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ANTIMICROBIAL SUSCEPTIBILITY TESTING OF *HELCOBACTER PYLORI* TO SELECTED AGENTS BY AGAR DILUTION METHOD IN SHIRAZ-IRAN

J Kohanteb, A Bazargani, M Saberi-Firoozi, *A Mobasser

**Abstract**

**Purpose:** To assess the pattern of antimicrobial susceptibility profile of *Helicobacter pylori* isolates from patients with gastritis, duodenal ulcer (DU) and gastroesophageal reflux disease (GERD) residing in Shiraz, Iran. **Methods:** One hundred and six *H. pylori* isolates from patients with gastritis, DU and GERD undergoing endoscopy at our university hospitals and clinics were analysed for their antimicrobial susceptibility to metronidazole, clarithromycin, amoxicillin, co-amoxiclav, tetracycline, ciprofloxacin and furazolidone. The minimum inhibitory concentrations were determined by agar dilution method. **Results:** Overall *H. pylori* resistance rate was 72.6% to metronidazole, 9.4% to clarithromycin and furazolidone, 20.8% to amoxicillin and 4.7% to tetracycline and ciprofloxacin. No resistance to co-amoxiclav was detected among *H. pylori* isolates. No significant differences between antimicrobial resistance and clinical outcome were detected. **Conclusions:** With regard to the increasing resistance of *H. pylori* isolates to various antibiotics, susceptibility testing of *H. pylori* isolates prior to the treatment of infection must be performed to achieve better eradication and to reduce the risk of selection of *H. pylori* resistant strains.

**Key words:** Agar dilution, antimicrobial susceptibility test, *Helicobacter pylori*, Iran

*Helicobacter pylori* is a gram-negative rod which is responsible for a spectrum of diseases in alimentary canal including chronic superficial gastritis, chronic atrophic gastritis, gastric and duodenal ulcers (GU & DU), gastric cancer and mucosa-associated lymphoid tissue lymphoma.1 In general, combined therapy is used to eradicate *H. pylori* infection.2 However, increase in the resistant isolates highlights the need for susceptibility testing of *H. pylori* isolates prior to the eradication of infection.3

There are several methods for susceptibility testing of *H. pylori* isolates including agar dilution, micro dilution, disc diffusion and E-test methods. Agar dilution is a gold standard method for susceptibility testing of *H. pylori*. This method is not always practical to perform in routine laboratories, but it is relatively inexpensive and *H. pylori* grows readily on solid media. The broth micro dilution method is limited by the difficulty in growing *H. pylori* in liquid media. Disc diffusion is easy to perform but does not give actual minimum inhibitory concentrations (MICs). The E-test is expensive and has been reported to give discrepancies in the interpretation of ‘susceptible’ or ‘resistant’ when testing for metronidazole.4

The objective of present investigation was to determine *in-vitro* antimicrobial susceptibility testing of *H. pylori* isolates from patients with gastritis, DU and gastroesophageal reflux disease (GERD) to the seven routinely used antimicrobial agents by agar dilution method and to determine their MICs. Statistical analysis was performed to determine significant differences between antimicrobial resistance patterns and clinical outcome.

**Materials and Methods**

*H. pylori* isolates

One hundred and six *H. pylori* isolates were recovered from gastric biopsies of patients with gastritis, DU and GERD undergoing endoscopy at the university hospitals and clinics, Iran, from September 2004 to August 2005. None of the patients had received antimicrobial therapy for at least 1 week prior to endoscopy. The specimens were placed in the sterile screw-capped tubes containing 3-4 mL brain heart infusion (BHI) broth (Merck Co, Germany) and transported to the microbiology laboratory at Shiraz Medical School, Iran, for isolation, diagnosis and antibiotic susceptibility tests within 4 h. The biopsies were ground in a tissue grinder. Using a pasture pipette, about two drops of homogenates were inoculated into Brucella agar (Merck Co, Germany) containing 10% horse blood, vancomycin, polymyxin B and amphotericin B and streaked with bacteriological loop. The inoculated culture media were transferred into the anaerobic jar (Merck Co, Germany) in which microaerophilic environment was achieved using grade C gas pack (Merck Co, Germany) and incubated at 37 °C for 7 days. Isolates that exhibited gram negative curved rods on Gram stain reaction and were positive for catalase, oxidase and urease tests were considered as *H. pylori*. The isolates were suspended in the Eppendorf tubes containing BHI broth with 30% glycerol and stored at –70 °C until used.

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Susceptibility testing

The MICs were carried out by agar dilution method using Muller-Hinton agar (Merck Co, Germany) containing 5% heparinized horse blood and various concentrations of drugs. The ranges of antibiotic concentrations that were used in this study were as follows: Clarithromycin (CLA, 0.125-16 µg/mL), metronidazole (MTZ, 0.5-64 µg/mL), tetracycline (TET, 0.125-16 µg/mL), ciprofloxacin (CIP, 0.0625-8 µg/mL), amoxicillin (AXM, 0.03125-4 µg/mL), co-amoxiclav (CO-AMX, 0.03125-4 µg/mL) and furazolidone (FZD, 0.03125-4 µg/mL). All antimicrobial agents were purchased from Pakhsh-e-Hejrat, Iran.

Isolates were removed from freezer, thawed and subcultured on Brucella agar containing 10% horse blood without antibiotics and incubated under microaerophilic conditions at 37 ºC for 3 days. After a lawn of growth appeared, bacterial colonies were suspended in sterile saline at a density equivalent to no. 3 McFarland’s standard and 5 µL (106 colony forming unit/spot) of bacterial suspension was spot inoculated into susceptibility testing media. The spot inoculated plates were incubated under microaerophilic conditions. The MIC was defined as the lowest concentration of the antibiotic, which completely inhibited visible bacterial growth at 37 ºC after 72 h. Resistance cutoff for the antibiotics were as follows: metronidazole (MIC > 8 µg/mL), clarithromycin (MIC > 1 µg/mL),6-10 tetracycline and ciprofloxacin (MIC > 2 µg/mL) amoxicillin, co-amoxiclav and furazolidone (MIC > 0.5 µg/mL).

Statistical analysis

The significance of the antibiotic resistance patterns between two groups of patients (GA and DU) was determined by Chi-square and Fisher’s exact tests. SPSS, version 11.5 was used to perform statistical analysis.

Results

In this study, 106 patients with positive culture for H. pylori, 66 male, 40 female with the age range of 9-89 years were enrolled. Sixty-five (61.3%) of these patients suffered from gastritis, 33 (31.1%) had DU and 8 (7.5%) had gastroesophageal reflux disease. Table 1 summarizes the results of susceptibility of H. pylori isolates to the antimicrobial agents tested. Among 106 H. pylori isolates, 77 (72.6%) showed resistance to metronidazole, 10 (9.4%) to clarithromycin and furazolidone, 22 (20.8%) to amoxicillin, 5 (4.7%) to tetracycline and ciprofloxacin and none of the 106 isolates exhibited resistance to co-amoxiclav.

Table 2 summarizes the pattern of multi-drug resistant strains of H. pylori isolates, 25 (23.5%) elicited double, 4 (3.7%) triple and 1 (0.94%) quadruple drugs resistance and the overall multidrug resistance of H. pylori isolates was 28.1%. No significant differences were found between antimicrobial resistance patterns and the two groups of GA and DU patients (P > 0.05). Since only eight patients were in GERD group they were not included in statistical analysis.

Discussion

The efficacy of the treatment of gastric H. pylori infection can be reduced by the occurrence of primary or acquired resistance to various drugs.11 Therefore, susceptibility testing of H. pylori has been increasingly important for the eradication of this organism. An additional advantage of susceptibility testing is that it may reduce the risk of H. pylori resistance.12

In the present investigation, we tested the sensitivity and resistant pattern of H. pylori isolated from patients residing in Shiraz, Fars province, Iran, to seven routinely used drugs including amoxicillin, metronidazole, clarithromycin, co-amoxiclav, furazolidone, tetracycline and ciprofloxacin.

Worldwide resistance of H. pylori to metronidazole has been reported, with rates ranging from 0 to 98%.9,10,13 In the three studies carried out by Siavoshi et al.,7 Nariman et al.14 and Falsaﬁ et al.,15 in Tehran, Iran, the prevalence of metronidazole resistance were reported to be 37.5, 72 and 72-79%, respectively. We found 72.6% resistance among H. pylori isolates in Shiraz southern Iran. Metronidazole is a frequently used drug in Iran and therefore it is not unexpected to find such a high level of resistance. Metronidazole has been widely prescribed for the other infections like parasitic or genital infections and the use or abuse of this inexpensive drug may contribute to the increase in metronidazole resistance. The differences between the resistance rates may reflect the variation in metronidazole usage between countries.

<table>
<thead>
<tr>
<th>Antibiotic agent</th>
<th>Range (µg/mL)</th>
<th>MIC50* (µg/mL)</th>
<th>R** breakpoint (µg/mL)</th>
<th>No. of R isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metronidazole</td>
<td>2-64</td>
<td>32</td>
<td>&gt;8</td>
<td>77 (72.6)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.25-4</td>
<td>1</td>
<td>&gt;1</td>
<td>10 (9.4)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>0.0625-2</td>
<td>1</td>
<td>&gt;0.5</td>
<td>22 (20.8)</td>
</tr>
<tr>
<td>Co-amoxiclav</td>
<td>0.03125-0.5</td>
<td>0.125</td>
<td>&gt;0.5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>0.625-1</td>
<td>0.5</td>
<td>&gt;0.5</td>
<td>10 (9.4)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.25-8</td>
<td>1</td>
<td>&gt;2</td>
<td>5 (4.7)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.125-4</td>
<td>0.5</td>
<td>&gt;2</td>
<td>5 (4.7)</td>
</tr>
</tbody>
</table>

*MIC50 represents the MIC at which 90% of isolates were inhibited. **R - resistant
Table 2: Pattern of multiple antimicrobial resistance of *H. pylori* isolates

<table>
<thead>
<tr>
<th>Pattern of resistance</th>
<th>No. of resistant strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double drug</td>
<td></td>
</tr>
<tr>
<td>MTZ + AMX</td>
<td>25 (23.5)</td>
</tr>
<tr>
<td>MTZ + FZD</td>
<td>10 (9.4)</td>
</tr>
<tr>
<td>MTZ + CLA</td>
<td>7 (6.6)</td>
</tr>
<tr>
<td>MTZ + CIP</td>
<td>5 (4.7)</td>
</tr>
<tr>
<td>MTZ + TET</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>MTZ + TET</td>
<td>1 (0.94)</td>
</tr>
<tr>
<td>Triple drug</td>
<td></td>
</tr>
<tr>
<td>MTZ + AMX + CLA</td>
<td>4 (3.7)</td>
</tr>
<tr>
<td>MTZ + CLA + CIP</td>
<td>1 (0.94)</td>
</tr>
<tr>
<td>MTZ + AMX + TET</td>
<td>1 (0.94)</td>
</tr>
<tr>
<td>AMX + CIP + TET</td>
<td>1 (0.94)</td>
</tr>
<tr>
<td>Quadruple drug</td>
<td></td>
</tr>
<tr>
<td>MTZ + AMX + CIP + TET</td>
<td>1 (0.94)</td>
</tr>
</tbody>
</table>

MTZ - Metronidazole, CLA - Clarithromycin, AMX - Amoxicillin, FZD - Furazolidone, TET - Tetracycline, CIP - Ciprofloxacin

Amoxicillin is the only β-lactam used to treat *H. pylori* infection and it is included in most current therapeutic regimens. Until recently, resistance to amoxicillin was considered to be absent or very rare; however, amoxicillin-resistant *H. pylori* strains have now been identified in different countries.8,9 The World-wide prevalence of resistance to amoxicillin is 0-41%.10,14,16 In the two previous studies in Iran, investigators reported 7 and 27% amoxicillin resistance among *H. pylori* isolates.7,15 In the present study, the amoxicillin resistance was found to be 20.8% of isolates. The reason for this high level of amoxicillin resistance remain unclear, however, since no pharmaco-epidemiological data regarding amoxicillin use in Iran exists, it may be speculated that this drug is used in a disproportional manner.

All *H. pylori* isolates tested in this investigation were susceptible to co-amoxiclav. This high rate of susceptibility has also been reported by Piccolomini et al., in Italy.6 Co-amoxiclav is not used routinely in the common *H. pylori* infection therefore finding such a high level of susceptible *H. pylori* strains is not unexpected. With regard to high rate of amoxicillin resistance of *H. pylori* isolated from patients residing in Shiraz, Iran, co-amoxiclav may produce better eradication results if used in preference to amoxicillin. We recommend clinical trials with this antibiotic in our region.

Clarithromycin is a macrolide widely used in combination with a proton pump inhibitor with or without a second antibiotic. The worldwide rate of clarithromycin resistance ranges from 0 to 44.7%.10,13 In three separate studies performed in Iran, clarithromycin resistance of *H. pylori* isolates were reported 14.5, 21 and 23% of the isolates.7,14,15 In our study, only 9.4% of the isolates were resistant to clarithromycin. Since high cost of clarithromycin limits the use of this drug in Iran, finding such resistant isolates may be partially explained by the primary resistance of *H. pylori* to clarithromycin.

The differences between the resistance rates may reflect the variation in clarithromycin usage between countries.

Furazolidone has been used as an alternative to overcome metronidazole resistance strains. This compound has a good *in vitro* activity against *H. pylori*. Because of the high rates of metronidazole resistance in *H. pylori* in many areas and the preserved activity of furazolidone against metronidazole-resistant strains, this compound has been recommended as a possible alternative in *H. pylori* eradication regimens. Resistance to furazolidone was considered to be rare, but different studies showed resistance rates ranging from 0 to 13% among various countries.7,9 Siavoshi et al.,7 reported furazolidone resistance among 5% of *H. pylori* isolates in Tehran, Iran, however in our study, we found 9.4% resistance rate. Although furazolidone is cost effective and has good effect against *H. pylori* infection, a major problem with furazolidone at the standard dose of 200 mg BD is the high rate of severe adverse effects which limit the use of this drug.

Tetracyclines are currently used for treatment of *H. pylori* infection as part of quadruple therapy. Overall resistance of *H. pylori* to tetracycline has been found to be low and is estimated to be less than 2%.17 However, higher resistance rates, up to 20%, have been reported in other studies from different countries.8,15 The prevalence of tetracycline resistance of *H. pylori* isolates in Iran is 20%,17 but our study shows a low resistance of 4.7% indicating the importance of this drug in eradicating *H. pylori*. We found no resistance to tetracycline among *H. pylori* isolates from patients with DU. This drug is not routinely used in *H. pylori* eradication regimens, therefore finding such a low resistance rate is not unexpected.

Ciprofloxacin is a fluoroquinolone that inhibit A subunit of the DNA gyrase. Although ciprofloxacin is not the drug of choice for *H. pylori* infection, 0-20% resistance to this antibiotic has been reported in different countries.6,14 We found only 4.7% ciprofloxacin resistance among *H. pylori* isolates. Falsafi et al.,15 have previously reported 20% resistance to ciprofloxacin among *H. pylori* isolated from patients in Tehran. The higher level of resistance among *H. pylori* isolates in Tehran in comparison with *H. pylori* isolates from Shiraz shows that our *H. pylori* isolates may be more susceptible than those in Tehran.

Data obtained in this investigation revealed that 23.5% of *H. pylori* strains were resistance to two antibacterial agents, 3.7% isolates to three drugs and 0.94% to four antibiotics. Torres et al.,18 from Mexico have reported 30.7% double resistance and 8.7% triple resistance among *H. pylori* isolates. *H. pylori* resistance to these antimicrobials may be partially explained by the high prevalence of these bacteria in our population. In our study, similar to that reported by Wolle et al.,17 we found no significant differences in the prevalence of seven antibiotics resistance between *H. pylori* isolates from patients with gastritis and DU.
Metronidazole and amoxicillin in combination with a proton pump-inhibitor were used for treatment of *H. pylori* infection with eradication rate of 80% in Europe. However, this triple therapeutic regimen was found to be less than 60% efficient among Iranian population. In this study, 72.6% of *H. pylori* isolates were resistance to metronidazole and therefore, recurrence of the infection with *H. pylori* could be expected among patients receiving the above mentioned triple therapy. Considering the low percentage resistance of *H. pylori* to ciprofloxacin (4.7%), one may substitute metronidazole by ciprofloxacin to achieve a better eradication rate.

We suggest that culture and antimicrobial susceptibility testing of *H. pylori* must be performed in patients who have failed the metronidazole based triple therapy.

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**References**


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