Antimicrobial effectiveness of furazolidone against metronidazole-resistant strains of *Helicobacter pylori*

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فعالية الفورازوليدون المضادة للمكروبات ضد ذراري الـمَلْوِيَّة البوابيَّة المقاومة للمتـرونيدازول

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الخلاصـــة: إن ظهـور الـذراري المقاومـة للمتــرونيدازول، يـؤدِّي إلى إخفـاق نظـام استئـصال الــمَلْوِيَّة البوابيَّـة *Helicobacter pylori* بالأدوية الأربعة، في جمهورية إيـران الإسـلامية. وقـد تم في هـذه الدراسـة مقارنـة النجاعـة المختبرية للفورازوليدون مع نجاعة كل من المتـرونيدازول، والكلاريثروميسين، والأموكسيسيلين، والتتــراسيكلين، في معالجة 70 مـستفرَدة مـن الــمَلْوِيَّة البوابيَّـة، مـن مرضـى يعـانون مـن عـسر الهـضم. وقـد كـان ثلـث (33٪) المستفرَدات مقاومة للمتـرونيدازول، في حين كانت جميعاً شديدة الحساسية للفورازوليدون. ومن ثَـمَّ يمكن اعتبـار الفورازوليدون بديلاً ملائماً للمتـرونيدازول في معالجة العدوى بالـمَلْوِيَّة البوابيَّة.

ABSTRACT The occurrence of strains resistant to metronidazole is causing failure of the 4-drug regimen for eradication of *Helicobacter pylori* in the Islamic Republic of Iran. This study compared the *in vitro* efficacy of furazolidone with metronidazole, clarithromycin, amoxicillin and tetracycline in 70 *H. pylori* isolates from dyspeptic patients. Of the isolates, 33% were resistant to metronidazole but all were susceptible to furazolidone. Furazolidone could be considered as an appropriate substitute for metronidazole for *H. pylori* infections.

Efficacité antimicrobienne de la furazolidone contre les souches d'*Helicobacter pylori* résistantes au métronidazole

RÉSUMÉ L'apparition de souches résistantes au métronidazole cause l'échec du schéma associant quatre médicaments pour un traitement d'éradication d'*Helicobacter pylori* en République islamique d'Iran. La présente étude a comparé l'efficacité *in vitro* de la furazolidine avec le métronidazole, la clarithromycine, l'amoxicilline et la tétracycline pour 70 isolats de *H. pylori* provenant de patients dyspeptiques. Parmi ces isolats, 33 % présentaient une résistance au métronizadole, mais tous ont montré une sensibilité à la furazolidone. La furazolidone pourrait être considérée comme substitut approprié du métronidazole pour les infections à *H. pylori.*

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Introduction

Many studies on the chemotherapy of *Helicobacter pylori* infections have indicated that eradication of *H. pylori* from the human stomach is hard to achieve and recrudescence occurs as a result of a persistent residual population that survived the therapy or re-infection with new strains. The low efficacy of currently used antimicrobials, even as quadruple regimens, urges researchers to look for novel antimicrobials with higher efficacy.

Metronidazole has been included in quadruple therapies in the Islamic Republic of Iran, but due to suboptimal eradication rates, investigators have attempted to replace it with other antimicrobials such as furazolidone [*1*]. However, resistance to metronidazole has been reported with varying degrees (20%–30%), reaching up to 70% in some European countries [*2*]. There is evidence that most metronidazole-resistant strains have a mutation in the *RDXA* gene, which leads to an inability to reduce the active nitro- group in metronidazole [*3*].

Although clarithromycin is effective when used in combination with a bismuth salt, a proton pump inhibitor (PPI), and amoxicillin or metronidazole [*4,5*], worldwide reports also describe an increasing emergence of resistant strains [*6,7*], reaching up to 14% in France [*8*]. Clarithromycin resistance is believed to result from clonal selection of resistant variants rather than from reinfection with exogenous clarithromycin-resistant strains [*9*]. Resistance appears to be due to a single nucleotide mutation [*10*] or post-transcriptional methylation of the 23S rRNA [*11*]. Amoxicillin and tetracycline are the 2 most highly effective antimicrobials against *H. pylori in vitro* and there are very few reports of resistant strains emerging [*12–14*]. Furazolidone appears to be an effective antimicrobial agent against *H. pylori*, particularly in combination with other antimicrobials such as clarithromycin [*15,16*] or tetracycline [*17*]. Furthermore, there are few reports of *H. pylori* resistance to furazolidone [*18,19*].

H. pylori infection is highly prevalent in the population of the Islamic Republic of Iran (> 80% in one study [*20*]) and a considerable proportion of individuals suffer from dyspeptic diseases such as gastric ulcer [*20*] or cancer [*21*]. Furthermore, a high frequency (37%) of metronidazole-resistant strains in the country increases the risk of persistence of *H. pylori* infection [*22*]. Accordingly, this might be a plausible reason for incorporating furazolidone in quadruple regimens for the eradication of metronidazole-resistant strains of *H. pylori*.

In this study, epsilometer (E-test) and disk diffusion methods were used to compare the susceptibility of *H. pylori* isolates from Iranian patients to a range of antimicrobials including furazolidone. The minimum inhibitory concentration (MIC) of the antimicrobials was also determined.

Methods

The study group was 70 dyspeptic patients referred to the endoscopy unit at Shariaty Hospital in Tehran, Islamic Republic of Iran between 2001–03. The patients were diagnosed with ulcer (9), oesophagitis (18), Barrett's oesophagus (15) and gastritis (28).

Antral biopsies were cultured on selective Brucella blood agar (Merck), and plates were incubated under microaerobic conditions (5% CO_2) at 37 °C. The identity of bacterial isolates was confirmed by microscopy and positive catalase, oxidase and urease reactions. Three-day cultures were used to prepare bacterial suspensions in normal saline, with the turbidity equiva-

lent to McFarland standard no. 1. Volumes of 100 µL of bacterial suspensions were spread evenly over the Mueller–Hinton agar containing 7% defibrinated blood. E-test strips (AB Biodisk, Solna, Sweden) or blank paper disks were then deposited on the surface of the inoculated plates. Plates were incubated as mentioned earlier and examined after 2–5 days.

The E-test was used to assess the susceptibility of *H. pylori* isolates to metronidazole, clarithromycin, amoxicillin and tetracycline. The MIC obtained for amoxicillin, tetracycline and clarithromycin were within the same range of 0.016–0.25 µg/mL. Highly susceptible strains produced inhibition zones at MIC ≤ 0.016 mµg/mL. Susceptible isolates had MIC ranging from 0.016–0.25 µg/mL. Those *H. pylori* strains which were not inhibited by antimicrobial concentrations of ≥ 0.25 µg/mL were considered as resistant or highly resistant. For metronidazole, however, the MIC was different, ranging from 8–32 µg/mL. Highly resistant strains were not inhibited by concentrations of ≥ 32 µg/mL and resistant strains grew at metronidazole concentrations between 8–32 µg/mL. Susceptible strains produced inhibition zones at metronidazole concentrations of < 8 µg/mL.

The antimicrobial efficacy of furazolidone against *H. pylori* was assessed by the disk diffusion method. Serial dilutions of furazolidone (Sigma): 1, 0.75, 0.5, 0.25, 0.12 and 0.06 µg/mL were prepared in dimethylformamide. Then 10 µL volumes of furazolidone dilutions were introduced into paper disks on the surface-inoculated blood agar. The plates were examined after 2–5 days of microaerobic incubation. The MIC for furazolidone was determined as 0.12 µg/mL and susceptibility of *H. pylori* isolates was determined on the basis of the diameter of inhibition zones. Strains of *H. pylori* exhibiting the inhibition zones of 13–16 mm were considered as susceptible, and those producing inhibition zones of > 16 mm were considered as highly susceptible. Growth inhibition was not observed on plates deposited with blank discs containing dimethylformamide only.

Results

From 70 *H. pylori* isolates tested for susceptibility to amoxicillin, 61.4% were highly susceptible, 37.1% susceptible, and only 1 strain (1.4%) exhibited resistance (Table 1). The latter was highly susceptible to other antimicrobials. The majority of *H. pylori* isolates (72.8%) were highly susceptible to low concentrations of tetracycline, but 27.1% were inhibited by higher concentrations (0.016–0.25 mµg/mL), and are thus considered as susceptible. Resistance to tet-

Table 1 **Susceptibility of 70 isolates of *H. pylori* to amoxicillin, tetracycline and clarithromycin by epsilometer (E-test)**

Antimicrobial agent	Highly susceptible MIC ≤ 0.01			Susceptible 0.016 < MIC < 0.25			Resistant MIC ≥ 0.2		
	No.	%	95% CI	No.	%	95% CI	No.	%	95% CI
Amoxicillin	43	61.4	49.0–72.8	26	37.1	25.9–49.5	1	1.4	0.0–7.7
Tetracycline	51	72.9	60.9–82.8	19	27.1	17.2–39.1	0	0.0	
Clarithromycin	65	92.9	84.1–97.6	4	5.7	1.5–13.9	1	1.4	0.0–7.7

MIC = minimum inhibitory concentration (µg/mL).
CI = confidence interval.

racycline was not observed among *H. pylori* isolates (Table 1).

Clarithromycin showed a considerable efficacy in inhibiting *H. pylori*. Sixty-five out of 70 (92.9%) strains were highly susceptible, 5.7% susceptible, and only 1 strain (1.4%) showed resistance to clarithromycin (Table 1).

H. pylori isolates were also resistant to metronidazole: 21.4% were highly resistant, 11.4% resistant and 67.1% susceptible to metronidazole (Table 2). The frequency of metronidazole resistance in isolates from patients with Barrett's oesophagus (20.0%), ulcer (22.2%), and oesophagitis (27.8%) was higher than those from gastritis patients (17.9%), but *t*-test analysis showed it was not significant ($P = 0.56$) (Table 3).

Among 70 *H. pylori* isolates, 7 (10.0%; 95% CI: 4.1–19.5%) were susceptible and 63 (90.0%; 95% CI: 80.5–95.9%) were highly susceptible to furazolidone. None of the isolates exhibited resistance to furazolidone (Table 4). The number of highly susceptible *H. pylori* isolates to furazolidone was significantly more than to amoxicillin ($P < 0.001$; t-test), tetracycline ($P < 0.01$; *t*-test), and metronidazole ($P < 0.001$; *t*-test).

Discussion

Quadruple therapies have been proved to be the most effective antimicrobial regimens against *H. pylori* in the Islamic Republic of Iran [*1,4,23*], and recrudescence of infection occurs mainly due to the occurrence of strains resistant to metronidazole [*1*]. Metronidazole, although considered as one of the most suitable antimicrobials, induces the highest rate of resistance (15%–90%) in *H. pylori* populations [*24*]. In this study, resistance to metronidazole was 33%. It appeared that patients with ulcer, Barrett's oesophagus and oesophagitis were more often infected with resistant strains compared with gastritis patients. Previous study in the Islamic Republic of Iran showed that 37% of isolates were resistant to metronidazole [*22*]. Resistance to metronidazole is also prevalent in Japan with frequencies of 54.5% [*25*] and 26.5% [*26*], and in Netherlands with a rate of 24% [*27*]. Resistance as high as 61% is reported from Peru [*28*].

Clarithromycin efficacy, when used in combination therapies, has been reported [*4,5*], but the emergence of resistant strains plus its high cost, make its application limited. The frequency of resistance to clarithromycin in this study was 1.4%. This was close to the lower range of resistance (1%–13%) reported from different regions of the world, including Sweden, Poland and Spain [*2*].

Amoxicillin and tetracycline have applications against a wide range of pathogenic microorganisms and there are very few reports on the emergence of resistant strains. These 2 antimicrobials thus continue to be successfully used in combination therapies against *H. pylori* [*29,30*]. In this study, resistance to tetracycline was not observed, indicating its high efficacy against

Table 2 **Susceptibility of 70 isolates of *H. pylori* to metronidazole by epsilometer (E-test)**

Antimicrobial agent	Susceptible MIC > 8			Resistant 8 ≤ MIC ≤ 32			Highly resistant MIC > 32		
	No.	%	95% CI	No.	%	95% CI	No.	%	95% CI
Metronidazole	47	67.1	54.9–77.9	8	11.4	5.6–21.3	15	21.4	12.5–32.8

MIC = minimum inhibitory concentration (μg/mL).
CI = confidence interval.

Table 3 **Susceptibility of 70 isolates of *H. pylori* to metronidazole according to patient diagnosis**

Patient diagnosis	No. tested	Resistant No.	%	95% CI
Barrett's oesophagus	15	3	20.0	4.3–48.1
Ulcer	9	2	22.2	2.8–60.0
Oesophagitis	18	5	27.8	9.7–53.5
Gastritis	28	5	17.9	6.1–36.9

CI = confidence interval.

H. pylori. Bacterial isolates from Peruvian patients did not show resistance to tetracycline [*28*], although 4.9% resistant strains occurred in Korea and *6*.7% in Japan [*31*], and 58% in China [*32*]. Among 70 *H. pylori* isolates, 1 (1.43%) exhibited resistance to amoxicillin. The majority of studies reported no resistance to amoxicillin [*13,14*]; however, resistance as high as 71.9% was found in China [*32*].

Furazolidone with MIC of 0.12 μg/mL was comparable to amoxicillin, tetracycline and clarithromycin and showed a remarkable efficacy against *H. pylori*. None of the isolates exhibited resistance to this antimicrobial. Reports from different regions of the world also describe the high efficacy of furazolidone in eradication of *H. pylori* [*33*]. Furazolidone was effective in eradication of *H. pylori* when used in triple [*15,34*] or quadruple therapies [*35*]. Different MIC have been obtained in various laboratories, e.g. from < 0.006–0.2 μg/mL in China [*36,37*]. However, 4% furazolidone resistance and 42% metronidazole resistance were reported for *H. pylori* in Brazil [*19*]. A similar report from South Korea described 2% furazolidone and 52% metronidazole-resistant strains of *H. pylori* [*18*]. It was also found that metronidazole-resistant and -susceptible strains were both similarly inhibited by furazolidone [*33,38*]. These data are confirmed by other reports, indicating that there is no cross-resistance between metronidazole and furazolidone among *H. pylori* strains [*33*].

Furazolidone is an antimicrobial from the nitrofuran group. Like that of metronidazole, the bactericidal mechanism of action of this group of antimicrobials involves enzymatic reduction of the parent compound to electrophilic radicals [*39,40*]. In spite of the similarity in the mechanisms of action, it appears that development of bacterial resistance to metronidazole [*41,42*] is different from that of nitrofurans [*43*]. Furthermore, *H. pylori* does not appear to readily acquire resistance to nitrofurans [*44*]. Prescription of furazolidone in combination with amoxicillin or tetracycline, plus ranitidine and a bismuth salt has led to a higher eradication rate of *H. pylori* (82%) compared with metronidazole (56%) [*1*]. Furazolidone has been also effective in the clearance of *H. pylori* and resolution of acute gastric inflammation [*3,37*] and duodenal ulcer healing [*15*]. The results of this study suggest the recruitment of furazolidone as an effective, cheap and readily available antimicrobial [*33*] in quadruple therapy regimens especially in areas with high prevalence of metronidazole-resistant strains of *H. pylori*.

Table 4 **Susceptibility of 70 *H. pylori* strains to furazolidone with the minimum inhibitory concentration of 0.12 μg/mL**

Bacterial response	Diameter of inhibition zone (mm)	% resistant
Susceptible	13–16	10
Highly susceptible	> 16	90
Resistant	< 13	0

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