

## Original Article

## Antimicrobial Resistance of *Helicobacter pylori* Isolates from Iranian Adults and Children

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**Background:** *Helicobacter pylori* isolates from 84 adults and 51 children were assessed during the period of 2001 through 2004 to find out whether their susceptibilities to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone differ between the two groups or have changed compared with the results from our previous study done between 1997 – 2000.

**Methods:** Biopsies from 135 dyspeptic patients were cultured on Brucella blood agar. Pure cultures of *H.pylori* isolates were used for antibiotic susceptibility tests. Disk diffusion method was recruited to assess the susceptibility of *H.pylori* isolates to different dilutions of the above mentioned antimicrobials. Results obtained from the two groups were compared and minimum inhibitory concentrations determined.

**Results:** Thirty-five percent of *H.pylori* isolates from adults and 37% from children were resistant to metronidazole. Clarithromycin resistance rate was 2.4% and 5.9% in *H.pylori* isolates from adults and children, respectively. Amoxicillin resistance was 2.4% in isolates from adults and 5.9% in isolates from children. Tetracycline resistance rate was 0% in *H.pylori* isolates from adults and 2.0% in isolates from children. Resistance to furazolidone was not observed.

**Conclusion:** Resistance rates of *H.pylori* isolates from adults and children to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone are similar and not significantly affected by age and time.

Archives of Iranian Medicine, Volume 9, Number 4, 2006: 308 - 314.

**Keywords:** Adults • children • *Helicobacter pylori* • resistance

### Introduction

Accumulating evidence indicates that most adult infections of *Helicobacter pylori* are contracted during childhood.<sup>1</sup> Since signs and symptoms of the infection do not generally permit identification of the infected cases at the onset of the disease, *H.pylori* infection may develop into a chronic type, which can lead to gastric ulcer or cancer in later stages of life.<sup>2</sup>

In order to prevent such dramatic consequences,

*H.pylori* should be eradicated in early stages of life,<sup>3,4</sup> although eradication of *H.pylori* at a young age is hard mainly due to a low compliance of children, intrafamilial reinfection,<sup>5</sup> and emergence of resistant strains.<sup>6,7</sup>

On the other hand, most patients referred to endoscopy units are *H.pylori*-positive adults, indicating that persistent *H.pylori* infection establishes in later stages of life as a result of recurrence or recrudescence. Thus, to achieve successful eradication of *H.pylori*, resistance status of the bacterial isolates to the current antimicrobials within a population should be considered.

No strong evidence suggests that *H.pylori* strains, which colonize adults and children, are different in their main features such as genetic fingerprints, virulence factors, or susceptibility to antimicrobials. In this study, we compared the

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Accepted for publication: 8 March 2006

susceptibility of *H.pylori* isolates from Iranian adults and children to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone. We also evaluated the change in rates of resistance to the mentioned antimicrobials with time.

## Materials and Methods

We randomly collected 135 *H.pylori* isolates from dyspeptic patients including 51 children and 84 adults during the period of 2001 – 2004. The children consisted of 33 girls and 18 boys with the age range of 2 – 15 years (mean age of 8 years for girls and 9.7 years for boys) who were referred to Children's Medical Center of Tehran. The adults consisted of 50 women and 34 men with the age range of 15 – 75 years (mean age of 40.5 years for women and 42.7 years for men) who were referred to the Endoscopy Unit of Shariati Hospital.

Semisolid normal saline was used to transport urease-positive gastric biopsies to microbiology laboratory. Biopsy specimens were cultured on selective Brucella blood agar (Merck) containing defibrinated blood (7%), vancomycin (5 mg/L), trimethoprim (5 mg/L), and polymyxin B (2500 U/L). After microaerobic incubation at 37°C, bacterial cultures were purified and identified as *H.pylori* according to the microscopic observation of spiral morphology and catalase-, oxidase-, and urease-positive reactions.

The disk diffusion method was recruited to assess the susceptibility of *H. pylori* strains to different antimicrobials. Bacterial suspensions were prepared in normal saline, with the turbidity adjusted to No.1 McFarland standard. Surface of Brucella blood agar plates were inoculated with 100 µL of bacterial suspensions. Glass rods were used to spread the inocula. Blank disks were deposited on the plates and impregnated with 10 µL of different dilutions of metronidazole (32, 8, and 4 µg/mL) in methanol (Merck), clarithromycin (2, 1, 0.5, and 0.25 µg/mL) in ethanol (Merck), amoxicillin (1, 0.5, and 0.25 µg/mL) in dimethyl sulfoxide (Merck), tetracycline (1, 0.5, and 0.25 µg/mL) in ethanol, and furazolidone (1, 0.5, 0.25, and 0.12 µg/mL) in N, N-dimethyl formamide (Merck).

After 2 – 3 days of microaerobic incubation, the growth inhibition zone diameters were recorded. *H.pylori* strains were considered as susceptible when exhibited growth inhibition zones of  $\geq 20$  mm for metronidazole [minimum inhibitory

concentration (MIC) 8µg/mL], clarithromycin (MIC 2 µg/mL), amoxicillin (MIC 1 µg/mL) and, tetracycline (MIC 0.5 µg/mL), and  $\geq 13$  mm for furazolidone (MIC 0.5 µg/mL). No inhibition zone was observed on control plates deposited with blank disks containing solvents of antimicrobials.

SPSS software, version 12 was used for statistical analysis. The statistical tests applied were Student's *t* test, Chi-square test, and Fisher's exact test. Confidence intervals (CI) were calculated using binomial distribution. All tests performed were two tailed, and  $P < 0.05$  considered as significant.

## Results

Out of the 135 *H.pylori* isolates, 55 (40.0%; 95% CI, 31.6 – 48.4%) were resistant to at least one antibiotic. The highest rate of resistance belonged to metronidazole (36.3%, 28.1 – 44.5%). The resistance rates to clarithromycin, amoxicillin, and tetracycline were 3.7% (0.5 – 6.9%), 3.7% (0.5 – 6.9%), and 0.7% (0 – 2.1%), respectively. No resistance to furazolidone was observed.

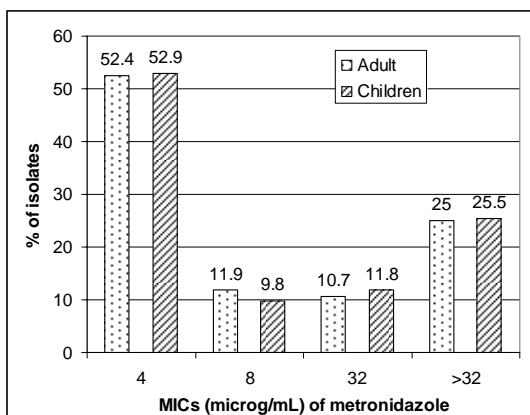
Resistance rates of *H.pylori* isolates from adults and children to recruited antimicrobials are shown in Table 1. Chi-square and Fisher's exact tests showed no significant difference between the resistance rates in adults and children ( $P > 0.30$ ).

The distribution of the MICs of metronidazole had an almost identical pattern in *H.pylori* isolates from children and adults (Figure 1). Chi-square test confirmed the similarity between the distributions ( $P = 0.98$ ). There was also no significant difference between the distribution of MICs of other antibiotics in *H.pylori* isolates from adults and children ( $P = 0.26$  for amoxicillin, 0.34 for clarithromycin, 0.43 for tetracycline, and 0.64 for furazolidone).

Resistance rates of all *H.pylori* isolates were determined at different dilutions of recruited antimicrobials. The highest rates were obtained at

**Table 1.** Resistance rates of *H. pylori* isolates from 84 adults and 51 children to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone.

Antimicrobial (MIC µg/mL)	Rates of resistance (%)	
	Isolates from adults	Isolates from children
Metronidazole (8)	30/84 (35.7%)	19/51 (37.3%)
Clarithromycin (2)	2/84 (2.4%)	3/51 (5.9%)
Amoxicillin (1)	2/84 (2.4%)	3/51 (5.9%)
Tetracycline (0.5)	0/84 (0%)	1/51 (2%)
Furazolidone (0.5)	0/84 (0%)	0/51 (0%)



**Figure 1.** Distribution of different MICs of metronidazole against 135 *H.pylori* isolates from 84 adults and 51 children. Distribution of different metronidazole MICs among *H.pylori* isolates from adults and children was similar. MIC of metronidazole for the majority of isolates (52.6%) was 4 µg/mL.

4 µg/mL of metronidazole (47.4%), 0.25 µg/mL of clarithromycin (9.6%), 0.25 µg/mL of amoxicillin (9.6%), 0.25 µg/mL of tetracycline (3%), and 0.12 µg/mL of furazolidone (17.8%) (Figure 2).

Resistance to multiple drugs was found in 6 isolates (4.4%; 0.9 – 7.9%), of them four (3.0%) were resistant to two antibiotics, and two isolates (1.5%) were resistant to three antibiotics. The prevalence of resistance to multiple drugs was 1.2% (1/84) in adults and 9.8% (5/51) in children ( $P = 0.029$ ).

Of the four isolates resistant to two antibiotics, three were resistant to metronidazole and clarithromycin and one was resistant to metronidazole and amoxicillin.

Of the two isolates resistant to three antibiotics, one was resistant to metronidazole, amoxicillin, and clarithromycin, and the other one was resistant to metronidazole, amoxicillin, and tetracycline.

*H. pylori* isolates from boys compared to girls, had a significantly higher resistance rate to metronidazole (55.5% vs. 27.3%,  $P = 0.046$ ) and clarithromycin (16.6% vs. 0.0%,  $P = 0.039$ ). A higher rate of resistance to both metronidazole and clarithromycin was also observed in boys (3/18, 16.6% vs. 0/33, 0.0%,  $P = 0.039$ ).

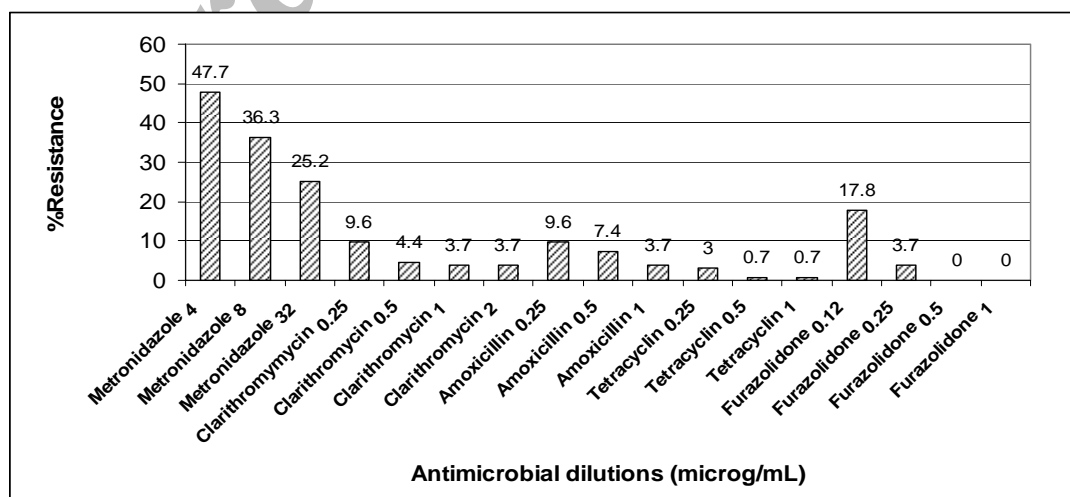
The resistance rates to amoxicillin and tetracycline were 16.6% and 5.55% in boys as compared to 0.0% and 0.0% in girls, respectively. The difference was significant for amoxicillin ( $P = 0.039$ ) but not for tetracycline ( $P = 0.39$ ). Resistance to furazolidone was not observed.

In *H.pylori* isolates obtained from women, the resistance rates to metronidazole, clarithromycin, and amoxicillin were 36.7%, 4.1%, and 2.1%, respectively, while in men these rates were 36.6%, 0.0%, and 3.4%, respectively. The differences between resistance rates in men and women were not significant.

The mean age of adults was 41.2 years and that of children was 8.6 years. The mean age of adults and children of whom resistant and susceptible strains were isolated did not differ significantly for metronidazole, clarithromycin, amoxicillin, and tetracycline (all  $P > 0.1$ ).

## Discussion

Susceptibility of *H.pylori* isolates from 84 adults and 51 children to metronidazole,



**Figure 2.** Resistance rates of 135 *H.pylori* isolates at different dilutions of antimicrobials.

**Table 2.** Metronidazole resistance rates of *H.pylori* isolates reported from Iran and other countries.

	Iran	Iran	Developed countries	Developing countries	Canada	Portugal	Germany	Belgium	Europe	Shanghai
Rates of resistance	32.8% (1997 – 2000)	35-37% (2001 – 4)	10 – 50%	80 – >90%	0%	32.3% (1990 – 99)	26.2%  (1995 – 2000)	30%  Increased to 48% over 5 years	20 – 30%  Increased to 50% (1993 – 98)	37.3%  Increased to 77.8% (1998 – 2000)
Change with time	–	NSCh	–	–	–	NSCh	NSCh			

NSCh = no significant change.

clarithromycin, amoxicillin, tetracycline, and furazolidone was assessed during the period of 2001 – 2004.

The results showed similar rates of resistance to metronidazole among *H.pylori* isolates from adults (35.7%) and children (37.3%). Studies from Portugal showed a higher rate of metronidazole resistance in *H.pylori* isolates obtained from adults (32.3%), compared to those from children (19%).<sup>8</sup> The rates of metronidazole resistance in this study were intermediate, compared to the range of rates reported worldwide for adults' isolates, from 10 – 50% in developed countries<sup>9–12</sup> to 80 – >90% in developing countries.<sup>8,9,13,14</sup> In contrast, no resistance has been reported from Canada.<sup>15</sup> The rates of metronidazole resistance in this study were close to the results of our previous study on *H.pylori* isolates from adults (32.8%), which was performed with the same MIC (8 µg/mL) of metronidazole, between the years 1997 to 2000.<sup>16</sup> These results indicated that metronidazole resistance among *H.pylori* isolates in Iran did not change significantly with time. Rates of resistance also remained constant among *H.pylori* isolates from Portugal<sup>8</sup> and Germany.<sup>17</sup> Although a considerable increment was observed in other European countries,<sup>9</sup> Belgium,<sup>18</sup> and Shanghai<sup>19</sup> (Table 2). Distribution of different metronidazole MICs among *H.pylori* isolates from adults and children showed similar patterns, and the MIC for the majority (52.6%) of isolates from adults and children was 4 µg/mL. This finding indicates that most of *H.pylori* isolates are highly sensitive to metronidazole (MIC<sub>≤</sub>4 µg/mL), although a considerable number (25.2%) are highly resistant to this antibiotic (MIC<sub>≥</sub>32 µg/mL).

The rate of resistance to metronidazole in *H.pylori* isolates from boys was significantly higher than those from girls, although a report

from France was different (Table 3).<sup>20</sup>

The rate of resistance to metronidazole was similar among *H.pylori* isolates from men and women. Similar results were reported from Portugal,<sup>8</sup> whereas isolates from women in Germany<sup>17</sup> and China<sup>21</sup> showed a higher resistance to metronidazole (Table 3).

The rate of resistance to clarithromycin in *H.pylori* isolates from adults (2.4%) and children (5.9%) were not significantly different. In Portugal clarithromycin resistance rate was lower in *H.pylori* isolates from adults (14.6%), compared to those from children (44.8%).<sup>8</sup> The range of clarithromycin resistance in *H.pylori* isolates from adults varies between 2 and 15% in developed countries<sup>8–11</sup> (Table 4). The results from this study, compared to the results from our previous study (1.43%, MIC 0.25 µg/mL),<sup>16</sup> showed no significant changes with time. Similarly, resistance rates of *H.pylori* isolates from adults in Germany<sup>17</sup> and France<sup>20</sup> did not show a significant change with time. Although reports from Belgium<sup>18</sup> and Portugal<sup>8</sup> describe significant change with time (Table 4).

Similar to metronidazole, the clarithromycin resistance rate was higher in *H.pylori* isolates from boys (16.6%) than in those from girls (0%). There was no significant differences between clarithromycin resistance in isolates from men (0%) and women (4.1%). Similar results have been reported from Germany<sup>17</sup> and Portugal<sup>8</sup> (Table 5).

The amoxicillin resistance rate was not

**Table 3.** Metronidazole resistance rates of *H. pylori* isolates reported from Iran and other countries, based on the age and gender.

Patients	Iran	France	Portugal	China	Germany
Boys	55%	40%	–	–	–
Girls	27.3%	60%	–	–	–
Men	36.6%	–	31.7%	69%	20.8%
Women	36.7%	–	30.8%	85%	32.2%

**Table 4.** Clarithromycin resistance rates of *H. pylori* isolates reported from Iran and other countries.

	Iran	Iran	Developed countries	Germany	France	Belgium	Portugal
Rates of resistance	1.43% (1997 – 2000)	2.4 % (2001 – 2004)	2 – 15%	2.2%	21%	1.7%	4.6%
Change with time	—	NSCh	—	NSCh (1995 – 2000)	NSCh (1994 – 99)	Increased to 10.5% (1989 – 94)	Increased to 22% (1990 – 99)

NSCh = no significant change.

significantly different in isolates from adults and children and did not change with time compared with our previous study (MIC 0.25 µg/mL)<sup>16</sup> (Table 6).

Tetracycline resistance was not observed in *H. pylori* isolates from adults in our present and previous (MIC 0.25 µg/mL) studies.<sup>16</sup> No significant resistance to amoxicillin and tetracycline has been reported from Germany,<sup>17</sup> France,<sup>20</sup> Bulgaria,<sup>22</sup> and Portugal.<sup>8</sup> However, considerable rates of resistance to both antimicrobials have been reported from China<sup>21</sup> and Italy<sup>23, 24</sup> (Table 6).

No resistance to furazolidone was observed in

**Table 5.** Clarithromycin resistance rates of *H. pylori* isolates reported from Iran and other countries based on the age and gender.

	Iran	Germany	Portugal
Boys	16.6%	—	—
Girls	0%	—	—
Men	0%	2%	16.9%
Women	4.1%	2.4%	20.1%

*H. pylori* isolates from adults and children. The MIC recruited in this study (MIC 0.5 µg/mL) was higher than the MIC used in our previous study (0.125 µg/mL),<sup>16</sup> but still similar to the MIC recruited in the other studies.<sup>25</sup> Here, we found 17.8% resistance rate with the MIC of 0.125 µg/mL. Furazolidone resistance has been reported from *in vitro* and *in vivo* studies in Korea (MIC 0.5 µg/mL)<sup>25</sup> and Brazil,<sup>26</sup> respectively (Table 6). Considering that furazolidone has been included in combination therapies as a preferred substitute for metronidazole,<sup>27 – 29</sup> further studies on higher number of isolates within time intervals will help to interpret the increase in the MIC of furazolidone as an indicative of emergence of resistance.

Resistance to multiple drugs was higher in *H. pylori* isolates from children compared to adults. The highest rate of resistance (50%) was to metronidazole and clarithromycin. Resistance to combination of metronidazole and clarithromycin has been also reported from France (9%),<sup>20</sup> Japan (10%),<sup>30</sup> Bulgaria (2.8%),<sup>22</sup> and Germany (58.3%).<sup>17</sup>

Resistance to combination of metronidazole, amoxicillin, and tetracycline was exhibited by one strain. However, 39.2% of isolates from Chinese children exhibited combined resistance to these antibiotics.<sup>21</sup>

Results from this study showed that age, sex, and time do not significantly affect the resistance of *H. pylori* isolates to different antibiotics. It appears that in spite of the current use of metronidazole and macrolides against parasitic, gynecologic, and respiratory infections, the rates of resistance to these antibiotics in many regions of the world have remained constant.<sup>17, 20</sup> This finding indicates that antibiotic resistance in *H. pylori*, like in other Gram-negative bacteria, is part of the evolutionary designed adaptation machinery. This includes poorly permeable outer membrane,<sup>31</sup> beta-lactamases,<sup>32</sup> and active efflux pumps,<sup>33</sup> which confer to bacteria the intrinsic resistance to environmental stresses. In other word, antibiotic resistance in bacteria is not necessarily the consequence of frequent exposure to certain antibiotics, although it could be due to the selection of resistant survivors and spread of resistant genes. Furthermore, lines of evidence indicate that efflux pumps in different bacteria have evolved long before the antibiotic era.<sup>34</sup>

Mutations either leading to over-expression of efflux pumps,<sup>35</sup> with an additive increase in MICs of antibiotics,<sup>36</sup> or to change in the target site(s) of

**Table 6.** Resistance rates of *H. pylori* isolates reported from Iran and other countries to amoxicillin, tetracycline, and furazolidone.

	Iran	Iran	Germany	France	Bulgaria	Portugal	China	Italy	Korea	Brazil
Amoxicillin	1.43% (1997 – 2000)	2.4%	0%	0%	—	0%	71.9%	31%	—	—
Tetracycline	0%	0%	0%	—	1.9%	0%	58.8%	6%	—	—
Furazolidone	0%	0%	—	—	—	—	—	—	2%	4%

antibiotics<sup>37</sup> could also confer resistance. These mutations are also the common consequences of bacterial responses to environmental stresses such as osmotic shock and starvation.<sup>38</sup> Concurrence of both types of mutations can lead to highly refractory bacterial infections.<sup>39</sup>

Since beta-lactamases are not present in *H.pylori*, it has been proposed that intrinsic resistance of *H.pylori* to antibiotics is due to the low permeability of bacterial lipopolysaccharide<sup>40</sup> and recently-recognized active efflux.<sup>41</sup> However, point mutations in the peptidyl transferase domain of the 23S rRNA lead to resistance to clarithromycin,<sup>42</sup> and mutations in *rdxA* or *fdxA* genes encoding NAD(P)H nitroreductase and NAD(P)H flavin oxidoreductase, respectively, could result in metronidazole resistance.<sup>43</sup>

In Iran, a considerable number of individuals (~ 90%) are infected with *H.pylori*<sup>44</sup> and there are regions in which the prevalence of peptic ulcers<sup>45</sup> and gastric cancer<sup>46</sup> is high. Since the use of antibiotics and eradication of *H.pylori* significantly decrease the recurrence rate of peptic diseases,<sup>47</sup> effective *H. pylori* eradication should be initiated especially in patients at risk.<sup>3, 4, 48</sup> Regarding the considerable rate of metronidazole resistance among *H.pylori* isolates (32.8 – 37%) in Iran, gastroenterologists recruit quadruple regimens in which metronidazole has been substituted with clarithromycin<sup>49</sup> or furazolidone.<sup>50</sup>

Based on the results of this study and several other reports,<sup>7, 17, 21, 22, 30</sup> and having considered that metronidazole is an antibiotic of choice against susceptible strains of *H.pylori*, performance of susceptibility tests is recommended for *H.pylori* isolates, in order to design an appropriate therapeutic regimen. Furthermore, comparison of geographic regions with low and high rates of resistance to metronidazole might reveal the cultural or environmental factors that play a role in the prevalence of resistance to metronidazole.

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