Introduction

*H. pylori*, once acquired early in the life of humans, could develop a persistent infection in the stomach with mild to severe consequences, including chronic gastritis, peptic ulcer, low grade gastric mucosa-associated lymphoid tissue lymphoma, atrophic gastritis and gastric adenocarcinoma. A large body of evidence emphasizes the crucial role of antimicrobials in eradication of *H. pylori* and thereby reducing the severity of gastric disease symptoms or complete recovery of patients. Furthermore, eradication of *H. pylori* has been strongly advised in asymptomatic first-degree relatives of cancer patients and in those who had early gastric cancer resection. However, treatment failure often occurs due to resistance of *H. pylori* to current antimicrobials.
Antimicrobials along with improved public knowledge of personal hygiene have played a crucial role in reducing the prevalence of *H. pylori* infection in developed populations. However, in developing countries, particularly in regions with a high prevalence of *H. pylori* infection, ignorance of standard hygiene and indiscriminate use of antimicrobials in frequent and short-interval courses have led to the increment of bacterial resistance. It is noteworthy that antimicrobials that are currently recruited for the eradication of *H. pylori* are commonly used for the treatment of a wide range of infections: metronidazole for parasite-related diseases, gynecological and dental infections; tetracycline: respiratory and bowel diseases, prophylaxis of traveler’s diarrhea and cholera; clarithromycin: respiratory infection; amoxicillin: streptococcal pharyngitis; urinary tract infection; and furazolidone: giardiasis. Accordingly, emergence of resistant strains of *H. pylori* to the above mentioned antibiotics cannot be an unexpected phenomenon.

Bacterial resistance to antimicrobials can be an intrinsic property related to chromosomal genes, although it can also be acquired through mutation in the chromosomal genes or by acquisition of foreign genes carried on mobile genetic elements (horizontal gene transfer) or the combination of both. It appears that *H. pylori*, while colonizing the stomach, could be affected by antimicrobials frequently used (selective pressure) for the eradication or killing of other pathogens. Although resistant strains that survive will serve as reservoirs of resistance genes. Normal microbiota in different sites of the human body could be similarly affected and the survivors could spread the resistance genes. It has been revealed that resistance genes occur frequently in the environment and can be spread by mobile genetic elements among bacterial populations. Horizontal gene transfer has been implicated in the transfer of resistant traits and in converting a bacterium to a multi-drug resistant pathogen.

To estimate the consequences of frequent use of antimicrobials in Iran, we examined the resistance rates of *H. pylori* isolates to currently used antimicrobials between 2005 and 2008. The results were compared to those from our previous studies (1997 – 2000 and 2001 – 2004).

**Materials and Methods**

**Patients and bacterial strains**

Strains of *H. pylori* were isolated from 160 dyspeptic patients who were referred to the Endoscopy Unit at Shariati Hospital, Tehran, Iran. Patients consisted of 82 women whose ages were 16 – 75 years old (mean age: 43 years) and 78 men whose ages ranged from 21 – 90 years old (mean age: 48 years). Patients were classified according to endoscopic diagnosis into individuals with gastritis (124, 77.5%), ulcers (32, 20%) and cancer (4, 2.5%; 2 esophageal cancer and 2 gastric cancer).

Antral biopsies with positive rapid urease tests were transported to the microbiology lab in semisolid (0.1% agar) normal saline. Selective medium containing brucella agar (Merck), 7% defibrinated sheep blood, vancomycin (5mg/L), trimethoprim (5mg/L), polymyxin B (50µg/L), and amphotericin B (4mg/L) was used for culturing the biopsies. Plates were incubated at 37°C under microaerobic conditions (CO2 incubator; Heraeus, Germany). Cultures were examined after 3 – 5 days for observation of pinpoint (1 – 2 mm) glistening colonies. Bacterial strains were identified as *H. pylori* on the basis of Gram stain and spiral microscopic appearance as well as positive activities of urease, oxidase, and catalase.

**Antimicrobial susceptibility test**

Antimicrobial susceptibility test was performed using disk diffusion method (DDM). Recruited antibiotics included metronidazole, tetracycline, clarithromycin, amoxicillin, and furazolidone. One hundred and ten strains were recruited in the first step of the susceptibility tests. The frequency of bacterial resistance to metronidazole and tetracycline appeared to be remarkably higher than our previous studies. Therefore, in the second step in order to increase the accuracy of the metronidazole resistance rate, an additional 50 strains were recruited for susceptibility testing with metronidazole (32, 16, 8, and 4 µg/mL). These 50 strains were also tested with 2, 1, and 0.5 µg/mL of tetracycline. Susceptibility tests were repeated twice for the strains which exhibited resistance to metronidazole or tetracycline. Serial dilutions of recruited antibiotics were prepared as follows: metronidazole (32, 16, 8, and 4 µg/mL) in dimethyl sulfoxide (DMSO; Merck), clarithromycin (2, 1, 0.5, and 0.25 µg/mL) in
ethanol (Merck), amoxicillin (1 μg/mL) in DMSO, tetracycline (2, 1 and, 0.5 μg/mL) in DMSO, and furazolidone (2, 1, and 0.5 μg/mL) in N, N-dimethylformamide (Merck).

Bacterial suspensions with the turbidity of MacFarland standard No.2 (equivalent to 6×10⁸ cell/mL) were prepared in normal saline. Non-selective blood agar plates were surface inoculated with 100 μL of each bacterial suspension. Bent glass rods were used for the even spreading of bacterial suspensions. Plates were allowed to dry at room temperature for about 10 minutes. Sterile blank disks were deposited on the surface of inoculated plates. A 10 μL volume of each antibiotic dilution was introduced into a blank disk. Control plates included those growth positive bacterial cultures with blank disks impregnated with 10 μL of the antibiotic solvents. Plates were incubated as mentioned earlier and examined after 3 – 5 days. The inhibition zone diameters (IZDs) were recorded and MICs were determined.

H. pylori isolates with IZDs of 20 mm for metronidazole, clarithromycin, amoxicillin, and tetracycline, and 13 mm for furazolidone were considered susceptible. MICs were determined as 8 μg/mL for metronidazole (160 isolates), 2 μg/mL for clarithromycin (110 isolates), 1 μg/mL for amoxicillin (110 isolates), 0.5 and 2 μg/mL for tetracycline (160 and 50 isolates, respectively), and 0.5 μg/mL for furazolidone (110 isolates).

Statistical analysis
SPSS software, version 13 was used for statistical analysis. The statistical tests applied were Student’s t-test and Chi-square test. Tests performed were two-tailed, and P<0.05 was considered as significant.

Results
Resistance rates of 110 H. pylori isolates to clarithromycin, amoxicillin, and furazolidone were 7.3%, 7.3% and 4.5%, respectively (Figure 1). Chi-square test showed no significant difference (P>0.05) between the resistance rates of these antimicrobials in H. pylori isolates from 54 men and 56 women with different age or disease status (Table 1).

Among 160 H. pylori isolates, 89 (55.6%) and 61 (38.1%) were resistant to metronidazole and tetracycline (MIC 0.5 μg/mL), respectively. Resistance rates of 50 isolates to tetracycline with MICs 1 and 2 μg/mL were 17% and 10%, respectively (Figure 1). Out of 160 H. pylori isolates, 78 isolates were obtained from men and 82 isolates from women. Chi-square test revealed no significant difference (P>0.05) between the resistance rates of metronidazole and tetracycline in isolates from men and women. Furthermore no difference was found in the rates of antibiotic resistance among isolates from patients of different gender, age or disease status (P>0.05) (Table 2).

Resistance rates of all H. pylori isolates were determined at different dilutions of recruited antimicrobials. The highest rates of resistance were obtained at 4 μg/mL of metronidazole (67.4%), 0.5 μg/mL of tetracycline (38.1%), 0.25 – 0.5 μg/mL of clarithromycin (26%) and 0.25 μg/mL of furazolidone (12%) (Figure 1).

Discussion
Triple34 and quadruple therapies35 have been effective in killing H. pylori and improving dyspeptic diseases, particularly in patients at high risk of developing peptic ulcer, atrophic gastritis and gastric cancer.9,35 However, the low eradication rate of H. pylori in developing countries13,36 such as Iran,37,38 compared to western countries9,39 indicates the failure of antimicrobial therapies. Fingerprinting studies have revealed that recurrence could be due to recrudescence of H. pylori.
colonization of the same strain) rather than reinfection (colonization of a new strain) in up to 80% of cases.\textsuperscript{36,40} Reports implicate metronidazole resistance as the main reason for chemotherapy failure.\textsuperscript{41}

Results of this study showed a significant increase ($P=0.023$) in the resistance rate of metronidazole (55.6%) compared to our two previous studies; 33\%\textsuperscript{42} and 36.3\%\textsuperscript{43} (Table 3; Figure 2). Metronidazole was considerably effective on the susceptible strains which produced IZDs of 48 – 80 mm at MICs of 4 – 32 $\mu$g/mL. However, 67.4\% of isolates exhibited resistance at MIC of 4 $\mu$g/mL, which was higher than our previous result (47.7\%).\textsuperscript{43} Increasing the MIC to 32 $\mu$g/mL resulted in a resistance rate of 43\%, which was also higher compared to 25.2\% in the previous study.\textsuperscript{43} Similar to reports from other countries such as China,\textsuperscript{44} resistance to metronidazole was not related to age, gender and type of gastric disease. Reports from Costa Rica\textsuperscript{45} and Mexico\textsuperscript{46} also demonstrated no significant difference in metronidazole resistance rates of $H. pylori$ isolates from men and women. However, in Brazil, the higher metronidazole resistance rate in $H. pylori$ isolates from women was associated with the treatment of gynecological infections.\textsuperscript{47} In Saudi Arabia\textsuperscript{48} and China\textsuperscript{44} metronidazole resistance rate showed a significant increase over time. High resistance to metronidazole has been reported from Nigeria,\textsuperscript{49} Mexico,\textsuperscript{46} and Hong Kong.\textsuperscript{50}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Status of patients} & \textbf{Clarithromycin resistance (\%)} & \textbf{P-value} & \textbf{Amoxicillin resistance (\%)} & \textbf{P-value} & \textbf{Furazolidone resistance (\%)} & \textbf{P-value} \\
\hline
\textbf{Gender} & & & & & & \\
Male ($n=54$) & 5 (9.3\%) & & 4 (7.4\%) & & 3 (5.6\%) & \\
Female ($n=56$) & 3 (5.4\%) & 0.431 & 4 (7.1\%) & 0.9765 & 2 (3.6\%) & 0.617 \\
\hline
\textbf{Age (years)} & & & & & & \\
15 – 40 ($n=37$) & 1 (2.7\%) & & 4 (10.8\%) & & 1 (2.7\%) & \\
41 – 65 ($n=64$) & 6 (9.4\%) & & 4 (6.3\%) & & 3 (4.7\%) & \\
66 – 90 ($n=9$) & 1 (11.1\%) & 0.414 & 0 (0\%) & 0.474 & 1 (11.1\%) & 0.522 \\
\hline
\textbf{Disease} & & & & & & \\
Gastritis ($n=88$) & 4 (4.5\%) & & 5 (5.7\%) & & 5 (5.7\%) & \\
Ulcer ($n=20$) & 4 (20\%) & & 3 (15\%) & & 0 (0\%) & \\
Cancer ($n=2$) & 0 (0\%) & 0.052 & 0 (0\%) & 0.520 & 0 (0\%) & 0.323 \\
\hline
\end{tabular}
\caption{Resistance rates of 110 $H. pylori$ isolates to clarithromycin, amoxicillin and furazolidone according to gender, age and disease status of patients.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
\textbf{Status of patients} & \textbf{Metronidazole resistance (\%)} & \textbf{P-value} & \textbf{Tetracycline resistance (\%)} & \textbf{P-value} \\
\hline
\textbf{Gender} & & & & \\
Male ($n=78$) & 46 (59\%) & 31 (39.7\%) & \\
Female ($n=82$) & 43 (52.4\%) & 0.406 & 30 (36.6\%) & 0.681 \\
\hline
\textbf{Age (years)} & & & & \\
15 – 40 ($n=53$) & 29 (54.7\%) & & 19 (35.8\%) & \\
41 – 65 ($n=91$) & 50 (54.9\%) & & 33 (36.3\%) & \\
66 – 90 ($n=16$) & 10 (62.5\%) & 0.843 & 9 (56.3\%) & 0.439 \\
\hline
\textbf{Disease} & & & & \\
Gastritis ($n=124$) & 69 (55.6\%) & & 44 (35.5\%) & \\
Ulcer ($n=32$) & 19 (69.4\%) & 0.427 & 15 (46.9\%) & \\
Cancer ($n=4$) & 1 (25\%) & & 2 (50\%) & 0.290 \\
\hline
\end{tabular}
\caption{Resistance rates of 160 $H. pylori$ isolates to metronidazole and tetracycline according to gender, age and disease status of patients.}
\end{table}
resistance rate of metronidazole has been reported from Italy\textsuperscript{51} and Bulgaria.\textsuperscript{52} The latter showed no significant change in resistance rate from 1996 – 2007 (Table 4). According to a considerable number of reports, increase in metronidazole resistance rates could be due to a widespread use of this antibiotic for the treatment of both \textit{H.pylori}\textsuperscript{13,14,53} and non-\textit{H.pylori} infections.\textsuperscript{18–24,54} Metronidazole has been currently prescribed against \textit{H.pylori}\textsuperscript{55,56} in Iran\textsuperscript{55,56} and other countries such as the USA,\textsuperscript{57} Sweden,\textsuperscript{58} Germany,\textsuperscript{59} China,\textsuperscript{44} and Mexico.\textsuperscript{46}

Most reports\textsuperscript{13,14,44,60} on antimicrobial therapy of \textit{H.pylori} propose that antibiotic overuse selects for resistant strains (selective pressure). It appears that antimicrobials, including metronidazole, could eradicate the susceptible bacterial population, however, resistant survivors could establish and emerge as a resistant majority.\textsuperscript{61} The resistance trait could be spread horizontally by plasmids to other bacteria, including human pathogens. Emergence of a resistance phenotype is a short-term phenomenon and takes 4 – 5 years rather than a decade.\textsuperscript{61} The driving force would be the indiscriminate, short-interval and frequent use of antibiotics.\textsuperscript{13,14,61}

Tetracycline resistance rates also showed a considerable increase (38.1\%) compared to our two previous

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<tr>
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<tbody>
<tr>
<td></td>
<td>MIC ((\mu g/mL))</td>
<td>Rate of resistance</td>
<td>MIC ((\mu g/mL))</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>8</td>
<td>33%</td>
<td>8</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.25</td>
<td>0%</td>
<td>0.5</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.25</td>
<td>1.4%</td>
<td>2</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>0.25</td>
<td>1.4%</td>
<td>1</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>0.12</td>
<td>0%</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* For metronidazole and tetracycline, an additional 50 strains were recruited for the susceptibility tests.
studies (0% – 0.7%; \(P=0.000\)) (Table 3; Figure 2). Similar to other countries such as China\(^{44}\) and Saudi Arabia\(^{48}\) age, gender, and type of disease did not affect the resistance rate. A resistance rate of 38.1% (MIC 0.5 \(\mu\)g/\(\mu\)L) could be reduced to 10% with a 4 fold increase in MIC (2/151g/mL). It has been revealed that a 2 – 8 fold increase in MIC for eradication of a bacterial pathogen is the indication of the emergence of resistance.\(^{62,63}\) Nigeria,\(^{49}\) Costa Rica,\(^{45}\) and China\(^{44}\) are among the countries with high tetracycline resistance rates. However, low rates have been reported from Bulgaria,\(^{52}\) Japan,\(^{64}\) and Portugal\(^{65}\) (Table 5). It appears that overuse due to low cost and over the counter purchase of the antibiotic have been implicated in the emergence of resistant strains. Tetracycline has been used for \(H.\) pylori eradication following failure of the first-line of treatment with amoxicillin+clarithromycin/ amoxicillin+metronidazole,\(^{66}\) or in order to reduce treatment costs.\(^{48,67}\) Resistance to tetracycline could arise by \textit{de novo} mutations in chromosomal genes as well as acquisition of the mutant 16S rRNA gene through horizontal transfer.\(^{68}\) It has been revealed that the gene \textit{tet} (\(O\)) which encodes tetracycline resistance in the \textit{Campylobacter spp.} has been acquired from gram positive bacteria.\(^{30}\) Increase in tetracycline resistance has been reported in bacteria that have originated from animals used for food consumption such as \textit{E. coli} (broiler chicken),\(^{69}\) \textit{Salmonella} (cattle),\(^{70}\) and \textit{Aeromonas} (fish).\(^{71}\) Tetracycline is among the antibiotics currently used for preventive therapy and growth promotion of animals used as food sources.\(^{20}\) Tetracycline has been frequently used in higher doses than standard for therapeutic purposes during outbreaks of diseases in aquaculturing of fish in order to prevent economic loss due to high mortality.\(^{71}\) Many reports indicate that long-term use of antibacterials selects for drug resistance and plasmids containing multiple resistance genes could be exchanged between bacterial pathogens of humans, fish, and other animals.\(^{72}\) A large body of evidence indicates that antibiotic-resistant bacteria from poultry, pigs, and cattle can enter the food supply, reach the human digestive tract and transfer resistance genes to human commensal microbiota.\(^{25,73}\) In Europe, to prevent the spread of resistance genes to human pathogens, there are tight regulations for application of antibacterials in food production.\(^{74}\) Accordingly, an increase in tetracycline resistance in certain countries might indicate the misuse and overuse of antibacterials.

Resistance rate of \(H.\) pylori isolates to clarithromycin (7.3%) did not show a significant change over time (\(P=0.105\)) when compared to our two previous studies; 1.4% and 3.7%\(^{43}\) (Table 3; Figure 2). Similar to other reports,\(^{31}\) resistance rates were not different among various ages, gender, or disease groups. Reports demonstrate lower resistance rates to clarithromycin from Spain\(^{75}\) and Costa Rica\(^{46}\) and higher rates from Bulgaria,\(^{52}\) Italy,\(^{31}\) Mexico,\(^{46}\) and Portugal\(^{65}\) (Ta-
ble 6). Clarithromycin is not frequently prescribed in Iran and developing countries due to its high cost.38 However, macrolide use in combination therapies against *H. pylori* has been increasing during recent years. This might select for stable macrolide-resistant *H. pylori* and indigenous microbiota.28 It has been demonstrated that clarithromycin-included therapies against *H. pylori* selected for resistant strains of *Enterococci*, the normal inhabitants of human colon and common causes of nosocomial infections, and *Staphylococcus epidermidis*, a member of the microbiota in human nostrils. The resistance was mediated by the plasmid-or transposon-carried *erm*(B)28,76 and *erm*(C)27 genes, respectively. A multi-drug resistance phenotype could often develop when an additional resistance gene joins the previously acquired genes, as reported in *E.coli* and *Salmonella*.31

Resistance to amoxicillin was 7.3% with no significant change over time (Table 3; Figure 2) or correlation with age, gender or disease status of patients. The resistance rate of furazolidone obtained in this study was an intermediate between Bulgaria (1.3%)52 and Brazil (13%)77 (Table 6). The resistance rate to furazolidone was the lowest among the rates of metronidazole, tetracycline, clarithromycin, and amoxicillin. The low cost and high efficacy of furazolidone have made it a good substitute for metronidazole and tetracycline in quadruple therapy regimens for eradication of *H. pylori* in Iran.80,81 Application of furazolidone has been recommended in developing countries with a high prevalence of *H. pylori* and increasing rate of metronidazole resistance.66 Furazolidone use has been recommended in the Latin America consensus conference.82

Worldwide increase in the failure of antimicrobial therapies against *H. pylori* indicates an increase in the emergence of resistant strains. Results of this study show that a remarkable increase in the resistance rates of *H. pylori* isolates to metronidazole and tetracycline has occurred during recent years. In countries like Iran, the high prevalence of *H. pylori* in addition to frequent, short-interval and indiscriminate use of antimicrobials for both the eradication of *H. pylori* or treatment of non-*H. pylori* infections could lead to the emergence of resistant bacterial pathogens as well as commensal microbiota. Accordingly, the frequency of *H. pylori* resistance to current antibiotics should be monitored periodically to determine the most appropriate regimen for treatment of gastric infections. The increase in *H. pylori* resistance rates to antimicrobials should be considered seriously as an indicator of misuse and overuse of antibiotics which ultimately limit their efficacy for the future generations.

### Table 5. Comparison of tetracycline resistance rates of *H. pylori* isolates from Iran with other countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of isolates</th>
<th>Susceptibility test method</th>
<th>Turbidity (McFarland standard)</th>
<th>MIC (µg/mL)</th>
<th>Resistant strains (%)</th>
<th>Change over time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nigeria</td>
<td>32</td>
<td>DDM</td>
<td>—</td>
<td>—</td>
<td>93.5%</td>
<td>—</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>41</td>
<td>DDM</td>
<td>—</td>
<td>—</td>
<td>80.4%</td>
<td>—</td>
</tr>
<tr>
<td>China</td>
<td>153</td>
<td>ADM</td>
<td>1</td>
<td>16</td>
<td>58.8%</td>
<td>—</td>
</tr>
<tr>
<td>Iran</td>
<td>160</td>
<td>DDM</td>
<td>2</td>
<td>0.5</td>
<td>38.1%</td>
<td>0%–0.7%→38.1% (1997→2008)</td>
</tr>
<tr>
<td>Bulgaria</td>
<td>779</td>
<td>E-testADM</td>
<td>1</td>
<td>4</td>
<td>5%</td>
<td>NSCh</td>
</tr>
<tr>
<td>Japan</td>
<td>565</td>
<td>E-test</td>
<td>—</td>
<td>2</td>
<td>4.9%</td>
<td>----</td>
</tr>
<tr>
<td>Portugal</td>
<td>473</td>
<td>E-test</td>
<td>—</td>
<td>8</td>
<td>0%</td>
<td>NSCh</td>
</tr>
</tbody>
</table>

ADM = agar dilution method; DDM = disk diffusion method; NSCh = No significant change.
Table 6. Comparison of clarithromycin, amoxicillin and furazolidone resistance rates of *H. pylori* isolates from Iran with other countries.

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</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>1.4%</td>
<td>3.7%</td>
<td>7.3%</td>
<td>17.9%</td>
<td>—</td>
<td>3.5%</td>
<td>10%</td>
<td>24%</td>
<td>16%</td>
<td>5.3%</td>
<td>19%</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>1.4%</td>
<td>3.7%</td>
<td>7.3%</td>
<td>0.9%</td>
<td>71.9%</td>
<td>—</td>
<td>—</td>
<td>18.5%</td>
<td>36%</td>
<td>3.8%</td>
<td>0%</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>0%</td>
<td>0%</td>
<td>4.5%</td>
<td>1.3%</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>13%</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

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