategies which led to successful adaptation of *H. pylori* to the human gastric niche.[12]

Resistance of pathogenic bacteria to different antibiotics has been reported from different parts of the world.[13] The minimum time needed for resistance to a new antibiotic to develop has been estimated to be three years which in many cases is associated with multidrug resistance (MDR).[14] Use of new drugs or prolonged antibiotic treatment of *H. pylori* infection has been recommended by several centers.[15,16] However, others

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indicate that antibiotic misuse and overuse against *H. pylori* infection is the main reason for the emergence of MDR.[17,18] Fingerprinting studies on *H. pylori* show that secondary infection (recrudescence) often happens as the consequence of failure in eradication of the primary infection.[6] High frequency of *H. pylori* antibiotic resistance in countries such as Iran.[19,20] is the main reason for failure in bacterial eradication. Accordingly, updated Knowledge of antibiotic resistance pattern is necessary for the selection of the most effective *H. pylori* eradication regimen by Iranian clinicians to prevent further increase in the frequency of bacterial resistance to antibiotics. In this study, *H. pylori* isolates from dyspeptic patients were examined for resistance to antibiotics currently used for bacterial eradication. *H. pylori* strains included 218 isolates that were cultured from gastric biopsies of dyspeptic patients during 2010–2017. Results were compared with those from our three previous studies to discover any change in frequency of antibiotic resistance over time.

Patients and Methods

Patients

The recruited patients in this study included 985 dyspeptic referrals to the endoscopy unit of Shariati hospital, Tehran-Iran, during 2010–2017. Out of 985 patients, 218 patients with positive rapid urease test (RUT) and positive culture of gastric biopsies; 188 (86.2%) with gastritis and 30 (13.8%) with peptic ulcer were recruited to this study. The 218 patients, 138 (63.3%) >40 years old and 80 (36.7%) <40 years old, included 100 male (45.9%) and 118 female (54.1%).

Isolation of *Helicobacter pylori* Strains

Two antral biopsies were taken from each patient. One was used for the RUT (Bahar afshan Co., Iran). The second biopsy was transported to a microbiology laboratory in semisolid normal saline for *H. pylori* culture. Brucella agar containing 10% defibrinated sheep blood and vancomycin (10 mg/L), trimethoprim (5 mg/L) and polymixin B (50 μg/L) was used for culturing of gastric biopsies. Plates were incubated in a CO_2 incubator (Heraeus, Germany) at 37°C and examined after 3–5 days. Bacterial isolates were identified as *H. pylori* according to growth of pinpointed (<2 mm) and glistening colonies, gram-negative spiral cells and positive results of catalase, oxidase, and urease tests. The identity of all isolates was confirmed by amplification of the *H. pylori*-specific *16S rRNA* gene and obtaining the amplicons with the size of 519 bp.

Antimicrobial Susceptibility Test by the Disk Diffusion Method

The enlisted antibiotics included MTZ, CLR, AMX, tetracycline (TET), furazolidone (FRZ), ofloxacin (OFX), ciprofloxacin (CIP) and LVX. The MICs for MTZ (8 μg/mL), TET (0.5 μg/mL), CLR (2 μg/mL), AMX (1 μg/mL) and FRZ (0.5 μg/mL) were the same as those used in previous studies.[21] MIC (1 μg/mL) for OFX, CIP and LVX, was determined using different dilutions (0.5, 1 and 2 μg/mL). Antimicrobial susceptibility test was performed according to the method recommended by the Clinical and Laboratory Standards Institute (CLSI).[22] All antibiotic solutions were prepared using dimethyl sulfoxide (DMSO). Fresh cultures of 218 *H. pylori* strains were used for preparation of bacterial suspensions, in normal saline, with the turbidity of MacFarland standard No.2 (6×10^8 cell/mL). A 100-μL volume of each bacterial suspension was surface inoculated on a non-selective Brucella blood agar plate. Sterile blank disks were deposited on the agar surfaces and used for introducing a 10-μL volume of each antibiotic solution. Blank disks impregnated with 10-μL of DMSO were deposited on the control plates. Plates were incubated as mentioned earlier and examined after 3–5 days for recording the inhibition zone diameters (IZDs). IZDs of ≥20 mm were considered as indication of susceptibility for all antibiotics except FRZ for which the IZD was estimated as ≥13 mm.[23]

Statistical Analysis

Nonparametric tests; chi-square, Fisher exact and Kruskal–Wallis tests were performed using SPSS software, version 20 for analysis the results and determination of significance of differences ($P<0.05$).

Results

Results of antimicrobial susceptibility tests showed that among 218 *H. pylori* isolates from dyspeptic patients, 79.4% were resistant to MTZ, 38.5% to TET, 34.4% to CLR, 27.1% to AMX, 23.9% to FRZ, 58.7% to OFX, 46.8% to CIP and 45% to LVX (Figure 1). The rates of resistance of *H. pylori* isolates from patients <40 years old to the enlisted antibiotics (22.5 %–81.3%) did not show a significant difference when compared with those of *H. pylori* isolates from patients >40 years old (24.6%–78.3%). Furthermore, no significant difference was found when resistance rates of *H. pylori* isolates from male (45.9 %) and female (54.1%) patients were compared ($P>0.05$). Similarly, *H. pylori* isolates from patients with gastritis (86.2%) and peptic ulcer (13.8%) exhibited no significant difference in their resistance to the recruited antibiotics ($P > 0.05$) (Table 1). Among 218 *H. pylori* isolates, 10 (4.6%) were susceptible to all of the 8 recruited antibiotics, 26 (11.9%) were resistant to one antibiotic, 33 (15.1%) were resistant to 2 antibiotics, 34 (15.6%) were resistant to 3 antibiotics, 35 (16.1%) were

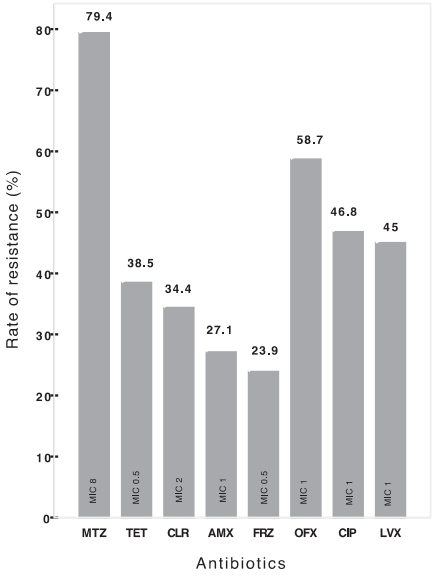

Figure 1. Frequency of Resistance Among 218 *Helicobacter pylori* Isolates to Currently Prescribed Antibiotics (MIC µg/mL). Metronidazole (MTZ 8), tetracycline (TET 0.5), clarithromycin (CLR 2), amoxicillin (AMX 1), furazolidone (FRZ 0.5), ofloxacin (OFX 1), ciprofloxacin (CIP 1), and levofloxacin (LVX 1).

resistant to 4 antibiotics, 38 (17.4%) were resistant to 5 antibiotics, 24 (11%) were resistant to 6 antibiotics, 13 (6%) were resistant to 7 antibiotics and 5 (2.3%) were resistant to all the 8 recruited antibiotics. Among the 26 single-antibiotic resistant *H. pylori* isolates, the highest rate of resistance was exhibited to MTZ (18, 8.3%). Altogether, 182 (79.9%) *H. pylori* strains were resistant to more than one antibiotic and thus were considered as MDR. The highest rates of resistance to combinations of 2–8 antibiotics were exhibited to MTZ + OFX (10, 4.6%), MTZ + OFX +TET (6, 2.8%), MTZ + OFX + CIP+ LVX (14, 6.4%) and MTZ + OFX + TET+ CIP+ LVX (11, 5%).

Discussion

Results of this study showed considerably high resistance rates of *H. pylori* isolates to currently used antibiotics in Iran. The highest resistance rate was to MTZ (79.4%), followed by OFX (58.7%), CIP (46.8%), LVX (45 %), TET (38.5%), CLR (34.4%), AMX (27.1%) and FRZ (23.9%). No significant correlation was found between the antimicrobial resistance rates of *H. pylori* isolates and patients' age, gender and type of peptic disease ($P > 0.05$). No male (75.5%) versus female (77.7%) difference in MTZ resistance was found in *H. pylori* isolates from Malaysian patients.[24] However, in Korea, MTZ resistance was significantly higher in *H. pylori* isolates from women (48.6%) compared with those from men (36.9%).[25] Higher rates of resistance to fluoroquinolones (FQs) in *H. pylori* isolates from females (55.8%) compared with males (28.6%) have been reported from Vietnam.[6] The positive association between aging and increase in resistance rates of *H. pylori* isolates to antibiotics such as CLR and LVX in Vietnam[6] or LVX in Europe[26] has also been reported.

In the present study, 79.4% of *H. pylori* isolates were resistant to MTZ. The frequencies of MTZ resistance of *H. pylori* isolates have been reported as 14.4% in Sweden,[27] 26.7% in Taiwan,[28] 67.1% in Germany,[29] 69.7% in China[30] and 75.5% in Malaysia.[24] Rate of resistance to CLR was 34.4% compared with 0% in Malaysia,[24] 10.6% in Taiwan,[28] 37.8% in China[30] and 67.1% in Germany.[29] High rates of MET (≥40%)[31,32] and CLR (>15%–20%)[4] resistance have been determined as the main reason for failure in *H. pylori* eradication. According to European guidelines, in areas with high resistance rates of MET (>40%) and CLR (>20%), these 2 antibiotics should not be prescribed and performance of antibiogram for designing an effective antimicrobial therapy for symptomatic patients is recommended.[15] FQs have been prescribed as substitutes in cases of CLR resistance, however, frequency of resistance to this group of antibiotics is now rapidly increasing.[6,29] In the present study, resistance rate of *H. pylori* isolates to FQs was within the range of 45%–58.7% compared with 0% in Malaysia,[24] 9.4% in Taiwan,[28] 24.9% in Germany[29] and 31.9% in the United States.[33]

Helicobacter pylori isolates showed the lowest rate of resistance to FRZ (23.9%) with the lowest MIC (0.5 µg/mL) among the recruited antibiotics. Including FRZ[34] in quadruple therapy in Iran led to successful eradication of *H. pylori* comparable with CLR.[35] FRZ-containing therapy has been recommended for the treatment of MET-resistant *H. pylori* infections in China[36] and

Table 1. Antibiotic Resistance Rate of 218 *Helicobacter* pylori Isolates From Patients Classified According to Age, Gender and Type of Peptic Disease

Patients classification		Number (%)	Number of Resistant Strains (%)							
			MTZ	TET	CLR	AMX	FRZ	OFX	CIP	LEX
Age (y)	<40	80 (36.7)	65 (81.3)	34 (42.5)	31 (38.8)	22 (27.5)	18 (22.5)	41 (51.2)	36 (45.0)	35 (43.8)
	>40	138 (63.3)	108 (78.3)	50 (36.2)	44 (31.9)	37 (26.8)	34 (24.6)	87 (63.0)	66 (47.8)	63 (45.7)
Gender	Male	100 (45.9)	77 (77.0)	42 (42.0)	34 (34.0)	28 (28.0)	23 (23.0)	61 (61.0)	44 (44.0)	45 (45.0)
	Female	118 (54.1)	96 (81.4)	42 (35.6)	41 (34.7)	31 (26.3)	29 (24.6)	67 (56.8)	58 (49.2)	49 (41.5)
Peptic disease	Gastritis	188 (86.2)	149 (79.3)	77 (41.0)	67 (35.6)	52 (27.7)	47 (25.0)	107 (56.9)	87 (46.3)	85 (45.2)
	Ulcer	30 (13.8)	24 (80.0)	7 (23.3)	8 (26.7)	7 (23.3)	5 (16.7)	21 (70.0)	15 (50.0)	13 (43.3)

Germany.[37] Resistance rate to TET was 38.5% compared with 0.3% in Sweden,[27] 2.3% in China[30] but higher (43.9%) in Cameroon.[38] Resistance rate to AMX was 27.1% compared with 0% in Malaysia[24] and Taiwan,[28] 1.1% in Vietnam,[6] <2% in European countries such as Germany,[39] 4.9% in China,[30] 38% in Korea[40] and 85.6% in Cameroon.[38]

In this study, among 218 *H. pylori* isolates, only 10 (4.6%) were susceptible to all 8 enlisted antibiotics and 26 (11.9%) were resistant to only one antibiotic. However, most of the isolates (182, 79.9%) were resistant to 2–8 antibiotics and thus considered as multidrug resistant strains. The highest rate of resistance to a single antibiotic was related to MTZ (18, 8.3%). The highest rates of MDR were to 2–5 antibiotics: 4.6% (MTZ+OFX), 2.8% (MTZ+OFX+TET), 6.4% (MTZ+OFX+CIP+LVX) and 5% (MTZ+OFX+TET+CIP+LVX). A report from Vietnam considered *H. pylori* strains resistant to 3 antibiotics (15.2%) or 4 antibiotics (1.1%) difficult to eradicate.[6] Reports indicate MDR rates of 15% in America, 8.3% in Asia and 8.9% in Europe.[41] Chromosomally-encoded and horizontally-acquired efflux pumps in *H. pylori*[42] and other bacteria[43] have been recognized as highly efficient systems involved in MDR.

Comparison of antibiotic resistance rates in *H. pylori* isolates recruited in this study with those obtained in our previous studies (1997–2008) revealed a remarkable increase in resistance rates by time; MTZ with highest increase in resistance from 33% to 36%, 55.6% and 79.4%, TET from 0 to 0.7%, 38.1% and 38.5%, CLR from 1.4% to 3.7%, 7.3% and 34.4% , AMX from 1.4% to 3.7%, 7.3% and 27.1%, FRZ from 0 to 0, 4.5% and 23.9% ($P < 0.001$). Among the tested antibiotics, MTZ showed the highest resistance and TET showed the lowest resistance (Figure 2). Furthermore, the resistance rates of *H. pylori* isolates to three FQS which have recently entered the Iranian market were considerably high; 45%–58.7%. Although FQS have rarely been used for *H. pylori* eradication in Iran, their frequent use

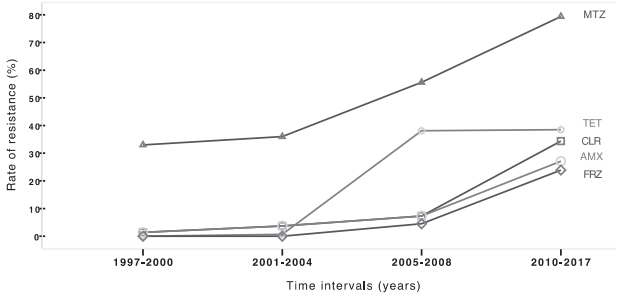

Figure 2. Increase in Resistance Rates of *Helicobacter pylori* Isolates by Time. Antibiotic resistance rates of H.pylori isolates in 2010-2017 were compared with those in 1997–2008. Metronidazole (MTZ), tetracycline (TET), clarithromycin (CLR), amoxicillin (AMX) and furazolidone (FRZ).

as first line therapy for urinary tract infection[44,45] could present a strong driving force for the emergence of *H. pylori* resistance to FQs in Iran. Increase in antibiotic resistance of *H. pylori* isolates by time has been reported from different parts of the world as the most important factor responsible for failure in antimicrobial therapy.[3,46] In Vietnam, resistance rates of *H. pylori* isolates to MTZ, CLR and LVX in 2014 were compared with those in 2012. MTZ resistance rate increased from 75.3% to 78.9%, CLR from 34.2% to 73.7% and LVX from 35.6% to 63.2%.[6] Over 10 years of study in Beijing, increase in resistance rates of *H. pylori* isolates was estimated as 14.8% to 65.4% for CLR, 38.9% to 78.8% for MTZ and 27.1% to 63.5% for FQs. Resistance rates to AMX and TET were 0.3% and 1.2%, respectively.[47] Overall, global increase in *H. pylori* resistance to MTZ, CLR and FQs is alarming. Accordingly, for prescribing these antibiotics, local resistance profile of *H. pylori* should be determined by antibiogram.

Increase in bacterial resistance by time reflects the worldwide increase in antibiotics misuse and overuse for treatment of infectious diseases.[2] It has been indicated that use of antibiotics in high concentrations or for a long time results in MDR.[17,18] Reports indicate that the majority of consumed antibiotics are excreted unchanged[48] and remain untreated[49] in natural environments and waste water streams, turning these environments into highly potent sources for exchange of antibiotic resistance genes between bacteria through HGT.[50,51] HGT has been implicated in the emergence of super MDR bacteria in communities and hospitals.[52] It is noteworthy that the mammalian body, especially the digestive tract provides an appropriate niche for the persistent or transient colonization of different bacteria and thus exchange of resistance genes through HGT.[53,54] The adverse side effects of antibiotics on human cells should not be overlooked. It has been demonstrated that long-term use or misuse of antibiotics can lead to mitochondrial dysfunction and arrest of cell metabolism and growth.[55]

Some investigators believe that maintenance of resistance genes is costly for bacteria and they lose these genes in the absence of antibiotics.[56] However, others propose that bacteria, by acquiring new resistance genes from the environment and adding it to their permanent resistance gene depository, broaden their antibiotic resistance spectrum and turn into super MDR bacteria.[57] In conclusion, to avoid the antibiotic resistance dead end, a global effort should begin to establish tighter regulations for antibiotic usage and save them for severe and deadly infections. Although the prevalence of *H. pylori* is considerably high in Iran (69%),[58] especially in the Northern region (89%) with high incidence of gastric cancer,[59] eradication must be performed for those who

are at risk of peptic ulcers or gastric cancer. Furthermore, local antibiotic resistance status of *H. pylori* should be monitored by performance of the susceptibility test.

Authors' Contribution

PS and FS designed the research project and wrote the paper. FH and SK did the research work. SKh did the data analysis.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interest.

Ethical Statement

All patients signed an informed consent and the study was approved by the research ethics committee of Tehran University of Medical Sciences.

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